TRANSCRIPTIONAL REGULATION OF HUMAN UDP-GLUCURONOSYLTRANSFERASES

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The UDP-glucuronosyltransferases (UGTs) are a superfamily of enzymes that glucuronidate small, lipophilic molecules, thereby altering their biological activity and excretion. In humans, important examples of UGT substrates include molecules of both endogenous and xenobiotic origin; thus, UGTs are considered essential contributors to homeostatic regulation and an important defence mechanism against chemical insult. In keeping with both roles, UGTs are most strongly expressed in the liver, a predominant organ involved in detoxification.

Rates of glucuronidation in humans are neither uniform among individuals, nor constant in an individual over time. Genetic determinants and non-endogenous signals are both known to influence the expression of UGTs, which in turn may affect the efficacy of certain pharmaceutical treatments or alter long-term risk of developing disease. Thus, this thesis focuses on the transcriptional regulation of UGT genes in humans, particularly on mechanisms that are likely to be relevant to their expression and variation in the liver. Two major approaches were used: firstly, extensive studies of several UGT promoters were performed to identify and characterise transcriptional elements that are important for UGT expression; and secondly, important hepatic transcription factors were investigated as potential regulators of UGT genes.

UGT1A3, UGT1A4 and UGT1A5 are a subset of highly related, but independently regulated, genes of the human UGT1 subfamily. UGT1A3 and UGT1A4 are expressed in the liver, whereas UGT1A5 is not. The presented analysis of the UGT1A3, UGT1A4 and UGT1A5 proximal promoters demonstrates that a hepatocyte nuclear factor (HNF)1-binding site common to all three promoters is important for
UGT1A3 and UGT1A4 promoter activity in vitro, but is insufficient to drive UGT1A5 expression. Two additional elements required for the maximal activity of the UGT1A3 promoter were also identified that may distinguish this gene from UGT1A4. UGT1A3 was investigated further, focusing on mechanisms that may contribute to interindividual variation in UGT1A3 expression. Polymorphisms in the UGT1A3 proximal promoter were identified and their functional consequences tested. Known variants of HNF1α were also tested for altered activity towards the UGT1A3 gene.

UGT1A9 is the only hepatic member of the UGT1A7-1A10 subgroup of UGT1 enzymes. Previous work had identified HNF1-binding sites in all four genes, and HNF4α as an UGT1A9-specific regulator. The work presented herein extends these findings to show that HNF1 factors and HNF4α synergistically regulate UGT1A9, and that HNF4α is not the only transcription factor responsible for the unique presence of UGT1A9 in the liver.

Liver-enriched transcription factors screened as potential UGT regulators were chosen from the HNF1, HNF4, HNF6, FoxA and C/EBP protein families. Functional interactions newly identified by this work were HNF4α with UGT1A1 and UGT1A6, HNF6 with UGT1A4 and UGT2B11, FoxA1 and FoxA3 with UGT2B11, UGT2B15 and UGT2B28 and C/EBPα with UGT2B17. Observations were also made regarding different patterns of interaction between each UGT and the transcription factors tested, particularly HNF1α.

These studies significantly advance the understanding of the transcriptional control of human UGT genes. In time, it is hoped that a detailed knowledge of UGTs will be useful in developing better therapeutic and prophylactic medical treatments.
DECLARATION

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Dione Gardner-Stephen
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incentive to finish this thesis and a very welcome distraction from the more mundane aspects of “writing-up”. I also want to recognise the contributions that my immediate and extended family, my friends and my church family have all made to my well-being over the course of my PhD. Thank you to you all. Finally, I willingly acknowledge the claim that Christ has on my life and praise Him for His unfailing provision for me; past, present and future.
PUBLICATIONS ARISING DIRECTLY FROM THIS THESIS


ADDITIONAL PUBLICATIONS RELATED TO THIS THESIS


effects of organic solvents, and inhibition by diclofenac and probenecid. *Drug Metab. Dispos.* **32**: 413-423.


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**AWARDS IN SUPPORT OF THIS THESIS**


### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADH</td>
<td>alcohol dehydrogenase</td>
</tr>
<tr>
<td>AF</td>
<td>activation function</td>
</tr>
<tr>
<td>AhR</td>
<td>aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>apo</td>
<td>apolipoprotein</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BAC</td>
<td>bacterial artificial chromosome</td>
</tr>
<tr>
<td>β-ME</td>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>bZIP</td>
<td>basic region leucine zipper</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT/enhancer binding protein</td>
</tr>
<tr>
<td>CAR</td>
<td>constitutive androstane receptor</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
</tr>
<tr>
<td>CDCA</td>
<td>chenodeoxycholic acid</td>
</tr>
<tr>
<td>Cdx</td>
<td>caudal-related homeodomain protein</td>
</tr>
<tr>
<td>CIP</td>
<td>calf intestinal alkaline phosphatase</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>CoA</td>
<td>coenzyme A</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP-response-element-binding protein</td>
</tr>
<tr>
<td>CYP</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>DBP</td>
<td>D-site binding protein</td>
</tr>
<tr>
<td>DCoH</td>
<td>dimerisation co-factor of HNF1</td>
</tr>
<tr>
<td>DD</td>
<td>dihydrodiol dehydrogenase</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotide-triphosphate</td>
</tr>
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<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>EMSA</td>
<td>electrophoretic mobility-shift assay</td>
</tr>
<tr>
<td>ERβ</td>
<td>oestrogen receptor β</td>
</tr>
<tr>
<td>fabp</td>
<td>fatty acid-binding protein</td>
</tr>
<tr>
<td>FoxA</td>
<td>forkhead box A</td>
</tr>
<tr>
<td>FXR</td>
<td>farnesoid X receptor</td>
</tr>
<tr>
<td>glut2</td>
<td>glucose transporter 2</td>
</tr>
<tr>
<td>GR</td>
<td>glucocorticoid receptor</td>
</tr>
<tr>
<td>GRIP</td>
<td>glucocorticoid receptor interacting protein</td>
</tr>
<tr>
<td>HAT</td>
<td>histone acetyltransferase</td>
</tr>
<tr>
<td>HCV</td>
<td>hepatitis C virus</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>HNF</td>
<td>hepatocyte nuclear factor</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>Inr</td>
<td>initiator</td>
</tr>
<tr>
<td>LAP</td>
<td>liver-enriched transcriptional activator protein</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
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<td>--------------</td>
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</tr>
<tr>
<td>LETF</td>
<td>liver-enriched transcription factor</td>
</tr>
<tr>
<td>LIP</td>
<td>liver-enriched transcriptional inhibitory protein</td>
</tr>
<tr>
<td>LXR</td>
<td>liver X receptor</td>
</tr>
<tr>
<td>MODY</td>
<td>mature onset diabetes of the young</td>
</tr>
<tr>
<td>MRP</td>
<td>multidrug resistance protein</td>
</tr>
<tr>
<td>NCoR</td>
<td>nuclear receptor co-repressor</td>
</tr>
<tr>
<td>NNAL</td>
<td>4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol</td>
</tr>
<tr>
<td>NRRE</td>
<td>nuclear receptor response element</td>
</tr>
<tr>
<td>NSAID</td>
<td>non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>OAT</td>
<td>organic anion transporter</td>
</tr>
<tr>
<td>Oct</td>
<td>octamer transcription factor</td>
</tr>
<tr>
<td>P/CAF</td>
<td>p300/CREB-associated factor</td>
</tr>
<tr>
<td>PBREM</td>
<td>phenobarbital response enhancer module</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>pBSII</td>
<td>pBlueScript II</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PGC</td>
<td>PPAR-gamma co-activator</td>
</tr>
<tr>
<td>PhIP</td>
<td>2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine</td>
</tr>
<tr>
<td>POU</td>
<td>Pit-1, Oct-1 and Oct-2, and Unc-86</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome-proliferator-activated receptor</td>
</tr>
<tr>
<td>PXR</td>
<td>pregnane X receptor</td>
</tr>
<tr>
<td>QPCR</td>
<td>quantitative real-time PCR</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
</tr>
<tr>
<td>rs</td>
<td>reference SNP</td>
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<tr>
<td>RXR</td>
<td>retinoid X receptor</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SHP</td>
<td>small heterodimer partner</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SMP</td>
<td>skim milk powder</td>
</tr>
<tr>
<td>SMRT</td>
<td>silencing mediator of retinoid and thyroid hormone receptor</td>
</tr>
<tr>
<td>SN-38</td>
<td>7-ethyl-10-hydroxycamptothecine</td>
</tr>
<tr>
<td>SNAP</td>
<td>S-nitroso-N-acetyl penicillamine</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>SRC</td>
<td>steroid receptor co-activator</td>
</tr>
<tr>
<td>TBE</td>
<td>tris-borate EDTA</td>
</tr>
<tr>
<td>TBST</td>
<td>tris-buffered saline/tween-20</td>
</tr>
<tr>
<td>TFII</td>
<td>transcription factor II</td>
</tr>
<tr>
<td>Tris</td>
<td>tris[hydroxymethyl]aminomethane</td>
</tr>
<tr>
<td>TSA</td>
<td>trichostatin A</td>
</tr>
<tr>
<td>TSS</td>
<td>transcription start site</td>
</tr>
<tr>
<td>UDP</td>
<td>uridine diphosphate</td>
</tr>
<tr>
<td>UGT</td>
<td>UDP-glucuronosyltransferase</td>
</tr>
<tr>
<td>XRE</td>
<td>xenobiotic response element</td>
</tr>
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</table>
1.1 Towards disease prevention and designer therapies

Human genetic variation, particularly in relation to disease, is becoming an increasingly important focus of medical research. The recent accomplishments of the Human Genome Project (International Human Genome Sequencing Consortium, 2001), the ongoing collection of single nucleotide polymorphism (SNP) data by the International HapMap Project and other groups (International HapMap Consortium, 2003; Johnson et al., 2005) and continuing advancements in bioinformatics, research methodologies and high-throughput technologies are providing an unprecedented wealth of information regarding human molecular biology. The ability to analyse our own genome on such a comprehensive scale is generating a collective belief that science can eventually tailor preventative and curative health-care right down to the level of an individual’s genetic shortcomings. The ultimate aspiration is to unravel the complex aetiology of the common diseases that are a major burden to Western society. These include cancer, cardiovascular disease and type 2 diabetes mellitus; ailments that were collectively responsible for over 18% of Australian medical expenditure in 2000-01 and 70% of deaths in 2001 (Australian Institute of Health and Welfare, 2005). Once the genetic susceptibility and causative environmental factors of such diseases are understood, it will then, at least theoretically, be possible to customise recommendations for a person’s health-care and life-style according to the risk factors that he or she has inherited (Haga et al., 2003; Ordovas and Mooser, 2004). For example, it is thought that many common clinical diagnoses, such as type 2 diabetes mellitus or hypertension, will be redefined into multiple subgroups based
on distinct molecular mechanistic causes: each subtype requiring its own treatment strategy for the best response (Heijmans et al., 2002; Lindpaintner, 2002). In addition, if relationships between genotype and cancer risk can be established, it may also be possible to develop measures to mitigate an individual’s probability of developing malignancies. Other multifactorial, polygenic disorders that are attracting such attention include psychiatric illness, stroke, obesity, neurodegenerative conditions including Parkinson and Alzheimer diseases, and inappropriate inflammatory/autoimmune responses including asthma and arthritis (Van Eerdewegh et al., 2002; El-Omar et al., 2003; de la Chapelle, 2004; Ordovas and Moos, 2004; Zee et al., 2004; Bernardini et al., 2005).

However, despite current progress, it will be many years or perhaps many decades, before personalised genomic medicine will have widespread usefulness for predicting an individual’s disease predispositions and metabolic limitations. This is due to a number of economic, ethical and technological hurdles that will not be considered in the scope of this thesis, but are discussed in detail by other authors (Issa, 2000; Lindpaintner, 2002; Haga et al., 2003; Paul and Roses, 2003). Nonetheless, in the meantime, information gleaned from studying basic gene regulation and genetic variation can better our comprehension of general biological concepts and lead to medical advances applicable to all humans, or to large genetic subgroups. For example, research focused on human drug metabolising enzymes is increasingly being taken into account at early stages of drug design and development, as well as in rational drug use, resulting in more targeted efforts in medical progress, and accordingly, improvements in safety and treatment outcomes. Genomic research is identifying new drugable targets, as well as revealing the mechanisms of previously observed drug-drug interactions (Lindpaintner, 2002;
Ross and Ginsburg, 2002; Evans and McLeod, 2003). Advances in molecular epidemiology are contributing to the identification of subpopulations (based on traits such as age, gender or genetic predisposition) that may have an elevated risk of developing cancer or other diseases following exposure to certain causative environmental factors (Perera, 2000). A better understanding of molecular targets and pharmacological parameters is also identifying specific patient genetic subgroups for whom certain drug regimes are most appropriate, or conversely, people for whom the use of specific therapies carry significant risk of harm. In time this will hopefully translate into improved prescribing, more accurate dosing and a reduction in adverse drug reactions (Weinshilboum, 2003; Ross et al., 2004), and is indeed already a reality for a select group of examples in some clinics, such as 6-mercaptopurine, irinotecan and several antidepressants (Kootstra-Ros et al., 2006; Maitland et al., 2006).

1.2 Genetic variation in metabolic pathways; implications for disease susceptibility and treatment

While the multifactorial causes of complex diseases are still poorly defined, it has long been evident that not all individuals exposed to a given set of external triggers will develop ill-health. Thus the chance that any particular individual will develop one or more such ailments is presumably determined not only by the environmental risk factors to which they are exposed, but also by intrinsic determinants such as age, gender and genetic disposition (Sanchez et al., 2001). Genetic risk for complex, multifactorial diseases is proving particularly difficult to characterise, as it is likely to be a product of seemingly trivial variations in multiple genes spread over the entire genome (Paul and Roses, 2003; Pharoah et al., 2004); however, variation in genes that affect an individual’s ability to detoxify and remove harmful chemicals
from the body, whether of endogenous or external origin, is one example where genetic variance may affect disease susceptibility. Many dietary constituents and environmental substances are potentially toxic or carcinogenic; therefore, enzymes and transporters that inactivate such compounds or facilitate their removal from the body minimise the damage incurred in the event of exposure (Perera, 2000; Perera and Weinstein, 2000; Hoffmann and Kroemer, 2004). In addition, metabolic enzymes and transport proteins affect the local and circulating levels of endogenous substances, many of which, such as steroids, have important homeostatic roles and must be maintained at appropriate levels (Nebert, 1994). Thus, sequence variation in the coding or regulatory regions of these genes, or in the factors that control their expression, may partially explain the observed variance in disease susceptibility between individuals (Perera and Weinstein, 2000; Desai et al., 2003; Leslie et al., 2005).

It is not only the likelihood of developing ill health or the severity of disease that can be affected by interindividual genetic variation and environmental stimuli. The degree to which standard medical intervention for a disease is successful also varies extensively between patients. The molecular mechanisms that determine the pharmacokinetic and pharmacodynamic properties of therapeutic drugs in humans can also be subject to genetic diversity and variation brought about by external cues. Thus, individuals can differ, or the same patient may vary temporally, in how effectively a drug will reach its target, the extent and type of modifications a drug will undergo, a drug’s clearance, the extent to which undesirable adverse effects are triggered and the clinical response obtained (Evans and Relling, 1999; Lindpaintner, 2002; Evans and McLeod, 2003; Weinshilboum, 2003). These issues extend not only to treatment of non-communicable disorders such as those mentioned above, but are
equally applicable to the treatment of infectious diseases and pharmaceutical management of pain.

It is evident that the more complete our understanding of human metabolism and its regulation becomes, the more effectively we will be able to devise and evaluate possible strategies for treating, or even preventing, disease and discomfit. Therefore, this thesis is designed to contribute further insights into the mechanisms regulating the metabolism of xenobiotic substances, endocrine signalling molecules and endogenous waste products in humans. In particular, this research investigates the regulation of the uridine diphosphate (UDP)-glucuronosyltransferase (UGT) genes, a subset of the many genes that participate in the biotransformation of lipophilic chemicals in humans and other higher organisms. General mechanisms, as well as those that may contribute to the differences that occur between individuals, are considered.

1.3 Biotransformation of small lipophilic molecules

The human body is constantly exposed to many potentially dangerous compounds, some arising from internal metabolic and catabolic processes, others being encountered directly through dietary intake, therapeutic and illicit drug use, or environmental contact (Wogan et al., 2004). Lipid-soluble chemicals of low molecular weight typically enter cells by passive diffusion across the membrane, those from external sources being readily absorbed via the gastrointestinal tract and lungs, and to a lesser extent, the skin (Artursson and Karlsson, 1991; Walle and Walle, 1999; Gunaratna, 2000). To avoid harm from carcinogens and toxins, or undesirable alterations in gene expression patterns by ligands, the body must regulate the intracellular concentration and biological activity of these substances through
chemical modification and/or active efflux through the cellular membrane. Members of the adenosine triphosphate (ATP)-binding cassette transporter protein superfamily, such as P-glycoprotein and the multidrug resistance proteins (MRP) can transport a large variety of unaltered lipophilic molecules out of the cell, returning them to the intestinal lumen or removing them from the systemic circulation for excretion via bile or urine. However, efficient efflux of small lipophilic chemicals, particularly by MRP transporters, normally requires or is significantly enhanced by prior biotransformation (Hoffmann and Kroemer, 2004).

Biotransformation is the act by which a chemical substrate is structurally modified by one or more enzyme-catalysed reactions. These reactions include such diverse processes as oxidation, reduction, conjugation and nucleophilic trapping, and are classically divided into two stages. However, although categorised as “Phase I/functionalisation” and “Phase II/conjugative” reactions, biotransformation events do not necessarily progress through the two stages sequentially, nor do they adhere strictly to the accepted generalisations for each class of reaction (Josephy et al., 2005). Therefore, for clarity, this thesis avoids the use of this nomenclature, referring specifically to the enzymes or reactions in question. Furthermore, while biotransformation enzymes also include oxygenases, dehydrogenases, reductases, deaminases, aminotransferases, methyltransferases and hydrolases, the description that follows concentrates primarily on the relevance of the cytochrome P450 (CYP) oxidoreductases and the glucuronosyltransferases, sulphotransferases, N-acetyltransferases and glutathione-S-transferases to human health.

Overall, biotransformation is considered to be protective in nature, as the terminal metabolic products of this process tend to be less biologically active than their parent compounds and more readily excreted. Oxidation, a common biotransformation
event predominately catalysed by the CYP superfamily, often results in the direct inactivation and elimination of a substrate. Moreover, with the exception of a small number of known examples, metabolites resulting from conjugative reactions are nearly always lacking in significant pharmacological or toxicological activity; glucuronides especially being renowned for their inert nature. In addition, many glucuronides, sulphates and glutathione-conjugates are excellent substrates for MRP transporters; thus they are more readily excreted from the cell than their parent compound. Elimination from the body is further enhanced by the inability of conjugated organic anions to passively re-enter cells by diffusion, due to their decreased lipid-solubility (Sanchez et al., 2001; Hoffmann and Kroemer, 2004).

It is important to note, however, that not all oxidations or conjugations generate inactive metabolites. Instead, metabolites from either type of biotransformation may have similar, increased or completely new actions compared with the original compound. One therapeutically relevant example is the requirement for codeine to be metabolised to morphine by CYP2D6 before an analgesic effect is experienced. Furthermore, a minor glucuronidation product of morphine, morphine-6-glucuronide, is believed to exhibit greater analgesic potency than morphine itself (Sawe et al., 1985; Osborne et al., 1992; Caraco et al., 1997). The glucuronides of certain steroids, retinoids and bile acids are also highly bioactive, with retinoyl beta-glucuronide having similar therapeutic benefit to retinoic acid but without the associated adverse effects (Ritter, 2000; Barua and Sidell, 2004). In contrast to these beneficial examples, oxidation and reduction reactions can also generate highly reactive electrophilic intermediates or nucleophilic radicals, capable of interacting deleteriously with cellular macromolecules such as proteins and DNA. Many environmental and dietary carcinogens, such as those found in tobacco smoke and
burnt meat have little or no mutagenic activity *per se*, but become highly reactive *in vivo* when metabolised by CYP or other enzymes. Examples of CYP-activated carcinogens include 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and other nitrosamines, polycyclic aromatic hydrocarbons such as benzo[a]pyrene, heterocyclic aromatic amines including 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), as well as aldehydes, phenols and aflatoxin (Nowell *et al.*, 1999; Guengerich, 2000; Fang *et al.*, 2002; Wiener *et al.*, 2004; Wogan *et al.*, 2004). A limited number of conjugates have also been shown to form DNA or protein adducts, enhance tumourigenesis, trigger toxic/immunological reactions or possess decreased water-solubility. Certain glutathione-S-transferase, *N*-acetyltransferase and sulphotransferase catalysed reactions have been found to contribute to the activity of environmental carcinogens, while there is some evidence that acyl glucuronides of carboxylic acid moieties such as non-steroidal anti-inflammatory drugs (NSAIDs) may trigger idiosyncratic adverse drug reactions. Furthermore, D-ring glucuronides of oestrogens such as oestradiol and ethinyl-oestradiol can cause cholestasis in rats, although ethinyl-oestradiol is used safely in human females as an oral contraceptive. Finally, while a given glucuronide may be inert, it may be subject to hydrolysis by β-glucuronidase to the parent aglycone, which may then be re-absorbed, or to a toxic intermediate as in the case of cleavage of 3-benzo[a]pyrene glucuronide. For some substances, this may actually lead to a prolonged exposure compared with alternative metabolic pathways. Acyl glucuronides are especially susceptible to hydrolysis and futile cycling, a factor that can be clinically important in patients with renal dysfunction (Kari *et al.*, 1984; Minchin *et al.*, 1992; Chou *et al.*, 1995; Sperker *et al.*, 1997; Grubb *et al.*, 1999; Bailey and Dickinson, 2003; Sanchez Pozzi *et al.*, 2003; Anders, 2004; Josephy *et al.*, 2005).
The actual impact of deleterious biotransformation events rests in the body’s ability to either circumvent them by directing parent compounds into alternative pathways that generate less harmful products, or by further metabolising reactive intermediates to inert substances. Glucuronidation is one such means by which cells are often protected from the effects of electrophilic metabolites. For example, PhIP is oxidised in the liver to $N$-OH–PhIP, a metabolite capable of forming DNA adducts that can also be further metabolised to even more highly reactive electrophilic species by acetyltransferases and sulphotransferases. Alternatively $N$-OH–PhIP can be inactivated by $N$-glucuronidation and safely excreted, avoiding the formation of the PhIP $N$-acetoxy and $N$-sulphonyoxy esters and their consequent DNA adducts, and hence the potential for carcinogenesis (see Figure 1.1) (Nowell et al., 1999; Hecht, 2003).

![Figure 1.1: Alternative metabolic pathways for 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) after CYP activation, resulting in DNA adducts or detoxification. Figure generated from information detailed by Nowell et al. (1999).](image-url)
Thus, the balance of biotransformation enzymes present in an individual controls the metabolic fate of many small lipophilic pharmaceutical drugs, xenobiotics and endogenous ligands, determining both their efficacy and toxicity. Genetic variations and environmental or inherent triggers that shift this balance will alter the individual’s response to drug therapies and change their likelihood of developing disease, whether for better or for worse. As glucuronidation is a key mechanism in the metabolism of many therapeutic compounds, dietary constituents, carcinogens, toxins, steroids, fatty acids, neurotransmitters and endogenous waste products such as bilirubin, an understanding of the factors that regulate this pathway is paramount to improved drug design and disease prevention strategies.

1.4 Glucuronidation and the UDP-glucuronosyltransferases

Glucuronidation is the process by which glucuronic acid, from the nucleotide sugar donor uridine 5’-diphosphoglucuronic acid, is transferred to a suitable functional group on an acceptor molecule. As a result, a β-D-glucopyranosiduronic acid derivative (glucuronide) of the original substrate is generated and UDP is released (see Figure 1.2). Multiple functional groups are amenable to glucuronidation, including hydroxyl (phenol and alcohol), carboxyl and thiol residues, as well as primary, secondary and tertiary amino groups. This diversity allows a large array of molecules from unrelated chemical classes to form glucuronides (Radominska-Pandya et al., 1999; King et al., 2000; Tukey and Strassburg, 2000). The glucuronidation reaction is thought to proceed through an acid/base bimolecular nucleophilic substitution (S_N2) reaction mechanism, as reviewed by Radominska-Pandya et al. (1999), and is catalysed by the members of a large enzyme family known as the UDP-glucuronosyltransferases (UGT; EC 2.4.1.17). The UGTs are in
members of the broader superfamily of UDP-glycosyltransferases, enzymes that transfer glycosyl groups to lipophilic substances from a variety of UDP-sugars. All UDP-glycosyltransferases, including UGTs, share a 44-amino acid characteristic “signature sequence” in their carboxyl-terminal half that is thought to be involved in the binding of the UDP moiety of the nucleotide sugar (Mackenzie et al., 1997; Mackenzie et al., 2005b).

Figure 1.2: Schematic representation of the glucuronidation reaction. Adapted from Timmers et al. (1997).
UGTs, or the glucuronides that they produce, have been identified in a diverse range of vertebrate species, including humans, other primates, other mammalian species (such as cow, dog, cat, sheep, pig and various rodents), birds and fish (Ohyama et al., 2004; Mackenzie et al., 2005b). For those species that have been investigated in detail, multiple UGT forms have been found, with each enzyme having its own distinct set of target substrates. Thus, each organism can metabolise an extensive range of lipophilic chemicals through the collective activity of its UGTs. Furthermore, many small compounds are recognised by more than one UGT enzyme within a species due to a considerable degree of overlap in UGT substrate selectivity. This allows for a valuable measure of redundancy in the glucuronidation system; as a result, glucuronidation is not only a versatile metabolic pathway, but also a relatively robust one. Accordingly, isolated mutations in single human UGT genes generally have no overt link to disease, with the notable exception of mutations in UGT1A1, as discussed in section 1.8.6.1. However, this cannot be taken to imply that UGTs other than UGT1A1 are unimportant in the prevention of disease. While over 1000 mutated genes have been linked to disorders caused by single gene aberrations, their incidence is low and they account for only a small proportion of the total disease burden in humans (van Ommen, 2002). Indeed, mutations in UGT1A1 that would be fatal without treatment or liver transplant only affect one in $1 \times 10^6$ newborns (Bosma, 2003). In contrast, conditions that have genetic predisposition as one component of a more complex aetiology are common, and it is certainly feasible that UGTs can influence the development of some such disorders through more subtle changes in metabolism.

The mammalian UGTs have been divided into two major families; UGT1 and UGT2, on the basis of amino acid sequence similarities (see Figure 1.3). In general,
members within each family share more than 45% amino acid identity but are ≤ 45% identical to UGTs of the alternative family. The UGT2 family has been further divided into subfamilies; UGT2A and UGT2B; the members of each sharing ≥ 70% amino acid identity. To date, humans are known to possess 19 potentially functional UGTs, as well as 9 pseudogenes (possessing at least one incomplete exon) and many gene remnants. The intact human genes include 9 members of the UGT1 family (known as UGT1A genes), three UGT2A genes and 7 members of the UGT2B subfamily (Mackenzie et al., 1997; Mackenzie et al., 2005b).

Figure 1.3: Dendogram depicting relationships between the primary amino acid sequences of human UGTs. Alignment of the amino acid sequences was performed with Clustal X v1.81 (Thompson et al., 1997) and visualisation of the dendogram was achieved using TreeView v1.6.6 (Page, 1996).
1.5 Genomic organisation of the human *UGT* gene family

1.5.1. The human *UGT1* locus

The human *UGT1A* coding sequences are found on a single locus located on chromosome 2q37, spanning approximately 200 kb. This locus contains 13 unique exons (exon 1), which encode the N-terminal portion of 13 potential UGT1A forms, and 4 exons (exons 2-5) that are shared by all full-length UGT1A transcripts as illustrated in Figure 1.4 (Ritter *et al.*, 1992b; Gong *et al.*, 2001). As a result, the UGT1A enzymes possess unique amino-terminal ends that provide functional diversity, while the 245 carboxyl-terminal amino acids of each are identical. Each *UGT1* unique first exon is preceded by a core promoter region that facilitates transcription of the corresponding UGT1A message, a process that is thought to occur independently of other UGT1A members. mRNA transcripts for specific UGT1A forms have been found to be initiated at transcription start sites (TSSs) located 16 to 112 nucleotides upstream of the initiation codon of their appropriate exon 1 (Ritter *et al.*, 1992b; Gong *et al.*, 2001; Gregory *et al.*, 2003). Each exon 1 sequence is followed by a donor splice site, allowing it to be joined to the first 5’ receptor splice site in the *UGT1A* locus, which precedes exon 2. As such, only the first exon 1 sequence on each mRNA transcript can be spliced to the shared exons, and alternative UGT1A transcripts are generated from alternative transcription initiation events followed by conventional splicing (Ritter *et al.*, 1992b). Although not strictly separate genes, the accepted convention for the *UGT1A* family is to consider the spliced transcripts as arising from such; thus they are named accordingly (Mackenzie *et al.*, 2005b).
It was only very recently that a fifth common exon was discovered in the human UGT1A locus (Levesque et al., 2007b). Usage of this new exon, named common exon 5b, results in truncated UGT mRNA transcripts such as UGT1A1_v2 (Figure 1.4). In this case, the resulting protein (UGT1A1_i1) behaves as an inhibitor of full-length UGT1A1 function. Truncated UGT1A mRNAs have been demonstrated in the liver, kidney, colon, oesophagus and small intestine, and it is anticipated that all UGT1A mRNAs can be spliced to form truncated variants (Levesque et al., 2007b).

Of the UGT1A first exons, four contain mutations that render them non-functional. These UGT1 pseudogenes are UGT1A2P, UGT1A11P, UGT1A12P and UGT1A13P. The remaining genes, UGT1A1, and UGT1A3 through to UGT1A10, are separated into clusters based on sequence relatedness (see Figure 1.3). The UGT1A3, UGT1A4
and UGT1A5 genes produce proteins of greater than 93% homology, while the enzymes of the UGT1A7-1A10 cluster share between 89 and 95% identity.

1.5.2. The human UGT2 locus

In contrast to the UGT1 gene, the UGT2 genes are almost all generated from discreet genes. This includes all of the UGT2B subfamily and UGT2A3. The only known exceptions are UGT2A1 and UGT2A2, which are generated through exon sharing in a similar manner to the UGT1A locus. All of the UGT2 genes are found on chromosome 4 at position 4q13, with the UGT2A and UGT2B members interdispersed as depicted in Figure 1.5. The UGT2B genes all consist of six exons and share similar intron/exon boundaries, although intron lengths vary between genes. Despite originating from separate genes, the carboxyl halves of the UGT2B enzymes are still highly conserved within the UGT2B family and, to a lesser extent, with the UGT1A subfamily (Turgeon et al., 2000; Tukey and Strassburg, 2001; Mackenzie et al., 2005b).

Figure 1.5: Schematic representation of the human UGT2 locus. Each UGT2B gene, consisting of six exons is represented by a coloured rectangle (not drawn to scale), except 2A1/2, which represents seven exons. The UGT2A1 and UGT2A2 genes contain unique first exons (2A1 and 2A2) and a shared set of five downstream exons (exons 2 to 6 in grey); their exon arrangement is depicted at the bottom of the figure. Pseudogenes are labelled with a “P”. The entire UGT2 locus extends over approximately 1.45 Mb. Figure adapted from the latest UGT nomenclature update as published by Mackenzie et al. (2005b).
Of the UGT2 family, disparately little is known about the role of the UGT2A forms in human health and disease. Human UGT2A1 has been found to be expressed mainly in olfactory tissue, and is known to conjugate phenolic, aliphatic and monoterpenoid odorants, as well as certain coumarins, flavonoids, therapeutic drugs and steroid hormones (Jedlitschky et al., 1999). UGT2A2 transcripts have been detected in liver and small intestine, but the substrate specificity of the protein is currently unknown (Tukey and Strassburg, 2001). UGT2A3 is the most recent member of the human UGT2A family to be recognised, being first reported by a project specifically designed to identify novel human secreted and transmembrane proteins in 2003 (Clark et al., 2003). Neither expression nor substrate data is currently available for this UGT form. Therefore, whilst presumably important in its own right, this subfamily will not be addressed further by this thesis.

1.6 UGT substrates

The known substrates of human UGTs are numerous, varied and continually increasing. Accordingly, the following summary of human UGTs and their substrates (Table 1.1) is not an exhaustive list, but rather aims to highlight some important target substances, or substrate classes, of each UGT form. More comprehensive lists of substrates are given in the appropriate chapters for those forms that have been investigated in detail in the presented work.

1.7 Location and distribution of human UGT gene products

Within the cell, UGT proteins are anchored in the membrane of the endoplasmic reticulum. This is achieved by virtue of a hydrophobic 17-amino acid domain located in the carboxyl-terminal portion of each enzyme. Only approximately 20 amino acids of the UGT proteins are located on the cytosolic side of the endoplasmic reticulum
Table 1.1: Human UDP-Glucuronosyltransferases and selected substrates.

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<td></td>
<td>Paracetamol</td>
<td>Analgesic; hepatotoxic and nephrotoxic</td>
<td>Court et al. (2001)</td>
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<td></td>
<td>SN-38</td>
<td>Chemotherapeutic agent</td>
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PhIP: 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; NSAID: non-steroidal anti-inflammatory drug; SN-38: 7-ethyl-10-hydroxycamptothecine; NNAL: 4-(methylnitrosoamo)-1-(3-pyridyl)-1-butanol; membrane, with the majority of each protein residing within the lumen. Nascent UGT protein molecules include a signal peptide that is cleaved after integration into the endoplasmic reticulum membrane, generating a mature protein of approximately 505 amino acids (Iyanagi et al., 1986; Mackenzie, 1986; Meech and Mackenzie, 1997a).
In their native environment, UGTs are thought to form dimers, including heterodimers, and may also form higher order complexes with other cellular proteins such as CYP enzymes. The formation of UGT heterodimers is potentially important in vivo, as it may increase the rate of glucuronidation and/or the breadth of substrates metabolised by co-expressed UGT enzymes. Conversely, UGT mutants that behave in a dominant negative manner may exacerbate disease (e.g. Crigler-Najjar syndrome type II) by reducing the effectiveness of remaining functional UGT protein through formation of inactive dimers (Koiwai et al., 1996; Ikushiro et al., 1997; Meech and Mackenzie, 1997b; Ghosh et al., 2001; Ishii et al., 2001; Kurkela et al., 2003; Ishii et al., 2004). Association with CYPs or other proteins may also modulate UGT activity (Taura et al., 2000; Taura et al., 2004).

Each UGT enzyme has been found to have its own unique tissue expression profile, which is subject to both developmental and cell-type determinants. Table 1.2 summarises the current understanding of adult human UGT mRNA distribution by tissue type. It should be noted that there is some disagreement within the literature about the presence of certain UGT forms in several organs (as indicated). Most of these discrepancies have likely arisen from either differing sensitivities of detection or polymorphic expression between individuals. For example, UGT1A1, UGT1A3 and UGT1A6 have all been demonstrated to only be expressed in the stomach of approximately one third of individuals (Strassburg et al., 1998b), while UGT1A10 transcripts were only detected in lung when amplified separately rather than in multiplex polymerase chain reaction (PCR) (Dellinger et al., 2006). The liver is considered to be the singly most important organ for glucuronidation in humans.
Table 1.2: Distribution of UDP-Glucuronosyltransferase mRNA in humans.

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Data compiled from Munzel et al. (1996), Strassburg et al. (1998a), Albert et al. (1999), King et al. (2000), Tukey and Strassburg (2000), Levesque et al. (2001), Tukey and Strassburg (2001), Turgeon et al. (2001), Vallee et al. (2001), Zheng et al. (2002), Belanger et al. (2003), Finel et al. (2005), Chouinard et al. (2006), Dellinger et al. (2006) and Kaivosaari et al. (2007). The majority of information presented was originally compiled by Dr. Philip Gregory. “+” UGT mRNA is present in tissue; “-” UGT mRNA is absent in tissue; “+/-” UGT mRNA presence is either known to be polymorphic or remains a subject of controversy. §Small amounts of UGT1A5 mRNA have been demonstrated in human liver (Finel et al., 2005) and duodenum (Tukey and Strassburg, 2001), but the functional relevance of these discoveries is still to be determined. ND = not determined.
1.8 Causes and consequences of variability in UGT expression and activity

Despite the robust nature of glucuronidation in humans, this metabolic pathway is still susceptible to factors that either change the activity of UGT enzymes or affect their expression profiles. Glucuronidation is a major route of elimination for many xenobiotics and endogenous molecules; therefore, deviation from the normal rate of glucuronidation can modulate the concentration and/or effect of compounds that are UGT substrates. In the case of pharmaceutical drugs, altered metabolism can have acute clinical consequences in the form of adverse drug reactions or lack of drug efficacy, particularly for drugs that have narrow therapeutic indices. On the other hand, changes in UGT activity may either improve or impair the body’s ability to protect itself against chemical insult and, over the longer term, protect against or predispose to cancer or other diseases caused by chronic chemical toxicity. It has been suggested that even small changes in glucuronidation may lead to disproportionately large increases in bioactivation for substances where the former is a quantitatively major pathway of elimination, especially if there are no alternative eliminating pathways, or those that exist are readily saturable (Wells et al., 2004). Known influences on glucuronidation rates in humans include developmental stage, gender, genetic variation, pregnancy, xenobiotic exposure and health status.

1.8.1. UGT expression during human development

Glucuronidation rates in the human foetus and neonate are significantly lower than the adult for most substrates, and increase during the first few months or years of life. The time frame required for glucuronidation to reach maturity depends on the substrate involved, presumably due to differential regulation of UGT genes. While
the significance of low UGT activity in the foetus is uncertain, poor understanding of neonatal glucuronidation has had fatal consequences (Robertson, 2003).

Several features of foetal development appear to contribute to inferior glucuronidation at this stage of life, including the structural immaturity of important metabolic organs, and the temporal control of UGT expression. Although UGT protein is expressed in an adult-like distribution in the metanephric kidney around 12 weeks gestation (Hume et al., 1995), the foetal kidney has low blood flow and excretes metabolites into the amniotic fluid, from which they can be re-absorbed (Morgan, 1997). On the other hand, the foetal liver lacks much of the UGT protein expressed in the adult organ. In one study, no UGT transcripts were detected in human foetal liver at 20 weeks gestation (Strassburg et al., 2002a). Studies using microsomes or homogenates from foetal liver of 15-27 weeks gestation have revealed low rates of glucuronidation for substrates such as bilirubin, 2-aminophenol, testosterone, morphine and 1-naphthol, but higher rates for oestrone and serotonin. The latter were found to be glucuronidated at 30% and > 100% of adult rates respectively, with the next highest rate of conjugation being for morphine at 10-16% (Kawade and Onishi, 1981; Pacifici et al., 1982; Leakey et al., 1987). Although outdated due to improvements in available human UGT anti-sera, an immunoblot analysis of foetal liver supports the hypothesis that most UGT forms are under-expressed before birth (Coughtrie et al., 1988).

During pregnancy, the foetus may be partially metabolically protected by the placenta. The human placenta expresses multiple UGT enzymes, and glucuronides formed in situ are preferentially transported into the maternal circulation (Collier et al., 2002b; Collier et al., 2004). Placental UGT expression appears to vary with gestational age; UGT1A and UGT2B forms are present in placenta during the first
trimester, however, only UGT2B forms are evident at term (Collier et al., 2002a; Collier et al., 2002b). Nonetheless, maternal metabolism is probably the major determinant of foetal exposure to potentially harmful UGT substrates (Morgan, 1997).

In human neonates, the rates of hepatic glucuronidation for many substances initially remain low (Leakey et al., 1987). As a result, newborns are particularly sensitive to the adverse effects of drugs that are glucuronidated or that inhibit glucuronidation, such as chloramphenicol and novobiocin, respectively (Robertson, 2003). Adult-like glucuronidation of most substrates appears to then develop steadily over the first few months of life. For example, at birth the elimination half-lives of extensively glucuronidated drugs such as morphine, naloxone, lorazepam and zidovudine exceed three times those observed when administered to adults, but these differences are lost between two and six months of age (de Wildt et al., 1999; Ginsberg et al., 2002; Bouwmeester et al., 2004). Correspondingly, at six months of age, UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT2B7, UGT2B10 and UGT2B15 mRNA transcript levels are comparable to those in adult liver, and UGT1A1, UGT1A6 and UGT2B7 protein levels in paediatric and adult liver samples are similar (Strassburg et al., 2002a). Furthermore, in liver microsomes, adult-level activities towards bilirubin and 2-aminophenol can be observed within three months of birth (Onishi et al., 1979). However, as mentioned earlier, the temporal postpartum acquisition of UGT activity does not occur equally swiftly for all UGTs. Trifluoperazine glucuronidation by liver microsomes does not plateau at adult levels until approximately 17 months of age (Miyagi and Collier, 2007), and at the molecular level, hepatic UGT1A9 and UGT2B4 mRNA content is still low in infants at 24 months relative to adults (Strassburg et al., 2002a). In addition, young children (6-24
months) may have much lower capacities than adults for glucuronidation of substances such as oestrone and buprenorphine (Strassburg et al., 2002a), although it should be noted that the functional integrity of the paediatric microsomal preparations used to generate these data were not adequately demonstrated.

1.8.2. Gender effects on human glucuronidation

In adulthood, gender has some influence on glucuronidation, but the effect is limited to a subset of UGT forms. UGT1A6 is the only UGT form that has been directly shown to be differentially expressed due to gender, being found at a higher level in male liver (Court et al., 2001). However, substrates known to be subject to sex-related differences in glucuronidation include paracetamol, propranolol, oxazepam and mycophenolic acid; all having higher clearances in men (Greenblatt et al., 1980; Mucklow et al., 1980; Abernethy et al., 1982; Miners et al., 1983; Walle et al., 1989; Bock et al., 1994; Court et al., 2001; Morissette et al., 2001; Court et al., 2004). The clinical relevance of these differences, at least in terms of immediate therapeutic outcome after drug administration, is currently thought to be minimal (Miners and Mackenzie, 1991).

1.8.3. Xenobiotic exposure and glucuronidation

A number of xenobiotics absorbed from the diet or the environment, or taken deliberately as pharmaceuticals, have been observed to affect UGT expression or activity in humans and other mammals. The UGT activity of freshly isolated or immortalised hepatocytes, liver microsomes, or microsomes from cells expressing heterologous UGT can also be altered by numerous compounds when treated in vitro. Severe acute adverse events due to altered glucuronidation in humans have been reported (Hirata-Koizumi et al., 2007), and lesser alterations in glucuronidation
that cause chronic under- or over-dosing of therapeutic substances may also be clinically relevant (Kiang et al., 2005; Weintraub et al., 2005).

Well documented examples of therapeutic agents that increase glucuronidation in vivo include the antibiotic rifampicin, the anticonvulsants phenytoin, carbamazepine and phenobarbital, and oral contraceptives (Miners and Mackenzie, 1991). Rifampicin treatment has been observed to increase the glucuronidation of bilirubin (Ellis et al., 2006), the anti-epileptic lamotrigine (Ebert et al., 2000), lorazepam (Chung et al., 2005) and the anti-retroviral zidovudine (Burger et al., 1993; Gallicano et al., 1999). Likewise, phenytoin, phenobarbital and carbamazepine can increase the clearance of drugs such as lamotrigine (Weintraub et al., 2005), paracetamol (Miners et al., 1984a) and oxazepam (Seideman et al., 1981), while phenobarbital can be used therapeutically to increase bilirubin conjugation in some patients with genetic deficiencies in UGT1A1 (Jansen, 1999). Oral contraceptive steroids have been shown to affect the metabolism of numerous drugs with which they are often co-administered, including lamotrigine, paracetamol and clofibric acid. All three examples exhibit decreased plasma levels or increased clearance in oral contraceptive users of approximately 50% that can be attributed to changes in glucuronidation (Miners et al., 1983; Miners et al., 1984b; Sabers et al., 2003). Finally, it has recently been suggested that induction of UGTs by antiepileptic drugs may be the cause of altered thyroid hormone homoeostasis seen in patients treated with phenobarbital, phenytoin, and carbamazepine (Benedetti et al., 2005).

Conversely, glucuronidation in humans can also be inhibited by administration of therapeutics. A clinically relevant example is the previously mentioned administration of novobiocin to neonates, where novobiocin competes for the limited UGT1A1 expressed in newborns and causes hyperbilirubinemia (Robertson, 2003).
Probenecid has also been implicated in the inhibition of numerous glucuronidated drugs in vivo (Miners and Mackenzie, 1991). Further examples where glucuronidation of one substance may be inhibited by the pre- or co-administration of another include: decreased glucuronidation of morphine in the presence of nortriptyline, amitriptyline, clomipramine or diazepam (Yue et al., 1990; Wahlstrom et al., 1994); decreased glucuronidation of testosterone in the presence of amitriptyline, imipramine or chlorpromazine (Sharp et al., 1992); decreased glucuronidation of paracetamol in the presence of propranolol (Baraka et al., 1990); and decreased glucuronidation or clearance of zidovudine, lamotrigine and lorazepam in the presence of valproic acid (Yuen et al., 1992; Lertora et al., 1994; Chung et al., 2005). It must be noted however, that the in vivo relevance of many interactions is still to be demonstrated, as much of the available data is from experiments utilising human liver microsomes in vitro.

Although in the majority, not all substances known to affect glucuronidation in humans are pharmaceuticals. Glucuronidation of paracetamol is increased in people on a diet of cruciferous vegetables (Pantuck et al., 1984), and the relative ratio of carbohydrate to protein in the human diet can also affect glucuronidation; with high carbohydrate intake being associated with increased glucuronidation of paracetamol and oxazepam at the expense of other metabolic pathways (Pantuck et al., 1991). A further dietary study reported a decrease in bilirubin levels in subjects who had a high intake of cruciferous vegetables, but that this effect was also restricted to individuals with a specific UGT1A1 genotype (Peterson et al., 2005). Acute ethanol consumption has been reported to slightly impair the clearance of lorazepam in humans (Hoyumpa et al., 1981), while UGT1A6 mRNA and protein levels have been found to be 2-fold higher in liver samples from patients with a history of excessive
alcohol usage than those without (Krishnaswamy et al., 2005a). Smoking of tobacco induces the glucuronidation of mexiletine and propranolol (Grech-Belanger et al., 1985; Walle et al., 1987), and has also been associated with increased glucuronidation or clearance of paracetamol (Mucklow et al., 1980; Bock et al., 1987; Bock et al., 1994), although several other studies have not supported this latter observation (Miners et al., 1984a; Scavone et al., 1990; Krishnaswamy et al., 2005a). Whether smoking habits (frequency, cigarette strength or co-consumption of substances such as alcohol), can explain the discrepancies between studies remains unclear. Interestingly, co-abuse of ethanol and tobacco by pregnant women synergistically increases UGT expression in placenta compared to either substance alone (Collier et al., 2002b), illustrating the complexities involved in delineating the relationships between UGT expression/activity and lifestyle. Other dietary components known to affect UGT expression or activity in human cell culture or rats include chrysin, quercetin, tumeric, curcumin, retinol, tannic acid, flavone and coumarin, although any relevance of these observations to human health is yet to be demonstrated (Galijatovic et al., 2000; Grancharov et al., 2001; Haberkorn et al., 2002; van der Logt et al., 2003; Naganuma et al., 2006).

There are a number of ways in which xenobiotics can alter glucuronidation in humans. The most extensively studied are increased glucuronidation through increased expression of one or more UGTs, and decreased glucuronidation via enzyme inhibition. Generally, where mechanistic studies have been performed to investigate the former, it has been found that increases in UGT expression caused by xenobiotic exposure are mediated at the transcriptional level by nuclear receptor transcription factors. These findings will be discussed at further length in section 1.9.3.2.
1.8.4. Pregnancy and UGT expression

During pregnancy there are many physiological changes that alter drug absorption, distribution and metabolism, including alterations in the expression of certain CYP and UGT enzymes (Anderson, 2005). Notably, a clinically relevant increase in clearance of the antiepileptic drug lamotrigine has been observed in pregnant women during all three trimesters (Pennell et al., 2004). While this effect has historically been postulated to be the consequence of an increase in UGT1A4 expression, a recent study shows that UGT2B7 is also a significant contributor to lamotrigine glucuronidation (Rowland et al., 2006). Additional evidence that expectant mothers may express more UGT2B7 than their non-pregnant counterparts comes from studies of zidovudine, morphine and oxazepam clearances. However, metabolism of these drugs by multiple UGT forms, and/or high basal interindividual variability in their observed clearances, have made the available data difficult to interpret (Anderson, 2005).

Paracetamol is another good example of a drug with increased clearance during human pregnancy, with both glucuronidation and oxidative pathways increased in the third trimester (Miners et al., 1986). Paracetamol is metabolised by multiple UGT forms including UGT1A1 and UGT1A6 (see Table 1.1). Interestingly, transgenic mice bearing the human UGT1A locus have been shown to express higher levels of UGT1A1, UGT1A4 and UGT1A6 mRNA during pregnancy, and UGT1A4 and UGT1A6 during postpartum lactation (Chen et al., 2005a). One suggested mechanism for the increase in lamotrigine and paracetamol clearances during human pregnancy is a transcriptional response of one or more UGT genes to increased hormone levels, as similar effects are seen in oral contraceptive users (Miners et al., 1983; Sabers et al., 2003; Anderson, 2005).
1.8.5. Health and glucuronidation

A number of common diseases and suboptimal body states are known to influence biotransformation pathways in humans. Whilst most research has focused on the effects of health on CYP-mediated drug metabolism, due to the magnitude of the changes observed and their immediate clinical significance, there is some evidence that UGT expression is also affected, albeit more subtly, by conditions such as inflammation, cancer and obesity.

Inflammation and sepsis have been shown to decrease hepatic glucuronidation in rodents (Strasser et al., 1998; Richardson et al., 2006), and may also have some effect in humans. The severity of inflammation during viral infection of the liver is associated with a reduction of UGT mRNA in human hepatocytes. This phenomenon differentially affects the UGT genes, with UGTIA4, UGTIA9 and UGT2B7 being particularly affected, and is specific to hepatitis, as expression of these UGTs in chronic liver fibrosis is maintained (Congiu et al., 2002). The one exception to the unaltered status of UGT expression in fibrotic liver may be UGT2B17 (Congiu et al., 2002). Proinflammatory cytokines such as interleukin (IL)-1β, IL-6 and tumour necrosis factor α, may be indirectly responsible for the loss of UGT expression in inflamed tissue, by altering the expression of key transcription factors (Assenat et al., 2004; Aitken et al., 2006; Richardson et al., 2006). Furthermore, while the basal expression levels of some UGTs do not appear to be greatly affected by inflammation, inducible expression may still be affected, as is the case for constitutive androstane receptor (CAR)-mediated induction of human UGTIA1 in the presence of IL-1β (Assenat et al., 2004). Furthermore, glucuronide hydrolysis may also be increased at inflamed sites, due to immune-mediated release of endogenous
β-glucuronidase and decreased pH. This may further diminish the net effectiveness of local UGT activity in inflammation (Shimoi et al., 2001).

Many malignant tissues have been reported to have decreased UGT expression, relative to healthy tissue procured from the same organ and donor. Examples include cancers of the urinary bladder, colon, liver, stomach and biliary tissue (Strassburg et al., 1997a; Strassburg et al., 1998b; Giuliani et al., 2005). However, the loss of UGT expression in tumours is not universal, even within cancer subgroups. Whether decreases in UGT expression can contribute to the progression of a cell to malignancy, or is simply a result of the dysregulation evident in cancerous cells, is currently unknown. However, when it occurs, the loss of UGT expression appears to be an early event in neoplastic transformation (Giuliani et al., 2005).

Another disease that may result in altered glucuronidation is hypothyroidism. The clearances of oxazepam and paracetamol are lower in patients with severe hypothyroidism than in those who have had their thyroid hormone levels corrected by pharmaceutical intervention (Sonne et al., 1990). However, the exact relationship between UGTs and thyroid hormones in humans is still unclear, and regulation of human UGT expression by these substrates is yet to be demonstrated, although is has been shown in rats (Haberkorn et al., 2002).

Obesity has been associated with an increased glucuronidation capacity in both males and females. Total metabolic clearances for lorazepam, oxazepam and paracetamol were all increased in obese subjects after adjustment for body weight (Abernethy et al., 1983). Obese men also have altered steroid hormone profiles compared with their lean counterparts, with lower androgen and higher oestrogen levels (Tchernof et al., 1999). These observations may be the consequence of altered
glucuronidation, as obese subjects have both an increased liver size (relative to body weight) and an expanded adipose mass, and both organs are sites of glucuronidation. In particular, UGT2B15, a steroid metabolising UGT form, is expressed in adipose tissue (Tchernof et al., 1999). In obese rats, a similar increase in glucuronidation capacity has been observed, with no concomitant increase in rates of sulphation or glutathione conjugation (Chaudhary et al., 1993).

1.8.6. Genetic variation and glucuronidation

Functional genetic variations have been found in many UGT coding regions and/or promoters, including those of UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B7 and UGT2B17 (Bosma et al., 1995; Jinno et al., 2003; Villeneuve et al., 2003; Bernard and Guillemette, 2004; Duguay et al., 2004a; Ehmer et al., 2004; Iwai et al., 2004; Wilson et al., 2004; Krishnaswamy et al., 2005b). As highlighted in sections 1.8.6.1 and 1.8.6.2, a number of polymorphisms in these genes have been associated with health outcomes in humans. Overall, it seems that UGTs represent good candidates for low-penetrance susceptibility genes that may contribute to disease risk by influencing homeostasis and altering the effects of carcinogen exposure.

So far, studies into the impact of genetic variation on glucuronidation in humans have largely been restricted to testing the effects of polymorphisms on UGT protein activity or mRNA levels. The latter is generally assumed to be a product of altered transcriptional rates, resulting from altered promoter function. However, it should be noted that genetic diversity can also potentially affect UGT expression through mechanisms not yet investigated for this gene set. Polymorphisms within genes may also cause differences in mRNA processing, pre-mRNA splicing, exon skipping,
mRNA stability, mRNA trafficking and production of regulatory RNA transcripts (reviewed in Johnson et al. (2005)), and may be relevant to UGT and other biotransformation enzyme genes. Furthermore, variation in trans-acting regulators (e.g. transcription factors) appears to be a major contributor to interindividual differences in mRNA profiles (Morley et al., 2004), and therefore present a potential source of variation for UGT expression. For example, the transcription factor hepatocyte nuclear factor (HNF)1α is a positive regulator of a number of UGT genes; thus, polymorphisms that alter the expression or activity of HNF1α may affect UGT expression (Ryffel, 2001; Toide et al., 2002; Mackenzie et al., 2005a). As regulatory factors for UGT genes are identified, the likely impact of their polymorphic activity or expression level on UGT expression can be assessed.

1.8.6.1. Genetic variability in UGTs: association with disease

The only UGT currently known to harbour genetic mutations that lead directly and unequivocally to disease in the absence of any xenobiotic exposure is UGT1A1. Severe UGT1A1 deficiency leads to the accumulation of the endobiotic bilirubin to toxic levels, as this is the only human UGT with any appreciable activity towards bilirubin (Bosma et al., 1994). The clinical outcome of an UGT1A1 deficiency relies on the degree to which UGT1A1 activity is compromised. Unconjugated hyperbilirubinemias range in severity from no detectable symptoms (most Gilbert syndrome patients), through to severe toxicity in Crigler-Najjar syndrome type II patients and fatal accumulation of bilirubin in Crigler-Najjar syndrome type I patients. Over 110 mutations have been described in the UGT1A1 gene, the majority of which are associated with Crigler-Najjar syndrome type I or type II phenotypes (UGT Nomenclature Committee, 2005). Several UGT1A1 mutants (UGT1A1 F83L, P229Q and R367G), and two UGT1A1 promoter polymorphisms (-3279(T>G) and
A(TA)$_6$TAA to A(TA)$_7$TAA) have also been associated with Gilbert syndrome. The former promoter polymorphism is located in a nucleotide element important for the $UGT1A1$ transcriptional response to xenobiotics (see section 1.9.3.2), while the latter is located in the TATA box of the $UGT1A1$ promoter and results in lower expression of UGT1A1 (Aono et al., 1995; Bosma et al., 1995; Sugatani et al., 2002; Sutomo et al., 2002).

Another significant group of diseases with aetiologies that may be influenced by variability in glucuronidation is cancer. The ability of UGTs to inactivate known carcinogens, as well as substances that support cell growth and survival (such as steroid hormones) suggests that UGTs may be protective against chemically-induced mutagenesis. A study that thoroughly illustrates this point measured the cytotoxicity of benzo[a]pyrene metabolites on lymphocytes from normal subjects. A 200-fold variation in UGT activities against benzo[a]pyrene metabolites was found between samples, and decreased UGT activity correlated with decreased protection against covalent binding of benzo[a]pyrene to cellular proteins and increased cytotoxicity of several benzo[a]pyrene metabolites (Hu and Wells, 2004).

Specific associations reported between genetic variation in $UGTs$ and cancer include: genetic variation in $UGT1A1$ with risk of breast, endometrial and colorectal cancer; genetic variation in $UGT1A7$ with risk of oral, gastrointestinal, colorectal, lung and liver cancer; genetic variation in $UGT1A10$ with risk of orolaryngeal cancer; genetic variation in $UGT2B15$ with risk of prostate cancer; and genetic variation in $UGT2B17$ with risk of lung and prostate cancer. It should be noted however, that most of these associations are weak, with odds ratios of less than three, meaning that this information is likely to be of most use when it can be considered in conjunction with other risk factors. There are also significant discrepancies between some studies
investigating the relationships of particular UGT polymorphisms with cancer risk, suggesting that a subset of the reported associations are likely to eventually be declared false positives. The following paragraphs detail the relationships between UGT genes and cancer reported so far.

For UGT1A1, the lower activity A(TA)7TAA TATA box allele (UGT1A1*28) was found to be associated with development of breast cancer in premenopausal African-American and Chinese women under 40 years old (Guillemette et al., 2000; Adegoke et al., 2004), but not in three other studies involving Greek Caucasian, African and postmenopausal American Caucasian women (Guillemette et al., 2001; Huo et al., 2007; Tsezou et al., 2007). In fact, the Nigerian study found that low activity UGT1A1 promoter alleles were protective against breast cancer, but only in premenopausal women (Huo et al., 2007). Yet, variation in the UGT1A1 gene at the TATA box has also been associated with breast cancer characteristics such as age at diagnosis and tumour grade in Caucasian women (Shatalova et al., 2005). Therefore, it remains unclear whether UGT1A1 genotype is a relevant risk factor for breast cancer in certain populations, and if so, how this risk is modified by age, environment and/or ethnicity.

Other studies investigating the links between UGT1A1 genotype and cancer risk have reported that the UGT1A1 G71R amino acid change may predispose to development of colorectal cancer in Taiwanese men (Tang et al., 2005), and conversely, that the A(TA)7TAA UGT1A1 allele may be protective against the development of endometrial cancer (Duguay et al., 2004b).

For UGT1A7, several alleles, UGT1A7*2, UGT1A7*3 and UGT1A7*4, have been shown to have low or very low activity towards PhIP and several benzo[a]pyrene
metabolites, all known carcinogens normally glucuronidated by UGT1A7 (Strassburg et al., 2002b). Allelic variants of UGT1A7 have been associated with higher risk of developing colorectal cancer in four studies (Strassburg et al., 2002b; van der Logt et al., 2004; Tang et al., 2005; Chen et al., 2006a), with some evidence suggesting that cigarette smokers who carry UGT1A7 polymorphisms are at a greater risk for colorectal cancer than smokers with wild-type UGT1A7 (Chen et al., 2006a). However, a fifth study found no association between low activity UGT1A7 genotypes and risk of developing colon cancer, except in individuals with a high exposure to heterocyclic amines (Butler et al., 2005), again suggesting that any associations between UGTs and cancer susceptibility are likely to be affected, even confounded, by other genetic and lifestyle factors that vary between populations.

UGT1A7*3 and other allelic variants have also been associated with higher risk of developing lung cancer (Araki et al., 2005), hepatocellular carcinoma (Vogel et al., 2001; Wang et al., 2004b; Tseng et al., 2005) and proximal digestive tract cancers (specifically orolaryngeal, but also possibly oesophageal) (Zheng et al., 2001; Vogel et al., 2002). Interestingly, the study by Zheng and co-workers also showed that UGT1A7 genotype was only predictive of cancer risk in patients who smoked (Zheng et al., 2001). Some questions remain over the likely mechanism of the association between UGT1A7 genotype and liver and lung cancers, as UGT1A7 is not expressed in these tissues, and the effect of UGT1A7 polymorphisms on circulating levels of relevant carcinogens is yet to be investigated. It may transpire that in such cases UGT1A7 is behaving as a biomarker rather than a causative risk factor. Finally, one study also reported an association between UGT1A7*3 and increased risk of chronic pancreatitis and pancreatic cancer (Ockenga et al., 2003), but two further studies have failed to replicate this result (Verlaan et al., 2005; Piepoli et al., 2006).
For *UGT1A10*, the only association between genotype and cancer risk to date is for orolaryngeal cancer and the UGT1A10 protein variant E139K (from *UGT1A10*2). Individuals with one or more *UGT1A10*2 alleles were found to have decreased risk of developing orolaryngeal cancer (Elahi *et al*., 2003); however, this variant has subsequently been shown to have less activity towards benzo[a]pyrene metabolites than wild-type UGT1A10 (Dellinger *et al*., 2006). Thus, the reason for this observation remains unknown.

For *UGT2B15*, two highly prevalent alleles (*UGT2B15*1 and *UGT2B15*2) exist, each occurring at frequencies of approximately 50% in Caucasians (Levesque *et al*., 1997; Gsur *et al*., 2002; Park *et al*., 2004c). The *UGT2B15*2 allele encodes a protein variant (UGT2B15 D85Y) that has approximately 2-fold increased activity towards dihydrotestosterone than the variant encoded by *UGT2B15*1 (Levesque *et al*., 1997). Since higher androgen exposure may predispose to prostate cancer, this UGT form has been extensively investigated for associations between genotype and risk of prostate cancer. Three studies have reported a positive association between the lower activity UGT2B15.1 variant and risk of developing prostate cancer in Asian and Caucasian ethnic groups (MacLeod *et al*., 2000; Park *et al*., 2004c; Okugi *et al*., 2006); however, three more studies disagree. Firstly, Gsur and colleagues found no association between *UGT2B15* genotype and prostate cancer in Austrians, while Hajdinjak and co-workers found no association between prostate cancer incidence and *UGT2B15* genotype in Slovenians, but that *UGT2B15* genotype was associated with pathological grade (Gsur *et al*., 2002; Hajdinjak *et al*., 2004). This second study is completely at odds with the similar study in Japanese men that showed an association of *UGT2B15* genotype with prostate cancer risk, but not pathological grade (Okugi *et al*., 2006). Thirdly, a study that examined 46 polymorphisms in the
androgen and oestrogen metabolic pathways found that there was no evidence of an association between UGT2B15 genotype and prostate cancer risk in sporadic or familial prostate cancer patients (Cunningham et al., 2007). Thus, the usefulness of this genotype as a risk marker for prostate cancer continues to be debated.

For UGT2B17, a major polymorphic variation exists in the form of a gene deletion, resulting in the complete absence of this UGT form in up to 85% of individuals, depending on ethnicity and study population (Wilson et al., 2004; Terakura et al., 2005; Jakobsson et al., 2006; Park et al., 2006). On the basis that similarly to UGT2B15, UGT2B17 also metabolises androgens (Jakobsson et al., 2006), one research group has recently studied the association of this gene deletion with prostate cancer risk. A positive association was reported (Park et al., 2006); however, since this deletion is known to be in high linkage disequilibrium with UGT2B15 genotype (Wilson et al., 2004), it is difficult to draw conclusions regarding the possible individual contribution of either gene to this association. A positive association between the complete absence of the UGT2B17 gene, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) glucuronidation and lung adenocarcinoma in Caucasian women, but not men, has also been recently reported (Gallagher et al., 2007).

Finally, genetic variation in UGT1A1 has also been associated with altered risk of cardiovascular and coronary heart disease. In a study of 1780 unrelated individuals, it was found that individuals homozygous for the UGT1A1 A(TA)7TAA promoter allele had approximately one third the risks for cardiovascular and coronary heart disease than carriers of the A(TA)6TAA allele (Lin et al., 2006). The protective effect may be due to higher circulating levels of bilirubin in homozygous carriers of the UGT1A1 A(TA)7TAA promoter allele, as bilirubin has antioxidant properties.
Several important relationships between the outcome of medical treatment and patient genotype have been reported for *UGTs*. The best known example is the association between *UGT1A1* and irinotecan disposition, which has now resulted in clinically relevant recommendations on irinotecan usage.

Irinotecan is an anticancer prodrug, primarily used to treat colorectal cancer. The active therapeutic, 7-ethyl-10-hydroxycamptothecin (SN-38), is generated by carboxylesterase metabolism and is eliminated mostly by glucuronidation (reviewed in Garcia-Carbonero *et al.* (2002)). SN-38 has a narrow therapeutic window, and over-dosing can cause life-threatening toxicities including diarrhoea and neutropenia.

*UGT1A1*, *UGT1A7*, *UGT1A9* and *UGT1A10* have been proposed to be the major catalysts of SN-38 glucuronide formation (Gagne *et al.*, 2002; Oguri *et al.*, 2004), and accordingly, *UGT1A1*, *UGT1A7* and *UGT1A9* genetic variations that decrease SN-38 glucuronidation have been associated with altered treatment outcomes. In particular, the *UGT1A1* A(TA)7TAA allele (*UGT1A1*^28*), -3156G>A promoter polymorphism (*UGT1A1*^93*) and *UGT1A1*^6* (*UGT1A1* G71R) have been associated with increased risk of irinotecan-induced toxicity, particularly neutropenia (Ando *et al.*, 2000; Innocenti *et al.*, 2004; Kitagawa *et al.*, 2005; Minami *et al.*, 2007), while *UGT1A7*^2* (*UGT1A7* N129K/R131K), *UGT1A7*^3* (N129K/R131K/W208R) and *UGT1A9*^1b* (*UGT1A9* -118(dT)9>10 promoter SNP) have been associated with decreased diarrhoea and increased efficacy (Carlini *et al.*, 2005). The association of *UGT1A1* A(TA)7TAA with irinotecan-mediated toxicity is sufficiently well established that the Food and Drug Administration has recently added recommendations for the testing of patient *UGT1A1* genotype prior to
irinotecan treatment to the drug label (Figure 1.6) (Maitland et al., 2006). The relative importance of the other implicated genotypes in irinotecan-treated patients remains unresolved; however, there is some evidence that the UGT1A1*28 allele is insufficient to predict severe toxicity in some populations, such as the Japanese and other Asian people groups (Minami et al., 2007; Sandanaraj et al., 2007).

**Patients with Reduced UGT1A1 Activity**  
Individuals who are homozygous for the UGT1A1*28 allele are at increased risk for neutropenia following initiation of CAMPTOSAR treatment. A reduced initial dose should be considered for patients known to be homozygous for the UGT1A1*28 allele (see DOSAGE AND ADMINISTRATION). Heterozygous patients (carriers of one variant allele and one wild-type allele which results in intermediate UGT1A1 activity) may be at increased risk for neutropenia; however, clinical results have been variable and such patients have been shown to tolerate normal starting doses.

**Hypersensitivity**  
Hypersensitivity reactions, including severe anaphylactic or anaphylactoid reactions, may occur.

Figure 1.6: Excerpt from the current label for Camptosar (Irinotecan HCl), as obtained from the Food and Drug Administration website. (Food and Drug Administration, 2005).

Other examples where UGT genotype may affect drug disposition in humans exist for UGT1A6, UGT1A9, UGT2B7 and UGT2B15. Two studies have shown that UGT1A6 genotypes modulate the protective effect of aspirin on the risk of developing colorectal adenoma, suggesting that aspirin use by individuals with wild-type UGT1A6 does not confer any chemopreventative benefits, but individuals with low activity UGT1A6 variants can reduce their risk of developing colorectal adenoma by regular aspirin consumption (Bigler et al., 2001; Chan et al., 2005). Yet, the results of two further studies conflict with these and each other; one showing that low activity UGT1A6 genotypes are protective against colorectal adenoma recurrence irrespective of aspirin intake (Hubner et al., 2006); the other that NSAIDs (including or excluding aspirin) are protective against colorectal adenoma regardless of UGT1A6 genotype. Furthermore, the importance of the UGT1A6 enzyme in
aspirin metabolism it is still under considerable debate (Miners and Day, 2007; van Oijen et al., 2007). Further research into the interaction between UGT1A6 genotype, aspirin use and colorectal adenoma is still clearly needed.

On the other hand, UGT1A9 and UGT2B7 are two enzymes important in the glucuronidation of mycophenolic acid, an immunosuppressant with a low therapeutic index and considerable interindividual variation in pharmacokinetics (Picard et al., 2005). In healthy volunteers, the UGT1A9 promoter -275T>A/-2152C>T, UGT1A9*3 and UGT2B7*2 (UGT2B7 H268Y) alleles have been associated with alterations in mycophenolic acid exposure, enterohepatic recycling and production of the toxic acyl-glucuronide metabolite (Levesque et al., 2007a). The clinical importance of these findings is still to be determined, but is certainly of interest. Similarly, UGT2B15 is an important enzyme for the metabolism of the anxiolytic drugs oxazepam and lorazepam, and the UGT2B15 D85Y (UGT2B15*2) variant has been associated with lower glucuronidation of oxazepam in human liver and lower clearance of lorazepam in healthy volunteers (Court et al., 2004; Chung et al., 2005). Whether this polymorphism has clinically relevant affects on lorazepam safety and/or efficacy in humans remains to be seen, but it seems probable, as this drug also has a relatively low therapeutic index (Chung et al., 2005).

Finally, the UGT2B17 gene deletion may be a risk factor for transplant-related mortality in recipients of haematopoietic stem cells. The UGT2B17 protein is immunogenic in individuals that are genetically devoid of the UGT2B17 gene, and may be responsible for a heightened risk of complications in recipients given transplants from donors mismatched for UGT2B17 (Terakura et al., 2005).
1.9 Regulation of human UGT genes

Even though differential UGT expression and activity between human individuals may be clinically important in multiple medical disciplines, our knowledge of the mechanisms that control UGT expression and interindividual variation in UGT levels remains limited. Therefore, this thesis was designed to expand the understanding of UGT gene regulation, based on the conviction that, in the future, such knowledge will be useful for identifying and understanding key points at which interindividual variation can occur, and ultimately, for identifying pathways that can be taken into account or manipulated for therapeutic benefit. The following sections discuss the extent of the knowledge base regarding the regulation of human UGT genes at the time this thesis was commenced. Additional advancements made during the period of this candidature are discussed in the following chapters as relevant to the work presented therein, and/or summarised in Chapter 7.

1.9.1. Transcriptional regulation: transcription factors and co-regulators

Proteins that bind to specific DNA sequences to control gene expression are collectively known as transcription factors. DNA sequences targeted by such proteins are referred to as regulatory elements and are typically found upstream of a gene’s coding region; however, they can also reside within coding regions, within introns, downstream of a gene’s coding region, and even within distant or interchromosomal DNA sequences that are not obviously part of the target gene (Brooks et al., 1994; Harrow et al., 2004; Patrinos et al., 2004; Kleinjan and van Heyningen, 2005). The general purpose of transcription factors is to facilitate appropriate interaction of the transcriptional machinery with each gene target, and as such, they may have a positive or negative role in this process. Furthermore, multiple
transcription factors bind and influence the expression of each gene, occurring in seemingly endless combinations that allow a high degree of control to be exerted over individual genes using a large but limited number of regulatory proteins. Differential expression of transcription factors (with some expressed ubiquitously, and others being tissue-restricted) allows further differentiation of gene expression profiles of different cell types. Additional complexity is provided by a second class of regulatory proteins known as co-regulators, which do not directly bind DNA, but are recruited by bound transcription factors and interact with the transcriptional machinery and/or the chromatin environment in which the target gene resides. Some proteins can also behave either as transcription factors or co-regulators, depending on the gene context. A comprehensive review of general gene regulation can be found in Schrem et al. (2002).

Gene expression requires a permissive DNA environment with a relatively open chromatin structure to proceed. Thus, the purpose of some transcription factors, co-regulators and other chromatin remodelling complexes is to modify chromatin to allow other regulatory proteins and the transcriptional machinery access to appropriate genes. The histones, around which DNA is wrapped to form chromatin, can be modified by processes such as acetylation, phosphorylation and ubiquitination, while the DNA itself can be methylated. Methylation and deacetylation of chromatin are two linked processes that contribute to the compaction of chromatin and the silencing of genes in vivo (Schrem et al., 2002). Of particular pertinence to work in this thesis, actively expressed genes are found in highly acetylated chromatin, and the acetylation status of chromatin is regulated by two groups of enzymes with opposing activities, the histone acetyltransferases (HATs) and histone deacetylases (HDACs). Many co-activators, including
p300/cAMP-response-element-binding protein (CREB)-binding protein (p300/CBP),
p300/CBP-associated factor (P/CAF), steroid receptor co-activator (SRC)-1 and
peroxisome-proliferator-activated receptor (PPAR)-gamma co-activator (PGC)-1, are
known to either possess or recruit HAT activity to target genes (Ogryzko et al.,
1996; Yang et al., 1996; Spencer et al., 1997; Puigserver and Spiegelman, 2003).
Some transcription factors also have intrinsic chromatin-opening activity such as the
forkhead box A (FoxA) proteins (Cirillo et al., 2002). Conversely, many co-
repressors, such as silencing mediator of retinoid and thyroid hormone receptor
(SMRT) and nuclear receptor co-repressor (NCoR), recruit HDAC activity (Ng and
Bird, 2000). In turn, many of these co-activators and co-repressors are known to
physically interact with transcription factors investigated in this thesis. The
properties of relevant transcription factors and their co-regulators are discussed in
detail in the chapters to which they are pertinent.

1.9.2. Gene regulatory elements: the core promoter, the proximal promoter
and enhancer elements

Immediately upstream of the initiation codon of a gene, is a region known as the core
promoter. This region typically contains the minimal DNA elements required for
RNA polymerase II and the other components of the transcriptional machinery to
bind a gene and initiate transcription. Common elements in the core promoter
include the TATA box, initiator (Inr) region, transcription factor II (TFII)B
recognition region and downstream core promoter element, located within a DNA
stretch of approximately 70 nucleotides and centred roughly around the TSS (see
Figure 1.7). Any particular core promoter may contain some, all or none of these
elements (Smale and Kadonaga, 2003; Buckland, 2006), and the human UGT gene
family contains examples of both TATA box-reliant and TATA-less promoters (see section 1.9.3.1).

**Figure 1.7: Idealised structure of an eukaryotic gene.** Common regulatory elements and regions found in eukaryotic genes (figure adapted from Smale and Kadonaga (2003)). TRE: TFIIB recognition element; Inr: Initiator region; DPE: Downstream core promoter element. Numbering is relative to the transcriptional start site. Consensus sequences for the four core promoter elements depicted are given in parentheses (S = G + C, R = A + G, W = A + T, Y = C + T, V = G + C + A).

The nucleotides immediately upstream of a gene’s initiation codon, encompassing and extending several hundred base pairs beyond the core promoter, are generally referred to as the proximal, or regulatory, promoter. Often this region will be sufficient to drive transcription in *in vitro* assays, and typically contains multiple transcription factor binding sites that are involved in recruiting and positioning the transcription machinery (Cooper *et al.*, 2006). Mutation of these sites *in vitro* can often demonstrate their importance for transcription, and even highly related sequences can vary significantly in function through relatively few nucleotide
substitutions (Buckland, 2006). However, when these sequences are integrated into the genome; i.e. in stable transfections or knock-in animal models, they are often insufficient to drive transcription, or cannot confer appropriate tissue-type specific and insertion-point independent expression patterns. Such behaviour typically requires longer sequences that contain additional transcription factor binding sites known as enhancers and locus control regions (Brooks et al., 1994; Harrow et al., 2004; Kleinjan and van Heyningen, 2005). There is no real distinction between proximal promoter and enhancer elements, except that the latter tends to be used to refer to elements that reside long distances from the TSS, up to many kilobases up- or downstream, and includes sites necessary for regulating the structure and nuclear localisation of chromatin (Blackwood and Kadonaga, 1998). Like the proximal promoter, enhancer regions often possess several transcription factor binding sites within close proximity of each other, facilitating the formation of protein complexes with specific functions. Combinatorial binding of transcription factors (in both enhancers and proximal promoters) is of sufficient importance that regulatory regions containing transcription factor site combinations conserved between orthologues, or genes of similar expression pattern within an organism, are considered highly likely to be functionally significant (Liu et al., 2003; Johnson et al., 2005).

1.9.3. Regulation of human UGT genes

At the commencement of this thesis, human UGT proximal promoters that had been cloned and subjected to some degree of functional analysis included UGT1A1, UGT1A6, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15 and UGT2B17.
1.9.3.1. Regulation of human UGT genes by transcription factors other than nuclear receptors

The first human UGT promoter clone to be published was UGT1A1, in work by Bosma and colleagues that showed that the length of the UGT1A1 TATA box affected promoter activity in transient transfections of HuH7 cells (Bosma et al., 1995). The UGT1A1 promoter containing the A(TA)$_7$TAA sequence associated with Gilbert syndrome was found to have only 18-33% of the activity achieved by the same promoter with the A(TA)$_6$TAA sequence (Bosma et al., 1995). Recently, it has been shown that increasing the number of thymine-adenine repeats in the UGT1A1 promoter causes a decrease in TATA-binding protein in vitro, providing a plausible mechanism for the observed concomitant decrease in promoter activity (Hsieh et al., 2007).

The only other UGT gene subsequently found to possess functional variants of the TATA box to date is UGT1A7, where a T to G transversion at nucleotide position -57 results in a 70% reduction in promoter activity in vitro (Lankisch et al., 2005). Indeed, some human UGT genes actually appear to be TATA-less (Figure 1.8). While canonical TATA boxes have been predicted for UGT1A1, UGT1A3, UGT1A4, UGT1A5, UGT1A6 and UGT1A7 (Ritter et al., 1992b; Bosma et al., 1995; Lankisch et al., 2005), UGT1A8, UGT1A9 and UGT1A10 have been shown to rely on an Inr-like region for initiation of transcription (Gregory et al., 2003) and are seemingly TATA-less. Likewise, the UGT2B7 gene does not have a canonical TATA box (Ishii et al., 2000), a feature shared by UGT2B4. In contrast, the remaining known human UGT2B genes contain the nucleotide sequence “TATAA”, predicted to be a TATA box, positioned at nucleotides -63 to -59 relative to the UGT2B17 initiation codon.
Accordingly, the TSS locations mapped for \textit{UGT2B7} and \textit{UGT2B17} are completely different (Gregory et al., 2000; Ishii et al., 2000) (see Figure 1.8).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.8.png}
\caption{The \textit{UGT1A} and \textit{UGT2B} core promoters showing putative TATA box and initiator-like elements. The putative elements are boxed, and experimentally demonstrated transcription start sites (TSSs) are indicated in bold and are marked by asterisks or dots. For the \textit{UGT1A7-1A10} cluster, conflicting explanations of transcriptional initiation have been published. Gong and colleagues postulated that the T-region shown behaves as the anchoring site for the transcriptional machinery and reported the TSSs marked by dots (Gong et al., 2001). In contrast, Gregory and co-workers reported an initiator (Inr)-like region further downstream and the TSSs marked by asterisks (Gregory et al., 2003). Two different cap sites have also been found for UGT1A6 by different research groups (Ritter et al., 1992b; Munzel et al., 1998). Such differences may indicate that UGT TSSs are at least partly determined by cell type (Munzel et al., 1998). The remaining data were obtained from Ritter et al. (1992b), Ishii et al. (2000), Lankisch et al. (2005) and Gregory et al. (2000).}

A large proportion of the initial studies addressing basic human \textit{UGT} promoter function concentrated on the role of HNF1 transcription factors. After a report that

HNF1α bound and activated the rat *UGT2B1* promoter was published (Hansen *et al.*, 1997), work by Bernard *et al.* (1999) showed that the *UGT1A1* promoter could also be regulated by HNF1α and HNF1β in HEK293 cells. This report was closely followed by three others that also implicated HNF1α in the regulation of two additional human *UGT* genes. Studies of the isolated *UGT2B7* promoter in HepG2 cells showed that HNF1α, but not HNF1β, could drive transcription from a proximal HNF1-binding site, and that octamer transcription factor-1 (Oct-1) could interact with HNF1α as a co-activator on this promoter to further increase transcription (Ishii *et al.*, 2000). Likewise, a proximal HNF1-binding element was found to confer *in vitro* responsiveness to HNF1α, but not HNF1β, to the *UGT2B17* gene promoter in HepG2 cells (Gregory *et al.*, 2000). Although the HNF1-binding element of the *UGT2B17* promoter is at the same position relative to the initiation codon as in *UGT2B7*, Oct-1 was found to have a negative effect on transcription from the *UGT2B17* promoter in HepG2 cells (Gregory *et al.*, 2000). Furthermore, it was found that binding of pre-B cell homeobox and related factors to a site immediately adjacent to the HNF1-binding site of *UGT2B17* modulated transcription *in vitro* by restricting access of HNF1α to the HNF1-binding site (Gregory and Mackenzie, 2002).

One further study published prior to 2003 regarding *UGT* regulation in humans identified two promoter elements in the *UGT2B15* promoter that were important for *in vitro* function in prostate-derived LNCaP cells, and that were not shared by the closely related *UGT2B17* promoter. The most proximal of these two elements was suggested to be a FoxA2 (HNF3β) binding site (Turgeon *et al.*, 2000); however, no experimental data was provided (then nor since) to confirm the identity of the binding sites or proteins important for these observations.
1.9.3.2. Regulation of human UGT genes by ligand-dependent nuclear receptors

Changes in the environment or health state of humans can alter the activity or level of many transcription factors, thus causing indirect alterations in the expression of their target genes. However, there is also a specific subset of transcription factors that overtly rely on xenobiotic and endogenous compounds as ligands to regulate their activity and consequent expression of target genes. These transcription factors are known as ligand-dependent nuclear receptors, and regulation of genes by these factors is generally considered “inducible” because the regulatory pathways that rely on these transcription factors are typically inactive or repressed in the absence of ligand. Such pathways are thought to be important for the appropriate expression of UGTs in response to chemical exposure, and in allowing co-ordinate expression of biotransformation enzymes from different stages of chemical metabolism and elimination (Xu et al., 2005; Trottier et al., 2006a).

The earliest evidence that ligand-dependent nuclear receptors were involved in the regulation of UGTs in humans was the discovery that the aryl hydrocarbon receptor (AhR) binds to a xenobiotic response element (XRE) in the human UGT1A6 promoter, and that the presence of an AhR agonist, 2,3,7,8-tetrachlorodibenzo-p-dioxin, increases transcriptional activity from this gene in vitro (Munzel et al., 1998). Further work demonstrated that UGT1A1, UGT1A9 and UGT2B7 expression could also be increased by exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin and polyaromatic hydrocarbons (AhR agonists) and/or antioxidant-type inducers that normally work through antioxidant response elements (Bock et al., 1998; Munzel et al., 1999; Ritter et al., 1999; Walle and Walle, 2002). The presence of a XRE in the UGT1A1 gene promoter was subsequently demonstrated by Yueh et al. (2003) and further studies of the UGT1A6 promoter indicated that the response of this particular
gene to antioxidants is through an atypical mechanism that probably involves AhR (Munzel et al., 2003).

Other nuclear receptors that were recognised as UGT regulators by 2003 include CAR, pregnane X receptor (PXR), PPARα, PPARγ and farnesoid X receptor (FXR). CAR is a constitutively active nuclear receptor that regulates UGT1A1 gene expression in response to agonists such as phenobarbital and antagonists such as androstenol, through a nuclear receptor response element (NRRE) that resides within a short DNA sequence known as the UGT1A1 phenobarbital response enhancer module (PBREM: UGT1A1 -3499 to -3210). The PBREM also consists of at least six other NRRE motifs that are functionally active (Sugatani et al., 2001; Sugatani et al., 2005b), and a polymorphism in one NRRE of the UGT1A1 PBREM (UGT1A1 -3279T>G) has been associated with Gilbert syndrome in Japanese patients (Sugatani et al., 2002). Other NRREs in the UGT1A1 PBREM include the XRE mentioned earlier (Yueh et al., 2003) and a PXR binding site (Xie et al., 2003). PXR activates UGT1A1 gene expression when liganded with xenobiotics such as rifampicin and can bind to three of the UGT1A1 PBREM NRREs. However, the majority of UGT1A1 gene activation appears to rely on one particular PBREM NRRE at nucleotide position -3430 to -3386, which can also be bound by CAR (Xie et al., 2003). PXR has also been shown to regulate the human UGT1A3, UGT1A4 and UGT1A6 genes, although the DNA sequences required for these effects have not been identified (Rae et al., 2001; Gardner-Stephen et al., 2004).

The closely related nuclear receptors PPARα and PPARγ were first recognised as human UGT regulators when Barbier and colleagues demonstrated that PPAR ligands could increase UGT1A9 expression in human hepatocytes, and that the UGT1A9 promoter contains a functional PPAR response element (Barbier et al.,
Similarly, the same research group demonstrated a role for PPARα in the regulation of UGT2B4 (Barbier et al., 2003a), and concurrently identified UGT2B4 as a gene target of FXR (Barbier et al., 2003b).

1.10 Experimental aims

Despite much evidence that UGTs are important in at least three major aspects of human health (homeostasis, xenobiotic defence and drug efficacy), the understanding of these enzymes and the factors that determine inter- and intra-individual variation in their expression remains limited. If the goal of personalised medicine is to become realised with widespread impact, it will be necessary to understand the biology of human UGTs (and other biotransformation enzymes such as the CYPs and sulphotransferases, N-acetyltransferases and glutathione-S-transferases) to a much greater extent than the current knowledge allows. Of particular concern, research into the basal regulation of human UGTs, and the interplay of this with genetic variation was almost nonexistent at the commencement of this PhD candidature, with the exception of limited studies into the role of HNF1 factors. Moreover, while a considerable amount of progress had been made on inducible expression of UGTs by nuclear receptors, there clearly remained much to be learnt. Therefore, the overall aim of this thesis was to substantially expand our knowledge of UGT regulation in humans, with an emphasis on how this may relate to the variability observed in UGT expression. To achieve this end, the specific aims of this thesis were to:

1. Identify DNA elements important for the promoter function of several lesser studied UGT1A genes; in particular, UGT1A3, UGT1A4 and UGT1A9;

2. Identify transcription factors involved in the regulation of human UGTs, with a specific focus on those that may be important for hepatic UGT expression;
3. Provide further information on the mechanisms by which selected transcription factors control \textit{UGT} promoter activity and how this may differ between \textit{UGTs} that share similar transcription factor sets;

4. Test the \textit{UGT1A3} promoter and HNF1\textalpha{} protein variants for effects that may be responsible for the observed variation in UGT1A3 expression levels between individuals.
CHAPTER TWO
MATERIALS AND METHODS

2.1 Materials

2.1.1. Chemicals and molecular biology reagents

The suppliers of all reagents and kits used throughout this thesis are listed in Table 2.1. All chemicals were of analytical reagent grade.

Table 2.1: Reagents used in experimental procedures.

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<th>Reagent</th>
<th>Supplier</th>
</tr>
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<tbody>
<tr>
<td><strong>Buffer Chemicals</strong></td>
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<tr>
<td>Acetic acid</td>
<td>BDH AnalaR (Merck), Kilsyth, VIC, Australia</td>
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<tr>
<td>Boric acid</td>
<td>BDH AnalaR (Merck)</td>
</tr>
<tr>
<td>Bovine serum albumin (BSA) solution (100 mg/ml)</td>
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<tr>
<td>Bromophenol blue</td>
<td>Sigma Chemical Co, St Louis, MO</td>
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<tr>
<td>CaCl₂cdot2H₂O</td>
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<td>Dimethyl sulfoxide (DMSO)</td>
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<tr>
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<td>Glycerol</td>
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<td>Glycine</td>
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Table 2.1 continued.

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<td>Sodium dodecyl sulphate (SDS)</td>
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<td>Amresco</td>
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<tr>
<td>Xylene cyanol FF</td>
<td>Pharmacia LKB Biotechnology, Bromma, Sweden</td>
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**Mammalian Tissue Culture**

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<td>Foetal calf serum</td>
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<tr>
<td>MEM non-essential amino acids</td>
<td>Invitrogen</td>
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<tr>
<td>MEM sodium pyruvate</td>
<td>Invitrogen</td>
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<tr>
<td>Rifampicin</td>
<td>Sigma Chemical Co</td>
</tr>
<tr>
<td>Tissue culture flasks and plates</td>
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<tr>
<td>Trypsin-EDTA</td>
<td>Invitrogen</td>
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<td>Trypan blue</td>
<td>Sigma Chemical Co</td>
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</table>

**Transfection and Reporter Gene Assays**

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<tr>
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Table 2.1 continued.

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<td>X-Omat Blue XB-1 autoradiographic film</td>
<td>Eastman-Kodak, Rochester, NY</td>
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2.1.2. General buffers

The following buffer formulae were obtained from Sambrook and Russell (2001).

1 × DNA gel loading buffer: 0.04% (w/v) bromophenol blue, 0.04% (w/v) xylene cyanol FF, 5% (v/v) glycerol.

1 × Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 2 mM KH₂PO₄, pH 7.4

1 × Tris-acetate EDTA electrophoresis buffer: 40 mM Tris-acetate, 1 mM EDTA

1 × Tris-EDTA buffer: 10 mM Tris-HCL, pH 7.5, 1 mM EDTA

0.5 × Tris-borate EDTA (TBE) electrophoresis buffer: 45mM Tris-borate, 1mM EDTA
2.1.3. **Eukaryotic and prokaryotic cell lines**

Human embryonic kidney (HEK293T), human hepatocarcinoma (HepG2) and human colorectal adenocarcinoma (Caco-2) cells were all obtained from the American Type Culture Collection (ATCC, Manassas, VA). The DH5α *Escherichia coli* (*E. coli*) strain was also bought from the ATCC. DH10B *E. coli* stocks containing clones from the RPCI-11 bacterial artificial chromosome (BAC) library (Osoegawa *et al.*, 2001) were obtained from BACPAC Resources (Children’s Hospital Oakland Research Institute, Oakland, CA). Top10 One-Shot chemically super-competent *E. coli* were purchased from Invitrogen.

2.1.4. **Mammalian reporter and expression vectors**

The reporter vectors pGL3-basic and pRL-Null were purchased from Promega. A schematic map of pGL3-basic showing the relevant restriction sites can be found in Appendix 1. The construction of pGL3 daughter plasmids carrying promoter inserts is described for each vector in the “Methods” sections of the appropriate chapters. The empty mammalian expression vector pCMX-PL2 was the kind gift of Dr. Ronald Evans (The Salk Institute for Biological Sciences, San Diego, CA) and is described in Umesono *et al.* (1991). pCMX vectors express high levels of recombinant protein through a cytomegalovirus (CMV) promoter and contain a T7 promoter sequence to facilitate *in vitro* transcription and translation.

pCMX-HNF1α (containing the human HNF1α-A variant cDNA) and subsequently derived pCMX-HNF1α WT+21, I27L, A98V, S487N and P291finsC mutant plasmids were constructed by Tamara Height (University of South Australia, Australia) (Mackenzie *et al.*, 2005a). The pBJ5-HNF1α and pBJ5-HNF1β expression vectors were kindly provided by Dr. Gerald Crabtree (Stanford University, Stanford,
CA) and express the murine orthologues of HNF1α and HNF1β. The pRB-HNF3α and pGEM-HNF3β plasmids, containing rat FoxA1 and FoxA2 cDNAs respectively, were the kind gift of Dr. Guntram Suske (Klinikum Der Philipps-Universitat Marburg, Germany). Expression vectors for the rat CCAAT/enhancer binding protein (C/EBP)α and C/EBPβ transcription factors were generously provided by Dr. Peter Johnson (National Cancer Institute, Frederick, MD). All vectors listed in this section confer ampicillin resistance.

2.1.5. Cloning vectors

The shuttle vectors pCR-blunt and pCR-2.1, used for cloning PCR products without prior restriction, were purchased from Invitrogen. pCR-blunt requires 50 μg/ml kanamycin for selection, whilst pCR-2.1-derived plasmids can be selected with 50 μg/ml kanamycin or 100 μg/ml ampicillin.

2.2 General methods

2.2.1. Maintenance of mammalian cell lines

All mammalian cell lines were cultured under sterile conditions in DMEM supplemented with 10% (v/v) foetal calf serum, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate. Cells were grown at 37°C in a humidified atmosphere of 5% CO2. All cells were PCR screened for mycoplasma infection by Anne Rogers before use and periodically thereafter. Cell stocks were preserved in foetal calf serum containing 10% (v/v) DMSO and stored in liquid nitrogen.

All cells were routinely passaged at approximately 80% confluence. HepG2 and Caco-2 cells were released from the surfaces of tissue culture flasks by incubation in 0.05% (w/v) trypsin at 37°C after washing in PBS. It was necessary to pass re-suspended HepG2 cells through a sterile stepper syringe (Nichiryō, Tokyo, Japan)
before re-plating, to disperse clumps and maintain correct morphology. Moderately vigorous pipetting without prior trypsin digestion was adequate to release and disperse HEK293T cells for re-plating. Cell cultures were replaced from frozen stocks after 15-25 passages.

2.2.2. Bacterial culture and preparation of competent cells

All bacterial strains were grown at 37ºC in LB liquid culture medium with vigorous shaking or on LB plates solidified with 15 g/L agar. Each culture was maintained under antibiotic selection if and as appropriate: bacteria carrying reporter or expression vectors were grown in 100 μg/ml ampicillin, with the exceptions of those harbouring BAC constructs (20 μg/ml chloramphenicol) and cells transformed with pCR-Blunt-derived plasmids (50 μg/ml kanamycin). Long term bacterial stocks were stored at -80ºC in LB containing 17.5% (v/v) glycerol.

To generate competent cells, DH5α E. coli were grown overnight to stationary phase in antibiotic-free LB and subsequently diluted 1:60 in fresh medium. The diluted cells were then grown to log-phase (an optical density of approximately 0.4 at 600 nm) and harvested by centrifugation at 2,000 × g, 4ºC for 15 minutes in a Sigma 4K15 centrifuge. The cell pellet was then re-suspended in ice-cold 50 mM CaCl₂ to 50% of their original volume and incubated on ice for 30 minutes. After a second centrifugation, the bacteria were re-suspended in one tenth of their original volume of ice-cold 50 mM CaCl₂ and glycerol was added to a final concentration of 15% (v/v). Competent DH5α were used immediately, or stored at -80ºC and thawed for 10 minutes on ice before use.
2.2.3. Extraction of total RNA and generation of cDNA

2.2.3.1. Extraction of RNA from HepG2 cells

HepG2 cells (untreated or transiently transfected) were harvested from single wells of 6-well tissue culture plates before they reached 80% confluence. To extract total RNA, the supernatant was aspirated, and the attached cells washed once with PBS and lysed by the addition of 350 μl Buffer RLT (Qiagen) containing 0.01% (v/v) β-ME. The lysate was then passed five times through a 21-gauge needle, and total RNA was isolated from each sample using the RNeasy Mini kit. To do this, one volume of 70% (v/v) ethanol was mixed with the homogenate and the mixture placed on an RNeasy spin column. The column was then centrifuged in a Beckman Microfuge 18 centrifuge at 8,000 × g for 15 seconds, and then washed by addition of 500 μl Buffer RW1 and centrifugation at 8,000 × g for 15 seconds, followed by two washes with 750 μl Buffer RPE under the same conditions. The column was dried by an additional centrifugation at 18,000 × g for 1 minute. Purified RNA was eluted from each column with 30 μl RNAse-free water and centrifugation at 8,000 × g for 1 minute. Finally, the eluant was passed through the column again to elute any RNA remaining on the column. All RNA samples were stored at -20°C.

2.2.3.2. Extraction of RNA from murine liver

Two hundred and fifty milligrams of murine liver (originating from a (CBAxC57Bl/6J)xC57Bl/6J hUGT1A8-7k transgenic mouse sacrificed under Flinders Medical Centre Animal Welfare Committee approval #574/04) was disrupted and homogenised immediately after collection in 16 μl/mg Buffer RLT (Qiagen) and 10 μl/ml β-ME using a hand micropestle followed by 10 passages through a 21-gauge needle. The homogenate was then frozen on dry ice and stored at -80°C until required for RNA extraction. Before extraction of RNA, the liver
homogenate was thawed at room temperature and then heated to 37°C for 20 minutes.

To extract total RNA from the murine liver, the homogenate was processed using an RNeasy Midi kit according to the manufacturer’s instructions. Briefly, the thawed homogenate was centrifuged at 5000 × g for 10 minutes in a Sigma 4K15 centrifuge and the supernatant transferred to a fresh tube. One volume of 70% (v/v) ethanol was then added to the homogenate, which was mixed vigorously and passed through an RNeasy midi column by centrifugation at 5000 × g for 5 minutes. The column was then washed once by addition of 4 ml Buffer RW1 (Qiagen) and centrifugation at 5000 × g for 5 minutes, and then twice by addition of 2.5 ml Buffer RPE (Qiagen) followed by centrifugation at 5000 × g for 2 minutes. After the third wash, the column was dried by a final centrifugation at 5000 × g for 10 minutes. Finally, the RNA was eluted with 250 μl RNase-free water, and the eluate passed through the column a second time to collect any remaining RNA. The yield obtained was approximately 0.5 μg RNA/mg of liver tissue.

2.2.3.3. DNase I treatment of RNA

Column-purified total RNA (2.5 μg) was treated with one unit amplification grade DNase I in 20 mM Tris-HCl (pH 8.4), 50 mM KCl and 2 mM MgCl₂ for 15 minutes at room temperature. EDTA was added to 2.3 mM and the sample was incubated at 65°C for 15 minutes to inactivate the DNase I.

2.2.3.4. Generation of cDNA

cDNA was generated from total HepG2 or mouse RNA in a random hexamer-primed SuperScript II reverse transcriptase reaction using the SuperScript First-Strand Synthesis System (Invitrogen). Briefly, one microgram of RNA was added to a 10 μl
reaction containing 1 mM dNTPs and 5 ng/µg random hexamer oligonucleotides, heated to 65°C for 5 minutes and cooled on ice for one minute. The reaction mix was then made up to a volume of 19 µl, such that when 1 µl of reverse transcriptase was also added later, the final composition was: 50 ng/µl RNA, 0.5 mM dNTPs, 2.5 ng/µl random hexamers, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 5 mM MgCl2, 0.01 mM DTT and 0.1 units/µl RNaseOUT™ Recombinant Ribonuclease Inhibitor. After pre-heating the reaction mix to 25°C for 2 minutes, 50 Units (1 µl) of Superscript II reverse transcriptase were added and the reaction incubated at 25°C for 10 minutes, then at 42°C for a further 50 minutes. cDNA synthesis was terminated at 70°C for 15 minutes, after which 2 units of *E. coli* RNase H were added and the RNA digested at 37°C for 20 minutes. Finally, the cDNA was diluted 1:5 in DNAse-free water before use. All DNA samples, whether genomic, cDNA, plasmid or PCR product, were stored at -20ºC.

### 2.2.4. Extraction of genomic DNA from HEK293T cells

HEK293T cells were grown to near-confluence in a 75 cm² tissue culture flask and washed twice in PBS. Cells were then lysed *in situ* by addition of 5 ml lysis buffer (10 mM Tris pH 8.0, 5 mM EDTA, 0.2% (w/v) SDS, 0.2 M NaCl and 0.1 mg/ml proteinase K) and incubation at 37°C for 2 hours with occasional shaking. After lysis, an equal volume of isopropanol was added to the lysate and the DNA collected by physically scooping the precipitate out of the supernatant. The DNA was then blotted on tissue paper and dissolved in 10 mM Tris pH 7.5, aided by incubation at 37°C for 2 hours.

To prepare the genomic DNA for use in PCR as template, 2.5 µg was digested with 20 Units *NotI* restriction endonuclease in a 50 µl reaction buffered with NEB buffer
3 (50 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl₂, 1 mM DTT, pH 7.9) plus 0.1 mg/ml BSA. Restriction was performed for 3 hrs at 37°C.

**2.2.5. Plasmid and BAC preparation**

Small scale plasmid DNA preparations (minipreps), suitable for use as PCR template, sequencing template or in restriction analysis, were generated from 3 ml overnight bacterial cultures using the QIAprep Spin Miniprep kit as per the manufacturer’s instructions. The bacterial pellet was collected by centrifugation of the overnight culture at 6,000 × g for 15 minutes in a Sigma 4K15 centrifuge and the supernatant discarded. The pellet was then resuspended in 250 μl Buffer P1 containing 100 μg/ml RNase A (Qiagen), lysed by addition of 250 μl Buffer P2, and the bacterial proteins and genomic DNA precipitated by the addition of 350 μl Buffer N3. This precipitate was then pelleted by centrifugation at 18,000 × g in a Beckman Microfuge 18 centrifuge for 10 minutes and the supernatant applied to a Qiaprep spin column attached to a Qiavac manifold. The DNA solution was drawn through the column by application of a vacuum. The column was washed with 500 μl Buffer PB, followed by 750 μl Buffer PE, both applied via the vacuum system. The column was then dried by centrifugation at 18,000 × g for 1 minute and the DNA eluted by application of 50 μl 10 mM Tris, pH 8.5 and a further centrifugation at 18,000 × g for 1 minute.

Large scale plasmid DNA preparations (midipreps), used for mammalian cell transfection, were generated from 50 ml overnight bacterial cultures using the QIAGEN Plasmid Midiprep kit as per the manufacturer’s instructions. The bacterial pellet was collected by centrifugation of the overnight culture at 6,000 × g for 15 minutes in a Sigma 4K15 centrifuge and the supernatant discarded. The bacteria
were then resuspended in 4 ml Buffer P1 containing 100 μg/ml RNase A (Qiagen), and 4 ml Buffer P2 (Qiagen) was added to facilitate lysis. After incubation at room temperature for 5 minutes, 4 ml ice-cold Buffer P3 (Qiagen) was added to the bacterial lysate, which was mixed vigorously and incubated on ice for 15 minutes. All precipitate was pelleted by two sequential centrifugations in a JM20 rotor/Beckman J2-21M/E ultracentrifuge at 20,400 × g, 4ºC, transferring the supernatant to a fresh tube in between. The initial centrifugation was for 30 minutes, and the second, 15 minutes. The cleared supernatant was then added to a Qiagen-tip 100 that had been pre-equilibrated with 10 ml Buffer QBT (Qiagen), and allowed to pass through the resin under gravity. The Qiagen-tip 100 was then washed twice with 10 ml Buffer QC (Qiagen) and the DNA eluted with 5 ml Buffer QF (Qiagen). 3.5 ml isopropanol was then added to precipitate the eluted DNA, and the DNA pelleted by centrifugation at 20,400 × g, 4ºC for 30 minutes. Finally the DNA was washed in 2 ml 70% (v/v) ethanol, recovered by centrifugation at 20,400 × g, 4ºC for 10 minutes and air-dried. Midiprep DNA pellets were re-suspended in 500 μl 10 mM Tris, pH 8.5.

BAC DNA was extracted from 3 ml overnight bacterial cultures by the following method. Overnight cultures were harvested by centrifugation in a Sigma 4K15 centrifuge at 6000 × g for 15 minutes and the pellets resuspended in 300 μl Buffer P1 and 100 μg/ml RNase A (Qiagen). The bacteria were then lysed by mixing the suspension with 300 μl Buffer P2 (Qiagen), followed by incubation at room temperature for 5 minutes. 300 μl Buffer P3 (Qiagen) was then added slowly to the cell lysate with gentle mixing, and the mixture placed on ice for 5 minutes. Following this second incubation, the mixture was centrifuged at 15,600 × g in an IEC Centra-M centrifuge at 4ºC for 10 minutes and the supernatant added to a fresh
tube containing 800 μl ice-cold isopropanol. After mixing, the DNA was allowed to precipitate by incubation on ice for 10 minutes and was collected by centrifugation at 15,600 × g, 4°C for 15 minutes. The DNA pellet was then washed with 500 μl 70% (v/v) ethanol, re-collected by centrifugation at 15,600 × g, 4°C for 5 minutes, air-dried until translucent, and resuspended in 40 μl 10 mM Tris, pH 8.5.

### 2.2.6. Polymerase chain reaction amplification

#### 2.2.6.1. Equipment

Non-quantitative PCR reactions were performed on Cetus (PerkinElmer), RoboCycler Gradient 96 (Stratagene) or iCycler (Bio-Rad) thermal cyclers. The particular instrument used is not specified for each reaction, except in instances where this altered the PCR outcome or an exact annealing temperature was required. For quantitative real-time PCR (QPCR) reactions, a Rotor-Gene™ 3000 (Corbett Life Science, Mortlake, NSW, Australia) thermal cycler was used. Analysis of real-time PCR results was performed using Rotor-Gene 6 software (Corbett Life Science).

#### 2.2.6.2. Primers

The nucleotide sequences of all oligonucleotides used for cloning and screening PCRs can be found in the “Methods” sections of each appropriate chapter, unless listed in Table 2.2. In addition, the sense sequences of the primers pairs used in site-directed mutagenesis reactions and electrophoretic mobility-shift assays (EMSAs) are also recorded in the relevant chapters. Oligonucleotides used for site-directed mutagenesis were polyacrylamide gel electrophoresis purified by Sigma-Genosys. Otherwise, primers were purchased desalted.
2.2.6.3. PCR for cloning

PCRs intended to generate DNA fragments for cloning were performed with the proof-reading \textit{PfuTurbo$^\text{\textregistered}$} DNA polymerase unless otherwise stated, to minimise the occurrence of introduced sequence errors. \textit{PfuTurbo} is a mixture of \textit{Pfu} DNA polymerase and the Archaemaxx$^\text{\textTM}$ polymerase-enhancing factor, and has an error rate of $1.3 \times 10^{-6}$ mutations per base pair per duplication. \textit{PfuTurbo} PCR reactions were performed in the supplied \textit{PfuTurbo} buffer (20 mM Tris-HCl pH8.8, 2 mM MgSO$_4$, 10 mM KCl, 10 mM (NH$_4$)$_2$SO$_4$, 0.1% (v/v) Triton-X 100 and 0.1 mg/ml BSA) with 0.05 Units/\textit{$\mu$L} \textit{PfuTurbo}, 200 nM dNTPs and 5 ng/\textit{$\mu$L} each primer. The templates, primer sequences and specific cycling conditions for each reaction are detailed in the appropriate sections. In cases where \textit{PfuTurbo} proved unsuitable for amplification, either the more robust \textit{Taq} DNA polymerase was used as described in section 2.2.6.4 or the Expand$^\text{\textTM}$ Long Template PCR system was utilised. For templates exceeding 2.5 kb where \textit{Taq} DNA polymerase was used, equal units of \textit{Taq Extender$^\text{\textTM}$} PCR additive and \textit{Taq} were added to the PCR to improve product yield. The Expand$^\text{\textTM}$ Long Template PCR System, which utilises a mixture of \textit{Taq} and \textit{Tgo} DNA polymerases to optimise amplification range and fidelity ($4.8 \times 10^{-6}$ mutations per base pair per duplication), was only used to clone the \textit{UGT1A3}-9.4kb promoter (Chapter 5, section 5.2.6)

After PCR, the remaining dNTPs, primers and salts were removed from the PCR products using the QIAquick PCR Purification kit. Five volumes of Buffer PB (Qiagen) were mixed with the PCR product and the entire mixture placed on a QIAquick spin column (Qiagen). After centrifugation in a Beckman Microfuge 18 centrifuge at 18,000 × g for 1 minute, the column was washed by addition of 750 \textit{$\mu$L} Buffer PE and centrifugation at 18,000 × g for 1 minute. The column was then dried
by centrifugation at 18,000 × g for 1 minute and the DNA eluted in 50 μl 10 mM Tris, pH 8.5.

If non-specific products persisted after optimisation of the PCR reaction, the PCR reaction was subjected to electrophoresis through an agarose gel (described in section 2.2.8) and the desired product excised. The DNA was then retrieved from the agarose slice using the QIAquick PCR Purification kit and Buffer QG (Qiagen) as per the manufacturer’s instructions (described in section 2.2.8).

2.2.6.4. **PCR for genotyping or screening**

In cases where fidelity was not critical, or if a target could not be amplified with *PfuTurbo*, *Taq* DNA polymerase was used. *Taq* DNA polymerase has an error rate of 1 × 10⁻⁴ to 2 × 10⁻⁵ mutations per base pair per duplication. *Taq* mediated-PCR reactions were performed in the supplied ThermoPol buffer (20 mM Tris-HCl pH8.8, 10 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄, and 0.1% (v/v) Triton-X 100) with 200 nM dNTPs and 5 ng/μl each primer. Again, the specific conditions for each reaction are detailed in the relevant sections of this thesis. If the resulting PCR product required purification before use downstream, this was performed as described in section 2.2.6.3. Generally, it was found that it was unnecessary to purify PCR products before sequencing, provided that the PCR product concentration was sufficiently high that the reaction mix could be diluted by at least 1:5 before submission to the DNA Sequencing Core Facility.

2.2.6.5. **Site-directed mutagenesis**

To insert mutations into plasmid constructs in a controlled manner, two complementary oligonucleotides were designed over the target site according to the rules established by Stratagene’s QuikChange® site-directed mutagenesis kit. Thus,
where practicable, the primers pairs were completely complimentary and contained the desired mutation(s) flanked by at least 10 bases of correct sequence on both sides. They also possessed melting temperatures of ≥ 78°C, a minimum GC content of 40% and one or more C or G terminating residues. Mutagenesis reactions were performed in a total volume of 50 µl containing PfuTurbo buffer, 200 nM dNTPs, 2.5 Units PfuTurbo DNA polymerase, 250 ng each oligonucleotide and 50 ng plasmid template. The default reaction conditions were 30 seconds of denaturation at 95°C, followed by 16 rounds of 30 seconds at 95°C, 1 minute annealing at 55°C and an extension time of 2 minutes per kb of target plasmid. Deviations from these conditions are recorded in the appropriate thesis sections. 10 Units of DpnI were added to the amplified vector and incubated at 37°C for 1 hour. This step degrades the wild-type parent vector, but not the mutated transcripts, as DpnI recognition of restriction sites is methylation sensitive and PCR-generated plasmids are not methylated. One microlitre of digested PCR product was then used to transform 100 µl competent DH5α E. coli as described in section 2.2.7. The presence of the desired mutation(s) was confirmed by sequencing of miniprep DNA prepared from representative colonies.

2.2.6.6. Quantitative real-time PCR

To quantify the levels of UGT mRNA transcripts and 18S ribosomal RNA (rRNA) present in RNA extracted from HepG2 cells, real-time PCR was used. The primer sets and annealing temperatures used are detailed in Chapter 5, section 5.2.5. However, the generic set-up used for all reactions were: 20 µl aliquots containing 1 × QuantiTect SYBR Green PCR master mix (comprised of HotStarTaq DNA polymerase, QuantiTect SYBR Green PCR buffer (Tris-HCl, KCl and (NH₄)₂SO₄, pH 8.7), dNTP mix, SYBR Green I, ROX passive reference dye and 2.5 mM MgCl₂),
0.5 μM each primer, and template cDNA equivalent to 40 ng input RNA. For 18S PCRs, the amount of template cDNA used per reaction was decreased to the equivalent of 1.25 ng input RNA. The ROX dye is not required for analysis using the Rotor-Gene 3000 and does not interfere with the system. The generic PCR cycling conditions used for quantitative analysis were: an activation period of 15 minutes at 95°C; 40 cycles of 95°C for 15 seconds, specific annealing temperature (see Table 5.1) for 20 seconds, and 72°C for 20 seconds; and a ramped melt analysis between 60 and 95°C with 5 second, 1°C steps. Data was acquired during the 72°C extension phase of each cycle.

2.2.7. Restriction digests, calf intestinal alkaline phosphatase treatment, ligation and transformation

Preparative restriction digests (for cloning) were done with 5 μg of starting material in 50 μl reactions. Each reaction also contained 1 × appropriate reaction buffer (see below), 100 μg/ml BSA and 0.2-0.4 Units/μl restriction enzyme. Digestion was performed at 37°C (unless otherwise stated) for 3 hours. Analytical restriction digests were performed with approximately 100 ng plasmid DNA in a reaction volume of 20 μl containing 100 μg/ml BSA and 0.1-0.2 Units/μl each restriction enzyme. DNA was digested at 37°C (unless otherwise stated) for 1 hour.

For KpnI digests, NEB Buffer 1 (10 mM Bis-Tris-Propane-HCl, 10 mM MgCl₂ and 1 mM DTT, pH 7.0) was used. For BamHI, BsrGI, EcoRI, HindIII, NheI, PvuII, SpeI, XbaI and XhoI digests, or double digests, NEB Buffer 2 (10 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl₂ and 1 mM DTT, pH 7.9) was used. For MluI and PstI digests, NEB Buffer 3 (50 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl₂ and 1 mM DTT, pH 7.9) was used. KpnI/MluI double digests were performed sequentially in their
respective buffers due to buffer incompatibility. *Mlu*I/*Xho*I double digestions were performed in NEB Buffer 3.

For vectors that were to be used in cloning after restriction with only a single enzyme, treatment with calf intestinal alkaline phosphatase (CIP) was employed to prevent self-ligation. CIP treatment was performed by addition of 10 Units CIP to the 50 μl preparative digestion after the initial 3 hour incubation (no buffer change necessary), followed by incubation at 37°C for an additional hour.

Ligations were performed using the NEB Quick Ligation kit. Twenty microlitre reactions were prepared with 50 ng plasmid DNA, a 3 to 10-fold molar excess of insert DNA and 1 × NEB Quick Ligase buffer (66 mM Tris-HCl, 10 mM MgCl₂, 1 mM DTT, 1 mM ATP and 7.5% (w/v) polyethylene glycol 6000, pH 7.6). One microlitre of Quick T4 DNA Ligase was then added to the mix and incubated for 5 minutes at room temperature. The reaction was chilled on ice before use in transformation.

All transformations were performed using chemically competent DH5α *E. coli* cells prepared as described in section 2.2.2, unless otherwise stated in the specific methods of each chapter. Competent cells were thawed on ice for 10 minutes before addition of up to 5 μl of ligation product. The cells were then mixed gently and incubated on ice for a further 30 minutes before exposure to heat shock. To facilitate uptake of the ligated DNA, the cells were incubated at 42°C for 45 seconds and then immediately placed on ice for 2 minutes. 400 μl of LB was then added to the shocked cells and they were allowed to recover at 37°C for one hour before being plated on LB/agar plates containing an appropriate antibiotic for selection. Colonies were allowed to form at 37°C overnight before being screened for desired clones.
2.2.8. Electrophoresis and quantification of DNA and RNA

DNA fragments and plasmids were analysed for relative size (based on mobility) and purity (from unwanted DNA fragments and contaminating RNA) by agarose gel electrophoresis. DNA samples were mixed with 6 × DNA gel loading buffer to give the final buffer concentration specified in section 2.1.2 and loaded onto 0.8-2% (w/v) agarose gels in Tris-acetate EDTA electrophoresis buffer (section 2.1.2) and 0.4 µg/ml ethidium bromide. Electrophoresis was performed in a Bio-Rad Mini-Sub Cell GT electrophoresis system by applying 5 volts/cm (between the electrodes) to the gel. The gel was then examined with either a Bio-Rad Gel Doc 1000 system with Molecular Analyst Software (Bio-Rad), or a Gene Genius Bio imaging system (Syngene, Cambridge, England) and GeneSnap version 6.04 software (Syngene).

To recover DNA samples, agarose slices containing the required DNA were swiftly excised under low-intensity UV irradiation and added to 3 volumes of buffer QG (Qiagen). After heating to 50°C for 10 minutes with occasional mixing, one gel volume of isopropanol was added to the sample, which was then mixed and added to a QIAquick column. After centrifugation at 18,000 × g in a Beckman Microfuge 18 centrifuge for 1 minute, the column was washed once with 500 µl Buffer QG and twice with 750 µl Buffer PE, with a 1 minute centrifugation step between each wash. The column was dried by centrifugation at 18,000 × g for 1 minute and the DNA eluted into a clean tube by addition of 50 µl 10 mM TrisCl, pH 8.5 to the column and centrifugation at 18,000 × g for 1 minute.

The concentration and purity of DNA samples were determined by spectrophotometry (optical absorbance at 260 nm versus 280 nm) using a GeneQuant II (Pharmacia Biotech (GE Healthcare), Buckinghamshire, England) spectrophotometer and/or estimation against a series of known standards after
electrophoresis into an agarose gel and staining with ethidium bromide as described earlier. Likewise, the concentration of RNA was determined by spectrophotometry.

2.2.9. Sequencing

All sequencing was performed by the DNA Sequencing Core Facility (Department of Haematology and Genetic Pathology, Flinders University) using Big Dye Terminator Cycle Sequencing Version 3.1 chemistry (Applied Biosystems, Foster City, CA) and an ABI 3100 Genetic Analyser sequencer (Applied Biosystems). Sequencing of pGL3-derived plasmids was performed with GL2 (5’ CTTATGTTTTTGGCGTCTTCC 3’) and RV3 (5’ GGGACAGCCTATTTTGCTAG 3’) primers. Sequencing of pCMX-derived plasmids was performed with a T7 primer (5’ TAATACGACTCACTATAGGGAGA 3’).

2.2.10. Transient transfection of HepG2, Caco-2 and HEK293T cells

HepG2, Caco-2 or HEK293T cells were re-suspended in growth medium before they reached 80% confluence and counted in the presence of 0.2% (w/v) trypan blue. Only cells that excluded the trypan blue dye were considered viable. For 24-well plates (Nunc, Kamstrupvej, Denmark), HepG2 cells were seeded at a density of 2 × 10^5 cells per well, Caco-2 at 7.5 × 10^4 cells per well and HEK293 at 1 × 10^5 cells per well in at total volume of 500μl. Where 6-well plates (Nunc) were used, HepG2 cells were plated a density of 1 × 10^6 cells per well in a total volume of 2 ml.

Twenty-four hours after plating, transfections were performed using Lipofectamine 2000. The amount and identity of the DNA used in each transfection was experiment-specific and is detailed in each appropriate chapter. For transfections in 24-well plates, the DNA for each well was added to 50 μl serum-free DMEM, for
experiments in 6-well plates, 250 μl of DMEM was used. Two or 10 μl of the Lipofectamine 2000 cationic lipid transfection agent was then mixed with 50 or 250 μl serum-free DMEM for 24 or 6-well plate experiments respectively, and allowed to stand at room temperature for 5 minutes. The DNA and Lipofectamine 2000 mixtures for each well were then combined and incubated at room temperature for a minimum of 20 minutes. One hundred microlitres (24-well plates) or 500 μl (6-well plates) of Lipofectamine 2000:DNA complexes were then added directly to each well without removing the plating medium. The cells were then incubated overnight before changing the medium for fresh culture medium with all additives, unless chemical treatment of cells was required to commence prior. In this case, cells were incubated with the Lipofectamine 2000:DNA complexes for a minimum of 6 hours before the transfection medium was replaced with culture medium containing the desired treatment. pRL-null, which constitutively expresses the renilla (Renilla reniformis) luciferase gene was added to transfections as an internal control for transfection efficiency, with the exception of transfections that were to be harvested for RNA.

2.2.11. Luciferase assay
Forty-eight hours post-transfection, cells were lysed by addition of passive lysis buffer (100 μl per well for 24-well plates; 500 μl per well for 6-well plates) followed by incubation at room temperature with continual rocking for 20 minutes. A 20 μl sample of lysate was then analysed sequentially for firefly (Photinus pyralis) and renilla luciferase activity using the Dual-Luciferase Reporter Assay system (Promega) and a TopCount luminescence and scintillation counter (Parkard, Mt Waverly, Victoria, Australia). Lysate was added to alternate wells of 96-well plates (to avoid crossover luminescence from neighbouring wells), and mixed with 50 μl of
firefly luciferase reagent (Luciferase Assay Reagent II, Promega) to measure the activity of firefly luciferase expressed from transiently transfected pGL3-derived vectors. The luminescence of each sample was quantified 100 seconds after addition of Luciferase Assay Reagent II. The samples were then quenched by addition of 50 μl of the Stop and Glo Reagent (Promega), which also contains the substrate for renilla luciferase. The luminescence from the renilla luciferase protein was also measured 100 seconds after addition of the relevant substrate. If the firefly luminescence reading exceeded $2 \times 10^6$ counts per second, the samples were diluted in passive lysis buffer to ensure that the results were within the linear range of the luminescence counter used.

Each transfection was performed in triplicate, and mean relative luciferase activities were calculated from the three resulting ratios of firefly to renilla luciferase activity. Results presented are the mean activity (plus one standard deviation) of each promoter-reporter vector relative to the promoterless pGL3-basic vector (with the activity of pGL3-basic being set arbitrarily to a value of one). Unless otherwise stated, all triplicate transfections presented were performed at least twice in independent experiments.

2.2.12. Preparation of nuclear extracts

Nuclear extracts were prepared by the following method adapted from Schreiber et al. (1989) as published in Gardner-Stephen and Mackenzie (2005). All wash and lysis buffers were pre-chilled on ice. HepG2 cells were grown to confluence in 175 cm$^2$ flasks, rinsed once in PBS and harvested in 10 ml PBS by scraping. The pellet from each flask was collected by centrifugation at 1500 × g for 5 minutes in a Sigma 4K15 centrifuge, washed in 1 ml PBS, pelleted in an IEC Centra-M centrifuge at
15,600 × g, 4°C for 1 minute and re-suspended in 800 μl buffer A (10 mM Tris-HCl pH 7.9, 10 mM KCl, 1 mM DTT, 1.5 mM MgCl₂ and 1 × Complete protease inhibitor cocktail) with 0.5% (v/v) Nonidet P-40. After 15 minutes on ice the nuclear fraction was pelleted at 15,600 × g for 1 minute (4°C) and washed in 800 μl buffer A. The washed nuclei from four flasks were pooled and re-suspended in 400 μl ice-cold buffer B (50 mM Tris-HCl pH 7.9, 500 mM KCl, 2mM DTT, 5 mM MgCl₂, 0.1 mM EDTA, 10% (w/v) sucrose, 20% (v/v) glycerol and 1 × Complete protease inhibitor cocktail). The tube containing the pooled sample was then buried in ice in a small beaker and shaken vigorously for an hour to facilitate lysis of the nuclei. The nuclear extract supernatant was separated from the remaining debris by centrifugation for 15 minutes at 15,600 × g at 4°C and transferred to a Slide-A-Lyzer® dialysis cassette. Dialysis was performed for at least 2 hours against 200 ml of buffer TM-1 (25 mM Tris-HCl pH 7.6, 100 mM KCl, 0.5 mM DTT, 5 mM MgCl₂, 0.5 mM EDTA and 10% (v/v) glycerol) at 4°C. The protein concentration of the dialysed nuclear extract was determined by comparison to BSA standards using the Bio-Rad Protein Assay reagent, before storage at -80 °C.

2.2.13. Labelling oligonucleotide probes
To generate double stranded DNA probes from two complementary oligonucleotides, 5 μg of each primer was denatured at 95°C for 2 minutes in 100 μl of annealing buffer (40 mM Tris-HCl pH 7.5, 20 mM MgCl₂ and 50 mM NaCl) in a dry block heater. The probes were then allowed to cool unassisted to room temperature in the heating block over the course of several hours. One hundred nanograms of annealed probe was then end-labelled by incubation with approximately 4 MBq γ³²P-ATP and 10 units of T4 polynucleotide kinase in the supplied reaction buffer (70 mM Tris-HCl pH 7.6, 10 mM MgCl₂ and 5 mM DTT) for one hour at 37°C. The labelled probe
was then diluted 1:10 in Tris-EDTA buffer (section 2.1.2) and separated from free \( \gamma^{32}\text{P-ATP} \) by purification through G25 columns according to the manufacturer’s instructions.

### 2.2.14. Electrophoretic mobility-shift assays

To determine whether nuclear proteins could bind to UGT promoter regions of interest, EMSAs were used. The mobility of a DNA oligonucleotide passing through a non-denaturing polyacrylamide gel is retarded if it forms a higher molecular weight complex with supplied proteins. To demonstrate the specificity of any protein-probe interactions formed, up to 500-fold excess unlabelled wild-type or unrelated probes were used to compete for protein binding. Mutations were also introduced into selected EMSA probes to demonstrate the importance of particular nucleotides in the binding of protein complexes. To confirm the identity of proteins bound to a probe, antibodies with reactivity towards the protein of interest were added to the reaction, thereby further increasing the molecular weight of the complex formed if the suspected protein was present (see section 2.2.15).

For HNF1 EMSAs, either 5 µg of HepG2 or Caco-2 nuclear extract, or 1 µl \textit{in vitro} synthesised HNF1\( \alpha \) or HNF1\( \beta \) protein (generated using the TNT Quick Coupled Transcription/Translation; see Chapter 4, section 4.2.10) were incubated with 1 µg poly(dIdC) for 10 minutes on ice, in a reaction mix made up to a total volume of 15 µl with buffer TM-1. If unlabelled competitor probes were required, they were also included in this 15 µl reaction mix. Fifty thousand counts per minute (cpm) of \( \gamma^{32}\text{P-ATP} \) end-labelled double-stranded DNA probe (as defined in each appropriate chapter) were then added to each reaction as an extra 1 µl aliquot. After the addition of radioactive probe, reactions were kept at room temperature for 30 minutes to
allow DNA-protein complexes to form. These were then resolved on a 4% (w/v) non-denaturing polyacrylamide gel made with 29:1 Acrylamide/Bis in 0.5 × TBE. After pre-running the gel at 4°C for 2 hours at 170 V, electrophoresis of samples was achieved by applying 250 V for 2 hours at 4°C using Dual Slab Gel kit electrophoresis equipment (CBS Scientific Company, Del Mar, CA). The gel was then dried under vacuum and exposed to X-Omat Blue XB-1 film at -80°C with the aid of intensifying screens.

For HNF4α EMSAs, 5 µg of HepG2 nuclear extract was incubated with 1 µg poly(dIdC) and 0.5 µg sonicated salmon sperm DNA for 10 minutes on ice in a reaction mix made up to a total volume of 15 µl with buffer TM-2 (10 mM Hepes-NaOH pH 7.8, 100 mM NaCl, 0.1 mM EDTA, 10% (v/v) glycerol, 1 mg/ml BSA and 0.5 mM DTT) (Pineda-Torra et al., 2002). Addition of unlabelled or labelled probe, incubation, electrophoresis and exposure to film were all performed as described for the HNF1 EMSA experiments.

2.2.15. Super-shift EMSA

For super-shift EMSAs, 2 µg of anti-HNF1α, anti-HNF1β or anti-HNF4α antibody was added to EMSA reactions immediately after the addition of labelled probe. Samples were then treated in the same way as described for standard EMSA experiments.

2.2.16. Western blot

Twenty micrograms of HepG2 or HEK293T total cell lysate or 1 µl in vitro synthesised HNF1α or HNF4α protein (see Chapter 4, section 4.2.10) were subjected to electrophoresis on 10% (w/v) SDS-polyacrylamide gel electrophoresis gels (made with 19:1 Acrylamide/Bis in 0.5 × TBE with a 4% (w/v) stacking gel).
Electrophoresis was performed at room temperature; at 70V until the samples cleared the stacking gel and then at 150V until the tracking dye reached the bottom of the gel using Mini-Protean II Cell equipment (Bio-Rad). The separated proteins were then transferred to Trans-Blot Transfer Medium (nitrocellulose membrane, 0.45 μm) for 1 hour at 100V, using an ice-cooled Mini Trans-Blot Cell apparatus (Bio-Rad).

To detect HNF1α or HNF4α proteins present on membranes after transfer, blots were blocked overnight at 4°C in TBST [Tris-buffered saline (10 mM Tris-HCl, pH 7.4 and 150 mM NaCl) plus 0.05% (v/v) Tween-20] containing 5% (w/v) skim milk powder (SMP). The membrane was then incubated for 2 hours with 1 μg/ml anti-HNF1α or anti-HNF4α antibody in TBST/SMP, and washed three times with TBST for 5 minutes each. The membrane was then incubated for a further hour in TBST/SMP containing 1:2,500 diluted rabbit anti-goat IgG-horseradish peroxidase-conjugated secondary antibody and washed three times with TBST for 5 minutes each. Finally, the blot was given a final wash for 5 minutes in Tris-buffered saline, was treated with ECL Western blotting detection reagent according to the manufacturer’s instructions and exposed to X-Omat Blue XB-1 autoradiographic film. All washes and antibody incubations were performed at room temperature with gentle rocking.

### 2.2.17. Statistical analysis

All statistical analyses reported in this thesis were performed using the SPSS software package version 12.0.1 (SPSS Inc., Chicago, IL) or Microsoft Office Excel 2003 software (Microsoft Corporation, WA).

The statistical significance of altered promoter activity in luciferase assays, or of altered levels of endogenous *UGT* mRNA transcripts in treated HepG2 cells, was
determined by independent-samples \( t \)-tests. Where results were subject to heteroscedasticity (nonconsistent variance), data were log-transformed before analysis. Results were considered statistically significant if \( P < 0.05 \).

Statistical analysis of \( UGT1A3 \) promoter variant frequencies and compliance with Hardy-Weinberg equilibrium (tested by Pearson \( \chi^2 \)) were performed using Microsoft Office Excel 2003 software (Microsoft Corporation, WA).

2.3 Cloning of liver-enriched transcription factors

The primers used to amplify the coding regions of each transcription factor are listed in Table 2.2. Details for the amplification of each PCR product are given below in each appropriate section.

2.3.1. Hepatocyte nuclear factor 1β (HNF1β)

pCMX-HNF1β was constructed as described in Gardner-Stephen and Mackenzie (2007a), using HepG2 cDNA as template for amplification of the HNF1β coding region, and primers HNF1βf and HNF1βr (Table 2.2). The following \( PfuTurbo \) PCR (see section 2.2.6.3) was performed on the cDNA equivalent of 0.8 ng/μl of input RNA. The amplification parameters used were: 4 minutes initial denaturation at 95°C; 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 3 minutes; completed by a 5 minute final extension at 72°C. The resulting PCR product was cleaned using the QIAquick PCR purification kit, digested with HindIII and BamHI (section 2.2.7), cloned into pCMX-PL2 and sequenced in full (section 2.2.9).
Table 2.2: Primers used for cloning transcription factor cDNAs.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Nucleotide Sequence (5'→3')</th>
<th>RE</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNF1βfor</td>
<td>AGCCATAAGCTTATGGTGTCCAAGCTCAGTGC</td>
<td>HindIII</td>
</tr>
<tr>
<td>HNF1βrev</td>
<td>AGCCATGGATCGCTCAACCAGTCGGTACACAC</td>
<td>BamHI</td>
</tr>
<tr>
<td>hHNF4α2for</td>
<td>AGCCATAAGCTTATGGTGTCCAAGCTCAGTGC</td>
<td>HindIII</td>
</tr>
<tr>
<td>hHNF4αrev</td>
<td>AGCCATGGATCGCTCAACCAGTCGGTACACAC</td>
<td>BamHI</td>
</tr>
<tr>
<td>QC hHNF4α1for</td>
<td>CACCTCAGCAACGGACAGATGAGCCACCTCAGA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QC hHNF4α1rev</td>
<td>AGCCATAAGCTTATGGTGTCCAAGCTCAGTGC</td>
<td>HindIII</td>
</tr>
<tr>
<td>rHNF4α1for</td>
<td>AGCCATAAGCTTATGGTGTCCAAGCTCAGTGC</td>
<td>HindIII</td>
</tr>
<tr>
<td>rHNF4α1rev</td>
<td>AGCCATAAGCTTATGGTGTCCAAGCTCAGTGC</td>
<td>HindIII</td>
</tr>
<tr>
<td>HNF6for</td>
<td>AGCCATAAGCTTATGGTGTCCAAGCTCAGTGC</td>
<td>HindIII</td>
</tr>
<tr>
<td>HNF6rev</td>
<td>AGCCATAAGCTTATGGTGTCCAAGCTCAGTGC</td>
<td>BamHI</td>
</tr>
<tr>
<td>hFoxA3for</td>
<td>AGCCATAAGCTTATGGTGTCCAAGCTCAGTGC</td>
<td>HindIII</td>
</tr>
<tr>
<td>hFoxA3rev</td>
<td>AGCCATAAGCTTATGGTGTCCAAGCTCAGTGC</td>
<td>BamHI</td>
</tr>
<tr>
<td>hPXRT1for</td>
<td>AGCCATAAGCTTATGGTGTCCAAGCTCAGTGC</td>
<td>HindIII</td>
</tr>
<tr>
<td>hPXRev</td>
<td>AGCCATAAGCTTATGGTGTCCAAGCTCAGTGC</td>
<td>XbaI</td>
</tr>
</tbody>
</table>

RE: restriction endonuclease site, as underlined. NA: not applicable. The position of the 30 nucleotides deleted from HNF4α2 to create HNF4α1 is indicated by the circumflex accent (^). The deliberate mutation in the hPXRT1for primer is highlighted in bold.

2.3.2. Hepatocyte nuclear factor 4α (HNF4α)

2.3.2.1. Human HNF4α splice variant 2

HNF4α2 transcripts were amplified by PCR from cDNA generated from HepG2 total RNA as described in Gardner-Stephen and Mackenzie (2007a). The 50μl PCR contained cDNA equivalent to 40 ng of input RNA and was performed using primers hHNF4α2for and hHNF4αrev (Table 2.2) and PfuTurbo DNA polymerase. The PCR
cycling parameters were: 95°C for 4 minutes; 30 cycles of 95°C for 30 seconds, 58°C for 30 seconds and 72°C for 2 minutes; and a final extension step of 72°C for 5 minutes. The resulting PCR product was cloned into the HindIII and BamHI sites of pCMX-PL2 utilising restriction sites incorporated into each primer. The HNF4α coding region was sequenced in full to verify that the wild-type sequence had been obtained, as HepG2 cells are known to be heterozygous for a single base-pair change at nucleotide 206 of HNF4α which results in a D69A mutation (Lausen et al., 2000). HNF4α2 is the most abundant HNF4α transcript in the adult liver (Hata et al., 1995).

2.3.2.2. Human HNF4α splice variant 1

The HNF4α1 splice variant differs from HNF4α2 by a 10 amino acid insertion present in the F domain of HNF4α2 relative to HNF4α1 (Hata et al., 1992; Chartier et al., 1994; Hata et al., 1995; Drewes et al., 1996; Sladek et al., 1999). To obtain an expression vector for human HNF4α1, pCMX-HNF4α2 was altered to contain an HNF4α1-identical cDNA region. This was achieved using the QuikChange site-directed mutagenesis protocol (section 2.2.6.5) and the primer pair QC hHNF4α1for and QC hHNF4α1rev, where QC hHNF4α1for is listed in Table 2.2 and QC hHNF4α1rev is perfectly complementary to QC hHNF4α1for. The specific QuikChange PCR parameters for this reaction were: 95°C for 30 seconds; 16 cycles of 95°C for 30 seconds, 55°C for 1 minute and 68°C for 14 minutes.

2.3.2.3. Rat HNF4α splice variant 1

The coding region of the rat HNF4α1 splice variant was amplified by PCR from the plasmid pSG5-HNF4α. The primers used were rHNF4α1for and rHNF4α1rev (Table 2.2). The following PCR was performed as a PfuTurbo reaction (section 2.2.6.3) using 0.6 ng/µl pSG5-HNF4α plasmid template. Cycling conditions were: 95°C for 4 minutes; followed by 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds and
72°C for 4 minutes; finished with an extra extension step of 72°C for 5 minutes. The resulting PCR product was cleaned with the Qiagen PCR purification kit, digested with HindIII and BamHI, and ligated into the corresponding sites of pCMX-PL2. The entire insert was then sequenced for nucleotide errors.

2.3.3. Hepatocyte nuclear factor 6 (HNF6, OneCut 1)

Attempts to clone HNF6 from HepG2 cDNA resulted in only truncated or mutated transcripts being obtained. Therefore, HNF6 was cloned from murine liver RNA.

HNF6 was amplified from the murine liver cDNA equivalent of 80 ng of input RNA using PfuTurbo. The primers used were mHNF6for and mHNF6rev (see Table 2.2), and the amplification conditions were: 95°C for 4 minutes; followed by 40 cycles of 95°C for 30 seconds, 55°C for 1 minute and 72°C for 5 minutes; finished with an extra extension step of 72°C for 5 minutes. The resulting PCR product was inserted into the HindIII and BamHI sites of pCMX-PL2, utilising the restriction sites incorporated into the PCR primers, and sequenced in full.

2.3.4. Forkhead box A factors (FoxA, hepatocyte nuclear factor 3 factors)

2.3.4.1. Rat FoxA1 (HNF3α) and FoxA2 (HNF3β)

Plasmids pRB-HNF3α and pGEM-HNF3β, containing rat FoxA1 and FoxA2 cDNAs respectively, were restricted with EcoRI to release their FoxA encoding inserts. The inserts were then separated from plasmid DNA by gel electrophoresis and purified using the QIAquick Gel Extraction kit. Each FoxA fragment was then non-directionally cloned into pCMX-PL2 pre-treated with EcoRI and CIP. The orientation of the FoxA1 insert, relative to the pCMX-PL2 promoter, was determined by digestion with NheI, followed by analysis of the restriction pattern by agarose gel electrophoresis. Likewise, the orientation of the inserted FoxA2 cDNA was
determined by double digestion with *Pvu*II and *Xho*I. Chosen clones were sequenced in full.

2.3.4.2. *Human FoxA3 (HNF3γ)*

FoxA3 was amplified from HepG2 cDNA using primers hFoxA3for and hFoxA3rev (Table 2.2) in a *PfuTurbo* DNA polymerase PCR reaction. The concentration of HepG2 cDNA present in the PCR reaction was the equivalent of 0.8 ng/μl of total RNA used to generate the cDNA. Amplification conditions for FoxA3 were as described for HNF1β (section 2.3.1), and the resulting PCR product was digested with *Bam*HI and *Hind*III and ligated into pCMX-PL2. Sequencing confirmed that a full-length FoxA3 clone without mutations had been obtained.

2.3.5. *CAATT enhancer binding protein factors (C/EBP)*

Plasmids containing rat C/EBPα and C/EBPβ cDNAs were used as starting material to construct the pCMX-CEBPα and pCMX-CEBPβ expression vectors. Similarly to the assembly of pCMX-FoxA1 and pCMX-FoxA2, the C/EBPα and C/EBPβ coding fragments were retrieved from their original vector by *Eco*RI digest followed by gel electrophoresis and excision/purification of the desired fragment. The rat C/EBPα and C/EBPβ cDNAs were then cloned non-directionally into the *Eco*RI and CIP treated pCMX-PL2 preparation as for FoxA1 and FoxA2, checked for insert orientation by *Pst*I digestion and sequenced.

2.3.6. *Pregnane X receptor (PXR)*

The T1 splice variant of human PXR was cloned as described in Gardner-Stephen *et al.* (2004). In detail, the PXR T1 transcript was amplified from HepG2 cDNA (at a concentration equivalent to 20 ng/μl reverse transcribed RNA) using the primers hPXRT1for and hPXRrev (Table 2.2) and *PfuTurbo* DNA polymerase. After an
initial 4 minutes at 95°C, 40 cycles of 95°C for 45 seconds, 60°C for 45 seconds and 72°C for 4 minutes were performed and the PCR finished with 5 minutes at 72°C. The PCR-amplified cDNA was then inserted into the XbaI and HindIII sites of pCMV5 (GenBank record AF239249, Andersson et al., 1989) and sequenced. To maintain consistency amongst the transcription factors investigated in this thesis, the PXR cDNA insert was then shuttled from the original pCMV5 vector into the HindIII and BamHI sites of pCMX-PL2 to create pCMX-PXR. It should also be noted that the native CTG initiation codon of the human PXR T1 mRNA transcript was replaced by the more conventional ATG initiation codon through primer mismatch in hPXRT1for.
CHAPTER THREE

IN VITRO CHARACTERISATION OF THE UGT1A3, UGT1A4 AND UGT1A5 PROXIMAL PROMOTERS

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3.1 Introduction

3.1.1. The UGT1A3, UGT1A4 and UGT1A5 gene cluster

UGT1A3, UGT1A4 and UGT1A5 are a triad of highly related UGT proteins encoded by the human UGT1A locus (see Chapter 1, Figure 1.3). They share greater than 90% identity in their primary amino acid sequences and more than 85% nucleotide sequence identity in their 1 kb proximal promoters (Green and Tephly, 1998; Gong et al., 2001). However, despite these similarities, they vary considerably in their substrate selectivities and in their expression patterns. Furthermore, all three genes are subject to considerable interindividual variation in expression (Strassburg et al., 1998b; Strassburg et al., 2000; Finel et al., 2005). This chapter describes a series of experiments designed to investigate the function of the UGT1A3, UGT1A4 and UGT1A5 proximal promoters in vitro; a first step in understanding the mechanisms that: a) allow these highly related genes to be independently regulated; and b) lead to differential expression of these UGTs between individuals.
3.1.2. Expression of UGT1A3, UGT1A4 and UGT1A5

UGT1A3 mRNA transcripts have been found in liver, biliary tissue, kidney, stomach, small intestine, colon, prostate, testis and breast. Interestingly, the stomach, duodenum, jejunum and ileum only express detectable levels of UGT1A3 transcripts in a subset of individuals (Mojarrabi et al., 1996; Strassburg et al., 1997b; Mojarrabi and Mackenzie, 1998; Strassburg et al., 1998b; Strassburg et al., 2000; Sabolovic et al., 2004; Chouinard et al., 2006). UGT1A4 mRNA has a similar expression pattern to UGT1A3, being found in liver, biliary tissue, breast and colon, and also having polymorphic expression along the small intestine (Strassburg et al., 1997b; Strassburg et al., 1998a; Strassburg et al., 2000; Chouinard et al., 2006). However, this enzyme is notably absent in the stomach (Strassburg et al., 1998b). Although there is weak evidence that UGT1A4 is more highly expressed in the liver and breast than UGT1A3 (Ritter et al., 1992b; Congiu et al., 2002; Chouinard et al., 2006), both proteins are readily detectable in liver extracts by enzyme-specific antibodies (Ikushiro et al., 2006). In contrast, UGT1A5 has not been found to be expressed to any significant extent in any tissues, although highly variable (but very low) expression in liver and gastrointestinal tract has been recently reported (Chen et al., 2005a; Finel et al., 2005).

As well as differing at the level of basal expression, UGT1A3 and UGT1A4 are differentially induced by various stimuli. Human UGT1A4, but not UGT1A3, has been found to have increased expression during pregnancy in Tg-UGT1 transgenic mice (Chen et al., 2005a). On the other hand, UGT1A3 expression is known to be increased by the nuclear receptor PXR in the presence of ligand to a much greater extent than UGT1A4 in HepG2 cells (Gardner-Stephen et al., 2004). UGT1A5 may also be a PXR-target gene, as rifampicin treated hepatocytes possess increased levels
of UGT1A5 mRNA (Finel et al., 2005). Most recently, UGT1A3 has been found to be responsive to the liganded liver X receptor (LXR)α in HepG2 cells and the liver of transgenic Tg-UGT1 mice, although it was not reported whether UGT1A4 is also regulated by LXRα (Verreault et al., 2006). Remarkably, despite their common sequences, the only regulators identified in common to UGT1A3 and UGT1A4 so far are HNF1 (this chapter, published by Gardner-Stephen and Mackenzie (2007b)), PXR (Rae et al., 2001; Gardner-Stephen et al., 2004) and PPARα (Senekeo-Effenberger et al., 2007).

3.1.3. Substrates of UGT1A3, UGT1A4 and UGT1A5

UGT1A3 and UGT1A4 are two UGTs involved in the catalysis of quaternary ammonium-linked glucuronides from tertiary amines, a substance class that includes many important pharmaceuticals and other bioactive molecules. Examples of substrates metabolised by both enzymes include ketotifen (an anti-allergic) (Breyer-Pfaff et al., 2000) and amitriptyline (an anti-depressant) (Green et al., 1998a; Breyer-Pfaff et al., 2000). Further substrates known to be common to UGT1A3 and UGT1A4 are predominantly primary and secondary amines, such as the muscle relaxant afloqualone (Kaji and Kume, 2005) and the carcinogen benzidine (Green and Tephly, 1996; Green et al., 1998a). Generally, amines are better substrates for UGT1A4 than UGT1A3 (Green and Tephly, 1996; Green et al., 1998a).

Substrates for UGT1A3, but not UGT1A4, include the cholesterol absorption inhibitor ezetimibe (Ghosal et al., 2004a), the pesticide methoxychlor (Hazai et al., 2004), oripavine opioids, coumarins such as 4-methylumbelliferone and scopoletin, the flavonoid quercetin, oestrogens, bile acids and carboxylic acid-containing pharmaceuticals such as the anti-inflammatory ketoprofen and ibuprofen (Green et
al., 1998a; Gall et al., 1999). In general, other UGTs also metabolise these chemicals and often, more efficiently (Tukey and Strassburg, 2000; Ghosal et al., 2004a; Hazai et al., 2004). However, UGT1A3 activity towards these shared substrates may be important under certain conditions. For example, UGT1A3-mediated glucuronidation of β-oestradiol may be important in individuals that have low UGT1A1 expression (Smith et al., 2005a). Furthermore, it is becoming increasing clear that UGT1A3 is an important biotransformation enzyme in its own right. UGT1A3 was recently reported to be the only enzyme catalyst of 26,26,26,27,27,27-F6-1α,23S,25-trihydroxyvitamin D3 glucuronidation in the treatment of hyperparathyroidism (Kasai et al., 2005); a major contributor to the metabolism of the anti-oestrogenic drug fulvestrant (Chouinard et al., 2006); and a major UGT form responsible for the glucuronidation of two bile acids, chenodeoxycholic acid (CDCA) (Trottier et al., 2006b) and lithocholic acid (Gall et al., 1999; Verreault et al., 2006; Senekoe-Effenberger et al., 2007). In particular, it has been suggested that UGT1A3 is an important regulator of bile acids in humans, and that drugs that increase the expression of this enzyme, such as fibrates, may be effective treatments for cholestasis (Trottier et al., 2006b; Senekoe-Effenberger et al., 2007).

One clinically relevant substrate for UGT1A4 is the anti-fungal agent posaconazole (Ghosal et al., 2004b). In addition, UGT1A4 is a catalyst for the N’-glucuronidation of trifluoperazine (an anti-psychotic) (Green and Tephly, 1996), imipramine (an anti-depressant) (Nakajima et al., 2002), tamoxifen (an oestrogen receptor antagonist) (Kaku et al., 2004), lamotrigine (an anti-epileptic) (Green et al., 1995), nicotine and cotinine (both stimulants) (Kuehl and Murphy, 2003) and NNAL (a nicotine-derived carcinogen) (Wiener et al., 2004). Some of these substrates, such as lamotrigine, tamoxifen, cotinine and nicotine, also undergo glucuronidation at functional groups
other than tertiary amines (with or without prior oxidation) or have an ammonium-linked glucuronidation component catalysed by other UGTs such as UGT1A9, UGT2B7 or UGT2B10 (Nishiyama et al., 2002; Nakajima and Yokoi, 2005; Rowland et al., 2006; Kaivosaari et al., 2007). Nonetheless, UGT1A4 has been postulated to be important in the elimination of each of the listed substrates.

In contrast to UGT1A3, but similarly to UGT1A4, UGT1A5 exhibits very low rates of 4-methylumbelliferone and scopoletin glucuronidation (Finel et al., 2005). In addition, UGT1A5 does not glucuronidate 4-aminobiphenyl, a good substrate for the highly homologous UGT1A4 and also a substrate of UGT1A3 (Green et al., 1998a; Finel et al., 2005). However, 1-hydroxypyrene, another substrate common to UGT1A3 and UGT1A4 (Luukkanen et al., 2005) is glucuronidated by UGT1A5 (Finel et al., 2005). Thus, if individuals or circumstances exist where UGT1A5 is expressed to any significant extent in any human tissues, it is expected that this enzyme will affect the glucuronidation of its own specific subset of substrates. Whether these individuals or circumstances actually exist is still unknown.

3.1.4. Regulatory controls of UGT1A3, UGT1A4 and UGT1A5

Although they may have important ramifications for drug, carcinogen and bile acid metabolism in humans, the factors that determine the expression levels and/or tissue specificity of the UGT1A3-1A5 cluster are currently not well understood. At the beginning of this PhD candidature, only PXR had been identified as a regulator of UGT1A3 and UGT1A4 (Rae et al., 2001; Gardner-Stephen et al., 2004), and little information regarding the molecular function of any of the UGT1A3, UGT1A4 or UGT1A5 promoters was available. The UGT1A4 TSS was identified by Ritter and colleagues as nucleotide -44C, relative to the initiation codon, and the TAATTA
sequence present at nucleotides -75 to -69 predicted to be a TATA box (Ritter et al., 1992b). The only other published observation regarding the regulation of any of these genes was that an element with high homology to the consensus HNF1-binding site, as defined by Tronche and Yaniv (1992), was present in the UGT1A4 proximal promoter (Tronche et al., 1997). This putative HNF1-binding element had not been tested for functional significance. However, the expression pattern of the UGT1A and UGT2B enzymes overlaps considerably with that of the HNF1 homeoproteins; the liver, and intestine are both important sites of glucuronidation, and both contain significant levels of HNF1. The kidneys and other regions of the gastrointestinal tract, such as the stomach, are further examples of tissues that contain at least one of the HNF1 isoforms and also express a subset of UGTs. It therefore seemed feasible that HNF1 proteins could play an important role in regulating the expression of UGT1A3 and UGT1A4.

3.1.5. **The hepatocyte nuclear factor 1 transcription factor family**

3.1.5.1. **Physical attributes of the HNF1 proteins**

The hepatocyte nuclear factor 1 transcription factor family is comprised of two closely related proteins, HNF1α and HNF1β. These transcription factors are considered to be divergent members of the POU (Pit-1, Oct-1 and Oct-2, and Unc-86) subgroup of the homeodomain protein superfamily, as both factors contain regions of homology with the POU-specific A-box but not B-box, and a large homeodomain region that contains 21 extra amino acids upstream of helix III when compared with other homeodomain proteins (Herr et al., 1988; Baumhueter et al., 1990; Rey-Campos et al., 1991). Although encoded by separate genes on separate chromosomes (Bach et al., 1991), HNF1α and HNF1β share substantial homology. Their amino-terminal dimerisation and internal DNA-binding domains have about
75% and 93% identity, respectively, in both rat and mouse (Mendel et al., 1991a; Rey-Campos et al., 1991; Tronche and Yaniv, 1992). Likewise, the same domains in the human HNF1α (Entrez Protein accession number AAJ04911) and HNF1β (AAH17714) proteins have 69% and 90% identity, respectively, as determined by alignment of their amino acid sequences with Clustal X (Thompson et al., 1997). These homologous regions allow HNF1α and HNF1β, which both bind DNA as dimers, to heterodimerise readily. In addition, homodimers and heterodimers all recognise the same inverted dyad DNA element with the consensus sequence GTTAATNATTAAC (Tronche and Yaniv, 1992; Tronche et al., 1997; Locker et al., 2002). HNF1 dimers are stabilised by the formation of tetramers containing two copies of the dimerisation co-factor of HNF1 (DCoH), also known as pterin-4a-carbinolamine dehydratase. HNF1 activity is especially dependent on DCoH when the homeoprotein concentration is low (Mendel et al., 1991b; Rhee et al., 1997).

In contrast to the highly homologous dimerisation and DNA-binding domains of HNF1α and HNF1β, the carboxyl-terminal activation domains are more divergent, with only approximately 47% identity between the two proteins. The activation domain of HNF1α is also considerably larger than that of HNF1β (Mendel et al., 1991a; Rey-Campos et al., 1991; Tronche and Yaniv, 1992), and because of this, these two proteins are not equal in their ability to transactivate genes containing HNF1-binding sites. However, there is significant overlap in their activity. Generally, of the two transcription factors, HNF1β is considered to be the weaker transactivator (De Simone et al., 1991; Mendel et al., 1991a; Rey-Campos et al., 1991; Liu and Gonzalez, 1995; Pontoglio et al., 1996; Song et al., 1998; Bernard et al., 1999; Kikuchi et al., 2006).
Due to alternative splicing, both HNF1α and HNF1β are expressed as multiple isoforms with varying activities. HNF1α has three known variants, HNF1α-A, HNF1α-B and HNF1α-C, with the latter two being 5-fold more active than the HNF1α-A isoform. However, in nearly all human tissues, and in HepG2 and Caco-2 cells, HNF1α-A is by far the most highly expressed (Bach and Yaniv, 1993). Likewise, HNF1β has three known variants, HNF1β-A, HNF1β-B and HNF1β-C, with HNF1β-A being the major mRNA species (Bach and Yaniv, 1993; Ringeisen et al., 1993). HNF1β-A has a higher transactivational potential than HNF1β-B (Ringeisen et al., 1993), while HNF1β-C is a dominant negative inhibitor of HNF1α (Bach and Yaniv, 1993).

3.1.5.2. Expression profiles of the HNF1 proteins

HNF1α and HNF1β have similar but distinct temporal and spatial expression profiles, with HNF1β expression preceding HNF1α gene activation in embryonic liver development (De Simone et al., 1991; Rey-Campos et al., 1991; Ryffel, 2001). Whereas both isoforms are found at comparable levels in the adult kidney, HNF1α is the predominant form in the liver and HNF1β is exclusively expressed in the lung. Tissues other than the kidney where both HNF1 variants are found include the intestine, stomach and pancreas (De Simone et al., 1991; Mendel et al., 1991a; Rey-Campos et al., 1991; Pontoglio et al., 1996). There is also some evidence that HNF1α expression increases in enterocytes as they differentiate from crypt to villous tip (Hu and Perlmutter, 1999). Like HNF1, expression of DCoH is tissue restricted, with considerable overlap in the localisation of the two tetramer constituents (Mendel et al., 1991b; Strandmann et al., 1998).
3.1.5.3. Gene targets of the HNF1 proteins

HNF1α has been implicated in the regulation of numerous genes in human liver, intestine, kidney and pancreas, such as glucose-6-phosphatase, albumin, cystic fibrosis transmembrane conductance regulator, α1-antitrypsin and the insulin receptor (Boj et al., 2001; Mouchel et al., 2004; Odom et al., 2004; Senkel et al., 2005; Gautier-Stein et al., 2006; Kikuchi et al., 2006). In addition, a number of human biotransformation enzymes and transporter proteins such as CYP1A2 (Chung and Bresnick, 1997), CYP7A1 (Chen et al., 1999), the class I alcohol dehydrogenase (ADH) gene locus (Su et al., 2006), UGT1A1 (Bernard et al., 1999), UGT1A8, UGT1A9 and UGT1A10 (Gregory et al., 2004), UGT2B7 (Ishii et al., 2000), UGT2B17 (Gregory et al., 2000), the organic anion transporter 3 (OAT3) (Kikuchi et al., 2006) and MRP2 (Qadri et al., 2006) have been identified as HNF1α-target genes. Likewise, HNF1β has also been shown to regulate the promoters of numerous kidney, pancreatic and foetal liver genes, including glucose-6-phosphatase, OAT3, dipeptidyl peptidase 4, angiotensin converting enzyme 2 and glucose transporter 2 (glut2) (Senkel et al., 2005; Gautier-Stein et al., 2006; Haumaitre et al., 2006; Kikuchi et al., 2006). However, of the UGT promoters shown to be responsive to HNF1α, only UGT1A1 has been shown to functionally interact with HNF1β. UGT1A8, UGT2B7 and UGT2B17 have all been specifically shown not to respond to HNF1β over-expression.

In the chromosomal setting, a single HNF1 site is insufficient to drive targeted expression of a gene. Rather, each gene is regulated by a transcription factor network that is specific both to that gene and to the cell types in which it is active. Promoters that are under the control of HNF1 generally have additional binding sites nearby for other transcription factors that participate in the overall activation of transcription,
and/or multiple HNF1 sites. Multiple HNF1 sites are particularly common among genes expressed in the liver (Frain et al., 1990; Song et al., 1998; Schrem et al., 2002; Costa et al., 2003). Other transcription factors known to interact with HNF1α in hepatocyte or enterocyte-derived cells include HNF4α (Miura and Tanaka, 1993), FoxA family members (Rouet et al., 1995; Cha et al., 2000), C/EBPα (Wu et al., 1994), Oct-1 (Ishii et al., 2000) and caudal-related homeodomain protein (Cdx)2 (Gregory et al., 2004). Furthermore, HNF1 factors are part of a complex network of liver-enriched transcription factors (LETFs) that are interdependent on each other for expression. HNF1α and HNF4α reciprocally bind the promoter of the other’s gene (Boj et al., 2001; Odom et al., 2004), targeted deletion of HNF1β in murine pancreas causes increased expression of HNF1α and decreased expression of HNF4α (Wang et al., 2004a), FoxA proteins have a weak, but positive effect on the rat and mouse hnf1α promoters (Kuo et al., 1992), foxA3 is a target gene of HNF1 (Hiemisch et al., 1997) and expression of HNF6 in the pancreas requires HNF1β (Poll et al., 2006).

The regulatory sequences upstream of the PXR gene also contain an HNF1-binding site (Uno et al., 2003).

3.1.5.4. HNF1α serves a dual purpose in gene transcription

HNF1α can influence transcription of its target genes at several levels; firstly by causing chromatin remodelling and secondly, by recruiting general transcription machinery. In the in vivo setting, HNF1α increases the accessibility of promoter elements to other transcription factors and nuclear receptors through modification of the chromatin environment. One study has shown that developmental demethylation of certain genes appears to be under the influence of HNF1α (Pontoglio et al., 1997). In addition, HNF1α is thought to induce repositioning or modification of nucleosomes through recruitment and activation of HAT proteins such as p300/CBP.
This HNF1α-mediated hyperacetylation of histones in target genes is cell-type specific. For example, while HNF1α interacts with the mouse glut2 promoter chromatin template in both liver and pancreatic islet cells, only the latter are dependent on HNF1α for hyperacetylation and transcriptional activity of the glut2 gene (Parrizas et al., 2001). Most recently, HNF1β has also been shown to interact with both the p300/CBP and P/CAF HAT factors (Barbacci et al., 2004; Hiesberger et al., 2005).

Apart from altering chromatin higher order structure, HNF1α and HNF1β can, in concert with appropriate combinations of other transcription factors and co-activators, increase the rate of transcription from promoters containing HNF1 sites. This is thought to be mediated by interaction, either directly or indirectly, with components of the general transcription machinery, providing recruitment and positioning services for the pre-initiation complex (Vorachek et al., 2000; Schrem et al., 2002). This may be particularly important in promoters that lack a TATA box but still have a well defined TSS, such as the mouse Ugt1a1 gene (Bernard et al., 1999).

Because this function can be observed in episomal DNA, in which nucleosomal organisation is considered relatively random compared to the highly organised nature of chromatin (Archer et al., 1992; Liu and Gonzalez, 1995; Smith and Hager, 1997; Soutoglou et al., 2000b; Akiyama and Gonzalez, 2003), it is often considered in isolation from the ability to direct histone acetylation. However, the two functions involve many of the same proteins, and in many cases are likely to be profoundly linked in vivo (Soutoglou et al., 2000b). HNF1α bound to sites in proximal promoters may direct the assembly of the pre-initiation complex by either interacting directly with components of the general transcription machinery such as TFIIB.
(Ktistaki and Talianidis, 1997), or through co-activator proteins that provide a bridge between the two. In addition to their intrinsic HAT activity mentioned earlier, CBP and P/CAF have well researched roles as co-activators, linking HNF1 dimers to the transcription apparatus (Dallas et al., 1997; Soutoglou et al., 2000b; Schrem et al., 2002; Dohda et al., 2004).

3.1.6. Aims
The work presented in this chapter arose from three central aims. These were to:

1. Clone the proximal promoters of the human UGT1A3-1A5 genes and investigate their relative abilities to drive reporter gene expression under basal conditions in cells of human hepatocyte and enterocyte origin;

2. Identify regions of the UGT1A3 and UGT1A4 proximal promoters important for transcriptional activity;

3. Test the functionality of the predicted UGT1A4 HNF1-binding element and of the homologous regions of UGT1A3 and UGT1A5.

3.2 Methods

3.2.1. Isolation of the UGT1A3-3.3k and UGT1A4-3.4k promoters
The proximal 3.3 and 3.4 kb of the UGT1A3 and UGT1A4 promoters, respectively, were amplified by nested PCR from NotI digested human genomic DNA. Briefly, 0.05 Units/μl PfuTurbo (see Chapter 2, section 2.2.6.3) were used to simultaneously amplify both promoters from 30 ng/μl genomic DNA using the primers 1A3/4prom-3.5k and 1A3/4rev-common (Table 3.1). Cycling conditions were: initial denaturation for 4 minutes at 95°C, 35 cycles of 95°C, 30 seconds; 50°C, 30 seconds; 72°C, 10 minutes, followed by a final extension at 72°C for 5 minutes. One tenth of
Table 3.1: Primers used for cloning the *UGT1A3-1A5* proximal promoters, and in generating deleted or mutated promoter-reporter constructs.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Nucleotide Sequence (5'→3')</th>
<th>Nucleotide Position on Target Gene</th>
<th>RE</th>
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</thead>
<tbody>
<tr>
<td>1A3/4prom-3.5k</td>
<td>GTTATCATTAATAATAATCCT</td>
<td><em>UGT1A3</em>: -3350 to -3329; <em>UGT1A4</em>: -3438 to -3417</td>
<td>NA</td>
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<td>1A3/4rev-common</td>
<td>GGCAGGGGAACCTGGAGTCCT</td>
<td><em>UGT1A3</em>: +29 to +9; <em>UGT1A4</em>: +29 to +9</td>
<td>NA</td>
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<td>1A3prom-3.3kHindIII</td>
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<td><em>UGT1A3</em>: -3315 to -3297</td>
<td>HindIII</td>
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<td>1A3UTRHindIII</td>
<td>AGCCATAAGCTTCTCAGCAGAAGACAGGACA</td>
<td><em>UGT1A3</em>: -1 to -20</td>
<td>HindIII</td>
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<td>Xhol</td>
</tr>
<tr>
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<td><em>UGT1A3</em>: -1 to -20</td>
<td>Xhol</td>
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<td>1A3/4prom-0.5k</td>
<td>AGCCATGCTAGCAAGCTTGTTAGCAATGTTGT</td>
<td><em>UGT1A3</em>: -507 to -487; <em>UGT1A4</em>: -506 to -486; <em>UGT1A5</em>: -508 to -488</td>
<td>Nhel</td>
</tr>
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<td>1A3/4prom-1.5k</td>
<td>AGCCATGCTAGCTAAGGGGGTTGGAGGAATGT</td>
<td><em>UGT1A3</em>: -1539 to -1519; <em>UGT1A4</em>: -1574 to -1554; <em>UGT1A5</em>: -1550 to -1530</td>
<td>Nhel</td>
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<td>1A3/4prom-2.5k</td>
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<td>1A3prom-165bp</td>
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<td><em>UGT1A3</em>: -165 to -148</td>
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Table 3.1 continued.

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<th>Oligonucleotide</th>
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<td>1A3prom-165s1 mut 4</td>
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<td>Nhel</td>
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<td>UGT1A3: -165 to -148</td>
<td>Nhel</td>
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<tr>
<td>1A4prom-130bp</td>
<td>AGCCATGCTAGCGGCACTTTGCTTTCCA</td>
<td>UGT1A4: -130 to -113</td>
<td>Nhel</td>
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<tr>
<td>1A4prom-150bp</td>
<td>AGCCATGCTAGCGGTAAATAGTAATGAGGA</td>
<td>UGT1A4: -150 to -130; UGT1A5: -150 to -129</td>
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<tr>
<td>1A4prom-165bp</td>
<td>AGCCATGCTAGCGATTTAGATTAATGCGGT</td>
<td>UGT1A4: -165 to -147</td>
<td>Nhel</td>
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<td>1A5rev</td>
<td>GCTCCACACAGACCTATGCTATG</td>
<td>UGT1A5: +391 to +367</td>
<td>NA</td>
</tr>
</tbody>
</table>

RE: restriction endonuclease site, as underlined. NA: not applicable. Mutations are highlighted in bold. Numbering is relative to the initiation codon of each respective gene.
the resulting PCR product was then used as template to specifically amplify each promoter in separate reactions. The primers used in the second round of amplification were 1A3prom-3.3kHindIII and 1A3UTRHindIII for the \textit{UGT1A3} promoter, or 1A4prom-3.4kNheI and 1A4UTRXhoI for \textit{UGT1A4} (Table 3.1). The amplification conditions were as for the first round PCR, but with 12 minutes extension during the cycling stage. Both promoters were cloned into pGL3-basic utilising the HindIII (\textit{UGT1A3}) or NheI/XhoI (\textit{UGT1A4}) restriction sites engineered into the second primer sets. The ends of each clone were sequenced to confirm their identity.

\textbf{3.2.2. Generation of UGT1A3 and UGT1A4 promoter deletion constructs and mutants}

The pGL3-1A3-3.3k and pGL3-1A4-3.4k vectors were used as templates to clone the required deletion fragments of each promoter. All PCRs were performed with \textit{PfuTurbo} as described in Chapter 2, section 2.2.6.3. The amplification reaction parameters for all fragments of 500 nucleotides or greater were: 95°C for 4 minutes, followed by 30 cycles of 95°C for 30 seconds, 50°C for 30 seconds and 72°C for 5 minutes, and completed with a single step of 72°C for 5 minutes. These conditions were also used for generating shorter promoter fragments (≤ 200 bp), but with a briefer extension step of one minute. All resulting PCR products were cloned into the \textit{NheI} and \textit{XhoI} sites of pGL3-basic and sequenced in full.

The antisense primer sequences for all \textit{UGT1A3} and \textit{UGT1A4} fragments were 1A3UTRXhoI and 1A4UTRXhoI, respectively. Both contain an \textit{XhoI} site, as marked by the underscored text (Table 3.1), while each of the sense primers contains a \textit{NheI} site. The sense primers 1A3/4prom-2.5k, 1A3/4prom-1.5k and 1A3/4prom-0.5k annealed to nucleotides -2541 to -2523, -1539 to -1519, and -507 to -487 of the
UGT1A3 promoter, respectively, relative to the translation start site (see Table 3.1). The three UGT1A4 promoter sub-fragments amplified with the same sense primers had lengths of 2610, 1574 and 506 nucleotides, respectively.

For the shorter UGT1A3 and UGT1A4 promoter fragments, the sense primers used annealed to nucleotides -200 to -184, -150 to -130 or -130 to -113 of their respective templates, -165 to -148 of the UGT1A3 promoter or -165 to -147 of the UGT1A4 promoter. A construct containing the proximal 180 nucleotides of the UGT1A3 promoter was also generated using the primer 1A3prom-180bp. To produce the mutated UGT1A3-150bp and UGT1A3-165bp constructs, the UGT1A3 promoter was re-amplified with primers containing the desired mutations (Table 3.1).

3.2.3. Isolation of the UGT1A5 promoter

The proximal 1.5 kb of the UGT1A5 promoter was also amplified using two sequential PfuTurbo reactions. The first round of PCR was performed on BAC 1308M2 from the human library RPCI-11 (BACPAC Resources) DNA using primers 1A5prom-2087bp and 1A5rev with the following cycling conditions: 95°C for 4 minutes, followed by 30 cycles of 95°C, 30 seconds; 65°C, 30 seconds; 72°C, 4 minutes and a final extension step of 72°C for 5 minutes. One fiftieth of the resulting products were used as template to amplify the UGT1A5-1550bp promoter using the same primers and conditions as for UGT1A4-1574bp. The 508 bp and 150 bp fragments of the UGT1A5 promoter were also amplified using the corresponding UGT1A4 primers defined above. All size fragments were ligated into the XhoI and NheI sites of pGL3-basic and sequenced in full.
3.2.4. **HNF1α and HNF1β expression vectors**

The cloning or acquisition of each HNF1 expression vector used in this chapter is detailed in Chapter 2, sections 2.1.4 and 2.3.1. The cDNAs encoded by the pCMX-HNF1α and pCMX-HNF1β expression vectors are human in origin and express the A-variants of each transcription factor. The proteins encoded by the pBJ5-HNF1α and pBJ5-HNF1β expression vectors are of murine origin and have 94% and 96% identity with their human counterparts at the amino acid level respectively.

3.2.5. **Transient transfection and luciferase reporter assay**

HepG2, Caco-2 and HEK293T cells were seeded into 24-well plates and transfected as described in Chapter 2, section 2.2.10. Each well was transfected with either 0.5 μg of empty pGL3-basic or a reporter vector carrying the indicated UGT1A3, UGT1A4 or UGT1A5 promoter sequences. For induction studies, 0.25 μg HNF1α or HNF1β expression vectors or empty pCMX vector were co-transfected with the pGL3 reporter constructs. pRL-null (0.025 μg) was added to all transfections as an internal control for transfection efficiency. After 48 hours, cells were lysed in passive lysis buffer and analysed for firefly and renilla luciferase activity using the Dual-Luciferase Reporter Assay System as detailed in Chapter 2, section 2.2.11.

3.2.6. **Electrophoretic mobility-shift assay**

HepG2 and Caco-2 nuclear extracts, prepared as described in Chapter 2, section 2.2.12 was used to perform EMSA and super-shift assays as detailed in Chapter 2, sections 2.2.13, 2.2.14 and 2.2.15. The sense sequences of the double-stranded DNA probes used are listed in Table 3.2. The anti-HNF1α antibody used in the super-shift assays was sourced from Santa Cruz Biotechnologies (sc-6547).
Table 3.2: Oligonucleotides used for EMSA and super-shift experiments.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Nucleotide Sequence (5’→3’)</th>
</tr>
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<tbody>
<tr>
<td>UGT1A3-HNF1</td>
<td>ATTAATGGTTAATAATTAACTAGAGG</td>
</tr>
<tr>
<td>UGT1A4/UGT1A5-HNF1</td>
<td>ATTAATGGTTAATAAGTAACCTGGTG</td>
</tr>
<tr>
<td>UGT1A3-HNF1mut</td>
<td>ATTAATGGCCCAACGCTTCACTAGAGG</td>
</tr>
<tr>
<td>HNF1-consensus</td>
<td>TCAGGTTAATCATTACGATCT</td>
</tr>
<tr>
<td>FXR-consensus</td>
<td>GATCTCAAGAGGTCATTGACCTTTTTG</td>
</tr>
</tbody>
</table>

Underlined text indicates the extent of the putative HNF1-binding sites in each probe and deliberate mutations are highlighted in bold. NB: Only the sense strand of each oligonucleotide pair is shown.

3.2.7. Statistical analysis

Statistical treatment of all reporter-promoter assay data was performed as described in Chapter 2, section 2.2.17 using independent-samples t-tests.

3.3 Results and discussion

3.3.1. Basal activities of the UGT1A3, UGT1A4 and UGT1A5 proximal promoters

In both liver (HepG2) and colon (Caco-2) derived cell lines, the UGT1A3 and UGT1A4-130bp promoters had minimal activity, exhibiting less than 3-fold increases over basal reporter gene expression by the promoter-less pGL3-basic vector. However, inclusion of a further 20 nucleotides of either promoter substantially increased luciferase expression in both cell types ($P < 0.001$) (Figure 3.1A and B). Further increases in promoter activity could be obtained in either cell line by inclusion of up to 500 base pairs of the UGT1A3 or UGT1A4 promoters ($P \leq 0.017$), with the exception of the UGT1A3 promoter in Caco-2 cells, which showed greatest activity at a length of 200 bp ($P = 0.001$) (Figure 3.1B). The largest increases obtained in promoter activity for UGT1A3 were 53-fold in HepG2 and 40-fold in Caco-2 cells; while the UGT1A4 promoter had maximal activities of 21 and 15 times
Figure 3.1: Successive deletion constructs of the UGT1A3 and UGT1A4 promoters reveal positive regulatory elements necessary for basal activity. A. HepG2 or B. Caco-2 cells were transfected in triplicate with 0.5 μg of pGL3 reporter vectors carrying the indicated lengths of the UGT1A3, UGT1A4 or UGT1A5 promoters and 25 ng of the promoter-less control vector pRL-Null. Forty eight hours post-transfection the cells were lysed and assayed for firefly and renilla luciferase reporter gene activities as described in “Methods”. Representative results of at least two independent experiments are presented as the mean firefly:renilla luciferase ratio relative to pGL3-basic (set arbitrarily to 1) plus one standard deviation. P values for the indicated comparisons are *$P < 0.001$ and †$P = 0.001$.

that of pGL3-basic in the same cell lines (Figure 3.1A and B). Increasing promoter length from 500 to 1500 nucleotides resulted in reduced promoter activity for both
UGT genes ($P \leq 0.01$), although this phenomenon was more marked in HepG2 cells than Caco-2. It was also found that, for all promoter lengths $\geq 200$ bp, the $UGT1A3$ gene had greater activity in vitro than $UGT1A4$ ($P \leq 0.005$) regardless of host cell type. In Caco-2 cells, the $UGT1A4$-150bp promoter also had less activity than the $UGT1A3$-150bp fragment ($P < 0.001$) (Figure 3.1B); but in HepG2 cells, the difference in the activity between these two constructs was statistically insignificant ($P = 0.069$) (Figure 3.1A).

Pair-wise comparison of the $UGT1A5$ promoter with the regulatory regions of $UGT1A3$ and $UGT1A4$ revealed that the former had the least activity for all three promoter lengths tested in HepG2 ($P \leq 0.005$) (Figure 3.1A), and for all but the $UGT1A4$ and $UGT1A5$-150 nucleotide promoter pair in Caco-2 cells, where equivalent activities were observed ($P = 0.144$) (Figure 3.1B). The $UGT1A5$ promoter also differed from $UGT1A3$ and $UGT1A4$ in that the shortest fragment tested, 150 bp, was the most active. Increasing the promoter length to 500 nucleotides decreased promoter function in both HepG2 and Caco-2 cells ($P = 0.001$ HepG2, and $P = 0.026$ Caco-2), a result in direct opposition to that obtained for $UGT1A3$ and $UGT1A4$ (Figure 3.1A and B). UGT1A5 expression has not been detected at a substantial level in any human tissue to date, and it has been suggested that this may be due to lack of a functional promoter (Tukey and Strassburg, 2001). These results support this hypothesis, and suggest that although the $UGT1A5$ core promoter is sufficient for assembly of a pre-initiation complex, one or more crucial regulatory elements between -150 and -500 bp are missing, and/or that the $UGT1A5$ promoter contains negative regulatory sequences not present in $UGT1A3$ and $UGT1A4$. 
3.3.2. HNF1 is required for basal activity of the *UGT1A3* and *UGT1A4* proximal promoters

For both *UGT1A3* and *UGT1A4*, it was found that nucleotides -130 to -150 were important for basal activity in HepG2 and Caco-2 cells. This region of the *UGT1A4* promoter has previously been predicted to contain an HNF1-binding site (Tronche *et al.*, 1997), based on the high identity (10 of 12 nucleotides) of this region with the HNF1-binding site consensus sequence. Furthermore, the equivalent region of the *UGT1A3* promoter contains a 100% match to the HNF1-binding site consensus, while the *UGT1A5* promoter is identical to *UGT1A4* over these nucleotides (Figure 3.2).

Before this present study, however, no experimental evidence had been presented to ascertain whether these putative HNF1-binding sites are functional in the context of their promoters. To investigate the influence of HNF1α on the activity of the *UGT1A3* and *UGT1A4* promoters, constructs containing 500 bp or less of each regulatory region were co-transfected with HNF1α into cells known to express HNF1 factors (HepG2 and Caco-2 cells) or a cell line devoid of HNF1 factors; namely HEK293T (Bernard *et al.*, 1999; Gardner-Stephen and Mackenzie, 2005). In HEK293T cells, it was found that *UGT1A3* and *UGT1A4* promoters of sufficient length to include the putative HNF1-binding site were highly responsive to heterologous expression of HNF1α (*P < 0.001*). Reporter gene expression under the control of the *UGT1A3* or *UGT1A4* promoters could be increased up to 22-fold over the basal expression from pGL3 (Figure 3.3A). In contrast, vectors containing only the most proximal 130 bp of the *UGT1A3* or *UGT1A4* promoters were completely unresponsive to the presence of HNF1α.
Figure 3.2: The human UGT1A3, UGT1A4 and UGT1A5 promoters contain putative HNF1-binding sites. The UGT1A3, UGT1A4 and UGT1A5 200-nucleotide proximal promoter regions are aligned, with the conserved putative HNF1-binding sites boxed in red. The initiation codons of each gene are labeled with bold italic text, the UGT1A4 transcription start site (TSS), as defined by Ritter and colleagues is marked in magenta (Ritter et al., 1992b). It has been predicted that the corresponding nucleotides of UGT1A3 and UGT1A5 are also their respective TSSs, based on sequence conservation. The sequence generally accepted to be the TATA box for these genes is bolded and underlined in green. Two UGT1A3 promoter regions found to contain positive transcriptional elements in work described in this chapter are boxed in blue. Asterisks indicate identity between all three promoters and the numbering is relative to the initiation codon of UGT1A3.

In HepG2 or Caco-2 cells, co-transfections of the UGT1A3 or UGT1A4 promoters with HNF1α resulted in no additional response, or only minor increases in luciferase expression respectively (Figure 3.3B and C). Since HepG2 and Caco-2 cells express HNF1 factors (Kuo et al., 1990; Rey-Campos et al., 1991), it seemed likely that the endogenous levels of HNF1α and/or HNF1β in these cells were sufficient to support expression of the reporter gene from the UGT1A3 and UGT1A4 promoters in vitro. Therefore, the putative HNF1-binding site in the UGT1A3-150bp promoter was mutated to abolish any binding of HNF1α. The functional result of this mutation was

<table>
<thead>
<tr>
<th></th>
<th>Site 2</th>
<th>Site 1</th>
<th>Site 3</th>
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<tbody>
<tr>
<td>A3</td>
<td>AGGTAGTATAGA</td>
<td>ATATTTTAA</td>
<td></td>
</tr>
<tr>
<td>A5</td>
<td>CAGTCTGAGTA</td>
<td>GTAGTTGTC</td>
<td></td>
</tr>
<tr>
<td>A4</td>
<td>CAGCTAGTAC</td>
<td>CTAGTTGTC</td>
<td></td>
</tr>
</tbody>
</table>

In HepG2 or Caco-2 cells, co-transfections of the UGT1A3 or UGT1A4 promoters with HNF1α resulted in no additional response, or only minor increases in luciferase expression respectively (Figure 3.3B and C). Since HepG2 and Caco-2 cells express HNF1 factors (Kuo et al., 1990; Rey-Campos et al., 1991), it seemed likely that the endogenous levels of HNF1α and/or HNF1β in these cells were sufficient to support expression of the reporter gene from the UGT1A3 and UGT1A4 promoters in vitro. Therefore, the putative HNF1-binding site in the UGT1A3-150bp promoter was mutated to abolish any binding of HNF1α. The functional result of this mutation was
Figure 3.3: The HNF1-binding site is required for maximal basal activity of the UGT1A3 and UGT1A4 proximal promoters. The HNF1-binding site of the UGT1A3-150bp promoter construct was mutated as described in section 3.2.2. A. HEK293T, B. HepG2 or C. Caco-2 cells were co-transfected with 0.5 μg pGL3-based vectors containing 500, 200, 150 or 130 nucleotides of the UGT1A3 or UGT1A4 promoters or 500 or 150 nucleotides of the UGT1A5 promoter, 25 ng pRL-Null and 0.25 μg of pCMX-HNF1α expression vector. The DNA concentration in control transfections was kept constant by addition of empty pCMX vector as necessary. The results of all experiments are the means of triplicate samples, expressed as a relative value of firefly luciferase activity to the internal renilla control, compared to the pGL3-basic control (set to 1). The error bars indicate one standard deviation. ND: Not done. P values for the indicated comparisons are *P < 0.001 and #P > 0.05 (not significant).
a loss of basal activity of the *UGT1A3*-150 bp promoter in both HepG2 and Caco-2 cells \((P < 0.001)\), and prevention of HNF1α-responsiveness in HEK293T cells \((P = 0.123)\). In all cases, the mutated *UGT1A3*-150bp promoter construct behaved in the same manner as the *UGT1A3*-130bp promoter that contains no recognised HNF1α-binding site (Figure 3.3). In support of the above evidence that the *UGT1A3* and *UGT1A4* promoter HNF1 sites are functional, binding of HNF1 factors from HepG2 and Caco-2 nuclear extracts to these sequences could be demonstrated by EMSA (Figures 3.4 and 3.5). Furthermore, the mutation used to abolish HNF1α-responsiveness of the *UGT1A3*-150bp promoter also prevented binding of HNF1 factors to this region *in vitro* (Figures 3.4 and 3.5), while neither the mutated nor unrelated (FXR) probes could interfere with HNF1 binding when added in 500-fold excess (Figure 3.4). Subsequently, Caillier *et al.* (2007) also confirmed that the putative *UGT1A3* HNF1-binding site interacts with HNF1α *in vitro* and is important for *UGT1A3* promoter function.

The *UGT1A5* promoter contains the same putative HNF1-binding site as *UGT1A4*. Therefore, this promoter was also tested for responsiveness to over-expressed HNF1α. The activity of the *UGT1A5*-500bp promoter was increased by HNF1α over-expression in all three cell lines tested \((P < 0.001)\), with the greatest response being observed in HEK293T cells (15.8-fold increase relative to pGL3-basic). Interestingly, even though the basal reporter gene expression from the pGL3-1A5-500 construct was only one quarter that of the equivalent *UGT1A4* vector in HepG2 cells, the overall activity of the *UGT1A5*-500bp promoter in the presence of excess HNF1α was 81% of that achieved for *UGT1A4* (Figure 3.3). The same phenomenon was observed in Caco-2 cells, although the initial contrast between the basal expression levels was not as severe.
Figure 3.4: HNF1α from HepG2 nuclear extracts binds to nucleotides -156 to -128 of the UGT1A3 and UGT1A4/UGT1A5 promoters. Electrophoretic mobility-shift assays were performed using 50,000 cpm of 32P end-labelled oligonucleotide probes encompassing the putative UGT1A3 or UGT1A4/UGT1A5 HNF1 sites. A. HNF1 complexes were super-shifted with 2 μg of HNF1α-specific antibody, or B. competed with between 10 and 500-fold excess cold probe. The positions of free probe and complexes containing HNF1α are indicated by parentheses. HNF1: consensus HNF1-binding sequence probe; WT: UGT1A3 or UGT1A4/IA5 wild-type probe; Mut: UGT1A3 mutant probe; FXR: consensus FXR-binding sequence probe.
Figure 3.5: HNF1α from Caco-2 nuclear extracts binds to nucleotides -156 to -128 of the UGT1A3 and UGT1A4/UGT1A5 promoters. Electrophoretic mobility-shift assays were performed using 50,000 cpm of 32P end-labelled oligonucleotide probes encompassing the putative UGT1A3 or UGT1A4/UGT1A5 HNF1 sites. HNF1 complexes were super-shifted with 2 μg of HNF1α-specific antibody. The positions of free probe and complexes containing HNF1α are indicated by parentheses. WT: UGT1A3 or UGT1A4/1A5 wild-type probe; Mut: UGT1A3 mutant probe.
The pGL3-1A5-150 reporter vector was also found to be responsive to HNF1α in HepG2 and HEK293T cells ($P < 0.001$), suggesting that it is the same HNF1-binding element driving the $UGT1A5$ response as for $UGT1A3$ and $UGT1A4$ (Figure 3.3). The presence of a homologous, functional HNF1-binding site in the $UGT1A5$ promoter indicates that, although the identified HNF1-binding elements are necessary for much of the $UGT1A3$ and $UGT1A4$ promoter activities in vitro, they are also insufficient to sustain maximal promoter activity. Since the $UGT1A4$ and $UGT1A5$ promoters possess identical HNF1-binding elements and TATA boxes, yet differ in their basal activity, it appears that one or more transcription factors other than HNF1 are also required to promote initiation of transcription. Given that $UGT1A5$ is not substantially expressed in liver while $UGT1A4$ is, this observation is consistent with the currently available information regarding transcriptional control of hepatic genes (see section 3.1.5.3). Interestingly, excess HNF1α overcomes much of the difference between the activity of the $UGT1A4$ and $UGT1A5$ promoters in all cell types tested, suggesting that this second factor may normally play a role in recruiting or activating HNF1 factors on the $UGT1A4$ promoter and/or be under the expressional control of HNF1α.

3.3.3. **HNF1β can transactivate the $UGT1A3$ promoter in vitro**

Since HNF1β can recognise the same nucleotide elements as HNF1α, but regulates an overlapping, yet separate set of genes, it was investigated whether this factor could also transactivate the $UGT1A3$ promoter. HNF1β activates the $UGT1A1$ promoter to the same extent as HNF1α (Bernard et al., 1999), but does not activate $UGT1A8$, $UGT2B7$ or $UGT2B17$, all known to be HNF1α target genes (Gregory et al., 2000; Ishii et al., 2000; Gregory et al., 2004). Since the published $UGT1A8$, $UGT2B7$ and $UGT2B17$ work, which was performed in our laboratory, was done...
with pBJ5 vectors expressing the mouse HNF1 homologues, these vectors were also 
used in this experiment so that the results would be directly comparable.

Murine HNF1α increased the expression of luciferase from the UGT1A3-500bp 
construct in HEK293T cells to 14-fold over the basal activity of the pGL3-basic 
control ($P < 0.001$) (Figure 3.6), a similar result as that obtained with human pCMX-
HNF1α expression vector (Figure 3.3). Likewise, HNF1β activated the UGT1A3-
500bp promoter in HEK293T cells ($P < 0.001$); however, the 9-fold increase in 
reporter gene expression produced by HNF1β over-expression was significantly less 
than that for HNF1α ($P < 0.001$) (Figure 3.6). These results are consistent with the 
observation that HNF1β is frequently a weaker transactivator than HNF1α when 
these proteins activate a common target (see section 3.1.5.1). Interestingly, the 
regulation of the UGT1A3 promoter by HNF1 factors is more similar to the UGT1A1 
gene than UGT1A8, UGT2B7 or UGT2B17 in that HNF1α and HNF1β can both 
function as positive transcriptional regulators for the UGT1A3 promoter and their 
respective HNF1 sites are essential for transcriptional activity in vitro.

### 3.3.4. The UGT1A3 and UGT1A4 proximal promoters differ in their HNF1 
responses

During the course of this study, two notable differences between the UGT1A3 and 
UGT1A4 HNF1 responses were observed. Firstly, whereas none of the UGT1A3 
promoter constructs exhibited any activity in HEK293T cells in the absence of 
HNF1α, UGT1A4 promoters of 150 bp or longer could support a small degree of 
basal activity ($P < 0.001$). This activity, which was 2 to 3-fold greater than the empty 
vector control, is presumably HNF1-independent and was also observed for the 
UGT1A5 promoter ($P < 0.001$) (Figure 3.3A). The second observation was that,
Figure 3.6: Activation of the UGT1A3-500bp promoter by HNF1β in HEK293T cells. HEK293T cells were transfected with 0.5 µg pGL3-basic or pGL3-1A3-500, and 0.25 µg pCMX, pBJ5-HNF1α or pBJ5-HNF1β as described in section 3.2.5. In addition, each transfection contained 25 ng of pRL-Null to serve as an internal control. The means of triplicate samples are expressed as a relative value of firefly luciferase activity to the internal \textit{renilla} control, compared to the pGL3-basic control (set to 1). The error bars indicate one standard deviation. \( P \) value for the indicated comparison is *\( P < 0.001 \).

whereas UGT1A3 promoter activity could not be increased in HepG2 cells by over-expression of HNF1α (\( P \geq 0.157 \)), UGT1A4 promoter activity was increased up to 2.3-fold by excess HNF1α for promoter fragments \( \geq 150 \) bp (\( P \leq 0.005 \)) (Figure 3.3B). One possible explanation is that the perfect UGT1A3 HNF1-binding element is fully occupied at physiological HNF1 concentrations, whereas the slightly flawed site of the UGT1A4 promoter is less efficient at competing with the multitude of genomic sites for limited HNF1. Therefore, addition of excess HNF1α into the system can only increase the occupancy rate of the UGT1A4 HNF1-binding site.

There is also likely a cell type-specific component to this second difference between the promoters, as it was only observed in cells of hepatic origin (Figure 3.3B).

To test whether the sequence differences between the UGT1A3 and UGT1A4 HNF1-binding elements were responsible for their different HNF1α-responses in HepG2
cells, the HNF1-binding site of the UGT1A3 promoter was mutated to the corresponding UGT1A4 sequence. When the mutated UGT1A3-150bp HNF1Δ4 construct was tested, it was found that it had 18% less basal activity than the UGT1A3 wild-type promoter ($P = 0.032$). However, in the presence of HNF1α over-expression it had the same activity as the wild-type UGT1A3-150bp promoter ($P = 0.861$), which is significantly less than that of the UGT1A4-150bp promoter under the same conditions ($P = 0.029$) (Figure 3.7). Thus, reducing the UGT1A3 HNF1-binding site’s similarity to the consensus sequence adversely affected the basal activity of the promoter, yet this loss of activity could be compensated for by a high HNF1α concentration. These results support the hypothesis that the UGT1A4 HNF1-binding site is less efficient than the corresponding UGT1A3 sequence at competing for limited HNF1 factors, and that a high HNF1α concentration increases promoter activity by increasing occupancy of the HNF1-binding element. However, the introduction of the UGT1A4 HNF1-binding site into the UGT1A3-150bp promoter did not cause its behaviour to become completely UGT1A4-like: the reporter activity emanating from the HNF1α-induced UGT1A3-150bp HNF1Δ4 construct was still significantly less than the similarly treated UGT1A4-150bp promoter. One feasible explanation for this is that HNF1 factors contribute less to the basal activity of the UGT1A4-150bp promoter than the UGT1A3-150bp promoter in HepG2 cells, but that a second stimulatory transcription factor bound downstream of the HNF1-binding site, on the UGT1A4 promoter only, causes the two promoters to have the same activity in these cells ($P = 0.069$) (Figure 3.1A). When the HNF1 concentration is increased, HNF1 binding to the UGT1A4 promoter increases, which in combination with the putative second factor, allows the UGT1A4 promoter activity to exceed that of UGT1A3. If this second putative factor is expressed in HepG2 cells but not Caco-2
cells, this hypothesis could also help explain why the difference between the basal activities of the *UGT1A3*-150bp and *UGT1A4*-150bp promoter constructs is much greater in the latter (Figures 3.1 and 3.3), and the cell-type specificity of the *UGT1A4* HNF1α-response noted earlier (Figure 3.3B).

**Figure 3.7:** Mutation of the *UGT1A3* HNF1-binding site to the equivalent *UGT1A4* sequence does not confer HNF1-responsiveness in HepG2 cells. The HNF1-binding site of the *UGT1A3* promoter was mutated to the corresponding *UGT1A4* sequence and tested for HNF1α-responsiveness in HepG2 cells. 0.5 μg of each reporter vector was co-transfected with 0.25 μg pCMX or pCMX-HNF1α and 25 ng of pRL-Null. Results are expressed as the mean (n = 3) relative value of firefly luciferase activity to the internal *renilla* control, compared to pGL3-basic (set arbitrarily to 1). The error bars indicate one standard deviation. *P* values for the indicated comparisons are ‡*P* = 0.029, ‡‡*P* = 0.032 and #*P* > 0.05 (not significant).

Therefore, it was concluded that the nucleotide differences of the *UGT1A3* and *UGT1A4* HNF1-binding site sequences are functionally significant, at least under the conditions tested *in vitro*. However, the differing behaviour of these promoters in the presence of excess HNF1α is also likely to be a consequence of further nucleotide discrepancies downstream of their HNF1-binding sites, and is more complex than
originally hypothesised. Further experiments will be required to elucidate the differences between the *UGTIA3* and *UGTIA4* promoters that cause their divergent behaviour in liver-derived cells.

When pGL3-1A3-165 and pGL3-1A3-180 reporter constructs were constructed and tested in HepG2 cells, two separate elements were found to be important for maximal basal activity. The proximal 165 bases of the *UGTIA3* promoter were more active than the *UGTIA3*-150bp promoter (*P* = 0.002), revealing the first positive element (site 1); and the *UGTIA3*-180bp promoter had the same activity as the pGL3-1A3-165 construct (*P* = 0.802), which is less than that for *UGTIA3*-200bp (*P* = 0.001), indicating that a second positive element (site 2) either resides between nucleotides -180 to -200 or overlaps the -180bp junction point (Figure 3.8).

To search the newly defined, functionally important regions of the *UGTIA3* promoter for known transcription factor binding sites, publicly available MatInspector v6.0 software (Cartharius *et al*., 2005; http://www.genomatrix.de) was used. The results indicated that, according to current knowledge, the guanine-rich region between nucleotides -187 and -180 constitutes the only likely transcription factor binding site that could account for the difference in activity between the *UGTIA3*-180bp and *UGTIA3*-200bp promoters. Candidate binding proteins were identified as Sp1/Sp3, Kruppel-like factors and MYC-associated zinc finger protein related transcription factor. No attempts have yet been made to confirm the importance of this guanine tract for *UGTIA3* activity, or to identify the transcription factors that bind to site 2.
Figure 3.8: The UGT1A3-200 to -150bp region contains at least two regulatory elements that can positively influence transcription in HepG2 cells. Firefly luciferase reporter constructs carrying the proximal 165 or 180 nucleotides of the UGT1A3 promoter were constructed and transfected into HepG2 cells as described in “Methods” to assess their basal activity. The means of triplicate samples are expressed as a relative value of firefly luciferase activity to the internal renilla control, compared to the pGL3-basic control (set to 1). The error bars represent one standard deviation. P values for the indicated comparisons are †P = 0.001, ††P = 0.002 and #P > 0.05 (not significant).

Similar analysis of the UGT1A3 promoter nucleotides -165 to -150 indicated a number of transcription factors that may bind site 1, the majority of which rely on the AT-rich region between nucleotides -156 and -151 for their high “core similarity” scores. To determine whether any of these putative transcription factors may be important contributors to UGT1A3 promoter activity, the AT-rich region was interrupted by mutating nucleotides -155 to -153 to the sequence ‘CGC’ (pGL3-1A3-165 s1mut CGC). However, when tested, the mutated promoter construct had greater activity than the wild-type sequence (P < 0.001) (Figure 3.9A). Since a decrease in activity was anticipated if these nucleotides formed the core of a biologically
Figure 3.9: Characterisation of the UGT1A3 promoter site 1 regulatory element. Reporter constructs containing A. mutated UGT1A3-165bp promoters or B. the wild-type UGT1A4-165bp promoter were constructed as described in section 3.2.2 and compared to existing constructs. HepG2 cells were transfected in triplicate with 0.5 μg of the indicated pGL3-derived reporter vectors and 25 ng of the promoter-less control vector pRL-Null. Forty eight hours post-transfection the cells were lysed and assayed for firefly and renilla luciferase reporter gene activities as described in “Methods”. Results are presented as the mean firefly:renilla luciferase ratio, relative to pGL3-basic (set to 1) plus one standard deviation. The experiment presented in panel A was only performed once. *P < 0.001 and #P > 0.05 (not significant).
relevant transcription factor binding site, it was deemed unlikely that any of the factors predicted to bind the AT-rich region were contributing to UGT1A3 activity. The only remaining transcription factor element predicted in this region with a core similarity of greater than 0.870 was a binding site for Pbx/Hox heterodimers. This site had a core similarity of 1.000 for nucleotide positions -162 to -159 against the Pbx/Hox matrix. This putative element is not conserved in the UGT1A4 promoter, as it encompasses the only nucleotide between positions -165 and -150 that is not identical between the UGT1A3 and UGT1A4 promoters (nucleotide -161). Since the UGT1A4 promoter does not change significantly in activity when its length is extended from 150 to 165 base pairs ($P = 0.063$), in contrast to UGT1A3 ($P = 0.001$) (Figure 3.9B), it was hypothesised that the identity of this nucleotide may be fundamental for site 1 function. Therefore, this base was mutated in the UGT1A3-165bp construct to match the UGT1A4 sequence (pGL3-1A3-165 s1mut Δ4) and was tested in HepG2 cells. Interestingly, instead of abolishing the fraction of UGT1A3 promoter activity attributable to the 15 nucleotides immediately upstream of the characterised HNF1-binding site, this nucleotide exchange substantially enhanced the activity of the UGT1A3-165bp promoter ($P < 0.001$) (Figure 3.9B). Therefore, it was concluded that Pbx-Hox factors are unlikely to be the transcription factors causing the 165-nucleotide UGT1A3 promoter to be more active than the 150 bp fragment.

The ability of the UGT1A4 -165 to -150 nucleotide region to drive the UGT1A3 promoter but not the UGT1A4 promoter is interesting. Given that there is only one nucleotide difference in this region, and that both sequences can activate the UGT1A3 proximal promoter, it seems highly likely that the same factors can bind the UGT1A3 and UGT1A4 sequences at this position. If this is true, this would mean that
the apparent functional difference that exists between the UGT1A3-165bp and UGT1A4-165bp promoters is actually due to elements downstream of the characterised HNF1-binding site. This result could be explained either by: a) factors bound downstream co-operating with the protein(s) bound to the -165 to -150 bp region in the case of UGT1A3; or b) factors bound downstream blocking access to the transcriptional machinery or otherwise inhibiting the factor(s) bound to the -165 to -150 bp region in the case of UGT1A4. These two mechanisms are not mutually exclusive. Data presented earlier (Figures 3.1 and 3.3) supports the notion that there are important functional differences in the proximal 150 nucleotides of the UGT1A3 and UGT1A4 promoters. The pGL3-1A4-150 reporter vector only has approximately half the activity of the corresponding UGT1A3 construct in Caco-2 cells; the UGT1A4-150bp vector is HNF1α-responsive in HepG2 cells, although the UGT1A3-150bp construct is not; while the UGT1A4-150bp promoter is the shortest vector to display the UGT1A4-specific, HNF1α-independent promoter activity noted in HEK293T.

3.3.5. Factors bound to at least two elements in the UGT1A3 and UGT1A4 promoters co-operate with HNF1α

In HEK293T cells, both the UGT1A3-200bp and UGT1A4-200bp promoters had greater HNF1α-responses than their 150 bp length counterparts (P < 0.001) (Figure 3.3). Therefore, it was hypothesised that there was a common element in the UGT1A3 and UGT1A4 promoters, within the -200 to -150 nucleotide regions that either binds a second HNF1 dimer, or a transcription factor that co-operates with the HNF1α bound to the previously characterised site. To narrow down the location of this putative element, reporter vectors carrying the UGT1A3-165bp and UGT1A4-
165bp promoters were tested for their activity in HNF1α-co-transfected HEK293T cells.

Interestingly, the results of this experiment showed that, for both genes, two separable elements were required to support the difference in activity between the HNF1α-induced 200 bp and 150 bp promoters (Figure 3.10). In both cases, the 165 bp proximal promoters had greater activities than the 150 nucleotide fragments in the presence of over-expressed HNF1α ($P \leq 0.001$), but less than the similarly treated 200 bp promoters ($P \leq 0.001$). Fold-activities over basal also increased as the promoters were extended. This behaviour closely resembles that of the UGT1A3 promoter in HepG2 cells, suggesting that the same elements may be involved in both systems and that the factors that bind and activate the UGT1A3 promoter through the two functional regions defined in HepG2 cells may be widely expressed rather than specifically liver-enriched. Whether the factors bound to the -200 to -180 and -165 to -150 bp regions of the UGT1A3 promoter also co-operate with HNF1α to drive UGT1A3 transcription in HepG2 cells remains to be tested.

The involvement of the site 1 region of both promoters in the HNF1α-response in HEK293T cells is noteworthy, since in HepG2 cells only the UGT1A3 site has observable function. Given that only one nucleotide substitution separates the two sequences at site 1, and the UGT1A3-165bp and UGT1A4-165bp promoters behave similarly, it would be reasonable to assume that, at least in HEK293T cells, the factors that bind the UGT1A3 and UGT1A4 site 1 regions are the same. If these factors are widely expressed and also bind this region in HepG2 cells, then the hypothesis that the activation of the UGT1A4-165bp promoter in HepG2 cells is specifically repressed by a tissue-restricted factor recruited by downstream
Figure 3.10: The UGT1A3 and UGT1A4 -200 to -150bp regions contain at least two regulatory elements that can positively influence their HNF1α-response in HEK293T cells. The HNF1α-responses of the UGT1A3-165bp and UGT1A4-165bp promoters were compared to the respective 200 and 165 nucleotide length promoters in HEK293T cells by co-transfecting triplicate cultures with 0.5 μg of each promoter reporter vector, 25 ng pRL-Null and 0.25 μg of either pCMX or pCMX-HNF1α. The results are expressed as a relative value of firefly luciferase activity to the internal renilla control, compared to the pGL3-basic control (set to 1). The error bars indicate one standard deviation. Transfection of HEK293T cells with the pGL3-1A3-165 and pGL3-1A4-165 constructs was only performed once. $P$ values for the comparisons indicated are *$P < 0.001$ and †$P = 0.001$.

nucleotides (see section 3.3.4) becomes the most probable. Considerable further work will be required to identify the factor(s) that interact with this 15 nucleotide fragment in each cell type and to elucidate the mechanism that prevents the UGT1A4 promoter from being activated in HepG2 cells. Further work is also required to characterise the second element that participates in the HNF1α-response of these promoters and to determine whether it is the same element as site 2 of the UGT1A3 promoter, as defined in HepG2 cells.
3.4 General discussion and summary

3.4.1. Achievement of aims

This chapter describes the cloning of the human UGT1A3 and UGT1A4 promoters to approximately 3.4 kilobases and the UGT1A5 promoter to 1.5 kb. Deletion, mutation and HNF1α/β-over-expression analyses established that the putative HNF1-binding sites of all three promoters are functional in vitro; while comparative studies revealed that although HNF1 factors are critical for UGT1A3 and UGT1A4 promoter activity they are also insufficient to drive high levels of transcription.

Further investigations into the function of the UGT1A3 promoter in HepG2 cells revealed two promoter regions between nucleotides -200 and -150 that were required for maximal promoter activity. Interestingly, these two functionally active regions of the UGT1A3 promoter identified in this series of experiments are considerably more conserved between UGT1A3 and UGT1A4 (site 1: 14/15 nucleotides, 93%; site 2: 10/11 nucleotides, 91%) than the intervening region (9/14 nucleotides, 64%) (Figure 3.1). Conservation of elements between promoters is often used as an indicator for functional importance (Cartharius et al., 2005); thus, despite the apparent lack of activity of these regions in the UGT1A4 promoter in HepG2 cells, it was not surprising to find that at least one of these regions is utilised in common in an alternative cell line. Finally, the results presented strongly suggest that there is at least one element in the proximal 150 bp of the UGT1A3 and UGT1A4 promoters that causes their activity to differ in vitro.

3.4.2. Future directions

Two major studies that would add value to the presented work are highlighted here. Firstly, it would be of interest to confirm the functional importance of the two newly
identified transcriptional elements of the \textit{UGT1A3} promoter and determine the factors that bind them. The former could be achieved by mutation of the nucleotides hypothesised to be involved, followed by comparison to the wild-type sequence in promoter-reporter assays or EMSA; however, the latter task would be more complicated. Super-shift EMSA studies could be used to assess whether any of the factors predicted by MatInspector can bind site 2, but as there are no satisfactory predictions associated with site 1, a screening method with higher throughput and much less bias would also need to be employed. One feasible option would be to use the predicted binding site in a yeast one hybrid screen against HepG2 cDNA, similar to that described by Catlow \textit{et al.} (2007), to identify potential transcription factors.

Secondly, it would be worthwhile to elucidate the element(s) in the proximal 150 nucleotides of the \textit{UGT1A3} and \textit{UGT1A4} promoters that cause their differential regulation. Because the HNF1-binding site at nucleotides -137 to -149 is crucial for promoter activity, further deletions could not be used to identify potential promoter elements; however, the construction of longer \textit{UGT1A3/UGT1A4} chimeric promoters through reciprocal nucleotide substitutions or overlap PCRs could be used instead. The latter would be useful for assessing groups of nucleotides for function, as the 14 bases that the \textit{UGT1A3} and \textit{UGT1A4} promoters differ by in this region are sufficiently well spread that producing combinatorial mutations by site-directed mutagenesis would be arduous. Additional information may also be gleaned by including the \textit{UGT1A5} promoter in these analyses, as the \textit{UGT1A4} promoter shares functional similarities with both \textit{UGT1A3} and \textit{UGT1A5}, suggesting that it may represent a mixture of the elements that functionally define these highly related sequences.
3.4.3. Relevance to pharmacogenetics

Because UGT1A3 and UGT1A4 are purported to be key contributors to the metabolism of many pharmaceuticals and endogenous molecules, it is of interest to understand the environmental and genetic conditions that control their expression and activity. This study has added a significant body of information to the current understanding of the *UGT1A3* and *UGT1A4* proximal promoters that, in combination with the suggested experiments in section 3.4.2, will be useful for understanding the interindividual and tissue-specific expression profiles of these proteins. The promoter regions identified as important can now be specifically screened for allelic variants with functional consequences and, as they are identified, the factors that bind these elements can also be screened for variation in activity or expression level. Work towards these ends is presented in Chapter 6.

Because it is expected that proteins bound at distal enhancer regions of a promoter exert their effects through interaction with the transcriptional complexes formed over the core and proximal promoter regions, differences in the proximal promoters between the *UGT1A3* and *UGT1A4* genes, and between individuals in the same gene, can be expected to affect both basal and inducible gene expression. A recent study (Senekeo-Effenberger *et al.*, 2007) highlights the need to elucidate the basic functions of the *UGT1A3* and *UGT1A4* promoters before their response to liganded nuclear receptors can be fully understood. In the humanised mouse line Tg-UGT1, *UGT1A3* and *UGT1A4* were both found to be up-regulated by activated PPARα, yet the *UGT1A4* hepatic response was only a small fraction of that seen for *UGT1A3*, even though the *UGT1A4* response in the small intestine was substantial. The authors’ conclusions that PPARα receptor abundance was not the sole factor dictating the PPARα-response (Senekeo-Effenberger *et al.*, 2007) is in agreement
with the findings of this study that regulatory pathways in common to these two genes can still diverge functionally due to the influence of other promoter elements.

3.4.4. Summary

This chapter describes the cloning and in vitro analysis of the *UGT1A3*, *UGT1A4* and *UGT1A5* proximal promoters. The results obtained highlight a critical role for HNF1 factors in *UGT1A3* and *UGT1A4* promoter activity and suggest a basis for the poor expression of UGT1A5 in humans. Two additional elements required for the maximal activity of the *UGT1A3* promoter in liver-derived cells were also identified, at least one of which appears to be shared by the *UGT1A4* promoter, but is only active in the context of *UGT1A3*. Further work suggested to elucidate the cause of this phenomenon may uncover an important fundamental difference between the *UGT1A3* and *UGT1A4* promoters that, at least in part, allows these highly related genes to be independently regulated in the liver.
CHAPTER FOUR
HNF1 TRANSCRIPTION FACTORS ARE ESSENTIAL FOR THE UGT1A9 PROMOTER RESPONSE TO HNF4α


4.1 Introduction

4.1.1. The UGT1A7, UGT1A8, UGT1A9 and UGT1A10 gene cluster

The UGT1A7, UGT1A8, UGT1A9 and UGT1A10 exon 1 sequences, known as the UGT1A7-1A10 cluster, share > 70% identity (Gong et al., 2001) (see Chapter 1, Figure 1.3). In addition, the proximal promoters of UGT1A7-1A10 share over 78% homology to approximately 400 bp upstream of their initiation codons (Figure 4.1); at which point the UGT1A7 promoter sequence diverges from the remainder due to the replacement of a 28 bp segment with 305 bp of Alu-like sequence. However, the UGT1A8-1A10 promoters continue to share > 75% identity to over 1 kb upstream of their TSSs (Gregory et al., 2003). Despite these similarities and much like the UGT1A3-1A5 cluster of the same UGT1A locus (Chapter 3), each enzyme of the UGT1A7-1A10 subfamily has its own unique, but overlapping, set of substrates and a gene-specific expression profile. Of particular interest, the expression patterns of UGT1A7, UGT1A8 and UGT1A10 are all strictly extrahepatic, in stark contrast to UGT1A9, which has a strong presence in the liver (Strassburg et al., 1997b; Mojarrabi and Mackenzie, 1998). This chapter presents a study designed to further
Figure 4.1: Alignment of the -391 bp UGT1A9 proximal promoter with the corresponding regions of UGT1A7, UGT1A8 and UGT1A10. The nucleotide sequence immediately upstream of human UGT1A9 exon 1 was aligned with the equivalent regions from the UGT1A7, UGT1A8 and UGT1A10 promoters using ClustalX software (Thompson et al., 1997) and GenBank record AF297093 (Gong et al., 2001). Periods indicate identity of each sequence with UGT1A9, while nucleotide substitutions are designated by the appropriate letter and deletions/insertions are indicated by hyphens. The UGT1A9 transcription start site is highlighted in bold underlined italic text, the initiation codon is indicated by bold italics and the 5’ boundaries of the UGT1A9-321bp and UGT1A9-184bp promoter inserts are shown with arrows. Mutations introduced into the UGT1A9 promoter constructs are denoted in bold under the wild-type sequence.
the understanding of the UGT1A9 proximal promoter function *in vitro*, with particular regard to mechanisms that may contribute to the unique hepatic expression of this enzyme among its most closely related family members.

### 4.1.2. Expression and substrates of UGT1A9

Human UGT1A9 is expressed primarily in the liver and kidneys, but has also been reported in the gastrointestinal tract (oesophagus, stomach and colon) and steroid responsive tissues such as prostate, breast, ovary and testes (Strassburg *et al.* 1997b; McGurk *et al.*, 1998; Strassburg *et al.*, 1998a; Albert *et al.*, 1999; Strassburg *et al.*, 1999). Substantial interindividual variation in the hepatic expression of UGT1A9 has also been unequivocally demonstrated, with expression in the liver varying at the levels of mRNA concentration (Congiu *et al.*, 2002), protein concentration (Girard *et al.*, 2004) and protein activity (Court *et al.*, 2001; Bernard and Guillemette, 2004; Girard *et al.*, 2004). In addition, conflicting reports have been published regarding the presence of UGT1A9 mRNA in gastric tissue, suggesting that expression in this organ also varies between individuals, or that UGT1A9 expression is not uniform throughout the stomach and that the location of biopsy is important (Strassburg *et al.*, 1997b; Albert *et al.*, 1999).

UGT1A9 contributes to the glucuronidation of a large variety of chemicals. These include planar and bulky phenols (such as 4-methyl phenol and 4-t-butyl phenol respectively (Albert *et al.*, 1999)), coumarins and flavonoids (including 4-methylumbelliferone, scopoletin, 5,7-dihydroxyflavone and naringenin (Albert *et al.*, 1999)), polychlorinated dibenzo-*p*-dioxins (*e.g.* 8-OH-2,3,7-trichloro-dibenzo-*p*-dioxin (Kasai *et al.*, 2004)), mycophenolic acid (an immunosuppressive prodrug (Bernard and Guillemette, 2004)), the anaesthetic propofol (Soars *et al.*, 2004), the
analgesic paracetamol (Court et al., 2001), NSAIDs such as etodolac (Tougou et al., 2004), anticancer agents such as SN-38, flavopiridol and bropirimine (Gagne et al., 2002; Ramirez et al., 2002; Wynalda et al., 2003; Yamanaka et al., 2004), fibrate hypolipidaemics (e.g. gemfibrozil, fenofibric acid, clofibrac acid and ciprofibrate acid (Barbier et al., 2003c)), catechol oestrogens (including 4-hydroxyoestradiol and 4-hydroxyoestrone (Albert et al., 1999)), fatty acid metabolites (such as 20-carboxy-leukotriene B4 (Turgeon et al., 2003b)) and dietary/tobacco procarcinogens (N-OH-PhIP and NNAL (Ren et al., 2000; Malfatti and Felton, 2001)). As these substances are bioactive and/or toxic at the concentrations readily encountered in everyday life, genetic variations in the coding or regulatory regions of UGT1A9 that result in a less active enzyme or lower protein expression levels are hypothesised to have clinically relevant outcomes (Court et al., 2001; Congiu et al., 2002; Gagne et al., 2002; Bernard and Guillemette, 2004; Girard et al., 2004). Therefore, it is desirable to understand more fully the mechanisms that contribute to the expression of this gene in order to understand its contribution to drug toxicity and efficacy, as well as its possible relevance to issues such as cancer risk.

4.1.3. Regulatory controls of UGT1A9

Published studies addressing the function of the UGT1A9 gene and the elements that distinguish it from the extrahepatic members of the UGT1A7-1A10 cluster are still limited in number, but include several reporter-promoter experiments, two SNP analyses and a clinical study involving patients receiving chemotherapy for treatment of metastatic colorectal cancer. Among the first studies investigating the molecular function of the UGT1A9 promoter was work showing that the UGT1A8 and UGT1A10 proximal promoters are up to 8 times more active than the equivalent UGT1A9 promoter construct when tested in the Caco-2 colon-derived cell line. This
difference was found to be partly dependent on a Sp1/Inr-like region conserved between \textit{UGT1A8} and \textit{UGT1A10}, but altered in \textit{UGT1A9} at two nucleotide positions (Gregory et al., 2003). In the same year, another study also linked the liver-enriched nuclear receptor PPAR\(\alpha\) with hepatic expression of human UGT1A9. PPAR\(\alpha\) agonists moderately increase the expression of UGT1A9 mRNA transcripts in human hepatocytes and HepG2 cells, an effect mediated by a PPAR\(\alpha\)-responsive element located at nucleotides -719 to -706 of the \textit{UGT1A9} promoter (Barbier et al., 2003c). Subsequently, evidence that the \textit{UGT1A8}, \textit{UGT1A9} and \textit{UGT1A10} genes share two promoter elements that may co-ordinately regulate the expression of each in the gastrointestinal tract was published. In each case, Cdx2 up-regulated their respective proximal promoters in transient assays, but only in the presence of a weak but functional HNF1 binding element (Gregory et al., 2004). This HNF1 site, which was able to facilitate a 2 to 3-fold up-regulation of the \textit{UGT1A8-1A10} genes in the presence of over-expressed murine HNF1\(\alpha\), but not murine HNF1\(\beta\), is also present in the promoter of the \textit{UGT1A7} gene (Gardner-Stephen and Mackenzie, 2005).

Another \textit{UGT1A9} promoter-reporter study published in 2004 reported that the length of the poly-deoxythymidine tract that starts at nucleotide -118 of the \textit{UGT1A9} promoter affects promoter activity \textit{in vitro}. In HepG2 cells, a 158 bp \textit{UGT1A9} promoter construct containing ten consecutive thymine residues was 2.6 times more active than the equivalent promoter with nine (Yamanaka et al., 2004). However, the function of this polymorphism \textit{in vivo} is still under debate. In support of the findings of Yamanaka et al. (2004), Carlini and colleagues found that the more prevalent \textit{UGT1A9} -118 \((dT)_{9/9}\) genotype was associated with less diarrhoea and better response than the \textit{UGT1A9} -118 \((dT)_{10/10}\) genotype in patients with metastatic colorectal cancer after treatment with irinotecan/capecitabine combination therapy.
The authors hypothesised that the lower activity UGT1A9 allele allows a higher systemic concentration of SN-38 to be achieved, while limiting the delivery of SN-38 to the gastrointestinal tract through biliary excretion of the SN-38-glucuronide and subsequent hydrolysis. However, an analysis correlating UGT1A9 promoter SNPs and hepatic UGT1A9 concentration by Girard and co-workers found no effect of the UGT1A9 -118 (dT)ₙ polymorphism on UGT1A9 expression levels in human liver microsomes, but that the identities of several other promoter nucleotides (at positions -275, -331/-440, -665 and -2152) predicted hepatic UGT1A9 expression (Girard et al., 2004). In their hands, the UGT1A9 -118 (dT)ₙ polymorphism also only altered promoter activity in vitro by 1.4-fold (Girard et al., 2006). Yet the same authors found that while the UGT1A9 -118 (dT)ₙ SNP did not predict UGT1A9 expression levels, it was associated with the glucuronidation rate of SN-38 (Girard et al., 2006). Another UGT1A9 SNP, the intronic polymorphism I339, was also associated with SN-38 glucuronidation rate and hepatic UGT1A9 and UGT1A1 content (Girard et al., 2006). The UGT1A9 promoter polymorphisms at positions -275 and -2152 have also been associated with mycophenolic acid exposure in renal transplant patients (Kuypers et al., 2005). To date, no mechanisms have been suggested for any of the reported associations of promoter sequence and activity.

Finally, in a recent study of UGT1A9 regulation, Barbier and co-workers have shown that HNF4α regulates the human UGT1A9 proximal promoter in vitro (Barbier et al., 2005). The ability to positively influence transcription from the UGT1A9 promoter in their experimental system required a weak HNF4α-response element positioned at nucleotides -372 to -360 that is, importantly, absent in the promoters of the other UGT1A7-1A10 cluster genes due to single or dual nucleotide substitutions. However, mutating the UGT1A8 promoter to create a UGT1A9-equivalent HNF4α-binding
element only partially bestowed HNF4α-responsiveness to the UGT1A8 promoter construct, indicating that further elements within the UGT1A9 promoter are also important for optimal HNF4α-mediated expression. This was not entirely unexpected, as detailed investigations of liver-specific regulatory elements reveal that hepatic expression of a gene generally requires association of numerous LETFs with the promoter, many of which may have multiple binding sites (Schrem et al., 2002; Costa et al., 2003). Therefore, as the HNF4α-binding site was the first and only element to be identified that exclusively activated the UGT1A9 gene of the UGT1A7-1A10 cluster, and because at least one other sequence difference between the promoters of these genes must co-operate in the HNF4α-mediated induction of UGT1A9, it was decided that the regulation of the UGT1A9 promoter by HNF4α warranted further investigation.

4.1.4. The hepatocyte nuclear factor 4 transcription factor family

4.1.4.1. Physical attributes of HNF4 proteins

Hepatocyte nuclear factor 4α is a 465 amino acid zinc-finger transcription factor belonging to the HNF4 subfamily of the nuclear receptor superfamily (Schrem et al., 2002). Other known members of the HNF4 subfamily are HNF4β, HNF4γ and DHNF4 (Zhong et al., 1993; Holewa et al., 1997), of which HNF4γ is the only other protein known to be found in mammals (Drewes et al., 1996). However, the function of HNF4γ remains poorly characterised, and only HNF4α was considered for the following investigations into the function of the UGT1A9 promoter. Yet it should be noted that HNF4γ is also a potential regulator of UGT1A9.

The known structural domains of HNF4α include an N-terminal region required for transactivation (activation function (AF)1; amino acids 1-49), a C-terminal repressor
domain (also known as the F domain; amino acids 368-465), and two domains conserved among nuclear receptors: a zinc-finger DNA-binding domain (amino acids 50-115) and a large hydrophobic region (amino acids 135-367) that functions as a second activation domain (AF-2) as well as the ligand-binding and dimerisation domains (Ryffel, 2001; Schrem et al., 2002). Like many nuclear receptors, HNF4α homodimers bind to DNA sequences that loosely match a pair of AGGTCA-like NRREs. In the case of HNF4α, these NRREs are arranged as direct repeats, separated by either one or two deoxyadenylate molecules, but with a very strong preference for only one spacer nucleotide. Thus the consensus sequence for known HNF4α-binding elements is RGGNCAAAGKTCR, where the “CAAAG” motif forms the essential core of the binding site, and R = A or G, K = G or T and N = A, C, G or T (Fraser et al., 1998). However, of the nuclear receptors that form dimers, HNF4α is among the minority in that it is not known to heterodimerise with any other members of the nuclear receptor superfamily and has been specifically shown not to heterodimerise with the most common nuclear receptor heterodimerisation partner, the retinoid X receptor (RXR)α, or with other nuclear receptors including RXRβ, RXRγ, retinoid acid receptor (RAR)α, RARβ2, RARγ, vitamin D receptor, PPARα and thyroid hormone receptor α (Jiang et al., 1995). HNF4α homodimers are stable in solution in the absence of DNA (Jiang et al., 1995).

Although human HNF4γ is encoded on a separate chromosome to HNF4α (chromosomes 8 and 20, respectively (Drewes et al., 1996)), the two genes are very similar. Overall, HNF4α1 (a splice variant of HNF4α; see section 4.1.4.2) and HNF4γ share 70% identity (Plengvidhya et al., 1999), and for the amino acids required for ligand binding, dimerisation and DNA recognition, this identity increases to 96%, 91% and 95% respectively (Drewes et al., 1996; Wisely et al., 1996).
2002). Thus, it has been suggested that heterodimerisation between HNF4α and HNF4γ may be the one exception to the observation that these proteins only form homodimers (Wisely et al., 2002). Support for this hypothesis comes from functional studies showing that excess HNF4γ in transient transfections can decrease the activity of co-transfected HNF4α to the levels achieved by HNF4γ alone (Drewes et al., 1996).

The search for ligands of HNF4α and HNF4γ has caused much controversy. Originally, HNF4α was considered an orphan nuclear receptor, as HNF4α activates transcription in the absence of exogenously added ligand (Sladek et al., 1990) and no activity-modulating ligands could be identified. However, in 1998, Hertz and colleagues showed that fatty acyl-coenzyme A (CoA) thioesters with chain lengths of 12 to 22 carbon atoms can be bound by amino acids 96 to 455 of HNF4α (the HNF4α ligand-binding and F domain), whereas free fatty acids are not (Hertz et al., 1998). They also showed that fatty acyl-CoA thioesters behave as HNF4α transactivational agonists or antagonists, depending on their length and degree of saturation. However, in 2002, two research groups crystallised the HNF4α (amino acid 132-382 or amino acid 103-465) and HNF4γ (amino acids 103-408) ligand-binding domains, finding that in crystallised protein the ligand-binding pocket is occupied by free fatty acids, is too small to accommodate the suggested thioesters, and that the bound fatty acids are held so tightly that they cannot be stripped out or exchanged for radiolabelled counterparts (Dhe-Paganon et al., 2002; Wisely et al., 2002; Duda et al., 2004). Therefore, they concluded that HNF4α and HNF4γ are constitutively bound and activated by fatty acids, which are most likely irreversibly incorporated into the protein structure during synthesis and folding. Despite this work, Hertz and colleagues continued to show that endogenous and xenobiotic acyl-CoAs alter the
activity of HNF4α in cell culture and that intracellular long-chain acyl-CoA synthases are required for fatty acids to exert an effect on HNF4α-mediated transcription (Hertz et al., 2001). Furthermore, collaborations with Petrescu and coworkers showed that fatty acyl-CoA binding to the HNF4α ligand-binding domain significantly alters its secondary structure, in a manner that is opposite between compounds known to be agonists and those that are antagonists (Petrescu et al., 2002). Finally, in 2005, the apparent discrepancies were resolved by publications showing that the C-terminal F domain of HNF4α has thioesterase activity and specifically binds and hydrolyses fatty acyl-CoA molecules, and that fatty acids trapped in the ligand-binding domain of the full length protein are exchangeable, but with a very strong preference for fatty acid molecules generated from hydrolysable fatty acyl-CoAs by the C-terminal thioesterase (Hertz et al., 2005). Thus, the fatty acids found in the ligand-binding pocket of HNF4α reflect the cellular pool of fatty acyl-CoA thioesters rather than the local free fatty acid constituents (Hertz et al., 2005; Schroeder et al., 2005). Therefore, the balance of current evidence suggests that HNF4α and HNF4γ bind the same or similar ligands, and that fatty acids are endogenous modulators of HNF4 activity via a pathway that involves esterification and hydrolysis, and an atypical relationship between the ligand-binding domain and receptor activation.

4.1.4.2. Expression profile of HNF4α

In humans, HNF4α expression has been demonstrated in the liver, kidney, pancreas, small intestine and colon (Sladek et al., 1990; Drewes et al., 1996). Liver and kidney are also major sites of expression in the mouse (Zhong et al., 1994). The HNF4α gene consists of 13 exons (Furuta et al., 1997; Torres-Padilla et al., 2001) that give rise to numerous splice variants in both humans and mice. Of particular note are
splice variants HNF4α1, HNF4α2 and HNF4α7. HNF4α1 is the originally identified HNF4α mRNA transcript (Sladek et al., 1990); however, HNF4α2 is actually the most abundant HNF4α mRNA in adult liver and HepG2 cells, and is a stronger transactivator than HNF4α1 in some circumstances due to enhanced interactions with co-activators. The HNF4α1 and HNF4α2 proteins differ by a 10 amino acid insertion present in the F domain of HNF4α2 relative to HNF4α1 (Hata et al., 1992; Chartier et al., 1994; Hata et al., 1995; Drewes et al., 1996; Sladek et al., 1999). On the other hand, HNF4α7 is an abundant splice variant in foetal liver, intestine, stomach and pancreas and, although also present in adult stomach, is very low in adult liver. This splice variant originates from an alternative promoter to HNF4α1/HNF4α2 and gives rise to a protein that has a different N-terminal domain to that of HNF4α1 (Nakhei et al., 1998; Torres-Padilla et al., 2001; Torres-Padilla et al., 2002; Briancon et al., 2004). HNF4α7 is a better transactivator of several promoters from genes known to be expressed early in development than HNF4α1 or HNF4α2 (Torres-Padilla et al., 2001). HNF4α8 is the HNF4α2-equivalent splice variant of HNF4α7, having the same C-terminal sequence as HNF4α2, and the same N-terminal region as HNF4α7 (Torres-Padilla et al., 2001). The remaining HNF4α splice variants are minor species and are yet to be properly characterised, although it is known that HNF4α3, which has a completely different, shorter C-terminal domain than HNF4α1 and HNF4α2, has similar activity to HNF4α1 in vitro (Kritis et al., 1996), and that HNF4α4, which has a 30 amino acid insertion in the N-terminal domain relative to HNF4α1, has very little activity in transient transfection assays (Drewes et al., 1996). HNF4α5 and HNF4α6 have the same N-terminal sequence as HNF4α4, but C-terminal ends equivalent to HNF4α2 and HNF4α3 respectively (Furuta et al., 1997).
Several external and endogenous factors or circumstances are known to influence either the expression or transactivational activity of HNF4α in humans or rodents. These include bile acids, cytokines, hypoxia, diet and exposure to drugs such as clofibratic acid analogues (Viollet et al., 1997; Hertz et al., 2001; Mazure et al., 2001; Zhang and Chiang, 2001; Li et al., 2006). Transcription factors that interact with the human HNF4α promoter include HNF1α, HNF1β, HNF6 and GATA6 (Hatzis and Talianidis, 2001), while HNF1β and FXR have been implicated in the expression of HNF4α in the mouse (Zhang and Chiang, 2001; Wang et al., 2004a).

HNF4γ was first identified in humans, and the corresponding mRNA is found in human liver, kidney, pancreas, small intestine, colon, testes, brain and lung (Plengvidhya et al., 1999). Although it has an overlapping expression and activity profile with HNF4α, HNF4γ cannot substitute for this transcription factor, as homozygous HNF4α-knockout mice die early in embyrogenesis (Chen et al., 1994).

4.1.4.3. Gene targets of HNF4α

The number of potential HNF4α gene targets in humans is enormous. The proportion of proximal promoters shown to be bound by HNF4α in human hepatocytes and pancreatic islet cells is 11-12% of the Hu13K DNA microarray; far greater than the equivalent results for HNF1α or HNF6 (approximately 1.6%) and any other transcription factor previously tested by the same research group (maximum 2.5%) (Odom et al., 2004). HNF4α is known to act as both a positive and negative regulator of gene expression, and is particularly important in determining the hepatic phenotype during development (Li et al., 2000).

A small selection of the many genes thought to be positively regulated by HNF4α in humans includes important examples involved in fatty acid, lipoprotein and lipid
metabolism (e.g. apolipoprotein (apo)AII, apoB, apoCIII, medium chain acyl-CoA dehydrogenase and fatty acid-binding protein (fabp)2 (Ladias et al., 1992; Carter et al., 1993; Klapper et al., 2007)), amino acid and protein metabolism (for example, α1-antitrypsin (Hu and Perlmutter, 1999)), haematopoiesis (erythropoietin and transferrin (Schaeffer et al., 1993; Galson et al., 1995)), blood coagulation (factors VII, IX, X and XI (Reijnen et al., 1992; Hung and High, 1996; Pollak et al., 1996; Tarumi et al., 2002)), biotransformation (CYP2A6, CYP2B6, CYP2C9, CYP2D6, CYP3A4, CYP3A5, CYP7A1, CYP8B1, UGT1A9, UGT2B11 and UGT2B15 (Jover et al., 2001; Zhang and Chiang, 2001; Odom et al., 2004; Barbier et al., 2005; Li et al., 2006) and this thesis) and organic ion transport (e.g. organic cation transporter 1 and OAT2 (Popowski et al., 2005; Saborowski et al., 2006)). HNF4α is also thought to be a central regulator of carbohydrate metabolism, as mutations in the human HNF4α gene cause the inherited autosomal dominant disease, mature onset diabetes of the young subtype 1 (MODY1) (Ryffel, 2001). However, most studies linking HNF4α with individual genes involved in carbohydrate metabolism have so far only investigated rodent homologues. Examples include insulin, aldolase B, phosphoenolpyruvate carboxykinase and human glucose-6-phosphatase (Hall et al., 1995; Gregori et al., 1998; Li et al., 2000; Wang et al., 2000; Bartoov-Shifman et al., 2002; Hirota et al., 2005). Finally, HNF4α is known to have a positive influence on the expression of several other transcription factors in humans and/or rodents, including HNF1α (rat and human) (Miura and Tanaka, 1993; Gragnoli et al., 1997; Li et al., 2000; Odom et al., 2004), PXR (mouse) (Li et al., 2000; Kamiya et al., 2003), HNF6 (rat) (Lahuna et al., 2000) and the small heterodimer partner (SHP; human) (Lai et al., 2003).
Conversely, a small number of genes are known to be repressed by HNF4α, including both alternative promoters of the *HNF4α* gene in humans and mice (Briancon *et al.*, 2004; Magenheim *et al.*, 2005). Other genes known to be repressed by HNF4α include rat mitochondrial acetyl-CoA synthase (Rodriguez *et al.*, 1998), rat arginase (Chowdhury *et al.*, 1996) and rat peroxisomal acyl-CoA oxidase (Nishiyama *et al.*, 1998). Rat apoAI, which is activated by HNF4α through one DNA element is also subject to negative regulation by HNF4α through another (Murao *et al.*, 1997). Repression may be caused directly by competition with other nuclear receptors that bind the same site but have a greater transactivational potential for that gene (Nakshatri and Chambon, 1994; Nishiyama *et al.*, 1998; Rodriguez *et al.*, 1998), or indirectly by inhibiting the function of other transcription factors, such as Sp1, without necessarily binding DNA (Chowdhury *et al.*, 1996; Magenheim *et al.*, 2005).

**4.1.4.4. HNF4α interacts with numerous co-factors and other proteins**

HNF4α has been shown to physically interact with numerous proteins that do not bind NRREs, including acyl-CoA-binding protein (Petrescu *et al.*, 2003), c-Jun (Li *et al.*, 2006) and Sp1 (Magenheim *et al.*, 2005). In addition, HNF4α has been shown to be able to synergistically regulate genes in combination with other transcription factors such as the androgen receptor (Chen *et al.*, 2005b), HNF6 (Beaudry *et al.*, 2006), the glucocorticoid receptor (GR) (Nitsch *et al.*, 1993) and C/EBPα (Pitarque *et al.*, 2005). Conversely, FoxA factors bound to the α-1-microglobulin/bikunin precursor gene promoter inhibit the positive effects of HNF4α (Rouet *et al.*, 1995), while PXR and HNF1α have both been shown to agonise or antagonise HNF4α-mediated transactivation, depending on the promoter context (Ktistaki and Talianidis, 1997; Hu and Perlmutter, 1999; Ozeki *et al.*, 2001; Divine *et al.*, 2003;
Bhalla *et al.*, 2004; Chen *et al.*, 2005b). In these listed instances, repression is caused by direct protein interactions or competition for co-factors; however, nuclear receptors such as chicken ovalbumin upstream promoter-transcription factors and apoAI regulatory protein 1 can also repress HNF4α-driven genes by competing for access to NRREs (Ladias *et al.*, 1992; Mietus-Snyder *et al.*, 1992).

Multiple transcriptional co-factors and components of the general transcription machinery have also been shown to interact with HNF4α. In particular, the HNF4α N-terminal acidic activation domain interacts with the general transcription factors TBP, TAFι31, TAFι80, TFIIB, TFIIH-p62, the co-activators CBP/p300 and PC4, and the transcriptional adaptor ADA2 (Yoshida *et al.*, 1997; Green *et al.*, 1998b). Functional interactions between HNF4α AF-2 and CBP, as well as between HNF4α AF-2 and p160 nuclear receptor co-factors containing LXXLL motifs have also been established. HNF4α activity is stimulated by p160 co-activators such as SRC-1, PGC-1, glucocorticoid receptor interacting protein (GRIP)-1 and amplified in breast cancer 1 (Wang *et al.*, 1998; Lee *et al.*, 2000; Yoon *et al.*, 2001). Other nuclear receptors, such as PXR and CAR can also bind these co-activators and thereby decrease HNF4α activity by sequestering its required co-factors (Bhalla *et al.*, 2004; Miao *et al.*, 2006). CBP/p300 and SRC-1 mediate target gene expression by recruiting and activating the basal transcriptional apparatus, and also by overcoming the inhibitory effects of chromatin structure through their intrinsic HAT activities (Dallas *et al.*, 1997; Soutoglou *et al.*, 2000b; Schrem *et al.*, 2002) (see also Chapter 3, section 3.1.5.4). In addition, CBP is known to acetylate HNF4α, a modification that is crucial for its nuclear retention and also important for DNA-binding (Soutoglou *et al.*, 2000a). On the other hand, HNF4α activity is repressed by the SMRT co-repressor, presumably through recruitment of HDACs to HNF4α/SMRT
complexes and/or by blocking positive interactions with GRIP1, CBP, or p300 (Ruse et al., 2002; Torres-Padilla et al., 2002), and by Prox1, which causes repression by competing for binding to the AF-2 domain with the co-activator PGC-1 (Song et al., 2006). Finally, SHP, which belongs to the nuclear receptor superfamily but lacks a DNA-binding domain (Seol et al., 1996), also interacts with the N-terminal AF-1 region, as well as the DNA-binding and AF-2 domains of HNF4α. Thereby SHP inhibits HNF4α-mediated transactivation of promoters through multiple mechanisms: by competing with p160 co-activator binding to HNF4α; by direct repression of HNF4α function; and by preventing HNF4α from binding to its cognate DNA elements (Lee et al., 2000; Shimamoto et al., 2004).

4.1.5. Aims

While work by Barbier and colleagues showed that HNF4α is a major contributor to the activity of the UGT1A9 promoter (Barbier et al., 2005), the identified element did not fully explain the difference between the response of the UGT1A9 promoter and the other members of the UGT1A7-1A10 cluster (section 4.1.3). Therefore, the aims of this study were to:

1. Further characterise the role of HNF4α in the hepatic expression of UGT1A9;

2. Identify additional promoter elements that contribute to the UGT1A9 gene response to HNF4α.

4.2 Methods

4.2.1. Construction of the pGL3+ reporter plasmid

In order to provide appropriate cloning sites for the UGT1A9-2k promoter in the pGL3-basic reporter vector, pGL3+ was created. A sticky-ended, double-stranded
DNA fragment containing the original sequence of the pGL3-basic multiple-cloning site plus additional *PstI* and *EcoRI* restriction sites was generated by kinase treatment and subsequent annealing of two oligonucleotides, pGL3insertF and pGL3insertR (see Table 4.1 for oligonucleotide sequences). The kinase reaction consisted of 10 μM each oligonucleotide, 70 mM Tris-HCl, 10 mM MgCl₂, 5 mM DTT, 1 mM ATP and 10 units T4 polynucleotide kinase (pH 7.6) and was incubated at 37°C for one hour before heating to 95°C for 5 minutes in a dry block heater. The heating element was then switched off and the oligonucleotides annealed by leaving the sample in the block, allowing it to return to room temperature without assistance. The newly annealed insert was then ligated into *MluI/XhoI* restricted pGL3-basic to generate pGL3+, and sequenced (see Chapter 2, section 2.2.7 for details of endonuclease restriction and ligation methods, and Appendix 2 for restriction map of pGL3+).

### 4.2.2. Generation of the *UGT1A9*-2k reporter construct

pGL3+ vector was prepared for insertion of the proximal 2 kilobases of the *UGT1A9* promoter by restriction with *PstI* and CIP treatment (see Chapter 2, section 2.2.7). The *UGT1A9*-2kb promoter fragment transferred into pGL3+ was released from pBS-2P by *PstI* digestion. (pBS-2P is a pBlueScript II (pBSII) clone containing a 2 kb fragment of the human *UGT1A9* promoter, constructed by Dr. Kim Duncliffe (Department of Clinical Pharmacology, Flinders University). This *UGT1A9* promoter insert was originally shuttled into pBSII from a lambda clone, which was isolated from a human placenta lambda library (Clontech, Mountain View, CA) by Dr. Duncliffe (Gregory *et al.*, 2003)). The desired *UGT1A9*-2kb DNA fragment was separated from the pBSII vector fragment by agarose gel electrophoresis, recovered
Table 4.1: Primers used in generating pGL3+, in cloning the \textit{UGT1A7}, \textit{UGT1A9} and \textit{apoCIII} proximal promoters, and in generating deleted and mutated promoter-reporter and transcription factor expression constructs.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Nucleotide Sequence (5’→3’)</th>
<th>Nucleotide Position on Target Gene</th>
<th>RE</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGL3insertF</td>
<td>CGCGTCTGCAGGAATTCGCTAGCCGGGC</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>pGL3insertR</td>
<td>TCGAGCCCAGGCTAGCAATTCCTGCAGA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>apoCIII-810for</td>
<td>AGCCATGCTACGACGAGAATCAGTCTCTGGT</td>
<td>apoCIII: -810 to -791</td>
<td>NheI</td>
</tr>
<tr>
<td>apoCIII+23rev</td>
<td>AGCCATCTCGAGCTGCCTCTAGGGATGAAC</td>
<td>apoCIII: +23 to +3</td>
<td>XhoI</td>
</tr>
<tr>
<td>1A7prom-5kb</td>
<td>AGCCATAAGCTTTCAACTGTAAGAAAAACAGA</td>
<td>UGT1A7: -5133 to -5112</td>
<td>HindIII</td>
</tr>
<tr>
<td>1A7rev</td>
<td>AGCCATAAGCTTTTCGCAATGGTGCCGTCAGC</td>
<td>UGT1A7: +310 to +290</td>
<td>HindIII</td>
</tr>
<tr>
<td>1A7rev2</td>
<td>AGCCATAAGCTTTACATATAGTGGAAGAGGCCA</td>
<td>UGT1A7: +41 to +21</td>
<td>HindIII</td>
</tr>
<tr>
<td>1A7prom-2.5kb</td>
<td>AGCCATAAGCTTTACTGTCATGAGTGGTGAT</td>
<td>UGT1A7: -2585 to -2565</td>
<td>HindIII</td>
</tr>
<tr>
<td>1A9prom-184bp</td>
<td>TTTTGGTACCTCAGAAAAAGTACTC</td>
<td>UGT1A9: -184 to -169</td>
<td>KpnI</td>
</tr>
<tr>
<td>1A9prom-321bp</td>
<td>AGCCATGGTAGCCTTCTGAACCTTCAAGGTCCA</td>
<td>UGT1A9: -321 to -300</td>
<td>KpnI</td>
</tr>
<tr>
<td>1A9prom-1kb</td>
<td>AGCCATGGTAGCCTTCTGGGCAAGCTTTCC</td>
<td>UGT1A9: -1038 to -1021</td>
<td>PstI</td>
</tr>
<tr>
<td>1A9UTRrevMluI</td>
<td>AGCCATACGCGAGAGAAGAGCTGGAGAGGAGC</td>
<td>UGT1A9: -1 to -21</td>
<td>MluI</td>
</tr>
<tr>
<td>1A9UTRrev</td>
<td>AGCCATCTCGAGAGAGAAGAGCTGGAGAGGAGC</td>
<td>UGT1A9: -1 to -21</td>
<td>PstI</td>
</tr>
<tr>
<td>QC1A9-HNF4s1mt</td>
<td>CTAAATTHTTTCGCTTGGAAGCTGGCCCTTGAAATGAGC</td>
<td>UGT1A9: -386 to -348</td>
<td>NA</td>
</tr>
<tr>
<td>Oligonucleotide</td>
<td>Nucleotide Sequence (5’→3’)</td>
<td>Nucleotide Position on Target Gene</td>
<td>RE</td>
</tr>
<tr>
<td>------------------</td>
<td>--------------------------------------------------------------------------------------------</td>
<td>-----------------------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>QC1A9-HNF4s2mt</td>
<td>GTTCTGCCCCAAGGCGGGGCCCTTTGTAAGCTACTGGTGTC</td>
<td>UGT1A9: -246 to -208</td>
<td>NA</td>
</tr>
<tr>
<td>QC1A9-HNF4s2mt8</td>
<td>GTTCTGCCCCAAGGCGAGACTATAAGCTACTGGTGTC</td>
<td>UGT1A9: -246 to -208</td>
<td>NA</td>
</tr>
<tr>
<td>QC1A9-HNF4s2cons</td>
<td>GTTCTGCCCCAAGGCAAGGTCATAAGCTACTGGTGTC</td>
<td>UGT1A9: -246 to -208</td>
<td>NA</td>
</tr>
<tr>
<td>QC1A9-HNF1s1mt</td>
<td>CTTGTTCTTTGGTGTCGCTCAGTCTAGTG</td>
<td>UGT1A9: -161 to -128</td>
<td>NA</td>
</tr>
<tr>
<td>QC1A9-HNF1s2mt</td>
<td>GGTCAAAAGCATTGGTCGCTAAATTCTGCTTTAAC</td>
<td>UGT1A9: -305 to -269</td>
<td>NA</td>
</tr>
<tr>
<td>QC1A9-HNF1s2mt8</td>
<td>CTTCAAGGTCGAAAACATTGGCTTAGTATTTTGCTTCTTAAACTTAA</td>
<td>UGT1A9: -311 to -264</td>
<td>NA</td>
</tr>
<tr>
<td>QC1A9-mtT-275A</td>
<td>GGTGTAATAATTCTGCTACTAAAATCTATAACATTGAGCGACAGGGC</td>
<td>UGT1A9: -291 to -249</td>
<td>NA</td>
</tr>
<tr>
<td>QC1A9-mtC-62T</td>
<td>GTGCTGATTTCTCCACTGATCTTAGTATAGGAGC</td>
<td>UGT1A9: -76 to -40</td>
<td>NA</td>
</tr>
<tr>
<td>QC1A9-mtA-59G</td>
<td>GTGCTGATTTCTCCACTGATCTTAGTATAGGAGC</td>
<td>UGT1A9: -76 to -40</td>
<td>NA</td>
</tr>
<tr>
<td>QC1A9-mtC-62T/A-59G</td>
<td>GTGCTGATTTCTCCACTGATCTTAGTATAGGAGC</td>
<td>UGT1A9: -76 to -40</td>
<td>NA</td>
</tr>
<tr>
<td>QC1A8-HNF4s2mt9</td>
<td>GGCATGATCTGTCCAAGGCCAAGGCTACTTATAG</td>
<td>UGT1A8: -264 to -222</td>
<td>NA</td>
</tr>
<tr>
<td>QC1A8-HNF1s2mt9</td>
<td>CAGGTCTAATAAGGCTATGTTAAATTCTGTTTCTAAACTCAAG</td>
<td>UGT1A8: -320 to -276</td>
<td>NA</td>
</tr>
<tr>
<td>T7</td>
<td>TAAATACGACTTATAGGAGA</td>
<td>T7 promoter of pCMX plasmid</td>
<td>NA</td>
</tr>
<tr>
<td>HNF1α-546Xrev</td>
<td>AGCCATGACCTTTAGTCTGGAGGTAAGACCTGCTT</td>
<td>HNF1α: +1636 to +1616</td>
<td>BamHI</td>
</tr>
</tbody>
</table>

*Table 4.1 continued.*
Table 4.1 continued.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Nucleotide Sequence (5’→3’)</th>
<th>Nucleotide Position on Target Gene</th>
<th>RE</th>
</tr>
</thead>
<tbody>
<tr>
<td>QCHNF4-mtR127W</td>
<td>CAGAATGAGCGGGACTGGATCAGCACTCAGAAG</td>
<td>HNF4α: +391 to +422</td>
<td>NA</td>
</tr>
<tr>
<td>QCHNF4-mtE276Q</td>
<td>GCAGATCGATGACAATCAGTATGCCTACCTCAAAGCC</td>
<td>HNF4α: +927 to +873</td>
<td>NA</td>
</tr>
<tr>
<td>QCHNF4-mtDG69/70AA</td>
<td>GGTGCCCTGAGCTGCGCGCTGCAAGGGCTTCTTC</td>
<td>HNF4α: +217 to +252</td>
<td>NA</td>
</tr>
<tr>
<td>QCHNF4-mtRR76/77AE</td>
<td>CTGCAAGGGCTTCTCGCGAGCGTGCAGGAAGAAC</td>
<td>HNF4α: +237 to +273</td>
<td>NA</td>
</tr>
</tbody>
</table>

RE: restriction endonuclease site, as underlined. NA: not applicable. Mutations are highlighted in bold. Nucleotide positions are numbered relative to the “A” of the initiation codon.
using the Qiagen gel extraction kit (see Chapter 2, section 2.2.8), and ligated into the
\textit{PstI} cut pGL3+ vector. Because the cloning of this promoter fragment was non-
directional, clones were screened for insert orientation by \textit{XhoI}/\textit{SpeI} digestion and a
representative from both the forward and reverse orientation clones was chosen and
sequenced.

\textbf{4.2.3. Generation of UGT1A9 deletion constructs and mutants}

A construct containing the \textit{UGT1A9}-184bp proximal promoter was generated by
PCR amplification from the \textit{UGT1A9} promoter lambda clone described above.
Amplification was achieved using \textit{Taq} DNA polymerase as described in Chapter 2,
section 2.2.6.4 with oligonucleotides 1A9prom-184bp and 1A9UTRrevMluI (Table
4.1). Cycling conditions were 95°C for 4 minutes, followed by 30 cycles of 95°C for
30 seconds, 50°C for 30 seconds and 72°C for one minute, and the reaction was
finished with a final extension step of 72°C for 10 minutes. To amplify the \textit{UGT1A9}-
321bp fragment, the 1A9prom-321bp sense primer (Table 4.1) was combined with
1A9UTRrevMluI in a \textit{PfuTurbo} PCR reaction on the pGL3+1A9-2k plasmid as
template of: 95°C for 4 minutes, 30 cycles of 95°C for 30 seconds, 55°C for 30
seconds and 72°C for 1 minute, and a final extension step of 72°C for 5 minutes.
Both PCR products were sequentially digested with \textit{MluI} and \textit{KpnI} and cloned into a
similarly digested sample of pGL3-basic vector. Both inserts were sequenced in full
to ensure that no PCR-generated errors had been introduced. To amplify the
\textit{UGT1A9}-1kb fragment, the primers 1A9prom-1kb and 1A9UTRrevPstI were used in
a \textit{Taq} DNA polymerase reaction (94°C for 4 minutes; 30 cycles of 94°C for 30
seconds, 55°C for 30 seconds and 72°C for 90 seconds; and 72°C for 10 minutes)
using pGL3+1A9-2k as the template. The \textit{PstI}-digested 1 kb PCR product was then
cloned into the PstI site of pGL3+, screened for insert direction by PvuII and XhoI digest, and sequenced in full.

To generate UGT1A9-2kb and UGT1A9-1kb proximal promoter-reporter clones carrying mutations as indicated by Figure 4.1, site-directed mutagenesis was performed as described in Chapter 2, section 2.2.6.5. The sense sequences for each oligonucleotide pair used for site-directed mutagenesis PCRs are listed in Table 4.1 with the mutated nucleotides highlighted in bold. UGT1A9-specific nucleotides were also substituted into the UGT1A8-2kb proximal promoter HNF1s2 and HNF4s2 regions in the same way.

4.2.4. Isolation of the UGT1A7-5k promoter and generation of pGL3-1A7-2.5k

To create the UGT1A7 promoter constructs, approximately 5 kb of the UGT1A7 promoter were cloned from human genomic DNA by nested PCR. NotI-digested HEK293T genomic DNA was used as template for two successive rounds of PCR using Taq DNA polymerase with Taq Extender PCR additive (see Chapter 2, sections 2.2.6.3 and 2.2.6.4). Firstly, the 1A7prom-5kb primer was combined with 1A7rev (Table 4.1) for the external PCR reaction, which contained 0.125 Units/μl Taq DNA polymerase, 0.125 Units/μl Taq Extender and 2.5 ng/μl NotI-digested gDNA, and was cycled through a single stage of 95°C for 4 minutes; 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 10 minutes; and finished with an additional extension step of 72°C for 10 minutes. One microlitre of the resulting PCR product was then used as template for a second round of amplification using primers 1A7prom-5kb and 1A7rev2 under the same conditions as the previous PCR. The ensuing 5 kb PCR product was cloned non-directionally into the HindIII site of pBSII, shuttled into the HindIII site of pGL3-basic and screened for direction of
insertion with EcoRI. Chosen clones were sequenced from each end to confirm the identity of the insert as the UGT1A7 promoter.

The pGL3-1A7-5k construct was then used as template to amplify approximately 2.5 kb of the UGT1A7 proximal promoter using the same Taq DNA polymerase/Taq Extender mix and PCR conditions as the 5 kb PCR reaction. The oligonucleotides used to amplify this region of the UGT1A7 promoter were 1A7prom-2.5kb and 1A7rev2 (Table 4.1). The resulting product was cloned directly into the HindIII site of pGL3-basic, and also screened for direction of insertion with EcoRI. Finally, chosen clones were screened for PCR errors in the proximal promoter region by sequencing.

4.2.5. UGT1A8 and UGT1A10 reporter constructs

The UGT1A8-2k and UGT1A10-2k constructs were created in our laboratory by Drs. Philip Gregory and Rikke Lewinsky respectively, as previously described (Gregory et al., 2003; Gregory et al., 2004). The pGL3-1A8-1k promoter-reporter vector was also created by Dr. Gregory (Gregory et al., 2003).

4.2.6. Isolation of the apoCIII-810/+23 promoter

To generate the pGL3-apoCIII-810/+23 reporter construct, the corresponding region was amplified from HEK293T genomic DNA using primers apoCIII-810for and apoCIII+23rev (Table 4.1), adapted from Fraser et al. (1998). Taq DNA polymerase was used for the amplification of the apoCIII promoter from human (HEK293T) genomic DNA with the cycling parameters: 95°C for 4 minutes; 35 cycles of 95°C for 30 seconds, 59°C for 1 minute and 72°C for 2 minutes; and 72°C for 5 minutes. The resulting PCR product was digested with NheI and XhoI and cloned into the same two respective sites of pGL3-basic.
4.2.7. Transcription factor expression vectors

The construction or acquisition of all expression plasmids used in this chapter, except pCMX-HNF1α 546X (which is described below), is detailed in Chapter 2, sections 2.1.4, 2.3.1 and 2.3.2.

The cDNAs encoded by the pCMX-HNF1α and pCMX-HNF1β expression vectors are human in origin and express the A-variants of each transcription factor. The human HNF1α 546X truncation mutant was generated by PCR amplification from the pCMX-HNF1α vector template with primers T7 and HNF1α-546Xrev (Table 4.1). The PCR was performed with PfuTurbo and the conditions used were: 95°C for 4 minutes; followed by 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 4 minutes; and finished with a final extension step of 72°C for 5 minutes. The resulting PCR product was restricted with HindIII and BamHI and cloned into these sites of pCMX-PL2.

cDNAs encoding the human HNF4α1, human HNF4α2 and rat HNF4α1 variants were also cloned into pCMX-PL2. Mutations in the human HNF4α2 coding region were introduced by site-directed mutagenesis as described in Chapter 2, section 2.2.6.5. The sense sequences of each oligonucleotide pair used for site-directed mutagenesis of the human HNF4α2 cDNA are listed in Table 4.1, with the mutated nucleotides highlighted in bold.

4.2.8. Transient transfection and luciferase reporter assay

HepG2 and HEK293T cells were seeded into 24-well plates and transfected as described in Chapter 2, section 2.2.10. Each well was transfected with either 0.5 μg of empty pGL3-basic or a reporter vector carrying the indicated UGT1A7, UGT1A8, UGT1A9, UGT1A10 or apoCIII promoter sequences. For induction studies, 0.25 μg
of each pCMX expression vector, as specified in the results, was co-transfected with the pGL3 reporter constructs. Empty pCMX-PL2 expression vector was added as required to maintain a total of 1 μg DNA per transfection and pRL-null (0.025μg) was added to all transfections as an internal control for transfection efficiency. After 48 hours, cells were lysed in passive lysis buffer and analysed for firefly and *renilla* luciferase activity using the Dual-Luciferase Reporter Assay System as detailed in Chapter 2, section 2.2.11.

### 4.2.9. Electrophoretic mobility-shift and super-shift assays

HepG2 nuclear extract, prepared as described in Chapter 2, section 2.2.12, or *in vitro* synthesised HNF1α and HNF4α protein was used to perform EMSAs and super-shift assays as detailed in Chapter 2, sections 2.2.13, 2.2.14 and 2.2.15. The sense sequences of the double-stranded DNA probes used are listed in Table 4.2. The anti-HNF1α and anti-HNF4α antibodies used in the super-shift assays were sourced from Santa Cruz Biotechnologies (sc-6547 and sc-6556).

### 4.2.10. In vitro synthesis of transcription factor proteins

*In vitro* synthesis of HNF1α and HNF4α protein was performed using the TNT Quick Coupled Transcription/Translation kit (Promega) according to the manufacturer’s instructions. Briefly, pCMX-HNF1α or pCMX-HNF4α expression plasmid was included at a concentration of 20 ng/μl in 50 μl reaction mixes that also consisted of 40 μl TNT Quick master mix (containing reticulocyte lysate, RNA polymerase, nucleotides, salts and RNasin ribonuclease inhibitor) and 20 mM methionine. The reaction was incubated at 37°C for 90 minutes to generate the desired protein and stored in aliquots at -20°C.
Table 4.2: Oligonucleotides used for EMSA and super-shift experiments.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Nucleotide Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>apoCIII-HNF4§</td>
<td>CAGCAGGTTGACCTTTGCCCAAGCC</td>
</tr>
<tr>
<td>UGT1A9-HNF4s1§</td>
<td>TTTGCTCTGGGACAAATTCCAATA</td>
</tr>
<tr>
<td>UGT1A9-HNF4s2</td>
<td>GCCCCCAAAGCAAGCATAAGCT</td>
</tr>
<tr>
<td>UGT1A9-HNF1s1</td>
<td>TTTGGGTAATTCATTGTCAGTAG</td>
</tr>
<tr>
<td>UGT1A9-HNF1s1mt</td>
<td>CTTGTTCTTTGGTGTCTATTGCTAGGTGACTG</td>
</tr>
<tr>
<td>UGT1A7-HNF1s2</td>
<td>GCATTGCTTTGTTCTCTTTTCTCTCT</td>
</tr>
<tr>
<td>UGT1A8-HNF1s2</td>
<td>GCATTGCTTTGTTCTCTTTTCTCTCT</td>
</tr>
<tr>
<td>UGT1A9-HNF1s2</td>
<td>GCATTGCTTTGTTCTCTTTTCTCTCT</td>
</tr>
<tr>
<td>UGT1A9-HNF1s2mt</td>
<td>GCATTGCTTTGTTCTCTTTTCTCTCT</td>
</tr>
<tr>
<td>UGT1A10-HNF1s2</td>
<td>GCATTGCTTTGTTCTCTTTTCTCTCT</td>
</tr>
</tbody>
</table>

Underlined text indicates the extent of the putative HNF1 or HNF4-binding sites in each probe and deliberate mutations are highlighted in bold. NB: Only the sense strand of each oligonucleotide pair is shown. §Probe sequences are the same as those used by Barbier et al. (2005).

4.2.11. Western blot

Joanna Treloar performed the Western blots presented in Figures 4.3B and 4.10B, using total cell lysates from HepG2 and HEK293T cells, as well as *in vitro* synthesised HNF1α and HNF4α proteins. The protocol used is detailed in Chapter 2, section 2.2.16.

4.2.12. Statistical analysis

Statistical treatment of all reporter-promoter assay data was performed as described in Chapter 2, section 2.2.17 using independent-samples *t*-tests.
4.3 Results and discussion

4.3.1. The UGT1A9 proximal promoter responds strongly to over-expression of human HNF4α2 in HepG2 cells

In a recent paper by Barbier and colleagues it was shown that the human UGT1A9 promoter was responsive to the presence of over-expressed HNF4α protein in vitro and, most interestingly, that the remaining UGT1A7-1A10 gene promoters were not stimulated by HNF4α (Barbier et al., 2005). To determine whether these findings could be replicated with my reporter constructs, the human UGT1A7-1A10 proximal promoters were co-transfected with a human HNF4α2 expression vector into the human hepatocellular carcinoma-derived cell line, HepG2. Figure 4.2 shows that, in these cells, over-expression of human HNF4α2 increased the UGT1A9-2kb promoter activity by 45-fold ($P < 0.001$), a response considerably larger than the 5 to 8-fold response reported by Barbier et al. (2005). In accordance with their work, however, the UGT1A7 and UGT1A8 promoter reporter constructs were unaffected ($P = 0.804$ and $P = 0.167$ respectively) and UGT1A10 was slightly inhibited ($P = 0.005$) by co-transfection with pCMX-HNF4α. Therefore, further experiments were designed to characterise in more detail the unique regulation of the UGT1A9 promoter by HNF4α.

4.3.2. The UGT1A9 promoter -372 to -360 HNF4α-binding site is not necessary for the UGT1A9 promoter response to human HNF4α2

The recent publication linking HNF4α expression with UGT1A9 transcription indicated that this was due to a weak but functional HNF4α-response element found only in the UGT1A9 promoter, located at nucleotide position -372 to -360 (HNF4 site 1, Figure 4.1) (Barbier et al., 2005). An oligonucleotide probe over this region bound HNF4α weakly in EMSA, and mutation of this site abolished pSG5-HNF4α-
Figure 4.2: Over-expression of human HNF4α in HepG2 cells strongly up-regulates the human UGT1A9 proximal promoter. HepG2 cells were transfected in triplicate with 0.5 μg of pGL3-based reporter vector, 25 ng pRL-Null and 0.25 μg pCMX-PL2 or pCMX-HNF4α2 as described under “Methods”. Mutations (HNF4s1mt, HNF4s2mt and HNF4s2cons) in the UGT1A9 HNF4 sites as defined by Figure 4.1 were introduced by site-directed mutagenesis. Cells were harvested 48 hours post-transfection and assayed for both firefly and renilla luciferase activity. Results shown are the mean firefly luciferase activities relative to the internal renilla controls, expressed as a fold induction over the promoterless pGL3-basic vector (set at a value of 1). Error bars represent one standard deviation. P values for the indicated comparisons are *P < 0.001, ‡P = 0.005, ‡‡P = 0.013 and #P > 0.05 (not significant). Also, UGT1A9 promoter deletion constructs have significantly greater activity than empty pGL3-basic vector (***P < 0.001, ¥P = 0.008) and pGL3-1A9-2kb HNF4s2cons has greater basal and inducible activity than pGL3-1A9-2kb (††P = 0.002).

mediated activation in HepG2 transfections. Therefore, I first targeted this region in my desire to further understand the UGT1A9 response to HNF4α. Figure 4.2 shows the results of human HNF4α2 co-transfections in HepG2 cells with UGT1A9 promoter constructs where this element (HNF4 site 1) is either mutated (pGL3-1A9-2k HNF4s1mt) or deleted (pGL3-1A9-321bp). It was found that, in the context of the
2 kb proximal promoter, mutation of the HNF4α-binding site caused only a modest reduction of less than 2-fold in the activation by human HNF4α2 ($P < 0.001$). In addition, the construct containing only the proximal 321 bp of the UGT1A9 promoter, where the putative HNF4α site is excluded, was activated to an even greater extent by HNF4α than the original 2 kb construct ($P < 0.001$). In contrast, activation of the UGT1A9 promoter by human HNF4α2 could be abolished by further 5’ deletion of the UGT1A9 sequence, leaving only 184 bp upstream of the initiation codon ($P = 0.472$) (Figure 4.2). Thus, I established that there was least one important element in the -321 to -184 region of the UGT1A9 promoter for HNF4α-responsiveness.

4.3.3. The UGT1A9 response to HNF4α in vitro is dependent on a second HNF4α-binding element

Examination of the nucleotide sequence of the UGT1A9 promoter between bases -321 and -184 revealed a CAAAG motif, known to be the core sequence required for HNF4α binding to DNA (Fraser et al., 1998). Therefore, I postulated that this element (nucleotides -235 to -223) may serve as the core of a second binding site for HNF4α, and may contribute to the HNF4α-responsiveness of the UGT1A9 promoter. Mutation of this putative binding site (HNF4 site 2, Figure 4.1) almost completely abolished the response of the UGT1A9-2kb promoter construct to over-expression of human HNF4α2 in HepG2 cells, although the 1.5-fold residual increase in luciferase expression that was retained in co-transfections was statistically significant ($P = 0.013$) (Figure 4.2). Furthermore, an oligonucleotide probe encompassing this site was able to bind HNF4α from HepG2 nuclear extracts (Figure 4.3A). It was also confirmed that HNF4 site 1 could bind HNF4α; however, under the assay conditions
Figure 4.3: Assessment of HNF4α binding to UGT1A9 promoter sequences in vitro. A. HNF4α binds weakly to both HNF4 sites of the UGT1A9 promoter in electrophoretic mobility-shift assays. $^{32}$P end-labelled double-stranded oligonucleotide probes (50,000 cpm) containing HNF4 sites of the human apoCIII or UGT1A9 promoters were incubated with 5 μg HepG2 nuclear extract and subjected to electrophoresis on a 4% (w/v) non-denaturing polyacrylamide gel. Binding of HNF4α to each probe was demonstrated by super-shift of the relevant complexes with 2 μg of anti-HNF4α antibody added immediately after the labelled oligonucleotides. The complexes pertinent to this study are indicated with arrows. B. HNF4α and HNF1α protein can be detected in HepG2 but not HEK293T total cell lysates. HepG2 and HEK293T total cell lysates were probed for the presence of HNF4α and HNF1α proteins by Western blot as described in Chapter 2, section 2.2.16. In vitro translated HNF4α and HNF1α proteins were utilised as positive controls.
used, this probe also bound a considerable quantity of protein that could not be super-shifted with anti-HNF4α antibody. Both UGT1A9 HNF4α sites were found to be extremely weak binders of HNF4α in comparison to a probe containing the most proximal HNF4α-binding element of the apoCIII gene (Figure 4.3A).

4.3.4. The UGT1A9 proximal promoter response to HNF4 requires HNF1α

When the response of the UGT1A9 proximal promoter to human HNF4α2 in HEK293T cells (which do not express endogenous HNF4α (Maeda et al., 2002) (Figure 4.3B)) was investigated, absolutely no increase in reporter activity was observed (\( P = 0.054 \)) (Figure 4.4A). In contrast, the apoCIII-810/+23 promoter, which is also regulated by HNF4α (Fraser et al., 1998), was increased by over 100-fold under the same conditions (\( P < 0.001 \)). This indicated that at least one additional component, missing from the transcription factor or co-factor profile of HEK293T cells, was required for up-regulation of the UGT1A9 promoter by HNF4α in vitro.

Furthermore, this factor was not required for the apoCIII promoter response to HNF4α.

The homeodomain transcription factor HNF1α has been reported to operate synergistically with HNF4α on several promoters (Hu and Perlmutter, 1999; Ozeki et al., 2001; Eeckhoute et al., 2004). In addition, HEK293T cells do not express HNF1 factors (Bernard et al., 1999) (Figure 4.3B), the UGT1A9 promoter is known to possess a functional HNF1 site located between nucleotides -148 and -136 (HNF1 site 1, Figure 4.1) (Gregory et al., 2004), and the apoCIII promoter does not contain any functional HNF1 sites (Kritis et al., 1993). Therefore, it was decided to investigate whether human HNF1α was able to affect the HNF4α-mediated regulation of the UGT1A9 promoter. When an HNF1α expression vector was
**Figure 4.4: HNF1α synergistically regulates the UGT1A9 promoter with HNF4α through a second HNF1-binding site.** A. Human HNF4α and HNF1α synergistically up-regulate the UGT1A9 promoter in HEK293T cells. HEK293T cells were transfected, according to the methods described in section 4.2.8, with 0.5 μg pGL3-basic or reporter vector containing the indicated lengths of the human UGT1A9 or apoCIII promoters. Each transfection also included 25 ng pRL-Null and 0.25 μg of the human HNF4α2 and/or HNF1α expression vectors. The DNA concentration in each transfection was kept constant by addition of empty pCMX-PL2 vector as necessary.

**B. A second HNF1-response element is a major requirement for the action of HNF4α on the UGT1A9 promoter.** Mutations were introduced into the known HNF1 response element (HNF1s1mt) and a putative HNF1 binding site (HNF1s2mt, HNF1s2mt8) in the UGT1A9-2k promoter by site directed mutagenesis (see Figure 1). HepG2 cells were transfected with the UGT1A9 wild-type and mutant promoter reporter constructs in the presence of pCMX-PL2 or either of the HNF4α or HNF1α expression plasmids, as described for HEK293T cells. The results of both the HEK293T and HepG2 experiments are the means of triplicate samples, expressed as a relative value of firefly luciferase activity to the internal renilla control, compared to the pGL3-basic control (set to 1). The error bars indicate one standard deviation. ND = not done. P values for the indicated comparisons are *P < 0.001, ‡P = 0.036 and #P > 0.05 (not significant). Also, pGL3-1A9-2k HNF1s1mt and pGL3-1A9-2k HNF1s2mt constructs are statistically less responsive to HNF1α than the wild-type UGT1A9 promoter in HepG2 cells (†††P = 0.003 and ‡‡P = 0.038).
included in HEK293T transfections of the UGT1A9 promoter, a modest increase in luciferase reporter activity of 8-fold ($P < 0.001$) was observed. Interestingly however, when co-transfections of pGL3-1A9-2k, pCMX-HNF4α2 and pCMX-HNF1α were performed, a synergistic response of 45-fold was obtained ($P < 0.001$) (Figure 4.4A). A similar response was also seen with the deletion construct pGL3-1A9-321 ($P < 0.001$). In stark contrast, HNF1α over-expression did not activate the apoCIII construct in HEK293T cells, but rather served to diminish the promoter’s response to the presence of excess human HNF4α2 ($P = 0.036$), as previously reported (Kritis et al., 1993). Interestingly, simultaneous over-expression of both HNF4α and HNF1α in HepG2 cells transfected with the UGT1A9-2kb promoter did not greatly increase luciferase expression (less that 2-fold) relative to that achieved by HNF4α alone ($P = 0.026$) (Figure 4.5). However, HepG2 cells are known to express both HNF1α and HNF1β (Song et al., 1998) (Figure 4.3B). Therefore, I postulated that the expression levels of endogenous HNF1 factors in these cells, particularly HNF1α, was adequate to support the observed HNF4α-response, and that mutation of the HNF1α binding site(s) involved would reveal the synergistic nature of the interaction.

Further evidence corroborating the importance of HNF1α in the HNF4α-mediated regulation of UGT1A9 expression was obtained by substituting the weak UGT1A9 HNF4 site 2 element with an optimal HNF4 consensus sequence (HNF4s2cons). Rowley and co-workers recently showed that HNF1α and HNF4α worked to increase the promoter occupancy rate of each other on the fabp1 gene (Rowley et al., 2006). As both putative HNF4-binding sites of the UGT1A9 promoter perform poorly in EMSA, the possibility that the requirement for HNF1α in HNF4α-mediated up-
Figure 4.5: HNF1α only moderately increases activation of the UGT1A9 proximal promoter by HNF4α in HepG2 cells. HepG2 cells were transfected with 25 ng pRL-Null, 0.5 μg pGL3-basic or pGL3+1A9-2k reporter plasmid and 0.25 μg of the human HNF4α2 and/or HNF1α expression vectors. The DNA concentration in each transfection was kept constant by addition of empty pCMX-PL2 vector as necessary. The results presented are the means of triplicate samples, expressed as a relative value of firefly luciferase activity to the internal renilla control, compared to the pGL3-basic control (set to 1). The error bars represent one standard deviation. ‡P = 0.026.

regulation of the UGT1A9 promoter may have been simply due to its ability to recruit HNF4α was considered. However, it was found that substituting UGT1A9 HNF4s2 for HNF4s2cons did not substantially alter the response of the UGT1A9 promoter to either HNF1α or HNF4α. While the basal activity of the UGT1A9 promoter and the magnitude of the response to over-expressed HNF4α in HepG2 cells were both marginally increased (P = 0.002 for both) (Figure 4.2), the absolute requirement for HNF1α was completely preserved (Figure 4.4A). Although this result by no means eliminates the possibility that HNF1α is involved in recruitment of HNF4α to the UGT1A9 promoter, it does indicate that this factor has a true transactivational role in the observed partnership.
4.3.5. A second HNF1 site is essential for synergistic regulation of the *UGT1A9* promoter by HNF1α and HNF4α

To test whether the known HNF1-binding element was responsible for the synergistic response of the *UGT1A9* promoter to HNF4α and HNF1α, this region was mutated using site-directed mutagenesis. Interestingly, disruption of the *UGT1A9* HNF1 site 1 element decreased the HNF1α/HNF4α synergism in HEK293T cells by 60% (*P* < 0.001) (Figure 4.6), but only reduced the HNF4α-responsiveness of the *UGT1A9* promoter in HepG2 cells by 26% (not significant, *P* = 0.179) (Figure 4.4B). However, it did completely prevent HNF1α-responsiveness of the promoter in both HepG2 and HEK293T cells, indicating that the mutagenesis was successful (Figure 4.4B, *P* = 0.003 and Figure 4.6, *P* < 0.001). Therefore, I reasoned that there was at least one additional element in the *UGT1A9* proximal promoter that was involved in its regulation by HNF1α and HNF4α.

Examination of the *UGT1A9* promoter DNA sequence between nucleotides -321 and -184 revealed a second possible HNF1-binding site at position -290 to -278 bp (HNF1 site 2, Figure 4.1, also mentioned in Girard *et al.* (2004)). Mutation of this site reduced the ability of HNF4α to activate the *UGT1A9*-2kb reporter construct in HepG2 cells by nearly 90% (*P* < 0.001) whilst only decreasing the activation seen with HNF1α alone by 35% (*P* = 0.006) (Figure 4.4B). In HEK293T cells, mutation of this putative HNF1-binding site also had a significant negative impact on the synergistic regulation of the *UGT1A9* promoter by HNF1α and HNF4α (*P* < 0.001), which was greater than the effect of mutating the HNF1 site 1 sequence (*P* = 0.001) (Figure 4.6). In addition, EMSA analyses established that a probe over the HNF1 site2 element was similarly capable of binding *in vitro* synthesised HNF1α protein as the previously defined *UGT1A9* HNF1-binding site 1 (Gregory *et al.*, 2003) (Figure
Figure 4.6: Mutation of a second putative HNF1-binding site decreases the cooperative activation of the UGT1A9 proximal promoter by HNF1α and HNF4α in HEK293T cells. Two HNF1-binding sequences in the pGL3-1A9-2k reporter vector were inactivated individually and together by site-directed mutagenesis. The resulting constructs, as well as the indicated wild-type UGT1A7, UGT1A8, UGT1A9 and UGT1A10 promoter-reporters (0.5 μg of each), were transfected into HEK293T cells with 25 ng of pRL-Null and 0.25 μg pCMX-HNF1α or pCMX-HNF4α2 as indicated, using empty pCMX-PL2 vector to equalise the DNA concentration between the combinations as required. Cells were harvested 48 hours post-transfection and assayed for both firefly and renilla luciferase activity. Results shown are the mean firefly luciferase activities relative to the internal renilla controls, expressed as a fold induction over the promoterless pGL3-basic vector (set at a value of 1). Error bars indicate one standard deviation. P values for the indicated comparisons are *P < 0.001 and †P = 0.001.

4.7). Importantly, when mutations were incorporated into both newly discovered transcription factor binding sites, HNF4 site 2 and HNF1 site 2, the statistically significant residual responses to HNF4α by either of the singly mutated promoters in HepG2 cells (Figures 4.2, P = 0.012 and 4.4B, P < 0.001) were completely abolished (P = 0.219) (Figure 4.4B). Furthermore, the 14.2-fold and 12.8-fold HNF1α/HNF4α synergistic responses of the 1A9-2k HNF1s1mt (P < 0.001) and 1A9-2k HNF1s2mt (P < 0.001) constructs in HEK293T cells were also inhibited by mutation of both sites in the one vector (pGL3-1A9-2k HNF1s1&s2mt). Although the residual activation of the 1A9-2k HNF1s1&s2mt promoter by co-expressed HNF1α and
Figure 4.7: The second UGT1A9 HNF1-response element binds HNF1α in vitro. Electrophoretic mobility-shift assays were performed using 50,000 cpm of a $^{32}$P end-labelled oligonucleotide probe encompassing the known UGT1A9 HNF1 site (HNF1s1) and in vitro synthesised HNF1α protein (see “Methods”). HNF1α protein was also incubated with probes containing UGT1A9 HNF1s2 or the corresponding regions of the UGT1A7, UGT1A8 and UGT1A10 promoters. For the HNF1α super-shifts, 2 μg of anti-HNF1α antibody were added to the incubations immediately following the labelled probe. Complexes were resolved on a 4% (w/v) non-denaturing polyacrylamide gel. Arrows indicate the positions of free probe and complexes containing HNF1α. Wt, wild-type; Mt, mutant. Note: the right-hand panel showing the super-shifted Wt UGT1A7 HNF1s2 probe-HNF1α complexes is vastly over-exposed relative to the left-hand panel.
HNF4α was still significant in HEK293T cells, it was less than 2-fold ($P < 0.001$) (Figure 4.6).

**4.3.6. Additional evidence for a direct interaction of HNF4α with UGT1A9**

As mentioned in the introduction to this chapter (section 4.1.4.4), co-operative interaction between the HNF1α and HNF4α transcription factors on gene regulatory promoters other than UGT1A9 has been observed previously. In fact, it is becoming increasingly evident that the transcriptional relationship between HNF1α and HNF4α is extremely complex. Examples of genes with binding sites for both factors found to be co-operatively regulated by HNF4α and HNF1α include the rat fabp1, and human dihydrodiol dehydrogenase (DD)4 and insulin genes (Ozeki et al., 2001; Bartoov-Shifman et al., 2002; Divine et al., 2003). Yet not all promoters subject to HNF4α/HNF1α co-regulation are HNF1α dependent as UGT1A9 is. The fabp1 gene promoter responds to HNF4α in HeLa cells in the absence of HNF1α (Rowley et al., 2006), while the insulin gene is independently up-regulated by HNF4α in HEK293 cells (Bartoov-Shifman et al., 2002). Furthermore, it has been found that it not always necessary for a promoter to possess binding sites for both HNF1α and HNF4α for regulatory interactions to occur between these two factors. For instance, HNF1α can interfere with HNF4α mediated transcription of certain promoters that contain binding sites for HNF4α, but not HNF1, such as the upstream regulatory region of the apoCIII gene (Kritis et al., 1993) (Figure 4.4A). Of particular relevance to this project, HNF4α has also been observed to act as a co-activator for HNF1α on a liver pyruvate kinase promoter fragment that contains an HNF1 binding site, but no HNF4 elements (Eeckhoute et al., 2004). Further to the myriad of possible physical interactions between these two transcription factors, the HNF1α/HNF4α relationship is complicated by considerable cross-talk between their respective genes (Odom et
In liver, both transcription factors are able to positively regulate the other’s expression level through interactions with response elements in their respective promoters, while HNF1α can repress its own expression (Kuo et al., 1992; Ktistaki and Talianidis, 1997; Hatzis and Talianidis, 2001).

Given that there are multiple possible transcriptional interactions between HNF4α and HNF1α, and that the demonstrated binding of HNF4α to the HNF4s1 and HNF4s2 elements of the UGT1A9 promoter in EMSA is weak and seems somewhat incongruent with the large effect that this factor has on UGT1A9 promoter activity, it is reasonable to question whether HNF4α genuinely affects the UGT1A9 promoter by binding to the identified element, or through another, indirect mechanism. The latter could possibly occur through alteration of the cellular gene expression profile by HNF4α or by over-expressed HNF4α behaving as a co-factor for HNF1α.

To test whether the DNA binding function of HNF4α is necessary for its ability to regulate the UGT1A9 promoter, two separate approaches were used. Firstly, four HNF4α mutants were assessed for their ability to regulate UGT1A9 – two MODY mutants and two artificial mutants. MODY mutant HNF4α R127W has previously been shown to have a lower DNA-binding potential than wild-type HNF4α, while MODY mutant HNF4α E276Q has a normal affinity for the HNF4-binding site of the HNF1α gene but has impaired function when required to perform as a co-factor for HNF1α (Suaud et al., 1999; Yang et al., 2000; Eeckhoute et al., 2004). The artificial mutants, HNF4α DG69/70AA and HNF4α RR76/77AE, were created based on homology with the oestrogen receptor β (ERβ). Within the zinc-finger DNA-binding domain of the ERβ, amino acid pairs 167/168 and 174/175 have been specifically shown to interact directly with DNA and can be mutated to disrupt DNA binding without affecting a known protein-protein interaction with Stat5b also mediated by
this domain (Schwabe et al., 1993; Bjornstrom and Sjoberg, 2002). When the zinc-finger domain of HNF4α was aligned with the same region of the ERβ, the HNF4α nuclear receptor amino acid pairs homologous with ERβ positions 167/168 and 174/175 were 69/70 and 76/77 (Figure 4.8). Therefore, HNF4α amino acid pairs 69/70 and 76/77 were mutated to match the DNA-binding-deficient ERβ mutant (Bjornstrom and Sjoberg, 2002).

Figure 4.8: Alignment of the oestrogen receptor β and HNF4α DNA-binding domains. A. Linear alignment of the oestrogen receptor β (ERβ) and HNF4α DNA-binding domains using Clustal X software (Thompson et al., 1997). B. Schematic illustration of the ERβ DNA-binding domain adapted from Bjornstrom and Sjoberg (2002) and corresponding representation of the HNF4α sequence. Amino acids mutated to specifically interrupt DNA-binding are marked in bold.

When the two HNF4α2 mutants HNF4α R127W and HNF4α E276Q were tested for their ability to up-regulate the UGT1A9-2k promoter in HepG2 cells, it was found that the activity of HNF4α R127W was impaired by 32% (P < 0.001), but that HNF4α E276Q was more transcriptionally competent than wild-type HNF4α (P = 0.001) (Figure 4.9A). Similarly, both mutants could co-operate with HNF1α to
Figure 4.9: The HNF4α R127W, DG69/70AA and RR76/77AE mutants have decreased ability to up-regulate the *UGT1A9* promoter *in vitro*. A. and C. HepG2 and B. HEK293T cells were transfected in triplicate with 0.5 μg of pGL3-based reporter vector, 25 ng pRL-Null and 0.25 μg pCMX plasmids expressing wild-type or mutant HNF4α2 and/or wild-type HNF1α as described under “Methods”. The DNA concentration in each transfection was kept constant by addition of empty pCMX-PL2 vector as necessary. Cells were harvested 48 hours post-transfection and assayed for both firefly and *renilla* luciferase activity. Results shown are the mean firefly luciferase activities relative to the internal *renilla* controls, expressed as a fold induction over the promoterless pGL3-basic vector (set at a value of 1). Error bars represent one standard deviation. *P* values for the indicated comparisons are *P* < 0.001, †*P* = 0.001, ‡*P* = 0.023 and ‡‡*P* = 0.039. Also, **pCMX-HNF4α DG69/70AA increases transcription from the *UGT1A9*-2k promoter to a greater extent than pCMX in HepG2 cells (*P* < 0.001) and #pCMX-HNF4α RR76/77AE has no effect on the *UGT1A9*-2k promoter (*P* > 0.05, not significant).
regulate the *UGT1A9* promoter in HEK293T cells, but HNF4α R127W was less active than wild-type HNF4α (*P* = 0.039) (Figure 4.9B). In contrast to the MODY HNF4α mutants, HNF4α DG69/70AA and HNF4α RR76/77AE were almost or completely inactive against the *UGT1A9* promoter, the former only increasing reporter expression by 6% of wild-type HNF4α (*P* = 0.001) (Figure 4.9C). These results correlate with the ability of these mutants to bind the consensus HNF4-binding site probe in EMSA (Figure 4.10A) and are not due to poor expression of the HNF4α R127W mutant in transfection (Figure 4.10C) or poor expression of any mutants in the *in vitro* synthesised samples (Figure 4.10C). Since the HNF4α E276Q mutant is known to be defective as an HNF1α co-factor (Eeckhoute *et al.*, 2004) but has wild-type activity towards the *UGT1A9* promoter, it is unlikely that HNF4α behaves as an HNF1α co-factor in *UGT1A9* regulation.

For the second investigation, transfected cells were treated with S-nitroso-N-acetyl penicillamine (SNAP), a nitric oxide donor known to decrease the ability of HNF4α to bind DNA in HepG2 cells without causing HNF4α degradation (de Lucas *et al.*, 2004). It was found that HNF4α-mediated regulation of the *UGT1A9* promoter was significantly decreased relative to the solvent control (*P* < 0.001) in HepG2 cells treated with 500 μM SNAP (Figure 4.11). Furthermore, the detrimental effect of SNAP treatment on the activation of the *UGT1A9* promoter by HNF4α was more profound than its effect on the *apoCIII* promoter, which did not reach statistical significance (*P* = 0.086) (Figure 4.11). This result is consistent with the affinity of HNF4α for its binding site in the *UGT1A9* promoter being significantly less than that of the *apoCIII* binding site, and therefore easier to disrupt. Taken together with the HNF4α mutant data, these results strongly suggest that the DNA-binding function of HNF4α is important for the *UGT1A9* response.
Figure 4.10: Expression and binding capacity of wild-type and mutant HNF4α proteins in vitro. A and B. The HNF4α R127W, DG69/70AA and RR76/77AE mutants have decreased DNA binding in EMSA. Wild-type and mutant HNF4α proteins were generated by in vitro transcription/translation and tested for DNA-binding activity to 50,000 cpm of apoCIII 32P end-labelled oligonucleotide probe as described under “Methods”. Complexes were resolved on a 4% non-denaturing polyacrylamide gel; arrows indicate the positions of free probe and complexes containing HNF4α. Note that panel B is the same experiment as panel A, but with a longer exposure. No binding of HNF4α RR76/77AE was detected against the apoCIII probe after either exposure period. C. Expression of wild-type and mutant HNF4α proteins in in vitro synthesised samples as well as in transfected HEK293T cells was confirmed by Western blot. One microlitre of in vitro translated protein mix or 25 μg of HEK293T total cell lysate was probed for the presence of HNF4α protein by Western blot as described in Chapter 2, section 2.2.16. The same HNF4α E276Q TNT reaction was included in both blots as a cross-reference.
A

TNT HNF4α reaction

Anti-HNF4α antibody

HNF4α super-shifted complexes

HNF4α complexes

Unidentified TNT protein complexes

Free probe

Region shown in 4.10B

B

HNF4α complexes

Unidentified TNT protein complexes

C

HNF4α

\( ^{\text{Ve}} \), WT, R127W, E276Q, E276Q, DG69/70AA, RR76/77AE

\( ^{\text{Ve}} \), WT, R127W, E276Q

TNT reaction

Transfected HEK293T lysate
Figure 4.11: $S$-nitroso-$N$-acetyl penicillamine (SNAP) treatment decreases the ability of HNF4α to up-regulate the UGT1A9 promoter in HepG2 cells. HepG2 cells were transfected with 25 ng pRL-Null, 0.5 μg promoter-reporter vector (pGL3-basic, pGL3-1A9-2k or pGL3-apoCIII-810/+23) and 0.25 μg HNF4α2 expression vector according to section 4.2.8. Six hours after transfection, cells were treated with 500 μM SNAP for an additional 42 hours, and harvested by lysis. Total cell lysates were analysed for firefly and renilla luciferase activity, and the mean ($n = 3$) firefly to renilla luciferase ratios (relative to pGL3-basic, which is set arbitrarily to 1) are reported. Error bars represent one standard deviation, and $P$ values for the indicated comparisons are *$P < 0.001$ and # $P > 0.05$ (not significant).

Since it seems that the ability of HNF4α to bind DNA is required for regulation of the UGT1A9 promoter, the remaining question is whether HNF4α binds the UGT1A9 promoter directly, or up-regulates the gene promoter of another factor that subsequently binds the region labelled UGT1A9 HNF4s2. There are several reasons to believe that the former is the most likely scenario. Firstly, chromatin immunoprecipitation experiments performed by Barbier et al. (2005) show that the UGT1A9 promoter can be enriched for by immunoprecipitation using anti-HNF4α.
antibody. Secondly, the interactions observed in this chapter in HepG2 cells were confirmed in HEK293T cells. HEK293 cells have no endogenous HNF1 or HNF4α expression (Bernard et al., 1999; Maeda et al., 2002) (Figure 4.3B), and over-expression of HNF4α in HEK293 cells only up-regulates 57 of the 18,400 genes represented by the Affymetrix HG_U133A DNA oligonucleotide microarray. Only 3 of these 57 up-regulated genes are known to be transcriptional regulators (Lucas et al., 2005). Thirdly, mutation of the UGT1A9 HNF4s2 element to the consensus HNF4-binding sequence either did not change (Figure 4.4A) or strengthened (Figure 4.2) the HNF4α-response, while mutating this element away from the HNF4-binding site consensus abolished the observed phenomenon (Figure 4.2). Thus the factor(s) that bind to the UGT1A9 HNF4s2 region have a preference for HNF4-binding site-like elements. Given that the HEK293 transcriptome is hardly affected by HNF4α over-expression (Lucas et al., 2005), and that the factor that binds the HNF4s2 region prefers HNF4-like sites and co-operates with HNF1α, the likelihood that HNF4α is not interacting directly with the UGT1A9 promoter seems slim. Finally, apparent lack of, or weak binding of a factor under EMSA conditions is not always a good reflection of in vivo binding and function (Mitchelmore et al., 2000). If the HNF4α-UGT1A9 interaction is stabilised by other factors brought into close proximity by other response elements surrounding HNF4s2 (i.e. interactions not replicable in an EMSA reaction), this would account for the apparent discrepancy between the EMSA and transfection data. Thus, in the absence of evidence to the contrary, it seems highly likely that HNF4α activates the UGT1A9 promoter through direct binding, the most common mechanism for HNF4α-mediated transcriptional activation. This hypothesis could be tested further using in vitro chromatin assays, where naked DNA templates are assembled into chromatin and transcribed in vitro.
If addition of HNF4α to such a system, where it cannot alter the expression of any other components, also improves transcription from the UGT1A9 promoter, then direct interaction with the UGT1A9 promoter would be strongly implicated.

4.3.7. Nucleotide substitutions in the second HNF4 and HNF1 sites may partly explain the uniqueness of the UGT1A9 promoter response to HNF4α

As the original UGT1A9 HNF4α-binding element (HNF4 site 1) was insufficient to explain the differing responses of the UGT1A7-1A10 cluster to human HNF4α in my hands, I examined whether the second HNF1 and HNF4α-response elements could be responsible. When the appropriate regions of the UGT1A7-1A10 promoters were aligned it was evident that there was at least one nucleotide substitution in each of the three non-responsive promoters that decreased the homology to the HNF1α consensus binding site GTTAATNATTAAC (Tronche et al., 1997). Likewise, the UGT1A9 HNF4s2 region was not completely conserved in any of the three non-responsive promoters (Figure 4.1). Of particular interest was that the UGT1A8 and UGT1A10 promoters both have nucleotide differences to the UGT1A9 sequence within the core region of the HNF4α-binding sequence.

Substitution of the second UGT1A9 HNF1α site with the corresponding UGT1A8 sequence had a similar effect on promoter responsiveness as the mutation designed to totally abolish HNF1α binding. The UGT1A9-2kb HNF1s2mt8 promoter construct could only be activated by HNF4α in HepG2 cells to approximately 7-fold; considerably less than the 34-fold increase in reporter activity obtained from the wild-type promoter under the same conditions (P < 0.001) (Figure 4.4B). In addition, binding of in vitro synthesised HNF1α to UGT1A8 or UGT1A10 probes corresponding to the second HNF1α-response element of the UGT1A9 promoter could not be demonstrated (Figure 4.7). Furthermore, an equivalent probe from the
UGT1A7 promoter bound only a small fraction of the HNF1α protein retarded by either of the UGT1A9 HNF1-binding site probes, which was confirmed to be HNF1α by super-shift (Figure 4.7).

Exchange of the UGT1A9-HNF4s2 element for the equivalent UGT1A8 sequence was also extremely effective in reducing the HNF4α-responsiveness of the UGT1A9 promoter. The very low basal activity of the pGL3-1A9-2kb HNF4s2mt8 reporter vector could only be increased 3-fold by over-expression of HNF4α2 (P < 0.001) (Figure 4.2). In addition, binding of HNF4α to the UGT1A8 HNF4s2 probe in EMSA could not be demonstrated (Figure 4.3A).

To investigate whether the UGT1A9 HNF1s2 and HNF4s2 elements were sufficient to confer HNF4α-responsiveness to the UGT1A8 promoter, the relevant UGT1A9 nucleotides were introduced into the pGL3-1A8-2kb construct by site-directed mutagenesis. The HNF4s2 element was introduced into the UGT1A8 promoter alone, and in combination with the HNF1s2 element. However, neither construct could respond positively to over-expressed HNF4α when co-transfected into HepG2 cells. Conversely, the pGL3-1A8-2k reporter vectors carrying the HNF1s2mt9 and HNF4s2mt9 sequence changes were repressed by HNF4α over-expression (P < 0.001 and P = 0.002, respectively), while the double mutant was unaffected (P = 0.134) (Figure 4.12). This result shows that at least three elements of the UGT1A9 promoter are involved in its unique regulation by HNF4α.

4.3.8. The UGT1A9 initiator-like region contributes to its HNF4α-response

Since it is evident that additional, unidentified differences between the UGT1A9 and UGT1A8 promoters control whether these genes respond to HNF4α or not, I began the search for extra transcriptional elements that may be involved. In the past, my
colleagues and I have established that the UGT1A8 and UGT1A9 genes are differentially regulated through their Inr-like regions (Gregory et al., 2003); therefore, I hypothesised that LETFs bound to the UGT1A9 HNF4s2 and HNF1s2 sites can only influence transcription when combined with proteins that specifically bind the UGT1A9 Inr-like sequence. Using the UGT1A9-1kb promoter-reporter constructs available from our published study (Gregory et al., 2003), it was found that altering nucleotide -59 to the corresponding UGT1A8 sequence (UGT1A9-1k A-59G) decreased the UGT1A9 response to HNF4α (P = 0.013) (Figure 4.13). This mutation has been shown to allow more Sp1-containing protein complexes to bind to
Figure 4.13: The Inr-like region of UGT1A9 is more supportive of HNF4α-responsiveness than the equivalent region of UGT1A8. HepG2 cells were transfected with 0.5 μg reporter constructs carrying the proximal kilobase of the UGT1A9 promoter, 25 ng pRL-Null and 0.25 μg empty pCMX-PL2 or pCMX-HNF4α2 expression plasmid. The HNF4α-response of the wild-type promoter was compared to the response of similar constructs carrying UGT1A8 nucleotides at promoter positions -59, -62 or both. All results are the mean ratios of the firefly to renilla luciferase activity of three replicates, presented relative to the pGL3-basic control. The standard deviation of each triplicate is indicated by the error bars. NB: This experiment was only performed once. P values for the comparisons indicated are ‡P = 0.013 and #P > 0.05 (not significant).

the Inr-like region than occurs over the wild-type site (Gregory et al., 2003). Mutation of the other nucleotide difference that was important for UGT1A8 and UGT1A10 activity (at position -62), had no effect on the HNF4α-responsiveness of the UGT1A9 promoter alone (P = 0.876) (Figure 4.13) or when used in combination with the A-59G mutation (P = 0.060). Although these results show that the UGT1A9 Inr-like region is optimised for interaction with HNF4α relative to the equivalent UGT1A8 sequence, there is no absolute requirement for this nucleotide combination in the UGT1A9 HNF4α-responsiveness. Thus, the nucleotide difference(s) between
the UGT1A8 and UGT1A9 promoters that prevent UGT1A8 from being HNF4α-responsive, other than those that reside within HNF1s2 and HNF4s2, remain unknown. Clearly, further work is required to fully elucidate the mechanisms that drive the hepatic expression of UGT1A9.

4.3.9. HNF1β can support activation of the UGT1A9 promoter by HNF4α

Although encoded by separate genes, HNF1α and HNF1β share highly homologous dimerisation and DNA binding domains. As a consequence, these factors readily heterodimerise and recognise the same DNA response elements (Rey-Campos et al., 1991). Numerous genes that are regulated by HNF1α have also been shown to be responsive to HNF1β, albeit in many cases, to a lesser extent. However, I was unable to find any reports that demonstrated that HNF1β is able to co-operate in any way with HNF4α to increase gene transcription. Therefore, the ability of HNF1β to synergistically up-regulate the UGT1A9 promoter in conjunction with HNF4α was investigated. When HEK293T cells were co-transfected with the UGT1A9-2kb reporter construct and an HNF1β expression vector, the UGT1A9 promoter was activated 3.6-fold ($P < 0.001$), approximately half the increase achieved by HNF1α (Figure 4.14A). In addition, a synergistic response was observed between HNF1β and HNF4α; while these factors were individually able to increase reporter expression by 3.6-fold ($P < 0.001$) and 1.3-fold ($P = 0.005$) respectively in HEK293T cells, a combined response of 15-fold ($P < 0.001$) was obtained. Furthermore, HNF1β did not interfere with the HNF4α-response of the UGT1A9 promoter in HepG2 cells. Over-expression of HNF1β and HNF4α in HepG2 cells gave 123% of the UGT1A9 promoter response observed with HNF4α alone (not significantly different, $P = 0.411$) (Figure 4.14B). Conversely, a truncated HNF1α
Figure 4.14: HNF1β facilitates regulation of the UGT1A9 proximal promoter by HNF4α. A and C. HEK293T or B. HepG2 cells were transfected in triplicate with 0.5 μg pGL3-basic, pGL3-UGT1A9-2k or pGL3-1A9-2k HNF1s1&s2mt reporter vectors, 25 ng pRL-Null and the appropriate combinations of 0.25 μg pCMX-HNF4α2, pCMX-HNF1α, pCMX-HNF1β or pCMX-HNF1α 546X. All methods are described in section 4.2 and the DNA concentration in each transfection was standardised by addition of empty pCMX vector as necessary. Transfected cells were harvested 48 hours post-transfection and assayed for firefly and renilla luciferase activity. The results are presented as the mean firefly:renilla luciferase ratio, relative to the pGL3-basic control which is set to 1. Errors bars indicate one standard deviation. P values for the indicated comparisons are: *P < 0.001, ††P = 0.002 and #P > 0.05 (not significant). Also, activity of ** marked transfections differs from pGL3-1A9-2kb/pCMX co-transfections (P < 0.001). ¥Activity of the pGL3-1A9-2k + pCMX-HNF1α 546X co-transfection differs from the pGL3-basic + pCMX-HNF1α 546X, pGL3-1A9-2k HNF1s1&2mt + pCMX-HNF1α 546X and pGL3-1A9-2k + pCMX-HNF1α co-transfections (P < 0.001 for all three comparisons).
construct, HNF1α 546X that possesses DNA-binding and dimerisation domains but no activation domain I, and is not able to co-operate strongly with HNF4α on the *UGT1A9* promoter in HEK293T cells (Figure 4.14C), significantly inhibits the HNF4α-response of the *UGT1A9* promoter in HepG2 cells (Figure 4.14B) (*P* = 0.002). Therefore, it seems likely that endogenous HNF1α/exogenous HNF1β heterodimers and/or HNF1β homodimers are also capable of interacting with HNF4α to synergistically activate the *UGT1A9* promoter in HepG2 cells.

This work shows for the first time that HNF1β is able to synergistically up-regulate a promoter in conjunction with HNF4α. In contrast to the interactions between HNF1α and HNF4α, the functional relationship between HNF4α and HNF1β has had little attention. The few studies that have investigated interactions of HNF1β with HNF4α have shown that HNF1β either has no role in co-operative initiation of transcription with HNF4α (Hu and Perlmutter, 1999; Hatzis and Talianidis, 2001), or is detrimental to HNF4α-mediated promoter activity, even though HNF1α exhibits synergy (Bartoov-Shifman *et al.*, 2002). Although not well expressed in the adult liver, HNF1β binds to HNF1 sites with an affinity equal to that of HNF1α, and is highly expressed in the kidney (Rey-Campos *et al.*, 1991), an organ that also contains high levels of UGT1A9. These findings that HNF1β can co-operatively regulate the *UGT1A9* promoter with HNF4α in HEK293T cells, and does not interfere with HNF4α-mediated regulation in cells that express HNF1α are consistent with HNF4α also having a co-operative role in renal expression of *UGT1A9*.

### 4.3.10. Differences between published work and this study are not explained by usage of HNF4α1 or the *UGT1A9* T-275A polymorphism

In the course of this investigation, several important mechanistic differences between this work and that previously published (Barbier *et al.*, 2005) were found. In my
hands, the HNF4 site 1 element was not necessary for the response of the *UGT1A9* promoter to HNF4α, and a much greater response of the *UGT1A9* promoter to HNF4α was also observed. This was despite the experimental systems in the two studies being very similar, using the same cell line and the same reporter vector system.

Several possible explanations exist for the conflicting results of the two studies. Firstly, differences in the HepG2 cells used or culture conditions could be to blame. Alternatively, the use of different HNF4α isoforms could be responsible, as the authors did not specify the origin or identity of their HNF4α cDNA. In this study the most abundant HNF4α transcript from the human liver, HNF4α2, was used, although much of the literature describes studies done with the rat HNF4α1 splice variant, now known to exhibit lower activity than HNF4α2 in some circumstances (Sladek et al., 1999). A final possibility is that the work of Barbier et al. (2005) was performed with a *UGT1A9* promoter clone that possesses one or more polymorphic differences relative to our construct, resulting in the changed response. Since the prospect of different HNF4α isoforms or *UGT1A9* alleles resulting in an alternative outcome has interesting ramifications for understanding interindividual variation in UGT1A9 expression, it was decided to investigate these avenues further.

To explore whether other HNF4α isoforms commonly used in other regulatory studies performed similarly to the human HNF4α2 variant used in my work, human HNF4α1 and rat HNF4α1 were chosen and cloned into pCMX-PL2. When these HNF4α expression vectors were tested for their ability to regulate the *UGT1A9* promoter in HepG2 cells, it was found that both human and rat HNF4α1 were similar to human HNF4α2 (*P* = 0.893 and *P* = 0.252 respectively) in their ability to activate the *UGT1A9* promoter (Figure 4.15A and B). In addition, in HEK293T cells, the rat
Figure 4.15: Comparison of human HNF4α2, human HNF4α1 and rat HNF4α1 pCMX expression vectors for effectiveness against the UGT1A9 promoter. Human HNF4α1 and rat HNF4α1 variants were cloned into pCMX-PL2 and compared with human HNF4α2 for their ability to regulate the pGL3-1A9-2k reporter construct. A and B. HepG2 cells and C. HEK293T cells were transfected in triplicate with 0.5 μg pGL3-basic or pGL3-1A9-2k, 25 ng pRL-Null and 0.25 μg each HNF expression vector as appropriate. The DNA concentration in each transfection was kept constant by addition of empty pCMX-PL2 vector as necessary. Forty eight hours post-transfection, total cell lysates were assayed for firefly and renilla luciferase activities. Each column represents the mean firefly:renilla ratio achieved for each treatment, relative to the pGL3-basic control, which is set to 1. Error bars represent one standard deviation and the P values for each indicated statistical comparison are ††† P = 0.003 and # P > 0.05 (not significant).

HNF4α1 expression construct had greater activity than the original human HNF4α2 expression vector against pGL3-1A9-2k (P = 0.003) (Figure 4.15C). Thus, the difference in activity between the human HNF4α2 and rat HNF4α1 constructs was
insufficient to explain the discrepancies between my results and those previously published. Since the rat HNF4α1 cDNA was obtained from one of the authors of the conflicting paper, and is presumably the variant used in their work, it was concluded that investigation of other possible HNF4α variants was unlikely be meaningful, and I therefore next considered the possibility that UGT1A9 genotype may be the determining factor.

In humans, the hepatic concentration of UGT1A9 has been correlated with promoter genotype by Girard et al. (2004). Furthermore, in addition to the obvious differences between the HNF4α-reponses of Dr. Barbier’s and my UGT1A9 promoter constructs, I noticed that the basal activity of my UGT1A9 promoter construct also differed from that of Barbier et al. (2005). In contrast to the results of Barbier and co-workers, which show no basal activity for the UGT1A9 promoter in HepG2 cells above that of the empty reporter vector (Barbier et al., 2005), my 2 kb, 321 bp and 184 bp UGT1A9 promoters increased luciferase expression over background by 2 to 5-fold ($P \leq 0.008$, see Figure 4.2). Therefore, I hypothesised that promoter polymorphisms may account for the different promoter behaviours. In the work by Girard et al. (2004), polymorphisms at positions -275, -331/-440, -665 and -2152 predicted hepatic UGT1A9 expression. Of these, I considered the deoxythymidine to deoxyadenosine substitution at nucleotide -275 the most likely candidate, as it was: a) one of the SNPs most strongly associated with gene expression (Girard et al., 2004); b) the only one to be covered by the UGT1A9-321bp reporter construct; and c) positioned only three nucleotides downstream of the HNF1s2 element required for the HNF4α-response. If the nucleotide substitution at this position creates/strengthens a binding site for a transcription factor that restricts access of HNF1 factors to the HNF1s2 element, it was conceivable that this could adversely
affect the HNF4α-response of the UGT1A9 promoter. Therefore, deoxyadenosine was substituted into the UGT1A9-2k promoter at position -275, and the alternative sequence tested for any effects on basal or inducible activity.

In HepG2 cells, it was found that the UGT1A9-2k promoter had the same basal activity regardless whether the -275 base was thymine or adenine ($P = 0.438$) (Figure 4.16). Therefore, this base substitution alone cannot explain why Dr. Barbier’s UGT1A9 promoter constructs have no basal activity (Barbier et al., 2005). Interestingly however, the HNF4α2-response of the UGT1A9-2k T-275A construct was only 55% that of the reference promoter ($P = 0.001$) (Figure 4.16). Again, this altered response is insufficient to explain the differences between my results and that of Barbier and colleagues. Nonetheless, the reduced HNF4α-responsiveness of the UGT1A9-2k T-275A construct is of interest. This polymorphism occurs in Caucasian populations at a frequency of 0.06 to 0.07, and is associated with a higher level of UGT1A9 expression in the liver (Girard et al., 2004). Yet, it was also noted by the authors that this polymorphism is in strong association with another SNP (-2152) and that it is impossible to differentiate the causative effect of these two polymorphisms on UGT1A9 levels. Thus, it may transpire that: a) the -2152 SNP can over-compensate for any detrimental effect of the -275 SNP on HNF4α-mediated regulation; b) in cells that express high levels of UGT1A9 (HepG2 cells have relatively poor UGT1A9 expression: see Chapter 5) other available factors make the HNF1s2 site less vital for HNF4α-mediated regulation; and/or c) HNF4α is not as important in the in vivo expression of UGT1A9 as Barbier, myself and our respective colleagues have postulated. It will be of interest to further investigate the relationship between the function of the UGT1A9 promoter in vivo versus in vitro, its co-
operative regulation by HNF4α and HNF1α, and any involvement of these two SNPs.

Figure 4.16: The UGT1A9 -275 SNP decreases stimulation of the UGT1A9 promoter by HNF4α2 in vitro. The UGT1A9 T to A SNP at promoter position -275 was introduced into the UGT1A9-2k promoter by site-directed mutagenesis as described in “Methods”. HepG2 cells were co-transfected with 25 ng pRL-Null, 0.25 μg pCMX-PL2 or pCMX-HNF4α2 and 0.5 μg pGL3-basic, pGL3-1A9-2k or pGL3-1A9-2k T-275A, and analysed 48 hours later for firefly and renilla luciferase activity. The mean (n = 3) firefly activity of each experimental group, relative to the internal renilla control and expressed as a proportion of basal pGL3-basic activity (set to 1), is presented. Error bars indicate one standard deviation. The P value for the indicated statistical comparison is †P = 0.001. NB: This experiment was performed only once.

4.4 General discussion and summary

4.4.1. Achievement of aims

It has long been recognised that UGT1A9 is the only hepatically expressed enzyme of the UGT1A7-1A10 gene cluster. Barbier et al. (2005) recently showed that, while the human UGT1A9 promoter is governed by HNF4α, similar regions of the UGT1A7, UGT1A8 and UGT1A10 promoters are unaffected by HNF4α over-
expression. Since the four promoters are highly conserved, it was suggested that HNF4α may contribute to the unique liver-specific expression of UGT1A9, either independently or in conjunction with other transcription factors. The aims of this work were to further investigate the relationship between HNF4α and UGT1A9 promoter activity, and to identify key elements that distinguish UGT1A9 from the remaining UGT1A7-1A10 genes. Accordingly, a major element through which HNF4α interacts with the UGT1A9 promoter was identified. It was also shown that the HNF4α-response of the UGT1A9 promoter is completely dependent on the presence of HNF1 factors, and that there are at least three major functional differences between the UGT1A8 and UGT1A9 promoters that allow HNF1 and HNF4α to co-operatively up-regulate only the latter in hepatocyte-derived cells. Additional elements specific to the UGT1A9 promoter, such as the HNF4s1 and the Inr-like regions also support the UGT1A9 promoter response.

4.4.2. Future directions

This work has helped identify several possibilities for further investigation into the function of the UGT1A7-1A10 gene promoters. Firstly, the nature of the HNF4α-HNF1α interaction on UGT1A9 remains largely uncharacterised. Synergistic regulation by HNF4α and HNF1α operates on a number of hepatic genes in humans and rodents; yet it is clear that there is not just one standard mechanism by which this occurs. As previously highlighted, HNF4α-HNF1α synergy is not necessarily coincident with HNF1α-dependence of HNF4α-mediated activation, or the presence of HNF4-binding sites. Furthermore, different HNF4α and HNF1α mutations have different effects in different systems. The HNF4α E276Q mutant is known to have decreased physical interaction with HNF1α (Eeckhoute et al., 2004), yet is at least as active as wild-type on the UGT1A9 promoter. On the other hand, HNF4α R127W has
wild-type-like activity on some promoters, but not all (Navas et al., 1999; Lausen et al., 2000; Yang et al., 2000), including UGT1A9. Indeed, the only human gene that I can ascertain as having a similar response profile to HNF4α and HNF1α as UGT1A9 encodes DD4, which interestingly is another biotransformation enzyme. DD4 is involved in the bioactivation of polycyclic aromatic hydrocarbons and metabolism of pharmaceutical drugs (Ozeki et al., 2001). However, very little has been reported on the mechanistic aspect of DD4 gene regulation by HNF4α and HNF1α, and the HNF4 and HNF1-binding sites of the DD4 and UGT1A9 are very differently located (relative to each other and the TSS) - so it is reasonable to expect that detailed investigations will uncover regulatory differences between these genes. Such mechanistic differences are important for understanding how genes that are apparently controlled by the same factors are independently regulated and not affected in a blanket manner by environmental and genetic influences.

One of the ways that genes regulated by the same factors are thought to be individually controlled is through recruitment of specific co-factor combinations (Torres-Padilla and Weiss, 2003). It may transpire that the interaction between HNF4α and HNF1α on the UGT1A9 promoter is mediated by co-factor bridges between these two transcription factors, as direct interaction seems to be insufficient to explain the observed synergy. Not only is the HNF4α E276Q mutant (with decreased direct interaction with HNF1α) 100% active towards the UGT1A9 promoter, the less active HNF4α R127W mutant was found to be able to bind HNF1α at least as well as wild-type HNF4α (Rowley et al., 2006), and the HNF1α 546X mutant cannot co-operate with HNF4α on UGT1A9 promoter-reporter constructs (Figure 4.14). The latter mutant, HNF1α 546X, possesses all of the domains currently known to interact directly with HNF4α (Ktistaki and Talianidis,
1897; Rowley et al., 2006). Further investigation into the transcriptional relationship between HNF4α and HNF1α on the UGT1A9 promoter compared to other similarly regulated promoters will allow a better understanding of how they remain independently regulated, and which changes in conditions are likely to most greatly affect which genes.

Another aspect of the role of HNF4α and HNF1α in the regulation of UGT1A9 requiring further attention is its importance in vivo. Approaches that could be taken include the use of a cell line/primary cells that express high levels of UGT1A9, or extracts from liver tissue. In the former, it would be hoped that the UGT1A9-2k promoter would have much higher basal activity than in HepG2 cells, making it possible to determine the role of the HNF4s2 and HNF1s2 elements in the basal activity of the UGT1A9 promoter by mutation. This is an ideal complementary method to over-expression of transcription factors, as it does not rely on changes to the cellular environment to produce results, and thus removes one level of possible artefacts. In the latter, it would be possible to measure the levels of HNF4α and HNF1α mRNA or protein expression and investigate whether levels of either factor correlate with the expression of UGT1A9.

Apart from further characterisation of the HNF4α-HNF1α-UGT1A9 relationship, there are several other worthwhile avenues for investigation of UGT1A9 proximal promoter function highlighted by this work. Of particular interest would be studies that continue to elucidate the functional differences between the UGT1A7, UGT1A8, UGT1A9 and UGT1A10 promoters. As discussed earlier, the HNF4s2 and HNF1s2 sites are not present in UGT1A8, but when introduced, are still insufficient to allow HNF4α-mediated regulation of the UGT1A8 promoter. This observation may be the result of either a third important UGT1A9 element, or active repression of the
UGT1A8 promoter. In addition, whilst the UGT1A7 HNF1s2 is much weaker than the corresponding UGT1A9 element, it can still interact with HNF1α to some extent, and the UGT1A7 HNF4s2 element does not have a nucleotide substitution in the core binding region as is the case for UGT1A8 and UGT1A10 (Figure 4.1). Therefore, it will be of interest to see whether either UGT1A7 sequence can function in the context of the UGT1A9 promoter. If so, unidentified important element(s) of UGT1A9 may turn out to be the “master switch” determining the uniqueness of the UGT1A9 HNF4α-response, and may be of relatively high importance. Like the HNF1s2 site, this putative element may not appear to have any function when not required for the HNF4α-response, so may be easier to find using cells that have high basal UGT1A9 expression.

Another interesting difference between the UGT1A7-1A10 proximal promoters that could be pursued is the nature of their HNF1α-only-responses. This is of interest because UGT1A7, UGT1A8 and UGT1A10 all share the same HNF1s1, which is one nucleotide different to the corresponding site of UGT1A9 (Figure 4.1); yet these promoters clearly fall into two groups when classified by HNF1α-only-response (Figure 4.6). When HNF1α is over-expressed in HEK293T cells, the UGT1A7 and UGT1A9 promoters respond similarly, while UGT1A8 and UGT1A10 do not respond at all. Mutation of UGT1A9 HNF1s1 abolishes the HNF1α-response of this promoter. Presumably the HNF1α-response of the UGT1A7 and UGT1A9 promoters is reliant on an unidentified factor that does not interact with the UGT1A8 and UGT1A10 genes, at least in the same way.

Further work also needs to be done to understand the effect of genotype on UGT1A9 expression, and whether this is at all related to the observed HNF4α/HNF1α regulation. The UGT1A9 T-275A SNP seems to decrease the HNF4α-response of the
UGT1A9 promoter, even though it is associated with higher UGT1A9 protein concentration. However, whilst this is counterintuitive, as previously discussed there are several possible explanations for such a result. To see whether the T-275A nucleotide substitution does adversely affect HNF1α binding to HNF1s2, an EMSA using a probe that encompasses both the HNF1s2 and -275 regions could be used to see whether there is an unidentified factor that can competitively exclude HNF1α by binding over -275 and surrounding nucleotides.

Finally, it would be of interest to determine whether HNF4γ can also participate in the regulation of UGT1A9, either as a substitute for HNF4α, or as a competitor. The most similarly regulated human gene known, DD4, can be regulated by either HNF4α or HNF4γ (Ozeki et al., 2001). Furthermore, use of HepG2 extracts in EMSA showed protein complexes formed on the HNF4-binding site of DD4 contained HNF4γ and had the same apparent mobility as those with HNF4α. Presumably the complexes on the probe were largely, but not exclusively HNF4α-HNF4γ heterodimers, as antibody against HNF4α super-shifted all complexes, but antibody against HNF4γ only super-shifted a majority of the retarded probe (Ozeki et al., 2001). Thus, it is possible that the complexes that are present on the UGT1A9 HNF4-site probes in the Figure 4.3 EMSA also contain HNF4γ.

4.4.3. Relevance to glucuronidation in humans

Although a major detoxification pathway, glucuronidation in humans is subject to considerable interindividual variation, even within organs where UGT expression is considered to be both strong and constitutive. Variable UGT activity between independent liver samples has been noted for numerous chemicals (Court et al., 2001; Wiener et al., 2004; Girard et al., 2005) and quantitative PCR techniques have
shown that this may be partly due to altered levels of UGT transcripts (Congiu et al., 2002). An increasing number of studies suggest that such interindividual disparities may have important clinical implications, determining personal therapeutic outcome or disease development in certain contexts (Ramirez et al., 2002; Girard et al., 2004; Kuypers et al., 2005). Many of the known substrates of UGT1A9 are toxins, carcinogens/procarcinogens or pharmaceutical agents with relatively narrow therapeutic windows (see section 4.1.2), thus interindividual variation in UGT1A9 activity or expression may be a determinant of drug toxicity/efficacy or a risk factor for developing cancer (Albert et al., 1999; Ren et al., 2000; Malfatti and Felton, 2001; Gagne et al., 2002; Ramirez et al., 2002; Bernard and Guillemette, 2004). Furthermore, co-expression of UGT1A9 with other UGT1A family members alters the glucuronidation kinetics of substrates that are specific for the latter (Fujiwara et al., 2007b). Thus, changes in the expression levels of UGT1A9 relative to other UGT1As may also affect the clearance and detoxification of substances that are not UGT1A9 substrates.

Interindividual differences in UGT1A9 expression, particularly in the liver, are well established (Court et al., 2001; Congiu et al., 2002; Bernard and Guillemette, 2004; Girard et al., 2004). However, the mechanisms that give rise to the observed variability are largely uncharacterised. A number of UGT1A9 allelic variants have been discovered (Girard et al., 2004), but those that are strongly associated with altered UGT1A9 protein levels are relatively rare and insufficient to fully explain the variability observed. Apart from genetic variation in the target gene, interindividual variation in expression can arise through variation in the levels or activity of important transcription factors or their co-factors. In turn, this variation can arise from genetic diversity in transcription factor gene promoters or coding regions, in
exposure to stimuli that alter the activity of transcription factors, or variation in the transcription factors that control expression of the transcription factors (a never-ending interconnected web of possibilities). Functional genetic variants of HNF4α are known. Rare mutations, such as HNF4α R127W and HNF4α E276Q, are associated with MODY1 (Ryffel, 2001), while several common variants have been postulated to be risk factors for type 2 diabetes mellitus, high serum lipid levels and metabolic syndrome (Love-Gregory et al., 2004; Silander et al., 2004; Weedon et al., 2004; Ek et al., 2006; Hara et al., 2006; Weissglas-Volkov et al., 2006; Lehman et al., 2007). Similarly, several rare mutations in the HNF1α and HNF1β genes have been associated with MODY3 and MODY5 respectively (Ryffel, 2001), while common HNF1α variants have been associated with type 2 diabetes mellitus, insulin resistance, insulin and serum C-peptide secretion during oral glucose tolerance tests, and high-density lipoprotein cholesterol levels (Urhammer et al., 1997; Urhammer et al., 1998a; Chiu et al., 2000; Babaya et al., 2003; Chiu et al., 2003; Holmkvist et al., 2006). Since common functional variants exist, it is feasible that HNF4α or HNF1α variants (coding or regulatory region) may partly determine the level of UGT1A9 expression in human liver.

In addition, there are a number of stimuli and conditions that have been associated with altered HNF4α levels or activity. Negative regulators of HNF4α levels and/or activity include: polyunsaturated fatty acyl-CoAs, the levels of which can be modulated in the liver by diet (Hertz et al., 1998; Kalderon et al., 2002); acyl-CoA hypolipodemic pharmaceuticals such as fibrates (interestingly, these are also UGT1A9 substrates) (Hertz et al., 2001; Kalderon et al., 2002); bile acids such as CDCA (Popowski et al., 2005; Li et al., 2006); the inflammatory mediator nitric oxide, and cytokines IL-1β and transforming growth factor β1 (de Lucas et al., 2004;
Li et al., 2006; pharmaceuticals that are PXR ligands (for example rifampicin-ligated PXR competes with HNF4α for the co-activator PGC-1) (Bhalla et al., 2004); hypoxia (Mazure et al., 2001); and phosphorylation (Viollet et al., 1997; Leclerc et al., 2001). Notably, phosphorylation of HNF4α is increased by fasting (Viollet et al., 1997). Known positive regulators of HNF4α levels and/or activity include: hepatic saturated fatty acyl-CoAs of length C14-C16, the levels of which again are related to diet (Hertz et al., 1998; Kalderon et al., 2002); differentiation, particularly in intestinal cells as they move from the crypt to villus (Stegmann et al., 2006); the transcription factor HNF1β (Wang et al., 2004a), hepatitis C virus (HCV) infection (Qadri et al., 2006); and increasing availability of acyl-CoA-binding protein (Petrescu et al., 2003).

A number of the stimuli and conditions that affect HNF4α also influence HNF1α. For instance, HCV infection increases HNF1α expression (Qadri et al., 2006), as does differentiation in Caco-2 enterocytes (Hu and Perlmutter, 1999); while the inflammatory cytokine IL-1β (Geier et al., 2003) and bile acids such as CDCA adversely affect HNF1α (Jung and Kullak-Ublick, 2003). The latter occurs through repression of HNF4α-mediated activation of the HNF1α gene promoter (Jung and Kullak-Ublick, 2003). Ceramide also reduces available active HNF1α protein (Park et al., 2004a), while lipopolysaccharide (Roe et al., 2001) and tumour necrosis factor α (Geier et al., 2003) decrease HNF1α-mediated transcription in treated cells.

Whether any of the listed factors affect UGT1A9 expression through modulation of HNF4α and HNF1α-mediated regulation remains unknown; however, this will depend on whether any affect HNF4α activity by mechanisms relevant to UGT1A9 transcription, and what effect they have on other transcription factors that also control the UGT1A9 promoter. For example, the inhibition of HNF4α by CDCA and
IL-1β is mediated through disrupted recruitment of PGC-1 (Li et al., 2006); so if PGC-1 is important in HNF4α-mediated regulation of UGT1A9, then UGT1A9 expression may be affected by changes in CDCA and IL-1β levels. Also, alternative cell types may be differentially affected by each stimulus, as genes expressed in more than one cell type are not necessarily driven by identical transcriptional complexes in each (Schaeffer et al., 1993; Navalon-Garcia et al., 2006).

Interesting possibilities for determinants of UGT1A9 expression via the HNF4α/HNF1α interaction include fasting and inflammation. Fasting not only decreases HNF4α activity, but also increases the risk of hepatotoxicity after paracetamol ingestion (Whitcomb and Block, 1994). Fasting depletes the availability of glutathione and UDP-glucuronic acid, required for the oxidation and glucuronidation of paracetamol (Whitcomb and Block, 1994; Zimmerman and Maddrey, 1995). However, it is conceivable that the effect of prolonged fasting on the hepatotoxicity of paracetamol may also be enhanced by decreased expression of relevant UGTs through phosphorylation of HNF4α. The three most active UGTs towards paracetamol are UGT1A9, UGT1A1 and UGT1A6 (Court et al., 2001), and all are potential HNF4α-target genes (see Chapter 5 for UGT1A1 and UGT1A6). On the other hand, inflammation decreases the hepatic expression of some human UGTs. In liver biopsies with increased IL-1β mRNA, UGT1A9 mRNAs tended to be reduced (although the trend failed to reach significance for the 5 samples tested, 4 of which were HCV infected) (Congiu et al., 2002). Given that HCV infection may partly compensate for the effect of inflammatory mediators on HNF4α and HNF1α by increasing their expression, it would be interesting to determine whether the likely loss of UGT1A9 expression is: a) related to the effects of inflammation on HNF4α and HNF1α; and b) greater in instances where hepatitis is not HCV-related.
4.4.4. Summary

This study highlights the synergistic role of HNF1α and HNF4α in regulation of the UGT1A9 promoter in vitro, and illustrates the potential for both promoter and transcription factor variants to alter UGT1A9 expression. The discovery of two new response elements of the UGT1A9 promoter enhances our understanding of the mechanisms that may contribute to hepatic, and possibly renal, expression of UGT1A9. Evidence presented in this chapter also points to the existence of at least one additional crucial element for hepatic UGT1A9 regulation that remains unidentified. Finally, this study shows that HNF1β can be substituted for HNF1α in the synergistic regulation of UGT1A9, the first time a positive HNF4α-HNF1β interaction has been reported.

HNF4 and HNF1 transcription factors are both expressed in tissues where UGT1A9 is found: in the liver, kidney and gastrointestinal tract (Sladek et al., 1990; Rey-Campos et al., 1991). Interestingly, HNF1α levels have already been correlated with UGT2B7 mRNA, while both HNF4α and HNF1α have been correlated with DD4 transcript abundance in human liver (Toide et al., 2002; Ozeki et al., 2003). Therefore, it seems feasible that a UGT1A9 promoter-specific interaction between HNF4α and HNF1 could contribute to the unique expression pattern of this protein amongst the UGT1A7-1A10 cluster, and that variation in the levels or activity of these factors could influence UGT1A9 expression.
5.1 Introduction

5.1.1. Promoter-reporter assays versus endogenous gene expression

The use of synthetic promoter-reporter constructs in transient-transfection cell-based assays is a common approach for investigating the function of eukaryotic gene promoters. As evidenced by earlier chapters, this methodology can yield many useful insights into the relationships between a promoter nucleotide sequence, the proteins that interact with this promoter, and the resulting mRNA synthesis - its major advantage being that the experimental system is relatively simple and amenable to manipulation. Not only can putative transcription factors be over-expressed in the host cell to assess their effect on reporter gene expression, but mutations and bona fide genetic variations can also be introduced into the promoter or the over-expressed transcription factors to further elucidate function. However, there are also a number of significant limitations to this approach. Noteworthy disadvantages of promoter-reporter assays include the following: a) each promoter must be cloned before any hypotheses can be tested (which can become quite arduous if multiple genes are to be investigated); b) the reporter plasmid is not incorporated into the genome and is therefore presumably not subject to the same degree of epigenetic control as the endogenous gene; and c) important enhancer and silencer elements may reside many kilobases upstream or downstream of the proximal promoter, or within introns, and therefore may be unintentionally excluded from analysis.
To help negate these concerns, changes in endogenous gene expression caused by chemical ligand exposure or exogenous transcription factor expression can also be studied. Quantification of endogenous gene expression can be achieved through a variety of methods, including biochemical enzyme assays, Western analysis of protein expression, or PCR detection of mRNA transcripts. This chapter describes the transcriptional response of human *UGT1A* and *UGT2B* genes to over-expression of ten LETFs in the human hepatocellular carcinoma cell line, HepG2. These experiments were performed in untreated cells, but also in cells treated with a histone deactetylase inhibitor, trichostatin A (TSA), to investigate any association of histone deacetylation with *UGT* gene repression in HepG2 cells. Finally, transient transfection assays were used to further investigate several of the interesting interactions between LETFs and *UGT* promoters discovered in this way, as there is still no convenient way of manipulating promoter sequences *in vivo*. Such comparisons are also useful to determine whether results obtained in the easily manipulated *in vitro* systems are likely to bear any relevance to the *in vivo* situation.

### 5.1.2. Aims

The aims of the work presented in this chapter were two-fold. These were to:

1. Identify new regulatory factors for the human *UGT* genes;

2. Further investigate known interactions between transcription factors and *UGT* promoters in a setting that may represent the *in vivo* situation better than transient plasmid transfections. Of particular interest were the HNF1 interactions with the *UGT1A1* (Bernard *et al.*, 1999), *UGT1A3*, *UGT1A4* (Gardner-Stephen and Mackenzie, 2007b), *UGT1A9* (Gregory *et al.*, 2004)
and UGT2B7 (Ishii et al., 2000) promoters, and the HNF4α interaction with UGT1A9 (Barbier et al., 2005; Gardner-Stephen and Mackenzie, 2007a).

There were several good reasons to expect to find new LETF-binding sites in at least some human UGT promoters. Firstly, human UGTs are not expressed ubiquitously, but most are expressed at moderate to high levels in the human liver (with the exception of UGT1A5, UGT1A7, UGT1A8 and UGT1A10). Of these exceptions, UGT1A7, UGT1A8 and UGT1A10 are expressed to high levels in at least one section of the gastrointestinal tract, an organ where many of the transcription factors known as LETFs are also expressed. In general, genes that have been found to be controlled by one or more LETFs generally possess multiple LETF-binding sites in their promoters. In contrast, only one or two LETF-binding sites have been discovered for most UGT genes; therefore, it seemed likely that a thorough investigation of human UGT genes would confirm them as targets of further LETFs.

The developmental profile of hepatic UGT expression (see Chapter 1, section 1.8.1) also indicates that the current understanding of UGT regulation is lacking. Much of the past work on human UGT promoters has focused on the role of HNF1 factors in driving transcription; however, HNF1 factors are expressed early in liver development (De Simone et al., 1991; Cereghini et al., 1992), while UGTs are not detected in early foetal liver and are not well expressed until after birth, even though most have been shown to possess HNF1-binding sites. This suggests that additional LETFs, expressed later in hepatic development, are also required for UGT expression.
5.1.3. Rationale

The transcription factors used in this experiment were PXR, HNF1α, HNF1β, HNF4α, HNF6, FoxA1, FoxA2, FoxA3, C/EBPα and C/EBPβ. Since the most important organ for UGT expression and activity is the liver, these proteins were chosen on the basis that they are all LETFs, and represent five separate protein families or superfamilies: zinc-finger nuclear receptors (HNF4 and PXR), POU homeodomain proteins (HNF1), onecut homeodomain proteins (HNF6), forkhead box proteins (FoxA) and basic-region leucine-zipper proteins (C/EBP) (Schrem et al., 2002; Schrem et al., 2004). Further considerations made in choosing these transcription factors were that PXR, HNF1α, HNF1β, HNF4α and C/EBPα have previously been identified as having a role in the expression of at least one UGT in humans or rodents (Hansen et al., 1998; Bernard et al., 1999; Ishii et al., 2000; Rae et al., 2001; Gardner-Stephen et al., 2004; Barbier et al., 2005; Gardner-Stephen and Mackenzie, 2007b; Gardner-Stephen and Mackenzie, 2007a), while HNF6, C/EBPβ and the FoxA family members, have been identified as regulators of other drug-metabolising enzymes in humans and rodents (Jover et al., 1998; Delesque-Touchard et al., 2000; Jover et al., 2002; Rodriguez-Antona et al., 2003; Bort et al., 2004). In particular, HNF1α, HNF1β and HNF4α were chosen because they have been shown to regulate the human UGT1A3, UGT1A4 and/or UGT1A9 promoters in vitro in work presented earlier in this thesis. The structural and functional features of HNF1α, HNF1β and HNF4α have been discussed at length in Chapters 3 and 4; therefore, the following literature review will only address these aspects in detail for the newly introduced transcription factors.
5.1.3.1. *Pregnane X receptor*

The pregnane X receptor (NR1I2) is a ligand-regulated nuclear receptor that contains two zinc fingers (constituting the DNA-binding domain) and a large ligand-binding domain of 293 amino acids. This ligand-binding domain is also required for dimerisation and transcriptional activation (Bertilsson *et al.*, 1998; Lehmann *et al.*, 1998; Kliewer *et al.*, 2002). PXR is expressed in the liver, and to a lesser extent, the colon and small intestine (Bertilsson *et al.*, 1998; Lehmann *et al.*, 1998), and heterodimerises with RXRα (Kliewer *et al.*, 1998) to activate genes through binding sites consisting of dual AGGTCA-like NRREs. These NRREs are arranged as direct or everted repeats, separated by three to eight nucleotides (Kliewer *et al.*, 2002).

On ligand binding, PXR undergoes a conformational change that facilitates interaction with p160 co-activators, such as SRC-1, PGC-1 and the receptor-associated co-activator 3 (Itoh *et al.*, 2006; Johnson *et al.*, 2006; Li and Chiang, 2006), while disrupting associations with co-repressors such as SMRT (Johnson *et al.*, 2006). Transcriptional activation of target genes by liganded PXR/co-activator complexes is then brought about through both histone acetylation and direct interactions with the basal transcription machinery. In addition to its associations with p160 proteins, PXR is also known to interact directly with other nuclear receptors and transcription factors to influence target gene transcription. PXR activity can be inhibited by the presence of the SHP nuclear receptor that lacks a conventional DNA-binding domain (Ourlin *et al.*, 2003). PXR has also been shown to recruit HNF4α to the CYP3A4 promoter but interferes with HNF4α regulation of CYP3A7. Both of these latter interactions may be mediated through a co-activator common to PXR and HNF4α, PGC-1 (Bhalla *et al.*, 2004; Li and Chiang, 2006). PXR also synergises with HNF4α to regulate the CYP2C9 gene (Chen *et al.*, 2005b).
Interestingly, binding of alternative ligands to PXR may change its interactions with other transcription factors: PXR-mediated induction of the \( \text{CYP3A4} \) promoter is variably reliant on interactions with Sp1, FoxA proteins or C/EBP\( \alpha \), depending on the ligand bound (Bombail et al., 2004).

The known ligands of PXR are a structurally diverse set of compounds that include several pregnenolone and progesterone derivatives, lithocholic acid, and many pharmacologically active substances such as paclitaxel, hyperforin, clotrimazole and rifampicin (Bertilsson et al., 1998; Lehmann et al., 1998; Moore et al., 2000; Staudinger et al., 2001; Synold et al., 2001). Whilst the murine homologue of PXR shares 96% identity with the human protein within the DNA-binding domain, there is only 77% homology within their ligand-binding domains. Thus, while murine PXR binds the same DNA targets as human PXR, the two homologues respond to a different, but overlapping, set of ligands (Bertilsson et al., 1998; Lehmann et al., 1998; Staudinger et al., 2001).

It has been proposed that PXR operates as a master regulator in the elimination of xenobiotics from the body, as liganded PXR regulates the expression of numerous biotransformation enzymes and transporter proteins, including \( \text{CYP3A4}, \text{CYP3A7} \) and \( \text{CYP2C9} \), (Bertilsson et al., 1998; Pascussi et al., 1999; Chen et al., 2004), \( \text{UGT1A1}, \text{UGT1A3}, \text{UGT1A4}, \text{UGT1A6} \) and \( \text{UGT1A9} \) (Rae et al., 2001; Gardner-Stephen et al., 2004; Soars et al., 2004; Sugatani et al., 2004; Bock and Kohle, 2005; Chen et al., 2005a), as well as MRP and perhaps glutathione-S-transferase family members (Kast et al., 2002; Maglich et al., 2002). PXR expression is itself subject to control by other ligand-activated nuclear receptors such as \( \text{PPAR}\alpha \) (Aouabdi et al., 2006) and possibly the oestrogen and glucocorticoid receptors (Gibson et al., 2006).
PXR may also be involved in a negative feedback loop that inhibits its own expression (Gibson et al., 2006).

As PXR has already been shown to up-regulate transcription of endogenous UGTs in HepG2 cells (Gardner-Stephen et al., 2004), this transcription factor was chosen to serve as the positive control for this study. Furthermore, whilst UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9 and UGT2B7 have all been identified as potential PXR targets by various research groups, their transcriptional responses have not been quantitatively assessed with methods as sensitive as real-time PCR. Finally, of the PXR-responsive UGT genes, a NRRE has only been identified for UGT1A1. Confirmation of other responsive genes would justify a search for further PXR-responsive elements within the UGT1A locus.

5.1.3.2. The hepatocyte nuclear factor 1 family

Of the transcription factors thought to be important in the constitutive expression of UGTs, HNF1α is, by far, the most thoroughly studied. Putative HNF1-binding sites have been identified in the proximal promoters of all human UGT genes (Figure 5.1) (Auyeung et al., 2001; Gardner-Stephen and Mackenzie, 2005), and function has been demonstrated for sites in the UGT1A1 (Bernard et al., 1999), UGT1A3, UGT1A4 (Gardner-Stephen and Mackenzie, 2007b and this thesis), UGT1A8, UGT1A9, UGT1A10 (Gregory et al., 2004; Gardner-Stephen and Mackenzie, 2007a and this thesis), UGT2B7 (Ishii et al., 2000) and UGT2B17 (Gregory et al., 2000) promoters. There is also substantial circumstantial evidence pointing to HNF1α as an important regulator of UGT expression: the expression of UGTs and HNF1α in adult tissues overlaps considerably (compare Chapter 1, Table 1.2 to Chapter 3, section 3.1.5.2); HNF1α knockout mice have reduced UGT expression (Pontoglio et al., 1996; Shih et al., 2001); many of the identified human UGT HNF1 sites are highly
Figure 5.1: All known human UGT1A and UGT2B promoters contain putative HNF1-binding sites. The UGT1A3-1A5, UGT1A7-1A10 and UGT2B proximal promoter regions containing HNF1-binding elements are aligned. The putative HNF1-binding sites are boxed, with the sites that have been confirmed experimentally indicated in bold. Numbering is relative to the initiation codon of UGT1A1, UGT1A3, UGT1A6, UGT1A9 and UGT2B7 as appropriate. This figure was adapted from Auyeung et al. (2001), Bernard et al. (1999), Gardner-Stephen and Mackenzie (2005), Gardner-Stephen and Mackenzie (2007b) and Gardner-Stephen and Mackenzie (2007a).

Conserved within UGT genetic sub-clusters (Figure 5.1), indicating likely functional importance; UGT2B7 expression levels have been correlated with HNF1α mRNA in human liver samples (Toide et al., 2002); and an extensive microarray study detected...
occupation of the \textit{UGT1A1}, \textit{UGT2B11} and \textit{UGT2B15} promoters by HNF1\textalpha in primary hepatocytes (Odom \textit{et al.}, 2004).

However, despite all this evidence, there is still some doubt about the relative importance of HNF1\textalpha in the expression of each UGT enzyme \textit{in vivo}. Reasons for this include; lack of correlation of UGT2B15 with HNF1\textalpha levels (Toide \textit{et al.}, 2002), residual bilirubin glucuronidation in HNF1\textalpha-knockout mice (Pontoglio \textit{et al.}, 1996), the aforementioned inability of either HNF1\textalpha or HNF1\textbeta to turn on UGT expression during early liver development, and the possibility that transcription factor sites found to be crucial for the activity of short promoter constructs may be less important in longer sections of the same promoter, as is the case for the proximal C/EBP\textalpha sites in the \textit{CYP3A4} gene (Martinez-Jimenez \textit{et al.}, 2005). Therefore, it was hoped that inclusion of HNF1\textalpha in this study would shed further light on the relative importance of this transcription factor for expression of each UGT enzyme.

Although HNF1\textbeta binds the same DNA response elements as HNF1\textalpha, the relevance of HNF1\textbeta to human UGT expression is not as well established. Potentially, HNF1\textbeta could also up-regulate UGT expression through the same sites as HNF1\textalpha. Alternatively, HNF1\textbeta may modulate the effects of HNF1\textalpha through competition or dimerisation, as it is generally accepted that HNF1\textbeta has a lower transactivation potential than HNF1\textalpha. However, functional interaction of HNF1\textbeta with human UGT promoters has, so far, only been demonstrated for \textit{UGT1A1} (Bernard \textit{et al.}, 1999) and \textit{UGT1A9} (Gardner-Stephen and Mackenzie, 2007a and this thesis).

\textbf{5.1.3.3. Hepatocyte nuclear factor 4\textalpha}

HNF4\textalpha is a zinc-finger nuclear receptor involved in the expression of numerous human enzymes involved in biotransformation, including CYP3A4, CYP3A5,
CYP2A6, CYP2B6, CYP2C9, CYP2D6 (Jover et al., 2001; Li and Chiang, 2006) and UGT1A9 (Barbier et al., 2005; Gardner-Stephen and Mackenzie, 2007a and this thesis, Chapter 4). Naiki and colleagues have also found that the UGT1A family of mRNA transcripts are increased in HepG2 cells infected with adenovirus expressing rat HNF4α (Naiki et al., 2004). Therefore, it seemed likely that HNF4α plays a role in regulating UGT genes other than UGT1A9. Strengthening this hypothesis, HNF4α was found to occupy the UGT2B11 and UGT2B15 promoters in human hepatocytes (Odom et al., 2004). Furthermore, in the mouse, HNF4α has been implicated in the regulation of both Ugt1a1 and Ugt1a9. Interestingly though, knockout of HNF4α expression in the mouse (Hayhurst et al., 2001) resulted in a decrease of murine Ugt1a9 transcripts by 73% (Barbier et al., 2005), while, in contrast, HNF4α−/− mice had 14-fold higher levels of Ugt1a1 mRNA than their wild-type litter mates (Ding et al., 2006).

A second reason for studying the effect of HNF4α on UGT expression comes from developmental observations. Although HNF4α is expressed early in hepatic development (Duncan et al., 1994), the ratio of the various HNF4α splice variants (see Chapter 4, section 4.1.4.2) present in embryonic liver changes as the hepatocytes mature. HNF4α7 transcripts are found at high levels in embryonic hepatocytes, but decrease around the time of birth to become only a minor fraction of the total liver HNF4α mRNA (Nakhei et al., 1998). In contrast, HNF4α1 and HNF4α2 are also expressed in embryonic liver, but are increased perinatally (Torres-Padilla et al., 2001). HNF4α1, HNF4α2 and HNF4α7 all share the same DNA-binding domain; however, HNF4α7 has a distinct N-terminal domain that allows it to interact differently with co-activators and co-repressors from HNF4α1 and HNF4α2, and thus regulate different genes (Torres-Padilla et al., 2001; Torres-Padilla et al., 2002).
of the major determinants thought to be responsible for the perinatal increase in HNF4α1 and HNF4α2 expression is the concomitant rise in glucocorticoids, which also induces several other hepatic genes at birth (Berger et al., 1996; Bailly et al., 2001). Moreover, GR and HNF4α have been shown to co-operatively regulate the hepatic tyrosine aminotransferase gene (Nitsch et al., 1993). Since expression of most human UGTs is initiated or increased around birth (see Chapter 1, section 1.8.1), it is possible that birth-related changes in LETF expression, such as the switch in HNF4α isoforms, are involved. Interestingly, UGT1A1 has already been shown to be a direct target of GR (Sugatani et al., 2005a), so the potential for a synergistic activation of UGT1A1 by GR and HNF4α also exists.

5.1.3.4. Hepatocyte nuclear factor 6

The transcription factor hepatocyte nuclear factor 6, also known as Onecut 1, possesses a single cut-domain and a divergent homeodomain, and binds to DNA sequences of consensus DRRTCVATND where D = A, G or T, R = A or G and V = A, G or C (Lemaigre et al., 1996; Lannoy et al., 1998; Jacquemin et al., 1999). The second, and only other known, human member of this transcription factor class is Onecut 2, which is expressed in liver and skin (Jacquemin et al., 1999). HNF6 is expressed in human liver and pancreas (Rausa et al., 1997) and regulates the hepatic expression of a number of proteins such as glucokinase (Lannoy et al., 2002) and the transcription factors FoxA2 and HNF4α (Samadani and Costa, 1996; Landry et al., 1997; Rausa et al., 1997; Hatzis and Talianidis, 2001; Briancon et al., 2004; Odom et al., 2004). In turn, the hnf6 gene is under the transcriptional control of HNF4α and C/EBPα, at least in the rat (Lahuna et al., 2000; Rastegar et al., 2000). HNF6 has been reported to physically interact with FoxA2 to both positively and negatively regulate gene promoters, depending on the promoter configuration (Delesque-
Touchard et al., 2000; Rausa et al., 2003; Rubins et al., 2005), while synergistic interactions have been identified with HNF1α, C/EBPα, HNF4α and another nuclear receptor, the retinoic-acid-receptor-related orphan receptor α (Hatzis and Talianidis, 2001; Nacer-Cherif et al., 2003; Beaudry et al., 2006; Yoshida et al., 2006). The co-activators recruited to a target gene by HNF6 depend on the target promoter and include CBP, P/CAF and PGC-1α (Lannoy et al., 2000; Rausa et al., 2003; Beaudry et al., 2006).

HNF6 has previously been reported to be bound to the UGT1A1, UGT2B11 and UGT2B15 promoters in liver when assessed by a chromatin immunoprecipitation experiment coupled with an extensive promoter microarray (Odom et al., 2004). However, no functional studies have yet been performed for these interactions.

5.1.3.5. The forkhead box A (FoxA, HNF3) family

The human FoxA transcription factor family, also known as the HNF3 family, is comprised of three isoforms: FoxA1 (HNF3α), FoxA2 (HNF3β) and FoxA3 (HNF3γ). Encoded by separate genes, these three proteins share a winged helix/forkhead box DNA-binding domain of 93-95% identity, through which they bind as monomers to DNA sequences of consensus VAWTRRTKRYTY (where V = A, G or C, W = A or T, R = A or G and K = G or T) (Overdier et al., 1994). FoxA1, FoxA2 and FoxA3 are able to interact with nucleosome core histones H3 and H4 to open compacted chromatin and alter nucleosome positioning, thereby facilitating the binding of other transcription factors to target promoters (McPherson et al., 1993; Shim et al., 1998; Chaya et al., 2001; Cirillo et al., 2002). In situations where this occurs, FoxA1 binding to its DNA elements is more stable on nucleosome-bound DNA than free DNA (Cirillo and Zaret, 1999). In addition, FoxA factors may also
contribute to target gene expression by enhancing the stability of the pre-initiation complex (Crowe et al., 1999).

Of the FoxA proteins, FoxA3 is the most widely and highly expressed. In the adult mouse, FoxA proteins are found in liver, lung, stomach, small intestine and colon. FoxA3 is the predominant FoxA species in these tissues; except the lung, from which it is absent. In particular, hepatic FoxA3 expression is approximately treble that of either FoxA1 or FoxA2, and may be a negative regulator of the latter two in the liver (Kaestner et al., 1998). FoxA3 expression is also detectable in mouse heart, adipose tissue, thymus, ovary and testes. In contrast, FoxA1 is found in the brain, pancreas, kidney and prostate, while FoxA2 is most highly expressed in the pancreas (Kaestner et al., 1994; Rausa et al., 1997; Besnard et al., 2004). FoxA factors are involved in the regulation of many hepatic genes, including gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase and aldolase B (Vallet et al., 1995; Friedman and Kaestner, 2006), and a number of human CYPs, including CYP3A4, CYP2C8, CYP2C9, CYP2C18 and CYP2C19 (Bombail et al., 2004; Bort et al., 2004).

Like most other LETFs, FoxA proteins regulate, and are regulated by, other LETFs. Hnf4α is a FoxA3 target gene (Bailly et al., 2001), and FoxA proteins have a weak, but positive effect on the hnf1α promoter (Kuo et al., 1992) – at least in rodents. In turn, foxA1 and foxA2 are subject to auto- and cross-regulation through FoxA-binding sites in their promoters (Pani et al., 1992; Peterson et al., 1997; Zhang et al., 2005), foxA2 is a target of HNF6 and C/EBP factors (Samadani et al., 1995; Samadani and Costa, 1996; Lahuna et al., 2000), while foxA3 is a target gene of HNF1 (Hiemisch et al., 1997). In addition, many functionally relevant, physical interactions between FoxA and other transcription factors or co-activators have been reported. For example, FoxA factors bound to the α-1-microglobulin/bikunin
precursor gene promoter support HNF1 driven transcription, yet inhibit the effects of HNF4α (Rouet et al., 1995); FoxA factors compete with HNF1 for binding on the rat aldolase B gene (Gregori et al., 1994); HNF1α, HNF6 and C/EBPα each interact synergistically with FoxA family members to activate transcription of various target genes (Cha et al., 2000; Delesque-Touchard et al., 2000; Rausa et al., 2003; Rodriguez-Antona et al., 2003); FoxA2 inhibits HNF6 stimulation of the murine glut2 promoter (Rausa et al., 2003); and SHP physically interacts with all three FoxA proteins to repress their DNA-binding activity and therefore, the activation of their target genes (Kim et al., 2004). The FoxA proteins have also been postulated to behave as “pioneer factors” for nuclear receptors such as the glucocorticoid, oestrogen and androgen receptors (Gao et al., 2003; Laganiere et al., 2005; Zhang et al., 2005) - proceeding and facilitating binding of the nuclear receptors to their respective NRREs (Friedman and Kaestner, 2006). Of particular interest is a recent report that small interfering RNA (siRNA)-mediated knockdown of FoxA1 transcripts in MCF-7 breast cancer cells results in a reduction of oestrogen-induced recruitment of the oestrogen receptor to the UGT2B17 promoter (Laganiere et al., 2005).

Despite the many similarities and apparent redundancies that have been observed for FoxA1, FoxA2 and FoxA3 in hepatic gene regulation, functionally distinct roles for the different proteins are emerging, particularly in embryonic development (Friedman and Kaestner, 2006). In addition, FoxA2 is the only isoform to interact with HNF6 to co-operatively regulate the foxA2 promoter (Rausa et al., 2003) although FoxA1 and FoxA2 can both synergistically enhance HNF6-mediated regulation of the rat CYP2C12 promoter (Delesque-Touchard et al., 2000). Furthermore, the co-activator PGC-1 only interacts with FoxA3 (Kim et al., 2004),
while FoxA1 expression is specifically diminished in hepatocytes during the acute-phase response (a condition that also results in reduced CYP activity) (Shedlofsky et al., 1994; Qian et al., 1995).

FoxA1, FoxA2 and FoxA3 were included in this study because there is substantial evidence for their involvement in regulating genes of biotransformation pathways, especially CYPs. Many transcription factors identified as CYP regulators have subsequently been shown to also regulate genes with conjugative functions; therefore, the FoxAs were considered important LETFs to test for involvement in UGT regulation. In addition, FoxA1 has been indirectly implicated in the expression of UGT2B17, at least in breast cells (Laganiere et al., 2005). A second reason to test these factors was the uncertainty that surrounds the ability of transient transfection systems to adequately assess the function of FoxA proteins. Although chromatin-mediated repression of transcription from transiently transfected promoter-reporter plasmids is relieved by FoxA expression in certain cases (Crowe et al., 1999), most data suggests that the importance of transcription factors with chromatin-altering functions may be underestimated in plasmid-based assays. Transiently transfected plasmids do not always acquire the same higher-order chromatin structure as observed with genomic DNA and may be inappropriately accessed by ubiquitous factors (Smith and Hager, 1997). In addition, inconsistent results between plasmid-reporter and endogenous responses to FoxA genes have been previously reported (Bort et al., 2004); therefore, it was considered prudent to assess the effects of FoxA over-expression on genomic UGT promoters.

5.1.3.6. The CCATT/enhancer binding protein family

The C/EBP transcription factor family is one subset of the basic region leucine zipper (bZIP) transcription factor family that also includes c-jun, c-fos and CREB.
Members of the bZIP family of proteins are characterised by a bipartite bZIP domain, consisting of a basic region for DNA binding and a leucine zipper region for dimerisation (Landschulz et al., 1989; Ramji and Foka, 2002). Sequential binding of two monomers to the palindromic repeat RTTGCYGAA (where R = A or G and Y = C or T) in target DNA allows the formation of a dimer, which then stabilises the DNA-protein complex (Hsu et al., 1994; Osada et al., 1996; Kohler et al., 1999). C/EBP factors can homodimerise, heterodimerise with each other, or heterodimerise with other bZIP proteins, such as those of the CREB protein family. C/EBPα and C/EBPβ heterodimers bind DNA with the same specificity as their respective homodimers; however, C/EBP heterodimers with other bZIP proteins generally bind asymmetric sequences composed of the consensus half-sites for each monomer (Williams et al., 1991; Hsu et al., 1994; Shuman et al., 1997).

The C/EBP transcription factor family consists of six members: C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε and C/EBPζ, although only C/EBPα and C/EBPβ are considered true LETFs (Lekstrom-Himes and Xanthopoulos, 1998; Schrem et al., 2004). In humans, C/EBPα is expressed in placenta, liver, intestine, lung, peripheral blood leukocytes, skeletal muscle, pancreas, heart, spleen, prostate and adipose tissue (Antonson and Xanthopoulos, 1995; Harp et al., 2001). Likewise, at least in rodents, C/EBPβ is expressed in liver, intestine, adipose tissue, lung, heart and spleen, but also kidney (Cao et al., 1991; Williams et al., 1991). Expression of C/EBPβ in human liver has been confirmed, although protein levels appear to be subject to considerable interindividual variation (Ferrini et al., 2001; Tomizawa et al., 2003). C/EBPγ and C/EBPζ are ubiquitously expressed (Roman et al., 1990; Ron and Habener, 1992; Thomassin et al., 1992), while C/EBPδ is constitutively expressed in a limited number of tissues (intestine, adipose tissue and lung), yet is strongly
induced in liver and many other tissues during the acute-phase response (Cao et al., 1991; Alam et al., 1992; Kinoshita et al., 1992; Yamada et al., 1997). C/EBPε is expressed in myeloid and lymphoid cell lineages, but not liver (Antonson et al., 1996).

The study of gene regulation by C/EBPα and C/EBPβ is complicated by the fact that both proteins are expressed as multiple isoforms, generated through use of internal methionines as alternative initiation codons. C/EBPα has two functionally distinct isoforms (p42 and p30) while C/EBPβ has up to four (p38, p35: also known as liver-enriched transcriptional activator protein (LAP), p21: also known as liver-enriched transcriptional inhibitory protein (LIP), and p14). Full-length and LAP-C/EBPβ can also be proteolytically cleaved in newborn liver to generate LIP-C/EBPβ. (Descombes and Schibler, 1991; Lin et al., 1993; Ossipow et al., 1993; Welm et al., 1999; Xiong et al., 2001; Ramji and Foka, 2002). The truncated isoforms p30-C/EBPα and LIP-C/EBPβ both possess full DNA-binding and dimerisation capabilities, but lack most or all of the N-terminal activation domains of their full-length counterparts (Ramji and Foka, 2002).

While full-length p42-C/EBPα acts as a transactivator for many hepatic genes and inhibits cell proliferation, the N-terminally truncated p30-C/EBPα lacks antimitotic activity and has attenuated transcription activation potential. p30-C/EBPα was found to activate the murine C/EBPα promoter; however, very little activity was demonstrated towards another known C/EBPα target, the mouse albumin promoter. p30-C/EBPα was also able to dramatically reduce p42-C/EBPα-driven transcription from the albumin promoter, but only when present in vast excess. At the p30/p42 ratios normally seen in mammalian liver, only a small degree of inhibition was
observed compared to activation by p42-C/EBP\(\alpha\) alone (Lin et al., 1993; Ossipow et al., 1993).

Similarly, LIP-C/EBP\(\beta\) does not possess the same activation potential as the full-length and LAP-C/EBP\(\beta\) proteins. However, in contrast to the C/EBP\(\alpha\) isoforms, LIP-C/EBP\(\beta\) usually behaves as a dominant-negative inhibitor of LAP-C/EBP\(\beta\) activity, inhibiting LAP-C/EBP\(\beta\)-mediated transactivation at low LIP/LAP ratios. It has been proposed that this is due to inactivation of LAP through heterodimerisation, combined with a higher affinity of the LIP homodimers and LIP-LAP heterodimers for the common DNA recognition sequences than LAP homodimers. The ratio of LAP-C/EBP\(\beta\) to LIP-C/EBP\(\beta\) in the hepatocyte is important in maintaining differentiation, and for controlling proliferation in response to liver damage. Pertinent to this, LIP-C/EBP\(\beta\) production can be regulated independently of LAP-C/EBP\(\beta\) translation through binding of the CUG repeat binding protein CUGBP1 to C/EBP\(\beta\) mRNA; favouring translation of the low-molecular-weight isoforms (Descombes and Schibler, 1991; Timchenko et al., 1999; Welm et al., 2000; Luedde et al., 2004; Timchenko et al., 2005). It should also be noted that, because the shorter C/EBP isoforms are produced by leaky ribosomal scanning, transient transfection with plasmids carrying the full-length C/EBP\(\alpha\) or C/EBP\(\beta\) coding regions results in the expression of the truncated as well as the full-length isoforms (Descombes and Schibler, 1991; Ossipow et al., 1993; Xiong et al., 2001).

In humans, C/EBP\(\alpha\) has been identified as a regulator of numerous hepatic genes including albumin, \textit{ADH2}, insulin-like growth factors (van Dijk et al., 1992; van Ooij et al., 1992; Jover et al., 1998), and \textit{CYP3A4}, \textit{CYP3A5}, \textit{CYP3A7}, \textit{CYP2B6}, \textit{CYP2C9}, \textit{CYP2D6} and \textit{CYP2A6} (Jover et al., 1998; Rodriguez-Antona et al., 2003; Bombail et al., 2004). In mice, many additional hepatic targets of C/EBP\(\alpha\) have been
identified, but one of particular interest is *Ugt1a1* (Lee *et al.*, 1997). Deletion of the *C/EBPα* gene in mice leads to severe jaundice due to an increase in unconjugated serum bilirubin. The rat *UGT2B1* promoter has also been proposed to be a *C/EBPα* target gene (Hansen *et al.*, 1998).

In regards to its participation in the LETF network, *C/EBPα* regulates its own expression in both humans and mice, although the mechanism involved varies between the two species. In humans, autoregulation of the human *C/EBPα* promoter is mediated indirectly through the ubiquitously expressed upstream stimulating factor, rather than through a direct *C/EBP*-binding site as found in mice (Timchenko *et al.*, 1995; Schrem *et al.*, 2004). *C/EBPα* also positively influences the rate of cleavage of *C/EBPβ* to LIP-*C/EBPβ* in mice and in human cells (Welm *et al.*, 1999), while other LETF target genes of *C/EBPα* include the murine *hnf4a1* promoter (Bailly *et al.*, 2001), the rat *hnf6* gene (Rastegar *et al.*, 2000) and rat *foxA2* (Samadani *et al.*, 1995; Yoshida *et al.*, 2006). As highlighted previously, *C/EBPα* is also known to be able to synergistically activate some of its target genes with other transcription factors such as PXR (Bombail *et al.*, 2004), HNF6 (Yoshida *et al.*, 2006) and FoxA3 (Rodriguez-Antona *et al.*, 2003). HNF4α (Pitarque *et al.*, 2005) and LAP-*C/EBPβ* (van Ooij *et al.*, 1992) are also able to co-operatively activate target genes with *C/EBPα*.

As is the case for *C/EBPα*, much more work has been done on the regulation of rodent genes by *C/EBPβ* than human genes. However, known human hepatic target genes of *C/EBPβ* include *MDR1, ADH1, ADH2, ADH3, CYP2A6* and *CYP3A4* (van Ooij *et al.*, 1992; Combates *et al.*, 1994; Martinez-Jimenez *et al.*, 2005; Pitarque *et al.*, 2005).
As C/EBPα and C/EBPβ bind the same DNA elements, there is substantial overlap in C/EBPα and C/EBPβ target genes. However, not all genes with C/EBP-binding sites are responsive to both. For example, the C/EBPα gene target UGT2B1 is not transactivated by C/EBPβ, whereas the ADH1 promoter responds specifically to C/EBPβ (van Ooij et al., 1992; Hansen et al., 1998). Similarly, synergistic interactions between C/EBPα and other LETFs, such as HNF6, are not necessarily supported by C/EBPβ (Yoshida et al., 2006), or may even be repressed. The normally activating C/EBPβ isoform, LAP-C/EBPβ, interferes with HNF4α-activation of the CYP2A6 promoter, even though C/EBPα co-operates with HNF4α on the same promoter (Pitarque et al., 2005).

In mice and rats, C/EBPβ regulates its own promoter and that of C/EBPα (Chang et al., 1995; Welm et al., 2000). The C/EBPβ promoter is also responsive to pro-inflammatory cytokines such as IL-6 and IL-1. Consequently, hepatic C/EBPβ expression is increased in inflammation, the acute-phase response and in response to mechanical liver damage. In addition, translation of the LIP-C/EBPβ isoform is increased under these conditions, and appears to cause the concomitant decrease in C/EBPα expression seen during the acute-phase response (Alam et al., 1992; Welm et al., 2000; Jover et al., 2002). Increases in C/EBPβ expression have also been associated with maintenance of FoxA2 expression during the acute-phase response in rats (Samadani et al., 1995). Synergistic interactions of C/EBPβ with other LETFs have been identified for HNF1α and HNF1β (Divine et al., 2003), C/EBPα (van Ooij et al., 1992) and at least one of the rat FoxA proteins (Pani et al., 1992).

C/EBPα and C/EBPβ were chosen for inclusion in this study for several reasons. Firstly, C/EBPα has a role in the expression of two non-homologous rodent UGTs, while both transcription factors regulate multiple human enzymes important for
metabolism and excretion of lipophilic compounds. Although there is no evidence for C/EBPβ-mediated regulation of mammalian UGTs as yet, these two proteins bind the same recognition sequences, heterodimerise, and regulate each other’s expression. Therefore, it was considered appropriate to include C/EBPβ as well as C/EBPα in this study. Secondly, the expression of C/EBPα and C/EBPβ during development is similar to that seen for many UGTs. Glucuronidation of most UGT substrates is absent or substantially lower in human foetal liver relative to adult, but increases directly after birth and reaches adult levels in the first few months or years of life (see Chapter 1, section 1.8.1). In rodents, C/EBPα expression is detectable in early liver development (Westmacott et al., 2006), but increases substantially late in foetal liver development, spikes around birth and is only found at high levels in fully differentiated cells (Birkenmeier et al., 1989). Likewise, C/EBPβ is more strongly expressed in late than early foetal liver development with a transient increase around birth, but importantly, the LAP/LIP ratio gradually increases by 5-fold between the period just before birth and adulthood (Descombes and Schibler, 1991). This relative increase in LAP-C/EBPβ has been associated with the gradual postnatal increase in expression of certain hepatic genes (van Ooij et al., 1992) and could potentially affect UGTs. Thirdly, expression of C/EBP proteins is altered in disease states that also affect human UGTs. During inflammation, both the levels of C/EBPα and the ratio of LAP/LIP-C/EBPβ in hepatocytes decrease, along with the expression of numerous CYP enzymes and, although to a lesser extent, several UGTs (Alam et al., 1992; Welm et al., 2000; Congiu et al., 2002; Aitken et al., 2006). C/EBPα levels have also been reported to be very low in human hepatocellular carcinoma, a condition that has also been associated with decreased expression of all hepatic UGT1A forms except UGT1A6 (Strassburg et al., 1997a; Xu et al., 2001). Given
that expression of C/EBP proteins is altered in several circumstances that are also associated with changes in UGT expression, it was of interest to determine whether either C/EBPα or C/EBPβ levels in HepG2 cells could affect the transcription of any endogenous UGT genes.

5.1.3.7. Choice of method

The effect of a given treatment on the expression of endogenous genes can be measured at several molecular levels. Ideally, it is best to measure final functional protein activity, as this is the outcome of most biological and clinical significance; however, protein and mRNA levels are two other common reference points that are used to assess changes in gene expression. The risk associated with measuring gene expression at points other than protein activity is that many genes are regulated through post-transcriptional and post-translational mechanisms. For example, proteins that are incorrectly folded or erroneously modified/unmodified (e.g. by leader sequence cleavage, phosphorylation or glycosylation) may be inactive, but will still usually be detected by Western blot. Furthermore, mRNA levels do not always correlate well with protein; for example, the tissue distribution of C/EBPα and C/EBPβ mRNA is far broader than that of detectable proteins (Williams et al., 1991).

However, for technical reasons, when it is of interest to study all known human UGTs, it is currently most appropriate to measure changes in UGT expression at the mRNA level. Assessment of the levels of each individual UGT by function would require unique probe substrates for each, and whilst substrates that are glucuronidated solely by one UGT have been identified, the substantial overlap in substrates between UGT proteins still precludes the measurement of all family members in this way. Furthermore, the effects of heterodimerisation between UGT
forms on function are poorly characterised, and may affect the interpretation of results in cells expressing multiple UGTs. Likewise, because of the high similarity in amino acid sequence between UGT family members, especially within the genetic sub-clusters, it is not possible to distinguish between all UGT forms with currently available antibodies. In contrast, there are sufficient dissimilarities between the coding sequences of each UGT to allow the design of oligonucleotide pairs that only amplify one target gene. Therefore, the current methods of choice for measuring UGT expression changes are PCR-based. In the case of UGTs, it is generally assumed that the resulting data is meaningful, as multiple studies imply that increases in UGT mRNA correspond with increases in UGT protein, and increases in UGT protein correspond with increases in glucuronidation (Girard et al., 2004; Sugatani et al., 2004; Harrington et al., 2006).

5.2 Methods

5.2.1. Generation of liver-enriched transcription factor expression plasmids
Each LETF was cloned into the pCMX-PL2 expression vector as described in detail in Chapter 2, section 2.3. In brief, PXR, HNF1α, HNF1β, HNF4α and FoxA3 were cloned directly from human cDNA, while the remaining transcription factor sequences were sourced from either rat or mouse orthologues, as attempts to procure them from human material were unsuccessful. HNF6 was cloned from mouse liver cDNA and has 99% homology to human HNF6 at the amino acid level. FoxA1 and FoxA2 were sub-cloned from vectors containing the rat sequences for each gene, which have 92% and 96% homology with the respective human amino acid sequences. Rat C/EBPα and C/EBPβ were also sub-cloned from previously
constructed vectors, and have 93% and 71% identity to their orthologous human amino acid sequences.

5.2.2. Transfection of HepG2 cells and extraction of total RNA

HepG2 cells cultured as described in Chapter 2.2.1 were plated in 6-well plates at a density of $1 \times 10^6$ cells per well, 24 hours before transfection. Transfection with 5 μg of expression plasmid was achieved using Lipofectamine 2000 as described in section 2.2.10. All cells were harvested for total RNA at 72 hours post-plating (48 hours post-transfection, 42 hours after addition of rifampicin or 24 hours after addition of TSA). RNeasy Mini spin-columns were used to purify the total RNA according to the manufacturer’s instructions as detailed in Chapter 2, section 2.2.3.1. All transfection/treatment combinations described were performed at least twice in independent experiments, with the exception of the 300 nM TSA-treated transfections, which were only performed once.

5.2.3. Trichostatin A treatment of HepG2 cells

Twenty-four hours post-transfection, the culture medium of each well was replaced with fresh medium containing 300 nM TSA, 3 μM TSA or vehicle (1:1000 diluted ethanol). Non-transfected cells were also treated with TSA or vehicle, 48 hours post-plating.

5.2.4. Rifampicin treatment of HepG2 cells

Six hours after transfection of cells with pCMX-PXR, the culture medium of each transfected or control well was replaced with fresh medium containing vehicle (DMSO diluted 1:1000) or 10 μM rifampicin. To ensure exposure to 10 μM rifampicin for the whole incubation period, the culture medium and DMSO/rifampicin treatments were replaced again at 30 hours post-transfection.
5.2.5. Reverse transcription and quantitative real-time PCR

Column-purified total RNA was treated with DNase I, and reverse-transcribed into cDNA using the random hexamer method detailed in Chapter 2, sections 2.2.3.3 and 2.2.3.4. After RNase H treatment, one 25th of each cDNA sample was used for quantification of UGT transcripts, or one 800th for quantification of 18S rRNA. The generic methods for QPCR can be found in Chapter 2, section 2.2.6.6, while the specific primers, annealing temperatures and templates used to generate standard curves are detailed in Table 5.1. Each pair of PCR primers was validated for specificity towards only the desired gene by visual inspection of the PCR product after gel electrophoresis and by sequencing, prior to the commencement of this PhD candidature. This work was performed by Anne Rogers, Dr. Takahito Nishiyama and me. Likewise, the plasmid templates used to generate standard curves for each gene, as listed below, were constructed before the commencement of this PhD candidature by Dr. Takahito Nishiyama and Anne Rogers. The pEF-IRES and pBS derived vectors, as well as pCR-blunt-2B28, contain the entire coding regions of the indicated genes. The remaining vectors each contain a single copy of the PCR product generated when the listed primers are used against the corresponding gene.

It should be noted that this application of the QPCR method determines the relative, not the absolute, concentration of target transcripts. This is due to at least three reasons. Firstly, the apparent mRNA copy number is dependent on whether cDNA synthesis is primed with gene-specific or random hexamer primers (Zhang and Byrne, 1999). Secondly, cDNA synthesis of the target and reference templates may not occur at the same efficiencies (Zhang and Byrne, 1997), and thirdly, the PCR amplification efficiencies of plasmid standards and their corresponding cDNA
<table>
<thead>
<tr>
<th>Gene</th>
<th>Nucleotide Sequence (5’→3’)</th>
<th>Nucleotide Position on Target RNA</th>
<th>Amplicon (bp)</th>
<th>Annealing temp (°C)</th>
<th>Standard</th>
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<td>HNF1α</td>
<td>F: CCAACACAGGTGCCTCCACCCCTGTT R: CCGTGTGGGTGTACTGGGCCACCT</td>
<td>HNF1α: +1280 to +1304</td>
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<td>pCMX-HNF1α</td>
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<td>UGT1A1</td>
<td>F: TTTTGTCTGGCTGTTCCTCCACT* R: GAAGGTCATGTGATCTGAATGAGA*</td>
<td>UGT1A1: +368 to +388</td>
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<tr>
<td>UGT1A4</td>
<td>F: ACCTGGGCTACACTCAAAG* R: TCATTATGCAGTAGCTCCACCAA*</td>
<td>UGT1A4: +277 to +296</td>
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<td>58</td>
<td>pBS-1A4</td>
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<td>UGT1A6</td>
<td>F: CTTTACACAGACCGCCCTTAC* R: TATCCACATCTCCTGGGACAG*</td>
<td>UGT1A6: +439 to +460</td>
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<td>F: TGGCTCGTGCAGGGGTGGACTG R: TTGCAGTTG GaGCTCCACAGC</td>
<td>UGT1A7: +2 to +22</td>
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<td>R: TCAGCCAGCAGCTCACCACAGGG*</td>
<td>UGT2B7: +485 to +463</td>
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<td></td>
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<td>UGT2B10: +583 to +562</td>
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<tr>
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<td>R: TCAGCCAGCAGCTCACCACAGGG*</td>
<td>UGT2B11: +485 to +463</td>
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Table 5.1 continued.

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<th>Amplicon (bp)</th>
<th>Annealing temp (°C)</th>
<th>Standard</th>
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<td>R: CAGGTACATAGGAAGGGAGGAA*</td>
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<tr>
<td></td>
<td>R: TCAGCCAGCAGCTCACCCACAGGG*</td>
<td>UGT2B28: +485 to +463</td>
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</table>

*Primer sequences obtained from Congui et al. (2002). #Primer sequence obtained from Strassburg et al. (1997b). Mismatches between mRNA and primers are indicated in bold text. F: forward primer; R: reverse primer.
targets are unlikely to be the same (due to differences in secondary or tertiary structure, as well as differences in the sensitivity of double versus single stranded standards (Overbergh et al., 1999)). To obtain precise mRNA copy numbers for each gene, it is necessary to spike the RNA preparations with synthetic RNA standards before cDNA synthesis (Bustin, 2000). Ideally, the target and reference genes should also be measured in the same tube to account for loading errors. However, as the purpose of this study was to measure change in UGT mRNA levels rather than the absolute concentration, it was deemed that the relative values this protocol would provide were both adequate and appropriate. Indeed, QPCR, as performed in this study, is currently the most common method used to measure the relative levels of specific mRNA transcripts between samples.

5.2.6. Cloning of the UGT1A3-9.4k and UGT1A4-5k promoters

Attempts to amplify the entire 9.4 kb DNA region separating the UGT1A3 and UGT1A4 first exons in one piece were unsuccessful. This was primarily due to the high similarity of the UGT1A3 primers to regions flanking the UGT1A4 promoter. Because the entire UGT1A4 promoter is only 5 kb, this sequence always amplified in preference to the longer UGT1A3 sequence. Therefore, the following strategy was used to obtain a pGL3-1A3-9.4k clone.

Firstly, 50 ng of BAC clone 1308M2 DNA was used as template to amplify the 6.4 kb immediately upstream of the UGT1A3 first exon using the Expand™ Long Template PCR System, which utilises a mixture of Taq and Tgo DNA polymerases to optimise amplification range and fidelity (4.8 × 10⁻⁶ mutations per base pair per duplication). The primer site 6.4 kb downstream of the UGT1A3 initiation codon was chosen to avoid repetitive DNA sequences in the UGT1A3 promoter as well as
regions of homology with the *UGT1A4* promoter. The PCR reaction was performed in 1× “Expand™ Buffer 3” with 500 µM dNTPs, 300 nM each primer (1A3prom-6.4kNhe1: 5’ AGCCATGCTAGCTCATTAAGTGGAAGTGGATCA 3’, and 1A3UTRNhe1: 5’ AGCCATGCTAGCTCAGCAGAGACACGGACA 3’) and 3.75 Units Expand™ DNA polymerase mix. The PCR parameters were: an initial step of 94°C for 2 minutes; followed by 10 cycles of 94°C for 10 seconds, 60°C for 30 seconds and 68°C for 10 minutes; followed by a further 25 cycles of 94°C for 10 seconds, 60°C for 30 seconds and 68°C for 10 minutes plus 15 seconds for each successive cycle; and a final extension at 68°C for seven minutes. The resulting PCR product was ligated into the TA cloning vector pCR-2.1 and transformed into TOP10 *E. coli* to generate pCR-1A3-6.4k.

The identity of the cloned PCR product was confirmed as the *UGT1A3*-6.4kb promoter by sequencing of both ends. The *UGT1A3*-6.4k insert was then excised from the vector backbone by *Nhe*I restriction digest (after the pCR plasmid sequence had been cut with *Xho*I and *Spe*I and CIP-treated to prevent it from participating in any downstream ligation reactions), ligated into *Nhe*I/CIP treated pGL3-basic and transformed into DH5α *E. coli*. This generated reporter vectors with inserts in the forwards and reverse orientations: pGL3-1A3-6.4kfor and pGL3-1A3-6.4krev.

To obtain the remaining 3 kb of the full *UGT1A3* promoter, the primers 1A3-9.4XbalNheI (5’ AGCCATTCTAGCTAGCTAGCGTGCTGTATTGGTGCTTC 3’) and 1A3-6.4revBsrGI (5’ GGAAGAAAGAATTTGATCTGTACAGAGAGACACGGACA 3’) were used in another Expand™ PCR reaction, using the same reaction conditions as described for the *UGT1A3*-6.4kb product, but with 55°C annealing. The resulting PCR product was restricted with *Xba*I and *Bsr*GI, ligated into *Xba*I/*Bsr*GI-cut pCR-1A3-6.4k and transformed into DH5α *E. coli* to generate pCR-1A3-9.4k. The full *UGT1A3*
promoter sequence was then excised from *XbaI/SpeI/CIP*-treated pCR-1A3-9.4k using *NheI*, ligated non-directionally into *NheI/CIP*-treated pGL3-basic and transformed into DH5α *E. coli*. To confirm the integrity of the constructed *UGT1A3*-9.4k promoters, forward and reverse orientation clones were tested for the expected restriction patterns after digestion with *NheI, HindIII* or *PstI*, and the ends of the inserts were sequenced.

To obtain a clone of the whole DNA sequence spanning the region between the *UGT1A4* and *UGT1A5* first exons, BAC DNA from clone 1308M2 was digested with *XbaI* and *NheI*, and subjected to electrophoresis through a 0.8% (w/v) agarose gel. All DNA fragments of length 5 to 6 kb were excised, purified using the QIAquick Gel Extraction kit and ligated into *XbaI/CIP*-treated pBSII. Clones containing BAC fragments that encompassed the *UGT1A4* promoter were detected by PCR, using primers 1A3/4rev-common and 1A3/4prom-0.5k (Table 3.1) as described in section 2.2.6.4, with cycling conditions of 95°C for 4 minutes, followed by 30 cycles of 95°C for 30 seconds, 50°C for 30 seconds and 72°C for 1 minute, and a final extension at 72°C for 5 minutes. A pBS-1A4-6k clone was then used as template for the following cloning PCR, rather than the 1308M2 BAC DNA, due to better yield and lack of non-specific amplification.

To clone the full *UGT1A4* promoter without the extra sequences still present in pBS-1A4-6k, 50 ng of pBS-1A4-6k was used as template in a *PfuTurbo* PCR reaction using primers 1A4UTRXhoI (Table 3.1) and 1A4prom-5kNheI (5’ AGCCATGCTAGCGTAGGTTATTGGGTGCGCTTT 3’), set up as per Chapter 2, section 2.2.6.3. The cycling conditions were: 95°C for 1 minute; 35 cycles of 95°C for 1 minute, 55°C for 1 minute and 68°C for 15 minutes; and a final extension at 68°C for 10 minutes. The resulting PCR product was then cloned directly into pCR-
Blunt and shuttled to pGL3-basic utilising the XhoI and NheI sites engineered into
the primers. Finally, both ends of the cloned fragment were sequenced to confirm its
identity as the region immediately upstream of the UGT1A4 first exon.

5.2.7. Transfection of HepG2 cells and dual-luciferase assay

Transient co-transfections of 0.5 μg of firefly luciferase reporter plasmid, 0.25 μg of
transcription factor expression plasmid and 25 ng pRL-Null were performed as
described in Chapter 2, section 2.2.10, using HepG2 cells seeded at a density of 2 ×
10^5 cells per well in 24-well plates. Cells were lysed 48-hours post-transfection using
passive lysis buffer, and the lysates assayed for firefly and renilla luciferase activity
as described in Chapter 2, section 2.2.11.

5.2.8. Hepatocyte nuclear factor 1α Western blot

Joanna Treloar performed the HNF1α Western blot presented in Figure 5.7, using
lysates from untreated and 3 μM TSA-treated HepG2 cells. The protocol used is
detailed in Chapter 2, section 2.2.16.

5.3 Results and discussion

5.3.1. Validation of 18S rRNA as a suitable internal control for HepG2 cells
over-expressing liver-enriched transcription factors and/or treated with
trichostatin A or rifampicin

When performing quantification of mRNA by reverse transcription and QPCR, it is
necessary to control for any inter-sample differences in amplifiable cDNA that are
inadvertently introduced during preparation. Significant sources of error are reported
to include variable quality of RNA after extraction (which can then significantly
affect both the determination of RNA concentration by spectrophotometric methods
and the efficiency of cDNA synthesis), and compounded operator error due to multi-
step protocols (Bustin, 2000; Bustin, 2002; Huggett et al., 2005). These errors are typically accounted for by normalising the gene(s) of interest against a gene whose expression (ideally) remains static between samples regardless of treatment, a so-called “house-keeping” gene. This can be effective because the reference transcript is subjected to all the steps of RNA extraction, quantification and reverse transcription as the genes to be investigated. However, the three most popular candidate reference genes, β-actin, GAPDH and 18S rRNA, have all been reported to vary significantly under certain experimental conditions and it is therefore imperative to determine the suitability of a chosen reference under the conditions used.

In our laboratory in the past, we have found that 18S rRNA typically varies less than β-actin or GAPDH mRNA in human cell lines subjected to transfection with transcription factor expression plasmids or treated with various chemicals. This observation is supported by several papers that have explored the effect of various experimental conditions on the expression of these three genes (Schmittgen and Zakrajsek, 2000; Selvey et al., 2001; Bas et al., 2004). Also, cell-cycle phase affects GAPDH expression (Mansur et al., 1993) and two factors used in this study, C/EBPα and C/EBPβ, can have profound effects on cell-cycle progression (Schrem et al., 2004). Furthermore, C/EBPα has been found to bind to the human GAPDH promoter (Claeyssens et al., 2003). With these factors in mind, 18S was chosen as the most likely suitable reference for the following LETF and TSA experiments.

To validate the choice of 18S rRNA as a suitable reference, 18S transcript levels were compared to total RNA concentration for all transfection, rifampicin-treatment and TSA-treatment combinations used in this study (Figure 5.2). When 18S rRNA
Figure 5.2: 18S rRNA levels in treated HepG2 cells as a proportion of total extracted RNA. A. HepG2 cells were treated with: 1:1000 ethanol, 300 nM trichostatin A (TSA) or 3 μM TSA for 24 hours; 1:1000 DMSO or 10 μM rifampicin for 42 hours; or transfected with plasmid expressing pregnane X receptor and subsequently treated with rifampicin or DMSO. B. HepG2 cells were transfected with control plasmid or one of nine vectors expressing the indicated liver-enriched transcription factors and subsequently incubated in the presence of 1:1000 ethanol, 300 nM or 3 μM TSA. Total RNA harvested from cells exposed to each treatment was analysed by quantitative PCR for 18S rRNA content and the results expressed as the mean 18S content/μg RNA relative to untreated cells (n = 3). Error bars indicate one standard deviation.
levels were analysed as a function of total RNA compared to untreated cells, it was found that there was a maximum of 12% change in apparent 18S rRNA levels in response to 300 nM or 3 μM TSA, or any combination of 10 μM rifampicin and PXR treatment (Figure 5.2A). Furthermore, it was found that the maximum change in 18S transcripts/μg total RNA, relative to untreated control cells, was less than 1.6-fold for LETF-transfected cells, regardless of TSA treatment (Figure 5.2B). In addition, no transcription factor or TSA treatment consistently altered 18S levels across all samples, leading to the assumption that 18S rRNA is not truly regulated by any of the experimental conditions tested and the small differences observed are most likely the result of genuine experimental variation. Similar levels of variation in the purity of RNA extracted with Qiagen RNeasy columns have been previously reported (Bustin, 2002); thus, the variation seen is within the known experimental error for similar systems. Therefore, 18S was shown to be an acceptable reference gene for this experimental system. However, it should be noted that these results do not indicate whether 18S would be a suitable reference gene for the same experiment in cell lines other than HepG2, as TSA was been shown to affect 18S rRNA levels in LNCaP cells and mouse prostate (Mogal and Abdulkadir, 2006).

5.3.2. Basal levels of UGT1A and UGT2B mRNA transcripts in HepG2 cells
HepG2 cells are considered to be one of the most differentiated human liver cell-lines available (Knowles et al., 1980; Ishiyama et al., 2003). However, the UGT content of these cells has not been well defined. Since immortalised cells never possess the exact same gene expression profile as the primary cells from which they were derived, (indeed HepG2s are known to be poor expressers of CYP enzymes (Rodriguez-Antona et al., 2002)) and the expression profile of HepG2 cells is likely to be dependent on culture conditions (as there is considerable disagreement in the
literature about the expression of certain genes in these cells), HepG2 RNA was assessed for UGT mRNA content under basal conditions. All UGT1A and UGT2B forms, barring UGT1A5, were tested for expression in HepG2 cells grown under the conditions prescribed by the ATCC (see Chapter 2, section 2.2.1). It was found that UGT2B7 and UGT2B10 were by far the most highly transcribed UGTs in HepG2 cells (approximately 5,000 mRNA copies per $1 \times 10^9$ 18S rRNA molecules), followed by UGT2B11, UGT1A1, UGT2B4 and UGT1A6, which were all readily detectable (200-800 mRNA copies per $1 \times 10^9$ 18S rRNA molecules). UGT1A9, UGT2B15, UGT2B17 and UGT1A3 were also detectable at low levels (20-50 mRNA copies per $1 \times 10^9$ 18S rRNA molecules), however, the basal levels of UGT1A4, UGT1A7, UGT1A8, UGT1A10 and UGT2B28 were below the reliable detectable limit of this assay at less than 10 mRNA copies per $1 \times 10^9$ 18S rRNA molecules (Figure 5.3). Three of these genes, UGT1A7, UGT1A8 and UGT1A10 are considered to be strictly extrahepatic in their expression (Strassburg et al., 1997b; Mojarrabi and Mackenzie, 1998; Strassburg et al., 1998a). Therefore, it is likely that the low levels detected by this sensitive technique reflect illegitimate transcription; the principle that transcription of any gene can be detected in any cell given sufficient sensitivity of detection (Chelly et al., 1989; Chelly et al., 1991). Consequently, it would seem that UGT1A4 and UGT2B28 are also not truly expressed in HepG2 cells under the basal culture conditions used.

This study shows that the UGT expression profile of UGTs in HepG2 cells differs significantly from that of primary hepatocytes. A similar study using primary tissue showed that UGT2B4, UGT2B7, UGT1A4 and UGT1A9 are the most prevalent UGTs in liver, followed by UGT2B15, UGT2B10, UGT1A1, UGT1A6 and UGT1A3. UGT2B11 and UGT2B17 were the most difficult hepatic UGTs to detect
Figure 5.3: Expression of UGT mRNA in HepG2 cells under basal culture conditions. Total RNA, extracted from untreated HepG2 cells, was analysed by QPCR for UGT1A and UGT2B mRNA content. Results are expressed as mean mRNA levels relative to 18S rRNA ($n = 3$) plus one standard deviation.

in primary hepatocytes (Congiu et al., 2002). UGT2B28 was not tested in the work of Congiu et al. (2002), but is also expressed in the liver (Levesque et al., 2001). It is not known how the absolute levels of UGT transcripts compare between HepG2 and hepatocytes. However, for the primer pairs and PCR conditions that are the same between the HepG2 study and the work of Congiu et al. (2002) (i.e. UGT1A1, UGT1A4, UGT1A9 and all UGT2Bs except UGT2B28), the relative efficiency of each PCR should be the same, making it possible to directly compare the rank orders of UGT expression in the two sample sets. Using UGT2B7 as the reference, HepG2 cells have lost substantial relative expression of all UGTs except UGT2B10 and
possibly UGT2B11, with the greatest losses suffered by UGT2B4, UGT1A4 and UGT1A9. Reasons for these changes to the ratios of UGT forms expressed could include changes in the transcription factor profile in the HepG2 hepatoma cells, or loss of exposure to hormones or other chemical signals that would normally be present in the whole organ/organism and may up-regulate UGT transcription \textit{in vivo}. However, this work shows that most hepatic \textit{UGT} genes are transcriptionally active in HepG2 cells, at least to some extent, with the exception of \textit{UGT1A4} and \textit{UGT2B28}. Therefore, it is reasonable to expect that most of the hepatic \textit{UGT} promoters are not situated in closed chromatin in HepG2 cells and are therefore likely to be accessible to over-expressed transcription factors.

\textbf{5.3.3. Effect of rifampicin-liganded pregnane X receptor on endogenous UGT expression in HepG2 cells}

To study the effect of rifampicin-liganded PXR expression on hepatic \textit{UGT} expression, HepG2 cells were transfected with either empty control plasmid or PXR-expressing plasmid, and subsequently treated with either vehicle or 10 μM rifampicin. QPCR analysis of total RNA from treated cells showed that UGT1A1 and UGT1A3 mRNA transcripts were increased 13-fold relative to 18S rRNA in cells transfected with PXR and then treated with rifampicin, when compared to untreated controls (\(P < 0.001\)). Rifampicin alone also increased the level of both of these transcripts in HepG2 cells by more than 2-fold (UGT1A1: \(P = 0.004\) and UGT1A3: \(P = 0.039\)) (Figure 5.4). This is consistent with the presence of endogenous PXR expression in HepG2 cells (Aninat \textit{et al.}, 2006). In contrast, the mRNA levels of all other hepatic \textit{UGT} genes were only increased 2-fold or less by the PXR/rifampicin combined treatment, with the only significant response being from \textit{UGT1A6} (2.0-
Figure 5.4: Endogenous UGT mRNA levels in HepG2 cells treated with 10 μM rifampicin and/or over-expressed pregnane X receptor. Total RNA, extracted from HepG2 cells treated with 1:1000 ethanol, 10 μM rifampicin and/or over-expressed PXR, was analysed by reverse transcription QPCR for altered mRNA levels of all hepatic members of the human UGT1A and UGT2B families. Results have been normalised to 18S content and are expressed as the mean change in mRNA level \( (n = 3) \), relative to untreated cells. Error bars indicate one standard deviation. \( P \) values for the indicated comparisons are \( *P < 0.001, \quad \ddagger P = 0.004, \quad \ddagger\ddagger P = 0.011 \) and \( \ddagger\ddagger\ddagger P = 0.039 \).

fold, \( P = 0.011 \) (Figure 5.4). The mRNA levels of UGT1A7, UGT1A8 and UGT1A10 also did not change (data not shown). This confirmed previous semi-quantitative PCR work in our laboratory that showed that UGT1A1 and UGT1A3 mRNAs were the most substantially increased UGT transcripts in HepG2 cells in the presence of over-expressed, rifampicin-liganded PXR (Gardner-Stephen et al., 2004). It also showed that the transfection, RNA extraction, reverse transcription and QPCR protocols had all been performed successfully.

As a model for the human hepatocyte, the HepG2 cells used herein are likely to be useful only for studying the well established effect of PXR on the regulation of UGT1A1 and UGT1A3. Although, increases in mRNA level or UGT activity have
been reported for *UGT1A4* (4.2-fold in primary hepatocytes, and present but not quantified in Tg-*UGTI* transgenic mice and another HepG2 line), *UGT1A6* (1 to 4-fold in primary hepatocytes, and present but not quantified in Tg-*UGTI* transgenic mice), *UGT1A9* (1 to 1.7-fold in primary hepatocytes, and present but not quantified in Tg-*UGTI* transgenic mice), *UGT2B7* (0.8 to 4-fold in primary hepatocytes), *UGT2B15* (1.2 to 1.5-fold in liver) and *UGT2B28* (1.6-fold in liver) in the presence of PXR ligands, particularly rifampicin and carbamazepine (Rae *et al.*, 2001; Gardner-Stephen *et al.*, 2004; Soars *et al.*, 2004; Chen *et al.*, 2005a; Oscarson *et al.*, 2006), there was no change in these genes, except *UGT1A6*, in this study. This was despite the presence of excess PXR protein, which exaggerates the *UGT1A1* and *UGT1A3* responses (compare 5.2 and 3.8-fold in primary hepatocytes respectively (Rae *et al.*, 2001), to 13-fold in PXR-transfected HepG2 cells (Figure 5.4)). One reason for the lack of response in HepG2 cells may include a deficiency in basal transcription factors required to support PXR-mediated activation, especially in the case of *UGT1A4* and *UGT2B28*, since both are essentially unexpressed in HepG2 cells. Alternatively, genotype may play a role in the PXR-responsiveness of some *UGT* genes. The UGT1A9 and UGT2B7 PXR-responses of primary cells have been reported to be highly dependent on the individual from whom the hepatocytes were isolated, with some hepatocyte preparations being completely unresponsive (Soars *et al.*, 2004). Therefore, it is possible that HepG2 cells are genetically incapable of a full UGT-response to PXR.

To further study the molecular mechanisms that confer PXR-responsiveness to *UGT* genes other than *UGT1A1* and *UGT1A3*, it will be necessary to develop additional models. Possibilities include other hepatic cell lines that better express UGTs (especially UGT1A4), or exhibit PXR-responsiveness for more *UGT* genes. One
possibility is the newly characterised human hepatoma cell line HepaRG, which exhibits hepatocyte-like expression of several CYPs, UGT1A1 and PXR when cultured in the presence of DMSO (Aninat et al., 2006). Methods that improve the differentiation state of HepG2 cells, such as exposure to retinoic acid (Falasca et al., 1999), may also induce more hepatocyte-like behaviour in response to PXR ligands. However, these results suggest that HepG2 cells are a suitable system for identifying and characterising the molecular determinants of the UGT1A3 PXR-response, in much the same manner as has been achieved for UGT1A1 (Sugatani et al., 2004). Further work towards identifying the mechanism by which PXR regulates UGT1A3 is presented below.

5.3.4. UGT1A3 promoter-reporter constructs lack ligand-dependent PXR responsiveness in HepG2 cells

To locate the PXR NRRE responsible for the PXR-responsiveness of the human UGT1A3 gene, the 3.3 kb UGT1A3 promoter-reporter construct described in Chapter 3 was initially tested for increased expression of the reporter gene in the combined presence of over-expressed PXR and 10 μM rifampicin. However, although a 3.5-fold increase in relative luciferase activity ($P < 0.001$, Figure 5.5) was observed when the UGT1A3-3.3k promoter was treated with PXR and rifampicin, a 2.6-fold ($P < 0.001$) increase was also observed with unliganded PXR. Therefore, the ligand-mediated PXR-response obtained for the UGT1A3-3.3k promoter was only 1.4-fold, which although statistically significant ($P < 0.001$), was insufficient to explain the response seen for the endogenous gene and was deemed unlikely to be biologically relevant.
Figure 5.5: PXR up-regulates the \textit{UGT1A3} and \textit{UGT1A4} promoters \textit{in vitro} in a ligand-independent manner. HepG2 cells were transfected with 25 ng pRL-Null plus 0.5 μg reporter plasmids carrying 3.3 kb, 6.4 kb or 9.4 kb of the \textit{UGT1A3} promoter, or 5 kb of the \textit{UGT1A4} promoter, with or without co-transfection of 0.25 μg pCMX-PXR. Transfected cells were also treated with solvent (1:1000 DMSO), or 10 μM rifampicin. Results presented are the mean (n = 3) increase in luciferase reporter activity, relative to the renilla internal control, over concurrent pGL3-basic transfections. Error bars indicate one standard deviation. \textit{P} values for the indicated comparisons are \(*P < 0.001\) and \(†P = 0.001\).

Since the \textit{UGT1A1} PXR NRRE is located over 3285 nucleotides upstream of the \textit{UGT1A1} start codon (Xie \textit{et al}., 2003), and PXR-binding sites had been predicted at -6930 and -8040 bp of the \textit{UGT1A3} promoter (Vyhlidal \textit{et al}., 2004), it was reasoned that the \textit{UGT1A3} PXR NRRE may lie upstream of the tested section of promoter. Therefore, two longer fragments of the DNA upstream of the UGT1A3 promoter were cloned: the proximal 6.4 kb, and the entire 9.4 kb region between the \textit{UGT1A3} initiation codon and the \textit{UGT1A4} first exon 3’ splice site. The entire 5 kb region between the \textit{UGT1A4} initiation codon and the \textit{UGT1A5} first exon was also cloned for comparative purposes. However, no ligand-dependent PXR response was
obtained for either gene, regardless of the construct tested (Figure 5.5). Although some increase in luciferase activity was observed in response to the presence of over-expressed PXR without exogenously supplied ligand for pGL3-1A3-3.3k (2.6-fold, \( P < 0.001 \)), pGL3-1A3-6.4k for (5.5-fold, \( P < 0.001 \)), pGL3-1A3-9.4k for (6.2-fold, \( P < 0.001 \)) and pGL3-1A4-5k (3.6-fold, \( P < 0.001 \)), the vectors containing reverse-orientation inserts had similar fold increases in reporter gene activity when co-transfected with pCMX-PXR (pGL3-1A3-6.4krev: 3.4-fold, \( P = 0.001 \) and pGL3-1A3-9.4krev: 4.5-fold, \( P = 0.001 \)) (Figure 5.5). Therefore, it would appear that this response is most likely an artefact of the experimental system.

The somewhat surprising absence of authentic response by the \( UGT1A3 \) promoter to PXR and rifampicin was consistent over several experiments and plasmid preparations. Because of good basal expression of the luciferase reporter, and the response of the endogenous gene (section 5.3.3), it is reasonable to assume that the transfections were effective, that no experimental components were degraded and that endogenous PXR ligands in HepG2 cell culture are at insufficient levels to explain the observed results. However, the unlikely possibility of experimental failure could, in future, be controlled for by the inclusion of a promoter known to be responsive in the same assay, such as the published \( UGT1A1 \) (Xie et al., 2003; Sugatani et al., 2005a) or \( CYP3A4 \) (Hustert et al., 2001) constructs. Interestingly, all three of these studies used either constitutively active PXR, or PXR-responsive enhancers ligated directly to proximal promoters, to demonstrate PXR-responsiveness. Thus, it may transpire that the combination of liganded PXR and full \( UGT1A3 \) promoter-reporter plasmid is unsuitable for detecting the expected PXR-response. Nevertheless, more interesting possible explanations for the lack of PXR-response by the \( UGT1A3 \) promoter constructs include that the PXR NRRE for
UGT1A3: a) is genuinely inactive in the cloned UGT1A3 allele (or has been inactivated by PCR mutation); b) requires an appropriate chromatin structural context to be effective; or c) still lies outside the cloned promoter region. The first scenario could simply be addressed by re-cloning the UGT1A3 promoter inserts from a different template (e.g. HepG2 genomic DNA) and repeating the experiment. The second possibility, as to whether the chromatin structure of the UGT1A3 promoter contributes to its regulation by PXR, could be further investigated by generating a stable HepG2 cell pool where the pGL3-1A3-9.4k reporter construct is integrated into the genome. Examination of the final option, however, would be more involved. Additional portions of the UGT1A locus could be screened for PXR-binding elements by the method of Xie et al. (2003) without the need for generating large promoter clones. However, if a PXR NRRE cannot be identified upstream of the UGT1A3 first exon, or immediately downstream, it may be necessary to consider whether the UGT1A1 PXR NRRE is also able to affect the UGT1A3 gene.

Enhancer elements, such as the UGT1A1 PBREM cluster of transcription factor binding sites, in which the UGT1A1 PXR NRRE resides, can exist up to tens of thousands of nucleotides upstream or downstream of their target promoter. They can also be present in either orientation in the DNA, and are sometimes shared between neighbouring genes. Enhancers can be prevented from promiscuous regulation of inappropriately receptive promoters in their vicinity by insulator and silencer elements – however, no studies have been done to determine whether the UGT1A1 PBREM functions in a uni- or bi-directional manner (Hatzis and Talianidis, 2002; de Laat and Grosveld, 2003; West and Fraser, 2005). If the UGT1A1 PBREM is necessary for the regulation of UGT1A3 by PXR, it presumably must interact physically with the UGT1A3 proximal promoter in PXR/rifampicin treated HepG2
cells. Chromosome conformation capture analyses could be used to determine whether the spatial proximity of the UGT1A1 PBREM and UGT1A3 promoter is affected by PXR/rifampicin treatment (Dekker, 2006), and hence, whether a functional interaction is likely. If it was found that the UGT1A1 PBREM has a role in the expression of UGT1A3 as well as UGT1A1, then functional polymorphisms in this region (Sugatani et al., 2002) may also be relevant to interindividual variation in UGT1A3 expression.

5.3.5. Effect of trichostatin A treatment on endogenous UGT expression in HepG2 cells

Histone acetylation, regulated by the opposing activities of HATs and HDACs, plays an important role in the control of gene transcription. Hyperacetylation of histones appears to create a permissive environment for gene expression by relaxing chromatin structure and making the DNA more accessible to modifying enzymes, transcription factors and RNA polymerase. Highly acetylated histones are generally associated with actively transcribed genes and genes poised for transcription, but not silent genes (Grunstein, 1997; Marks et al., 2000; Schrem et al., 2002).

Genes repressed by histone deacetylation are resistant to the influence of certain transcription factors that would otherwise activate their promoters (Honda et al., 2006). In tissue-specific expression of genes, this can be a legitimate mechanism to prevent their improper expression in the wrong cell types or at the wrong developmental stage. However, in immortalised cell lines, where some genes are inappropriately repressed, aberrant histone acetylation may interfere with what would otherwise be a genuine response to an exogenously supplied transcription factor. To assess whether any UGT genes are likely to be repressed by histone deacetylation in HepG2 cells, cultures were treated with TSA and checked for
changes in the levels of each UGT mRNA, except UGT1A5. TSA is a specific and potent HDAC inhibitor (Yoshida et al., 1990), which has previously been shown to change the expression profile of HepG2 cells (although no UGT data was reported) (Chiba et al., 2004). Initially, TSA was added at a concentration of 3 μM, to emulate the work of Bort and colleagues, which showed that TSA was necessary for FoxA3 to access the HepG2 endogenous CYP2C8 and CYP2C19 promoters (Bort et al., 2004). However, considerable cytotoxicity was observed at this TSA concentration after 24 hours, so the experimental protocol was altered to also include a lower TSA concentration of 300 nM.

Exposure of HepG2 cells to 300 nM TSA greatly increased the mRNA levels of three UGTs: UGT1A1 (18.7-fold, \( P < 0.001 \)), UGT1A3 (13.3-fold, \( P = 0.006 \)) and UGT2B17 (9.1-fold, \( P < 0.001 \)) (Figure 5.6). Other UGTs that had small responses (but greater than 2-fold) were: UGT1A4 (increased 2.2-fold, \( P = 0.001 \)), UGT1A9 (decreased 2.5-fold, \( P = 0.42 \)), UGT2B4 (increased 2.2-fold, \( P = 0.004 \)), UGT2B11 (increased 2.1-fold, \( P = 0.015 \)) and UGT2B15 (increased 2.1-fold, \( P = 0.001 \)).

Exposure to 3 μM TSA had a stronger positive effect on the levels of UGT1A1 and UGT1A3 mRNA than 300 nM TSA, giving rise to 46.4-fold (\( P < 0.001 \)) and 33.9-fold (\( P = 0.003 \)) increases respectively (Figure 5.6). Conversely, the increase in UGT2B17 transcripts seen after exposure to 300 nM TSA was muted for 3 μM TSA, dropping to only a 2.7-fold increase (\( P = 0.027 \)). Other UGT transcripts that were increased in HepG2 cells after treatment with 3 μM TSA were UGT2B11 (3.2-fold, \( P = 0.009 \)) and interestingly, three genes barely detectable in untreated HepG2 cells. UGT1A4 was increased 6.9-fold (\( P = 0.036 \)), UGT1A10 was increased 4.5-fold (\( P = 0.002 \)), and UGT2B28 was increased 5.8-fold (\( P = 0.024 \)). In all three cases, treatment with 3 μM TSA caused expression to exceed 30 mRNA copies per \( 1 \times 10^9 \).
Figure 5.6: Effects of trichostatin A on UGT mRNA levels in HepG2 cells. HepG2 cells were treated for 24 hours with either 1:1000 diluted ethanol, 300 nM TSA or 3 μM TSA and harvested for total RNA. The levels of all human UGT1A and UGT2B mRNAs, except UGT1A5, were assessed by QPCR, normalised to 18S rRNA content, and expressed as the mean change in UGT mRNA \((n = 3)\) when compared to untreated cells. Error bars indicate one standard deviation. P values for the indicated comparisons are \(* P < 0.001, † P = 0.001, †† P = 0.002, ††† P = 0.003, ‡‡ P = 0.006, ‡‡‡ P = 0.009\) and \(§ P > 0.02 \text{ but } < 0.05\).

18S rRNA molecules, which is comparable to the basal levels of UGT1A9, UGT2B15, UGT2B17 and UGT1A3 in HepG2 cells. There were also several significant losses of UGT expression in 3 μM TSA-treated HepG2 cells: UGT1A6 was decreased 6.7-fold \((P = 0.003)\), UGT1A9 was decreased 5-fold \((P = 0.001)\), UGT2B7 was decreased 3.4-fold \((P = 0.034)\) and UGT2B10 was decreased 30.3-fold \((P < 0.001)\).

The effects of TSA on UGT transcript levels in HepG2 cells indicate that several UGTs are repressed by the activity of HDACs. The most likely mechanism for this observation is through histone deacetylation, although it should be noted that HDACs can also directly deacetylate a few select transcription factor proteins such as GATA-1 and p53, decreasing their activity (Huo and Zhang, 2005). More importantly however, even assuming the involvement of histone acetylation, it must
be recognised that this data does not distinguish whether the substantial derepression of several UGT genes is a direct or indirect effect of TSA. TSA treatment relieves chromatin-mediated repression across the entire genome, altering the expression of many genes. One study using 670 nM TSA showed that of 4608 liver and gastric genes tested, 187 were increased and 63 were decreased in HepG2 cells (Chiba et al., 2004). Therefore, it is reasonable to expect that some affected genes will encode transcription factors, and that their downstream promoter targets could consequently also be altered. In support of this, cultured primary hepatocytes treated with 25 μM TSA retained expression of HNF4α and C/EBPα, two transcription factors that are usually lost over time in untreated hepatocyte cultures (Henkens et al., 2007). Furthermore, HepG2 cells treated with 250 nM TSA had modest increases (approximately 2-fold) in HNF4α and FoxA1 mRNAs, while HNF1α and C/EBPα levels were essentially unchanged (Yamashita et al., 2003). However, despite not knowing the exact mechanism by which TSA alters UGT transcript levels, this experiment showed that TSA treatment is a potentially useful tool for exploring the interaction of LETFs with endogenous UGT genes in HepG2 cells. Both concentrations tested created a more permissive environment for the transcription of at least three UGTs. Thus, TSA treatment may allow transcription factors better access to these UGT promoters, change the activity of important transcription factors, and/or cause the expression of one or more factors/co-factors required to co-operate in the regulation of UGT genes. Therefore, in the following experiments, LETFs were expressed both in the absence and presence of TSA to assess their potential as UGT regulators.

Although it could not be covered by the scope of this thesis, it would be valuable to further investigate the mechanisms behind the profound increase in UGT1A1,
UGT1A3, and UGT2B17 mRNA levels by TSA in HepG2 cells. Such a study would be likely to provide further insights into the transcriptional controls operating on these genes. Worthwhile experiments would include tests designed to separate the contributions of the local effects of chromatin deacetylation at the UGT loci from the effects caused by changes in transcription factor expression and activity, such as:

a) Nuclease sensitivity mapping (Aronow et al., 1995; Caslini et al., 2006) and anti-acetylated histone chromatin immunoprecipitation assays (Chiba et al., 2004) to assess the chromatin structure of HepG2 UGT promoters with and without TSA treatment. The former assesses the accessibility of DNA to endonucleases and is a measure of the degree of chromatin condensation; the latter tests the acetylation status of the nucleosomes integrated into the promoter of interest.

b) TSA treatment of HepG2 cells transiently transfected with UGT promoter-reporter plasmids (Kwon et al., 2006; see also section 5.3.7.1). A plasmid response to TSA that changed abruptly in a progressive promoter-deletion experiment would indicate specific target gene sequences that were required for the response. Successful site-directed mutagenesis of the responsible nucleotides would strengthen such a hypothesis. Identification of the target elements would help facilitate discovery of the required mediators, which could then be tested for changes in expression or activity.

c) DNase I footprinting to detect TSA-induced changes in the binding patterns of nuclear proteins to PCR-amplified UGT promoter segments. The nucleotide sequence of newly protected elements may convey sufficient information to identify altered transcription factor(s).
d) Comparison of untreated and TSA-treated HepG2 nuclear extracts for their ability to drive transcription in *in vitro* chromatin assays. Our laboratory is currently developing *in vitro* chromatin assays to study *UGT* promoter function: this system could also be used to study the effects of TSA-mediated changes in nuclear proteins on transcription from untreated chromatin. TSA-inhibition of HDACs has been shown to be reversible on dialysis (Kijima *et al.*, 1993); thus it would be possible to completely uncouple the direct and indirect effects of TSA treatment on *UGT* transcription.

e) QPCR or microarray studies to identify genes with altered mRNA levels in TSA-treated HepG2 cells. Although correlation does not indicate causality, transcription factors and co-factors that have altered expression in TSA-treated cells would make logical candidates for further investigation as *UGT* regulators. While QPCR is cheaper, and generally more sensitive and specific than microarrays (Lucas *et al.*, 2005; Senkel *et al.*, 2005), it is also a less efficient method of screening potential regulators of a gene, and is necessarily biased towards better characterised genes. To begin the process of investigating the effect of TSA on the expression of transcription factors in HepG2 cells, HNF1α levels in treated and untreated cells were investigated by quantitative PCR (see section 5.3.6).

In addition to these mechanistic studies, it would also be prudent to investigate whether TSA and other HDAC inhibitors are able to alter UGT expression in primary hepatocytes and hepatocellular carcinomas. Only a subset of genes induced by TSA in HepG2 cells is also up-regulated in human hepatocytes (Chiba *et al.*, 2004). However, the responses of *UGT1A1* and *UGT1A3* are approximately three times greater than any other response reported for HepG2 cells, and a response *in*
vivo could have clinical significance. Valproic acid, a relatively weak HDAC inhibitor (Phiel et al., 2001; Furchert et al., 2007), does not significantly increase UGT1A1 glucuronidation of the irinotecan metabolite SN-38 in patients receiving both pharmaceuticals (Raymond et al., 2003). However, more potent histone deacetylase inhibitors, such as suberoylanilide hydroxamic acid and MS-275 (a benzamide), are currently in clinical trials as anti-cancer therapeutics (Hess-Stumpp, 2005; Kelly et al., 2005; Duvic et al., 2007; Furchert et al., 2007), and suberoylanilide hydroxamic acid has been suggested for combined therapy with irinotecan for hepatocellular carcinoma (Ocker et al., 2005). So, the potential for clinically relevant HDAC effects on glucuronidation exists. It is also possible that HDACs could alter the local pharmacological properties of drugs within tumours. UGT1A1 mRNA is repressed in the majority of colorectal cancers, but is restored by treatment with HDAC inhibitors (Gagnon et al., 2006). Therefore, HDAC treatment could potentially increase the resistance of malignant cells to therapeutics that are inactivated by glucuronidation, such as irinotecan. UGT1A1 and UGT1A3 mRNA levels are also reduced in hepatocellular carcinoma (Strassburg et al., 1997a), and in light of the presented HepG2 data, may respond to HDACs. Therefore, further and more deliberate investigation is warranted into the effects of HDAC inhibitors on glucuronidation in humans.

5.3.6. Effect of trichostatin A treatment on endogenous expression of HNF1α in HepG2 cells

It has previously been shown, by Western blot and EMSA, that HepG2 cells cultured in our laboratory under the conditions described in Chapter 2, section 2.2.1 express HNF1α (Gardner-Stephen and Mackenzie, 2005; Gardner-Stephen and Mackenzie, 2007a and Chapter 4, section 4.3.3). Since HNF1α has been proposed to bind to the
proximal promoters of all human *UGT* genes, it was investigated whether TSA treatment had any effect on endogenous HNF1α expression.

Initially, the effect of 3 μM TSA treatment on the levels of HNF1α protein in HepG2 cells was investigated by Western blot (Figure 5.7A). As expected, it was found that HNF1α protein was readily detectable in lysates from untreated HepG2 cells. However, HNF1α levels in lysates from 3 μM TSA-treated cells were below the limit of detection. Although no attempt was made to demonstrate that the observed loss of HNF1α was not due to a general degradation of protein in TSA-treated HepG2 cells, it was shown that the loss of HNF1α expression in 3 μM TSA-treated HepG2 cells was also observable at the mRNA level. When HepG2 HNF1α mRNA concentrations were investigated by quantitative reverse transcription PCR in the same manner as discussed for UGT mRNA transcripts, and it was found that the solvent carrier, ethanol, had no effect on HNF1α mRNA levels (Figure 5.7B). Similarly, 300 nM TSA had no significant effect on HNF1α (1.4-fold increase, *P* = 0.059). In contrast, HNF1α mRNA levels were severely repressed in 3 μM TSA-treated cells (46.8-fold decrease, *P* < 0.001). Therefore, it would seem that the loss of HNF1α expression in 3 μM TSA-treated HepG2 cells was caused by either a specific increase in HNF1α mRNA degradation relative to 18S rRNA, or a decrease in *HNF1α* promoter activity, rather than degradation of protein. A TSA-induced decrease in *HNF1α* promoter activity would be the scenario most consistent with the known action of TSA.

Transfection of HepG2 cells with pCMX-HNF1α greatly enhanced HNF1α levels (17.4-fold, *P* < 0.001, Figure 5.7C). Co-treatment with either 300 nM or 3 μM TSA did not statistically affect HNF1α mRNA levels in pCMX-HNF1α transfected cells (*P* = 0.131 and 0.916, respectively; Figure 5.7C). Although this does not represent
Figure 5.7: Effect of ethanol, trichostatin A and pCMX-HNF1α on the levels of HNF1α in HepG2 cells. A. 25 μg protein lysate from untreated and 3 μM TSA-treated HepG2 cells were probed for HNF1α protein by Western blot, as described in Chapter 2, section 2.2.16. In vitro synthesised HNF1α (1 μl) was included in the Western blot as a positive control. B. and C. HepG2 cells were treated for 24 hours with 1:1000 diluted ethanol, 300 nM TSA or 3 μM TSA, with or without prior transfection with 0.5 μg pCMX-PL2 or pCMX-HNF1α as per “Methods”. The mean levels of HNF1α mRNA (n = 3) were assessed by QPCR and normalised to 18S rRNA content. Error bars indicate one standard deviation. P values for the indicated comparisons are *P < 0.001 and #P > 0.05 (not significant).
conclusive proof that TSA does not cause degradation of HNF1α transcripts, it is certainly consistent with the hypothesis that TSA affects HNF1α expression by specifically altering its regulation. Furthermore, these results show that treatment of HepG2 cells with TSA after transfection does not prevent mRNA synthesis from the CMV promoter present in pCMX-derived expression vectors, as no decreases in HNF1α mRNA levels were observed when pCMX-HNF1α-transfected cells were concurrently treated with TSA.

5.3.7. Effect of over-expressed liver-enriched transcription factors on endogenous UGT expression in HepG2 cells

Although UGT1A7, UGT1A8 and UGT1A10 are not expressed in hepatocytes, these genes were included in this study because many of the tested LETFs are also expressed in tissues where these UGTs are found, such as the intestine. In the presence of the chromatin-relaxing agent TSA, the mechanisms that normally silence these genes in hepatocytes might be bypassed and a response to LETFs may be seen if the UGT1A7, UGT1A8 or UGT1A10 promoters contain LETF binding sites that are normally functional in another cell type. Although any responses obtained for these genes would be biologically irrelevant for the liver, they would indicate potentially important interactions for further analysis in more appropriate cell lines. Furthermore, it was hoped that differences in the responses of UGT1A7, UGT1A8 and UGT1A10 to that of UGT1A9 would reveal important information about the mechanisms that cause UGT1A9 to be the only hepatically-expressed enzyme from the closely related UGT1A7-UGT1A10 gene cluster. However, since no responses to LETFs were obtained, with or without TSA treatment, for UGT1A8 or UGT1A10, only UGT1A7 data is included in the results presented below. The following figure (Figure 5.8A-M) summarises the responses of the HepG2 endogenous UGT genes to
Figure 5.8A-C: Effect of over-expressed liver-enriched transcription factors on endogenous UGT1A1, UGT1A3 and UGT1A4 expression in HepG2 cells. HepG2 cells were transfected with 0.5 μg empty pCMX-PL2 or pCMX-derived vectors expressing HNF1α, HNF1β, HNF4α, FoxA1, FoxA2, FoxA3, C/EBPα or C/EBPβ. Twenty-four hours after transfection, cells were also treated with 300 nM TSA or 3 μM TSA. After a further 24 hours, cells were harvested for total RNA and analysed for A. UGT1A1, B. UGT1A3 or C. UGT1A4 mRNA content by reverse transcription QPCR. Levels of each UGT were normalised against 18S rRNA and the results are presented as mean fold changes (n = 3) relative to the untreated, untransfected control (set arbitrarily to a value of one). Error bars indicate one standard deviation. P values for the indicated comparisons are *P < 0.001, †P = 0.001, †††P = 0.003, ‡P = 0.005, ‡‡P = 0.006 and §P > 0.02 but < 0.05.
A

UGT1A1

Fold Change (mRNA level)

No treatment
300 nM TSA
3 μM TSA

B

UGT1A3

Fold Change (mRNA level)

No treatment
300 nM TSA
3 μM TSA

C

UGT1A4

Fold Change (mRNA level)

No treatment
300 nM TSA
3 μM TSA
Figure 5.8D-F: Effect of over-expressed liver-enriched transcription factors on endogenous UGT1A6, UGT1A7 and UGT1A9 expression in HepG2 cells. HepG2 cells were transfected with 0.5 μg empty pCMX-PL2 or pCMX-derived vectors expressing HNF1α, HNF1β, HNF4α, FoxA1, FoxA2, FoxA3, C/EBPα or C/EBPβ. Twenty-four hours after transfection, cells were also treated with 300 nM TSA or 3 μM TSA. After a further 24 hours, cells were harvested for total RNA and analysed for D. UGT1A6, E. UGT1A7 or F. UGT1A9 mRNA content by reverse transcription QPCR. Levels of each UGT were normalised against 18S rRNA and the results are presented as mean fold changes (n = 3) relative to the untreated, untransfected control (set arbitrarily to a value of one). Error bars indicate one standard deviation. *P values for the indicated comparisons are *P < 0.001, †P = 0.001, †††P = 0.003, ‡‡P = 0.006, ‡‡‡P = 0.009 and §P > 0.02 but < 0.05.
Figure 5.8G-I: Effect of over-expressed liver-enriched transcription factors on endogenous UGT2B4, UGT2B7 and UGT2B10 expression in HepG2 cells. HepG2 cells were transfected with 0.5 μg empty pCMX-PL2 or pCMX-derived vectors expressing HNF1α, HNF1β, HNF4α, FoxA1, FoxA2, FoxA3, C/EBPα or C/EBPβ. Twenty-four hours after transfection, cells were also treated with 300 nM TSA or 3 μM TSA. After a further 24 hours, cells were harvested for total RNA and analysed for G. UGT2B4, H. UGT2B7 or I. UGT2B10 mRNA content by reverse transcription QPCR. Levels of each UGT were normalised against 18S rRNA and the results are presented as mean fold changes (n = 3) relative to the untreated, untransfected control (set arbitrarily to a value of one). Error bars indicate one standard deviation. P values for the indicated comparisons are *P < 0.001, †P = 0.001 and §P > 0.02 but < 0.05.
Figure 5.8J-L: Effect of over-expressed liver-enriched transcription factors on endogenous UGT2B11, UGT2B15 and UGT2B17 expression in HepG2 cells. HepG2 cells were transfected with 0.5 μg empty pCMX-PL2 or pCMX-derived vectors expressing HNF1α, HNF1β, HNF4α, FoxA1, FoxA2, FoxA3, C/EBPα or C/EBPβ. Twenty-four hours after transfection, cells were also treated with 300 nM TSA or 3 μM TSA. After a further 24 hours, cells were harvested for total RNA and analysed for J. UGT2B11, K. UGT2B15 or L. UGT2B17 mRNA content by reverse transcription QPCR. Levels of each UGT were normalised against 18S rRNA and the results are presented as mean fold changes (n = 3) relative to the untreated, untransfected control (set arbitrarily to a value of one). Error bars indicate one standard deviation. P values for the indicated comparisons are *P < 0.001, †P = 0.001, ††P = 0.002, †††P = 0.006, ††††P = 0.009, ¥P = 0.013, ¥¥P = 0.014, ¥¥¥P = 0.015 and §P > 0.02 but < 0.05.
Figure 5.8M: Effect of over-expressed liver-enriched transcription factors on endogenous UGT2B28 expression in HepG2 cells. HepG2 cells were transfected with 0.5 μg empty pCMX-PL2 or pCMX-derived vectors expressing HNF1α, HNF1β, HNF4α, FoxA1, FoxA2, FoxA3, C/EBPα or C/EBPβ. Twenty-four hours after transfection, cells were also treated with 300 nM TSA or 3 μM TSA. After a further 24 hours, cells were harvested for total RNA and analysed for UGT2B28 mRNA content by reverse transcription QPCR. Levels of each UGT were normalised against 18S rRNA and the results are presented as mean fold changes (n = 3) relative to the untreated, untransfected control (set arbitrarily to a value of one). Error bars indicate one standard deviation. P values for the indicated comparisons are *P < 0.001, †P = 0.001, $P = 0.007, $$$P = 0.012 and §P > 0.02 but < 0.05.

Each transcription factor tested (HNF1α, HNF1β, HNF4α, FoxA1, FoxA2, FoxA3, C/EBPα or C/EBPβ), as measured by changes in mRNA concentrations. Changes in each UGT mRNA concentration was only considered to be of likely biologically relevance if the change was greater than 2-fold and reached statistical significance (P < 0.05).

5.3.7.1. Hepatocyte nuclear factor 1α

When HNF1α was over-expressed in HepG2 cells, no effect was seen on the mRNA levels of the following UGT genes: UGT1A1 (Figure 5.8A), UGT1A3 (Figure 5.8B), UGT1A4 (Figure 5.8C), and all of the UGT2B forms (Figure 5.8G-M). In contrast, UGT1A6 mRNA was increased 5.6-fold (P = 0.003) (Figure 5.8D), UGT1A7 mRNA
was increased by 9.1-fold \((P < 0.001)\) (Figure 5.8E), and UGT1A9 mRNA was increased 3.1-fold \((P = 0.03)\) (Figure 5.8F).

In the presence of 300 nM TSA, the HNF1α response of UGT1A6 was increased from 5.6-fold to 10.6-fold \((P = 0.002)\) (Figure 5.8D), while the UGT1A7 and UGT1A9 responses were both diminished such that they lost statistical significance. The remaining UGT genes were also not significantly altered by combined HNF1α/300 nM TSA treatment, relative to their expression in pCMX-PL2-transfected cells treated with 300 nM TSA.

In contrast, exogenous over-expression of HNF1α in the presence of 3 μM TSA caused a great deal of change in UGT mRNA levels in HepG2 cells. Statistically significant increases in mRNA, greater than 2-fold relative to pCMX-PL2-transfected cells treated with 3 μM TSA, were observed for UGT1A4 (8.6-fold, \(P < 0.001\)), UGT1A6 (26.8-fold, \(P < 0.001\)), UGT1A7 (45.5-fold, \(P < 0.001\)), UGT1A9 (16.0-fold, \(P < 0.001\)), UGT2B4 (18.3-fold, \(P = 0.001\)), UGT2B11 (3.3-fold, \(P = 0.002\)), UGT2B15 (4.9-fold, \(P = 0.006\)), UGT2B17 (3.4-fold, \(P = 0.014\)) and UGT2B28 (12.0-fold, \(P = 0.001\)) transcripts (Figure 5.8C-G and J-M).

At least one potential HNF1-binding site has been identified in the proximal promoter of every known human UGT1A and UGT2B gene (Figure 5.1). As a result, HNF1α is the most thoroughly studied transcriptional regulator of human UGTs to date. However, until now, no attempts have been made to compare the effects of HNF1α on each individual gene in one experimental system. Therefore, the relative importance of this transcription factor in the expression of each UGT has remained unexplored. This experiment begins to address this issue, highlighting the variety of functional interactions that HNF1α may have with different UGT genes, and the
importance of not only identifying relevant transcription factors, but also understanding how they fit into the hierarchy of a gene’s transcriptional control.

The first notable observation from this experiment is that the endogenous levels of UGT1A1, UGT1A3 and UGT1A4 mRNAs do not change with HNF1α over-expression in HepG2 cells. Furthermore, UGT1A1 and UGT1A3 mRNA levels are highly increased in the presence of 3 μM TSA, a condition that has been shown to cause dramatic loss of HNF1α expression (Figure 5.7). Yet, all three corresponding genes have previously been identified as HNF1α target genes in reporter-plasmid assays, and each contains an experimentally confirmed HNF1α-binding site in its proximal promoter (10/12, 12/12 and 10/12 consensus nucleotides respectively, although the UGT1A4 element does not contain any perfect half-sites). There are at least two explanations that would reconcile these seemingly contradictory results. The first is that HNF1α is not required for the expression of these UGT1A forms in vivo, and that the dependence of the naked proximal promoters on this transcription factor is an artefact of studying short promoter sequences. However, the weight of evidence suggests that a more complex reason lies behind these observations. Excess HNF1α does not greatly increase the activity of the UGT1A3 or UGT1A4 promoters in vitro in HepG2 cells (the effect of HNF1α on the naked UGT1A1 promoter in HepG2 cells is unreported), and the importance of HNF1α for the function of the UGT1A3 and UGT1A4 promoters is only evident if their respective HNF1-binding sites are destroyed or if HNF1-factors are initially absent in the host cell and subsequently supplied (see Chapter 3). In regards to these latter properties, the UGT1A1 promoter behaves similarly (Bernard et al., 1999). This has led to the hypothesis that the strong HNF1-binding elements in the UGT1A1, UGT1A3 and UGT1A4 promoters are fully occupied, or nearly so, by HNF1 factors at the
concentrations at which they naturally occur in HepG2 cells (Chapter 3). If this premise is true, and holds true for the endogenous genes also, then excess HNF1α would not be expected to greatly increase endogenous gene expression either. Therefore, a lack of response does not preclude a regulatory role for HNF1α. However, a loss of HNF1α, as seen after the 3 μM TSA exposure would be expected to decrease expression of HNF1α-dependent genes, while instead, the UGT1A1 and UGT1A3 promoter activities were greatly increased. Yet, this unexpected response could also be explained if the presence of TSA makes the function of HNF1α redundant for these genes. As reviewed in Chapter 3, section 3.1.5.4, HNF1α has a dual purpose in gene regulation; chromatin remodelling and recruitment of the transcription machinery. The former is achieved through recruitment and activation of proteins with HAT activity such as p300/CBP and P/CAF (Pontoglio et al., 1997; Rollini et al., 1999; Soutoglou et al., 2000b; Parrizas et al., 2001; Soutoglou et al., 2001), and HNF1α has been proposed to only play an obligate role in transcriptional activation when it is required to recruit HAT activity (Parrizas et al., 2001). Since treatment with HDAC inhibitors has a similar net effect on gene acetylation as HAT recruitment, TSA may be able to replace the function of HNF1 factors on the UGT1A1 and UGT1A3 promoters by counteracting any recruitment of HDACs that would normally occur. Interestingly, unliganded and antagonist-loaded nuclear receptors can recruit HDAC activity to their target genes through co-repressors such as SMRT and NCoR (Ng and Bird, 2000; Karvonen et al., 2006), and UGT1A1 is a known target for numerous nuclear receptors (PXR, CAR, GR, AhR and PPARα) (Sugatani et al., 2004; Usui et al., 2006a;Seneko-Effenberger et al., 2007). UGT1A3 is also a target for PPARα and PXR (Gardner-Stephen et al., 2004; Seneko-Effenberger et al., 2007). Furthermore, TSA-mediated relief of nuclear
receptor-mediated gene repression, through inhibition of nuclear receptor-associated HDACs, is a recently recognised phenomenon (Huang and Hung, 2006; Karvonen et al., 2006; Kwon et al., 2006; Qi and Ratnam, 2006). Therefore, if the literature and this experiment are considered collectively, the data suggests that HNF1α may be more important in directing histone acetylation than recruitment of the transcription machinery to UGT1A1 and UGT1A3 in the hepatic chromosomal setting, as was found for the murine glut2 and L-type pyruvate kinase genes in pancreatic cells (Parrizas et al., 2001).

Since increasing HNF1α in HepG2 cells did not have a corresponding effect on the UGT1A1 or UGT1A3 genes, the importance of HNF1α in maintaining their basal expression could, in the future, possibly be demonstrated through knock-down experiments. Indeed, unpublished studies performed by Anne Rogers in our laboratory show that siRNA-mediated knock-down of HNF1α mRNA in Caco-2 cells results in similar fold decreases in HNF1α and UGT1A1 mRNA levels (personal communication, results not shown). However, HepG2 cells would not be a suitable experimental system for such an investigation of the UGT1A3 gene, due to insufficient basal expression of UGT1A3 mRNA. Instead, primary hepatocytes could be used to study the effect of HNF1α-knock-down on UGT1A3 (and all other hepatic UGTs), but are resistant to conventional transfection methods and would require treatment with an adenoviral-mediated HNF1α-antisense targeting vector system like that described for HNF4α knock-down by Jover et al. (2001), or similar. Several donors would also be required to account for interindividual differences in response.

Evidence that genomic UGT1A4 may also be a genuine HNF1α-target gene, as previously suggested by transient transfection assays (Gardner-Stephen and Mackenzie, 2007b and Chapter 3), was only obtained when cells were co-treated
with 3 μM TSA and pCMX-HNF1α. This combined treatment resulted in an increase in UGT1A4 transcripts of 8.7-fold (Figure 5.8C). The simplest explanation of these results is that HNF1α plays a role in recruiting, or positioning, the transcription machinery to the UGT1A4 gene, but only if permitted by other more important chromatin remodelling factors, and/or in co-operation with other transactivator(s) normally unavailable in HepG2 cells. Treatment of HNF1α with 1 μM TSA has been shown to inhibit its interaction with HDAC-1, allowing constitutive activation through its co-factors p300/CBP and P/CAF (Soutoglou et al., 2001). If the HNF1α dimers that normally reside on the integrated UGT1A4 promoter require the assistance of other factors to facilitate a switch in bound co-factors from NCoR/HDAC to p300/CBP and P/CAF, this would explain why only the high concentration of TSA had a profoundly positive effect on UGT1A4 transcription by HNF1α. Additional evidence that HNF1α may be relatively low in the hierarchy of proteins required for UGT1A4 expression comes from transient transfections in HEK293T cells that showed that the UGT1A4 proximal promoter has some basal activity in the complete absence of HNF1 factors (Chapter 3, Figure 3.3A). What is certain, however, is that HNF1α and the other factors normally expressed in HepG2 cells are insufficient to drive UGT1A4 promoter activity when this gene is chromatin bound.

The stark contrast between the responses of the UGT1A3 and UGT1A4 genes to HNF1α, with or without TSA exposure, was not anticipated due to the high similarity in primary sequence of their proximal promoters (over 88% identity over 1kb) and their comparable responses to HNF1α and HNF1-site mutation in transient transfection assays (Chapter 3). Thus, this new data illustrates the value of performing studies on endogenous genes, and suggests that distal enhancer elements
may play a significant role in the differential expression of \textit{UGT1A3} and \textit{UGT1A4}. However, this work also raises questions regarding how many of these differences would be apparent if the \textit{UGT1A4} gene was already expressed at a more appropriate basal level. Comparison of the results obtained in HepG2 cells with a cell line that expresses UGT1A4 may be useful for further characterising the role of HNF1α in \textit{UGT1A4} promoter activity. However, since such a model was unavailable, the full \textit{UGT1A3} and \textit{UGT1A4} promoters, which are both active in HepG2 transient transfection assays, were tested for responsiveness to HNF1α in the presence of 3 μM TSA to determine whether any further information could be gleaned regarding the differential expression of these genes.

The result of treating cells transfected transiently with the full \textit{UGT1A3} and \textit{UGT1A4} promoters with 3 μM TSA (Figure 5.9) was surprisingly similar to the effect seen with the endogenous genes (Figure 5.8B and C). The \textit{UGT1A3}-9.4kb promoter was highly responsive to the presence of TSA, giving an 11.4-fold increase in luciferase reporter activity over solvent treated cells (\(P < 0.001\)). In contrast, the response of the \textit{UGT1A4}-5kb promoter was only 1.3-fold (\(P = 0.014\)) (Figure 5.9). This observation reflects the much greater increase in UGT1A3 mRNA levels in 3 μM TSA-treated HepG2 cells over UGT1A4 (Figure 5.6). Furthermore, HNF1α overexpression in TSA-treated cells only had a mild effect on the \textit{UGT1A3} promoter, resulting in a further 2.4-fold increase in reporter activity (\(P < 0.001\)), but a drastic effect on the \textit{UGT1A4} promoter (Figure 5.9), as seen for the endogenous gene (Figure 5.8C). Reporter gene expression from the \textit{UGT1A4}-5kb promoter in the presence of both HNF1α and 3 μM TSA was increased 58.6-fold over TSA alone (\(P < 0.001\)). Interestingly, the combined effects of HNF1α-over expression and 3 μM
TSA-treatment on the *UGT1A3* and *UGT1A4* promoters resulted in similar overall reporter gene expression (Figure 5.9).

![Graph showing luciferase activity](image)

**Figure 5.9:** The *UGT1A3* and *UGT1A4* promoter responses to TSA and HNF1α. HepG2 cells were transiently transfected with 25 ng pRL-Null, 0.5 μg pGL3-basic, pGL3-1A3-9.4k or pGL3-1A4-5k and 0.25 μg pCMX-PL2 or pCMX-HNF1α. After 24 hours, each transfection was exposed to 1:1000 diluted ethanol, or 3 μM TSA for a further 24 hour period. The luciferase reporter activity of each cell lysate was then normalised to renilla activity and mean ratios (*n* = 3) are presented relative to ethanol-treated pGL3-basic transfections. Error bars indicate one standard deviation. *P* values for the indicated comparisons are *P* < 0.001 and ¥¥*P* = 0.014. NB: this experiment was only performed once.

The discovery that the transiently transfected *UGT1A3* promoter is specifically activated by TSA in HepG2 cells will allow some of the questions raised earlier in this thesis to be investigated. From the current data, it is still impossible to determine whether the effect of TSA on *UGT1A3* is due to direct changes in histone acetylation or a secondary effect of changed protein expression in HepG2 cells. However, TSA-treatment of promoter deletion constructs, TSA treatment of promoter constructs with specific mutations (particularly in the known HNF1-binding site), *in vitro* chromatin assays and histone acetylation studies could all be combined to determine the mechanisms responsible for the differences in the *UGT1A3* and *UGT1A4* TSA response. Similar studies would also determine whether the synergistic effect of
HNF1α and TSA on the UGT1A4 promoter is the result of HDAC-inhibition, an interaction of HNF1α with TSA-induced factors, or possibly, both.

Interestingly, of all the human UGT1A and UGT2B genes, the UGT1A6, UGT1A7 and UGT1A9 promoters were the only sequences to respond to excess HNF1α in HepG2 cells without TSA. This result is consistent with the presence of putative HNF1 sites in these sequences (10/12, 9/12 and 9/12 consensus nucleotides respectively, with no perfect half-sites), and with the functional data published for the UGT1A9 promoter (Gregory et al., 2004; Gardner-Stephen and Mackenzie, 2007a). Elements that diverge significantly from the HNF1-binding consensus, whilst still remaining capable of binding HNF1 factors, could be expected to mediate a response to increased levels of HNF1α. Such sites are unlikely to compete efficiently for HNF1 factors at physiological levels; therefore, increasing HNF1α levels may increase binding-site occupancy. To my knowledge, this is also the first actual report of HNF1α-responsiveness for human UGT1A6 and UGT1A7, although both outcomes have been predicted (Auyeung et al., 2003; Gardner-Stephen and Mackenzie, 2005).

In addition to their increased expression in response to increased HNF1α, UGT1A6 and UGT1A9 mRNA levels are both severely diminished by 3 μM TSA treatment. Since their expression can be restored by exogenous HNF1α in the presence of TSA, but only to similar levels as seen for pCMX-HNF1α-transfected cells not treated with TSA, it appears that the relationship between HNF1α and expression of UGT1A6 and UGT1A9 is one of complete dependence. Furthermore, the response is independent of any other intracellular changes TSA may cause. Since histone hyperacetylation by 3 μM TSA does not functionally compensate for the loss of HNF1α that it triggers, the predominant role of HNF1α in the expression of UGT1A6
and UGT1A9 appears to be the recruitment or activation of other transcription factors or the transcription machinery. This observation is consistent with the discovery that the HNF4α response of the UGT1A9 promoter is completely reliant on HNF1 factors (Gardner-Stephen and Mackenzie, 2007a and Chapter 4). Interestingly however, HNF1α does not stimulate transcription from the UGT1A6-3kb promoter in transient transfections (Figure 5.10), even though this construct contains the predicted HNF1-binding site. This is in direct contrast with the effect of HNF1α on the proximal UGT1A9 promoter (Chapter 4, Figure 4.4B) and again highlights the value of testing endogenous gene responses. As an explanation, it is possible that the HNF1-binding site of the transiently transfected UGT1A6-3k promoter is fully occupied by HNF1 factors under basal conditions, as hypothesised for the UGT1A4 promoter, which likewise has a HNF1-binding site with 10/12 matches with

![Figure 5.10](image_url)

**Figure 5.10: Response of the UGT1A6 proximal promoter to HNF1α and HNF4α in HepG2 cells.** HepG2 cells were transfected with 25 ng pRL-Null, 0.5 μg pGL3-basic or pGL3-1A6-3k and 0.25 μg pCMX-PL2, pCMX-HNF1α or pCMX-HNF4α. After 48 hours, cells were lysed and assayed for luciferase and renilla activity. Mean luciferase:renilla ratios (n = 3) are presented relative to the result for pGL3-basic (set arbitrarily to one). Error bars represent one standard deviation. The *P* value for the indicated comparison is *P* < 0.001.
consensus but no perfect half-site. However, the data from the endogenous gene belies this suggestion, unless the structural chromatin environment reduces the affinity of the *UGT1A6* HNF1-binding site for HNF1 factors without completely precluding HNF1 binding. Other possible explanations are that the observed *UGT1A6* response to HNF1α is not mediated through the published HNF1-binding site, but through another site not included in the cloned fragment; or that the transfected promoter fragment is sufficiently more accessible than the endogenous gene to cause the (still theoretical) role of HNF1α in recruiting additional transcription factors or the transcriptional machinery to the *UGT1A6* promoter to become superfluous. Longer clones of the *UGT1A6* promoter and site-directed mutagenesis of the putative HNF1-binding site will be required to test these hypotheses.

Although the *UGT1A7* gene is expected to be HNF1α-responsive in the appropriate context, as its proximal promoter structure is most like that of *UGT1A8*, *UGT1A9* and *UGT1A10*, the response of *UGT1A7* to over-expressed HNF1α in HepG2 cells in the absence of chromatin-altering agents was unforeseen. Like *UGT1A8* and *UGT1A10*, which did not respond to HNF1α in HepG2 cells (data not shown), *UGT1A7* is only extrahepatically expressed. Yet, the unaided response of *UGT1A7* to HNF1α indicates that this gene is poised for transcription in liver-derived HepG2 cells. Whether this is true in human hepatocytes, or is a function of the slightly dedifferentiated state of HepG2 cells is unknown, but an interesting question. Given that *UGT1A7* has a weak HNF1 site, perhaps this gene is not expressed in hepatocytes partly because in these cells it cannot recruit enough HNF1α when this factor is only available at physiological levels. On the other hand, UGT1A7 mRNA transcription may be driven by HNF1α in non-hepatic tissues where different factors
are available to bind to the UGT1A7 promoter, thereby recruiting HNF1α to its binding site. Over-expression of HNF1α in primary hepatocytes would help determine whether UGT1A7 is normally more tightly suppressed in the liver than in HepG2 cells. Promoter studies combined with TSA treatment, as suggested for the UGT1A3 and UGT1A4 promoters, may also be worthwhile for comparing the promoters of UGT1A7 and UGT1A9. Despite having very similar proximal promoters and basal responses to HNF1α, HNF1β and HNF4α over-expression (see also sections 5.3.7.2 and 5.3.7.3), UGT1A7 and UGT1A9 have very different reactions to the combined TSA/HNF1α treatments. Elucidation of the cause may reveal important functional elements that differ between these genes, and contribute to our understanding of the mechanisms causing their distinct tissue-specific expression patterns.

Interestingly, the UGT2B genes all have one known HNF1-binding site (9/12 consensus nucleotides each, no perfect half site) in their proximal promoters (Figure 5.1), yet none of these genes were up-regulated by excess HNF1α alone in HepG2 cells. However, all but two, UGT2B7 and UGT2B10, were responsive to HNF1α in the presence of 3 µM TSA, identifying their promoters as potential HNF1α targets. Why no HNF1α-response was seen for the UGT2B7 and UGT2B10 genes under any conditions is a mystery, particularly as both genes are transcriptionally active in HepG2 cells (Figure 5.3), the UGT2B7 promoter has been shown to be HNF1α-responsive in vitro (Ishii et al., 2000; Gregory et al., 2006) and UGT2B7 mRNA expression levels have been correlated with HNF1α mRNA levels in human liver (Toide et al., 2002). Perhaps the HNF1-binding elements of these genes are fully occupied at physiological levels, even though they are expected to be of relatively low affinity, through recruitment or stabilisation by other proteins. HNF1α-mediated
regulation of the *UGT2B7* gene is already known to be enhanced by co-operation with Oct-1 in HepG2 cells and Cdx2 in Caco-2 cells (Ishii *et al.*, 2000; Gregory *et al.*, 2006). Another possibility is that a factor required for co-operation with HNF1α is limited in HepG2 cells, and the system is already relatively saturated with HNF1α. Either way, the hypothetical need for at least one other factor for HNF1α to effectively regulate the *UGT2B7* and *UGT2B10* promoters is supported by the observation that over-expression of HNF1α did not rescue their expression after repression by 3 μM TSA, and thus the profound loss of UGT2B7 and UGT2B10 expression in 3 μM TSA-treated cells is not entirely due to the physical absence of HNF1α. A third possibility is that HNF1α has no real role in the expression of the endogenous *UGT2B7* and *UGT2B10* genes in hepatocytes, and the relationship noted between HNF1α and UGT2B7 mRNAs by Toide *et al.* (2002) is not a causative association. Once again, siRNA-mediated knockdown of HNF1α in HepG2 cells would add valuable information to the apparently contradictory observations between this experiment and previous results.

The responses of the remaining *UGT2B* genes to over-expressed HNF1α and/or TSA treatment were very similar in pattern to *UGT1A4*. Therefore, the arguments presented earlier for the involvement of HNF1α in the expression of *UGT1A4* are also pertinent for these genes, except that it is unknown whether any of these promoters have residual activity in HNF1-negative cells. One additional piece of information we do have however, is that siRNA-mediated knockdown of HNF1α in Caco-2 cells results in a decrease in UGT2B15 transcripts (Anne Rogers, unpublished observations), providing further support for the hypothesis that HNF1α genuinely interacts with this gene in at least one cell type.
5.3.7.2. Hepatocyte nuclear factor 1β

Similarly to HNF1α, HNF1β over-expression had significant positive impact on the levels of UGT1A6, UGT1A7 and UGT1A9 mRNA in HepG2 cells, increasing them by 2.5-fold \((P = 0.036)\), 8.1-fold \((P < 0.001)\) and 2.9-fold \((P = 0.044)\) respectively (Figure 5.8D-F). However, on co-treatment with 300 nM TSA, none of these responses to HNF1β over-expression were retained. On the other hand, UGT2B28 mRNA levels, which were unaffected by HNF1β alone, or in combination with 300 nM TSA, were increased 4.8-fold \((P = 0.007)\) by HNF1β over-expression in the presence of 3 μM TSA.

Although HNF1β is not expressed to high levels in the adult liver (Rey-Campos et al., 1991), it was of interest to compare its effect with that of HNF1α because these two proteins recognise the same nucleotide sequences and heterodimerise readily, but possess functionally divergent activation domains (see Chapter 3, section 3.1.5.1). Of the three genes transactivated by HNF1α alone, UGT1A7 and UGT1A9 were equally responsive to HNF1β as HNF1α \((P > 0.05)\) (Figure 5.8E and F), whereas the third HNF1α-responsive gene, UGT1A6, was also increased by HNF1β, but only to half the extent \((P = 0.014)\). In general, HNF1β has been considered to be a less potent transactivator than HNF1α (Senkel et al., 2005), and this assumption has held true for transient transfections against the human UGT1A3 (Chapter 3), UGT1A8 (Gregory et al., 2004), UGT1A9 (Gardner-Stephen and Mackenzie, 2007a and Chapter 4, section 4.3.9), UGT2B7 (Ishii et al., 2000), UGT2B17 (Gregory et al., 2000), rat UGT1A6 (Auyeung et al., 2003) and rat UGT2B1 (Hansen et al., 1997) promoters; with human and rat UGT1A1 and rat UGT1A7 being the only known exceptions (Bernard et al., 1999; Metz et al., 2000). However, Senkel and colleagues have recently postulated that HNF1β may be a more effective transactivator in the
chromosomal context than in transient transfections (Senkel et al., 2005), a hypothesis that appears relevant to at least UGT1A9. The discovery that HNF1β may be as effective as HNF1α in regulating the UGT1A7 and UGT1A9 genes suggests that HNF1β may have a more important role in non-hepatic UGT expression than previously thought.

In the presence of TSA, the functional differences between HNF1α and HNF1β were more apparent. UGT1A6, the only gene to show a further increase in response to HNF1α in the presence of 300 nM TSA, was not affected by the HNF1β/300 nM TSA combination (in comparison to HNF1β alone, P > 0.05). Furthermore, the UGT gene responses to HNF1α observed in the presence of 3 μM TSA were either significantly reduced or completely absent for HNF1β. This result indicates that the mechanism responsible for the widespread synergistic effect of HNF1α and 3 μM TSA on the expression of many HepG2 endogenous UGT genes is not simply the loss of HDAC-1 recruitment by HNF1α, because HNF1β also interacts with HDAC-1 and has greatly increased activity in the presence of 1 μM TSA (Barbacci et al., 2004). Therefore, the specific up-regulation of UGT transcription by HNF1α and TSA is likely to also involve interactions with proteins that have increased expression and/or activity after TSA exposure, and that differ in their ability to cooperate with the two HNF1 proteins.

Interestingly, HNF1β over-expression did not inhibit the transcription of any UGT genes, even those that have been shown to be exclusively responsive to HNF1α in HepG2 transient transfections, such as UGT2B7 (Ishii et al., 2000) and UGT2B17 (Gregory et al., 2000). In the event of HNF1β over-expression, HepG2 endogenous HNF1α protein would be expected to exist largely in heterodimers with HNF1β, and would be competing with HNF1β homodimers for DNA-binding sites. As a
consequence, it is expected that where HNF1β cannot substitute functionally for HNF1α, HNF1β over-expression would prevent gene activation by HNF1α. Thus, it seems that either: a) HNF1α is not supporting basal expression of the human UGT genes; or b) HNF1α/HNF1β heterodimers have similar activity towards the UGT promoters as HNF1α homodimers and specific protein-protein interactions between HNF1α and other promoter-bound proteins cause only HNF1α-containing heterodimers to be recruited.

5.3.7.3. Hepatocyte nuclear factor 4α

Over-expression of HNF4α in HepG2 cells had no effect on any UGT2B forms, whether expressed in the presence or absence of TSA. In contrast, HNF4α alone increased the mRNA levels of UGT1A1 by 7.0-fold, \((P < 0.001)\), UGT1A6 by 5.9-fold \((P = 0.001)\), UGT1A7 by 12.7-fold \((P < 0.001)\) and UGT1A9 by 21.8-fold \((P < 0.001)\) (Figure 5.8A and D-F).

Addition of 300 nM TSA to HNF4α over-expressing cells enhanced the UGT1A9 response \((74.0\text{-fold}, P < 0.001)\) and decreased the apparent response of UGT1A1 \((2.4\text{-fold}, P = 0.001)\), UGT1A6 \((2.6\text{-fold}, P = 0.049)\) and UGT1A7 \((9.3\text{-fold}, P = 0.006)\) (Figure 5.8A and D-F). However, in all three latter cases, the decrease in fold change was caused by increased UGT expression in the 300 nM TSA/pCMX controls, rather than a decrease in the total transcripts induced by HNF4α.

Addition of 3 μM TSA to HNF4α over-expressing cells had a variable effect, depending on the UGT studied. UGT1A1 expression continued to be enhanced by HNF4α at this TSA concentration \((2.8\text{-fold}, P = 0.005)\), however, the UGT1A6 and UGT1A7 responses were completely inhibited, while the UGT1A9 response was severely suppressed, being reduced to 7.6-fold \((P = 0.001)\) (Figure 5.8A and D-F).
As was found for the HNF1 factors, over-expressed HNF4α had multiple UGT targets and at least two distinct activation pathways in HepG2 cells. Of the four UGT1A genes that responded to HNF4α, UGT1A9 was the only previously known target, although the expression patterns of UGT1A1 and UGT1A6 (Table 1.2) are both consistent with possible HNF4α-mediated regulation. The discovery of an unaided response of UGT1A7 to another LETF in HepG2 cells was, once again, surprising, as the only currently known overlap in HNF4α and UGT1A7 expression occurs in the stomach. Other major sites of HNF4α expression - the liver, kidney and intestine - are all free of UGT1A7 mRNA. Furthermore, in transient transfections, the UGT1A7 proximal promoter is completely unresponsive to HNF4α (Barbier et al., 2005; Gardner-Stephen and Mackenzie, 2007a and Chapter 4). These observations previously founded the hypothesis that HNF4α-responsiveness is one mechanism that affords UGT1A9 its unique hepatic status among the UGT1A7-1A10 genetic cluster; however this theory must now be questioned. Clearly there is more work required, both to characterise the UGT1A7 promoter elements that allow HNF4α to increase UGT1A7 transcript levels, and to identify further DNA elements that cause UGT1A9 to be specifically expressed in hepatocytes. None of the remaining LETFs investigated in this experiment provided any leads towards the second challenge.

The four HNF4α-responsive UGT1A genes can be divided on the basis of the combined effect of TSA and excess HNF4α on their transcript levels. 300 nM TSA synergistically increased the transactivational activity of HNF4α towards UGT1A1 (Figure 5.8A), but had little or no effect for UGT1A6, UGT1A7 and UGT1A9 (Figure 5.8D, E and F) over HNF4α alone. Exposure to 3 μM TSA further accentuated the response of UGT1A1 to HNF4α, but abolished the responses of UGT1A6 and
UGT1A7, and reduced the UGT1A9 response by 90%. Therefore, it would be reasonable to postulate that the mechanisms that drive HNF4α-mediated transcription from UGT1A1 are different from those of the other three genes. HNF4α activation of the UGT1A9 proximal promoter in HepG2 cells is dependent on the co-expression of at least one of the HNF1 factors (Gardner-Stephen and Mackenzie, 2007a and Chapter 4). Since 3 μM, but not 300 nM, TSA exposure is known to cause a loss of HNF1α mRNA in these cells, this mechanism alone could explain the significant loss of UGT1A9 activity, and by association, also that of UGT1A6 and UGT1A7. Once the HNF4α-responsive elements of the UGT1A6 and UGT1A7 promoters have been identified, this hypothesis could be tested by transient transfection in HEK293 cells as was demonstrated for UGT1A9. Towards this end, the UGT1A6-3kb promoter has been shown to be HNF4α-responsive in HepG2 cells (P < 0.001) (Figure 5.10).

The residual effect of HNF4α on the UGT1A9 gene in the presence of 3 μM TSA, and hence in the absence of HNF1α, is also interesting. This weaker activation could represent co-operation with HNF1β, as the effect of 3 μM TSA on expression of HNF1β is unknown, or with residual levels of HNF1α too low to be detected by Western blot. Alternatively, the residual activation observed may represent a minor HNF1-independent effect of HNF4α on UGT1A9. The latter possibility may be mediated through mechanisms that do not operate on transiently transfected DNA, or may be the result of HNF4α-binding site(s) positioned beyond the currently tested promoter fragments.

The synergistic increase in UGT1A1 mRNA levels at both tested concentrations of TSA indicates that HNF4α probably does not require HNF1α for recruitment to the UGT1A1 promoter or interaction with the transcriptional machinery. However, if these two factors would ordinarily synergistically affect acetylation of the UGT1A1
promoter, this effect would be masked by the TSA treatment, so cannot be ruled out. How TSA increases the effect of HNF4α on the UGT1A1 promoter is unknown, but possibilities include: a) increased accessibility of the promoter through inhibition of HDACs recruited by other factors; b) increased acetylation of the HNF4α protein itself, which increases its nuclear retention and DNA binding (Soutoglou et al., 2000a); c) increased activity of HNF4α through prevention of its known associations with HDAC through SMRT (Torres-Padilla et al., 2002); or d) increased co-operation with other transcription factors or co-factors normally limited/unavailable in untreated HepG2 cells. Further work, as described for HNF1α in section 5.3.7.1, will be necessary to further characterise the role of HNF4α in UGT1A1 expression. In particular, it will be interesting to determine the relative importance of HNF4α in the constitutive and inducible expression of UGT1A1, as HNF4α was found to be necessary for the CAR-response of the mouse Ugt1a1 gene, but not basal expression (Ding et al., 2006).

5.3.7.4. Hepatocyte nuclear factor 6

HNF6 was unable to affect the expression of any human UGT gene in HepG2 cells without the assistance of TSA. In combination with 300 nM or 3 μM TSA however, UGT1A4 mRNA transcript levels were increased by 5.7-fold \((P = 0.010)\) and 4.4-fold \((P < 0.001)\) respectively (Figure 5.8C). An increase in UGT2B11 mRNA levels (2.9-fold, \(P < 0.001\)) was also observed at the higher TSA concentration (Figure 5.6J).

Acetylation of HNF6 protein by CBP has been shown to increase its half-life, allowing it to accumulate to higher levels in HepG2 cells co-transfected with HNF6 and CBP, than those transfected with HNF6 alone (Rausa et al., 2004). Therefore, although it is unknown whether HDACs can deacetylate HNF6, thereby reducing the
protein’s stability, it is conceivable that TSA could protect HNF6 from degradation, and hence increase its apparent activity. However, it seems unlikely that this is the mechanism responsible for the TSA-mediated HNF6-responses of the UGTIA4 gene. The first reason for this conclusion is that expression of HNF6 from a transiently transfected, CMV-promoter-driven construct has previously been shown to saturate an HNF6-responsive reporter-promoter without exogenously supplied mediators of acetylation (Rausa et al., 2004). Secondly, while HNF6 is only able to transactivate the endogenous UGTIA4 gene in the presence of TSA, the transiently transfected UGTIA4 promoter is increased 10.3-fold ($P < 0.001$) by HNF6 in TSA-naive cells (Figure 5.11). Moreover, 3 μM TSA is inhibitory to this interaction, reducing the ability of HNF6 to activate the exogenous promoter by two thirds ($P = 0.001$). In

![Figure 5.11](image_url)

**Figure 5.11:** HNF6 regulates the transiently transfected UGTIA4 promoter in HepG2 cells. HepG2 cells seeded into 24-well plates were transiently transfected with 25 ng pRL-Null, 0.5 μg pGL3-basic, pGL3-1A4-5k and 0.25 μg pCMX-PL2 or pCMX-HNF6 per well. Twenty-four hours post-transfection, cells were treated with 1:1000 diluted ethanol or 3 μM TSA. After a further 24 hours, cell lysates were assayed for luciferase and renilla activity. Mean luciferase:renilla ratios ($n = 3$) are presented relative to the result for pGL3-basic (set arbitrarily to one). Error bars represent one standard deviation. $P$ values for the indicated comparisons are *$P < 0.001$ and †$P = 0.001$. NB: the ethanol and TSA-treated triplicates of this experiment were only performed once.
light of these results, it seems unlikely that TSA is increasing HNF6 activity, or causing the expression of co-factors, or other transcription factors, required for HNF6 to have a functional impact on the UGT1A4 promoter. Therefore, the most likely mechanism through which HNF6 increases the levels of UGT1A4 mRNA in HepG2 cells is through improved accessibility of the promoter. Whether a similar argument will hold true for the UGT2B11 promoter is uncertain, as this gene is already relatively active in HepG2 cells (Figure 5.3). However, TSA alone had a similar effect on UGT2B11 as UGT1A4 (Figure 5.6), so it is both conceivable and of interest to investigate.

5.3.7.5. Forkhead box proteins FoxA1, FoxA2 and FoxA3

When FoxA1 was over-expressed in HepG2 cells, the most responsive gene was UGT2B15, with an increase in mRNA levels of 4.0-fold ($P = 0.002$) (Figure 5.8K). An increase in UGT1A1 transcripts of 2.9-fold ($P = 0.032$) was also observed (Figure 5.8A). In the presence of 300 nM TSA, the UGT2B15 mRNA response to FoxA1 was still apparent (2.2-fold, $P = 0.034$), although it was stronger when the cells were co-treated with 3 μM TSA (10.1-fold, $P = 0.013$). Concomitant 3 μM TSA exposure also allowed FoxA1 to increase UGT2B11 transcripts by 8.9-fold ($P = 0.001$) and UGT2B28 mRNA by 8.4-fold ($P = 0.001$) (Figure 5.8J and M). No UGT1A responses were observed to FoxA1 in the presence of TSA.

No changes in HepG2 UGT mRNA levels greater than 2-fold were recorded for cells transfected with FoxA2 expression plasmid alone, or cells that received the combined FoxA2 expression/300 nM TSA treatment. Furthermore, the only response to FoxA2 over-expression in the presence of 3 μM TSA that was greater than two-fold and reached statistical significance was from UGT2B28 (2.7-fold, $P = 0.046$).
The effect of FoxA3 over-expression on the UGT mRNA profile of HepG2 cells was most similar to that of FoxA1. Alone, FoxA3 over-expression increased levels of UGT2B15 mRNA by 4.2-fold (P < 0.001) (Figure 5.8K). In addition, although the increase of UGT2B15 transcripts dropped to less than two-fold with simultaneous 300 nM TSA treatment, 3 μM TSA restored UGT2B15 activation by FoxA3 to 5.4-fold (P < 0.001). The results obtained with FoxA3 further resembled the effects of FoxA1 in that FoxA3 was able to cause the accumulation of UGT2B11 (8.9-fold increase, P < 0.001) and UGT2B28 (9.9-fold increase, P = 0.001) transcripts in HepG2 cells treated with 3 μM TSA (Figure 5.8 J and M). Because the responses of the human UGT genes to FoxA1 and FoxA3 are essentially the same, they will be discussed together.

Two studies that have collectively investigated FoxA3 regulation of five human CYP genes have identified five independent patterns of response (Rodriguez-Antona et al., 2003; Bort et al., 2004). Similarly, the study presented in this thesis has identified four human UGTs as potential FoxA1 and/or FoxA3 gene targets, with three distinguishable response patterns. UGT1A1 most resembled CYP2C9 with a weak response to FoxA1 that was not synergistically increased by TSA. The UGT2B11 and UGT2B28 genes only responded to FoxA1/FoxA3 in the presence of 3 μM TSA, much like CYP2C8 and CYP3A4. And akin to the CYP2C19 gene, the endogenous UGT2B15 responses to FoxA1 and FoxA3 were both strengthened by the addition of TSA. Thus, the observation that UGT gene family members are regulated by many of the same transcription factors, yet independently of each other through different mechanisms, is a recurring theme that also extends to the regulation of other biotransformation-enzyme superfamilies. Furthermore, comparing the current literature and the work presented in this chapter, there appear to be more similarities
in the FoxA regulation of genes from different enzyme superfamilies than within genetic clusters, suggesting a potential mechanism for the co-ordinate regulation of enzymes required for different stages of chemical metabolism and elimination that would be worthy of further investigation.

FoxA proteins can stably bind to their target sequences within compacted, hypoacetylated chromatin to promote the assembly of enhancer complexes, and the acetylation status of histones does not affect DNA binding by FoxA1 (Cirillo and Zaret, 1999). Furthermore, to date, FoxA factors have not been reported to physically associate with HDACs. Therefore, it is unlikely that TSA greatly enhances the accessibility of FoxA target sites or increases the inherent activity of these proteins. Yet, addition of 3 μM TSA improved the UGT responses to FoxA1 and FoxA3 in all but one example. One possible explanation for these results is that although over-expressed FoxA proteins bind to the identified genes, and perhaps even remodel their respective promoters, these interactions are insufficient to efficiently recruit and activate the basal transcription machinery. Other transcription factors are presumably required. These may be limited in HepG2 cells and supplied or replaced by TSA; or in the case of repressors, removed or inactivated by TSA. In support of this hypothesis, Rodriguez-Antona and colleagues found that FoxA3 alone did not affect CYP3A4 promoter activity in the context of either plasmid or genomic DNA in HepG2 cells (Rodriguez-Antona et al., 2003). However, FoxA3 was highly co-operative with C/EBPα, activating the endogenous promoter in a hepatocyte-specific manner.

A logical extension of this theory is that it is possible for any of the UGT genes that were completely unresponsive to FoxA factors in this assay to still be FoxA targets, either in liver or other tissues; provided that none of the conditions tested supplied all
of the factors required for their FoxA-mediated activation. Indeed, it is not expected that FoxA factors are independently capable of driving UGT expression, as FoxA factors are expressed early in development (Friedman and Kaestner, 2006), but UGTs are not. One potential example is UGT2B17, a gene that requires FoxA1 for its oestrogen-responsiveness in MCF-7 cells (Laganiere et al., 2005), but did not respond to FoxA in this experiment. It will be relatively difficult to identify such genes, if they exist, especially if FoxA factors are only required for the inducible component of their expression. siRNA-mediated knockdown of FoxA1 did not affect the basal levels of UGT2B17 transcripts (Laganiere et al., 2005), and it is unknown whether this is because FoxA factors are entirely unnecessary for basal expression, or whether FoxA2 or FoxA3 can compensate for FoxA1 in constitutive transcription but cannot interact with the oestrogen receptor. Certainly, for the currently identified UGT targets of FoxA, FoxA1 and FoxA3 have very similar abilities to drive expression. Expression of a truncated FoxA protein in hepatocyte-derived cells has been shown to decrease expression of certain FoxA-dependent genes (Vallet et al., 1995), and presumably competes with all three FoxA factors by occupying all binding sites. Consequently, this mutant may be a better tool for assessing the requirement of human UGT genes for FoxA factors in HepG2 or primary cells than siRNA knockdown studies.

The lack of responses to the FoxA2 expression plasmid in this experiment may have several causes, including a genuine lack of involvement of FoxA2 in UGT expression, deficiencies in the HepG2 proteome such that FoxA2 cannot support transcription from its target genes, insufficient homology of rat FoxA2 with the human protein to allow functional equivalence (unlikely, but possible at 96% identity), or a vector error that prevents FoxA2 expression. Since no sensible
conclusions can be drawn from the presented data if FoxA2 protein was not expressed, the FoxA2 expression vector was validated against a published gene target (see section 5.3.8). The vector was found to be functional; therefore, it is reasonable to conclude that FoxA2 is sufficiently different from the remaining two FoxA family members that it does not participate in UGT regulation, at least in the same way. This is an interesting observation, as FoxA1 and FoxA2 are the more closely related in sequence, being 39% identical and 51% similar outside the highly conserved forkhead domain, while FoxA3 is only weakly similar to FoxA1 and FoxA2 (Friedman and Kaestner, 2006).

5.3.7.6. CCATT/enhancer binding protein α

C/EBPα over-expression in HepG2 cells did not alter UGT expression in the absence of TSA, or when performed in conjunction with 300 nM TSA treatment. However, addition of 3 μM TSA to C/EBPα-expressing cells increased the levels of UGT1A7, UGT2B17 and UGT2B28 mRNA. UGT1A7 mRNA levels increased in pCMX-CEBPα/3 μM TSA-treated cells to 8.5 times the levels in pCMX/3 μM TSA-treated controls ($P = 0.009$) (Figure 5.8E). Likewise, UGT2B17 mRNA levels were increased by 3.3-fold ($P = 0.015$) and UGT2B28 mRNA levels by 2.4-fold ($P = 0.027$) (Figure 5.8L and M).

Of the UGT genes that responded to C/EBPα in HepG2 cells, the most responsive was an extrahepatic gene. C/EBPα was the fourth LETF found to affect UGT1A7 mRNA levels in HepG2 cells, but the only one requiring 3 μM TSA to do so. The action of this LETF on UGT1A7 was somewhat surprising, as there is no overlap in the known expression patterns of UGT1A7 and C/EBPα (see Table 1.2 and section 5.1.3.6). It seems therefore, that either C/EBPα does not normally access the UGT1A7 promoter in tissues such as the liver, lung and intestine, where C/EBPα is
highly expressed, or it does not drive transcription from the UGT1A7 promoter in these tissues when it does bind. This raises the question as to whether C/EBPα could actually be a repressor of UGT1A7 promoter activity. It is possible that C/EBPα can genuinely bind to the UGT1A7 promoter, but normally does so in a repressive capacity. If TSA treatment interferes with co-repressor recruitment by C/EBPα on the UGT1A7 gene, allowing inappropriate association with co-activators, it is conceivable that this experimental system could return a false positive signal for a bound repressor. Generally, C/EBPα is regarded as a positive transcription factor, which can mediate gene transcription through direct interactions with the basal transcription machinery and by recruiting p300/CBP and other chromatin remodelling factors to target promoters. However, a small number of promoters that are repressed by C/EBPα have been reported, including the rat hnf6 gene (Rastegar et al., 2000). Most recently, a report by McFie and colleagues has shown that the human transcriptional elongation factor CA150 can physically interact with C/EBPα, but only on target genes negatively regulated by C/EBPα (McFie et al., 2006). CA150 is expressed in liver and lung (while gastrointestinal tissues remain untested for CA150 content), so it is feasible that such an interaction could be relevant for the UGT1A7 promoter in these tissues. C/EBPα can also repress target gene expression through recruitment of HDAC-1 (Di-Poi et al., 2005), a mechanism that is clearly vulnerable to TSA treatment. PPARβ is one such target gene, and its expression pattern in keratinocytes was found to be mutually exclusive with that of the C/EBPs (Di-Poi et al., 2005). Interestingly, despite all its other regulatory similarities with UGT1A7, UGT1A9 did not respond to C/EBPα, with or without TSA treatment. If UGT1A7 is indeed normally repressed by C/EBPα, this difference could partially explain why UGT1A9 is hepatically expressed, while UGT1A7 is not. To investigate
whether C/EBPα is involved in the regulation of the UGT1A7 gene (and if so, how), further cloning and in vitro experiments will be necessary. In addition, it would be useful to ascertain whether C/EBPα occupies the UGT1A7 promoter in hepatocytes and other primary cells through ChIP analyses.

UGT2B17 and UGT2B28 are both hepatic genes, yet respond to C/EBPα in a very similar manner to the non-hepatic UGT1A7. Whether this is coincidental or implies functional similarity remains to be seen. It is possible that C/EBPα represses the expression of these genes, despite their hepatic nature, as rat hnf6 is a hepatic gene negatively regulated by C/EBPα. Alternatively, C/EBPα may genuinely positively affect these genes, but in a manner that requires TSA, either to provide gene access or additional transcription factors/co-factors absent from the HepG2 nuclear environment. This is also feasible, as synergistic activation of another gene, human CYP3A4, by C/EBPα and 3 μM TSA has been previously reported (Rodriguez-Antona et al., 2003). In the published case, TSA was postulated to replace the role of FoxA3 in relaxing the chromatin surrounding the CYP3A4 C/EBP-binding site, allowing stronger activation by C/EBPα.

UGT2B17 is unique among the human hepatic UGT genes in that UGT2B17 mRNA levels showed a tendency to decrease with increasing fibrosis/cirrhosis scores in liver biopsies (Congiu et al., 2002) (it should be noted that UGT2B28 had not been discovered at this time and was not included in the study). Therefore, transcription factors that exclusively affect UGT2B17 (or UGT2B17 and UGT2B28 only) are potential mediators of this effect. Since C/EBPα levels decrease when significant hepatocyte proliferation is occurring (Mischoulon et al., 1992), which is the case in one third of cirrhotic livers (Donato et al., 2001), it would be of interest to explore whether there is any relationship between C/EBPα and UGT2B17 or UGT2B28
levels in damaged liver tissue. If C/EBPα does control UGT2B17 expression in human liver, it may also play important roles in other tissues where these proteins are co-expressed, such as prostate and lung (Antonson and Xanthopoulos, 1995; Beaulieu et al., 1996)

5.3.7.7. CCATT/enhancer binding protein β

C/EBPβ was ineffective as a transactivator for endogenous UGT genes in HepG2 cells. Like FoxA2, no significant responses over two-fold were observed for any UGT genes exposed to C/EBPβ over-expression in the absence of TSA, or after treatment with 300 nM TSA. Combined C/EBPβ expression and 3 μM TSA treatment did cause a 2.9-fold increase in UGT2B28 transcripts ($P = 0.012$) (Figure 5.8M), but no other responses were observed.

Since the only response to C/EBPβ was weak, and from UGT2B28, a gene that exhibited marginal responses to nearly all of the tested LETFs in the presence of 3 μM TSA, it seemed wise to test the integrity of the C/EBPβ expression vector. Co-expression with a fabp1 promoter-reporter plasmid (see section 5.3.8) gave a weak, but positive response. Therefore, it was concluded that over-expressed rat C/EBPβ has no effect on human UGT mRNA levels in HepG2 cells under the experimental conditions used. However, some further technical points are worthy of consideration before C/EBPβ is completely discarded as a potential regulator of human UGTs.

Although rat C/EBPβ is often used to study human promoters, whether or not this result can be directly extrapolated to human C/EBPβ requires further investigation as the human and rat C/EBPβ proteins share only 71% identity. Also, it would be of interest to determine the ratios of LAP-C/EBPβ to LIP-C/EBPβ produced in the transfected cells. If LIP-C/EBPβ is produced to significant levels from the pCMX-C/EBPβ expression vector, this could potentially decrease or completely mask any
positive effects that C/EBPβ may otherwise have on endogenous target genes (Descombes and Schibler, 1991). Repetition of the experiment with an expression vector designed to express only the human LAP-C/EBPβ protein, by substituting the human gene in the vector described in Descombes and Schibler (1991), would confirm the inability of C/EBPβ to independently increase UGT expression in HepG2 cells.

The failure of human UGTs to respond in any substantial way to C/EBPβ, and for the most part, also C/EBPa, implies that the changes seen in UGT expression around the time of birth, and during inflammation are driven by factors other than C/EBPs. In addition, these observations offer a possible explanation as to why conditions such as cirrhosis and inflammation affect human CYPs to a much greater extent than UGTs, resulting in acute, clinically relevant, losses of CYP-mediated drug metabolism.

Unlike most human UGT genes, many human CYP genes have been identified as targets of C/EBPa, C/EBPβ or both (Jover et al., 1998; Rodriguez-Antona et al., 2003; Bombail et al., 2004; Martinez-Jimenez et al., 2005; Pitarque et al., 2005). Thus, inflammatory stimuli may be able to preferentially affect CYP expression through C/EBP-mediated regulatory pathways.

5.3.8. Validation of the FoxA2 and C/EBPβ expression vectors

To assess whether an expression vector is functional, host cells can be tested for the presence of relevant mRNA by PCR or protein by Western blot; however, neither positive result guarantees that the exogenous protein is folded correctly or that it is capable of normal function. Therefore, the functionality of the pCMX-FoxA2 and pCMX-C/EBPβ plasmids was assessed by testing for FoxA2 and C/EBPβ activity against an appropriate reporter-promoter construct. The reporter vector chosen,
pTS388, contains 617 nucleotides of the proximal rat \textit{fabp1} gene promoter, inserted into the \textit{Bgl} II and \textit{Kpn} I sites of pGL3-basic (Rowley \textit{et al.}, 2006). This promoter was previously shown to be responsive to HNF1\(\alpha\), FoxA2 and C/EBP\(\beta\) in HepG2 cells (Divine \textit{et al.}, 2003).

When pTS388 was co-transfected into HepG2 cells with pCMX-HNF1\(\alpha\) or pCMX-FoxA2, increases in firefly luciferase expression relative to the \textit{renilla} luciferase control were observed of 9.1-fold and 4.9-fold respectively (Figure 5.12), confirming that active FoxA2 was expressed from the pCMX-FoxA2 plasmid. On the other hand, no apparent increase in reporter gene activity was detected for pTS388 and pCMX-C/EBP\(\beta\) co-transfections. However, a closer inspection of the raw data revealed that firefly luciferase expression was actually increased from the \textit{fabp1}

![Figure 5.12: The pCMX-FoxA2 and pCMX-C/EBP\(\beta\) expression plasmids are active in HepG2 cells.](image)

Transient co-transfections of 0.25 \(\mu\)g of pCMX-PL2, pCMX-HNF1\(\alpha\), pCMX-FoxA2 or pCMX-C/EBP\(\beta\) expression plasmids with 0.5 \(\mu\)g of pTS388 and 25 ng pRL-Null were performed as described in Chapter 2, section 2.2.10. Results are presented as fold increases over the pTS388/pCMX transfections plus one standard deviation. Firefly:\textit{renilla} luciferase ratios, as well as individual changes in firefly and \textit{renilla} luciferase expression are included. NB: This experiment was only performed once.
promoter by pCMX-C/EBPβ, but that renilla luciferase expression from the pRL-Null transfection-efficiency control was also increased, and to an even greater extent (Figure 5.12). Therefore, it appears that the pCMX-C/EBPβ plasmid is indeed active, but that pRL-Null is an inappropriate vector to control for transcription efficiency in this situation because it is also C/EBPβ-responsive. In future experiments involving C/EBPβ, better results may be obtained using phRL-Null (Promega) as the internal control, a pRL-Null-derived vector that has had numerous transcription factor binding sites removed in an effort to prevent outcomes such as the one presented. The transcription factor binding sites removed from pRL-Null included multiple C/EBP binding elements (Zhuang et al., 2001).

5.4 General discussion and summary

5.4.1. Achievement of aims

This study achieved both of its aims: to identify new potential regulators of the human hepatic UGT genes; and to further investigate the effects of HNF1 and HNF4α on endogenous UGT expression in a liver cell line. Previously unreported interactions between UGTs and LETFs identified by this work and warranting further investigation were HNF4α with UGT1A1 and UGT1A6, HNF6 with UGT1A4 and UGT2B11, FoxA1 and FoxA3 with UGT2B11, UGT2B15 and UGT2B28 and C/EBPα with UGT2B17. In addition, HNF1α, HNF1β, HNF4α and C/EBPα were identified as potential regulators of the UGT1A7 gene, and these possibilities should be pursued in a more appropriate cell line.

A significant number of observations were also made regarding different patterns of interaction between the LETFs and each UGT target, especially for HNF1α and HNF4α. Such differences are likely to:

a) stem from differing combinations of LETF
interactions with co-factors and other transcription factors; and b) be related to the body’s ability to independently regulate expression of each UGT despite high nucleotide sequence similarities and shared transcription factors.

Finally, it was observed that no two UGT genes were identically regulated by the combinations of LETFs and TSA used in this study, and that the UGTs with the most similar LETF responses were not necessarily those considered most closely related. Regardless of whether the human UGTs are grouped based on their amino acid sequences or the nucleotide sequences of their proximal promoters, several gene clusters are evident. These are: UGT1A3, UGT1A4 and UGT1A5; UGT1A7, UGT1A8, UGT1A9 and UGT1A10; UGT2B15 and UGT2B17; and UGT2B11 and UG2B28. Yet these groupings were poorly predictive of promoter function. UGT2B11 and UGT2B28 are closely related (94% amino acid identity) and behaved similarly; yet the only response observed in common for UGT2B15 and UGT2B17, which are also 94% identical, was to HNF1α. There were also some very striking functional similarities between UGT1A1 and UGT1A3, but no overlap between the responses of UGT1A3 and UGT1A4, even though the latter belong to the same UGT cluster. Many of the UGT proteins encoded by the genes of each cluster have substantial overlap in substrate specificity, leading to a considerable level of redundancy within the glucuronidation system. The independent regulation of such genes may be a further safe-guard that ensures continued glucuronidation, at least to some extent, in the event that one gene is adversely affected at the regulatory level.

5.4.2. Broader directions for future investigations

Apart from the various experiments already suggested in section 5.3, there are a number of other investigations that would also add value to the presented results.
These include:

a) A detailed characterisation of the chosen experimental system. To further understand the mechanisms behind the changes in UGT expression in response to LETF over-expression and TSA treatment, it would be beneficial to know what the basal levels of each LETF are under the conditions in which HepG2 cells are grown in our laboratory, and how they are affected by TSA treatment. Additional experiments incorporating TSA concentrations between 300 nM and 3 μM are also recommended, as these two TSA concentrations produced opposite effects for a number of UGTs. It is necessary to specifically characterise the HepG2 cells under the conditions used in this experiment, because although the expression levels of each LETF used have been previously reported in the literature for HepG2 cells, many of the accounts are conflicting. For example: HNF1β was found in HepG2 cells by Auyeung et al. (2003), but not Kikuchi et al. (2006); FoxA1 was expressed in HepG2 cells when grown by Qian et al. (1995), but not in HepG2 cells cultured by Rodriguez-Antona et al. (2002); C/EBPα was found to be absent in HepG2 cells by Buck et al. (1994) but present by Ishiyama et al. (2003); and both major C/EBPβ isoforms were found to be absent from HepG2 cells in a report by Descombes and Schibler (1991), yet Rodriguez-Antona et al. (2002) found that LAP-C/EBPβ but not LIP-C/EBPβ was expressed in HepG2 cells. It is likely that different culture conditions used between laboratories, or inadvertent selection of HepG2 sub-populations, accounts for many of these inconsistencies. In particular, insulin is a popular additive for HepG2 cultures, although not used in our laboratory, and has been found to alter expression of numerous genes in HepG2 cells and other hepatoma cell
lines, including albumin, phosphoenolpyruvate carboxykinase, LIP-C/EBPβ, the co-factor PGC-1, and several CYPs (Campos and Baumann, 1992; Duong et al., 2002; Martinez-Jimenez et al., 2006a; Martinez-Jimenez et al., 2006b). Different detection methods may also account for some differences in perceived expression of a gene, particularly for the C/EBPs where transcription is not well correlated with translation (Williams et al., 1991).

b) An investigation addressing the relationship between UGT mRNA levels and UGT protein expression in treated cells. Where possible, specific antibodies or substrates could be used to determine whether the LETF/TSA-induced increases in UGT mRNA are translated into corresponding increases in protein. In light of the extremely recent discovery that UGT1A1 and presumably other UGT1A family members can be alternatively spliced into active and inactive forms (Levesque et al., 2007b), it would be pertinent to determine whether the balance of full-length (isoform 1, i1) to the shorter i2 isoform transcripts is altered, particularly with TSA treatment. The real-time assays used in this work will have detected both the UGT1A i1 and i2 mRNAs.

c) Repetition of the experiment in cell lines derived from other human tissue-types. Differences in the UGT gene responses to LETFs between cell types may give further insight into the mechanisms behind the tissue-specific patterns of UGT expression seen in humans. In particular, it will be of interest to determine whether UGT1A8 and UGT1A10 are responsive to the LETFs that are also expressed in the gastrointestinal tract when tested in a more appropriate cell line, such as Caco-2.
d) Experiments designed to test further transcription factors and co-factors, or combinations of LETFs for involvement in UGT regulation. Other regulatory proteins that have potential to regulate UGT promoters include members of a sixth family of LETFs, the proline and acidic amino acid residue-rich family of bZIP transcription factors, especially D-site binding protein (DBP). DBP is expressed only after birth (Mueller et al., 1990), and is important in the expression of several hepatic genes including albumin, CYP7A genes and CYP2C6 (Schrem et al., 2004). DBP can also synergise with HNF1 on target promoters (Babajko and Groyer, 1993). Other potential transcription factors include GATA family members, GR, ubiquitous proteins such as Sp1 and CCAAT box binding factors, and co-factors such as PCG-1. These factors are either known to synergise with LETFs identified as UGT regulators, or have been shown to be involved in TSA-mediated responses of other genes. Therefore, they are logical choices to pursue. GATA-4 occupancy of the albumin gene enhancer requires FoxA1 (Cirillo and Zaret, 1999), and several UGT genes were found to be FoxA1 targets. GR has already been shown to affect UGT1A1 (Sugatani et al., 2005a) and may synergise with HNF4α on this or other UGT genes (Nitsch et al., 1993). Sp1 and CCAAT box binding factor binding to promoters are commonly found to be enhanced in the presence of TSA (Kwon et al., 2006; Qi and Ratnam, 2006). PCG-1 has been found to be low in HepG2 cells compared to liver, and its relative absence retards the expression of a subset of HNF4α target genes in these cells (Oberkofler et al., 2004; Martinez-Jimenez et al., 2006b). Therefore, expression of PGC-1 in HepG2 cells may enhance HNF4α-mediated transcription of UGT1A1, UGT1A6, UGT1A7 or UGT1A9, but more
importantly, may reveal more *UGT* genes that are HNF4α-responsive, but have different co-factors requirements to those already discovered. PGC-1 is also a co-factor for HNF6, so potentially affects the expression of HNF6-target *UGT* genes as well (Beaudry *et al.*, 2006).

Since many LETFs have been shown to interact synergistically when recruited to the same promoter, it would be astute to test them in combination for their effects on endogenous gene expression in HepG2 cells. Known combinations of LETFs that produce synergistic interactions in the correct context, which could be tested with our current clones, include: HNF1 with HNF4α; HNF4α with PXR, HNF6 or C/EBPα; HNF6 with HNF1, FoxA1, FoxA2 or C/EBPα; FoxA factors with HNF1 or C/EBPα; and C/EBPα with PXR or C/EBPβ.

e) An assessment of the effects of DNA methylation on UGT expression. Methylation is another common mechanism whereby genes are silenced (Schrem *et al.*, 2002) and may prevent genuine interactions of the LETFs with their target *UGTs*. Furthermore, chromatin acetylation and DNA methylation are dynamically linked, and HDAC inhibitors such as TSA and valproate can trigger the demethylation of a small subset of genes (Cervoni and Szyf, 2001; Milutinovic *et al.*, 2007). The direct effect of methylation on gene expression can be assessed through bisulphite mapping and treatment of cells with DNA demethylating agents such as 5-aza-2’-deoxy-cytidine.

f) Use of additional models to assess the importance of the newly identified transcription factor interactions with *UGT* promoters. Although primary human hepatocytes are subject to variability in patient drug history and
genetics, as well as being difficult to procure and transfec
t, they may be required to detect some LETF interactions with human *UGT* genes. For example, C/EBPα barely increased expression from an aldolase B gene promoter-reporter construct in HepG2 cells, but strongly enhanced reporter expression in primary hepatocytes (Gregori *et al.*, 1993).

Another option is the construction of stable cell lines to test LETF interactions with *UGT* promoters. If synthetic *UGT* promoter fragments integrated into the HepG2 genome behave similarly to the endogenous gene, this system has the advantage that the promoters can be manipulated as required before integration, allowing study of the relative importance of various promoter elements. Also, reporter gene assays are less arduous and costly than real-time PCR. Integrated promoters may be more appropriate than transient transfections when chromatin structure is important in gene regulation: for example, the human β-globin gene is only expressed correctly in transgenic mice when all four DNase-1 hypersensitive sites are included, even though transient transfections only reveal enhancer function for one of these sites (Ellis *et al.*, 1996); and a stably integrated *c-jun* promoter is 20-times more responsive to retinoic acid treatment than the same construct in transient transfections, while activation proceeds through entirely different elements (Kitabayashi *et al.*, 1992).

Finally, rodent models of human UGT expression will be increasingly useful for testing the role of each LETF in UGT expression. A transgenic mouse strain, Tg-*UGT1*, harbouring the entire known human *UGT1A* locus except much of the *UGT1A8* promoter and the *UGT1A11p* and *UGT1A12p* first exons is now available. These mice exhibit a tissue-restricted expression
pattern of human UGT mRNAs that bears many similarities to 
bona fide
human UGT expression (Chen et al., 2005a). A mouse model of the UGT1A8
promoter is also being developed by our laboratory. Cross-breeding of either
mouse model with LETF-knockout animals has the potential to clarify which
factors are indispensable for UGT expression. Despite some cross-species
limitations, enough functional similarities exist between the human and
murine orthologues for the LETFs investigated in this chapter that such
experiments should be extremely informative. Constitutive or conditional
knockout animals are available for hepatic expression of HNF1α (Pontoglio
et al., 1996; Lee et al., 1998), HNF4α (Hayhurst et al., 2001), HNF6
(Jacquemin et al., 2000), FoxA1 (although animals die within 10-14 days of
birth) (Kaestner et al., 1999), FoxA2 (Sund et al., 2000), FoxA3 (Kaestner et
al., 1998), C/EBPα (Lee et al., 1997) and C/EBPβ (Tanaka et al., 1995) but
not HNF1β. In particular, it would be of interest to determine whether
UGT1A1 expression is decreased in Tg-UGT1/HNF4α−/− mice. Expression of
the mouse Ugt1a1 homologue is increased when HNF4α is absent, indicating
that HNF4α is not a crucial factor for transcription of this gene. It was
postulated that an increase in bile acid accumulation in HNF4α−/− mice caused
the increased Ugt1a1 expression, through stimulation of PXR and FXR rather
than HNF4α being a repressor of Ugt1a1 (Ding et al., 2006). If a similar
scenario occurs for the human UGT1A1 gene, comparisons with the UGT1A3,
UGT1A4, UGT1A6 and UGT1A9 responses will make it possible to refine this
hypothesis, as they have different combinations of PXR- and HNF4α-
responsive behaviours.
5.4.3. Relevance to pharmacogenetics and disease

As the mechanisms that control the expression of human UGTs become better understood, it will be possible to identify more of the factors that lead to interindividual variation in glucuronidation. The work presented in this chapter identifies a number of transcription factors that may be instrumental in controlling human UGT expression, and accordingly, should be further investigated in the context of interindividual variation. Polymorphisms in the genes coding for these transcription factors or their co-factors, or in their cognate binding sites, may affect UGT expression. Indeed, there are several non-coding polymorphisms in human UGT genes that have been associated with altered promoter activity, UGT expression or adverse drug events (Acuna et al., 2002; Girard et al., 2004), but have not yet been allocated any function. Potentially, these SNPs could alter transcription factor binding sites. Furthermore, stimuli that alter the expression or activity of these transcription factors may also change the balance of UGTs relative to each other, and to other metabolic pathways. As discussed in Chapter 1, all of these parameters may influence drug efficacy or toxicity in an individual, or their vulnerability to diseases caused by xenobiotic exposure or protracted disturbances in homeostasis.

Mutations and polymorphisms exist in the HNF1α, HNF1β and HNF4α genes, as discussed in Chapter 4, and vary in consequential severity from being primary causes of MODY, to being a potential risk factor for atherosclerosis or type II diabetes, or having no apparent effect (Ryffel, 2001; Babaya et al., 2003; Love-Gregory et al., 2004; Holmkvist et al., 2006). In addition, it is known that mutations in HNF1α affect different genes to different extents, presumably due to the diversity of roles that HNF1α can have in driving transcription from different promoters (Soutoglou et al., 2001). Different functional variants of PXR affect the interactions of PXR with
the CYP3A4 promoter and the MDR1 gene differently (Hustert et al., 2001; Zhang et al., 2001) and variation in PXR expression has been linked to inflammatory bowel disease (Dring et al., 2006). Therefore, it is conceivable that variants of some LETFs could affect the expression of UGT proteins, and in a manner that preferentially affects a subset of their UGT targets according to the mechanisms by which they interact. In contrast, C/EBPα appears to be free of frequent functional polymorphisms, but mutations in this gene are associated with haematologic cancers (Gombart et al., 2002). Very little work has been done to identify polymorphisms of the other LETFs that elicited UGT responses, although multiple alleles are known to exist for HNF6, FoxA1 and FoxA3 (Vaisse et al., 1997).

Transcription factor levels are also known to vary between individuals. For example, the level of HNF1α mRNA in human liver varies up to 10-fold (Toide et al., 2002). Accordingly, target genes may be expressed at levels that are directly related to the concentrations of their most important transcription factors (Toide et al., 2002), or alternatively, may be subject to threshold effects, meaning that relatively small changes in transcription factor concentration can result in relatively large changes in target gene expression (Beaudry et al., 2006). Examples of compounds known to alter transcription factors in human tissues include chenodeoxycholate (decreases HNF1α and HNF4α expression in liver) (Jung et al., 2007), lipopolysaccharide/proinflammatory cytokines (decrease CAR, PXR, RXR, and PGC-1α expression in kidney-derived cells or liver) (Assenat et al., 2004; Wang et al., 2005), insulin (increases LIP-C/EBPβ and represses PGC-1α expression in liver-derived cells) (Duong et al., 2002; Martinez-Jimenez et al., 2006b), retinoic acid (increases HNF4α expression in liver-derived cells) (Hatzis and Talianidis, 2001) and genistein (increases HNF4α activity in liver-derived cells) (Ktistaki et al., 1995).
In addition, diet and signalling molecules such as thyroid hormones, growth hormones, retinoic acid, glucocorticoids, insulin, ceramide and cytokines modulate HNF1α, HNF4α, HNF6, C/EBPα and/or C/EBPβ expression or activity in rodents (Viollet et al., 1997; Lahuna et al., 2000; Park et al., 2004a; Park et al., 2004b; Schrem et al., 2004). Thus, there is plenty of scope for investigating the role of LETF variability in interindividual variation in glucuronidation.

5.4.4. Summary

In short, the work presented in this chapter has identified a number of LETFs that may be instrumental in the expression of human hepatic UGTs, and shown that not all UGT targets of a particular transcription factor are co-regulated. Identification of polymorphisms and mutations in the genes encoding these LETFs, as well as in their UGT gene binding sites, will improve our understanding of the mechanisms that cause interindividual variation in UGT expression. Therefore, further investigations into the effect of common transcription factor variants on UGT expression are warranted, as are studies that aim to identify more SNPs in human UGT promoters. Both types of studies are represented in Chapter 6, where potential causes of interindividual variation in UGT1A3 expression are explored.
6.1 Introduction

6.1.1. Genetic variation in human UGT genes

There are a large number of genetic variations known to be present in human UGT genes, some of which are already known to alter UGT function or expression, and/or to occur in association with increased risk of developing disease, particularly cancer. As of July 2007, there were 62 officially recognised alleles of \textit{UGT1A1}, 7 of \textit{UGT1A3}, 17 of \textit{UGT1A4}, 7 of \textit{UGT1A5}, 22 of \textit{UGT1A6}, 10 of \textit{UGT1A7}, 4 of \textit{UGT1A8}, 19 of \textit{UGT1A9}, 13 of \textit{UGT1A10}, 24 of \textit{UGT2B4}, 20 of \textit{UGT2B7}, 6 of \textit{UGT2B15}, 2 of \textit{UGT2B17} and 3 of \textit{UGT2B28} (UGT Nomenclature Committee, 2005). The vast majority of these variants are located in the coding region of the \textit{UGT} genes, although some promoter and intronic variants are also included in this list. However, not all distinct alleles with coding region polymorphisms lead to changes in UGT protein, as some represent silent mutations. The known associations between \textit{UGT} polymorphisms, disease and metabolism of pharmaceuticals are detailed in Chapter 1, section 1.8.6.

In addition to the officially recognised \textit{UGT} alleles, there are a growing number of polymorphisms identified in \textit{UGT} promoter regions, which have not all been allocated allele designations because their linkage with coding region variants has not been fully determined. In particular, a thorough study of the \textit{UGT1A9} proximal promoter has identified 15 promoter alleles, several of which are associated with altered expression levels of UGT1A9 protein (Girard \textit{et al}., 2004). In contrast, at the
time the study reported in this chapter was performed, there was very little information regarding the presence of polymorphisms in the promoters of the $UGT1A3$-$IA5$ cluster. Specifically, the only information available for $UGT1A3$ was that five promoter SNPs had been identified from the human genome sequencing project and had been allocated reference SNP (rs) identification numbers. However, no information was available regarding their linkage or functionality. Yet, interindividual hepatic UGT1A3 mRNA levels have been reported to be among the most variable for human liver $UGTs$ (Congiu et al., 2002). Furthermore, UGT1A3 mRNA has been reported to be expressed in a polymorphic fashion in the human small intestine, being undetectable in a subset of the population screened (Strassburg et al., 2000). Therefore, I decided to investigate whether polymorphisms in the $UGT1A3$ proximal promoter could be at least partly responsible for the variation observed in $UGT1A3$ mRNA levels between individuals. In addition, since I had previously identified an HNF1-binding site in the $UGT1A3$ promoter that is essential for activity in vitro (Chapter 3), the possibility that polymorphisms in the $HNF1\alpha$ gene coding region could affect $UGT1A3$ promoter activity was also explored.

6.1.2. $HNF1\alpha$ gene polymorphisms in humans

Ever since it was established that mutations in the human $HNF1\alpha$ gene lead to an autosomal dominant form of diabetes mellitus known as MODY3, genetic variation in this gene has been of interest (Ryffel, 2001). It is now known that, apart from rare MODY3-causing $HNF1\alpha$ mutations, there are also several relatively common polymorphisms of $HNF1\alpha$ that lead to changes in the HNF1\alpha amino acid sequence and have more subtle functional consequences. These include polymorphisms that produce the HNF1\alpha variants HNF1\alpha I27L, HNF1\alpha A98V and HNF1\alpha S487N, chosen for inclusion in this study.
The leucine amino acid substitution in HNF1α I27L occurs in the dimerisation domain, and has a frequency of 32.3% in healthy Danish Caucasians (Urhammer et al., 1997) and 48.2% in healthy Japanese men (Babaya et al., 2003). The valine amino acid substitution in HNF1α A98V lays two amino acids to the N-terminal side of the POU domain, and has an allelic frequency of 4.2% in healthy Danish Caucasians (Urhammer et al., 1997). The asparagine amino acid substitution in HNF1α S487N is positioned in the C-terminal HNF1α activation domain, and has an allelic frequency of 29.3% in healthy Danish Caucasians (Urhammer et al., 1997). Two of these HNF1α variants may be associated with disease risk in humans. Two studies have suggested that the I27L HNF1α variant is associated with insulin resistance (Urhammer et al., 1997; Chiu et al., 2000), although this result could not be replicated in a third, larger group of subjects (Urhammer et al., 1997). In contrast, the I27L HNF1α variant was found to be associated with high levels of serum high-density lipoprotein-cholesterol, and therefore may be protective against atherosclerosis (Babaya et al., 2003). The HNF1α A98V variation is also associated with disease in humans, being a risk factor for poor pancreatic β-cell function during glucose challenge (Urhammer et al., 1997; Urhammer et al., 1998a). However, no significant functional changes have been attributed to the S487N HNF1α amino acid substitution (Urhammer et al., 1998b).

The remaining two HNF1α variants used in this study were HNF1α P291fsinsC and HNF1α WT+21. HNF1α P291fsinsC, an HNF1α MODY3 mutant that occurs in several independent family lines, was included in this study as a negative control, as it has been shown to be a dominant negative inhibitor of HNF1α function (Yamagata et al., 1998; Ryffel, 2001). On the other hand, HNF1α WT+21 arises from an mRNA transcript discovered in HepG2 cells by Tamara Height in the process of cloning.
HNF1α from these cells. This transcript contains an additional 21 nucleotides (AGGCTGCCTCTGCTCCCCCAGG) - derived from intron 8 of HNF1α and inserted at position +1647, resulting in a 7-amino acid insertion in the carboxyl-terminal activation domain (Ryffel, 2001). It was unknown whether the resulting protein would have altered activity, but since HepG2 cells were derived from a human hepatocellular carcinoma (suggesting that this transcript may be present in this or other cancer types) it was of interest to investigate.

6.1.3. Aims

The work presented in this chapter was designed to investigate whether genetic variations in the UGT1A3 proximal promoter or the HNF1α coding region were significant contributors to interindividual differences in UGT1A3 expression in humans. Therefore, the aims were to:

1. To sequence the proximal promoter of the UGT1A3 gene from human genomic DNA samples in order to identify UGT1A3 promoter nucleotide polymorphisms and investigate their segregation into alleles;

2. To clone the distinct UGT1A3 promoter alleles identified in Aim 1 and to investigate their transcriptional activities in vitro;

3. To compare the chosen HNF1α variants HNF1α WT+21, HNF1α I27L, HNF1α A98V, HNF1α S487N and HNF1α P291fsinsC with the reference HNF1α protein for the ability to transactivate the UGT1A3 promoter in vitro.
6.2 Methods

6.2.1. Amplification of UGT1A3 sequences from genomic DNA

Genomic DNA samples isolated from unrelated patients of German descent with colon cancer \((n = 50)\) and matched controls \((n = 51)\) were kindly provided by Prof. J. Abel (Medical Institute of Environmental Hygiene, Dusseldorf, Germany). The UGT1A3-883bp promoter was amplified from 100 ng of genomic DNA in a two-round nested PCR using 0.5 Units Taq DNA polymerase in a reaction volume of 20 \(\mu\)l (see Chapter 2, section 2.2.6.4). The first round of PCR was performed using primers 1A3/4prom-1.5k and 1A3/1A4/1A5+619rev (see Table 6.1), designed to amplify all three promoters of the UGT1A3-IA5 cluster. This was a deliberate design feature, intended to allow sequence analysis of all three promoters without requiring addition genomic DNA material for each; thus preserving a limited resource. The PCR conditions were: initial denaturation at 95°C for 4 minutes; 35 cycles of 95°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 1 minute; and a final extension step at 72°C for 5 minutes.

The second round of amplification was specific for the UGT1A3 promoter and utilised primers 1A3prom-884bp and 1A3CDS+352 (Table 6.1). The PCR regime was as for the first round of amplification, with two exceptions: an annealing temperature of 60°C, and only 32 cycles of amplification. After sequencing, identified alleles were reamplified from the first round PCR product with primers 1A3prom-884NheI and 1A3UTRXho1 and cloned into the NheI and XhoI sites of pGL3-basic.
Table 6.1: Primers used in amplifying and cloning the human UGT1A3 proximal promoter from genomic DNA samples.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Nucleotide Sequence (5’→3’)</th>
<th>Nucleotide Position on Target Gene(s)</th>
<th>RE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A3/4prom-1.5k</td>
<td>AGCCATGCTAGCTAAGGGGTTGAGGAATAGT</td>
<td>UGT1A3: -1539 to -1519; UGT1A4: -1574 to -1554; UGT1A5: -1550 to -1530</td>
<td>NheI</td>
</tr>
<tr>
<td>1A3/1A4/1A5+619rev</td>
<td>ATGTCATGGTGTCGATGTTG</td>
<td>UGT1A3: +619 to +599; UGT1A4: +619 to +599; UGT1A5: +619 to +599</td>
<td>NA</td>
</tr>
<tr>
<td>1A3prom-884bp</td>
<td>GCCTGGATGACTGAAATAAAG</td>
<td>UGT1A3: -884 to -864</td>
<td>NA</td>
</tr>
<tr>
<td>1A3CDS+352</td>
<td>TCAACATGGCCATACCTTCTGA</td>
<td>UGT1A3: +352 to +332</td>
<td>NA</td>
</tr>
<tr>
<td>1A3prom-884Nhel</td>
<td>AGCCATGCTAGCGCTTGAGTGACTGAAATAAAG</td>
<td>UGT1A3: -884 to -864</td>
<td>Nhel</td>
</tr>
<tr>
<td>1A3UTRXho1</td>
<td>AGCCATCTCGAGCTCAAGGCAAGGACA</td>
<td>UGT1A3: -1 to -20</td>
<td>XhoI</td>
</tr>
<tr>
<td>1A3prom-108bp</td>
<td>CACGTTGATTTGCTAAGTGG</td>
<td>UGT1A3: -108 to -89</td>
<td>NA</td>
</tr>
<tr>
<td>1A3intron1rev</td>
<td>TGGATGAAGGCCACCAATACA</td>
<td>UGT1A3: +890 to +871 (intronic)</td>
<td>NA</td>
</tr>
</tbody>
</table>

RE: restriction endonuclease site, as underlined. NA: not applicable.
Amplification of the UGT1A3 exon 1 sequence from genomic DNA samples was achieved using the PCR primers and conditions reported by Iwai et al. (2004). These were primers 1A3prom-108bp and 1A3intron1rev (Table 6.1) and initial denaturation at 95°C for 2 minutes; 32 cycles of 95°C for 1 minute, annealing at 62°C for 1 minute and extension at 72°C for 2 minutes; and a final extension step at 72°C for 8 minutes.

6.2.2. HNF1α expression vectors
The wild-type and variant HNF1α expression vectors used in this study are detailed in Chapter 2, section 2.1.4.

6.2.3. Transient transfection and luciferase reporter assay
Transient co-transfections of 0.5 μg of pGL3-1A3-promoter-reporter plasmid, 0.25 μg of HNF1α expression plasmid and 25 ng pRL-Null were performed as described in Chapter 2, section 2.2.10, using HepG2 cells seeded at a density of $2 \times 10^5$ cells per well in 24-well plates. Cells were lysed 48-hours post-transfection using passive lysis buffer, and the lysates assayed for firefly and renilla luciferase activity as described in Chapter 2, section 2.2.11.

6.2.4. Statistical analysis
Statistical analysis of UGT1A3 promoter variant frequencies and compliance with Hardy-Weinberg equilibrium (tested by Pearson $\chi^2$) were performed using Microsoft Office Excel 2003 (Microsoft Corporation, WA) and SPSS (SPSS Inc., IL) software.

6.3 Results and Discussion

6.3.1. The UGT1A3 promoter contains multiple SNPs, resulting in five alleles
Sequence analysis of the proximal 884 nucleotides of the UGT1A3 promoter in 101 individuals uncovered 10 SNPs and one insertion/deletion event, as detailed in Table
6.2. By comparison to the published *UGT1A3* promoter sequence, I deduced that seven of these SNPs segregated into five alleles that were found to be in Hardy-Weinberg equilibrium (df(10) $\chi^2 = 13.19$, $P = 0.213$) in the tested population. Five of these seven SNPs had been previously reported by the HapMap project (see Table 6.2), but the two rarest (-148Y and -553R) were novel. The remaining SNPs occurred only as heterozygous changes in a single sample each, and were therefore designated mutations, as their allelic frequencies were $\leq 0.5\%$. It was not determined whether these mutations were present in the original genomic template or were a product of the first round of PCR amplification, but they were certainly not introduced in the second PCR amplification reaction, as all were retrieved by re-amplification and cloning from first-round PCR products. Of the five genuine *UGT1A3* promoter alleles, the most common (frequency = 0.55) was the reference sequence. The next most regularly observed allele was *UGT1A3* Promoter 2 (-66C/-204G/-581T/-751C/-758G), with a frequency of 0.32. The remaining three alleles were rare, found at frequencies of less than 0.05 (5%). The functional integrity of the *UGT1A3* Promoter 3 allele (-66C/-148C/-204G/-581T/-751C/-758G) was of particular interest, as the T-148C SNP is located within the HNF1-binding site of the *UGT1A3* promoter.

After the completion of this project and during preparation of this thesis, Caillier et al. (2007) published a study of the *UGT1A3* promoter that produced similar findings to those reported here. Caillier’s study of 249 Caucasians from the Québec Family Study identified the exact same seven polymorphisms in the *UGT1A3* promoter, although they found that these segregated into six alleles rather than five. Five of the *UGT1A3* promoter alleles identified by Caillier et al. (2007) were identical to those described in this chapter, with similar frequencies as found in the German population (Reference sequence = 55\% in both studies; Promoter 2/H2 = 32\% in Germans
Table 6.2: Single nucleotide polymorphisms detected in a German population. The frequencies of ten single nucleotide polymorphisms (SNPs) and one insertion/deletion event are reported in relation to the reference sequence published as Genbank entry AF297093 (Gong et al., 2001). SNPs of frequency less than 0.01 were defined as mutations and excluded from the haplotype analysis. The remaining SNPs were found to segregate into five independently inherited alleles. Five of the identified SNPs have previously been allocated rs identifiers (http://www.hapmap.org/).

<table>
<thead>
<tr>
<th>Alleles</th>
<th>Nucleotide position</th>
<th>n</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-66^a</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-148</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-204^b</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-553</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-581^c</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-751^d</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-758^e</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference</td>
<td></td>
<td>111</td>
<td>0.55</td>
</tr>
<tr>
<td>Promoter 2</td>
<td></td>
<td>65</td>
<td>0.32</td>
</tr>
<tr>
<td>Promoter 3</td>
<td></td>
<td>7</td>
<td>0.035</td>
</tr>
<tr>
<td>Promoter 4</td>
<td></td>
<td>7</td>
<td>0.035</td>
</tr>
<tr>
<td>Promoter 5</td>
<td></td>
<td>8</td>
<td>0.04</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mutations</th>
<th>Description</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promoter A</td>
<td>Reference allele with C to G mutation at position -48</td>
<td>1</td>
</tr>
<tr>
<td>Promoter B</td>
<td>Reference allele with T to C mutation at position -99</td>
<td>1</td>
</tr>
<tr>
<td>Promoter C</td>
<td>Promoter 2 with G to C mutation at position -207</td>
<td>1</td>
</tr>
<tr>
<td>Promoter D</td>
<td>10 bp deletion with 13 bp insertion. Replace: UGT1A3-200 to -191 (TCGGTCTTTT) with “AAAACTGTGGCC”</td>
<td>1</td>
</tr>
</tbody>
</table>

^a rs3806596; ^b rs3806597; ^c rs2008595; ^d rs1983023; ^e rs2008584
versus 28.7% for Cailler et al. (2007); Promoter 3/H3 = 3.5% versus 4.6%; Promoter 4/H5 = 3.5% versus 4.0%; and Promoter 5/H4 = 4% versus 6%, respectively). The sixth allele, not present in the German population studied, was formed by the -581T polymorphism occurring in isolation from the remaining polymorphisms, and was only present in the Québec Family Study at a frequency of 1.6%. Thus, it remains unclear whether this allele is not present in people of German descent, or whether sequencing a larger cohort of German individuals would reveal its occurrence at a low frequency. Although the sequences identified by Cailler et al. (2007) have since been allocated UGT1A3* allele names, unfortunately these names cannot be adopted in this work because the linkage between the promoter alleles identified herein and UGT1A3 coding region variants was not determined.

6.3.2. Activities of the newly defined UGT1A3 promoter alleles in vitro

The four new UGT1A3 promoter alleles and four mutated sequences (Table 6.2) were re-amplified, cloned into the pGL3 reporter vector and used in transient transfections to establish whether their activities were altered, relative to the published sequence. This experiment revealed a small amount of variation between the basal activities of the UGT1A3 promoter alleles in HepG2 and Caco-2 cells, the extent of which was less than 1.4-fold (Figure 6.1A and B). Of the differences observed, only the mutant UGT1A3 Promoter A and D alleles had statistically altered activity relative to the reference allele in HepG2 cells ($P = 0.009$ and $P = 0.003$ respectively). In Caco-2 cells, the UGT1A3 Promoter 3, B, C and D alleles also had statistically altered activity relative to the reference allele ($P = 0.016$, $P = 0.019$, $P = 0.003$ and $P = 0.018$ respectively). The alternative UGT1A3 promoter sequences also all responded
Figure 6.1: Alternative *UGT1A3* promoter alleles vary less than 1.4-fold in basal activity. Five alternative *UGT1A3*-884bp promoter alleles, including the reference sequence, were cloned into the pGL3 reporter vector and transfected into A. HepG2 or B. Caco-2 cells. A further four promoter constructs containing the mutations reported in Table 6.2 were also tested. All transfections contained 25 ng of the pRL-Null control vector, were performed in triplicate and were assayed for luciferase and renilla activity 48 hours post-transfection as described in “Methods”. Results are presented as the mean firefly luciferase activities relative to the internal renilla control, plus one standard deviation. The values of the promoter-less pGL3-basic control transfections are set to 1 (indicated by the white bar). Dark grey bars indicate the activities of genuine *UGT1A3* promoter alleles; light grey bars indicate the activities of the *UGT1A3* reference promoter sequence containing observed mutations. $P$ values for the indicated comparisons are †††$P = 0.003$, ‡‡‡$P = 0.009$ and §$P \geq 0.016$ but $\leq 0.019$. 

![Figure 6.1](image-url)
strongly to over-expression of HNF1α in HEK293T cells, in a similar manner to the reference construct. None of the effects of HNF1α on the variant promoters differed to the wild-type response by more than 2-fold (Figure 6.2), although all but UGT1A3 Promoter allele B were statistically increased relative to the reference promoter sequence. In particular, the UGT1A3 Promoter 3 allele (-66C/-148C/-204G/-581T/

![Figure 6.2: Alternative UGT1A3 promoter alleles are not decreased in their ability to respond to over-expressed HNF1α.](image)

Five alternative UGT1A3-884bp promoter alleles, including the reference sequence, were cloned into the pGL3 reporter vector and transfected into HEK293T cells. A further four promoter constructs containing the mutations reported in Table 6.2 were also tested. All transfections contained 25 ng of the pRL-Null control vector and 0.25 μg pCMX-PL2 or pCMX-HNF1α, were performed in triplicate and were assayed for luciferase and renilla activity 48 hours post-transfection as described in “Methods”. Results are presented as the mean firefly luciferase activities relative to the internal renilla control, plus one standard deviation. The values of the promoter-less pGL3-basic control transfections are set to 1. Dark grey bars indicate the activities of genuine UGT1A3 promoter alleles with HNF1α; light grey bars indicate the activities of the UGT1A3 promoter mutants with HNF1α. P values for the indicated comparisons are *P < 0.001, †P = 0.001, ‡‡P = 0.006 and §P = 0.01.
-751C/-758G) that contains the 148C polymorphism within the HNF1α-binding site was increased by 1.3-fold \( (P = 0.006) \), even though this nucleotide change theoretically slightly decreases the integrity of the HNF1-binding element. Therefore, it seemed that genetic differences in the \( UGT1A3 \) proximal promoter are unlikely to be a major cause of interindividual variation in \( UGT1A3 \), although it cannot be ruled out that the identified polymorphisms may have a greater effect in the genomic context.

Interestingly, the results of this study disagree with those reported in the recent publication by Caillier \textit{et al.} (2007). While I found that there were no statistically significant differences in the activities of any of the four genuine promoter variants (Promoters 2-5) relative to the activity of the reference \( UGT1A3 \) promoter sequence in HepG2 cells (Figure 6.1A), Caillier \textit{et al.} (2007) found that the activity of these same four promoter alleles (H2-H5) were decreased by 2 to 2.5-fold, and the promoter not identified in my study (H6) was also decreased by 30%. The cause of these differing results is unknown, but may be due to differences in cell culture conditions for the HepG2 cells used, or the length of the promoter constructs used. The constructs used in the Québec study contained 1144 bp of \( UGT1A3 \) promoter, rather than 884 bp. Yet, Caillier and colleagues found that there were no further polymorphisms in the \( UGT1A3 \) promoter region between nucleotides -1144 and -884 (Caillier \textit{et al.}, 2007): so, if it transpires that promoter length is important, this would indicate that the relationship between the allelic variants and promoter activity is more complex than just the altered binding of one or more transcription factors over the polymorphic regions, and it would be of importance to test much greater lengths of the \( UGT1A3 \) promoter for allele-determined function.
6.3.3. Regulation of the UGT1A3 promoter by HNF1α variants

HNF1α is subject to polymorphic variation in humans and is an important transcription factor for the UGT1A3 promoter in vitro. Therefore, because the differences in basal activities of the UGT1A3 promoter alleles were insufficient to explain the extent of UGT1A3 mRNA variation observed in human tissues, it was investigated whether variants of HNF1α could affect the rate of transcription of this gene. The UGT1A3-500bp promoter was co-transfected into HEK293T cells with pCMX vectors expressing three polymorphic HNF1α variants discovered in humans (I27L, A98V and S487N), HNF1α WT+21, and the HNF1α mutant P291fsinsC that is associated with MODY3. It was found that the three constructs encoding proteins with single amino acid substitutions all had 20-25% lower activity towards the UGT1A3 promoter than wild-type HNF1α (Figure 6.3). In contrast, the P291fsinsC mutant could not support any transcription from the reporter construct in HEK293T cells and the HNF1α WT+21 variant was slightly more active than the reference HNF1α construct (Figure 6.3A). In HepG2 cells, the P291fsinsC HNF1α mutant was found to behave in a dominant negative manner (Figure 6.3B) as previously reported (Yamagata et al., 1998). These results suggest that the functional effects of the HNF1α I27L, A98V and S487N polymorphisms are unlikely to account for much of the observed interindividual variation of UGT1A3 expression in vivo. However, this experiment was limited in scope in that the HNF1α variants could only be tested as over-expressed protein; lower concentrations of the less active HNF1α variants may cause non-linear losses of promoter transcription. Furthermore, no adjustments were made for any possible variance in the levels of HNF1α expressed from the alternative expression constructs.
Figure 6.3: Known HNF1α protein variants are insufficient to explain the variability of UGT1A3 mRNA levels in humans. HNF1α variants as previously described by Mackenzie and colleagues (2005a) were tested for their ability to regulate the reference UGT1A3-500bp promoter in A. HEK293T cells or B. HepG2 cells. Transfections were performed as per “Methods” and contained 0.5 μg of pGL3 or pGL3-1A3-500, 0.25 μg of empty pCMX-PL2 or pCMX vectors encoding the HNF1α variants and 25 ng pRL-Null. Results are the means obtained from triplicate samples, expressed as a relative value of firefly luciferase activity to the internal renilla control, compared to the pGL3-basic control (set to 1). Error bars indicate one standard deviation. WT: wild-type. P values for the indicated comparisons are *P < 0.001, †††P = 0.003, ‡P = 0.005, ‡‡P = 0.007 and §P = 0.024.
The *UGT1A3* Promoter 2 and Promoter 3 alleles were also tested for activity in combination with the HNF1α variants in HEK293T cells, but the results were as found for the reference *UGT1A3* promoter, with no *UGT1A3* promoter/HNF1α variant combinations resulting in changes in reporter gene expression of greater than 1.4-fold (Figure 6.4). Promoter 2 was chosen because it is the most prevalent allele other than the *UGT1A3* reference sequence, while Promoter 3 was chosen due to the presence of the nucleotide difference within the identified HNF1-binding site.

**Figure 6.4:** Two UGT1A3 promoter variants interact with the tested HNF1α protein variants similarly to the UGT1A3 reference promoter sequence. HNF1α variants as previously described by Mackenzie et al. (2005a) were tested for their ability to regulate the UGT1A3-883bp Promoter 2 (P2) and Promoter 3 (P3) reporter constructs in HEK293T cells, relative to the UGT1A3-883bp reference promoter (Ref)/wild-type (WT) HNF1α combination. Transfections were performed as per “Methods” and contained 0.5 μg of pGL3 or pGL3-1A3-883 reporter constructs, 0.25 μg of empty pCMX-PL2 or pCMX vectors encoding the HNF1α variants and 25 ng pRL-Null. Results are the means obtained from triplicate samples, expressed as a relative value of firefly luciferase activity to the internal renilla control, compared to the pGL3-basic control (set to 1). Error bars indicate one standard deviation. P values for the indicated comparisons are *P < 0.001, §P = 0.034 and §§P = 0.041.
6.3.4. The *UGT1A3* Promoter 2 allele is under-represented in a colon cancer cohort and is associated with the W11R/V47A protein variant

When the genomic DNA donors were categorised according to *UGT1A3* promoter genotype and colon cancer status, it was found that while the control population continued to obey Hardy-Weinberg equilibrium (df(10) $\chi^2 = 1.004, P = 1.000$), the cancer patient population deviated from the expected genotype frequencies (df(10) $\chi^2 = 20.73, P = 0.023$). Analysis of the genotypes present in each sub-population revealed a significant reduction ($P = 0.004$) in the occurrence of the *UGT1A3* Promoter 2 homozygous genotype in the cancer patients relative to the control population (Table 6.3). All other genotypes were equally distributed ($P \geq 0.298$).

Since functional assays did not suggest any mechanism by which the Promoter 2/Promoter 2 *UGT1A3* promoter genotype could be protective against colon cancer, it was postulated that it was, instead, behaving as a biomarker. Therefore, it was investigated whether the *UGT1A3* Promoter 2 allele was associated with any known *UGT1A3* protein variant. Six of the eight genomes that were homozygous for the *UGT1A3* Promoter 2 allele were sequenced over the *UGT1A3* exon 1 sequence using the method of Iwai et al. (2004). The *UGT1A3* first exon was also sequenced from six individuals known to be homozygous for the published promoter sequence, for comparison. All genomes that were homozygous for the reference *UGT1A3* promoter were also found to be homozygous for the *UGT1A3* exon 1 sequence *UGT1A3*\(^*1\). However, all samples homozygous for the *UGT1A3* Promoter 2 allele were found to be homozygous for the *UGT1A3*\(^*2\) allele, which encodes the W11R/V47A UGT1A3.2 protein variant reported by Iwai et al. (2004).

The finding that individuals homozygous for the *UGT1A3* Promoter 2 allele were under-represented among German colon cancer patients, similar to the previously
Table 6.3: Association of $UGT1A3$ promoter genotype with colon cancer.

<table>
<thead>
<tr>
<th>$UGT1A3$ promoter genotype$^a$</th>
<th>Control ($n = 51$)</th>
<th>Colon cancer ($n = 50$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$UGT1A3$ Reference/Reference</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>$UGT1A3$ Reference/Promoter 2</td>
<td>19</td>
<td>21</td>
</tr>
<tr>
<td>$UGT1A3$ Reference/Promoter 3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>$UGT1A3$ Reference/Promoter 4</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>$UGT1A3$ Reference/Promoter 5</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>$UGT1A3$ Promoter 2/Promoter 2</td>
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<td>0$^*$</td>
</tr>
<tr>
<td>$UGT1A3$ Promoter 2/Promoter 3</td>
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<td>0</td>
</tr>
<tr>
<td>$UGT1A3$ Promoter 2/Promoter 4</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>$UGT1A3$ Promoter 2/Promoter 5</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>$UGT1A3$ Promoter 3/Promoter 3</td>
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</tr>
<tr>
<td>$UGT1A3$ Promoter 3/Promoter 4</td>
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</tr>
<tr>
<td>$UGT1A3$ Reference/Promoter A</td>
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<tr>
<td>$UGT1A3$ Promoter 2/Promoter B</td>
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</tr>
<tr>
<td>$UGT1A3$ Reference/Promoter C</td>
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<td>1</td>
</tr>
<tr>
<td>$UGT1A3$ Reference/Promoter D</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

$^a$ Allelic sequences as defined in Table 6.2. $^*$ $P = 0.004$, compared with control by Pearson $\chi^2$.

reported reduction in the homozygous $UGT1A7*1$ genotype among patients with colorectal cancer (Strassburg et al., 2002b), was unexpected. Since all 6 individuals homozygous for the $UGT1A3$ Promoter 2 allele tested were also homozygous for $UGT1A3*2$, it seems likely that the $UGT1A3$ Promoter 2 sequence is part of the $UGT1A3*2$ allele, or at least strongly linked to the W11R and V47A polymorphisms. Evidence supporting the latter hypothesis is found in the recent publication by Caillier et al. (2007), where 98.6% of the $UGT1A3$ H2 promoter (Promoter 2) haplotypes also contained the W11R and V47A coding region polymorphisms. The remaining two H2 promoter alleles only occurred once each in 498 chromosomes.
and, therefore, would be considered mutations by the criteria used in this study and are indeed designated “hypothetical alleles” by Caillier and colleagues. Of the provably genuine UGT1A3 alleles in the Québécan study containing the H2 promoter, 92.9% would result in the UGT1A3.2 (W11R/V47A) protein and the remainder would produce UGT1A3.6 (W11R/V47A/M270V).

The in vivo functional consequences of possessing one or more copies of the UGT1A3*2 allele remain unknown. Iwai and colleagues found that the W11R/V47A UGT1A3.2 protein variant had 369% of UGT1A3.1 activity towards oestrone (Iwai et al., 2004). On the basis of these results, it was hypothesised that UGT1A3.2 may be protective for diseases that have serum oestrone levels as a risk factor, such as osteoporosis and colon carcinoma (Iwai et al., 2004). However, the only subsequent study (Caillier et al., 2007) to investigate the enzymatic activity of UGT1A3.2 towards oestrone found that this variant had only 61% of UGT1A3.1 activity towards this substrate (difference not significant). Furthermore, it was found that UGT1A3.6 was an extremely low activity enzyme, with only 0.1% of UGT1A3.1 oestrone-glucuronidating activity (Caillier et al., 2007). As discussed by Callier et al. (2007), there are a number of possible reasons for the observed differences between the two studies, including dissimilarities in the expression, assay and analytical methods used, and it is likely that the second study is the more accurate assessment of UGT1A3 activity. However, further investigations are necessary to definitively characterise the relationship between polymorphisms in UGT1A3 and its ability to glucuronidate oestrone, and ideally, between the presence of UGT1A3 variants and oestrone levels in humans in vivo.

If it transpires that differences in oestrone glucuronidation between UGT1A3 variants is not a likely risk factor for colon cancer, another possibility is that
UGT1A3.2 has increased activity towards its known carcinogenic substrates (such as primary aromatic amines and benzo[a]pyrene metabolites) (Mojarrabi et al., 1996; Green et al., 1998a) or is able to metabolise a carcinogen(s) that is not a substrate of the wild-type protein, and is consequently better able to protect the colonic mucosa from chemical damage. It is already becoming evident that different UGT1A3 protein variants are affected differently in their ability to metabolise various UGT1A3 substrates. For instance, UGT1A3.4, which has an unchanged or lowered capacity to glucuronidate oestrone (Iwai et al., 2004; Caillier et al., 2007), has a greatly increased ability to glucuronidate the flavonoids quercetin, luteolin and kaempferol relative to UGT1A3.1, with a changed preference in regioselectivity (Chen et al., 2006b). Thus, it is clear that it will be necessary to better characterise common variants of all UGT enzymes for their preferred substrates before we can fully understand how genetic variation in these proteins is likely to affect human health.

One final possible explanation for the observed absence of Promoter 2 homozygotes among colon cancer patients worth discussing is that, although this association is statistically significant, that it is the result of a statistical type I error. As the presented study is limited in scope, only encompassing 101 individuals, it is possible that the association seen is a product of chance, and that inclusion of more individuals will cause the association to lose significance. Similar difficulties have been reported for other studies, including one that attempted to identify an association between amino acid variants of HNF1α and pancreatic beta-cell function. In this instance, a study of 74 individuals returned a statistically significant association of the HNF1α I27L variant with poor beta-cell function in an oral glucose tolerance test, but a second, larger study of 230 individuals failed to replicate these
findings (Urhammer et al., 1998b). It would certainly be wise to further investigate the potentially important association of UGT1A3 gene variants with colon cancer risk, and determine whether the findings of this study hold true in a larger cohort.

6.4 General discussion and summary

6.4.1. Achievement of aims

This study confirmed the presence of 5 known UGT1A3 promoter polymorphisms in a German population and detected a further two. These polymorphisms were able to be allocated to 5 distinct alleles, fulfilling the first stated aim of this work. The remaining two aims, to determine whether these polymorphisms or variations in HNF1α affected UGT1A3 promoter activity were also carried out successfully, with the results indicating that neither are likely to be major contributors to interindividual variation in UGT1A3 expression in vivo.

6.4.2. Future directions

There are a number of improvements and extensions that could be made to this study to further investigate the impact of genetic variation on the expression of UGT1A3. These include:

a) Sequencing longer segments of the UGT1A3 proximal promoter in order to identify nucleotide polymorphisms further upstream and investigate their relationship with promoter function.

b) Testing a larger colon cancer case-control cohort for evidence that the UGT1A3 Promoter 2 allele is associated with a protective effect against the development of colon cancer.
c) Further investigating the relationship between HNF1\(\alpha\) and \textit{UGT1A3} transcription; in particular, titration of the HNF1\(\alpha\) variants against the \textit{UGT1A3} promoter to determine whether any HNF1\(\alpha\) variants are less able to activate \textit{UGT1A3} transcription when present at low concentration. Also, variation in HNF1\(\alpha\) levels may be more important in determining UGT1A3 expression than the presence of HNF1\(\alpha\) variants. Therefore, it would be of interest to investigate whether HNF1\(\alpha\) mRNA/protein levels correlate with UGT1A3 levels, in a similar manner as was found for UGT2B7 (Toide \textit{et al.}, 2002).

d) Identifying additional regulators of the \textit{UGT1A3} promoter and investigating whether variation in their activity or expression level affects UGT1A3 expression. Candidates include PXR (Rae \textit{et al.}, 2001; Gardner-Stephen \textit{et al.}, 2004) and AhR (Chen \textit{et al.}, 2005a).

e) Investigating genetic variation in other \textit{UGT} genes, particularly the closely related gene \textit{UGT1A4}. As there is some overlap in UGT1A3 and UGT1A4 substrates (see Chapter 3, section 3.1.3), it may be that the combination of \textit{UGT1A3} and \textit{UGT1A4} alleles inherited is more informative than either gene considered in isolation. Such a hypothesis would be reasonable, as certain combinations of \textit{UGT1A1} and \textit{UGT1A9} alleles have already been proposed to be important in predicting patient response and the likelihood of suffering toxicity when treated with irinotecan (Innocenti \textit{et al.}, 2005; Girard \textit{et al.}, 2006).
6.4.3. Relevance to pharmacogenetics

Although it has been known for some time that UGT1A3 is expressed in a polymorphic manner along the gastrointestinal tract (Strassburg et al., 2000), and with a significant degree of interindividual variation in the liver (Congiu et al., 2002), it has only more recently become clear that different human populations have very different frequencies of the various UGT1A3 variants. The second most common UGT1A3 variant, UGT1A3.2, occurs with a frequency of 35.9% in Caucasians from Québec (Caillier et al., 2007), but only 12.5% in people of Japanese extraction (Iwai et al., 2004) and 14% in a Chinese Han population (Chen et al., 2006b). The data presented in this chapter indicates that it is likely that the UGT1A3.2 protein variant is also approximately twice as common in German Caucasians as in Japanese/Chinese people; a result supported by two earlier studies of the UGT1A3 V47A polymorphism (which coincides with UGT1A3.2 at a frequency of 93.2%, at least in Caucasians (Caillier et al., 2007)). The first showed that in German people, alanine occurs in UGT1A3 amino acid position 47 at a frequency of 35% (Ehmer et al., 2004); the second showed that UGT1A3 47A occurs at a frequency of 37.9% in Caucasians and 16.3% in Asians (Thomas et al., 2006).

Therefore, it would be prudent to not only continue investigating the determinants of interindividual variation in UGT1A3 expression, but also to comprehensively characterise the catalytic activity of UGT1A3.2 and other UGT1A3 variants. It is clear that not all UGT1A3 variants are equally able to glucuronidate the same substrates, and that there may be substances that are better substrates for UGT1A3.2 or the other variants, than they are for the widely tested UGT1A3.1.

Whether interindividual variation in UGT1A3 expression has a significant impact on human health and will therefore be useful as a pharmacogenetic target remains to be
seen. In the past, UGT1A3 has not been regarded highly as an important contributor to human glucuronidation. This opinion was largely based on the observation that for many of the originally recognised UGT1A3 substrates, other UGT enzymes appeared to be more relevant in vivo (Tukey and Strassburg, 2000). However, UGT1A3 was recently reported to be important in the metabolism of 26,26,26,27,27,27-F6-1α,23S,25-trihydroxyvitamin D3 (Kasai et al., 2005), the anti-oestrogenic drug fulvestrant (Chouinard et al., 2006) and the bile acids CDCA and lithocholic acid (Verreault et al., 2006). As such, it is probable that other unique or major substrates exist. Now, with the discovery that there is at least one highly prevalent UGT1A3 protein variant other than UGT1A3.1 present in humans, particularly in Caucasians, the possibility that UGT1A3 is an important polymorphic contributor to human glucuronidation is an interesting prospect. In particular, further work should be done to investigate the possible link between UGT1A3 and the risk of colon cancer.

Although no mechanisms were identified that can adequately explain the absence of UGT1A3 expression in the colon of some individuals (Strassburg et al., 2000) or the hepatic interindividual variation seen in humans (Congiu et al., 2002), this study adds to our knowledge of genetic variation within the UGT1A locus. If the glucuronidative capacity of an individual is eventually going to become a useful predictor/tool in personalised medicine, it is likely that it will be necessary to consider genetic variation within the UGT1A locus, and possibly all UGT genes, as a single entity (although the importance of particular variants may also need to be weighted towards the most relevant enzymes depending on the disease being studied). It is already known that linkage between distant UGT1A genes occurs in the human genome (Innocenti et al., 2005; Thomas et al., 2006). In addition, UGT enzymes can affect the activity of each other when present in the same cell (Fujiwara
et al., 2007a; Fujiwara et al., 2007b); therefore, the combination of enzymes present in a tissue is likely to be more important than the sum of the activities of each. Furthermore, the UGT1A locus is subject to regulation through alternative splicing of the common exons that results in the production of inactive, truncated enzyme (Levesque et al., 2007b). Thus, it seems likely that some polymorphisms of UGT genes not directly involved in the glucuronidation of a particular substrate will nonetheless be found to be important in pharmacogenetic predictions for that substrate. As such, the study presented in this chapter is one small but important step towards understanding the UGT1A locus, and thus, the greater goal of achieving effective personalised medical treatment.

6.4.4. Summary

Single nucleotide polymorphisms in UGT regulatory regions have previously been correlated with altered expression of UGT1A1 and UGT1A9 (Girard et al., 2004; Girard et al., 2005). Therefore, I chose to investigate whether SNPs in the UGT1A3 promoter could also account for the variability in hepatic mRNA levels observed by Congiu et al. (2002) and Mojarrabi et al. (1996), and/or the polymorphic intestinal expression reported by Strassburg et al. (2000). Sequencing of 101 unrelated individuals of German descent revealed seven SNPs that occurred with sufficient frequency to be considered true polymorphisms. It was also determined that these polymorphisms could be accounted for by five alleles; which were subsequently cloned and tested for altered promoter activity. However, it was found that the basal activities of each promoter allele were comparable to the UGT1A3 reference sequence in both Caco-2 and HepG2 cells, and that all promoters were similarly responsive to HNF1α in HEK293T cells. Likewise, testing of the functional HNF1α I27L, A98V and S487N variant proteins against the UGT1A3 promoter only revealed
variations in activity of up to 25%. These results imply that genetic polymorphisms in the UGTIA3 promoter and the transcription factor HNF1α only contribute a small proportion of the observed variance in vivo.
7.1 Towards disease prevention and designer therapies

As discussed in Chapter 1, there are many incentives for thoroughly understanding the biology of human UGTs, including a better appreciation of their role in maintaining human health, and likely improvements in pharmaceutical drug design and usage. Indeed, clinically relevant outcomes have already been achieved from research into the \textit{UGT1A1} gene. The usefulness of such information will further increase once we can consider haplotype structures across both the \textit{UGT1} and \textit{UGT2} gene loci, as well as \textit{UGT} variation in combination with other biotransformation enzyme variants. As it becomes realistic to consider whole metabolic networks rather than individual enzymes, it is reasonable to expect that it will be possible to make health care improvements such as safely developing and prescribing therapeutics that would ordinarily be discarded after causing severe adverse reactions in a small minority of patients, as high-risk genotypes could be identified and excluded from treatment (Thomas \textit{et al.}, 2006). For other drugs, where the ratio of two UGT forms, or of UGT to alternative metabolic enzyme, determines the efficacy or toxicity of a drug, an intimate knowledge of regulatory mechanisms may allow the development of strategies to temporally alter the relative expression of the relevant enzymes, achieving a better outcome from the original therapeutic. Variation in UGT activity and expression in humans is prevalent, and importantly, potentially manipulable; many \textit{UGT} genes are now known to be targets of nuclear receptors whose activity can be altered by xenobiotic exposure. Thus, it is clear that research into the activity
and regulation of human biotransformation enzymes is an important investment in future medical practice.

### 7.2 Summary of the research findings presented in this thesis

The overall aim of this thesis, to substantially expand the knowledge of UGT regulation in humans, was achieved through four independent pieces of work, summarised as follows.

#### 7.2.1. Chapter 3: In vitro characterisation of the UGT1A3, UGT1A4 and UGT1A5 proximal promoters

The UGT1A3, UGT1A4 and UGT1A5 genes are highly related, sharing greater than 85% nucleotide sequence identity in their 1 kb proximal promoters. Yet, they vary considerably in their expression patterns; to the extent that while UGT1A3 and UGT1A4 are considered key hepatic enzymes, UGT1A5 expression in humans is currently thought to be negligible. Furthermore, it is clear that the UGT1A3 and UGT1A4 genes are independently regulated, despite their extensive similarities. To explore the mechanisms responsible for these observations, the UGT1A3, UGT1A4 and UGT1A5 promoters were cloned and analysed by deletion, mutation and HNF1α/β-over-expression experiments. The ensuing work established that putative HNF1-binding sites present in all three promoters are functional in vitro, but that while HNF1 factors are critical for UGT1A3 and UGT1A4 promoter activity, they are also insufficient to drive high levels of transcription. Two additional elements required for the maximal activity of the UGT1A3 promoter in liver-derived cells were also identified, at least one of which appears to be shared by the UGT1A4 promoter, but is only active in the context of UGT1A3. The discussion of this work highlights the likely functional relevance of these findings with respect to the
independent regulation of these genes, the lack of UGT1A5 expression in humans, and the implications for further pharmacogenomics research into these three genes.

7.2.2. Chapter 4: HNF1 transcription factors are essential for the UGT1A9 promoter response to HNF4α

Of the closely related UGT1A7, UGT1A8, UGT1A9 and UGT1A10 genes, UGT1A9 is the only member expressed in the liver. A study published during the course of this PhD candidature showed that, of this gene cluster, HNF4α regulated only the UGT1A9 gene in a positive manner (Barbier et al., 2005). The work presented in this chapter extends and refines these observations: identifying a major element through which HNF4α interacts with the UGT1A9 promoter; showing that the HNF4α-response of the UGT1A9 promoter is completely dependent on the presence of HNF1 factors; and establishing that there are at least three major functional differences between the UGT1A8 and UGT1A9 promoters that allow HNF1 and HNF4α to co-operatively regulate only the latter in hepatocyte-derived cells. The discussion of this work explores the differences between the regulation of UGT1A9 and other human genes by HNF1 and HNF4 transcription factors, and how variability in the expression and activity of such transcription factors could contribute to the variability of UGT1A9 expression in humans.

7.2.3. Chapter 5: Regulation of endogenous UGT expression in HepG2 cells by liver-enriched transcription factors

To overcome some of the potential drawbacks of studying gene regulation using transiently transfected promoters, and to identify new transcriptional regulators of the human hepatic UGT genes, liver-enriched transcription factors were over-expressed in HepG2 cells and the endogenous UGT mRNA levels subsequently measured. This experiment was performed both in the absence and presence of a
chromatin-relaxing agent, TSA. A number of interesting interactions between the
chosen transcription factors and endogenous UGT transcription were observed,
including previously unreported interactions of HNF4α with UGT1A1 and UGT1A6,
HNF6 with UGT1A4 and UGT2B11, FoxA1 and FoxA3 with UGT2B11, UGT2B15
and UGT2B28 and C/EBPα with UGT2B17. In addition, although UGT1A7 is not
hepatically expressed, HNF1α, HNF1β, HNF4α and C/EBPα were identified as
potential regulators of the UGT1A7 gene.

Another important set of observations presented and discussed in Chapter 5 were of
the differences in the way that HNF1α and HNF4α interact with each of their target
genes. In particular, HNF1α has been proposed as an important regulator of all
human hepatic UGT genes on the basis of DNA sequence and in vitro promoter-
reporter data, yet there is currently little information about the relative importance of
this transcription factor in the expression of each gene in vivo. The second purpose of
this study was, therefore, to investigate the hypothesis that HNF1α is not equally
important in the transcriptional hierarchy of each UGT gene. In support of this
hypothesis, the hepatic UGT genes could be separated into four distinct groups based
on their responses to HNF1α and TSA. Four UGT genes showed no direct evidence
of regulation by HNF1α (UGT1A1, UGT1A3, UGT2B7 and UGT2B10), two
responded to the over-expression of HNF1α alone (UGT1A6 and UGT1A9) and six
responded to HNF1α over-expression in the presence of TSA (UGT1A4, UGT2B4,
UGT2B11, UGT2B15, UGT2B17 and UGT2B28). Of the four genes that did not
respond to HNF1α over-expression in this system, two (UGT1A1 and UGT1A3) were
increased in the presence of TSA despite a concomitant loss of HNF1α expression,
and two (UGT2B7 and UGT2B10) were decreased after TSA treatment.
Thus, this chapter extends our knowledge of the transcription factors likely to be important in the regulation of human \textit{UGTs}, and shows that even when genes share conserved transcription factor binding sites, the mechanisms involved in their regulation may be significantly divergent. Knowing new potential transcriptional regulators for the human \textit{UGTs} opens up fresh avenues for research into the determinants of interindividual variation in UGT expression.

\textbf{7.2.4. Chapter 6: Genetic determinants of human UGT1A3 expression}

UGT1A3 is expressed in a polymorphic manner along the gastrointestinal tract, and with a significant degree of interindividual variation in the liver. However, the cause of these variations remains unknown. The study presented in this chapter examined the genetic variation present in the \textit{UGT1A3} promoter of people of German descent, finding seven polymorphisms that segregated into five alleles. These alleles were tested for promoter function \textit{in vitro}, and were found to have similar activities to the reference sequence (within 1.4-fold activity of the reference allele). Similarly, none of the identified polymorphisms severely affected the ability of the \textit{UGT1A3} promoter to respond to HNF1\(\alpha\) over-expression \textit{in vitro}. However, when the \textit{UGT1A3} genotypes were grouped according to whether the donor was a colon cancer patient or matched control, it was found that there was a significant difference in the genotype distribution. Possible mechanisms for this association were discussed and further work was recommended to investigate the possible link between \textit{UGT1A3} genotype and the risk of colon cancer.

Since HNF1\(\alpha\) was identified as an important regulator of \textit{UGT1A3} promoter activity \textit{in vitro}, the studies presented in Chapter 6 were also extended to investigate the effect of several common \textit{HNF1\(\alpha\)} coding polymorphisms on \textit{UGT1A3} promoter
function. It was found that the chosen HNF1α variants were still highly active towards the UGT1A3 promoter in transient transfections.

Overall, the results presented in Chapter 6 imply that genetic polymorphisms in the UGT1A3 promoter and the transcription factor HNF1α are likely to contribute only a small proportion of the observed variance in UGT1A3 expression *in vivo*. Further research into the regulation of the UGT1A3 promoter will be required to discover why UGT1A3 expression is so inconsistent between individuals.

### 7.3 Relevant work published over the duration of this PhD candidature

Apart from the work presented in this thesis, there have been other significant advancements made into the research of human UGT regulation since the commencement of this PhD candidature in 2004, the majority of which has been in the understanding of the inducible expression of UGTs. However, a new focus of identifying functional polymorphisms in UGT gene promoters has also recently emerged.

Recent advancements regarding the inducible regulation of human UGTs include recognition that: a) multiple NRREs of the UGT1A1 PBREM are responsible for the activation of the UGT1A1 gene by flavonoids, with the greatest contribution from the PBREM XRE (Sugatani *et al.*, 2004); b) the UGT2B7 promoter possesses a NRRE that allows negative regulation by FXR (*Lu et al.*, 2005); c) oxidants such as tert-butylhydroquinone can increase UGT1A1 transcription through binding of Nrf2 to an anti-oxidant response element that flanks the UGT1A1 PBREM XRE (*Yueh and Tukey*, 2007); d) liganded PPARα also activates the UGT1A1 promoter through a NRRE located within the PBREM (*Seneko-Effenberger et al.*, 2007); e) bile acids
can up-regulate the UGT1A3 promoter through a NRRE that binds LXRα (Verreault et al., 2006); f) the UGT2B15 gene is unique among the UGT2B genes (although UGT2B28 was not tested) in being oestrogen-responsive (Harrington et al., 2006); and g) the UGT1A1 promoter responds to GR ligand exposure in vitro. Two alternative mechanistic models have been proposed for the induction of UGT1A1 expression by glucocorticoids. Firstly, the research group lead by Dr. Mizutani found that induction of UGT1A1 transcription by dexamethasone or cortisol is reliant on GR and the UGT1A1 HNF1α-binding site, but is independent of the PBREM and appears to be mediated indirectly (Kanou et al., 2004; Usui et al., 2006a; Usui et al., 2006b; Kuno et al., 2007). Conversely, Sugatani and colleagues found that the UGT1A1 PBREM contains functionally important glucocorticoid response elements and that liganded GR enhances the PXR- and CAR-mediated transactivation of the UGT1A1 promoter through the co-activator GRIP-1 (Sugatani et al., 2005a). Further work will be needed to resolve these apparently conflicting results.

In addition to the above research, the construction of a transgenic mouse model (Tg-UGT1) that carries a large portion of the human UGT1A locus has allowed further research into the induction of UGT1A genes by xenobiotics and hormones. AhR or PXR ligands were able to induce the transcription of all human UGT1A mRNAs in the gastrointestinal tract, and all but UGT1A5, UGT1A7 and UGT1A8 in the liver of Tg-UGT1 mice. Furthermore, these effects were enhanced in the presence of glucocorticoid (Chen et al., 2005a). The in vitro responses of the human UGT1A1 gene to oxidative stress and a PPARα agonist, and of UGT1A3 to bile acids, were also confirmed in Tg-UGT1 mice (Verreault et al., 2006; Senekeo-Effenberger et al., 2007; Yueh and Tukey, 2007).
Recent research addressing the basic regulation of human \(UGT\) genes or the effect of promoter polymorphisms on \(UGT\) promoter function is still limited, but has shown that: a) \(UGT1A9\) is an HNF4\(\alpha\) target gene (Barbier et al., 2005); b) the \(UGT1A9\) promoter contains polymorphisms at positions -275, -331/-440, -665 and -2152 that can be correlated with \(UGT1A9\) expression levels in the liver (although the mechanisms responsible are yet to be elucidated) (Girard et al., 2004); c) Cdx2 and HNF1\(\alpha\) co-operatively regulate the \(UGT2B7\) promoter (Gregory et al., 2006); d) \(UGT1A3\) promoter variants have significantly lower transcriptional activity than the accepted reference promoter sequence (Caillier et al., 2007) (contrary to the work shown in this thesis); and e) a \(UGT2B7\) promoter polymorphism at nucleotide position -840 affects morphine glucuronidation in sickle cell disease patients (Darbari et al., 2007). Finally, and somewhat controversially, \(UGT1A8\) and \(UGT1A10\) transcripts have recently been reported to be present in primary human hepatocytes (Li et al., 2007), despite the widely held view that these \(UGT\) forms are strictly extrahepatic.

The work presented in this thesis complements the current bias that exists in human \(UGT\) research towards exploring inducible regulation. This is an important contribution, as a thorough understanding of inducible regulation will also ultimately require an understanding of the underlying constitutive control of \(UGT\) expression.

### 7.4 Modelling human \(UGT\) gene regulation

This thesis demonstrates the value of using different experimental models to investigate human \(UGT\) regulation. Unfortunately, there is no naturally occurring, convenient model of human \(UGT\) expression. Standard laboratory animals, such as rats and mice, do have similar \(UGT\) loci to humans (Mackenzie et al., 2005b), but
many of the human UGT genes exhibit a higher similarity to each other than to any UGT genes in rodents, so meaningful orthologous relationships cannot be reliably determined. Maturation of glucuronidation during development is also very different between rats and humans (Ring et al., 1999) and there are significant gender-related differences in UGT expression in mice not seen in humans (Buckley and Klaassen, 2007). Even the humanised Tg-UGT1 mouse, which is being used very successfully to provide much information about inducible expression of human UGT1A forms, is likely to be subject to significant limitations due to differences that exist between mice and humans in regulatory protein networks and in the activation of nuclear receptors by ligands. On the other hand, human cell lines usually exhibit very different gene expression patterns to the tissues from which they were derived, and their metabolism of xenobiotics can be very different from primary tissues (Smith et al., 2005b; Bonzo et al., 2007). Yet, primary cells, such as hepatocytes, are difficult to procure, don’t transfect well, and undefined genetic variation or history of xenobiotic exposure between donors can confound experiments (McCarver and Hines, 2002). DNA introduced into primary or immortalised cells for transient reporter assays is not moderated by its normal chromosomal context, with unknown consequences. Finally, there are obvious ethical limitations to the extent of the data that can be collected using human subjects. However, as demonstrated by this thesis and the recent literature, significant progress can be made when multiple, complementary models are used to explore human UGT regulation.

### 7.5 Final remarks

The design of this thesis was influenced by two important concepts. Firstly, it has been estimated that, although nonsynonymous changes in gene coding regions are
the most commonly studied genetic alterations, they are outnumbered by functional
\textit{cis}-acting regulatory polymorphisms that remain largely uncharacterised (Johnson \textit{et al.}, 2005). Secondly, polymorphisms that alter the expression or activity of \textit{trans}-acting factors have been predicted to be dominant determinants of gene expression patterns (Morley \textit{et al.}, 2004). Thus, the intent and achievement of the work presented in this thesis was to provide significant new insight into the regulatory control of human \textit{UGT} genes, and to identify mechanisms that can be further explored as potential contributors to interindividual variation in UGT expression.
APPENDIX ONE
pGL3-BASIC REPORTER VECTOR MAP

![Diagram of pGL3-basic vector with various cloning sites and functional elements labeled.](image-url)
APPENDIX TWO
pGL3+ REPORTER VECTOR MAP

pGL3+
4830 bp


hydroxycamptothecin glucuronidation levels in the liver. *Drug Metab. Dispos.* **34**: 1220-1228.


