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ISOLATION OF THE UDP GLUCURONOSYLTRANSFERASE 1A3 AND 1A4
PROXIMAL PROMOTERS AND CHARACTERIZATION OF THEIR DEPENDENCE ON
THE TRANSCRIPTION FACTOR HEPATOCYTE NUCLEAR FACTOR 1 ALPHA

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Running Title: Regulation of *UGT1A3* and *UGT1A4* by HNF1 α

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The abbreviations used are: UGT, UDP-glucuronosyltransferase; HNF, hepatocyte nuclear factor; EMSA, electrophoretic mobility shift assay

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ABSTRACT

The *UGT1A3-1A5* genes are a highly related UDP-glucuronosyltransferase (UGT) cluster, exhibiting high levels of coding and regulatory region homology. However, the ensuing proteins have both differing substrate specificities and differing expression patterns. The expression profile of each enzyme also varies considerably from one individual to the next. Differences in UGT expression have been predicted to contribute to an individual's response to pharmaceuticals, and to predisposition towards cancer in the event of carcinogen exposure. Therefore, it is desirable to elucidate the mechanisms that drive the transcription of *UGT* genes and identify the factors responsible for their variable expression. To this end we have isolated the *UGT1A3*, *UGT1A4* and *UGT1A5* proximal promoters and begun to investigate the regulatory elements necessary for activity *in vitro*. We have established that the nucleotide sequence upstream of the *UGT1A5* exon 1 is an ineffective promoter, correlating with the lack of substantial expression of this UGT in human tissues. In contrast, the *UGT1A3* and *UGT1A4* proximal promoters are both highly active in hepatic and colonic cell lines, with maximal activity being encoded by the proximal 500 bp. However, the *UGT1A3* and *UGT1A4* promoters exhibit low activity in the human embryonic kidney cell line HEK293, unless co-expressed with hepatocyte nuclear factor (HNF) 1 α . Furthermore, mutation of the consensus-like HNF1-binding site in the *UGT1A3* promoter abolishes promoter function in all cell types. This study suggests an important role for HNF1 α in the transcriptional regulation of the human *UGT1A3* and *UGT1A4* genes.

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Many small lipophilic molecules require conjugation with a polar moiety before they can be efficiently excreted from the body. Catalyzed by members of the UDP-glucuronosyltransferase (UGT) enzyme superfamily, glucuronidation is a predominant example of this metabolic process, and one that usually results in a loss of bioactivity of the parent compound. Therefore, the efficacy and/or toxicity of many xenobiotics (pharmaceuticals, environmental contaminants and dietary compounds) and the fate of numerous endogenous signaling molecules (such as steroid hormones and neurotransmitters) can be regulated through glucuronidation (Radomska-Pandya et al., 1999; Tukey and Strassburg, 2000). Differences in UGT expression have been predicted to contribute to an individual's response to pharmaceuticals, and to their predisposition towards developing certain cancers. Thus it is desirable to elucidate the mechanisms that drive the transcription of *UGT* genes and identify the factors responsible for their variable expression.

UGT1A3, UGT1A4 and UGT1A5 are a trio of highly related proteins encoded by the human *UGT1A* locus. They share greater than 90% identity in their primary amino acid sequences and more than 85% nucleotide sequence identity in their proximal promoters to one kilobase (Green and Tephly, 1998; Gong et al., 2001). However, despite these similarities, this gene cluster varies considerably in both substrate specificity and expression pattern. While *UGT1A3* and *UGT1A4* mRNA transcripts have been found in many tissues including liver, biliary tissue, colon, and breast (Strassburg et al., 1997; Strassburg et al., 1998a; Chouinard et al., 2006), *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 (Finel et al., 2005). Furthermore, there are inherent differences in the expression of *UGT1A3* and *UGT1A4*; for example, *UGT1A3* is found in the stomach of some individuals, while *UGT1A4* is not (Strassburg et al., 1998b). Because the mechanisms that determine the expression levels and/or tissue specificity of the *UGT1A3-1A5* cluster are currently not well understood, we have isolated their respective proximal promoters and begun to investigate the regulatory elements necessary for their activity *in vitro*.

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METHODS

Isolation of the *UGT1A3* and *UGT1A4* 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, *PfuTurbo* (Stratagene, CA) was used to simultaneously amplify both promoters using the primers GTTATCATTAATAATAATCCT and GGCAGGGGAACCTGGAGTCCT, and the resulting PCR product used as template to specifically amplify each promoter in separate reactions. The second round PCR primers were AGCCATAAGCTTATTGGATACCAGTATTGCT and AGCCATAAGCTTCTCAGCAGAAGACACGGACA for the *UGT1A3* promoter, or AGCCATGCTAGCATCTGATATCAGTAATGTG and AGCCATCTCGAGCTCAGCAGAAGCCACCGACA for *UGT1A4*. Both promoters were cloned into pGL3-basic (Promega, WI) and their ends sequenced to confirm their identity.

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. The antisense primer sequences for all *UGT1A3* and *UGT1A4* fragments were AGCCATCTCGAGCTCAGCAGAAGACACGGACA and AGCCATCTCGAGCTCAGCAGAAGCCACCGACA respectively. The sense primers annealed to bases -2541 to -2523, -1539 to -1519, -507 to -487, -200 to -184, -150 to -130 or -130 to -113 of the *UGT1A3* promoter, relative to the translation start site. The three longest *UGT1A4* promoter sub-fragments were amplified with the same sense primers as *UGT1A3*, giving lengths of 2610, 1574 and 506 nucleotides. For constructs containing less than 500 bases of the *UGT1A4* promoter, PCR primers annealing to *UGT1A4* nucleotides -200 to -184, -150 to -130 or -130 to -113 were used. To generate the mutated *UGT1A3*-150bp construct, the *UGT1A3*-150bp promoter was re-amplified with the sense primer AGCCATGCTAGCGGCCAACGCTTCACTAGAGGA containing the desired mutations (in bold). All resulting PCR products were cloned into pGL3-basic and sequenced in full.

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 bacterial artificial chromosome 1308M2 from the human library RPCI-11 (BACPAC Resources, CA) using primers GAGGTCTTTAGACCACTTAGTC and GCTCCACACAAGACCTATGTATGAT. The resulting products were used as template to amplify the *UGT1A5*-1550bp promoter using the same primers as for *UGT1A4*-1574bp. The 508bp and 150bp fragments of the *UGT1A5* promoter were also amplified using the corresponding *UGT1A4* primers defined above. All fragments were ligated into pGL3-basic and sequenced in full.

Transient transfection and luciferase reporter assay

HepG2, Caco-2 and HEK293 cells were obtained from the American Type Tissue Collection. All transfections were performed using Lipofectamine 2000 (Invitrogen) in 24-well plates seeded with 2×10^5 HepG2, 7.5×10^4 Caco-2 or 1×10^5 HEK293 cells the previous day. Either 0.5 μ g of empty pGL3-basic or a reporter vector carrying *UGT1A3*, *UGT1A4* or *UGT1A5* promoter sequences was co-transfected with 0.25 μ g of pCMX-HNF1 α (Mackenzie et al., 2005) or empty pCMX vector. pRL-null (0.025 μ g) was added to all transfections as an internal control for transfection efficiency. The reporter and expression plasmid concentrations were optimized to maximize promoter response without exceeding the recommended DNA:Lipofectamine ratio or inducing cytotoxicity (Gardner-Stephen and Mackenzie, 2005). After 48 hours, cells were lysed in passive lysis buffer (Promega) and analyzed for firefly and renilla luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) and a TopCount luminescence and scintillation counter (Parkard, Australia). As a minimum, all triplicate transfections were performed twice in independent experiments.

Electrophoretic mobility-shift assay (EMSA)

HNF1 α EMSA, super-shift and competition assays were performed with Caco-2 and HepG2 nuclear extracts as described in Gardner-Stephen and Mackenzie (2005). The DNA probes utilized were: *UGT1A3*-HNF1 (ATTAATGGTTAATAATTAACTAGAGG); *UGT1A4*-

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HNF1 (ATTAATGGGGTAATAAGTAACTGGTGG); *UGT1A3*-HNF1mut
(ATTAATGGCCAACGCTTCACTAGAGG) and the unrelated probe sequence FXR-
consensus (GATCTCAAGAGGTCATTGACCTTTTTG). Underlined text indicates the
extent of the putative HNF1-binding sites in each probe and mutations are highlighted in bold.

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RESULTS AND DISCUSSION

Relatively little is currently known about the transcriptional regulation of the *UGT1A3-1A5* genetic cluster. Accordingly, we have initiated a detailed analysis of the *UGT1A3-1A5* promoters and hereby describe a number of fundamental functional similarities and differences between these highly related sequences.

Basal activities of the *UGT1A3*, *UGT1A4* and *UGT1A5* proximal promoters

In both liver and colon-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 (Figure 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, with the exception of the *UGT1A3* promoter in Caco-2 cells, which showed greatest activity at a length of 200bp (Figure 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 that of pGL3-basic in the same cell lines (Figure 1A and B). Increasing promoter length beyond 500 nucleotides resulted in reduced promoter activity for both *UGT* genes, although this phenomenon was more marked in HepG2 cells than Caco-2. It was also found that, for all promoter lengths, the *UGT1A3* gene had greater activity *in vitro* than *UGT1A4*, regardless of host cell type. Much of the increased activity of the *UGT1A3* promoter over that of *UGT1A4* was found to require nucleotides -150 to -200, although the transcription factors mediating this effect remain unidentified.

Comparison of the *UGT1A5* promoter with the regulatory regions of *UGT1A3* and *UGT1A4* revealed that the former had the least activity. This was true for all three promoter lengths tested, in both HepG2 and Caco-2 cells (Figure 2A and B). 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, a result in direct opposition to that obtained for *UGT1A3* and

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UGT1A4 (Figure 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*.

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 Caco2 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 (11 of 13 nucleotides) of this region with the HNF1-binding site consensus sequence GTTAATNATTAAC. Furthermore, the equivalent region of the *UGT1A3* promoter contains a 100% match to the HNF1-binding site consensus. As to date, however, no experimental evidence has been presented to ascertain whether these sites are functional in the context of their promoters. To investigate the influence of HNF1 α on the activity of the *UGT1A3* and *UGT1A4* promoters, constructs containing 500 bp or less of each regulatory region were co-transfected with HNF1 α into cells known to express HNF1 factors (HepG2 and Caco-2 cells) or a cell line devoid of HNF1 factors; namely HEK293 (Bernard et al., 1999; Gardner-Stephen and Mackenzie, 2005). In HEK293 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 HNF1 α . Reporter gene expression under the control of the *UGT1A3* or *UGT1A4* promoters could be increased up to 22-fold over basal levels (Figure 2C). In contrast, vectors containing only the most proximal 130 bp of the *UGT1A3* or *UGT1A4* promoters were completely unresponsive to the presence of HNF1 α .

In HepG2 or Caco-2 cells, co-transfections of the *UGT1A3* or *UGT1A4* promoters with HNF1 α resulted in little or no additional response (Figure 2A and B). Since HepG2 and Caco-2 cells express HNF1 factors (Kuo et al., 1990; Rey-Campos et al., 1991), we postulated that

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the endogenous levels of HNF1 α and/or HNF1 β in these cells were sufficient to support expression of the reporter gene from the *UGT1A3* and *UGT1A4* promoters *in vitro*. Therefore, we mutated the putative HNF1-binding site in the *UGT1A3*-150bp promoter to abolish any binding of HNF1 α . The functional result of this mutation was a loss of basal activity of the *UGT1A3*-150 bp promoter in both HepG2 and Caco-2 cells, and prevention of HNF1 α -responsiveness in HEK293 cells. In all cases, the mutated *UGT1A3*-150bp promoter construct behaved in the same manner as the *UGT1A3*-130bp promoter that contains no recognized HNF1 α -binding site (Figure 2). In support of the above evidence that the *UGT1A3* and *UGT1A4* promoter HNF1 sites are functional, we were able demonstrate that HNF1 factors from Caco-2 and HepG2 nuclear extracts can bind these sequences in EMSA (Figure 3). Furthermore, the mutation used to abolish HNF1 α -responsiveness of the *UGT1A3*-150bp promoter also prevented binding of HNF1 factors to this region *in vitro*, while neither the mutated or unrelated (FXR) probes could interfere with HNF1 binding when added in 500-fold excess (Figure 3).

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, while none of the *UGT1A3* promoter constructs exhibited any activity in HEK293 cells in the absence of HNF1 α , *UGT1A4* promoters of 150 bp or longer could support a small degree of basal activity. This activity, which was 2 to 3-fold greater than the empty vector control, is presumably HNF1-independent (Figure 2C). The second observation was that while *UGT1A3* promoter activity could not be increased in HepG2 cells by over-expression of HNF1 α , *UGT1A4* promoter activity was increased up to 2.3-fold by excess HNF1 α for promoter fragments ≥ 150 bp (Figure 2A). One possible explanation is that the perfect *UGT1A3* HNF1-binding element is fully occupied at physiological HNF1 concentrations, while 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 HNF1 α 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

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second difference between the promoters, as it was only observed in cells of hepatic origin (Figure 2).

Summary

In summary, we have shown that while the nucleotide sequence upstream of the *UGT1A5* first exon is ineffective as a promoter, the *UGT1A3* and *UGT1A4* proximal promoters are transcriptionally active *in vitro*. We have also shown that HNF1 α can interact functionally with both the *UGT1A3* and *UGT1A4* promoters, and that the HNF1 site of the *UGT1A3* gene is essential for transcriptional activity *in vitro*. In this regard, the *UGT1A3* promoter appears to be most similar to *UGT1A1* (Bernard et al., 1999), and distinct from that of the human *UGT1A8-1A10* gene cluster (Gregory et al., 2003). Since HNF1 factors are present in cell types where *UGT1A3* and *UGT1A4* are expressed (Kuo et al., 1990; Rey-Campos et al., 1991), it would be reasonable to expect that HNF1 α is important in *UGT1A3* and *UGT1A4* transcription *in vivo*.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 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 μ g 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) \pm standard deviation.

Figure 2: HNF1 α is required for maximal basal activity of the *UGT1A3* and *UGT1A4* proximal promoters.

Mutations were introduced into the HNF1-binding site of the *UGT1A3*-150bp promoter by PCR as described in “Methods”. A. HepG2, B. Caco-2 or C. HEK293 cells were co-transfected with 0.5 μ g pGL3-based vectors containing 500, 200, 150 or 130 nucleotides of the *UGT1A3* or *UGT1A4* promoters, 25 ng pRL-Null and 0.25 μ g of pCMX-HNF1 α 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.

Figure 3: HNF1 α binds to nucleotides -156 to -128 of the *UGT1A3* and *UGT1A4* promoters

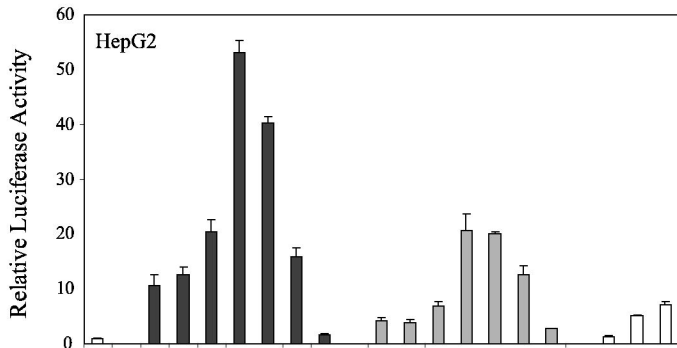
Electrophoretic mobility-shift assays were performed using 50,000 cpm of 32 P end-labelled oligonucleotide probes encompassing the putative *UGT1A3* or *UGT1A4* HNF1 sites. HNF1 complexes were competed with 500-fold excess cold probe, or super-shifted with 2 μ g HNF1 α -specific antibody (Santa Cruz Biotechnology, CA). The positions of free probe and

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complexes containing HNF1 α are indicated by arrows. WT: *UGT1A3* or *UGT1A4* wild-type probe; Mut: *UGT1A3* mutant probe; FXR: consensus FXR-binding sequence probe.

Figure 1

A



B

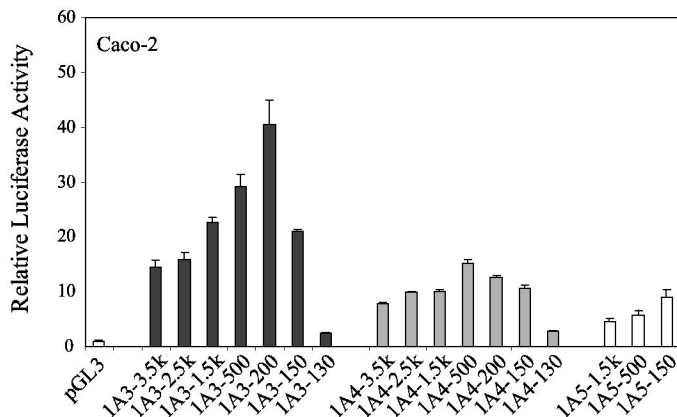
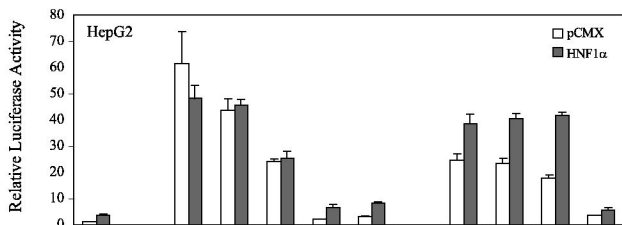
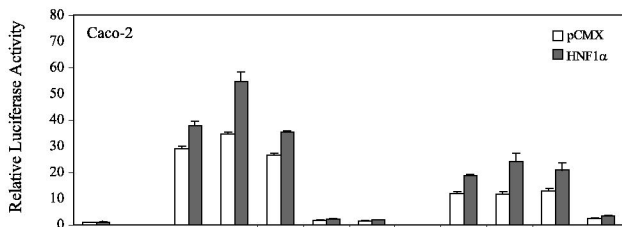


Figure 2

A



B



C

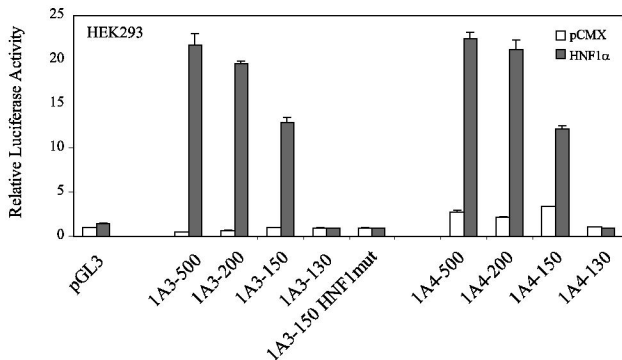


Figure 3

