

# Activation of the Mouse TATA-less and Human TATA-Containing UDP-Glucuronosyltransferase 1A1 Promoters by Hepatocyte Nuclear Factor 1<sup>1</sup>

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## ABSTRACT

UDP-glucuronosyltransferase (UGT) 1A1 (UGT1A1) catalyzes the glucuronidation of bilirubin in liver. Among all UGT isoforms identified to date, it is the only relevant bilirubin-glucuronidating enzyme in human. Because glucuronosylation is the major route of bilirubin elimination, any genetic alteration that affects bilirubin glucuronosyltransferase activity may result in a more or less severe hyperbilirubinemia. In this study, we report the cloning and characterization of the transcriptional regulation of the mouse *UGT1A1* gene. Primary-structure analysis of the mouse Thymidine A device promoter revealed marked differences with its human homolog. First, the mouse promoter lacks the highly polymorphic thymidine/adenine repeat occurring in the human promoter, which has been associated with some

forms of hyperbilirubinemia. Second, an L1 transposon element, which is absent in the human promoter, is found 480 bp upstream of the transcription start site in mouse. Using the electromobility shift and DNase I footprinting experiments, we have identified a hepatocyte nuclear factor 1-binding site in the mouse *UGT1A1* promoter that confers responsiveness to both factors HNF1 $\alpha$  and HNF1 $\beta$  in HEK293 cells. Furthermore, we show that this element, which is conserved in the human promoter, also confers strong HNF1 responsiveness to the human *UGT1A1* gene. Together, these results provide evidence for a major regulatory function of this liver-enriched transcription factor in UGT1A1 activity in both rodents and human.

In mammals, detoxification of the hydrophobic bilirubin occurs mainly in the liver via its conjugation with uridine diphosphate glucuronic acid. This reaction, which is catalyzed by microsomal UDP-glucuronosyltransferases (UGTs) (EC 2.4.1.17) produces hydrophilic bilirubin-mono- and di-conjugates that are excreted at high rates in the bile (Hauser and Gollan, 1990; Jansen et al., 1992). UGTs are produced by a multigene superfamily with specificity for a variety of endogenous substrates and xenobiotics. Mammalian UGTs are subdivided into two groups, the *UGT1* and the *UGT2* families. UGT1 family members are encoded by a particularly complex gene of about 500 kb ("the *UGT1* cluster," Mackenzie et al., 1997). It consists of multiple related homologous unique first exons (exon 1), which encode the isoform specific N terminus of the enzymes. The C-terminal part is encoded

in a single set of four exons (exons 2–5), which is common to all UGT1 isoforms (Ritter et al., 1992; Emi et al., 1995; Mackenzie et al., 1997). A promoter region with its regulatory elements is thought to be located upstream of each unique first exon, but, for several of them, this region has not yet been characterized (Ritter et al., 1992).

The UGT1A1 isoform is likely to be the major isoform involved in bilirubin detoxification in human (Bosma et al., 1994). Inherited disorders that decrease or suppress *UGT1A1* expression result in unconjugated bilirubin accumulation. Mild forms of unconjugated hyperbilirubinemia are known as Gilbert and Crigler-Najjar type II syndromes, whereas in more severe forms, the Crigler-Najjar type I disease can be fatal (Aono et al., 1995; Bosma et al., 1995). Some of the mutations causing these disorders are localized in the common exons 2 to 5, leading to an alteration of all isoforms produced by the *UGT1* locus (reviewed in Mackenzie et al., 1997). Others are in exon 1A1 and thus alter only UGT1A1 activity. Thus far, the *UGT1A1*\*28 polymorphism is the only genetic variation reported in a regulatory region of the gene. It is responsible for most Gilbert's disease cases (Bosma et

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al., 1995). This polymorphism concerns the six-thymidine/adenine (TA) track in the wild-type *UGT1A1* promoter, which is replaced by five, seven, or eight TA repeats in the altered genes, whose activity is inversely correlated with the length of the track (Beutler et al., 1998). Expression of the human *UGT1A1* mRNA is primarily in the liver but can also be detected in biliary and colon epithelia (Ritter et al., 1992; Strassburg et al., 1997, 1998). In rat, the tissue distribution is similar in liver and intestine. In addition, *UGT1A1* is also expressed at low levels in kidney, which is not the case in human (Ritter et al., 1992; Emi et al., 1995).

Surprisingly, only the promoter of the human *UGT1A1* gene has been sequenced thus far. However, with the exception of the TA track, no functional promoter characterization has been performed (Brierley et al., 1996). The broad use of mice and rats as laboratory animals for pharmacological studies has prompted us to investigate *UGT1A1* transcriptional regulation in the mouse, not least because the gene knockout technology is now well mastered for this animal and might be applied later to the *UGT1* locus once characterized. Moreover, studies using this technology provided evidence for a role of liver-enriched transcription factors in *UGT1A1* expression. The inactivation of the HNF1 $\alpha$  gene resulted in a mild hyperbilirubinemia, which, according to the above-mentioned observations, might reflect an altered *UGT1A1* gene expression (Pontoglio et al., 1996). Similarly, knock-out of C/EBP $\alpha$  in mouse liver affects *UGT1A1* gene expression resulting in a severe toxic hyperbilirubinemia (Lee et al., 1997). These observations, together with *UGT1A1* being the main isoform from the *UGT1* locus expressed in rat liver (Emi et al., 1995), argue strongly for a crucial role of the *UGT1A1* isoform in bilirubin detoxification in rodents. Recently, other studies have highlighted the important role of HNF1 $\alpha$  and C/EBP $\alpha$  in regulating the transcription of the rat *UGT2B1* isoform (Hansen et al., 1997, 1998). In contrast, nothing is presently known of the mechanisms of transcriptional regulation of the *UGT1A1* gene by these factors, although their involvement has been suggested, as mentioned above.

To better understand the physiological role of the *UGT1A1* isoform in the mouse, we have isolated the *UGT1A1* gene. Its promoter was then compared with that of the human gene, which revealed the lack of a TATA box in the mouse promoter. Furthermore, evidence is provided for a direct involvement of HNF1 in the regulation of the mouse and human *UGT1A1* promoters.

## Experimental Procedures

**Materials.** The expression plasmids pRSV-HNF1 $\alpha$  and pRSV-HNF1 $\beta$  were a kind gift from Dr. Moshe Yaniv (Unité des Virus Oncogènes, Institut Pasteur, Paris, France). The  $\alpha$ -HcT-284 polyclonal antiserum directed against HNF1 $\alpha$  (Chouard et al., 1997) was a kind gift from Dr. Marco Pontoglio (Unité des Virus Oncogènes, Institut Pasteur, Paris, France) and Dr. Moshe Yaniv. All *UGT1A1* nucleotide sequences used were numbered relative to the major transcription start site determined in this study.

**Isolation and Characterization of Genomic and cDNA Clones.** A portion ( $1 \times 10^6$  plaque-forming units) of a mouse L129/SVJ genomic DNA library in  $\lambda$ FIXII (Clontech Laboratories, Palo Alto, CA) were screened with two oligonucleotides complementary to the mouse *UGT1A1* cDNA (Chu et al., 1997) as probes. Two clones ( $\lambda$ MPGT1A1-1,  $\lambda$ MPGT1A1-2) were isolated, plaque-purified, and

characterized by polymerase chain reaction (PCR) and restriction enzyme analysis. From the  $\lambda$ MPGT1A1-1 clone, a 2.4-kb *Xba*I restriction fragment containing the *UGT1A1* first exon was subcloned in the pBluescript KSII vector, yielding the pKS-1A1 genomic subclone. DNA was sequenced on both strands by the dideoxy termination method using T7 DNA polymerase (T7 sequencing kit; Pharmacia Biotech Europe, Dübendorf, Switzerland). The cDNA clones were obtained by screening  $1 \times 10^6$  plaque-forming units of an adult mouse liver 5'-extended cDNA library in  $\lambda$ gt10 with the same oligonucleotides as indicated above, yielding 10 independent clones. Similarly, a 15-day-old embryonic mouse 5'-extended cDNA library in  $\lambda$ gt10 was screened following the same procedure, yielding one clone. After PCR amplification using phage-specific oligonucleotides, the 11 independent clones were subcloned into the pBluescript vector for subsequent sequencing. The human *UGT1A1* promoter was cloned by PCR on human genomic DNA and subcloned into the pBLCAT3 vector yielding the -617/+15 hUGT1A1-chloramphenicol acetyltransferase (CAT) construct. The construct was sequenced on both strands. Its sequence is identical with that published previously (Brierley et al., 1996) except for the length of the TA repeat in the polymorphic TATA box, which is (TA)<sub>5</sub>TAA instead of (TA)<sub>6</sub>TAA (Beutler et al., 1998).

**RNA Isolation and Primer Extension Analysis.** Total RNA was isolated from mouse liver by the phenol and guanidine isothiocyanate method following the supplier's instructions (Trizol reagent; Life Technologies, Basel, Switzerland). An end-labeled antisense oligonucleotide primer complementary to nucleotides +95/+120 of the *UGT1A1* gene was hybridized to 50  $\mu$ g of total RNA for 1 h at 42°C in 50 mM Tris-HCl (pH 8.3), 8 mM MgCl<sub>2</sub>, 30 mM KCl, 10 mM DTT in a final volume of 50  $\mu$ l. The primer-annealed RNA was then subjected to extension by adding 2 mM each dNTP and 25 units of avian myeloblastosis virus-reverse transcriptase (Pharmacia Biotech Europe, Dübendorf, Switzerland) for 1 h at 42, 44, or 46°C. The reaction was stopped by addition of 50 mM EDTA and 15  $\mu$ g of Rnase Avian myeloblastosis virus (Boehringer Mannheim, Rotkreuz, Switzerland) and incubated for 1 h at 37°C. The reaction products were resolved on a 7 M urea, 8% polyacrylamide-1 $\times$  Tris-borate-EDTA sequencing gel.

**RNase Protection.** A DNA fragment corresponding to nucleotide -76/+336 of the *UGT1A1* gene was generated by PCR (Expand High Fidelity PCR system; Boehringer Mannheim, Rotkreuz, Switzerland) using the pKS-1A1 genomic subclone as a template and cloned into the pBluescript vector to obtain the pKS-RPA-1A1 construct. A 293-bp radiolabeled antisense riboprobe was generated from this plasmid. The riboprobe ( $0.5 \times 10^5$  cpm) was mixed with 20  $\mu$ g of total mouse liver RNA in 30  $\mu$ l containing 40 mM PIPES (pH 6.4), 1 mM EDTA, 400 mM NaCl, and 80% formamide. After overnight hybridization at 42°C, the samples were digested with 300  $\mu$ l of RNase mix containing 300 mM NaCl, 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 2  $\mu$ g/ml RNase T1 (Life Technologies, Inc., Basel, Switzerland), and 40  $\mu$ g/ml RNase A (Boehringer Mannheim) for 30 min at 30°C. The reaction was stopped by addition of 100  $\mu$ g of proteinase K (Boehringer Mannheim). The samples were resolved on a 7 M urea, 8% polyacrylamide-1 $\times$  Tris-borate-EDTA sequencing gel.

**Generation of *UGT1A1* Promoter Deletion Constructs and Site-Directed Mutagenesis.** Four reporter genes encompassing different lengths of the *UGT1A1* promoter were generated by PCR using the pKS-1A1 genomic subclone as a template. The 5'-sense oligonucleotide primers used were complementary to the nucleotides -455 to -436, -93 to -74, and -44 to -25 of the cloned genomic sequence. The longest construct (from -1,074) was obtained using a vector-specific oligonucleotide primer. The common 3'-antisense oligonucleotide used was complementary to nucleotides -2 to +19 of the cloned genomic sequence. The PCR products were subcloned into the promoterless vector pBLCAT3, yielding the following plasmids: -1074/+19-mUGT1A1-CAT; -455/+19-mUGT1A1-CAT; -93/+19-mUGT1A1-CAT; and -44/+19-mUGT1A1-CAT. Point mutations were introduced into the

–455/+19 mUGT1A1-CAT plasmid (designated WT-mUGT1A1-CAT in Fig. 8), at specific regulatory elements by PCR using the QuikChange site-directed mutagenesis kit (Stratagene, Basel, Switzerland) following the manufacturer's instructions. The three mutant plasmids were designated  $\Delta$ HNF1-mUGT1A1-CAT,  $\Delta$ Sp1-mUGT1A1-CAT, and  $\Delta$ Sp1/HNF1-mUGT1A1-CAT; the integrity of each was validated by sequencing. The same procedure was used to mutate the HNF1 site of the –617/+15-hUGT1A1-CAT plasmid (designated WT-hUGT1A1-CAT in Fig. 8), yielding  $\Delta$ HNF1-hUGT1A1-CAT plasmid.

**Preparation of Nuclear and Whole-Cell Extracts (WCEs).** Nuclear extracts from mouse liver were prepared as described (Dignam et al., 1983). Briefly, the final suspension buffer containing 20 mM HEPES (pH 7.9), 20 mM KCl, 2 mM  $MgCl_2$ , 0.2 mM EDTA, 0.5 mM DTT, 20% glycerol, and protease inhibitors (Complete; Boehringer Mannheim) was aliquoted, frozen in liquid nitrogen, and stored at  $-70^\circ\text{C}$ . WCE was prepared from transfected and nontransfected human embryonic kidney (HEK) 293 cells by disrupting the cell membrane with three steps of freezing/thawing. The residual debris were pelleted by centrifugation for 10 min at 12,000g at  $4^\circ\text{C}$ . The extracts were aliquoted and stored at  $-70^\circ\text{C}$ .

**DNase I Footprint Analysis.** The DNA fragment from –253 to +81 of the proximal *UGT1A1* promoter sequence was subcloned into the pBLCAT3 vector. This plasmid possesses restriction sites to generate probes, with either the coding or noncoding strands labeled. End-labeled DNA fragments ( $3 \times 10^4$  cpm) were incubated with 10 to 50  $\mu\text{g}$  of mouse liver nuclear extracts (MLNEs) or with 10  $\mu\text{g}$  of whole HEK293 cell extracts, and when indicated, a 100-fold molar excess of oligonucleotide competitor was added. After 30 min of incubation on ice, the mixtures were digested with 1.5 U of RQ1DNase I (Promega, Madison, WI) for 1 min at room temperature. The reaction was stopped by addition of 200  $\mu\text{l}$  of 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 2% SDS, 0.4 mg/ml proteinase K, and 100  $\mu\text{g}/\text{ml}$  glycogen. Samples were separated on a 7 M urea, 8% polyacrylamide-1 $\times$  Tris-borate-EDTA sequencing gel. The position of specific DNase I-protected fragments was determined by chemical sequencing of the probe (Maxam and Gilbert, 1977).

**Electrophoretic Mobility-Shift Assay (EMSA).** EMSAs were performed as described (Ijpenberg et al., 1997) with either 10  $\mu\text{g}$  of MLNEs or 2.5  $\mu\text{g}$  of whole HEK293 cell extracts. For supershift experiments, 1  $\mu\text{l}$  of HNF1 $\alpha$ -specific antibody was used. Competition assays were performed by adding a 100-fold excess of unlabeled

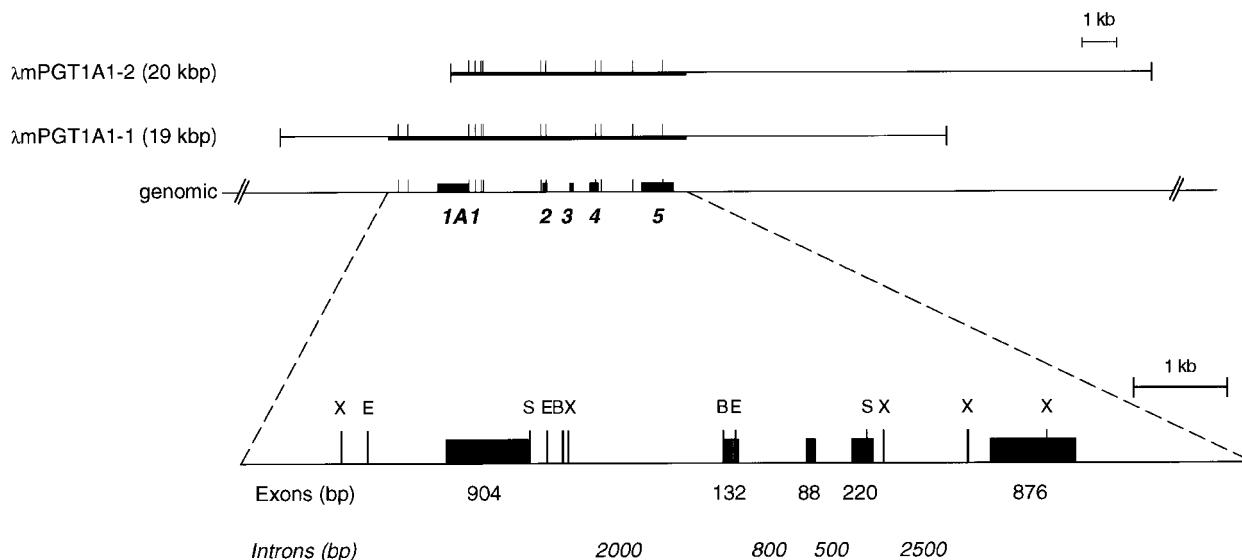
double-stranded oligonucleotide to the reaction 10 min before the addition of labeled probe.

**Cell Culture and Transfection.** HEK293 cells were seeded at  $3 \times 10^5$  cells/well in six-well plates, cultured overnight, and transfected with plasmid DNAs by the calcium phosphate-DNA coprecipitation method (Jordan et al., 1996). The reporter deletion constructs were at 250 ng/well, and expression plasmids for HNF1 $\alpha$  (pRSV-HNF1 $\alpha$ ) and HNF1 $\beta$  (pRSV-HNF1 $\beta$ ) were each at 500 ng/well. Cytomegalovirus- $\beta$ -galactosidase expression vector (50 ng/well; Stratagene, Basel, Switzerland) was included as an internal standard for transfection efficiency. After 12 h, the DNA precipitates were removed, and the cells were further incubated in fresh culture medium for 24 h. The CAT activity was assayed using  $^{14}\text{C}$ -labeled chloramphenicol (Amersham Pharmacia Biotech, Dübendorf, Switzerland) and *n*-butyryl coenzyme A substrates. The  $\beta$ -galactosidase activity was determined as described (Sambrook et al., 1989). All transfections were performed in duplicate. Activities reported are an average  $\pm$  S.D. of three independent experiments.

## Results

**Cloning of the Mouse *UGT1A1* Gene.** A mouse genomic DNA library was screened using two oligonucleotides derived from the previously reported mouse *UGT1A1* cDNA (Chu et al., 1997). Two clones ( $\lambda$ mPGT1A1–1,  $\lambda$ mPGT1A1–2) were isolated and characterized (Fig. 1). Together, they form a contig of about 25 kb, but only clone  $\lambda$ mPGT1A1–1 (19-kb insert) contains the entire gene, including several kb of 5'- and 3'-flanking sequences. The 5'-end of clone  $\lambda$ mPGT1A1–2 (20-kb insert) lies within the isoform specific exon 1A1. This cloned genomic DNA fragment also contains all common exons and extends far into the 3'-flanking region of the gene. The common exons 2, 3, 4, and 5 of the *UGT1* locus are localized about 2 kb downstream of exon 1A1, and their position relative to each other as determined by PCR analysis (Fig. 1) is similar to that observed in rat and human (Ritter et al., 1992; Emi et al., 1995).

A 2.4-kb *Xba*I restriction fragment from the  $\lambda$ mPGT1A1–1 clone, which contains exon 1A1 and its 5'-flanking region, was subcloned and sequenced. The insert is 2424 bp long,



**Fig. 1.** Structure of the mouse *UGT1A1* locus. Schematic representation of the mouse *UGT1A1* gene deduced from the characterization of the two genomic DNA clones  $\lambda$ mPGT1A1–1 and  $\lambda$ mPGT1A1–2 that are shown. Restriction enzyme cleavage sites were mapped only in the region given in bold. The position of the exons was determined by restriction enzyme mapping and PCR analysis of both clones. Only the  $\lambda$ mPGT1A1–1 clone contains the entire gene with the complete isoform-specific exon 1A1 and promoter sequences. X, *Xba*I; S, *Stu*I; E, *Eco*RI; B, *Bam*HI.



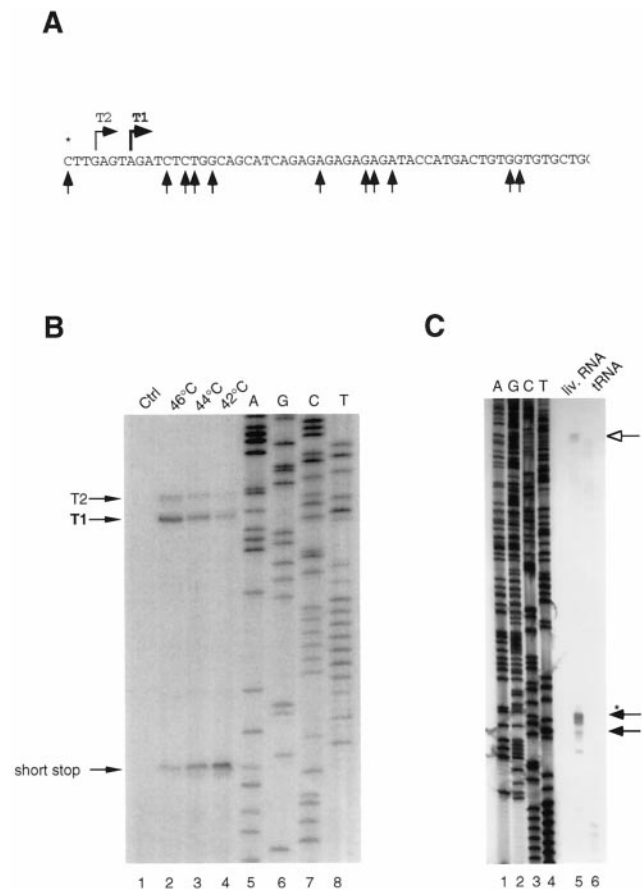
with 1075 bp of 5'-flanking region, 904 bp of exon 1A1 sequence, and 445 bp of intron I. The coding region of exon 1A1 is 99% identical in terms of amino acids to that of the reported cloned mouse cDNA (Y → S change at position 25; Chu et al., 1997). Surprisingly, the 5'-untranslated region (UTR) sequence of this previously reported cDNA is completely different from that of our genomic subclone. To clarify this point and to determine the real 5'-UTR upstream of the ATG translation start codon in exon 1A1, we screened a 15-day-old mouse embryonic cDNA library and an adult mouse liver cDNA library using the same procedure as for the genomic screening. All 11 independent clones obtained in this experiment had a 5'-UTR sequence matching that of the genomic subclone, albeit of different length, reflecting most likely incomplete cDNA 5'-extension. The 5'-end nucleotide of these cDNAs is indicated in Fig. 2A. The reason for the 5'-UTR structural discrepancy between our 11 cDNA clones and the previously reported clone remains unclear.

**Determination of the Mouse *UGT1A1* Transcription Start Site.** The transcription start site of the mouse *UGT1A1* gene was identified by primer extension and RNase protection assays. Primer extension using mouse liver RNA as template was performed at 42, 44, and 46°C (Fig. 2B). Three extension products were obtained in different relative amounts, depending on the reaction temperature. The upstream end of the short-stop product is in the coding sequence in a region with potential secondary structure, which explains a decrease in the amount of this product at higher temperature in favor of the longer extension products. The upstream end of these latter products is 34 and 38 bp upstream of the ATG translation start codon at sites we named T1 and T2, respectively. T1 is considered the major transcription start site (+1) because it appears stronger than T2 at all three temperatures used in the extension assay (see Fig. 3A). The 5'-end of the longest cDNA clone we have obtained is three nucleotides upstream of T2 and most likely corresponds to a minor transcription start site. To confirm the position of transcription initiation, we carried out RNase protection analysis with mouse liver RNA and a radiolabeled antisense riboprobe, which corresponds to the region between nucleotides -76 and +336 with respect to the major transcription start site T1. The protected fragments also indicate transcriptional start sites at positions T1 and T2 (Fig. 2C). The bands between these positions in Fig. 2C most likely reflect an incomplete digestion of the riboprobe-RNA duplexes (as indicated by the presence of residual nondigested riboprobe; open arrow). A minor fragment indicating the same start site as that of the longer cDNA clone could be seen after longer exposure (\* in Fig. 2, A and C). All three initiation start sites (\*, T1, T2) are shown with respect to the 5'-ends of the 11 cDNA clones mentioned above (Fig. 2A) and in the context of the promoter sequence in Fig. 3A.

**Structural Features of the Mouse *UGT1A1* TATA-Less Promoter.** Analysis of the promoter region of the mouse *UGT1A1* gene revealed several structural features of interest, some of them highlighted for comparison with the human sequence (Fig. 3, A and B). The mouse promoter has neither a TATA box (Matsui et al., 1980) nor an identifiable initiator-type sequence, often found in eukaryotic TATA-less promoters at the transcription start site (Emami et al., 1998). In contrast, the human promoter has a TATA box-like element associated with the polymorphic TA repeat responsible

for some of the cases of Gilbert's disease (Clarke et al., 1997). Otherwise, this TATA box has not yet been characterized functionally.

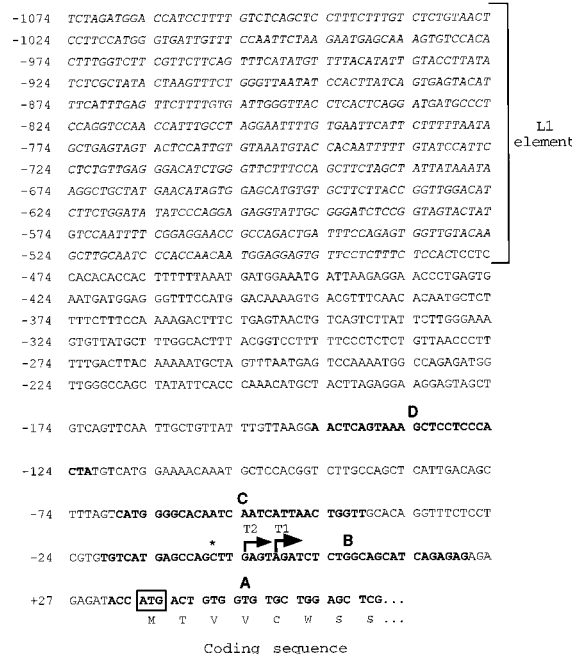
In addition to this particular feature, an L1 retrotransposon element (Smit et al., 1995) is present starting at position -480 in the mouse promoter (Fig. 3A). This insertion was confirmed by PCR analysis and Southern blot of uncloned mouse genomic DNA, which excludes a possible cloning artifact in clone  $\lambda$ MPGT1A1-1 (data not shown). Such an element is absent in the human promoter. Between the human and mouse promoter, there are two conserved regions within



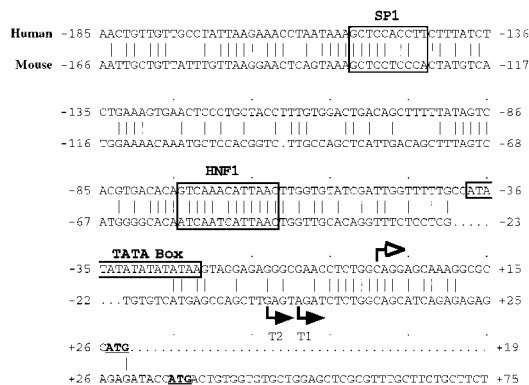
**Fig. 2.** Determination of the transcription start sites of the mouse liver *UGT1A1* gene. A, sequence of the 5'-UTR of the *UGT1A1* gene with the transcription initiation sites (T1, T2, \*) and the position of the 5'-end of 11 cDNA clones (vertical arrows). B, primer extension analysis was carried out at three different temperatures using a  $^{32}$ P-end-labeled primer complementary to nucleotides +95/+120 of the *UGT1A1* exon annealed to either 50  $\mu$ g of total mouse liver RNA (lanes 2–4) or yeast tRNA as a control (lane 1). The primer-extended products were analyzed on an 8% denaturing polyacrylamide gel in parallel with sequencing reactions (lanes 5–8) carried out on the genomic subclone using the same primer as above. Arrows indicate the extended products. T1 and T2 indicate the major and minor transcription initiation site, respectively; Short-stop, prematurely ended extension products (see Results). C, RNase protection assay using an antisense riboprobe corresponding to nucleotides -76/+336 of the *UGT1A1* gene annealed to 20  $\mu$ g of total mouse liver RNA (lane 5) or yeast tRNA (lane 6) as control. After RNase digestion, protected fragments were resolved on an 8% polyacrylamide gel together with sequencing reactions (lanes 1–4). T1, T2, and \* indicate the major, minor, and weak transcription initiation site, respectively. In positioning these initiation sites on the sequencing ladder, a correction of 1 base has been made because of the lower mobility of RNA than DNA of the same length (Sambrook et al., 1989). The open arrow indicates residual nondigested riboprobe.

the 170 bp upstream of the transcription-initiation site corresponding to HNF1- and Sp1-binding sites (Fig. 3B). We thus concentrated on the function of these elements in the regulation of the *UGT1A1* promoter.

# A

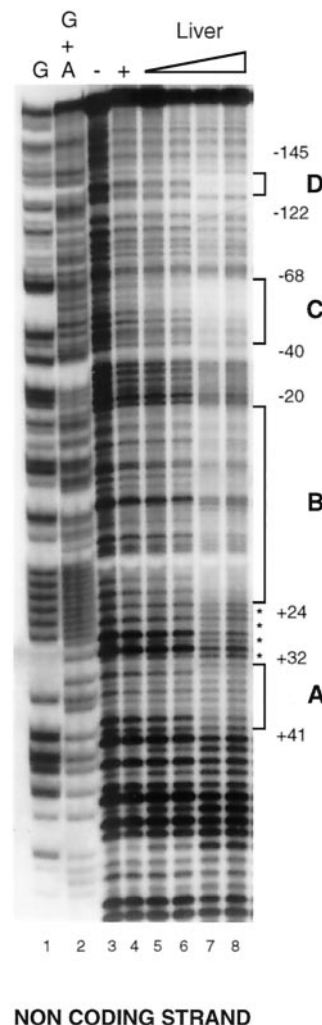


# B



**Fig. 3.** Characteristics of the mouse and human *UGT1A1* promoter. A, nucleotide sequence of the mouse *UGT1A1* promoter. Nucleotides are numbered with respect to the major transcription start site as determined herein (T1). The minor and a very weak transcription start sites are indicated with a small arrow (T2) and an asterisk, respectively. The translation-initiation codon ATG is boxed, and the first eight amino acids are given. The nucleotide sequence corresponding to the L1 retrotransposon element is indicated in italics. Footprinted regions A, B, C, and D obtained in Fig. 4 are indicated in bold letters. B, comparison of human (Brierley et al., 1996) and mouse *UGT1A1* promoter sequences. The human *UGT1A1* nucleotide sequence (top) was aligned with its mouse counterpart (bottom) using the Gap program from the GCG package (Wisconsin Package Version 9.1; Genetics Computer Group, Madison, WI). The transcription initiation site (T1) of the mouse gene and the conserved HNF1 and Sp1 sites are indicated on both mouse and human sequence. The putative human TATA box is indicated. The transcription-initiation site of the human promoter (open arrow) has been determined previously (Ritter et al., 1992).

**Liver Nuclear Proteins Bind to the Mouse *UGT1A1* Promoter.** *UGT1A1* has a major detoxification function in the liver; therefore, it was of interest to test whether a factor present in MLNEs is able to bind to the putative HNF1 and Sp1 sites described above. For this purpose, we performed a DNase I footprinting analysis. As shown in Fig. 4, a total of four independent protected areas, designated A to D, were identified in the proximal promoter region (-253/+81; non-coding strand). Footprints A (+32/+41) and B (-20/+24) are close to and comprise the transcription initiation site, respectively; they are separated by DNase I-hypersensitive sites (\*). It is reasonable to speculate that these footprints result from the binding of factors belonging to the machinery responsible for transcription initiation, and therefore they were not further characterized herein. Footprint C (-68/-40) corresponds to the putative HNF1 recognition site and footprinted region D between nucleotides -145 to -122, comprising the putative Sp1 site to which purified Sp1 can indeed bind in



**Fig. 4.** Liver nuclear protein-binding sites within the mouse *UGT1A1* promoter. DNase I footprinting experiment carried out with a -253/+81-proximal *UGT1A1* promoter fragment (noncoding strand) and increasing amounts of MLNEs. -, DNase I digestion of the probe without added protein; +, DNase I digestion of the probe with 50 μg of purified bovine serum-albumin. Amounts of nuclear extracts used are 10 μg (lane 5), 20 μg (lane 6), 30 μg (lane 7), and 40 μg (lane 8). Protected areas are indicated on the right of the autoradiogram. DNase I-hypersensitive sites present between the A and B regions are indicated by \*. Also shown are chemical sequencing ladders G and G+A (lanes 1 and 2).

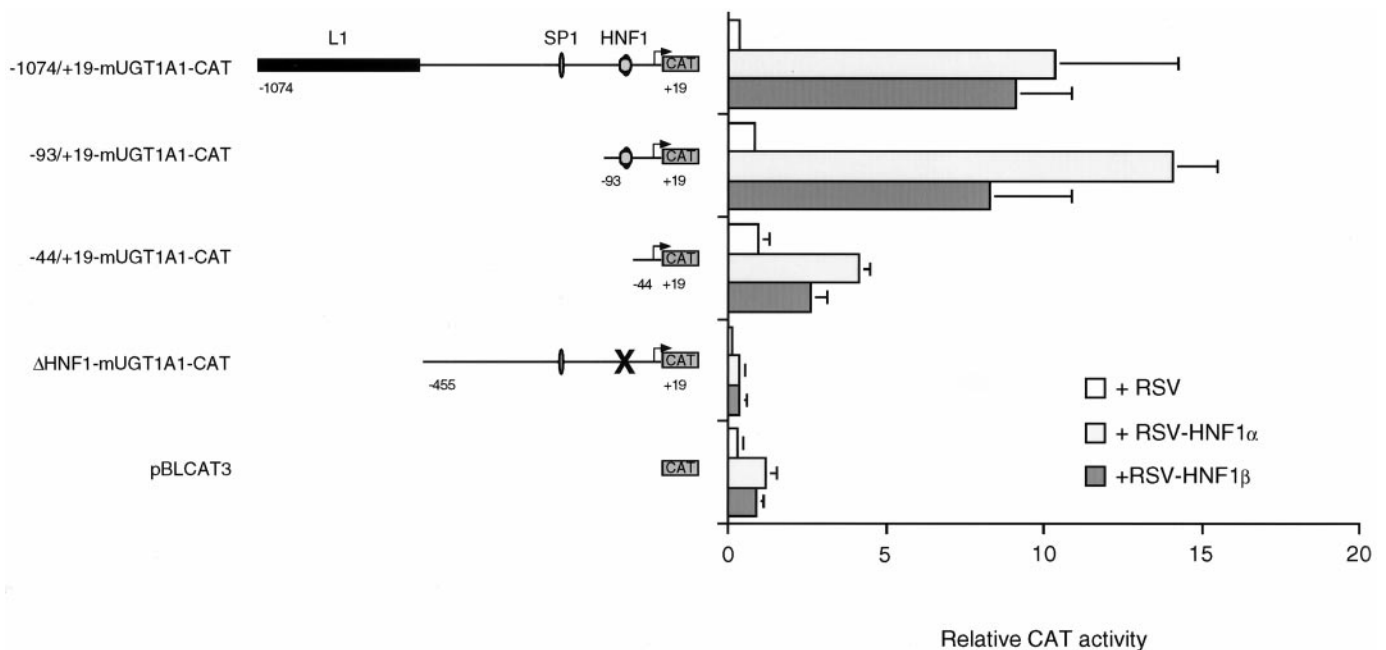
gel-retardation assays, as will be shown later. The same results were obtained with the coding strand (data not shown). These data, together with the observation that these binding sites are well conserved in the mouse and human promoters, suggested that these factors may play a role in *UGT1A1* regulation.

**The Mouse *UGT1A1* Promoter Is Transactivated by HNF1.** The functional role of HNF1 was assayed by cotransfection assays performed with HNF1 $\alpha$  and HNF1 $\beta$  expression vectors and a CAT reporter gene driven by the mouse *UGT1A1* promoter comprising decreasing lengths of 5'-upstream sequences (-1074/+19, -93/+19, -44/+19). The empty expression vector (Rous sarcoma virus) and promoterless reporter CAT vector (pBLCAT3) were used as controls. The recipient cells were the HEK293 cells in which HNF1 cannot be detected. The results show that both HNF1 $\alpha$  and HNF1 $\beta$  are able to transactivate both the -1074/+19 mUGT1A1-CAT reporter gene and the -93/+19 mUGT1A1-CAT construct, both of which contain the HNF1 site (Fig. 5). The minimal promoter -44/+19 still has a relatively high basal promoter activity, a noteworthy feature for a TATA-less promoter. Some residual HNF1-dependent stimulatory activity was seen with this minimal promoter that is thought to be due to vector sequences brought close to the promoter as a result of upstream promoter deletion (see results with the promoterless CAT-vector). Instead of analyzing this effect further, we performed site-directed mutagenesis of the HNF1 site in the context of a relatively large promoter region (-455/+19). A complete loss of HNF1-dependent stimulation of the mutated promoter confirmed that the HNF1 element is responsible for mediating induction by the liver-enriched transcription factor (Fig. 5,  $\Delta$ HNF1-mUGT1A1-CAT). We conclude that HNF1 is a transactivator of the *UGT1A1* gene

and that the stimulatory effect is mediated by the HNF1 binding site located at -68/-40. It is noteworthy that removal of L1 element sequences did not influence HNF1 responsiveness.

**HNF1 Binds to the *UGT1A1* Promoter.** To assess whether the HNF1-mediated transactivation is due to a direct binding of the factor to the HNF1-binding site in the promoter, as suggested by the results presented above, we carried out EMSAs using the *UGT1A1*-HNF1 site as a probe. We first confirmed that a factor in the MLNE binds to this site, as shown previously by the DNase I footprinting experiment. A major retarded complex (C1) was detected in the presence of the nuclear extract (Fig. 6A, lane 2). This complex was competed away by a molar excess of a HNF1-specific oligonucleotide added to the binding reaction (lane 5), whereas binding sites for other transcription factors were unable to displace the complex (lanes 3, 4, 6, and 7). We also observed a faster-migrating minor complex (C2). This C2 complex most likely contains HNF3, because it was specifically displaced by a molar excess of HNF3 sites, which is in accordance with previously reported results indicating that HNF3 may bind to HNF1 sites (Gregori et al., 1993). Figure 6B shows that the C1 complex was disrupted by a polyclonal antibody directed against HNF1 $\alpha$  (lane 3), allowing a reinforcement of C2 complex formation. The nonspecific band observed with the antibody alone (lane 4) has been previously reported (Chouard et al., 1997). Together, these results provide strong evidence that C1 is due to the occupancy of the *UGT1A1*-HNF1 site by HNF1 $\alpha$ , which is abundant in liver (Rey-Campos et al., 1991).

To strengthen the evidence for specific binding of HNF1 $\alpha$ , we used the mutated *UGT1A1*-HNF1 site depicted in Fig. 6C as competitor. Figure 6D shows that a 100- and 500-fold

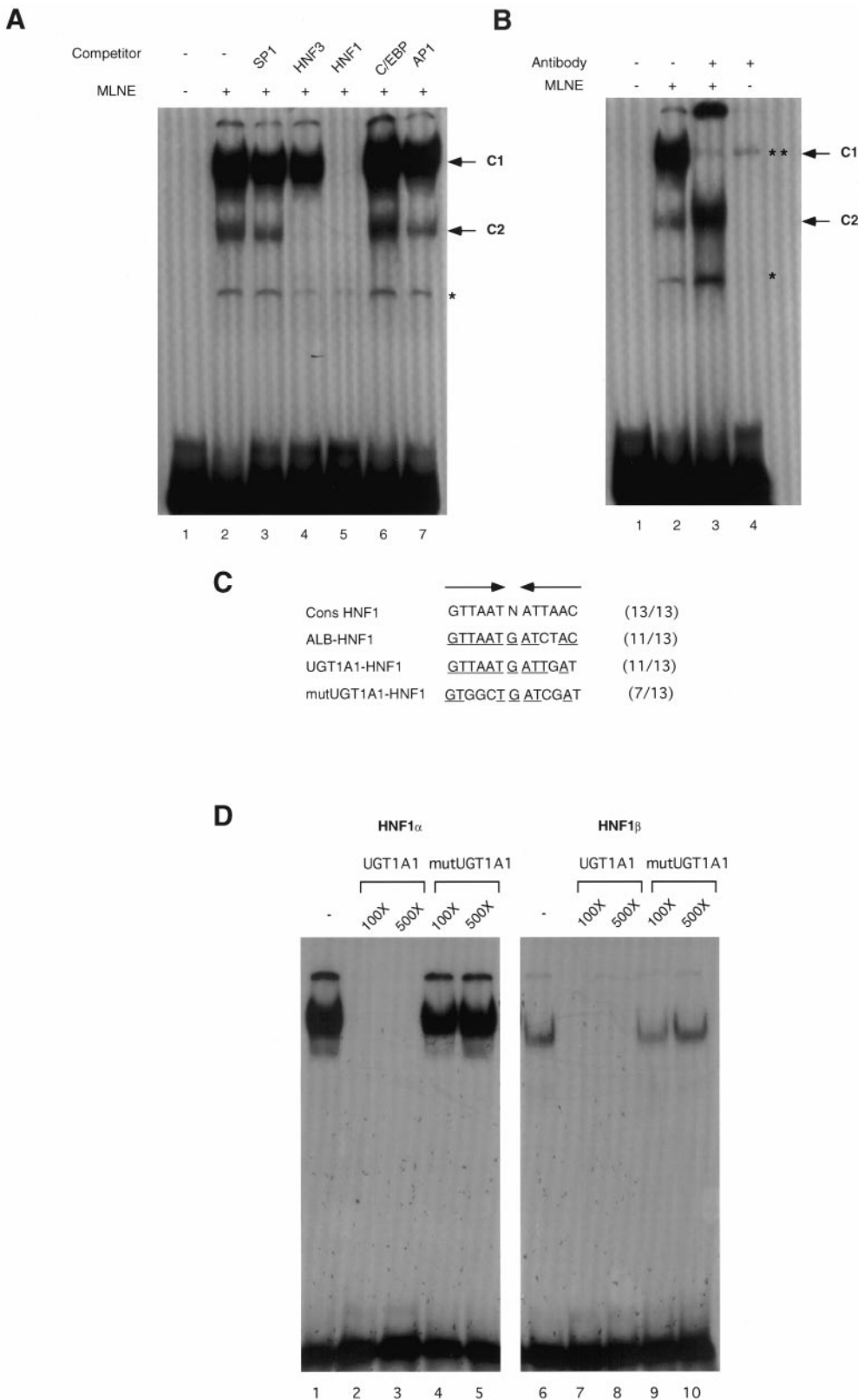


**Fig. 5.** HNF1 $\alpha$  and HNF1 $\beta$  transactivate the mouse *UGT1A1* promoter. HEK293 cells were cotransfected in six-well plates with 0.25  $\mu$ g per well of the different deletion and mutant reporter constructs and 0.5  $\mu$ g of expression vector for HNF1 $\alpha$  (RSV-HNF1 $\alpha$ ), HNF1 $\beta$  (RSV-HNF1 $\beta$ ), or empty vector (RSV) as a control. A pCMV $\beta$ -galactosidase (50 ng per well) expression vector was included as an internal control of transfection efficiency. Results are the mean of at least three independent experiments  $\pm$  S.D. Deletion constructs are depicted, with the L1 element indicated as a black bar, and the position of the Sp1 and HNF1 sites are given according to the results of Fig. 4. In  $\Delta$ HNF1-mUGT1A1-CAT, the mutated HNF1 site is represented by a cross; this result is taken from Fig. 8. pBLCAT3 was used as a promoterless control reporter gene.

molar excess of the mutant oligonucleotide was unable to displace the HNF1 $\alpha$ -retarded complexes (lanes 4 and 5).

Finally, we have demonstrated that HNF1 $\beta$  is also able to bind this element, as suggested by the transfection result shown in Fig. 5. Indeed, overexpressed HNF1 $\beta$  binds to the

UGT1A1-HNF1 site (Fig. 6D, lane 6). The complex is displaced by a 100- and 500-fold molar excess of the probe (lanes 7 and 8) but not by the mutant oligonucleotide (lanes 9 and 10). Altogether, these results provide evidence for a specific interaction of HNF1 with the *UGT1A1* promoter at a site



**Fig. 6.** HNF1 proteins bind to the C site of the mouse *UGT1A1* promoter. A, EMSA experiments using a MLNE were performed with a radiolabeled oligonucleotide corresponding to site C (HNF1-binding site) of the *UGT1A1* promoter in the presence or absence of a 100-fold excess of different competitor oligonucleotides as indicated at the top of the autoradiogram. For the identification of C1 and C2 complexes, see text. \* is an unspecific band. B, supershift experiments in which EMSA with the radiolabeled site C oligonucleotide was carried out with MLNE in the presence or absence of a specific antibody directed against HNF1 $\alpha$  (Chouard et al., 1997). \* is an unspecific band, as in A. \*\* is an unspecific band generated by the antibody, as described (Chouard et al., 1997). C, sequence of the different wild-type and mutated HNF1-binding sites comprised in the oligonucleotides used in EMSA, as compared with the consensus HNF1-binding site. Nucleotides corresponding to the consensus binding site are underlined in ALB-HNF1, UGT1A1-HNF1, and mut UGT1A1-HNF1. The matching index is given in brackets. D, EMSA using overexpressed HNF1 $\alpha$  or HNF1 $\beta$  in HEK293 cells was performed with radiolabeled site C as probe (see above) in the presence of 100- or 500-fold excess of mutant site C (mutUGT1A1) or wild-type site C (UGT1A1) competitor oligonucleotides.



that we demonstrated to be responsible for the transcriptional activation mediated by this transcription factor.

**HNF1 and Sp1 Do Not Cooperate for the Control of the *UGT1A1* Promoter.** The footprinting experiment using MLNEs revealed a binding site for Sp1 in the mouse *UGT1A1* promoter, which is conserved in the human promoter, 54 bp upstream of the HNF1 site. This observation was confirmed in EMSA experiments with whole HEK293 cell extracts as well as with purified Sp1 for both the mouse and human Sp1 sites (data not shown).

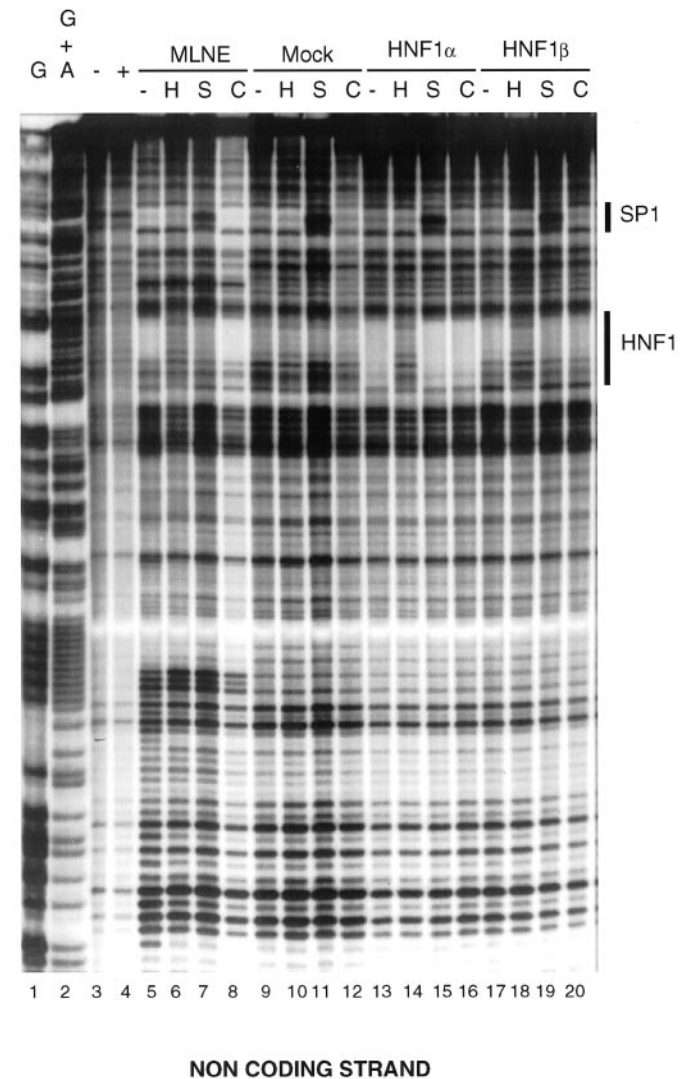
The proximity of HNF1- and Sp1-binding sites on the *UGT1A1* promoter prompted us to test whether the binding of the two factors was independent or cooperative. Indeed, it has been observed that Sp1 can bind cooperatively to DNA with transcription factors such as C/EBP $\alpha$  (Lee et al., 1994). For this purpose, we carried out oligonucleotide competition in DNase I footprint experiments using MLNEs or whole HEK293 cell extracts from cells transfected with either HNF1 $\alpha$  or HNF1 $\beta$  as well as mock-transfected cell extracts as control. HEK293 cells contain sufficient amounts of endogenous Sp1-binding activity to produce a footprint over the *UGT1A1*-Sp1 site (Fig. 7, lanes 9, 13, and 17). Competitive inhibition of binding to the Sp1 site did not influence binding to the HNF1 site (lanes 7, 15, and 19). Similarly, competitive inhibition of binding to the HNF1 site did not affect protein interaction with the Sp1 site (lanes 6, 14, and 18). Competition with an unrelated control oligonucleotide (lanes 8, 16, and 20) had no effects on either of these two sites. These results indicate that, in this assay, binding of HNF1 on the promoter occurs independently of Sp1 binding.

The fact that cooperative binding to the HNF1 and Sp1 sites could not be detected in competition footprinting experiments does not necessarily preclude functional interaction between the two proteins during transcriptional stimulation. This kind of functional cooperation has indeed been demonstrated between Sp1 and nuclear hormone receptors (Krey et al., 1995). To test this possibility, cotransfection experiments were performed using a mouse promoter construct mutated at either the Sp1 site ( $\Delta$ Sp1-m*UGT1A1*-CAT) or HNF1 site ( $\Delta$ HNF1-m*UGT1A1*-CAT). The mutation introduced in the HNF1 site corresponds to that shown previously in Fig. 6C (mut*UGT1A1*), which hinders HNF1 binding. The wild-type promoter or the promoter simultaneously mutated at the two sites under analysis ( $\Delta$ HNF1/Sp1-m*UGT1A1*-CAT) were used as positive and negative controls, respectively. As shown previously, invalidation of the HNF1 site abolished transcriptional activation of the promoter by HNF1, whereas mutation of the Sp1 site had no effect on transactivation by HNF1 (Fig. 8). This indicates that there is no functional interaction between the Sp1 and HNF1 sites in our experimental system. However, mutation of the Sp1-binding site alone reduced strongly the basal activity of the promoter in absence of cotransfected HNF1. This observation suggests that Sp1 plays a role in the basal activity of the mouse *UGT1A1* promoter. Because the human and mouse *UGT1A1* promoters display a very well conserved HNF1 site, we tested whether HNF1 is able to mediate transactivation of the human promoter. As shown in Fig. 8, the basal activity of the human promoter (−617 to +15) is similar to its mouse counterpart in HEK293 cells. HNF1 $\alpha$  and HNF1 $\beta$  activated the human *UGT1A1* promoter even more strongly than the mouse promoter. Similarly to what has been observed with

the mouse promoter, disruption of the HNF1 binding site ( $\Delta$ HNF1-h*UGT1A1*-CAT) abolished HNF1-mediated transactivation. In conclusion, both the mouse and human *UGT1A1* promoters can be transcriptionally regulated by HNF1 transcription factors, despite important structural differences between them.

## Discussion

Glucuronidation of bilirubin is essential for its excretion from the liver into the bile. In human, *UGT1A1* is the major UGT isoform involved in this process (Bosma et al., 1994). Consequently, alteration in *UGT1A1* gene expression or mutations in the coding region of this gene can lead to a com-



**Fig. 7.** Noncooperative binding between Sp1 and HNF1 within the proximal mouse *UGT1A1* promoter. DNase I footprinting was carried out using a −253/+81 *UGT1A1* promoter fragment (noncoding strand) and protein extracts from mouse liver and HEK293 cells as indicated at the top of the gel in the presence of 100-fold excess of different competitor oligonucleotides. Mock, WCE from mock-transfected HEK293 cells; HNF1 $\alpha$ , WCE from HNF1 $\alpha$ -transfected HEK293 cells; HNF1 $\beta$ , WCE from HNF1 $\beta$ -transfected HEK293 cells. −, DNase I digestion of the probe without added protein; +, DNase I digestion of the probe with 50  $\mu$ g of purified BSA; H, HNF1 consensus oligonucleotide; S, Sp1 consensus; C, C/EBP consensus oligonucleotide. Protected areas corresponding to the Sp1- and HNF1-binding sites are indicated on the right of the gel. The sequencing ladder tracks G and G+A are indicated.



plete or partial absence of bilirubin glucuronidation activity, which results in toxic accumulation of unconjugated bilirubin (Hauser and Gollan, 1990). One of the ways to study and better understand bilirubin metabolism in human is to compare it to other mammalian species, such as the increasingly used mouse model. For this purpose, we have cloned and functionally characterized the mouse *UGT1A1* promoter. Important structural differences with the human promoter were found. Surprisingly, the mouse *UGT1A1* promoter is TATA-less and consequently does not contain any of the TA repeats found in human. In contrast, both human and mouse promoter activities are stimulated by HNF1 $\alpha$  and HNF1 $\beta$  via a functional HNF1 site particularly well conserved between the two promoters.

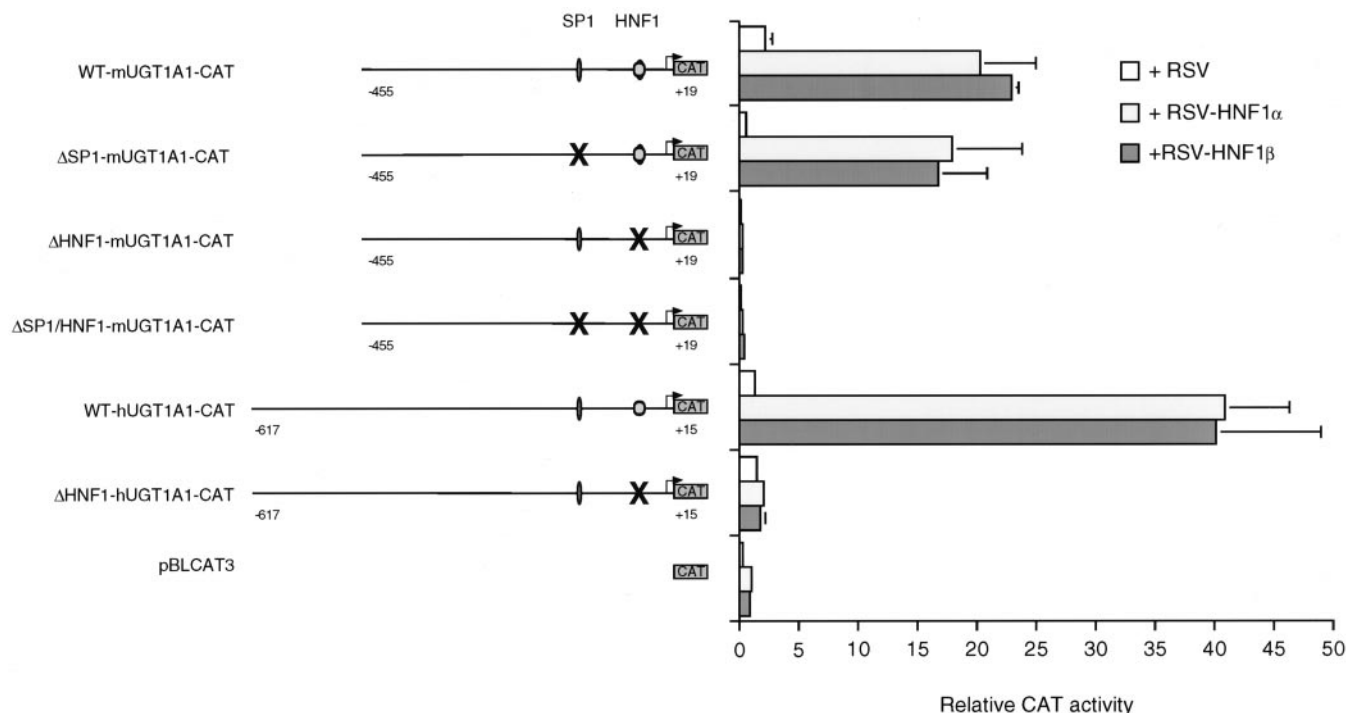
#### The Mouse *UGT1A1* Promoter Lacks a TATA Box.

Two major new features occur only in the mouse *UGT1A1* promoter in comparison to the human promoter. First, the mouse *UGT1A1* promoter has no apparent TATA box, as observed previously for the rat *UGT1A7* promoter (Metz and Ritter, 1998). In contrast, other UGTs belonging to family 1 (Emi et al., 1996) as well as to family 2 (Mackenzie and Rodbourn, 1990) possess a TATA-like element, indicating that there is no common promoter structure for all UGT isoforms. It is noteworthy that the rat *UGT1A7* promoter has multiple transcription start sites clustered within 45 bp, whereas the mouse *UGT1A1* has only two independent well detectable transcription start sites clustered in less than 10 bp, which can be detected by analysis of liver transcripts. Despite the lack of a TATA-binding protein binding site, it is clear that the mouse *UGT1A1* transcriptional start site is well defined, suggesting that binding of transcription factors to this promoter directly interacts with the basal transcrip-

tion machinery to help in positioning RNA polymerase II. HNF1 indeed is one of the transcription factors that have been shown to interact directly with components of the general transcription apparatus, such as transcription factor IID or transcription factor IIB (Ktistaki and Talianidis, 1997). The second characteristic feature of the mouse promoter is the presence of a retrotransposon-like sequence element, which belongs to the L1 family, at 480 bp upstream the transcription start site. The L1 element is found at more than 100,000 copies in mouse and represents about 10% of the genome (Smit et al., 1995). However, the majority of the L1 elements are truncated and not active in transposition (Kolosha and Martin, 1995). This is the case for the element identified in the mouse *UGT1A1* promoter because it lacks the 5'-long terminal repeat. The recent characterization of the apolipoprotein(a) enhancer in mouse, which resides within a L1 element, illustrates the possibility of a regulatory action of these elements on neighboring promoters (Yang et al., 1998). However, further studies are needed, especially in hepatocytes rather than in HEK293 cells, to evaluate the relevance, if any, of this L1 element insertion for tissue-specific activity of the mouse *UGT1A1* gene.

#### HNF1 Binds to and Activates the Mouse *UGT1A1*

**Promoter.** We have demonstrated that both HNF1 $\alpha$  and HNF1 $\beta$  bind to the mouse *UGT1A1* promoter and are able to transactivate it. However, because HNF1 $\beta$  is less expressed in adult mouse liver than HNF1 $\alpha$ , it is very likely that it is the latter factor that plays a regulatory role in this organ. Our results suggest that HNF3 also binds to the HNF1 site, although the signal of HNF1 is much stronger than that of HNF3 in liver nuclear extracts. It will be interesting to investigate whether there is a competition between these two



**Fig. 8.** Absence of functional interaction between HNF1 $\alpha$  and Sp1 in transactivation of the mouse *UGT1A1* promoter. HEK293 cells were cotransfected in six-well plates with 0.25  $\mu$ g per well of different mutant reporter constructs and 0.5  $\mu$ g of expression vector for HNF1 $\alpha$  (RSV-HNF1 $\alpha$ ), HNF1 $\beta$  (RSV-HNF1 $\beta$ ), or empty vector as control (RSV). A pCMV $\beta$ -galactosidase (50 ng per well) expression vector was included as an internal control for transfection efficiency. Results are the mean of at least three independent experiments  $\pm$  S.D. The reporter constructs used are depicted; mutated Sp1, and HNF1 sites are indicated by a cross. pBLCAT3 was used as promoterless control reporter.

factors relevant for expression of this gene in liver. The HNF1-binding site of the mouse *UGT1A1* promoter is very similar to that of the rat albumin promoter, which displays a very well conserved half-site and a more divergent one with respect to the consensus sequence (Cereghini, 1996). Consistent with previous studies on the albumin-HNF1 site, HNF1 proteins failed to bind and transactivate the mouse *UGT1A1* promoter when the conserved half-site is mutated. Our study provides strong evidence for HNF1 $\alpha$  and HNF1 $\beta$  being involved in the expression of this member of the UGT superfamily in rodents. In adult mouse, HNF1 $\alpha$  and HNF1 $\beta$  are expressed in the same tissues (kidney, liver, intestine, and pancreas; Rey-Campos et al., 1991) but at different relative levels. In liver, HNF1 $\beta$  is less expressed than HNF1 $\alpha$ , whereas both are present at similar levels in kidney. This pattern of expression correlates with that of the rat *UGT1A1*, which is predominantly found in liver, and less in kidney (Emi et al., 1995). Furthermore, HNF1 $\alpha$  and HNF1 $\beta$  play an important role during development. It has been shown that HNF1 $\beta$ , which systematically precedes HNF1 $\alpha$  expression, is involved in early events of liver and kidney morphogenesis, whereas HNF1 $\alpha$  is required for maintenance of the differentiated state (Cereghini, 1996). Further studies are required to elucidate to what extent these two factors contribute to the developmental expression of the *UGT1A1* gene in mouse.

**Is HNF1 Crucial for the *UGT1A1* Gene Expression in Human and Mouse?** The structural differences between human and mouse *UGT1A1* promoters raised the question as to whether they are subjected to the same regulatory process. Comparison of the two promoters revealed conserved HNF1- and Sp1-binding sites, and our study also indicates that HNF1 $\alpha$  and HNF1 $\beta$  transactivate both the human and mouse *UGT1A1* promoter in the same manner. However, an extrapolation from cell culture experiments to the in vivo situation in human is not possible without further consideration. For instance, neither bilirubin glucuronidation nor *UGT1A1* mRNA can be detected in human kidney (McGurk et al., 1998) in which HNF1 proteins are expressed and active. This indicates that HNF1 is not sufficient for the human *UGT1A1* expression in this organ.

Recent studies using the knockout technology have revealed a role of hepatic transcription factors in mouse *UGT1A1* expression. The inactivation of the HNF1 $\alpha$  gene by homologous recombination resulted in a complex phenotype with impaired functions in liver and kidney (Pontoglio et al., 1996). The HNF1 $\alpha$ -/- mice lack expression of the HNF1 target gene phenylalanine hydroxylase, whereas the expression of other HNF1 target genes such as albumin,  $\alpha$ -antitrypsin, and  $\alpha$ - and  $\beta$ -fibrinogen was only partially reduced. Interestingly, these mutant mice exhibit a mild hyperbilirubinemia. Based on the results reported herein, it is likely that it is caused by an altered expression of the *UGT1A1* gene. Furthermore, our results suggest that the residual expression of *UGT1A1* indicated by the mild hyperbilirubinemia might be caused by HNF1 $\beta$ , which is expressed in the liver (see above) and is also a transcriptional regulator of the *UGT1A1* promoter. HNF1 $\beta$  may partially rescue the HNF1 $\alpha$  deficiency, as was proposed for other HNF1-target genes (Pontoglio et al., 1996). Although this is the most plausible explanation, we cannot exclude the possibility that other bilirubin UGTs isoforms maintain bilirubin glucuronidation in the HNF1 $\alpha$ -/- mice.

Often, tissue-enriched factors cooperate with ubiquitous factors, for instance C/EBP $\alpha$  with Sp1 (Lee et al., 1994). Herein, we show that Sp1, which binds upstream of HNF1 on the *UGT1A1* promoter, seems to have no influence on HNF1 binding or on its transcriptional activity in HEK293 cells. However, we have also demonstrated that Sp1 alters the mouse *UGT1A1* promoter activity independently of HNF1. Further analysis of this promoter, which may identify additional regulatory factors, is necessary to assess novel interactions between the transcription factors involved in its regulation.

In conclusion, we have identified HNF1 as the first important transcriptional regulator of *UGT1A1* gene expression in mouse and human. It will now be the aim of further studies to determine the extent of its regulatory contribution in *UGT1A1* expression with respect to the species- and tissue-specific characteristics of bilirubin glucuronidation.

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