

Xenobiotic Responsive Element-mediated Transcriptional Activation in the UDP-glucuronosyltransferase Family 1 Gene Complex*

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We have isolated genomic DNA clones containing rat UDP-glucuronosyltransferase family 1 (UGT1) sequences and have shown drug-responsive and tissue-specific alternative expression of multiple first exons (Emi, Y., Ikushiro, S., and Iyanagi, T. (1995) *J. Biochem. (Tokyo)* 117, 392–399). The *UGT1* locus encodes at least nine UGT1 isoforms. UGT1A1 is a major 3-methylcholanthrene (MC)-inducible form in rat liver. In this report, we have identified a *cis*-acting element necessary for transcriptional activation of UGT1A1 in hepatocytes. A promoter region was fused to a chloramphenicol acetyltransferase gene, and the resultant construct was transiently transfected into hepatocytes. A DNA fragment carrying 1,100 nucleotides derived from the 5'-flanking region of the UGT1A1 gene was enough for MC induction. Unidirectional deletion of this region revealed that there existed one xenobiotic responsive element (XRE), TGCCTG, between –134 and –129. When a single base substitution was introduced into the XRE, MC-induced expression of the UGT1A1 gene was completely abolished. In addition, an XRE-deleted construct failed to respond to MC. Gel mobility shift assays showed MC-inducible binding of the nuclear aromatic hydrocarbon receptor-ligand complex to this motif. Gel shift-coupled DNase I protection analyses revealed that the GCGTG-core sequence was a target site of the liganded aromatic hydrocarbon receptor. These results suggest that the XRE participates in induction of the rat UGT1A1 gene by MC.

UDP-glucuronosyltransferases (UGTs)¹ (EC 2.4.1.17) are a family of isoenzymes that catalyze transfer of the glucuronic acid moiety of UDP-glucuronic acid to a wide variety of substrates including bilirubin, steroids, and fat-soluble vitamins. UGTs also participate in the metabolism and activation of various foreign compounds such as drugs, morphine, and carcinogens (1, 2). Based on sequence similarities, UGT forms can be divided into two families, UGT1 and UGT2, consisting of drug-glucuronidating forms and steroid-glucuronidating forms,

respectively (3). cDNA clones encoding several isoforms of the UGT1 family were isolated from rat (4–6) and human (7–9). Their 3'-halves are all identical in nucleotide sequence. Recently, genomic clones encoding UGT1 were isolated from human (10, 11) and rat (12). Characterization of these clones demonstrated that the *UGT1* locus consists of multiple first exons encoding isoform-specific sequences that are located at intervals of 10–15 kb apart and followed by a single set of commonly used exons (exons II, III, IV, and V) encoding the sequence that is identical in all UGT1 forms. Each UGT1 is made from the complex gene by the splicing of a unique first exon to the commonly used exons. We investigated the mRNA expression of nine isoforms identified in the rat *UGT1* locus and showed drug-responsive and tissue-specific alternative utilization of the multiple first exons (12). UGT1B1² was a major bilirubin-glucuronidating form in livers of untreated rats, and its expression was stimulated by administrations of clofibrate and dexamethasone to rats. UGT1A1 is identified as a 4-nitrophenol-glucuronidating form and is induced in the livers of 3-methylcholanthrene (MC)-treated rats. An additional MC-inducible form, UGT1A2, was identified in rat liver for the first time by our RT-PCR study.

Polychlorinated aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and polycyclic aromatic hydrocarbons such as MC are effective transcriptional inducers of several genes including cytochromes P-450 (CYP1A1 and CYP1A2) (13, 14), NAD(P)H:quinone oxidoreductase (15, 16), glutathione *S*-transferase Ya subunit (17), and UGT1s (UGT1A1 and UGT1A2) (12). These inducers bind to aromatic hydrocarbon (Ah) receptors in the cytoplasm, and the Ah receptor-ligand complex translocates into the nucleus, depending upon the presence of the Ah receptor nuclear translocator. The nuclear-localized Ah receptor-ligand complex interacts with a specific *cis*-acting DNA sequence, termed the xenobiotic responsive element (XRE), and stimulates transcription of these genes (18, 19).

The Ah receptor-mediated transcriptional activation has

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) D63585.

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¹ The abbreviations used are: UGT, UDP-glucuronosyltransferase; Ah, aromatic hydrocarbon; CAT, chloramphenicol acetyltransferase; CYP, cytochrome P-450; kb, kilobase pairs; MC, 3-methylcholanthrene; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic responsive element; RT-PCR, reverse transcriptase-polymerase chain reaction; BTE, basic transcription element.

² A recommended nomenclature system proposed by Burchell *et al.* (3) is a convenient classification for the UDP-glucuronosyltransferase super gene family. According to this nomenclature, the UGT1 family is composed of drug-glucuronidating forms, and the UGT2 family contains steroid-glucuronidating forms. Recently, several groups including us reported that the UGT1 gene is a single locus with multiple first exons followed by only one set of commonly used exons and that each UGT1 isoform was generated through alternative splicing (10–12). Because of ambiguities of the unified naming system for the UGT1 family, a different nomenclature system for the UGT1 family must be derived by consensus. Consequently, we tentatively divided UGT1 isoforms into a Phenol cluster (cluster A) and a bilirubin cluster (cluster B) according to their preferential substrates, that is "A" and "B" represent the initials of aromatic compounds and bilirubin, respectively (12). Numbering of each isoform indicates the exact locations of the first exons from the commonly used exons of the *UGT1* locus (see Fig. 1A). For example, UGT1B1 is made by an alternative combination of exon B1 with the commonly used exons.

been well documented in the CYP1A1 gene, which is a phase I drug-metabolizing enzyme. Transcriptional regulation of genes for phase II drug-metabolizing enzymes has received little attention until recently. In this paper, we investigated MC induction of UGT1A1 mRNAs in primary cultures of rat hepatocytes and assayed CAT enzyme expression in cultures transfected with CAT expression vectors containing the rat UGT1A1 promoter. Deletion constructs of the UGT1A1 promoter were prepared to identify *cis*-acting elements responsible for MC induction. Additional mutations were generated by site-directed mutagenesis to study functions of the XRE and flanking sequences of the UGT1A1 gene. We also show a specific interaction of the XRE with nuclear-localized Ah receptor-ligand complex by gel mobility shift assays and DNase I protection analyses.

MATERIALS AND METHODS

Plasmid Construction for CAT Assay—A 1.6-kb fragment containing a CAT gene and an SV40 terminator was excised from pSV00CAT (20) by *Hind*III plus *Bam*HI double digestion and ligated to pBlueScriptII SK(+) to generate a basal plasmid pBlueII/CAT. A genomic clone COS4-1 (12) was digested with *Hind*III, and a resultant 1.4-kb fragment containing the UGT1A1 promoter region was recloned into the *Hind*III site of pBlueII/CAT to make a fusion plasmid pA1-CAT. For preparation of 5'-progressive deletions, pA1-CAT was linearized with *Xho*I (5'-protruding) and *Kpn*I (3'-overhang). Deletions were carried out from the *Xho*I site using exonuclease III and mung bean nuclease digestion (TaKaRa, Kyoto, Japan) following the manufacturer's protocol. The resultant deleted plasmids were sequenced to determine their deletion end points. Seven unidirectional deletion plasmids at -1002, -855, -692, -606, -337, -94, and -8 from a transcription start site were selected and designated pA1-CAT/ Δ 1, pA1-CAT/ Δ 2, pA1-CAT/ Δ 3, pA1-CAT/ Δ 4, pA1-CAT/ Δ 5, pA1-CAT/ Δ 6, and pA1-CAT/ Δ 7, respectively. A point mutation and removal of specified sequences were accomplished using the SculptorTM *in vitro* mutagenesis system (Amersham, UK) as described in the instruction manual. Primers for site-directed mutagenesis were made using ABI model 394 DNA synthesizer. A single-stranded template for mutagenesis was prepared by infection of helper phage (M13KO7) into *Escherichia coli* TG1 harboring pA1-CAT/ Δ 5.

Transfection of Plasmids into Primary Cultures of Rat Hepatocytes and CAT Assay—Male Wistar rats weighing 180–210 g were used in all experiments and fed *ad libitum* with commercial rat chow. Isolated hepatocytes were prepared by the two-step collagenase perfusion method as described (21). Supercoiled plasmids were transfected into hepatocytes by electroporation using Cell-Porator (Life Technologies, Inc.) following the instruction manual with minor modifications. Viable freshly isolated hepatocytes (5×10^6 cells) were suspended in 0.8 ml of ice-cold phosphate-buffered saline and transferred into a 0.4-cm electroporation chamber. All manipulations for transfection were carried out at 4 °C. The cells were mixed with 200 μ g of sonicated salmon sperm DNA plus 20 μ g of plasmid and in cotransfection experiments with an additional 2 μ g of pmiwZ (22) as an internal standard for normalization of transfection efficiencies. After mixing DNAs, the cells were allowed to sit for 5 min at 4 °C and then exposed to a single pulse of 800 microfarads and 300 V. The transfected cells were allowed to sit for 5 min at 4 °C before being suspended in 8 ml of Williams' medium E supplemented with 5% bovine serum, 10^{-6} M dexamethasone, 10^{-6} M insulin, and kanamycin at 40 μ g/ml. 4-ml portions of the cell suspension were separately plated onto two culture dishes (6 cm in diameter) coated with type I collagen and incubated at 37 °C in 95% air, 5% CO₂. 4 h after plating, the culture medium was changed, and it was changed daily thereafter.

16 h after electroporation, the cells plated onto two separated culture dishes were treated with MC or 0.1% dimethyl sulfoxide (Me₂SO) for 48 h. Cells were scraped off with a rubber policeman, and cell extracts were prepared as described by Gorman *et al.* (23). CAT enzyme expression was assayed with ¹⁴C-labeled chloramphenicol (Amersham, UK) following the standard protocol (24). Acetylated products were separated using a silica gel TLC plate (Whatman, UK), and their radioactivities were measured using BAS2000 imaging analyzer (Fuji, Tokyo). The induction rate was calculated from the ratio of CAT activity of induced cells to that of uninduced cells. All measurements presented here are averages from three individual transfections of hepatocyte cultures prepared from three different rats. In some cotransfection experiments,

normalization of transfection efficiency was performed by measuring β -galactosidase activities in different cell extracts by a spectrophotometric assay using *o*-nitrophenyl- β -D-galactopyranoside as a substrate (22).

Preparation of Total RNA and Analysis of mRNA—The isolated hepatocytes (5×10^6 cells) were plated onto a 10-cm culture dish as described above. 4 h after plating, the culture medium was changed. After an additional incubation for 12 h, cells were treated with MC or 0.1% Me₂SO for 48 h. Total RNA from cultured hepatocytes was prepared as described (21). Expression of mRNA species for UGT1 isoforms was analyzed by RT-PCR as described (12). The transcription start site of UGT1A1 gene was determined by primer extension analysis as described (25). An oligonucleotide primer, 5'-ATCTGGAAGTCCGTTTTTCAGT-3', complementary to the sequence from +56 to +77 of the exon A1*, was synthesized and used.

Gel Mobility Shift Assay and Gel Shift-coupled DNase I Protection Analysis—Complementary synthetic oligonucleotides containing XRE sequences of rat UGT1A1 and rat CYP1A1 (13) were synthesized and purified by acrylamide gel electrophoresis. Their sequences are as follows: UGT-XRE, 5'-GAGAATGTGCGTGACAAGGTCTGG-3'/3'-CTCT-TACACGCACTGTTCCAGACC-5'; UGT-XRE40, 5'-TGTGGAATGTGAGAATGTGCGTGACAAGGTCTGG-3'/3'-ACACCTTACACTCTTACACGCACTGTTCCAGACC-5'; CYP-XRE1, 5'-GCTCTTCTCAGCAACTCCGG-3'/3'-CGAGAAGAGTGCCTTGAGGCC-5'. UGT-XRE and UGT-XRE40 oligonucleotides were end-labeled with [γ -³²P]ATP using T4 polynucleotide kinase, and unincorporated nucleotides were removed using Sephadex G-50 minicolumns. Single-stranded oligonucleotides were annealed at 75 °C for 10 min and allowed to cool gradually to room temperature.

Nuclear extracts were prepared from untreated and MC-treated hepatocyte cultures (7.5×10^7 cells) as described by Dignam *et al.* (26). Cells were treated with 5 μ M MC for 1 h prior to preparation. 10 μ g of nuclear extract was mixed with 2 μ g of poly(dI-dC) in a 24- μ l buffer containing 30 mM Hepes-KOH (pH 7.9), 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 14% glycerol. The reaction mixture was preincubated for 15 min at 4 °C and then was followed by adding the probe DNA (2×10^4 cpm, about 50 fmol). Incubation was continued for another 15 min at 4 °C. Competition reactions were carried out under the same conditions with addition of unlabeled oligonucleotides or DNA fragments generated by polymerase chain reaction. Protein-DNA complexes were resolved through a 4% polyacrylamide gel using $1 \times$ TAE buffer (7 mM Tris-HCl, 3.3 mM sodium acetate, 1 mM EDTA, pH 7.5). The gel was then dried and subjected to autoradiography with an intensifying screen at -80 °C overnight.

Gel shift-coupled DNase I protection analysis was performed as essentially described (27) using a double-stranded UGT-XRE40 probe. The binding reaction was carried out as above, after which 3 μ l of DNase I solution (80 mM MgCl₂, 0.35–0.7 units/ μ l DNase I; note that CaCl₂ was omitted from the reaction) was added. The partial cleavage reaction was conducted at 25 °C for 1 min and was stopped by the addition of 1 μ l of 0.5 M EDTA. The resulting mixture was immediately electrophoresed. The shifted DNA fragments were excised from the gel and eluted by soaking in a buffer containing 0.5 M ammonium acetate, 1 mM EDTA, 0.1% SDS, 10% methanol, and 50 μ g/ml proteinase K. The eluted DNAs were extracted with phenol/chloroform (1:1) and precipitated with ethanol. An equal amount of radioactive material was resolved in a sequencing gel containing 16% acrylamide and 7 M urea. The G+A chemical sequencing reaction (28) was carried out to identify the protected nucleotides.

RESULTS

Induction of UGT1A1 in Primary Cultures of Rat Hepatocytes—As indicated in Fig. 1A, all first exons except for that of UGT1A1 encode both 5'-untranslated regions and protein-coding regions. The UGT1A1-coding specific sequence is separated by an intervening sequence of about 4 kb. The exon A1* encodes a 5'-untranslated region and has not been identified in the human *UGT1* locus. To examine mRNA expression of each UGT1 isoform in cultured hepatocytes, total RNAs were prepared from untreated and MC-treated cells and analyzed by RT-PCR. Fig. 1B shows a dose-dependent induction of UGT1A1 by MC. UGT1A1 mRNA was scarcely detected in untreated cells while a significant induction was observed in cells treated with 1 μ M MC. When cells were treated with 5 μ M MC, expression of UGT1A1 was elevated more than 40-fold as compared

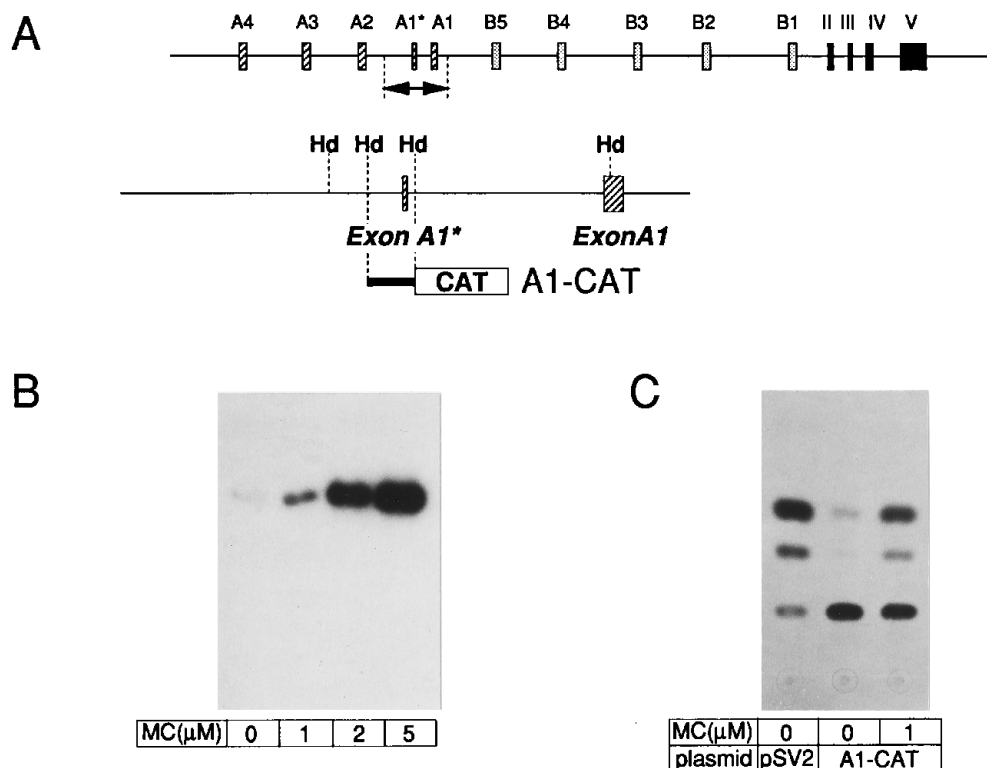


FIG. 1. Induction of *UGT1A1* in primary cultures of rat hepatocytes. **A**, structure of the rat *UGT1* gene and *UGT1A1*-CAT reporter gene. Structure of the rat *UGT1* locus is illustrated on the top line. The hatched and the stippled boxes indicate isoform-specific first exons. The closed boxes represent commonly used exons. Detailed locations of exon A1*, exon A1, and their flanking regions are shown on the middle line with *Hind*III restriction sites. Schematic illustration of the *UGT1A1*-CAT fusion gene is shown on the bottom line. **B**, dose-dependent induction of *UGT1A1* mRNA by MC. Total RNAs were prepared from hepatocyte cultures treated with various concentrations of MC for 48 h and analyzed by RT-PCR using *UGT1A1*-specific primers. **C**, activation of the *UGT1A1*-CAT fusion gene in MC-treated hepatocytes. pA1-CAT and pSV2CAT were transfected into rat hepatocytes by electroporation. Basal (without MC) and induced (with 1 μ M MC) CAT activities were monitored by TLC, and a representative autoradiogram is shown.

with untreated cells. *UGT1A2* mRNA was also induced in hepatocyte cultures treated with MC at 5 μ M (data not shown). *UGT1B1* is a major bilirubin-glucuronidating form in the liver of untreated rats (29). *UGT1B1* mRNA was also detected in cultured hepatocytes as well as in the liver (data not shown). These results suggest that expression of the *UGT1* gene in hepatocyte primary cultures is similar to that *in vivo* and that primary cultures are a good model to study *UGT1* gene expression.

To identify potential regulatory *cis*-elements that participate in the MC-triggered transcriptional activation of the *UGT1A1* gene, a DNA fragment containing its promoter region was excised from a COS4-1 genomic clone (12) and fused to the CAT reporter gene (Fig. 1A). A plasmid, pA1-CAT, containing the exon A1*, a 1.1-kb promoter region, and a 0.17-kb 3'-flanking sequence, was transiently transfected into freshly isolated rat hepatocytes by electroporation to assess whether this construct was sufficient for MC-mediated transcriptional activation in transfected cells. As shown in Fig. 1C, a basal level of CAT enzyme expression was observed in untreated hepatocyte cultures harboring pA1-CAT. When the cells were treated with 1 μ M MC, a 7.8-fold induction of CAT expression was observed, indicating that the pA1-CAT construct expressed CAT activities in response to MC-mediated transcriptional activation. In addition, treatment of the cells with 2 μ M MC showed approximately 11.2-fold induction as compared with untreated cells (data not shown). These results obtained from CAT assays are in good agreement with the mRNA expression shown in Fig. 1B.

Identification of a *cis*-Acting Element Required for Induction of the *UGT1A1* Gene by MC—To identify the *cis*-acting ele-

ments in pA1-CAT necessary for MC induction, the 1.1-kb 5'-flanking region of the *UGT1A1* gene was deleted progressively in the 5' to 3' direction in the pA1-CAT plasmid. These deleted constructs were transfected into cultured hepatocytes, and transient expression of CAT enzyme driven by the deleted sequences was determined in the absence or presence of 1 μ M MC (Fig. 2). Roughly equivalent fold induction of CAT activities by MC was observed with a series of unidirectional deleted constructs containing 1078, 1002, 855, 692, 606, and 337 nucleotides of the *UGT1A1* 5'-flanking region. In contrast, a further deletion to 94 nucleotides (pA1-CAT/ Δ 6) failed to respond to MC, identifying a region that contains important MC-responsive sequences. pA1-CAT/ Δ 7, which contains eight nucleotides from the transcription start site but lacks a TATA box, showed only background CAT activity in MC-treated cells. These observations established that one or more XREs reside in the proximal 244 nucleotides (between -337 and -94) of the 5'-flanking region and participate in the induction of the *UGT1A1* gene by MC.

The DNA sequence of the 1.1-kb 5'-flanking region was analyzed, and the proximal 400 nucleotides are shown in Fig. 3A. A putative TATA box, TATTA, is present at -29 nucleotides upstream from the transcription start site preceding exon A1*. Just in front of the TATA box, there is a GC-rich region at position -51 to -38. Similar GC-rich sequence has been identified in several genes including *CYP1A1* and has been designated BTE for basic transcription element (30). A characteristic repetitive sequence showing four AATGTG motifs was found between -161 and -133. A C/EBP-recognition site, CCAAT-box (31), was present at position -163 to -159. A consensus XRE core sequence, GCGTG, was found between -133 and

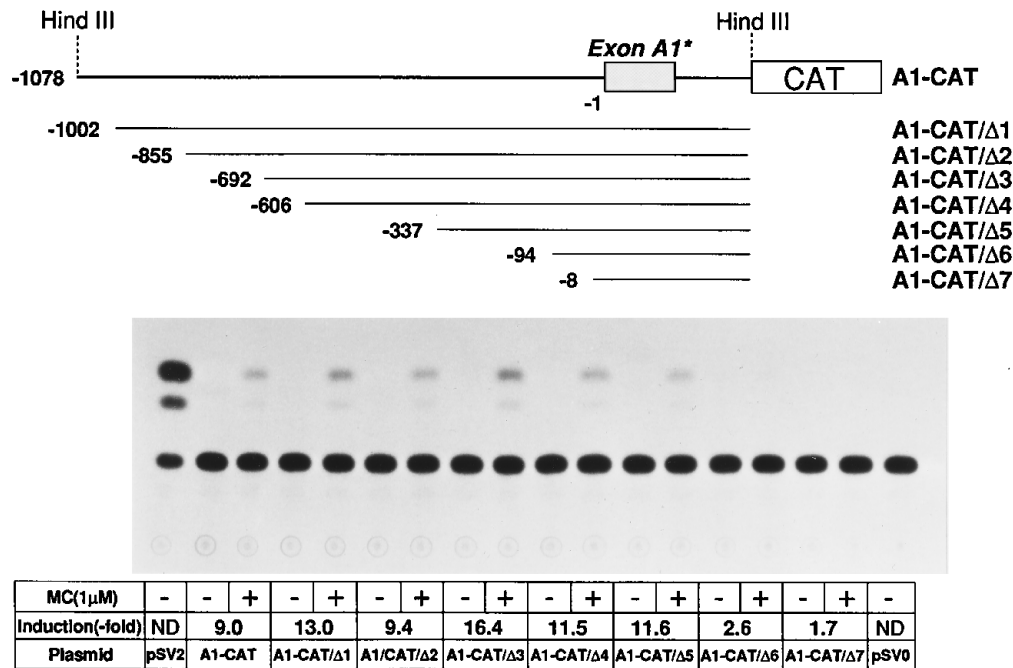


FIG. 2. Structure of successive 5'-deletion mutants and their respective CAT activities. Illustration shows the pA1-CAT fusion construct and several 5'-deletions. Numbers given to the left sides of each plasmid indicate deletion end points from the transcription start site of the UGT1A1 gene. These plasmids were used in transient transfection studies. All assays were done in triplicate. Basal (without MC) and induced (with 1 μM MC) CAT activities were monitored by TLC and a representative autoradiogram is shown. The -fold induction was calculated from the ratio of CAT activity of induced cells to uninduced cells. pSV2CAT and pSV0CAT were used as positive and negative controls, respectively.

A

CAATATAAACTCACAAGAATCCAAATAAAACATAAGGAAACACTCCTTACCCCCCATGATGCCTCACCCAGCTTGGGCTGAGTGGAGTCTTTGTCA -301
 GCCTTAACAAAGAACTTTCAGCTTGGCTCGTGTTCCTGGGGCCAGGTTCCTTCAATCCTGCTTTCCTGAGCCCCAGTCCACTA -201
 GGGTGCCCAAGGTTAATGCAAGCGCTGGCATGCTGGTTCCTTCCAAATGTGGAATGTGAGAATGTGCGTGACAAGGCTCGGGTGAGCCAGTCTTTTGA -101
 TGCAAGAACAACACTCTCTCCCTCAGCTGCCTGATTTCCACATCCATCACATCCCCCGCCCTTCAGCAGCTATTATTAACAACAGAACAAATGTAACCCGC -1
 CCTCTTTGGTGGTGGTTAGGAAACCCGGAATTCAGATGGTGGCTGAGGGTGGCTGACTGAAAAACGGACTTCCAGATTCTTACTCTTCCACAAGGGCCC
 Exon A1*
 TCATCACGTGCGCAGGTCTCTAACCTTACTCTCTGAAAGGCAA gtaaaaaaattatgtgtgtgagagagcaatacagaatttgaaagataggatctt
 ttgtctcccatccagagcttcacaaatgtctgtggttttatgagggcgggagggaacactgtactggttccttatttgcaatgggtgcttttacttt
 ttttttttttaagctt

B

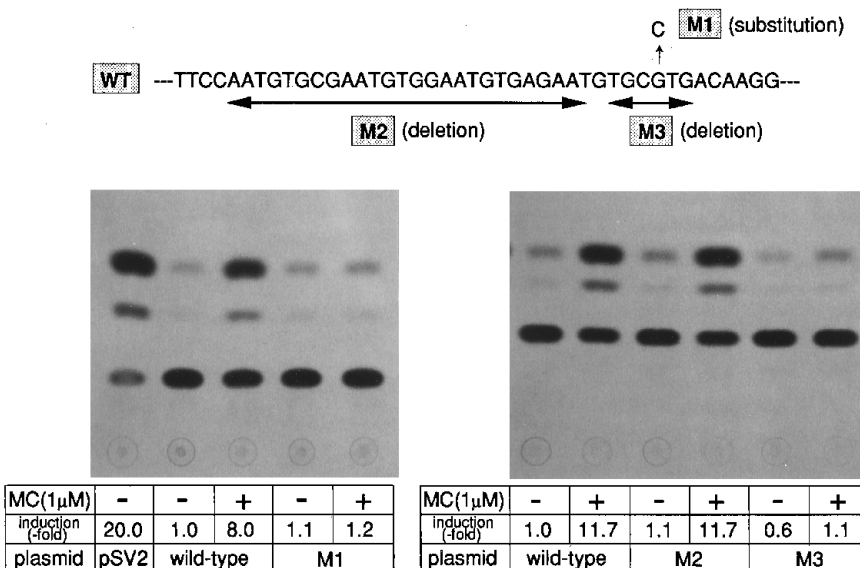
rat UGT1A1	GTGAGAATGTGCGTGACAAGGTCTGGG
human CYP1A2 (X1)	CTAAGGTAGTGCGTGTTCAGGTCTCTTC
rat CYP1A1 (XRE-1)	CCCCGGAGTTGCGTGAGAAGAGCCTGG
rat CYP1A1 (XRE-2)	GGGGATCCTAGCGTGACAGCACTGGGA
rat GST Ya subunit	CAGGCATGTTGCGTGATCCCTGAGGC
rat QR	CCTTCCCCTTGCGTGCAAAGGCGATTT

FIG. 3. Nucleotide sequence of the UGT1A1 gene and comparison of XREs identified in several genes. A, the nucleotide sequences of hepatocyte nuclear factor-4, CCAAT, XRE, GC-rich region (BTE), and TATA box are enclosed in boxes. Four AATGTG motifs are indicated by arrows. The sequence underlined with a straight line indicates exon A1*, and the first C residue is the transcription start site determined by the primer extension analysis (see "Materials and Methods"). The lowercase script shows part of the 3'-flanking region. The sequences underlined with double broken lines represent primer sequences used in PCR. B, XREs and their flanking regions of the rat UGT1A1 (this report), human CYP1A2 (14), rat CYP1A1 (13), rat glutathione S-transferase (GST) Ya subunit (17), and rat NAD(P)H:quinone oxidoreductase (QR) (15) genes are aligned. The consensus XRE core sequence is enclosed in a box.

-129 and compared with the XRE sequences identified in other MC-inducible genes (Fig. 3B). Detailed analyses of the XREs of the CYP1A1 gene revealed recognition motifs for the Ah receptor-ligand complex as 5'-(A/T)NGCGTG-3' (32). In the rat UGT1A1 gene, a variant sequence, TGCGTG, was identified. The same variant XRE was found in the human CYP1A2 gene (14).

To determine whether this variant XRE sequence participates in the transcriptional activation of the UGT1A1 gene by MC, we prepared mutated constructs by site-directed mutagenesis and compared them with a wild-type gene for their ability to respond to MC (Fig. 4). When a single base substitution was introduced to alter TGCGTG to TGCGTG (M1), MC induction of a CAT enzyme activity was completely abolished. Removal of

FIG. 4. Structure of mutants and their respective CAT activities. The nucleotide sequence of wild type (WT), one single base substitution (M1), and two deletions of the AATGTG repeat (M2) and the XRE (M3), respectively, are shown on the top. Constructs containing these mutations were transiently transfected into hepatocyte cultures, and CAT activity was measured in extracts prepared from untreated or MC-treated (1 μ M, 48 h) cells. The -fold induction is expressed as a relative value as compared with CAT activity obtained in untreated cells carrying the wild-type fusion gene. All assays were done in triplicate, and a representative autoradiogram is shown.



the TGGGTG motif also led to loss of the response to MC (construct M3). In contrast, mutant M2, which has the intact TGGGTG motif but lacks the AATGTG-repeated sequence, had the same inducible activity as the original fusion gene (wild type), suggesting that this characteristic repetitive motif does not influence the induction by MC. Therefore, these results confirm that the TGGGTG motif acts as an XRE and plays a pivotal role in the MC-mediated transcriptional activation of the UGT1A1 gene.

Interaction of the UGT-XRE with Liganded Ah Receptor—The presence of XRE in the MC-inducible UGT1A1 gene suggested that the TGGGTG motif may serve as a target site for the Ah receptor. Gel mobility shift assays were carried out by using a double-stranded synthetic oligonucleotide as a probe to assess whether the nuclear-localized Ah receptor-ligand complex interacted with the XRE of the UGT1A1 gene (Fig. 5A). When a nuclear extract prepared from MC-treated hepatocyte cultures was incubated with a 32 P-labeled UGT-XRE probe, one prominent DNA-protein complex was observed. In contrast, an extract from uninduced cells gave faint shifted bands. The amount of MC-inducible shifted band was completely diminished by adding excess amounts of unlabeled UGT-XRE oligonucleotides, indicating the specificity of this protein-DNA interaction. In addition, a 10-fold molar excess of unlabeled rat CYP-XRE1 (13) competed effectively for the binding, and a 100-fold molar excess completely abolished the shifted band, strongly suggesting the presence of the Ah receptor-ligand complex.

Further competition experiments were carried out to investigate functional effects of mutations described above, and the results are shown in Fig. 5B. Competitor DNA fragments were generated by polymerase chain reaction using UGT1A1 promoter-specific primers (their sequences are indicated in Fig. 3A) and plasmid templates (designated WT, M1, M2, and M3; shown in Fig. 4). When WT and M2, which contained the intact XRE sequence, were used as competitors for binding of the liganded Ah receptor to the UGT-XRE, the shifted band was completely eliminated. Competitor M1, with a single base substitution in the XRE, did not inhibit the shifted band. Competitor M3 lacking the XRE sequence also failed to compete with the labeled UGT-XRE probe. The loss of the capacities of these mutated XREs to bind to the liganded Ah receptor shown in the gel mobility shift assays (Fig. 5B) coincided with their failure to respond to the MC-triggered transcriptional activation observed in the CAT assays (Fig. 4).

To determine the contact site of protein binding in more detail, we performed DNase I protection analyses using UGT-XRE40 oligonucleotides (Fig. 6A). As obvious footprinting patterns were not obtained by usual methods (lanes 5 and 6), the gel shift-coupled DNase I protection analysis was performed. This is a modified protocol for footprinting and has the advantage of showing weak or partial interactions between nucleotides and proteins (27). The labeled DNA was incubated with a nuclear extract prepared from MC-treated hepatocytes and digested partially with DNase I, after which the digest was subjected to preparative electrophoresis as described in the gel shift assays. The partially cleaved DNA fragments associated with proteins were recovered from the shifted band and analyzed by electrophoresis through a sequencing gel (lanes 3 and 4). When 20,000 cpm of the labeled oligonucleotides were used, approximately 400 cpm of radioactive materials were recovered. Because of poor recovery of small-sized DNA fragments from the shifted band, their corresponding bands on the autoradiogram were faint or disappeared. Therefore, the actual protection site was determined from overlapping the footprint regions of the upper strand and the lower strand. As indicated in Fig. 6B, the GCGTG core motif was found to be protected from DNase I digestion. This core motif was not protected in the absence of nuclear proteins (Fig. 6A, lanes 1 and 2). These results are in good agreement with previous reports showing that methylation of guanine residues of the core sequence eliminated the interaction between the XRE of CYP1A1 and the Ah receptor-ligand complex (33, 34).

DISCUSSION

The levels of CYP1A1 and UGT1A1 mRNAs were markedly elevated in the liver of rats treated with MC or TCDD. These two enzymes are localized in the endoplasmic reticulum (4) and play important roles in phase I and phase II reactions of a hepatic drug-metabolizing system, respectively. Simultaneous transcriptional activation of the two genes by MC or TCDD would be very advantageous when the metabolism of specific chemicals occurs by the sequential action of phase I and phase II reactions. Genetic evidence obtained from detailed studies using inbred strains of mice suggested that co-induction of CYP1A1 and UGT1 isoenzymes is regulated by a single genetic locus designated the *Ah* locus (35). The molecular basis of the induction of the CYP1A1 gene has been investigated in detail, while there is limited information on regulation of the UGT1A1 gene. Recently, Lamb *et al.* (36) demonstrated that induction of

FIG. 5. Gel mobility shift assays. A, nuclear-localized Ah receptor-ligand complex interacts with the UGT-XRE. ³²P-labeled UGT-XRE probe was incubated with nuclear extracts prepared from untreated and MC-treated (5 μ M, 1 h) hepatocyte cultures. Unlabeled UGT-XRE and CYP-XRE1 are used as competitors in 10- and 100-fold molar excess over the probe DNA. The shifted band is indicated with an arrow head. B, mutations introduced into UGT-XRE fail to interact with liganded Ah receptor. Gel mobility shift assays were done as described above. Schematic illustration of wild-type (WT) and mutated (M1, M2, and M3) competitors are shown on the top. These competitor DNAs were generated by PCR and used in 10-fold and 100-fold molar excess over the probe DNA. Their nucleotide sequences are presented in Figs. 3 and 4. The shifted band is indicated with an arrow head.

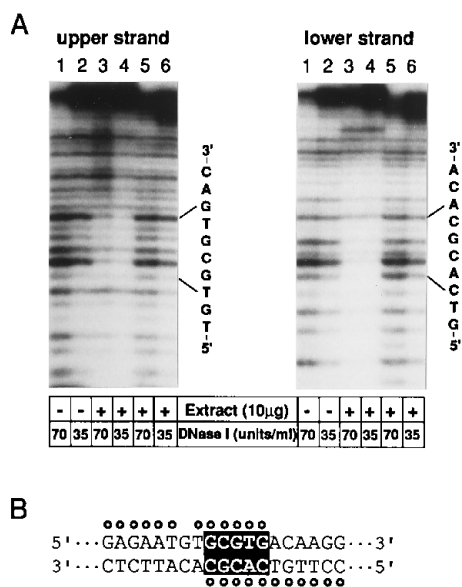
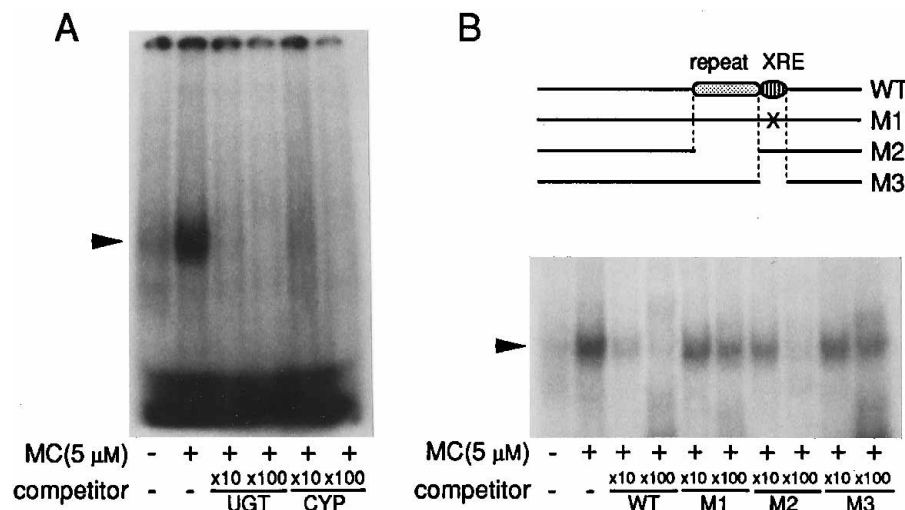


FIG. 6. Gel shift-coupled DNase I protection analysis. A, autoradiogram of DNase I protection analysis. UGT-XRE40 oligonucleotides were labeled at either 5'-end and annealed. The labeled probe was incubated with the crude nuclear extracts and subjected to partial digestion with the indicated amounts of DNase I. Immediately after digestion, the DNA-protein complex was separated by gel electrophoresis, purified from the shifted band, and analyzed by a sequencing gel (lanes 3 and 4). Samples on lanes 1, 2, 5, and 6 were prepared according to a usual method for DNase I footprinting. B, summary of the interactions between the UGT-XRE40 probe and the liganded Ah receptor. Nucleotides corresponding to the bands that disappear on the autoradiogram shown in panel A are denoted by open circles. The actual protein binding site (enclosed in a box) was determined by overlapping the results obtained with the upper strand and the lower strand.

mRNA encoding mouse Ugt1.6 (corresponds to rat UGT1A1) was dependent upon the nuclear-localized Ah receptor complex using wild-type mouse hepatoma cells and class II variant cells defective in translocation of the liganded Ah receptor into the nucleus (36). As hepatocyte primary cultures are suitable for investigations of liver-specific functions in contrast to other hepatoma cell lines such as HepG2, we decided to perform transient transfection studies using this culture system to precisely demonstrate the presence of an XRE in the promoter region of the UGT1A1 gene and the specific binding of the liganded Ah receptor to this element.

The nucleotide sequence of the UGT1A1 promoter region predicted that other regulatory elements participate in regulation of tissue-specific, basal, and inducible expression (Fig. 3A).

Two possible target sites of hepatocyte nuclear factor-4 were identified from -251 to -236 and from -314 to -302 based on their sequence similarities to consensus recognition sequences of hepatocyte nuclear factor-4 found in several liver-specific genes (37). Another regulatory element, designated BTE, was found as a GC-rich region just in front of the TATA box. BTE was identified in the CYP1A1 gene from its ability to produce a synergistic effect on XRE-mediated transcriptional enhancement (30). Constitutive expression of the CYP1A1 gene with a GC box is suppressed by BTE-binding protein but activated by Sp1 (38). These positive and negative regulatory elements may affect the expression of the UGT1A1 gene and their participation in the transcriptional regulation remain to be resolved.

The mechanisms regulating the production of UGT1 mRNA by alternative usage of multiple promoters and first exons are complex (12). UGT1A1 and UGT1A2 are induced by MC. UGT1B1 is a constitutively expressed form, and its transcription is stimulated by administration of dexamethasone or clofibrate to rats. These accumulative data suggest that the isoform-specific promoters exist in the 5'-flanking regions of each first exon. Our present results clearly demonstrate that the XRE of the UGT1A1 promoter is involved in MC induction of this gene. From sequence analysis of the UGT1A2 promoter, a plausible XRE sequence represented by ATGCGTG was identified.³ It will be of interest to determine whether the UGT1A2 gene can also be activated by the liganded Ah receptor. We are presently attempting to analyze the promoter region that is required for MC induction of the UGT1A2 gene. Further research is needed to define the mechanisms that regulate alternative usage of multiple first exons in the UGT1 gene complex.

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