Identification and Functional Characterization of a Conserved, Nuclear Factor 1-like Element in the Proximal Promoter Region of CYP1A2 Gene Specifically Expressed in the Liver and Olfactory Mucosa

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CYP1A2 is a major cytochrome P-450 isoform in the liver and the olfactory mucosa but is essentially not expressed in other tissues. A nuclear factor 1 (NF-1)-like element was identified in the proximal promoter region of rat, mouse, rabbit, and human CYP1A2 genes through data base analysis. In vitro DNase I footprinting with a −211 to +81 probe from the rat CYP1A2 gene and nuclear extracts from rat liver and olfactory mucosa revealed a single protected region corresponding to the NF-1-like element at −129 to −111. Protein binding to this NF-1-like element was tissue-selective and was confirmed by in vivo footprinting in native chromatin from rat liver. Multiple DNA-binding complexes were detected in gel-shift assays using the CYP1A2 NF-1-like element and nuclear extracts from liver and olfactory mucosa, all of which were supershifted in the presence of an anti-NF1 antibody. The NF-1-like element was essential for transcriptional activity of the CYP1A2 gene in an in vitro transcription assay using nuclear extracts from the two tissues. Thus, members of the NF-1 family of transcription factors may play an important role in the tissue-selective expression of the CYP1A2 gene in the liver and olfactory mucosa.

Tissue-selective gene expression, which is a common feature for most mammalian members of the CYP1 gene superfamily, leads to important organ-selective functions and plays a critical role in tissue-selective toxicity of drugs and other xenobiotic compounds. Each tissue expresses a subset of xenobiotic-metabolizing P450 genes which determines, to a large extent, sensitivity of that organ to the toxicity of a given chemical compound. Variation in the levels of expression of P450 genes is one of the major contributing factors in interindividual and interspecies differences in susceptibility to environmental toxicants. Changes in P450 expression may result from alterations in those regulatory mechanisms, such as the transcriptional regulatory elements and the protein factors, which are potential targets for genetic polymorphism and cytotoxic events. However, despite recent progress in our understanding of xenobiotic-induced CYP gene expression, very limited advance has been made in identifying the elements and factors involved in the constitutive tissue- or cell-type-selective expression of xenobiotic-metabolizing P450s.

CYP1A2 is constitutively expressed preferentially in the liver (1) and the olfactory mucosa (2, 3) in mammals. In the liver, CYP1A2 is inducible by a number of xenobiotic compounds through AhR-mediated pathways (4–8) as well as by other mechanisms (9–12), but significant induction of CYP1A2 has not been found in the olfactory mucosa (13–15). CYP1A2 metabolizes several endogenous substances such as retinoic (16), arachidonic acid (17), and the sex steroids (18, 19) as well as numerous xenobiotic compounds, including many environmental procarcinogens and therapeutic agents, such as polycyclic aromatic hydrocarbons, heterocyclic amines, arylamines, caffeine, acetalenaphen, and aflatoxin B1 (20).

Tissue-specific gene expression may be regulated by cell-type-specific as well as ubiquitous transcription factors (21). Previous studies on the liver-selective expression of P450 genes in the CYP2 family suggested that each P450 gene may be controlled by unique regulatory mechanisms (22). Little is known of the mechanisms involved in tissue-selective, constitutive expression of the CYP1A2 gene. An early study found that a 1.8-kilobase pair mouse Cyp1a2 5’-flanking sequence (−1843 to +52) was insufficient for constitutive expression of a reporter gene in mouse hepatoma Hepa-1 cells (23). However, a recent study (6) identified two regions in the 5’-flanking sequence of the human CYP1A2 gene that were required for induction by 3-methylcholanthrene in transiently transfected human hepatoma cell line HepG2. One (−2532 to −2423) binds the AhR and the other (−2259 to −1987), which was subsequently shown to function as an enhancer (12), contains an AhR-binding site as well as two conserved AP-1 sites and a TATA box. In a similar study (24), a proximal 42-bp (−72 to −31) DNA and a distal 259-bp (−2352 to −2094) DNA of the human CYP1A2 gene were found to be important for the constitutive expression of a reporter gene construct in transiently transfected human hepatoma cell line HepG2. The distal sequence contained three protein-binding sites, including an AP-1 site and a site for the liver specific transcription factor HNF-1 (25). The proximal sequence contains GC, CCAAT, and TATA boxes, but protein binding to these potential sites was not demonstrated (24). Interestingly, recent studies with the AhR−/− mice indicated that targeted disruption of the AhR gene led to decreased consti-
tutive expression of CYP1A2 in mouse liver (7, 8), although it is not yet clear whether the partial dependence of constitutive CYP1A2 expression on the AhR resulted from direct or indirect mechanisms and whether it is unique to the liver.

Recently, we have identified a conserved NF-1-like binding site (named the NPTA element) potentially involved in transcriptional activation and tissue-selective expression of rat CYP2A3, mouse Cyp2a5, and human CYP2A6 genes in the olfactory mucosa (26). The NPTA element interacts with unique proteins detected only in the olfactory mucosa. Interestingly, a survey of available CYP gene 5'-flanking sequences revealed that an NF-1-like sequence was also present in the proximal promoter region of rat, mouse, rabbit, and human CYP1A2 genes. Because CYP1A2 is also expressed selectively in the olfactory mucosa, in addition to the liver, the present study was conducted to determine whether this NF-1-like sequence is also important for its transcriptional activation and tissue-selective expression. Thus, the proximal promoter region of rat CYP1A2 gene was analyzed using a number of approaches to demonstrate tissue-specific binding of nuclear proteins to this NF-1-like sequence and consequent transcriptional activation. Furthermore, olfactory mucosal and hepatic proteins bound to this NF-1-like sequence in the CYP1A2 gene were compared with the olfactory mucosal NPTA-binding proteins to determine whether the two genes share common regulatory pathways.

**EXPERIMENTAL PROCEDURES**

**Preparation of Intact Nuclei and DNase I Treatment for in Vivo Footprinting**—Intact nuclei were isolated from liver of 2-month-old rats essentially as described elsewhere (26–28) with modifications. Fresh tissues were dissected and immediately immersed in 100 volumes of cold 0.9% NaCl solution for 1 min. The tissues were minced into small pieces with a razor blade, washed sequentially in 10 volumes of an ice-cold washing buffer (10 mM Tris-HCl buffer, pH 7.7, containing 150 mM NaCl and 15 mM sodium citrate) and 5 volumes of a homogenization buffer (10 mM Tris-HCl buffer, pH 7.7, containing 10 mM NaCl, 0.1 mM EDTA, 0.5 mM spermine, 0.15 mM spermine, 0.5% Tergitol NP-10, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) and homogenized on ice in 3 volumes of the homogenization buffer, applied to a 10-ml cushion of 2 M sucrose in homogenization buffer and spun at 24,000 rpm for 60 min at 2 °C in a prechilled SW28 rotor. The resultant nuclear pellet was resuspended in 10 volumes of a DNase I digestion buffer (15 mM Tris-HCl buffer, pH 7.7, containing 0.5 mM spermine, 0.15 mM spermine, 80 mM KCl, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 5% glycerol, 5 mM MgCl2, and 5 mM CaCl2) and spun again at 3000 rpm for 5 min in an Eppendorf microcentrifuge. The purified nuclei were resuspended in the DNase I digestion buffer at about 5 absorbance unit (at 260 nm/ml) and treated with 4–30 unit/ml DNase I (Promega) on ice for 5 min. The reaction was terminated by incubating with 1/5 volume of a DNase I stop buffer (30 mM Tris-HCl, pH 7.7, containing 200 mM NaCl, 30 mM EDTA, 1.2% SDS, and 1 mg/ml protease K) at 50 °C for 50 min. The samples were then treated with DNase-free RNase A (40 μg/ml) at 42 °C for 1 h and extracted once with an equal volume of phenol/chloroform/picric acid (25:24:1). The isolated DNA was further digested with HindIII (which does not cut in the CYP1A2 proximal promoter region) to reduce viscosity, purified again by extraction, and used as templates in ligation-mediated PCR as described below.

**Ligation-mediated PCR for in Vivo Footprinting**—Ligation-mediated PCR was performed essentially as described by Mueller and Wold (29), with some modifications. A nested set of three primers specific to the rat CYP1A2 gene were used for amplifying the antisense strand (Primers 1–3, see Fig. 2C), with the same linker primers and ligation adaptors as described previously (29). First strand DNA synthesis with primer 1 and denatured genomic DNA from DNase I-treated nuclei, PCR amplification with primer 2 and the linker primer, and labeling reaction with 32P-labeled primer 3 were all performed with use of Vent DNA polymerase (New England Biolabs). The PCR products were extracted, and the footprints were analyzed by electrophoresis through a 6% polyacrylamide, 7 μM urea DNA sequencing gel as described (26).

DNA sequence G ladder was produced by ligation-mediated PCR (29) of purified rat liver genomic DNA that had been treated with 1% dimethyl sulfate and pipidine according to Maxam and Gilbert (30). The footprint of the DNA sample was then analyzed by gel electrophoresis in 20% polyacrylamide gel, using a 1.5 mM stock solution of the dye (20 μg) in 200 μl of a digestion buffer (10 mM Tris-HCl, pH 7.7, containing 10 mM MgCl2, 5 mM CaCl2, and 1 mM dithiothreitol) to be treated with 1–3 × 10–4 units of DNase I at room temperature for 5 min. The reaction was stopped by mixing with an equal volume of a stop solution (200 mM NaCl, 30 mM EDTA, 1% SDS, and 100 μg/ml RNA). The DNA sample was purified by extraction prior to ligation-mediated PCR.

**In Vivo Transcription Assays**—Procedures for in vitro transcription have been described recently (26), as modified by Ueno and Gonzalez (31) and Tamura et al. (32). Plasmids used as templates in in vitro transcription assays were constructed in pCR-Script vector (Stratagene). Plasmid pLIA2–412 was constructed by inserting a −412 to +293 fragment of the rat CYP1A2 gene at the SrFI site of the vector; this fragment was obtained by double digestion of the plasmid with the enzymes XbaI and XhoI. Plasmid pLIA2–412/M was made by site-directed mutation of pLIA2–412 to convert a “CA” dinucleotide (−115 and −114) to an “AG” using the Transformer Site-directed Mutagenesis Kit from CLONTECH. The mutagenesis experiment was performed as described previously (31). With use of the two primer nucleotide primers: one containing the intended sites of mutation in the CYP1A2 promoter (5′-gggatctgatgaagaagagtc3′) and the other containing a mutation in the unique EcoRI site in the vector (5′-ttgatatcgaagtcctgcagcc3′). A control plasmid, pLIA3–254 (26), was used in experiments with olfactory mucosa, and another control plasmid, pLTR-3000/2A6, containing a full-length CYP2A6 cDNA driven by a 3-kilobase pair TTR promoter fragment (34), was used with use of the TTR promoter with human hepatic RNA and the TTR promoter fragment (3000 to +20, lacking the AGT codon) was derived as a SulI/HindIII fragment from a pTTR-CAT plasmid obtained from Dr. Robert H. Costa of the University of Illinois at Chicago. The structures of all promoter constructs were verified by sequencing. The oligonucleotide probes used for detecting CYP1A2, CYP2A3, and CYP2A6 transcripts in S1 nuclease protection experiments were complementary to one unit end-labeled primer of the complementary strand and primers (sense, 5′-ccctatctgctgttgggaa3′; antisense, 5′-ggtgtggtgctgtcagcagc3′) in 200 μl of a digestion buffer (10 mM Tris-HCl, pH 7.7, containing 10 mM MgCl2, 5 mM CaCl2, and 1 mM dithiothreitol) to be treated with 1–3 × 10–4 units of DNase I at room temperature for 5 min. The reaction was stopped by mixing with an equal volume of a stop solution (200 mM NaCl, 30 mM EDTA, 1% SDS, and 100 μg/ml RNA). The DNA sample was purified by extraction prior to ligation-mediated PCR.

**Preparation of Oligonucleotide and cDNA Probes**—All oligonucleotides were synthesized by the Molecular Genetics Core of the Wadsworth Center. Detailed procedures for the construction of 32P-labeled double-stranded oligonucleotide probes for gel-shift assays, [32P]ATP end-labeled PCR primers, and single-stranded DNA probes for S1 nuclease digestion were described previously (26). 32P-labeled, double-stranded cDNA probes for DNase I footprinting experiments were prepared by PCR with one 5′-end-labeled primer (coding strand, 5′-gtatctgatgaagaagagtc3′; or noncoding strand, 5′-cggatctgatgaagaagatgc3′) and a control probe (1A2pm). DNA sequence G ladder was produced by ligation-mediated PCR (29) of purified rat liver genomic DNA that had been treated with 1% dimethyl sulfate and pipidine according to Maxam and Gilbert (30). The footprint of the DNA sample was then analyzed by gel electrophoresis in 20% polyacrylamide gel, using a 1.5 mM stock solution of the dye (20 μg) in 200 μl of a digestion buffer (10 mM Tris-HCl, pH 7.7, containing 10 mM MgCl2, 5 mM CaCl2, and 1 mM dithiothreitol) to be treated with 1–3 × 10–4 units of DNase I at room temperature for 5 min. The reaction was stopped by mixing with an equal volume of a stop solution (200 mM NaCl, 30 mM EDTA, 1% SDS, and 100 μg/ml RNA). The DNA sample was purified by extraction prior to ligation-mediated PCR.
Fig. 1. NF-1-like elements in the proximal promoter region of the CYP1A2 genes and comparisons with the NPTA element in the CYP2A3 gene. The promoter region sequences were from the following references: adenovirus type 2 (67), rat CYP1A2 (33), mouse Cyp1a2 (37), rabbit CYP1A2 (38), human CYP1A2 (39), and CYP2A3 (68). Numbers in parentheses indicate nucleotide positions relative to the transcriptional start site. Nucleotides critical for protein binding to the NPTA element in the CYP2A3 gene are underlined. The boxed nucleotide in human CYP1A2 sequence was absent in another report (1).

Approximately 1.2 kilobase pairs of rat CYP1A2 promoter and 5′-flanking sequences have been reported previously (33). A single NF-1-like element, located at -129 to -111, was found by scanning the TRANSFAC data base (36) using the TFSEARCH program, with the threshold set at 85.0 (Fig. 1). Similar sequences were also found in the proximal promoter region of mouse Cyp1a2, at -150 to -112 (37), rabbit CYP1A2, at -122 to -104 (38), and human CYP1A2, at -129 to -111 (39). In all four genes, the NF-1-like element is located about 80 bp upstream of a conserved TATA box. However, the NF-1-like element is not present in the human CYP1A2 proximal promoter sequence reported by another group (1), as a result of a single nucleotide deletion (Fig. 1). Nevertheless, sequence analysis of human CYP1A2 proximal promoter region with genomic DNA derived from 100 individuals (50 each Caucasian and African American) did not detect the variant allele at the NF-1-like element (data not shown), suggesting that it is rare. A comparison of the CYP1A2 NF-1-like element with the NPTA element in the CYP2A3 gene revealed significant homology at sites critical for NPTA binding (26).

The presence of a highly conserved NF-1 site in the proximal promoter region of rat, mouse, and rabbit CYP1A2 genes suggests potential functional importance in gene regulation, because the three orthologous genes are all known to be expressed tissue selectively and abundantly in the liver and olfactory mucosa. To determine whether this NF-1-like element interacts with DNA-binding proteins, in vitro DNase I footprinting analysis was performed using a -211 to +81 probe from the rat CYP1A2 gene with nuclear extracts from different rat tissues. As shown in Fig. 2A, a single, unambiguous DNase I footprint (−109 to −131) was identified on the noncoding strand with nuclear extracts from rat liver and olfactory mucosa, but not with those from testis and kidney. However, a partial protection of the same site was observed with extracts from the lung. Similar results were observed with a probe labeled on the coding strand, with the protected region at −129 to −107 (Fig. 2B). The specificity of protein binding to this site was confirmed by using an unlabeled double-stranded oligonucleotide, 1A2pm (corresponding to −133 to −107, Fig. 2C), which comprises the protected sequence, and two other oligonucleotides, 1A2pmmut1 and 1A2pmmut2 (Fig. 2D), which contain dinucleotide mutations in the critical regions for NF-1 (40, 41) and NPTA binding (26). As shown in Fig. 2, A and B, the footprint was completely abolished when 1A2pm was added at 80× excess but was only partially removed or not affected by the addition of 1A2pmmut1 and 1A2pmmut2, respectively.

Binding of nuclear proteins to the NF-1-like element in the rat CYP1A2 gene was confirmed by in vivo DNase I footprinting experiments. The sequence of the proximal promoter region (33) and the location of the three primers used for ligation-mediated PCR are shown in Fig. 2C. In contrast to a single protected region (−129 to −107) detected in in vitro experiments, several footprints were revealed by in vivo footprinting (Fig. 3) on the noncoding strand in the native chromatin of rat liver, as compared with the DNase I digestion pattern of protein-free, purified rat genomic DNA. The footprints shown were reproducible, even with different DNase I concentrations. The most prominent footprint (−95 to −131 bp) contained the NF-1-like sequence, but the boundaries extended beyond those detected in in vitro footprinting. Additional footprints were detected both upstream and downstream of the NF-1-like element, including one (−21 to −34 bp) corresponding to a TATA box (42) and a weaker one (−87 to −65) containing sequences similar to GATA-1- or GATA-2-binding sites (43). Highly conserved binding sites for the Nkx-2.5 factor (44) and the AP-4 protein (45) are also present in this region, as shown in Fig. 2C, but corresponding footprints were not found. No unambiguous in vivo footprints were detected in rat olfactory mucosa or lung (data not shown) most likely because of the heterogeneous cell types present in these tissues and the use of the highly sensitive PCR procedure.

The tissue-selective expression of nuclear proteins bound to the CYP1A2 NF-1-like binding site was investigated in gel-shift experiments with nuclear extracts from rat liver, olfactory mucosa, testis, kidney, and lung. As shown in Fig. 4A, with the 1A2pm probe, prominent protein-DNA complexes were detected with nuclear extracts from liver and olfactory mucosa but not in the other tissues examined. Weak signals were observed with lung extracts, which had much slower mobilities than the major bands detected in liver and olfactory mucosa. These observations are consistent with the results from DNase I footprint assays and with CYP1A2 being predominantly expressed in liver and olfactory mucosa but not the other tissues.

Multiple bands were detected with nuclear extracts from both liver and olfactory mucosa, but there were clear differences in the banding patterns between the two samples (Fig. 4A, lanes 1 and 2); the most predominant complex formed with olfactory proteins, which had the highest mobility of all bands detected, was undetectable in the liver. The other bands also had subtle differences in mobilities between the two tissues. Binding specificity was demonstrated by nearly complete inhibition of binding activities of liver (Fig. 4B) and olfactory (Fig. 4C) nuclear extracts by unlabeled 1A2pm probe but not by two mutated oligonucleotides (1A2pmmut1 and 1A2pmmut2) or unrelated oligonucleotide competitors (data not shown). These results indicate that liver and olfactory nuclear proteins interacting with the NF-1-like element in rat CYP1A2 gene may be different.

To determine potential interactions between CYP2A3 NPTA-binding proteins and proteins bound to the CYP1A2 NF-1-like element, competition experiments were performed with labeled 1A2pm and 2A3pm probes, respectively, and nuclear extracts from liver and olfactory mucosa. Unlabeled 2A3pm probe only partially blocked protein binding to 1A2pm in either liver or olfactory nuclear extracts at an 80× molar excess (Fig. 4B, lane 8, and Fig. 4C, lane 7). Unlabeled 1A2pm also blocked protein binding to labeled 2A3pm probe in olfactory nuclear extracts (Fig. 4D), apparently at a higher efficiency; a complete blockade of binding to the labeled 2A3pm probe was achieved at a 40× molar excess of 1A2pm (Fig. 4D), whereas an 80× excess of unlabeled 2A3pm failed to completely block the binding to labeled 1A2pm probe (Fig. 4C). However, as reported previously (26) and confirmed in Fig. 4E, liver proteins did not bind

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Furthermore, when comparing the banding patterns of olfactory complexes formed with the two different probes (Fig. 4, lanes 3 and 4), it was evident that the major bands formed with 2A3pm probe migrated slightly faster than those formed with 1A2pm probe, although the 2A3pm probe itself is larger and migrated slower than 1A2pm probe. This implies that, despite the cross competition between the two probes, they bind to different proteins in the olfactory mucosa.

The multiple bands detected in gel-shift assays in Fig. 4 suggest that the CYP1A2 NF-1-like element may bind multiple transcription factors, either directly or through interactions.

Experimental Procedures.” The nucleotides are numbered to the left, with the transcriptional start site (angled arrow) as +1 (33), and the sequence of the 1A2pm oligonucleotide competitor is underlined. The positions of several other putative regulatory elements identified by searching the TRANSFAC data base are also shown (boxed), including a TATA box and the binding sites for GATA-1/2, AP-4, and the homeodomain factor Nkx-2.5. D, the sequences of 1A2pm, 1A2pmmut1, 1A2pmmut2, and 2A3pm oligonucleotide probes are shown with the changed nucleotides in the mutant probes underlined.
Gel-shift assays were performed with 32P-labeled double-stranded 1A2pm (A, B, C, and E) or 2A3pm (D and E) oligonucleotide probe and crude nuclear extracts prepared from liver, olfactory mucosa, testis, kidney, or lung of 2-month-old rats. Binding reaction mixtures contained 20 μg of nuclear proteins, 1 ng of labeled 1A2pm or 2A3pm, and other components as described under “Experimental Procedures.” The reactions were carried out in the presence or absence of various competitors as indicated at the top of each lane. The sequences of the competitors are shown in Fig. 2D. Nuclear extracts were omitted in the lanes labeled Probe alone. In A, the positions of DNA-protein complexes in liver and olfactory mucosa are indicated by arrowheads and the weak signals detected in lung are indicated by an arrow.

To determine which of the 1A2pm-binding complexes contain an NF-1 factor, supershift assays were performed with a polyclonal anti-NF-1 antibody, named anti-CTF1 (35), which has been used in a number of studies to detect NF-1-related proteins (26). Liver and olfactory mucosa nuclear extracts were exposed to the antibody prior to incubation with labeled 1A2pm probe. As shown in Fig. 5, the addition of anti-NF-1 to the reaction mixture resulted in the appearance of a supershift band as well as a strong well shift in both nuclear extracts, which was accompanied by decreases in the intensities of all bands detected in either liver or olfactory mucosa. This result is in contrast to previous findings obtained with the 2A3pm probe that addition of the same anti-NF-1 antibody affected the intensities of only the three upper bands but not the most prominent band detected in olfactory mucosal nuclear extracts (26). The supershifted bands were not detected when a preimmune serum was used (Fig. 5). These observations indicate that all of the 1A2pm-binding complexes formed with liver or olfactory mucosal nuclear extracts contained an NF-1 factor and suggest that the multiple bands were derived from binding either with different NF-1 factors (as monomers, homodimers, or heterodimers) or with complexes of an NF-1 factor with other proteins of different sizes. In addition, they provide further support that, in the olfactory mucosa, the major proteins bound to the NPTA element of the CYP2A3 gene are different from the 1A2pm-binding proteins.

To determine whether the NF-1-like element plays a role in transcriptional activation of the CYP1A2 gene in the liver and the olfactory mucosa, in vitro transcription assays were performed. The promoter constructs used in in vitro transcription analyses are shown in Fig. 6A. Transcription from the truncated CYP1A2 gene (p1A2−412) was detected by S1 nuclease protection assay using a 24-mer antisense oligonucleotide probe corresponding to the 5′-end of the CYP1A2 mRNA. A mutant, p1A2−412/M, containing the same dinucleotide substitution as in 1A2pmmut2 (Fig. 2D), which abolished the binding activity of the 1A2pm probe in gel-shift assays (Fig. 4), was used to determine the functions of the proteins that bind to this NF-1-like element. Transcription activities were also determined with p2A3−254 (26) and TTR-3000/2A6 plasmids, which were used as internal controls for transcription efficiency in olfactory mucosa and liver, respectively; these promoter sequences have previously been shown to be capable of directing olfactory- and liver-selective expression of the CYP2A3 (26) and TTR (34) genes, respectively. CYP1A2 transcripts were abundant in reactions with p1A2−412 promoter construct using nuclear extracts from either liver (Fig. 6B, lanes 2 and 4) or olfactory mucosa (Fig. 6B, lanes 6 and 8) but were barely detectable with p1A2−412/M (Fig. 6B, lanes 3 and 7). In contrast, the transcription rates from the control plasmids were about the same in the reactions with wild type (p1A2−412) or the mutated (p1A2−412/M) construct, indicating equal transcription efficiency in these reactions. With olfactory nuclear extracts, transcription from the CYP2A3 promoter construct appeared to be slightly less active than from the CYP1A2 promoter, which was consistent with the relative binding activities of 1A2pm and 2A3pm probes seen in the competitive gel-shift assays (Fig. 4, C and D). However, inclusion of p2A3−254 plasmid at equal molar amounts with p1A2−412 plasmid did not cause an inhibition of the transcription activity from the CYP1A2 promoter, and vice versa, despite the
cross competition seen in gel-shift assays with excess amounts of unlabeled probes (Fig. 4C), which is in agreement with the notion that different proteins interact with these two elements. These results indicate that the NF-1-like element is necessary for the transcriptional activation of the CYP1A2 gene in the olfactory mucosa and the liver and that the two nucleotides at −115 and −114 are critical for both protein binding and transcription.

**DISCUSSION**

Several interesting observations were made in this study. First, in vitro footprinting and gel shift experiments demonstrated that protein binding to a previously unrecognized, highly conserved NF-1-like element in the CYP1A2 gene was tissue-selective and consistent with its tissue-selective expression in liver and olfactory mucosa. Second, this sequence was essential for transcriptional activation of the CYP1A2 gene in vitro and was occupied by DNA-binding proteins in vivo in native chromatin. Third, results from supershift experiments indicated that all of the DNA-binding complexes formed with the CYP1A2 NF-1-like element contained NF-1 factors. These data strongly suggest that members of the NF-1 family of transcription factors may play an important role in the tissue-selective expression of the CYP1A2 gene in the liver and olfactory mucosa.

A number of NF-1 transcription factors have been reported, which are derived from a family of at least four genes, i.e. NFI-A, NFI-B, NFI-C, and NFI-X (46, 47). NF-1 factors have unique organ and cell type-specific expression and can function in either transcriptional activation or repression (46, 48–50). NF-1-like elements containing the highly conserved TGG motif have been found in the promoters and enhancers of many genes. Of particular interest, an NF-1 site is present in the phenobarbital-responsive enhancer module in mouse Cyp2b10 and rat CYP2B2 genes and is important for the magnitude of the induction response (51–53). An NF-1 element was also found to be a functional component of the mouse Cyp1a1 promoter in a study with mouse hepatoma cells (54); a similar site is present in rat and human CYP1A1 promoter and was found to be involved in the down-regulation of CYP1A1 gene expression by oxidative stress (55). Moreover, we have recently identified an NF-1-like positive regulatory element (NPTA element) in the proximal promoter region of rat CYP2A3 gene (26), which may be important for the tissue-selective expression of the gene in the olfactory mucosa.

The NPTA element of the CYP2A3 gene and the NF-1-like element of the CYP1A2 gene competed for protein binding in gel-shift assays, with the CYP1A2 NF-1-like element apparently having a greater affinity in both liver and the olfactory mucosa. However, the NPTA element did not bind nuclear proteins in liver and the hepatic and olfactory mucosal proteins bound to the CYP1A2 NF-1-like element were apparently different in size from the olfactory NPTA-binding proteins. This is consistent with the previous finding that not all NPTA-binding complexes were supershifted by the same antibody used in the present study (26). Thus, different NF-1 factors may be involved in the regulation of these two CYP genes leading to the nasal predominant expression of the CYP2A3 gene and the equally abundant, but highly selective expression of the CYP1A2 gene in the liver and the olfactory mucosa.

Multiple NF-1 isoforms have been detected in the liver and the olfactory mucosa (56, 57). The anti-NF-1 antibody used in this study was prepared against a bacterial fusion protein of unlabeled probes (Fig. 4C), which is in agreement with the notion that different proteins interact with these two elements. These results indicate that the NF-1-like element is necessary for the transcriptional activation of the CYP1A2 gene in the olfactory mucosa and the liver and that the two nucleotides at −115 and −114 are critical for both protein binding and transcription.

**FIG. 6. Role of the NF-1-like element in in vitro transcription of the CYP1A2 gene with nuclear extracts from rat liver and olfactory mucosa.** A, CYP1A2, CYP2A3, and TTR promoter constructs used for in vitro transcription. Exons are shown as solid bars, and the antisense oligonucleotide probes used for S1 nuclease protection assays (S1 probe) are shown as open boxes. Transcription initiation sites are indicated by angled arrows. The approximate positions of the NF-1-like element in the CYP1A2 5' flanking region, the NPTA element in the CYP2A3 gene, and the proximal promoter and enhancer in the pTTR-3000/2A6 construct are indicated, and the mutated sequence in the pIA2–412M construct is also shown. B, in vitro transcription was performed as described under “Experimental Procedures” using nuclear protein extracts prepared from 2-month-old rats. The transcription reactions contained 60 μg of the liver or olfactory nuclear proteins and 1 μg of each plasmid as indicated. RNA transcripts were directed by S1 nuclease protection assay using 32P-labeled, single-stranded oligonucleotide probes of different sizes (24-mer for CYP1A2, 52-mer for CYP2A3, and 33-mer for TTR/2A6) and visualized by autoradiography. The positions of the protected in vitro transcripts from the CYP1A2 gene (*), the CYP2A3 gene (**), and the TTR/2A6 fusion gene (***) are shown.
appropriate for specific protein binding to these sites.

The CYP1A2 NF-1-like element was partially protected by lung nuclear proteins in in vitro footprinting experiments. However, gel-shift analysis suggested that the DNA-binding proteins from rat lung were apparently different from those of the liver or olfactory mucosa. A recent report indicated that CYP1A2 was detected in human peripheral lung tissue (59), and CYP1A2 mRNA was also detected in mouse lung following induction by 3-methylcholanthrene (60). However, the very low abundance of the DNA-protein complexes detected in rat lung nuclear extracts and the very low levels of CYP1A2 expression make it difficult to examine the possible role of the NF-1 factors in the regulation of CYP1A2 expression in the lung.

The CYP1A2 NF-1-like element is highly conserved in rats, mice, and rabbits, in which the tissue-selective expression of CYP1A2 in the liver and olfactory mucosa has been documented. In humans, CYP1A2 is one of the major P450 isofoms in the liver, but its expression in olfactory mucosa has not been characterized. Sequences of the human CYP1A2 gene have been reported by two groups (1, 39); they differ at a critical site in the NF-1-like element characterized in this study. One of these sequences (1) contains a single nucleotide deletion that shortens the distance between the two conserved palindromic TGG half-sites, which would render the element nonfunctional. However, sequence analysis of 100 genomes detected only the sequence corresponding to the conserved NF-1-like element but not that of the deletion variant. Thus, this NF-1-like element is also conserved in humans, although its functional significance needs to be confirmed and it remains a possibility that a genetic polymorphism occurs at this site in a subpopulation. Notably, large interindividual variations in hepatic CYP1A2-dependent metabolic activities have been reported (20). Genetic polymorphism of the human CYP1A2 gene was not found until recently (61–64), and only one, involving a single nucleotide mutation at −2964, was found to be functionally significant (62). This mutation was suggested to be a causal factor of decreased CYP1A2 inducibility in Japanese smokers.

The regulation of the constitutive hepatic expression of the human CYP1A2 gene has been examined in a previous study in transiently transfected HepG2 cells (24). Interestingly, deletion of 5′-flanking sequences between −2093 and −72 did not cause a decrease in reporter gene expression, but further deletion of −72 to −30 sequences almost completelyabolished reporter gene activity. It was suggested that this 42-bp sequence (−72 to −30), containing a GC box, a CCAAT box, and a TATA box, is essential for constitutive expression of the human CYP1A2 gene. Whereas multiple regulatory elements may be necessary for promoter activity, this result appears to be in contrast to the present finding with the rat CYP1A2 gene that the conserved NF-1-like element at −129 to −111 is important for the promoter activity. Nevertheless, the sequence of the proximal promoter region of the human CYP1A2 genomic clone used in that study (24) was not reported. Therefore, it is not known whether it had the conserved NF-1-like element at −129 to −111. Furthermore, the immortalized HepG2 cell line expresses CYP1A2 at a low level only detectable by RNA-PCR (65). Thus, some critical transcriptional regulators may not be available or may be expressed at levels too low to activate the endogenous or transfected CYP1A2 gene, as suggested (24). On the other hand, the contrasting results may represent species differences in CYP1A2 regulation between humans and the animal species, because the CCAAT box (−52 to −48) found in the human CYP1A2 gene is not found at similar positions in rat, mouse, or rabbit CYP1A2 genes (33, 37, 38).

The decreased basal expression of hepatic CYP1A2 in AhR−/− mice suggested that this transcription factor may be directly or indirectly involved in the constitutive expression of the CYP1A2 gene (7, 8). Of interest, treatment of rats with 3-methylcholanthrene, a known inducer of hepatic CYP1A2, did not increase the abundance or alter the profile of the hepatic or olfactory mucosal DNA-protein complexes with the CYP1A2 NF-1-like element,4 suggesting that the NF-1-related factors are not directly regulated by the AhR-mediated pathways. Thus, although the AhR-binding sites are located in the distal region of the CYP1A2 gene (6), far from the NF-1-like element, constitutive expression of CYP1A2 may involve interactive events between the two regulatory elements; a similar interaction has recently been demonstrated in the down-regulation of CYP1A1 expression by H2O2 (66).
Identification and Functional Characterization of a Conserved, Nuclear Factor 1-like Element in the Proximal Promoter Region of CYP1A2 Gene Specifically Expressed in the Liver and Olfactory Mucosa
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