Transcriptional Regulation of Human CYP2C Genes: Functional Comparison of CYP2C9 and CYP2C18 Promoter Regions

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ABSTRACT: The cytochrome P4502C subfamily comprises a group of constitutive microsomal hemoproteins which are expressed primarily in liver. In humans, this subfamily is responsible for metabolism of a variety of therapeutic drugs such as warfarin, mephenytoin, omeprazole, and antiinflammatory drugs. In the present study, we analyzed the promotor activity of the 5′-flanking region of two human *CYP2C* genes, *CYP2C9* and *CYP2C18*. The ability of the 2.2-kb 5′-flanking region of the *CYP2C9* gene to direct expression of a luciferase reporter gene in HepG2 cells was 25 times greater than that of the 1.3-kb 5′-flanking region of *CYP2C18*. Deletional analysis of *CYP2C9* indicated that the minimal promotor was located between the translation start site and nucleotide −155, and an HPF-1 domain consensus sequence was identified in this region. Gel shift analysis demonstrated that nuclear proteins from HepG2 cells had a high binding affinity for a 20-bp oligonucleotide containing the HPF-1 site of *CYP2C9*. Antiserum to rat HNF-4 supershifted this DNA−protein complex, and an oligonucleotide derived from an HNF-4 motif present in the human apolipoprotein CIII promotor competed for the supershifted complex. Cotransfection with an HNF-4 expression plasmid increased transcriptional activity of the *CYP2C9* minimal promotor (~2-fold) in HepG2 cells and elevated activity more substantially in nonhepatic NIH3T3 cells (26-fold) and Cos 1 cells (9-fold). A possible HPF-1 motif was identified 661 to 641 bases upstream of the translational start site of *CYP2C18* which differed from the HPF-1 consensus sequence by the substitution of three cytosines for purines at positions 4−6 and one adenine residue at position 15. An oligonucleotide containing the *CYP2C18* HPF-1 motif bound nuclear proteins from HepG2 cells only weakly in gel shift assays, but replacement of the three tandem cytosine residues in the HPF-1 site by guanines using site-directed mutagenesis caused the formation of a complex whose mobility was supershifted by anti-HNF4. Similarly, mutation of the three guanines in the *CYP2C9* HPF-1 site to cytosines prevented the formation of the specific DNA−protein complex seen with this motif. However, cotransfection with an HNF-4 expression plasmid did not increase transcriptional activity of *CYP2C18* promotor constructs containing the original *CYP2C18* HPF-1 motif or the mutated motif containing guanine residues in any of the cell lines tested. We conclude that the HPF-1 site is an important *cis*-acting element directing hepatic expression of the *CYP2C9* promoter but does not contribute to the weak transcriptional activity of the 1.3-kb upstream region of *CYP2C18*.

Cytochromes P450 are a diverse group of heme-containing enzymes which catalyze the biotransformation of endogenous and foreign substances. This multigene array of enzymes are classified into families, of which CYP2 is the largest (Guengerich, 1989; Gonzalez, 1990; Nelson et al., 1993). Expression of many CYP2 proteins occurs primarily in the hepatocytes, where they function in drug oxidation through selective activation of groups of chemically related agents (Okey, 1990). Although members of this gene family are closely related, they exhibit a developmental and sex-dependent expression profile in rodents (Gonzalez, 1990). *CYP2E1*, for instance, is transcriptionally activated immediately after birth in both male and female rats, whereas *CYP2C6* and *CYP2C7* are activated only at the onset of puberty (Ueno, 1990; Zaphiropoulos et al., 1989). In contrast, *CYP2C11* and *CYP2C13* are exclusively expressed in adult male rats, while *CYP2C12* is activated only in females (Zaphiropoulos et al., 1989).

There is abundant evidence that control of gene expression is exerted primarily at the level of transcription and depends largely on the interaction of a diverse network of transcription proteins with specific DNA elements in gene promoters (Mitchell & Tjian, 1989). The human CYP2C subfamily of cytochrome P450 consists of at least seven genes and pseudogenes (Ged et al., 1988) clustered on chromosome 10 (Meehan et al., 1988). Although structurally related, little is known about the mechanisms by which the members of the human 2C subfamily are differentially regulated. Four members of the subfamily, *CYP2C8*, *CYP2C9*, *CYP2C18*, and *CYP2C19*, have been isolated and shown to be differentially expressed in human liver (Umbenhauer et al., 1987; Okino et al., 1987; Kimura et al., 1987; Romkes et al., 1991; Goldstein et al., 1994). CYP2C9 is the major protein in human liver and plays an important role in the metabolism of tolbutamide, S-warfarin, and nonsteroidal antiinflammatory drugs (Brian et al., 1989; Relling et al., 1990; Yasumori et al., 1991, 1993; Rettie et al., 1992; Srivastava et al., 1992). In comparison to CYP2C9, CYP2C18 is expressed at low levels in human liver (Goldstein et al., 1994). Several *cis*-

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acting DNA elements with putative tissue-specific regulatory functions have been identified from a number of hepatic genes (de Simone & Cortese, 1992; Lai & Darnell, 1991) and include ARP-1, COUP transcription factors, C/EBP, DBP, HNF-1, HNF-3, and HNF-4. Some of these elements have been reported in the upstream sequence of *CYP2C9* and *CYP2C18* genes (Ged & Beanue, 1992; de Morais et al., 1993). However, the role of these elements in the transcriptional regulation and tissue-specific expression remain uncertain. In this paper, we have examined the functional elements of the human *CYP2C9* and *CYP2C18* gene promoters. To understand the basic mechanisms regulating the tissue-specific and differential expression of *CYP2C* genes in the liver, we analyzed the ability of various promoter deletion constructs to activate luciferase expression in the homologous HepG2 (Knowles et al., 1980) cell line. Transcriptional activity of constructs was also analyzed in heterologous cell systems (NIH3T3 and Cos-1) as examples of cell types which do not express specific hepatic nuclear transcription factors. Finally, gel shift assays and methylation interference assays were applied to identify putative motifs involved in the basic transcriptional regulation.

EXPERIMENTAL PROCEDURES

Materials. Molecular biological reagents were purchased from Life Technologies Inc. and New England Biolabs, and tissue culture media and reagents were from Life Technologies Inc. The firefly luciferase plasmids pGL2-enhancer and pGL2-control and the β-galactosidase control plasmid were purchased from Promega Corporation. Luciferase assay reagents, β-galactosidase reagents, dimethyl sulfate, and piperidine were obtained from Promega Corporation, Sigma Chemical Company, and Aldrich. [γ-^{32}P]ATP (6000 Ci/mmol) was purchased from Amersham Corporation. Antibody to rat HNF-4 was kindly provided by Dr. Francis Sladek, University of California, Riverside.

Synthetic Oligonucleotides. Oligonucleotides for site-directed mutagenesis, probes and competitors used in gel shift analysis, and their complements were synthesized with an Applied Biosystem 380B DNA synthesizer (Applied Biosystem, Foster City, CA). The following oligonucleotides were synthesized for probes and competitors for gel shift analysis: 2C9-1991F, 5′-TCAGTGGGTCAAAGTCCTTT-3′; 2C9-1987M, 5′-TCTATCAGTCCCTCAAAGTCATTT-CAG-3′; 2C18-649F, 5′-GATTGCCCTCAAAGTCATATTT-CC-3′; 2C18-649M, 5′-GATTGGGGTCAAAGTCATATTT-CC-3′; and apoCIII, 5′-CGAGCGCTGGGCAAAGGTCAC-CTGC-3′.

DNA Cloning and Preparation of 5′ Deletion Mutants. A 2.2-kb *Sal*I−*Sst*I fragment of the *CYP2C9* 5′-flanking region extending ~59-bp into exon 1 and 1.3-kb of the *CYP2C18* 5′-flanking region previously cloned in the pBluescript vector were excised and cloned into the *Xho*I−*Bgl*II and *Hind*III sites respectively of the promoterless luciferase reporter vector pGL2-enhancer. The fragments were blunt ended with T4 DNA polymerase and force cloned. Subsequently, the 59-bp of coding exon in the *CYP2C9* recombinant plasmid referred to as p2.2Luc/E was removed by exchanging the 3′ *Eco*RI-*Hind*III terminus of *CYP2C9* insert with a polymerase chain reaction (PCR) fragment amplified with a reverse primer (5′-TACCCAAGCTTGAAGCCTTCTCTTCTT-3′) which began at position −1, eliminating sequences downstream of the translation initiation codon. These plasmids were constructed to eliminate ATG codons 5′ of the luciferase translation start site.

To construct the primary deletion mutants of the *CYP2C9* gene, the restriction enzyme recognition sites at *Stu*I (−1877), *Hind*III (−1454), *Pst*I (−990), *Eco*RI (−362), and *Avr*II (−114) were used to progressively trim the 5′ end of p2.2Luc/E and generate the mutant plasmids p2.2/−1877, p2.2/−1454, p2.2/−990, p2.2/−362, and p2.2/−114. Three additional deletion constructs, p2.2/−155, p2.2/−140, and p2.2/−98, designed to identify the minimal promoter and the functions of an overlapping C/EBP and an AP-I site, an HPF-I motif, and a canonical CCAAT box were cloned by PCR amplification of the appropriate short DNA segments and religated into the *Xho*I−*Hind*III site of pGL2-enhancer. For *CYP2C18* deletions, the recombinant plasmid p1.3Luc/E was sequentially shortened at the *Sal*I (−989), *Stu*I (−523), and *Pst*I (−227) sites to yield p1.3/−989, p1.3/−523, and p1.3/−227. The *Xho*I and *Nhe*I sites 5′ of the inserts in the luciferase vector served as anchors to facilitate the progressive deletions of plasmids p2.2Luc/E and p1.3Luc/E, respectively.

To mutate the variant cytosine residues in the putative HPF-1 binding domain of the *CYP2C18* promoter to the guanine residues present in the *CYP2C9* promoter and the consensus sequence for HNF-4/LF-1 (Ramji et al., 1991), we used a site-directed mutagenesis method which employs two mutagenic primers: one that contains the desired mutation and one that contains a mutation in any unique nonessential restriction site in the target plasmid (Deng & Nickoloff, 1992). Fidelity of all chimeric plasmid constructs was confirmed by restriction endonuclease digestion and dideoxy chain termination sequencing.

Cell Transfections. HepG2 human hepatoma, NIH3T3 mouse fibroblast, and Cos-1 monkey kidney fibroblast cell lines were maintained at 37 °C under 6% CO_2 in Dulbecco's modified Eagle's high-glucose medium supplemented with 50 μg/mL penicillin−streptomycin solution and 10% fetal bovine serum. Confluent cells were trypsinized and seeded at a density of ~2 × 10^5 cells/35-mm plate for HepG2 and ~1 × 10^4 cells/35-mm plate for NIH3T3 and Cos-1 cells 18 to 24 h before transfection. DNA from recombinant luciferase plasmids (5 μg) and the β-galactosidase expression plasmid pSV-β-galactosidase control (1 μg) coprecipitated with calcium phosphate were used in cellular transfections (van der Eb & Graham, 1980). The β-galactosidase plasmid served as an internal control for differences in transfection efficiencies. After 16 h the medium was changed, and 48 h thereafter the cells were washed twice in phosphate-buffered saline (PBS) and harvested. For cotransfection studies, 1.0 μg of the HNF-4 expression plasmid pLEN4S in the sense orientation or pLEN4A in the antisense orientation was added to the recombinant luciferase and β-galactosidase plasmids before transfection. The transfections were done in triplicate and repeated at least three times.

Luciferase and β-Galactosidase Assays. Luciferase assays were performed with the Promega luciferase assay system. Transiently expressed cells were washed with PBS and lysed with 200 μL of reporter lysis buffer (Promega Corporation). A 20-μL aliquot of each plate lysate was transferred to a 5-mL Sarstedt polycarbonate tube, and the luciferase reaction was started by injecting 100 μL of luciferase assay reagent [20 mM tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2{\cdot}5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM dithiothreitol (DTT), 270 μM coenzyme A, 470 μM luciferin, and 530 μM ATP].

Luminescence was measured for 25 s at room temperature in an Omptocomp II luminometer (MGM Instruments Inc., Hamden, CT). For β-galactosidase assays, 50 μL of diluted cell lysates and 50 μL of 2× β-galactosidase assay buffer (120 mM Na_2HPO_4, 80 mM NaH_2PO_4, 2 mM $MgCl_2$, 100 mM β-mercaptoethanol, and 1.33 μg/mL *o*-nitrophenyl β-D-galactopyranoside) were added to labeled wells of a 96-well microtiter plate. The reaction mixtures were incubated at 37 °C until color development, the reactions were stopped by addition of 150 μL of 1 M sodium carbonate, and absorbance of the samples was read at 405 nm in a Thermomax microplate reader (Molecular Devices). The luciferase activities were normalized to the β-galactosidase expression values for each transfection.

Preparation of Nuclear Extracts. Nuclear extracts were prepared by the method of Dignam et al. (1983) as modified by Dent and Latchman (1993). Cells (5×10^7) were washed twice in PBS (1.2 mM KH_2PO_4, 2.7 mM KCl, 138 mM NaCl, and 8.1 mM $Na_2HPO_4 \cdot 7H_2O$) and incubated on ice in 5 vol of buffer A containing 10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.5 mM phenylmethanesulphonyl fluoride (PMSF) for 10 min. The cells were pelleted, resuspended in 3 vol of buffer A with 0.05% Nonidet P-40 (NP40), and homogenized in a tight-fitting Dounce homogenizer. Released nuclei were pelleted at 250 *g* for 10 min and resuspended in 1 mL of buffer C [5 mM Hepes (pH 7.9), 26% glycerol (v/v), 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF]. After a 30-min incubation on ice, the nuclear extracts were spun at 24000 *g* for 20 min at 4 °C, and the supernatants were aliquoted, quickly frozen in liquid nitrogen, and stored at −70 °C. Protein content was quantitated by the Bradford assay (1976).

Electrophoretic Mobility Shift Assays. PCR fragments and double-stranded oligonucleotides were used as probes for protein binding assays. The probes were labeled by T_4 polynucleotide kinase using [γ^{32}-P]ATP and purified of unincorporated nucleotides by chromatography through a Sephadex G-25 column. Nuclear extracts (5−10 μg) were preincubated at 4 °C for 5 min with 2 μg of poly(dI-dC), 10 mM Tris (pH 7.5), 50 mM NaCl, 2.5 mM $MgCl_2$, 0.5 mM DTT, and 4% glycerol in a volume of 20 μL. Labeled probes (1×10^4 cpm) were added, and incubation was continued on ice for 30 min. When needed, cold competitor oligonucleotides or DNA fragments were included in the reactions. For supershift assays, α-445 rat HNF-4 antiserum was diluted 5-fold in 3% bovine serum albumin (BSA), and 2 μL was added to the binding reactions, midway in the incubation. The complexed and unbound probes were then resolved by electrophoresis on a 5% acrylamide gel in 0.5× Tris borate−EDTA (TBE), pH 8.0: the gel was then dried and exposed to Kodak XAR film.

Methylation Interference Footprinting. Probes for methylation interference analysis were prepared by independently labeling each strand of a duplex DNA fragment by PCR amplification. Approximately 0.5 pmol of each labeled forward oligonucleotide primer and 0.5 pmol of cold reverse primer were included in a 50-μL PCR mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 1 mM deoxynucleotide triphosphates, 10 ng of plasmid DNA, and 0.25 unit of Taq DNA polymerase (Perkin Elmer, Norwalk, CT). The mixture was denatured for 2 min at 94 °C and processed for 25 cycles consisting of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C with a final extension at 72 °C for 10 min. Unincorporated nucleotides were removed by Sephadex G-50 column chromatography. Methylation interference assays were performed according to the method of Atchinson et al. (1990). The labeled probe (2×10^6 cpm) diluted in 50 mM sodium cocodylate and 1 mM EDTA, was partially methylated by incubation in dimethyl sulfate (DMS) for 2 min, precipitated, and resuspended in TE (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). Band-shift reactions were conducted as outlined previously with 1×10^5 cpm of probe and 50 μg of HepG2 nuclear extract in a 100-μL volume. The protein−DNA complexes and free probes were then subjected to electrophoretic separation on a 5% non-denaturing acrylamide gel in 0.5× TBE and recovered from the gel slices by elution. Cleavage of the protected and unprotected DNA strands with piperidine formate solution (1 M) was performed at 90 °C for 1 h followed by ethanol concentration and electrophoretic analysis on 8% denaturing (42% urea, 1× TBE) polyacrylamide gel.

RESULTS AND DISCUSSION

Measurement of CYP2C9 and CYP2C18 Promoter Function. We recently reported the presence of a number of transcriptional control elements in the regulatory region of two human *CYP2C* genes, *CYP2C9* and *CYP2C18*, which include canonical TATA and CCAAT boxes, glucocorticoid response elements (GRE), the ubiquitous AP-1, SP-1, and SP-2 domains, and consensus sequences for liver-enriched factors including C/EBP, DBP, HNF-1, and HPF-1 (de Morais et al., 1993). To compare the functional characteristics of human *CYP2C9* and *CYP2C18* promoters, DNA segments from the 5′-flanking region of these genes were inserted upstream of the luciferase expression vector (pGL2-enhancer) and analyzed by transient transfection in HepG2 hepatoma cells. The results show that the transcriptional activity of *CYP2C9* (p2.2Luc/E), normalized for β-galactosidase expression, was ~25-fold greater than that of *CYP2C18* (p1.3Luc/E) (Figure 1A,B) and 5-fold greater than the activity of a control plasmid driven by viral SV40 promoter. The relative activity of the two promotors is consistent with the low concentrations of CYP2C18 protein in human livers (<5 pmol/mg) relative to CYP2C9 (Goldstein et al., 1994) and the low abundance of *CYP2C18* cDNAs in human liver libraries compared to *CYP2C9* cDNAs (4% of CYP2C cDNAs vs 60%, respectively) (Romkes et al., 1991).

The *CYP2C9* promoter was further analyzed for *cis*-acting DNA elements required for initiation of gene transcription by deletional analysis (Figure 1A). Deletion of a 270-bp fragment of DNA from the 5′ end of p2.2Luc/E (p2.2/−1877) consistently resulted in ~4-fold increase in activity, suggesting the presence of a domain which may negatively modulate promoter function. Additional deletion to nucleotide −1454 (p2.2/−1454) decreased luciferase activity to that observed with p2.2Luc/E. Further trimming of downstream DNA between −1454 and −362 did not significantly alter luciferase expression; however, luciferase activity was decreased by ~80% when deletion was extended to residue −114 (p2.2/−114). The marked reduction in promoter function indicates the presence of transcription regulatory elements in this region which may play a major role in the hepatic expression of human *CYP2C9*. Nucleotide sequence analysis demonstrates that the fragment from −362 to −114

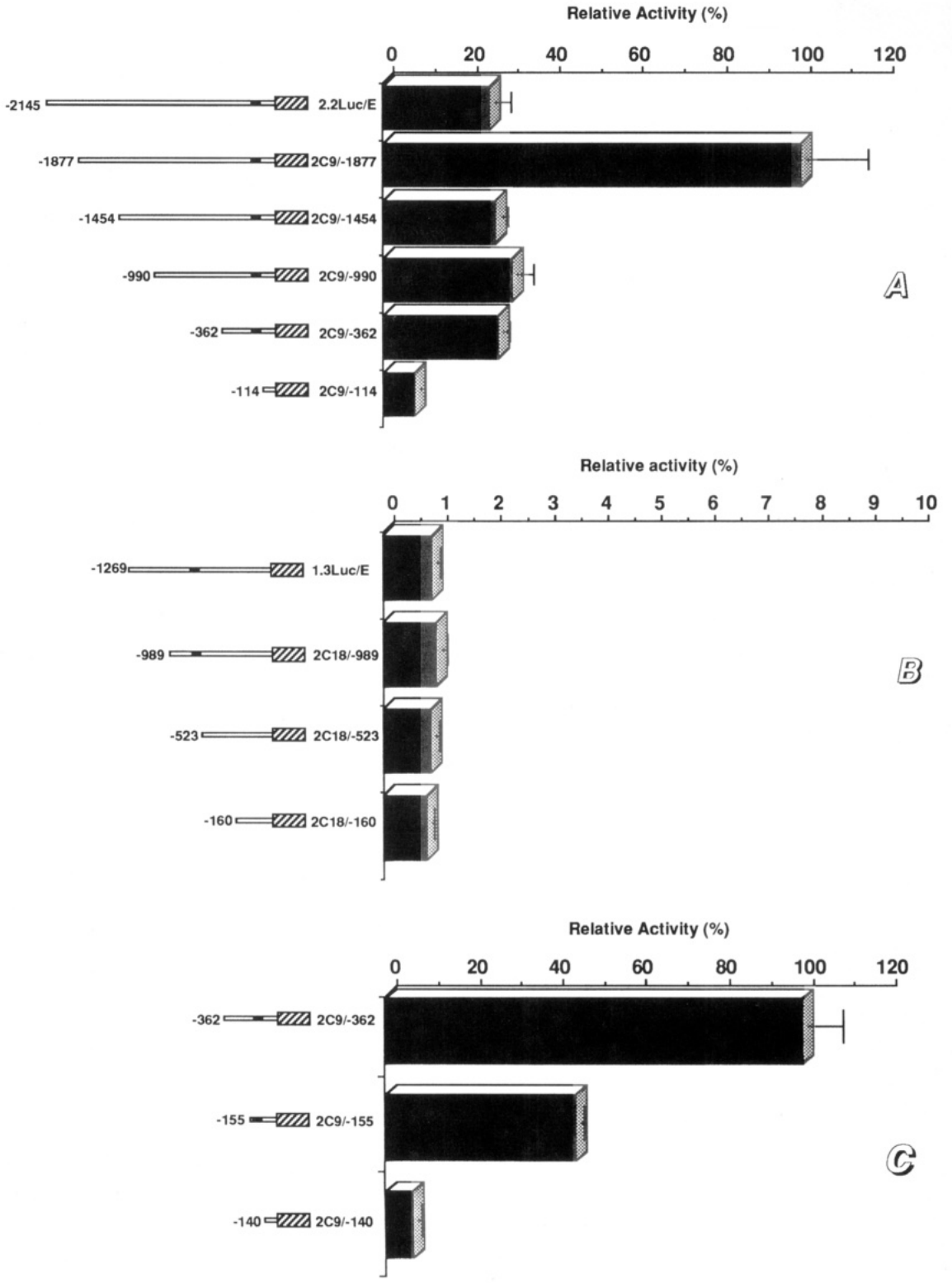

FIGURE 1: Deletional analysis of human *CYP2C9* and *CYP2C18* promoter constructs in HepG2 cells: (A) 5′ deletion of the *CYP2C9* promoter; (B) 5′ deletion of the *CYP2C18* promoter; (C) HPF-1 sequence-dependent activity of the *CYP2C9* promoter. Schematic representations of the promoter and deletion constructs are shown to the left of each panel. The promoter sequence is indicated by an open box; numbers at the left edge of the boxes refer to the upstream position relative to the translation start site for the endogenous gene. The luciferase gene is indicated by a stippled box. Each construct was cotransfected with pSV-β-galactosidase, and transfection efficiencies were normalized to β-galactosidase activity. The normalized average activity of each construct, from at least three experiments, is shown. Each average and the standard error are expressed as a percentage of the activity in cells transfected with p2C9/−1877 in panels A and B.

contains potential binding sites for the transcription factors AP-1, C/EBP, and HPF-1 (de Morais et al., 1993).

A *cis*-acting transcriptional regulatory element (HPF-1 site) involved in the liver-enriched expression of the rabbit *CYP2C1* and *CYP2C2* genes has been characterized (Venepally et al., 1992) and recently identified as a functional binding site for the orphan receptor HNF-4 (Chen et al., 1994a), a member of the nuclear hormone receptor family of transcriptional activators (Sladek et al., 1990). In an attempt to locate the *cis*-acting elements for the minimal expression of human *CYP2C9* gene, two deletion mutants were constructed from p2.2/−362. The first, p2.2/−155,

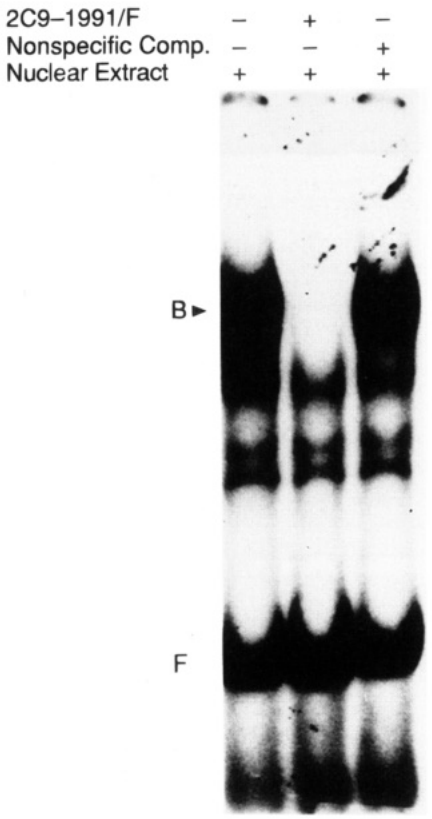

FIGURE 2: Binding of the *CYP2C9* promoter to HepG2 nuclear proteins in vitro. A 40-bp end-labeled DNA fragment (−155 to −115) spanning the HPF-1 domain of *CYP2C9* promoter (2C9-1991F) was incubated with nuclear extract from HepG2 cells. The unlabeled specific oligonucleotide competitor 2C9-1991F and a nonspecific DNA fragment (Nonspecific Comp.) derived from the 5′-terminal region of *CYP2C9* (−2145 to −1875) were added in 100-fold excess as indicated above the lanes. Electrophoretic mobility shift assays were performed as described in the text. The arrowhead indicates the position of specific DNA−protein complex. (−) indicates the omission of a component; (+) indicates the addition of a component.

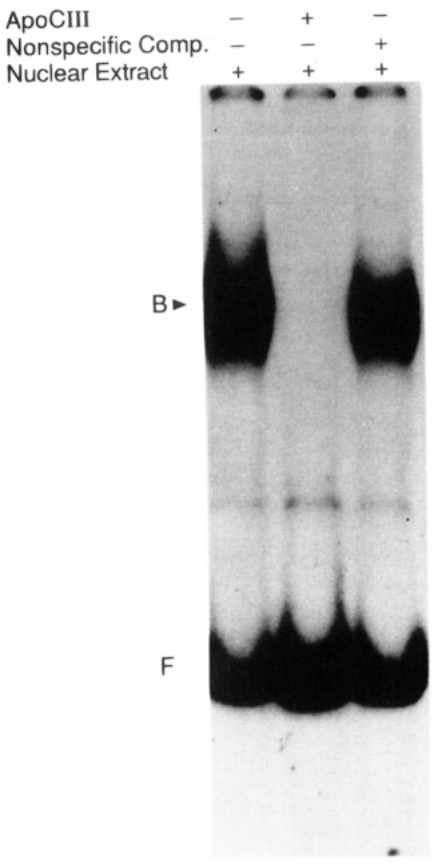

FIGURE 3: Abolishment of binding of HepG2 nuclear extract to the *CYP2C9* HPF-1 site by the human apolipoprotein CIII gene HNF-4 site. A double-stranded 20-bp oligonucleotide (2C9-1991F) corresponding to the *CYP2C9* HPF-1 site was radiolabeled and incubated with HepG2 nuclear extract. Unlabeled oligonucleotide, apoCIII, containing the HNF-4 site of the human apolipoprotein CIII gene and a DNA fragment of *CYP2C9* (Nonspecific Comp.) (−2145 to −1875) were used as specific and nonspecific cold competitors. Gel shift assays were performed as described in Experimental Procedures. The arrowhead points to the position of the complex formed with the 2C9-1991F probe. (−) indicates the omission of a component; (+) indicates the addition of a component.

contained the core consensus sequence for HPF-1 but was missing the AP-1 and C/EBP sites, and the second, p2.2/−140, was missing all three sites. Expression data with these two mutant chimeric clones showed that deletion of the C/EBP and AP-1 sites (−362 to −155) resulted in approximately a 2-fold reduction in promoter activity in HepG2 cells, whereas loss of the HPF-1 site (−155 to −140) resulted in an additional 7−8-fold reduction in activity (Figure 1C). Additional deletions to residues −95 (p2.2/−95) and −50 (p2.2/−50) which removed the CCAAT and TATA boxes, resulted in no further changes in promoter activity. These data suggest that the HPF-1 site is an important *cis*-acting element involved in the function of *CYP2C9* promotor. However, the 2-fold difference in the promotional activities of the −362 and the −155 constructs suggests the possibility that additional *cis*-acting elements exist in this region. It is noteworthy that this region also contains AT stretches up to 13-bp. Chen et al. (1994b) suggested that long AT tracts in the *CYP2C3* promotor might function as transcriptional activation elements (Winter and Varshavsky, 1989).

In contrast, the sequential deletion of the *CYP2C18* 5′-flanking region (1.3/−989, 1.3/−523, and 1.3/−227) did not significantly alter the minimal promotional activity seen with the 1.3-kb construct (Figure 1B). Moreover, unlike 2C9, the removal of a potential HPF-1 site at position −661 to −641 of the *CYP2C18* promoter had no consequence on the functional activity of the promoter in HepG2 cells. This observation suggests that the HPF-1 element may be cryptic in *CYP2C18* and hence might not participate in the regulation of the gene.

Presence of an HNF-4 Binding Motif in the Promoters of CYP2C9 and CYP2C18. The promoter regions of the *CYP2C9* and *CYP2C18* genes contain motifs related to the consensus sequence for HPF-1. Since the HPF-1 element was earlier reported to be essential for the liver-specific expression of rabbit *CYP2C* promoters (Venepally et al., 1992), we examined the ability of nuclear proteins from HepG2 cells to bind fragments of the two human *CYP2C* promoters in gel shift assays. The mobility of a 40-bp *Nhe*I−*Avr*II fragment (−155 to −115) of p2C9/−155 containing the HPF-1 site was retarded after incubation with HepG2 nuclear proteins (Figure 2). The formation of a complex between the fragment and nuclear proteins was specifically inhibited by a synthetic double-stranded oligonucleotide designated 2C9−1991F corresponding to the consensus HPF-1 binding region of *CYP2C9* but not by a 270-bp 5′ terminal fragment of p2.2Luc/E. Three other minor bands of faster mobilities were detected on the gel shift, and these bands were not eliminated by the addition of the cold competitor, indicating that other proteins in the crude extract are interacting with other regions of the DNA fragment. A

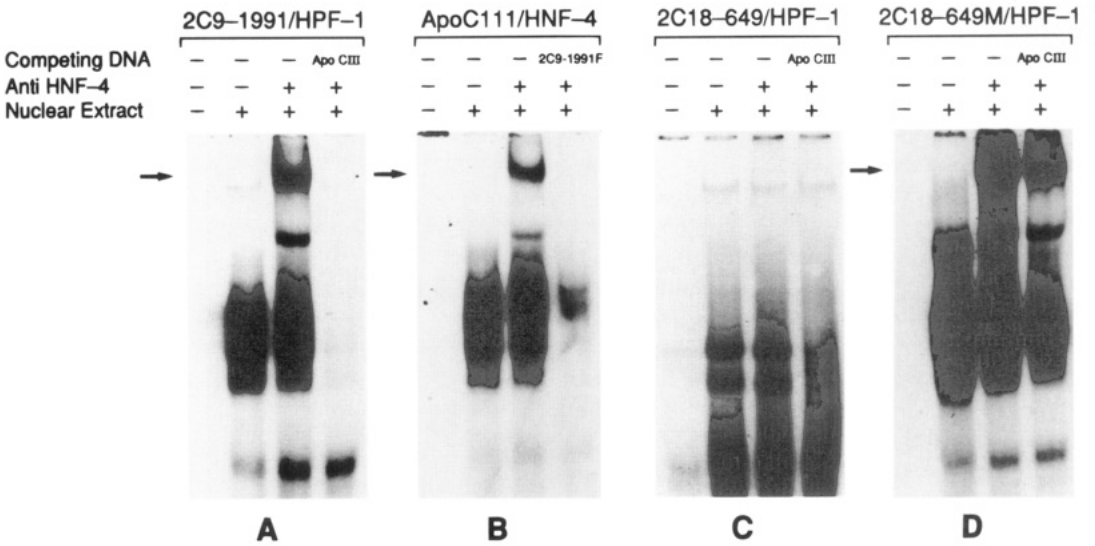

FIGURE 4: Effect of antibody to rat HNF-4 on formation of DNA−protein complexes with HepG2 cell extracts and oligonucleotides derived from the HPF-1 site of *CYP2C9*, *CYP2C18* and the HNF-4 site of apolipoprotein CIII (apoCIII). Double-stranded oligonucleotides 2C9-1991/HPF-1 (A), human apoCIII/HNF-4 (B), 2C18-649/HPF-1 (C), and 2C18-649M/HPF-1 (D) were radiolabeled to similar specific activities and used in the *in vitro* binding reaction with HepG2 nuclear extract. Oligo 2C18-649M was constructed by site-directed mutagenesis of 2C18-649M by the replacement of three cytosine residues with guanines found in the HPF-1 consensus sequence. Antisera to α-445 rat HNF-4 were added to the binding reaction halfway into the 30-min incubation period. Gel shift analysis and detection of products were as described in Experimental Procedures. Panels A and B show the protein complex formation with 2C9-1991F and apoCIII oligonucleotides, respectively; competition with apoCIII and 2C9-1991F oligonucleotides; and supershifting of the complexes with antibody to rat HNF-4 (arrows). Panel C shows minor protein−DNA complexes formed with CYP2C18-649 which are neither competed by apoCIII oligo nor supershifted after incubation with antibody to HNF-4. Panel D shows the enhancement of binding of the *CYP2C18* oligo after mutation of three cytosines to guanines and supershifting of the complex after incubation with antibody to HNF-4. (−) indicates omission of a component; (+) indicates addition of a component.

complex was also seen with a 246-bp fragment (−382 to −136) containing the HPF-1 site but not with a 114-bp *Eco*RI−*Avr*II fragment (−362 to −249) upstream of the HPF-1 domain (data not shown).

The HPF-1 motif has been reported earlier to resemble the consensus sequence of the binding site for hepatic nuclear factor 4 (HNF-4) of the human apolipoprotein CIII gene (Venepally et al., 1992; Ramji et al., 1991), and in a recent report the rabbit *CYP2C1* and *CYP2C2* HPF-1 motifs were shown to bind proteins with properties very similar to HNF-4 transcription factor (Chen et al., 1994a). To determine whether the HepG2 nuclear factor which binds to the upstream region of human *CYP2C9* is also similar to HNF-4, the ability of the HNF-4 binding domain of the human apolipoprotein CIII gene to compete with ^{32}P-labeled 2C9-1991F was analyzed in gel mobility assays. As shown in Figure 3, HepG2 nuclear extracts formed a complex with 2C9-1991F, and the binding of proteins to this oligonucleotide was effectively inhibited by the addition of excess apoCIII competitor oligonucleotide corresponding to the human apolipoprotein CIII HNF-4 site. Furthermore, coincubation of 2C9−1991F with crude nuclear extracts and antibody to α-445 rat HNF-4 supershifted the DNA−protein complex (Figure 4A). Again, the apoCIII competitor oligonucleotide effectively eliminated formation of the supershifted ternary complex. The above results were confirmed by using the apoCIII oligonucleotide as the labeled probe and cold *CYP2C9* double-stranded oligonucleotide as the competitor, although binding of nuclear extracts to apoCIII was weaker than that of *CYP2C9* (Figure 4B). These data suggest that the promoter activity of *CYP2C9* gene is modulated by HNF-4 or a closely related protein.

In contrast to the results with the *CYP2C9* promotor, gel shift assays showed that an HPF-1 like motif found in the upstream region of *CYP2C18* (oligo 2C18-649F) formed only two relatively minor bands and a lower nonspecific band with HepG2 nuclear extract in gel shift assays (Figure 4C). Neither band was supershifted by anti HNF-4, although the upper two bands were lost with the addition of apoCIII competitor DNA. The HPF-1 site of *CYP2C18* (5′-TTG*C-CC*TCAAAGTC*A*TATTTC-3′) differs from the consensus HPF-1 sequence (5′-XXX*RRR*XCAAAGTX*C*AXYYXX-3′) proposed by Venepally et al. (1992) and the HPF-1 site of *CYP2C9* (5′-AGT*GGG*TCAAAGTC*C*TTTCAG-3′) by the substitution of three cytosines at positons 4−6 and an adenine at position 15. We thus postulated that the variant cytosines may relatively weaken the HPF-1 protein binding configuration, thus preventing its recognition by nuclear proteins. Interestingly, mutation of the three cytosines to guanines in a 24-mer containing the 2C18 HPF-1 motif (oligo 2C18-649M) dramatically enhanced the binding of the nuclear proteins to the DNA in gel shift assays (Figure 4D). Incubation with antibody to rat HNF-4 supershifted the position of the mutated *CYP2C18* DNA−protein complex, and excess cold apoCIII oligo partially inhibited the strong binding of nuclear proteins to this modified sequence. Similarly, mutation of three guanines to cytosines in a short *CYP2C9* fragment containing the HPF-1 site abolished binding to HEPG2 nuclear extracts in gel shift assays (data not shown). These data indicate that the sequence of the HPF-1 like motif in the upstream region of *CYP2C18* differs sufficiently from the consensus HPF-1 sequences of *CYP2* genes to prevent it from functioning as a liver-specific element. Similarly, the transcriptional activities of rabbit *CYP2C1*, *CYP2C2*, and *CYP2C3* genes have been reported to correlate with their relative binding affinity for HNF-4 factor and appear to be dependent on a single nucleotide change in the HNF-4 motif (Chen et al., 1994b). Finally, nuclear extracts from two nonhepatic cell lines (NIH3T3 and Cos-1) produced no DNA−protein complexes in gel shift

assays when incubated with oligonucleotides corresponding to the HPF-1 site of *CYP2C9*, *CYP2C18*, or the modified *CYP2C18*, which is consistent with the liver-specific expression of *CYP2C9* (Wang et al., 1983).

The DNA contact point of the *CYP2C9 trans*-activator protein was evaluated by methylation interference analysis using a 95-bp PCR-amplified DNA fragment spanning the HPF-1 motif. The cleavage patterns of the gel-retarded and free DNA probes indicate that the methylation of guanine residues at −142, −148, and −149 on the sense strand and nucleotides −140 and −146 on the antisense strand completely interfered with protein binding (Figure 5). Partial interference was evident at −133 and −150 on the sense strand and on the antisense strand at nucleotide −139. The results further confirm that the binding site for the *CYP2C9 trans*-activator protein is at the HPF-1 site.

Evidence for trans-Activation of CYP2C9 but not CYP2C18 Promoter in Vivo by HNF-4. The importance of HNF-4 in *trans*-activation of the human *CYP2C9* promoter was tested by cotransfecting HepG2, NIH3T3, and Cos-1 cells with the *CYP2C9* promoter−luciferase constructs and an HNF-4 expression plasmid, pLEN4S, or its antisense counterpart pLEN4A (Sladek et al., 1991). Coexpression of p2C9/−155 with pLEN4S increased luciferase activity ~2-fold in HepG2 (Figure 6). In contrast, a 26-fold increase in activity was observed in NIH3T3 cells, and a 9-fold increase in Cos-1 cells, after cotransfection of p2C9/−155 with pLEN4S. No effect of the HNF-4 expression plasmid was observed in any of the three cell lines with the shorter p2C9/−140 construct, which lacks the HPF-1 site. A larger construct, p2C9/−362, exhibited higher promotor activity than p2C9/−155 in the extrahepatic cell lines. Cotransfection with the HNF-4 expression plasmid produced only a small increase in promotional activity with this construct. The reason for this anomaly is unclear; however, this plasmid contains additional transcriptional regulatory elements, including C/EBP and ubiquitous AP-1 factor sites, which may account for the promotional activity of the construct in these cells. In summary, the coexpression data suggest that HNF-4 or a similar protein is a transactivating protein contributing to the hepatic expression of the *CYP2C9* promoter.

As described above, mutation of the three cytosines to guanines in the inactive HPF-1 like motif of *CYP2C18* restored binding to an HNF4 like factor in HepG2 nuclear extracts in gel shift assays. However, when these bases were modified in the 1.3-kb *CYP2C18* promoter−luciferase construct and the modified construct 1.3Luc/hpfM was transfected into HepG2 cells, no change in promoter strength was observed (data not shown). Moreover, coexpression of the HNF-4 plasmid, pLEN4S, with the unmodified *CYP2C18* promotor (1.3Luc/E) or the modified 1.3Luc/hpfM did not increase promotional activity. The reason for the low promotional activity of this construct is not clear. However, the lack of a functional HPF-1 site in the *CYP2C18* promotor and the low promotional activity of the 5′-flanking region of this gene are consistent with the low expression of CYP2C18 protein in human liver and the low representation of *CYP2C18* cDNAs in human liver libraries compared to *CYP2C9* (Romkes et al., 1991; Goldstein et al., 1994).

In conclusion, the 5′-flanking region of the human *CYP2C9* gene has strong promoter activity in HepG2 cells compared to that of the 1.3-kb 5′-flanking region of the *CYP2C18* gene. An HPF-1 like motif present in the 2C9 promoter appears to be an important *cis*-acting element

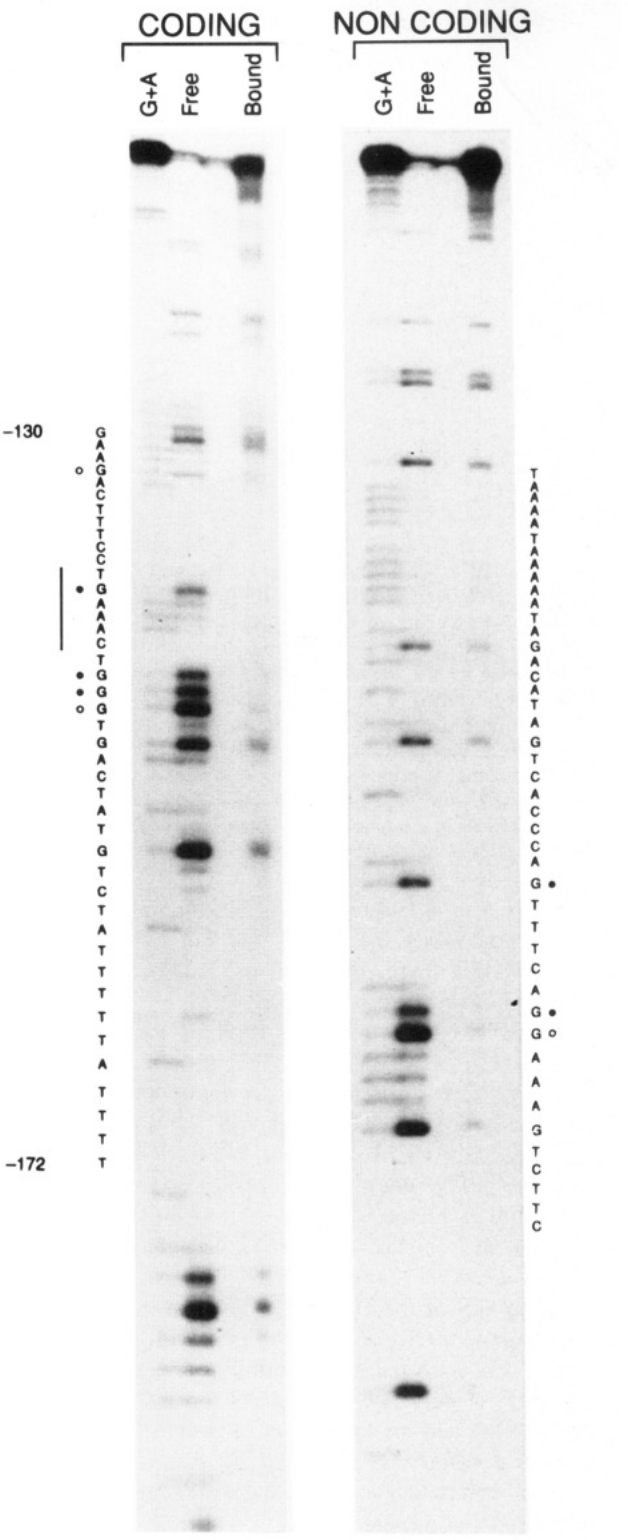

FIGURE 5: Localization of the protein binding site of *CYP2C9* by methylation interference footprinting. The coding and noncoding strands of *CYP2C9* DNA spanning the HPF-1 site were labeled at the 5′-end with ^{32}P, partially methylated by DMS, and used for binding with HepG2 nuclear extract as described in Experimental Procedures. The protein-associated and free DNA were resolved on polyacrylamide gel, recovered, and cleaved with piperidine. The cleavage products were separated on 6% sequencing gels. A Maxam−Gilbert sequence reaction for guanine and adenine residues was electrophoresed alongside the footprint reactions. Closed circles indicate residues that strongly interfered with DNA−protein interaction, and open circles show residues that partially interfered with protein binding.

contributing to the liver-specific expression of this gene. This was demonstrated by both deletional analysis and gel

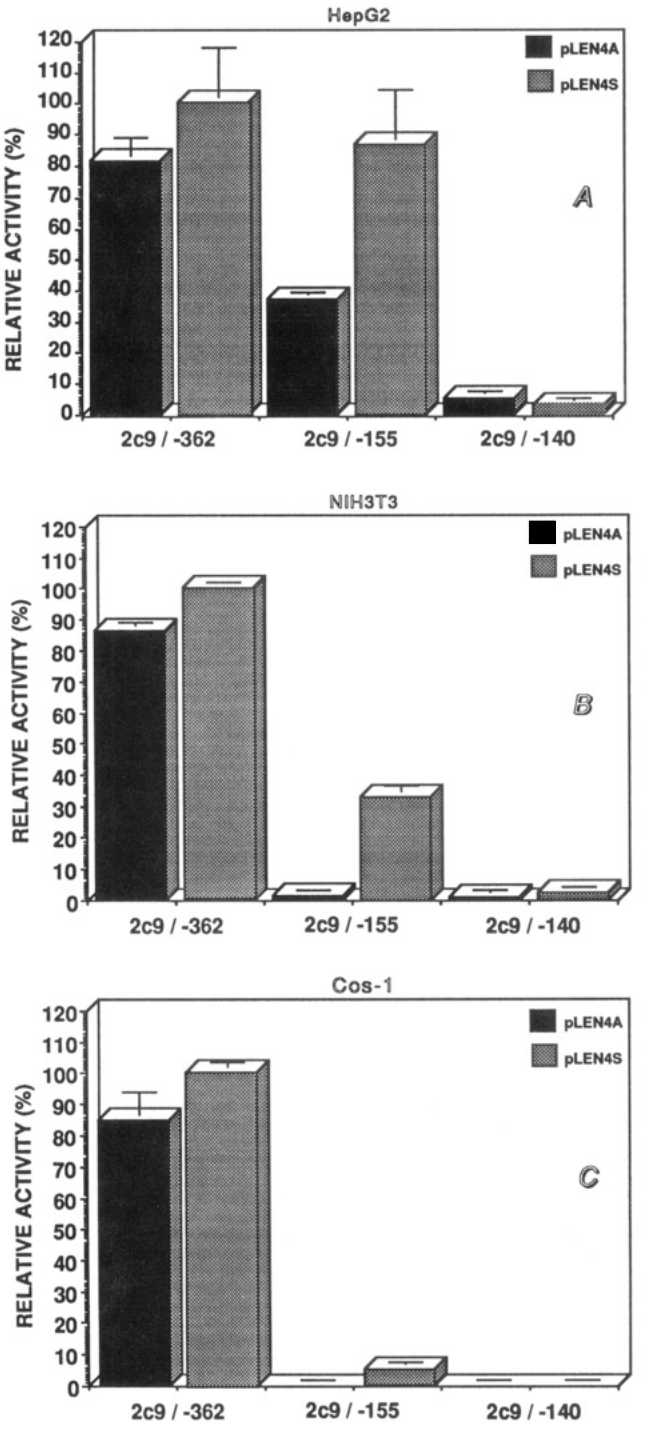

FIGURE 6: Coexpression of HNF-4 transcriptionally activates *CYP2C9* promoter constructs in hepatic and non-hepatic cell lines. (A) Luciferase activity in HepG2 cells. (B) Luciferase activity in NIH3T3 fibroblast cells. (C) Luciferase activity in Cos-1 cells. *CYP2C9* promoter-luciferase plasmids containing the HPF-1 domain (p2C9/−362, p2C9/−155) or missing the HPF-1 site (p2C9/−140) were coexpressed with HNF-4 plasmids cloned in the sense (pLEN4S) or antisense (pLEN4A) orientation. Transfection efficiency was normalized to β-galactosidase activity of pSV-β-galactosidase. The transcriptional activities shown are expressed relative to the activity of p2C9/−362 and pLEN4S in each cell line. Solid bars indicate activity obtained with pLEN4S cotransfection. Stippled bars represent activity obtained by cotransfection with the antisense plasmid, pLEN4A.

retardation assays. Antibody to HNF-4 produced a supershift of the complex formed between the HPF-1 domain and HepG2 nuclear extract. The HNF-4 consensus sequence of human apolipoprotein CIII gene effectively competed with the HPF-1 site in gel shift assays, and cotransfection of extrahepatic cells with HNF-4 increased promotor activity. These data indicate that HNF-4 or an identical protein is important in the liver-specific expression of *CYP2C9* gene. The substitution of three guanines to cytosines in a putative HPF-1 site of the upstream region of *CYP2C18* appears to prevent binding of liver nuclear proteins to this site and may contribute to the low expression of *CYP2C18* in human liver compared to *CYP2C9*.

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