MOLECULAR AND CELLULAR BIOLOGY, Oct. 1995, p. 5355–5362 Vol. 15, No. 10
0270-7306/95/$04.00+0

A DNA Methylation Site in the Male-Specific P450 (*Cyp* 2d-9) Promoter and Binding of the Heteromeric Transcription Factor GABP

NORIHIKO YOKOMORI,[1] RYUJI KOBAYASHI,[2] RICK MOORE,[1]
TATSUYA SUEYOSHI,[1] AND MASAHIKO NEGISHI[1]*

Pharmacogenetics Section, Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709,[1] and Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724[2]

Received 21 April 1995/Returned for modification 17 May 1995/Accepted 24 July 1995

The *Cyp* 2d-9 gene encodes the male-specific steroid 16α-hydroxylase in mouse liver and shares a conserved regulatory element ($^{-100}$TTCCGGGC^{-93}) with another male-specific Slp promoter. As shown with the Slp promoter (N. Yokomori, R. Moore, and M. Negishi, Proc. Natl. Acad. Sci. USA 92:1302–1306, 1995), the male-preferential demethylation also occurs at CpG/−97 in the *Cyp* 2d-9 promoter. The transcription factor which specifically binds to the demethylated element has been purified. The peptide sequences reveal that the factor consists of GABPα and GABPβ1 with Ets and Notch motifs, respectively. Both DNase I footprinting and gel shift assays indicate that the bacterially expressed glutathione *S*-transferase–GABP fusion proteins bind to the regulatory element only when CpG/−97 is demethylated. Moreover, *Cyp* 2d-9 promoter is *trans*-activated by coexpression of GABP proteins in HepG2 cells. Given the additional results that CpG/−50 of the female-specific steroid 15α-hydroxylase (*Cyp* 2a-4) promoter is preferentially demethylated in the females, the sex-specific expressions of the P450 genes correlate very well with DNA demethylation. We also conclude that GABP is a methylation-sensitive transcription factor and is a potential transcription activator of the male-specific *Cyp* 2d-9 promoter.

Sexually dimorphic gene expression in nonreproductive organs is one of the many basic biological processes observed from brain functions to hepatic steroid and drug metabolism. Of particular interest here are a number of the hepatic sex-specific genes such as P450s, sex-limited protein (Slp), mouse major urinary protein, and prolactin receptor. Among those, the sex-specific P450 genes often encode steroid hydroxylases, which implies their possible role in maintaining hormonal homeostasis. Recent studies have shown that the expression of the mouse sex-specific P450 genes (*Cyp* 2d-9 and *Cyp* 2a-4) is regulated transcriptionally (1). However, only limited information is now available to understand sex-specific transcription with respect to its regulation by a *cis*-acting DNA element and *trans*-acting factor because of the lack of an in vitro system which mimics functionally the sex-specific phenotypes.

Mouse *Cyp* 2d-9 and *Cyp* 2a-4 genes encode the male-specific steroid 16α- and female-specific steroid 15α-hydroxylases, respectively (20, 24, 32). We have previously defined a regulatory element (called SDI) in the *Cyp* 2d-9 promoter by in vitro transcription assay (37). Although its role as a sex-specific regulatory element has not been substantiated by a functional assay, our phylogenetic studies show that SDI is a unique element in the male-specific *Cyp* 2d-9 gene in mouse livers. The other subfamily members such as *Cyp* 2d-10 which are expressed in both sexes do not contain this element in their promoter sequences. Within the SDI, there is a regulatory sequence ($^{-100}$TTCCGGGC^{-93}) which is also found in another male-specific Slp gene. Interestingly, a potential CpG methylation site resides within this conserved sequence (CpG/−97 for *Cyp* 2d-9 and CpG/−121 for Slp). The female-specific *Cyp* 2a-4 promoter also has a GC-rich sequence ($^{-53}$GTCCG GCC^{-46}) with CpG/−50 as a potential methylation site. Our previous report has demonstrated that CpG/−121 of the male-specific Slp promoter is preferentially demethylated in the livers of male mice (36). These CpG sites in the sex-specific P450 genes, however, have not been investigated with respect to sexually dimorphic demethylation in accordance with their expression in mouse livers.

DNA methylation is known to regulate cellular physiology by altering gene expression. It is programmed in the growth and developmental processes (2, 6, 27, 29). In general, methylation is inversely associated with gene expression. For instance, cell transformation results in a high degree of DNA methylation (34), whereas demethylation of *myo* D or another regulatory gene leads to myogenesis (9). The parental imprinting of the H19 and Igf 2 receptor genes is regulated by allele-specific methylation; the promoters are methylated in the inactive paternal genes and demethylated in the active maternal copies (19). In addition, we have recently suggested DNA demethylation as a regulatory mechanism for sex-specific transcription (36). The CpG/−121 site in the regulatory element of the Slp promoter undergoes sexually dimorphic demethylation in accordance with its male-specific expression. Moreover, there appear to be two distinct nuclear factors which specifically bind to the methylated or demethylated element.

This paper extends our studies to examine whether the sex-specific *Cyp* 2d-9 and *Cyp* 2a-4 genes can also be regulated by DNA methylation. For this purpose, we first investigated methylation patterns of CpG sites of these P450 genes in male and female mice. We then purified and cloned the nuclear factor which binds to the demethylated regulatory element (TTCC GGGC) in the male-specific *Cyp* 2d-9 gene. These proteins were expressed in bacterial cells and used to characterize their

* Corresponding author. Mailing address: Pharmacogenetics Section, Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709. Phone: (919) 541-2404. Fax: (919) 541-0696.

binding to the element. Furthermore, the functional role of the proteins was examined by transient-transfection assay in HepG2 cells.

MATERIALS AND METHODS

Animals. Male and female CD-1 mice were obtained from Charles River Breeding Co. (Raleigh, N.C.).

Sequencing of the sodium bisulfite-treated promoter. Genomic DNAs were prepared from the livers of 2-day-old neonatal and 2-month-old CD-1 (Charles River) males and females. Sequencing of sodium bisulfite-treated DNA was performed as previously described (10). The following oligonucleotide primers were used to amplify the *Cyp* 2a-4 and *Cyp* 2d-9 promoters. For the −186 to −1 region of the *Cyp* 2d-9 promoter, 5′-GGGTCTAGATATTTGTTTATTATGT TTAGTTTA and 5′-GGGTCTAGAAAAACCCCAAACTTAAATCCTATC were used as 5′ and 3′ primers, respectively. For the −107 to +44 region of the *Cyp* 2a-4, 5′-GGGTCTAGAGGTGTATAATAGTGGGTAGTT and 5′-GGGT CTAGAAAAAATCCTAAAATCAACATA were used as the 5′ and 3′ primers, respectively. The underlined regions indicate the added *Xba*I site at ends of the amplified DNAs.

Construction of plasmids. To construct a series of p16αCAT plasmids, we amplified various sizes of the *Cyp* 2d-9 gene promoter with PCR kits (Perkin-Elmer Cetus), the 3′ primer (5′-GGGTCTAGAAACACTCAATACAACACC TA) in which the *Xba*I site (underlined) was created, and the following 5′ primers: p16α(−110)CAT, 5′-GGGGGCATGCCTCCTCCCTATTCCGGGC CA; p16α(−92)CAT, 5′-GGGGGCATGCCAAAACAAACACATATTTGG; and p16α(−110)/m(−97)CAT, 5′-GGGGGCATGCCTCCTCCCTATTCaGGG CCAAAACAAACAC. The newly created *Sph*I sites at the 5′ ends of the 5′ primers are underlined, while the mutated nucleotides at position −97 are shown in lowercase letters. We also amplified the *Cyp* 2a-4 promoter by using the specific primers to create the *Hin*dIII site at the 5′ end. We designed the 3′ primer (5′-AGACATCAAAACCAGGACGCTGAGGAA) so as to utilize the internal *Pst*I site at position +50 for a cloning site. The following were used as the 5′ primers: p15α(−71)CAT, 5′-GGGAAGCTTTGCATAATCAAGACCA AAGTC, and p15α(−45)CAT, 5′-GGGAAGCTTTTCTGTCTCTGGATGTAT AAA. Newly created *Hin*dIII sites are underlined. The amplified promoter DNAs of 16α were digested by *Sph*I and *Xba*I. The amplified promoter DNAs of 15α were digested by *Hin*dIII and *Pst*I. Then, these DNAs were ligated into the corresponding cloning sites of pCAT Basic vectors (Promega, Madison, Wis.). The plasmids were purified by CsCl centrifugation and verified by DNA sequencing.

Purification of GABP from mouse liver nuclear extracts. Liver nuclear proteins were prepared from 200 CD-1 male mouse livers by the method of Gorski et al. (13). About 100 μg of nuclear proteins was applied to a heparin-Sepharose column (1.6 cm [diameter] by 10 cm) and eluted with a gradient of NaCl from 0.05 to 1.0 M. The active fractions were determined by a gel mobility shift assay and concentrated with an Amicon YM-10 membrane. Further purification was done by DNA affinity magnetic beads (Dynabeads M-280 Sterptavidine; Dynal, Oslo, Norway) conjugated with the concatenated TTCCGGGC sequence. gatct $(TTCCGGGC)_5$g was annealed with 5′-a$(AAGGCCCG)_5$cctag and ligated into PSL1180 to prepare the concatenated DNA containing the 80 repeats. Purified proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The protein bands, first and third from the top, were excised from the gel and incubated with the buffer containing lysylendopeptidase. Peptides from this digestion were separated with a reverse-phase high-pressure liquid chromatography (HPLC) column, and amino acid sequences were analyzed with an ABI 477 protein sequencer. GABPα and GABPβ1 cDNAs were obtained from the mouse liver by reverse transcriptase-PCR. The following primers were used to amplify the GABPα and the GABPβ1 cDNAs. For the GABPα, 5′-ATGACTAAGAGAGAAGCAGA and 5′-AACCTTAAGACTCC CCAGGA were used as 5′ and 3′ primers, respectively. For the GABPβ1, 5′-ATGTCCCTGGTAGATTTGGG and 5′-CTAAACGGCTTCTTTGTTGG were used as the 5′ and 3′ primers, respectively. The amplified fragment was cloned into pCRII (Invitrogen) and sequenced. The amplified fragment of GABPα or GABPβ1 described above was also cloned into pCR3 (Invitrogen), used for cotransfection assay.

Bacterial expression and antibody production. The pCRII vector that has GABPα or GABPβ1 coding sequence was digested with the *Eco*RI site and ligated with *Eco*RI-digested pGEX-2T (Pharmacia Biotech). Ten milliliters of overnight culture of transformed bacteria was inoculated in 1 liter of Luria broth supplemented with ampicillin (100 μg/ml) and glucose (0.2%). IPTG (isopropyl-β-D-thiogalactopyranoside) was added to the final concentration of 0.1 mM after 3 h of incubation at 37°C. After incubation for 2 h, bacterial cells were harvested to purify glutathione *S*-transferase (GST) fusion protein with a glutathione-Sepharose 4B column (Pharmacia Biotech). Two hundred micrograms of each GST fusion protein was repeatedly injected into rabbits to raise antibodies.

DNase I footprinting. We performed DNase I footprinting with the Sure Track Footprinting kit (Pharmacia Biotech). The DNAs containing the proximal promoters and the 157-bp fragments (−142 to +15) from the P45016α gene were generated, end labeled with [γ-^{32}P]ATP (>5,000 Ci/mmol [Amersham, La Jolla, Calif.]) with T4 polynucleotide kinase, and purified by agarose gel electrophoresis. Methylated DNA was prepared with *Hpa*II methylase according to the supplier's protocol (New England Biolabs, Beverly, Mass.) and then end labeled. Labeled DNAs (30,000 cpm) were incubated with recombinant mouse GABPα and/or GABPβ1 in 50 μl of 10 mM Tris-HCl buffer (pH 7.5) containing 2.5 μg of poly(dI-dC), 50 mM NaCl, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM EDTA, and 5% glycerol for 30 min at room temperature. Then the DNAs were digested by 1 U of DNase I for 30 s, extracted with phenol-chloroform, and precipitated with ethanol. As the sequence marker, the corresponding DNA fragment was chemically cleaved at nucleotides G and A by the method of Maxam and Gilbert (21). Finally, the digested DNA samples were electrophoresed on an 8% polyacrylamide–7 M urea gel, dried, and exposed to X-ray films (Kodak XAR-5).

Gel shift assay. Fifty adult CD-1 mice were used to obtain nuclear extracts as described by Gorski et al. (13). Each oligonucleotide was annealed to its com-

Promoter	Sequence	Sex specificity
Slp:	$^{-133}$TCACGTGGTTTCCGGGC^{-117}	Male
Cyp 2d-9:	$^{-110}$CTCCTCCCTATTCCGGGC^{-93}	Male
Cyp 2a-4:	$^{-61}$AGACCAAAGTCCGGCC^{-46}	Female

FIG. 1. Sequence comparisons. The 5′-flanking sequences in the genes Slp (23), *Cyp* 2d-9 (33), and *Cyp* 2a-4 (20) are compared. The conserved sequence in the Slp and *Cyp* 2d-9 promoters and its corresponding sequence in the *Cyp* 2a-4 gene are underlined. The potential CpG sites are in boldface.

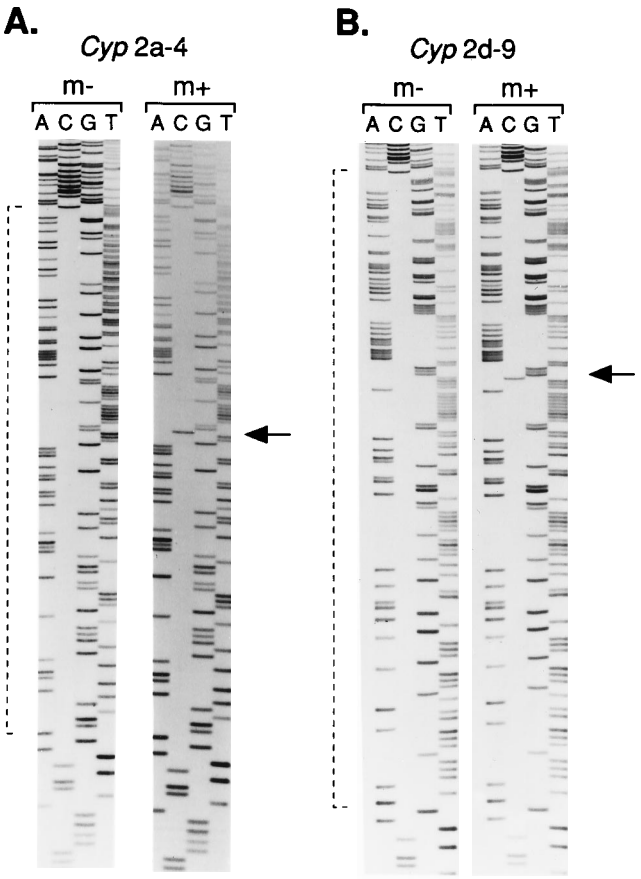

FIG. 2. DNA sequences of bisulfite-treated promoters. The promoter sequences with different methylation patterns are shown. They were amplified from the deaminated DNAs, cloned into M13 vectors, and sequenced. Methylated C remained as C on the sequencing gel, while demethylated C appeared as T. Accordingly, symbol m− or m+ indicates either methylated or demethylated promoter sequence, while the arrows denote CpG/−50 and CpG/−97 in the *Cyp* 2a-4 and *Cyp* 2d-9 promoter sequences, respectively, in Fig. 1. A bracket with a broken line represents the corresponding promoter sequences including these in Fig. 1.

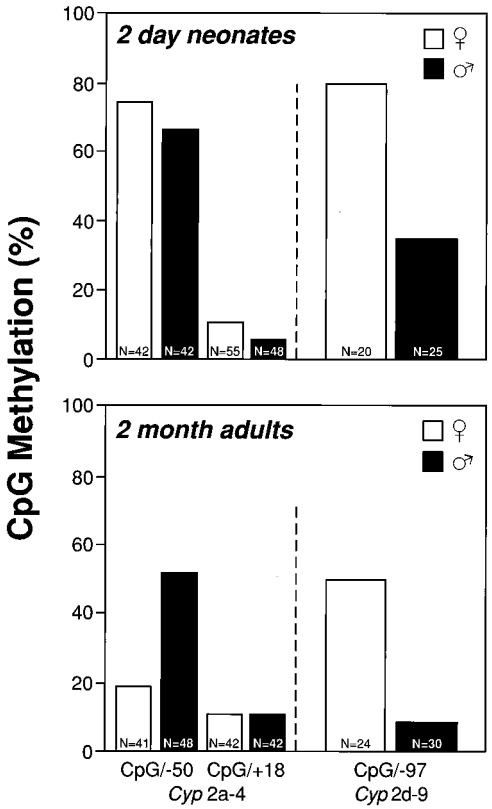

FIG. 3. Level of CpG methylation. The levels of methylation are determined as percentages of the total number of sequences (indicated by N at the bottom of each bar). The values were generated from the two independent bisulfite treatments and the two separate amplifications from each bisulfite-treated DNA sample.

plement and labeled by using [α-^{32}P]dATP (>6,000 Ci/mmol [Amersham]) and DNA polymerase Klenow fragment. Methylated oligonucleotides were prepared by including 5-methyl deoxycytidine (β-cyanoethyl-*N*, *N*-diisopropylamino) phosphoamidite (Pharmacia Biotech) during the appropriate cycle of synthesis. Each radioactive probe was incubated with 5 μg of nuclear proteins or GST fusion proteins of GABPα and GABPβ1 in 10 μl of 20 mM Tris-HCl buffer (pH 7.5) containing 1 μg of poly(dI-dC), 50 mM NaCl, 0.1 mM dithiothreitol, and 10% glycerol at room temperature. The following oligonucleotides were used in the studies as the probes: $(TTCCGGGC)_2$, $(TTCm^5CGGGC)_2$, $^{-112}$GGCTCCTCC CTATTCCGGGC^{-92} (for 2d-9 promoter), and $^{-61}$AGACCAAAGTCCGGCC TTCT^{-42} (for 2a-4 promoter).

Cell culture, transfection, and chloramphenicol acetyltransferase (CAT) activity. HepG2 cells were cultured in modified Eagle's medium (Gibco, Grand Island, N.Y.) supplemented with 10% fetal bovine serum, 100 U of penicillin per ml, and 100 ng of streptomycin per ml at 37°C. At 24 h prior to transfection, HepG2 cells were transferred to plastic plates (6-cm diameter). With the Cellphect transfection kit (Pharmacia Biotech), each p16α-CAT (2 μg) or p15α-CAT (2 μg) or pCAT Basic (2 μg) DNA was transfected or cotransfected with pCR3-α (1 μg) and/or pCR3-β1 (1 μg) into the HepG2 cells at 37°C for 12 h. The total amount of transfected DNA was adjusted to 4 μg by adding a carrier DNA. Transfected cells were then washed twice with phosphate-buffered saline solution and cultured in the medium at 37°C for 40 h. To measure CAT activity, the cells were lysed by freezing and thawing and the cell extract (5 μg of protein) was incubated with [^{14}C]chloramphenicol according to the method of Gorman et al. (12).

RESULTS

Potential methylation sites in the sex-specific P450 promoters. Promoter sequences of the male-specific *Cyp* 2d-9 and Slp genes and the female-specific *Cyp* 2a-4 genes are compared in Fig. 1. The male-specific *Cyp* 2d-9 gene contains a GC-rich element (TTCCGGGC) which is also conserved in the other male-specific Slp promoter. This conserved element provides a potential methylation site: CpG/−97 and CpG/−121 in the *Cyp* 2d-9 and Slp promoters, respectively (23, 32). Similarly, the GC-rich sequence (GTCCGGCC) of the female-specific *Cyp* 2a-4 promoter also includes a CpG site at position −50 (20). Given our previous finding that the CpG/−121 of the Slp promoter undergoes demethylation preferentially in males (36), we have here examined whether the CpG sites of the P450 promoters also exhibit sexually dimorphic demethylation.

Developmental and sexually dimorphic demethylation. For this purpose, genomic DNAs were prepared from mouse livers and treated with sodium bisulfite. Subsequently, the bisulfite-treated P450 promoters were amplified and sequenced to determine the methylation levels at the CpG/−97 and CpG/−50 sites by the method of Frommer et al. (10). The sequences are depicted in Fig. 2, and Fig. 3 summarizes the methylation levels at each site in the neonatal and adult CD-1 males and females. In accordance with male-specific expression, the CpG/−97 site of the *Cyp* 2d-9 promoter was preferentially demethylated in the livers of male mice. Note that more than 50% of the site was already demethylated in male neonates and the demethylation was nearly complete in adult males. On the other hand, demethylation of the CpG/−97 site occurred to a much lesser degree in both neonatal (less than 20%) and adult (45%) females compared with males. As a result, the level of methylation became sevenfold lower in adult males than females. This male-preferential demethylation of CpG/−97 in the *Cyp* 2d-9 gene is reminiscent of that of the corresponding CpG/−121 in the male-specific Slp gene. In contrast, the CpG/−50 site in the female-specific *Cyp* 2a-4 promoter exhibited a female-preferential demethylation (Fig. 2). The heavily and equally methylated CpG/−50 in both female and male neonates underwent developmental demethylation, and the methylation resulted in a threefold lower level in adult females than in males. CpG/+18 of the *Cyp* 2a-4 gene, however, was always demethylated heavily (over 90%) regardless of sex and age, which therefore, provided an excellent internal control for the demethylations at the CpG/−50 and CpG/−97 sites. The results indicate that the sex-specific P450 promoters contain a

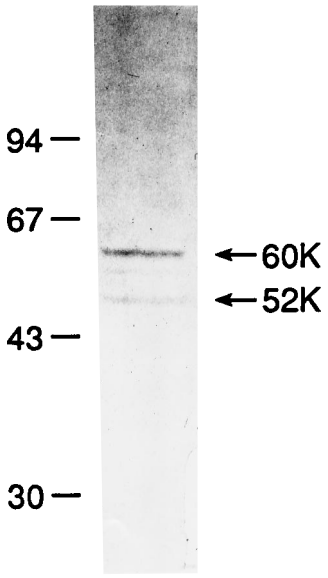

FIG. 4. Purified fraction on SDS-polyacrylamide gel. A 10% polyacrylamide gel was used. Arrows and numbers indicate the migrations and sizes (in kilodaltons) of the maker proteins (obtained with a Molecular Weight Calibration LMW kit from Pharmacia Biotech).

TABLE 1. Sequences of peptides derived from the purified proteins[a]

Protein sample	Sequence	Corresponding residues in:	
		GABPα	GABPβ1
60K	REAEELIEIEIDG	4–16	
	LNQPELVAQK	49–359	
52K	VDRTPLHMAA		70–80
	EQEAE		359–363

[a] Lysylendopeptidase-digested peptides were separated by HPLC, sequenced, and compared with those reported by LaMarco et al. (18).

CpG site which undergoes sexually dimorphic demethylation in accordance with their expression.

Purification, cloning, and expression of methylation-sensitive factor. Both CpG sites, CpG/−121 and CpG/−97 in the conserved regulatory element (TTCCGGGC) of the male-specific *Cyp* 2d-9 and Slp promoters, respectively, exhibited the male-preferential demethylation. Our previous studies showed that there are two distinct nuclear factors which bind specifically either to the demethylated or to the methylated element (36). In order to investigate the roles that the regulatory element and the CpG methylation play in transcription, we purified nuclear factor which binds to the demethylated element (TTCCGGGC). A DNA affinity-purified factor appeared as two major bands (60 and 52 kDa) and an additional faint band (58 kDa) on SDS gel electrophoresis (Fig. 4). The amino acid sequences of tryptic peptides derived from the 60K and 52K proteins were identical to the corresponding regions of the heteromeric factors GABPα and GABPβ1, respectively (Table 1), indicating that the purified factor is a mixture of GABPα

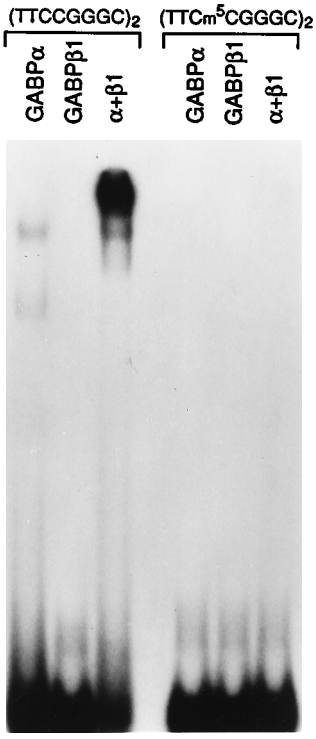

FIG. 5. Binding of bacterially expressed GABP. The demethylated probe or the methylated probe was incubated with 100 ng of the bacterially expressed GST-GABP fusion protein.

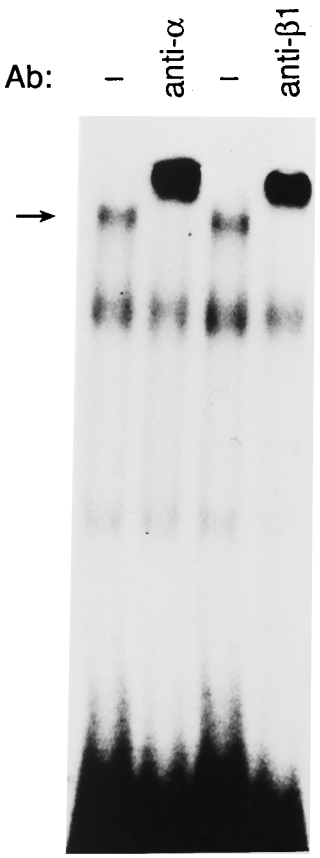

FIG. 6. Supershift by anti-GABP of DNA-protein complex. Liver nuclear extract was incubated with antiserum (1 μl) prior to the addition of radioactive probe $(TTCCGGGC)_2$. Preimmune serum (1 μl) was added to the reaction mixture for control, indicated by a minus sign on the top of wells. The arrow shows the specific complex which was supershifted by antibody (Ab). The band below is a nonspecific complex.

(60K) and GABPβ1 (52K). In order to examine whether GABP can bind to the promoters and regulate transcription, using reverse transcriptase-PCR, we cloned cDNAs encoding the α and β1 subunits, ligated them into pGEX vectors, and expressed GABP as GST hybrids in bacterial cells.

Direct binding of bacterially expressed GABP proteins. Figure 5 shows gel shift assays using the bacterially expressed GABP and the demethylated or the methylated $(TTCCGGC)_2$ as a probe. The bacterially expressed GST-GABPα bound to the demethylated element weakly alone and strongly in the presence of GST-GABPβ1. As expected, this binding was sensitive to DNA methylation as it was not observed with the methylated element. The results indicate that GABP can bind to the *Cyp* 2d-9 promoter depending on the demethylation. Supershift assays using anti-GABPα and anti-GABPβ1 verified that the binding proteins in nuclear extracts were, in fact, GABPα and GABPβ1 (Fig. 6). Subsequently, a direct binding of GABP to the *Cyp* 2d-9 promoter was also examined by DNase I footprinting (Fig. 7A). In the P450 promoter, the bacterially expressed GST-GABP protected the −110 to −93 region in which the conserved element ($^{-100}$TTCCGGGC^{-92}) is included at the 3′ end; the protected sequence was extended 11 more bp upstream from the conserved element. When the methylated promoter (at CpG/−97) was used as a DNA template, however, this protection was not observed. In addition to the footprintings, gel shift assay also showed that

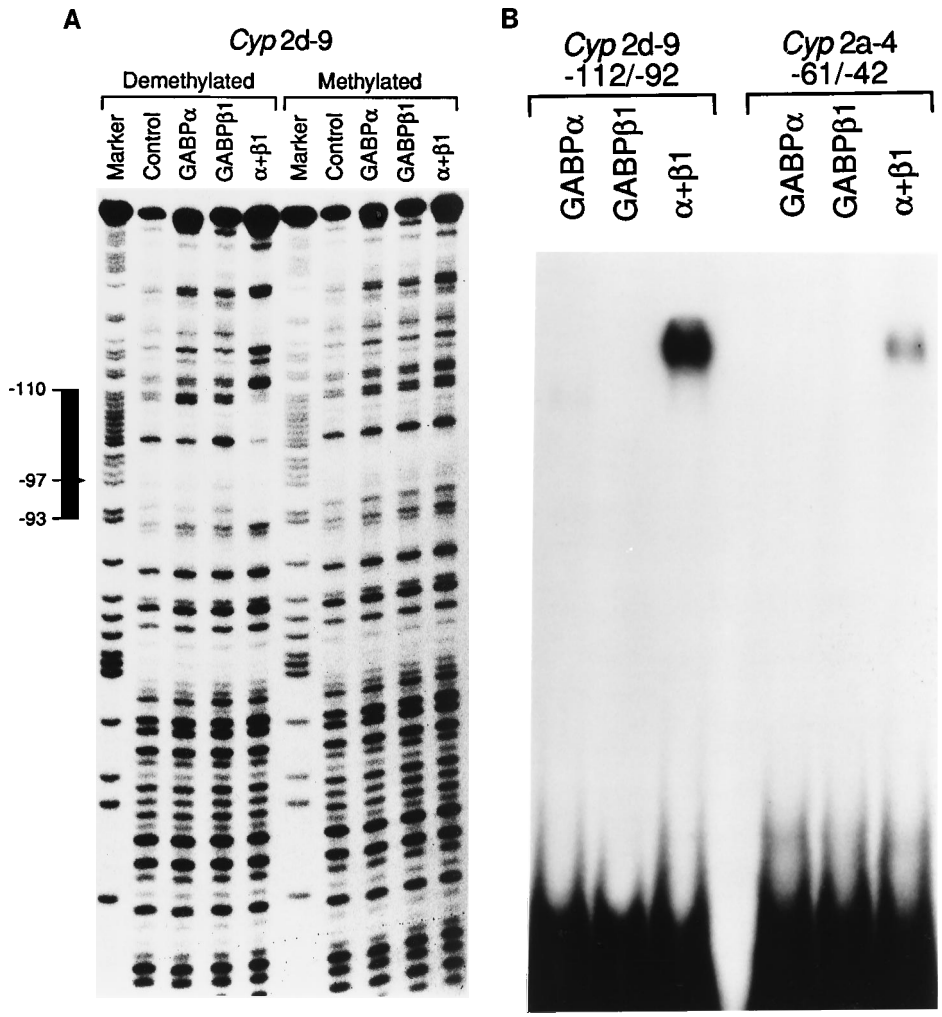

FIG. 7. Direct binding of GABP to *Cyp* 2d-9 promoter. (A) Footprints were obtained by DNase I digestion of the demethylated or the methylated DNA template in the presence or absence of the bacterially expressed GST-GABP fusion proteins (1 μg each). The protected region is indicated by a closed bar with the 5′ and 3′ positions. The arrow shows nucleotide C at position −97. (B) A gel shift assay was performed with oligonucleotides derived from the P450 promoter sequences and the bacterially expressed GST-GABP fusion proteins (100 ng each).

the sequence (−112 to −92) bound to the bacterially expressed GABP (Fig. 7B). The GABP, on the other hand, exhibited only a weak binding to a *Cyp* 2a-4 promoter sequence (−61 to −42). The results, therefore, indicate that GABP is a sequence-specific and methylation-sensitive transcription factor which specifically binds to the *Cyp* 2d-9 promoter.

Activation by GABP proteins of the *Cyp* 2d-9 promoter. By transient transfection assay, *trans*-activation by GABP proteins of the *Cyp* 2d-9 and the *Cyp* 2a-4 promoters was examined in HepG2 cells. For that, we constructed plasmid p16α(−110) CAT, which contained the whole region protected by GABP including the conserved element. The two additional plasmids p16α(−110)mCAT and p16α(−92)CAT were constructed to eliminate from the promoter sequence the binding ability for GABP. In the former plasmid, the nucleotide at position −97 was mutated from C to A, while in the latter one the conserved element was deleted. Transcriptional activity of p16α(−110) CAT was increased approximately 12-fold by simultaneous cotransfections of both GABPα and GABPβ1 (Fig. 8A). On the other hand, neither p16α(−110)mCAT nor p16α(−92)CAT was *trans*-activated to the same degree as was p16α(−110) CAT. Only three- to fourfold activity increases were observed with these constructs. Inherent GABP-binding sequences in the expression vector might have caused the low activation as transcriptional activity of pCAT-Basic was also increased approximately threefold by the coexpressions (Fig. 8B). As expected from its weak binding to GABPs, the female-specific *Cyp* 2a-4 promoter was not activated higher than the control levels (Fig. 8B). The results indicate that GABPs can activate the male-specific *Cyp* 2d-9 but not the female-specific *Cyp* 2a-4 promoter.

DISCUSSION

Sexual dimorphism of some of the hepatic steroid metabolism in rodents was first reported more than 30 years ago by Yates et al. (35). Gustafsson and his associates proposed pituitary-liver axis as a regulatory mechanism of the sex-specific metabolism and showed that growth hormone is one which directly regulates expression of sex-specific P450 genes which catalyze steroid hydroxylation in rat livers (15, 22, 38). Our studies indicate that growth hormone transcriptionally regulates sex-specific P450 in mouse livers (1). Furthermore, neonatal androgen exposure, a developmental process called neo-

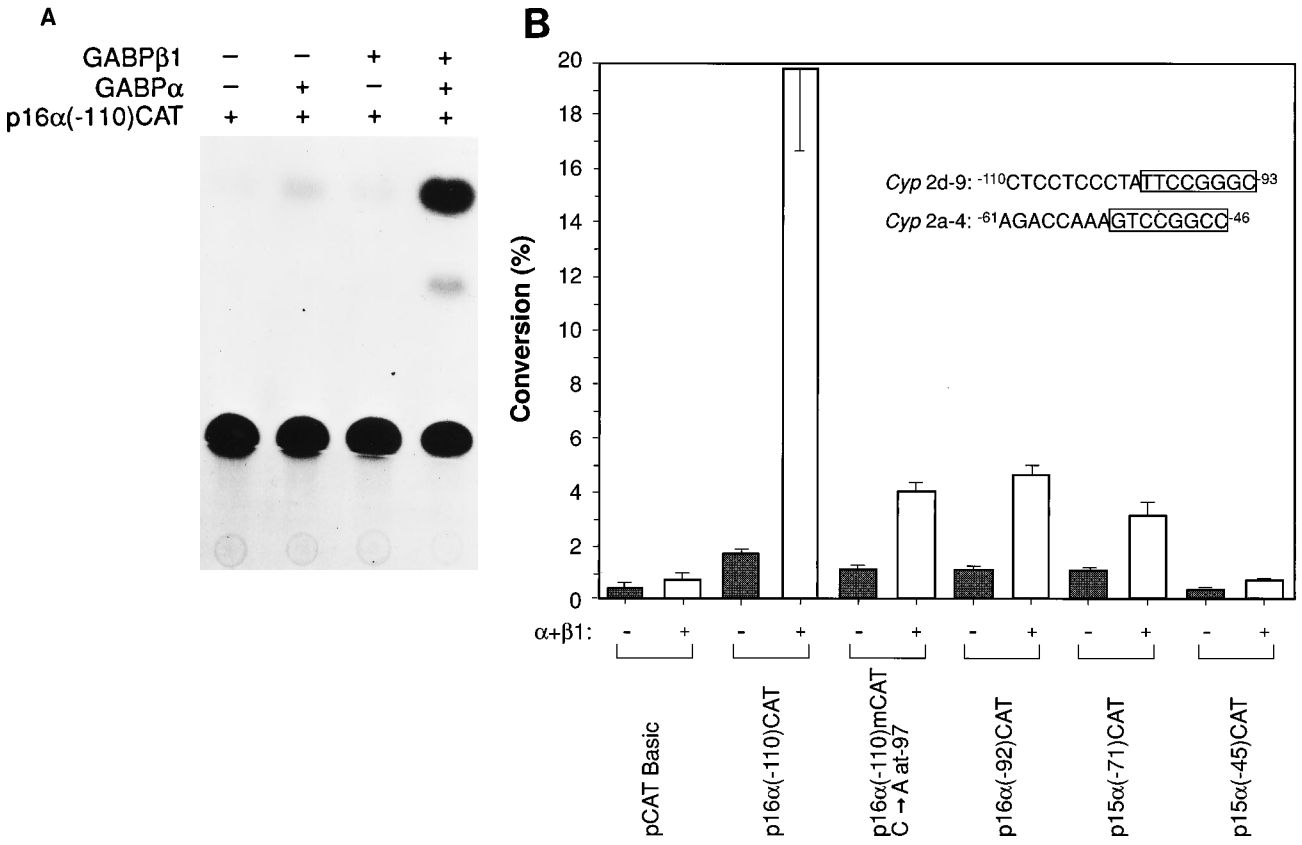

FIG. 8. *trans*-Activation by GABP of *Cyp* 2d-9 but not *Cyp* 2a-4 promoter. (A) HepG2 cells were cotransfected by various combinations of the expression vectors (pCR3-α and pCR3-β1). CAT activity of the transfected cells was measured by thin-layer chromatography. (B) The CAT plasmids containing 5′-progressive deleted or mutated promoters were cotransfected in the presence (open bar) and the absence (closed bar) of both pCR3-α and pCR3-β1. The promoters of *Cyp* 2d-9 and *Cyp* 2a-4 are represented by symbols p16α and p15α, respectively. Boxes indicate the conserved element in the *Cyp* 2d-9 promoter and the similar sequence in the *Cyp* 2a-4 promoter.

natal imprinting predetermines, at least in part, the sex specificity of P450 gene expression in adulthood (8, 14, 33). The mechanism of the imprinting is virtually unknown.

Although no consensus exists at this point with respect to *cis*-acting elements which regulate sex-specific transcription, there have been intensive studies conducted to define them. Sundseth et al. (26) have recently reported that there are sex-specific hypersensitive sites in the proximal region of the male-specific CYP2C11 and the female-specific CYP2C12 promoters in rat livers. Most recently, Strom et al. (25) have observed a sex-dependent binding of nuclear protein to a region (positions −107 to −95) of the CYP2C11 promoter. Interestingly, the previously defined SDI element specific to the *Cyp* 2d-9 gene is located in the region corresponding to these sex-specific sites of the rat P450 genes (36). Unlike those of the rat P450 genes, however, sex-specific binding was not observed reproducibly with the mouse *Cyp* 2d-9 gene. Besides a sex-specific nuclear factor, DNA demethylation can provide an additional mechanism for sex-specific transcription, as with the Slp promoter (36). Our present findings indicate that the sex-specific mouse P450s, *Cyp* 2d-9 and *Cyp* 2a-4, also exhibit the sexually dimorphic demethylations at their CpG sites. Most significantly, the male-specific *Cyp* 2d-9 promoter is preferentially demethylated in the males, while the female-specific *Cyp* 2a-4 promoter is preferentially demethylated in the females. These CpGs which exhibit the differential demethylations are sequence specific. Unlike the CpG/−50, CpG at position +18 of the *Cyp* 2a-4 gene is consistently demethylated regardless of the sex and age of mice. The most striking observation is the difference in CpG/−121 and CpG/−119 of the Slp and C4 genes in the demethylation pattern (36). The nucleotide sequences around these CpG sites differ by only one base: C^{-121}CGGGC and CCC^{-119}GGC in the Slp and C4 genes, respectively. In spite of this remarkable sequence similarity, CpG/−121 exhibits sexually dimorphic demethylation, while CpG at position −119 does not. Thus, the sequence-specific DNA demethylation is generally correlated with the expression of the sex-specific P450 (*Cyp* 2a-4 and *Cyp* 2d-9) genes as well as that of the male-specific Slp promoter. In addition to SDI, our previous in vitro transcription studies identified another regulatory element (designated CTE) in the *Cyp* 2d-9 promoter (35). While SDI is a strong element which is unique to the male-specific *Cyp* 2d-9 gene, CTE is a weak one which is also present in the non-sex-specific *Cyp* 2d-10 gene. Taking the fact that SDI is the major regulatory element for the *Cyp* 2d-9 promoter into consideration, finding the putative CpG methylation and GABP binding sites within the SDI sequence thus strongly suggests a possibility that those sites may be essential for male-specific transcription. It will be extremely interesting and important to delineate a role that the sexually dimorphic DNA demethylation may play in the growth hormone-dependent transcription and neonatal imprinting.

We have demonstrated that the heteromeric transcription factor GABP can bind to the sequence of the *Cyp* 2d-9 pro-

moter only when CpG at position −97 within the conserved element (TTCCGGGC) is demethylated. GABP consists of the two subunits α and β1; GABPα is a weak DNA-binding protein with an Ets domain (28). GABPβ1 contains a Notch-related structural motif and forms a heterodimer with the α subunit. Although GABPβ1 is not capable of binding DNA directly, it dramatically increases the DNA binding affinity of GABPα by forming a heterodimer. McKnight and his associates determined the pentanucleotide 5′-CGGA(A or T) as the core of the DNA binding site of the transcription factors with an Est motif, including GABP (4). Moreover, the binding affinity also depended on the type of nucleotide at the 3′ outside of this core. For example, the hexanucleotide 5′-CGGAA(A or G)-3′ was identified as the GABP binding site of the herpes simplex virus promoter (18, 28). Its reverse complement ($^{-101}$ATTCCG^{-96}) is present in a regulatory element of the *Cyp* 2d-9 promoter, except that the 5′-variable nucleotide (T or C) is substituted with an A. The corresponding GABP-binding sequence in the other male-specific Slp promoter contains a hexanucleotide ($^{-125}$TTTCCG^{-120}) the exact reverse of that in the herpes simplex virus promoter. Comparison of DNase I footprinting on the *Cyp* 2d-9 promoter with that on the Slp promoter has indicated that this 5′ nucleotide A weakens GABP binding to the former promoter (unpublished observation). In addition, the hexanucleotide is not repeated in a regulatory element of these promoters, although it appears to be essential for GABP binding to the herpes simplex virus promoter (28). The binding sequences are also not repeated in either the adenovirus early-region 4 (E4) promoter or the E1A core enhancer (3, 30, 31). Additionally, GABP is known to bind to various cellular promoters including the mitochondrial COXIV gene (5) and the ribosomal protein genes, rpL30 and rpL32 (11). GABP may, therefore, play diverse roles in transcriptional regulation of sex-specific and housekeeping genes depending on the binding sequence.

There are various methylation-sensitive transcription factors such as AP-2, CREB/ATF, and NF-κB (27). We have demonstrated that GABP does not bind to the methylated element of the *Cyp* 2d-9 promoter. This methylation sensitivity is not due to its lower affinity for the $^{-101}$ATTCCG^{-96} sequence in the promoter, because GABP is also not able to bind to the sequence $^{-125}$TTTCCG^{-120} in the Slp promoter, which is a high-affinity binding site (unpublished observation). Our present study, therefore, has added GABP to the list of factors sensitive to methyl-CpG. Although the role of a single CpG methylation in transcription remains enigmatic, there are some reports to support this. AP-2, for example, regulates the cyclic AMP-dependent transcription of the human proenkephalin gene depending on the demethylation of its cognate element (7, 16). Myeloid-specific transcription of the mouse M lysozyme gene is also reported to be regulated by a single CpG methylation within the enhancer (17). Our present results show that CpG methylation provides a novel mechanism for regulating the GABP-dependent transcriptional activation of genes such as *Cyp* 2d-9 and Slp which contain the putative methylation sites such as that of the factor binding motif. It should, however, be noted that since GABP binding motifs do not always contain CpG, methylation is not a general mechanism to regulate the GABP-dependent transcriptional activation.

In conclusion, the CpG sites in promoters of the sex-specific P450 genes exhibit the sex-preferential demethylation which is correlated with their expression in the livers of mice. The heteromeric transcription factor GABP can *trans*-activate the male-specific *Cyp* 2d-9 promoter through the regulatory element (TTC^{-97}CGGGC) in HepG2 cells. Moreover, the binding of GABP is sensitive to methyl-CpG at position −97. GABP, on the other hand, neither binds nor activates the female-specific *Cyp* 2a-4 promoter in HepG2 cells.

REFERENCES

1. **Aida, K., and M. Negishi.** 1993. A trans-acting locus regulates transcriptional repression of the female-specific steroid 15α-hydroxylase ($P450_{15\alpha}$) gene in male mice. J. Mol. Endocrinol. **11:**231–240.
2. **Bird, A.** 1992. The essentials of DNA methylation. Cell **70:**5–8.
3. **Bolwing, G. M., J. T. Bruder, and P. Hearing.** 1992. Different binding site requirements for binding and activation for the bipartite enhancer EF-1A. Nucleic Acids Res. **20:**6555–6564.
4. **Brown, T. A., and S. T. McKnight.** 1992. Specificities of protein-protein and protein-DNA interaction of GABPα and two newly defined est-related proteins. Genes Dev. **6:**2502–2512.
5. **Carter, R. S., and N. G. Avadhani.** 1994. Cooperative binding of GA-binding protein transcription factors to duplicated transcription initiation region repeats of the cytochrome c oxidase subunit IV gene. J. Biol. Chem. **269:** 4381–4387.
6. **Cedar, H.** 1992. DNA methylation and gene activity. Cell **90:**3–4.
7. **Comb, M., and H. M. Goodman.** 1990. CpG methylation inhibits proenkephalin gene expression and binding of the transcription factor AP-2. Nucleic Acids Res. **18:**3975–3982.
8. **Dannan, G. A., E. P. Guengerich, and D. J. Waxman.** 1986. Hormonal regulation of rat liver microsomal enzymes. Role of gonadal steroids in programming, maintenance, and suppression of delta 4-steroid 5 alpha-reductase, flavin-containing monooxygenase, and sex-specific cytochromes P450. J. Biol. Chem. **261:**10728–10735.
9. **Davis, R. L., H. Weintraub, and A. B. Lassar.** 1987. Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell **51:**987–1000.
10. **Frommer, M., L. E. McDonald, D. S. Millar, C. M. Collis, F. Watt, G. W. Grigg, P. L. Molloy, and C. Paul.** 1992. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc. Natl. Acad. Sci. USA **89:**1827–1831.
11. **Genuario, R. R., D. E. Kelley, and P. P. Perry.** 1993. Comparative utilization of transcription factor GABP by the promoters of ribosomal protein genes rpL30 and rpL32. Gene Exp. **3:**279–288.
12. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2:**1044–1051.
13. **Gorski, K., M. Carneiro, and U. Schibler.** 1986. Tissue-specific in vitro transcription from the mouse albumin promoter. Cell **47:**767–776.
14. **Gustafsson, J.-A., G. Mode, G. Norstedt, and P. Skett.** 1983. Sex steroid induced changes in hepatic enzymes. Annu. Rev. Physiol. **45:**51–60.
15. **Gustafsson, J.-A., and A. Stenberg.** 1976. On the obligatory role of the hypophysis in sexual differentiation of hepatic metabolism in rats. Proc. Natl. Acad. Sci. USA **73:**1462–1465.
16. **Iguchi-Ariga, S. M. M., and W. Schaffner.** 1989. CpG methylation of the cAMP-responsive enhancer/promoter sequence TGACGTCA abolishes specific factor binding as well as transcriptional activation. Genes Dev. **3:**612–619.
17. **Klages, S., B. Mollers, and R. Renkawitz.** 1992. The involvement of demethylation in the myeloid-specific function of the mouse M lysozyme gene downstream enhancer. Nucleic Acids Res. **20:**1925–1932.
18. **LaMarco, K., C. C. Thompson, B. P. Byers, E. M. Walton, and S. T. McKnight.** 1991. Identification of Ets- and Notch-related subunits in GA binding protein. Science **253:**789–792.
19. **Li, E., C. Beard, and R. Jaenisch.** 1993. Role of DNA methylation in genomic imprinting. Nature (London) **366:**362–365.
20. **Lindberg, R., B. Burkhart, T. Ichikawa, and M. Negishi.** 1989. The structure and characterization of type I $P450_{15\alpha}$ gene as major steroid 15α-hydroxylase and its comparison with type II $P450_{15\alpha}$ gene. J. Biol. Chem. **264:**6465–6471.
21. **Maxam, A. M., and W. Gilbert.** 1977. A new method for sequencing DNA. Proc. Natl. Acad. Sci. USA **74:**560–564.
22. **Mode, A., G. Norstedt, B. Simic, P. Eneroth, and J.-A. Gustafsson.** 1981. Transcriptional and posttranscriptional regulation of sexually differentiated rat liver cytochrome P-450 by growth hormone. Endocrinology **108:**2103–2108.
23. **Nonaka, M., H. Kimura, Y. D. Yeul, S. Yokoyama, K. Nakayama, and M. Takahasi.** 1986. Identification of the 5′-flanking regulatory region responsible for the difference in transcriptional control between mouse complement C4 and Slp genes. Proc. Natl. Acad. Sci. USA **83:**7883–7887.
24. **Noshiro, M., and M. Negishi.** 1986. Pretranslational regulation of sex-dependent testosterone hydroxylases by growth hormone in mouse liver. J. Biol. Chem. **261:**15923–15927.
25. **Strom, A., H. Eguchi, A. Mode, C. Legraverend, P. Tollet, P.-E. Stromstedt, and J.-A. Gustafsson.** 1994. Characterization of the proximal promoter and two silencer elements in the CYP2C11 gene expressed in rat liver. DNA Cell Biol. **13:**805–819.
26. **Sundseth, S. S., E. T. Alberta, and D. J. Waxman.** 1992. Sex-specific, growth hormone-regulated transcription of the cytochrome P450 2C11 and 2C12 genes. J. Biol. Chem. **267:**3907–3914.
27. **Tate, P. H., and A. P. Bird.** 1993. Effects of DNA methylation on DNA-

binding proteins and gene expression. Curr. Opin. Genet. Dev. **3:**226–231.
28. **Thompson, C. C., T. A. Brown, and S. T. McKnight.** 1991. Convergence of Ets- and Notch-related structural motifs in a heteromeric DNA binding complex. Science **253:**762–768.
29. **Tilghman, S.** 1993. DNA methylation: a phoenix rises. Proc. Natl. Acad. Sci. USA **90:**8761–8762.
30. **Watanabe, H., J. Sawada, K. Yano, K. Yamaguchi, M. Goto, and H. Handa.** 1993. cDNA cloning of transcription factor E4TF1 subunits with Est and Notch motifs. Mol. Cell. Biol. **13:**1385–1391.
31. **Watanabe, H., T. Wada, and H. Handa.** 1990. Transcriptional factor E4TF1 contains two subunits with different functions. EMBO J. **9:**841–847.
32. **Wong, G., T. Itakura, K. Kawajiri, L. Skow, and M. Negishi.** 1989. Gene family of male-specific testosterone 16α-hydroxylase (C-P-$450_{16\alpha}$) in mice: organization, differential regulation, and chromosome localization. J. Biol. Chem. **264:**2920–2927.
33. **Wong, G., K. Kawajiri, and M. Negishi.** 1987. Gene family of male-specific testosterone 16α-hydroxylase (C-P-$450_{16\alpha}$) in mouse liver: cDNA sequence, neonatal imprinting and reversible regulation by androgen. Biochemistry **26:**8683–8690.
34. **Wu, J., J.-P. Issa, J. Herman, D. E. Bassett, Jr., B. Nelkin, and S. B. Baylin.** 1993. Expression of an exogeneous eukaryotic DNA methyl-transferase gene induces transformation of NIH 3T3 cells. Proc. Natl. Acad. Sci. USA **90:** 8891–8895.
35. **Yates, F. E., A. L. Herbst, and J. Urquhart.** 1958. Sex difference in rate of ring A reduction of Δ^4-3-keto-steroids *in vitro* by rat liver. Endocrinology **63:**887–902.
36. **Yokomori, N., R. Moore, and M. Negishi.** 1995. Sexually dimorphic DNA demethylation in the promoter of the Slp (sex-limited protein) gene in mouse liver. Proc. Natl. Acad. Sci. USA **92:**1302–1306.
37. **Yoshioka, H., M. Lang, G. Wong, and M. Negishi.** 1991. A specific cis-acting element regulates *in vitro* transcription of sex-dependent mouse steroid 16α-hydroxylase (C-P$450_{16\alpha}$) gene. J. Biol. Chem. **265:**14612–14617.
38. **Zaphiropoulos, P. G., A. Mode, G. Norstedt, and J.-A. Gustafsson.** 1989. Regulation of sexual differentiation in drug and steroid metabolism. Trends Pharmacol. Sci. **10:**149–154.