*Proc. Natl. Acad. Sci. USA*
Vol. 82, pp. 2024–2028, April 1985
Biochemistry

# Sex-dependent expression of mouse testosterone 16α-hydroxylase (cytochrome P-450$_{16\alpha}$): cDNA cloning and pretranslational regulation

(sexual dimorphisms/recombinant DNA/hybrid protein)

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*Communicated by Paul J. Kramer, December 6, 1984*

**ABSTRACT** **By using both double-colony hybridization and an *in situ* immunostaining assay for transformants, 39 cDNA clones (clone p-16α) encoding mouse liver microsomal testosterone 16α-hydroxylase (cytochrome P-450$_{16\alpha}$) were isolated from a cDNA library constructed in the cloning vector pUC-9 with poly(A)$^+$ RNA immunoenriched from total liver polysomes of male 129/J mice. mRNA selected by hybridization with clone p-16α translated the P-450$_{16\alpha}$ apoprotein *in vitro*. Total cellular proteins, which were prepared from immunopositive transformant *Escherichia coli* cells, were conjugated with Sepharose 4B. Antibody purified with the Sepharose 4B conjugate from mixed antiserum to P-450$_{16\alpha}$ and P-450$_{15\alpha}$ specifically inhibited testosterone 16α-hydroxylase activity in microsomes. The cDNA insert of one recombinant plasmid (clone P-16α-1) was 1.75 kilobases in size and contained one or more internal restriction sites for *Hin*dIII, *Bam*HI, *Bgl* I, *Pst* I, *Alu* I, *Hin*pI, and *Rsa* I. $^{32}$P-labeled clone p-16α-1 hybridized with a single mRNA (2000 bases) that was 10 times more concentrated in liver cells from male 129/J mice than in female mice. This result was consistent with the finding that poly(A)$^+$ RNA from male mice translated 10 times as much P-450$_{16\alpha}$ *in vitro* as did the poly(A)$^+$ RNA from females. Thus, the predominant expression of testosterone 16α-hydroxylase in male 129/J mice is regulated pretranslationally, presumably at the transcriptional level of the P-450$_{16\alpha}$ gene.**

Many liver proteins and enzymes such as $\alpha_2$-microglobulin, mouse major urinary protein, steroid hormone and prolactin receptors, monoamine oxidase, aldehyde oxidase, drug oxidase, and steroid hydroxylase activities show considerable sexual dimorphism (1).

These sexual dimorphisms of liver enzymes and proteins are considered to be regulated reversibly or irreversibly by multiple hormones such as androgens, growth hormone, and thyroxine (2, 3). Liver microsomal testosterone 16α-hydroxylase activity in adult rats is one example of an enzyme whose expression is irreversibly predetermined by neonatal androgens, a process called "neonatal imprinting" (4). Microsomal testosterone 16α-hydroxylase (cytochrome P-450$_{16\alpha}$) activity is expressed predominantly in certain strains of male inbred mice such as 129/J (5, 6). On the other hand, testosterone 15α-hydroxylation activity is expressed predominantly in female 129/J mice (5, 6). These hydroxylases are believed to be involved in degradation of steroid hormones in liver cells. The results from offspring obtained by interbreeding the appropriate parental strains have demonstrated that sex-dependent expression of the 16α-hydroxylase activity is under the control of a single sex-related gene between 129/J and C57BL/6J mice. In C57BL/6J mice the 16α-hydroxylase activity is expressed in female as well as male mice (5). It is not yet understood how the sex-dependent developmental regulation of testosterone hydroxylase occurs at the molecular level.

To investigate sexual regulation of testosterone hydroxylase activities in mice, we have previously purified liver microsomal cytochrome P-450s specific for the 16α- or 15α-hydroxylation based on the specific hydroxylase activity in eluates from chromatographic columns (6, 7). Throughout the purification procedure, we demonstrated that sex-dependent differences in testosterone 16α- and 15α-hydroxylase activity in inbred mice (129/J) can be explained by the existence of highly specific cytochrome P-450s for 16α- and 15α-hydroxylation activities in male and female mice, respectively. A specific antibody to purified P-450$_{16\alpha}$ or P-450$_{15\alpha}$ fraction was able to inhibit nearly 100% of either 16α- or 15α-hydroxylase activity in untreated male or female 129/J mice (6, 7).

To extend further our knowledge of the mechanism of sexual regulation of testosterone 16α-hydroxylase in 129/J mice, we used the specific antibody to P-450$_{16\alpha}$ to measure indirectly the level of translatable P-450$_{16\alpha}$ mRNA in male and female mouse livers. In addition, we used the specific antibody to isolate and characterize the cDNA encoding P-450$_{16\alpha}$ mRNA. Finally, we quantitated directly the level of P-450$_{16\alpha}$ mRNA in liver of male and female mice by hybridization with the isolated cDNA.

## EXPERIMENTAL PROCEDURES

**Preparation of Anti-P-450$_{16\alpha}$.** In all experiments 2- to 3-month-old mice (The Jackson Laboratory) were used. The purification of P-450$_{16\alpha}$ and P-450$_{15\alpha}$ and the preparation of specific anti-P-450$_{16\alpha}$ and anti-P-450$_{15\alpha}$ were carried out by methods described previously (6, 7). Rabbit antiserum to the purified P-450$_{16\alpha}$ fraction was purified by antigen-affinity chromatography (7). The purified P-450$_{16\alpha}$ antibody was further chromatographed on DEAE-cellulose adsorbed with RNasin to remove RNase in the antibody solution (29).

**Isolation of Liver Polysomes and Immunoenrichment.** Livers from 80 male 129/J mice were homogenized to isolate total liver polysomes from the postmitochondrial fraction according to the procedure of Gough and Adams (8). All buffers that were used for isolation of polysomes contained 10 mM vanadyl-riboside complex. The polysome–antibody complexes were precipitated by adsorption onto *Staphylococcus aureus* protein A ghosts, as described by Gough and Adams (8). One-hundred units of RNasin were included in all buffers used for immunoprecipitation of polysomes. Poly(A)$^+$ RNAs were extracted by phenol from the total or im-

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Abbreviations: iPrSGal, isopropyl β-D-thiogalactopyranoside; DBM, diazobenzyloxymethyl.

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munoenriched polysomal fractions and selected by oligo(dT)-cellulose chromatography (9).

**Construction of cDNA Library.** Immunoenriched poly(A)$^+$ RNA was reverse-transcribed into single-strand DNA by using reverse transcriptase (SeikagakuAmerica) (10). Alkaline-treated single-stranded cDNAs were primed to synthesize double-stranded cDNAs with DNA polymerase I Klenow fragment (P-L Biochemicals) (11). Double-stranded cDNAs were digested with S1 nuclease (P-L Biochemicals) (12) and tailed by terminal transferase. C-tailed double-stranded cDNAs were annealed to G-tailed plasmid pUC-9 (P-L Biochemicals) (13, 14). Finally, annealed plasmids were used to transform *E. coli* (JM-103) by the calcium/rubidium method (15) and transformants were selected on LB plates containing ampicillin (30 $\mu$g/ml). All experiments were carried out in accordance with National Institutes of Health guidelines.

**Identification of Positive Recombinants.** Transformant colonies were triplicated on three pieces of nitrocellulose paper. Two pieces of the paper were hybridized *in situ* with $^{32}$P-labeled single-stranded cDNAs synthesized from either enriched or nonenriched poly(A)$^+$ RNA. By this double-colony hybridization procedure (16), 39 transformants, which reacted strongly and selectively with the radioactive probe from enriched poly(A)$^+$ RNA, were selected.

The 39 transformants were grown on nitrocellulose paper, induced by 100 mM isopropyl $\beta$-D-thiogalactopyranoside (iPrSGal) (Sigma), and subjected to *in situ* immunostaining by the procedure of Young and Davis (17) with modifications. The transformants on nitrocellulose paper were treated with chloroform vapor. The treated nitrocellulose paper was placed on Whatman filter paper wetted with 25 mM Tris·HCl, pH 8.0/0.15 M NaCl/0.01% NaDodSO$_4$ (buffer A) for 1 hr and rinsed three times with buffer A. This step was repeated with buffer A containing 10 $\mu$g of DNase I per ml for 15 min and then with buffer A containing 3% bovine serum albumin for 20 min. This sequentially treated nitrocellulose paper was washed twice with buffer A containing 1 mM EDTA, 0.2% 3-(1-tetradecyl-1,1-dimethylammonia)-1-propane sulfonate, and 0.05% NaDodSO$_4$. The washed paper was incubated with P-450$_{16\alpha}$ antibody (100 $\mu$g/ml) in buffer A containing 0.01% NaDodSO$_4$, 0.05% Triton X-100, 1 mM EDTA, and 1% bovine serum albumin for 1 hr. Unbound antibody was removed by shaking the paper in buffer A containing 0.01% NaDodSO$_4$, 0.05% Triton X-100, and 1 mM EDTA three times. Finally, the paper was incubated with $^{125}$I-labeled protein A ($10^7$ cpm; New England Nuclear) in buffer A containing 0.01% NaDodSO$_4$, 0.05% Triton X-100, 1 mM EDTA, and 1% bovine serum albumin for 1 hr at room temperature. After washing with buffer A containing 0.01% NaDodSO$_4$, 0.05% Triton X-100, and 1 mM EDTA five times, the nitrocellulose paper was exposed to x-ray film (Kodak). All of the reactions were carried out at room temperature.

**Purification of Antibody by Sepharose 4B Conjugated with Cellular Proteins Synthesized by Positive Transformants.** Positively and negatively expressing transformants for P-450$_{16\alpha}$ determined by the *in situ* immunostaining assay were grown in 100 ml of LB medium and induced with iPrSGal. *E. coli* cells were collected by centrifugation and lysed in 10 ml of 50 mM Tris·HCl (pH 7.5) containing 10 mg of lysozyme, 50 mM EDTA, 7% sucrose, and 1% Triton X-100. Supernatant fractions were obtained by centrifugation at 35,000 rpm for 1 hr and dialyzed against 500 ml of 0.1 M NaHCO$_3$. The dialyzed supernatants were incubated with 10 g of CNBr-activated Sepharose 4B to synthesize Sepharose 4B conjugated with the cellular proteins by a method described previously (7).

**Hybrid Selection of mRNA and *in Vitro* Translation.** Poly(A)$^+$ RNA was translated in a reticulocyte lysate system (New England Nuclear) (18) by using [$^{35}$S]methionine as the radioactive precursor. Hybrid selection of P-450$_{16\alpha}$ mRNA with clone P-16$\alpha$-1 plasmid was carried out by the procedure reported by Parnes *et al.* (19). Immunoprecipitation from translational products and analysis of immunoprecipitate were carried out by the method of Negishi and Nebert (20).

**Electrophoresis.** NaDodSO$_4$/polyacrylamide gel electrophoresis was performed by using the method of Maizel (21). Methylmercury (5 mM)-agarose gels of RNAs were made by the method of Bailey and Davidson (22), and RNAs were transferred to diazobenzyloxymethyl-paper (DBM-paper) (23). All DNA probes were nick-translated by using [$^{32}$P]-dCTP (Amersham) as the radioactive precursor (24).

**Assay of the 16$\alpha$-Hydroxylase.** Testosterone 16$\alpha$-hydroxylation activity in microsomes was measured by the method described by Harada and Negishi (6).

## RESULTS

***In Vitro* Translation of P-450$_{16\alpha}$ mRNA.** Total liver poly(A)$^+$ RNAs isolated from male and female 129/J mice were translated in rabbit reticulocyte lysate, and total translation products and immunoprecipitable product with anti-P-450$_{16\alpha}$ were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis. As shown in Fig. 1, >10-fold as much P-450$_{16\alpha}$ was immunoprecipitated by anti-P-450$_{16\alpha}$ when the translation assay was programed by male rather than by female poly(A)$^+$ RNA. The result indicates that the about 10 times higher testosterone 16$\alpha$-hydroxylase activity in male microsomes compared to female microsomes (6) is due to higher levels of translatable P-450$_{16\alpha}$ mRNA in male liver cells of 129/J mice. Therefore, it is most likely that the sex-dependent expression of liver microsomal testosterone 16$\alpha$-hydroxylase is the result of sex-dependent regulation of the P-450$_{16\alpha}$ mRNA.

**Isolation of cDNA Clone Encoding P-450$_{16\alpha}$.** Polysomes bearing P-450$_{16\alpha}$ mRNA were enriched by immunoprecipitation with P-450$_{16\alpha}$ antibody. Total translation products us-

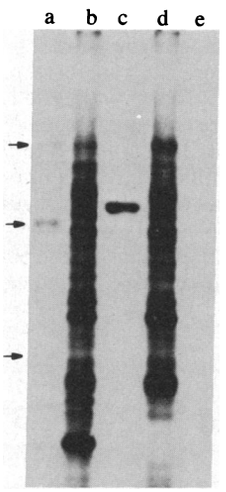


FIG. 1. *In vitro* translation of P-450$_{16\alpha}$ programed by male or female liver total poly(A)$^+$ RNA. Five-hundred nanograms of poly(A)$^+$ RNA prepared from total liver polysomes of male or female 129/J mice was translated in rabbit reticulocyte lysate (25-$\mu$l reaction volume). About one-fifth of the reaction mixture was used for immunoprecipitation of P-450$_{16\alpha}$ apoprotein with P-450$_{16\alpha}$ antibody. A 9% polyacrylamide gel was used, and electrophoresis was from top to bottom. The gel was soaked with Enlightning (New England Nuclear), dried, and exposed to x-ray film. Lane a, molecular markers (New England Nuclear) [serum albumin ($M_r$ 68,000), ovalbumin ($M_r$ 45,000), and carbonic anhydrase ($M_r$ 30,000)]; lane b, total translation products of male poly(A)$^+$ RNA; lane c, immunoprecipitate from lane b; lane d, total translation products of female poly(A)$^+$ RNA; lane e, immunoprecipitate from lane d. The arrows show the positions of marker proteins.

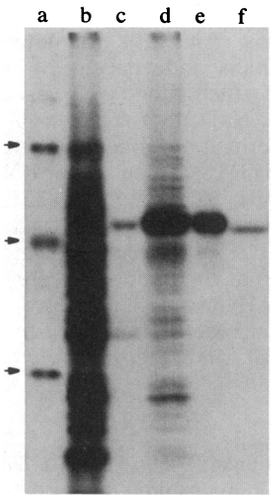


FIG. 2. Immunoenrichment of liver polysomes containing P-$450_{16\alpha}$ mRNA. Polysomes bearing P-$450_{16\alpha}$ mRNA were precipitated from the total polysomal fraction of male 129/J mice. Five-hundred or 100 ng of total or immunoenriched poly(A)$^+$ RNA, respectively, was translated *in vitro* by reticulocyte lysate, and total translation products and immunoprecipitable proteins were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis and fluorography. Lane a, radioactive molecular markers (see Fig. 1); lane b, total translation products of nonadsorbed poly(A)$^+$ RNAs; lane c, immunoprecipitate from lane b; lane d, total translation products of immunoenriched poly(A)$^+$ RNA; lane e, immunoprecipitate from lane d; lane f, no exogenous mRNA added. A 9% polyacrylamide gel was used, and electrophoresis was from top to bottom. The arrows show the positions of marker proteins.

ing the enriched mRNA fraction gave a single major product on $NaDodSO_4$/polyacrylamide gel electrophoresis, which comigrated with purified P-$450_{16\alpha}$ and was precipitated with P-$450_{16\alpha}$ antibody (Fig. 2). By using differential double-colony hybridization with $^{32}$P-labeled single-stranded DNA synthesized from immunoenriched mRNA versus the nonadsorbed mRNA fraction, 39 transformants reacting strongly with the $^{32}$P-labeled probe synthesized from the enriched mRNA fraction were selected from 4000 transformants. From the 39 recombinants subjected to *in situ* immunostaining with P-$450_{16\alpha}$ antibody, we identified 7 positively expressing recombinants (Fig. 3). The sizes of insert cDNA of the 39 recombinant plasmids were analyzed by agarose gel electrophoresis, and they ranged from 600 to 1750 base pairs. The recombinant, clone p-16$\alpha$-1, indicated by an arrow in Fig. 1, contained the largest insert DNA (1750 base pairs)

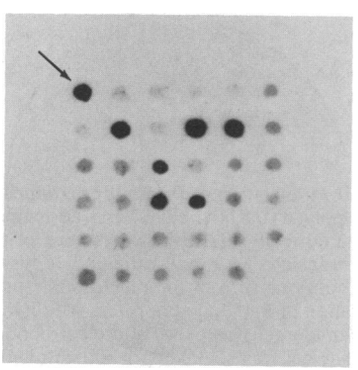

FIG. 3. Screening of transformants with the *in situ* immunostaining assay. Thirty-nine transformants selected by double-colony hybridization were grown on nitrocellulose paper and induced by iPrS-Gal and the *in situ* immunostaining assay was carried out. The transformant indicated by the arrow was named clone p-16$\alpha$-1 and was used for all experiments.

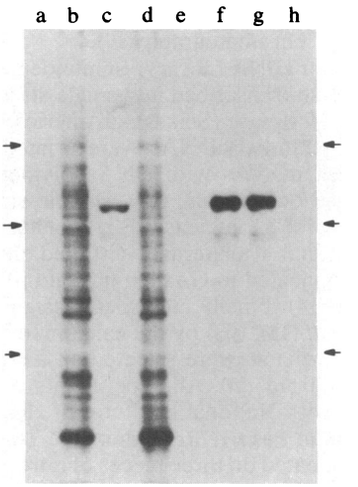


FIG. 4. Hybrid selection of P-$450_{16\alpha}$ mRNA with clone p-16$\alpha$-1 plasmid. About 300 $\mu$g of clone p-16$\alpha$-1 was bound to a 2.5-cm diameter of nitrocellulose disc (S and S) by filtration. About 150 $\mu$g of total poly(A)$^+$ RNA from male livers were incubated with the nitrocellulose paper to select P-$450_{16\alpha}$ mRNA. The experimental RNAs eluted from the nitrocellulose paper were dissolved in 5 $\mu$l of 10 mM dithiothreitol solution containing 5 units of RNasin, and 1 $\mu$l was used for the *in vitro* translation assay. Lanes a and h, radioactive molecular markers (see Fig. 1); lane b, total translation products of total poly(A)$^+$ RNA from male livers; lane c, immunoprecipitate from lane b; lane d, total translation products of nonadsorbed poly(A)$^+$ RNA; lane e, immunoprecipitate from lane d; lane f, total translation products of the hybrid-selected poly(A)$^+$ RNA; lane g, immunoprecipitate from lane f. The arrows indicate the positions of marker proteins.

and was nick-translated with [$^{32}$P]dCTP and hybridized with cDNA inserts of all 39 recombinant plasmids. All 39 recombinants were strongly cross-hybridized (data not shown), suggesting that they were the same or very closely related genes.

**Characterization of Clone p-16$\alpha$-1.** With the use of recombinant plasmid clone p-16$\alpha$-1, P-$450_{16\alpha}$ mRNA was selected from total liver poly(A)$^+$ RNA isolated from male 129/J mice. Total translation products and immunoprecipitable product programed by total poly(A)$^+$ RNA before the selection, after the selection, and that selected by hybridization with clone p-16$\alpha$-1 were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 4). Under the conditions of hybridization used, nearly 100% of P-$450_{16\alpha}$ mRNA was arrested (lane e). Total translational products of selected mRNA gave a single major band on $NaDodSO_4$/polyacrylamide gel electrophoresis. This major product comigrated with purified P-$450_{16\alpha}$ and was precipitated with anti-P-$450_{16\alpha}$ (lane g). An antibody was purified from mixed antisera containing anti-P-$450_{16\alpha}$ and anti-P-$450_{15\alpha}$ by Sepharose 4B conjugated with total cellular proteins from positively expressing transformants of *E. coli*. The purified antibody was shown to be anti-P-$450_{16\alpha}$ by the specific inhibition of testosterone 16$\alpha$-hydroxylase activity in microsomes (Fig. 5). This provided further evidence that clone p-16$\alpha$-1 indeed codes for the apoprotein of P-$450_{16\alpha}$. A Sepharose 4B conjugate with cellular proteins from immunonegative transformant *E. coli* did not purify either P-$450_{16\alpha}$ or P-$450_{15\alpha}$ antibody from the mixed antisera.

**Restriction Map of cDNA Insert of Clone p-16$_\alpha$-1.** Fig. 6 shows a restriction map of clone p-16$\alpha$-1 containing 1.75 kilobases of cDNA insert. It has four internal *Hin*pI sites, two *Pst* I, *Alu* I, *Bgl* I, and *Rsa* I sites, one *Bam*HI site, and one *Hin*dIII site. 5′ and 3′ orientations were determined based on the direction of transcription in the lacZ protein of expression vector pUC-9.

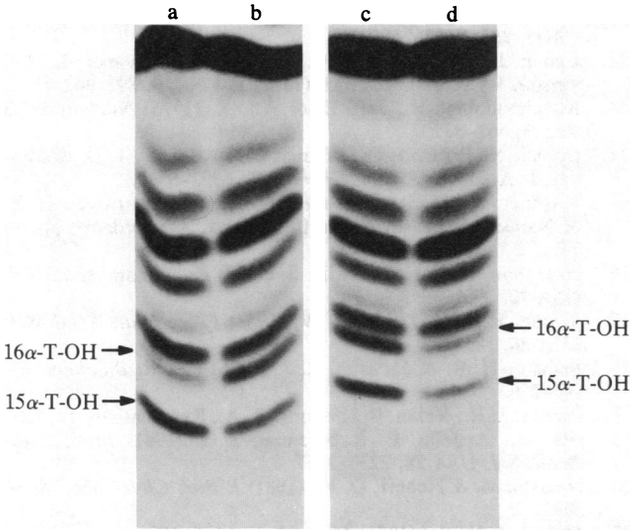


FIG. 5. Inhibition of testosterone 16α-hydroxylase activity by an antibody purified by cellular proteins prepared from positively expressing transformants of *E. coli*. Total cellular proteins were isolated from positively and negatively expressing transformants and were covalently coupled with Sepharose 4B. An antibody fraction purified from a mixture of anti-P-450$_{16\alpha}$ and P-450$_{15\alpha}$ sera by either the positive or negative transformant was tested for inhibition of testosterone hydroxylase activities in liver microsomes of untreated male 129/J mice. Lane a, thin-layer chromatogram of testosterone metabolites formed by microsomes in the presence of the antibody purified by the positive transformants; lane b, testosterone metabolites formed by microsomes with the presence of the antibody purified by the negative transformant; lane c, testosterone metabolites formed by microsomes alone; lane d, testosterone metabolites formed by microsomes in the presence of P-450$_{16\alpha}$ and P-450$_{15\alpha}$ antibodies. 16α-T-OH designates 16α-hydroxytestosterone and 15α-T-OH designates 15α-hydroxytestosterone.

**Hybridization of $^{32}$P-Labeled Clone P-16α-1 with Total Liver Poly(A)$^+$ RNA.** Total liver poly(A)$^+$ RNA was isolated from male and female 129/J mice. After electrophoresis, the RNAs were transferred to DBM-paper and hybridized with nick-translated clone p-16α-1. As shown in Fig. 7, a single size of mRNA was hybridized with the clone p-16α-1, and its size was about 2.0 kilobases. Based on the intensities of hybridization, >10-fold as much P-450$_{16\alpha}$ mRNA is present in male liver compared to female liver.

## DISCUSSION

In this report we describe the isolation of clone p-16α-1 encoding testosterone 16α-hydroxylase (P-450$_{16\alpha}$) and pretranslational regulation of male predominant expression of this hydroxylase in 129/J mice. We have used a specific antibody to precipitate polysomes bearing P-450$_{16\alpha}$ mRNA for construction of the cDNA library. The antibody used was absolutely specific for inhibiting only the formation of 16α-hydroxytestosterone in microsomes. More than 30 testosterone metabolites were formed in liver microsomes (6). Transformants for P-450$_{16\alpha}$ were screened by double-colony hybridization and an *in situ* immunostaining assay. The screened recombinants were positively shown to encode P-450$_{16\alpha}$ mRNA by a hybrid selection of P-450$_{16\alpha}$ mRNA with the recombinant plasmid (clone p-16α-1). The purification of inhibitory antibody to P-450$_{16\alpha}$ from mixed antisera to P-450$_{16\alpha}$ and P-450$_{15\alpha}$ with Sepharose 4B coupled to total cellular proteins of positively expressing transformant determined by *in situ* immunostaining provided definitive immunochemical proof that clone p-16α encodes P-450$_{16\alpha}$ mRNA.

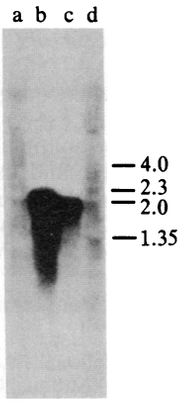


FIG. 7. Hybridization analysis of clone p-16α-1 with total poly(A)$^+$ RNA. Total poly(A)$^+$ RNA was prepared from male and female livers and electrophoresed on a 1% methylmercury-agarose gel. After mRNA transfer to DBM-paper, hybridization with $^{32}$P-labeled clone p-16α-1 was carried out. *Hin*dIII-digested λ DNA and *Hae* III-digested φX174 DNA were electrophoresed and hybridized with nick-translated digested λ and φX174 DNA for molecular markers. Molecular weights are shown as $M_r \times 10^{-3}$. Lanes a and d, molecular marker DNAs; lane b, total poly(A)$^+$ RNA from male; lane c, total poly(A)$^+$ RNA from female.

It cannot be absolutely ruled out that clone p-16α encodes some other cytochrome P-450 that may be cross-reacting with the P-450$_{16\alpha}$ antibody. However, unlike the case of 3-methylcholanthrene- or phenobarbital-induced cytochrome P-450s (21, 25, 26), the possibility of sharing a common antigenic site to the P-450$_{16\alpha}$ antibody used here is remote for the following reasons. First, the P-450$_{16\alpha}$ antibody precipitated only one band from total translation products programed by either male or female liver mRNA; second, all 39 recombinant plasmids strongly cross-hybridized, and the restriction maps of the several recombinant cDNA inserts analyzed showed their identity, suggesting that only one kind of mRNA in liver cells of untreated male 129/J mice was enriched by the antibody.

Hepatic microsomal testosterone 16α-hydroxylase activity is 10-fold higher in male than in female 129/J mice, and the 16α-hydroxylase activity in liver microsomes of male and female mice can be fully accounted for by the purified P-450$_{16\alpha}$ (7). The results obtained from the *in vitro* translation of male and female mRNA and from hybridization of clone p-16α-1 with the mRNA conclusively showed that male specific expression of the 16α-hydroxylase activity is regulated by pretranslational control. Presumably this is at the transcription level, as judged by the fact that an increasing level of 2-

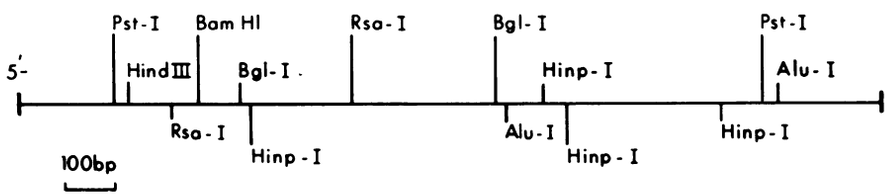


FIG. 6. Restriction map of cDNA insert of clone p-16α-1. Restriction sites were determined by partial digestions of end-labeled DNA with the appropriate restriction enzymes. bp, Base pairs.

kilobase P-$450_{16\alpha}$ mRNA in male mice was concomitant with increasing levels of large precursors (multiple bands) of P-$450_{16\alpha}$ mRNA.

Mouse major urinary proteins consist of a multigene family, and their male predominant expression is regulated transcriptionally by the action of multiple hormones, including testosterone, growth hormone, and thyroxine (27, 28). We have also recently isolated cDNA clones encoding female predominant testosterone 15α-hydroxylase (P-$450_{15\alpha}$) in liver microsomes of 129/J mice. With the use of the isolated cDNA clone as a hybridization probe, >10-fold as much P-$450_{15\alpha}$ mRNA was recovered from female as from male liver cells (unpublished data).

We thank Drs. Jack Bend (National Institute of Environmental Health Sciences) and Ida Owens (National Institute of Child Health and Human Development) for critical reading of this manuscript and Debbie Garner for typing the manuscript.

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