Skou, J. C. (1965) *Physiol. Rev. 3*, 596–617.
Skou, J. C., & Esmann, M. (1979) *Biochim. Biophys. Acta 567*, 436–444.
Thomas, D. D. (1985) in *The Enzymes of Biological Membranes* (Martonosi, A. N., Ed.) 2nd ed., Vol. 1, pp 287–312, Plenum Press, New York.
Thomas, D. D., & Hidalgo, C. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5488–5492.
Thomas, D. D., Dalton, L. R., & Hyde, J. S. (1976) *J. Chem. Phys. 65*, 3006–3024.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.

Gene Family of Male-Specific Testosterone 16α-Hydroxylase (C-P-450$_{16\alpha}$) in Mouse Liver: cDNA Sequences, Neonatal Imprinting, and Reversible Regulation by Androgen

Garry Wong, Kaname Kawajiri, and Masahiko Negishi*

Laboratory of Pharmacology, National Institute of Environmental Health Sciences, National Institutes of Health, P.O. Box 12233, Research Triangle Park, North Carolina 27709

Received June 10, 1987; Revised Manuscript Received July 23, 1987

ABSTRACT: The cDNA clone p16α-1 for the male-specific isozyme (C-P-450$_{16\alpha}$)[1] of testosterone 16α-hydroxylase in livers of 129/J mice [Harada, N., & Negishi, M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2024–2028] and two additional full-length cDNAs overlapping with p16α-1 (p16α-2 and p16α-16) were sequenced. p16α-2 contained a single open reading frame of 1512 nucleotides, consisting of 71 base pairs of the 5′-noncoding region and 63 base pairs of the 3′-noncoding region with an additional poly(A) tract. From this DNA sequence, C-P-450$_{16\alpha}$ was deduced to contain 504 amino acids with a calculated molecular mass of 56 948 daltons. p16α-1 showed a nucleotide sequence identical with that of p16α-2 but lacked nine amino acid residues from the N-terminus. Another cDNA clone, p16α-16, also exhibited the same coding sequence with the exception of a 142 base pair deletion spanning from nucleotide 853 to nucleotide 994 of p16α-2. This deletion seems to be a whole exon of this gene, resulting in a shift of reading frame and an early termination codon at 10 amino acid residues from the deletion. The expected translation product of this mRNA is calculated to be 294 amino acids and 33 300 daltons. The putative poly(A) addition signal AATAAA is present for all three clones, but there are polymorphisms in the start sites of polyadenylation. Amino acid alignment with P-450b, P-450 M1, and P-450c revealed that P-450$_{16\alpha}$ is 39.8%, 40.7%, and 31.5% homologous, respectively. Five highly homologous genes were selected from a genomic DNA library of BALB/cJ mice by plaque hybridization to the cDNA and characterized. An oligonucleotide only specific for p16α-1 and its gene was prepared from p16α-2 and its gene. The p16α-1 and the specific oligonucleotide were hybridized with liver poly(A)+ RNA from control and testosterone propionate treated adult 129/J mice that had been castrated at day 1 of birth or at adult age. The results indicated that p16α-1 recognizes at least two mRNAs in terms of their regulation by androgen; one is reversibly regulated and the other is neonatally imprinted in expression in adult livers. The two differentially regulated C-P-450$_{16\alpha}$-dependent testosterone 16α-hydroxylase activities in the liver microsomes from these mice were also detected and associated with the hybridizable mRNA levels. A hybridization of the specific oligonucleotide probe with the poly(A)+ RNA concluded that p16α-1 and p16α-2 encode C-P-450$_{16\alpha}$, which is reversibly regulated.

As with other liver enzymes and proteins, such as drug oxidases, monoamine oxidase, prolactin receptor, and major mouse urinary protein (MUP), steroid hydroxylase activities in microsomes exhibit a marked sexual dimorphism in rodents (Colby, 1980; Roy & Chatterjiee, 1983). For instance, testosterone 16α-hydroxylase activity in mouse liver microsomes is known to be male predominant in some inbred strains, such as 129/J, whereas the activity is expressed at a high level in females as well as in males of other inbred strains, such as BALB/cJ and C57BL/6J (Ford et al., 1979; Harada & Negishi, 1984a; Harada & Negishi, 1984b; Noshiro et al., 1986). In inbred mice, such as BALB/cJ, apparent 16α-hydroxylase activity in microsomes is the sum of at least two sexually differentiated isozymes (forms of P-450); one (called C-P-450$_{16\alpha}$)[1] is predominantly expressed in male mice and the other (called I-P-450$_{16\alpha}$) is female specific (Harada & Negishi, 1984b; Devore et al., 1985; Noshiro et al., 1986). The strain differences in the activity among the inbred strains of mice

* Address correspondence to this author at the Pharmacogenetics Section, Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709.

[1] When the form of P-450 specific for testosterone 16α-hydroxylase activity was purified from 129/J male mice, we named this form P-450$_{16\alpha}$ (Harada & Negishi, 1984a). Subsequently, another P-450 was purified from phenobarbital-treated 129/J female mice, which is also specific for testosterone 16α-hydroxylase activity (Devore et al., 1985), and the immunochemical evidence suggested that this P-450 is constitutively female specific. Therefore, in order to distinguish the two isozymes, the P-450$_{16\alpha}$ was renamed C-P-450$_{16\alpha}$ (Noshiro et al., 1986a).

have been used to establish a genetic model for the sex-dependent expression of testosterone 16α-hydroxylase activity (Pasleau et al., 1984; Noshiro et al., 1986; Ford et al., 1979). Noshiro and co-workers (Noshiro et al., 1986; Noshiro & Negishi, 1986a) demonstrated that this strain difference in the 16α-hydroxylase activity is due to a lack of expression of the female-specific isozyme (I-P-450$_{16\alpha}$) in 129/J mice and is inherited as an autosomal recessive trait. This sex-dependent regulation of liver enzymes and proteins is known to be developmentally regulated and is affected by a variety of hormones, including androgen, estrogen, thyroxine, and growth hormone (Gustafsson et al., 1983). Noshiro and Negishi (1986b) recently demonstrated that the expression of C-P-450$_{16\alpha}$ is regulated by episodic secretion of growth hormone in male mice. In terms of developmental regulation, a "neonatal imprinting" has been proposed for a mechanism of male-predominant expression of testosterone 16α-hydroxylase activity (Conney et al., 1969; Einarson et al., 1973; Levin et al., 1975; Chung & Chao, 1980). In this mechanism, the expression of the activity in adult male rats is irreversibly predetermined by neonatal androgen. If this hypothesis is correct, a castration of adult animals should not effect the level of testosterone hydroxylase activity in livers.

In order to investigate the molecular mechanism of sex-dependent hormonal regulation of male-specific C-P-450$_{16\alpha}$ gene, our laboratory has isolated cDNA clone p16α-1 for this P-450 (Harada & Negishi, 1985). In this paper, we isolated two additional overlapped clones to p16α-1, and the complete DNA sequences were obtained to elucidate a primary structure of C-P-450$_{16\alpha}$. With cDNA and a specific oligonucleotide as hybridization probes, at least two differently regulated mRNAs for C-P-450$_{16\alpha}$ were demonstrated in livers of adult male 129/J mice; one is reversibly regulated by androgen and the other is neonatally imprinted.

Experimental Procedures

Animals. Mice strains 129/J, BALB/cJ, and C57BL/6J were purchased from Jackson Laboratory (Bar Harbor, ME). Adult (2-month-old) male mice were anesthetized by ip injection of 0.2 mL of Nembutal solution (diluted to 15% with saline) before castration. A small incision was made in the scrotal sac, and testes were gently squeezed out, ligated with silk thread, and then removed. The incision was closed with a 9-mm wound clip. Sham-operated mice were also anesthetized, and testes were exposed to air and then returned to the body. The mice that were operated upon were housed for at least 2 weeks in order to remove any effect of Nembutal and endogenous testosterone. Testosterone propionate (10 mg/kg body weight) was dissolved in corn oil and injected ip once a day for five consecutive days. One-day-old neonates of 129/J were anesthetized by cooling their bodies on ice and castrated. The castrated neonates were fostered to CD female mice and subsequently treated with a dose of testosterone propionate from 50 days of age for five consecutive days.

Enzymes and Chemicals. [γ-^{32}P]ATP (5000 Ci/mmol), [α-^{32}P]ATP (400 Ci/mmol), [α-^{32}P]dCTP (3000 Ci/mmol), and [α-^{35}S]dATPαS (>1000 Ci/mmol) were purchased from Amersham Corp. All restriction enzymes used were products of New England Biolabs (Beverly, MA). Reverse transcriptase was from Seikagaku America. *Eschericia coli* RNase H, DNA ligase, DNA polymerase I, T4 DNA polymerase, T4 polynucleotide kinase, and *Eco*RI methylase were obtained from Pharmacia P-L Biochemicals. Promega supplied λgt-11 and packing solution. The cycloning system was purchased from International Biotechnologies, Inc. (New Haven, CT). Nitrocellulose paper and Nytran were purchased from Schleicher and Schuell (Keene, NH). Testosterone propionate and corn oil were obtained from Sigma Chemical Co. All other reagents used for the present work were of the highest quality available from major chemical suppliers.

Preparation of Poly(A)+ RNA and Microsomes from Mouse Livers. Livers from mice were pooled and minced. The minced livers were separated into two parts. From one part, total RNA was extracted by using 8 M guanidine hydrochloride (Cox, 1968) and subsequently enriched for poly(A)+ RNA by oligo(dT) cellulose chromatography (Aviv & Leder, 1972). The remaining minced liver was homogenized and centrifuged to isolate liver microsomes as described previously (Harada & Negishi, 1984a). The microsomal pellets were homogenized in 50 mM potassium phosphate buffer, pH 7.25, containing 1 mM ethylenediaminetetraacetic acid (EDTA), 2 μg/mL leupeptin, and 20% sucrose for the enzyme assay.

Construction of the cDNA Library. Total liver poly(A)+ RNA from untreated 129/J male mice was used to synthesize double-stranded cDNAs. Single-strand cDNA was synthesized by reverse transcriptase, and then the method of Okayama and Berg (1982) was used to make double-strand cDNA. The cDNAs were ligated into λgt-11 vector by using the *Eco*RI linker according to the procedures described by Young and Davis (1983). The library was screened by plaque hybridization with the ^{32}P-labeled p16α-1 cDNA insert to select the overlapped cDNA clones (p16α-2 and p16α-16).

Construction of the Genomic DNA Library. The spleen of the BALB/cJ mouse was homogenized in 10 mL of 0.25 M sucrose containing 50 mM EDTA and overlaid on 10 mL of 0.35 M sucrose containing 50 mM EDTA. The nuclear pellet was recovered by centrifugation at 3000 rpm for 10 min and subsequent suspension in 10 mL of 25 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer (pH 7.5) containing 50 mM EDTA. Sodium dodecyl sulfate (SDS) (final concentration 1%) and proteinase K (final concentration 50 μg/mL) were added to the suspension, which was incubated at 52 °C for 1 h. Finally, genomic DNA was extracted with phenol, phenol–chloroform, and then chloroform and precipitated by ethanol. The DNA pellet was dried and dissolved in 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA. The genomic DNA was partially digested with *Sau*3A and ligated to the EMBL3 vector, packaged, and transfected to host *E. coli* 2392 by the method described by Frishauf et al. (1983). The transfected cells were plated on LB plates. Only the primary library was used for screening in this work. Recombinant phages (2×10^6) were screened by plaque hybridization with ^{32}P-labeled p16α-1 insert DNA (Benton & Davis, 1977). The positive recombinant phage in *E. coli* 2392 was grown in a 200-mL culture, and its DNA was purified by the procedure previously described (Nakamura et al., 1983).

Northern and Southern Hybridization. Poly(A)+ RNA was denatured with 2.2 M formaldehyde and 7.2% formamide and electrophoresed on a 0.8% agarose gel containing 2.2 M formamide as described by Thomas (1980). The RNA was then transferred to Nytran and hybridized with the ^{32}P-labeled 3′ *Pst*1 fragment (350 bp) of p16α-1 in a solution containing 5 × standard saline citrate (SSC), 1 × Denhart's solution, 0.1% SDS, and sonicated herring sperm DNA (250 μg/mL) at 70 °C overnight. The oligonucleotide (5′-TGA-AGCTGCTCTCAGGAT) was incubated with [γ-^{32}P]ATP and T4 polynucleotide kinase to 5′ end label and then used to probe with the RNA transferred to Nytran in the solution containing 5 × SSC, 1 × Denhart's solution, 0.1% SDS, and 250 μg/mL sonicated herring sperm DNA at 42 °C overnight. The hybridized Nytran was washed with the proper solution

(0.1 × SSC containing 0.1% SDS at 52 °C or 2 × SSC contaning 0.1% SDS at room temperature for Nytran hybridized with the *Pst*1 fragment or with the oligonucleotide as probes, respectively). Cloned genomic DNAs were digested by restriction enzyme, electrophoresed on agarose gel, and transferred to Nytran as described by Southern (1975). The Nytran papers were hybridized with the radioactive probes in 5 × SSC containing 1× Denhart's solution, 0.1% SDS, and 250 μg/mL sonicated herring sperm DNA at 70 or 42 °C depending upon the probes and washed with the same solution mentioned above. The washed papers were allowed to air-dry and were exposed to X-ray films.

DNA Sequence. DNA fragments generated from the three cDNA inserts by restriction digests were ligated with the appropriate M13 vectors digested with the corresponding restriction enzymes. Single-strand DNA was isolated from recombinant *E. coli* JM103 as described previously (Messing et al., 1977) and sequenced by chain termination according to the methods of Sanger et al. (1980) and Biggin et al. (1983). Cycloning of the DNA fragment was performed as described by Dale et al. (1985). Most fragments were sequenced in both directions, and each fragment was sequenced at least twice.

Computer Analysis of DNA Sequences. Analysis of the DNA sequences was performed by using programs from the Microgenie software package (Queen & Korn, 1984).

Other Analytical Methods. Testosterone 16α-hydroxylase activities were measured by methods previously reported (Harada & Negishi, 1984a). Purification of C-P-450$_{16\alpha}$ and preparation of the antibody to C-P-450$_{16\alpha}$ were described in a previous paper (Harada & Negishi, 1984b). The assay conditions with the antibody to estimate C-P-450$_{16\alpha}$-dependent activity in liver microsomes were also described previously (Harada & Negishi, 1984b). Protein concentration was determined by the method of Bradford (1976).

RESULTS

Isolation of cDNA Clones and Nucleotide Sequence Analysis. By use of a specific antibody elicited to C-P-450$_{16\alpha}$ purified from 129/J male mice, the cDNA (p16α-1) was previously isolated and characterized (Harada & Negishi, 1985). The cDNA has been used to demonstrate that C-P-450$_{16\alpha}$ mRNA is expressed specifically in 129/J male mice and regulated by episodic secretion of growth hormone (Harada & Negishi, 1985; Noshiro & Negishi, 1986b). The nucleotide sequence obtained from p16α-1 indicated the lack of an initiation Met in this cDNA clone.[2] We therefore constructed a new cDNA library from liver poly(A)+ RNA of 129/J male mice using λgt-11 as the cloning vector and screened it by plaque hybridization with the nick-translated p16α-1 insert DNA. Of the 50 positives from the first screening of approximately 35 000 recombinants, 10 recombinant phage DNAs were purified. Two clones, 16α-2 and 16α-16, were subsequently subcloned into pBR322, and their restriction maps were determined. Figure 1 shows the comparison of restriction maps of p16α-1, p16α-2, and p16α-16 with the nucleotide sequence strategies. The restriction maps of p16α-1, p16α-16 are identical except that a deletion of 142 bp occurred within p16α-p16.

The complete nucleotide sequences of all three cDNAs and the deduced amino acid sequence are shown in Figure 2.

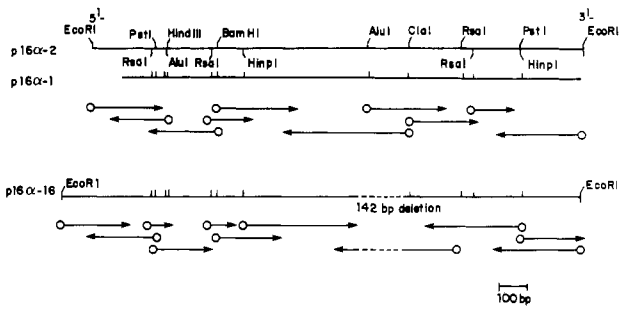

FIGURE 1: Restriction maps of cDNAs and sequence strategy. The restriction sites and nucleotide sequence strategies are shown. The sizes of restriction fragments were estimated from the migrations on agarose gel by using *Hin*dIII-digested λDNA and *Hae*III-cut φX174 DNA as molecular markers. The number of nucleotides (142 bp) deleted in p16α-16 was later determined from the nucleotide sequence analysis. 5′- and 3′-*Eco*RI sites are the linker sequences for cDNA cloning. The arrows indicate the direction and extent of nucleotide sequence of the fragment.

p16α-2 consisted of 1662 base pairs (bp) and contained an open reading frame spanning 1512 nucleotides, which begins 79 base pairs downstream from the 5′ *Eco*RI cloning site. The nucleotide sequences of p16α-1 and p16α-2 were identical. Both cDNAs contain the same poly(A)+ adenylation signal AATAAA in the identical locations, yet polyadenylation actually starts at 15 bp earlier in p16α-2 than in p16α-1. The p16α-16 had the same nucleotide sequence as the p16α-2 with the following exception: the deletion of 142 bp in p16α-16 spanning nucleotides 853–994 of p16α-2. The portion of deletion is bracketed in Figure 2.

Amino Acid Sequence and Comparison with Other P-450s. The amino acid sequence of C-P-450$_{16\alpha}$ predicted from p16α-2 is 504 residues long with a calculated molecular weight of 56 984. The nucleotide sequence surrounding the initiation Met is GCCATGG and is in excellent context for that of a translation start site according to Kozak (1986). Alignment of the amino acid sequence of C-P-450$_{16\alpha}$ to the known sequences of P-450 M1, and P-450c (Fujii-Kuriyama et al., 1982; Yabusaki et al., 1984; Yoshioka et al., 1987) revealed overall homologies to be 39.8%, 40.7%, and 31.5%, respectively. These sequences were chosen for comparison on the basis of their respective isozyme abilities to catalyze testosterone 16α-hydroxylation (P-450b, P-450 M1) and male-specific expression (P-450 M1), and P-450c was selected as an unrelated form. Figure 3 shows three relatively conserved regions; one is from Pro-37 to Gly-55 with 73.7% homology to P-450b, the second is from Met-356 to Pro-374 with 78% and 68% homology to P-450b and P-450 M1, respectively, and the third is from Lys-432 to Gln-465 with 73.5% homology to both P-450b and P-450 M1. The second conserved region was first proposed by Ozols et al. (1981), and the Cys in the third conserved region was first proposed by Fujii-Kuriyama et al. (1982) to be the fifth ligand for heme binding. The homologies of all three areas to P-450c are 40–47%. In the case of p16α-16, the reading frame is changed by the deletion of 142 bp, resulting in the stop (TAG) codon 29 bp downstream from the deletion. The predicted amino acid sequence from p16α-16 is 294 amino acids long with a molecular weight of 33 300.

Gene Family of C-P-450$_{16\alpha}$. Twenty independent genomic clones hybridized with the ^{32}P-labeled p16α-1 cDNA insert were isolated. On the basis of restriction analysis and degrees of hybridization, five highly homologous, but different genes were identified, which are shown in the top panel of Figure 4. The nucleotide sequences of portions of these genes revealed that genomic clone 52 codes p16α-2 mRNA. It was also found that the portions of DNA corresponding to nu-

[2] A portion of this work was previously presented for the FABC meeting (Wong et al., 1986). We reported in that abstract that the p16α-1 contains the initiation Met. However, this was due to misreading of the nucleotide sequence gel. The sequence ladders were disturbed by the presence of dG-dC polymers in the end of cDNA (p16α-1).

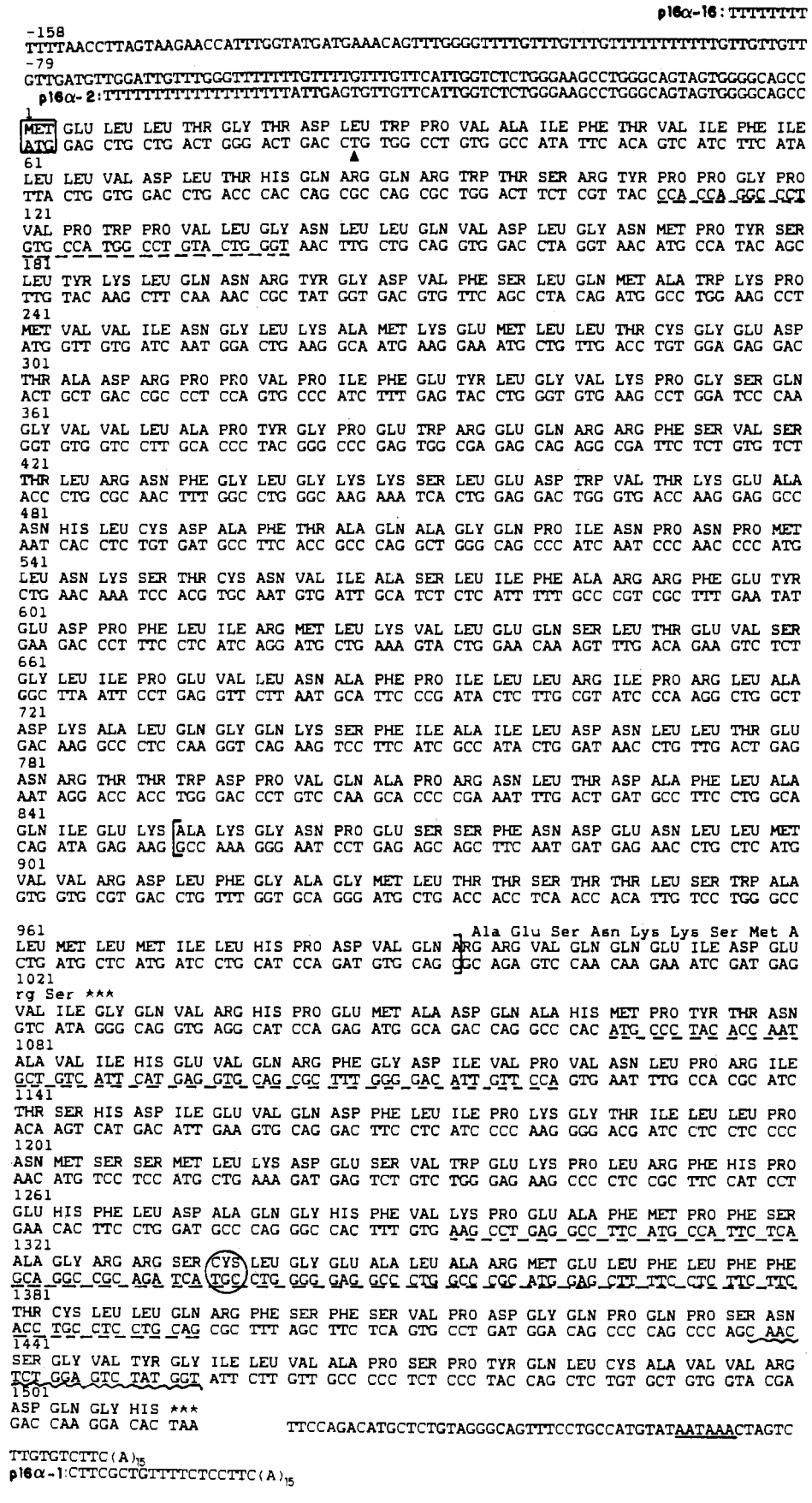

FIGURE 2: Nucleotide sequences and deduced amino acid sequence of C-P-450$_{16\alpha}$. The sequences are constructed on the basis of the results from p16α-2. The initiation codon is enclosed by the solid line. The three stars show the termination codons. The three conserved regions are shown by dotted underlines. The Cys, which is the proposed fifth ligand of heme binding, is circled. The poly(A) signal is marked by a solid underline. The sequence of p16α-1 starts at the position indicated by the arrow. The deleted nucleotide sequence in p16α-16 is bracketed, and the changes of amino acid sequence after the deletion are written at the top of the C-P-450$_{16\alpha}$ amino acid sequence. The op-1 sequence for the specific probe is underlined by a wavy line.

```
                37                                                  55
C-P-45016α:  P P G P V P W P V L G N L L Q V D L G
   P-450b:           R   L   L               L   R
  P-450M1:           T   L   I I       I     I Y M K
   P-450c:           W G L   F I   H V   - - T

               356                                                 374
 M P Y T N A V I H E V Q R F G D I V P
           D                 I       S   L
           D       V         I       Y I   L
         L E   F   L   T F   H S S F

               432
 K P E A F M P F S A G R R S C L G E A
   S                   T   K   I           G
   S D Y                   K   I   A
 H L S K V I L   G L   K   K     I       T
                                   465
 L A R M E L F L F F T C L L Q
 I     N                 T I
       T                 T I
 I G   L   V       L A I
```

FIGURE 3: Comparisons of conserved regions of C-P-450$_{16\alpha}$, P-450b, P-450 M1, and P-450c. Only these three regions are picked out by the sequence homology program from Microgenie, with a criteria of 60% minimum homology. The numbers show amino acids of the starts and ends of the regions.

cleotides 1437–1454 of p16α-2 were diversed in all of these genes. When synthetic oligonucleotide of the reverse complement to the 18 bases (op-1) was used for a hybridization probe, it hybridized only to the cDNA and genomic clone 52 in spite of the fact that the 3′ *Pst*1 fragment, in which the specific oligonucleotide sequence is located, hybridized to all of the genes. The results indicated the presence of multiple genes for C-P-450$_{16\alpha}$. In addition to these five genes, there was an additional undetermined number of genes that were less homologous to the cDNA. Recently, Itakura in this laboratory isolated a cDNA clone encoded by one of the four genes other than clone 52. However, the structures of five genes should be determined before we conclude that all five are active genes.

Effect of Castration on C-P-450$_{16\alpha}$-Dependent Hydroxylase Activity. Liver microsomes were prepared from testosterone propionate treated and nontreated adult 129/J mice castrated at day 1 for neonates or at adulthood. Testosterone propionate was administered once daily for 5 days prior to killing. The values of testosterone 16α-hydroxylase activities estimated by the antibody inhibition are shown in Figure 5. The activity in 129/J mice castrated at adulthood was decreased to ~65% of that seen in liver microsomes from sham-operated adult mice. On the other hand, the activity in neonatally castrated adult mice was only ~3% of that seen in control mice and was the same level as in normal adult female mice. When testosterone propionate was injected into 129/J males castrated at adulthood, the activity was increased to the level seen in sham-operated males. It was, however, found that testosterone propionate treatment increased the activity in adult males that have been neonatally castrated to only ~35% of that in the control males.

Northern Hybridization of Liver Poly(A)+ RNA from Castrated Mice with P16α-1 and Specific Oligonucleotide (op-1). Liver poly(A)+ RNA was isolated from the same 129/J mice used for preparing the microsomes. The results from Northern hybridization with p16α-1 cDNA or with specific oligonucleotide (op-1) as probe are seen in Figure 6a. The relative amounts of hybridizable mRNA were estimated by laser scanning of the X-ray films and summarized in Figure 6b. The changes induced by castration and hormone treatment in the amount of mRNA hybridized with p16α-1 correlated with those of C-P-450$_{16\alpha}$-dependent activity. In the adult males that had been castrated neonatally, the hybridizable mRNA to the cDNA probe was repressed to the level seen in adult female mice, which is approximately 3% of control adult males,

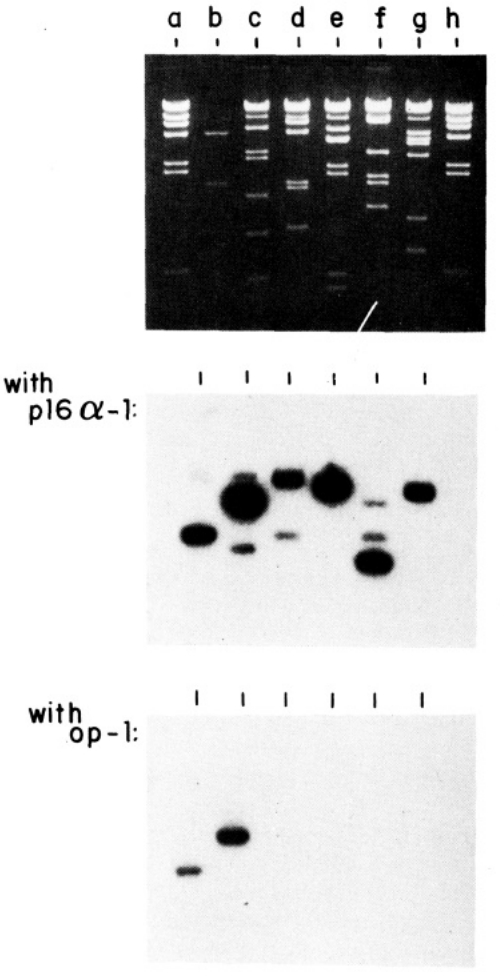

FIGURE 4: Southern hybridization of p16α-1 cDNA and specific oligonucleotides (op-1) with five isolated genomic DNA clones. The p16α-2 was digested with *Eco*RI, and the five genomic DNAs were digested with *Bam*H1 and *Sal*1. All digested DNAs were then electrophoresed on 1% agarose gel and transferred to Nytran paper. The hybridizations with the ^{32}P-labeled 3′ *Pst*1 fragment of p16α-1 cDNA or op-1 took place under the conditions described under Experimental Procedures. The top is ethidium bromide stained agarose gel with the restriction fragments generated from clone 52 (c), clone 5 (d), clone 21 (e), clone 54 (f), and clone 58 (g). Lane b contained *Eco*RI-digested p16α-2 with ~1.8 kb of insert, and lanes a and h contained *Hin*dIII-cut λDNA. The middle and bottom are pictures of X-ray films exposed to Nytran paper hybridized with p16α-1 and op-1, respectively.

whereas the males castrated at their adulthood retained the level of mRNA at one-third of that seen in normal adult males. Testosterone propionate returned the mRNA level in mice castrated at adulthood to that in normal adult male, but it increased the mRNA level in neonatally castrated adult mice to only ~40% of that in normal adult males. On the other hand, the expression of mRNA hybridized to op-1 probe depended upon a constant supply of androgen; neonatal castration or castration at adulthood decreased the mRNA level to the female level, and testosterone propionate treatment of both castrated males recovered fully the mRNA to the control level.

Discussion

In this paper, we have sequenced three overlapping cDNA clones for male-specific mouse C-P-450$_{16\alpha}$. The deduced amino acid sequence indicates that mouse C-P-450$_{16\alpha}$ is not a homologue of P-450 M1 (Yoshioka et al., 1987). P-450 M1 is a male-specific rat P-450 that catalyzes 16α-hydroxylation of testosterone (Matsumoto et al., 1986) and is believed to be the same rat P-450 as the other forms purified by different

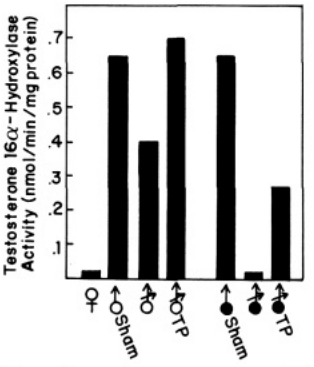

FIGURE 5: C-P-$450_{16\alpha}$-dependent testosterone 16α-hydroxylase activities in liver microsomes from castrated 129/J mice. Liver microsomes were prepared from adult female mice and adult male mice castrated on the first day for neonates or at adulthood. Testosterone propionate was administered always at adulthood. As the control, the sham-operated male mice were also used to isolate liver microsomes. Testosterone 16α-hydroxylase activities were measured with the presence or absence of antibody to C-P-$450_{16\alpha}$ under conditions previously described (Harada & Negishi, 1984b; Noshiro et al., 1986). The C-P-$450_{16\alpha}$-dependent activities were estimated as the activity inhibited by the antibody. The symbols used are (♀) female, (δ_{sham} and δ_{sham}) sham-operated, (δ and δ with arrow) castrated, and (δ_{TP} and δ_{TP} with arrow) testosterone propionate injection. The closed or open symbols indicate that the operations were done on the first day or at adulthood (~50 days old), respectively. Values were averaged from two separate experiments. Variation of activities in these experiments was less than 10%.

laboratories (P-450 RLM5, Cheng & Shchenkman, 1982; UT-A, Guengrich, 1982; P-450 male, Kamataki et al., 1983; 2C, Waxman, 1984; h, Ryan et al., 1984; P-$450_{16\alpha}$, Morgan et al., 1986). This implies that mouse and rat have developed a different P-450 gene family for establishing male-specific testosterone 16α-hydroxylase activity in liver microsomes.

The deletion of 142 bp in p16α-16 creates an early termination codon 29 bp downstream. This protein would not contain the conserved Cys peptide for the fifth ligand and would differ by only 10 amino acids from the corresponding amino acid sequence of C-P-$450_{16\alpha}$. It is interesting to note that C-P-$450_{16\alpha}$ was originally purified as a complex of two proteins: one is 49 500 daltons and the other is about 25 000 daltons (Harada & Negishi, 1984b). The molar ratio of the two proteins was about 1 to 1 in the purified fraction. When comparing the values estimated from SDS–polyacrylamide gel electrophoresis of purified P-450, it is not unusual to find that the molecular weights are considerably larger when they are calculated from the amino acid sequences deduced from nucleotide sequences of P-450 cDNAs. For instance, 48 000 was reported for the molecular weight of rabbit P-450 1 by SDS–polyacrylamide gel electrophoresis (Dieter et al., 1983), yet the obtained molecular weight from the P-450 1 cDNA sequence is 55 200 (Tukey et al., 1986). There is therefore a distinct possibility that the 25 000-dalton protein copurified with the 49 500-dalton protein is a translation product from p16α-16. More study will be required to prove this possibility.

The recent results from nucleotide sequences of the isolated genomic clone 52 indicated that the deleted 142-bp DNA is organized exactly as one exon, most likely the sixth exon of the gene (Wong et al., unpublished observation). When all gene structures are elucidated, it will be possible to determine whether or not the p16α-16 mRNA is transcribed from a gene without that exon or transcribed from a gene with that exon and processed alternatively.

At least two possible mechanisms could explain how the expressions of male-specific P-450 and its activity are regulated; one mechanism is the irreversible fixation (imprinting)

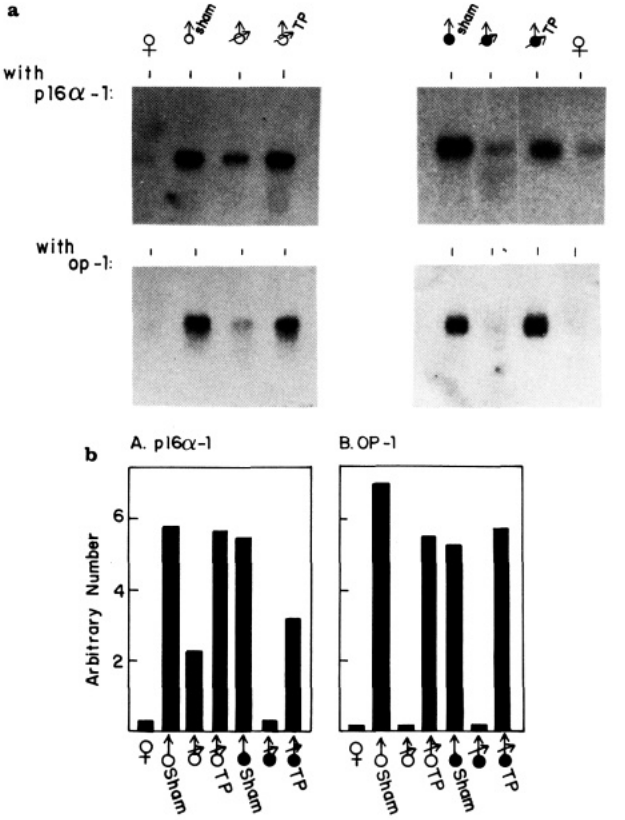

FIGURE 6: (a) Northern hybridization of p16α-1 cDNA and specific oligonucleotide (op-1) with liver poly(A)+ RNA from castrated 129/J mice. Liver poly(A)+ RNA was isolated from the same mice as those used for preparing liver microsomes in Figure 4. One microgram of each poly(A)+ RNA was electrophoresed on a 0.8% agarose gel containing formamide and transferred to Nytran paper. The paper was hybridized with the ^{32}P-labeled 3′ *Pst*1 fragment of p16α-1 cDNA and exposed to X-ray films. For the hybridization of op-1, 4 μg of each poly(A)+ RNA was used for the electrophoresis and the Nytran paper was incubated with op-1 labeled by the kination reaction with T4 polynucleotide kinase and [γ-^{32}P]ATP under the conditions described under Experimental Procedures. The symbols used are the same as those presented in Figure 5. (b) Effects of castration on mRNA levels in 129/J mice. The arbitrary numbers are representations of the intensities of the bands hybridized with the probes and were obtained by laser scanning of the negatives. The symbols used are the same as those in Figure 5.

of the expression at adulthood by neonatal androgen; the other is a reversible regulation by androgen at adulthood (Gustaffson et al., 1983). If the expression of testosterone 16α-hydroxylase activity is neonatally imprinted, the activity level in adult liver should not be influenced by withdrawing androgen by castration. The activity in neonatally castrated adult mice is as low as that in adult female mice and should not be increased by androgen treatment at their adulthood. In the case of mouse C-P-$450_{16\alpha}$-dependent activity, approximately 35% of control activity is lost by the castration at adulthood and is fully restored by the injection of testosterone propionate. On the contrary, the activity in liver microsomes from neonatally castrated adult mice is decreased to the level seen in normal adult female mice (only 3% of normal adult male) and is increased only to about 40% of the level in normal adult males by testosterone propionate treatment at their adulthood. This strongly indicates the presence of at least two differentially regulated C-P-$450_{16\alpha}$'s in liver microsomes from adult male 129/J mice. One (which contributes approximately 65% of total activity in microsomes) is neonatally imprinted, and the other (consisting of approximately 35% total activity in microsomes) is reversibly regulated. The Northern hybridizations of the p16α-1 cDNA and the specific oligonucleotide (op-1)

with liver poly(A)+ RNA from these mice also proved the presence of two different types of C-P-450$_{16\alpha}$ mRNAs. The Northern hybridization with the op-1 probe indicated that C-P-450$_{16\alpha}$ mRNA encoded by p16α-2 is reversibly regulated by androgen. Recently, Waxman et al. (1985) and Dannan et al. (1986) reported that male-specific rat P-4502c/UT-A is fully induced to the normal adult male level when the neonatally castrated adult rats are treated by testosterone. In contrast, the same hormone treatment increases in the C-P-450$_{16\alpha}$-dependent activity in the neonatally castrated adult mice to only about 40% of the level in normal adult mice. In terms of the definition of imprinting, rat P-4502c/UT-A may not be a form of P-450 that is neonatally imprinted. An explanation of the differences in the regulation of male-specific expressions between mice and rats is not certain at the present time.

In conclusion, we have found that at least two differentially regulated C-P-450$_{16\alpha}$'s and their mRNAs exist in livers of 129/J male mice: one is reversibly regulated by androgen and the expression of the other is neonatally imprinted. The cDNA of reversibly regulated C-P-450$_{16\alpha}$ was sequenced and was found to be a product from the family that consisted of at least five genes. Increasing attention has recently been given to the role of pituitary hormones (particularly growth hormone) in the sex-dependent regulation of P-450s and their activities in liver microsomes. We reported previously that the male-specific expression of C-P-450$_{16\alpha}$ is controlled by episodic secretion of growth hormone (Noshiro & Negishi, 1986a). Further study should elicidate the mechanism by which gonadal and pituitary hormones act cooperatively to regulate reversible and irreversible expression of the C-P-450$_{16\alpha}$ gene family in male mice.

Acknowledgments

We thank Drs. Mitsuhide Noshiro and Toru Ohyama for help in the construction of the genomic and cDNA libraries, respectively. We are grateful to Drs. Yukio Kato and Nobuhiro Harada for their contribution to the early stage of this work. We appreciate the technical assistance of Rickie Moore and James Clark and we thank Debbie Garner for typing the manuscript.

References

Aviv, H., & Leder, P. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1408–1412.

Benton, W. D., & Davis, R. W. (1977) *Science (Washington, D.C.) 196*, 180–182.

Biggin, M. D., Gibson, T. J., & Hong, G. F. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3963–3965.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Cheug, K.-C., & Schenkman, J. B. (1982) *J. Biol. Chem. 257*, 2378–2385.

Chung, L. W. K., & Chao, H. (1980) *Mol. Pharmacol. 18*, 543–549.

Colby, H. D. (1980) *Adv. Sex Horm. Res. 4*, 27–71.

Conney, A. H., Levin, W., Jacobson, M., & Kuntzman, R. A. (1969) in *Microsomes and Drug Oxidations* (Gillette, J. R., Conney, A. H., Cosmides, G. J., Estabrook, R. W., Fouts, J. R., & Mannering, G. J., Eds.) pp 299–302, Academic, New York.

Cox, R. A. (1968) *Methods Enzymol. 12*, 120–129.

Dale, R. M. K., McClure, B. A., & Houchins, J. P. (1985) *Plasmid 3*, 31–40.

Dannan, G. A., Guengerich, F. P., & Waxman, D. J. (1986) *J. Biol. Chem. 261*, 10728–10735.

Devore, K., Harada, N., & Negishi, M. (1985) *Biochemistry 24*, 5632–5637.

Dieter, H. H., Müeller-Eberhard, U., & Johnson, E. F. (1982) *Biochem. Biophys. Res. Commun. 105*, 515–520.

Einarsson, K., Gustafsson, J. A., & Stenborg, A. (1973) *J. Biol. Chem. 248*, 4987–4997.

Ford, H. C., Lee, E., & Engle, L. L. (1979) *Endocrinology (Baltimore) 104*, 857–861.

Frischauf, A.-M., Lehrach, H., Poustka, A., & Murray, N. (1983) *J. Mol. Biol. 170*, 827–842.

Fujii-Kuriyama, Y., Mizukami, Y., Kawajiri, K., Sogawa, K., & Muramatsu, M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2793–2797.

Guengrich, F. P., Dannan, D. A., Wright, S. T., Martin, M. V., & Kaminsky, L. S. (1982) *Biochemistry 21*, 6019–6039.

Gustafsson, J. Å., Mode, Norstedt, G., & Skett (1983) *Annu. Rev. Physiol. 45*, 51–60.

Harada, N., & Negishi, M. (1984a) *J. Biol. Chem. 259*, 1265–1271.

Harada, N., & Negishi, M. (1984b) *J. Biol. Chem. 259*, 12285–12290.

Harada, N., & Negishi, M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2024–2028.

Kamataki, T., Maeda, K., Yamazoe, Y., Nagai, Y., & Kato, R. (1983) *Arch. Biochem. Biophys. 225*, 758–770.

Kozak, M. (1986) *Cell (Cambridge, Mass.) 44*, 283–292.

Levin, W., Ryan, D., Kuntzman, R., & Conney, A. H. (1975) *Mol. Pharmacol. 11*, 190–208.

Matsumoto, T., Emi, Y., Kawabata, S., & Omura, T. (1986) *J. Biochem. (Tokyo) 100*, 1359–1371.

Messing, J., Gronenborn, B., Muller-Hill, B., & Hotschneider, P. H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3642–3646.

Morgan, E. T., MacGeoch, C., & Gustafsson, J. Å. (1985) *J. Biol. Chem. 260*, 11895–11898.

Nakamura, M., Negishi, M., Altieri, M., Chen, Y. T., Ikeda, T., Tukey, R. H., & Nebert, D. W. (1983) *Eur. J. Biochem. 134*, 19–25.

Noshiro, M., & Negishi, M. (1986a) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 45*, 2174.

Noshiro, M., & Negishi, M. (1986b) *J. Biol. Chem. 261*, 15923–15927.

Noshiro, M., Serabjit-Singh, C. J., Bend, J. R., & Negishi, M. (1986) *Arch. Biochem. Biophys. 244*, 857–864.

Okayama, H., & Berg, P. (1982) *Mol. Cell Biol. 2*, 161–170.

Ozols, J., Heinemann, F. S., & Johnson, E. F. (1981) *J. Biol. Chem. 256*, 11405–11408.

Pasleau, C., Kolodcizi-Mehaignoul, C., & Gielen, J. E. (1984) *Endocrinology (Baltimore) 115*, 1371–1379.

Queen, C., & Korn, L. J. (1984) *Nucleic Acids Res. 12*, 581–599.

Roy, A. K., & Chatterjiee, B. (1983) *Annu. Rev. Physiol. 45*, 37–50.

Ryan, D. E., Iida, S., Wood, A. W., Thomas, P. E., Leiber, C. S., & Levin, W. (1984) *J. Biol. Chem. 259*, 1239–1250.

Sanger, F., Coulson, A. R., Barrel, B. G., Smith, A. J. H., & Roe, B. A. (1980) *J. Mol. Biol. 143*, 161–178.

Southern, E. M. (1975) *J. Mol. Biol. 98*, 503–517.

Thomas, P. S. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5201–5205.

Tukey, H. R., Okino, S., Barnes, H., Griffin, K. J., & Johnson, E. F. (1985) *J. Biol. Chem. 260*, 13347–13354.

Waxman, D. J. (1984) *J. Biol. Chem. 259*, 15481–15490.

Waxman, D. J., Dannan, G. A., & Guengerich, F. P. (1985) *Biochemistry 24*, 4409–4417.

Wong, G., Kawajiri, K., Kato, Y., Harada, N., & Negishi, M. (1986) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 45*, 2176.
Yabusaki, T., Shimizu, M., Murakami, H., Nakamura, K., Oeda, K., & Ohkawa, H. (1984) *Nucleic Acids Res. 12*, 2929–2938.
Yoshioka, H., Norohashi, K., Sogawa, K., Miyata, T., Kawajiri, K., Hirose, T., Inayama, S., Fujii-Kuriyama, Y., & Omura, T. (1987) *J. Biol. Chem. 262*, 1706–1711.
Young, R. A., & Davis, R. W. (1982) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1194–1198.

Characterization and Purification of a Protease in Serum That Cleaves Proatrial Natriuretic Factor (ProANF) to Its Circulating Forms†

Jerome B. Zisfein, Robert M. Graham, Stephen V. Dreskin, Gary M. Wildey, Alan J. Fischman, and Charles J. Homcy*

Cellular and Molecular Research Laboratory, Cardiac Unit, Massachusetts General Hospital, Boston, Massachusetts 02114

Received April 15, 1987; Revised Manuscript Received June 30, 1987

ABSTRACT: Atrial natriuretic factor (ANF) is synthesized and stored in atrial cardiocytes as a 17-kilodalton (kDa), 126 amino acid polypeptide, proANF, but circulates as smaller, 24 and 28 amino acid peptide fragments of the carboxy terminus of proANF. It has previously been shown that proANF is secreted intact from cultured atrial cardiocytes and can be cleaved by a serum protease to smaller, 3-kDa peptides believed to be the circulating forms. This report describes the purification and characterization of this proANF-cleaving protease from rat serum. The cleavages both of ^{35}S-labeled proANF derived from rat atrial cell cultures, as assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)/autoradiography, and of a synthetic *p*-nitroanilide-containing substrate were used as assays for the detection of enzyme activity. ProANF-cleaving activity was found in rat serum, with no such activity detectable in rat plasma. Cleavage in serum was not dependent on the presence of platelets or other cellular elements. Complete inhibition of proANF cleavage was obtained with the protease inhibitors benzamidine, leupeptin, phenylmethanesulfonyl fluoride, and diisopropyl fluorophosphate (DFP) but not with aprotinin, soybean trypsin inhibitor, pepstatin, or hirudin. Unlike the vitamin K dependent plasma proteins, the proANF-cleaving protease did not adsorb to barium sulfate. With the sequential application of ion-exchange, hydroxylapatite, lectin affinity, and gel filtration chromatography, a 5000–6000-fold purification of the enzyme from rat serum was achieved. Fractionation of either whole serum or the purified enzyme by gel filtration chromatography revealed a single peak of activity corresponding to a protein with a Stokes radius of 45 Å. The p*I* of the enzyme was found to be approximately 5.6. Incubation of the purified enzyme with [^{3}H]DFP followed by SDS–PAGE and autoradiography revealed a specifically labeled 38-kDa peptide, the substrate binding subunit. Analysis by high-performance liquid chromatography of the 3-kDa products resulting from the cleavage of ^{35}S-labeled proANF by the purified enzyme revealed, as previously described with whole serum, two radiolabeled peptides which coeluted with the 28 and 24 amino acid C-terminal peptides. Moreover, a time-dependent increase in the abundance of the latter peptide was found. These observations imply a precursor–product relationship, with the initial cleavage of proANF to the 28 amino acid peptide, which is then cleaved to the 24 amino acid peptide. These studies indicate that the majority of proANF cleavage activity found in rat serum is represented by that of a distinct serine protease whose properties are different from a variety of well-characterized proteases, such as kallikrein, plasmin, and the vitamin K dependent plasma proteins. The role of this protease in the in vivo processing of proANF remains to be defined.

Atrial natriuretic factor (ANF) is synthesized in atrial cardiocytes as a 152 amino acid "preprohormone" (Seidman et al., 1984; Yamanaka et al., 1984; Maki et al., 1984) and is stored in atrial granules as a 126 amino acid polypeptide, proANF (Kangawa et al., 1984; Glembotski et al., 1985; Vuolteenaho et al., 1985; Zisfein et al., 1986a; Miyota et al., 1985). Analysis of immunoreactive and bioactive ANF in blood has revealed that the major circulating forms are the 24 and 28 amino acid peptides, corresponding to the carboxy-terminal fragments of proANF (Sugarawa et al., 1985; Yamaji et al., 1985; Schwartz et al., 1985; Thibault et al., 1985). There is some evidence that cleavage of proANF to these smaller peptides is necessary for full biological activity (Currie et al., 1984a,b). The site of cleavage of proANF to these smaller forms is unknown.

Recently, we have demonstrated by intrinsic labeling studies that proANF, identified as a peptide of approximately 17 kilodaltons (kDa) by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), is both stored and secreted by rat atrial cardiocytes in culture (Bloch et al., 1985; Zisfein et al., 1986b). Incubation of the 17-kDa proANF derived from these cells with rat serum results in cleavage to two major fragments: a cysteine-containing 3-kDa peptide and a 14-kDa methionine-containing peptide (Bloch et al., 1987). High-performance liquid chromatography (HPLC) and radiosequence analysis of the 3-kDa fragment revealed the presence of two major peptide products corresponding to the 28 and 24 amino acid carboxy-terminal ANF peptides (Bloch et al.,

† This work was supported by a grant from the National Institutes of Health (HL-35642-01) and by a grant from the R. J. R. Nabisco Co. R.M.G. is an Established Investigator, American Heart Association (AHA 82-240).

* Address correspondence to this author.

0006-2960/87/0426-8690$01.50/0