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Rip Locus: Regulation of Female-Specific Isozyme (I-P-450$_{16\alpha}$) of Testosterone 16α-Hydroxylase in Mouse Liver, Chromosome Localization, and Cloning of P-450 cDNA†

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ABSTRACT: The constitutive expression of phenobarbital-inducible mouse cytochrome P-450 (I-P-450$_{16\alpha}$) at the mRNA level and its associated testosterone 16α-hydroxylase activity in liver microsomes was a female characteristic in many inbred mice, including BALB/cJ, A/HeJ, and C57BL/6J. This sex-dependent constitutive expression of the mRNA and enzyme activity was severely reduced in females of mouse strain 129/J. The distribution patterns of the mRNA and activity levels in individual offspring of F1, F2, and F1 backcrosses to progenitors, generated from crosses between 129/J and BALB/cJ mice, indicated that the female-specific expression of I-P-450$_{16\alpha}$ is an autosomal dominant trait under the regulation of a sex-limited single locus. It was found that the genotypes of this locus exhibited concordance with that of the coumarin hydroxylase locus (Coh locus) in eight out of nine 9×A recombinant inbred strains, suggesting the localization of this sex-limited locus on chromosome 7. We propose Rip (*r*egulation of sex-dependent, constitutive expression of phenobarbital-*i*nducible *P*-450) as the name of this sex-limited locus. With the use of the rat P-450e cDNA probe, a cDNA library from liver poly(A+) RNA of BALB/cJ was screened, and three distinct cDNAs (pf3, pf26, and pf46) were selected on the basis of their restriction patterns. Nucleotide sequences of the cDNAs revealed that pf3 and pf46 are clones overlapped, with the exception that the 27-bp DNA is inserted in the coding region of pf46. The nucleotide sequence (named pf3/46) obtained from the overlapping sequences of pf3 and pf46 contained 1473 or 1500 bp of open-reading frame, and the deduced amino acid sequence shared 93% similarity with those of rat P-450b. The 27-bp insertion resulted in nine extra amino acids just in front of the cysteine residue, the fifth ligand for heme binding. The mRNA with 27-bp insertion was ubiquitously present in other inbred mice such as A/HeJ and C57BL/6J, but not in 129/J. S-1 nuclease analysis estimated a ratio of p46 and pf3 to be 1:50. Nucleotide and deduced amino acid sequences of the 1473-bp open-reading frame in pf26 possessed 83% similarity to those of pf3/46. Hybridizations of oligonucleotide probes (pf26-cu and pf3/46-cu) specific to either pf26 or pf3/46 with liver poly(A+) RNA from males and females of BALB/cJ, 129/F, and F1 offspring demonstrated that the expression of pf26, but not pf3/46, mRNA was associated with the autosomal dominant inheritance of I-P-450$_{16\alpha}$. The levels of the hybridization of pf26cu to mRNA from F1 backcross to 129/J correlated well with those of the high and the low I-P-450$_{16\alpha}$-dependent testosterone 16α-hydroxylase activities at a 1:1 ratio. Therefore, it was concluded from the genetic evidence that pf26 represents cDNA encoding of testosterone 16α-hydroxylase I-P-450$_{16\alpha}$.

Cytochrome P-450 (P-450)[1] represents a group of terminal oxidases of a membrane-bound monooxygenase system that consists of NADPH–cytochrome P-450 reductase, cytochrome b_5, and NADH–cytochrome b_5 reductase (Sato & Omura, 1978). This monooxygenase system in hepatic microsomes is involved in metabolic inactivation of many endogenous compounds, such as steroid hormones. But in some cases, toxicity and carcinogenicity of certain xenobiotics are enhanced by P-450-dependent metabolism. Since metabolism is influenced by the presence of multiple forms of P-450 (Lu & West,

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[1] Abbreviations: Rip, *r*egulation of sex-dependent, constitutive expression of phenobarbital-*i*nducible *P*-450; PB, phenobarbital; P-450, cytochrome P-450; 20× SSC, 3 M sodium chloride and 0.3 M sodium citrate; 6× NET, 0.9 M sodium chloride, 6 mM ethylenediaminetetraacetate, and 0.09 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.0; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; 5× Denhardt's solution, 0.1% each of poly(vinylpyrrolidone), bovine serum albumin, and Ficoll 400.

1980; Nebert & Negishi, 1982), the sex-specific forms of P-450 may be critical for sex-dependent toxicity and carcinogenicity of certain drugs and chemicals, such as aflatoxin and 2-(acetylamino)fluorene in rodents. Thus, it is important to understand the molecular mechanism(s) by which sex-, age-, and tissue-specific expression of individual forms of P-450 is (are) regulated.

Testosterone 16α-hydroxylase activity in liver microsomes is at least 10 times higher in male than in female 129/J mice. On the other hand, the activity is expressed at equally high levels in females as well as in males of many other inbred mice such as BALB/cJ. There are at least two isozymes (forms of P-450) of testosterone 16α-hydroxylase that are constitutively expressed either in female or in male mice (Noshiro et al., 1986b; Harada & Negishi, 1984b). The lower testosterone 16α-hydroxylase activity in 129/J compared to BALB/cJ female mice is found to be primarily due to the reduced levels of a female-specific isozyme (called I-P-450$_{16\alpha}$) in 129/J mice (Noshiro et al., 1986). The other isozyme, C-P-450$_{16\alpha}$, which is male specific, is expressed normally in both 129/J and BALB/cJ mice.

By taking advantage of this strain difference, the expression of I-P-450$_{16\alpha}$ in livers of 129/J and BALB/cJ progenitors and offspring from [129/J × BALB/cJ]F1 and F2 and the F1 backcross to progenitors was studied in this report in order to elucidate the genetic basis of the female-specific expression of this cytochrome.

In order to explore further details of the genetic regulation of sex-specific expression of I-P-450$_{16\alpha}$ in mouse liver, the isolation and characterization of the I-P-450$_{16\alpha}$ gene was inevitable. Since we have demonstrated previously that rat P-450e cDNA (R17) cross-hybridizes to mouse I-P-450$_{16\alpha}$ mRNA and that the hybridization of R17 to mouse liver poly(A+) RNA is an accurate reflection of the amount of I-P-450$_{16\alpha}$ mRNA present in poly(A+) RNA sample (Noshiro et al., 1986b), in this paper, therefore, we have isolated and sequenced mouse cDNAs selected after hybridization of a radioactive rat cDNA (R17) probe from a cDNA library constructed from liver poly(A+) RNA of untreated BALB/cJ female mice. The specific oligonucleotide probes based on the nucleotide sequences of isolated mouse cDNAs were subsequently prepared and used to identify cDNA for I-P-450$_{16\alpha}$.

Experimental Procedures

Animals and Drug Treatments. In all of the experiments, 6–9-week-old mice were used. Male and female 129/J, BALB/cJ, and A/HeJ mice were purchased from Jackson Laboratory. [129/J female × BALB/cJ male]F1 (129CF1/J), [BALB/cJ female × 129/J male]F1 (C129F1/J), and the F1 backcross to 129/J offspring were produced by the Comparative Medicine Branch, National Institute of Environmental Health Sciences (NIEHS). Recombinant inbred strains, nine strains of 9×A (129/sv-sl crossed to A/HeJ), were kindly supplied by Dr. Leroy Stevens, and four strains of N×129 (NZB/BINJ crossed to 129/J) were from Dr. Benjamin Taylor at Jackson Laboratory.

Preparation of Microsomes and Poly(A+) RNA from Mouse Livers. Isolation of microsomes from liver homogenates, extraction of liver RNA by guanidine hydrochloride, and purification of poly(A+) RNA by oligo(dT)–cellulose column chromatography were performed as described in previous papers (Harada & Negishi, 1984a; Cox, 1968; Aviv & Leder, 1972).

Isolation of cDNAs and Nucleotide Sequencing. Liver poly(A+) RNA from untreated BALB/cJ female was treated with avian reverse transcriptase (Seikagaku) to make single-strand cDNA, which was subsequently converted to double-stranded cDNA with DNA polymerase I and RNase H as described by Okayama & Berg (1982). The double-stranded cDNA was blunt-ended by T4 DNA polymerase and ligated to the λgt11 vector by using *Eco*RI linkers according to the method of Young and Davis (1982). The recombinant phages were transferred to *Escherichia coli* Y1090 and screened by nick-translated R17 cDNA (a generous gift from Dr. Milton Adesnik). For nucleotide sequencing of the cDNAs, DNA fragments were generated by digestions with proper restriction enzymes and cloned into M13 vectors. Single-strand recombinant M13 phage DNA was prepared (Messing et al., 1977) and sequenced by the dideoxy chain termination method (Sanger et al., 1980).

Northern Hybridization with Oligonucleotide Probe and Dot Hybridization with cDNA Probe. The cDNA (R17) was nick-translated with [α-^{32}P]dCTP and hybridized to dotted poly(A+) RNA on nitrocellulose paper. The hybridization was done by using the conditions previously described (Tukey et al., 1981; Norstedt & Palmiter, 1984). The following oligonucleotides were prepared: $^{5'}$GCATAACTAGCTGAGCTG$^{3'}$ (pf26-cu) and $^{5'}$ACAACAGTAGAAGGAAGG$^{3'}$ (pf26-u4) specific to pf26; $^{5'}$TGAAGGTTGGCTCAACGA$^{3'}$ (pf3/46-cu) and $^{5'}$GGAGACATGCAATAGGAGTA$^{3'}$ (pf3/46-u4) specific to pf3/46; $^{5'}$TCCCACAGACTTTTGATCAAAAATTTG$^{3'}$ (pf46-i) specific to pf46. Poly(A+) RNA (4 μg) was denatured by 2.2 M formaldehyde and 50% formamide, electrophoresed on agarose gel containing 2.2 M formaldehyde as described by Thomas (1980), and then transferred to nylon membrane (Nytran; Schleicher & Schuell), with 20× SSC as transfer buffer. The Nytran paper was baked at 80 °C for 1 h and incubated in prehybridization solution containing 5× Denhardt's, 10 mM ethylenediaminetetraacetate, pH 8.0, and 0.5% sodium *N*-lauroylsarcosinate at 42 or 50 °C, depending upon the probes. The hybridization solution consisted of 6× NET, 5× Denhardt's (Dehardt, 1966), 250 μg/mL sonicated herring sperm DNA, and 0.1% sodium *N*-lauroylsarcosinate at 42 or 50 °C, depending upon the probes. Oligonucleotides were labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (Maxam & Gilbert, 1980). After being hybridized, the Nytran paper was washed with 6× NET containing 0.5% sodium *N*-lauroylsarcosinate at room temperature and exposed to X-ray film.

S-1 Nuclease Mapping. pf46 plasmid was digested with *Sca*I to generate the 200-bp fragment containing the 27-bp insertion sequence, and then the fragment was subcloned into the *Eco*RV site of pBR322. This recombinant plasmid (named p46asca) was purified by two rounds of centrifugation in CsCl gradients to remove any contamination with RNA and subsequently was linearized by digestion with *Mst*II, which exists only in the 200-bp insert DNA. The linearized p46asca was dephosphorylated by bacterial alkaline phosphatase and then labeled by [γ-^{32}P]ATP and T4 polynucleotide kinase according to the method of Maxam and Gilbert (1980), except that 20 mM potassium phosphate (pH 9.2) was added to the reaction mixture to inhibit dephosphorylation. The ^{32}P-labeled pf46asac (10^6 cpm/μg of DNA) was purified by Bio-Gel P-6DG column chromatography, coprecipitated with 5 μg of yeast tRNA in 70% ethanol and 3.5 M ammonium acetate, and finally dried. The dried DNA was dissolved in 20 μL of 80% formamide containing 100 mM HEPES, pH 6.5, and 0.5 M NaCl. Liver poly(A+) RNA (12 μg) from A/HeJ female mice was precipitated with ethanol and then dried under vacuum. This RNA was dissolved in the formamide solution containing the radioactive p46asac. After incubation in a siliconized mi-

crotube at 80 °C for 5 min and then at 45 °C overnight, the RNA–DNA mixture was transferred into 160 μL of cold water, and then 20 μL of 10× concentrated S-1 buffer (300 mM sodium acetate, pH 4.6, and 10 mM $ZnSO_4$ in 50% glycerol) and 10 μg of sonicated herring sperm DNA were added to it. The mixture was then equally divided into Eppendorf tubes and digested with 5 or 12.5 units of S-1 nuclease (BRL) at 37 °C for 30 min. The DNA–RNA hybrids that remained were precipitated with ethanol, dried, and denatured in sample loading buffer (90% formamide, 10 mM Tris-HCl, pH 7.5, 1 mM ethylenediaminetetraacetate, and 0.2% bromophenol blue and xylene cyanol). After incubation at 90 °C for 2 min, the denatured sample was applied on a 7.5% polyacrylamide gel under the alkaline-denatured condition. The polyacrylamide gel was dried and exposed to X-ray film to analyze sizes of DNA protected from S-1 nuclease digestion.

Other Analytical Methods. Testosterone 16α-hydroxylase activity was measured by the methods of Harada and Negishi (1984). I-P-$450_{16\alpha}$-dependent testosterone 16α-hydroxylase activity in liver microsomes was determined as the activity inhibited by antibody to I-P-$450_{16\alpha}$ as described previously (Devore et al., 1985; Noshiro et al., 1986b). Protein contents were measured according to the method of Bradford (1976). Analysis of the DNA sequences was performed by programs from the MICROGENIE software package (Queen & Korn, 1984).

RESULTS

Regulation of Female-Specific Expression of I-P-$450_{16\alpha}$ at mRNA Levels by a Single Locus. Liver poly(A+) RNA was prepared from females of individual 129/J and BALB/cJ progenitors, 129CF1/J [129/J female × BALB/cJ male], C129F1/J [BALB/cJ female × 129/J male], and 129CF2/J and 129CF1/J backcrosses to 129/J and BALB/cJ offspring, and subjected to dot hybridization analysis with the ^{32}P-labeled cDNA (R17). The radioactivities recovered from the areas hybridized are summarized as histograms in Figure 1. The highest value of radioactivity in the 129/J mice was about 4 times lower than the lowest value in the BALB/cJ mice (first panel). Consistent with our previous observations (Noshiro et al., 1986b), the mRNA levels were as high in all of the F1 offspring as in the BALB/cJ females (second panel). The ratios of segregation of the high and the low mRNA levels in the individuals from 129CF2/J and 129CF1/J backcrosses to 129/J offspring were 18:4 and 12:10, respectively (third and fourth panels). These ratios were not significantly different ($p < 0.5$) from the theoretical ratios of 3:1 and 1:1, respectively, for associating the inheritance with regulation at a single locus. As expected, all individuals from the 129CF1/J backcross to BALB/cJ offspring showed the high mRNA levels (last panel). Therefore, we concluded that the expression of I-P-$450_{16\alpha}$ at mRNA as well as at activity levels in female mice is under an autosomal dominant regulation by a single locus in this pair.[2] The levels of mRNA hybridizing to the cDNA probe R17 in 129/J and BALB/cJ male mice and their offspring (F1, F2, and F1 backcrosses to progenitors) were almost $^1/_{10}$ of those seen in the males, and no differences in the mRNA levels were detected (data not shown); thus, the action of this locus was sex-limited. We propose Rip (*r*egulation of sex-dependent constitutive expression of phenobarbital-*i*nducible *P*-450) as the name of this locus.

Chromosome Localization of Rip Locus. The distribution of the hybridizable mRNA and enzyme activity levels in nine

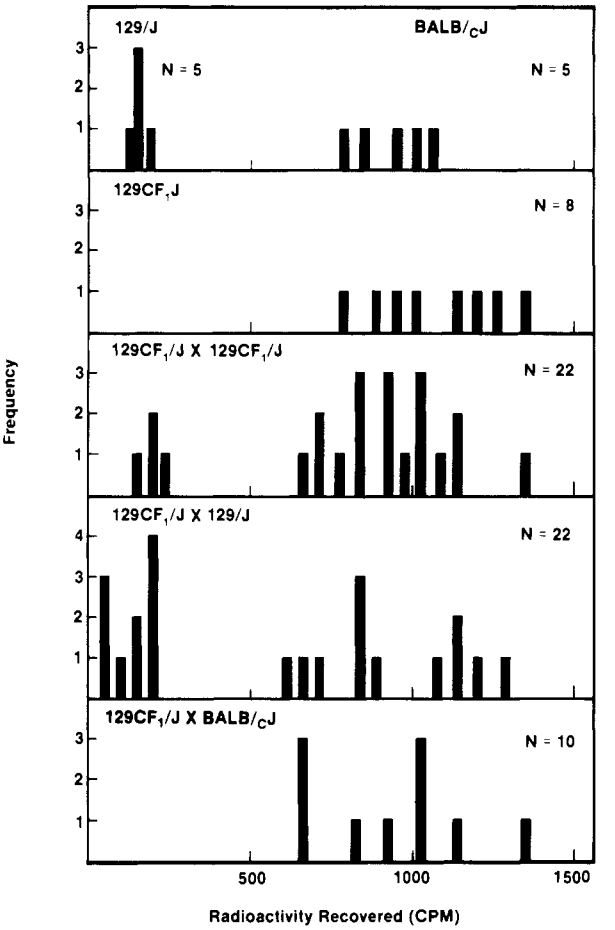

FIGURE 1: Histogram of levels of mRNA hybridizable with cDNA (R17) in females of 129/J and BALB/cJ progenitors and their offspring. In all of the experiments, 6–9-week-old mice were used. Strains 129/J and BALB/cJ were purchased from Jackson Laboratory. Total liver poly(A+) RNA (1 μg) prepared from individual mice (progenitors and offspring) was denatured by 5 mM methylmercury hydroxide, dotted on nitrocellulose paper, and hybridized with ^{32}P-labeled R17 cDNA. after exposure to X-ray films, dotted areas were cut out and their radioactivity was measured by liquid scintillation counting. The radioactivity averaged from four different experiments was used to construct the histogram. In the range of poly(A+) RNA dotted (up to 5 μg), the hybridized radioactivity was linearly correlated with the amount of the RNA (Noshiro et al., 1986b).

recombinant inbred strains (9×A) are summarized in Figure 2. The four mouse strains (9×AA, 9×AD, 9×AE, and 9×AK) possessed the higher levels of I-P-$450_{16\alpha}$, both at the mRNA and activity levels such as those seen in A/HeJ. The other five inbred mice demonstrated the reduced levels of I-P-$450_{16\alpha}$ such as those seen in 129/J. Perfect correlation was observed between the levels of the mRNA and I-P-$450_{16\alpha}$-dependent activity in all nine recombinant inbred strains. The same correlation was also found in four N×129 recombinant inbred strains (data not shown). The linkage of Rip locus to Coh locus on mouse chromosome 7 was tested by using nine strains of 9×A inbred mice (Table I). The Coh locus represents an induction of liver microsomal coumarin hydroxylase activity by PB and encodes P-450 catalyzing of coumarin hydroxylase activity on mouse chromosome 7 (Wood & Taylor, 1979). It was found that eight out of nine recombinant inbred strains, the genotypes of Rip and Coh loci, are concordant. The strain 9×AM exhibited the recombination between the two loci.

Association of I-P-$450_{16\alpha}$-Dependent Activity with mRNA Levels and with Rip Locus. In orde to confirm an association of I-P-$450_{16\alpha}$-dependent activity with amounts of mRNA

[2] Our result is consistent with that concluded by Ford et al. (1979) but not by Pasleau et al. (1984).

Strains	Radioactivity Recovered [CPM]	"I"-P-450$_{16\alpha}$-Dependent Activity [nmol/min/mg protein]
A/HeJ	1650	0.35
129/$_{SvJ}$	68	0.02
9XAA	1400	0.40
9XAB	35	0.00
9XAD	740	0.30
9XAE	865	0.22
9XAF	100	0.00
9XAI	32	0.00
9XAJ	16	0.00
9XAK	530	0.26
9XAM	80	0.00

FIGURE 2: Distribution of the mRNA and enzyme activity levels in nine recombinant inbred strains (9×A). Poly(A+) RNA and microsomes were prepared from livers of three female mice of each nine recombinant inbred strains. The contents of hybridizable mRNA with ^{32}P-labeled R17 cDNA and I-P-450$_{16\alpha}$-dependent testosterone 16α-hydroxylase activity in the microsomes were measured. The picture showed dot hybridization of the poly(A+) RNA samples on nitrocellulose paper. The radioactivity recovered from the dotted area was averaged from two hybridization experiments.

Table I: Coumarin Hydroxylase Activity and Linkage to Rip Genotype in Female Mice of Recombinant Inbred Strain (9×A)[a]

strains	coumarin hydroxylase activity [nmol min^{-1} (mg of protein)$^{-1}$]	locus genotype Coh		Rip
9×AA	1.29	A		A
9×AB	9.00	9		9
9×AD	1.86	A		A
9×AE	1.12	A		A
9×AF	8.26	9		9
9×AI	15.0	9		9
9×AJ	9.1	9		9
9×AK	1.22	A		A
9×AM	1.00	A	×	9

[a] PB (50 mg/100 g of body weight) was administered ip once a day for 3 days. Liver microsomes were prepared from the drug-treated female mice of each recombinant inbred strain. Coumarin hydroxylase activity in the microsomes was measured. The letter A is used to represent the Coh genotype of A/HeJ, and the number 9 represents 129/J. The Coh genotypes of the inbred mice are taken from the previous work by Wood and Taylor (1979). The Rip genotype was determined from the data obtained in Figure 2.

hybridized to R17, the mRNA and the activity were measured in the same individuals from 129CF1/J backcrossed to 129/J offspring. Eight out of eighteen female offspring showed the low mRNA and activity levels, and the remaining ten females had the high mRNA and activity levels (Figure 3). Similarly, all F1 offspring showed the high rates of testosterone 16α-hydroxylase activity, and the F2 offspring that possessed the low mRNA levels also demonstrated the low hydroxylase activity (data not shown). It was therefore concluded that I-P-450$_{16\alpha}$-dependent activity is regulated by the Rip locus at

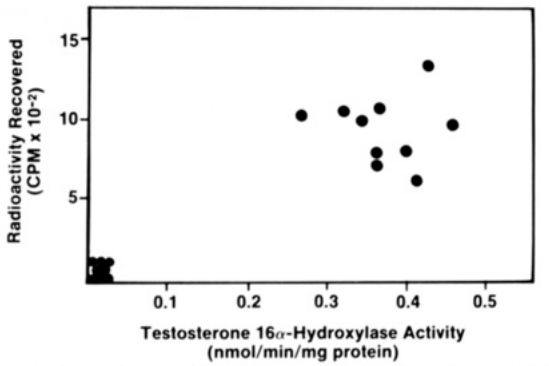

FIGURE 3: Association of testosterone 16α-hydroxylase activity in hepatic microsomes with the mRNA level in individual female offspring from 129CF1 × 129/J mice. Testosterone 16α-hydroxylase activity in microsomes (250 μg) was measured in the presence and absence of anti-I-P-450$_{16\alpha}$. The activity catalyzed by I-P-450$_{16\alpha}$ was taken to be the rate of activity that was inhibited by the antibody. The mRNA levels were determined by the same dot hybridization procedure as those in Figure 1.

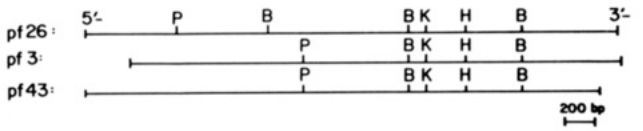

FIGURE 4: Restriction maps of three types of cDNAs. pf26, pf3, and pf46 represented the three different types of cDNA based on restriction analysis. pf26 differed from pf3 and pf46 by the position of *Pst*I and by extra *Bgl*II. pf3 and pf46 showed identical restriction patterns, except that a slightly larger *Kpn*I–*Bgl*II (5′–3′) fragment was reproducibly generated from pf46 but not from pf3. Symbols: P, *Pst*I; B, *Bgl*II; K, *Kpn*I; and H, *Hin*dIII. All cDNA were started and ended by *Eco*RI cloning sites.

the mRNA level. We contructed the cDNA library from liver poly(A+) RNA of BALB/cJ females and isolated the cDNA-encoded female-specific P-450 regulated by the Rip locus in order to extend the research.

Isolation and Characterization of cDNAs. Twenty-one independent cDNA clones were purified and subcloned into pBR322 and categorized into three distinct groups on the basis of the differences in their restriction maps (Figure 4). pf3 (1.8 kbp), pf46 (1.9 kbp), and pf26 (2.0 kbp) were the largest clones in each categorized group. pf3 and pf46 exhibited an identical map, except that the *Kpn*I–*Bgl*II (5′–3′) fragment from the pf46 clone is slightly longer than that from pf3. Figure 5 shows the nucleotide sequence of pf26 cDNA, with underwritten different nucleotides of pf3/46 cDNA. pf26 consisted of 1473 bp of open-reading frame, and 1 and 400 bp of 5′- and 3′-noncoding regions, respectively. The nucleotide sequence of pf3/46, which also contained the 1473- or 1500-bp open-reading frame, was constructed from the overlapping sequences of pf3 and pf46. The nucleotide sequences of additional clones (pf40 and pf11) verified that all of these clones (pf3, pf46, pf40, and pf11) overlapped. The overall sequence similarity between pf26 and pf3/46 was 83%.

Polymorphism in pf3/46 cDNA. The nucleotide sequences of pf3 and pf46 overlapped, except for possessing two different types of polymorphism; one was the 27-bp insertion in the coding region of pf46. It was of interest that the nucleotide sequence of the 27-bp insert has potential to form a loop structure. The 18-base reverse complement (pf16-i) of the nucleotide sequence from the 27-bp insertion was synthesized, labeled by T4 polynucleotide kinase and [γ-^{32}P]ATP, and hybridized with liver poly(A+) RNA from 129/J, A/HeJ, BALB/cJ, and C57BL/6J female mice. The results indicated that pf46-type mRNA is ubiquitously present in livers of adult

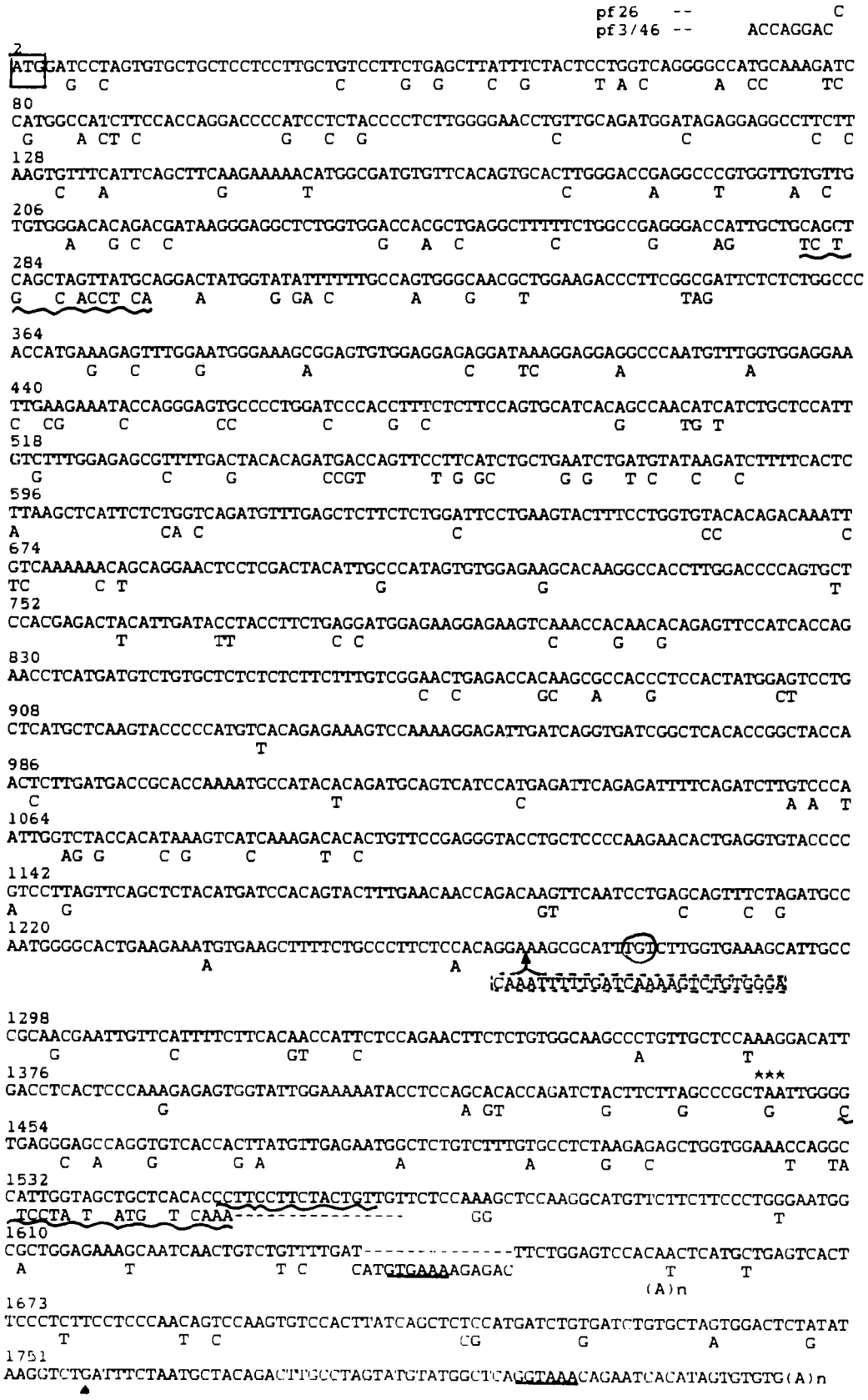

FIGURE 5: Nucleotide sequences of cDNA pf26 and pf3/46. The 1875 bp of the nucleotide sequence for pf26 are shown. The different nucleotides from pf26 are underwritten for pf3/46. The sequence for pf3/46 was generated by overlapping the sequences obtained from pf3 and pf46. The 5′-noncoding region of pf3/46 was extended eight more bases from the 5′-end of pf26. The arrow indicates the 3′-end of pf3/46. The position of the poly(A) tract in pf46 is also underwritten. The 27-bp fragment is boxed with a broken line with the arrow indicating the position of this insertion. The initiation codon is boxed with a solid line, and termination codons are indicated with stars. The triplet coding the cysteine that has been predicted to be the fifth ligand for heme binding is circled. The putative poly(A) additional signals are underlined. The nucleotide sequences from which the specific oligonucleotide probes are prepared are shown by wavy lines: the oligonucleotides from coding regions were named pf26-cu and pf3/46-cu, and those from 3′-noncoding regions were named pf26-u4 and pf3/46-u4.

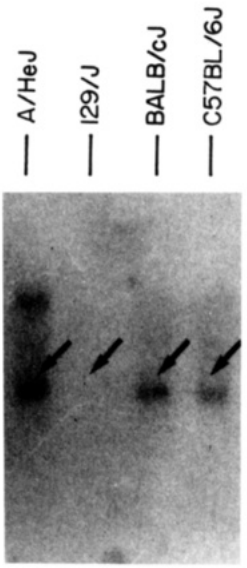

FIGURE 6: Northern hybridization of specific oligonucleotide probe (pf46-i) with total liver poly(A+) RNA from A/HeJ, 129/J, BALB/cJ, and C57BL/6J female mice. The reverse complement (pf46-i) of the 27-bp DNA inserted in pf46 was prepared and labeled by T4 polynucleotide kinase and [γ-^{32}P]ATP. Total liver poly(A+) RNA (4 μg) from A/HeJ, 129/J, BALB/cJ, and C57BL/6J female mice were electrophoresed on formamide–agarose gel and transferred to nitrocellulose paper as described under Experimental Procedures. The nitrocellulose paper was hybridized with ^{32}P-labeled pf46-i under the conditions as described under Experimental Procedures.

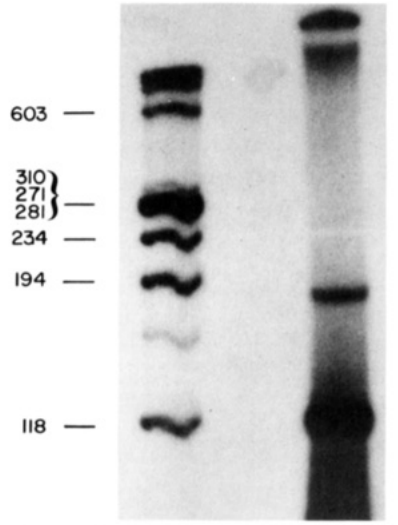

FIGURE 7: S-1 nuclease analysis to determine the ratio of mRNAs encoded pf3 and pf46 in cDNAs. The recombinant plasmid pf46asca was constructed as described under Experimental Procedures and was digested with *Mst*II. 5′-Ends of the linear pf46asca were labeled by T4 polynucleotide kinase and [γ-^{32}P]ATP and then hybridized with total liver poly(A+) RNA from A/HeJ female mice according to the method described under Experimental Procedures. After being digested with S-1 nuclease, RNA–DNA hybrids were electrophoresed on polyacrylamide gel under the denatured conditions, and then the polyacrylamide gel was dried and exposed to X-ray films. HaeIII-cut ϕX174 cDNAs were labeled by [γ-^{32}P]ATP and T4 polynucleotide kinase and used as the size markers. The arrows indicate the two bands corresponding to the protected DNAs. The expected sizes of DNAs should be 221 and 97 bases.

mice, except for 129/J (Figure 6). S-1 nuclease analysis of DNA–mRNA hybrids confirmed the presence of mRNA with the 27-bp insert in liver cells and estimated a ratio of pf46-type and pf3-type mRNA in liver poly(A+) RNA of 1:50 (Figure 7). In addition to the polymorphism with the presence or absence of the 27-bp insertion, pf46 and pf11 differed as their polyadenylation sites. The 3′-noncoding region of pf11 was extended 99 bases further downstream from the poly(A) starting site of pf46, and the nucleotide sequence of the extended part was identical with the corresponding portion of pf3. The modified polyadenylation signal GTGAAA was found at 21 bases upstream from the poly(A) track in pf46. The same polyadenylation signal was present at the same positions of the pf11 and pf3. However, this signal was not utilized in these mRNAs. Therefore, it appeared that at least four different cDNAs for pf3/46 might exist, which are created from the presence or absence of the 27-bp insertion and the multiple polyadenylation sites. pf26 consisted of a 400-bp 3′-noncoding sequence, which is 85% similar to the corresponding 3′-noncoding regions of the 350 bp of pf3 and pf11. It was of interest that the 15-bp DNA fragment containing a poly(A) signal GTGAAA in pf3/46 was deleted in pf26. The poly(A) adenylation signal GGTAAA is located at 20 bases upstream from the poly(A) tract of pf26.

Deduced Amino Acid Sequences. Both pf26 and pf3/46 without the 27-bp insertion contained 1473-bp open-reading frames translated to 491 amino acids with molecular masses of 56 740 and 56 856 daltons, respectively. Figure 8 shows the amino acid sequence deduced from pf26 with underwriting of different amino acid residues of pf3/46 and of rat P-450b. There were only two amino acid substitutions found in the first 50 amino acids of the pf3/46 when its sequence was aligned with rat P-450b. The overall sequence similarity between mouse pf3/46 and rat P-450b was 95%. The insertion of the 27-bp DNA resulted in an additional nine amino acids in pf46 ending at four residues before the cysteine that is the fifth ligand for heme binding (Fujii-Kuriyama et al., 1982). The open-reading frame of pf46 was not altered either before or after the insertion.

The deduced amino acid sequence from pf26 was 85% similar to that from pf3/46. Although the amino acid substitutions were scattered throughout the molecules, the 20 amino acids beginning at position 101 exhibited only 35% similarity between them. The similarities of the deduced amino sequence from pf26 to that of mouse C-P-450$_{16\alpha}$ (Wong et al., 1987) and P-450$_{15\alpha}$ (Squires & Negishi, 1988) were 38% and 50%, respectively. The highest sequence similarity to rat P-450s was 82% to P-450b (Suwa et al., 1985). The similarities of this amino acid sequence were also 49%, 48%, 46%, 36%, and 28% to rat P-450a (Nagata et al., 1987), -M1 (Yoshioka et al., 1987), -f (Gonzalez et al., 1986), -c (Yabusaki et al., 1987), and -PCN (Gonzalez et al., 1985), respectively. The highest sequence similarity to rat P-450s was 82% to P-450b (Suwa et al., 1985). The similarities of this amino acid sequence were also 49%, 48%, 46%, 36%, and 28% to rat P-450a (Nagata et al., 1987), -M1 (Yoshioka et al., 1987), -f (Gonzalez et al., 1986), -c (Yabusaki et al., 1984), and -PCN (Gonzalez et al., 1985), respectively.

Although the total similarities in each amino acid sequence were in the range of 45–50%, there were conserved regions in pf26, P-450$_{15\alpha}$, P-450a, -f, and -M1. The five regions with 70–100% similarity are shown in Figure 9. Besides these regions, the sequence started by Met at position 346 and the amino acid sequence containing the cysteine that is the fifth ligand for heme binding were also well conserved. These two regions were previously described (Ozols et al., 1981; Fujii-Kuriyama et al., 1982). Such high regional sequence similarities suggested that these are critical areas of the molecules that are basic to the function of these P-450s.

Hybridization of Specific Oligonucleotide Probes with Liver Poly(A+) RNA. The oligonucleotides of two separate portions in pf26 and the corresponding portions in pf3/46, which are

```
pf26:     MDPSVLLLLA VLLSLFLLLV RGHAKIHGHL PPGPHPLPLL GNLLQMDRGG
pf3/46:    E         L VGFL   A    P SR NF     R
P-450b:    E  I      L VGFL   A    P SR VF     R

          LLKCFIQLQE KHGDVFTVHL GPRPVVVLCG TQTIREALVD HAEAFSGRGT
             S    R   Y               M     D       G Q
            NS   MR   Y               M     D  K    G Q  D

          IAAAGLVMQD YGIFFASGQR WKTLRRFSLA TMKEFGMGKR SVEERIKEEA
          V VVEPTFKE   VI  N E
          I VIEPIFKE   VI  NE     A

          QCLVEELKKY QGVPLDPTFL FGCITANIIC SIVFGERFDY TDDQFLHLLN
                R S   A                 V           E    R   R  E
                R S   A                                  R   R  E

          LMYKIFSLLS SFSGQMFELF SGFLKYFPGV HRQIVKKQQE LLNYIAHSVE
           F QT   I     S                VA     SNNL     D  G
           F RT          S V               A     S NL   I D  G I

          KHYATLDPSA PRDYIDTYLL REMKEKSNHN TEFHHQNLMN SVLSLFFAGT
            R       V   F          MD     Q    K
            R       V   F          ME             E   I  L

          ETTSATLHYG VLLMLKYPHV TETVQKEIDQ VIGSHRLPTL DDRTKMPYTD
            S TR R   F          A K        L
            S TR T   F          A K                     S

          AVIHEIQRFS DLVPIGLPHK VIKDTLFRGY LLPKNTEVYP VISSALHDPQ
                       I   V     T                    IL
                           V     T                    IL

          YFEQPDKFNP EHFLDANGAL KKCEAFLPFS TGKRICLGES IARNELFIFF
                S    D             S                        L
                                   GQIFDQKSV
            DH  S                  S                        L

          TTILQNFSVA SPVAPKDIGL TPKESGIGKI PPAHQIYFLA R
           S         AH                      TY  C
                     AHL                     TY  C S
```

FIGURE 8: Comparison of deduced amino acid sequences. The amino acid sequence deduced from pf26 is shown here, and the amino acid residues deduced from pf3/46 and for rat P-450b (Fujii-Kuriyama et al., 1982) were also underwritten. The extra nine amino acids inserted in pf46 are boxed with the arrow pointing to the position of insertion.

indicated by wavy lines in Figure 5 and named pf26-cu, pf26-u4, pf3/46-cu, and pf3/46-u4, were prepared as the specific oligonucleotide probes to either pf26 mRNA or pf3/46 mRNA. Liver poly(A+) RNA was prepared from females and males of 129/J, BALB/cJ, 129CF1/J, and C129F1/J and hybridized with the ^{32}P-labeled oligonucleotide probes in order to investigate an association of their expression with the Rip locus.

Northern hybridizations of pf26-cu or pf3/46-cu with the liver poly(A+) RNA are shown in Figure 10. In BALB/cJ mice, both probes hybridized preferentially with the mRNA from females, but not from males, indicating that the expression of both mRNAs is female specific. On the other hand, the liver poly(A+) RNA from either 129/J female or male mice did not show significant hybridization with these oligonucleotide probes, indicating the repression of these two mRNAs not only in 129/J male but also in female mice. A dominant inheritance of the expression of pf26 mRNA in BALB/cJ mice was suggested by the high levels of pf26 mRNA in the F1 offspring such as those seen in the BALB/cJ progenitor, whereas the hybridization experiments with the pf3/46-cu probe demonstrated a recessive inheritance for the expression of pf3/46 mRNA in female mice. Northern hybridizations with the other oligonucleotide probes, pf26-u4 and pf3/46-u4, have resulted in the same conclusions about the inheritances (data not shown).

Since the dominant inheritance of pf26 mRNA expression in the 129/J and BALB/cJ pair was consistent with that of I-P-$450_{16\alpha}$-dependent testosterone 16α-hydroxylase activity, the association of pf26 mRNA levels with the Rip locus and with I-P-$450_{16\alpha}$-dependent testosterone 16α-hydroxylase activities was further confirmed by offspring from the 129CF1/J backcross to 129/J progenitor (Figure 11). The results from the hybridization exhibited a 1:1 ratio of offspring with the high (such as in BALB/cJ) and the low (such as in 129/J) levels of pf26 mRNA. Furthermore, the offspring possessing the high (or low) levels of pf26 mRNA exhibited the high (or low) I-P-$450_{16\alpha}$-dependent activities in liver microsomes.

Discussion

In this report, the strain difference in I-P-$450_{16\alpha}$-dependent testosterone 16α-hydroxylase activity and mRNA levels between 129/J and BALB/cJ mice was used to establish the mouse genetic model of female-specific expression of this isozyme in livers. The distribution patterns of the levels of the isozyme in F1, F2, and F1 backcross to progenitors between the two strains indicated that the isozyme expression in this pair is an autosomal dominant trait and regulated by a single locus named Rip. One recombination between Rip and Coh loci was found in nine recombinant inbred strains, 9×A. This means that the recombinant frequency between the two loci is 3 ± 0.3% based on the calculation by the formula, $R = 4r(1 + 6r)$ described by Haldane and Waddington (1931). Thus, it can be concluded that the Rip locus is located approximately 3 cm from the Coh locus on mouse chromosome 7.

The action of the sex-limited Rip locus is to regulate female-specific constitutive expression of I-P-$450_{16\alpha}$ in mouse livers. Although it is difficult to determine the nature of the

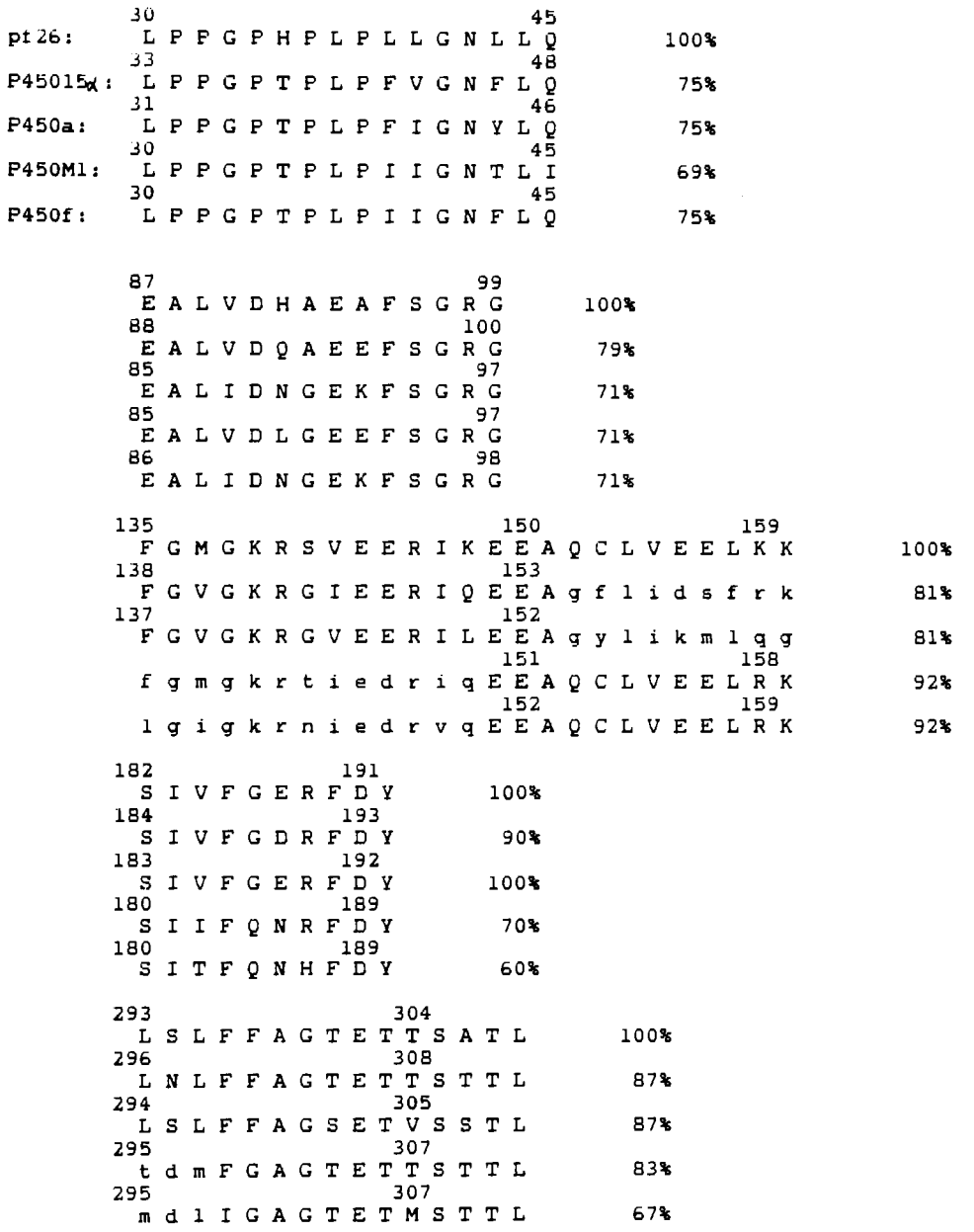

FIGURE 9: Conserved amino acid sequences among pf26, P-450$_{15\alpha}$, P-450a, P-450M1, and P-450f. With the use of the MICROGENIE program, the amino acid sequences of these P-450s were aligned in the way that the maximum homology to the pf26 is obtained. The regions possessing over 70% homology in at least 10 consecutive residues were selected from all 4 P-450s with a few exceptions. The amino acids typed with small letters are not included in the calculation of homology. The numbers written on the sequences show the positions starting and ending the conserved regions of each P-450.

regulation of the Rip locus at this time, the dominant inheritance of the locus leads us to favor the idea that the Rip locus encodes a trans-acting regulatory element. For the first step in finding the regulatory mechanism of the Rip locus, we began to isolate cDNA-encoded I-P-450$_{16\alpha}$ from BALB/cJ mice.

Three different cDNAs pf26, pf3, and pf46 encoding mouse P-450s were identified from a cDNA library constructed from liver poly(A+) RNA of untreated BALB/cJ female mice. pf3 and pf46 were overlapped cDNAs, except for the 27-bp insertion in pf46. The deduced amino acid sequence obtained from the overlapped cDNA (named pf3/46) contained 491 residues and showed 95% sequence similarity to that of rat P-450b and P-450e. Thus, it can be concluded that pf3/46 is the encoded mouse homologue of rat P-450b or P-450e. The translation product from pf46 mRNA, which has an extra 27 bases in its coding region, should consist of 491 plus 9 extra amino acids. These nine amino acids are located just in front of the Cys that is the fifth ligand for heme binding. To data there is no report suggesting or isolating a P-450b or -e homologue possessing nine extra amino acids, so it remains to be seen whether the P-450 protein translated from pf46 mRNA actually exists in liver microsomes. Since pf46 mRNA is ubiquitously present in many inbred mice, a possible biological meaning of the nine amino acid insertion should not be ruled out.

It is not known whether pf46 and pf3 mRNAs are transcribed from one gene and processed alternatively. Recently, rat P-450b and -e gene structures were elucidated by Fujii-Kuriyama (1982) and his associates (Suwa et al., 1985). On the basis of the structures of the rat genes, it is concluded that the insertion of nine amino acids in pf46 occurs exactly at the junction of the eighth and ninth exons. If the two mRNAs are transcribed from one gene, the mechanisms of alternative splicing might be similar to those of fibronectin (Schwarzbauer et al., 1983), human growth hormone (De Noto et al., 1981), colony-stimulating factor CSF (Nagata et al., 1986), and

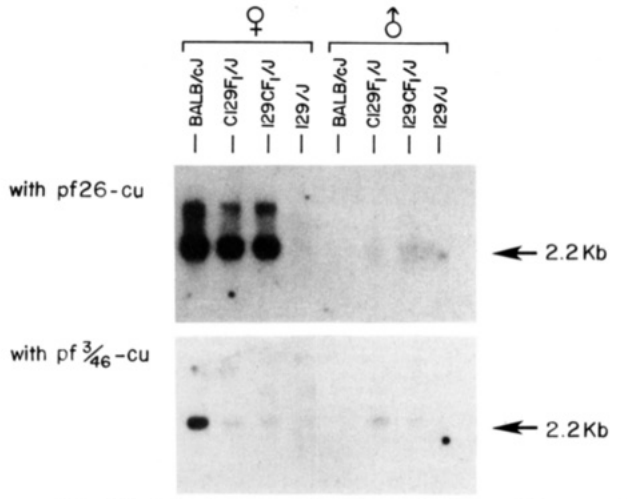

FIGURE 10: Hybridization of specific oligonucleotides, pf26-cu, and pf3/46-cu with total liver poly(A+) RNAs from males and females of BALB/cJ, 129/J, and F1 mice. Liver poly(A+) RNAs (4 μg) from untreated males and females of BALB/cJ, C129F1/J, 129CF1/J, and 129/J mice were denatured with formaldehyde, electrophoresed on agarose gel containing formamide, and transferred to Nytran. The Nytran papers were hybridized with ^{32}P-labeled oligonucleotide probe (pf26-cu and pf3/46-cu) as described under Experimental Procedures. Approximately the same amounts (1×10^7 cpm) of the probes were used for the hybridizations. The paper with pf26-cu was exposed for 5 h and that with pf3/46-cu for overnight.

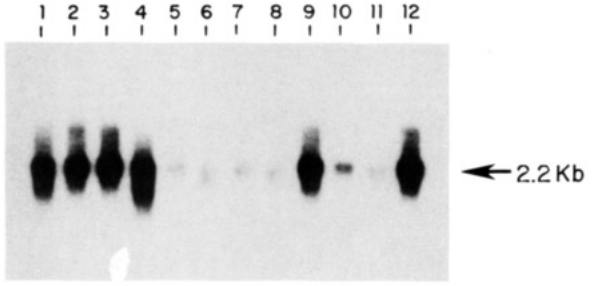

FIGURE 11: Hybridization of ^{32}P-labeled pf26-cu with liver poly(A+) RNAs from 129CF1/J backcross to 129/J offspring. Total liver poly(A+) RNA was prepared from individual offspring from 129CF1/J × 129/J, denatured by formaldehyde, and electrophoresed on agarose gel containing formamide. The RNA was transferred to Nytran paper and hybridized with ^{32}P-labeled pf26-cu. I-P-450$_{16\alpha}$-dependent testosterone 16α-hydroxylase activities in liver microsomes from these mice (1–12) were 0.46, 0.445, 0.351, 0.350, 0.01, 0.04, 0.02, <0.01, 0.325, 0.02, 0.01, and 0.465 nmol min^{-1} (mg of protein)$^{-1}$, respectively.

pp60c-src (Martinez et al., 1987). In these cases, more than one 3′- or 5′-splicing sites generate multiple mRNAs from their genes.

As indicated by Northern hybridization of pf3/46-cu with liver poly(A+) RNA, the mouse homologue of PB-inducible rat P-450 is female specific in BALB/cJ mice, which is in contrast with the rather male-predominant expression of the rat counterpart in untreated animals (Yamazoe et al., 1987). A reason for this species difference in the constitutive expressions of the P-450 is not certain at the present time.

pf26 consists of one open-reading frame with 1473 bp or 491 amino acids, which are the same as those from pf3/46 without the nine amino acid insertion. The amino acid sequence deduced from pf26 exhibits 82% and 85% similarities to rat P-450b and its mouse homologue (pf3/46), respectively. When compared with pf3/46 mRNA, pf26 mRNA is a major constituent in the poly(A+) RNA from livers of untreated BALB/cJ female mice. The hybridization of the specific oligonucleotides and of total cDNA (pf26 or pf3/46) probes provided the indirect estimation of a ratio of pf26 and pf3/46 mRNAs to be approximately 9:1. The relative amounts of mRNA hybridized with pf26-cu have always agreed with those hybridized with the total cDNA probe. The expression of the pf26 mRNA hybridized with pf26-cu was female specific in BALB/cJ mice, while repressed in 129/J female mice. These expressions and repressions of pf26 mRNA in the two inbred mice are consistent with those of mRNA obtained previously by using R17.

As indicated from the high levels of hybridization of pf26-cu with the liver poly(A+) RNA from C129F1/J and 129CF1/J, the expression of pf26 mRNA in female livers was inherited as a dominant trait in 129/J and BALB/cJ pairs. In contrast, pf3/46 mRNA is repressed in the F1 offspring at levels as low as in the 129/J progenitor, suggesting a recessive inheritance of the expression of pf3/46 mRNA in this pair. The dominant inheritance of the expression of pf26 mRNA was consistent with that of I-P-450$_{16\alpha}$-dependent testosterone 16α-hydroxylase activity in liver microsomes from female mice.

The pattern of hybridization of pf26-cu with liver poly(A+) RNA from individual offspring from the F1 × 129/J progenitor resulted in a 1:1 ratio of the high (like BALB/cJ) and the low (like 129/J) levels of pf26 mRNA. Furthermore, all of the offspring with the high pf26 mRNA levels possessed the high I-P-450$_{16\alpha}$-dependent activity representative of the Rip^h/Rip^h phenotype, and similarly, low enzyme activities (Rip^l/Rip^l phenotype) were associated with the low pf26 mRNA levels in the F1 offspring. Thus, it was concluded from these geneticstudies that pf26 represents a cDNA encoding I-P-450$_{16\alpha}$, which is a female-specific isozyme of testosterone 16α-hydroxylase regulated by the Rip locus.

In conclusion, we have determined a locus by which the female-specific I-P-450$_{16\alpha}$ is regulated. This locus, named Rip, is near the Coh locus on mouse chromosome 7. On the basis of the genetics, cDNA encoding I-P-450$_{16\alpha}$ was identified and characterized. This mouse genetic model will be useful for studying the mechanism of female-specific expression of P-450 in livers.

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Registry No. P-450, 9035-51-2; testosterone 16α-hydroxylase, 37364-16-2; DNA (clone pf26 P-450 mRNA complementary), 115461-76-2; P-450 (clone pf26 protein moiety reduced), 115461-72-8; DNA (clone pf3/46 P-450 mRNA complementary), 115461-78-4; P-450 (clone pf3/46 protein moiety reduced), 115461-73-9; DNA (clone pf46 P-450 mRNA complementary), 115461-77-3; P-450 (clone pf46 protein moiety reduced), 115461-74-0.

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