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Gene Structure of Cytochrome P-450(M-1) Specifically Expressed in Male Rat Liver†

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Received May 6, 1987; Revised Manuscript Received July 31, 1987

ABSTRACT: Cytochrome P-450(M-1) [P-450(M-1)] is specifically expressed in adult male rat liver [Yoshioka, H., Morohashi, K., Sogawa, K., Miyata, T., Kawajiri, K., Hirose, T., Inayama, S., Fujii-Kuriyama, Y., & Omura, T. (1987) *J. Biol. Chem. 262*, 1706–1711]. Isolation and analysis of the gene for P-450(M-1) revealed that the coding region of the gene is interrupted by eight introns and is dispersed over a 35-kilobase pair region of chromosomal DNA. Intron insertion sites of the P-450(M-1) gene are located at equivalent positions to those of cytochrome P-450b and P-450e, which are phenobarbital-inducible. Sequence analysis of the 5′-upstream region of the P-450(M-1) gene shows that there is a homologous sequence to glucocorticoid regulatory elements (GRE) identified in glucocorticoid-responsive genes.

Cytochrome P-450 (P-450)[1] is a group of monooxygenases that catalyze oxidation of a variety of both endogenous and exogenous substrates (Sato & Omura, 1978; Lu & West, 1980). These enzymes contain a heme moiety as a prosthetic group and are related with one another as revealed by the comparison of their amino acid sequences (Gotoh et al., 1983). The sequence similarity of P-450s suggests that they have diverged from a common ancestral enzyme in the course of evolution. In addition to diversity of substrate specificity, constituents of the P-450 superfamily exhibit various modes of expression of their own, for example, in temporal, tissue-specific, sex-dependent, or inducer-specific manners (Sato & Omura, 1978; Lu & West, 1980). Because of the variety in the mode of expression, P-450 should provide a suitable system for the study on the regulation of gene expression.

P-450(M-1) is present in microsomes of adult rat livers and catalyzes testosterone 16α-hydroxylation (Matsumoto et al., 1986; Morgan et al., 1985a). The expression of the enzyme is male- and age-specific. Recently, cDNA clones for P-450(M-1) were isolated in our laboratory (Yoshioka et al., 1987). Although sequence analysis of the cDNA clones revealed that P-450(M-1) has a high degree of sequence similarity to the coding sequence of phenobarbital-inducible P-450s, Northern blot analysis clearly showed that P-450(M-1) mRNA is specifically synthesized in adult male rat livers as a constitutive form. Morgan et al. (1985b) have demonstrated that the expression of the male-specific P-450 of rat livers is under the control of the growth hormone secretion pattern, a highly pulsatile secretion of the hormone in the male and a more constant level of the hormone in the female rats. The regulation mechanism of the hormonal axis (pituitary–liver) mediated by the growth hormone, however, has not yet been fully unraveled at a molecular level. As an essential step toward investigating the regulation mechanism of male- and age-specific expression of P-450(M-1), we have isolated and characterized the P-450(M-1) gene.

Experimental Procedures

Materials. Restriction endonucleases were purchased from Takara Shuzo Co. (Kyoto, Japan), New England Biolabs (Beverly, MA), and Bethesda Research Laboratories (Rockville, MD). *Escherichia coli* DNA polymerase I (large fragment), T4 DNA ligase, bacterial alkaline phosphatase, and polynucleotide kinase were obtained from Takara Shuzo Co.

† This work was supported in part by grants-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, research grants from the Ministry of Health and Welfare of Japan, and funds obtained under the Life Science Project from the Institute of Physical and Chemical Research.
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[1] Abbreviations: P-450, cytochrome P-450; kb, kilobase pair(s); bp, base pair(s); SSC, 0.15 M NaCl containing 15 mM sodium citrate; GRE, glucocorticoid regulatory elements.

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Nuclease S1 was from P-L Biochemicals (Milwaukee, WI). [α-^{32}P]dCTP (3000 Ci/mmol) and [γ-^{32}P]ATP (5000 Ci/mmol) were from the Radiochemical Centre (Amersham, England) and ICN Radiochemical (Irvine, CA). [α-^{35}S]dATP (650 Ci/mmol) was from the Radiochemical Centre. All other reagents were of analytical grade.

Plaque Screening. A rat genomic library of Charon 4A bacteriophage, which was made from partial *AluI*/*Hae*III digests of rat genomic DNA, was kindly provided by Drs. L. L. Jagodzinsky and J. Bonner. Approximately 1.2 × 10^6 plaques from the library were screened by the procedure of Benton and Davis (1977) with the P-450(M-1) cDNA fragment [1-kb *Hpa*I/*Xba*I fragment of pcP-450(M-1)-1; Yoshioka et al., 1987]. The 1-kb fragment was radiolabeled with [α-^{32}P]dCTP to a specific radioactivity of about 4 × 10^8 cpm/μg by nick translation (Rigby et al., 1977). Filter hybridization was performed overnight at 65 °C (Benton & Davis, 1977), with the ^{32}P-labeled DNA (6 × 10^4cpm/filter) used as a probe. The filters were washed twice at 65 °C for 30 min in 0.1× SSC (SSC, 0.15 M NaCl containing 15 mM sodium citrate) and 0.1% sodium lauroylsarcosine solution. Phage DNAs were prepared essentially according to Maniatis et al. (1982).

Southern Blot Analysis. Rat chromosomal DNA or cloned phage DNA was digested with restriction enzymes, and digestion products were subjected to agarose gel electrophoresis for Southern blot analysis (Southern, 1975). The blotted filters were hybridized to nick-translated cDNA or cloned genomic DNA probe at 65 °C overnight and washed twice with 0.1× SSC (stringent conditions) or 5× SSC (relaxed conditions) as described above.

Subcloning of DNA Fragments for Restriction Mapping and Sequencing. For the structural analysis of cloned genomic DNAs, appropriate DNA fragments that had been prepared by digestion of cloned phage DNA with restriction enzymes were subcloned into plasmid pBR322, by use of *Escherichia coli* HB101 as the host cell (Hanahan, 1983).

DNA Sequencing. Restriction fragments containing a part of the P-450(M-1) gene and its flanking regions were subcloned into phage M13mp18 or mp19 for sequencing by the chain-termination method (Sanger et al., 1977; Messing et al., 1981). All the DNA fragments containing exon sequences were sequenced in both directions.

Nuclease S1 Mapping. Nuclease S1 protection mapping was carried out as described previously (Berk & Sharp, 1977). At first, we tried to prepare a *Nhe*I/*Ava*II fragment (−36 to +116, see Figure 4) as a source of a complementary strand to P-450(M-1) mRNA, but digestion experiments with *Ava*II showed that the *Ava*II site in the first exon was resistant to the enzyme, probably due to *dcm* methylation. For demethylation of the *Ava*II site, a 1.6-kb *Sph*I/*Eco*RI fragment which contained the first exon (Figure 3) was subcloned into plasmid pBR322 with *E. coli* GM31 (*dcm*$^-$) (kindly provided by Dr. H. Masaki) as a host cell. The plasmid was isolated according to the method of Maniatis et al. (1982) and cleaved with *Sph*I and *Eco*RI. The *Sph*I/*Eco*RI fragment was purified by agarose gel electrophoresis and then cleaved with *Ava*II. The internal *Ava*II/*Ava*II fragment was labeled at the 5′-termini with [γ-^{32}P]ATP and then the approximately 152-bp fragment containing the upstream half of the putative first exon and its 5′-flanking sequence was isolated after cleavage with *Nhe*I. The *Nhe*I/*Ava*II fragment was hybridized with 10 μg of poly(A+) RNA from adult male rat livers (Yoshioka et al., 1987), and the mixture was treated with nuclease S1 (200 units) at 37 °C for 30 min as described (Sogawa et al., 1984). As the size markers, the *Nhe*I/*Ava*II fragment was degraded by the base-specific chemical modification method (Maxam & Gilbert, 1977), and degradation products were electrophoresed together with the S1 nuclease digests on a 6% polyacrylamide/8 M urea gel.

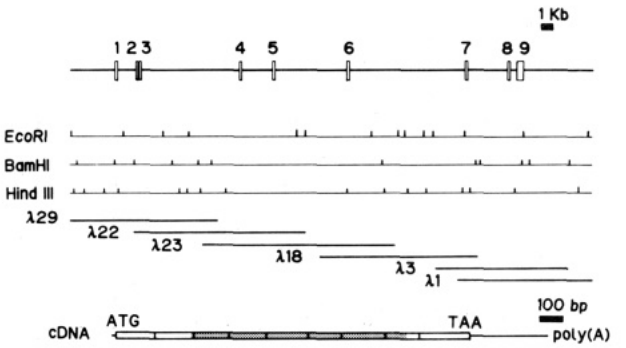

FIGURE 1: Gene organization of cytochrome P-450(M-1) and its isolated genomic clones. Chromosomal DNA containing the P-450(M-1) gene is represented by a thick line. Open boxes show the locations of exonic regions of the gene. The lengths of the numbered lines show DNA sequences which are covered by each cloned genomic DNA. The outline of cDNA structure is also presented, where the boxes from left to right correspond to exons 1–9 and the solid lines on both ends of the cDNA represent nontranslated leader and trailer sequences which are included in the first and the last exon, respectively. A stippled part represents a cDNA fragment used as a probe for screening of rat genomic library.

RESULTS

Isolation of the Cytochrome P-450(M-1) Gene. At the initial stage of this study, we obtained a P-450(M-1) cDNA clone that contained a DNA insert of approximately 1.1 kb [pcP-450(M-1)-1] corresponding to an internal part of a 1.9-kb message (Yoshioka et al., 1987). We used the 0.9-kb *Hpa*I/*Xba*I fragment of the DNA insert as a probe to isolate the P-450(M-1) gene. From about 1.2 × 10^6 recombinant phages of rat genomic library, 29 positive clones were selected. Restriction cleavage mapping and Southern blot analysis with cDNA probes (*Hpa*I/*Xba*I fragment and its subfragments generated by further cleavages) showed that 24 out of 29 clones could be aligned as overlapping clones which covered a stretch of approximately 45 kb of chromosomal DNA as shown in Figure 1. We selected six typical clones (Figure 1) for further analysis as described below. The remaining five clones were hybridized to the *Hpa*I/*Nco*I fragment (codons 319–631). Four out of five clones were identical in restriction cleavage pattern (*Bam*HI, *Eco*RI, *Hind*III) with one another. These 5 clones, however, had no overlapping region to any of the other 24 clones. We, therefore, tentatively concluded that these genomic fragments derived from a differnt gene with high sequence similarity to the P-450(M-1) gene and did not further analyze these clones.

To examine whether the cloned DNAs covered the entire region of the P-450(M-1) gene, the DNAs were digested by restriction enzymes and analyzed by Southern blot analysis with about 200-bp fragments derived from various parts of the cloned P-450(M-1) cDNAs [pcP-450(M-1)-3 and pcP-450(M-1)-4 (Yoshioka et al., 1987)] as probes. The two cDNA insert altogether contained the full-length cDNA copy for P-450(M-1) mRNA. The Southern blot analysis showed that all of the fragments of the cDNA were hybridized to any one or two of the restriction fragments of the cloned genomic DNAs (data not shown). In addition, when the total rat DNA was analyzed by Southern blot analysis (Figure 2A) with the 1.6-kb *Bam*HI/*Bam*HI fragment of pcP-450(M-1)-3 as a probe, only the hybridizable bands, which had the expected

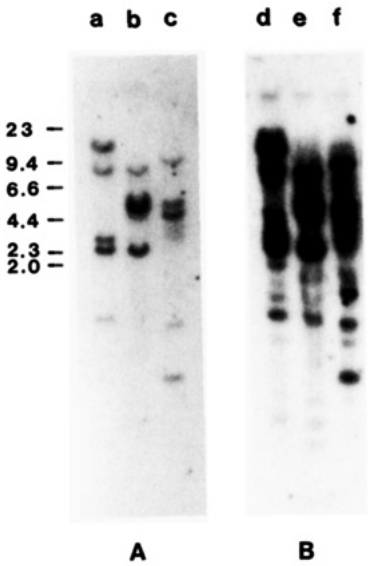

FIGURE 2: Blot-hybridization analysis of total DNA of rat. Rat liver DNA (30 μg) was digested with *Bam*HI (a and d), *Eco*RI (b and e), and *Hin*dIII (c and f) and then subjected to 0.8% agarose gel electrophoresis. The digests were blotted onto a nitrocellulose filter paper and then hybridized with [^{32}P]cDNA fragments prepared from pcP-450(M-1)-3 (Yoshioka et al., 1987). The autoradiographs of the hybridization bands are presented. (A) Washed with 0.1× SSC; (B) washed with 5× SSC at 65 °C. The length (in kilobase pairs) of the size markers is indicated at the left of the panel.

size from a restriction cleavage map of the 45-kb DNA region, were detected. Taken together, these analyses showed that the cloned DNAs covered almost the entire sequence of the P-450(M-1) gene. We subcloned the restriction fragments of the cloned DNA, which were hybridizable to the cDNA probes to facilitate the sequencing of exons.

Although the constituents of the P-450 gene family have high degree of sequence homology to one another (Gotoh et al., 1983; Mizukami et al., 1983a; Atchison & Adesnik, 1983), the data mentioned above suggest that the observed bands on the Southern blot of rat total DNA were generated from a single locus of P-450(M-1) gene extending over about 45 kb since the Southern blot analysis was performed under the same stringent conditions as those for the screening of the recombinant DNA. When the Southern analysis was performed under less stringent conditions (see Experimental Procedures), extra bands were detected in addition to the fragments of the P-450(M-1) gene by use of the same cDNA probe (Figure 2B). It is plausible that these additional bands were generated from an other (other) P-450 gene(s), probably of the phenobarbital-inducible type (see Discussion).

Nucleotide Sequence of the Cytochrome P-450(M-1) Gene. The regions for the exons of the P-450(M-1) gene within the cloned genomic DNA were located by Southern blot analysis with the labeled fragments of the cDNA clones (data not shown). Appropriate genomic DNA fragments which had been shown to contain exonic sequences were subcloned into plasmid pBR322 (Figure 3), with *E. coli* HB101 as host cells (Hanahan, 1983).

We constructed fine restriction maps of the subcloned fragments and determined the nucleotide sequence of exons and their flanking sequences in the introns. The strategy for sequencing is outlined in Figure 3.

Coding nucleotide sequences were determined in reference to the cDNA sequence and the consensus sequence of the exon–intron boundary (Figure 4). The exonic sequence was split into nine parts and distributed on approximately 35-kb of cloned genomic DNA (Figure 1). The determined sequence,

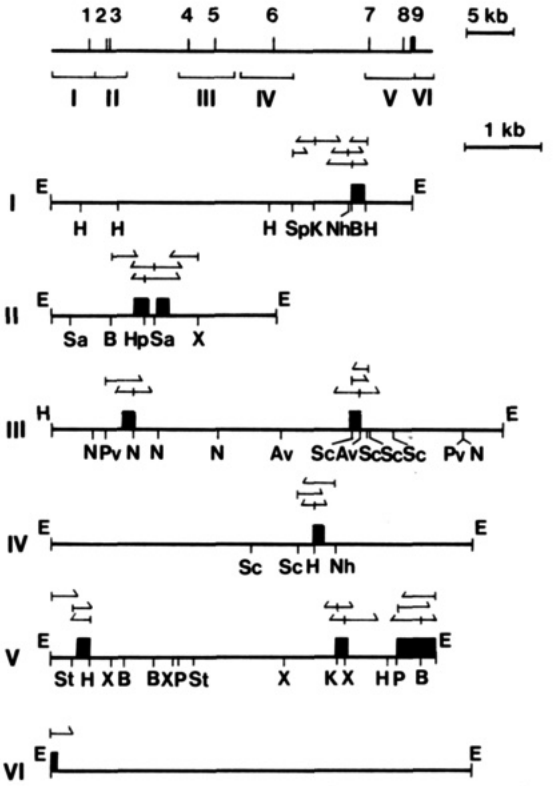

FIGURE 3: Restriction cleavage maps of the regions containing the exonic sequences of the cytochrome P-450(M-1) gene and the sequencing strategy. (Top) The region of the cloned chromosomal DNA is represented by a solid line. Exonic sequences are shown by closed boxes. The regions indicated by Roman numerals are subcloned into pBR322 at appropriate restriction sites. (Bottom) fine restriction maps of the subcloned regions and sequencing strategy. The arrows above each subcloned region indicate the direction and length of the fragments sequenced by the chain termination method. Closed boxes show exonic sequences. Fragments I and II were prepared from clone 29, fragments III and IV were from clone 23, and fragments V and VI were from clone 1. (A) *Ava*II; (B) *Bam*HI; (E) *Eco*RI; (H) *Hin*dIII; (Hp) *Hpa*I; (K) *Kpn*I; (N) *Nco*I; (Nh) *Nhe*I; (P) *Pst*I; (Pv) *Pvu*II; (Sa) *Sac*I; (Sp) *Sph*I; (St) *Stu*I; (X) *Xba*I.

which encodes 501 amino acids, contained the complete P-450(M-1) cDNA sequence with a single nucleotide substitution at the second letter of codon 989 (Ado for Guo in the cDNA sequence), causing an amino acid change [His (CAT) for Arg (CGT)]. This substitution could be due to allelic polymorphism rather than sequencing error, because the two sequences were confirmed by bidirectional determination. Taken together with the fact that all the hybridizable bands in the Southern blot analysis of total DNA (Figure 2A) could be explained as the fragments generated from the cloned region of the genome, we concluded that the cloned genomic DNA was the gene for P-450(M-1).

Nuclease S1 Mapping. The boundary of the gene was determined by nuclease S1 protection mapping (Berk & Sharp, 1977). The ^{32}P-labeled *Nhe*I/*Ava*II fragment (151-base antisense strand) was chosen as a probe for the nuclease S1 analysis because the fragment spanned from the nucleotide about 35 bp upstream of the 5′ end of the cDNA sequence (the putative transcription-initiation site) to the first nucleotide of the 32nd codon in the putative first exon. When the radiolabeled fragment was hybridized with the poly(A+) RNA fraction of adult male rat livers and then subjected to nuclease S1 digestion, three major protected bands were produced (indicated by an arrow and arrowheads in Figure 5). The largest band (indicated by an arrow) corresponds to C in the sequence ladder of the anticoding strand, which is located 22

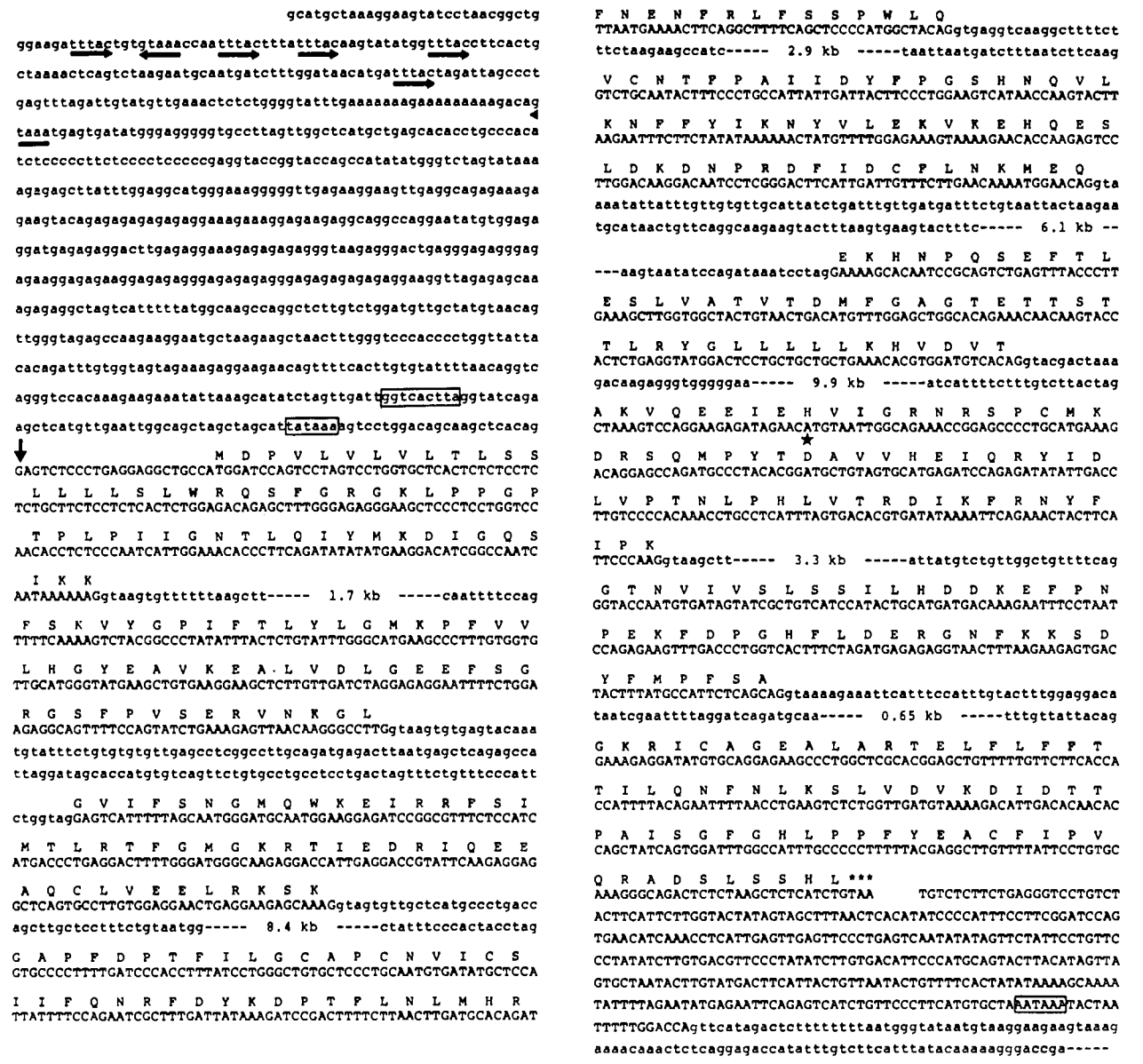

FIGURE 4: Nucleotide sequence of the rat cytochrome P-450(M-1) gene and its flanking sequence. The deduced amino acid sequence and the nucleotide sequence for P-450(M-1) are shown. Amino acids are represented by single-letter symbols. Capital letters represent exonic sequence, and small letters show intron and 5′ and 3′ flanking sequences. The determined cap site is indicated by a vertical arrow. The sequences GGTCACTTA and TATAAA in the 5′ flanking regions and the sequence AATAAA in the 3′ noncoding region are indicated by enclosures. Repeated sequences are indicated by horizontal arrows. An asterisk (*) shows a nucleotide substitution from the sequence of P-450(M-1) cDNA, which causes an amino acid change (see the text). A termination codon is indicated by (***).

bp upstream from the translation-initiation codon and is also 2 bp upstream from the 5′ end of the cDNA sequence. Since it has been proved that transcription of most eukaryotic mRNAs begins with purine (Breathnach & Chambon, 1981), the result indicates that the corresponding G in the coding strand is most probably the transcription-initiation site of the P-450(M-1) mRNA. Twenty-nine base pairs upstream from this G, a typical TATA sequence (Goldberg, 1979), TATAAA, was found. A possible equivalent CAAT sequence, GGTCACTTA, was also found 78 bp upstream from the G. The other two bands in Figure 5 (indicated by arrowheads) correspond to G at 3 bp upstream and T at 16 bp downstream from the initiation codon. We doubt, however, that these two nucleotides are really the transcripton start sites. It is possible that the DNA fragment tended to have secondary structure around these sites, which prevented the mRNA from further hybridizing to the upstream region of the DNA, since immediately downstream from these sites palindromic sequences (CCATGG and CCTGG) are present. From their positions in the sequence, it seems unlikely that these two protected bands represent the actual transcription start sites but rather they seem to be artifactual products due to the intrinsic nature of the DNA probe.

Discussion

It has been shown that constituents of the P-450 family have structural relatedness with one another (Gotoh et al., 1983; Mizukami et al., 1983a; Atchison & Adesnik, 1983). To discriminate and isolate the P-450(M-1) gene from the other members of the P-450 family, plaque hybridization for screening of rat genomic library was done under stringent conditions. Most (24 out of 29) of the positive phage clones isolated were overlapping in part with one another to cover a single stretch of about 45 kb of chromosomal DNA with consistent restriction mapping (Figure 1). In Southern blot analysis of total rat DNA, all the expected hybridization bands from the restriction map of the cloned genomic DNA and no more extra band were detected under stringent conditions with

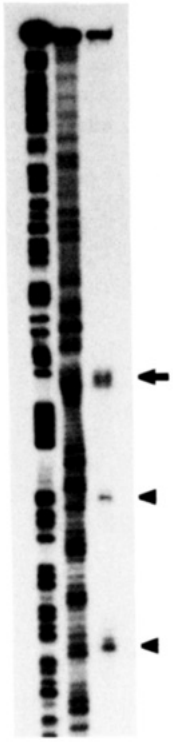

FIGURE 5: Nuclease S1 mapping of the 5′ terminus of the cytochrome P-450(M-1) mRNA. The *Nhe*I/*Ava*II fragment (151 bases) which contained a part of the first exon and its 5′ flanking sequence was labeled at the 5′ end with ^{32}P and then hybridized with 10 μg of poly(A+) RNA from adult male rat liver (Yoshioka et al., 1987). The reaction mixture was treated with nuclease S1 (200 units) at 37 °C for 30 min. The nuclease digest was analyzed on a 6% polyacrylamide gel together with the base-specific degraded fragments of the anticoding strand as size markers. From right to left, nuclease S1 digest and G+A and C+T degradation products. Arrow and arrowheads indicate the bands of fragments protected against nuclease S1 treatment.

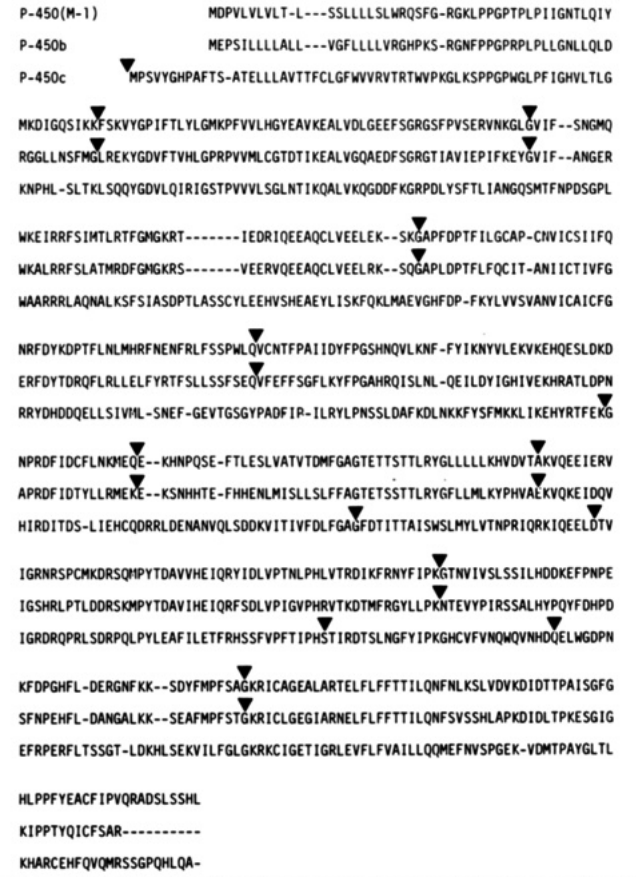

FIGURE 6: Intron insertion sites in various forms of cytochrome P-450 genes in relation to the amino acid sequences. Amino acid sequences of P-450(M-1), phenobarbital-inducible P-450b (Mizukami et al., 1983b), and methylcholanthrene-inducible P-450c (Sogawa et al., 1984) are shown by single-letter symbols. Location of the introns in the amino acid sequences are indicated by filled triangles. The first intron of the P-450c gene is localized 15 bp upstream from the initiation codon, and its location is indicated by a triangle in front of the initiation methionine. Phenobarbital-inducible P-450e and methylcholanthrene-inducible P-450d have equivalent positions of intron insertion with P-450b and P-450c, respectively.

P-450(M-1) cDNA as a probe (Figure 2A), suggesting that there is most likely a single gene for P-450(M-1) in rat genome. When the stringency of hybridization was reduced, many additional bands were observed (Figure 2B). Since the P-450(M-1) cDNA has a relatively high degree of sequence similarity (52%) with phenobarbital-inducible P-450 cDNA (Yoshioka et al., 1987) and, therefore, is considered to belong to the phenobarbital-inducible P-450 family, the extra signals in the Southern blot analysis are interpreted to mean the presence of many genes or gene-like DNA sequences which are classified into the P-450 family of phenobarbital-inducible type.

The structural analysis of the cloned gene enables us to compare the gene organization of the P-450(M-1) gene with those of two drug-inducible P-450s. Figure 6 shows the comparison of the intron insertion sites in relation to the amino acid sequence for these P-450s. It is found that all the insertion sites of introns in the P-450(M-1) gene are located exactly at equivalent positions to those of phenobarbital-inducible P-450 genes (P-450b and P-450e), while they are totally different from those of the methylcholanthrene-inducible P-450 gene, which is split into seven exons. The similarity of intron–exon arrangement and sequence similarity pointed out previously give sufficient evidence to suggest that P-450(M-1) and phenobarbital-inducible P-450s evolved from a common ancestor and belong to the same P-450 family.

Although there is the structural similarity demonstrated above between P-450(M-1) and phenobarbital-inducible P-450 genes, they differ from each other in their modes of expression as well as substrate specificities (Matsumoto et al., 1986; Yoshioka et al., 1987; Ryan et al., 1982). P-450(M-1) is not induced by the treatment of phenobarbital but expressed constitutively in adult male rat livers (Matsumoto et al., 1986; Yoshioka et al., 1987; Andersson & Jornval, 1986). The divergence of the substrate specificity could be attributed to a consequence of amino acid change in the substrate binding and/or active sites in the enzymes as discussed in a previous paper (Gotoh et al., 1983). As for the mode of the gene expression, the difference between the two genes may be due to the variation in the sequences of the 5′ upstream region of the genes, because the 5′ flanking sequences of many other genes are reported to function as cis-acting control elements for their regulated expression. In this context, it seems useful to compare the 5′ upstream sequence of the P-450(M-1) gene with those of other P-450 genes, especially phenobarbital-inducible P-450 genes. We have determined the 5′ upstream sequence of the P-450(M-1) gene and found essentially no homology with those of drug-inducible P-450s (P-450b, P-450c, P-450d, P450e) (Suwa et al., 1985; Sogawa et al., 1984, 1985; Mizukami et al., 1983b) as well as the steroidogenic P-450-(C-21) gene of humans (Higashi et al., 1986). A stretch of purine–pyrimidine sequence, which was observed in drug-inducible P-450 genes, is absent within the 869-bp region upstream from the transcription-initiation site. The features of the 5′ upstream region of the P-450(M-1) gene include the

MMTVI	-184	GTTACAAACTGTTCT	-170
MMTVIIa	-127	GTATCAAA*TGTTCT	-114
ch-LYS	-52	GATTC*CTCTGTTCT	-65
h-MT IIa	-261	GT*ACACTGTGTTCT	-148
P-450(M-1)	-141	GTGA*AAACTGTTCT	-153

FIGURE 7: Comparison of the 5′ upstream sequence of the cytochrome P-450(M-1) gene with the nucleotide sequence of the binding sites for the glucocorticoid receptor. The nucleotide sequence of AGAACA and its flanking sequence in the 5′ upstream region of the P-450(M-1) gene is compared with the nucleotide sequences of the binding sites for the glucocorticoid receptor in the promotor regions of mouse mammary tumor virus (MMTVI and MMTVIIa; Scheidereit et al., 1983), chicken lysozyme (ch-LYS; Renkawitz et al., 1984), and human metallothionein IIa (h-MT IIa; Karin et al., 1984) genes. For comparison, the sequences of antisense strands of P-450(M-1) and chicken lysozyme gens are presented. The essential hexameric sequences are underlined. Gaps (*) are introduced to maximize homology among these sequences.

following: (i) typical TATA sequence ($^{-29}$TATAAA) and modified CAAT sequence ($^{-78}$GGTCACTTA); (ii) repeated sequence of TTTAC and its inversed complement of GTAAA (in the region of −834 to −657); (iii) a G/C-rich region (−646 to −569; (iv) a purine-rich region (−519 to −294). It remains to be studied how these features are involved in the expression of the P-450(M-1) gene as cis-acting control elements.

Within the region at about 60–250 bp upstream from the transcription-initiation site, glucocorticoid-inducible genes such as mouse mammary tumor virus (Scheidereit et al., 1983), chicken lysozyme (Renkawitz et al., 1984), and human metallothionenin IIa (Karin et al., 1984) have a hexameric sequence of $\frac{AGAACA}{TCTTGT}$, which has been shown to be essential for the glucocorticoid receptor binding. It is worth noting that at the equivalent position between the modified CAAT sequence and the purine-rich region of the P-450(M-1) gene there is a significantly homologous sequence to the glucocorticoid receptor binding sequence or glucocorticoid regulatory element (GRE). Figure 7 shows the GRE sequence of the glucocorticoid-inducible genes and the sequence of P-450(M-1). In the compared region of the P-450(M-1) gene (hexamer and flanking sequence), the matching nucleotides to those of the GRE sequences are 53–87%. The comparison for one set is on the sense strand of GRE of mouse mammary tumor virus and human metallothionein IIa genes, and for the other set [chicken lysozyme and P-450(M-1)] the sequence is on the antisense strand. The matching is the most notable with mouse mammary tumor virus gene (87% with binding site I). Although it has not yet been known whether glucocorticoid really affects the expression of the P-450(M-1) gene, the striking similarity of the sequence tempts us to infer that the AGAACA region of the P-450(M-1) gene may be involved in the regulation of the gene as a binding site for the glucocorticoid or other steroid hormone receptor. Although testosterone, a substrate for P-450(M-1), is reported to regulate positively the level of P-450(M-1) in rat livers (Gustafsson et al., 1983), it is suggested by the experiments using hypophysectomized and/or castrated animals that the effect of testosterone is not direct but rather is mediated by growth hormone through a novel endocrine axis, hypothalamic–pituitary–liver system (Morgan et al., 1985b). However, a regulatory mechanism involving testosterone and growth hormone remains unknown at the molecular level. It would be interesting to investigate whether or not the sequence with high homology to the GRE sequence in the upstream region of the P-450(M-1) gene actually provides the binding site for the androgen receptor complex in the regulation process of the gene.

ACKNOWLEDGMENTS

We thank Drs. L. L. Jagodzinsky and J. Bonner for the gift of rat genomic library. We also thank Dr. H. Masaki for providing *E. coli* GM31.

Registry No. Cytochrome P-450, 9035-51-2; DNA [rat liver cytochrome P-450(M-1) gene coding region], 111159-29-6; RNA [rat liver male-specific cytochrome P-450(M-1) specifying messenger], 111159-31-0; cytochrome P-450 (rat liver male-specific isoform M-1 protein moiety reduced), 111159-26-3.

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Reactions of Hydroxylamine with the Electron-Donor Side of Photosystem II†

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Received May 20, 1987; Revised Manuscript Received August 11, 1987

ABSTRACT: The reaction of hydroxylamine with the O_2-evolving center of photosystem II (PSII) in the S_1 state delays the advance of the H_2O-oxidation cycle by two charge separations. In this paper, we compare and contrast the reactions of hydroxylamine and *N*-methyl-substituted analogues with the electron-donor side of PSII in both O_2-evolving and inactivated [tris(hydroxymethyl)aminomethane- (Tris-) washed] spinach PSII membrane preparations. We have employed low-temperature electron paramagnetic resonance (EPR) spectroscopy in order to follow the oxidation state of the Mn complex in the O_2-evolving center and to detect radical oxidation products of hydroxylamine. When the reaction of hydroxylamine with the S_1 state in O_2-evolving membranes is allowed to proceed to completion, the S_2-state multiline EPR signal is suppressed until after three charge separations have occurred. Chemical removal of hydroxylamine from treated PSII membrane samples prior to illumination fails to reverse the effects of the dark reaction, which argues against an equilibrium coordination of hydroxylamine to a site in the O_2-evolving center. Instead, the results indicate that the Mn complex is reduced by two electrons by hydroxylamine, forming the S_{-1} state. An additional two-electron reduction of the Mn complex to a labile "S_{-3}" state probably occurs by a similar mechanism, accounting for the release of Mn(II) ions upon prolonged dark incubation of O_2-evolving membranes with high concentrations of hydroxylamine. In *N*,*N*-dimethylhydroxylamine-treated, Tris-washed PSII membranes, which lack O_2 evolution activity owing to loss of the Mn complex, a large yield of dimethyl nitroxide radical is produced immediately upon illumination at temperatures above 0 °C. The dimethyl nitroxide radical is not observed upon illumination under similar conditions in O_2-evolving PSII membranes, suggesting that one-electron photooxidations of hydroxylamine do not occur in centers that retain a functional Mn complex. We suggest that the flash-induced N_2 evolution observed in hydroxylamine-treated spinach thylakoid membrane preparations arises from recombination of hydroxylamine radicals formed in inactivated O_2-evolving centers.

The mechanism of photosynthetic O_2 evolution involves the catalysis of the four-electron oxidation of H_2O by a polynuclear Mn complex in the O_2-evolving center of photosystem II (PSII)[1] [for a recent review, see Babcock (1987)]. Electron paramagnetic resonance (EPR) spectroscopy (Dismukes & Siderer, 1981; Zimmermann & Rutherford, 1984) and X-ray absorption experiments (Goodin et al., 1984) have shown that Mn oxidation state changes occur as the O_2-evolving center advances through its five oxidation states S_i, i = 0–4, suggesting that the Mn complex functions to store the required oxidizing equivalents for the H_2O-oxidation reaction. The Mn complex appears to consist of four exchange-coupled Mn ions (Dismukes et al., 1982; de Paula et al., 1986a) that are proposed to be arranged in the S_2 state in a Mn_4O_4 cubane-like configuration (Brudvig & Crabtree, 1986).

Considerable effort has been expended in the past few years toward understanding the inhibition of photosynthetic O_2 evolution by primary amines, which might coordinate to Mn in the O_2-evolving center (Sandusky & Yocum, 1983, 1984, 1986). When the S_2-state multiline EPR signal was used as a probe for ligand-substitution reactions at the Mn complex,

†This work was supported by the National Institutes of Health (GM32715). G.W.B. is the recipient of a Camille and Henry Dreyfus Teacher–Scholar Award and an Alfred P. Sloan Fellowship.

[1] Abbreviations: DCBQ, 2,5-dichloro-*p*-benzoquinone; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EPR, electron paramagnetic resonance; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; kDa, kilodalton; MES, 2-(*N*-morpholino)ethanesulfonic acid; PSII, photosystem II; P680, primary electron donor in PSII; Q_A, primary electron acceptor in PSII; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/87/0426-8285$01.50/0