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A GENE STRUCTURE OF TESTOSTERONE 6β-HYDROXYLASE (P450IIIA)

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SUMMARY: Genomic clones of a rat testosterone 6β-hydroxylase have been isolated and characterized as the first gene (P450/6βA) among P450IIIA subfamily. This gene spans about 25Kb and consists of 13 exons, which is the largest number of exons among cytochrome P-450 genes reported previously. The nucleotide sequence of the exon region showed high similarity to those of P450PCN2 and P450PCN1 cDNA (Gonzalez, F.J. et al. (1987) Mol. Cell. Biol. 2969-2974), but several replacements and deletions of nucleotide were found between the P450/6βA gene and both cDNAs, indicating the existence of multiple P450IIIA genes in rats.

Cytochrome P-450 (P-450) catalyzes oxidation of exogenous chemicals with diverse structures as well as endogenous substrates, and constitutes a gene superfamily (1). The enzymes included in P450III family distribute widely and share as major forms in livers of experimental animals and human. Amounts of P450IIIA proteins are also known to be increased by the treatment of experimental animals and humans with various types of chemicals including barbiturates, glucocorticoids and macrolide antibiotics (2,3). We have recently purified four different forms of P450IIIs ($P\text{-}450_{6\beta\text{-}1\text{-}4}$) from male rats treated with phenobarbital or dexamethasone (4). Although these proteins showed high catalytic activities of testosterone 6β-hydroxylation in their reconstituted systems and immunochemical similarity, these proteins were induced in different degrees by these chemicals (4). We also have shown that these forms are regulated by pituitary growth hormone (5,6) and thyroid hormone (7) as male dominant expression in rats. Recently, it has been suggested that certain DNA elements in the regulatory gene are

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associated with the regulation of protein by hormones and xenobiotics. For understanding of the regulation of P450IIIA genes at molecular levels with endocrinal hormones and xenobiotics, it is essential to analyze the genomic DNA.

In the present study, we have determined a genomic structure of a rat P450III as the first characterized gene among this family.

MATERIALS AND METHODS

Cloning and Sequencing of P450/6βA Gene

A rat genomic library in the vector EMBL 3 (8) was constructed from partial Sau3AI-digested genomic DNA of a Sprague-Dawley rat. Approximately $5x10^5$ plaques from the library were screened by plaque hybridization with the P450PCN1 cDNA (6,9) fragments (upstream ApaI fragment and downstream EcoRI fragment) as a probe. Phage clones that gave a positive signal were purified and digested with EcoRI. The DNA fragments hybridized with the P450PCN1 cDNA were subcloned into pUC19. These pUC19s were directly subjected to shotgun cloning and dideoxy sequencing using the method of Deininger (10) and Sanger et al.(11), respectively. Sequence data were analyzed by using the Beckman Microgenie program.

Determination of the Transcription Start Site

The transcription start site was determined by the method of primer extension and S1 mapping. S1 mapping was performed essentially as reported (12). A 151bp BamHI-AvaII fragment (positions from -56 to +95) containing the 5'-flanking region and the upstream half of the putative first exon was isolated (Fig.2). The BamHI-AvaII fragment was labeled at the 5'-termini with α-[^{32}P]dCTP. The labeled fragment was hybridized at 52°C with 10 μg of poly(A)$^+$ RNA isolated from the liver of rat treated with phenobarbital and the mixture treated with S1 nuclease at 37°C for 30 min. Primer extension was performed essentially as reported (13). A specific 20-mer oligonucleotide, 5'-GTCCATCCCTGCTTGTCTGT-3'(positions from +76 to +95) was synthesized on an Applied Biosystems model 380B DNA synthesizer. The oligonucleotide was labeled at the 5'-termini and hybridized with 10 μg of poly(A)$^+$ RNA isolated from the liver of rat treated with phenobarbital at 37°C. The mixture was treated with reverse transcriptase at 37°C for 2 h.

RESULTS AND DISCUSSION

Screening of P450/6βA Gene

A rat genomic library constructed in EMBL 3 was screened using a P450PCN1 cDNA fragment (upstream ApaI fragment) as a probe, and twenty hybridized clones were isolated to sequence. The nucleotide sequences and EcoRI and BamHI restriction profiles of these clones suggest the existence of multiple forms of P450III. A clone (λ6βA1) whose nucleotide sequence of the exon region was similar to that of the P450PCN2 cDNA (14) was selected and characterized. Since the λ6βA1 clone did not contain full information on the cDNA (Fig.1), the genomic library was

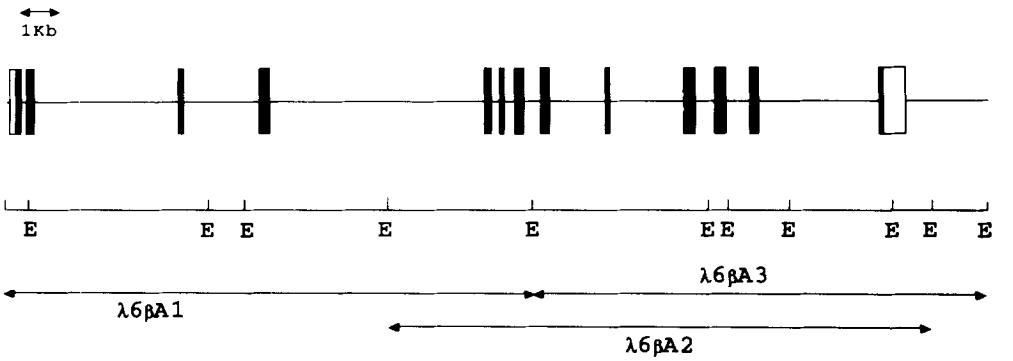

Fig. 1. Gene structure and restriction map of P450/6βA gene. The boxes represent exons; closed boxes are coding sequence and open boxes are noncoding sequence. Restriction sites for EcoRI(E) are indicated. Isolated genomic clones are shown below the gene structure.

rescreened using a P450PCN1 cDNA fragment (downstream EcoRI fragment). As results, two different clones (λ6βA2 and λ6βA3) were obtained (Fig.1). The nucleotide sequences of the exon region and restriction fragment pattern in the overlapping region of these three clones were completely identical to each other, indicating that these three clones are derived from a single gene (P450/6βA).

Sequence and Structure of P450/6βA Gene

The sequence of the P450/6βA gene spanned about 25 Kbp and was divided by 12 introns. Each exon contained a translational region in the sequence. Gene structures of many P-450 forms have been characterized. The P450I, II, VII, XI, XVII, XIX and XXI families are shown to contain 7, 9, 6, 8, 10, 10 and 10 exons, respectively (1,15). The exon-intron organization of P-450 genes is generally known to be conserved within the same P450 family. In an exceptional case, the P450IV family contains two genes which consist of 12 and 13 exons (16), although the thirteenth exon does not contain a coding region. Our present study of the P450/6βA gene indicates that P450IIIA has the largest number of exons with coding regions among P-450 genes reported previously.

The nucleotide sequence of the exon region of the P450/6βA gene showed a high similarity to those of the P450PCN2 (99.4%) and P450PCN1 cDNA (89.4%). Although the nucleotide sequence of the P450/6βA gene was very similar to that of the P450PCN2 cDNA, 3 nucleotide replacements were present in coding region and 9 nucleotide replacements or deletions were present in noncoding region. The comparison of the deduced amino acid sequence

Table 1. Sequences of exon-intron junctions of P450/6βA gene

	Exon	Intron	Exon	
			TGTGTGGT	I
I	CTCTACCG	GTGAGTGA....CTTTATAG	ACTTGGAA	II
II	ACTACAAG	GTGAGTGT....CTTTTCAG	GGCTTAGG	III
III	ATATGGGG	GTGAGTAT....CCACACAG	GTTGTTTG	IV
IV	ACCGGCGG	GTAGGTCA....GACTCTAG	GATTTTGG	V
V	TCAAGGAG	GTGAGTGA....TTCTTTAG	ATGTTCCC	VI
VI	ATGAAAAA	GTGAGTGC....CTTCTCAG	AGTGTTTG	VII
VII	GTCAGTAG	GTATGTGG....TTTTCCAG	TACTCTTT	VIII
VIII	AGCATAAG	GTAGTAAG....GCTTCCAG	CACCGAGT	IX
IX	TCATAAAG	GTAAGCAA....ACTTCCAG	CCCTGTCT	X
X	CGAGTAAG	GTGAGAGG....CTTCTCAG	GCACCTCC	XI
XI	CCAGAAAG	GTACCAGA....GTCTCCAG	GTTTAGCA	XII
XII	AAACACAG	GTGAGAGA....TATTGTAG	ATACCTCT	XIII
XIII	TGGAAAGT			
Consensus sequence	AAG	GTGAGTGA.... TTTCCAG		

Nucleotides which are identical with consensus sequence are underlined.

between the P450PCN2 cDNA and P450/6βA gene showed 2 amino acid replacements. His was replaced by Asp and Asp was replaced by Gly in the P450/6βA gene at the 429th and 445th position, respectively. Since His and Asp correspond to a basic and acidic amino acid, respectively, this replacement might affect the protein structure. A nucleotide sequence of a P-450 cDNA which we isolated from a Sprague-Dawley rat cDNA library with the same probe as described above was completely identical with that of the exon region of P450/6βA gene (data not shown). These results suggest that the P450/6βA gene is actually transcribed to the corresponding mRNA in rat liver, although further analyses are required to elucidate the relationship between the P450/6βA gene and P450PCN2 cDNA. The exon-intron junction of the P450/6βA gene is shown in Table 1. The P450/6βA gene followed the GT/AG rule at the splice junctions. Sequences of the 5′ and 3′ ends of introns were considerably conserved as GTGAGTGA and TTTCCAG, respectively. The sequence on the 5′-flanking region in the P450/6βA gene was shown in Fig.2. The consensus sequence of a TATA box, TATAAG, was found 31bp upstream from the transcription start site.

Determination of Transcription Start Site in P450/6βA Gene

To determine precisely the transcription start site in the P450/6βA gene, primer extension and S1 mapping were performed

-60 GATCCCTGGTGCCACCCATCTTTCCAGCATATAAGTACTGCAGGCTGGCCTCAGTGCAGC

+1 TGTGTGGTCAGGAGGGAAGACCTGCAGAGTATCAGAGGCCCAGCTAGAGGGAGAACACCG

+61 AGGAGTAATTTGCTGACAGACAAGCAGGGATGGACCTGCTTTCAGCTCTCACACTGGAAA

+121 CCTGGGTCCTCCTGGCAGTCATCCTGGTGCTTCTCTACCGGTGAGTGACCTCGGAAGTTC

+181 CTCCTTGTGACCAGGTTCTT

Fig. 2. Nucleotide sequence of the 5'-flanking region of P450/6βA gene. The transcription start site is designated as +1. The "TATA box" (-31 to -26) and the initiating methionine (+90 to +92) are boxed. The first exon is underlined. The primer used for primer extension is designated by the arrow.

(Fig.3). Primer extension yielded one fragment, while S1 mapping yielded a major protected fragment which was the same size (95bp) as that of the primer extension and four minor

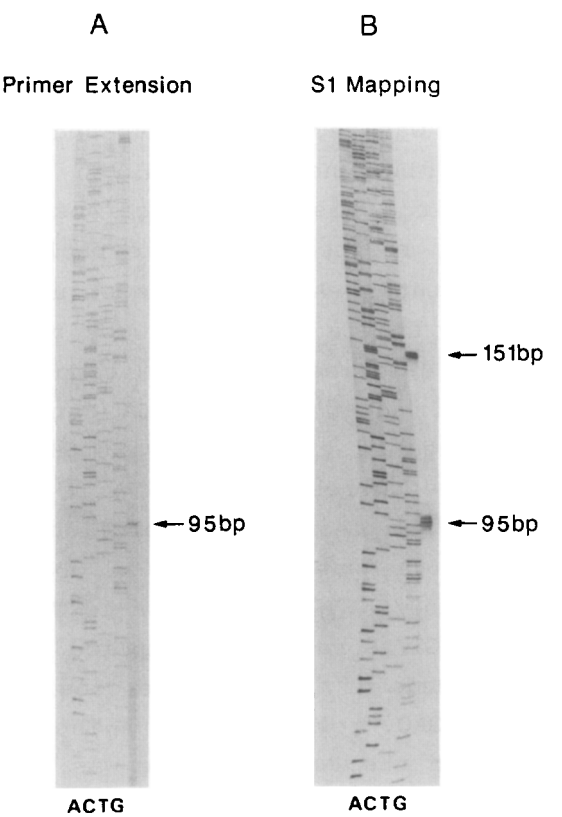

Fig. 3. Determination of the transcription start site by primer extension and S1 mapping. (A) primer extension and (B) S1 mapping were carried out as described in "Materials and Methods." Lanes, ACTG, show a dideoxy sequencing ladder of the P450/6βA gene with the same primer as primer extension. The size of the fragments is shown.

fragments located above and below the major fragment. Another long fragment(151bp) of S1 mapping should result from the exceeding BamHI-AvaII fragment. These results suggest that the fragment (95bp) which primer extension yielded, corresponding to a T residue, was assigned as the transcription start site[at +1 in Fig.2].

Finally, we have determined the gene structure of a rat P450III family (P450/6βA) as the first gene in this family and characterized it to consist of the largest numbers [13] of exons among P-450s reported.

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