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ISOLATION OF A CYTOCHROME P-450e GENE VARIANT AND CHARACTERIZATION OF ITS 5' FLANKING SEQUENCES

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SUMMARY: A cytochrome P-450e gene variant has been isolated from the rat liver genomic library. It is a typical e gene clone but unique in having b-like single base substitutions at specific sites in the 5' flanking region. It also appears to have certain additional restriction sites in the introns. When compared with the cytochrome P-450b gene, the e gene has some of the repetitive motifs interrupted in the 5' flanking region. In addition, this region is characterized by the presence of alternating pyrimidine-purine stretch, steroid hormone regulatory elements, consensus eukaryotic enhancer sequence and sequences involved in general amino acid regulation.

The prototype drug phenobarbitone induces essentially two species of cytochrome P-450, b and e, in rat liver(1,2). The b and e species are highly homologous but are distinct gene products. It has been suggested that the cytochrome P-450e gene family may contain 9-11 members, although some of them may turn out to be psuedogenes(3). In the present study, a cytochrome P-450e gene clone has been isolated from the rat liver EcoRI-genomic library and its 5'flanking sequence upto -800 base pairs has been characterized. This clone appears to be a cytochrome P-450e gene variant, having some b-like single base substitutions in the 5'flanking region and it also contains many potential regulatory sequences.

MATERIALS AND METHODS

A charon 4A-rat liver genomic library (kindly provided by T.D.Sargent, R.B.Wallace and J.Bonner) was screened with nick-translated cytochrome P-450e probe, pP-450e91 covering exons six to nine(4), by Benton-Davis procedure(5). About 300,000 plaques were analysed and two prominent signals were obtained. One of them, λ P-450e5, was plaque purified and the DNA was isolated from lysed E.coli (LE392) cultures. The DNA was digested with EcoRI and a 14 kb fragment migrating between the left and right arms was eluted from 0.5% agarose gels. This fragment was further characterized by southern blotting and restriction mapping(6). The DNA fragments were subcloned in M13mp10 and M13mp11 and sequenced by dideoxy procedure(7).

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RESULTS AND DISCUSSION

The 14 kb insert isolated from EcoRI digests of λP-450e5 DNA, lights up in southern hybridization with the nick-translated cDNA probe,pP-450e91 (Fig.1a). BamHI digestion of the fragment and analysis on 0.8% agarose gels reveal three fragments of size 7.0 kb, 4.5 kb and 2.5 kb (Fig.1b). Gross restriction analysis of the fragments and partial sequence analysis reveal λP-450e5 to be a typical cytochrome P-450e clone and it is very similar to the clone pgP-450pb6 described by Mizukami et al(8). The clone contains exons one to six and about 5 kb of the 5' flanking sequence. A detailed analysis of the 2.5 kb BamHI-EcoRI fragment of λP-450e5 covering exons four, five and six reveals additional sites for Sau96I, HpaII, BglII and AvaII in the introns in addition to having all the sites reported for pgP-450pb6(Fig.2).

The 1.0 kb BamHI-SstI fragment, covering the first exon, a portion of the first intron and about 800 nucleotides of the 5' flanking region was sequenced and the data are presented in Fig.3. A comparision of this sequence with the 5' flanking sequence information available upto -450 base pairs in the case of cytochrome P-450b and e genes(9) reveals sequence identity except that λP-450e5 has typical b-like single base substitutions at four sites in the 5'flanking region. The cytochrome P-450e

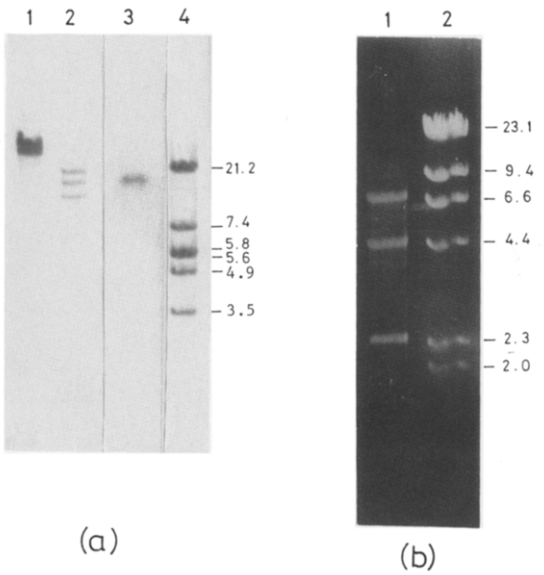


Fig. 1. Restriction enzyme digestion and southern analysis of λP-450e5 DNA.(a) lane1-uncut DNA; lane2-EcoRI digested DNA; lane3-Southern analysis with nick-translated cytochrome P-450e cDNA probe; lane4-Size markers. (b) lane1-BamHI digestion of the 14 kb insert; lane2-size markers.

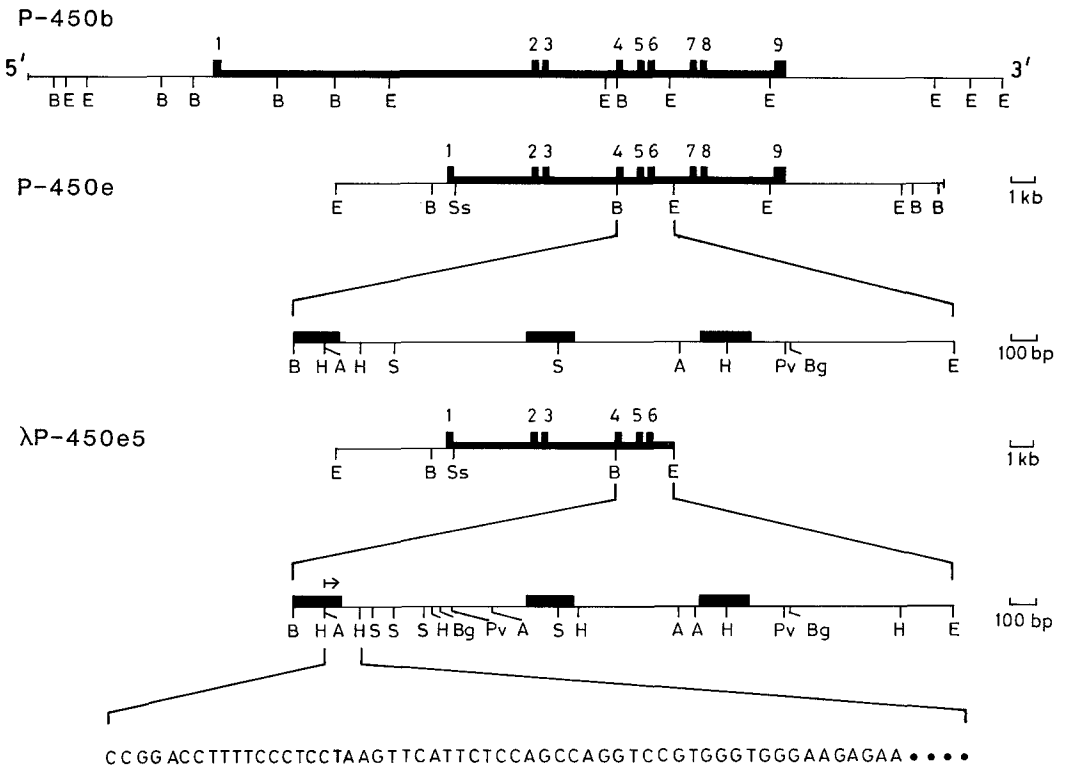
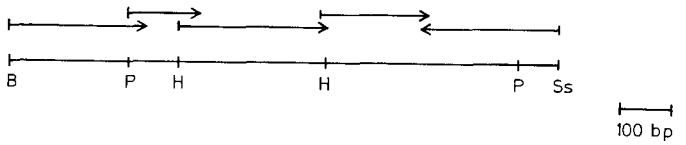


Fig. 2. Restriction map analysis of λP-450e5 in comparision with those of cytochrome P-450b and e genes. The restriction map of the BamHI-EcoRI fragment covering exons four, five and six(presented in line three from the top) is from the cytochrome P-450e gene clone, pgP-450pb6,described by Mizukami et al(9). Restriction sites: B-BamHI; E-EcoRI; Ss-SstI; H-HpaII; A-AvaII; S-Sau96I; Pv-PvuII; Bg-BglII.

gene variants are reported to show a high homology over the region spanning exons seven and eight, with the remaining exons showing significant but substantially less homology to the corresponding regions of the cytochrome P-450e gene(3). λP-450e5 described in the present study appears identical to the e gene but is characterized by having typical b-like single base substitutions in the 5'flanking region. It also appears to have additional restriction sites in the introns, although some of these sites in the cytochrome P-450e gene may not have been depicted earlier.

There are two interesting features in the 5'flanking region, which may contribute to the differences in the efficiency of expression of the cytochrome P-450b and e genes in response to phenobarbitone. As already reported by Suwa et al(9), the length of alternating pyrimidine-purine sequence in the region -255 to -290 which can assume a Z-DNA conformation, is much longer in the cytochrome P-450e gene $[(ca)_{19}]$ than in the P-450b gene $[(ca)_5]$. This is true of λP-450e5 as well. In addition, certain



-800
ggatccatttctcaattcaagttctctcctctcaaatgactctagcttgtatcatgttgacacaaaagttagacccgggg

-700
ccccaacttcttctctgccaggtttcagcctctctctccatttgactcctgagcctcactgctcctgaatctcttgccttt

-600
cctttcttttcctccccattctcccacctgtggctatcatgggccagagagtgaaatggggactctgcagaaattgcatc

-500
tggtagacttttcaaagacagggacaggaaccaacagacggagacaagcacaggaatcatgggatactattcttgtcaa

b b
ctcaaacataatcacatgtacccaggacacaaaaaacatacagagaagccccaataatttaagattatacatgtaaata

-400 b b
taccctagacatgcaagaaaagaccacccagtgcatctagactcagacaaagaaatttacatcggtacgtttatatcag

-300
aaatgatctttcacataggaaaagcatatagaacacgcacacacacacacacacacacacacacacacacacacacaat

-200
cccatgccctagtaagtaaacagagctgacaaaactgagttgacaagtgcacacccatttacataaaacaagaggcct

-100
aagtcccagtgcccttttgtgtcctgtgtatctgtttcgtggtgtcctgccaacatctatggtgtgggtaagggaatgagga

gtgaatagccaaagcaggaggcgtgaacatctgaagttgcataactgagtgtaggggcagattcagcataaaagatcct

1
gctggagagcatgc ACTGAAGTCTACCGTGGTTACACCAGGACCATGGAGCCCAGTATCTTGCTC

100
CTCCTTGCTCTCCTTGTGGGCTTCTTGTTACTCTTAGTCAGGGGACACCCAAAGTCCCGTGGC

AACTTCCCACCAGGACCTCGTCCCCTTCCCCTCTTGGGGAACCTCCTGCAGTTGGACAGAGGA

200
GGCCTCCTCAATTCCTTCATGCAGgtgagatattcacagggcctggagctc

Fig. 3. DNA sequence analysis of the BamHI-SstI fragment. The 1.0kb fragment covering the first exon, a portion of the first intron and about 800 nucleotides of the 5'flanking region was sequenced by the dideoxy chain termination method. The sequencing strategy is indicated by arrows. Restriction sites: B-BamHI; P-PstI; H-HincII; Ss-SstI. The cytochrome P-450b-like single base substitutions are underlined. The steroid recognition elements are boxed. The eukaryotic core enhancer-like sequence and the putative heme-regulatory domain are indicated by a wavy line. The dashed lines indicate general aminoacid regulatory sites.

repetitive motifs depicted in Table I are seen in cytochrome P-450b gene but are interrupted by single base substitutions in the cytochrome P-450e gene. Repetitive motifs in the 5'flanking region offering sites for the

Table 1

Repetitive motifs in the 5'flanking region of the cytochrome P-450e and b genes. The numbers indicate the first nucleotide positions in the 5'flanking region of the cytochrome P-450e gene

Cytochrome P-450e gene	Cytochrome P-450b gene
-170 -352 CCCAGTGC, CCCAGCGC	CCCAGTGC, CCCAGTGC
-123 -419 -474 ACATCT, ACATGT, ACATGT	ACATGT, ACATGT, ACATGT
-359 -457 AAGAA, AAAAA	AAGAA, AAGAA

binding of regulatory proteins are considered to contribute to the total strength of promoter elements(10). These differences may contribute to the higher level of expression of the cytochrome P-450b gene than that of the e gene in response to phenobarbitone.

There are a few other potential regulatory sites in the 5' flanking region of λP-450e5. The sequence TGTCCT figuring at -134 and -157 is a steroid hormone regulatory element detected in human growth hormone and metallothionin genes(11). This sequence can also be seen elsewhere in the 5' flanking regions of the cytochrome P_1-450 and P-450(C21) genes(12,13). The sequence CTTT-CACA(-322 to -315) resembles in the opposite orientation, the core enhancer consensus sequence $TGTGG^{AAA}_{TTT}G$ seen in immunoglobulin and viral systems(14). This region in λP-450e5 also contains the sequence TTTATATCAGAATGATCTTTCACA(-339 to -315) which can be examined for a heme regulatory site, since the upstream activation sequence in yeast involved in the heme regulation of isocytochrome C gene is characterized by TTT repeats seperated by spacers containing short direct repeats(15). It has been shown in this laboratory that heme, the prosthetic group of cytochrome P-450, regulates this hemoprotein gene transcription(4,16). Finally, the sequence TGACTC implicated in general aminoacid regulation(10) is also seen at -685 and -772 and may provide binding sites for regulatory proteins. Studies are underway to identify specific transcription factors and their sites of binding in the 5'flanking region of cytochrome P-450 genes.

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