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ISOLATION AND CHARACTERIZATION OF A NOVEL CYTOCHROME P-450-LIKE PSEUDOGENE

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SUMMARY: A rabbit liver P-450-like pseudogene has been isolated from a λ phage genomic library. Sequence analysis revealed structural homology with respect to the rat P-450b and P-450e genes as well as a similar intron-exon organization. A 5'-proximal TATA box-like sequence and two 3'-distal putative polyadenylation signals were identified, and all putative intron-exon boundaries except at the 3'-splice site of intron 2 were found to follow the GT/AG rule. With allowance for apparent deletions and insertions, the structural homology of the amino acid sequence deduced from the pseudogene with respect to rabbit P-450 isozyme 2 is lower for exons 1 through 4 (18-28%) than for exons 5 through 9 (42-65%). S_1 nuclease mapping showed that mRNAs complementary to the DNA sequence of exon 9 are expressed. However, due to the alterations in the pseudogene, it appears that functional P-450 would not be produced from such mRNAs. © 1986 Academic Press, Inc.

Cytochrome P-450, an enzyme found widely in nature, is involved in the oxygenation and other metabolic reactions of a large variety of drugs, carcinogens, and other xenobiotics as well as naturally occurring substances such as steroids and prostaglandins (1-4). Over fifty isozymes of P-450 have been purified to electrophoretic homogeneity from various species, mostly from rabbit, rat, and mouse, and the amino acid sequence is known in about ten instances by direct determination or by prediction from cDNA sequences (5). Two classes of P-450 inducers have been widely studied, one represented by the drug phenobarbital and the other by the chemical carcinogen 3-methylcholanthrene (6), but other classes apparently exist as well, such as the ethanol type (7). On the other hand, several hepatic P-450's appear to be constitutive, since no inducer has as yet been identified (2).

A number of investigators have recently undertaken the task of isolating and sequencing P-450 genes (5,8-14). Of the gene families already partially characterized, those in the phenobarbital family have 9 exons, and those in the methylcholanthrene family have 7 exons. Most such studies have been done in the rat and the mouse, but the isolation of several rabbit liver cDNA clones of the phenobarbital family has been reported (15). We

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screened a λ phage rabbit liver genomic library to search for clones of the phenobarbital type (16), and in the present report we describe the first example of a DNA segment having the properties of a P-450 pseudogene.

MATERIALS AND METHODS

A λ phage rabbit liver genomic library (17) was kindly provided by Dr. R. C. Hardison, Pennsylvania State University, a rat P-450e cDNA clone (18) by Dr. M. Adesnik, New York University, and a rat P-450b cDNA clone (19) by Dr. Y. Fujii-Kuriyama, Japanese Foundation for Cancer Research. New Zealand White male rabbits were given an intraperitoneal injection of phenobarbital (100 mg/kg) 18 hr before sacrifice or isosafrole (150 mg/kg) 3 hr prior to sacrifice. Hepatic polysomal RNA was prepared (20), and mRNA was isolated by oligo-dT column chromatography (21). DNA polymerase from *Escherichia coli* and the Klenow fragment of DNA polymerase were obtained from Boehringer Mannheim, DNA ligase and restriction enzymes were from New England Biolabs, and S_1 nuclease was from New England Nuclear. Deoxy- and dideoxynucleotides and polyd(A) were obtained from Pharmacia, salmon sperm DNA was from Sigma, and [γ-^{32}P]ATP, [α-^{32}P]dATP, and [^{35}S]dATPαS were from Amersham.

The λ phage rabbit liver genomic library was plated on petri dishes at a density of approximately 10,000 plaques per dish. Two nitrocellulose filters were sequentially layered on top of each dish, and the DNA on the filter was denatured and baked (22). The P-450e cDNA fragment was labeled by nick-translation (23) to a specific activity of 10^8 cpm/µg of DNA, boiled for 10 min, and added immediately to the hybridization mixture. Low stringency hybridizations were carried out as follows. The filters were washed at 55 °C with 4X SET (1X SET is 0.15 M NaCl, 2 mM EDTA, and 30 mM Tris buffer, pH 8.0) containing 0.1% $NaDodSO_4$ and 10X Denhardt's solution (24) for 3 hr and then incubated in 4X SET containing 0.1% $NaDodSO_4$, 0.1% Na pyrophosphate, 50 µg/ml denatured salmon sperm DNA, 10 µg/ml polyd(A) and 10X Denhardt's solution for 1 hr. The denatured probe was added, and incubation was continued for 18 to 24 hr. Four 30-min washes were done, each in a total volume of 500 ml. The first wash consisted of 4X SET, 0.1% $NaDodSO_4$, and 0.1% Na pyrophosphate, while for the other three the concentration of SET was reduced to 3X. The filters were then blotted dry and exposed to film for 1 to 7 days. Plaques from a region which gave a signal on duplicate filters were picked and rescreened at a lower density until well isolated plaques gave a clear positive signal.

DNA from the hybrid λ phage was prepared (25) and digested with restriction enzyme EcoRI. The three EcoRI inserts generated were subcloned into pUC-12 vectors, and single, double, and partial restriction digestions were done with use of enzymes whose sites are present in the polylinker region of M13 mp10. In certain cases the technique of Smith and Birnstiel (26) was used to confirm the location of the restriction sites. For S_1 nuclease mapping (27), the EcoRV-PstI fragment containing part of the 3'-terminal exon was end-labeled, annealed with liver mRNA from phenobarbital- or isosafrole-treated rabbits, and subjected to S_1 nuclease digestion (28). The mixture was boiled and applied to a 6% acrylamide-8 M urea sequencing gel.

The DNA restriction fragments identified as hybridizing with the P-450b and e cDNA probes were subcloned into the M13 sequencing vectors mp10 and mp11. DNA sequencing was done (29,30) with use of either the 15-bp or the 17-bp universal primer. Exon 5 was found to be in the middle of a 1.3-kb PstI fragment with no convenient restriction sites nearby. A 15-base oligonucleotide (GGTCCTGTCCCCGGA) was used to prime the sequencing reactions.

RESULTS AND DISCUSSION

Isolation and Characterization of Genomic Clones. Screening of the λ phage rabbit liver genomic library with the rat P-450e cDNA probe showed a number of positive clones, and the one giving the strongest signal was further characterized. The rabbit DNA in this clone was found to be composed of three EcoRI restriction fragments of about 8, 6, and 2.5 kb. These were subcloned into pUC-12 vectors, and restriction maps were generated, with the results shown in Fig. 1. Hybridization of EcoRI restriction digests of the genomic clone with nick-translated rat P-450e cDNA showed that both the 8- and 6-kb fragments gave strong signals. To determine the orientation of the gene on the genomic clone, the rat P-450e cDNA probe was cut into two fragments by the use of HindIII. These two fragments were used to probe Southern blots (31) of various restriction digests of the subcloned EcoRI fragments. The results indicated that the 3'-end probe hybridized to a 1.1-kb SstI-PstI region on the 6-kb EcoRI fragment, while the 5'-end probe hybridized to a 0.8-kb SmaI-PstI region on the 8-kb EcoRI fragment. Sequencing indicated a region homologous to exon 9 of the rat P-450e gene on the SstI-PstI fragment and regions homologous to exons 6 and 7 on the SmaI-PstI fragment. The DNA sequences located between these two fragments showed a region homologous to exon 8 of the rat P-450e gene.

Since the rat P-450e cDNA clone used contained sequences only for exons 6 through 9, a full length rat P-450b cDNA clone was used to localize the remaining upstream exons. Hybridization experiments with Southern blots made from restriction digests of the 8-kb EcoRI fragment revealed exon sequences on the 1.3-kb PstI, the 0.9-kb SstI-PstI, the 0.8-kb SmaI, and the 1.8-kb SstI fragments. The homologous regions were localized more precisely by digesting the above-mentioned fragments with Sau3A and HpaII. After

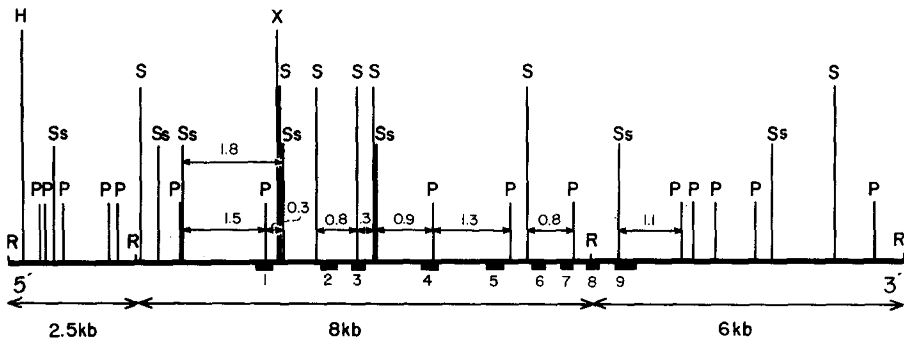

Fig. 1. Restriction map of the genomic clone with the location of the exons indicated by numbered black rectangles. The restriction sites are abbreviated as follows: R, EcoRI; H, HindIII; P, PstI; S, SmaI; Ss, SstI; and X, XbaI. The size of the restriction fragments is given in kb.

Southern transfer the blots were hybridized with nick-translated rat P-450b cDNA. From the low intensity of the signals, it was apparent that the homology of the 5'-end exons of the gene was weaker than that of the 3'-end exons with respect to the rat probe. Sequence analysis supported this conclusion and also revealed the likely locations: exon 5 in the 1.3-kb PstI fragment, exon 4 in the 0.9-kb SstI-PstI fragment, exon 3 overlapping in the 0.8-kb and 0.3-kb SmaI fragments, exon 2 in the 0.8-kb SmaI fragment, and exon 1 most likely in the 0.3-kb PstI-SstI fragment with a 5'-untranslated region extending into the 1.5-kb PstI fragment. Analysis of the DNA regions corresponding to the exons of the pseudogene indicated that allowance had to be made for apparent deletions and insertions in order to calculate maximum homology with respect to rat P-450 isozymes b and e or to rabbit isozyme 2. This is particularly evident at the 3'-end of the gene where it is very clear from the high degree of homology that insertions or deletions of one or two nucleotides cause a shift in the translational reading frame. Two such shifts occur in exon 7, two in exon 8, and one in exon 9, while exons 5 and 6 have continuous reading frames. The 5'-end exons also have such frameshifts but, because of the lower homology, their presence is less apparent. Such shifts are characteristic of a number of pseudogenes for non-P-450 proteins (32-34). Intron 2, in contrast to the other introns, may have a modified 3'-splice site (35), AA instead of AG.

Overall Sequence Comparisons. Figure 2 shows the nucleotide sequence of the pseudogene as well as the amino acid sequence deduced with use of translation reading frames which gave the highest homology with P-450 isozyme 2. The homology calculated in this manner for exons 1-9 is 28, 24, 20, 18, 42, 65, 56, 46, and 56%, respectively. The values obtained are thus much lower for exons 1 through 4 than for exons 5 through 9.

Nuclease S_1 Mapping. In order to gain insight as to whether mRNAs closely homologous to the pseudogene are expressed, S_1 nuclease mapping was performed using as a probe the EcoRV-PstI fragment containing part of the 3'-end terminal exon (see Fig. 3). When mRNA from livers of rabbits treated with phenobarbital or isosafrole was used, the size of the two protected fragments (about 235 and 210 bp) corresponded to that predicted from the DNA sequence of the gene. This finding showed that transcription had ended 15 to 20 bp downstream from the two putative polyadenylation signals, AATAA. Also worth noting is the fact that the 3'-end nontranslatable region has a very high GC content.

We believe that this pseudogene is part of the phenobarbital P-450 family. The high degree of structural homology and the similar genomic organization with respect to the genes for rat P-450 isozymes b and e support this hypothesis. On the other hand, the rat P-450 b and e genes are

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AGGAAGGGAACCGATGTTCAAATAGGCTGCTGGGAGCCGGCCAGGGAGGGGGCGGAGGTCTGGACGGGAGAACTT
                  ******
GGGGACAGTGAGGTCAAGAGGGTTTGGCCTTGTTGATGAGCTCAGTGCCTGCAGCACTGGCATCTCATTTGGGCA
                                     MetSerSerValProAlaAlaLeuAlaSerHisLeuGlyT

CTGGTTCAAGTCCCGGCTGCTCCACTTCCGATCCAGCTCTCTGCTATGGCCTGGGAAAGCAGTAGAAGGTGGCCC
hrGlySerSerPro  LeuLeuHisPheArgSerSerSerLeuLeuTrpProGlyLysAlaValGluGlyGlyPr

AAGTCCTTGGGCCTGCACCCAGGTGGGAGGCCCGGAAGAACCCCTTGGCTTCGGATTGGTGCAGCTCCAGCCAGT
oSerPro  GlyLeuHisProGlyGlyArgProGlyArgThrProTrpLeuArgIleGlyAlaAlaProAlaL

GTG......GGAGGAGAGTCCCCGGAGAAGCCTGGGGAAGCTGGGGCCACCCAGCACGCCGGCCGCTTCCTTCCT
                ysSerProGluLysProGlyGluAlaGlyAlaThrGlnHisAlaGlyArgPheLeuPro

GGGGTCCCCCTCACCGTAGCCTTTGAAGAGCCAGTCGAAGGTGGCCAGCTCGCCACGCCCGCTGAAATCCTCAGC
GlyValProLeuThrValAlaPheGluGluProValGluGly AlaSerSerProArgProLeuLysSerSerAl

CTGGTCCACCAGCGCCTCCTTTACTGCCTCCTGTCCG......AGGCTGGACTTTGATCCTGGTTATACAATGGT
aTrpSerThrSerAlaSerPheThrAlaSerL                                         euVa

TTCATTTGACCTCCCAACTACACCCCTTGCCCTGCACCTCCCTTGCTTGGGGTACAGCCAGCAAGGGGGTTGGGG
lSerPheAspLeuProThrThrProLeuAlaLeuHisLeuProCysLeuGlyTyrSerGlnGlnGlyGlyTrpGl

ACACGGGGTACCCGGGGGCGGCTTAGCTGCTGCCCCCACACTTCCGCCTCACGTGAGGGTTTTGAAGCAGACCCT
yHisGlyValProGlyGlyGlyLeuAlaAlaAlaProThrPheProProHisValArgValLeuLysGlnThrLe

GCGGCGGGAACACCGTGCGTGGAGTTCCAAA......CCCAGCAGAGCCAGCCCCCACTCTTGGAACACTGGACA
uArgArgGluHisP                                 roSerProHisSerTrpAsnThrGlyGl

GTTGCTCCCAAACCTGGACAGGTGTCCCAATTCAGTCTCCAGGGACATCGGGGTATCTCAGCAGATGCCCCCCAA
nLeuLeuProAsnLeuAspArgCysProAsnSerValSerArgAspIleGlyValSerGln ArgCysProProL

AACAGAGCTGCAGGCAAGCTACATGTCTTCAACTCCGCCTTCAACTTGAGGTGGTGCTCACCTGTAACATC....
ysGlnSerCysArgGln LeuHisValPheAsnSerAlaPheAsnLeuArgTrpCysSerPro

..CCCTCAGCTCTATGAGATGTTCTACTCAGTGATGAAGCACCTGCCAGGGCCGCAGCAACAGGCGTTCAAGGAG
          LeuTyrGluMetPheTyrSerValMetLysHisLeuProGlyProGlnGlnGlnAlaPheLysGlu

CTGGAGGGGCTGAGAGACTTCATAGCCAAGAAGGTGGAGCGGACACAGCGCACGCTGGACCCCAACTCCCCGCGG
LeuGluGlyLeuArgAspPheIleAlaLysLysValGluArgThrGlnArgThrLeuAspProAsnSerProArg

GACTTCATCGACTCTTTCCTCATCCGCATGCAGGAGGTGCAGCAG......TTCTTGCAGGAGAAGAAGGACCCC
AspPheIleAspSerPheLeuIleArgMetGlnGlu                          GluLysLysAspPro

AAGTCGGAGTTCCACATGAAGAACCTGGTGATGACCACGCTCAACCTCTTCTTTGCGGGCACAGAGACCGTCAGC
LysSerGluPheHisMetLysAsnLeuValMetThrThrLeuAsnLeuPhePheAlaGlyThrGluThrValSer

ACCACCATGCGCTATGGCTTCCTGCTGCTCATGAAACACCCGGATGTGGAGGGTGAGGCG......CCCCCAGCC
ThrThrMetArgTyrGlyPheLeuLeuLeuMetLysHisProAspValGluA                      la

AAGGTCCACGAGGAGATCGACCGAGTGATTGGCAGGAACCGGCAGCCCAAGTTCGAGGACGGCAAAGATGCCCTA
LysValHisGluGluIleAspArgValIleGlyArgAsnArgGlnProLysPheGluAsp AlaLysMetProTy

CACAGAGGCGGTGATCCACGAGATCCAGAGATTCACAGACATGATCCCCATGGCCTGGCCACAGGGTCACCAGGG
rThrGluAlaValIleHisGluIleGlnArgPheThrAspMetIleProMetAlaTrp HisArgValThrArgA

ACACCAAGTTCCGGGACTTCCTCCTCCCCAAGGTGCTGCCC......CCCCAGGGCACCGAAGTGTTCCCATGCT
spThrLysPheArgAspPheLeuLeuProLys                      GlyThrGluVal  ProMetLe

GGGCTCTGTGCTGAAAGACCCCAAGTTCTTCTACCAAGCCCCGGAATTCTACCCCCAGCACTTCCTGGATGAGAA
uGlySerValLeuLysAspProLysPhePheTyrGlnAlaProGluPheTyrProGlnHisPheLeuAspGluLy

GGGGCAGTTTAAGAAGAGCGACGCTTTTCATGCCCTTTTCCGTCGGTAA......GCTCCAGGAAAGCGCTACTG
sGlyGlnPheLysLysSerAspAlaPhe MetProPheSerValG                   lyLysArgTyrCy

TCTCGGAGAAGGCCTGGCCAGAATGGAGCTCTTCCTCTTCTTCACCACCATCATGCAGAACTTCCGCTTCCGCTC
sLeuGlyGluGlyLeuAlaArgMetGluLeuPheLeuPhePheThrThrIleMetGlnAsnPheArgPheArgSe

TCAGCAGGCGCCCCAGGATATCGACGTGTCCCCCAAGCACGTGGGCTTCGCACGATCCCGCGAACCTACACCATG
rGlnGlnAlaProGlnAspIleAspValSerProLysHisValGlyPheAlaArg  ProArgThrTyrThrMet

AGCTTCGTGCCGCGCTAGGCAGGGGCGGGGCTAGCAGGAGGGCGGGGCTGCGAGAAGGGGCGGGGCTGCGGGAGG
SerPheValProArgEND

GGCGGGGCGAGGGCAGGGCTGGGGGTCGGCCTTGCTGGAGATGCGCGCACCGATTCTAATAACAGCACACCACAT

TCTAATAACAGCL
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Fig. 2. Nucleotide sequence of the P-450-like genomic clone and corresponding amino acid sequence. Reading frames which gave the highest homology with P-450 isozyme 2 were used for this purpose. Underlined amino acids are identical to those in isozyme 2. Dotted lines indicate intron sequences not shown. A TATA box-like sequence is indicated by asterisks and the two polyadenylation signal-like sequences are underlined twice. The 5′- and 3′-splice site dinucleotides (GT/AG rule) are enclosed in rectangles. The presence of a modified 3′-splice site in intron 2 should be noted.

23 and 14 kb in length, respectively, while this rabbit gene is shorter, about 6.5 kb. Although S_1 nuclease mapping experiments indicated that an mRNA complementary to this gene may be expressed in liver, the DNA sequence indicates that functional P-450 could not be synthesized due to shifts in the reading frame that would result in premature termination.

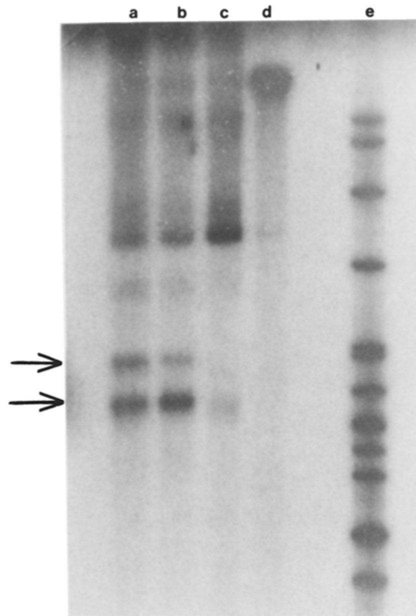

Fig. 3. S_1 nuclease mapping of the 3'-terminal exon of the gene. The EcoRV-PstI fragment starting 66 bp downstream from the SstI site in exon 9 was 3'-end-labeled by use of the Klenow fragment of DNA polymerase, hybridized to liver polysomal mRNA, and, after S_1 digestion, submitted to gel electrophoresis. The arrows indicate the protected fragments, about 235 (upper) and 210 bp (lower). Individual lanes are as follows: a, phenobarbital-induced rabbit liver mRNA hybridized with the 3' end-labeled probe; b, same but with isosafrole-induced rabbit liver mRNA; c, yeast tRNA hybridized with the 3' end-labeled probe as a control; d, 3' end-labeled EcoRV-PstI genomic probe; and e, HpaII restriction fragments of plasmid pBR322 as m. wt. markers.

The phenobarbital family of P-450 genes appears to have many more members than the methylcholanthrene family. At least nine distinct phenobarbital-type genomic clones have already been characterized in the rat (36). Two of these genes were found to code for P-450's b and e, while the remaining isolated genomic clones have not yet been fully characterized. In one case the genomic clone was shown to correspond to an isolated cDNA (M. Adesnik and A. Anderson, personal communications), and another genomic clone was found to be expressed in the preputial gland but not in liver (36). Whether or not the remaining isolated genomic clones are expressed remains to be seen. Although several rabbit cDNAs coding for proteins with a high degree of homology to isozyme 2 have been characterized (15), none correspond to the genomic clone described in this report.

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