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ISOLATION AND PARTIAL CHARACTERIZATION OF THE GENE FOR CYTOCHROME P-450 3a (P-450$_{ALC}$) AND A SECOND CLOSELY RELATED GENE

Shahrokh C. Khani, Todd D. Porter, and Minor J. Coon

Department of Biological Chemistry, Medical School
The University of Michigan, Ann Arbor, MI 48109

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SUMMARY: Two genes that hybridize to the cDNA for alcohol-inducible cytochrome P-450 form 3a (P-450$_{ALC}$) have been isolated from a rabbit genomic library and characterized by restriction mapping, hybridization, and partial sequence analysis. The genes show extensive sequence similarity as judged by hybridization at high stringency to the coding region of P-450 3a cDNA. However, only gene 1 hybridizes under these conditions to the 3' nontranslated segment of P-450 3a cDNA. The hybridizing fragments derived from both cloned genes were found to be present in the genome of all rabbits examined by Southern blot analysis, indicating that the genes represent separate loci and are not polymorphic alleles. Partial sequence analysis indicated that gene 1 encodes P-450 3a. Gene 2, if transcribed, would encode a protein with greater than 96% sequence identity with P-450 3a in the NH_2-terminal region.

Cytochrome P-450s constitute an array of structurally related monooxygenases responsible for biotransformation of xenobiotics, including alcohols (1,2), and endobiotics. Determination of the primary structure of these enzymes has provided ample evidence for the existence of a cytochrome P-450 gene superfamily (3). The diversification in this family appears to be a dynamic phenomenon involving, in part, gene duplication followed by mutational divergence. Examples of very closely related cytochromes having >90% sequence identity within the same species include rat P-450 b and e (4) and PCN 1 and 2 (5), and rabbit P-450 1 and 1-88 (6). The extensive homology within these pairs of isozymes is thought to result from recent duplication and to be maintained by gene conversion (7).

Recently, this laboratory isolated a set of overlapping cDNA clones spanning nearly the entire nucleotide sequence of the mRNA for rabbit cytochrome P-450 3a (P-450$_{ALC}$) (8), the alcohol-inducible P-450 which exhibits a high turnover number in the oxidation of alcohols and the demethylation of nitrosamines

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(2,9). cDNAs have also been cloned (10) for the orthologous cytochrome (P-450 j) found in rat (11) and man (12). The induction of P-450s 3a and j by a variety of foreign compounds appears to be largely nontranscriptional, as only a small increase in the levels of the mRNAs is observed upon exposure to these compounds (8,10,12). On the other hand, a comparable increase in the levels of P-450 j mRNA and protein is observed during fasting or soon after birth (10,13,14).

To study the different mechanisms of regulation of the alcohol-inducible P-450s, it was necessary to determine the number of genes that are expressed in this family. Preliminary data reported by Song *et al.* (10) suggest the presence of a single gene in rat and man. In rabbits, however, the pattern of bands observed on Southern blot analysis of total genomic DNA suggests the presence of two or more genes (8). Furthermore, two RNA transcripts in rabbit liver hybridize to the 3a cDNA (8), making the presence of a second gene highly homologous to the 3a gene likely. We have therefore screened a rabbit genomic library with P-450 3a cDNA, isolated the gene for form 3a and a closely related gene, and shown them to be non-allelic loci with a high degree of homology.

METHODS

Screening of the Genomic Library. A *Bam* HI-*Pst* I restriction fragment derived from the 5' end of the p3a-1 cDNA (8) and a mixture of 400-bp *Pst* I fragments from the p3a-2 cDNA (8) were radiolabelled with [^{32}P]dCTP by nick-translation (15) and used to screen 10^6 plaques of a New Zealand White rabbit genomic library in λ Charon 4a (16) as described by Maniatis *et al.* (16). The positive clones were plaque-purified and the DNA was isolated by standard techniques (17). Restriction sites were mapped by single or multiple digestions with various enzymes and the fragments containing exonic sequences were identified by hybridization with a mixture of nick-translated p3a-1 and p3a-2 plasmid probes. Transcriptional orientation of the clones was determined by hybridization of phage DNA restriction fragments to nick-translated m13mp10 plasmids containing either a *Bam* HI-*Pst* I fragment from the 5' end of p3a-1 or a 3' non-coding *Pst* I fragment from p3a-2. Selected fragments of phage DNA subcloned into m13mp10 and m13mp11 were sequenced by the chain termination method (18).

Genomic DNA Hybridization. Total genomic DNA was isolated from the livers of New Zealand White rabbits by a modification of the procedure described by Gross-Bellard *et al.* (19). Finely minced liver tissue was gently shaken overnight at 37°C with 10 volumes of 10 mM Tris-Cl buffer, pH 7.4, containing 10 mM NaCl, 25 mM EDTA, 0.5% $NaDodSO_4$, and 200 μg/ml of proteinase K. The resulting suspension was extracted twice with phenol and once with phenol/chloroform by gentle inversions and centrifuged at 10,000 x *g* for 30 min. The nucleic acid in the supernatant fraction was precipitated with ethanol, spooled out, and washed

repeatedly with 70% ethanol. The DNA was dried and resuspended in water, and aliquots were incubated with restriction enzymes at 37°C overnight. The digested DNA was fractionated on a 1.0% agarose gel and transferred to nitrocellulose filters according to the method of Southern (20). The filters were prehybridized in 6X SSC (1X SSC is 150 mM NaCl in 15 mM sodium citrate buffer, pH 7.0), 10X Denhardt's (1X Denhardt's is a solution of 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, and 0.02% Ficoll), and 0.1% $NaDodSO_4$ at 68°C for several hours. Hybridization was carried out at 68°C overnight in fresh prehybridization mixture containing 100 μg/ml of sheared and denatured salmon sperm DNA, 10% dextran sulfate, and 5-10 ng/ml of probe radiolabelled by the hexanucleotide priming method (21) to a specific activity of 10^8 to 10^9 cpm/μg. The filters were washed for 1.5 hours with three changes of 1.5X SSC containing 0.1% $NaDodSO_4$ and once for one hour with 0.5X SSC containing 0.1% $NaDodSO_4$ at 68°C. The hybridizing bands were visualized by autoradiography.

RESULTS AND DISCUSSION

Approximately one million recombinants from a λphage rabbit liver genomic library were screened using probes from various regions of cytochrome P-450 3a cDNA (8). Seven positive clones with distinct restriction maps were recovered. The DNA from one of the clones appeared grossly rearranged and another clone hybridized only under reduced stringency conditions. Careful restriction mapping and hybridization analysis of the DNA derived from the remaining five clones revealed the presence of two highly similar yet distinct sets of overlapping clones. As shown in Fig. 1, one set of recombinants includes clones 16, 28, and 7, all of which span gene 1 and its flanking regions. The other set includes the remaining two overlapping clones, 23 and 11, which encompass gene 2. Genes 1 and 2 are very similar, but not identical, as judged by restriction analysis.

To determine the limits of the hybridizing regions and the transcriptional orientation of genes 1 and 2, phage DNA was digested with various restriction enzymes, transferred to nitrocellulose, and hybridized with a mixture of p3a-1 and p3a-2 cDNA clones (complete probe) or subcloned restriction fragments derived from the 5' (5' probe) or the 3' nontranslated (3' probe) region of the 3a cDNA (8). The hybridizing fragments from the two genes correspond to all the major bands observed by Southern blot analysis of the restriction digests of New Zealand White rabbit genomic DNA (8). As shown in Fig. 1, the total hybridizable DNA of gene 1 spans 10.4 kb, while the hybridizable segment of gene 2 spans 9.2 kb. The 5' 3.3- and 0.2-kb *Bam* HI fragments from both genes hybridize with the 5' cDNA probe, although the intervening 0.9-kb *Bam* HI fragment in gene 1 does

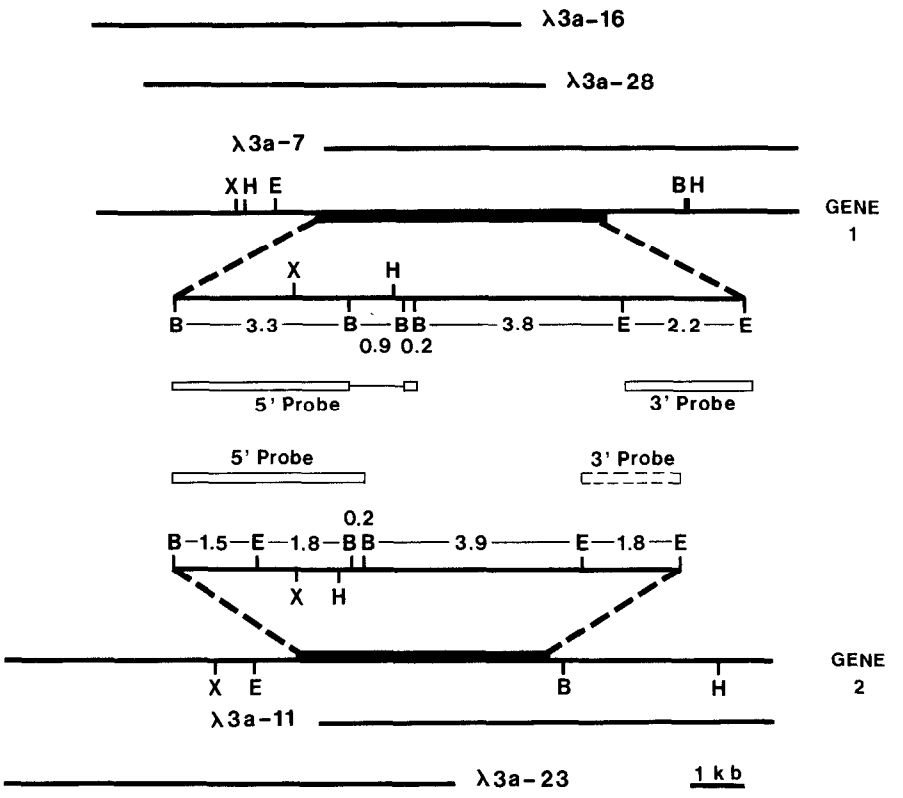

Fig. 1. Genomic clones and restriction maps for P-450 3a genes 1 and 2. The genomic clones spanning each gene are shown above and below the composite restriction maps. The *filled boxes* indicate the extent of hybridization of each gene to the complete 3a cDNA probe; the *unfilled boxes* indicate segments which hybridize to the 5' or 3' probes, as indicated. The 3' probe (unfilled box enclosed by dashed lines) hybridizes to gene 2 only at low stringency. B, *Bam* HI; E, *Eco* RI; H, *Hind* III; X, *Xba* I. Distances between restriction sites are indicated in kb.

not, and therefore appears to contain only intronic sequences. The *Bam* HI-*Eco* RI segments in the middle of both genes and the 3' *Eco* RI fragments hybridize with the full-length probe. The 3' 2.2-kb *Eco* RI fragment from gene 1 also hybridizes with the 3' nontranslated probe. In contrast, the 1.8-kb *Eco* RI fragment from the 3' end of gene 2 hybridizes with this probe only under reduced stringency conditions, indicating significant divergence in this segment between the two genes. Increasing the stringency of the washes to 0.1X SSC did not cause selective melting of the complete probe from either gene, indicating greater than 90% identity of the protein-coding sequences in the two genes (22).

In order to examine whether the two homologous genomic segments represent distinct genetic loci or polymorphic alleles

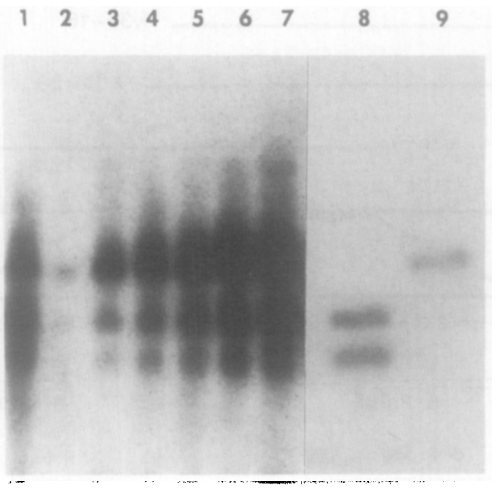

Fig. 2. Southern blot analysis of rabbit genomic DNA with the 5' P-450 3a cDNA probe. Genomic DNA (1-10 μg) isolated from seven outbred New Zealand White rabbits (lanes 1-7) was digested with *Bam* HI and *Eco* RI, fractionated on a 1% agarose gel, and transferred to nitrocellulose. The blots were hybridized and washed as described under *Methods*. Lanes 8 and 9 contained 1 μg of *Bam* HI-*Eco* RI-digested DNA from clone λ23 (gene 2) and clone 16 (gene 1), respectively.

of the 3a locus, genomic DNA from a number of out-bred rabbits was digested with *Bam* HI and *Eco* RI, fractionated on an agarose gel, and hybridized with a subcloned 5' *Bam* HI-*Pst* I cDNA fragment. As indicated in Fig. 1, digestion of gene 1 with *Bam* HI and *Eco* RI would be expected to generate two *Bam* HI fragments, 0.2 and 3.3 kb in length, which would hybridize with the 5' probe; as noted above,the 0.9-kb fragment is apparently intronic. In contrast, digestion of gene 2 with the same restriction enzymes would be expected to generate a 0.2-kb fragment and two *Bam* HI-*Eco* RI fragments, 1.5 and 1.8 kb in length, all of which would hybridize to the 5' probe. If the differences between genes 1 and 2 represent an allelic polymorphism, different patterns of hybridizing restriction fragments should be observed in genomic DNA from different rabbits. In other words, homozygotes and heterozygotes should display hybridization patterns characteristic of one or both genes, respectively. On the other hand, observation of all the hybridizing fragments in the genome of all rabbits would indicate that each gene represents a separate locus. The results of such an experiment are shown in Fig. 2. All the expected hybridizing restriction fragments (except the 0.2-kb fragment, which ran off the gel) were present in the genome of each of the seven rabbits examined.

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   AlaValLeuGlyIleThrValAlaLeuLeuGlyTrpMetValIleLeuLeuPheIle    19
ATGGCTGTTCTGGGCATCACCGTCGCCCTGCTGGGGTGGATGGTCATCCTCCTGTTCATA   60
                                  T          C
                                             Ala

SerValTrpLysGlnIleHisSerSerTrpAsnLeuProProGlyProPheProLeuPro   39
TCCGTCTGGAAGCAGATCCACAGCAGCTGGAACCTGCCCCCAGGACCTTTCCCACTGCCC   120

IleIleGlyAsnLeuLeuGlnLeuAspLeuLysAspIleProLysSerPheGlyArg      58
ATCATCGGGAATCTTCTCCAGTTGGATTTGAAGGATATTCCCAAGTCCTTTGGCAGG      177
  T                           C                  A
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Fig. 3. Nucleotide and derived amino acid sequence of the first coding exon of genes 1 and 2 with the substitutions in gene 2 indicated. The sequences were determined from the *Pvu* II site underlined.

It is therefore highly unlikely (<1% probability) that gene 1 and gene 2 are allelic variants of the 3a gene. They probably represent two loci that have arisen by a recent duplication in rabbits of the ancestral 3a gene.

Further evidence for the homology between the two genes was provided by sequence comparison of the 5' regions of gene 1 and gene 2. Corresponding 5' restriction fragments were isolated from the two genes and subcloned into m13mp10 and m13mp11, and the nucleotide sequence of the first coding exon from each gene was determined (Fig. 3). The nucleotide sequence of gene 1 and the deduced amino acid sequence of the encoded polypeptide are in complete agreement with the sequence of the 3a cDNA (8) and the NH_2-terminal sequence of P-450 3a protein as determined by Edman degradation (1,8). Therefore, cytochrome P-450 3a appears to be encoded by gene 1. Examination of the sequence alignment shown in Fig. 3 reveals a striking similarity between genes 1 and 2, with only five differences in 177 nucleotides. Three of these differences are in the third position of codons 11, 41, and 56, and would conserve the amino acid sequence in the putative gene product at those positions. The remaining two differences occur in the second position of codon 15 and the third position of codon 50. These would substitute alanine for valine and phenylalanine for leucine in the NH_2-terminal sequence of the putative protein encoded by gene 2. From these limited data we are presently unable to determine if gene 2 encodes a functional protein.

Previously, two mRNA species differing in size were observed when the p3a-2 cDNA was hybridized at high stringency to electrophoretically fractionated poly(A)$^+$ RNA from rabbit liver (8). As only a single mRNA, presumably the gene 1 transcript,

was detected with the 3' nontranslated probe, it is evident that a second mRNA is present that hybridizes to the coding region of 3a cDNA but not the 3' probe. This other mRNA may be the product of gene 2. Definitive evidence for the production of functional mRNA and protein from gene 2 awaits further investigation.

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