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cDNA and derived amino acid sequence of ethanol-inducible rabbit liver cytochrome P-450 isozyme 3a (P-450$_{ALC}$)

(immunoscreening/hybridization-selection/mRNA hybridization/genomic hybridization/homology of P-450 cytochromes)

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ABSTRACT **Administration of ethanol to rabbits is known to induce a unique liver microsomal cytochrome P-450, termed isozyme 3a or P-450$_{ALC}$, which is responsible for the increased oxidation of ethanol and other alcohols and the activation of toxic or carcinogenic compounds such as acetaminophen and *N*-nitrosodimethylamine. To further characterize this cytochrome P-450 we have identified cDNA clones to isozyme 3a by immunoscreening, DNA hybridization, and hybridization-selection. The cDNA sequence determined from two overlapping clones contains an open reading frame of 1416 nucleotides, and the first 25 amino acids of this reading frame correspond to residues 21–45 of cytochrome P-450 3a. The complete polypeptide, including residues 1 to 20, contains 492 amino acids and has a molecular weight of 56,820. Cytochrome P-450 3a is approximately 55% identical in sequence to P-450 isozymes 1 and 3b and 48% identical to isozyme 2. Hybridization of clone p3a-2 to electrophoretically fractionated rabbit liver poly(A)$^+$ RNA revealed multiple bands, but, with a probe derived from the 3′ nontranslated portion of this cDNA, only a 1.9-kilobase band was observed. Treatment of rabbits with imidazole, which increases the content of isozyme 3a, resulted in a transient increase in form 3a mRNA, but this was judged to be insufficient to account for the known 4.5-fold increase in form 3a protein. Genomic DNA analysis indicated that the cytochrome P-450 3a gene does not belong to a large subfamily.**

Chronic ethanol consumption by animals and humans is associated with a variety of diseases and toxicities, many of which may be related to the induction of one or more forms of cytochrome P-450. This laboratory previously isolated and characterized a unique cytochrome P-450, designated form 3a or P-450$_{ALC}$, that is elevated in rabbit liver after ethanol administration (1, 2). More recently we have provided immunochemical evidence that this isozyme is also induced by a variety of other apparently unrelated compounds, including imidazole, acetone, isoniazid, trichloroethylene, and pyrazole (3, 4). The corresponding rat cytochrome, called P-450j, is also induced by ethanol and isoniazid (5). Isozyme 3a exhibits high specific activity in O_2-dependent oxidation of compounds such as ethanol, other alcohols, and aniline (1, 2), the carcinogen *N*-nitrosodimethylamine (6), and *p*-nitrophenol (7), as well as in the conversion of acetaminophen (8) and carbon tetrachloride (9) to reactive metabolites. The enhanced toxicity of the latter two compounds in animals pretreated with ethanol, as well as in human alcoholics, is believed to be due to the induction of isozyme 3a in the alcoholic state (8, 9).

A physiological role of isozyme 3a was recently described in which this cytochrome catalyzes the first two steps in a proposed gluconeogenic pathway from acetone with the formation of acetol and then methylglyoxal (10, 11). These reactions may become significant in conditions of elevated blood acetone levels, such as during fasting, in diabetic ketoacidosis, and possibly in the alcoholic state (10, 12). The presence of elevated blood acetone levels may induce the synthesis of isozyme 3a in the liver, and thereby increase the conversion of acetone to these glucogenic intermediates. This substrate-mediated induction is an interesting example in which an endogenous compound induces the cytochrome P-450 responsible for its further metabolism.

To more precisely characterize this form of cytochrome P-450 and establish its structural relationship to other P-450 isozymes, we have used monospecific antibodies to identify a cDNA for isozyme 3a in a rabbit liver cDNA library. Using this cDNA as a probe, we have identified additional cDNAs, and we now report the entire amino acid sequence of P-450 3a. Sequence comparison shows that the enzyme is most similar to P-450 isozymes 1 (13) and 3b (14). Genomic DNA analysis indicates that the P-450 3a gene is not a member of a large subfamily.

MATERIALS AND METHODS

Screening of the cDNA Library. A pBR322 library constructed from New Zealand White rabbit liver poly(A)$^+$ RNA (13) was kindly provided by R. H. Tukey (University of California at San Diego). Approximately 10^4 tetracycline-resistant recombinants were screened by the method of Helfman *et al.* (15), as follows. Lysed colonies on duplicate filters were incubated with monospecific anti-3a IgG (5 μg/ml) raised in sheep (16). The filters were then incubated with a 1:300 dilution of rabbit anti-sheep IgG (Cappel Laboratories, Cochranville, PA), washed with Tris-buffered saline, and incubated with ^{125}I-labeled staphylococcal protein A (10^5 cpm/ml). A single colony, p3a-2, which gave a strong positive signal by autoradiography, was purified, and the derived plasmid was characterized by restriction mapping. The *Pst* I fragments that correspond to the 5′ and 3′ ends of p3a-2 were nick-translated with [^{32}P]dATP and used to rescreen the cDNA library, and the positive clones obtained were purified and mapped.

Hybridization-Selection. Hybridization-selection was performed as described (13), with the following modifications. Plasmid DNA (30 μg) from the immunopositive clone p3a-2 was denatured by boiling in 0.3 M NaOH for 5 min, and ammonium acetate was added to a final concentration of 2 M. The denatured DNA solution was slowly filtered through 25-mm nitrocellulose filters (HATF, Millipore) that had been washed with 6× SSC (1× SSC is 150 mM NaCl in 15 mM sodium citrate buffer, pH 7.0) at 60°C and then with 1 M ammonium acetate. After several washes with 6× SSC, the

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Abbreviations: P-450 3a or form 3a, rabbit liver microsomal cytochrome P-450 form 3a; bp, base pair(s); kb, kilobase(s).

bound denatured DNA was fixed to the filters by baking under reduced pressure at 80°C for 2 hr. Rabbit liver poly(A)$^+$ RNA was hybridized to the immobilized plasmid DNA, nonbound RNA was removed by washing as described (13), and specifically bound mRNA was eluted with 90% (vol/vol) formamide. The selected mRNA was translated in a reticulocyte lysate (Bethesda Research Laboratories) in the presence of [^{35}S]methionine, and the synthesized protein was immunoprecipitated with anti-P-450 3a monoclonal antibody linked to Affi-Gel 10 (Bio-Rad) (17). The protein bound to this antibody-linked agarose was eluted with sample dilution buffer and subjected to electrophoresis on a NaDodSO$_4$/7.5% polyacrylamide gel (18) and then visualized by fluorography.

DNA Sequencing. Restriction fragments were subcloned in M13mp10 or mp11 phage and sequenced by the chain-termination method (19). In regions that lacked convenient restriction sites, appropriate clones were generated by BAL-31 nuclease digestion (20); in some cases polymerization reactions were initiated by use of synthetic primers complementary to regions of DNA with known sequence. Each position was determined an average of six times, and 98% of the coding sequence was determined from both strands.

RNA Hybridization. Total liver RNA was isolated by the guanidine hydrochloride method (21) from male New Zealand White rabbits that were untreated or treated with imidazole (200 mg/kg of body weight) or isosafrole (150 mg/kg of body weight) 3 hr prior to sacrifice (1, 4). Poly(A)$^+$ RNA was isolated by one cycle of oligo(dT)-cellulose chromatography (22), subjected to electrophoresis at 20 V on 1% agarose/2.2 M formaldehyde gels (23), and transferred to GeneScreen (New England Nuclear). Filters were hybridized with ^{32}P-labeled nick-translated probes at 68°C as described (24) and washed at 68°C sequentially with 4×, 3×, and 1× SET containing 0.1% NaDodSO$_4$ and 0.1% sodium pyrophosphate (1× SET is 150 mM NaCl and 2 mM EDTA in 30 mM Tris·HCl buffer, pH 8.0) (24). RNA levels were quantified by scanning densitometry; size determinations were made with RNA standards from Bethesda Research Laboratories.

Genomic DNA Hybridization. Genomic DNA was prepared from the liver of a male New Zealand White rabbit by digestion with proteinase K and extraction with phenol (25). Isolated high molecular weight DNA (20 μg) was digested to completion with *Bam*HI, *Hin*dIII, or *Eco*RI and subjected to electrophoresis at 15 mA on a 20-cm 1% agarose gel. The fractionated DNA was transferred to GeneScreen*Plus* (New England Nuclear) under alkaline conditions (26) and hybridized in 5× SSPE (1× SSPE is 180 mM NaCl, 10 mM sodium phosphate buffer at pH 7.0, and 10 mM EDTA) and 10% dextran sulfate to 5 × 10^6 cpm/ml of ^{32}P-labeled nick-translated probe at 68°C for 36 hr. The filters were washed at 68°C three times with 1.5× SSC containing 0.1% NaDodSO$_4$ for 20 min and once with 0.5× SSC containing 1% NaDodSO$_4$ for 45 min.

RESULTS AND DISCUSSION

Identification of P-450 3a Clones. A cDNA library constructed from rabbit liver poly(A)$^+$ RNA and cloned in pBR322 was screened with antibody monospecific for P-450 form 3a, and a single clone (p3a-2) that gave a strong signal on repeated rounds of screening was isolated. Preabsorption of the antibody to purified form 3a reduced the signal obtained with this clone to background levels (data not shown). To provide further evidence that the p3a-2 cDNA contained sequence encoding at least part of form 3a, rabbit liver poly(A)$^+$ RNA was hybridization-selected with the p3a-2 plasmid and the *in vitro* translation products were immunoprecipitated with monoclonal antibody that recognizes only form 3a (17). As shown in Fig. 1, from the

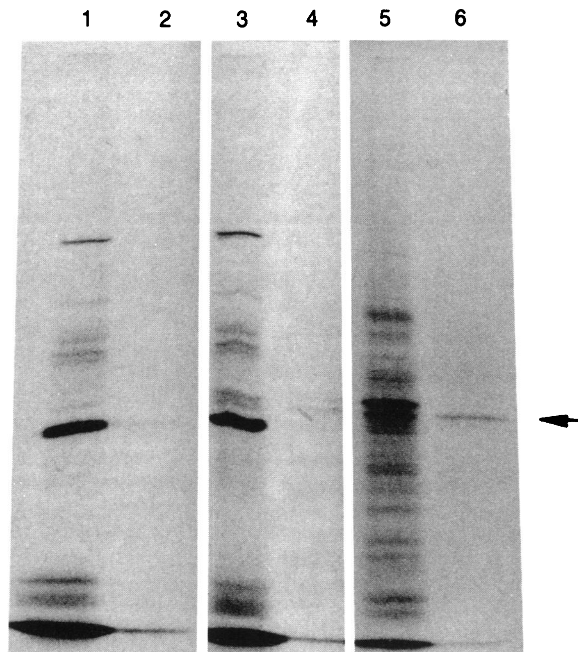

FIG. 1. Autoradiogram of products of hybridization-selected RNA with clone p3a-2. Products of *in vitro* translation were subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis followed by fluorography. Lane 1, no added RNA; lane 2, no added RNA, with products immunoprecipitated with monoclonal antibody to P-450 3a; lane 3, RNA selected by hybridization to p3a-2; lane 4, hybridization-selected RNA, with products immunoprecipitated with monoclonal antibody to P-450 3a; lane 5, total poly(A)$^+$ RNA from rabbit liver; lane 6, total poly(A)$^+$ RNA, with products immunoprecipitated with monoclonal antibody to P-450 3a. The arrow indicates the position of purified form 3a as determined by staining with Coomassie blue.

translation products of rabbit liver total poly(A)$^+$ RNA (lane 5), the antibody precipitated a polypeptide (lane 6) with the same electrophoretic mobility as authentic form 3a (arrow). Similarly, when the translation products of total poly(A)$^+$ RNA previously hybridization-selected with the p3a-2 plasmid (lane 3) were immunoprecipitated with the monoclonal antibody, a polypeptide with the same mobility as form 3a was obtained (lane 4). Although a control translation mixture without added RNA (lane 1) yielded products similar to those obtained with hybridization-selected RNA (lane 3), immunoprecipitation of these endogenous translation products (lane 2) did not yield a polypeptide with the same electrophoretic behavior as form 3a. These results demonstrate that p3a-2 selects an RNA species that encodes a protein immunochemically and electrophoretically indistinguishable from P-450 3a and indicate that the p3a-2 cDNA contains sequence complementary to form 3a mRNA.

The sequence of clone p3a-2 showed that it contained a

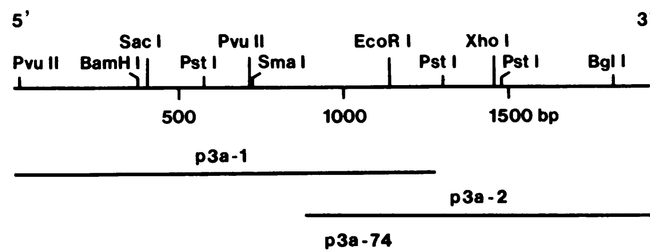

FIG. 2. Restriction map of cDNA clones encoding P-450 3a. Clone p3a-2 was identified by immunoscreening; clones p3a-1 and p3a-74 were subsequently identified by rescreening the library by hybridization with nick-translated p3a-2 restriction fragments.

1040-base-pair (bp) cDNA insert, which was smaller than the expected size needed to encode the entire P-450 3a polypeptide (1). To identify longer cDNAs, two restriction fragments isolated from p3a-2 were used to rescreen the cDNA library by DNA hybridization. From 10^4 recombinants approximately 40 positive clones were isolated; of these clones, 5 with large cDNA inserts were characterized by restriction mapping; these appeared to represent a single mRNA species. Clone p3a-74 contained the largest insert, while p3a-1 contained the greatest amount of 5′ sequence and shared a 390-bp overlap with p3a-2 (Fig. 2). The nucleotide sequences and derived amino acid sequences of both p3a-1 and p3a-2 were determined; the total sequence is shown in Fig. 3. Together these two cDNAs contain 1923 nucleotides, including a poly(A)$^+$ stretch of 24 nucleotides and an open reading frame that begins at position 1 and extends through position 1416. The first 25 amino acids of this reading frame correspond to residues 21–45 of P-450 3a determined by Edman degradation (ref. 1 and unpublished data). Thus, the complete polypeptide would contain 492 amino acids and have a molecular weight of 56,820. This is larger than the value of 51,000 estimated for form 3a by $NaDodSO_4$/polyacrylamide gel electrophoresis (1), but similar differences have been noted for other P-450 isozymes and are ascribed to unusually high binding of the detergent per unit weight of polypeptide in these hydrophobic proteins, resulting in greater mobility during electrophoresis (27).

Comparison of Form 3a to Other P-450 Isozymes. The amino

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                                                                                GTC TGG AAG CAG ATC CAC AGC AGC TGG AAC     30
Ala Val Leu Gly Ile Thr Val Ala Leu Leu Gly Trp Met Val Ile Leu Leu Phe Ile Ser Val Trp Lys Gln Ile His Ser Ser Trp Asn
                                    10                                      20                                      30

CTG CCC CCA GGA CCT TTC CCA CTG CCC ATC ATC GGG AAT CTT CTC CAG TTG GAT TTG AAG GAT ATT CCC AAG TCC TTT GGC AGG CTG GCA    120
Leu Pro Pro Gly Pro Phe Pro Leu Pro Ile Ile Gly Asn Leu Leu Gln Leu Asp Leu Lys Asp Ile Pro Lys Ser Phe Gly Arg Leu Ala
                                    40                                      50                                      60

GAG CGC TTT GGG CCG GTG TTC ACT GTG TAC CTG GGC TCC AGG CGT GTT GTG GTT CTG CAC GGC TAC AAG GCG GTG AGG GAG ATG CTG TTG    210
Glu Arg Phe Gly Pro Val Phe Thr Val Tyr Leu Gly Ser Arg Arg Val Val Val Leu His Gly Tyr Lys Ala Val Arg Glu Met Leu Leu
                                    70                                      80                                      90

AAC CAC AAG AAC GAG TTC TCT GGG CGT GGC GAG ATC CCT GCT TTC CGG GAG TTT AAG GAC AAG GGG ATC ATT TTC AAC AAT GGA CCC ACC    300
Asn His Lys Asn Glu Phe Ser Gly Arg Gly Glu Ile Pro Ala Phe Arg Glu Phe Lys Asp Lys Gly Ile Ile Phe Asn Asn Gly Pro Thr
                                    100                                     110                                     120

TGG AAG GAC ACT CGG CGG TTC TCC CTG ACC ACC CTC CGG GAC TAT GGG ATG GGG AAA CAG GGC AAC GAG GAC CGG ATC CAG AAG GAG GCC    390
Trp Lys Asp Thr Arg Arg Phe Ser Leu Thr Thr Leu Arg Asp Tyr Gly Met Gly Lys Gln Gly Asn Glu Asp Arg Ile Gln Lys Glu Ala
                                    130                                     140                                     150

CAC TTC CTG CTG GAG GAG CTC AGG AAG ACC CAG GGC CAG CCC TTC GAC CCC ACC TTT GTC ATC GGC TGC ACA CCC TTC AAC GTC ATC GCC    480
His Phe Leu Leu Glu Glu Leu Arg Lys Thr Gln Gly Gln Pro Phe Asp Pro Thr Phe Val Ile Gly Cys Thr Pro Phe Asn Val Ile Ala
                                    160                                     170                                     180

AAA ATC CTC TTC AAT GAC CGC TTT GAC TAT AAG GAC AAG CAG GCT CTG AGG CTG ATG AGT TTG TTC AAC GAG AAC TTC TAC CTG CTC AGT    570
Lys Ile Leu Phe Asn Asp Arg Phe Asp Tyr Lys Asp Lys Gln Ala Leu Arg Leu Met Ser Leu Phe Asn Glu Asn Phe Tyr Leu Leu Ser
                                    190                                     200                                     210

ACT CCT TGG CTG CAG GTT TAC AAT AAT TTT TCA AAC TAT CTA CAG TAC ATG CCT GGA AGT CAC AGG AAA GTA ATA AAA AAT GTG TCT GAA    660
Thr Pro Trp Leu Gln Val Tyr Asn Asn Phe Ser Asn Tyr Leu Gln Tyr Met Pro Gly Ser His Arg Lys Val Ile Lys Asn Val Ser Glu
                                    220                                     230                                     240

ATA AAA GAG TAC ACA CTC GCA AGA GTG AAG GAG CAC CAC AAG TCG CTG GAC CCC AGC TGC CCC CGG GAC TTC ATT GAC AGC CTG CTC ATA    750
Ile Lys Glu Tyr Thr Leu Ala Arg Val Lys Glu His His Lys Ser Leu Asp Pro Ser Cys Pro Arg Asp Phe Ile Asp Ser Leu Leu Ile
                                    250                                     260                                     270

GAA ATG GAG AAG GAC AAA CAC AGC ACG GAG CCC CTG TAC ACG CTG GAA AAC ATT GCT GTG ACT GTG GCG GAC ATG TTC TTT GCG GGC ACG    840
Glu Met Glu Lys Asp Lys His Ser Thr Glu Pro Leu Tyr Thr Leu Glu Asn Ile Ala Val Thr Val Ala Asp Met Phe Phe Ala Gly Thr
                                    280                                     290                                     300

GAG ACC ACC AGC ACC ACG CTG CGA TAT GGG CTC CTG ATC CTG CTG AAG CAC CCC GAG ATC GAA GAG AAA CTT CAT GAA GAA ATC GAC AGG    930
Glu Thr Thr Ser Thr Thr Leu Arg Tyr Gly Leu Leu Ile Leu Leu Lys His Pro Glu Ile Glu Glu Lys Leu His Glu Glu Ile Asp Arg
                                    310                                     320                                     330

GTG ATT GGG CCG AGC CGA ATG CCT TCT GTC AGG GAC AGG GTG CAG ATG CCC TAC ATG GAC GCT GTG GTA CAT GAG ATT CAG CGA TTC ATC   1020
Val Ile Gly Pro Ser Arg Met Pro Ser Val Arg Asp Arg Val Gln Met Pro Tyr Met Asp Ala Val Val His Glu Ile Gln Arg Phe Ile
                                    340                                     350                                     360

GAT CTC GTG CCC TCC AAT CTG CCG CAC GAA GCC ACA CGG GAC ACC ACC TTC CAA GGA TAC GTC ATC CCC AAG GGC ACT GTT GTA ATC CCG   1110
Asp Leu Val Pro Ser Asn Leu Pro His Glu Ala Thr Arg Asp Thr Thr Phe Gln Gly Tyr Val Ile Pro Lys Gly Thr Val Val Ile Pro
                                    370                                     380                                     390

ACT CTG GAC TCC CTT TTG TAT GAC AAG CAA GAA TTC CCT GAT CCC GAG AAG TTC AAA CCA GAG CAC TTT CTG AAT GAG GAG GGG AAG TTC   1200
Thr Leu Asp Ser Leu Leu Tyr Asp Lys Gln Glu Phe Pro Asp Pro Glu Lys Phe Lys Pro Glu His Phe Leu Asn Glu Glu Gly Lys Phe
                                    400                                     410                                     420

AAG TAT AGC GAC TAC TTC AAG CCG TTT TCC GCA GGA AAA CGC GTG TGT GTT GGA GAA GGC CTG GCT CGC ATG GAG TTG TTT CTG CTC CTG   1290
Lys Tyr Ser Asp Tyr Phe Lys Pro Phe Ser Ala Gly Lys Arg Val Cys Val Gly Glu Gly Leu Ala Arg Met Glu Leu Phe Leu Leu Leu
                                    430                                     440                                     450

TCT GCC ATT CTG CAG CAT TTT AAC CTC AAG CCT CTC GTT GAC CCA GAG GAC ATT GAC CTT CGC AAT ATT ACG GTG GGC TTT GGC CGT GTC   1380
Ser Ala Ile Leu Gln His Phe Asn Leu Lys Pro Leu Val Asp Pro Glu Asp Ile Asp Leu Arg Asn Ile The Val Gly Phe Gly Arg Val
                                    460                                     470                                     480

CCA CCA CGC TAC AAA CTC TGT GTC ATT CCC CGC TCG TAA ACCCAAGGGCAGCACCCAGAGGCCACTCTTCTCCTCGAGTGCCCCTGGGGAGGCTCTGCCTGCAGCC   1486
Pro Pro Arg Tyr Lys Leu Cys Val Ile Pro Arg Ser End
                                    490     492

CTGATGCCCTCCACCTTTGGGTGCCACCCCTGGCCCACTCTGGGATCATTGTTGAGATGAAGATAGGTCTGAGAAGGCAGCACACCCGTCTGCTCTTCTCTTAGCGTCAGGACACGGAG   1605

AGCCCCAGCTTATATGGAGCAAGGGTAAAATCCTTCCAGTGAACTTAGCATTAATGTTGCAAAACAGGAGTTTGTTGCAAGACTCCTGTCCCAGTGCGGCCAGCCCTCAGTGGGCTCAG   1724

TGAGCGCTGTCTCTGTGCCTAGCCTCCCCTTGCACCGTGGAGACGATGCAGCCTGTCCACCGTGTCTGCATGAGGGCCGAGTTTGCCCGGCCTGCATGGCCTTTGGAACATTCACAGCA   1843

CAGGGTCACCCTGTGTCCATGTTGCCTGGTAAAATAAACAGTAACCTTTCAAGCCCAAAAAAAAAAAAAAAAAAAAAAAA                                          1923
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FIG. 3. Nucleotide sequence encoding P-450 3a with derived amino acid sequence. The NH_2-terminal sequence, determined by the Edman method, was previously reported through residue 25 (1) and in the present work has been extended through residue 45. Nucleotides are numbered to the right of each line and amino acids are numbered below the corresponding residues.

acid composition of form 3a is similar to that of other sequenced P-450 isozymes: 38% hydrophobic (Phe, Ile, Leu, Met, Val, Trp, and Tyr), 13% basic (Arg and Lys), and 12% acidic. The protein contains five Trp and four Cys residues, but, like P-450scc (28) and P-450pcn (29), lacks the generally conserved cysteine located in the NH_2-terminal region, as found at position 152 of rabbit form 2 (30). Several regions are strongly conserved between form 3a and all other sequenced P-450s, including the region in form 3a surrounding the likely fifth heme ligand, Cys-436, and the proline cluster at residues 32–39, whose function remains to be established (27). Isozyme 3a shows 57% sequence identity with rabbit form 1 (13), 55% with form 3b (14), and 48% with form 2 (30). Forms 1 and 3b are progesterone hydroxylases constitutively expressed in the liver, and they share 70% sequence identity. Form 2 is a phenobarbital-inducible liver isozyme and is 50% identical to forms 1 and 3b. Thus, while all four isozymes may be considered members of the same gene family (having approximately 50% or greater sequence identity), forms 1 and 3b may be considered members of the same subfamily (having 70% or greater sequence identity), while forms 3a and 2 each belong to separate subfamilies. Isozyme 3a exhibits less than 30% sequence identity with pregnenolone-16α-carbonitrile-inducible rat P-450 (29), with isosafrole-inducible rabbit isozyme 4 and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-inducible rabbit isozyme 6 (31), and with mitochondrial P-450scc (28).

RNA Hybridization Analysis. Hybridization of p3a-2 to electrophoretically fractionated rabbit liver poly(A)$^+$ RNA revealed a multiple banding pattern (Fig. 4*A*). To discriminate between differential processing of the 3a gene transcript (i.e., alternate splicing or polyadenylylation) and hybridization to mRNAs for closely related P-450s, a duplicate filter was hybridized with the 3′ *Pst* I restriction fragment (nucleotides 1480–1923) of p3a-2. This fragment contains only 3′ nontranslated sequence and thus should show greater specificity for form 3a mRNA than for other P-450 mRNAs. As shown in Fig. 4*B*, a single band, approximately 1.9 kilobases (kb), was observed with this probe; presumably this corresponds to the P-450 3a mRNA. The sequence GGTAAA, which serves as a polyadenylylation signal in the rat P-450b and -e transcripts (32), is present at position 1629 of the form 3a nontranslated region, but evidently it is not used as a polyadenylylation signal in this transcript, as a correspondingly shortened RNA is not detected with the 3′ probe. These results indicate that the lower molecular weight RNAs evident in Fig. 4*A* probably encode other P-450s homologous to form 3a, rather than alternately processed 3a transcripts.

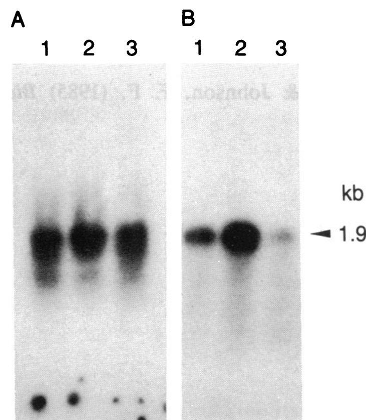

FIG. 4. Hybridization of rabbit liver mRNA complementary to p3a-2. Poly(A)$^+$ RNA (2 μg) from untreated animals or animals treated with imidazole or isosafrole was fractionated by electrophoresis, transferred to nylon filters, and hybridized with p3a-2 (*A*) or the 3′ nontranslated *Pst* I fragment of p3a-2 (*B*). In both *A* and *B* the RNAs are as follows: lane 1, untreated animals; lane 2, imidazole-treated animals; and lane 3, isosafrole-treated animals.

Treatment of rabbits with imidazole increases the content of liver P-450 3a protein 4.5-fold over that in untreated animals (4). To determine if this is due to increased levels of form 3a message, RNA was prepared from rabbits at various times after treatment with imidazole and hybridized with the 3′ probe that is specific for form 3a message. The results of one such experiment are illustrated in Fig. 4*B*; the differences in RNA levels are less pronounced when the p3a-2 cDNA is used (Fig. 4*A*), probably because the coding portion of the cDNA hybridizes to other P-450 mRNAs. A 2-fold elevation of 3a mRNA was found 3 hr after treatment with imidazole (Fig. 4*B*, lane 2), after which levels rapidly declined to those of untreated controls (data not shown). To ensure that this brief increase was not a nonspecific result of drug treatment, RNA was prepared from animals 3 hr after treatment with isosafrole, which does not affect P-450 3a enzyme levels (1). No significant difference in form 3a mRNA levels was found between untreated and isosafrole-treated animals (cf. lanes 1 and 3 in Fig. 4*B*), indicating that the increase after imidazole treatment was not an artifact of drug treatment. It is unlikely, however, that this modest and short-lived increase in form 3a message after imidazole treatment is sufficient to account for the increase in form 3a protein; this induction more likely results from either enhanced translational efficiency or decreased enzyme turnover. Whether other inducers of form 3a, including ethanol, act nontranscriptionally remains to be determined.

Genomic DNA Hybridization Analysis. To examine the genomic hybridization pattern associated with the P-450 3a gene, electrophoretically fractionated rabbit liver DNA was hybridized to p3a-74 (Fig. 5*A*) or to the 3′ nontranslated *Pst* I fragment from p3a-2 (Fig. 5*B*). The stringency of hybridization in these experiments was such that only sequences with greater than 80% identity would hybridize. With the p3a-74 probe three bands representing a total of at least 18 kb of DNA hybridized strongly in each digest (Fig. 5*A*), and an additional 7 kb in each digest hybridized weakly. With the 3′ probe a single strongly hybridizing band in the range between

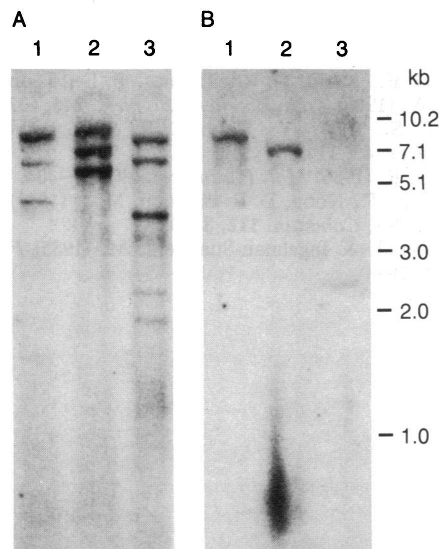

FIG. 5. Hybridization of genomic DNA to the P-450 3a cDNA. Restriction-endonuclease-digested genomic DNA was fractionated by electrophoresis, transferred to nylon filters, and hybridized with p3a-74 (*A*) or an M13 clone containing the 3′ nontranslated *Pst* I fragment of p3a-2 (*B*). In each panel the DNA was digested with *Bam*HI (lane 1), *Hin*dIII (lane 2), or *Eco*RI (lane 3). The positions of selected fragments of the 1-kb ladder from Bethesda Research Laboratories are indicated.

2.3 and 8 kb was present in each digest (Fig. 5*B*), and additional faint bands were present in the *Bam*HI and *Hin*dIII digests (Fig. 5*B*, lanes 1 and 2). The presence of both strongly and weakly hybridizing sequences in these digests when probed with either p3a-74 or the 3′ nontranslated fragment suggests that the rabbit genome may contain several P-450 genes that hybridize to P-450 3a cDNA sequences under conditions of high stringency; the presence of multiple RNA species that hybridize to p3a-2 (Fig. 4*A*) supports this view. The nature of these other putative P-450 3a-like genes is presently unclear. Of the P-450s sequenced to date, forms 1 and 3b are the most similar to form 3a (55% at the protein level), but the coding nucleotide sequences for the two isozymes are less than 65% identical to the coding nucleotide sequence for form 3a (13, 33). The high stringency of these genomic hybridizations makes it unlikely that the genes for these P-450s are hybridizing to the form 3a cDNA probes. Although the P-450 3b gene has not been characterized by hybridization analysis, evidence based on catalytic activities indicates that two distinct nonallomorphic forms of isozyme 3b are present in New Zealand White rabbits (34, 35). Furthermore, hybridization of a form 1 cDNA to genomic DNA revealed a multiplicity of hybridizing fragments, and it was estimated that a minimum of four genes encoding P-450 form 1-like proteins were present in the rabbit genome (13). It is possible that one or more of these genes may be similar enough to the form 3a gene to hybridize to our P-450 3a cDNA probes. A more complete characterization of this diverse P-450 gene family is clearly needed.

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