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Primary Structures of Multiple Forms of Cytochrome P-450 Isozyme 2 Derived from Rabbit Pulmonary and Hepatic cDNAS

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SUMMARY

Rabbit pulmonary and hepatic mRNA was used to construct cDNA libraries that were screened with a cDNA probe (pf 3/46) to murine cytochrome P-450 homologous with rat cytochrome P-450b. Three types of cDNA clones were identified on the basis of restriction analysis with Bst EII. Two types of clones (B0 and B1) were present in a library constructed from pulmonary mRNA. The nucleotide sequence of B0 cDNA encodes a protein of 491 amino acids having a sequence identical to one reported for isozyme 2. The sequence derived from the b1 cDNA also contains 491 amino acids but differs from the B0 sequence at six positions. B1 clones were also obtained from a library constructed from hepatic mRNA isolated 12 hr after a single treatment with phenobarbital. A third type of clone (B2) was also obtained from this library, but no B0 clones were found. The sequence derived from the B2 cDNA contains 491 amino acids and differs from the B0 and B1 sequences at 11 and 15 positions, respectively. A second hepatic cDNA library, constructed from mRNA from the liver of a rabbit treated with phenobarbital daily for 4 days, contained B0 clones. The sequence of one of these was found to be identical to that of the pulmonary B0 clones.

The cytochrome P-450 monooxygenase system (P-450 system) of rabbit lung is capable of metabolizing a wide variety of exogenous substrates, including a number that exhibit pulmonary-selective toxic effects (1). It is reasonably clear that the toxic effects of these compounds are a consequence of cytochrome P-450-catalyzed formation of reactive metabolites, but the role metabolism plays in the determination of selectivity is not well understood. In the case of 4-ipomeanol, however, evidence for a direct relationship between metabolism and selectivity does exist. The toxicity of 4-ipomeanol, which is directed primarily towards a single type of pneumocyte (2), appears to stem from the relatively high concentration of two specific isozymes of cytochrome P-450 in the target cells (3–5).

Several of the unanswered questions about the pulmonary P-450 system concern cytochrome P-450 isozyme 2. This isozyme, which comprises about 50% of the total pulmonary cytochrome P-450, has been purified and its properties have been compared with those of isozyme 2 from liver. On the basis of immunochemical (6, 7), catalytic (6), structural (7, 8), and spectroscopic characteristics (9), the isozymes from the two tissues appear to be identical. However, there are several differences between liver and lung with respect to the regulation and properties of isozyme 2. First, the microsomal concentration of isozyme 2 in lung, particularly in the Clara cell, is higher than in liver (5). Second, the hepatic (10) but not the pulmonary (11) concentration of this isozyme increases following treatment of rabbits with PB. Third, purified hepatic isozyme 2 can be separated into three fractions by chromatography on DEAE-cellulose, a behavior not observed with isozyme 2 from lung (7). These findings indicate that the regulation of isozyme 2 in liver and lung differs, that the isozymes from the two tissues may not be identical, and that multiple forms of isozyme 2 may be present in liver. The latter possibility is consistent with the different amino acid sequences that have been reported for isozyme 2 (12, 13), and with the presence of two homologs of isozyme 2 (P-450b and P-450e) induced by PB in rat liver (14).

As a first step in establishing the identities of rabbit pulmonary and hepatic isozyme 2, we have characterized cDNA clones derived from the mRNA of both tissues. The results show that lung contains a minimum of two populations of mRNA encoding different forms of isozyme 2 and that the liver contains at least three, two of which are apparently identical to those present in lung.

Materials and Methods

Animals. Adult, male New Zealand White rabbits (Dutchland Farms, Denver, PA) were used for all experiments.

Isolation of RNA for construction of cDNA libraries. Lungs from 10 rabbits were pooled for the isolation of RNA for the construc-

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ABBREVIATIONS: P-450 system, cytochrome P-450 monooxygenase system; PB, phenobarbital; EDTA, ethylenediaminetetraacetate; SSC, sodium chloride/sodium citrate concentration: 1× = 0.15 M sodium chloride, 15 mM sodium citrate; kb, kilobase; DBM, diazobenzyloxymethyl.

tion of a cDNA library (Lg library). RNA was obtained from a polysomal preparation by the method of Gough and Adams (15), and polyadenylated mRNA was purified by chromatography on oligo(dT)-cellulose (16). Livers from individual rabbits were used as the source of mRNA for the construction of hepatic libraries (Lv-1 and Lv-4). The mRNA for the Lv-1 library was isolated by extraction with guanidine hydrochloride and precipitation with ethanol (17) from the liver of a rabbit treated once with PB (60 mg/kg) for 12 hr; mRNA for the Lv-4 library was isolated by the method described below from the liver of a rabbit treated daily with PB (60 mg/kg) for 4 days.

Isolation of RNA for hybridization (Northern) analysis. Pulmonary mRNA isolated by the methods noted above gave unsatisfactory results when analyzed by hybridization with cDNA for isozyme 2 (see Results). However, we were able to obtain intact pulmonary mRNA by a modification of the methods reported by Chirgwin *et al.* (18) and Glisin *et al.* (19). Lungs were removed from the rabbits as quickly as possible, frozen by submersion in liquid nitrogen, and stored at −70°. The frozen tissue (10 g) was crushed and then powdered with a mortar and pestle cooled with liquid nitrogen. The powdered tissue was quickly added to a solution (150 ml, room temperature) of Tris-HCl buffer (100 mM, pH 6.5), guanidine isothiocyanate (4 M), sodium sarcosinate (2%), and β-mercaptoethanol (100 mM) and homogenized immediately for 1 min with a Polytron (Brinkmann Instruments, Westbury, NY) at the highest setting. The homogenate was centrifuged at 10,000 × *g* for 10 min at −10°, and CsCl (1 g/2.5 ml) was added to the supernatant fraction after it was filtered through cheesecloth. The sample was then layered on a cushion of CsCl (5.7 M; 0.25 ml/ml sample) and centrifuged in a swinging bucket rotor (SW28, Beckman Instruments, Palo Alto, CA) at 25,000 rpm for 20–24 hr. Following centrifugation the supernatant fraction was removed by aspiration and the insides of the tubes were wiped clean without disturbing the pellets. The RNA was dissolved in homogenization buffer (1.5 ml/g of tissue) adjusted to pH 7.0 and containing EDTA (20 mM), gently shaken for 2 hr, and then centrifuged to remove any insoluble material. Dissolved RNA was then precipitated overnight at −20° by the addition of sodium acetate (3 M, pH 5.5; 0.6 ml/ml sample) and ethanol (2.2 ml/ml sample). The precipitate was collected by centrifugation, washed sequentially with 70% and absolute ethanol, and dried in a vacuum chamber prior to isolation of mRNA by chromatography on oligo(dT)-cellulose (16). Complete separation of polyadenylated mRNA from the sample requires that the chromatography step be repeated. Preparations of pulmonary and hepatic mRNA used for hybridization experiments, and hepatic mRNA for the Lv-4 library, were obtained by this procedure.

Cloning of cDNA derived from pulmonary and hepatic mRNA. Double-stranded cDNA was synthesized from mRNA by sequential incubation with avian myeloblastosis virus-reverse transcriptase and DNA polymerase I (20, 21), coupled to synthetic *Eco R1* linkers (22), size fractionated on Sepharose 4B to remove species <1500 bases in length, and ligated into λgt11 (23, 24). About 6 × 10^6 recombinant clones were obtained per μg of cDNA. The Lv-1 library was screened on nitrocellulose by plaque hybridization (25) with nick-translated murine cDNA (pf 3/46) that encodes an isozyme of cytochrome P-450 homologous to rat P-450b.[1] Hybridization was allowed to proceed for 12–16 hr at 37° in a solution containing formamide (50%), SSC (5×), Denhardt's solution (1×), SDS (0.1%), and denatured salmon sperm DNA (0.1 mg/ml), after which the nitrocellulose was washed at room temperature with a solution of SSD (2×) and SDS (0.1%). Positive clones were purified and λDNA isolated as described by Yamamoto *et al.*, (26) and Vande Woude *et al.*, (27). Clone Lv-1:B2-1 (see Results), which contained a single 2kb *Eco R1* insert, was labeled by nick-translation with ^{32}P-dCTP (Amersham, Arlington Heights, IL) and used as a probe for further screening of the Lv-1 library and for screening of the Lg and Lv-4 libraries. The temperature for hybridization with Lv-1:B2-1 and subsequent washing was 42°. The solutions used were the same as with the murine cDNA probe except that the concentration of SSC in the wash was lowered to 0.1×. Clones of interest were restricted with *Eco R1*; then, the cDNA inserts were isolated by chromatography in low melting point agarose (28), purified on Elutip-d (Schleicher and Schuell, Keene, NH) columns (28), and subcloned (22) into plasmid pGEM-2 (Promega Biotech, Madison, WI).

Nucleotide sequence analysis. Libraries of cDNA inserts were prepared by subcloning various restriction fragments into M13 (mp 18 replicative form) and transformed into *Escherichia coli* JM103 (29, 30). Plaques containing inserts were amplified in liquid culture and assessed for size by gel electrophoresis. In the absence of suitable restriction sites, single strand M13 recombinants were annealed to a synthetic primer, restricted with *Hind III*, and treated with T4 DNA polymerase in order to generate sequential deletions (31). Sequences were determined by the dideoxy chain termination method (32, 33) for which the 17-base universal primer was used. The data were analyzed by standard computer programs (34–37).

Analysis of mRNA. Preparations of mRNA (polyadenylated) were electrophoresed for 4–6 hr at 60 V in agarose (1%) containing methylmercury (5 mM) (38). The gels were then treated with NaOH (0.5 N), neutralized with potassium phosphate buffer (0.2 M, pH 6.5), and equilibrated with sodium acetate (1 M, pH 4.0) prior to overnight transfer of the mRNA to DBM paper (39). Next the DBM paper was treated for 4–6 hr at 42° with a solution of SSC (5×), Denhardt's solution (5×), sodium phosphate buffer (50 mM, pH 6.5), denatured salmon sperm DNA (0.25 mg/ml), glycine (10 mg/ml), dextran sulfate (10%), and formamide (50%). The mRNA was then allowed to hybridize with nick-translated cDNA (Lg:B0-1; described under Results) at 42° for 16–22 hr in a solution of SSC (5×), Denhardt's solution (5×), sodium phosphate (20 mM, pH 6.5), denatured salmon sperm DNA (0.1 mg/ml), dextran sulfate (10%), and formamide (50%). Following hybridization, the DBM paper was washed several times at 42° with a solution of SSC (0.1×) and SDS (0.1%), dried, and subjected to autoradiography.

Materials. All reagents not specifically noted above were obtained at the highest purity possible from commercial sources.

Results

Analysis of cDNA clones derived from hepatic mRNA isolated 12 hr after treatment with PB. Because it has been demonstrated that a single treatment of rats with PB markedly increases mRNA for the homolog of rabbit isozyme 2 (40), mRNA from a rabbit treated in a similar manner was used for the synthesis of cDNA. Recombinant clones (approximately 100,000) in this library (Lv-1) were screened with murine cDNA (pf 3/46) under conditions of stringency suitable for the approximately 70% homology. Sixty-nine positive clones were isolated and one of approximately 2 kb was subcloned into a plasmid vector (pGEM-2) for use as a cDNA probe. Fifty-five of the clones identified with the murine cDNA hybridized with the rabbit cDNA when highly stringent conditions were used. DNA was then isolated from 49 clones that were selected arbitrarily from the 55 positives. Twenty-seven of the clones were chosen for further study on the basis of *Eco R1* restriction (one fragment) and size (>1.4 kb). The remaining clones included four that produced no *Eco R1* fragments, three that produced two *Eco R1* fragments, and 15 that were less than 1.4 kb in length. The largest clone selected for further study was found to contain a total of 2077 bases, an open reading frame of 1473 bases, a 3'-flanking region of 572 bases, and a 5'-flanking region of 32 bases. A polyadenylation signal (AGATAAA) was found 21 bases from the start of the polyadenylation tract. (The sequence of this clone, Lv-1:B2-1, can be found in Table 1 under the heading "B2"; the strategy used is shown in Fig. 1.) The number of amino acids (491) encoded by the

[1] M. Noshiro and M. Negishi, manuscript in preparation.

TABLE 1

Nucleotide sequences of cDNAs encoding for B0, B1, and B2 variants of cytochrome P-450 isozyme 2, and amino acid sequence derived for variant B0[a]

```
B0                                        5′ END       -32          (N)n-CTGACGGCCGGGACC   -1
B1                                                        (N)n-GAGGTGGACAG
B2                                                  (N)n-TGGAAG            C   A

B0    1 MET GLU PHE SER LEU LEU LEU LEU LEU ALA PHE LEU ALA GLY LEU LEU LEU LEU LEU PHE   20
B0    1 ATG GAG TTC AGC CTG CTC CTC CTC CTG GCT TTC CTC GCA GGC CTC CTG CTG CTT CTG TTC   60
B1
B2

B0   21 ARG GLY HIS PRO LYS ALA HIS GLY ARG LEU PRO PRO GLY PRO SER PRO LEU PRO VAL LEU   40
B0   61 AGG GGC CAC CCC AAG GCC CAC GGC CGC CTC CCC CCG GGA CCC TCC CCT CTG CCC GTC CTG  120
B1                                           T       C                       +A+
B2                                         T   T       C        +C+

B0   41 GLY ASN LEU LEU GLN MET ASP ARG LYS GLY LEU LEU ARG SER PHE LEU ARG LEU ARG GLU   60
B0  121 GGG AAC CTT CTG CAG ATG GAC AGG AAG GGC CTG CTC CGC TCC TTC CTG CGG CTC CGA GAG  180
B1                                                                 +A+
B2                    A

B0   61 LYS TYR GLY ASP VAL PHE THR VAL TYR LEU GLY SER ARG PRO VAL VAL VAL LEU CYS GLY   80
B0  181 AAA TAC GGG GAC GTG TTC ACG GTG TAC CTG GGA TCC AGA CCC GTG GTC GTG CTG TGT GGG  240
B1
B2

B0   81 THR ASP ALA ILE ARG GLU ALA LEU VAL ASP GLN ALA GLU ALA PHE SER GLY ARG GLY LYS  100
B0  241 ACG GAT GCC ATC CGC GAG GCC CTC GTG GAC CAA GCG GAG GCC TTT TCT GGC AGG GGG AAG  300
B1                                                 A
B2        A                                        T

B0  101 ILE ALA VAL VAL ASP PRO ILE PHE GLN GLY TYR GLY VAL ILE PHE ALA ASN GLY GLU ARG  120
B0  301 ATC GCC GTG GTG GAT CCG ATC TTC CAG GGA TAC GGA GTG ATC TTT GCC AAC GGG GAG CGC  360
B1
B2                                                    +T+                    +A+

B0  121 TRP ARG ALA LEU ARG ARG PHE SER LEU ALA THR MET ARG ASP PHE GLY MET GLY LYS ARG  140
B0  361 TGG CGG GCC CTT CGG AGA TTC TCC CTG GCC ACC ATG CGG GAC TTC GGC ATG GGG AAG CGG  420
B1
B2

B0  141 SER VAL GLU GLU ARG ILE GLN GLU GLU ALA ARG CYS LEU VAL GLU GLU LEU ARG LYS SER  160
B0  421 AGC GTG GAG GAG CGC ATT CAG GAG GAG GCC CGG TGT CTG GTG GAG GAG CTG CGG AAA TCC  480
B1
B2

B0  161 LYS GLY ALA LEU LEU ASP ASN THR LEU LEU PHE HIS SER ILE THR SER ASN ILE ILE CYS  180
B0  481 AAG GGA GCC CTC CTG GAC AAC ACC TTG CTG TTC CAC TCA ATC ACC TCC AAC ATC ATC TGC  540
B1                                                    G+G+
B2                                                    G+G+

B0  181 SER ILE VAL PHE GLY LYS ARG PHE ASP TYR LYS ASP PRO VAL PHE LEU ARG LEU LEU ASP  200
B0  541 TCC ATT GTC TTT GGA AAA CGC TTT GAC TAC AAG GAC CCC GTG TTC CTG CGG CTG CTG GAC  600
B1                                  C                                 A
B2                                                                    A
```

[a] The complete nucleotide and derived amino acid sequences for B0 are shown. For B1 and B2, nucleotides are shown only at positions where they differ from those in the B0 sequence. Nucleotides marked by plus signs (+N+) indicate changes in the amino acid encoded for (see Table 2 for amino acid differences). Positions marked with a dash (-) indicate apparent deletions. Nucleotides in the 5′-flanking region have been assigned negative numbers and those in the coding and 3′-flanking regions positive numbers.

open reading frame of Lv-1:B2-1 was the same as that reported for isozyme 2 (13), but the derived sequence differed at 11 positions from the sequence reported by Tarr *et al.* (13) and at 16 positions from the sequence reported by Heinemann and Ozols (12). (Amino acid differences for all sequences reported are shown in Table 2.) Extensive restriction analysis (Fig. 2) showed that two of these differences, at amino acids 174 and 370, coincided with the only *Bst EII* restriction sites present (Table 3). Because the *Bst EII* site associated with residue 174 would not exist in the nucleotide sequence corresponding to the amino acid sequences reported for the protein (12, 13), and the site associated with residue 370 would be questionable (Table 3), we analyzed the remaining 26 positive clones from the Lv-1 library by restriction with *Bst EII*. Seventeen of the clones contained two *Bst EII* sites (B2 clones). Digestion of the B2 clones with additional restriction enzymes produced patterns that were identical to those formed from the B2 clone (Lv-1:B2-1) that had been sequenced. The remaining 9 clones each contained a single *Bst EII* restriction site (B1 clones). The B1 clones, which behaved uniformly with a number of restriction enzymes, differed from the B2 clones in response to several enzymes in addition to *Bst EII* (Fig. 2).

Analysis of clone Lv-1:B1-1 showed that the *Bst EII* site present was associated with residue 174, and that the derived

TABLE 1—*Continued*

```
B0   201 LEU PHE PHE GLN SER PHE SER LEU ILE SER SER PHE SER SER GLN VAL PHE GLU LEU PHE  220
B0   601 TTG TTC TTC CAG TCC TTC TCC CTC ATC AGC TCC TTC TCC AGC CAG GTG TTC GAG CTC TTC  660
B1       C                                                             T
B2       C

B0   221 PRO GLY PHE LEU LYS HIS PHE PRO GLY THR HIS ARG GLN ILE TYR ARG ASN LEU GLN GLU  240
B0   661 CCG GGC TTC CTA AAG CAC TTT CCT GGC ACG CAC AGG CAG ATC TAC AGG AAC CTG CAG GAG  720
B1
B2                                     C       A

B0   241 ILE ASN THR PHE ILE GLY GLN SER VAL GLU LYS HIS ARG ALA THR LEU ASP PRO SER ASN  260
B0   721 ATC AAC ACT TTC ATC GGC CAG AGC GTA GAG AAG CAC CGC GCA ACC CTG GAC CCC AGC AAC  780
B1
B2                                   +C+

B0   261 PRO ARG ASP PHE ILE ASP VAL TYR LEU LEU ARG MET GLU LYS ASP LYS SER ASP PRO SER  280
B0   781 CCC AGG GAT TTC ATC GAC GTC TAC CTG CTC CGC ATG GAA AAA GAC AAG TCC GAC CCA AGC  840
B1
B2                                                             T               G

B0   281 SER GLU PHE HIS HIS GLN ASN LEU ILE LEU THR VAL LEU SER LEU PHE PHE ALA GLY THR  300
B0   841 AGC GAG TTC CAC CAC CAG AAC CTC ATC CTC ACC GTG CTC TCG CTC TTC TTC GCC GGC ACC  900
B1                                     +A+
B2                           +G+                       +A+

B0   301 GLU THR THR SER THR THR LEU ARG TYR GLY PHE LEU LEU MET LEU LYS TYR PRO HIS VAL  320
B0   901 GAG ACC ACC AGC ACC ACC CTC CGC TAC GGC TTC CTG CTC ATG CTC AAG TAC CCA CAC GTC  960
B1                                                 +C+
B2

B0   321 THR GLU ARG VAL GLN LYS GLU ILE GLU GLN VAL ILE GLY SER HIS ARG PRO PRO ALA LEU  340
B0   961 ACA GAG AGA GTC CAG AAG GAG ATT GAG CAG GTG ATC GGC TCC CAC CGC CCT CCG GCC CTC 1020
B1                                   C
B2                                   C

B0   341 ASP ASP ARG ALA LYS MET PRO TYR THR ASP ALA VAL ILE HIS GLU ILE GLN ARG LEU GLY  360
B0  1021 GAT GAC CGA GCC AAA ATG CCC TAC ACG GAC GCG GTC ATC CAC GAG ATC CAG CGG CTC GGG 1080
B1
B2

B0   361 ASP LEU ILE PRO PHE GLY VAL PRO HIS THR VAL THR LYS ASP THR GLN PHE ARG GLY TYR  380
B0  1081 GAC CTC ATC CCC TTC GGG GTG CCC CAC ACG GTC ACA AAA GAC ACA CAG TTC CGA GGC TAT 1140
B1                                                           G
B2            T+G+            T +C+         +T+       C           G

B0   381 VAL ILE PRO LYS ASN THR GLU VAL PHE PRO VAL LEU SER SER ALA LEU HIS ASP PRO ARG  400
B0  1141 GTC ATC CCC AAG AAC ACG GAA GTG TTC CCC GTC CTG AGC TCG GCT CTC CAT GAC CCG CGC 1200
B1
B2

B0   401 TYR PHE GLU THR PRO ASN THR PHE ASN PRO GLY HIS PHE LEU ASP ALA ASN GLY ALA LEU  420
B0  1201 TAC TTT GAA ACA CCG AAC ACC TTC AAC CCC GGC CAC TTT CTG GAT GCC AAC GGG GCA CTG 1260
B1                   G           T                                                 C+A+
B2                   G                                                   +G+
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amino acid sequence must differ from those reported (12, 13), but might be the same as that derived from clone Lv-1:B2-1 (Table 3). However, complete analysis showed that the sequences of Lv-1:B1-1 and Lv-1:B2-1 differed at 82 positions (31 in the coding region) and encoded different amino acids at 15 positions (Tables 1 and 2). The sequence of Lv-1:B1-1 contains 2063 bases, an open reading frame of 1473 bases, a 3'-flanking region of 564 bases, and a 5'-flanking region of 26 bases. A polyadenylation signal (AGATAAA) is located 23 bases from the start of the poly-A tract. The 3′-flanking region has a deletion of 12 bases, with respect to the Lv-1:B2-1 sequence, starting 27 bases from the stop codon. Partial sequences (1850 and 720 bases) of two additional clones, Lv-1:B1-2 and Lv-1:B2-2, showed 100% identity with Lv-1:B1-1 and Lv-1:B2-1, respectively (Fig. 1).

Analysis of cDNA clones derived from pulmonary mRNA. Pulmonary mRNA was purified from the lungs of 10 rabbits and used for the synthesis of cDNA and construction of a library (Lg library). The recombinants formed (approximately 30,000) were screened with hepatic cDNA (Lv-1:B2-1) and 22 positive clones that contained single *Eco R1* inserts were isolated. Restriction analysis of the eight inserts greater than 1.5 kb in length produced uniform results that differed in several respects from those obtained with either the B1 or B2 clones (Fig. 2). The absence of *Bst EII* restriction sites in these inserts (B0 clones) was particularly noteworthy. The sequence of one B0 clone (Lg:B0-1) contained 2044 bases, an open reading frame of 1473 bases, a 3'-flanking region of 556 bases, and a 5'-flanking region of 15 bases (Table 1). A putative polyadenylation signal (AAATAAA) was found 23 bases from the start of the polyadenylation tract. The base sequence of Lg:B0-1 differed from those of Lv-1:B1-1 and Lv-1:B2-1 at 56 positions (19 in the coding region) and 86 positions (31 in the coding region), respectively. Relative to the sequence for B2-1, the 3'-flanking region of Lg:B0-1 contained a deletion of 19 bases starting 18 bases from the stop codon (Table 1). The

TABLE 1—*Continued*

```
B0   421 LYS ARG ASN GLU GLY PHE MET PRO PHE SER LEU GLY LYS ARG ILE CYS LEU GLY GLU GLY  440
B0  1261 AAG AGG AAT GAA GGC TTT ATG CCC TTC TCC CTG GGG AAG CGC ATT TGT CTG GGC GAA GGC 1320
B1
B2                                        T

B0   441 ILE ALA ARG THR GLU LEU PHE LEU PHE PHE THR THR ILE LEU GLN ASN PHE SER ILE ALA  460
B0  1321 ATC GCG CGG ACC GAG CTG TTC CTC TTC TTC ACC ACC ATC CTG CAG AAC TTC TCC ATC GCC 1380
B1
B2

B0   461 SER PRO VAL PRO PRO GLU ASP ILE ASP LEU THR PRO ARG GLU SER GLY VAL GLY ASN VAL  480
B0  1381 AGC CCC GTG CCT CCC GAG GAC ATC GAC CTC ACT CCC CGG GAG AGT GGC GTG GGC AAC GTG 1440
B1
B2

B0   481 PRO PRO SER TYR GLN ILE ARG PHE LEU ALA ARG  491  *OP
B0  1441 CCC CCG AGC TAC CAG ATC CGC TTC CTG GCC CGC 1473  TGA  1476
B1
B2

B0  1477 AGGGGCCACAGGGACCCC------------------TGGTCATTAGGTGTCCCCGCCTCTGTAGAGAATGGCCCCAG 1535
B1  1477                  CCCAGCCCA------------   G                                    1542
B2  1477               A  CCCCCCCACCGCACCGCCC     G C                                  1554

B0  1536 ACTCCTCCCGCATCTTCCTGCCTCTCAGACCTGGGGCCAGCCGGGACCCTCCCCTTGGCCTCCTCGGCGTGGGGAGTC 1613
B1  1543                                        A                     T               1620
B2  1555         -                              A                  AA T               1631

B0  1614 CTCTTT-GTGGTCCCATCTCCTTCCACTAGGCTGTGGCTTTTCCAAAGACTCAGGGAACTGTTCTCTTCCTCTCCTCC 1690
B1  1621       -         C          G     C       G                              G    G  1697
B2  1632     G G                    T     C                                      G    G  1709

B0  1691 CTGCGTGTGGCACCAGCCCCAGCCGCTAGGGCAAGGATTATACCCAGTTTGCAGTTGAGGGACAGAGGCCAGGCAAGG 1768
B1  1698        C                                                                      1775
B2  1710        C     TG        T      T                                   G          1787

B0  1769 TGCCAGCAGTCTGAGATCACTGTGCCCCTGAGTGGAGGAGAGAGAGCCACGATGTCGC-ACAGCCCACTCTTTGCAGT 1845
B1  1776                                                        G  C                   1853
B2  1788                                                           -                   1864

B0  1846 CACGTAGTTAGGTTAAGATGCAGCCCACACAGCACGCACGAAGCTGTCCATTTAATGGGATGGTTTTGTGCCTTTTCG 1923
B1  1854   T                                        C          A    C      A         T 1931
B2  1865                                       T     C       C  A              A-       1941

B0  1924 CTCCTCCACAAGGTCCTCACAACCACAGGATCTGTGCCATTGCCCCAGGGGAAACCCCAAACCTACCAAGCTGTTGAT 2001
B1  1932                  T G                                 A          G    C          G    2009
B2  1942                  T                                                  C    CA         2019

B0  2002 GTCCCAAATAAACGGGGTCACACCTCTG(A)n  2029    3' END
B1  2010       G       T             (A)n  2037
B2  2020       G                   --(A)n  2045
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amino acid sequence (491 residues) derived from Lg:B0-1 differed from those of Lv-1:B1-1 and Lv-1:B2-1 at 6 and 11 positions, respectively, but was identical to the sequence reported by Tarr *et al.* (13) for the protein (Table 2). None of the differences among the sequences derived from the B0, B1, and B2 clones coincided with any of the 16 differences between the sequences reported by Tarr *et al.* (13) and Heinemann and Ozols (12) for isozyme 2 (Table 2).

Six clones from the pulmonary library with inserts less than 1.5 kb but greater than 1.0 kb in length were also analyzed by restriction with *Bst EII*. Although B2 clones of this size would be expected to contain one *Bst EII* site, none were found. We then used *Bgl I* restriction to determine if these cDNAs behaved in a manner consistent with that expected for either B0 or B1 inserts; three restriction sites would be present in B0 inserts of this size, whereas the B1 inserts would contain a single site. Restriction of four of these clones produced fragments consistent with B0 inserts; the patterns of the remaining two indicated the presence of B1-like inserts. Partial sequences (1091 and 1074 bases) of these B1 clones (Lg:B1-1 and Lg:B1-2) were identical to those of Lv-1:B1-1 and Lv-1:B1-2 (Fig. 1). In addition, the partial sequence (860 bases) of a second pulmonary B0 clone (Lg:B0-2) was found to be identical to that of Lg:B0-1 (Fig. 1).

Analysis of cDNA clones derived from hepatic mRNA following multiple treatments with PB. Because we were unable to obtain a B0 clone from the Lv-1 library, we constructed a second hepatic library (~30,000 recombinants). However, in this case the mRNA was isolated from the liver of a rabbit treated with PB daily for 4 days. Two clones that hybridized with Lv-1:B2-2 contained inserts of greater than 1500 bases, neither of which contained any *Bst EII* restriction sites. One of these (Lv-4:B0-1) was sequenced and found to be identical to Lg:B0-1 for the 1735 bases present (Fig. 1).

Detection of total pulmonary and hepatic mRNA for isozyme 2. Our initial attempts to isolate intact pulmonary

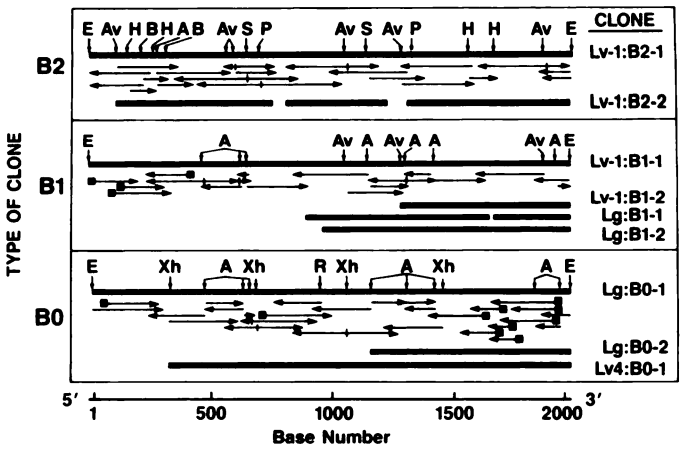

Fig. 1. Fragments used for the determination of the sequences of cDNAs encoding for different forms of cytochrome P-450 isozyme 2. At the top of each section (B2, B1, and B0) is a graphical representation of the clones (Lv-1:B2-1, Lv-1:B1-1, and Lg:B0-1) that were initially sequenced. The fragments from which the sequences were obtained are shown *below* each clone; the direction of sequencing is indicated by the *arrows*. Fragments were obtained by restriction (——) or by sequential deletion (——■). The restriction enzymes used were: *Eco R1* (E), *Ava II* (Av), *Hae III* (H), *Bam HI* (B), *Alu I* (A), *Sst I* (S), *Pst I* (P), *Rsa I* (R), and *Xho II* (Xh). Sequences obtained for other B2 (LV-1:B2-2), B1 (Lv-1:B1-2, Lg:B1-1, and Lg:B1-2), and B0 (Lg:B0-2 and Lv-4:B0-1) clones are depicted graphically at the *bottom* of each section.

mRNA in reasonable yields were unsuccessful. Recoveries of mRNA from polysomes isolated in the presence of RNase inhibitors (vanadyl-ribonucleotide complex, heparin, glutathione, β-mercaptoethanol, and cycloheximide) were consistently low (less than 5 μg of polyadenylated mRNA/g of tissue). RNA isolated by extraction with guanidine hydrochloride (8 M) and precipitation with ethanol was contaminated with a viscous, water-insoluble, material that could not be eliminated from the preparations. Again, low yields of mRNA were encountered. Marked degradation of mRNA was observed with both of these methods. Next, we tried a number of approaches, based on different combinations of steps used in published procedures (18, 19) and devised a suitable method. Several aspects of this procedure (see Materials and Methods) appear to be essential for the preparation of intact pulmonary mRNA. First, the lungs must be frozen in liquid nitrogen upon removal from the animal; all attempts to extract RNA from fresh tissue gave marginal results at best. Second, guanidine isothiocyanate in the homogenization buffer gives much better results than other guanidine compounds. Third, it is essential that sodium sarcosinate (2%) be included in the homogenization and extraction buffers. Fourth, the CsCl centrifugation step must be carried out prior to precipitation; insoluble contaminants and low yields were encountered when the order was reversed. Fifth, two passes through the oligo-dT column are required in order to obtain pure mRNA. Yields of 20–30 μg mRNA/g of lung, with little or no apparent degradation, are produced by this method. The advantage of this method is shown by the results of hybridization experiments carried out with pulmonary mRNA and cDNA (Lg:B0-1) to isozyme 2 (Fig. 3).

The extent of hybridization of Lg:B0-1 with pulmonary and hepatic mRNA as determined by blot (Northern) analysis is shown in Fig. 4, A and B. A single band (~2.4 kb) was observed with mRNA from either tissue. The extent of hybridization was greater with mRNA from the lung than from the liver of an untreated rabbit (Fig. 4A), a difference observed with three different animals. However, treatment of rabbits with PB markedly increased the content of hepatic mRNA related to isozyme 2; the increase 20 hr after a single treatment was greater than 12 hr after, but less than after treatment daily for 4 days (Fig. 4A). In contrast, treatment of rabbits with PB had no obvious effect on the amount of mRNA related to isozyme 2 in lung (Fig. 4B). Pulmonary RNA that hybridized with Lg:B0-1 was found only in the polyadenylated fraction (Fig. 4B), a result that was also obtained with pulmonary mRNA isolated from rat lung (not shown).

Discussion

We have examined 43 cDNA clones related to rabbit cytochrome P-450 isozyme 2. Three types of cDNAs (B0, B1, and B2), as defined by restriction analysis with *Bst EII*, account for these clones. Results of sequence analyses indicate that the cDNA clones were derived from three distinct mRNA species, each of which encodes a different form of isozyme 2. The amino acid sequence determined for the B0 form is identical to the sequence reported for purified isozyme 2 by Tarr *et al.* (13). The sequences of the B1 and B2 forms differ from that of the B0 form at 6 and 11 positions, respectively, and from each

TABLE 2

Amino acid substitutions among sequences for isozyme 2 determined from cDNAs and protein

Sequence	Amino acid position[a]															
	35	39	57	91	95	96	99	100	114	120	135	136	141	174	193	221
Lv-1:B2-1	pro	VAL	ARG	GLN	PHE	SER	GLY	LYS	phe	his	PHE	GLY	SER	val	PRO	PRO
Lv-1:B1-1	SER	ile	gln	GLN	PHE	SER	GLY	LYS	ILE	ARG	PHE	GLY	SER	val	PRO	PRO
Lg:B0-1	SER	VAL	ARG	GLN	PHE	SER	GLY	LYS	ILE	ARG	PHE	GLY	SER	ILE	PRO	PRO
Protein[b]	SER	VAL	ARG	GLN	PHE	SER	GLY	LYS	ILE	ARG	PHE	GLY	SER	ILE	PRO	PRO
Protein[c]	SER	VAL	ARG	glu	ser	phe	—	—	ILE	ARG	gly	tyr	gly	ILE	lys	ser
	248	286	290	294	303	314	363	367	370	417	420	461	462	463	464	465
Lv-1:B2-1	thr	arg	LEU	thr	THR	MET	val	ala	met	asp	LEU	SER	PRO	VAL	PRO	PRO
Lv-1:B1-1	SER	GLN	ile	SER	THR	leu	ILE	VAL	THR	ASN	met	SER	PRO	VAL	PRO	PRO
Lg:B0-1	SER	GLN	LEU	SER	THR	MET	ILE	VAL	THR	ASN	LEU	SER	PRO	VAL	PRO	PRO
Protein[b]	SER	GLN	LEU	SER	THR	MET	ILE	VAL	THR	ASN	LEU	SER	PRO	VAL	PRO	PRO
Protein[c]	SER	GLN	LEU	SER	ala	MET	ILE	VAL	THR	ASN	LEU	gly	asn	leu	ser	leu

[a] With the exception of position 174, substitutions are confined to a single sequence; these substitutions are shown in lower case letters.
[b] Data from the sequence reported by Tarr *et al.* (13).
[c] Data from the sequence reported by Heinnemann and Ozols (12). A second sequence for isozyme 2 reported by this laboratory (45) was the same except for the presence of glycine and lysine at positions 99 and 100, respectively. This difference was not commented upon by the authors.

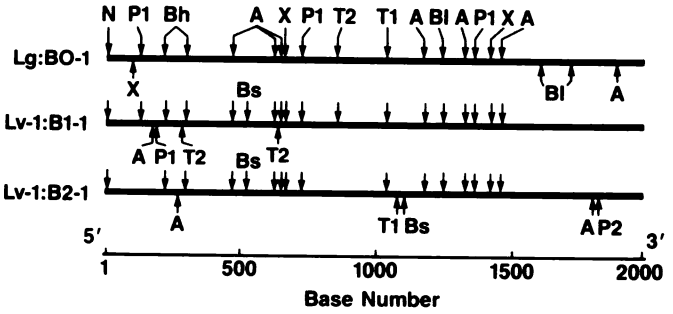

Fig. 2. Restriction sites present in cDNAs encoding for different forms of cytochrome P-450 isozyme 2. Restriction sites are shown for three different cDNAs, Lg:B01, Lv-1:B1-1, and Lv-1:B2-1. ↓, restriction sites present on more than one cDNA; ↑, restriction sites that are unique for one cDNA. The restriction sites are marked as follows: N (*Nco I*), P1 (*Pst I*), Bh (*Bam HI*), A (*Alu I*), X (*Xma I*), *T2* (*Tth 111 II*), *T1* (*Tth 111 I*), Bl (*Bgl I*), BS (*Bst EII*), and P (*Pvu II*). With the exception of X, T1, and T2, all sites were confirmed experimentally. Additional restriction sites are shown in Fig. 1.

TABLE 3

Analysis of cDNA clones for isozyme 2 by restriction with *Bst EII*

Source	Sequence	*Bst EII* restriction sites (GGTNACC)[a]							
		Amino acid number						Restriction	
		173	174	175	370	371	372	Site 1	Site 2
Protein[b]	Nucleotide	?	AT?	AC?	?	GT?	AC?	No	?
	Amino Acid	SER	ILE	THR	THR	VAL	THR		
cDNA Lv-1:B2-1	Nucleotide	G	GTC	ACC	G	GTC	ACC	Yes	Yes
	Amino Acid	SER	VAL	THR	MET	VAL	THR		
cDNA Lv-1:B1-1	Nucleotide	G	GTC	ACC	G	GTC	ACA	Yes	No
	Amino Acid	SER	VAL	THR	MET	VAL	THR		
cDNA Lg:B0-1	Nucleotide	A	ATC	ACC	G	GTC	ACA	No	No
	Amino Acid	SER	ILE	THR	THR	VAL	THR		

[a] The sequence recognized by *Bst EII* is GGTNACC and cleavage takes place between the guanidine residues. The designation "N" means that any base can be present. Bases that could not be derived from the protein sequences are indicated by question marks.

[b] The amino acid sequences are those reported by Tarr *et al.* (13) and Heinemann and Ozols (12), and aligned by Tarr *et al.* (13).

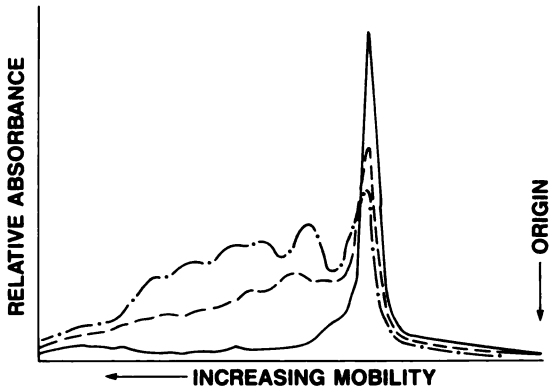

Fig. 3. A comparison of the hybridization of cDNA (Lg:B0-1) encoding isozyme 2 with mRNA isolated from rabbit lung by three methods. Pulmonary mRNA (5 μg) prepared by isolation from polysomes (—•—), by extraction with guanidine chloride and precipitation with ethanol (— — —), and by the procedure described in detail under Materials and Methods (——) was electrophoresed in agarose gels, transferred to DBM paper, and hybridized with cDNA (Lg:B0-1) encoding isozyme 2. The figure shows laser scans of the resulting autoradiograms.

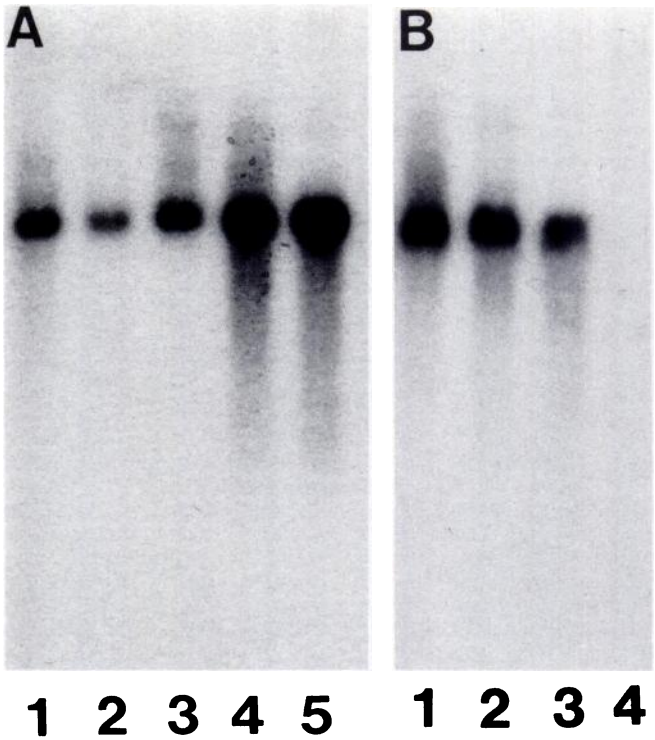

Fig. 4. Hybridization of cDNA (Lg:B0-1) encoding isozyme 2 with mRNA from lungs and livers of untreated rabbits and rabbits treated with PB. Pulmonary and hepatic mRNA was electrophoresed in agarose gels, transferred to DBM paper, and hybridized with Lg:B0-1 labeled by nick-translation with ^{32}P. The autoradiograms shown were developed after 16 hr of exposure. The mRNA samples (5 μg) used to obtain the results shown in A were from untreated liver (*lane 1*) and lung (*lane 2*) of the same rabbit, and from livers of rabbits treated with PB 12 hr after a single treatment (*lane 3*), 20 hr after a single treatment (*lane 4*), and 24 hr after four daily treatments (*lane 5*). The mRNA samples (5 μg) used to obtain the results shown in B were from untreated lung (*lane 1*) and from lungs of rabbits treated with PB 12 hr after a single treatment (*lane 2*) and 24 hr after four daily treatments (*lane 3*). *Lane 4* contained 50 μg of RNA from which the polyadenylated RNA had been removed.

other at 15 positions. None of these positions of variance coincide with any of the differences between the sequences reported by Tarr *et al.* (13) and Heinemann and Ozols (12).

The relative proportions of the B0, B1, and B2 clones in libraries constructed with mRNA from different sources suggest that the three forms of isozyme 2 may differ with respect to their tissue distributions and responses to PB. Of the 27 clones obtained from the Lv-1 library, 18 were B2 clones and 9 were B1 clones; no B0 clones were identified. In contrast, 12 of the 14 clones identified in the Lg library were B0, 2 were B1, and none were B2. Because the Lg library was constructed with pulmonary mRNA from 10 rabbits, it is likely that lung expresses little or no B2 mRNA.[2]

The presence of B0 clones in the Lg library, but not in the Lv-1 library, appeared to be anomalous since B0 mRNA encodes a protein with a sequence identical to that of isozyme 2 purified from rabbit liver (13). However, the rabbits used by Tarr *et al.* (13) were treated with PB (via their drinking water) for 5 days (13), whereas the rabbit we used (Lv-1 library) was killed 12 hr after a single dose of PB. This difference between treatments suggested the possibility that a single administra-

[2] Results obtained with specific oligonucleotide probes are consistent with this conclusion (R. Gasser and R. M. Philpot, manuscript in preparation).

tion of PB might not be sufficient to induce B0 mRNA. The identification of B0 clones in the Lv-4 library, derived from a rabbit treated with PB daily for 4 days, is consistent with this possibility.[3] If this is the case, induction of B1 and/or B2 mRNA must account for the marked increase in total isozyme 2 mRNA observed 12 hr after a single treatment with PB.

The lack of induction by PB of mRNA related to isozyme 2 in lung is consistent with our previous conclusion that pulmonary isozyme 2 content is not increased by PB (11). Whether or not PB alters the relative proportions of the two mRNA populations in lung remains to be determined. In any case, it is clear that the effects of PB on isozyme 2 in lung and liver are different, even though at least two identical mRNA populations are expressed in each tissue. It has been reported that pulmonary mRNA for rat P-450b, unlike the hepatic mRNA, does not bind to oligo(dT)-cellulose, and that this may reflect differences important to tissue-specific expression (41). However, we have found that hybridization of cDNA for isozyme 2, a homolog of P-450b, occurs exclusively with the polyadenylated fraction of pulmonary or hepatic RNA isolated from either rabbits or rats. It is likely that differences between the methods used for the isolation of pulmonary mRNA account for these conflicting results.

The induction by PB of highly related forms of cytochrome P-450 has been thoroughly documented for rat liver and also appears to be the case with hamster. In rat liver the concentrations of both P-450b and B-450e increase following treatment with PB. The primary sequences of these isozymes, which are synthesized from different mRNAs (42) transcribed from different genetic loci (43), differ at only 14 of 491 positions (14). (The differences among the sequences of the B0, B1, and B2 forms of isozyme 2 are not related to the differences between P-450b and P-450e in any obvious way.) In spite of their similarities, P-450b and P-450e have different mobilities on polyacrylamide gels in the presence of SDS. This difference has been used to demonstrate the presence of an immunochemically related protein with the mobility of P-450e in untreated liver, and one in lung with the mobility of P-450b (44). A tentative identification of the pulmonary form as P-450b has been made on the basis of mRNA hybridization with a specific oligonucleotide probe (41). We have also observed that immunoblots of hamster hepatic microsomal preparations stained with antibodies to isozyme 2 show two bands of distinctly different mobilities. The intensities of both bands are markedly increased by PB, and only one band is observed with pulmonary microsomal preparations.[4] Unfortunately, the presence of multiple forms of isozyme 2 in rabbit liver cannot be ascertained on the basis of mobility differences. However, preliminary results with specific oligonucleotides indicate that it will be possible to determine the distribution of different forms of isozyme 2 at the level of mRNA.

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