Isolation and Characterization of cDNA Clones for Cytochromes P-450 Immunochemically Related to Rat Hepatic P-450 Form PB-1†

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ABSTRACT: Rat hepatic cytochrome P-450 PB-1 is a prominent constitutive P-450 form whose levels increase approximately 2–3-fold upon phenobarbital administration. Antibodies raised against this protein recognized two major proteins in immunoblots of rat liver microsomal proteins and precipitated comparable amounts of two electrophoretically separable hepatic mRNA translation products. The levels of the two mRNAs encoding these polypeptides were increased substantially upon phenobarbital administration. The anti-PB-1 antibodies were used to screen a cDNA library, and two distinct cDNA clones, pTF-1 and pTF-2, were isolated. These clones contain inserts of 1227 and 410 base pairs, respectively, and show 80% nucleic acid sequence homology in their region of overlap. The DNA sequences of these clones show 54% sequence homology to the corresponding portions of the mRNA encoding P-450 PB-4, a major phenobarbital-inducible form of rat liver P-450, and can be optimally aligned with the PB-4 sequence without introducing insertions or deletions. The level of hepatic mRNA which hybridizes to clone pTF-2 increases approximately 2–4-fold after phenobarbital treatment, whereas mRNA which hybridizes to pTF-1 does not change in concentration after this treatment. mRNA, which hybridizes to pTF-1, is, however, 4-fold more abundant in livers of female rats than in livers of male rats. Differential thermal elution of liver mRNA from DNA–RNA hybrids formed with the two plasmid DNAs followed by in vitro translation indicated that pTF-2 very likely corresponds to the mRNA for P-450 PB-1 whereas pTF-1 corresponds to that encoding a PB-1-related P-450 form not yet identified. On the basis of the available sequence data, it is concluded that the two clones represent members of a gene subfamily which is part of the P-450 PB-4/PB-5 gene family. The complexity of genomic blots obtained by using pTF-1 and pTF-2 as probes indicates that this P-450 PB-1 gene subfamily is likely to include several additional members.

The cytochrome P-450[1]-dependent mixed function oxidase system of mammalian liver is active in the metabolism of a wide variety of endogenous substrates, including steroid hormones and prostaglandins, as well as structurally diverse foreign compounds, including many drugs, carcinogens, and environmental pollutants. This broad substrate specificity of the P-450 system results in part from the existence of at least a dozen distinct forms of P-450, many of which individually exhibit broad, albeit distinguishable, specificity profiles [reviewed by Coon & Koop (1983)]. Biochemical and immunochemical studies of P-450s isolated from rat and rabbit liver have established that some forms are virtually undetectable in hepatic tissue unless the animals are pretreated with monooxygenase inducers such as phenobarbital (PB) or 3-methylcholanthrene while other forms are present in significant amounts in uninduced rat liver, i.e., are expressed constitutively. Structural studies have established that the two major phenobarbital-inducible forms of rat liver cytochrome P-450, PB-4[2] and PB-5 (Waxman & Walsh, 1982; Ryan et al., 1982; Guengerich et al., 1982), are 97% identical in their primary structures (Fujii-Kuriyama et al., 1982; Kumar et al., 1983; Yuan et al., 1983), whereas the two major polycyclic-inducible forms P-450c and P-450d (Ryan et al., 1982; Guengerich et al., 1982; Goldstein et al., 1982), are 70% homologous to each other, with greater than 90% homology found within localized segments of the polypeptide chain (Kawajiri et al., 1984; Sogawa et al., 1984). Sequence comparisons indicate limited, yet significant, overall homology (approximately 28%) between members of the phenobarbital- and polycyclic-inducible P-450 families.

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[1] Abbreviations: P-450, cytochrome P-450; PB, phenobarbital; ELISA, enzyme-linked immunoabsorbent analysis; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl and 15 mM sodium citrate, pH 7.0; bp, base pairs.

[2] The nomenclature used by this laboratory for designation of rat hepatic P-450 forms and its rationale have been described in detail previously (Waxman, 1984, 1986). Other designations used for the P-450 forms discussed in this report include the following: P-450 PB-1 = P-450 PB-C; P-450 PB-4 = P-450b, P-450 PB-B; P-450 PB-5 = P-450e, P-450 PB-D; P-450 2c = P-450 h, P-450 UT-A, P-450 RLM5, P-450 male; P-450 2d = P-450i, P-450 UT-I, P-450 female; P-450 3 = P-450a, P-450 UT-F; P-450 2a = P-450p, P-450 PCN-E. [For a complete listing of references, see Waxman (1984, 1986).]

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Although only limited structural information is available for constitutive rat hepatic P-450s, immunochemical studies suggest that several of these forms are structurally distinct from each other as well as from the major phenobarbital- and polycyclic-inducible P-450 forms [e.g., see Ryan et al. (1984) and Waxman (1984)]. In many cases, however, antisera used to demonstrate the absence of immunochemical cross-reactivity are first adsorbed with covalently immobilized heterologous P-450 fractions [e.g., see Thomas et al. (1981)], a procedure which might, in principle, remove antibodies cross-reactive with other P-450 forms. In addition, it has been observed that antibodies shown to be monospecific for a given P-450 form by one technique, e.g., Ouchterlony immunodiffusion analysis, may exhibit cross-reactivity with other P-450s when other more sensitive analytical methods are used such as enzyme-linked immunosorbent analysis (ELISA) (Waxman, 1984). These findings indicate that more detailed studies are required to evaluate the structural relationships among these constitutive P-450 forms as well as their relationship to the major phenobarbital- and polycyclic-inducible P-450s studied previously.

Rat hepatic cytochrome P-450 PB-1 (also termed P-450 PB-C) is a prominent constitutive P-450 whose microsomal levels increase approximately 2–3-fold upon phenobarbital administration (Waxman et al., 1982; Waxman & Walsh, 1983; Guengerich et al., 1982). This P-450 exhibits a broad specificity for xenobiotic substrates, and its activity is stimulated substantially by cytochrome b_5 in reconstituted systems. P-450 PB-1 is also activated by metyrapone, a heme binding ligand which is inhibitory for many other P-450 enzymes (Waxman & Walsh, 1983). In this report, we describe the isolation and characterization of two cDNA clones encoding fusion proteins recognized by affinity-purified anti-P-450 PB-1 antibodies. One of these clones is shown to hybridize at high stringency to mRNA inducible aproximately 2–4-fold upon phenobarbital administration and probably encodes P-450 PB-1. The second clone is 80% homologous to the first in their region of overlap and hybridizes to mRNA which is not inducible by phenobarbital; this latter clone probably corresponds to a PB-1-related P-450 form not yet identified. Finally, both cDNA clones are shown to exhibit about 54% nucleotide sequence homology to the mRNAs encoding P-450s PB-4 and PB-5, the major phenobarbital-inducible P-450 forms of rat liver. Thus, the P-450 PB-4/PB-5 gene family is now shown to include members which encode P-450s expressed in rat liver at substantial levels in uninduced animals and which were not previously recognized by cross-hybridization to P-450 PB-4 and PB-5 cDNA probes (Atchison & Adesnik, 1983; Mizukami et al., 1983).

Materials and Methods

Antibody Preparation and Specificity. The preparation and affinity purification of rabbit antiserum against purified P-450s PB-1 and PB-4 were described previously (Waxman, 1984). The anti-PB-1 and anti-PB-4 antibodies used in this report were affinity purified by adsorption to covalently immobilized solubilized microsomes isolated from livers of phenobarbital-induced, 4-week-old male rats. Affinity-purified anti-PB-4 antibodies were shown to recognize P-450 PB-4 and the highly homologous P-450 PB-5 specifically as judged by ELISA and Ouchterlony immunodiffusion analysis (Waxman, 1984) as well as by immunoblotting (not shown). In Ouchterlony immunodiffusion analysis, affinity-purified anti-PB-1 antibodies were found to specifically immunoprecipitate P-450 PB-1 and showed no cross-reactivity with six other distinct purified P-450 forms. However, cross-reactivities of P-450 PB-1 with other rat hepatic P-450s (most notably P-450s 2c and 2d and, to a lesser extent, P-450 3) could be demonstrated by the ELISA technique, which provides a more sensitive assay for shared, minor antigenic determinants (Waxman, 1984). These cross-reactivities almost certainly result from inherent immunochemical homologies between these P-450s rather than from the presence of P-450 contaminants in the PB-1 immunogen or heterologous antigen preparations. Thus, P-450 form-specific steroid hydroxylase assays (Waxman et al., 1983) demonstrated a less than 3% cross-contamination of P-450 PB-1 by P-450 2c or P-450 3. Moreover, P-450 PB-1 used in these studies was isolated from phenobarbital-induced adult male liver microsomes, which do not contain detectable levels of the female-specific P-450 2d (Waxman et al., 1985). In addition, affinity purification of the anti-PB-1 antibodies on covalently immobilized solubilized microsomes isolated from either uninduced adult male rat liver or phenobarbital-induced immature male rat liver (which are essentially devoid of the female-specific P-450 2d and the male-specific P-450 2c, respectively) did not remove these cross-reactivities (data for P-450 2c shown in Figure 1). Finally, these cross-reactivities could also be detected after resolution of the purified P-450s on SDS gels followed by Western blot analysis, thus establishing that they do not result from contamination of the heterologous P-450s by P-450 PB-1 (see, e.g., Figure 1).

Immunoblotting Analysis. Samples of total microsomes and various purified rat hepatic P-450 forms (Waxman et al., 1983; Waxman, 1984) were analyzed by electrophoresis on 10% SDS–polyacrylamide gels (Laemmli, 1970) and transferred to nitrocellulose membranes by electroblotting (Burnette, 1981). After a preincubation with bovine serum albumin, the blots were incubated successively with affinity-purified anti-PB-1 antibodies (diluted to an ELISA titer of 1/15 in phosphate-buffered saline containing 1% bovine serum albumin and 0.1% Triton X-100) and peroxidase-conjugated goat anti-rabbit IgG (Cappel Laboratories, 1/1000 dilution) essentially as described (Thomas et al., 1984) followed by development with diaminobenzidine hydrochloride (Sigma) and hydrogen peroxide according to the procedure provided by Cappel Laboratories.

RNA Purification and in Vitro Translation. Total RNA was prepared from livers of starved Sprague-Dawley rats (Taconic, Inc.) by the guanidine isothiocyanate–CsCl procedure (Chirgwin et al., 1979), and poly(A+) mRNA was isolated by oligo(dT)–cellulose chromatography (Aviv & Leder, 1972). Where indicated, animals were injected with PB (100 mg/kg) 9–18 h prior to sacrifice. Poly(A+) mRNA was translated in the wheat germ cell-free system, and the translation products were then analyzed by immunoprecipitation, SDS–polyacrylamide gel electrophoresis, and autoradiography as described previously (Kumar et al., 1983). The antibodies used to immunoprecipitate PB-4 + PB-5 were described previously (Kumar et al., 1983).

Construction and Screening of a cDNA Library. A cDNA library was constructed essentially as described by using poly(A+) mRNA from livers of 150-g male rats killed 9 h after phenobarbital treatment as template for reverse transcription, the Klenow fragment of DNA polymerase to effect second-strand synthesis, and the dG-dC tailing procedure to insert the cDNA into the *Pst*I site of pBR322 (Maniatis et al., 1982). Transformation was carried out according to the procedure of Dagert and Ehrlich (1979), and approximately 8000 independent clones were obtained from 40 ng of tailed double-stranded cDNA. The clone collection was scraped off the plates into LB medium and stored as a glycerol stock

culture. For immunoscreening, cells were plated onto nitrocellulose filbers (~5000 colonies/150-mm diameter filter) which were screened with affinity-purified anti-PB-1 IgG (diluted to an ELISA titer of 1/100) essentially as described (Young & Davis, 1983). Bound antibody was detected by incubation with goat anti-rabbit IgG coupled to horseradish peroxidase (Cappel Laboratories, 1/1000 dilution) followed by reaction with diaminobenzidine and H_2O_2 as described by the supplier of the second antibody. Three positive clones were identified, all of which proved to be positive on a second and third screening with anti-PB-1 antibodies. Two of these had inserts of identical size, and therefore, only one of the two, designated pTF-2, was studied further.

Hybridization Selection of mRNA. Hepatic poly(A+) mRNA (10 μg) from male rats injected with PB 17 h prior to sacrifice was hybridized to 20 μg of plasmid DNA immobilized on a 3 mm^2 piece of nitrocellulose paper as described previously (Maniatis et al., 1982). The mRNA was eluted by heating the filter at 100 °C for 1 min in 300 μL of sterile water containing 6 μg of yeast tRNA. For differential thermal elution, the filter was incubated successively in aliquots of the same solution for 4 min at 48, 52, 56, and 70 °C. Twenty microliters of 3 M sodium acetate, pH 4.8, was added to each eluate before precipitation with 1.2 mL of ethanol at −70 °C for 30 min. The RNA was then translated in a 50-mL reaction volume in the wheat germ cell-free system.

Slot Blotting Analysis of mRNA Levels. Twofold serial dilutions of total liver RNA were treated with formaldehyde and bound to nitrocellulose filters (Schleicher & Schuell; Keene, NH) using the Schleicher & Schuell slot-blotting apparatus as described (White & Bancroft, 1982). The filters were hybridized overnight to ^{32}P-labeled nick-translated plasmid DNA at 65 °C in 2 × SSC (0.3 M NaCl and 0.03 M sodium citrate, pH 7.0) and washed in 0.1 × SSC before air drying and autoradiography at −70 °C using preflashed Kodak XR-5 X-ray film with an intensifying screen.

DNA Sequencing. The intact inserts obtained by *Pst*I digestion of the recombinant plasmids and restriction fragments generated from them by digestion with *Hin*fI or *Alu*I were purified by electrophoresis on a 5% polyacrylamide gel, cloned into M13mp8, and sequenced by the Sanger dideoxy method as previously described (Atchison & Adesnik, 1983). The *Hin*fI subfragments were made blunt by treatment with the Klenow fragment of DNA polymerase before blunt-end ligation into the *Sma*I site of the vector in order to obtain subclones with inserts in both orientations. All sequences were determined 2–4 times, and both DNA strands were sequenced over the entire length of both cDNA clones except for the 111 bp within pTF-1 extending from residues 830 to 940 in the sequence. The DNA sequence in this region was determined from two distinct overlapping subclones which contained inserts in the same orientation.

Southern Blotting. Rat liver DNA was purified by the procedure of Blin and Stafford (1976). Restriction endonuclease digestion and Southern hybridization to nick-translated inserts from the recombinant plasmids were carried out as previously described (Atchison & Adesnik, 1983).

Results

Anti-P-450 PB-1 Antibodies Recognize Two Polypeptides in Rat Liver Microsomes. Affinity-purified anti-PB-1 antibodies were used to probe nitrocellulose blots of electrophoretically resolved rat liver microsomal proteins as well as several purified P-450 forms (Figure 1). These antibodies recognized two bands of comparable intensity in microsomes isolated from untreated rats, with the more rapidly migrating

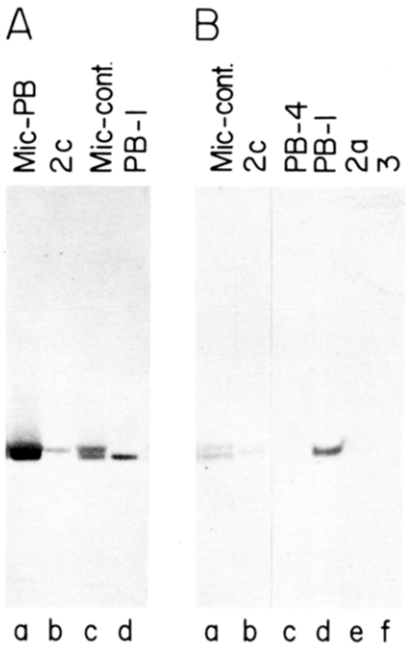

FIGURE 1: Characterization of anti-P-450 PB-1 by immunoblotting analysis. Samples of SDS-solubilized total microsomal protein and purified preparations of cytochrome P-450 forms were analyzed by SDS gel electrophoresis and immunoblotting using affinity-purified anti-PB-1 antibodies as probe as described under Materials and Methods. (Panel A) (a) Two micrograms of total microsomal proteins from the livers of mature (300 g) male rats treated with three successive injections of PB (100 mg/kg) and killed 16 h after the last injection; (b) purified P-450 2c, 0.1 μg; (c) microsomal protein from untreated mature (300 g) male rats, 2 μg; (d) purified PB-1, 0.025 μg. In a separate experiment (panel B), the following samples were analyzed: (a) total microsomal protein from mature (270 g) female rats, 5 μg; (b–f) 0.1 μg of various purified P-450s as indicated above each lane. The intensities of the bands in panels A and B cannot be compared since they depend not only on the amount of each protein but also on the incubation time for the peroxidase reaction.

species displaying the same electrophoretic mobility as purified P-450 PB-1 (Figure 1A, lanes c and d, and Figure 1B, lanes a and e). Phenobarbital pretreatment of rats led to a significant but somewhat variable increase in the more rapidly migrating component (Figure 1A, lanes a vs. lane c). The anti-PB-1 antibodies showed a weak (approximately 10-fold less) reactivity with purified P-450 2c (Figure 1A, lane b, and Figure 1B, lane b) which exhibits an electrophoretic mobility intermediate to the major microsomal bands detected by these antibodies. No cross-reactivity with purified P-450 forms PB-4, 2a, and 3 (Figure 1B, lanes c, e, and f, respectively) or microsomal epoxide hydrase (data not shown) was detected at the level of sensitivity of these experiments.

Phenobarbital Induces Two mRNAs Whose Translation Products Are Recognizable by Anti-P-450 PB-1 Antiserum. To assess the level of PB-1 mRNA and its inducibility by phenobarbital, mRNA from both untreated and PB-treated male and female rats was translated in a wheat germ system, and the resultant translation products were then immunoprecipitated with anti-PB-1 antibodies. Two electrophoretically separable translation products were thus detected (Figure 2A, lane a), with the more rapidly migrating one exhibiting the same electrophoretic mobility as purified P-450 PB-1 (Figure 2A, lane b). The mRNAs encoding both anti-PB-1-precipitable polypeptides were induced severalfold and to similar extents by phenobarbital treatment, with equivalent results obtained by using mRNA preparations from both male and female rats (Figure 2B). Both polypeptide products were readily distinguished from the PB-4 and PB-5 translation

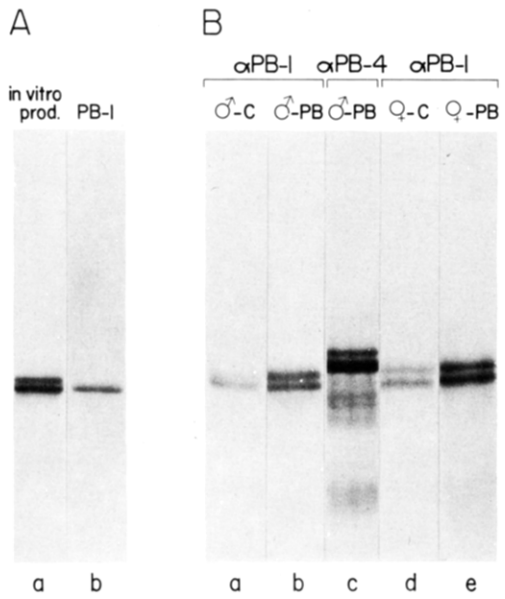

FIGURE 2: Immunoprecipitation of two in vitro translation products with anti-PB-1 antibodies: phenobarbital induction of translatable PB-1 mRNA. (Panel A) Poly(A+) mRNA (1 μg) from livers of PB-treated rats (9 h) was translated in the wheat germ cell-free system, and an aliquot (8×10^6 cpm) was immunoprecipitated with affinity-purified anti-PB-1 IgG. After elution from the protein A–agarose beads, the immunoprecipitate was mixed with 5 μg of purified P-450 PB-1. The sample was then electrophoresed on a 10% SDS–polyacrylamide gel which was stained with Coomassie blue, then dried, and autoradiographed. Lane a, autoradiographic pattern obtained with anti-PB-1 antibodies. Lane b, Coomassie blue stained pattern of the anti-PB-1 immunoprecipitate coelectrophoresed with purified P-450 PB-1. (Panel B) Hepatic poly(A+) mRNA from untreated male (lane a), PB-treated (17 h) male (lanes b and c), untreated female (lane d), and PB-treated female rats (lane e) was translated in vitro, and 4×10^6 trichloroacetic acid precipitable counts were immunoprecipitated with anti-PB-1 (lanes a, b, d, and e) or anti-P-450 PB-4/PB-5 (lane c) antibodies, and the immunoprecipitates were analyzed by SDS gel electrophoresis and fluorography.

products immunoprecipitated by using anti-PB-4 antibody (Figure 2B, lane c). Significantly, the two mRNAs encoding PB-1-related peptides were present in livers of PB-treated rats at levels comparable to those of the mRNAs encoding the major phenobarbital-inducible P-450s PB-4 and PB-5 (Figure 2B, lanes b and e vs. lane c).

Isolation of cDNA Clones by Immunoscreening with Anti-PB-1 Antibodies. A cDNA library containing about 8000 independent clones was constructed from mRNA isolated from livers of PB-induced rats by using the GC-tailing procedure and insertion into the *Pst*I site of pBR322 (Villa-Komaroff et al., 1978). Clones found in this library are expected to yield β-lactamase fusion proteins if the cDNA is inserted in the proper orientation and with the correct reading frame (corresponding to only one out of six clones). The library was screened for PB-1-immunoreactive clones by using affinity-purified rabbit anti-PB-1 antibodies, followed by horseradish peroxidase coupled to goat anti-rabbit IgG as a detection reagent. Two distinct immunoreactive clones, designated pTF-1 and pTF-2, containing inserts of approximately 1250 and 450 bp, respectively, were thus identified. These clones hybridized weakly to each other under conditions of moderate stringency (2 × SSC at 65 °C) but not at all to clone R17 (Kumar et al., 1983) which contains DNA sequences corresponding to the 3′ half of the P-450 PB-5 mRNA (not shown).

The clones were further characterized by the hybridization selection translation procedure. Hybridization of mRNA from livers of PB-induced rats to both cloned cDNAs led to a marked enrichment of the two mRNAs encoding the polypeptides precipitated by the anti-PB-1 antibodies (Figure 3). These polypeptides, which are the most prominent products greater than 20000 daltons in size found in the total translation products of the hybrid-selected mRNA, are not at all discernible within the highly complex pattern [e.g., see Kumar et al. (1983)] of total translation products of the input mRNA used for the hybridization selection procedure (not shown). The fact that the two cDNAs cross-hybridize to each other only weakly suggested that the mRNAs hybridizing to the two clones might be differentially eluted from the recombinant plasmid DNAs by varying the temperature or ionic strength of the elution buffer. Indeed, the mRNA encoding the more rapidly migrating polypeptide (which comigrates with PB-1) was efficiently eluted from pTF-1 at 48 °C whereas elution at 52 °C was required for elution of this mRNA from pTF-2, the clone containing the shorter insert (Figure 4). In contrast, the mRNA for the more slowly migrating translation product was eluted from pTF-1 at 52 °C and not at 48 °C. It may

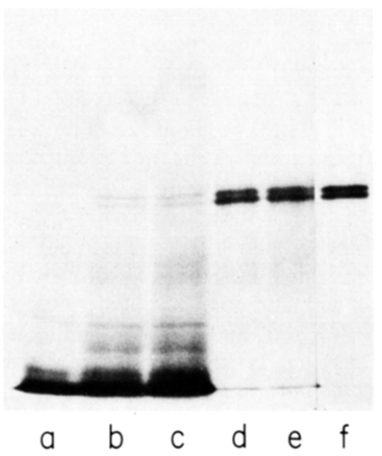

FIGURE 3: Translation of mRNAs purified by hybridization to recombinant plasmid DNAs. mRNA selected by hybridization to pTF-1 DNA (b and d) or pTF-2 DNA (c and e) or total poly(A+) mRNA (f) was translated in vitro, and the total translation products (b and c; 5 μL) and immunoprecipitated products (d and e; from 40 μL) were analyzed by SDS gel electrophoresis and fluorography. (a) Total endogenous wheat germ translation products labeled in the absence of added mRNA.

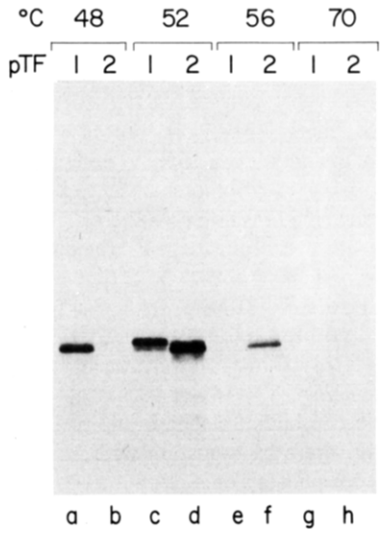

FIGURE 4: Differential thermal elution of mRNAs which hybridize to the recombinant plasmids. mRNA hybridized to pTF-1 (a, c, e, and g) or pTF-2 (b, d, f, and h) was eluted by successive incubations in elution medium at 48 °C (a and b), 52 °C (c and d), 56 °C (e and f), and 70 °C (g and h) and translated in vitro before immunoprecipitation with anti-PB-1 antibodies, gel electrophoresis, and fluorography.

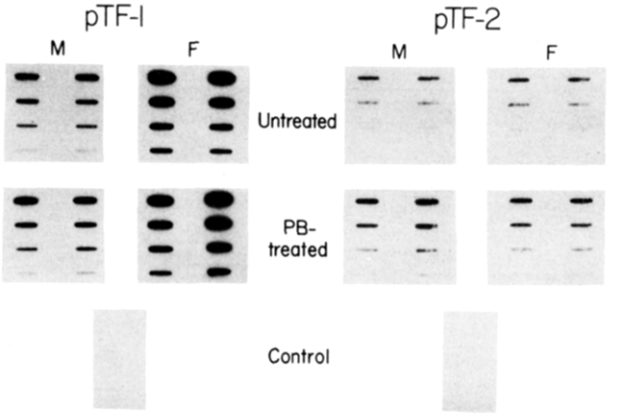

FIGURE 5: Relative levels of mRNA hybridizable to pTF-1 and pTF-2 in untreated and phenobarbital-induced male and female rats. Serial dilutions of total RNA (10, 5, 2.5, and 1.25 μg) isolated from livers of untreated and phenobarbital-treated rats (200 g) of both sexes were applied to nitrocellulose filters and hybridized to the nick-translated plasmid DNAs, as indicated. Analyses of RNA isolated from two individual animals from each group are shown. The control RNA sample, kindly provided by Dr. D. Colman (NYU), was rat brain RNA, partially depleted of poly(A+) mRNA by passage through oligo(dT)–cellulose.

be noted that in this experiment, pTF-2 selected the mRNA encoding the more rapidly migrating polypeptide much more efficiently than it did the other mRNA. These data demonstrate clearly that pTF-1 is not derived from the PB-1 mRNA and strongly suggest that pTF-2 corresponds to the mRNA encoding PB-1 or a very closely related P-450 form.

Responsiveness of pTF-2 and pTF-1 mRNA Levels to Phenobarbital Administration. As noted above, hepatic levels of two translatable mRNAs encoding PB-1-related polypeptides increase severalfold after PB treatment. It was therefore of interest to determine if the levels of the mRNAs which hybridize to pTF-1 and pTF-2 increase after PB administration. Samples of total RNA from livers of untreated and PB-induced male and female rats were analyzed by a slot-blotting procedure using the pTF-1 and pTF-2 plasmids as probes. This hybridization experiment (Figure 5), which was carried out under relatively stringent conditions, revealed that mRNA hybridizing to pTF-2 increases approximately 2–4-fold following PB treatment whereas the mRNA which hybridizes to pTF-1 does not show a significant increase. Although this increase in mRNA hybridizing to pTF-2 was reproducibly observed, it is far less than the 30-fold increase observed for P-450 PB-4 and P-450 PB-5 mRNAs after PB administration (Adesnik et al., 1981; Omiecinski et al., 1985). Interestingly, the levels of mRNA hybridizing to pTF-1 are approximately 4 times higher in female rats than in male rats. Essentially the same results were obtained when the probes used were ^{32}P-end-labeled, 22-residue-long, oligonucleotides with sequences specific for each clone (see below) and differing in 8 residues from each other. Northern blotting analysis demonstrated that the mRNAs which hybridize to the two cDNA probes have electrophoretic mobilities in formaldehyde–agarose gels identical with those of the P-450 PB-4 and P-450 PB-5 mRNAs which hybridize to plasmid R17, a P-450 PB-5 cDNA clone (Kumar et al., 1983) (not shown).

Sequence Analysis of the Inserts in Clones pTF-1 and pTF-2. The cDNA inserts in clones pTF-1 and pTF-2 were sequenced by the Sanger dideoxy method in order to compare the sequences to each other and to those of other P-450 forms. Their DNA sequences, aligned with the previously reported P-450 PB-4 cDNA sequence (Fujii-Kuiryama et al., 1982), are shown in Figure 6. The polypeptide sequences encoded

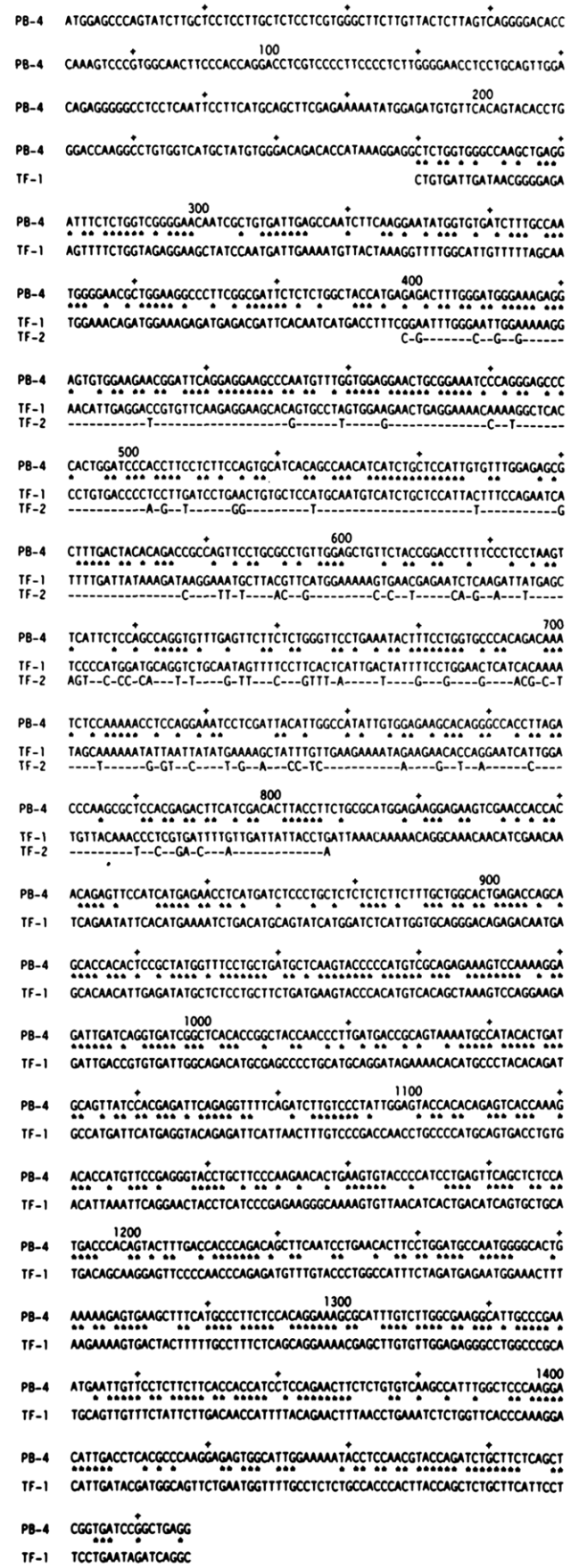

FIGURE 6: DNA sequences of the cDNA inserts in pTF-1 and pTF-2: comparison to that of P-450 PB-4 mRNA. The sequences for pTF-1 (second line) and pTF-2 (third line) are aligned with each other and with the coding sequence for p-450 PB-4 cDNA (Fujii-Kuriyama et al., 1982). Asterisks mark residues where pTF-1 and P-450 PB-4 are identical, and dashes mark residues where pTF-2 and pTF-1 are invariant.

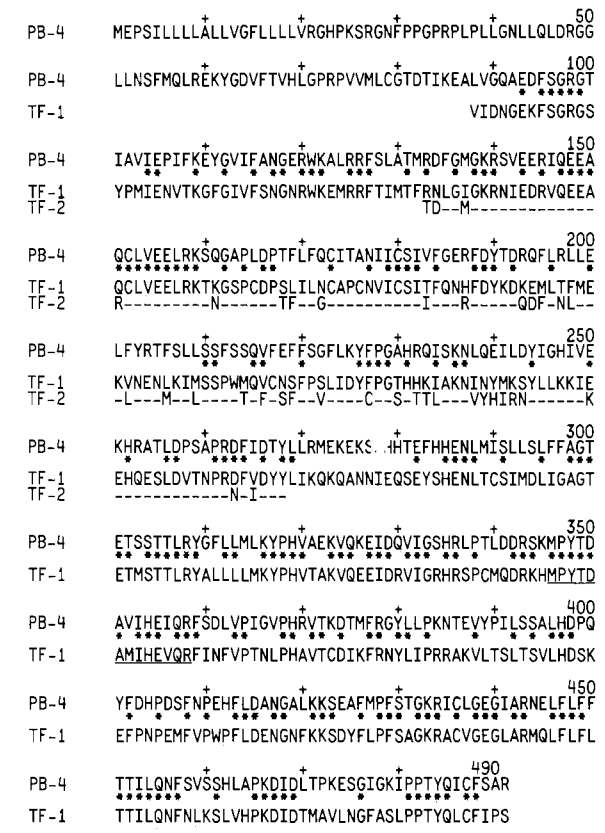

FIGURE 7: Amino acid sequences for polypeptide segments encoded by pTF1 and pTF2: comparison to P-450 PB-4. The amino acid sequences derived by translation of the DNA sequences in Figure 6 are aligned as in that figure. Residues where pTF-1 and P-450 PB-4 are identical are marked with asterisks, and residues where pTF-2 and pTF-1 are invariant are marked with dashes. The conserved tridecapeptide (Met_{346}–Arg_{358}) is underlined. The relatively high sequence divergence in the region extending approximately from residues 200 to 300 is apparent. Thus, in this region, the pTF-1-encoded sequence shows only 36% homology to PB-4 compared to 55% in the remainder of the sequence. In the same region, pTF-2 shows 35% homology (24/69 matches) to P-450 PB-4 and 68% homology (47/69 matches) to pTF-1 as compared to 61% (41/67 matches) and 78% (52/67 matches) homology, respectively, in the remainder of the sequence.

by the two cloned cDNAs are compared to the deduced amino acid sequence of P-450 PB-4 in Figure 7. It is very striking that the pTF-1 and pTF-2 sequences can be optimally aligned with that of P-450 PB-4 without deleting or inserting a single codon. The cDNA clone pTF-1 encompasses 1227 bp of sequence beginning at the position equivalent to codon 88 in the P-450 PB-4 sequence and extends past the termination codon including 14 bp of 3′-untranslated sequence. Clone pTF-2 contains 409 bp of sequence and begins at the position equivalent to codon 133 in P-450 PB-4 and extends to codon 268. Clones pTF-1 and pTF-2 are 79% and 73% homologous to each other at the DNA and protein levels, respectively. The overall DNA and protein sequence homologies between these two cDNA clones and P-450 PB-4 are 55% and 49% for pTF-1 and are 53% and 47% for pTF-2, respectively. However, the distribution of homology within the various sequences is not random. The first 60 amino acids encoded by pTF-2 (corresponding to codons 133–192 in P-450 PB-4) show an 83% homology to pTF-1 and a 63% homology to P-450 PB-4. Over the remaining 76 amino acids encoded by pTF-2 (corresponding to residues 193–268 in P-450 PB-4), one observes only 64% homology to pTF-1 and 36% homology to P-450 PB-4. Most strikingly, an identical, cysteine-containing tridecapeptide (Gln_{147}–Lys_{159}) is found in P-450 PB-4 and in the

Table I: Homology of the Polypeptide Sequences Encoded by pTF-1 and pTF-2 to the Polypeptide Segments Encoded by Each Exon of the P-450 PB-4 Gene[a]

exon	residues	pTF-1 (%)	pTF-2 (%)
3	112–162	62	66
4	162–215	35	46
5	216–274	37	36
6	275–322	53	
7	322–384	60	
8	385–432	45	
9	432–491	56	
9A	432–457	76	
9B	458–491	41	

[a] The exonic organization of the P-450 PB-4 gene is taken from Suwa et al. (1985). The exon 9 encoded peptide segment is divided into two parts (designated 9A and 9B) to reveal the highly conserved region surrounding Cys_{436}.

protein encoded by pTF-1 which includes Cys_{152} in the former protein. The corresponding peptide encoded by pTF-2 deviates by only one amino acid residue from the sequence present in the other two proteins. This cysteine-containing peptide has been found to be conserved in many different P-450s including the bacterial $P\text{-}450_{cam}$ (Black & Coon, 1986).

Comparison of the amino acid sequences encoded by the pTF-1 and pTF-2 cDNA clones to the sequence of P-450 PB-4 on an exon by exon basis [using the intron–exon organization determined for the P-450 PB-4 gene (Suwa et al., 1985)] revealed homologies ranging from as low as 35% and 37% for exons 4 and 5, respectively, to as high as 62% and 60% for exons 3 (which contains the cysteine residues at position 152 mentioned above) and 7, respectively (Table I). The polypeptide segment encoded by the region in pTF-1 which corresponds to the 5′ end of exon 9 of the P-450 PB-4 gene shows 76% homology to the P-450 PB-4 sequence and contains a cysteine residue at the position equivalent to Cys_{436} in cytochrome P-450 PB-4 (and P-450 PB-5) (exon 9A in Table I). This conserved cysteine is believed to supply the fifth ligand to the heme iron (Gotoh et al., 1983; Kimura et al., 1984). The extraordinarily high homology of this region, as well as the region surrounding Cys_{152}, provides strong evidence that these two segments of the P-450 molecule play important structural or functional roles.

The highly homologous region within pTF-1 and P-450 PB-4 which encompasses sequences corresponding to exon 7 of the P-450 PB-4 gene is worthy of special mention. This region encodes an analogous tridecapeptide sequence (residues Met_{346}–Arg_{358}) first observed in two ostensibly unrelated forms of rabbit cytochrome P-450 (Ozols et al., 1981). Indeed, the polypeptide encoded by clone pTF-1 also contains a tridecapeptide which matches 11 out of 13 of the amino acid residues of the corresponding segment in P-450 PB-4. It should be noted, however, that because different segments within the same portion of mRNA molecules show substantial differences in their degrees of homology to other P-450s, an assessment of the overall homology using incomplete clones is clearly tentative.

Southern Blotting Analysis of Rat Genomic DNA Using pTF-1 and pTF-2 as Probes. Previous studies have demonstrated that the rat genome contains approximately 10 genes which hybridize to P-450 PB-4/PB-5 cDNA probes (Atchison & Adesnik, 1983; Mizukami et al., 1983). The genes encoding the mRNAs represented by cDNA clones pTF-1 and pTF-2 can be considered members of this same gene family (see Discussion for a more thorough analysis of this question). To obtain some idea of the number of genes in the rat genome which contain sequences homologous to pTF-1 and pTF-2, rat

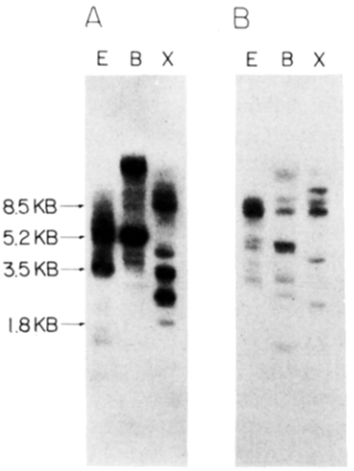

FIGURE 8: Hybridization of cloned cDNAs to Southern blots of genomic DNA. Rat liver DNA (10 μg) was digested with *Eco*RI (E) *Bam*HI (B), or *Xba*I (X) and, after agarose gel electrophoresis and blotting, hybridized to the nick-translated inserts of pTF-1 (A) and pTF-2 (B).

genomic DNA was fragmented with several restriction endonucleases and analyzed by the Southern blotting technique using pTF-1 and pTF-2 cDNAs as probes (Figure 8). The patterns obtained with the two probes are rather complex and suggest that multiple distinct genes are homologous to the two cDNAs. It should be noted that since the gene corresponding to the pTF-2 cDNA is in the same family as the PB-4 gene, these two genes are expected to have a very similar intron–exon organization. The pTF-2 cDNA probe would, therefore, be expected to hybridize to genomic fragments containing only exons 3–5 of the corresponding gene (Table I). The complexity of the pattern of genomic fragments hybridizing to the pTF-2 insert, which does not contain cleavage sites for the enzymes used in the blots in Figure 8, is therefore indicative of a family of related genes. Indeed, we have isolated[3] a third rat liver cDNA clone by immunoscreening with antibodies raised to P-450 2c, a male-specific P-450 form which is only weakly cross-reactive with PB-1 (Waxman, 1984, and Figure 1). This clone hybridizes to portions of pTF-1 and pTF-2, but partial sequence analysis clearly shows that it is distinct from both of them and represents a third member of the PB-1 gene subfamily.

Discussion

Two proteins are said to be in the same subfamily if they have greater than 70% amino acid sequence homology, in the same family if they show greater than 50% homology, and in the same superfamily if there is less than a 1 in 10^6 probability that their limited homologies occurred by chance (Dayhoff, 1976). Since clones pTF-1 and pTF-2 are incomplete, the overall homology between the corresponding proteins and between them and P-450s PB-4 and PB-5 cannot yet be definitively determined. However, on the basis of the 80% homology between the segments encoded by pTF-1 and pTF-2 and the approximately 50% homology between both these clones and the corresponding segments of PB-4 and PB-5, it is reasonable to assign the corresponding pairs of proteins to distinct subfamilies within the same gene family. The familial relationship between the members of these two subfamilies is only apparent from direct DNA sequence comparisons and cannot be demonstrated by cross-hybridization. Clones pTF-1 and pTF-2 clearly do not correspond to any of the genes belonging to the P-450 PB-4/PB-5 gene subfamily which were previously identified by hybridization to P-450 PB-4/PB-5 cDNAs (Atchison & Adesnik, 1983; Mizukami et al., 1983). Indeed, the DNA sequences for exons 7 and 8 of the four P-450 PB-4 and PB-5 subfamily members cloned in our laboratory show approximately 90% homology to the corresponding segments of the P-450 PB-4 and PB-5 genes (Atchison & Adesnik, 1985) whereas cDNA pTF-1, which contains the corresponding exonic region, shows only 53% homology over this domain.

The cytochrome P-450 PB-1/PB-4 gene family thus consists of at least two gene subfamilies. The P-450 PB-4/PB-5 subfamily contains approximately 10 distinct members most of which have been cloned from a rat liver genomic library in this laboratory (Atchison & Adesnik, 1983) or by other (Mizukami et al., 1983). In addition to the PB-4 and PB-5 genes, at least two of the other four PB-4/PB-5 subfamily members described by us are functional genes. One, gene 4, is expressed in the rat preputial gland but not in the liver[4] whereas a second gene, gene 5, appears to be expressed in the liver since a cDNA clone whose DNA sequence perfectly matches the limited available sequence for exons 7 and 8 of this gene has been recently isolated from a liver cDNA library (Affolter et al., 1986). The P-450 PB-1 gene subfamily contains at least three functional members represented by cDNA clones pTF-1 and pTF-2 described in this paper as well as by clone pAM-7, identified by immunoscreening with an antibody raised against P-450 2c (see below).[3] Since hybridization patterns obtained in genomic Southern blots using pTF-1 and pTF-2 as probes (Figure 8) are as complex as those obtained with a P-450 PB-4 cDNA probe (Atchison & Adesnik, 1983; Mizukami et al., 1983), it appears likely that this gene subfamily contains several additional members. One of these is likely to be that for P-450 2d, a female-specific P-450 form, since antibodies raised against P-450 PB-1 cross-react strongly with P-450 2d and vice versa (Waxman, 1984).

The pTF-1- and pTF-2-encoded polypeptides do contain limited segments which show exceptionally high sequence homology to the corresponding portions of the P-450 PB-4 and PB-5 sequences, most notably in the vicinities of Cys_{152} and Cys_{436} as well as in the segments corresponding to the analogous tridecapeptide, residues 346–358. The fact that the peptide region surrounding Cys_{152} is even more conserved than is the heme binding cysteine-containing peptide centered at Cys_{436} is quite striking. The function of this peptide segment is yet to be determined although the absence of the corresponding cysteine residue in P-450-PCN (Gonzalez et al., 1985b) and in the bovine adrenal $P\text{-}450_{scc}$ (Morohashi et al., 1984) clearly estabishes that it is not essential for the catalytic properties common to all P-450 enzymes.

It is worth emphasizing that pTF-1 and pTF-2 showed unusually high sequence divergence when compared to each other or to P-450 PB-4 in the region comprising amino acid residues 200–300 (Figure 7). A relatively high divergence in approximately the same region can be noted when comparing the two 3-methylcholanthrene-inducible hepatic P-450 enzymes of rat (P-450c and P-450d) or mouse (P_1-450 and P_3-450) to each other or to the sequences of the major PB-inducible P-450 forms (Yabusaki et al., 1984; Kawajiri et al., 1984; Sogawa et al., 1984; Kimura et al., 1984; Gonzalez et al., 1985a). These observations suggest that the precise amino acid sequence in this portion of the molecule is not critical for the

[3] Morville et al. (unpublished results).

[4] Friedberg et al. (unpublished results).

cytochromes's overall structure or catalytic capacity and therefore that this region may contribute to the unique substrate specificities of the various P-450 forms.

The sequence relationships of pTF-1 and pTF-2 to each other and to PB-4 are highly reminiscent of those exhibited by a group of three rabbit liver cDNA clones isolated by Leighton et al. (1984). These latter clones show 55–60% DNA sequence homology to the rat P-450 PB-4 cDNA and encode proteins with 49–51% homology to rat P-450 PB-4 and 46–49% homology to P-450 LM-2, the major phenobarbital-inducible P-450 enzyme of rabbit liver. P-450 LM-2 shows 76% amino acid homology to rat P-450 PB-4 and is believed to be the orthologous gene product, although given the complexity of relationships between various P-450s in a single species it is difficult to demonstrate this conclusively. Indeed, if rat PB-4 and rabbit LM-2 are truly orthologous gene products, their 24% divergence places them among the most rapidly evolving vertebrate proteins (Leighton et al., 1984). The fact that the pTF-1-encoded protein for which a major portion of the sequence is presented shows only 59–63% homology to the polypeptides encoded by the three rabbit cDnA clones could imply that this polypeptide has evolved significantly more rapidly than the major PB-induced P-450 forms or perhaps that it does not correspond to any of these rabbit cDNA clones.

We have recently isolated a third cDNA clone, designated pAM-7 and related in sequence to pTF-1 and pTF-2, by immunoscreening of the same cDNA library used in this study with antibodies raised against P-450 2c, a male-specific constitutive rat liver P-450 form. The anti-P-450 2c antibodies give a very weak but detectable reaction with colonies expressing the pTF-1- and pTF-2-encoded products and cross-react weakly with purified PB-1 in an ELISA or immunoblotting assay. Sequence analysis of a portion of this cDNA clone (corresponding to nucleic acid residues 555–933 of PB-4) revealed that it is more homologous (75% and 79%, respectively) to rabbit cDNA clones pP-450PBcl and pP-450PBc2 (Leighton et al., 1984) than it is to pTF-1 (66%) or pTF-2 (70% over the region of overlap).[3] Clone pAM-7 thus appears to represent a third member of the PB-1 gene subfamily.

The existence of subfamilies of immunochemically related cytochromes P-450 encoded by genes that are sufficiently similar in sequence as to cross-hybridize to each other clearly makes it difficult to definitively associate a specific cDNA clone with a specific characterized P-450 form. These difficulties are exemplified by the experimental results reported in this paper. First, the antibody raised against purified P-450 PB-1 recognized to a comparable extent two proteins in rat liver microsomes and immunoprecipitated comparable amounts of two electrophoretically separable mRNA translation products, one of which comigrated with purified PB-1. It was perhaps not suprising, therefore, that these antibodies recognized two distinct cDNA-encoded proteins expressed in *Escherichia coli* and that in a hybridization reaction the two cDNAs selected the mRNAs which encode the same two translation products that are immunoprecipitated by the anti-PB-1 antibody. One clone (pTF-2), however, preferentially selects the mRNA which encodes the polypeptide which comigrates with PB-1 whereas the other clone preferentially hybridizes to the mRNA encoding the second, more slowly migrating polypeptide. On the basis of these data, we can conclude that pTF-2 corresponds to PB-1 mRNA or to an mRNA which encodes a very closely related protein, one which is more closely related to PB-1 than is the protein encoded by pTF-1. pTF-1, on the other hand, is likely to correspond to the second as yet unidentified P-450 polypeptide recognized by the anti-PB-1 antibody in immunoblots of rat liver microsomes. Definitive identification of these clones will require comparison of the encoded protein sequences to experimentally determined amino acid sequences which at this time are limited to the amino-terminal region in the case of PB-1 (Waxman & Walsh, 1983) and several other rat liver P-450s (Black & Coon, 1986).

The mRNAs corresponding to cDNA clones pTF-1 and pTF-2 do not appear to be coordinately regulated: mRNA hybridizable to pTF-2 increased 2–4-fold after phenobarbital treatment, whereas mRNA hybridizable to pTF-1 did not show any increase. Moreover, the levels of mRNA hybridizable to pTF-1 were 4 times higher in female as compared to male rats, whereas the levels of mRNA hybridizable to pTF-2 showed no sex differences. Although these hybridization measurements were carried out at relatively high stringency (0.1 × SSC at 65 °C), it is conceivable that the increased hybridization of pTF-1 to mRNA from livers of female rats reflects the presence of a distinct, very closely related female-specific mRNA such as that encoding P-450 2d, a protein which cross-reacts rather strongly with the anti-PB-1 antibodies used in this study (Waxman, 1984). On the other hand, equivalent differences in hepatic mRNA levels in male vs. female rats were observed when an oligonucleotide 22 residues long, specific for the pTF-1 sequence (and differing in 8 residues from the corresponding segments in the pTF-2 and pAM-7 sequences), was used as a specific hybridization probe. These hybridization data conflict somewhat with the mRNA translation results which indicated that the mRNAs encoding both translation products immunoprecipitable with anti-PB-1 antibodies increase after PB treatment and that there were no major sex differences in the relative levels of the mRNAs for the two polypeptides (shown most convincingly for the mRNAs from the phenobarbital-treated rats) (Figure 2). These apparent contradictions may very well reflect the complexity of the P-450 system in which the PB-1 gene subfamily may contain as yet unrecognized expressed members, and where limited regions of extraordinarily high sequence conservation within related genes may not necessarily correspond to the immunodominant determinants responsible for the specificity of a particular preparation of polyclonal antiserum.

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