0026-895X/90/060810-10$02.00/0

MOLECULAR PHARMACOLOGY, 37:810-819

P450IIB Gene Expression in Rat Small Intestine: Cloning of Intestinal P450IIB1 mRNA Using the Polymerase Chain Reaction and Transcriptional Regulation of Induction

PETER G. TRABER, WEI WANG, MICHAEL MCDONNELL, and JORGE J. GUMUCIO

Department of Internal Medicine, Gastroenterology Division, Ann Arbor Veterans Administration Medical Center, and the University of Michigan Medical School, Ann Arbor, Michigan 48109

Received August 22, 1989; Accepted February 9, 1990

SUMMARY

Intestinal cytochromes P450 (P450) may function in the "first pass" metabolism of drugs, the detoxification of xenobiotics, or the activation of carcinogens. However, little is known about the expression of specific P450 genes in intestinal mucosa. We have previously shown that a P450 mRNA that is homologous to rat liver P450IIB1 (P450b) is expressed in rat small intestine and is inducible by phenobarbital, polyhalogenated biphenyls, and organochlorine pesticides. However, there are multiple highly homologous genes in the IIB subfamily and, therefore, studies using liver-derived cDNAs or oligonucleotides based on those cDNAs cannot definitively establish the identity of the intestinal mRNA(s). The polymerase chain reaction was used to enzymatically amplify cDNA synthesized from intestinal and hepatic RNA, and the amplified segments were identified by Southern blot analysis. These studies demonstrated that the amplified segment of the phenobarbital-inducible P450 mRNA in intestine was identical to this same segment of the hepatic P450b mRNA; furthermore, this analysis showed that P450e was not expressed in intestine. To definitively establish the identity of the intestinal mRNA, the full coding sequence of the P450b mRNA was cloned from intestinal and hepatic RNA and sequenced. The sequences of the intestinal and hepatic cDNA were identical and coded for P450b; the deduced protein sequence in the F344 rat differed in one amino acid from the reported sequence in Sprague-Dawley rats and, thus, represents a different allele of the same gene. An increment in intestinal P450b mRNA was detected as early as 1 hr following a single intraperitoneal injection of phenobarbital; this prompt rise in mRNA suggested that transcriptional activation may be the primary mechanism for induction. Nuclear run-on experiments were performed using nuclei isolated from intestinal mucosa 3 and 6 hr following treatment with phenobarbital. The rate of transcription of the P450IIB1 gene was increased approximately 6-fold 6 hr following phenobarbital; this was very similar to the increment in P450b mRNA as measured by quantitative dot blot analysis. Therefore, the predominant mechanism for the induction of P450b mRNA in intestine in response to phenobarbital was an increase in gene transcription. These studies indicate that the same member of the P450IIB subfamily, P450IIB1 or P450b, is expressed and inducible by similar mechanisms in small intestine and liver. Although putative P450b mRNA and apoprotein have been identified in lung and testes, the capacity for induction by phenobarbital, and presumably other xenobiotics, is unique to liver and intestine. In contrast, P450e or P450IIB2, which is expressed in liver and induced by phenobarbital in parallel with P450b, is not expressed in small intestine and, therefore, the tissue-specific expression of these two closely linked genes appears to be regulated by separate mechanisms. Comparison of transcriptional regulation of these genes in liver and small intestine may provide a model to study tissue-specific P450 gene expression.

The intestinal mucosa is exposed to multiple ingested natural and man-made chemicals that include pharmaceutical agents and environmental toxins and carcinogens. The enterocytes lining the small intestinal mucosa contain enzymes capable of metabolizing many of these compounds (1–5). Therefore, the intestine may act as a metabolic organ in series with the liver, the main organ in the body involved in xenobiotic metabolism. As in liver, the microsomal monooxygenase system may be the major pathway for the oxidative metabolism of xenobiotics in small intestinal mucosa. This enzyme system consists of phospholipid, NADPH P450 reductase, and multiple hemoproteins called the P450s that provide for a broad substrate specificity (6–8). The P450 apoproteins are encoded for by a superfamily of related genes that, at present, contains eight families of mammalian genes (9–11). The complement of P450 enzymes that are expressed in any given tissue will, to a large extent, determine the phenotypic metabolic capacities of that tissue. Therefore, the elucidation of the metabolic importance and

This work was supported by National Institutes of Health Grant DK 41393-01 and a Veterans Administration Associate Investigatorship Award to P.G.T. and National Institutes of Health Grant DK 32842-05 and a Veterans Administration Merit Review to J.J.G.

ABBREVIATIONS: P450, cytochrome P450; DTT, dithiothreitol; bp, base pair; DMBA, 7,12-dimethylbenz[*a*]anthracene; TES, *N*-tris[hydroxymethyl] methyl-2-aminoethanesulfonic acid; SDS, sodium dodecyl sulfate.

tissue-specific regulation of extrahepatic P450s will depend initially on identification of the specific P450 forms expressed in that tissue. However, because of overlapping substrate specificities and the marked homology of P450 genes in certain subfamilies, extrahepatic P450s cannot be definitively identified using enzyme assays, antibodies to hepatic P450 apoproteins, or, in some cases, cDNA probes isolated from liver (11).

The P450IIB gene subfamily in rat appears to contain 9–11 highly homologous genes (12). In liver, it has been firmly established that P450IIB1 (P450b) and P450IIB2 (P450e) mRNAs and functional hemoproteins are expressed and are induced by phenobarbital as well as multiple other xenobiotics (11, 12); these two P450 genes are 97% similar in both amino acid and cDNA nucleotide sequence (12). Labbe and co-workers (13) have cloned a full length cDNA, designated P450IIB3, that is constitutively expressed in male and female rat liver and is not inducible by phenobarbital; a portion of this cDNA is identical to gene 5 described by Atchison and Adesnik (14). Recently, another member of this gene subfamily, P450IIB4, was cloned from rat liver; the 4.3-kilobase mRNA was expressed at low levels in liver and was induced by phenobarbital approximately 6-fold (15). As yet, the apoproteins encoded for by either P450IIB3 or P450IIB4 mRNA have not been isolated from rat liver, nor have the cDNAs been expressed in yeast or mammalian cell lines to determine whether they code for functional P450s. Although these four genes are the only P450IIB genes that have been demonstrated to be expressed in liver, it is possible that other genes in the P450IIB subfamily are expressed in small intestine or other extrahepatic tissues.

We previously reported that members of the P450IIB subfamily were expressed in rat small intestine, based on molecular hybridization analysis using cDNA and oligonucleotide probes (16). P450b-like mRNA was identified in rat small intestinal mucosa and was inducible following treatment with phenobarbital, polyhalogenated biphenyls, and organochlorine pesticides (16). P450e-like mRNA appeared by Northern blot analysis to be present constitutively in small amounts but was not inducible (16) (the studies described in this report were unable to confirm the low level constitutive expression of P450e in small intestine). Therefore, our previous work suggested that these two closely linked genes, which are induced by phenobarbital in parallel in liver, are regulated differently in small intestine. However, because of the number of highly homologous genes in the P450IIB subfamily, the identity of mRNAs expressed in intestine cannot be definitively established using liver-derived cDNAs or oligonucleotide probes based on those cDNAs. In fact, the oligonucleotide probe used to identify P450b-like mRNA in our previous study (16) has the identical sequence as three other rat genes identified by Atchison and Adesnik (genes 2, 3, and 4) (14) and the newly described P450IIB4 (15).

The purpose of this study was to definitively identify the P450IIB genes that are expressed in rat small intestine and then to investigate the molecular mechanisms responsible for induction of expression by phenobarbital. To accomplish this, we used the polymerase chain reaction (17) to amplify specific sequences of either P450b or P450e mRNAs from small intestinal RNA samples. Oligonucleotide primers were used to enzymatically amplify cDNA that had been synthesized from intestinal RNA (18). The identity of the amplified segments was confirmed by Southern blot analysis, using oligonucleotides homologous to DNA sequences internal to the amplified segments. Following this analysis, the full coding sequence of the P450IIB cDNA was amplified from intestinal and hepatic mRNA, cloned, and sequenced. From these studies it was concluded that P450b mRNA is expressed and inducible in small intestine and that P450e mRNA is not expressed in intestinal mucosa. Nuclear run-on studies demonstrated that the predominant mechanism for the induction of P450b mRNA was an increase in gene transcription.

Experimental Procedures

Materials

Guanidinium isothiocyanate, cesium chloride, T_4 polynucleotide kinase, Klenow enzyme, and T_4 ligase were obtained from Bethesda Research Laboratories (Gaithersburg, MD). [^{32}P]dCTP (>3000 Ci/mmol), [^{32}P]ATP (>5000 Ci/mmol), ^{35}S-dATP (1000 Ci/mmol), Hybond-N nylon membranes, and Hybond-M polyuridine chromatography paper were obtained from Amersham Corporation (Arlington Heights, IL). Avian myoblastosis virus (AMV) reverse transcriptase, all restriction enzymes, oligothymidylate 15, and dideoxynucleotides were obtained from Boehringer Mannheim Biochemicals (Mannheim, FRG). Taq polymerase was purchased from Perkin Elmer-Cetus Corporation (Norwalk, CT). Oligonucleotides were synthesized by the University of Michigan Gastrointestinal Peptide Hormone Center's oligonucleotide synthesis facility. Nu Sieve, Se Kem, and Se Plaque agaroses were obtained from FMC Biochemical (Rockland, ME). *Escherichia coli* RNA was purchased from Calbiochem. All other chemicals were obtained from Sigma Chemical Company (St. Louis, MO).

Animals

Male F344 rats weighing 200–250 g were used in these studies. In some experiments, as indicated in the text, Sprague-Dawley rats were used. The animals were housed in wire-floored cages and had free access to standard laboratory rat chow and water. In our previously reported work (16), we used only Sprague-Dawley rats. In this study, we used Fisher 344 rats, a genetically more homogeneous strain of rat, to facilitate comparisons between animals. Studies were performed to establish that F344 rats expressed P450IIB genes in the same fashion as previously reported for Sprague-Dawley rats.[1] Animals were treated with phenobarbital (80 mg/kg) administered as a single intraperitoneal dose and were killed at the times specified for each experiment. Control animals (or "untreated" rats) were injected with 0.15 M NaCl.

RNA Extraction

Livers and small intestines were removed from rats that had been anesthetized by ether inhalation. A portion of each liver was immediately frozen in liquid nitrogen. Each small intestine was thoroughly washed with ice-cold 0.15 M NaCl and opened longitudinally, and the mucosa was removed by scraping with a glass slide, as previously described (16). Total RNA was extracted from the tissues using a modification of the technique of Chirgwin *et al.* (19), as previously described (16). In some experiments, RNA was extracted as described by Chomczynski and Sacchi (20). Polyadenylated RNA was isolated from aliquots of total RNA using polyuridine paper chromatography (21), as previously described (16).

DNA Probes

A full length P450b cDNA, clone pSP450, and a P450e cDNA corresponding to exons 6–9 of the P450e gene, clone R17 (22), were gifts of M. Adesnik (New York University). A P450c cDNA probe, p210, that was cloned from rat liver was gift of Dr. J. Fagan [Fairfield, IA (23)]. A full length P450 reductase cDNA, pSP65-OR, that was

[1] Unpublished data.

cloned from rat liver was a gift of Dr. C. Kasper [Madison, WI (24)]. Control cDNAs used in nuclear run-on experiments included a human β-actin cDNA, pHFBA-1 (25), and an 18 S ribosomal RNA cDNA (26), both gifts of Dr. Craig Thompson (University of Michigan). The cDNAs that were used in Northern or dot blot analyses were isolated from their plasmid vectors and radiolabeled with [^{32}P]dCTP using a random-primed oligonucleotide labeling method (27), as previously described (16). Oligonucleotides were 5′-labeled with [^{32}P]ATP using T_4 polynucleotide kinase, as previously described (16). Unincorporated nucleotides were removed from the labeled DNA using Sephadex G-25 or G-50 chromatography (Nick columns; Pharmacia, Uppsala, Sweden).

Northern Blot Analysis

Samples of total or polyadenylated RNA were size separated by electrophoresis in 2.2 M formaldehyde-agarose gels. The RNA was transferred to Hybond-N (Amersham) nylon membranes by an electroblotting method and cross-linked to the membranes using UV radiation (28). The membranes were prehybridized, hybridized, and washed according to the membrane manufacturer's recommendations.

Quantitative Dot Blot Hybridizations

Dot blots were performed as previously described (16), except that Hybond-N membranes were used and the RNA was fixed to the membranes by UV radiation cross-linking. Following autoradiography, the blots were quantitated using a Betascope 603 Blot Analyzer (Betagen Corporation).

Enzymatic Amplification and Identification of mRNA

cDNA synthesis. cDNA was synthesized from either total or polyadenylated RNA using oligothymidylate (15) (1 μg) as a primer or random hexamer primers (Promega Biotech, Madison WI) and AMV reverse transcriptase (13 units/reaction), in a total reaction volume of 50 μl containing 50 mM Tris·HCl (pH 8.3), 50 mM KCl, 8 mM $MgCl_2$, 10 mM DTT, and 0.5 mM levels each of dATP, dCTP, dGTP, and dTTP. The reaction mixture, without the reverse transcriptase, was incubated at 41° for 15 min; the reverse transcriptase was then added and the reaction was allowed to proceed for 1 hr at 41°.

Polymerase chain reaction. Segments of the P450b or P450e cDNAs were enzymatically amplified using oligonucleotide primers and thermostable DNA polymerase isolated from the bacterium *Thermus aquaticus* (Taq polymerase), as described by Saiki *et al.* (17). Twenty microliters of the cDNA synthesis reaction mixture were amplified using 1 μM levels of each oligonucleotide primer (20-mers) and 2.5 units of Taq polymerase, in a total reaction volume of 100 μl containing 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 2 mM DTT, 0.01% gelatin, and 200 μM levels each of dATP, dCTP, dGTP, and dTTP. The reaction mixture was overlayed with 100 μl of mineral oil and subjected to 30 cycles of amplification. The samples were heated to 94° for 1 min in a programmable heating block (Perkin Elmer-Cetus), cooled to a specific annealing temperature, and held there for 2 min; temperatures of annealing will be presented in Results. The temperature was then increased to 72° and held there for 3 min to allow DNA synthesis by the polymerase to proceed. Following the final cycle, the temperature was held at 72° for an additional 10 min to allow for complete extension of primed DNA segments.

Southern blot analysis of polymerase chain reaction products. Aliquots of the polymerase chain reaction mixture were electrophoresed in 3% Nu Sieve and 1% Sea Kem agarose gels containing ethidium bromide (0.5 μg/ml), using a Tris/borate/EDTA buffer. After photography, the DNA was electrophoretically transferred to Hybond-N nylon membranes and the DNA was bound to the nylon using UV radiation cross-linking. The membranes were prehybridized in a solution containing 5% sodium dodecyl sulfate, 5× standard saline citrate, and 100 μg/ml sonicated salmon sperm DNA. Hybridizations were performed in the same solution that contained 1.5×10^6 cpm/ml of 5′-labeled oligonucleotide probe. The hybridization was performed at the hypothetical temperature of dissociation of DNA-DNA hybrids (29) for the oligonucleotide to maximize the specificity of hybridization. The membranes were washed in 1× standard saline citrate and 5% sodium dodecyl sulfate at the temperature of hybridization, and autoradiography was performed with Kodak X-Omat film between two intensifying screens.

Ligation Reactions

In some experiments, the polymerase chain reaction primers contained restriction endonuclease sites to facilitate the cloning of amplified segments into plasmid vectors. The polymerase chain reaction product was digested with the appropriate endonucleases and then separated in a low melting point agarose gel (Sea Plaque). The plasmid vector was also digested and separated in a low melting point agarose gel. Ligations were performed "in gel" using T_4 ligase (BRL), as described by Crouse *et al.* (30). The ligations were incubated for 4 hr at room temperature, diluted 5-fold with water, and then transformed into competent bacteria (XL1-Blue; Stratagene).

DNA Sequencing

Double-stranded DNA templates were sequenced using the dideoxynucleotide termination method (31). Purified plasmid DNA was stably denatured, as described by Hattari and Sakaki (32), and sequencing was performed using modified T_7 DNA polymerase (Sequenase 2.0; United States Biochemical, Cleveland, OH) and ^{35}S-dATP, as described by the manufacturer.

Nuclear Run-on Assay

The method used was adapted from several published reports (33–36). Nuclei were isolated by modifying and combining the methods of Thompson *et al.*, (33), McDonald and Goldfine (34), and Bloor *et al.*, (36). Mucosal scrapings were washed twice in ice-cold 0.15 M NaCl, 5 mM DTT, and resuspended in nuclei lysis buffer (10 mM Tris·HCl, pH 7.4, 10 mM NaCl, 5 mM DTT, 2% Nonidet P-40). This suspension was homogenized in a Dounce homogenizer, using 10 strokes of the loose pestle, and centrifuged at 800 × *g* and 4° for 10 min. The pellet was resuspended in nuclei lysis buffer, homogenized, and centrifuged again in the same fashion. The resulting pellet was resuspended in sucrose buffer (2.4 M sucrose, 1 mM $CaCl_2$, 5 mM DTT) by vortexing and was centrifuged at 50,000 × *g* at 4° for 1 hr. The pellet containing the purified nuclei was resuspended in nuclei storage buffer (40% glycerol, 50 mM Tris·HCl, pH 8.3, 0.1 mM EDTA, 5 mM $MgCl_2$) and an aliquot was counted in a hemacytometer and frozen at −80°. This method yielded intact nuclei by phase contrast microscopy.[2]

Transcription reactions were initiated by addition of 60 μl of 5× run-on buffer (25 mM Tris·HCl, pH 8.0, 12.5 mM $MgCl_2$, 750 mM KCl, 1.25 mM levels, each of ATP, GTP, and CTP) and 30 μl of [^{32}P]UTP (>3000 Ci/mmol, 10 μCi/μl) to 210 μl of nuclei storage buffer containing 5×10^7 nuclei (33, 34, 36). Following incubation at 30° for 30 min, the labeled RNA transcripts were isolated as described by Celano *et al.* (37).

Plasmids containing the cDNAs of interest were denatured by heating in 0.2 M NaOH and were applied to nitrocellulose, using a Minifold II slot blot device (Schleicher and Scheull) as described (33). The following cDNAs were used: pSBF1 (full coding sequence of P450b, cloned as described in this manuscript), p210 (P450c cDNA probe), pSP450OR (P450 reductase cDNA), pHFBA-1 (human β-actin cDNA), prA (human 18 S ribosomal RNA cDNA), and the bacterial vector pGEM, which served as a negative control for nonspecific binding. The nitrocellulose containing the bound cDNAs were prehybridized at 60° for 8 hr in a solution containing 10 mM TES, pH 7.4, 0.2% SDS, 10

[2] Since the completion of these studies, the method of nuclei isolation has been altered. Although both methods yielded nuclei that were competent in nuclear run-on assays, the revised method provides for a significantly improved yield of nuclei. In the revised method, Nonidet P-40 was omitted from the nuclei lysis buffer and the homogenization was changed to 10 strokes with the loose pestle and 10 strokes with the tight pestle (the homogenization was performed only once). The remainder of the protocol was not altered.

mM EDTA, 0.3 M NaCl, 1 × Denhardt's solution, and 5 μg/ml *E. coli* RNA. This solution was replaced with 2 ml of the same solution containing 1 × 10^7 cpm/ml labeled RNA transcripts and hybridization continued for 36 hr (cpm were equalized for each sample of nuclei). The membranes were washed in three changes of 0.1× standard saline citrate and 0.1% SDS, at the hybridization temperature. Following autoradiography, the blots were quantitated using a Betascope 603 Blot Analyzer.

Results

Specificity of the polymerase chain reaction primers. The oligonucleotide primers used for the polymerase chain reaction were designed using oligonucleotides described by Omiecinski *et al.* (38) and sequences published by Kumar *et al.* (22) for P450e and by Suwa *et al.* (39) for P450b. The amplified segments of either P450b or P450e were 200 bp in length and were located in exons 7 and 8 of the respective genes (Fig. 1). The location of the primers was chosen to include intron 7 so that an amplified segment of genomic DNA, which might contaminate the RNA samples, could be differentiated from amplified mRNA by its larger size (500 bp versus 200 bp). The primer located in exon 8, designated B/E, had the same sequence for both P450b and P450e and, thus, was a common primer used in each amplification reaction. The primers located at the 5′ end of the amplified segment, located in exon 7, were those designed by Omeicinski *et al.* (38); the sequence of the oligonucleotide primer for P450b (B) differed from the sequence of the primer for P450e (E) by four nucleotides (Fig. 1). The primer for P450b was homologous to sequences located in genes 2, 3, and 4 described by Adesnik and Atchison (12) and to P450IIB4 (15); therefore, the B primer would also amplify these gene products if expressed in the tissue from which the RNA was extracted. Neither of the 5′ primers would hybridize to the P450IIB3 mRNA described by Labbe *et al.* (13).

The specificity of the polymerase chain reaction primers was tested using known cDNA templates for P450b and P450e. The P450b (pSP450) and P450e (R17) cDNAs were isolated from their plasmid vectors and used as polymerase chain reaction templates to investigate the specificity of the primers and to define the optimum reaction conditions. Ten picograms of template cDNA were mixed with 1 μg of sonicated salmon sperm DNA and amplified by the polymerase chain reaction. When annealing of primers was performed at 55–65° and 30 cycles of the polymerase chain reaction were completed, there was evidence that the P450b cDNA was amplified to a small extent by the P450e-specific primer (data not shown). On the other hand, at these annealing temperatures the P450b primer was completely discriminatory and did not amplify the P450e cDNA template. When the annealing step was performed at 72°, both B and E primers were highly specific for P450b and P450e templates, respectively (Fig. 2). Therefore, the amplifications for the remainder of the experiments were performed by denaturation at 94° for 1 min, followed by primer annealing and extension at 72° for 3 min. Primer annealing of 20-mers at such a high temperature possibly diminishes the efficiency of the amplification reaction but was necessary for the required specificity.

Amplification of hepatic and intestinal mRNA. For these experiments, amplified hepatic and intestinal RNA samples were compared on the same agarose gel with amplified P450b and P450e cDNA templates and amplified rat genomic DNA. Total and polyadenylated RNA was isolated from rat liver and from the proximal third of the small intestinal mucosa 12 hr following a single intraperitoneal injection of phenobarbital (80 mg/kg). Aliquots of polyadenylated RNA from liver and small intestine were used to synthesize cDNA and were then amplified with the primers as described above. Fig. 3A shows the agarose gel of the polymerase chain reaction products of rat genomic DNA, the P450b and P450e cDNA templates, and the liver and small intestinal cDNA. When rat liver DNA was amplified with either the B or E primer, a single band of 500-bp size was found (Fig. 3A, *lanes 2* and *4*, respectively). As expected, a product of 200-bp length was seen when liver cDNA was amplified with either the B or the E primer. This indicated that both P450b and P450e mRNA were expressed in liver, as previously described by multiple investigators. In small intestine, a product of 200 bp was also amplified using the B primer; when the E primer was used, no reaction product was evident.

The amplified product was identified using oligonucleotide probes that were designed to hybridize to sequences of either P450b or P450e nested within the amplified segments (sequences and location of oligonucleotides, designated B.2 and E.2, are shown in Fig. 1). The B.2 oligonucleotide was designed to differentiate P450b from P450IIB3, P450IIB4, and genomic clones 5, 16, and 25. As seen in Fig. 3B, the B.2 oligonucleotide hybridized to the B-amplified genomic sequences, the amplified P450b cDNA template, and the B-amplified product of both

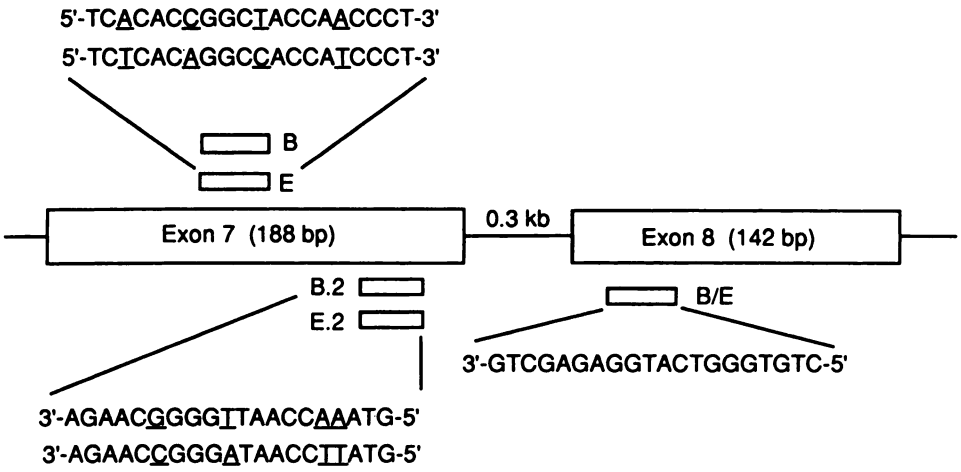

Fig. 1. Description of the polymerase chain reaction primers and oligonucleotide probes for P450b and P450e. This diagram depicts exons 7 and 8 of the P450b and P450e genes. The primer designated B/E is located in exon 8 and has the same sequence as both P450b and P450e; thus, this oligonucleotide was a common primer used in each polymerase chain reaction. The 5′ primers located in exon 7 differed by four base pairs (*underlined*); B and E for P450b and P450e, respectively. The predicted size of the polymerase chain reaction product from either P450b or P450e mRNA was 200 bp. In contrast, the predicted size of amplified genomic DNA was 500 bp. The oligonucleotides B.2 and E.2 were designed to be homologous to sequences in exon 7 and within the amplified segments for P450b and P450e, respectively.

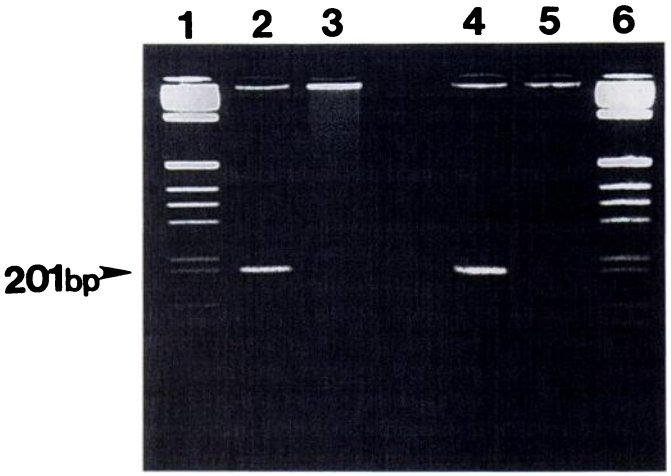

Fig. 2. Amplification of P450b and P450e cDNAs. The insert cDNAs of pSP450 (full length P450b cDNA) and R17 (cDNA of exons 6–9 of P450e) were isolated from the plasmid vectors and amplified using the polymerase chain reaction (see Experimental Procedures) and the primers described in Fig. 1. Ten picograms of cDNA were mixed with 1 μg of salmon sperm DNA and subjected to 30 cycles of the polymerase reaction as follows: denaturation at 94° for 1 min and annealing and extension at 72° for 3 min. Eight microliters of the products were separated in a 1% Sea Kem, 3% Nu Sieve agarose gel containing ethidium bromide and were photographed under UV illumination. *Lanes 1* and *6*, 1-kb DNA ladder (BRL, Bethesda, MD); *lane 2*, P450b cDNA amplified with the B and B/E primers; *lane 3*, P450b cDNA amplified with the E and B/E primers; *lane 4*, P450e cDNA amplified with the E and B/E primers; *lane 5*, P450e cDNA amplified with the B and B/E primers. *201 bp*, size of the marker in the DNA ladder.

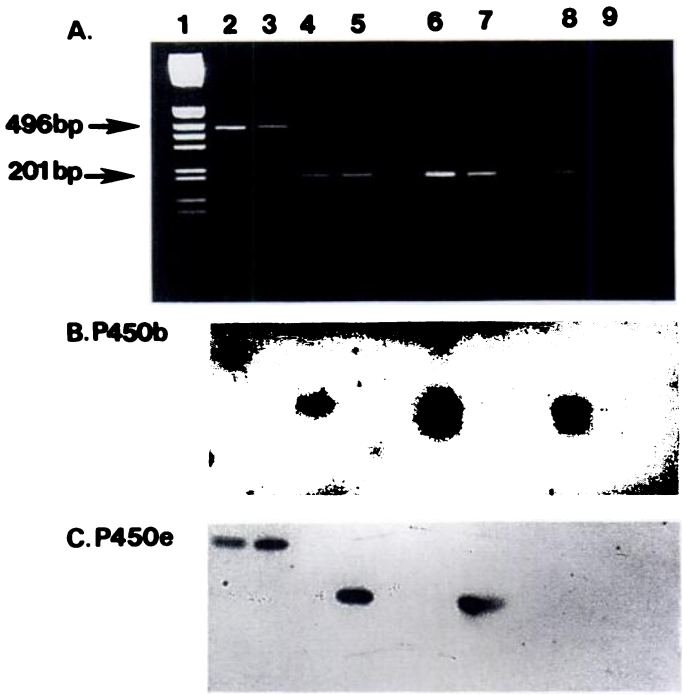

Fig. 3. Amplification of P450b and P450e mRNA. Polyadenylated RNA was extracted from liver and proximal small intestinal mucosa of rats treated with a single dose of phenobarbital (80 mg/kg) and killed 12 hr later. DNA was synthesized from 1 μg of polyadenylated RNA, as described in Experimental Procedures, and then amplified using the polymerase chain reaction primers described in Fig. 1. In addition, rat liver genomic DNA was amplified. A, The polymerase chain reaction products were separated in a 1% Sea Kem, 3% Nu Sieve agarose gel that contained ethidium bromide and were photographed under UV illumination. *Lane 1*, 1-kb DNA ladder (BRL); *lane 2*, rat liver genomic DNA (1 μg) amplified with B and B/E primers; *lane 3*, rat liver genomic DNA (1 μg) amplified with the E and B/E primers; *lane 4*, P450b cDNA amplified with the B and B/E primers; *lane 5*, P450e cDNA amplified with the E and B/E primers; *lane 6*, rat liver polyadenylated RNA (1 μg) from rats treated with phenobarbital 12 hr before sacrifice, reverse transcribed and amplified with the B and B/E primers as described in Experimental Procedures; *lane 7*, rat liver polyadenylated RNA (1 μg) from rats treated with phenobarbital 12 hr before sacrifice, reverse transcribed and amplified with the E and B/E primers as describe din Experimental Procedures; *lane 8*, rat small intestinal polyadenylated RNA (1 μg) from rats treated with phenobarbital 12 hr before sacrifice, reverse transcribed and amplified with the B and B/E primers as described in Experimental Procedures; *lane 9*, rat small intestinal polyadenylated RNA (1 μg) from rats treated with phenobarbital 12 hr before sacrifice, reverse transcribed and amplified with the E and B/E primers as described in Experimental Procedures. B, The gel in A was transferred to a nylon membrane and hybridized with ^{32}P-labeled B.2 oligonucleotide (Fig. 1). C, The membrane used in the hybridization of B was stripped of the probe and rehybridized with ^{32}P-labeled E.2 oligonucleotide (Fig. 1).

liver and small intestinal RNA. There was no cross-hybridization with the amplified polymerase chain reaction products using the E primer. The filter shown in Fig. 3B was stripped to the B.2 oligonucleotide probe and rehybridized with the E.2 oligonucleotide (Fig. 3C). The E.2 probe hybridized to both B- and E-amplified genomic sequences, the amplified P450e cDNA template, and the E-amplified polymerase chain reaction product of liver. Even after extended periods of exposure, no hybridization was detected in Fig. 3C, *lane 9*, indicating that no P450e mRNA was present in the small intestinal RNA samples. The hybridization of the E.2 oligonucleotide to genomic sequences amplified by the B primer was expected, because the E.2 oligonucleotide is also homologous to gene 3 described by Atchison and Adesnik (14), which would also be amplified by the B primer.

When the B.2 oligonucleotide was hybridized to the polymerase chain reaction products in *lanes 6* and *8* Fig. 3B, two bands were obtained, one at the expected 200-bp location and another band representing a lower molecular weight DNA. Hybridization with strand-specific oligonucleotides indicated that this was due to synthesis of both double- and single-stranded DNA, because asymmetric amounts of primers had been inadvertently added to the polymerase chain reaction (Ref. 40 and data not shown).

Because of the high temperature of annealing of the primers and the resultant potential decrease in efficiency of amplification, it was possible that a small amount of P450e mRNA was present and yet not detected. Therefore, the annealing reaction was performed at 55° and the amplification was continued for 30 cycles. Southern blot analysis of the polymerase chain reaction products still failed to identify any P450e mRNA amplified in small intestinal mRNA (data not shown.).

Further experiments were conducted to determine whether P450e may be expressed in the small intestine in a nonpolyadenylated form. Fig. 4 shows an agarose gel of products of a polymerase chain reaction that amplified cDNA-generated total RNA that was reverse transcribed using random hexamer primers rather than oligothymidylate. The RNA used was from both Sprague-Dawley and F344 rats treated with three daily doses of phenobarbital, to determine whether there were either strain differences or effects of more prolonged induction. P450e mRNA was amplified from liver cDNA, but there was no amplified DNA present in the reactions containing cDNA from the intestine of either rat strain. From these experiments, one

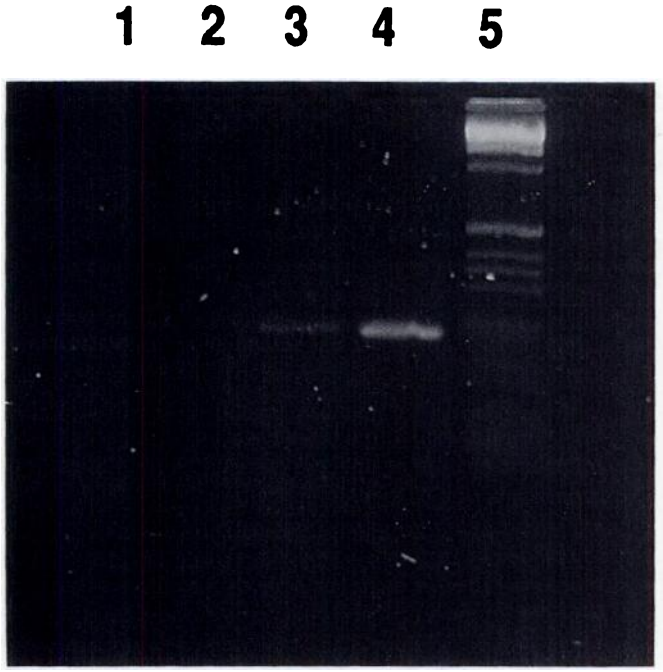

Fig. 4. Amplification of P450e using cDNA that was synthesized from total RNA using random primers. This an ethidium bromide-stained 3% Nu Sieve, 1% Sea Kem agarose gel of P450e amplified products. *Lane 1*, intestinal RNA from Sprague-Dawley rat that was treated with three daily doses of phenobarbital, reverse transcribed with random primers and amplified with the E-specific primers; *lane 2*, intestinal RNA from a F344 rat that was treated with three daily doses of phenobarbital, reverse transcribed with random primers and amplified with the E-specific primers; *lane 3*, liver RNA from a F344 rat that was treated with three daily doses of phenobarbital, reverse transcribed with random primers and amplified with the E-specific primers; *lane 4*, R17 cDNA that was amplified with the E-specific primers; *lane 5*, DNA size markers.

can conclude that P450e is not expressed in small intestinal tissue in either a polyadenylated or nonpolyadenylated form.

Cloning and sequencing of the full length intestinal P450IIB and mRNA. The analysis described above strongly suggested that P450b mRNA was expressed in small intestine; however, the amplified segment could conceivably have been derived from another, as yet unidentified, member of the P450IIB gene family. To definitively identify the intestinal P450IIB mRNA, primers were designed to amplify the nearly full length mRNA containing the entire coding sequence of the P450b apoprotein. The primer at the 5′ end of the mRNA contained a *Not*1 restriction endonuclease site and the primer on the 3′ end of the mRNA contained a *Xba*I restriction site.

5′ primer: 5′-
GTGGCGGCCGCACCGTGGTTACACCAGGACCATGGAG
CCCA-3′

3′ primer: 5′-
CACTCTAGAAGGCCATTCCCAACAGAACTGGGGCAC
ATG-3′

Intestinal and hepatic RNA (1 μg) was reverse transcribed as described under Experimental Procedures and 30 cycles of the polymerase chain reaction were completed (94° for 1 min and 72° for 3 min). The reaction product from liver and intestinal RNA contained a single fragment of DNA, approximately 1600 bp in size (data not shown). The 1600-bp fragment was ligated into a pBluescript KS−plasmid (Stratagene, La Jolla, CA) and sequenced. Both strands were sequenced using a series of oligonucleotide primers (17-mers). Eleven full length cDNAs from two separate animals were cloned from intestinal RNA and sequenced. In addition, nine full length cDNAs were cloned from hepatic RNA obtained from the same animals. Multiple clones from different animals were sequenced to help identify potential mistakes in base incorporation by the polymerase chain reaction. Each cDNA sequenced from the liver and small intestine had the identical nucleotide sequence, which differed in only one base from the published sequence of the P450b cDNA (41).[3] The full length cDNA encodes a protein of 491 amino acids in length that is identical to P450b, except it codes for a glycine in position 192 instead of aspartate as originally reported (41, 42). Because there is no protein sequence data published for this amino acid (42), it is difficult to determine whether there was a cloning or sequencing error in the original report (41) or whether this represents an allelic difference between strains of rats. Because it has been shown that there are minor allelic differences in certain strains of rats (43), this represents the most likely possibility.

Time course of induction of intestinal P450b mRNA. In our previous studies on the induction of intestinal P450b mRNA in response to chemical inducers, rats were administered the compounds in four daily doses and studied approximately 20 hr following the final dose (16). To begin investigations into the mechanism for the increase in steady state intestinal P450b mRNA levels, a time course of P450b mRNA induction was performed following a single dose of phenobarbital. Rats were administered a single intraperitoneal injection of phenobarbital (80 mg/kg) and were killed 1, 3, 6, and 12 hr later. RNA was extracted from liver and the proximal 20 cm of the small intestinal mucosa (beginning 10 cm past the pylorus) from each rat, and Northern blots were performed using total RNA and RNA hybridized with ^{32}P-labeled R17 cDNA. The Northern blot of total intestinal RNA shown in Fig. 5A demonstrates that a 2.1-kb mRNA species, corresponding to the expected size of mature P450b mRNA (16), was increased as early as 1 hr following injection of phenobarbital and was markedly induced by 6 hr.

Quantitative dot blots were performed and quantitated by directly counting β emmisions from the membrane using a Betascope 603; results were recorded as number of counts over 30 min (minus background counts) per μg of RNA. The number

[3] It should be noted that there are several discrepancies between the sequence of the P450b cDNA (30) and the sequence of the P450b gene (28) as reported by the same investigators using the same strain of rat. These discrepancies are also duplicated in the Genbank data files. The sequence of the cDNA correlates with the protein sequence data (31) in those areas where the protein has been sequenced. In addition, our full length cDNA differs in only one nucleotide from the published cDNA sequence (30). The published genomic sequence (28) has differences when compared with the cDNA; some of the differences encode for different amino acids and this is not indicated in the manuscript. The differences are at the following locations in the sequence:

Base number	cDNA	Amino acid	Gene	Amino acid
66	C	Leu	G	Leu
662	C	Thr	G	Arg
663	C	Thr	G	Arg
1030	T	Ile	C	Val
1119	C	Gln	A	His
1223	T	Leu	G	Arg
1292	T	Asp	C	Asp
1295	C	Ala	T	Val
1296	C	Ala	T	Val
1299	T	Asn	C	Asn
1349	G	Arg	A	His

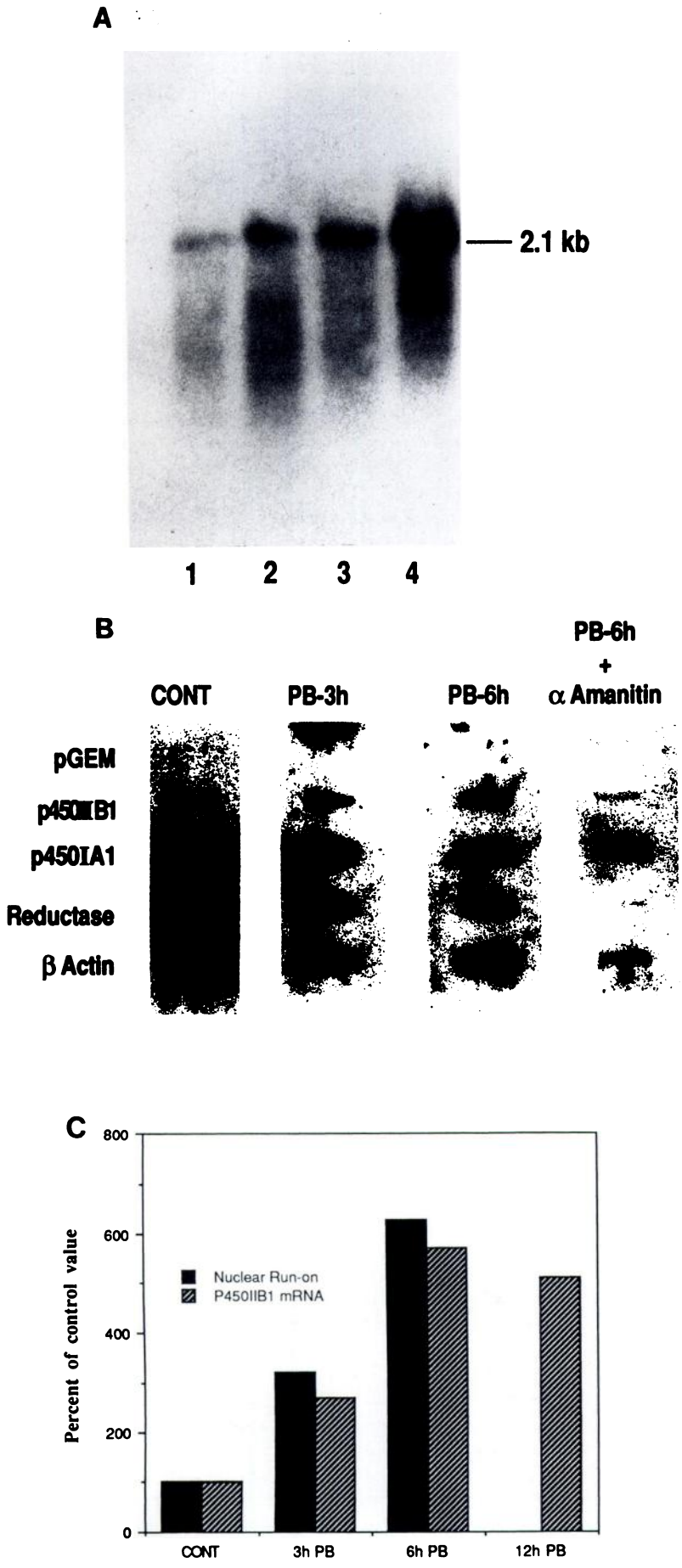

Fig. 5. A, Northern blot analysis of time course for induction of P450b mRNA in small intestinal mucosa. Ten micrograms of total RNA isolated from proximal small intestine were separated in a 1% agarose gel, transferred to nylon membrane, and hybridized with ^{32}P-labeled P450IIB1 cDNA that was cloned as described in Results. *Lane 1*, untreated rat; *lane 2*, rat treated with phenobarbital (80 mg/kg intraperitoneally) and killed 1 hr later; *lane 3*, rat treated with phenobarbital (80 mg/kg intraperitoneally) and killed 3 hr later; *lane 4*, rat treated with phenobarbital (80 mg/kg intraperitoneally) and killed 6 hr later. B. Nuclear run-on assay. Untreated rats and rats treated with phenobarbital (80 mg/kg intraperitoneally) were used to isolate nuclei from jejunal mucosa. These nuclei were used for *in vitro* transcription and the labeled transcripts were hybridized to bound plasmids containing cDNAs, as described in Experimental Procedures. *CONT*, nuclei from untreated rats; *PB-3h*, nuclei from rats treated with a single intraperitoneal dose of phenobarbital and killed 3 hr later; *PB-6h*, nuclei from rats treated with a single intraperitoneal dose of phenobarbital and killed 6 hr later; *PB-6h + αamanitin*, same nuclei as PB-6h, pretreated with 2 μg/ml amanitin before transcription reaction. *pGEM*, plasmid vector without cDNA insert; p450IIB1 (P450b), pSBF-1, full coding sequence of P450b, as described in Results; *p450IA1*, p210, P450c-specific probe; *Reductase*, pSP65 OR, full length P450 reductase cDNA; *βActin*, pHFBA-1, human actin cDNA. C, Quantitative comparison of nuclear run-on with mRNA levels. The nuclear run-on blots that are shown in B were quantitated using a Betascope 603 and the results were normalized for actin activity; the induced levels are expressed as a percentage of the values found in untreated nuclei. The levels of P450b (P450IIB1) mRNA determined by quantitative dot blot analysis, as reported in Results, have been normalized for values in untreated animals (for each time point $n = 3$ animals).

of counts bound to small intestinal RNA isolated from untreated animals was 265 ± 8 counts/μg of RNA (three experiments). Following treatment with phenobarbital this increased to 709 ± 98 counts/μg of RNA at 3 hr ($p < 0.011$ versus untreated), 1516 ± 142 counts/μg of RNA (three experiments) at 6 hr ($p < 0.0001$ versus untreated), (three experiments), and 1342 ± 237 counts/μg of RNA (three experiments) at 12 hr ($p < 0.011$ versus untreated). Hence, P450b mRNA was induced approximately 6-fold at 6 hr following a single dose of phenobarbital and did not increase further by 12 hr. Furthermore, this degree of induction was not appreciably greater than that observed after 4 consecutive days of phenobarbital treatment (16).

Nuclear run-on assays. The results of the time course of induction suggested that the increment in P450b mRNA might be due to an increase in gene transcription. Therefore, nuclei were isolated from intestinal mucosa of untreated rats and of rats 3 and 6 hr following treatment with phenobarbital (80 mg/kg); initiated transcripts were labeled and hybridized to membrane-bound cDNAs as described in Experimental Procedures. As seen in Fig. 5B, there was no appreciable difference between control and 3- and 6-hr-treated nuclei in the transcription rate of β-actin, P450 reductase, or P450IA1 genes. In contrast, there was a marked increase in the transcription of the P450IIB1 gene in the nuclei from the phenobarbital-treated rats. Treatment of the nuclei with α-amanitin (2 μg/ml), which inhibits RNA polymerase II activity, markedly inhibited labeling of the transcripts; it is unknown why P450IA1 gene transcription was only moderately inhibited. The plasmid used for binding P450IA1 (P450c) has been shown to be highly specific for P450c and does not cross-react with the other member of the P450I family, P450IA2 (P450d). In addition, the relatively high level of transcription of the P450c gene, as noted in these experiments, correlates with constitutive expression of P450c mRNA in untreated rats on standard rat chow diets.[1] The filters were counted using the Betascope 603 and the degree of induction of transcription, as measured by nuclear run-on, was compared with the induction of mRNA measured using dot blot analysis (Fig. 1C). This demonstrated that the increment in transcription closely paralleled the increase in P450b mRNA.

Discussion

These studies demonstrated that the major phenobarbital-inducible P450IIB mRNA in rat small intestinal mucosa is P450b or P450IIB1, the same P450 that is inducible in liver. P450b-like mRNA has been identified in other rat extrahepatic tissues including lung and testes, using oligonucleotide probes (44), as we had shown for intestine (16). However, the oligonucleotide used to identify P450b mRNA in our previous work (16) as well as in the other study (44) had sequences that would cross-hybridize with at least four other P450IIB genes (14, 15). The direct sequencing of intestinal P450b mRNA as presented here represents the first definitive proof that P450b is expressed in extrahepatic tissues. In addition, P450b mRNA is markedly induced in intestine by phenobarbital and other inducers and is predominantly polyadenylated (16). In contrast, the putative P450b mRNA (44) and P450b apoprotein (45) identified in other tissues are not inducible by phenobarbital (44, 45) and the RNA is not extensively polyadenylated (44).

These experiments further indicate that P450e is not expressed in small intestine and, combined with data from other tissues as reported by Omiecinski (44), lend support to the concept that P450e is liver specific. In previous work, it was suggested that P450e-like mRNA was expressed in small intestinal mucosa in constitutive levels but was not inducible by phenobarbital or other chemical inducers (16). The hybridization found on Northern blots in the previous work may have represented hybridization to another member of the P450IIB family or hybridization of the oligonucleotide probe to residual ribosomal RNA (polyadenylated RNA was purified with one binding cycle to polyuridine paper). Because there was binding to RNA that corresponded to the size of both 28 S and 18 S ribosomal RNAs and the band intensity was in the same relative proportions, we feel that the latter is the more likely possibility. These findings suggest that Northern blot analyses using oligonucleotide probes and long autoradiographic exposure times should be interpreted with care.

The observed pattern of P450IIB gene expression in intestinal mucosa substantiates the findings of studies that have attempted to identify P450IIB apoproteins in intestine. Bonkovsky *et al.* (46) used Western blot analysis and a monoclonal antibody to P450b (recognizes both P450b and P450e apoproteins) and found that a protein the size of P450b was present in microsomes from rats treated with phenobarbital; however, a band appropriate for the size of the P450e apoprotein was not identified. Christou and co-workers (47) obtained identical results using a polyclonal antibody that recognized both P450b and P450e in rat liver microsomes. However, these same investigators found contradictory results when they examined the enzymatic activity of intestinal microsomes. In these studies, Christou *et al.* (47) used the metabolism of DMBA to identify P450IIB functional holoenzymes in extrahepatic microsomes. This analysis indicated that phenobarbital altered the regioselectivity of DMBA metabolism to a pattern consistent with induction of P450e in small intestinal microsomes (47); the inability to detect P450e apoprotein by Western blot was attributed to the insensitivity of the technique. However, because of the high sensitivity of the polymerase chain reaction (18), it is unlikely that the DMBA metabolic activity found by these investigators was due to P450e. Of interest, Sesardic *et al.* (48) have suggested that P450 enzymes may exhibit a broader range of substrate specificities in extrahepatic tissues than the same P450 enzymes in liver. Using immunoinhibition studies, these investigators found that the P450IA1 gene product (rat P450c) in intestine was able to catalyze benz[*a*]pyrene hydroxylation and phenacetin *O*-deethylation; in contrast, P450c in liver microsomes appeared to catalyze only benz[*a*]pyrene hydroxylation and not the deethylation of phenacetin (48). It is possible that a similar situation occurs with the enzymatic activity of P450b in intestinal mucosa.

Because of substrate overlap and close homology within many P450 subfamilies, the methods used in this study may prove useful for the evaluation of other P450 subfamilies in extrahepatic tissues. Several approaches were available to definitively identify the intestinal P450IIB mRNA, 1) Northern blot analysis with multiple oligonucleotide probes to evaluate all possible genes, 2) direct cloning from an intestinal cDNA library of the cDNAs that cross-hybridize with liver-derived DNA probes, and 3) enzymatic amplification of specific sequences of intestinal cDNA as well as amplification of full length cDNA, using primers derived from known P450 sequences. Enzymatic amplification was chosen for these exper-

iments for the following reasons: 1) the amplified product can be directly analyzed by hybridization analysis with oligonucleotide probes, 2) the sensitivity of this method allows one to demonstrate the absence of expression of a specific mRNA, and 3) full length cDNAs with known sequences can be rapidly cloned and sequenced to definitively identify the expressed mRNA in the tissue. The data presented in this study indicate that, for highly homologous multigene families, enzymatic amplification of cDNA can be used to rapidly assess the presence or absence of expression of specific mRNA in tissues and can be used to definitively identify the expressed mRNA by sequencing analysis.

The inducibility of P450 genes has been an area of intense research, in an attempt to understand the molecular mechanisms by which xenobiotics can induce gene expression. The mechanisms of P450IIB gene induction in rat liver following treatment with phenobarbital have been partially elucidated. Using nuclei isolated from rat liver and a cDNA probe that recognizes both P450b and P450e mRNAs, nuclear run-on studies have demonstrated that the predominant mechanism for the induction of P450IIB mRNA in liver is due to transcriptional activation of the genes (49, 50). The prompt and marked induction of P450b mRNA in small intestinal mucosa following phenobarbital treatment suggested that transcriptional activation may be one mechanism by which P450b was induced in small intestine. Nuclear run-on experiments confirmed this impression, and quantitative analysis demonstrated that virtually all of the increase in P450 mRNA could be accounted for by an increment in transcription. In liver, transcription of P450IIB genes was previously shown to be induced 23-fold over control levels 3–12 hr following treatment with phenobarbital (50). In our studies we found a 6-fold induction of P450b gene transcription in proximal small intestinal mucosa. This degree of induction in both studies is in close agreement with our previously reported data following treatment with four daily oral doses of Aroclor, where P450IIB mRNA was induced 6-fold in proximal small intestine and 19-fold in liver (16); the induced levels in intestine were 30% of induced levels in liver (16). The cDNA probes used in the nuclear run-on experiments (Ref. 50 and this manuscript) as well as the mRNA quantitation in small intestine and liver (16) would be expected to hybridize to both P450b and P450e transcripts and possibly to other P450IIB gene products as well. However, because we have shown that P450e, as well as four other P450 genes, is not expressed in small intestine, this assay most likely detects predominantly P450b transcripts in intestine. Because P450e as well as P450b is inducible in liver, this may partially account for the lower degree of transcriptional induction in small intestine versus liver.

Although P450b and P450e gene transcription has not been separately evaluated in liver, it has been assumed that induction of expression by phenobarbital of both genes occurs in parallel and is regulated at the level of transcription (11, 12). In contrast, the data presented in this study demonstrate that transcriptional activation of these genes is not regulated coordinately in the small intestine. The tissue-specific differences in the expression of these two closely linked genes may provide a model for experimentally examining transcriptional mechanisms responsible for induction by phenobarbital.

In previous studies, we have shown that there is a heterogeneous intratissue distribution of P450IIB gene expression (51, 52). In liver, P450IIB genes are constitutively expressed and induced by phenobarbital (51) and other inducers (52) predominantly in zones 2 and 3 of the liver acinus; this has been confirmed by other investigators using oligonucleotide probes (53). In intestine, there is differential expression of P450b gene expression along the horizontal and vertical axes of the intestine. In both untreated and xenobiotic-treated rats, P450b mRNA was found predominantly in the proximal intestine, with relatively less in the distal intestine (horizontal gradient of expression) (16). Experiments employing *in situ* hybridization demonstrated that P450IIB mRNA was induced predominantly in the more mature enterocytes located in the intestinal villus, with little mRNA detected in crypt cells (16). Therefore, the expression of P450IIB genes not only is regulated in a tissue-specific fashion but also varies within the same tissue. Study of these genes may also provide a model to investigate mechanisms for differential intratissue gene expression in liver and small intestine.

In summary, P450b (P450IIB1) mRNA, but not P450e (P450IIB2) mRNA, is induced in the intestinal mucosa of rats following treatment with xenobiotics known to induce this P450 in liver. The P450b mRNA is rapidly induced following administration of phenobarbital and this induction is mediated by an increment in the transcription of the gene. The rapid induction of P450b in intestinal mucosa may have implications for the adaptive response of the intestine to metabolize ingested xenobiotics.

References

1. Burke, M. D., and S. Orrenius. Isolation and comparison of endoplasmic reticulum membranes and their mixed function oxidase activities from mammalian extrahepatic tissues. *Pharmacol. Ther.* **7**:549–599 (1979).
2. Chhabra, R. S., and W. C. Eastin. Intestinal absorption and metabolism of xenobiotics in laboratory animals, in *Intestinal Toxicology* (C. M. Schiller, ed.). Raven Press, New York 145–160 (1984).
3. Combes, R. D. Cytochrome P450, mixed function oxidases and formation of genotoxic metabolites by the intestinal tract, in *Intestinal Metabolism of Xenobiotics* (A. S. Koster, E. Richter, F. Lauterbach and R. Hartmann, eds.). Gustav Fischer Verlag, Stuttgart, 119–141 (1989).
4. Grafstrom, R. C. Metabolism of chemical carcinogens by intestinal tissue, in *Experimental Colon Carcinogenesis* (H. Autrup and G. M. Williams, eds.). CRC Press, Inc., Boca Raton, FL, 64–79 (1983).
5. George, C. F. Drug metabolism by the gastrointestinal mucosa *Clin. Pharmacokinet.* **6**:259–274 (1981).
6. Lu, A. Y. H., and S. B. West. Reconstituted mammalian mixed function oxidase: requirements, specificities and other properties. *Pharmacol. Ther.* **2**:337–358 (1978).
7. Black, S. D., and M. J. Coon. Comparative structures of P-450 cytochromes, in *Cytochrome P-450* (P. R. Oritz de Montellano, ed.). Plenum Press, New York, 161–216 (1986).
8. Lu, A. Y. H., and S. B. West. Multiplicity of mammalian cytochromes P450. *Pharmacol Rev.* **31**:277–295 (1980).
9. Nebert, D. W., M. Adesnik, M. J. Coon, R. N. Estabrook, F. J. Gonzalez, F. P. Guengerich, I. C. Gunsalus, E. F. Johnson, B. Kemper, W. Levin, I. R. Phillips, R. Sato, and M. R. Waterman. The P450 gene superfamily: recommended nomenclature. *DNA* **6**:1–11 (1987).
10. Nebert, D. W., D. R. Nelson, M. Adesnik, M. J. Coon, R. W. Estabrook, F. J. Gonzalez, F. P. Guengerich, I. C. Gunsalus, E. F. Johnson, B. Kemper, W. Levin, I. R. Phillips, R. Sato, and M. R. Waterman. The P450 superfamily: updated listing of all genes and recommended nomenclature for the chromosomal loci. *DNA* **8**:1–13 (1989).
11. Nebert, D. W., and F. J. Gonzalez. P450 genes: structure, evolution, and regulation. *Annu. Rev. Biochem.* **56**:945–993 (1987).
12. Adesnik, M., and M. Atchison. Genes for cytochrome P450 and their regulation. *Crit. Rev. Biochem.* **56**:945–993 (1987).
13. Labbe, D., A. Jean, and A. Anderson. A constitutive member of the rat cytochrome P450IIB subfamily: full-length coding sequence of the P450IIB3 cDNA. *DNA* **7**:253–260 (1988).
14. Atchison, M., and M. Adesnik. Gene conversion in a cytochrome P450 gene family. *Proc. Natl. Acad. Sci. USA* **83**:2300–2304 (1986).
15. Giachelli, C. M., J. Lin-Jones, and C. J. Omiecinski. Isolation and characterization of rat cytochrome P450IIB gene family members: use of the polymerase chain reaction to detect expression of a novel P450 mRNA. *J. Biol. Chem.* **264**:7046–7053 (1989).

16. Traber, P. G., J. Chianale, R. Florence, K. Kim, Wojcik, and J. J. Gumucio. Expression of cytochrome P450b and P450e genes in small intestinal mucosa of rats following treatment with phenobarbital, polyhalogenated biphenyls, and organochlorine pesticides. *J. Biol. Chem.* **263**:9449–9455 (1988).
17. Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science (Wash. D. C.)* **239**:487–494 (1988).
18. Rappolee, D. A., D. Mark, M. J. Banda, and Z. Werb. Developmental expression of PDGF, TGF-*alpha*, TGF-*beta* genes in preimplantation mouse embryos. *Science (Wash. D. C.)***241**:708–712 (1988).
19. Chirgwin, H. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochem. J.* **18**:5294–5299 (1979).
20. Chomczynski, P., and N. Sacchi. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **244**:261–272 (1987).
21. Werner, D., Y. Chemla, and M. Herzberh. Isolation of poly(A$^+$) RNA by paper affinity chromatography. *Anal. Biochem.* **141**:329–336 (1984).
22. Kumar, A., C. Raphael, and M. Adesnik. Cloned cytochrome P-450 cDNA: nucleotide sequence and homology to multiple phenobarbital-induced mRNA species. *J. Biol. Chem.* **260**:3247–3250 (1983).
23. Fagan, J. B., J. V. Pastewka, S. C. Chalberg, E. Gozukara, F. P. Guengerich, and H. V. Gelboin. Noncoordinate regulation of the mRNAs encoding cytochromes P-450$_{BNF/MC-B}$ and P-450$_{IBF/BNF-G}$. *Arch. Biochem. Biophys.* **244**:261–272 (1986).
24. Porter, T. D., and C. B. Kasper. Coding sequence of rat NADPH-cytochrome P-450 oxidoreductase cDNA and identification of flavin-binding domains. *Proc. Natl. Acad. Sci. USA* **86**:3963–3967 (1989).
25. Gunning, P., P. Ponte, H. Okayama, J. Engel, H. Blau, and L. Kedes. Isolation and characterization of full-length cDNA clones for human α-, β- and γ-actin mRNAs: skeletal but not cytoplasmic actins have an amino-terminal cysteine that is subsequently removed. *Mol. Cell. Biol.* **3**:787–795 (1983).
26. Wilson, G. N., B. A. Hollar, J. R. Waterson, and R. D. Schmickel. Molecular analysis of cloned human 18S ribosomal DNA segments. *Proc. Natl. Acad. Sci. USA* **75**:5367–5371 (1978).
27. Feinberg, A. P., and B. Vogelstein. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**:6–13 (1983).
28. Khandjian, D. W. UV crosslinking of RNA to nylon membrane enhances hybridization signals. *Mol. Biol. Rep.* **11**:107–115 (1986).
29. Zeff, R. A., and J. Geliebter. Oligonucleotides for genomic DNA blots. *Focus* **9**:1–2 (1987).
30. Crouse, G. F., A. Frischauf, and H. Lehrach. An integrated and simplified approach to cloning into plasmids and single-stranded phages. *Methods Enzymol.* **101**:78–89 (1983).
31. Sanger, R., S. Nicklen, and A. R. Coulson. DNA sequencing with chain-terminated inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467 (1977).
32. Hattari, M., and Y. Sakaki. Dideoxy sequencing method using denatured plasmid templates. *Anal. Biochem.* **152**:232–238 (1986).
33. Thompson, C. B., P. B. Challoner, P. E. Neiman, and M. Groudine. Expression of the c-*myb* proto-oncogene during cellular proliferation. *Nature (Lond.)* **319**:374–380 (1986).
34. McDonald, A. R., and I. R. Goldfine. Glucocorticoid regulation of insulin receptor gene transcription in IM-9 cultured lymphocytes. *J. Clin. Invest.* **81**:499–504 (1988).
35. Bloor, J. H., A. Dasmahapatra, M. Weiser, and W. D. Klohs. Kinetics of subceullar distribition in rat intestine of 1,25-dihydroxycholecalciferol admininstered *in vivo*. *Biochem. J.* **208**:567–575 (1982).
36. Linial, M., N. Gunderson, and M. Groudine. Enhanced transcription of c-*myc* in bursal lymphoma cells requires continuous protein synthesis. *Science (Wash. D. C.)* **230**:1126–1132 (1985).
37. Celano, P., C. Berchtol, and R. A. Casero. A simplification of the nuclear run-off transcription assay. *Biotechniques* **7**:942–944 (1989).
38. Omiecinski, C. J., R. G. Waltz, and G. P. Vlasuk. Phenobarbital induction of rat liver cytochrome P-450b and P-450e: quantitation of specific RNAs by hybridization to synthetic oligodeoxyribonucleotide probes. *J. Biol. Chem.* **260**:3247–3250 (1985).
39. Suwa, Y., Y. Mizukami, K. Sogawa, and Y. Fujii-Kuriyama. Gene structure of a major form of phenobarbital-inducible cytochrome P450 in rat liver. *J. Biol. Chem.* **260**: 7980–7984 (1984).
40. Gyllensten, U. B., and H. A. Erlich. Generation of single-stranded DNA by the polymerase chain reaction and its application to direct sequencing of the HLA-DQA locus. *Proc. Natl. Acad. Sci. USA* **85**:7652–7656 (1988).
41. Fujii-Kuiryama, Y., U. Mizukami, K. Kawajiri, K. Sogawa, and M. Muramatsu. Primary structure of a cytochrome P450: coding nucleotide sequence of phenobarbital-inducible cytochrome P450 cDNA from rat liver. *Proc. Natl. Acad. Sci. USA* **79**:2793–2797 (1982).
42. Yuan, P.-M., D. E. Ryan, W. Levin, and J. E. Shively. Identification and localization of amino acid substitutions between two phenobarbital-inducible rat hepatic microsomal cytochromes P450 by micro sequence analyses. *Proc. Natl. Acad. Sci. USA***80**:1169–1173 (1983).
43. Rampersaud, A., and F. G. Walz. At least six forms of extremely homologous cytochromes P450 in rat liver are encoded at two closely linked genetic loci. *Proc. Natl. Acad. Sci. USA* **80**:6542–6546 (1983).
44. Omiecinski, C. J. Tissue-specific expression of rat mRNAs homologous to cytochromes P450b and P450e. *Nucleic Acids Res.* **14**:1525–1539 (1986).
45. Imaoka, S., Y. Terano, and Y. Funae. Expression of four phenobarbital-inducible cytochorme P450s in liver, kidney, and lung of rats. *J. Biochem.* **105**:939–945 (1989).
46. Bonkovsky, H. L., H. Hauri, U. Marti, R. Gasster and U. A. Meyer. Cytochrome P450 of small intestinal epithelial cells: immunochemical characterization of the increase in cytochrome P450 caused by phenobarbital. *Gastroenterology* **88**:458–467 (1985).
47. Christou, M., N. M. Wilson, and C. R. Jefcoate. Expression and function of three cytochrome P450 isozymes in rat extrahepatic tissues. *Arch. Biochem. Biophys.* **258**:519–534 (1987).
48. Sesardic, D., A. Boobis, and D. Davies. Tissue specific inducibility of members of the P450IA gene sub-family in rat and man. in *Xenobiotic Metabolism and Disposition* (R. Kato, R. Estabrook, and M. Cayne, eds.). Taylor and Franceis, Philadelphia, 45–52 (1989).
49. Adesnik, M., S. Bar-Nun, F. Maschio, M. Zunich, A. Lippman, and E. Bard. Mechanism of induction of cytochrome P450 by phenobarbital. *J. Biol. Chem.* **256**:10340–10345 (1981).
50. Hardwick, J. P., F. J. Gonzalez, and C. B. Kasper. Transcriptional regulation of rat liver epoxide hydratase, NADPH-cytochrome P450 reductase, and cytochrome P450 genes by phenobarbital. *J. Biol. Chem.* **258**:8081–8085 (1983).
51. Wojcik, E., C. Dvorak, J. Chianale, P. G. Traber, D. Keren, and J. J. Gumucio. Demonstration by *in situ* hybridization of the zonal modulation of rat liver cytochrome P-450b and P-450e gene expression after phenobarbital. *J. Clin. Invest.* **82**:658–666 (1988).
52. Traber, P. G., P. Maganto, E. Wojcik, D. Keren, and J. J. Gumucio. Induction of P450IIB genes within the rat liver acinus is not dependent on the chemical inducer or on the acinar organization. *J. Biol. Chem.* **264**:10292–10298 (1989).
53. Hassett, C., D. L. Luchtel and C. J. Omiecinski. Hepatic expression of rat P450 mRNA assessed by *in situ* hybridization to oligomer probes. *DNA* **8**:29–37 (1989).

Send reprint requests to: Peter G. Traber, M.D., 6520A Medical Science Research Building I, Box 0682, 1150 West Medical Drive, Ann Arbor, Michigan 48109.