Tissue-specific expression of rat mRNAs homologous to cytochromes P-450b and P-450e

Curtis J.Omiecinski

Department of Environmental Health, School of Public Health and Community Medicine, University of Washington, Seattle, WA 98195, USA

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ABSTRACT

The tissue-specific expression of cytochrome P-450b and P-450e mRNAs was examined with synthetic 18-mer oligomer probes in the liver, lung, kidney, and testis of control and inducer pretreated adult rats. RNAs homologous to the P-450e probe were detected in trace amounts in control and 3-methylcholanthrene (MC) induced livers and at high levels in livers from phenobarbital (PB) induced animals. P-450e mRNA levels were below detection limits in the other tissues examined, regardless of pretreatment. In contrast, mRNAs homologous to the P-450b oligomer were detected at low levels in control and inducer pretreated lung and testis, and at high levels in PB induced liver. No P-450b mRNAs were detected in these assays in RNA isolates from the kidney or from control or MC pretreated liver. Solution hybridization data indicated that the rat lung contained 9-12%, and the testis, 6-9%, respectively, of the levels of P-450b mRNA measured in the PB induced liver. Results from oligo(dT)-cellulose and poly(U)-affinity experiments indicated that the hepatic mRNAs for P-450b and P-450e were present predominantly in the bound, polyadenylated fraction, whereas the homologous lung and testes P-450b mRNAs predominated in the flow-thru fractions.

INTRODUCTION

The cytochrome P-450 mixed-function oxygenases (P-450s) are a family of hemoenzymes that catalyze a large variety of biotransformation reactions. Substrates include endogenous compounds, such as steroids and prostaglandins, and xenobiotic compounds, such as the procarcinogenic polycyclic aromatic hydrocarbons and mycotoxins (1-3). The liver contains the highest specific content of total P-450, but P-450s are present in extrahepatic tissues as well (4,5). Most P-450s possess broad but overlapping substrate specificities with the overall metabolic capacity of a particular tissue dependent upon its composite of individual P-450 isozymes and other metabolizing enzymes. Extra-

hepatic tissues are often target sites for bioactivation mediated toxicities, including cancers, mutations, and birth defects (6). Consequently, the tissue-specific regulation of P-450 gene expression may represent an important parameter determining the susceptibility of individual tissues to the toxic effects of chemicals undergoing bioactivation.

Transcription of individual P-450 genes can be markedly stimulated by a variety of agents such as 3-methylcholanthrene (MC) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; 7-8), phenobarbital (PB; 9-11), and potentially many other compounds (12). The rat P-450s, P-450b and P-450e, are extremely homologous proteins that are induced markedly in the liver by PB via transcriptional activation (10-11). These cytochromes are distinct gene products (13,14), yet are immunochemically indistinguishable with polyclonal antisera (14), and share >97% homology in their amino acid (15) and nucleic acid (16) sequences. In addition, the hepatic microsomal protein concentrations for P-450b and P-450e appear to correspond well to their respective tissue mRNA levels (17).

We have reported previously a method employing 18-mer synthetic oligodeoxyribonucleotide hybridization probes that provided a highly sensitive and discriminating means of analyzing mRNA levels for each of the cytochromes, P-450b and P-450e (17). In the present study the oligomer probes have been utilized to determine the tissue-specific regulation of the mRNAs corresponding to the P450b and P450e isozymes in the rat lung, kidney, testis, and liver. The hybridization data demonstrate that the regulation of the P450b and P450e genes in extrahepatic tissues is markedly distinct from the patterns of gene expression as observed in the liver.

METHODS

Animals. Male Sprague-Dawley rats, 125-150 g (Tyler Labs, Bellevue, WA) were employed for these studies. PB pretreatments consisted of one intraperitoneal injection (75 mg/kg, prepared in normal saline) 16 h before sacrifice. Pretreatment with 3-methylcholanthrene was similar except that the vehicle was corn oil. Untreated control animals were also employed. Treatment with vehicle alone did not significantly affect tissue RNA levels for P-450b or P-450e (unpublished observations).

Hybridizations. RNA was isolated as described previously (17). Poly(A)+ RNA was purified by 2 cycles of

oligo(dT)-cellulose (Type III, Colaborative Research, Waltham, MA) chromatography, as described by the manufacturer. Poly(U)-affinity experiments were performed with mRNA affinity paper (Orgenics, Ltd., Yavne, Israel) as reported previously (18). Northern blot experiments were performed as described (19) with the following modifications: after electrophoresis in submerined 2.2 M formaldehyde/agarose gels, the RNA was transferred to GeneScreen Plus nylon membranes (New England Nuclear, Boston, MA) in 10X SSC buffer (10X SSC: 0.15M NaCl, 0.015M Na Citrate, pH 7.0) and processed according to the manufacturer's directions. Hybridization were performed at 52° in 5X SSC/1% SDS/25 mM Na phosphate, pH 6.5/1X Denhardt's/10 µg/ml of denatured salmon sperm DNA, and 1 X 10⁶ cpm of oligomer probe. Washings consisted of 2 room temperature washes in 5X SSC/0.1% SDS for 10 min each followed by two 10 min washes at 52° in the same buffer. Solution hybridizations were performed as reported previously (17).

Oligomer probes. The 18-mer synthetic oligonucleotides, 3'-d(AGTGTGGCCGATGGTTGG)-5' for P-450b, and 3'-d(AGAGTGTCGGTGGTAGG)-5' for P-450e, were synthesized and isolated as previously discussed (17); and correspond to the hypervariable region of genomic exon 7, i.e., amino acid positions 334 through 339 (...Ser-His-Arg-Leu-Pro-Thr... for P-450b, and ...Ser-His-Arg-Pro-Pro-Ser... for P-450e). 5'-end labeling of the oligomer probes was performed as reported (17) except that 10 pmol of oligomer was added to each reaction along with 15 pmol of [γ -32P]ATP (ICN, Irvine, CA; 4500 Ci/mmol) and the reactions were conducted at 37° for 30 min. Under these conditions, nearly complete (>95%) labeling was achieved and resulted in specific activities of approximately 9 X 10⁶ cpm/pmol for each oligomer. All other materials and procedures were as described (17,19).

RESULTS

In the upper portion of Figure 1, the results of a Northern blot experiment with the P-450b oligomer probe are presented. The probe was hybridized to total RNA isolated from control, PB and MC pretreated hepatic and extrahepatic rat tissues. Panel A illustrates a shorter exposure of the corresponding lanes 1-3 of panel B. These results clearly show the PB-inducibility of the hepatic P-450b mRNAs and the lack of any hybridization with RNA from control or MC induced liver. In contrast, PB or MC pretreatments produced only minimal effects on P-450b mRNAs in the lung or testis. Also in contrast to the hepatic results is the lack of observable hybridization in any of the RNA fractions isolated from the kidney. No P-450b mRNA was detectable in this tissue even after more lengthy exposures. Low levels of hybridization signal were occasionally observed in the kidney preparations but following low stringency washing conditions

(5X SSC/0.1% SDS, room temperature). The resulting autoradiograms indicate that the isolated lung and testis RNAs contained much less P-450b mRNA than the PB liver preparations. No striking alterations in the extrahepatic tissue mRNA levels for P-450b were observed in these experiments as a consequence of inducer pretreatment.

In the lower portion of Figure 1 the P-450e oligomer hybridization data are presented. P-450e mRNA was clearly present in PB induced liver (lane 2), but not observable in any of the extrahepatic tissues studied, regardless of pretreatment. Traces of P-450e mRNA were apparent however in both control and MC induced liver preparations, although the faint levels did not reproduce well from the original autoradiogram.

Solution hybridization experiments were performed to further quantitate P-450b and P-450e mRNA levels in the various tissues. These data are presented in Table 1. The rat lung possessed approximately 9-12% of the amounts of mRNA present in the PB induced liver, and the testis, approximately 5-7% of the induced hepatic levels. No mRNA for P-450b was detected in the kidney. Although some apparent alterations in the extrahepatic mRNA levels were observed after pretreating animals with PB and MC, the effects were slight relative to the effects of PB on the corresponding hepatic P-450 mRNAs. Solution hybridization data obtained with the P-450e oligomer were consistent with the results presented in Figure 1 and demonstrate that P-450e mRNA concentrations were beyond the detection limits of the assay in any of the lung, testis, or kidney preparations. Trace quantities of P-450e mRNA were apparent in the control and MC

Figure 1. Northern blot hybridization of P-450b and P-450e oligomer probes with total RNA preparations from rat liver, lung, testes, and kidney. 10 µg of total RNA per lane was subjected to electrophoresis in 1.25% formaldehyde gels and transferred to Genescreen Plus nylon membranes for hybridization. The designations are as follows: C, control tissue; P, phenobarbital pretreated tissue; M, 3-methylcholanthrene pretreated tissue. The size, in kilobases (kb), of the major hybridization product is indicated to the left of the figure. Upper Figure: Data obtained with the P-450b oligomer probe. Panel A shows a 4 hr exposure of a portion of the membrane in panel B (3 day exposure). Lower Figure: Data obtained with the P-450e oligomer probe (3 day exposure).

TABLE 1
Tissue Levels of P-450b and P-450e mRNAs Quantitated
by Solution Hybridization

Tissue	Pretreatment	Probe	Relative Abundance Fraction (RNA/PB Liver P-450b mRNA)X100
Lung	None	P-450b	9
	PB		11
	MC		12
Testis	None		7
	PB		6
	MC		5
Kidney	None		ND*
	PB		ND
	MC		ND
Liver	None		ND
	PB		100
	MC		ND
Liver	None	P-450e**	<1
	PB		20
	MC		<1

* ND = Not detectable.

** No detectable hybridization occurred when the P-450e probe was incubated with RNA from any of the lung, kidney, or testis preparations.

Each reaction was performed in triplicate and contained 10 µg of total RNA and 4500 cpm of either 32P-P450b or 32P-P450e oligomer probe. Hybridizations and filtering of TCA precipitates were conducted as previously described (17).

induced liver extracts but comprised less than 1% of the PB induced hepatic P-450b levels and could not be quantitated with confidence.

To ascertain the extent of polyadenylation of the mRNAs hybridizing to P-450b and P-450e oligomer probes, total RNA preparations from various tissues were subjected to two cycles of oligo(dT)-cellulose chromatography. The resulting RNAs were then Northern hybridized as above. The results are shown in the upper portion of Figure 2. In these experiments, 5 µg of poly(A)+ RNA was applied to each lane versus 10 µg for the total RNA lanes. Considering that only 1-2% of the total cellular RNA is polyadenylated (20), these loadings should represent an approximate 25-fold enrichment of poly(A)+ RNA relative to the amounts contained in the adjacent lanes for each tissue. Lanes 1-4 contain liver RNA and demonstrate that P-450b mRNA is

detected only in the PB induced preparation (lanes 3-4). The polyadenylated RNA (lane 3) is highly enriched in P-450b sequences relative to the total RNA (lane 4). The converse was observed in the lung RNA preparations and in RNA fractions from control and PB induced testes (lanes 5-8) within the 1.8 kb regions. In these tissues, the P-450b mRNA was observed predominantly in the total RNA lanes. A faint smear of radioactivity appeared in the high molecular weight region of lane 5, but was attributed to non-specific background associated with this individual blot since it was not present upon subsequent repetitions of the experiment, including that shown in Figure 3. Again, P-450b mRNA was not detected with the oligomer probe in any of the kidney RNA preparations (lanes 9-12).

The lower portion of Figure 2 depicts the similar experiment utilizing the P-450e oligomer probe. The P-450e mRNA from the PB induced liver was present predominantly in the polyadenylated RNA fraction (lane 3 versus lane 4). No P-450e mRNA was detected in poly(A)+ RNA or total RNA fractions from testes or kidney preparations (lanes 5-12).

In separate experiments, with RNA subfractions from control lung and testes, similar results were obtained (Figure 3). Included in these blots are lanes containing the oligo(dT) flow-thru fractions (poly(A)- RNAs). To obtain a more representative picture of normally occurring RNA abundances, only 1 μ g of poly(A)+ RNA was run on these gels, versus 10 μ g per lane for both the total and poly(A)- RNAs. Even upon extreme over-exposures, the P-450b mRNAs from the lung and testis were only minor components of the bound, poly(A)+ RNA fraction.

To further examine the apparent differences in polyadenylation between the hepatic and extrahepatic mRNAs for P-450b, poly(U)-affinity experiments were performed with messenger affinity paper (mAP), according to procedures described recently (18). The poly(U)-matrix was found to be slightly more effective than the oligo(dT)-matrix in the selection of testes and lung P-450b mRNAs (data not shown) but the respective mRNAs for these tissues were still found predominately in the non-poly(U)-associated fractions.

Figure 2. Hybridization of poly(A)+ RNA and total RNA from control and phenobarbital pretreated rat livers, testes, and kidneys to the P-450b and P-450e oligomer probes. Poly(A)+ RNA was prepared by two binding/elution cycles of total RNA through an oligo(dT)-cellulose matrix. 5 μ g of poly(A)+ RNAs or 10 μ g of total RNAs were applied to the respective lanes. The odd-numbered lanes contain RNA enriched in poly(A)+ sequences; the even-numbered lanes contain total tissue RNA. The following lanes contain the indicated RNAs: Lanes 1 and 2, control rat liver; lanes 3 and 4, phenobarbital induced liver; Lanes 5 and 6, control rat testis; lanes 7 and 8,

phenobarbital induced testis; Lanes 9 and 10, control rat kidney; lanes 11 and 12 phenobarbital treated kidney. Upper Figure: Data obtained with the P-450b oligomer probe. Lanes 1-4 show a 1 h autoradiograph exposure and lanes 5-12 show a 5 day exposure of the same membrane. Lower Figure: Data obtained with the P-450e oligomer probe (5 day exposure).

DISCUSSION

Tissue-specific gene regulation forms the basis of differentiation and specialization of complex organisms. In order to understand the mechanisms through which genes in various tissues are controlled, and to determine which tissues, chemical exposures, and developmental stages predispose an animal to the toxic effects of chemicals undergoing bioactivation, it is important to understand the expression of genes that code for the requisite enzyme systems. Since the P-450s also catalyze numerous tissue-specific reactions with critical endogenous substrates (21), this system represents an area in need of further investigation. The studies described herein employed synthetic 18-mer hybridization probes of defined sequence in the assessment of two major PB inducible hepatic forms of P-450 mRNA, P-450b

Figure 3. Northern hybridization of poly(A)+, poly(A)-, and total RNA isolates from control rat lungs and rat testes. 1.0 μ g of poly(A)+ RNA; or 10 μ g of poly(A)- RNA or total (T) RNA, was applied to the indicated lanes. Experiments were performed as described in the legend to Figure 1. The autoradiography was for 3 weeks.

and P-450e. These studies were conducted with liver, lung, testis, and kidney RNA subfractions from control, PB, and MC pretreated adult male rats. The results indicate that the mRNAs for these cytochromes are regulated in a complex tissue specific manner.

The P-450b and P-450e oligomer probes differ from one another in 4 of 18 positions and under stringent reaction conditions hybridize to their respective mRNAs with extremely high specificity (17). We have determined that even in the presence of 1000-fold molar excess of unlabelled competing oligomer, no significant alterations were detected on the specific hybridization of the individual radiolabelled oligomers (17). The melting temperature (T_m) of the perfectly matched P-450b/P-450e oligomer/RNA hybrid can be calculated to be 20-40° greater than the T_m of the corresponding mismatched hybrid (22,23). Indeed, with the hybridization conditions employed here (T_m -5°; 24), we have observed that the P-450b/P-450e oligomer probes hybridize specifically and without cross-hybridization to their counterpart cDNA derivatives (manuscript in preparation).

In the present study, P-450b mRNAs were not detectable in livers of control rats or in livers from MC pretreated rats (Figures 1 and 2). In hepatic RNA preparations from PB pretreated rats however, P-450b mRNA levels increase to >600 molecules per cell (17), and these mRNAs are predominantly polyadenylated (Figure 2). In the lung and testis, P-450b message is present constitutively, at levels approximately 10% and 6%, respectively, of the levels present in PB induced liver. PB or MC pretreatments exhibited only slight effects on the corresponding P-450b mRNAs in these tissues (Figure 1 and Table 1). The kidney did not contain detectable P-450b mRNA, even after PB or MC pretreatments. The kidney may, however, possess a related isozyme, since other PB inducible P-450 variants do exist (13,25,26), and since some evidence of hybridization signal was apparent in the Northern blot experiments prior to the last high stringency wash of the membranes. Hybridizations with isolated P-450b and P-450e cDNA probes should reveal whether related P-450s exist in this tissue.

Only very small quantities of P-450e mRNA, near our detection limits with the present methodology, exist in control

hepatic tissues (Figures 1, 2; Table 1). Pretreatment with MC did not appear to affect these levels whereas treatment with PB markedly induced the mRNA levels in this tissue. Hepatic P-450e mRNA was associated with the polyadenylated mRNA fraction predominantly (Figure 2). In contrast to the findings with P-450b mRNA, P-450e mRNA was not detected in any of the extrahepatic tissues studied (Figures 2, 3; Table 1). The use of a probe labelled to higher specific activity will be necessary to determine whether the P-450e mRNAs are present in these tissues at extremely low levels. Interestingly, the finding of only P-450b and not P-450e mRNAs in the lung are in agreement with assessments of the respective protein constituents in this tissue as obtained by other investigators using Western blotting techniques (N. Wilson, C. Jefcoate, personal communication) and two dimensional gel analyses of rat lung microsomes (F.G. Walz, Jr., personal communication). It appears then, at least for the rat lung and liver, a good correlation exists between mRNA and protein levels for P-450b and P-450e. Such findings also agree with previous reports (10-11) showing that PB induction of P-450b+e enzyme in the liver is a consequence of increased gene transcription.

Previous studies have indicated that P-450b and P-450e proteins (27) and messages (17, 24) were coordinately induced by PB in the rat liver. The results reported here are consistent with these observations with regard to mRNA expression in the liver but clearly demonstrate that, 1) neither mRNA is appreciably affected by inducer pretreatment in the lung, testis, or kidney; and, 2) that P-450e mRNA is not detectable currently in any of these extrahepatic tissues. Furthermore, small amounts of P-450e message are observed in control liver, while no P-450b mRNA is detected in this tissue. These findings indicate that the genes coding for the respective messages are not subjected to coordinate controls in tissues other than the PB induced liver. Of the tissues studied here, the hepatic genes for P-450b and P-450e thus appear to be uniquely sensitive to the inducer, PB. The actual mechanisms regulating the tissue-specific expression and differential inducibility of the P-450b and P-450e genes remain to be elucidated.

The PB inducible cytochrome P-450 gene family is complex, consisting of at least 6 and perhaps more than 20 genes (28,29). Adesnik and Atchison (30) have reported that, within this gene family, sub-families of P-450b-like genes and P-450e-like genes each possess hypervariable regions that distinguish each from the other, yet these same hypervariable regions appear to be conserved within each P-450b or P-450e sub-family. Since the hypervariable regions of the the PB incucible P-450 genes include exon 7, targeted by the oligomer probes employed here, it remains to be demonstrated whether the lung and testis mRNAs that hybridized to the P-450b probe are, in fact, identical to the corresponding P-450b mRNA expressed in the liver. Preliminary data indicate that the P-450b and P-450e oligomers each hybridize to multiple, but unique genes based on restriction endonuclease patterns obtained in Southern blot analyses of rat DNA. The P-450b probe hybridized, under conditions of very high stringency, to DNA fragments apparently from 3-4 separate genes while the P-450e probe demonstrated similar high homologies to potentially 2 genes. The cloning and sequencing of the homologous extrahepatic P-450b mRNAs should provide the more detailed information necessary to assess the potential complexities of tissue specific expression for this gene family.

The poly(A)+ tract on mRNAs has been postulated as playing a role in RNA stabilization (31) and in translation (32). The finding that only the liver contains oligo(dT)-selectable polyadenylated mRNA for both P-450b and P-450e whereas the lung and testis P-450b mRNAs are difficult to select on the basis of poly(A)+ affinity procedures is striking. In all cases where P-450b or P-450e mRNAs were detected in Northern hybridizations, the size of the message appeared identical, being approximately 1.8 kb in length. Based on the migrations of size standards, our gel system can readily distinguish RNAs differing by 50 bases. Explanations for the lack of a detectable difference in size between the polyadenylated mRNAs and those apparently more lacking in polyadenylation include the following: 1) the poly(A)+ tract on the hepatic P-450b mRNAs is sufficiently short such that no difference in size is observed in the Northern yet its length is sufficient to bind to the oligo(dT)- or poly(U)- affinity

matrices; or, 2) the hepatic P-450b mRNAs have an average length (approximately 200 bp) poly(A)+ tract, which is shorter or lacking in the extrahepatic messages but replaced by additional 3' or 5' untranslated sequence. The latter possibility would be of interest since tissue specific expression of other unrelated mRNAs has been postulated to arise as a result of differential lengths of 3' or 5' untranslated information (33,34). Exact size determinations of the poly(A)+ tracts of each message in addition to sequence determination of the respective cDNAs will be necessary to test these possibilities. Reports have shown that the P-450b and P-450e genes and cDNAs contain an unusual polyadenylation signal, GGTAAG, rather than the expected AATAAG signal (11,16). It may be that tissue differences exist with regard to their respective abilities to recognize or utilize unusual polyadenylation signals.

Leighton and Kemper (36) have reported that mRNAs coding for members of a PB inducible P-450 family in the rabbit also demonstrate differential tissue induction and tissue-specific expression. However, none of their clones corresponded to the major rabbit PB inducible form of P-450, LM2, which appears to be the rabbit counterpart to the rat P-450b isozyme (37). Consequently it is not yet clear whether the related LM2 isozyme in the rabbit also shares the properties of tissue-specific expression, differential induction or polyadenylation. Their data (36), together Adesnik's (30) and that presented here, indicate that in different animal species, the genes for at least for some closely related members of the PB inducible family of cytochromes P-450 are under complex tissue specific control.

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