GENES FOR CYTOCHROME P-450 AND THEIR REGULATION

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I. INTRODUCTION

Microsomal monooxygenase systems containing cytochrome P-450 play an important role in the metabolism of endogenous substrates such as steroids and fatty acids, in the detoxification of many drugs and xenobiotics, and in the activation of environmental agents to toxic, mutagenic, and carcinogenic forms.[28,56] The extraordinarily broad substrate specificity of these enzyme systems results from the multiplicity of distinct molecular forms of the terminal oxidase, cytochrome P-450*, which have different, but overlapping, broad substrate specificities (reviewed in References 125 and 148). This has become apparent in recent years as a result of the identification, purification, and characterization of various molecular forms of rat and rabbit liver cytochrome P-450.

A second very important property of several of the forms of cytochrome P-450 is that they are inducible. This inductive response plays a significant role in increasing the rate of metabolism of foreign compounds to detoxified forms or in some cases to harmful intermediates. This may be clinically important since in some cases treatment with one drug can, in fact, either potentiate the action or diminish the efficacy of a different drug which is subsequently administered (see Reference 28).

Much of the current research on the molecular biology of the cytochrome P-450 systems is concerned with the elucidation of the genetic, molecular, and evolutionary mechanisms responsible for the existence of a multiplicity of forms of cytochrome P-450 as well as for the induction of distinct forms by specific inducing agents. The tools of molecular biology are also beginning to be applied to the problems of tissue specificity and endocrine and developmental regulation of cytochrome P-450 expression.

A. Multiplicity of Cytochrome P-450 Proteins

Approximately a dozen distinct forms of the monooxygenase have been purified from rat liver,** several of which have low basal levels which are substantially increased (30- to 50-fold) after treatment of animals with the classic specific inducing agents such as phenobarbital (PB), 3-methylcholanthrene (3-MC), or Aroclor® 1254, a mixture of polychlorinated biphenyls.[30,62,190,206]

Many of the well-characterized forms of cytochrome P-450 such as the major PB

* The proper name of these enzymes is "multisubstrate monooxygenase". "P-450" is acceptable as the trivial name (compare Reference 150).

** It is not our intention in this article to review the characterization of all known cytochrome P-450s from different organs and different species. We provide, however, a more detailed, but still not complete discussion of the P-450s identified in rat liver since the major recombinant DNA studies on cytochrome P-450 have dealt with the rat liver proteins. See Reference 148 for an attempt to compare P-450s from rat, mouse, and rabbit.

and 3-MC induced forms are very different in their enzymatic, structural, and immunological properties[16,62,188,206] and certainly represent products of different, distantly related genes which are probably members of the same gene superfamily.* The major forms of cytochrome P-450 induced by 3-MC (P-450c) and isosafrole (P-450d), however, show significant immunological cross-reactivity[183,211] as well as some similarities in their peptide fragmentation patterns[189] which suggests that they represent products of different members of a single gene family. In addition, these two P-450s show an important similarity in their regulation in that the isosafrole induced form is also markedly induced by 3-MC.[62,137,210]

The two major PB-induced forms of cytochrome P-450, P-450b and P-450e (designated PB-4 and PB-5 by Waxman and Walsh [227] and PB-B and PB-D by Dannan et al.[30],) are even more closely related. They are immunochemically indistinguishable[190,222,227] and have almost identical substrate specificity profiles with P-450b being about five times more active than P-450e with all but one of the substrates examined. On the basis of amino acid sequence data for tryptic peptides representing 75% of the sequences of these two enzymes,[244] they show only 13 amino acid differences all of which are clustered in the carboxy terminal halves of the polypeptide chains. As will be discussed in greater detail below, these two proteins appear to be encoded by two very closely related genes.

Synthetic steroids such as pregnenolone 16 α-carbonitrile (PCN) and dexamethasone[45,62,85] induce a distinct form of cytochrome P-450 designated P-450$_{PCN}$. Other rat liver P-450 forms such as those designated PB-1**[228] and P-450a,***[209] are present at substantial levels in microsomes from untreated animals and increase only two- to fourfold after treatment with various classical inducing agents.[62,209,228] Cytochrome P-450 2c[226†] is yet another form of cytochrome P-450 which is present at substantial levels in livers of untreated mature male rats and is responsible for the testosterone 16 α-hydroxylase activity which is developmentally induced in male rats after puberty.[225] Another constitutive†† P-450 present in liver microsomes of female but not male rats has also been described.[93,187,225] Two other distinct constitutive enzymes,P-450f and P-450g,††† have been isolated from male rat liver, with the latter being undetectable in livers of females.[187] A constitutive rat liver P-450 active in the 25-hydroxylation of C_{27}-steroids and vitamin D_3 has also been purified,[4] but its relationship to the other purified constitutive isozymes remains to be determined.

The total number of distinct forms of cytochrome P-450 which are produced in various tissues of the body is not known. In rat liver, as just noted, nearly a dozen forms have been identified and characterized. Several different P-450s have also been isolated from rabbit lung. Two of these appear to correspond to liver forms which are induced by phenobarbital.[184] A third form of lung P-450 induced by TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin), a compound which in liver induces the same forms as 3-

* In the nomenclature of Dayhoff,[32] proteins within a superfamily have a low probability (less than 10^{-6}) of being related by chance. Proteins within the same family have less than 50% difference in their amino acid sequences and proteins in the same subfamily have less than 20% difference in their sequences. We apply the equivalent definitions for genes in the same subfamily, family, and superfamily.

** Equivalent to PB-C of Guengerich et al.[62] See References 62 and 187 for a discussion of relationships between enzyme preparations named and characterized by different laboratories.

***Equivalent to PB-3 of Waxman et al.[226] and Ut-F of Guengerich et al.[62]

† Equivalent to UT-A of Guengerich et al.[62] and probably equivalent to P-450h of Ryan et al.,[187] the male-specific P-450 of Kamataki et al.[93] and RLM$_5$ of Cheng and Schenkman.[25]

†† The term "constitutive" is used here to refer to enzymes present at substantial levels in microsomes from untreated rats. Some of these may yet be found to be inducible by certain xenobiotics.

††† The amino terminal peptide sequence of this protein[77] and of RLM$_3$ of Cheng and Schenkman[25] suggest that these P-450s are closely related, if not identical.

MC, appears to be equivalent to form 6 of rabbit liver, which is responsible for the bulk of TCDD-induced benzo[*a*]pyrene hydroxylase in that organ.[122] Four distinct cytochrome P-450s which function in the biosynthesis of corticosteroids from cholesterol (reviewed in Reference 224; and see below) have been isolated from the adrenal cortex, an organ whose major function is the regulated synthesis of steroid hormones. Two of these enzymes, the cholesterol side chain cleavage enzyme and the 11-deoxycortisol 11β-hydroxylase, are located in the inner mitochondrial membrane, whereas the other two enzymes, the progesterone 17α-hydroxylase and the 17α-progesterone-21-hydroxylase, are contained within endoplasmic reticulum membranes. The relationships between the adrenal P-450s and those of the liver have not been examined in detail, although it has been found that bovine liver microsomes contain a protein immunochemically indistiguishable by Oucherlony immunodiffusion from the adrenal 21-hydroxylase, in amounts comparable to that found in microsome fractions from the adrenal gland.[139] In addition, antibodies against a purified bovine liver cytochrome P-450 showed immunological cross-reactivity with the purified adrenal 21-hydroxylase.[86]

II. APPLICATION OF RECOMBINANT AND METHODS TO STUDIES ON THE DIVERSITY AND STRUCTURAL RELATEDNESS OF CYTOCHROME P-450 ISOZYMES

It has been proposed[142] that hundreds or even thousands of forms of cytochrome P-450 account for the broad substrate specificities of the detoxifying systems induced by a wide variety of xenobiotics. Several laboratories, including ours, have initiated a recombinant DNA approach to the question of the number of cytochrome P-450 forms and the extent of their structural similarities. In this approach one isolates cDNA and genomic clones for specific P-450s and determines the number of related genes in the genome and the extent of homology of the different genes and their encoded polypeptides. It is expected that most, if not all, of the distinct cytochrome P-450 enzymes are encoded by genes derived from a single ancestral gene by a process of gene duplication and evolutionary divergence. Many of these genes, because of their evolutionary history, are expected to show a common organization of introns and exons with substantial sequence conservation in exonic regions when the DNA sequences are directly compared.[91]

The determination of the DNA sequences of cloned cDNAs or genomic segments corresponding to different characterized forms of P-450 is also providing insights into the structural features of the proteins responsible for common function. In addition, as will be illustrated below, the application of recombinant DNA techniques is leading to the isolation and characterization of genes and cDNAs for P-450s which have not yet been isolated and characterized by the classical biochemical techniques. Ultimately, the possibility for the expression of cloned P-450 cDNA in either bacterial or eukaryotic cell hosts should permit the application of site-directed mutagenesis procedures as well as methods for constructing chimeric genes to the analysis of the structural features of the enzymes involved in their catalytic properties, in particular, the differences in their substrate specificites.

A. Genes For the Major Phenobarbital-Inducible Isozymes of Cytochrome P-450

1. Characterization of cDNA Clones

A number of laboratories have isolated cDNA clones derived from messenger RNAs encoding liver cytochrome P-450s inducible with PB. These include cDNA clones prepared from rat,[1,3,51,52,59,80,113,173] rabbit,[119] chicken[22], and mouse[200] liver mRNA.

In our initial report on the cloning of the cDNA for phenobarbital-induced cytochrome P-450, we noticed that translation of messenger RNA selected by hybridization

to a single cloned cDNA yielded two electrophoretically separable polypeptides which were both efficiently immunoprecipitated by a polyclonal antibody raised against PB-induced cytochrome P-450.[1] We did not pay much attention to this observation at that time since it had not yet been reported that phenobarbital actually induces two immunochemically indistinguishable P-450 forms in Long-Evans rats[190,221,222] and three such proteins in some colonies of Holtzman rats.[221] Subsequently, we found that, indeed, depending on the rat strain examined, two or three in vitro translation products separable by one-dimensional SDS gel electrophoresis are precipitated by either monoclonal or polyclonal antibodies to PB-induced P-450 and that the mRNAs for all these polypeptides are purified with equal efficiency by hybridization to P-450e cDNA.[113] More recently, we have found that using certain induction protocols, four electrophoretically separable products immunoprecipitable by anti-P-450 antibodies are found among the translation products of mRNA selectable by hybridization to cloned P-450e cDNA (T. Friedberg, A. Kumar and M. Adesnik, unpublished observations).•

cDNA clones for PB-induced cytochrome P-450 were also isolated by Fujii-Kuriyama and associates.[52] Soon thereafter, these workers reported the cDNA sequences for two overlapping cDNA clones corresponding to nearly the entire coding region of the mRNA encoding one form of P-450 as well as one cDNA clone covering codons 52 to 358 of a very closely related mRNA.[51] The two mRNAs showed an astonishing organization of sequence similarities and dissimilarities. Their nucleotide sequences were identical from the codons encoding amino acid residues 52 to 302. At this point the nucleotide sequences began to diverge, there being 13 nucleotide differences within the remaining 167 nucleotides of overlapping sequence. Although these cDNA clones did not contain the sequence encoding the amino terminus of the cytochrome P-450 isozymes, this sequence was obtained by a primer extension experiment employing a small restriction fragment from the 5′ end of these clones to prime cDNA synthesis from total mRNA from liver of PB-induced rats. This sequence was in agreement with that reported for P-450b.[16] With the report of amino acid sequence data for tryptic peptides representing 75% of the sequences of cytochromes P-450e and b,[244] it became apparent that the longer cDNA sequence corresponded to that for P-450b mRNA, whereas the shorter sequence corresponded to that for P-450e.••

Subsequent to this report, the sequence for the 3′ half of P-450e mRNA[113-173] as well as exonic sequences for the entire P-450e gene[7,134] were reported. These data permitted a comparison of nearly the entire sequences of the P-450b and e mRNAs and revealed the existence of two small regions of high divergence within the two mRNAs. The highly nonrandom distribution of nucleotide differences between P-450b and P-450e mRNA is schematically depicted in Figure 1. While the 5′ terminal 936 nucleotides of the 2 cDNAs are apparently identical, there are 37 nucleotide differences over the remaining kilobase of cDNA sequence, and 15 of these 37 differences are clustered within 2 short regions of the cDNAs which flank a segment encoding a tridecapeptide se-

• Two of these polypeptides clearly correspond to P-450b and P-450e, but the identity of the other two polypeptides is still problematic. Although one of these may be a polymorphic variant of either P-450b or P-450e, this does not appear to be the case for the fourth polypeptide.

•• The cDNA clone identified by Omiecinski et al.[164] as one derived from an mRNA encoding a major phenobarbital-induced P-450 (P-450b or e) hybridizes to an mRNA approximately 4 Kb long and apparently not to a shorter mRNA 2 Kb in length, the size expected for P-450b and e mRNA from DNA sequence data.[61,113,134] Moreover, a bona fide clone to P-450e, identified by DNA sequence analysis, hybridizes only to an mRNA 2 Kb in length and not at all to a larger mRNA.[137] It appears that the clone of Oniecinski et al.[164] was either erroneously identified or corresponds to a very rare mRNA with an extended 3′ untranslated region but which is nevertheless a product of the P-450b or e genes or an as yet unidentified very closely related gene.

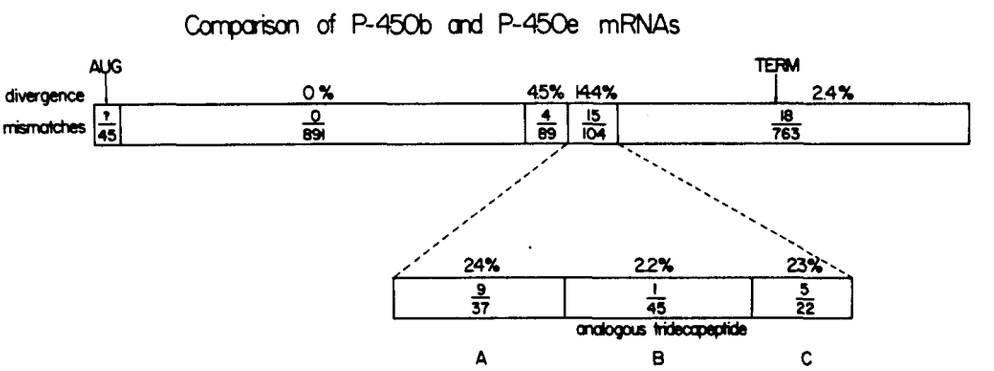


FIGURE 1. Segmented regions of homology and dishomology within cytochrome P-450b and P-450e mRNA's. The P-450b and e mRNA sequences are compared over segments chosen to emphasize the nonrandomness of their dishomologies. The position of the initiation codon is indicated by AUG, while the location of the termination codon is indicated by TERM. The fraction in each rectangle represents the number of mismatches (numerator) over the number of residues within the segment which are being compared (denominator). The first 45 nucleotides in the 2 mRNAs are apparently identical based on primer extension studies by Fujii-Kuriyama et al.[51] However, direct confirmation of this awaits the sequencing of this region in the P-450b gene. While the next 891 nucleotides are identical between these 2 mRNA's, the 3' 956 nucleotides diverge by 3.8%, with 1 segment of 104 nucleotides diverging by 14.4%. This segment of 14% divergence can be split into 2 regions (A and C) of 23 to 24% divergence flanking a region (B) of 2.2% divergence which encodes the analogous tridecapeptide observed by Ozols et al.[167]

quence which has been observed in two unrelated forms of rabbit liver cytochrome P-450.[167] Amino acid sequence data obtained for the purified cytochrome P-450b and P-450e proteins confirm this highly nonrandom distribution of variable residues within them.[244]

2. Mechanisms for Generating mRNA Molecules with Segmented Regions of Homology and Dishomology

The existence of identical (5' half) and divergent (3' half) portions within the two mRNA molecules encoding cytochromes P-450b and P-450e may be explained in several ways. Firstly separate "constant" and "variable" genes may rearrange somatically to produce a functional gene by a mechanism similar to that involved in the formation of functional immunoglobulin genes.[19] This mechanism does not appear to account for the production of cytochromes P-450b and e, however, since comparisons of sperm and liver DNA by the southern blotting procedure have yet to reveal a difference in banding pattern when probed with a P-450e cDNA clone (M. Atchison unpublished). In an alternative mechanism a single transcription unit which includes a "constant region" and two "variable" regions encoding the variable polypeptide segments contained within cytochromes P-450b and e could yield transcripts which are differentially processed to produce either mature P-450b or P-450e mRNAs.* This alternative is also unlikely since there is no evidence for a duplication of the exons responsible for the variable region of P-450b within 10 Kb downstream of the P-450e gene.[7] In addition, we have obtained evidence that for this transcription unit transcriptional termination occurs approximately 1.2 Kb downstream from the last exon encoded by the P-450e gene (E. Rivkin and M. Adesnik, unpublished). This would make it impossible to in-

* This differential processing involves not only differential splicing, but the selection of one of two or more alternative poly (A) addition sites which ultimately define the 3' end of the mRNAs (see References 41 and 124 for examples of how such a mechanism is employed to yield two or more mRNAs from a single gene).

clude any downstream sequences within the P-450e gene primary transcript. Finally, the identical 5′ halves of the P-450b and P450e mRNAs could have resulted from concerted evolution of the corresponding genes. This evolutionary mechanism[5,87] accounts for the fact that members of a multigene family within a single species are generally much more closely related to each other than to equivalent genes in other species despite the fact that they have had an equally long period of evolutionary time to diverge from each other. The molecular mechanism responsible for concerted evolution can involve unequal crossing over between tandemly linked related genes, a double reciprocal recombination event, or gene conversion. The latter term refers to a process which may occur between related genes whereby a segment of one gene replaces the corresponding sequences of the other (see Reference 9). This differs from a reciprocal recombination event in that segments of DNA are not exchanged between genes. Rather, sequences from one gene replace those within another gene, with the apparent loss of the replaced sequences. Gene conversion can, therefore, serve to "homogenize" all or parts of duplicated genes which have already undergone substantial sequence divergence during evolution (see, for example, References 6, 130, 132, 197, and 240). On the other hand, gene conversion may also produce short regions of high diversity within closely related members of a multigene family resulting from the incorporation of DNA segments from more distantly related members of the gene family.[111,129,238] We propose that such gene conversion events are responsible for the nonrandom distribution of dishomologies within the cytochrome P-450b and e genes. In this view distinct P-450b and e genes formed by a relatively recent duplication of a common ancestral gene evolved by acquiring point mutations as well as by one or more gene conversion events. One conversion event* would be responsible for making the 5′ halves of the 2 genes identical, while 1 or more events would be responsible for producing the 15 exonic sequence differences observed between the genes, which are clustered to 2 short regions. The evidence to support this evolutionary scenario results from DNA sequence analysis of other genes related to the P-450e gene.

3. The Cytochrome P-450e Gene

The gene encoding cytochrome P-450e has been cloned and characterized in detail.[7,134] This gene, which encodes a 2-Kb mRNA, spans approximately 14 Kb of genomic DNA and is split into 9 exons (Table 1). While the size of the introns of the P450e gene varies from 180 to 3.7 kb, the exons are all approximately 150 to 200 bp long with the exception of the last exon which is 565 bp in length. This exon, however, contains a coding region of only 179 bp with the remaining 386 nucleotides comprising 3′ untranslated sequences. Nineteen nucleotides upstream from the poly A tail in the P-450e mRNA is the sequence GGUAAA**, a putative polyadenylation signal, which deviates from the canonical poly A addition signal of AAUAAA observed in most other eukaryotic mRNAs.[179] At the 5′ end of the gene, the nucleotide corresponding to the first residue in the mRNA (cap site) lies 30 bp upstream from the initiation codon,[134] and 20 bp upstream from the cap site is a possible "TATA"[20] sequence, CATAAA, which is thought to function in selecting the site of transcriptional initiation.

* As previously noted, the apparently identical 5′ halves of the two genes could also result from an unequal crossover event.

** This unusual polyadenylation signal is found in both P-450b and e cDNAs. The P-450e-like cDNA described by Phillips et al.[173] has a different noncanonical poly (A) signal, GUUAAA.

Table 1
INTRON-EXON ORGANIZATION OF THE CYTOCHROME P-450e GENE

Exon	Acceptor site (5′ end)	Donor sites (3′ end)	Approximate intron size (base pairs)
	CAP		
1	gagcatgcACTGAA -30	ATGCAG gtgagata Gln^{67}	3200
2	tttggcagTTTCGC Phe^{58}	AATATG gtaagact Gly^{112}	260
3	tttgtcagGTGTGT Gly^{112}	CCCAGG gtgagctc Gly^{162}	2800
4	atccccagGAGCCC Gly^{162}	AGCCAG gtcgtggg Gln^{215}	650
5	ccttacagGTGTTT Val^{216}	GAGAAA gtgagtcc Lys^{274}	400
6	gcaaccagGAGAAG Glu^{275}	TCACAG gtatatca Glu^{322}	1300
7	ctacgcagAGAAAG Glu^{322}	CCCAAG gtgaggc Lys^{384}	150
8	tctcccagAACACT Asn^{385}	CCACAG gtgaggca Gly^{432}	3700
9	gtccacagGAAGC Gly^{432}	GGTAAA CAGAAT-CACAG AGTGTGTG agcttcggtg	—

Legend to Table 1: The intron-exon organization of the cytochrome P-450e gene is presented from the data of Atchison and Adesnik[7] and Mizukami et al.[134] Capital letters indicate sequences within exons, while lower case letters represent those within introns, before the cap site or after the site of poly A addition. The codon at each splice junction is shown in brackets and indicated below is the encoded amino acid and codon number. The sequences presented for exons 2 through 9 come from Reference 7. It should be noted, however, that at codons 58, 274, and 322, Mizukami et al.[134] observed the sequences CTT encoding a Leu substitution, AAG encoding a silent A to G change, and GTG encoding a Val substitution, respectively. The CAP site, which represents the 5′ end of exon 1, is indicated with an arrow at position -30. The poly A addition sequence in exon 9 is underlined. It should be noted that in the P-450e cDNA sequence determined by Phillips et al.,[173] the poly A addition sequence is represented by the sequence GTTAAA.

4. A Cytochrome P-450e Gene Family: Evidence for Gene Conversion

Recombinant bacteriophage-containing portions of approximately nine* distinct genes which hybridize to P-450e or P-450b cDNA have been isolated and characterized.[7,133] Genomic southern blots probed with a P-450e cDNA clone encoding exons 6 through 9 reveal the existence of two restriction fragments not contained in the cloned genes.[7] It therefore appears that the P-450e gene family contains 9 to 11 members.

From hybridization studies it became apparent that the other cross-hybridizing genes were highly homologous to the P-450e gene only over the region spanning exons 7 and 8 with the remaining exons showing significant, but substantially less homology to the corresponding regions of the P-450e gene[7] (M. Atchison, unpublished observation). In addition, heteroduplex experiments performed between the P-450e gene and genomic

* When the genomic clones isolated by Mizukami et al.[133] are compared to those isolated in our laboratory,[7] it appears that three of their clones may not represent genes corresponding to those represented by our clones. Similarly, their clone collection is missing two of the genes represented in our collection. There is some difficulty in comparing the two sets of genomic clones which may contain nonoverlapping segments of the same gene.

clones encoding two other members of the P-450e gene family showed homology only over a small region of DNA (Figure 2). The high sequence conservation within exon 7 of the P-450e-related genes was of particular interest since that exon encoded both short regions of high divergence observed between cytochromes P-450b and e. Since this region was so highly related among all the P-450e related genes, it became attractive to hypothesize that one of them may have donated this segment of DNA to the P-450b or e gene in a gene conversion event. This would thus account for the greater than expected number of differences observed between the very closely related P-450b and e (genes) in this region. To test this hypothesis, this region was sequenced in all of the genes cloned in our laboratory and these sequences were compared to the corresponding region of the P-450e gene. As indicated in Figure 3, one of the cloned genes, represented by clones 19 and 25 (see Reference 7), did indeed match precisely one of the hypervariable sequences encoded by the P-450e gene. Furthermore, these data show that the region of identity between this gene and the P-450e gene covers 241 pb, including 120 bp in the intron separating exons 7 and 8. Upstream from this region of identity and further downstream (especially downstream of exon 8), the sequence homology dropped off dramatically further emphasizing the uniqueness of this stretch of identity. A gene conversion event between these two genes, therefore, appears highly probable. It is interesting to note that while all of the P-450e-related genes differ in DNA sequence at the 3′ hypervariable region (with the exception of P-450e and clones 19 and 25), all of the genes except the P-450e gene are very similar to the P-450b sequence at the 5′ hypervariable region. This implies that an additional gene, not represented by our phage collection, has donated DNA sequences by a gene conversion event into the P-450e gene at the 5′ hypervariable region.

Comparison of the DNA sequences from exons 7 and 8 as well as from intron 7 and portions of introns 6 and 8 from all of the related genes cloned in our laboratory (Figure 3) produced another striking observation. Generally it has been found that the exons of related genes retain high sequence homology, while intronic sequences diverge much more rapidly.[91] However, intron 7 of all of the cloned P-450e-related genes appeared to be just as homologous (approximately 90%) to the corresponding P-450e gene sequences as are the flanking exons 7 and 8. On the other hand, upstream from exon 7 into intron 6 and downstream of exon 8 into intron 8, the DNA sequence homology was close to random when compared to the P-450e gene. Limited sequence data from exon 9 from two of the P-450e-related genes have confirmed that the coding region of this exon is much less homologous (~70 to 80%) to the P-450e gene than are exons 7 and 8, as well as intron 7 (M. Atchison, unpublished). This is in spite of a highly conserved region (see below) in the 5′ portion of exon 9 encoding a peptide which has been implicated as being involved in supplying the fifth (axial) ligand to the heme iron via Cys 436 of P-450e.[51,61a] In summary, there appears to be a region of DNA within each of the P-450e-related genes spanning approximately 500 bp which is highly homologous to the corresponding sequences found in the P-450e gene. These results could be interpreted to be due to frequent gene conversion events in this region. Since from a mechanistic point of view, gene conversion is essentially a recombination event (see, for example, Reference 210), this may imply that a DNA sequence is contained within or at the boundaries of this region which represents a hot spot for recombination. As will be discussed later, this same region of DNA may also be involved in gene conversion events between genes encoding two related forms of rabbit liver cytochrome P-450.

5. Functionality of the P-450e Related Genes

Presently, little is known concerning whether the genes which cross hybridize with the P-450e gene, other than the P-450b gene, function to express protein products. It

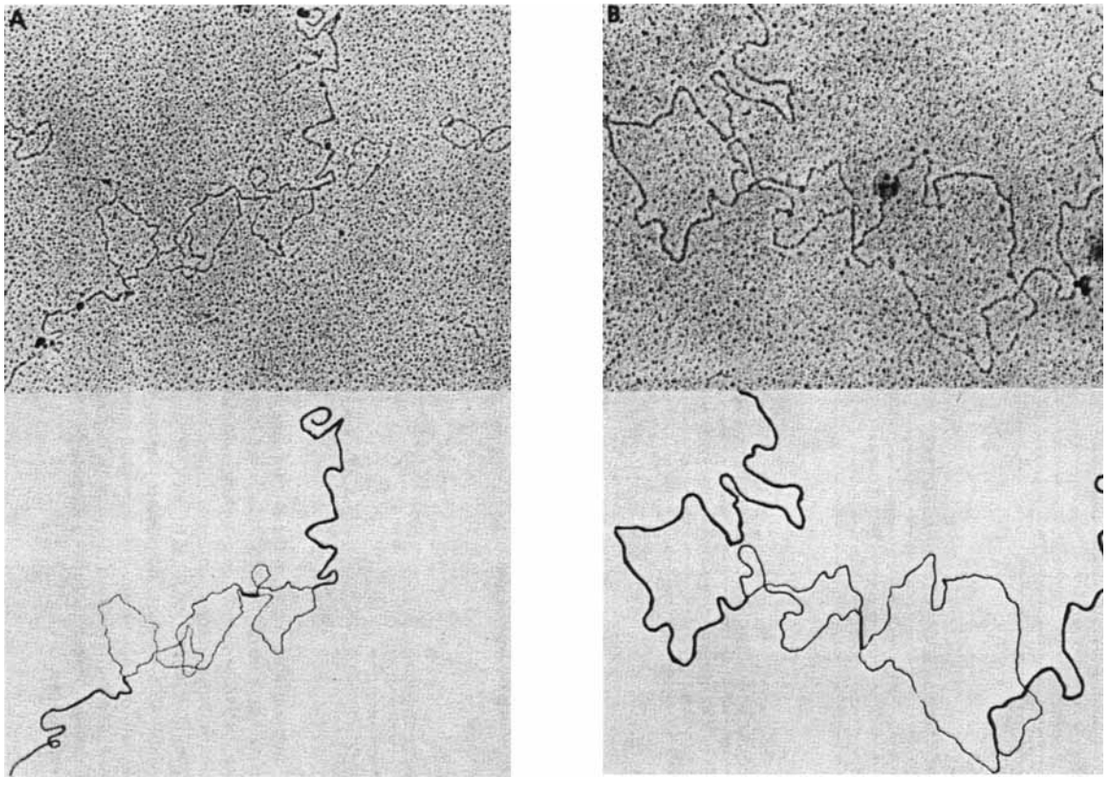


FIGURE 2. Heteroduplex analysis of homologies between the P-450e gene and two related genes. Heteroduplexes were formed between recombinant bacteriophage clone 1 DNA, representing the P-450e gene, and DNA from clones 19 (A) and 9 (B), representing distinct P-450e-related genes.[7] The heteroduplexes show only a short region of high homology between the P-450e gene and the two other genes. The two long regions of duplex on either end of the heteroduplexes represent the bacteriophage arms.

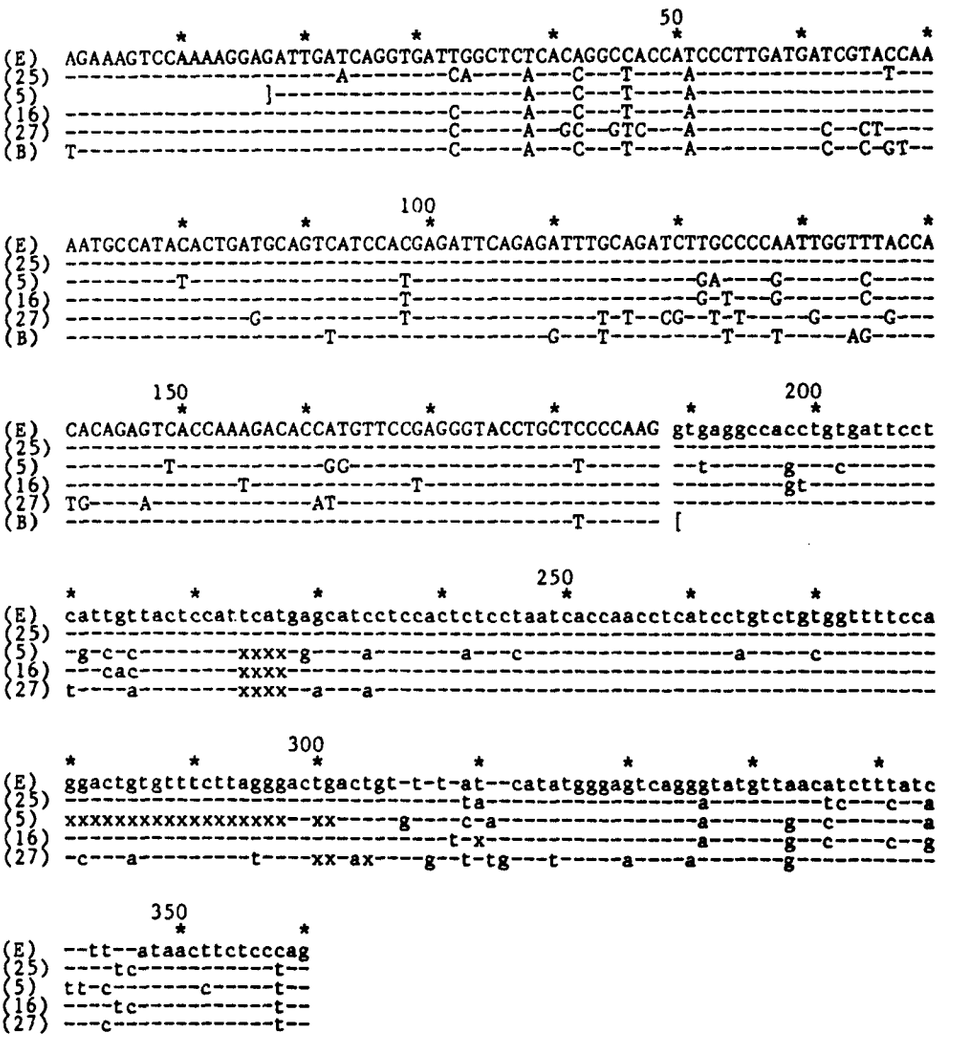


FIGURE 3. The 3′ hypervariable region in the P-450e gene is the result of a gene conversion event. The DNA sequences for exon 7 and intron 7 of the cytochrome P-450e (E) and four related genes (25, 5, 16, and 27) as well as the exon 7 DNA sequence for cytochrome P-450b (B) determined from clones of cDNA by Fujii-Kuriyama et al.[51] are shown. Exonic sequences are indicated by capital letters, while intronic sequences are represented by lower case letters. Residues in teh P-450-related genes which are identical to the corresponding residues in the P-450e gene are indicated by dashes, while nucleotide differences are represented by the substituted nucleotides. X's in the P-450e-related gene sequences and dashes in the P-450e gene sequences represent deletions and insertions, respectively, which are introduced to maximize homology. The crucial observation is that the gene in clone 25 is identical to the P-450e gene for 241 bp starting at residue 68 and extending to residue 308.

is, indeed, possible that one or more of the genes may be nonfunctional pseudogenes as has been observed with members of other multigene families.[115,123,198] Hybridization of in vitro labeled nuclear RNA from livers of control and PB-treated rats to southern blots of the different P-450e-related genomic clones indicated that only the P-450e gene was markedly induced by PB.[7] This methodology was not sufficiently sensitive to determine if the other genes are transcribed at all. Using synthetic oligonucleotides specific for each of the P-450e-related genes, to probe northern blots of liver mRNA, none of the corresponding mRNAs were detectable. Since the P-450b and P-450e specific

oligonucleotide probes easily detect the low levels of these mRNAs in livers from uninduced animals, we can conclude that the other genes are not expressed at levels higher than the basal levels of P-450e and b mRNA (~ 0.02% of total mRNA). The mRNA product of the gene represented by clones 5 and 33 (see Reference 7) was, however, found to be present in mRNA isolated from rat preputial glands, but was not detected in RNA isolated from liver, kidney, lung, testis, adrenal, or brain (T. Friedberg and M. Atchison, unpublished. Phenobarbital and estradiol appeared to induce this mRNA, which is approximately 3000 nucleotides in length, but only by a factor of 2 or 3. Since the preputial gland contains substantial steroid metabolizing activities,[73] it is possible that this form of cytochrome P-450 functions in steroid metabolism. It is worth noting that P-450b has a relatively high activity in the hydroxylation of testosterone and androstenedione at several different positions on the steroid nucleus.[225,226,236a] In addition, using gene-specific oligonucleotide probes we have also detected P-450b and e mRNAs in the adrenal gland. On emay speculate, therefore, that the endogenous substrates for these two forms of cytochrome P-450 may be steroid hormones.

The observation of the P-450e-related product in the preputial gland has several important implications. First, it emphasizes the existence of related tissue-specific forms of cytochrome P-450 which may carry out tissue-specific functions. In addition, it demonstrates the power of recombinant DNA technology to identify functional genes for which no protein product has yet been identified. This is particularly relevant in this case since the gene in question has not been found to be expressed in the adrenal, lung, or liver, the three organs in which cytochrome P-450 has been most extensively studied.

B. Genes For Rat Liver Constitutive Cytochrome P-450 Isozymes Marginally Induced By Phenobarbital

1. Characterization of cDNA Clones

Using immunoscreening methodologies,[242] we have isolated two cDNA clones from a rat liver cDNA library, which react strongly with an antiserum prepared against P-450 PB-1. It should be noted that the western blotting data of Dannan et al.[30] indicate that PB-C (equivalent to PB-1) is only induced twofold by PB administration. The antiserum used to detect these clones recognizes two electrophoretically separable proteins in liver microsomes from untreated and PB-treated animals, one of which comigrates with PB-1 (D. Waxman and M. Adesnik, unpublished). Similarly, the antiserum precipitates two translation products from cell-free systems programmed with mRNA from livers of PB-treated or untreated rats and the more rapidly migrating cell-free product comigrates with the immunizing antigen. The level of both mRNAs increased severalfold after phenobarbital treatment. The two cloned cDNAs contain inserts approximately 400 and 1200 bp in length which have been sequenced and found to encode distinct polypeptides. These show approximately 50% sequence homology to P-450b and P-450e and 72% homology to each other over their regions of overlap which encompasses amino acid residues analogous to 134 to 269 of P-450b (M. Atchison and M. Adesnik, unpublished observation). In hybridization selection experiments, both cloned cDNAs select mRNAs encoding both immunoprecipitable translation products although the clone with the shorter insert preferentially selects the mRNA encoding the translation product which comigrates with PB-1. Furthermore although the clone with the longer insert appears to select both mRNAs equally, the hybridization of the mRNA encoding the polypeptide which comigrates with PB-1 is clearly weaker since it can be eluted from the hybrid at lower temperatures than the mRNA encoding the other polypeptide.

Recently, Kemper's group has cloned from PB-treated animals three distinct rabbit liver cDNAs encoding proteins which bear sequence homology to P-450 LM2, the major PB-inducible form of P-450 in rabbit liver.[119] Two of these cDNAs are 88% homologous to each other, but only 72% homologous to the third. All 3 of the encoded polypeptides are 50% homologous to P-450 LM2 and to rat P-450b. The levels of the mRNAs corresponding to pP-450PBcl and c2 increase substantially after PB administration, whereas pP-450PBc3 mRNA levels are not affected by this treatment.[119a] On the basis of this fact, and the finding that the tridecapeptide sequence in rabbit P-450 LM3b is identical to that encoded by clone pP-450PBc3, it has been suggested[119a] that the latter clone corresponds to LM3b or a very closely related protein. The encoded amino acid sequence of the shorter of our 2 PB-1-like cDNAs is 76, 72, and 58% homologous to the rabbit cDNA clones pP-450PBc1, c2, and c3, respectively, of Leighton et al.[119] while the longer clone is 65, 69, and 58% homologous to the same 3 cDNAs over the 299 amino acids of sequence determined thus far. In addition, the encoded polypeptides of the two cDNA clones isolated by Leighton et al.[119] which extend furthest to the 5′ direction bear substantial sequence homology to the N terminal sequences obtained by Waxman and Walsh[228] for rat cytochrome P-450PB-1. It is therefore reasonable to propose that the two most homologous rabbit liver cDNA clones isolated by Leighton et al.[119] are analogous to the two PB-1 related cDNA clones which we have isolated from rat liver. If this is true, there may exist an additional isozyme in rat liver which is analogous to the rabbit clone pP-450PBc3 isolated by Leighton et al.[119] Genomic southern blots probed with the larger of the two PB-1 related cDNA clones are comparable in complexity to those obtained with P-450e cDNA probes (M. Atchison unpublished) and suggest the existence of additional family members. It is therefore possible that additional forms of P-450 related to PB-1 exist in the liver or other organs which have not yet been characterized.

2. More Gene Conversion in Cytochrome P-450 Genes

The DNA sequence data for the three cDNA clones characterized by Leighton et al.[119] also appear to show a segmented organization of regions of homology and dishomology. While the similarity between the 2 most related cDNAs, pP-450PBc1 and c2, is quite high, there is 1 region of homology which is extraordinarily high with there being only 1 mismatch over 140 bp. Interestingly, this region of extraordinary homology, spanning residues 924 to 1063 of the Leighton et al.[119] sequence, corresponds to most of exon 7 of the P-450e gene. In addition to this region of extraordinary homology, there are several other relatively long segments of low divergence. For example, there are two differences in residues 2 to 92, four differences in residues 472 to 611, and two differences in residues 1244 to 1349. It is tempting to speculate that these regions of low divergence may represent the results of multiple segmented gene conversion events.

In contrast to these segments of low divergence there is a short segment (residues 1351 to 1384) near the 3′ ends of the coding regions of the cDNA clones P-450PBc1 and c2 which is hgihly divergent (14 differences out of 35 residues). However, the DNA sequence in this region in cDNA clone pP-450PBc3, which shows only 72% overall homology to the other 2 cDNAs, shows only 3 differences with the sequence found in clone pP-450PBc1 (Figure 4). These data strongly suggest that the genomic segment encompassing residues 1351 to 1384 of the mRNA corresponding to cDNA clone pP-450PBc1 was introduced into that gene by a gene conversion event with the gene corresponding to clone pP-450PBc3 being the donor for this conversion.

In summary, while gene conversion in some cases may act to maintain sequence homogeneity within a gene family, it can also produce segmented regions of divergence within related genes as in the P-450b and e case, as well as within the genes encoding

```
P450PBc2  CCCGAAACCTCTGGTCAACCCAAACAATGTTGATGAAAATCTATTCTCCAGTGGAATTGT
P450PBc1  ------G--------TG-------GG-CA----CACC-CC-C-C-TGT------GC-A-G
P450PBc3  --T---G--C-----TG-------GG--A-----CCC-CC-C-C-GGAA-A---GT----
```

FIGURE 4. Evidence for gene conversion between two rabbit cytochrome P-450 genes. Portions of the nucleotide sequences determined by Leighton et al.[119,119a] for rabbit cDNA clones pP-450PBc2, c1, and c3 are listed. Residues identical to pP-450PBc2 are indicated by dashes, while nucleotide differences are shown by the substituted nucleotides. A region of potential gene conversion covering 35 bp between clones pP-450PBc1 and c3 (residues 1351 to 1385 in their numbering system) is overlined. Clones c1 and c2 show 88% homology over the remainder of their sequences.

the cDNAs cloned by Leighton et al.[119] Since the cytochrome P-450 (super)family of isozymes recognizes such a huge number of substrates and catalyzes numerous enzymatic reactions, it might have been advantageous from an evolutionary standpoint for these genes to evolve quickly.* Gene conversion may greatly assist in this process by allowing a gene to procure a segment of DNA from a distantly related gene thus producing a gene encoding a protein with novel function. The genes resulting from such conversion events can then be fixed in the population (or eliminated) by natural selection.

C. Genes For 3-Methylcholanthrene-Inducible Isozymes Of Cytochrome P-450

Microsomal cytochrome P-450 associated monoxygenase activities inducible by 3-MC and other polycyclic aromatic compounds have been studied with intense interest for quite some time. This is primarily because the cytochrome P-450 mediated metabolism of aromatic hydrocarbons is often a key step in the conversion of these compounds to mutagenic and carcinogenic derivatives. Secondly, as will be discussed later in great detail, the induction phenomenon was itself of considerable interest and, indeed, substantial progress has been made in the elucidation of the molecular details of the induction mechanism.

In rat liver, two major species of cytochrome P-450 designated cytochrome P-450c and P-450d** are induced by 3-methylcholanthrene treatment.[137,210] As previously noted, the latter corresponds to the major isosafrole induced form of cytochrome P-450.[189] These proteins, which have very different amino terminal peptide sequences, are easily separated by SDS gel electrophoresis and are encoded by distinct, electrophoretically separable mRNAs, 3 and 2 Kb in length, respectively, which do not hybridize to P-450e DNA sequences.[137]

1. Characterization of cDNA Clones for the 3-MC Induced Isozymes of Cytochrome P-450

Recently, the complete nucleotide sequences of the coding regions within cDNA clones for rat liver P-450c [239] and P-450d[98] and for mouse P_3-450[100,101] and P_1-450,[101] the equivalents of rat P-450d and P-450c, respectively, were reported. These sequences showed only approximately 30% homology to those of the major PB-inducible isozymes. The clone for P-450c was identified as such on the basis of complete agreement of the polypeptide sequence encoded by the first 18 codons of the cDNA with the amino terminal 18 residues of the MC-induced P-450 isolated in that laboratory. The same amino terminal sequence was also reported by others.[113a] Although this amino

* In yeast, where the immediate products of conversion events can be detected directly, it has been shown that this process occurs quite frequently. Thus, when a second polymorphic copy of a yeast gene is incorporated into the genome of a haploid yeast cell adjacent to the original gene, it was found that after sexual reproduction approximately 1% of the tetrads produced show evidence of a meiotic gene conversion event.[103]

** These correspond to P-450$_{BNF\text{-}MC\text{-}B}$ and P-450$_{ISF\text{-}BNF\text{-}G}$ of Guengerich et al.[42]

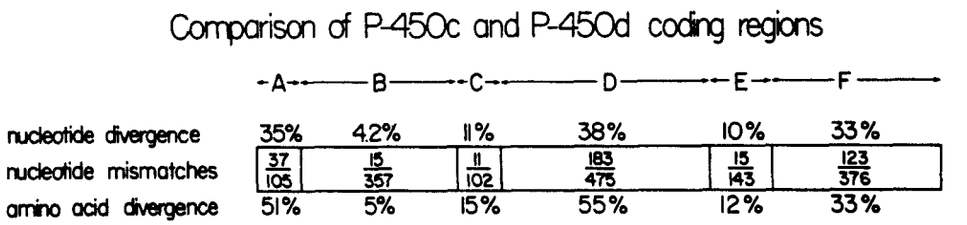


FIGURE 5. Segmented regions of high homology within the P-450c and P-450d coding regions. The P-450c and d cDNA coding sequences have been divided into six segments (A through F) to emphasize the nonrandom distribution of their homologies. The extent of DNA sequence divergence for each segment and the amino acid sequence divergence for the peptide segment encoded by each region are shown. While segments A, D, and F show 33 to 38% dishomology, segments B, C, and E show only 4 to 11% dishomology. The peptide encoded by segment C has been implicated as possibly supplying the fifth ligand to the heme iron and sequences in this region have been found to be well conserved in many P-450 isozymes (Figure 7). Likewise, segment E encodes the analogous tridecapeptide observed by Ozols et al.[167] and has also been observed to be well conserved in many cytochromes P-450. Segment B shows extraordinarily high homology over 357 nucleotides and this homology may be the result of a recent gene conversion event.

terminal sequence differed in six residues from that previously reported for cytochrome P-450c,[16] it is now clear that the latter was incorrect and that the true sequence corresponds to that encoded by the cloned cDNA.[77] Furthermore, all the cysteine containing peptides of P-450c, which contain a total of approximatly 90 residues, have been sequenced and these sequences agree at all but 2 residues to the cDNA encoded sequence.[78]

The P-450d cDNA clone[98,99] was identified by virtue of the fact that the amino terminal 30 residues encoded by this clone are in perfect agreement with those determined for the purified protein.[17] This assignment is confirmed by the finding that the sequence of an internal cysteine containing peptide, 15 residues in length,[78] is found to be present in the cDNA encoded protein sequence.

A comparison of the cDNA sequences for the two related isozymes, P-450c and P-450d, again shows a striking, nonrandom distribution of divergent residues (Figure 5). Whereas the total sequences show approximtely a 30% divergence, there is one exceptionally long segment 357 bp in length which has only 4% divergent residues and encodes a peptide segment with only 6 amino acid differences. This homologous segment is presumably responsible for the hybridization of a cloned P-450d cDNA to both P-450c and P-450d mRNAs in hybridization selection and northern blotting experiments. (J. Fagan, personal communication). Two other short regions which show 90% conservation of sequence are present adjacent to regions with only 60% sequence conservation. The immunological determinant(s) shared by these two P-450 isozymes[183,211] is presumably contained within the more highly conserved segments. It is reasonable to propose that the longer segments showing 96% sequence conservation result from a gene conversion event similar to the one which may have homogenized the 5′ halves of the P-450b and P-450e mRNAs, whereas the shorter regions of 90% sequence homology may simply reflect evolutionary conservation of common functions. A comparision of the sequences of P-450c and P-450d cDNAs and those of their encoded proteins (as well as those for mouse P_3-450) to each other and to those for the PB-induced forms of P-450 is discussed below in the context of attempts to identify functionally important domains of these related proteins.

A cDNA clone isolated by Kimura et al.[100] from a partial library constructed from mRNA purified from polysomes immunoadsorbed to immobilized affinity purified antibody to mouse liver cytochrome P_3-450 was shown to encode the mouse equivalent of P-450d: the first 25 residues encoded by this cDNA clone are identical to those

found at the amino terminus of rat P-450d.* Comparison of the total sequence to that of the P-450d clone of Kawajiri et al.[98] confirms that the two proteins are very closely related. Mouse cytochrome P_3-450[217] has been defined as the form induced in mouse liver by 3-MC and other polycyclic hydrocarbons which is most closely associated with acetanilide-4-hydroxylase activity.** It is distinct from the P-450 which is specifically associated with either arylhydrocarbon hydroxylase activity, P_1-450,[66] or that associated with isosafrole metabolite complex formation, P_2-450***.[158] It should be noted that these three isozymes have been purified and used to obtain antisera each of which specifically inhibits the catalytic activity associated with the heterologous antigens.[158,159] It is clear, as was pointed out in the report describing these findings,[158] that the inability of an antibody to inhibit catalytic activity need not be correlated with an inability to immunoprecipitate the corresponding protein. Similarly, an antibody might precipitate a protein without blocking its catalytic activity. The cytochrome P_3-450 clone isolated and sequenced by Kimura et al.[100] was identified as such by its increased hybridization to mRNA isolated from polysomes purified by immunoadsorption to immobilized anti-P_3-450 antibody relative to the hybridization to mRNA from the nonimmunoabsorbed polysomes. The definitive association of this clone with a specific P-450 isozyme,† however, ultimately depends on the monospecificity of this antibody in polysome immunoadsorption which has not been demonstrated. Furthermore, since the mRNAs for P_1-450 and P_3-450 contain segments of sufficient sequence homology to cross-hybridize to cDNA clones for the heterologous mRNA (see below), in principle the differential hybridization should have identified clones for both proteins.

Although no data are yet available on the structure of the genes for rat liver P-450c†† and P-450d and their relationship to each other and possibly to other genes in the rat genome, some important information has been obtained on the genes for mouse liver P-450s induced by 3-MC. Firstly, a cloned cDNA, clone 46, which hybridizes to a single gene in mouse DNA, was obtained which was preliminarily concluded to be derived from cytochrome P_1-450 mRNA, primarily on the basis of hybridization-selection experiments.[153] The identification of this clone as a P_1-450 cDNA was confirmed by the demonstration[88] that it hybridizes to a 23s mRNA (and not to a 2-Kb mRNA), the levels of which correlate with arylhydrocarbon hydroxylase activity and not with levels of P_2-450 or P_3-450 or their associated enzymatic activities. Subsequently, genomic clones for the P_1-450 gene which hybridized to the clone 46 were isolated and

* The marked amino acid dishomology between the P_3-450 and P-450d sequences found between codons 27 to 47 is the result of a sequencing error in the P_3-450 sequence involving an insertion of a G residue at nucleotide 139 of the Kimura et al.[100] sequence and a deletion of a G residue after nucleotide position 201. [Nucleic Acids Research *12:* 4810 (1984)].

** This form was originally designated P-448 on the basis of the difference spectrum for its CO reduced form.[66] In rat liver, all of the forms of cytochrome P-450 purified by Guengerich and associates have substantial acetanilide-4-hydroxylase activity (3 to 6 nmol/min/nmol P-450) although $P\text{-}450_{BNF\text{-}B2}$ (P-450c) has a 10-fold higher activity and accounts for 70% of this activity in microsomes from BNF-treated rats.[62] It should be noted, however, that rat P-450c has been considered the equivalent of mouse P_1-450 because of their association with 3-MC-induced arylhydrocarbon hydroxylase activity.[149]

*** P_2-450 has been defined as the form of isosaffrole induced cytochrome P-450 in DBA/2N mouse liver most specifically correlated with isosaffrole metabolism.[158] The relationship between P_2-450 and P_3-450 is uncertain and in the paper on the nucleotide sequence of the putative p_3-450 cDNA clone it is, in fact, suggested that P_2-450 of DBA/2N mice may represent a "protein polymorphism" (i.e., an allelic variant) of P_3-450 in C57BL/6N mice.[100] It is not clear, however, how this could explain all the features of this isozyme. Nevertheless, this conclusion seems quite reasonable since extensive southern blotting analysis of mose genomic DNA reveals the existence of only two closely related genes.[60]

† As will be apparent from subsequent discussion, it clearly doesn't correspond to P_1-450, the mouse equivalent of rat P-450c, since it is derived from an mRNA approximately 2100 bp long, whereas the P_1-450 mRNA is approximately 2.9 Kb in length.

†† See notes added in proof for a discussion of the intron-exon organization of the rat P-450c gene and the mouse P_1-450 and P_3-450 genes based on the complete DNA sequences of these genes.[59a,197a]

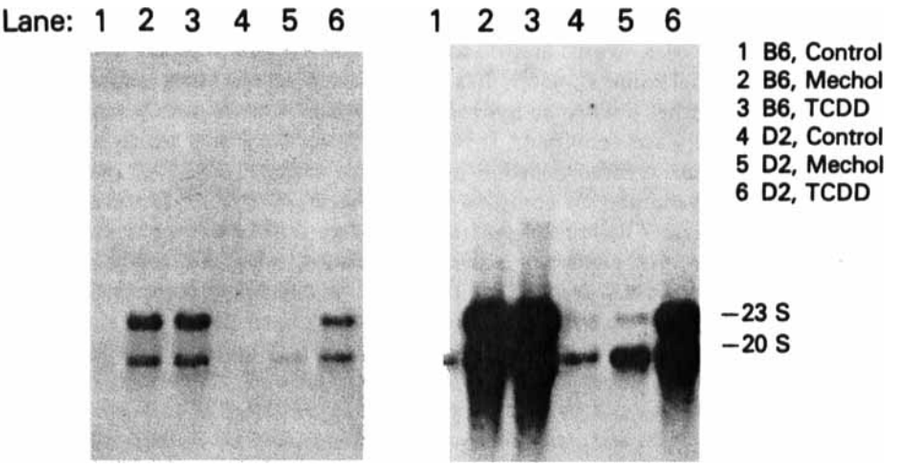


FIGURE 6. A 5′ fragment of the mouse P_1-450 gene hybridizes to two electrophoretically separable mRNAs both induced by 3-methylcholanthrene. A 2.9-Kb Eco R1 restriction fragment derived from the 5′ portion of the cloned mouse P_1-450 gene[141] was labeled by nick translation and hybridized to northern blots containing electrophoretically fractionated poly (A)* mRNA from livers of responsive (B6) or nonresponsive (D2) mice treated with 3-MC, TCDD, or with corn oil (controls). The electrophoretic migration is from top to bottom. The autoradiograms obtained after a 3- (left) or 24-hr (right) exposure are shown. The 23s RNA band is the only one obtained when a similar blot is hybridized to the P_1-450 cDNA clone 46. The relative intensities of the two bands in each lane do not accurately reflect the relative amount of each mRNA since only the 23S band is perfectly homologous to the exonic regions in the probe. Notice that even though the induction of both mRNAs by 3-MC is defective in D2 mice, the 20s mRNA is induced more than the 23s mRNA in these mice. This correlates with the fact that in B6 mice, the 20s mRNA is induced by lower concentrations of 3-MC than induce the 23s mRNA. These data are from Ikeda et al.[88]

partially characterized.[141] As a result of these studies, it became clear that this cDNA clone, which carried an insert approximately 1.1 Kb long, was derived primarily from the 3′ untranslated region of the P_1-450 mRNA. Whereas cDNA clone 46 only recognized a single genomic fragment in southern blotting analysis, a 2.9-Kb subfragment of a P_1-450 genomic clone, estimated to cover the 5′ portion of the P_1-450 gene, hybridizes to 2 or 3 bands.[88] More importantly, this fragment hybridizes both to the 23S mRNA representing the P_1-450 mRNA and to a 20S band (Figure 6) which contains the P_3-450 mRNA.[217] This clearly indicates that there is a homology between these mRNAs in their 5′ portions. This homology was confirmed in hybridization experiments in which 5′ subfragments of a putative P_3-450 cDNA clone were shown to hybridize to the 23s P_1-450 mRNA and to the cloned P_1-450 gene.[217]

Very recently, essentially full length cDNA clones and genomic clones for mouse liver cytochrome P_1-450 and P_3-450 were isolated in Nebert's laboratory.[60] DNA sequence analysis of the 2 cDNAs confirms the existence of highly homologous segments near the 5′ ends of the 2 mRNAs, segments 425 bp long in the 2 mRNAs which contain only 10 nucleotide differences[100,101] and it was suggested that this homology could have resulted from a gene conversion event.[101] This region of extraordinary homology is similar to that observed in the corresponding regions of the rat P-450c and P-450d cDNAs, which, based on the DNA sequence data now available, clearly represent the rat genes which are orthologous* to mouse P_1-450 and P_3-450, respectively.

Analysis of heteroduplexes formed between each cDNA clone and the corresponding genomic clone indicated that the P_1-450 and the P_3-450 genes are approximately 6 and

* Two different types of homology have been defined.[49] Two different genes whose difference is a consequence of independence arising from speciation are said to be orthologous. Paralogous genes in a single organism result from a gene duplication and evolved side by side in parallel in a single line of descent.

8 Kb in length, respectively, and that both genes contain 7 exons and 6 introns.* The sizes of the exons in the two genes are very similar except for the 3′ terminal exon which contains the 3′ untranslated regions and it is not unreasonable to expect that the exonic DNA is, in fact, interrupted at equivalent locations within the two genes. The intron-exon organization of these genes, however, appears to be substantially different from that of the P-450b and e genes. In particular, exon 2 of the P_1-450 and P_3-450 genes which is certainly within the coding regions of these genes is approximately 900 bp long and is likely to include segments equivalent to those contained within 4 to 6 exons in the P-450b and e genes. The exact location of the introns within the P_1-450 and P_3-450 genes can, of course, only be determined by a direct comparison of genomic and cDNA sequences.**

D. Sequence Comparisons of Different Cytochrome P-450 Isozymes: Functional Domains of Cytochrome P-450

The catalytic function of the cytochromes P-450 involves the acceptance of electrons from NADPH via an interaction with NADPH-cytochrome P-450 reductase and the transfer of these electrons to molecular oxygen such that one oxygen atom is introduced into a specific substrate, whereas the second oxygen atom is used to form water. It is reasonable to presume that the different multiple forms of cytochrome P-450 contain homologous domains reflecting their common function. These would include a domain involved in heme binding and another involved in the interaction with a single molecular species of the reductase.[165] In addition, although the various P-450s would be expected to contain different active sites involved in substrate recognition, these too may very well show substantial homology, given the overlapping substrate specificities of the different isozymes.

One of the goals of studying the sequences of the various forms of cytochrome P-450 is to determine which domains of the proteins participate in common functions such as heme binding and interaction with cytochrome P-450 reductase and which domains are involved in isozyme specific functions such as substrate recognition. The former may be identified by comparing amino acid sequences of distantly related isozymes which have diverged substantially but still function as a monooxygenase catalyst. In contrast, domains involved in the determination of isozyme-specific substrate specificities may be recognizable when comparing the amino acid sequences of closely related isozymes which show considerable overall homology, but which possess distinguishable substrate specificity profiles.

Comparison of the available cytochrome P-450 sequences reveals at least three regions which appear to be related among all cytochromes P-450, including the most distantly related P-450 proteins. A cysteine residue has been implicated [232] in supplying the fifth ligand to the heme iron and in the case of P-450cam this appears to correspond to Cys_{134}.[40] Peptides centered on Cys_{152} in P-450b and e, and Cys_{150} in P-450 LM2, are homologous to the peptide centered on Cys_{134} in P-450cam. In this region, there is a stretch of 13 identical amino acids in P-450b and the somewhat divergent (48% homology) protein encoded by the PB-1 related cDNA clone isolated and sequenced in our laboratory (Figure 7). Two closely spaced regions in P-450c, P-450d, and P_3-450 show homology to these conserved peptides (Figure 7). These are centered on Cys_{158} and His_{170} in P-450d and P_3-450 and Cys_{160} and Tyr_{172} in P-450c. However, even

* The previously described genomic clone for P_1-450 [141] was isolated from a mouse plasmacytoma genomic library. R-loop analysis using that clone indicated that the P_1-450 gene was 4.6 Kb long and contained 5 exons. This gene differs at its 5′ end from the one isolated from the normal mouse liver library and it is now believed that the plasmacytoma clone represents the result of a chromosomal rearrangement within the tumor cell genome.[40]

** Such an analysis has recently been reported[59a] and is discussed in notes added in proof.

A

P450b	PHE	SER	THR	GLY	LYS	ARG	ILE	436 CYS	LEU	GLY	GLU	GLY	ILE	ALA	ARG	ASN	GLU	LEU	PHE	LEU	PHE									
P450e	PHE	SER	THR	GLY	LYS	ARG	ILE	436 CYS	LEU	GLY	GLU	GLY	ILE	ALA	ARG	ASN	GLU	LEU	PHE	LEU	PHE									
LM2	PHE	SER	LEU	GLY	LYS	ARG	ILE	434 CYS	LEU	GLY	GLU	GLY	ILE	ALA	ARG	THR	GLU	LEU	PHE	LEU	PHE									
PTF-1	PHE	SER	ALA	GLY	LYS	ARG	ALA	CYS	VAL	GLY	GLU	GLY	LEU	ALA	ARG	MET	GLN	LEU	PHE	LEU	PHE									
PBc2	PHE	SER	THR	GLY	LYS	ARG	VAL	CYS	VAL	GLY	GLU	ALA	LEU	ALA	ARG	MET	GLU	LEU	PHE	LEU	PHE									
PBc1	PHE	SER	THR	GLY	LYS	ARG	VAL	CYS	VAL	GLY	GLU	ALA	LEU	VAL	ARG	MET	GLU	LEU	PHE	LEU	PHE									
PBc3	PHE	SER	ALA	GLY	LYS	ARG	ALA	CYS	VAL	GLY	GLU	GLY	LEU	VAL	ARG	MET	GLU	LEU	PHE	LEU	LEU									
$P_3$450	PHE	GLY	LEU	GLY	LYS	ARG	ARG	456 CYS	ILE	GLY	GLU	ILE	PRO	ALA	LYS	TRP	GLU	VAL	PHE	LEU	PHE									
P450d	PHE	GLY	LEU	GLY	LYS	ARG	ARG	456 CYS	ILE	GLY	GLU	ILE	PRO	ALA	LYS	TRP	GLU	VAL	PHE	LEU	PHE									
P450c	PHE	GLY	LEU	GLY	LYS	ARG	LYS	460 CYS	ILE	GLY	GLU	TYR	ILE	GLY	ARG	LEU	GLU	VAL	PHE	LEU	PHE									
C21	GLY	ALA	ALA	GLY	SER	ARG	VAL	CYS	LEU	GLY	GLU	PRO	LEU	ALA	ARG	LEU	GLU	LEU	PHE	VAL	VAL									
CAM	PHE	GLY	HIS	GLY	SER	HIS	LEU	355 CYS	LEU	GLY	GLN	SER	LEU	ALA	ARG	ARG	GLU	ILE	ILE	VAL	TYR									

FIGURE 7. Amino acid sequences of two highly conserved regions observed in all cytochrome P-450 isozymes. The peptides centered on Cys 436 (A) and Cys 152 (B) in rat cytochrome P-450b have been compared to analogous segments in all of the known cytochrome P-450 sequences. Amino acid residues which are found in at least five of the peptide sequences are boxed. Peptides marked by an asterisk in B represent a second region within cytochromes P_3-450, P-450d, and P-450c which shows homology to the peptide centered on Cys 152 in P-450b. The rat cytochrome P-450b and e sequences have been taken from Fujii-Kuriyama et al.[51] and Yuan et al.;[244] rabbit P-450 LM2 from Heinemann and Ozols[83] and Tarr et al.;[203] rat pTF-1 and pTF-2 from our unpublished results for two proteins recognized by antibodies to PB-1;[228] rabbit P-450 PBc2, c1, and c3 from Leighton et al.;[119] mouse P_3-450 from Kimura et al.;[100] rat P-450d from Kawajiri et al.;[98] rat P-450c from Yabusaki et al.[229] and Haniu et al.;[77,78] bovine P-450$_{c21}$ from Yuan et al.;[243] and bacterial P-450$_{cam}$ from Haniu et al.[76]

though the homology between P-450b and c in this region is higher than the overall homology between these two isozymes, this homology is not as high as that observed in the region surrounding Cys_{436} in P-450b. Furthermore, in this region P-450cam is more closely related to rat P-450b than the latter is related to P-450c and P-450d. A second more highly conserved cysteine-containing peptide region centered on Cys_{436} in rat P-450b and e, Cys_{456} in mouse P_3-450 and rat P-450d, Cys_{460} in rat P-450c, Cys_{434} in rabbit P-450 LM2, and Cys_{355} of bacterial P-450cam is present in all P-450s which have been studied to date (Figure 7). It has also been proposed that these peptides provide the heme binding site.[51,61a,101] The greater hydrophobic pocket in these C terminal conserved cysteine-containing peptides as compared to the amino terminal cysteine-peptides favor this cysteine as the more likely candidate for providing the thiolate ligand to the heme iron at the enzyme active site.[61a] In any case, the high conservation of both peptide segments suggests that they play an important structural or functional role.

An additional region of high homology is the analogous tridecapeptide observed by Ozols et al.[167] within two unrelated forms of rabbit P-450, LM-2 and LM3b.This tridacapeptide is 54% homologous between cytochromes P-450b and c, while the overall homology between these two isozymes is only 29%. In fact, a region encompassing 49 residues spanning amino acids 337 to 385 of the P-450b sequences (including the analogous tridecapeptide) is 47% homologous between P-450b and c. Although the se-

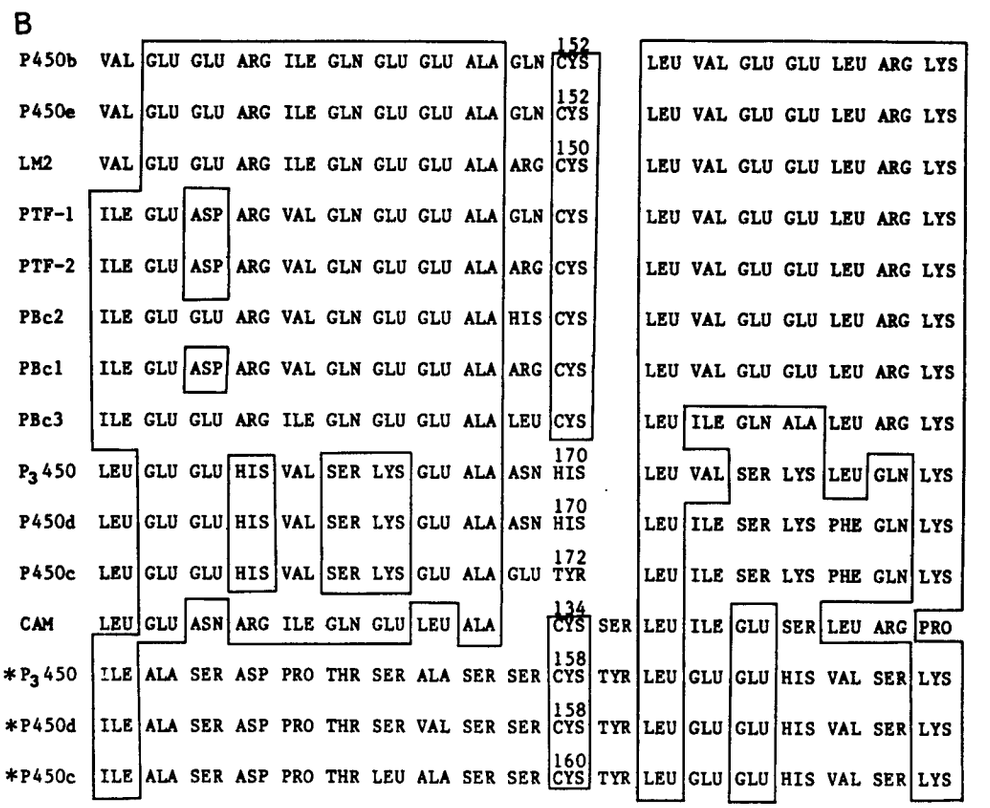


FIGURE 7 B

quence data is incomplete, the small segment spanning residues 337 to 362 in the PB-1 related cDNA clone mentioned above shows 68% homology to P-450b, while the overall homology between these two isozymes is only 48% (M. Atchison, unpublished). Similarly, the overall homology between rabbit P-450 LM2 and the proteins encoded by cDNA clones described by Leighton et al.[119] is 50%, whereas the tridecapeptides in the latter proteins show 11 of 13 or 12 out of 13 matches with that of LM2. The high conservation of this region observed in all eukaryotic cytochrome P-450 isozymes sequenced to date suggests that it must be involved in a functional or structural feature common to all these enzymes. Finally, it should be noted that there is a region of 54% homology between cytochromes P-450b and d (as well as P-450c) spanning residues 72 to 98 in the P-450b sequence.

While the above discussion has concentrated on similarities between the various molecular forms of cytochrome P-450, what can be said of the differences? Here, we would like to propose that residues involved in substrate specificity lie in the region corresponding to 200 and 300 in the P-450b protein sequence. For example, it is rather striking that cytochromes P-450c and P-450b which have very different substrate specificity profiles[188,190] show very little (<15%) amino acid homology over this region. Indeed, this is the region of lowest sequence homology within the two isozymes. Similarly, the cytochrome P-450 PB-1-like clone encodes a protein, which is 48% homologous to P-450b and e, but shows only 30% homology to P-450b over residues 200 to 300.

The same comparison can be made between the related proteins P-450c and d. While these 2 enzymes are clearly related immunologically[183,211] and, as previously noted, are

approximately 70% conserved in amino acid sequence, they exhibit distinguishable substrate specificity profiles.[188,189] However, once again, the region of highest divergence between these 2 isozymes lies between residues 200 to 300 where they show only 38% homology. Cytochromes P-450b and e which are identical in this region possess nearly identical substrate specificities. On the other hand, the enzymatic activity of P-450e with nearly all substrates is approximately five times lower than that of P-450b.[190] Could the difference in enzymatic activity observed between these two P-450s be related to the numerous differences in the "hypervariable regions" flanking the analogous tridecapeptide[167] which may, for instance, affect the interaction of cytochrome P-450e with P-450 reductase in such a way as to make P-450e less active? One can further ask, if the fact that the hypervariable region upstream from the analogous tridecapeptide is conserved among P-450b and the other genes we have cloned but not P-450e (Figure 3) reflects evolutionary conservation of a domain required for high catalytic activity which may have been incorporated into these genes as a result of gene conversion events followed by natural selection.

While the interpretation presented above is clearly speculative, this hypothesis may soon be testable now that cDNA and genomic clones are available for a number of distinct forms of P-450. Chimeric genes can be constructed in which the exonic regions encoding the amino acid residues spanning 200 to 300 are inserted into another cloned cytochrome P-450 gene and the substrate specificity of the resulting gene product, synthesized in a suitable eukaryotic or prokaryotic host, can be determined. In addition, in vitro site directed mutagenesis can be carried out in an attempt to locate functional regions in the cytochrome P-450 protein.

E. How Many Cytochrome P-450 Genes Are There?

Recombinant DNA and protein purification studies are rapidly expanding the number of identified forms of cytochrome P-450. It now appears likely that the total number of cytochrome P-450 genes far exceeds the number of distinct P-450 proteins which have been purified to date. It is first worth noting that each cytochrome P-450 which has been studied at the gene level has been found to be encoded by a member of a multigene family with a minimum of 2 members of each family being expressed. In fact, in several cases, related genes were detected even before the existence of related proteins was firmly established. This is true for the two PB-1-like cDNA clones isolated in our laboratory and the set of similar clones from rabbit liver which were characterized by Leighton et al.[119] In the cytochrome P-450e gene family, cytochromes P-450b and e are known to be expressed in the liver, and an additional member is now known to be expressed in the preputial gland. Hence, at least three of the members of this multigene family are expressed. Rat cytochromes P-450c and d are encoded by members of the same multigene family as are their mouse equivalents P_1-450 and P_3-450. A comparison of all the cytochrome P-450 gene sequences studied to date indicates that they can be grouped into gene subfamilies and families which together constitute a large gene superfamily.

As previously noted, molecular cloning studies have identified 8 to 11 genes which cross-hybridize with cytochrome P-450e and b sequences,[7,133] although it is not known how many of these genes are functional. The cDNA clones encoding the cytochrome P-450 PB-1 related sequences cloned in our laboratory do not cross-hybridize with a P-450e cDNA clone (A. Kumar and M. Adesnik, unpublished), but based on their overall sequence homology of approximately 50% with the latter cDNA do, indeed, belong to the same gene family. The same relationship holds for the gene encoding rabbit LM2 and the subfamily corresponding to the cDNAs sequenced by Leighton et al.[119] One of the rat PB-1-like clones has been shown to hybridize with six to eight bands in the rat genome (M. Atchison, unpublished). The gene family containing the

P-450b, e, and PB-1 genes appears, therefore, to possibly contain 20 (or even more) family members.

Cytochromes P-450c and P-450d (and mouse P_1-450 and P_3-450) are approximately 70% homologous to each other and thus are on the border of fitting the definition of being members of the same subfamily (80% homology). However, they are clearly encoded by members of the same family (50% homology) which is distinct from the P-450b, P-450e, and PB-1 gene family since members of these 2 families show only 30% homology. These data, therefore, define at least two multigene families within the cytochrome P-450 superfamily of genes. Despite the exceptionally simple genomic blotting pattern which indicates that the P_1-450, P_3-450 gene family contains few, if any, additional cross-hybridizing members,[66,88] other members of the gene family may exist but not be detectable by the hybridization procedure which requires at least 70% nucleic acid homology. Indeed, the familial relationship between P-450b,e, and PB-1 genes could only be recognized when the corresponding cDNAs were cloned by independent procedures and their sequences compared.

Recently, Hardwick et al.[80] have cloned the cDNA for P-450_{PCN} and found that this cDNA hybridizes to six to eight bands in the rat genome. This cDNA does not cross-hybridize with the P-450b or e mRNAs* and recognizes a set of genomic restriction fragments distinct from those recognized by P-450e cDNA. If the cDNAs encoding P-450 PB-1 related proteins and P-450 PCN do not cross-hybridize, the number of P-450 genes so far recognized begins to approach 30. On the basis of the limited sequence data obtained for adrenal microsomal steroid 21-hydroxylase,[231,243] these do not belong to the cytochrome P-450b, e or P-450c, d gene families. It is not unreasonable to speculate that the total number of P-450 genes could easily exceed 50.

F. The Genetics of Cytochrome P-450: Structural, Functional, and Regulatory Polymorphisms and Chromosomal Localization of Cytochrome P-450 Genes

1. Coumarin Hydroxylase

Liver microsomes from certain inbred strains of mice have been shown to have higher 2.5-1 to 10-fold) PB-induced coumarin hydroxylase activity than liver microsomes from other strains.[236,237] Analysis of F1 progeny obtained from genetic crosses between members of the various strains exhibiting high or low coumarin hydroxylase activity suggested that there was a single autosomal locus residing on mouse chromosome 7 which contained the coumarin hydroxylase gene.[237] Additional crosses established the location of this gene to a position very near the glucose phosphate isomerase (Gpi-1) locus.[237] Recently, Simmons and Kasper[194] have used a cytochrome P-450b (or e) cDNA clone to probe southern blots of DNA isolated from a number of mouse strains exhibiting either high or low courmarin hydroxylase activity, as well as southern blots of DNA isolated from crosses between these two classes of mouse strains. In each case, certain restriction fragment length polymorphisms showed a 100% concordance with the courmarin hydroxylase polymorphism, indicating that they were very closely linked to the coumarin hydroxylase locus. The simplest explanation of the data was that a deletion had occurred in the low responding strain relative to the high responding mouse strain. Since, as discussed above, this locus has been mapped to mouse chromosome 7,[237] it suggested that at least one member of the P-450e-related multigene family is encoded on mouse chromosome 7.

2. Debrisoquine Hydroxylase

Genetic differences in specific cytochrome P-450-mediated drug metabolizing activ-

* The hybridization was not, however, carried out at a sufficiently low stringency to reveal weak homologies if they existed.

ities have also been observed in humans. While most individuals metabolize the antihypertensive drug debrisoquine to 4-hydroxy debrisoquine, approximately 5 to 10% of the Caucasian population is deficient in this activity.[46,126,213] Based on large population and family studies, these differences in enzymatic activity result from a polymorphism in a single autosomal, recessive Mendelian gene.[46,126] The enzyme responsible for debrisoquine hydroxylation appears to be a form of cytochrome P-450 since the activity is NADPH-dependent and is inhibited by carbon monoxide.[94] In addition, other substrates for which there is in vivo evidence for association of their metabolism with the debrisoquine hydroxylation polymorphism are good inhibitors of the reaction in vitro.[15]

The debrisoquine hydroxylase polymorphism has important implications in drug therapy since homozygous recessive individuals are more sensitive to and experience more serious side effects from certain pharmacologic agents due to their inability to metabolize them. Recently, a constitutive form of rat cytochrome P-450 with debrisoquine hydroxylase activity has been purified and partially characterized.[114] While this isozyme, designated P-450 UT-H, was found to represent approximately 6% of the microsomal cytochrome P-450 present in Sprague-Dawley rats, it was present at less than 5% of this level in female rats of the DA strain, which had previously been shown to have greatly reduced debrisoquine hydroxylase activities. The defect in the DA strain is likely to be in a regulatory locus since the low level of enzyme activity correlates with a low level of the corresponding protein as detected by immunochemical methods. Cytochrome P-450 UT-H was found not to be inducible by PB, BNF, PCN, isosafrole, or Aroclor® 1254. Characterization of the rat debrisoquine hydroxylase isozyme and its gene may lead to the ability to detect the mutant human allele by using restriction site polymorphisms detectable on southern blots of DNA isolated from the blood of patients. This could supersede the present method of detection of the polymorphism which involves administration of low doses of the drug and monitoring the patients for side effects.

3. 17α-Progesterone 21-Hydroxylase of the Adrenal Cortex

Genetic defects in specific cytochrome P-450 catalytic activities have been implicated as the cause of hereditary deficiencies in cortisol biosynthesis by the adrenal gland (for a review see Reference 154). While a deficiency in the mitochondrial cytochrome P-450 responsible for the side chain cleavage of cholesterol ($P\text{-}450_{scc}$) has been implicated in some cases of decreased cortisol biosynthesis,[104] a deficiency in the microsomal 17α-progesterone-21 -hydroxylase is more commonly observed, occurring in 1 out of 5000 people.[154] Family studies have established that this C_{21} hydroxylase deficiency is an autosomal recessive trait closely linked to the HLA complex.[39] In confirmation of this, the gene encoding the mouse P-450 C_{21} hydroxylase has been recently localized to a position on chromosome 17 near the region encoding the MHC class III genes.[231a] This was accomplished by hybridization of cloned bovine $P\text{-}450_{c21}$ cDNA[231] to cosmid clones containing DNA segments from the mouse MHC locus.

4. Rabbit LM3b

Strain differences in the catalytic activity of rabbit cytochrome P-450-3b have been reported.[35] Microsomes isolated from most outbred NZW rabbits catalyze the hydroxylation of progesterone at the 6β and 16α positions. However, microsomes from the inbred rabbit strain IIIVO/J exhibit a greatly diminished 6β hydroxylase activity. Cytochrome P-4503b isolated from NZW rabbits catalyzes the 6β and 16α-hydroxylation of progesterone, whereas an equivalent preparation from IIIVO/J rabbits catalyzes only the 16α-hydroxylation reaction and has a greatly diminished 6β hydroxylase activ-

ity. Enzyme kinetic data for the enzyme isolated from NZW rabbits suggest that it is a mixture of two very closely related subforms, only one of which catalyzes the 6β hydroxylation. Analysis of complete tryptic digests of the two purified protein preparations by reverse phase high pressure liquid chromatography revealed only one distinguishing peptide. Although genetic studies were not performed to establish definitively that these differences result from allelic variation, the high degree of similarity of enzyme preparations from the two rabbit strains supports such an interpretation.

5. The Major Phenobarbital-Induced P-450s of Rat Liver

Polymorphisms have also been detected by Rampersaud and Walz[182] using two-dimensional isoelectric focusing-SDS polyacrylamide gel electrophoresis to separate cytochrome P-450b and e isozymes from rat liver microsomes from four inbred and five outbred rat strains/colonies as well as the F1 progeny from ten crosses. The results obtained were consistent with there being two closely linked loci encoding cytochromes P-450b and e with at least six alleles at the P-450b locus and at least two alleles at the P-450e locus.

Presently, there are four cDNA[3,51,113,173] and two genomic[7,134] DNA sequences available which encode cytochrome P-450e* or highly related isozymes. None of these DNA sequences are identical, although the number of differences observed between any two sequences is very small and the encoded polypeptides remain over 99% homologous. There are a total of 15 positions in the protein coding portion of each DNA sequence in which 1 or more of the sequences has been found to differ, and 8 of the 15 changes result in an amino acid replacement. One might imagine that each of these published DNA sequences represents a distinct P-450 gene resulting from the existence of six P-450e-related genes of remarkable homology. Alternatively, these differing sequences may only reflect a high degree of polymorphism present in the P-450e gene locus. Indeed, if polymorphism is the cause of the differing P-450e DNA sequences, it would suggest that the number of P-450e alleles in the rat population greatly exceeds six since none of the P-450e DNA sequences obtained thus far in various laboratories have been found to be identical.

6. Chromosomal Localization of Cytochrome P-450 Genes

Since at least one cytochrome P-450 gene (i.e., the coumarin hydroxylase gene) is located on mouse chromosome 7, it is of interest to know whether genes encoding other P-450s lie on this chromosome as well. Little data is available on the chromosomal location of cytochrome P-450 genes. Work from Nebert's laboratory has established that the Ah** regulatory gene resides on mouse chromosome 17,[118] while the P_1-450 and P_3-450 structural genes have been localized to chromosome 9.[215] Thus, the regulatory and structural genes of the Ah locus appear to reside on distinct chromosomes, and the location of these genes differs from the one mapped by Simmons and Kasper[194] and Wood and Taylor[237] for a phenobarbital-inducible P-450 gene. As mentioned above, the gene for one other P-450 isozyme, the mouse adrenal 17α-progesterone-21 hydroxylase, has been found to reside on mouse chromosome 17 near the MHC class III genes.[231a] On the basis of numerous studies with other multigene families (for a

* A gene is identified as a P-450e-like gene if it encodes at the "hypervariable" regions the amino acid sequence corresponding to that of P-450e.

** As discussed in greatdetail below, the inducibility of both P_1-450 and P_3-450 by polycyclic hydrocarbons is regulated by the Ah gene which appears to encode a protein, the Ah receptor, which, when complexes with an inducing ligand, can activate transcription of the Ah structural genes. In particular, C57BL/6 (i.e., B6) mice are responsive (inducible by 3-MC) to 3-MC, whereas DBA/2 (i.e., D2) mice are nonresponsive. Inducibility in these strains is dominant and strictly correlates with the presence of the responsive C57BL/6 allele. Even nonresponsive strains are inducible my more potent inducers such as TCDD.

review see Reference 87), it is reasonable to propose that the Ah structural genes are all clustered to mouse chromosome 9 and all of the P-450e-related genes are clustered on chromosome 7. If this is the case, the close proximity of related P-450 genes to one another may enhance gene conversion events between nonallelic genes. This could promote extensive polymorphism of allelic genes, as has been observed in the immunoglobulin 2a and 2b genes.[163] This may partially explain the high degree of apparent polymorphism observed at the P-450e and b loci. In this respect, it is interesting to note[3] that several of the polymorphic residues observed in the P-450e cDNA and genomic DNa sequences are identical to the corresponding residues in the highly homologous cytochrome P-450b cDNA sequences (see Table 1) determined by Fujii-Kuriyama et al.[51]

G. Evolution of Cytochrome P-450

Genes which arise by duplication represent one of the most important raw materials for evolution since one copy of the duplicated gene is free to diverge rapidly without jeopardizing the ability of the organism to carry out the original function of the genes. The process of unequal crossing over which generates gene duplications is clearly a reversible one in the evolutionary time frame so that gene families may expand and contract as evolution proceeds.[202] The ultimate size of a gene family within a species, however, appears to reflect to a considerable extent the result of natural selection. Some families, such as those encoding ribosomal RNAs, transfer RNAs, and histones, are quite large since multiple gene copies offer the selective advantage of providing high rates of synthesis of those cellular components which are required in unusually large amounts (see Reference 87). Gene families which encode proteins are usually much smaller,[87] indicating that gene dosage doesn't generally provide a sufficient evolutionary advantage to perpetuate multiple genes. Rather, the establishment of gene families with multiple members generally reflects some functional divergence of the encoded proteins and/or the evolutionary advantage of having differential regulation of expression of the family members. The properties of the β-globin gene family exemplify these principles since, in humans, for example, different β-like genes are expressed in embryonic, fetal, and adult erythroid cell lineages, and these divergent β-polypeptide chains confer on the tetrameric hemoglobin different capacities to bind and release oxygen.[121]

As previously noted, it appears that the cytochrome P-450 genes represent a relatively large gene superfamily and that the family containing the genes encoding P-450b and e and PB-1 may contain 20 or more members, at least 5 of which are certainly functional. These expressed genes appear to be differentially regulated as well as to encode enzymes with different catalytic activities. The expansion of at least this cytochrome P-450 gene family has apparently been promoted by the evolutionary advantages associated with sequence divergence of both structural and regulatory gene elements.

Estimates have been made for several forms of P-450 for their time of divergence from a common ancestor. For example, cytochromes P-450b and P-450e have been estimated to have diverged 8 million years ago and both have diverged from P-450d approximately 380 million years ago.[98] Since rabbits and rats diverged approximately 60 million years ago,[50] both would be expected to have P-450s equivalent to members in the P-450b and P-450d gene families. However, since the P-450b and P-450e genes diverged only 8 million years ago, it would not be unexpected if rabbits did not have isozymes equivalent to both rat P-450b and P-450e. Rabbit P-450 LM2 is 76% homologous to rat P-450b and these may represent evolutionarily orthologous genes, although the high degree of dishomology between them makes it difficult to determine whether LM2 is equivalent to P-450b or to P-450e. The 30% divergence of rat P-450c

and P-450d suggests that these two proteins diverged more than 60 million years ago, the time when rats and rabbits diverged from a common ancestor. This is consistent with the fact that rabbits have P-450s equivalent to both rat P-450c and P-450d (LM6 and LM4, respectively).

Leighton et al.[119] have proposed that the three rabbit cDNA clones they isolated corresponded to genes which are the products of three gene duplication events. The first duplication, leading to a contemporary sequence divergence of approximately 40 to 45%, separated LM2 from the common ancestor of these genes about 140 million years ago. A second duplication separated clone c3 from clone c2 (approximately 23% divergence) about 85 million years ago, while a third duplication separated c1 and c2 (approximately 12% divergence) about 30 million years ago. It should be stressed, however, that all of the estimates discussed above assume a constant substitution rate throughout evolution and do not take into consideration the mechanisms of concerted evolution and segmented gene conversion which may cause the actual evolutionary divergence times to differ substantially from the estimated times. This is especially true for the rat P-450b and P-450e gene duplication where the extensive regions of sequence identity and small regions of high divergence appear to have arisen by these mechanisms.

III. REGULATION OF EXPRESSION OF CYTOCHROME P-450 GENES

Studies on the regulation of cytochrome P-450 gene expression fall into four distinct categories. The major category, of course, deals with induction of specific forms of cytochrome P-450 by specific chemical agents. Not only does the induction phenomenon have profound pharmacological consequences (reviewed in Reference 28), but it has been relatively easy to document and quantify, initially by pharmacological studies on drug metabolism and subsequently by measurements of specific monooxygenase catalytic activities in tissue microsome fractions. More recently this has been achieved by immunochemical measurements using monospecific antibodies against individual purified cytochromes P-450 to quantitate apoprotein levels as well as their rates of synthesis. The isolation of cDNA and genomic clones for specific forms of P-450 has recently permitted the analysis of the induction phenomenon at the gene level. As will be documented below, in general, increased levels of isozymes result from increased rates of *de novo* synthesis of the P-450 apoprotein which can be accounted for by accumulation of the corresponding mRNA within the induced cell. In the two cases where it has been studied, it is now clear that the latter is primarily a consequence of transcriptional activation of the corresponding genes. In these cases, at least, it is apparent that ultimate analysis of the molecular mechanism of cytochrome P-450 induction will require the identification and characterization of interactions between regulatory proteins, small molecule allosteric effectors, and specific genomic DNA segments or chromatin domains which mediate the induction process.

A phenomenon which is somewhat related to cytochrome P-450 induction by xenobiotics is the regulation of specific isozyme levels by endogenous effectors. The secretion of growth hormone by the pituitary appears to play a major role in determining the sex differences in specific microsomal steroid hydroxylase activities of rat microsomes (for review see Reference 71; see below). Similarly, ACTH and FSH appear to regulate the levels of cytochrome P-450 isozymes involved in steroid biosynthesis by the adrenal cortex (for reviews see References 195 and 224) and ovarian granulosa cells.[54]

A third important category of cytochrome P-450 gene regulatory phenomena relates to the tissue-specific expression of specific cytochrome P-450 isozymes. This too may have some important pharmacological consequences, for example, in determining the

susceptibility of different organs such as the lung and prostate to toxicity and carcinogenesis induced by polycyclic aromatic hydrocarbons.[75] From a more basic perspective, the existence of families of related genes, the members of which show characteristic tissue specificities of expression, may provide excellent model systems for identifying and characterizing macromolecular elements responsible for these specificities.

The fourth regulatory mechanism which operates in the P-450 system is the developmental regulation of levels of specific P-450 enzymes. For example, in rats, the PB-induced isozymes begin to be synthesized postnatally.[149a] In mouse embryos, only P_1-450 is substantially induced by 3-MC and other polycyclic hydrocarbons, whereas after birth P_3-450 is also induced.[150] A most interesting developmental system is the appearance of androgen hydroxylating activities in the liver of male rats after puberty (c.f. reviewed in Reference 71) which can be attributed to the isozyme P-450 2c.[225,228] As will be described in detail below, this developmental program requires the presence of androgen early in the neonatal period which appears to act by programming the changes in hypothalamic regulation of growth hormone secretion by the pituitary which occur after puberty (reviewed in References 67 and 71).

The application of recombinant DNA techniques is likely to contribute very substantively to the analysis of the molecular bases for these regulatory phenomena. Firstly, the availability of hybridization probes to measure mRNA levels and transcription rates should clarify the level at which regulation is effected, providing very accurate direct measurements of the changes in levels of gene expression. These probes will also permit analysis of changes in chromatin structure[229] or DNA methylation[36] which may be associated with the activation or inactivation of specific cytochrome P-450 genes. In addition, the introduction of natural or modified cloned cytochrome P-450 genes into cultured cells or transgenic mice may identify DNA sequences important in induction, tissue specificity, or developmental regulation of gene expression. Such sequences may be, for example, transcriptional enhancer elements which can activate transcription from adjacent promoters only in certain cell types[10,57,181,223] or at certain stages of development. Ultimately, rigorous analysis of the molecular details of the induction mechanism will require the development of cell-free systems capable of initiating transcription of specific genes and responding to the presence of physiological regulatory molecules.

A. Mechanism of Induction of Cytochrome P-450

1. Protein and Enzymological Studies

It is not our purpose in this section to provide comprehensive documentation of the nearly innumerable studies in which specific xenobiotics or pharmacological agents have been shown to enhance specific microsomal monooxygenase activities or even the levels of specific forms of P-450 measured by immunochemical methods (for reviews see References 27, 28, and 145). Rather we will concentrate on those examples which provide insights into aspects of the induction mechanism and, in particular, with those in which information is available on molecular biological features of the induction process.

Initially, inducers of drug metabolism were divided into two classes, phenobarbital type or 3-MC type, based on the enzymatic activities[27] and spectral properties of hepatic microsomes after treatment of rats with these xenobiotics. Clearly the PB-type inducers correspond to those which induce high levels of P-450b and e in rats (or the forms with equivalent activities in other species), whereas the 3-MC-type inducers lead to high levels of activity associated with P-450c and d or their equivalents in other species. Recently, however, it was found that certain polychlorinated biphenyls have both 3-MC-type and PB-type inducing properties[169,170] and induce cytochromes P-

Table 2
MICROSOMAL LEVELS OF EIGHT DISTINCT LIVER P-450 ISOZYMES AFTER TREATMENT OF RATS WITH VARIOUS INDUCERS

Form of P-450	Treatment					
	None	PB	BNF	PCN	ISF	Aroclor® 254
2c	1.20	0.49	0.33	0.33	0.33	0.27
P-450$_{PCN}$	0.39	1.06	0.33	1.32	0.48	0.77
PB-1	0.36	0.69	0.26	0.31	0.34	0.36
P-450a	0.15	0.10	0.12	0.08	0.11	0.15
P-450b	0.03	1.27	0.04	0.10	0.12	1.29
P-450e	0.07	0.92	0.04	0.09	0.18	1.46
P-450c	0.04	0.04	1.41	0.06	0.13	1.45
P-450d	0.03	0.03	0.57	0.03	0.97	1.23

Legend to Table 2: These data are taken from the work of Guengerich and associates.[64] The nomenclature used is consistent with what we have used throughout this review rather than that used by these investigators, which is perhaps more rational since their name for each isozyme includes the abbreviation of its major inducers. In this regard, it should be noted that P-4502c = UT-A; P-450$_{PCN}$ = PB/PCN-E; PB-1 = PB-C; P-450a = UT-F; P-450b = PB-B; P-450e = PB-D; P-450c = βNF-B; P-450d = βNF/ISF-G. The data, expressed as nanomole of each protein per milligram of total microsomal protein, were obtained for microsomes of adult male rats by densitometry of peroxidase-stained immunoblots employing each purified protein to generate a standard curve for that isozyme. Errors in the estimation of the concentration of each purified protein do not affect the accuracy of the determination of the relative levels of each isozyme in the different microsome samples, but would affect the estimation of the absolute levels of each isozyme in any one sample and hence the relative levels of two different isozymes.

450a,b,c,d, and e.[171] Finally, PCN and related natural and synthetic corticosteroids appear to belong to a completely different class of inducer which induces a unique profile of microsomal drug oxidizing activities[208] and a single unique form of P-450, P-450$_{PCN}$.[45,62,85,222] It is now clear that many structurally diverse compounds can induce the same form of cytochrome P-450. Thus, phenobarbital, α-chlordane, SKF-525A, *trans*-stilbene oxide,[209,222] and several halogenated biphenyls[30,169,170] induce cytochromes P-450b and e. P-450$_{PCN}$ is not only induced by corticosteroids, but also to some extent by phenobarbital, isosafrole, SKF-525A, Aroclor® and *trans*-stilbene oxide.[62,222] The compounds which induce a single form of P-450 appear to be as structurally diverse as its potential substrates. Although it is conceivable that a single receptor protein could interact with these diverse inducers to mediate the activation of specific P-450 genes, it has been proposed that the induction phenomenon could actually result from interaction of these compounds with constitutive P-450 isozymes.[168] According to this view, inducers of P-450 are poor substrates of the constitutive P-450 enzymes which uncouple the monooxygenation reaction and lead to the accumulation of activated molecular oxygen which represents the true inducer. This model can only account for the differential induction of specific forms of P-450 by postulating that there are distinct populations of hepatocytes with different complements of constitutive P-450s which interact with the inducers to mediate the induction process.

The data in Table 2 taken from the work of Guengerich and associates[62] indicate the levels of eight distinct forms of rat liver cytochrome P-450 after treatment of rats with various inducing agents. Similar data for 4 of these forms, with P-450b and P-450e not being distinguished, after treatment with 12 structurally diverse inducers, have been

obtained by a different immunochemical procedure.[210] The noteworthy points of these data are

1. In untreated male animals, P-4502c, PB-1 and P-450$_{PCN}$ account for a very large fraction (75 to 85%) of the total microsomal P-450. In this work, P-450 2c was found to be present at a three times higher concentration than in a subsequent paper,[30] where the three major constitutive isozymes were reported to be present at approximately equal levels. It should be noted that the high basal level of P-450$_{PCN}$ which can only be increased threefold by administration of PCN is characteristic of male rats (see below).
2. The level of P-4502c is decreased severalfold by all treatments. Even larger decreases (greater than tenfold) were observed when animals were treated with several polybrominated biphenyls which are exceptionally potent inducers of both P-450c and P-450d.[30] This may not be a true genetic regulatory phenomenon, but may, in fact, represent a competition for available heme and may parallel the reduction in the magnitude of P-450b and e induction by PB which results from simultaneous administration of 3-MC.[30]
3. P-450$_{PCN}$ is induced marginally by isosafrole and approximately 2-fold, 2.5-fold, and 3-fold by Aroclor® 1254, PB, and PCN, respectively. The maximal induced level is comparable to the levels attained by other inducible P-450s after treatment with their optimal inducer. Studies with female rats, however, have revealed that control levels of P-450$_{PCN}$ are very low (<3% of total P-450) and are increased by factors of approximately 13, 20, and 30 after treatment with PB, PCN, and dexamethasone, respectively.[85] A systematic comparison of the levels of this P-450 in adult male and female rats confirms that basal levels are much lower in females, whereas induced levels are comparable in both sexes.[31] From a mechanistic point of view, it is worth noting that PCN and other steroidal inducers appear to act by a process which is distinct from that which involves interaction of these hormones with the classical glucocorticoid receptor.[85,191] Thus, the dose of dexamethasone required for P-450$_{PCN}$ induction was at least ten times higher than that required for maximal induction of tyrosine aminotransferase. Secondly, PCN actually suppresses the levels of the latter corticosteroid inducible enzyme. Finally, other well-established glucocorticoids such as hydrocortisone and cortisone do not induce P-450$_{PCN}$.
4. The level of PB-1 increases twofold after PB treatment, but is unaffected by other inducing agents, including Aroclor® 1254, which do induce the major PB-inducible forms.
5. The levels of P-450a are not substantially affected by any of the inducers. On the other hand, measurements of this P-450 in microsomes of immature and adult female and male rats treated with PB or 3-MC indicate that it is induced in immature male rats and immature as well as adult female rats approximately fourfold by 3-MC and slightly less by PB.[209] In mature males, 3-MC leads to a very slight induction and PB does not induce at all.
6. P-450b and e are induced only by phenobarbital and Aroclor® 1254, with both isozymes being present at comparable levels. Although in this work, the ratio of P-450b/P-450e was 1.4, in subsequent work it was 0.8.[30] The low levels of these proteins in livers of uninduced animals and in animals treated with weak inducers[30] make it difficult to decide if their levels are coordinately regulated. On the other hand, somewhat qualitative data obtained for the induction of these P-450s by isosaffrole, SKF-525A, Aroclor,® α-chlordane, and *trans*-stilbene oxide which gave levels of P-450b and P-450e which varied over a threefold range suggested that the levels of these proteins are indeed coordinately regulated.[222]

7. P-450c is strongly induced only by BNF and Aroclor® 1254 and more weakly by isosafrole. On the other hand, P-450d is induced markedly by BNF and to 1.5-fold and 2-fold higher levels by Aroclor® 1254 and isosafrole, respectively. These and subsequent data from the same laboratory[30] as well as those of others[210] with a wider variety of inducers indicate that although there is a significant correlation between levels of these two P-450s, they are clearly not coordinately regulated. The correlation in levels of these two proteins presumably reflects the fact that they are encoded by somewhat closely related genes. The lack of coordinate regulation would then reflect some evolutionary divergence of the genetic segments which regulate these genes. A similar correlation was obtained for the levels of PB-1, P-450b, P-450e, and P-450$_{PCN}$.[30] As previously noted, our DNA sequence data clearly show that the PB-1 gene is in the same gene family as P-450b and P-450e genes which might account for the correlation between the levels of these isozymes. It is tempting to speculate that P-450$_{PCN}$ will turn out to be much more closely related to P-450b and e and PB-1 than to P-450c and d.

2. mRNA Accumulation and Transcriptional Activation of Specific Cytochrome P-450 Genes

a. Genes Inducible with Phenobarbital

Early studies on the mechanism of induction of cytochrome P-450 using inhibitors of RNA and protein synthesis indicated that enzyme induction required both *de novo* protein synthesis and gene transcription.[55,166] Metabolic labeling studies and immunoprecipitation with specific antibodies demonstrated that the large increase in the cytochrome P-450 after PB treatment resulted, at least in part, from an increase in the rate of synthesis of the apoprotein.[13] Soon thereafter it was shown that administration of PB led to a marked increase in hepatic levels of mRNA encoding PB-inducible cytochrome P-450(s) as monitored by in vitro translation and immunoprecipitation of the cell free products by specific antibodies.[1,14,37,112] The substantial differences in P-450b mRNA levels in induced and untreated rats permitted the isolation of a specific cDNA probe which was used to measure physical (and not functional) levels of the mRNA and also permitted the identification of specific cDNA clones by a differential colony hybridization procedure.[1,52,59,173] Using cloned cDNA probes to measure the sum of P-450b and e mRNAs, enhanced levels were detectable within 3 hr after PB treatement and peaked at approximately 16 hr, resulting in a 25-to 100-fold increase (to a level corresponding to approximately 1% of the total liver poly A$^+$ mRNA) as compared to the levels in untreated animals.[1,81*]

It should be noted that to date the relative levels of the two closely related P-450b and P-450e mRNAs have not been measured directly, although we have been able to show that the levels of the two mRNAs are not very different using synthetic oligonucleotides containing the "hypervariable region" sequences downstream from the conserved tridecapeptide as discriminating hybridization probes (unpublished observations). Furthermore, of 17 cDNA clones obtained by screening a cDNA library with a previously isolated P-450e cDNA, 10 were shown to be P-450b clones and the rest were P-450e clones (T. Friedberg, unpublished). This suggests that the corresponding mRNAs are present in a ratio of approximately 1.5/1 after PB administration.

The demonstration that high molecular weight (4.8 Kb) poly A$^+$ nuclear RNA (i.e., putative mRNA precursors) isolated from livers of PB-treated, but not control rats, hybridized to a P-450b (or e) cDNA clone suggested that the increase in P-450b and e mRNA was due to transcriptional activation of the P-450b and e genes.[59] Direct con-

* The fourfold increase in mRNA hybridizable to a P-450b cDNA clone originally reported by Fujii-Kuriyama et al.[82] probably results from a high basal level of these mRNAs in the animals studied.

firmation of this conclusion was obtained by hybridization of in vitro labeled nascent nuclear transcripts[34] to a vast excess of immobilized cloned P-450 cDNA. Increased transcription from these genes was found to be detectable within 30 min after PB treatment of rats, peaked at approximately 4 hr, declined to about 50% of this level after 16 to 24 hr, and returned to control levels after 38 hr.[2,81] This increase in the rate of transcription after induction has been estimated to be approximately 25- to 50-fold.[2,81] In addition, the transcription rate across the entire P-450e gene has been measured and proceeds in an equimolar fashion along the gene, with transcription terminating or decreasing markedly approximately 1.2 Kb downstream from the poly A addition signal (E. Rivkin and M. Adesnik, in preparation. The transcription rate of the P-450b and e genes is between 100 and 200 ppm/Kb of DNA which is nearly the same as that of the albumin gene whose mRNA product represents about 10% of rat liver mRNA.[204] It can therefore be concluded that the dramatic increase of cytochromes P-450b and e observed after PB treatment appears to be largely due to transcriptional activation of the corresponding genes.

Transcriptional studies on the NADPH cytochrome P-450 oxidoreductase and epoxide hydratase genes, both of which are also activated by PB treatment, have provided evidence for differential regulation of these two genes as compared to the genes for cytochrome P-450b and e.[81] Similar to the P-450b and e genes, increased transcription from the reductase and epoxide hydratase genes is detectable within an hour after PB treatment. However, while the transcription rate from the P-450b and e genes peaks at 4 hr and then slowly declines, the transcription rates from the reductase and epoxide hydratase genes peak at 1 hr and then decline rapidly.

b. Genes Inducible with Polycyclic Hydrocarbons

Studies in several laboratories have indicated that administration of 3-MC to rats leads to a large increase in translatable mRNAs encoding the 3-MC-induced cytochromes P-450.[21,112,137,174] Our translation studies demonstrated marked increases in the levels of 2 mRNAs, approximately 2 and 3 Kb in length, which encoded P-450d and P-450c, respectively.[137] Using a cDNA transcript of highly purified P-450c mRNA isolated from immunoprecipitated polysomes, it was found that there is greater than a 100-fold increase in P-450c mRNA after 3-MC injection with the maximal level attained at 15 hr.[21] Hybridization studies with a cloned P-450d cDNA probe[99] indicated a marked increase after 3-MC treatment of a single mRNA species with the same electrophoretic mobility as that of P-450b, e mRNAs (~2 Kb). More recently, Fagan and associates[47] have demonstrated that after 3-MC treatment there is greater than a 100-fold increase in the levels of both P-450c and P-450d mRNAs, 2.9 and 2.0 Kb in length, respectively. The levels of the two mRNAs were comparable, although the P-450c mRNA increased more rapidly, reaching half maximum at 4 hr after drug administration, whereas the P-450d mRNA reached half maximum at 12 hr and peaked at 24 hr. The finding that the P-450d mRNA level dropped by approximately 70% within 24 hr after attaining the maximal level, whereas the P-450c mRNA only dropped marginally, reflects an important difference in the regulation of these two mRNAs which can only be definitively interpreted after the kinetics of transcriptional activation for the two genes are determined.

Extensive studies have been carried out, primarily by Nebert and associates, on the molecular mechanisms responsible for the increased synthesis of specific forms of cytochrome P-450 after treatment of mice with polycyclic hydrocarbons. Induction by 3-MC of mouse cytochrome P_1-450 mRNA was first demonstrated by in vitro translation of mRNA isolated from drug-treated and control responsive (B6) and nonresponsive (D2) mice, followed by immunoprecipitation of the translation products with anti-P_1-450 antisera.[152] Northern blotting and solution hybridization kinetic experiments using

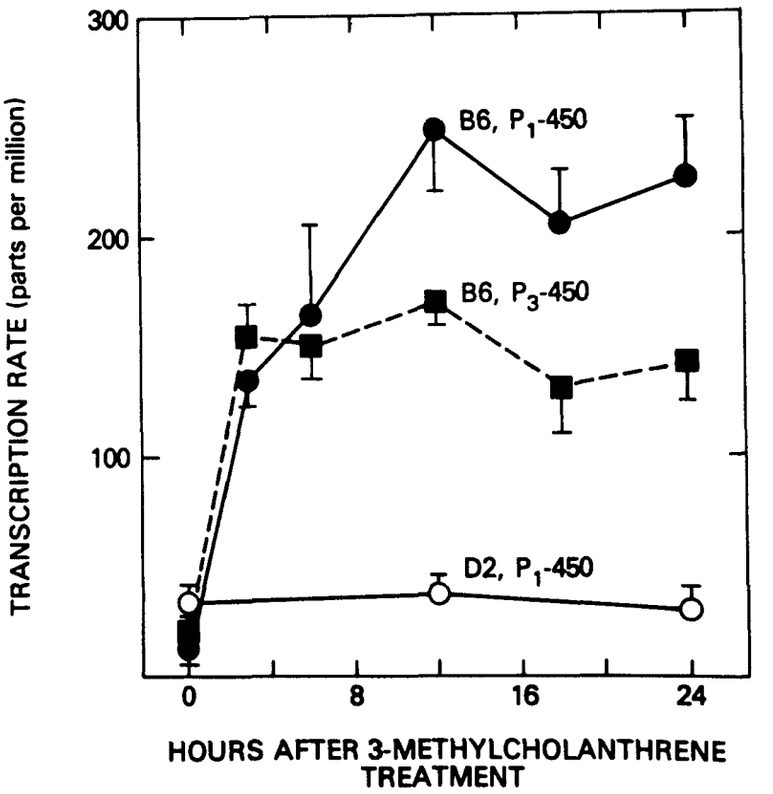
a 3′ untranslated P_1-450 cDNA probe (clone 46) to the P_1-450 mRNA demonstrated the existence of a 23s mRNA, highly inducible with 3-MC (greater than 100-fold), the levels of which strikingly correlated with that of induced aryl hydrocarbon hydroxylase in both responsive and non responsive mice after treatment with various inducers.[216,218] Using a DNA probe derived from the 5′ portion of the P_1-450 gene, it was shown that both a 23s and 20s mRNA accumulate after 3-MC induction (Figure 6). THe 23s mRNA is the same one recognized by clone 46 cDNA, whereas the 20s mRNA was identified as P_2-450 mRNA* on the basis of the observation that it was induced to the same extent by both isosafrole and TCDD in both B6 (3-MC responsive) and D2 (3-MC nonresponsive) mice.[88] This is in accord with the defining feature of P_2-450, i.e., it is an isozyme induced equally by isosafrole in the different mouse strains. The 23s mRNA, on the other hand, is only marginally induced by isosaffrole.[88] One very striking observation was that the dose response curves for accumulation of the 23s and 20s mRNAs as a function of 3-MC doses were very different, with the 20s mRNA being induced at much lower levels of drug.[88] This finding emphasizes the fact that these two mRNAs are not coordinately regulated despite the fact that their induction by 3-MC is mediated by the product of the same regulatory gene.

The first suggestive evidence that enhanced transcription was the main factor responsible for increased P_1-450 mRNA levels after induction was the observation in liver of induced mice of a high molecular weight RNA band which hybridized with cDNA clone 46, but was absent from cytoplasmic poly A^+ mRNA.[218] This large mRNA was concluded to represent a nuclear precursor to P_1-450 mRNA.[218] Increased transcription of the P_1-450 gene after 3-MC or TCDD treatment has recently been directly demonstrated with whole animals[61] as well as cultured cells.[89] Thus, the transcription rate of the P_1-450 gene was measured using in vitro labeled nascent nuclear transcripts and found to be induced approximately 20-fold after 3-MC treatment of responsive B6 mice (Figure 8), while no increase was observed in nonresponsive D2 mice. As expected, the transcription rate did increase (four- to sixfold) in D2 mice after treatment with TCDD.

The availability of clones pP_3-450-21 and pP_1-450-57, each of which hybridizes primarily to distinct polycyclic hydrocarbon-induced, Ah-regulated mRNAs encoding P_3-450 and P_1-450, respectively, permitted a direct comparison[61] of the rates of transcription of the two corresponding genes to the accumulated levels of the corresponding mRNAs (Figure 8). The transcription rate plateau for the P_1-450 gene was 1.5 to 2 times higher than that for the P_3-450 gene, although it took somewhat longer to reach the plateau. On the other hand, the maximal P_3-450 mRNA level was four to five times higher than that of P_1-450 mRNA. Furthermore, the uninduced level of P_3-450 mRNA is slightly greater than the induced level of P_1-450 mRNA, yet the transcription in induced liver of the P_1-450 gene is much higher (eight- to tenfold) than that of the P_3-450 gene in untreated animals. One can conclude, therefore, that disproportionately high levels of P_3-450 mRNA as compared to P_1-450 mRNA result from post-transcriptional regulatory factors such as either a higher cytoplasmic stability of P_3-450 mRNA, a more efficient processing of its hnRNA precursors, or more efficient nucleocytoplasmic transfer of the mature mRNA. In this regard, the kinetics of accumulation of P_1-450 mRNA after 3-MC treatment were also determined and the level of this mRNA was shown to reach a maximum at approximately the same time that the transcription rate reached a maximal constant level indicating that the P_1-450 mRNA has a relatively short half-life.

Although the transcriptional activation of the P_1-450 gene after administration of 3-MC to mice was somewhat delayed as compared to more rapid transcriptional activa-

* Given the likelihood that P_2-450 represents a D2-specific polymorphic variant of P_3-450 of B6 mice,[60,100] the 20s band in B6 mice corresponds, in fact, to P_3-450 mRNA.



A

FIGURE 8. Transcriptional activation of the mouse P_1-450 and P_3-450 genes and accumulation of the corresponding messenger RNAs after 3-methylcholanthrene treatment. (A) Nuclei isolated from the livers of responsive (B6) and nonresponsive (D2) mice treated with 3-MC for various lengths of time were incubated in vitro with ^{32}P-UTP to label nascent transcripts. The in vitro synthesized RNA was hybridized to a vast excess of immobilized recombinant plasmids containing P_1-450 (plasmid pP_1-450-57) and P_3-450 (pP-450-21) cDNA inserts of approximately equal size (1700 to 1750 bp). The background of this hybridization assay, approximately 5 to 15 ppm, measured by incubation of each RNA sample with vector DNA lacking an insert, does not affect the accuracy of the measurement of induced transcription rates, but in some cases does generate a significant uncertainty in the measurement of the basal transcription rate. Despite the region of very strong sequence homology (~500 bp) within the P_1-450 and P_3-450 mRNAs, hybridization to pP_1-450-57 essentially measures only P_1-450 mRNA since the cDNA is missing approximately 1000 bp from the 5′ portion of the mRNA sequence which contains this homologous segment and RNAse treatment of the DNA-RNA hybrids ensures that other regions of weak homology do not contribute significantly to the hybridization signal. On the other hand, although the P_3-450 cDNA clone does presumably contain a larger portion of the region of strong homology between the two mRNAs, the cross-hybridization of P_1-450 transcripts represents perhaps 10 to 20% of the hybridization to the P_1-450 cDNA. Data are from Reference 61. (B) Poly (A)$^+$ mRNA from livers of B6 and D2 mice sacrificed after various times of treatment with 3-MC was fragmented by alkaline hydrolysis and uniformly labeled with ^{32}P using polynucleotide kinase and ^{32}P-ATP as substrate. The mRNA samples were hybridized to the cloned P_1-450 and P_3-450 cDNAs as described in (A). Data are from Reference 61.

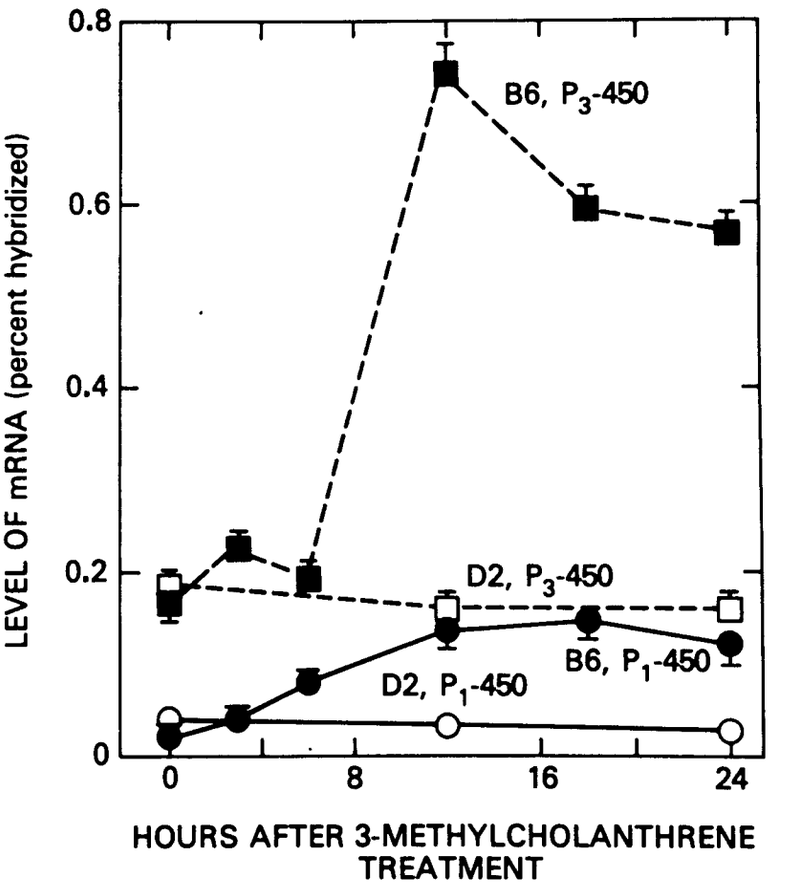


FIGURE 8B.

tion of the P_3-450 gene by 3-MC or the genes encoding the PB induced isozymes,[2,81] treatment of cultured hepatoma cells with TCDD, led, within 30 min, to a 20-fold increase in the rate of transcription of RNA which hybridizes to the P_1-450 cDNA clone 46 to a rate which persists for at least 18 hr in the presence ot TCDD.[89] No increase in transcription was observed in TCDD-treated variant cells which are benzo(*a*)pyrene resistant and have a defective Ah receptor which after complexing with inducer binds only weakly to the nucleus (see below). The increased transcription rate appears to be a primary response to inducer since it is so rapid and is not blocked by inhibition of protein synthesis by cycloheximide. It is noteworthy that the rapidity of induction is similar to that observed with steroid hormone activation of transcription of the mammary tumor virus genome, where evidence is beginning to accumulate for discrete sequences within or adjacent to the gene to which the hormone-receptor complex binds.[96]

c. Induction with PCN

The rat cytochrome $P450_{PCN}$ gene has not been studied at the transcriptional level, although increases in this isozyme after PCN treatment are also likely to be largely due to transcriptional activation of the corresponding gene. Firstly, after PCN treatment of female rats, an increase in in vitro translatable $P450_{PCN}$ mRNA has been observed.[44] Subsequently, studies using cloned $P450_{PCN}$ cDNA as a hybridization probe have demonstrated a sevenfold increase in specific mRNA after PCN treatment of male rats.[80]

This increase is detectable within 3 hr after PCN treatment, with mRNA levels reaching a peak at 12 hr and remaining elevated for at least 24 hr. Phenobarbital treatment leads to a fourfold increase in the P-450$_{PCN}$ mRNA levels which is not unexpected given the substantial increase in this protein which occurs after PB treatment of male rats.[62] Presumably a greater relative increase in mRNA levels would be obtained with female rats which have a lower basal level of this P-450 and show a 14-fold induction of P-450$_{PCN}$ by PB.[85] PCN treatment leads to only a marginal increase of P-450b and e mRNA as expected from the observation that levels of P-450b and e are not significantly affected by PCN treatment.[210] The peak level of P-450$_{PCN}$ mRNA corresponds to approximately 0.7% of total mRNa which is comparable to the level of P450b and e mRNAs which accumulate after PB treatment.[80]

3. The Ah receptor: Molecular Interactions in the Transcriptional Activation of Cytochrome P-450 Genes

Although convincing data for the involvement of transcriptional activation of cytochrome P-450 genes have been obtained for the regulation of both the phenobarbital and 3-MC induced forms of cytochrome P-450, only in the latter case has it been possible to characterize, albeit in an indirect manner, at least one *trans*-acting regulatory gene and its encoded protein product. This success results primarily from the existence of mutant mice which are defective in the induction by 3-methylcholanthrene. This genetic system and its biochemical correlates are described extensively in several recent reviews.[43,149] In this section, we will briefly summarize the salient features of this genetic regulatory system, especially the results obtained in the last 2 years, and critically evaluate the model proposed for the regulation of specific P-450 genes which are activated by polycyclic hydrocarbons.

a. Genetic Differences in Inducibility of Cytochrome P-450 by 3-MC

It was reported nearly 15 years ago[143,146] that the induction of arylhydrocarbon (benzo[*a*]pyrene) hydroxylase activity by treatment with 3-methylcholanthrene (3-MC) is defective in certain inbred strains of mice which differ from other responsive strains at what has been designated the Ah (aromatic hydrocarbon) genetic locus. Most wild mice and inbred strains such as the prototype strain C57BL/6 (B6, genotype Ah^b/Ah^b) show a substantial (fivefold) induction in liver benzo[*a*]pyrene hydroxylase activity after injection of 3-MC, whereas the nonresponsive strains such as DBA/2 (D2, genotype Ah^d/Ah^d) show a sevenfold lower AHH level which is not significantly affected by 3-MC treatment).[149]

Originally a single form of cytochrome P-450 designated P$_1$-450, which is responsible for the inducible arylhydrocarbon hydroxylase activity in responsive animals, was shown to be associated with the Ah locus.[82] Cytochrome P$_1$-450 cannot be detected in livers of control responsive mice,[150] and quantitative hybridization experiments[216] using cloned P$_1$-450 cDNa (clone 46) as a probe indicate approximately a 200-fold increase in P$_1$-450 mRNA after treatment of responsive mice with potent inducers like TCDD, 3-MC and benzo[*a*]anthracene. Although, inexplicably, nonresponsive mice contain higher basal levels of P$_1$-450 mRNA than do responsive mice, maximal doses of 3-MC only lead to a 4-fold increase in P$_1$-450 mRNA to a level which is perhaps 25-fold lower than the induced level in responsive animals.[216] D2 nonresponsive mice are clearly not defective in the structural gene for cytochrome P$_1$-450 since they can be induced to nearly maximal levels by doses of TCDD which are 12 to 18 times higher than those required to induce B6 mice.[176,178] It should be noted that TCDD is the most potent known inducer of AHH, being effective at doses which are 30,000-fold lower than the paradigmatic inducer, 3-MC.[175]

The Ah^b/Ah^d heterozygote is inducible for AHH by 3-MC indicating that inducibility is inherited as an autosomal dominant trait.[149,207] This finding suggests that the Ah^b allele encodes a positive *trans*-acting regulatory element which is required for AHH inducibility. The genetics of arylhydrocarbon responsiveness is, however, more complicated than this (reviewed in Reference 149). Other nonresponsive (AKR/N and AKR/J) as well as responsive strains (C57BL/6J) are known. The F1 heterozygote (C57BL/6J)(AKR/N) is Ah nonresponsive, whereas the F1 derived from either B6 strain (C57BL/6N or C57BL/6J) crossed with AKR/J and from either AKR strain crossed with C57BL/6J is Ah responsive. Even more complicated is the fact that among the F2 progeny when certain nonresponsive strains are the progenitors, some mice are responsive.[185] Furthermore, in crosses between the responsive C3H/HeJ and nonresponsive DBA/2J mice AHH inducibility is inherited as an additive trait.[205] It has been proposed that AHH induction must involve at least two independent loci with at least three alleles each.[185]

As previously discussed, more recent studies have indicated that in addition to cytochrome P_1-450, the protein responsible for most of the AHH activity in microsomes from polycyclic hydrocarbon-induced mice, a second distinct form of cytochrome P-450, P_3-450, is induced by these agents and its induction also appears to be governed by the Ah locus. Firstly, induction by 3-MC doesn't occur in D2 mice, but does occur in B6 mice and in the Ah^b/Ah^d heterozygotes.[150] This was originally demonstrated in measurements which involved quantitative immunoprecipitation of the radioactive in vitro labeled apoprotein.[151] More recently, the correlation of expression of the P_3-450 structural gene with the Ah regulatory locus was confirmed in experiments which measured P_3-450 mRNA levels or gene transcription by hybridization to cloned P_3-450 cDNA.[61,217] P_3-450 and not P_1-450 appears to represent the major polycyclic hydrocarbon-induced isozyme of mice since anti-P_3-450 precipitates four to ten times more radioactivity from in vitro labeled microsomes than does anti-P_1-450.[150] Similarly, as previously noted, after TCDD administration, P_3-450 mRNA reaches levels four to five times that of P_1-450 mRNA.[61]

b. The Ah Receptor Protein

Considerable evidence has accumulated to support the notion that the Ah regulatory gene encodes a receptor protein with similar properties to the steroid hormone receptors, which, upon binding an inducing ligand, can activate expression of specific structural genes. The classical formulation of this model includes the ligand-induced translocation of receptor molecules from the cytoplasm to the nucleus where the ligand-receptor complex has its effect.

The receptor model was first proposed on the basis of the early finding that both responsive and nonresponsive mouse strains (not inducible by 3-MC) could be induced by extremely potent inducers such as TCDD.[175,178] This indicated that both types of strains do have the structural and regulatory genes required for arylhydrocarbon hydroxylase induction. The notion that TCDD and 3-MC participate in a common inducing mechanism came from the observations that the two agents produce parallel log-dose response curves for induction and evoke the same maximal response which is the same even if maximally inducing doses of both drugs are administered simultaneously.[175] It was proposed that the defect in 3-MC nonresponsive mice, which is reflected by their inability to respond to less potent inducers, results from the diminished binding affinity of a site to which inducer binds to mediate the induction process. The fact that nonresponsive strains require 14 to 18 times more TCDD for maximal induction than does the B6 responsive strain and that the B6/D2 F1 heterozygote responsive mice had a sensitivity to TCDD intermediate to those of the responsive and nonresponsive parental strains[176] supported this proposal.

The first direct demonstration of the existence of a cellular receptor protein which binds highly radioactive ^{3}H-TCDD was obtained by Poland et al.,[177] using a charcoal-dextran suspension to remove ligand which was not complexed to the putative receptor. As in most receptor-ligand binding assays, specific binding of ligand was determined by subtracting the nonspecific binding which occurs in the presence of a large (200-fold) excess of nonradioactive ligand. In the first experiments, ^{3}H-TCDD was added to total liver homogenate, with 16% of the label partitioning into the cytosolic fraction. The amount of label bound by cytosolic proteins was markedly diminished by pretreating the mice with nonradioactive ligand and the amount of binding to cytosolic fractions from B6 mice was much higher than for D2 mice. The binding component in the cytosol appeared to be a protein since it was largely destroyed by trypsin treatment, but not by DNAse or RNAse. Scatchard plots indicated that there were 84 fmol of binding sites per milligram of cytosolic protein (5000 sites per cell) with a binding affinity of 2.7×10^{-10} *M*. The notion that the hepatic cytosolic component that binds [^{3}H] TCDD is, in fact, the receptor which mediates the induction process was supported by the finding that the binding affinity of various TCDD analogues for the receptor as monitored by their ability to compete for specific ^{3}H-TCDD binding correlated reasonably well with their potency as in vivo AHH inducers.[177] On the other hand, the polycyclic hydrocarbons, e.g., 3-MC and BNF, showed a surprisingly high avidity (1/25 to 1/2 of TCDD) compared to their inductive potency (1/30,000 in animal studies;[175] 1/250 to 1/950 in tissue culture studies[155]). It was proposed that the diminished biological potency of 3-MC compared to TCDD as a AHH inducer results from the metabolic inactivation of 3-MC by basal levels of AHH. Strong data to support this conjecture are not available, but it should be noted that problems of metabolic inactivation could be minimized if the immediate effects of the inducer, i.e., transcriptional activation, were monitored and correlated with inductive capacity of the various compounds. It is conceivable, however, that the TCDD-receptor complex is more effective in interaction with its putative receptor in the nucleus than are the complexes formed with other less potent inducers.

In this early work on the Ah receptor, the dextran-charcoal adsorption procedure employed to remove uncomplexed ligand measured a large proportion of nonsaturable binding sites: at subsaturating levels of ligand, at most 75% of binding could be competed by saturating levels of cold competitor. In recent work, a sucrose density gradient fractionation is employed after dextran-charcoal absorption. This effectively separates a class of high-affinity, low-capacity binding sites from nonsaturable sites for binding ^{3}H-TCDD.[160] In fact, data in the latter paper suggest that no reliable data can be obtained by the dextran-charcoal assay alone. On the other hand, the more refined procedure did not permit Scatchard plot analysis to estimate the dissociation constant since at low values of ligand bound, the curves deviated substantially from linearity. Because of the extremely low background of this assay it was possible to measure the number of binding sites per cell. The 60 fmol of ligand bound per milligram of cytosolic protein corresponds to approximately 5500 binding sites per hepatocyte. With this sensitive assay, in which the saturable receptor fraction sediments at 8 to 10s, more rapidly than the bulk of the nonspecific binding, essentially no specific binding could be found in hepatic cytosol of nonresponsive mice, and the level of binding for different inbred strains of responsive mice varied from 12 to 60 fmol/mg protein. It is, perhaps, of interest that the low level of receptor (12 fmol/mg protein) in C3H/HeJ responsive mice could possibly account for the fact that in C3H/D2 heterozygotes AHH inducibility by 3-MC is half as great as in the responsive C3H parent.[43]

With the sucrose gradient assay, it was first possible to assay for nuclear and cytosolic ^{3}H-ligand receptor complex in livers from mice injected with radioactive ligand.[161] A peak of nuclear-binding complex (5.7s) was found in nuclei from B6 mice, but not

in D2 mice, as expected. The B6 cytoplasm contained a binding peak at 8 to 10S which was perhaps 75% of the amount in the nuclear peak. The fact that greater quantities of receptor-ligand complex were found in nuclei at 18 hr after TCDD administration than at 2 hr provided the first evidence for translocation of the receptor-ligand complex from the cytosol to the nucleus. The small amount of nuclear receptor in D2 mice (after in vivo injection of ^{3}H-TCDD) was considered to be insignificant at this time, but in later work[214] it was shown to be sufficient to account for the TCDD mediated induction of these mice (see below).

The apparent translocation of the receptor from the cytoplasm to the nucleus was directly demonstrated using continuous cell culture lines incubated with ^{3}H-TCDD.[161] The key observation in this regard was that incubation of Hepa-1 cells, a permanent clonal line of rat hepatoma cells highly inducible for AHH, with ^{3}H-TCDD at 37°C for 1 hr, revealed a major peak of receptor-ligand complex in the nuclear fraction and very little in the cytosol. On the other hand, if the cells were incubated with labeled ligand for 1 hr at 4°C, only the cytosolic peak and not the nuclear peak was observed. However, when these cells were transferred from 4 to 37°C, the appearance of labeled receptor in the nucleus occurred in parallel with a commensurate decline in cytosolic level of receptor-ligand complex. It is perhaps worth noting that in experiments of this sort less than 1% of the total ^{3}H-TCDD retained by the cells was associated with specific high affinity binding sites in the cytosol or nucleus. The notion that the labeled receptor-ligand complex found in the nucleus did not result from the direct association of ligand with unoccupied receptors indigenous to the nucleus was supported by the inability to demonstrate a receptor-ligand complex after in vitro incubation of nuclear extracts with ^{3}H-TCDD.

It is interesting that certain cell lines not inducible for AHH by TCDD nevertheless have normal levels of Ah receptors which appear to translocate normally to the nucleus.[161] This indicates that the presence of a functional regulatory gene for the Ah receptor is necessary but not sufficient for AHH inducibility. It was proposed that the defect in these cultured cells might be similar to the situation with New Zealand white rabbit liver where AHH induction occurs only in the neonate and not in the adult despite adequate receptor levels in both conditions.[93] Similarly, the thymus contains substantial levels of receptor, but does not contain inducible AHH.[127]

In recent work, a quantitative correlation between the intranuclear appearance of inducer-receptor complex and level of induced cytochrome P_1-450 mRNA was obtained which represented the most direct evidence that the Ah receptor is directly involved in P_1-450 induction[214] in both B6 and D2 mice. It should first be recalled that in previous work from this laboratory, cytosolic receptor could not be found in livers of D2 mice, although low, ostensibly insignificant levels of nuclear receptor were found. In this work, more attention was paid to this low level of nuclear receptor in D2 mice which had also been observed by others.[127] A very well-defined sucrose gradient peak with the same sedimentation velocity but approximately five times lower in magnitude was found in D2 mouse liver as compared to B6 mouse liver after in vivo administration of ^{3}H-TCDD. Curiously, the kinetics of appearance of labeled ligand-receptor complexes in nuclei following a single injection of ^{3}H-TCDD were substantially different in B6 and D2 mice. In B6 mice a peak was reached at around 4 hr which began to decrease after around 10 hr. In D2 mice the level of receptor was relatively constant from 4 to 18 hr (increasing approximately 20% between 4 and 12 hr). As a result of these kinetic differences, the relative levels of nuclear receptor in B6 and D2 mice would vary from twofold to perhaps sixfold, depending on the time it was assayed. In this work an excellent correlation was found between the level of induced P_1-450 mRNA and the level of nuclear-receptor complex in B6 and D2 mice treated with different doses of ^{3}H-TCDD when these were measured at 18 hr after injection of the

inducer. Given the different kinetics of nuclear receptor accumulation and decline in B6 and D2 mice, it is not clear why data from this time point yielded such a good correlation. In fact, one would expect the transcription rate of the P_1-450 gene to correlate with the nuclear receptor level at each time so that the accumulated level of mRNA should correlate with the integral of the receptor accumulation curve.

c. Somatic Cell Genetic Studies on Arylhydrocarbon Hydroxylase Induction

Studies with cultured cells have recently become increasingly useful for analyzing the role of the Ah receptor in AHH induction. Two groups have taken advantage of the fact that inducibility of AHH is required in order for cells to be susceptible to the toxicity of moderate levels of benzo[*a*]pyrene (BP). As a result, cell lines with defective Ah receptor can be identified among BP-resistant derivatives of the Hepa-1 cell line. BP-resistant mutants were originally isolated by Hankinson.[78a,b] A minority of the mutants were subsequently found to be dominant, whereas the others were recessive, but fell into three complementation groups.[79] Representative mutants from each class were characterized in collaboration with Nebert's group.[117] The dominant mutant, and the two members of complementation group A that were studied had normal Ah receptor levels, displayed normal kinetics for translocation of the receptor-inducer complex to the nucleus yet exhibited very low or undetectable basal and inducible AAH. The two group B mutants studied had no more than 10% of the wild-type receptor levels, showed normal kinetics of nuclear translocation, and had no more than 20% of normal AHH. The clone in complementation group C was of particular interest since it had normal levels of the receptor, but apparently could not translocate receptor-inducer complex to the nucleus. This clone had no detectable basal or induced AHH activity. Only one mutant in complementation group C was identified among the 59 BP-resistant mutants that were subjected to complementation analysis.

Recently, the TCDD inducibility of P_1-450 mRNA in the various classes of BP resistant mutants was studied.[79a] Group B and group C mutants, both of which appear to be defective in the Ah receptor, lack or have very low P_1-450 mRNA levels after TCDD treatment. Dominant mutants as well as dominant x wild-type hybrids are also noninducible for the mRNA, suggesting that the dominant mutants synthesize a *trans*-acting repressor of P_1-450 mRNA transcription. Group A mutants were heterogeneous, with some lacking P_1-450 mRNA and other having levels as high as the wild type. This suggests that gene A is the P_1-450 structural gene. Furthermore, the mutants with high mRNA levels, which also have low but detectable AHH activities, are likely to be missense mutations in the P_1-450 gene, whereas the mutants with low mRNA levels are likely to be defective in a *cis*-acting transcriptional regulatory element or perhaps in the post-transcriptional process (splicing or polyadenylation) of the P_1-450 gene transcripts. Several cases of human thalassaemia have been shown to result from such transcriptional or post-transcriptional defects.[212a]

Van Gurp and Hankinson[219] have developed an ingenious procedure for isolation of revertants of the benzo[*a*]pyrene resistant cell lines which have regained AHH activity. This method takes advantage of the fact that certain polycyclic hydrocarbons such as benzo(*g,h,i*)perylene [B(*ghi*)P] are rendered highly cytotoxic when illuminated with near UV light. Cells which have taken up B(*ghi*)P, but can eliminate it by virtue of their AHH activity, are resistant to subsequent exposure to near UV light, whereas AHH deficient cells exposed to B(*ghi*)P are killed by such illumination. This procedure was used to isolate revertants of the four groups of benz[*a*]pyrene-resistant variants.[220] The revertants isolated from the two groups of mutants which appear to have a defective receptor were found to be normal with respect to in vivo temperature sensitivity of AHH induction and ED_{50} for the inducer, TCDD. It was anticipated that if the original mutations were in the structural gene(s) for the Ah receptor, then some of the rever-

tants would result from second site intragenic mutations which might restore enzyme activity, but lead to the production of an altered receptor protein which might show temperature sensitivity or a difference in affinity for the inducer. Thus, the finding of normal phenotypes of these revertants represents a failure to provide evidence that the original mutations were actually in the structural genes for the Ah receptor protein. The class of benzo[*a*]pyrene mutants which had no defect in receptor and hence could have been defective in the structural gene for the AHH yielded revertants which were identical to the wild type with respect to both in vivo and in vitro stability of the enzyme as well as Km for the substrate, benzo[*a*]pyrene. On the basis of an argument similar to that presented above, this provided suggestive evidence that the original mutations were not in the structural gene for the enzyme. An interesting revertant of one of the putative receptor deficient mutants was isolated which was constitutive for AHH activity in the absence of inducer and was not further inducible by TCDD. The fact that this constitutive mutation is recessive to wild-type inducibility in somatic cell hybridization experiments indicates that it is not analogous to operator constitutive mutations of bacterial operons.

BP-resistant variants were also isolated more recently by Whitlock and associates.[131] The mutants fell into two classes, those with no detectable AHH activity and those with low activity, less than 10% of that of the wild-type Hepac1c7 cells. For those with measurable AHH levels, it was possible to conclude that the defect was likely to be in a regulatory gene rather than in the structural gene since an inducer dose-response curve indicated a tenfold higher concentration of inducer was required for maximal induction than in the wild type. Receptor assays were carried out on the different mutants by assaying nuclear and cytosolic receptor-ligand complexes after incubating cells with ^{3}H-TCDD. Variants with low AHH activity showed low cytosolic and nuclear receptor levels (<10% of wild type), whereas the variants with no detectable activity showed normal levels of cytosolic receptors, but no nuclear receptor. For the low activity variants, one cannot determine whether the number of receptors is lower or if the affinity for receptor is lower. A genetic analysis indicated that both phenotypes are recessive to the wild-type allele and that the lesions are in different genes. The complementation between the two variant classes proves that at least one variant class has an intact structural gene for AHH. It should be noted that whereas in whole animals TCDD at high concentrations can induce nonresponsive strains to the same levels as responsive strains, in the cell culture variants, high doses of TCDD were still ineffective. It was proposed that in these variants the number of receptors, rather than their affinity for inducer is decreased or that there possibly could be a problem with the insolubility of TCDD in aqueous media.

The existence of a class of mutants which is apparently defective in nuclear translocation of receptor appears to support the basic model for receptor action according to which receptor is normally located in the cytosol, but translocates to the nucleus after binding ligand. Recently, however, Whitlock and Galeazzi[233] have provided evidence which suggests that even the unoccupied receptor is normally located in the nucleus from which it is artifactually leached out during cell fractionation in large volumes of buffer. Their data indicate that when the receptor is complexed to ligand, it appears to undergo a temperature-dependent conversion to a configuration which shows strong binding to a nuclear component leading to its retention in the nuclear fraction. The mutants which were originally described as being unable to accumulate receptor in the nucleus appear still to be somewhat defective in the retention of the receptor-ligand complex in the nucleus suggesting a weaker association with their nuclear recognition sites. These data still favor a two-step model for TCDD action in which after receptor-ligand complex formation in the nucleus, a second step occurs which is either a conformational change, an enzymatic modification of the receptor, an association or dis-

sociation of a subunit, or some other process which is responsible for the temperature-dependent event.[233]

The more recent work from Okey's laboratory,[33a] however, raises some doubt about the conclusion of Whitlock and Galeazzi[233] that the unoccupied Ah receptor resides in the nucleus. Thus, it was also found[33a] that the Ah receptor appears to shift out of the nuclear fraction and into the cytosolic fraction as the volume of the homogenization buffer or its ionic strength is increased. However, because the distribution of the Ah receptor was identical to that of three standard cytosolic marker enzymes,[33a] it appears that the nuclear location of the Ah receptor under certain homogenization conditions actually represents the experimental artifact.

It is worth noting that recent studies with steroid receptors have also begun to raise doubts about the general validity of the prevalent model that cytosolic-nuclear translocation of a receptor ligand complex is a key step in mediating steroid hormone action. Thus, two separate groups used completely different methodologies to demonstrate the nuclear localization of unoccupied estrogen receptors. One group[102] used monoclonal antibodies and immunocytochemical staining to localize the receptor in the nuclei of several different tissues or tumor cells. In an independent set of experiments, the estrogen receptor of rat pituitary tumor cells was shown to remain associated with the nucleoplast fraction and not with the cytoplast fraction when these were prepared by cytochalasin B-induced enucleation of the cells.[230]

The biochemical purification of the Ah receptor has thus far seemed rather elusive. There is serious doubt that the 3-MC binding protein identified and partially purified by Bresnick and associates from rat liver cytosol[212] is the rat equivalent of the Ah receptor. The recent work of Okey and Vella[162] demonstrates that ^{3}H-3-MC and ^{3}H-TCDD bind to a common receptor site in mouse and rat cytosol which sediments at 8 to 10S and not at 4.5S as does the putative receptor identified by Bresnick's group.

It is reasonable to expect that detailed analysis of the receptor protein and its mechanism of action will come after recombinant DNA technology is applied to this problem sooner than will information be obtained by purifying the receptor protein directly.[144] Thus, the ease with which receptor defective cell lines can be identified among BP-resistant mutants suggests that the gene encoding the receptor can be identified via its ability to restore AHH inducibility to these defective variants. Presumably, this can be accomplished using the selection scheme devised by Van Gurp and Hankinson[219] to identify revertants of the BP-resistant lines. With this assay for the Ah gene, it will be possible to clone it by methodologies applied in other systems where the sole assay for a gene was the phenotype it conferred on cells after DNA mediated transfection.[172] Once cDNA clones for the receptor protein(s) are obtained (using genomic segments as probes), it may be possible to obtain the proteins in large quantities after expression of the cloned cDNA in either prokaryotic or eukaryotic hosts. These proteins could, in principle, be used in in vitro transcription systems to directly study the mechanism of transcriptional activation.

B. Expression of Extrahepatic Cytochrome P-450: Evidence For Tissue Specificity

Recently, several studies have attempted to correlate the cytochrome P-450s present in mammalian liver with those present in extrahepatic tissues. These studies have usually compared the levels of specific P-450s before and after treatment of animals with various inducing agents by exploiting the immunological, electrophoretic, catalytic, and physical properties of the various cytochromes P-450. The results have led to the realization that there is often a tissue specificity for the presence or inducibility of certain forms of P-450.

In rabbit lung, there are at least two constitutive forms of cytochrome P-450, designated P-450 I and II. These each constitute approximately 30% of the pulmonary P-

450 in untreated animals and are distinct proteins based on amino acid composition and immunological, structural, and catalytic properties.[184,192,196,235] P-450$_I$ appears to be identical to P-450 LM2, the major PB-inducible form found in rabbit liver. No difference can be detected between these two P-450s in their peptide maps, NH_2-terminal sequences, subunit molecular weight, and immunological reactivity with anti-P-450 LM2 antisera.[196,234] Of particular interest is the fact that P-450 P-450$_I$ is present at relatively high constitutive levels in rabbit lung microsomes as compared to the uninduced level in the liver, but is not inducible by PB.[192] Similarly, rabbit lung P-450$_{II}$ appears to be equivalent to a liver microsomal P-450, designated form V, which is also markedly induced (10- to 12-fold) in the liver by phenobarbital treatment.[184] The pulmonary content of this isozyme is also not affected by phenobarbital treatment.[196] Though less extensively characterized, a similar situation appears to exist in the rat, where a constitutively expressed non-PB-inducible form of P-450 is present in lung microsomes, which is immunologically identical to cytochrome P-450b.[63,64] This form of P-450 was not detectable in kidney, brain, heart,[63] and prostate microsomes.[75]

Although treatment of rabbits with TCDD induces two distinct liver microsomal cytochromes P-450 designated forms 4 and form 6, which are likely to correspond to rat P-450d and P-450c, respectively, this treatment only induces form 6 in lung and kidney.[122] Similarly, both P-450c and P-450d are induced by TCDD in rat liver, but only P-450c is inducible in rat kidney, lung, intestine, spleen, and testis.[58] In addition, hybridization studies suggest that in mice only P_1-450 mRNA and not P_3-450 mRNA is induced in the kidney by 3-MC administration.[88] The differential induction in these extrahepatic tissues of the aromatic hydrocarbon inducible forms of cytochrome P-450 clearly indicates that the Ah receptor may be necessary but not sufficient for induction.

Tissue-specific expression of the three closely related members of the PB-inducible rabbit cytochrome P-450 gene family isolated by Leighton et al.[119] was recently reported.[119a] None of the corresponding mRNAs was detectable in lung tissue and only one of the mRNAs, corresponding to clone P-450 PBc2, was expressed in the kidney. The basal level of this mRNA in the kidney was 15% of that in the liver, and PB administration led to a severalfold increase in both organs.

Recently, we have observed, as previously noted, that a P-450e-related gene cloned in our own laboratory is expressed in a tissue-specific manner. Thus, this gene appears to be expressed in the rat preputial gland, but not in the liver, kidney, lung, adrenal, brain, or testis. On the other hand, mRNA which hybridizes with a P-450e cDNA probe is expressed in the liver, lung, and adrenal but not in the preputial gland (M. Atchison, T. Friedberg, M. Adesnik, unpublished). These mRNAs are, however, expressed at rather low levels in the latter two organs. For the adrenal this level, expressed as a fraction of total tissue mRNA, is comparable to the uninduced level in the liver. For the lung it is at least tenfold lower. This is not surprising since the level of the corresponding immunoreactive protein in the lung is approximately tenfold lower than in liver of PB-treated rats when expressed per milligram of microsomal protein.[64] Furthermore, the amount of microsomal protein in lung is likely to be substantially less than in liver. Thus, the steady state level of P-450b and e in the lung, expressed as a fraction of total protein, may very well correlate with the level of the specific mRNAs, expressed as a fraction of total tissue mRNA. In addition, it is now clear that not all cells within the lung express cytochrome P-450 recognizable with antisera specific for P-450 LM2. Early suggestions by Boyd[18] have been confirmed by immunofluorescence studies indicating that only nonciliated bronchiolar epithelial cells (Clara cells) express this cytochrome P-450.[193] Thus, only a small percentage of the cells present in the rabbit lung (2 to 3%) actually express cytochrome P-450, in contrast to the relatively diffuse, albeit somewhat nonhomogeneous expression seen in the liver.[11,12] Our hybridization data, suggest, therefore, that the P-450e gene is expressed in the Clara cells at

a level comparable to that in uninduced hepatocytes, but that it is not activated by phenobarbital administration.

C. Regulation of Cytochrome P-450 Isozymes In the Adrenal Cortex

The production of the glucocorticoids cortisol and corticosterone in the adrenal cortex is dependent upon several cytochrome P-450 isozymes (reviewed in References 195 and 225). Two of these are located in the mitochondrial inner membrane, while one or two, depending on the species involved, reside in the endoplasmic reticulum. The adrenal glucocorticoid biosynthetic pathway involves the side chain cleavage of cholesterol by P-450$_{scc}$ in mitochondria to form pregnenolone, the production of progesterone from pregnenolone by the 3β-hydroxysteroid dehydrogenase-isomerase enzyme system in the ER, the formation of 17α-hydroxy-progesterone from progesterone by P-450$_{17\alpha}$ in the ER, the hydroxylation of 17α-hydroxyprogesterone at the C21 position by P-450$_{C21}$ to produce 11-deoxy-cortisol in the ER, and finally the conversion of 11-deoxycortisol to cortisol by P-450$_{11\beta}$ in the mitochondria.

The mitochondrial P-450 isozymes are somewhat different from microsomal cytochrome P-450 in that they accept reducing equivalents from NADPH through the interaction of an iron-sulfur protein, adrenodoxin, and a flavoprotein, adrenodoxin reductase. In contrast, microsomal cytochromes P-450 only interact with a flavoprotein, NADPH-cytochrome P-450 reductase. In addition, mitochondrial P-450 isozymes are synthesized as higher molecular weight precursors which are cleaved post-translationally to produce the mature protein product, while the microsomal isozymes are not synthesized as higher molecular weight precursors.[38,108,138,140]

Adrenocorticotropin (ACTH) stimulates steroidogenesis in the adrenal cortex by two distinct mechanisms. Firstly, it has short-term actions which apparently involve an activation of the production of pregnenolone by the mitochondrial side chain cleavage system (reviewed in Reference 195). Secondly, ACTH has a long-term effect in maintaining the steroidogenic capacity of the cortex by stimulating the synthesis of several of the enzymes of the corticosteroid biosynthetic pathway. Thus, more than a decade ago it was shown that the biosynthesis of glucocorticoids in the adrenal is dependent upon the continued presence of ACTH since hypophysectomy of rats results in a rapid decrease in the activities of P-450$_{scc}$, P-450B$_{11\beta}$, and P-450$_{C21}$ which can be restored to nearly control levels by administration of ACTH.[180] Recently, it has become possible to culture primary bovine adrenocortical cells and thus examine the effects of ACTH treatment on cytochrome P-450 synthesis in more detail.

Treatment of such cultures with ACTH leads within 36 hr to approximately a 3-fold increase in adrenodoxin synthesis,[107] a 9-fold increase in the synthesis of P-450$_{scc}$,[38] a 7-fold increase in the synthesis of P-450$_{11\beta}$,[110] and a 15-fold increase in the synthesis of P-450$_{C21}$.[53] ACTH treatment also leads to a marked increase in the levels of P-450$_{17\alpha}$ which accounts for a shift in the pattern of corticosteroid secretion by these cells from approximately equal amounts of cortisol and corticosterone to nearly exclusively cortisol.[128] In vitro translation studies indicated that the enhanced rates of synthesis of the different cytochrome P-450 correlated with somewhat lesser increases in the levels of the corresponding mRNAs.[38,53,107,110,195]

Recently, cDNA clones for bovine P-450$_{scc}$ have been isolated and used to study aspects of the ACTH regulatory mechanism.[92] In RNA-DNA hybridization experiments the increase in P-450$_{scc}$ mRNA is detectable within 8 hr of ACTH treatment and by 36 hr reaches a level 7-fold higher than in untreated cultures. The P-450$_{scc}$ mRNA, 2 Kb in length, is detectable in both adrenal cortex and corpus luteum, but not in RNA samples prepared from bovine heart, liver, and kidney. Similar studies with cDNA clones for the other adrenal cytochromes P-450 as well as direct studies on rates of transcription of the various genes can be expected in the near future.

The action of ACTH on increased cytochrome P-450 synthesis appears to be mediated by cyclic AMP since increases in cytochrome P-450$_{scc}$, P-450$_{11\beta}$, and adrenodoxin can be obtained by treatment of cultured adrenocortical cells with cyclic AMP or cyclic AMP analogs.[109] A similar situation appears to exist in ovarian granulosa cells where treatment with follicle stimulating hormone and cyclic AMP analogs leads to an increase in P-450$_{scc}$ and adrenodoxin protein and mRNA levels.[54] A detailed review of the regulation of adrenal and gonadal microsomal mixed function oxygenases of steroid biosynthesis has recently appeared.[48]

D. Expression of Cytochrome P-450 Genes During Development

1. Cytochrome P-450 Levels and Inducibility During Ontogeny

Numerous studies indicate that cytochrome P-450 levels are very low in livers of fetuses, but increase rapidly after birth (e.g., see References 8, 29, 65, and 149a). These studies employed spectral measurements of microsomal P-450, assays of monoxygenase activities with various substrates, staining of SDS gel electrophorograms, and immunochemical measurements of P-450 recognized by antibody raised against cytochrome P-450b and e. It has also been observed that administration of PB to pregnant females does not induce cytochrome P-450 in fetal liver, but is effective within the first day or two after birth.[29,65,149] On the other hand, 3-MC does appear to be able to induce cytochrome P-450 when administered prenatally, although it is less effective than in adult animals.[8,29,65,146]

There does, however, appear to be a significant difference in the relative inducibility of the different 3-MC inducible forms of P-450 in fetal and adult liver. Studies with New Zealand white rabbits[8] demonstrated that induction of *n*-acetylarylamine hydroxylase activity, which correlates with a microsomal polypeptide of Mr = 54,000, is only detectable after 5 days of age. The inducibility (i.e., ratio of induced to control levels) reaches a maximum at 25 days after birth, remaining constant throughout adulthood. On the other hand, the inducibility of arylhydrocarbon hydroxylase activity which correlates with a microsomal protein of Mr = 57,000 is maximal 5 days prior to birth. Although substantial levels of this activity are present in animals older than 12 days, no increase in activity is observed after 3-MC treatment. Tissue specificity in the inducibility of this enzyme was observed, however, since arylhydrocarbon hydroxylase was inducible in the kidney of rats of all ages.[8] Similar results were obtained in the livers of rat and mice treated with TCDD,[66] although in these species marked inducibility of liver AHH persisted in adult animals. In rats, the 56,000-dalton microsomal protein induced in fetuses almost certainly corresponds to P-450c, whereas a 54K protein inducible at parturition, 22 days postconception, is likely to correspond to P-450d. Using antibodies prepared against mouse P_1-450 and P_3-450 (P-448), it was demonstrated that TCDD induces comparable levels of a protein immunoprecipitable by anti-P_1-450 in fetuses (20 days postconception) as well as in adult animals.[151] On the other hand, approximately 12-fold higher levels of a protein immunoprecipitable with anti-P_1-450 are found in treated adult mice as compared to fetuses.[151]

More recently, cytochrome P-450 mRNAs inducible with TCDD have been quantitated in fetal and adult mouse liver by northern blotting experiments using P_1-450 cDNA or segments of the P_1-450 gene as probes. A 23s mRNA correlating with P_1-450 activity and detectable with a P_1-450 cDNA clone (clone 46) was found to be inducible by TCDD as early as 15 days of gestation.[24] However, consistent with microsomal protein and enzymatic activity data, a 20s mRNA detected by cross-hybridization with a portion of the P_1-450 structural gene and presumably correlating with P_3-450 (although identified as P_2-450 in this study) was not induced prenatally (15 days), but became inducible by TCDD treatment 7 days later in development.[88] Thus, although inducibility of P_1-450 and P_3-450 is controlled by the Ah receptor, other factors must be involved in regulating the expression of the corresponding genes.

2. Androgenic "Imprinting" Of Sex Specific Hepatic Steroid Metabolism: Developmental and Hormonal Regulation of Specific Cytochrome P-450 Isozymes

It has been known for some time that male and female rats show large differences in the hepatic metabolism of drugs and steroid hormones (reviewed in References 26, 71, and 97). Male animals, in general, show higher steroid hydroxylating activities than females,[27a] whereas females exhibit a higher 5α-reductase activity.[241] Male and female patterns of hepatic steroid metabolism can be defined in terms of the levels of various specific enzymatic activities. In particular, the cytochrome P-450 catalyzed 16α-hydroxylation of testosterone or 4-androstene-3,17-dione by liver microsomes of female rats is essentially undetectable, whereas mature males have quite high levels of this activity.[73,84,90,120] In males, the level of this enzyme activity is developmentally regulated, with the enzyme first being detectable at around 30 days of life, at puberty.[84,90,120] Similarly, livers of female rats contain a developmentally induced 15β-hydroxylase which is active on steroid sulfates and is present in adult males at levels less than 0.03% of the level in females.[68] On the basis of extensive experimental data, the salient points of which are summarized below, Gustafsson and associates have proposed the existence of a novel endocrine axis, the hypothalamic-pituitary-liver system which regulates the sex-dependent state of hepatic steroid metabolism (reviewed in References 67 and 71). In this regulatory pathway, at birth, testicular androgens irreversibly program brain centers in the hypothalamus which regulate the pituitary control of hepatic sex-dependent steroid and drug metabolism. This irreversible programming, which is termed imprinting, results in activation of a hypothalamic secreting center which is turned on in males just before puberty to produce a "feminostatin". This factor inhibits the secretion of a pituitary "feminizing factor" that sets the female pattern of hepatic steroid metabolism. Thus, the basic state of differentiation of rat liver is masculine and the secretion of the feminizing factor by the pituitary is required to induce the feminine state of steroid metabolism. In adult males, the masculine state is reestablished by blocking secretion of the feminizing factor. The critical pieces of evidence for this model are

1. Castration of males at birth leads to female pattern of steroid metabolism in adulthood, in particular the absence of 16α-hydroxylase activity[73] and the presence of normal female levels of 15β-steroid sulfate hydroxylase levels.[68] Castration of adult males does not induce this female-specific activity[68] and leads to only a modest reduction in the 16α-hydroxylase activity which can be restored to normal levels by treatment with appropriate androgens.[73] Castration at 2 weeks of life, however, or administration of a single injection of appropriate androgens on the second day life to males castrated at birth, results in 16α-hydroxylase levels during adulthood comparable to those in livers of males castrated as adults.[73]* Furthermore, testosterone treatment of adult animals castrated at 2 weeks or later leads to normal levels of this male-specific enzyme activity. These data indicate that the presence of androgen in the early postnatal life of male rats irreversibly programs the liver to display in adult life the male pattern of steroid metabolism. This pattern can also be imprinted in female rats by a single injection of testosterone at birth.[199] In this case the male pattern becomes manifest only after ovariectomy which removes the suppressing affect of circulating estrogen.

* It should be noted that although castration of adult male rats and ovariectomy of adult females does not affect the levels of the female-specific steroid sulfate hydroxylase activity, treatment of females with high doses of androgen markedly suppresses that activity, whereas treatment of postpubertally castrated males with estrogens induces a substantial level of the female specific activity[68] and a marked reduction in male-specific hydroxylase activities.[72]

2. Hypophysectomy of female animals leads to the male pattern of steroid metabolism, i.e., the abolition of steroid sulfate hydroxylase activity[69] and the appearance of 16α-hydroxylase activities comparable to those in castrated males.[33,72] This experiment indicates that the female pattern is dependent on an intact pituitary which is therefore thought to produce a feminizing factor. It is also important to note that the effects of androgens and estrogen on the levels of sex-specific steroid hydroxylase activities in gonadectomized males and females are not observed in hypophysectomized animals.[69,74] This suggests that the effects of these hormones on liver steroid metabolism are mediated through the pituitary gland.
3. Implantation of a pituitary gland under the kidney capsule of a hypophysectomized male or female rat results in feminization of steroid metabolism.[33,74] This indicates that the ectopic pituitary gland (which is not under hypothalamic control) secretes a factor into the general circulation that is normally only secreted by the female pituitary *in situ*.
4. Electrothermal lesion of the hypothalamus of male rats leads to feminization of steroid metabolism.[70] This suggests that the male hypothalamus produces a factor which is responsible for the male pattern of hepatic steroid metabolism. Together with the previous experiments on the effects of hypophysectomy on females or implantation of an ectopic pituitary in males, it is reasonable to propose that the hypothalamic factor inhibits secretion of the pituitary feminizing factor, hence its name feminostatin.[67]
5. Recently, evidence has been presented that the feminizing factor is growth hormone (GH) (or a variety of growth hormone). Continuous infusion of growth hormone into male rats for 7 days leads to feminization of steroid metabolism, i.e., the nearly complete abolition of 16α-hydroxylase activity.[136] Furthermore, purification of the feminizing factor from rat pituitaries on the basis of an assay for its feminizing activity upon continuous infusion into male rats led to a highly purified preparation of growth hormone.[135] In addition, the hypothalamic feminostatin appears to be somatostatin since treatment of male rats with antibodies to somatostatin leads to a partial feminization of hepatic steroid metabolizing activities.[156] It should be noted that in adult male rats there are regular surges in blood levels of growth hormone ($\sim$200 ng/ml) every 3 to 4 hr, with low, essentially undetectable levels ($<$1 ng/ml) between peaks, whereas in females the periodicity is irregular with somewhat lower peak heights and higher levels between peaks than in male animals.[42] In immature rats of both sexes, serum levels of growth hormone are much lower than in adults and the temporal pattern of secretion is irregular.[42] Nothing is yet known about the mechanism(s) by which the subtle differences in the temporal patterns of circulating growth hormone could account for the extreme differences in specific enzyme levels in the livers of adult male and female rats. It would not be surprising if both transcriptional and post-translational regulatory mechanisms are utilized to establish and maintain the sex-specific pattern of steroid metabolizing enzymes.

Several laboratories have reported the partial or complete purification of a cytochrome P-450 from livers of control or imprinted rats (i.e., castrated at 4 weeks of age or later) which has substantial testosterone 16α-hydroxylase activity.[23,187,226] Rabbit antisera raised against P-450 2c[226] specifically inhibits most (85%) of the testosterone 16α-hydroxylase activity in liver microsomes from untreated rats.[225] Furthermore, six different microsomal steroid hydroxylase activities characteristic of purified cytochrome P-450 2c which are induced at puberty in male but not female liver are similarly sensitive to inhibition by antibody to the purified enzyme. Ouchlertony double diffusion analysis demonstrates that P-450 2c is not detectable in liver microsomes from

livers of sexually immature male rats. These studies convincingly demonstrate that cytochrome P-450 2c corresponds to the male-specific, developmentally regulated steroid 16α-hydroxylase of rat liver.* A direct comparison of P-450 2c with P-450 UT-A isolated in Guengerich's laboratory[30] using structural, immunochemical, and enzymatic criteria has revealed no differences between these two enzyme preparations (D Waxman, unpublished). On the basis of their catalytic properties, cytochrome P-450 2c also appears to be equivalent to P-450h of Ryan et al.[187] The male-specific cytochrome P-450 described by Kamataki et al.[95] is also likely to correspond to the same protein — its level in liver microsomes of male rats is reduced approximately 40% by postpubertal castration and returns to normal after testosterone treatment.

The same three laboratories which purified the male-specific P-450s also purified a distinct female-specific P-450 by applying the identical chromatographic procedures to solubilized microsomal preparations from female rats.[95,187,225] Immunochemical measurements indicate that levels of the female-specific enzyme obtained by Kamataki et al.[95] are not affected by ovariectomy of female rats, but are reduced to undetectable levels by testosterone treatment of ovariectomized females. This sensitivity to gonadal steroids is identical to that observed for the female-specific steroid sulfate 15β-hydroxylase activity.[68] These three preparations of female specific cytochrome P-450 are likely to correspond to the same or closely related proteins as is the partially purified steroid sulfate hydroxylase described by Lefevre et al.[116] In fact, of nine distinct forms of purified cytochrome P-450, only P-450i, the female-specific form, catalyzed the hydroxylation of the steroid sulfate 5α-androstane -3α,17β-diol-3,17-disulfate.[186a] It is interesting to note that the male- and female-specific P-450s may very well represent closely related gene products. This is not only based on their identical chromatographic properties. The two proteins purified by Waxman show substantial immunological cross-reactivity and similar partial chymotryptic cleavage patterns.[225] It is conceivable, therefore, that the sexually differentiated states of hepatic steroid metabolism may involve somewhat unique regulatory mechanisms in which closely related genes with similar regulatory elements are affected in opposite ways by the same effector molecules. The availability of antibodies to the purified proteins ensures that cDNA and genomic clones for these isozymes will be obtained in the near future. These will provide critical reagents for studying the molecular genetic aspects of the developmental and hormonal regulation of the corresponding genes. Although such studies are of considerable interest from the perspective of the biology of cytochrome P-450, they also have broader implications since a sizable number of other genes in the livers of male and female rats appear to be regulated by similar mechanisms.[186]

IV. SUMMARY

The microsomal cytochromes P-450 are encoded by a superfamily of genes which can be subdivided into subfamilies of very closely related genes and families of somewhat more distantly related genes. DNA sequence data for cloned cDNAs and genomic fragments corresponding to different members of the same family or subfamily indicate that extensive gene conversion has occurred during evolution to generate genes which encode proteins with a segmented arrangement of regions of extraordinary ho-

* The testosterone 6β-hydroxylase activity which is present at much higher levels in liver microsomes from mature male as compared to mature female rats and is also imprinted[120] is associated with P-450$_{PCN}$.[31] The regulation of this enzyme is more complicated since it is present in immature males as well as females at relatively high levels (approximately 40% of the level in mature males), but the female level decreases approximately 7-fold after puberty.[120] Furthermore, dexamethasone treatment of mature females leads to levels of P-450$_{PCN}$ comparable to those found in males treated with that synthetic glucocorticoid.[31]

mology or divergence. Analysis of the sequence data obtained to date for various cytochrome P-450s reveals the existence of highly conserved domains within the proteins which are presumed to participate in common functional properties of the various monooxygenases.

Cloned genes or cDNAs for various forms of P-450 have provided important reagents for studying their regulation. Using such probes it has been demonstrated that induction of specific P-450s results from the accumulation of the corresponding mRNA which, in the case of induction by phenobarbital and 3-methylcholanthrene, can be accounted for by a marked rapid transcriptional activation of the corresponding genes.

Recent studies confirm earlier proposals that the Ah gene, which regulates the inducibility of mouse cytochrome P_1-450 and P_3-450 by polycyclic hydrocarbons, encodes a receptor protein which binds the inducing ligand. The availability of hepatoma cell lines defective in induction of arylhydrocarbon hydroxylase which appear to have lesions in the Ah receptor protein coupled with a new procedure for isolating inducible revertants should soon permit the cloning of gene(s) encoding this protein.

Further studies on the regulation of cytochrome P-450 are likely to focus on mechanisms determining the tissue specificity and hormonal and developmental regulation of expression of specific genes as well as the interactions between regulatory molecules and DNA segments which are involved in the induction of specific isozymes by xenobiotics.

V. NOTES ADDED IN PROOF

The complete nucleotide sequence of the methylcholanthrene inducible cytochrome P-450c gene was recently reported.[197a] The gene is approximately 6.0 kbases in length and is split into 7 exons. The first exon contains only 5′ untranslated sequences, whereas the second exon corresponds to amino acid sequences homologous to those encoded by all of the first four exons and most of the fifth exon of the P-450e gene. Indeed, when the amino acid sequences of P-450c and P-450e were aligned by inserting deletions or additions to maximize homology, it appears that the structures for the two P-450 genes are completely different with not a single intervening sequence located in exactly equivalent positions relative to the amino acid sequences. The only ways these 2 genes could be derived from a common ancestral gene would be if that gene contained 14 or more introns and different introns were precisely deleted during the course of evolution of the duplicated copies of that gene. Alternatively, introns in these genes could have arisen by random insertion of transposon-like elements.

The complete nucleotide sequences of the mouse P_1-450 and P_3-450 genes and substantial segments of upstream and downstream flanking regions were also recently reported.[59a] The introns of these genes interrupt the exonic sequence at equivalent positions and all the exons of the P_1-450 gene, except for the last one (which is 25 bp shorter in the P_1-450 gene) are exactly equal in length to those in the orthologous rat P-450c gene. Exonic regions of the mouse P_1-450 gene are 86 to 96% homologous to the rat P-450c gene (overall exonic homology is 93%), whereas intronic regions show 80 to 90% homology.

The segments of extraordinarily homology near the 5′ ends of the P_1-450 and P_3-450 mRNAs are wholly contained within exon 2 which roughly corresponds to exons 1 through 5 of the P-450e gene. It is pointed out that exon 3 of the P_1-450 and P_3-450 is similar to P-450e exon 6, P_1-450 and P_3-450 exons 4 and 5 are similar to P-450e exon 7, and P_1-450 and P_3-450 exon 6 is similar to P-450e exon. It was proposed that the intron-exon organizations of the 3-MC and PB-inducible genes are sufficiently similar to conclude that the ancestral P-450 gene had a minimum of 14 exons of which differ-

ent subsets were removed during the evolution of the 3-MC inducible and PB inducible gene families.

Of considerable interest was the finding of three short (11 or 12 bp) highly homologous regions in the region between -280 and -530 upstream from the cap sites of the P_1-450 and P_3-450 genes which represent candidates for segments involved in the common aspects (e.g., TCDD and 3-MC inducibility) of the regulation of these genes.

Recently, Montisano and Hankinson[136a] have found that the AHH^- phenotype in complementation class A mutants can be corrected by transfection with DNA from wild-type Hepa-1 cells, rat hepatoma cells (line H4II EC3), or from a class A/human hybrid in which the A^- mutation is complemented by the corresponding human gene. All transfectants were unstable in culture, and in one transfected with rat DNA, southern blot analysis showed that AHH activity segregated together with the rat P_1-450 gene. These results demonstrate that the A gene is either the structural gene for P_1-450 or another very closely linked gene.

The complete sequence for the coding region of the mRNA corresponding to the precursor of the mitochondrial cytochrome P-450(SCC) of bovine adrenal cortex was recently reported.[136a] This precursor consists of 520 amino acids with the amino terminal 39 residues constituting the extra peptide which is removed upon incorporation of the protein into the mitochondrion. This P-450 shows higher homology (18 to 20%) to the various microsomal P-450s than to the bacterial P-450cam (13.6%). It contains only two cysteine residues, both in the carboxy terminal half of the protein. The region around Cys_{461} shows substantial homology to the conserved cysteine-containing peptides in the carboxy terminal halves of all the other P-450s which have been sequenced (Figure 7A). This absence of a conserved cysteine-containing peptide in the amino terminal half of the protein provides strong support that the cysteine residues in the conserved peptides in the carboxy-terminal halves of the proteins depicted in Figure 7A provides the thiol group which coordinates to the heme iron in the various P-450s.

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