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The Molecular Biology of Cytochrome P450s

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I. Introduction

A. Background and History

THE MICROSOMAL multisubstrate mixed function monooxygenase system has been the subject of intense study for over 30 yr. This system has evolved to handle the constant onslaught of foreign chemicals that organisms ingest and absorb every day. Early studies recognized the complexity of NADPH/O_2-dependent oxidation and the fact that many chemicals induce their own metabolism. The key enzymatic components of this system are the flavoprotein NADPH-cytochrome P450-oxidoreductase and cytochrome P450. A reduced pigment that had an absorption band with a λ_{max} at 450 nm after binding to carbon monoxide was first identified by Klingenberg (227) and Garfinkle (82) in 1958. This pigment was further characterized as a P450 hemoprotein in 1961 by Omura and Sato (327). More extensive characterization on a solubilized fraction was carried out in 1964 (328, 329). The role of P450 in the microsomal mixed function monooxygenase system was also established at this time (45). Over the next 20 yr it became evident through protein purification efforts that multiple forms of P450 existed in mammals and other species. Studies using systems of purified P450s reconstituted with lipid and NADPH-cytochrome P450-oxidoreductase (146, 254) revealed that individual forms of P450 can exhibit either highly specific or less specific overlapping substrate positional or stereospecificities (119). For example, a single form of P450 can possess a low K_m activity toward one or more substrates and a high K_m activity toward other substrates. These latter substrates may be more actively metabolized by other forms of P450. Furthermore, a single substrate such as testosterone can be metabolized at numerous ring positions. Certain P450s are active toward hydroxylating testosterone in a stereospecific manner at one or more ring positions, while a second form of P450 will hydroxylate this substrate at other positions (450). Among the compounds metabolized by P450s are (*a*) endogenous substances, such as fatty acids, prostaglandins, steroids, and ketones; (*b*) carcinogens, including polycyclic aromatic hydrocarbons, nitrosamines, hydrazines, and arylamines; and (*c*) drugs, such as nifedipine, mephenytoin, codeine, midazolam, and cyclosporine.

Most P450-mediated metabolism results from the insertion of a single atom of oxygen, derived from O_2, into a substrate. Depending on the particular reaction and the nature of various unstable intermediates, different reactions can occur. These include oxidative and reductive dehalogenation; N-hydroxylation and N-oxidation; oxidative deamination; S-, N-, and O-dealkylation; and aliphatic and aromatic hydroxylation. The net result of the multiplicity of P450s and their diverse and overlapping substrate specificities is the ability to metabolize scores of chemicals. In general, the microsomal P450s, particularly those found in the liver, function to convert hydrophobic substances to more hydrophilic derivatives that can be easily eliminated from the body. For instance, a lipophilic compound can be hydroxylated by a P450, and subsequently this hydroxyl group can be a substrate for transferase enzymes that attach either sulfate or glucuronic acid. The hydrophilic conjugated material is then easily passed from the body via the urine or bile. With certain compounds, however, this defense mechanism goes astray, and the high energy intermediates of oxygenation reactions, frequently epoxides, attack the cellular biomolecules DNA, RNA, and protein, producing toxicity, cell death, mutations, and cell transformation. This property of P450s underscores the importance of completely understanding their multiplicity, substrate specificities, and regulation. Earlier reviews contain more complete discussions of the protein multiplicity (20, 55, 119, 122, 256, 448), enzymatic mechanisms (127, 461), and the role of P450s in carcinogenesis (40, 41, 85, 120, 198, 337).

Not all P450s are associated with catabolic processes. Some of the most important enzymes in steroid biogenesis are P450s located in the adrenal gland. These include two microsomal enzymes, steroid 17α-hydroxylase and steroid 21-hydroxylase, and two mitochondrial P450s, the cholesterol side chain cleavage enzyme and steroid 11β-hydroxylase. The mitochondrial P450s are quite distinct from their microsomal counterparts in that they are synthesized with a leader peptide and they collect electrons via adrenodoxin and adrenodoxin reductase. Further, these enzymes are associated with severe genetic defects in cortisol biosynthesis. The biology, enzymology, and regulation (44, 69, 131, 188, 280) of the adrenal enzymes and their relationship to human disease (281, 282, 315) have been reviewed.

As mentioned earlier, numerous laboratories have described the purification and characterization of P450s (20, 55, 119, 256, 261, 382, 448). In addition, polyclonal (419) and monoclonal (87, 419) antibodies have been used to quantitate levels of P450s in rats given inducing agents and to study the contribution of specific forms of P450 to the metabolism of various compounds in crude microsomal preparations. Antibodies were also instrumental in the isolation and characterization of many P450 cDNAs and their genes.

B. Methodologies

Two major approaches were used to isolate the first cDNAs, and both relied on antibodies generated against

P450s. One property of the P450 system that was exploited in the early work on purification is that some forms of P450 can be specifically induced at high levels by treating animals with such agents as phenobarbital and 3-methylcholanthrene. It was further shown, through in vitro translation technology, that these agents induce enzymes by increasing levels of their mRNAs (96, 97). Therefore by administering certain chemicals to rats, it is possible to produce a pool of mRNA that contains an increased level of certain P450 mRNAs relative to mRNA from untreated rats. cDNA libraries are then constructed from the induced mRNA, and clones from these libraries were differentially screened with cDNA probes synthesized from induced and uninduced mRNAs. In some cases, polysome immunoadsorption was used to construct P450 mRNA-enriched cDNA libraries and to isolate enriched cDNA probes for specific P450 mRNAs (99, 141). Clones that hybridized specifically with the induced probe were then characterized by hybrid-arrest and hybrid-select translation. Using this methodology, the first cDNA clones were isolated for rat phenobarbital-induced (2, 77, 99), rat steroid-induced (141), and mouse 3-methylcholanthrene-induced (102, 311) P450s. These procedures were adequate for isolating cDNA to abundant inducible mRNAs; however, the bulk of P450s are not significantly inducible, and their mRNAs are present in low or moderate amounts. To circumvent this problem, antibodies were used to screen the libraries. For instance, a plasmid library was screened with antibody to rabbit P450 1 resulting in the first instance of the isolation of a cDNA clone for a constitutively expressed P450 (427). Constitutive expression is used to refer to a P450 that is expressed in the absence of an inducer. Some inducible P450s are also constitutively expressed, and some constitutively expressed enzymes are developmentally regulated and/or exhibit sex-specific expression (see below). Antibody screening procedures were further exploited with the development of the novel bacteriophage cDNA expression vector, λgt11 (492), which allowed the isolation of cDNA clones to low abundance mRNAs. Using this vector, several cDNAs have been isolated (15, 101, 105, 142, 298, 404, 431). Other investigators have used oligonucleotide probes generated against partial peptide sequences to screen for P450 cDNAs (28, 294, 491). Finally, as will be discussed later, there are subfamilies of P450s with considerable homology at the amino acid and nucleotide levels. Libraries of cDNAs can be screened with specific probes to effectively isolate cDNAs for several members of a particular P450 gene subfamily (106, 108, 213, 218, 219).

C. Advantages of the Molecular Biology Approach

There are several difficulties inherent in the biochemical approach to the study of P450s. First, these intrinsic membrane proteins are extremely difficult to purify, particularly those forms that are present in low abundance in liver and extrahepatic tissues. The study of human P450s has been further hampered by the limited availability of human tissue specimens in some laboratories. Genetic variability between human samples also complicates the development of a purification scheme that can be used by various laboratories to isolate the same P450 from different sources of human tissue. For instance, purification procedures must be tailored to a single liver. Despite these limitations, several laboratories have successfully isolated multiple forms of human P450 (56, 129, 130, 208, 389, 476, 479, 480). Even when P450s can be purified, it is difficult to demonstrate homogeneity. The most common criteria for homogeneity have been the detection of single polypeptides on sodium dodecyl sulfate-polyacrylamide gels and amino-terminal protein sequence. However, P450s can have identical gel mobilities and similar or identical amino-terminal sequences. For instance, using cDNA cloning technology, two distinct forms of P450 were found that share the same mobility on gels and identical amino-terminal sequences (108). Furthermore, antibodies raised to a single homogeneous preparation of enzyme can potentially cross-react with members of the same gene family, and this problem can only be resolved by extensive immunoadsorptions (11, 419). Yet, even this procedure is unlikely to work with very closely related P450s, such as rat IIB1 and IIB2, which display 97% amino acid similarity. Monoclonal antibodies have, however, been developed that are specific for IIB1 (419), but it can never be certain whether a particular antibody only recognizes a single protein. Because of the property of cross-reactivity, studies using antibodies as probes to examine protein levels and enzyme inhibition must be cautiously interpreted. Finally, the most critical limitation to the biochemical approach is that it may not be possible to isolate minor forms of P450 in humans and rodents.

Molecular biology has offered a valuable adjunct to the biochemical and immunological approach to P450 research. It is now possible, via molecular cloning, to isolate, identify, and characterize practically all of the P450s in a range of organisms and tissues. Among the problems investigated with recombinant DNA technologies are (*a*) the evolution and phylogenetic comparison of P450 and the roles of P450s within a given organism in chemical carcinogenesis and drug metabolism and (*b*) the catalytic activities of P450s generated through cDNA expression systems. Molecular cloning has made it now feasible to study the catalytic specificities of P450s that are difficult to purify to homogeneity. Furthermore, studies of structure-function relationships in the catalytic cycle of P450s can be accomplished by using site-directed mutagenesis and preparing of chimeric enzymes. The regulation of P450s is also being examined in detail, with the aid of isolated P450 genes. The nature of the receptors and other factors that control the levels of transcription of P450 genes can now be examined. Finally, the

study of human P450s can be expanded using cDNA and genomic clones. The multiplicity, catalytic activities, and expression of human P450s can be studied, and the search for relationships between disease, cancer susceptibility and resistance, and P450 expression can be intensified.

Several authors have reviewed the molecular biology of P450s during the past few years (1, 306). Adesnik and Atchison (1) nicely summarize the early work on the isolation and sequencing of the first P450 cDNAs and genes and critically review experiments on the mechanism of regulation of P450 genes. A more recent review (306) describes the P450 gene superfamily and integrates the new recommended P450 gene nomenclature system (301). The mechanism of regulation of the "Ah locus"-associated P450 genes has been reviewed in detail (306, 308, 462). The evolution and structural comparison of P450s have also been considered (112, 307, 312). In the remaining portion of this article, the structure and evolution of the P450 genes will be examined, with emphasis on the catabolic P450s. The molecular biology of the anabolic steroid-synthesizing P450s has been covered in earlier reviews (282, 306). Recent work on cDNA-direct expression, P450 gene regulation, and human P450 catalytic activities, structure, and polymorphisms will be discussed in more detail.

II. Structure and Evolution of P450 Genes

A. Summary of P450 Gene Nomenclature

One of the hallmarks of P450 research has been the confusing nature of P450 nomenclature. Virtually every laboratory involved in P450 purification has developed its own system of nomenclature. For instance, the P450IA1 has been designated P-450c (369), P-450BNF-B (123), P-448_2 (170), P-450 MC-1 (244), P-450MC (483), PCB P-448-L (201), P-450 isozyme 6 (194), P_1450 (310), and P-450_{MC1} (442). The isolation and sequencing of many P450 proteins, cDNAs, and genes have facilitated the development of a P450 classification system based on primary amino acid sequence alignment data (301, 309). In this system, the P450s are grouped into a gene superfamily that is further subdivided into gene families and finally into gene subfamilies. The limits of amino acid similarity that define families and subfamilies are somewhat arbitrary and were determined by examining all 65 P450 protein sequences available at the end of 1986. To date, 13 gene families have been found. A protein in one gene family is ≤36% similar to the P450s in another gene family, and within a family a protein in a given subfamily is about 40 to 65% similar to a protein in another subfamily. With few exceptions, a particular P450 can accurately be assigned to a family or subfamily only if its full amino acid coding sequence is known.

A few problems exist with a P450 nomenclature system that is based solely on sequence similarities. As expected, due to the complexity of the P450s, their polymorphic nature, the fact that they are rapidly diverging, and the prevalence of gene conversions, it has been difficult, in many cases, to determine whether a given P450 in one species is orthologous to a P450 in another species. For instance, does a specific form of P450 have a counterpart, or orthologue, in another species, and can this be determined on the basis of sequence similarities? For certain families of P450s, the assignment of orthologues is straightforward; however, in other families the designation of orthologues is virtually impossible. The nomenclature system is further complicated by the marked overlapping and diverse substrate specificities of P450s, which again are more of a problem in certain gene families. Some P450s have very distinct, high levels of catalytic activity toward specific substrates while other P450s have broad substrate specificities. The possibility exists that orthologous P450s will have different substrate specificities and, conversely, that nonorthologous P450s or even P450s in different subfamilies will have similar substrate specificities. An example of these complexities will be illustrated below. In any case, as long as the shortcomings are kept in mind, the P450 nomenclature system, based on primary sequence data, should be adequate.

The "nuts and bolts" of the nomenclature system are as follows. Gene families are designated by Roman numerals beginning with I. Gaps are left in the family numbers to allow for the addition of newly discovered gene families. For example, the primary hepatic drug-metabolizing enzymes currently comprise four gene families designated I through IV. A P450 gene from a housefly is classified within gene family VI (70). The extrahepatic P450s involved in steroid biosynthesis fall within families XVII (steroid 17α-hydroxylase), XIX (P450 aromatase), and XXI (steroid 21-hydroxylase) (301). The mitochondrial, yeast, and bacterial P450 gene families are referred to as XI, LI/LII, and CI/CII, respectively. Subfamilies are indicated with sequential capital letters, and genes within a subfamily are denoted by sequential Arabic numbers. For instance, there are currently eight subfamilies in the P450II gene family, designated A through H, and the next subfamily discovered would hence be called III. The individual genes are usually numbered depending on when sequence data are published. Once a P450 sequence has been determined, if a clear orthologous counterpart cannot be identified from another species that has previously been sequenced, then the P450 is assigned the next consecutive number.

The P450I and P450III gene families provide examples of straightforward and confusing nomenclature assignments, respectively. In the P450I gene family, two genes have been identified in man, rat, mouse, and rabbit and designated IA1 and IA2. These are easily distinguished by enzyme activities and sequence. For instance, rabbit IA1 is much more similar to mouse, rat, and human IA1 than mouse, rat, and human IA2, and vice versa. This

type of comparison is not as simple in other families, however. For example, two cDNAs in the rat P450III family were sequenced and named PCN1 and PCN2 (105, 108). Two human P450 cDNAs were then sequenced and designated $P450_{NF}$ (15) and P450p (288). A member of the rabbit P450III family, designated P450 3c, was also sequenced (48). The net result is that rats have P450s designated PCN1 and PCN2, rabbits have P450 3c, and humans have $P450_{NF}$ and P450p. A comparison of the primary amino acid sequences of these five P450s does not reveal a strong similarity between one of the rat P450s and one of the human P450s or the rabbit P450. The reason for this is that recent gene duplication and gene conversion events make it impossible to identify orthologous counterparts between species in the P450III family. The only plausible way to deal with the species variability is to name each P450 gene as a distinct entity within a species, i.e., to designate five distinct P450 genes in the P450III family. Species variability and nomenclature assignments are also a problem in the P450III gene family, as will be illustrated in later sections of this review.

B. The P450 Gene Superfamily

I. Overview of the superfamily. The P450s are presumed to have been present in the earliest organisms over 1.4 billion yr ago (305, 312). The gene superfamily presently consists of 13 gene families, including two bacterial, two yeast, one insect, and eight vertebrate families (307). Families I through IV primarily code for catabolic microsomal enzymes found in liver and to some extent in extrahepatic tissues, whereas families XVII, XIX, XXI, and XXII code for P450s involved in steroid biosynthetic pathways. These latter four gene families are expressed in specialized extrahepatic tissues. The mitochondrial P450s, which use adrenodoxin and adrenodoxin reductase for electron transfer, fall into the P450XI gene family. These enzymes are made in the adrenal cortex and are also involved in steroid synthesis. A new P450 gene family, designated P450VI, has been identified in the domestic house fly (70). Finally, the yeast LI and LII families code for a lanosterol demethylase and an alkane-inducible P450, respectively, and the bacterial P450CI family, found in *Pseudomonas*, codes for a camphor-metabolizing P450 (127, 435). A second bacterial gene from *Bacillus megaterium*, which codes for a M_r 120,000 fusion protein of a P450 moiety and NADPH-P450-oxidoreductase (300), represents the CII gene family (458).

Several other P450s have been identified or isolated and characterized, but their cDNAs or genes have not been cloned. These include (*a*) hepatic cholesterol 7α-hydroxylase (30, 321, 449); (*b*) $P450_{C\text{-}M/F}$, an estrogen 2- and 16α-hydroxylase constitutively expressed in rat liver (410); (*c*) a rat brain estrogen 2-hydroxylase P450 (283); (*d*) a rat kidney lauric acid hydroxylase designated P450K-5 (173); (*e*) a rat kidney mitochondrial 25-hydroxy-vitamin D_3 1α-hydroxylase (158); (*f*) a leukotriene B_4 ω-hydroxylase found in polymorphonuclear leukocytes (386, 387); (*g*) rat lanosterol 14α-methyl demethylase (425); (*h*) a platelet thromboxane synthase (147); (*i*) a P450 expressed in rat prostate (411); (*j*) a human arachidonic acid epoxygenase (249); and (*k*) several P450s found in plants, including an obtusifoliol 14α-demethylase (355) and a cinnamic acid hydroxylase in elicitor-treated bean cells (22). It is quite possible that some of these P450s may fall into existing gene families.

The evolutionary relationships between various P450s can be estimated by comparing primary amino acid sequence data from all known P450 sequences. Fortunately, the sequences of many different forms of P450s have been determined in certain species such as rat. In addition, the same P450, or orthologous P450s, have been sequenced in a variety of species. Using computer alignment programs and estimated species divergences that are based on fossil evidence, phylogenetic trees can be constructed (112, 306, 312). These trees can be used to predict the unit evolutionary period (UEP)* of P450s and to estimate whether a given species will contain a given cDNA. The UEP, or the time in millions of years required for a 1% change in amino acid sequence, ranges from 2 to 4 for P450s (312). This value is quite distinct in different gene superfamilies and is thought to reflect both the structural constraints of a particular protein and the need for genetic diversity. The best demonstration for the importance of these two factors in determining the evolution of multigene families is provided by histone and immunoglobulin gene families. The histones, which are some of the most stable proteins, with a UEP of about 400, require a highly conserved tertiary structure for their function as the building blocks of nucleosomes. The immunoglobulins, on the other hand, are rapidly evolving proteins with less rigid structural requirements. These proteins, with UEPs of about 0.7, have flexible tertiary structures for their diverse antigen-binding properties. P450s are also diverging rapidly, and this may be due to less rigid constraints in their membrane-bound environment. In fact, current thinking is that only a small portion of the P450 is imbedded in the membrane (see below). Another factor that may govern P450 divergence is that they must evolve to metabolize a wide variety of substances. Flexibility and rapid evolution of these enzymes were probably necessary to cope with changes in plant fauna and dietary habits of organisms over time, as noted below.

For the purpose of comparing enzymes in a superfam-

*Abbreviations used are: UEP, unit evolutionary period; PCN, pregnenolone 16α-carbonitrile; DPLC, dilaurylphosphatidylcholine; RFLP, restriction fragment length polymorphism; SV40, simian virus 40; MC, 3-methylcholanthrene; TCDD, 2,3,7,8-tetrachlorodibenzoparadioxin; AHH, aryl hydrocarbon hydroxylase; DRE, drug regulatory elements; XRE, xenobiotic regulatory elements; GRE, glucocorticoid responsive element; TAO, triacetyloleandomycin; ALV-S, 5-aminolevulinate synthase; poly (A), polyadenylation.

ily, phylogenetic trees are constructed. Briefly, these trees are generated using amino acid sequence comparisons and certain assumptions, for example, a constant evolutionary change rate. Several methods can be used to construct a phylogenetic tree, and some of these have been discussed (112, 312). An evolutionary tree of P450s is displayed in fig. 1; the major branch points of P450 divergence are taken from Nelson and Strobel (312). Several conclusions concerning the evolution of P450s can be drawn by examining of the phylogenetic tree (305, 306, 312). First, early P450s may have evolved to metabolize cholesterol and its derivatives. For instance, the oldest P450s are the mitochondrial cholesterol-metabolizing enzymes in the P450XI family. Related to these P450s are those that metabolize fatty acids such as the P450IV lauric acid hydroxylases. The cholesterol- and fatty acid-metabolizing P450s may have been involved in the maintenance of membrane integrity of early eucaryotes (305). A later evolutionary event was the formation of the endogenous steroid-synthesizing P450s (P450XVII, XIX, and XXI) and the catabolic enzymes about 900 million yr ago. The latter group of P450s diverged into the major drug- and carcinogen-metabolizing enzymes of the P450I and P450II families. A tremendous expansion in the P450II gene family has occurred within the past 400 million yr. Estimating the time when each P450 diverged within the P450II gene subfamilies is difficult, because of the preponderance of gene conversion events, as will be discussed below.

The reason for the increasing numbers of P450 genes during the past several hundred million years is unclear, although it has been suggested that this diversification may have coincided with the emergence of aquatic vertebrates onto land and the consequent changes in their diets which contained toxic poisons found in land vegetation (312). It is becoming quite clear, however, that various species have been, and are, developing their own specialized battery of P450s, particularly those in the P450II subfamilies. This undoubtedly relates to the evolution of distinctive dietary habits. Although humans have recently been exposed to a tremendous number of man-made chemicals, it seems unlikely that our P450s are evolving rapidly enough to cope with this onslaught. This may be a factor in the increase in chemically induced diseases such as cancer, a point that will be elaborated below.

One of the most interesting features of P450 gene evolution is the occurrence of multiple genes that, in many cases, are present in some species but not in others. These arise through the process of gene duplication and the fixation of duplicated genes through natural selection. This phenomenon probably accounts for some of the major interspecies differences in drug and carcinogen metabolism that have been noted over the years. An example of this phenomenon is depicted in fig. 2. Rabbits, rats, and humans diverged about 75 million yr ago. The P450IIE subfamily in rats and humans contains a single gene (404, 432, 434); however, two highly similar IIE genes are present in rabbits (210–212). Based on the sequence similarity of the two rabbit proteins, we can predict that a gene duplication occurred in this species about 10 million yr ago, long after the rabbit-rat-human speciation. However, a similar gene duplication did not occur in rats or humans. These recent gene formations are particularly evident within the individual P450II gene subfamilies.

In the sections below, brief descriptions are given for

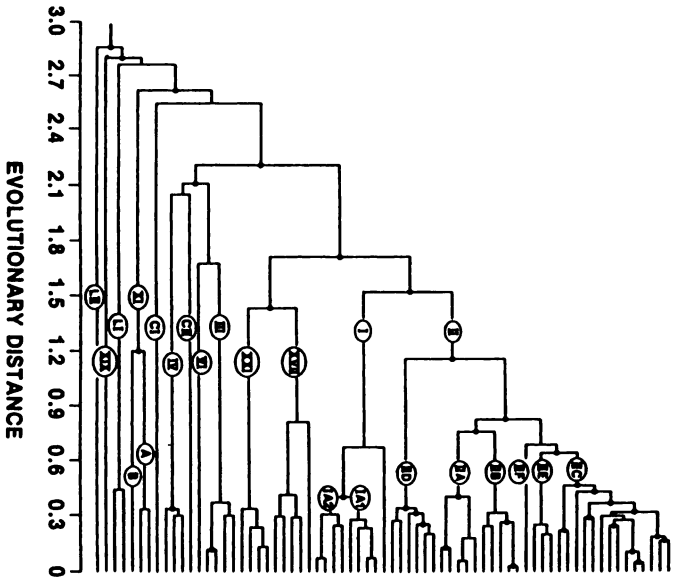

FIG. 1. A P450 protein phylogenetic tree. The divergence times were calculated as described by Nelson and Strobel (312). A total of 69 P450 sequences were compared.

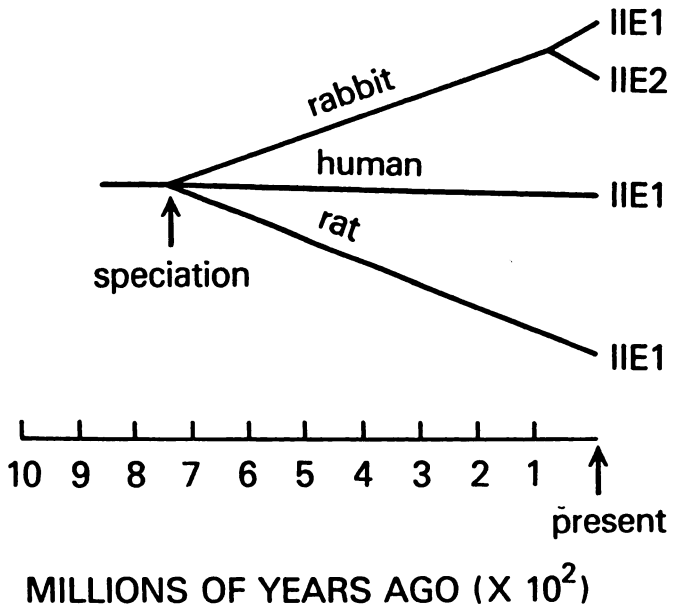

FIG. 2. Evolution of the P450IIE genes in rats, rabbits, and humans. The speciation point is taken from ref. 259, and the approximate time of the gene duplication event in the rabbit subfamily was calculated from the percentage of amino acid similarities of the two rabbit proteins and the UEP of the P450II subfamily.

TABLE 1
List of sequenced P450s

Family/subfamily*	Species trivial name† (ref.)
P450I	
IA1	Man P_1, form 6, mouse P_1, rat c, rabbit form 6, fish P_1
IA2	Man P_3, form 4, mouse P_1, rat d, rabbit form 4
P450II	
IIA1	Rat a (298)
IIA2	Rat IIA2, RLM2 (264)
IIA3	Human P450(1), mouse 15α type I and type II, rat IIA3 (218, 338, 407)
IIB1	Human MP, rat b (276, 413)
IIB2	Rat e (6)
IIB3	Rat IIB3 (245)
IIB4‡	Rabbit LM2, B0, B1, b15, b46, b54 (83, 151, 230, 416)
IIB5‡	Rabbit HP1, B2, b52 (83, 230)
IIB7	Human IIB7§
IIB8	Human IIB8§
IIB9	Mouse pf26 (316)
IIB10	Mouse pf3/46 (316)
IIC1	Rabbit PBc1 (252)
IIC2	Rabbit PBc2, K, pHP2 (171, 252)
IIC3	Rabbit PBc3, 3b (252, 334)
IIC4	Rabbit p1-88, PBc4 (191, 496)
IIC5	Rabbit form 1 (427)
IIC6	Rat PB-1, PB-C, k (101, 213)
IIC7	Rat f (101, 213)
IIC8	Human form 1 (84, 219, 323)
IIC9	Human MP-4 (219, 273, 431, 489)
IIC10	Human MP-8 (84)
IIC11	Rat h, M-1, 2c, 16α (287, 491)
IIC12	Rat i, 2d, 15β (495)
IIC13	Rat g‖
IIC14	Rabbit pHP (171)
IIC15	Rabbit b32-3 (172)
IID1	Rat db1 (103)
IID2	Rat db2 (103)
IID3	Rat IID3§
IID4	Rat IID4§
IID5	Rat IID5§
IID6	Human db1 (110)¶
IID7	Human IID7§
IID8	Human IID8§
IID9	Mouse 16α (474)
IIE1	Human j, rat j, rabbit 3a (212, 404)
IIE2	Rabbit gene 2 (211)
IIF1	Human IIF1§
IIG1	Rat olf1‖
IIH1	Chicken PB15 (159)

TABLE 1—Continued

Family/subfamily*	Species trivial name† (ref.)
P450III	
IIIA1	Rat PCN1 (105)
IIIA2	Rat PCN2 (108)
IIIA3	Human p (288)
IIIA4	Human nf (15, 106)
IIIA5	Human PCN3§
IIIA6	Rabbit 3c (48)
P450IV	
IVA1	Rat LAω (142)
IVA2	Rat IVA2§
IVA3	Rat IVA3‡
IVA4	Rabbit p-2 (262)
IVA5	Rabbit LAω 1‖
IVA6	Rabbit LAω 2‖
IVA7	Rabbit LAω 3‖
IVB1	Rabbit, rat, form 5‖, human IVB1§

* These names have been assigned in Nebert et al. (301, 309). A newer nomenclature system for gene locus symbols has also been established (309).

† The references for IA1 and IA2 are in the text. This list does not include all known trivial names for the various P450s.

‡ Author's laboratory, unpublished data.

§ Some of these P450s could be either allelic variants or very closely related genes. However, they have been considered as allelic variants since they were isolated from outbred animals and their cDNAs share only a few nucleotide differences (309).

‖ Taken from ref. 309.

¶ Referred to in the text as IID1.

each P450 cDNA isolated from several species. For simplicity, the P450I, IIA, IIB, IID, IIE, IIF, and IV genes are named according to the classical nomenclature system, and the names referred to in the individual references are disregarded. The P450s and genes in the P450IIC subfamily and the P450III family are designated by trivial names taken from the individual references. This is because of the complex nature and uncertainty about the orthologous genes in the P450IIC subfamily and P450III family. Table 1 lists the current status of the P450s cloned and sequenced to date and some of their trivial names.

2. The P450I gene family. There are two P450I genes in each species so far examined. The cDNA and genes were isolated, and their sequences have been determined for P450IA1 and P450IA2 in mice (100, 102, 215, 217, 311), rats (156, 206, 401, 402, 482, 483), rabbits (322), and humans (178, 180, 181, 183, 184, 207, 354). The IA1 cDNA sequence has also been determined in fish (150). P450IA genes each contain seven exons and have been localized on mouse chromosome 9 (153, 426), hamster chromosome 4 (155), and human chromosome 15 (154, 184). Unlike the other P450 genes sequenced to date, this family also contains noncoding first exons.

The IA1 and IA2 genes are ubiquitous in mammals, and for the most part these enzymes have similar catalytic activities in several mammalian species. More interestingly, benzo(*a*)pyrene hydroxylase activity, which is usually associated with P450IA1, is present and can be induced in many organisms, ranging from fungi to insects and birds. Direct evidence for the presence of IA2 in insects, fish, birds, and other nonmammalian organisms is, however, lacking.

On the basis of the evolutionary conservation of the IA1 and IA2 genes in mammals and the ubiquitous nature of IA1, it can be suggested tentatively that these enzymes are crucial for survival and may play a key role in the metabolism of critical endogenous substances. It was postulated that IA1 is important at an early stage of

development (214). To date, however, IA1 has not been found to metabolize an endogenous substrate. IA1 actively metabolizes benzo(*a*)pyrene and other substrates (94, 123, 170, 194, 201, 244, 310, 369, 376), while IA2 exhibits a high level of catalytic activity toward many arylamine compounds, including 2-acetylaminofluorine (95, 193, 271), in addition to its ability to metabolize potent promutagens derived from pyrolysates of amino acids and proteins (201, 269, 270). Mouse IA2 also displays aflatoxin B_1-4-hydroxylase activity (67, 236). Both enzymes in the IA family could therefore potentially play very important roles in chemically induced human cancer. In this connection, it is also noteworthy that IA1 is induced by 3-methylcholanthrene in many tissues (216, 371) and by cigarette smoke in humans (403). It has not been clearly established whether IA1 is inducible in human liver even though the enzyme has been found in a single liver specimen (476). The role of IA1 in carcinogenesis has been reviewed (85, 337).

3. The P450IIA gene subfamily. Highly specific polyclonal antibody against rat P450a was used to isolate the IIA1 cDNA from a λgt11 expression library (298). During the course of experiments designed to analyze the regulation of IIA1 mRNA, it was noted that a second mRNA transcribed from a related gene was expressed only in adult male rats in a manner distinct from the expression of IIA1 mRNA (298). A cDNA library constructed from an adult male rat was screened with the IIA1 cDNA, anda second clone, designated IIA2, was isolated (264). The IIA2 protein was also purified (264) and found to correspond to P450 RLM2 (185). IIA2 shared 93% of the nucleotide sequence and 88% of the deduced amino acid sequence of IIA1 cDNA. Analysis of the expression of IIA2 and IIA1 mRNAs using oligonucleotide probes confirmed that they are regulated quite differently during development and after administration of the carcinogen 3-methylcholanthrene (see below). Both genes have been sequenced and shown to contain nine exons (author's laboratory, unpublished data). In contrast to the IA genes, the IIA genes contain a coding first exon.

Although IIA1 and IIA2 proteins demonstrate considerable amino acid sequence similarity, they show distinct positional specificities toward the hydroxylation of the prototype substrate testosterone (264). IIA1 hydroxylates testosterone and other steroids at the 7α position with a minor metabolite resulting from hydroxylation at the 6α position (264, 298, 453, 475). In contrast, IIA2 exhibits a high level of testosterone 15α-hydroxylase activity, and only about 5% of the total hydroxylated metabolites are due to hydroxylation at the 7α position. In addition, a second major metabolite (originally thought to be 7β-hydroxy testosterone; ref. 185) was also produced by IIA2; however, it has not been identified (264). The rat IIA1 and IIA2 are, therefore, differentially regulated and have distinct substrate specificities.

A third gene in the rat IIA subfamily was also recently isolated (218). This P450, designated IIA3, is expressed only in rat lung and is induced by 3-methylcholanthrene. The cDNA-deduced amino acid sequence of IIA3 demonstrates 71% and 73% similarity to the IIA1 and IIA2 proteins, respectively. Using the average UEP of 3.0 calculated for P450s, these data indicate that a single gene duplication event occurred in the IIA subfamily about 75 to 80 million years ago, resulting in the IIA3 gene and the precursor to the IIA1/IIA2 genes, followed by another duplication event approximately 25 million yr ago, which produced the IIA1 and IIA2 genes. These three genes have since diverged to the extent that they are differentially regulated. The IIA gene cluster is located on mouse chromosome 7 (author's laboratory, unpublished data).

Recently, a cDNA corresponding to developmentally regulated testosterone 15α-hydroxylase, designated $P450_{15\alpha}$ subtype I, and a related cDNA, designated type II, were isolated from the mouse (407). Type I mRNA is expressed in the male kidney, whereas type II mRNA is preferentially expressed in female kidneys. Both mRNAs are expressed to a similar extent in male and female livers. This differential regulation strongly suggests the existence of two separate genetic loci for type I and type II P450s as opposed to their being allelic variants. The deduced amino acid sequences of these P450s are 98% similar, indicating that they diverged about 4 to 12 million yr ago. Most interestingly, these mouse cDNAs are in the IIA gene subfamily and show 90% deduced amino acid similarity with IIA3 and only 70% and 75% amino acid similarity with IIA1 and IIA2, respectively. These data indicate that the mouse $P450_{15\alpha}$ type I and type II genes are orthologous to the rat IIA3 gene. This finding is very surprising given that IIA3 is expressed only in the rat lung, while the two mouse cDNAs are expressed in both liver and kidney (407). However, $P450_{15\alpha}$ expression in mouse lung has not been analyzed. Although the enzymatic specificity of rat IIA3 is still unclear, it cannot be ruled out that this P450 is also a testosterone 15α-hydroxylase. Taken together, these data suggest that the IIA gene subfamily has evolved quite differently in rats and mice.

A P450IIA gene subfamily member, designated P450 (1), was the first cDNA cloned and sequenced from humans (338). This subfamily may contain up to three genes having from 85 to 95% amino acid similarity (339). The human IIA gene cluster is localized on an approximately 50-kilobase segment of chromosome 19 at position 19q 13.1–13.3 on the long arm (339, 340). The partially deduced amino acid sequence of P450 (1) is 74% similar to mouse $P450_{15\alpha}$ type I and 60%, 58%, and 75% similar to rat IIA1, IIA2, and IIA3, respectively. A full-length human IIA cDNA was recently isolated, and an identical cDNA was found in cDNA libraries made from three separate livers. Its cDNA-deduced amino acid sequence was 88% similar to the partial sequence of P450

(1), suggesting that this P450 may be derived from a different gene (author's laboratory, unpublished data). Interestingly, this full-length sequence shares 83% of the deduced amino acid sequence of mouse 15α and 69%, 65%, and 85% of the amino acid sequences of rat IIA1, IIA2, and IIA3, respectively. These data indicate that this second human P450IIA gene is orthologous to rat IIA3, even though the rat gene is expressed in lung and the human gene in liver. Further comparisons between the rodent and human IIA gene subfamilies await data on the tissue-specific expression of these genes in humans and the enzymatic characterization of the IIA gene products. In any case, it seems quite possible that the IIA genes have evolved differently in rodents and humans.

4. The P450IIB gene subfamily. The cDNAs for the P450b and P450e in rats were among the first to be isolated and completely sequenced because they are strongly induced by phenobarbital, and specific polyclonal antibodies were available. A detailed review of this early work and a through comparison of the P450b and P450e genes (IIB1 and IIB2, respectively) have been published (1). Briefly, both genes contain nine exons, and only 40 nucleotide differences exist between the two mRNAs (6, 76, 240, 285, 413).

The IIB1 and IIB2 proteins exhibit 97% amino acid similarity (14 substitutions of 491 amino acids) and have distinct chromatographic and electrophoretic properties (123, 356, 369, 455, 470). Furthermore, it was established that these two P450s are encoded by separate genetic loci and are not allelic variants of the same gene from an outbred rat strain (356, 439). These P450s have similar substrate specificities; however, the purified IIB1 has about a 5-fold higher catalytic activity than IIB2 for certain substrates, including benzphetamine (123, 369, 455, 470), and testosterone (453), while only a 2-fold to 3-fold difference is detected with benzo[a]pyrene and 7,12-dimethylbenz[a]anthracene as substrates (470). Interestingly, the positional selectivities of IIB1 and IIB2 for testosterone are similar (453), whereas IIB2 favors 12-methyl hydroxylation while IIB1 favors 7-methyl hydroxylation of 7,12-dimethylbenz[a]anthracene (470). For certain analogues of resorufin, up to a 100-fold higher activity is displayed by IIB1 relative to IIB2 (472). However, for some substrates, these reconstituted activities are dependent on the parameters of the in vitro system [for example, NADPH-P450-oxidoreductase concentration (203, 470) and lipid composition] and may not reflect the activities of these enzymes in microsomes or the intact cell. For instance, IIB1 mediated metabolism of 7,12-dimethylbenz[a]anthracene by lung and liver microsomes is much lower than expected from their content of the enzyme and the activity of the purified proteins (31, 32, 469, 470). In contrast, IIB2 activities in microsomal preparations were comparable to that exhibited by the pure enzyme (31). Finally, as will be discussed below, the IIB1 and IIB2 genes are differentially regulated.

It was first determined several years ago by Southern blotting experiments and the isolation and analysis of genomic clones that the rat IIB subfamily contains multiple genes (7, 284). Sequence data and published restriction maps suggest the presence of greater than six genes with considerable nucleotide similarities (6, 7, 284). It remains unclear, however, whether all of these genes are expressed in the rat.

A third cDNA in the rat IIB subfamily, designated IIB3, was also isolated from a liver cDNA library, and its cDNA-deduced amino acid sequence was only 77% similar to IIB1 and IIB2 (245). Furthermore, the IIB3 nucleotide sequence corresponded to the partial sequence of gene 5 derived from a rat genomic library (7). Unlike the IIB1 and IIB2 genes, IIB3 is constitutively expressed as a minor form in male and female liver and is not induced by phenobarbital. Expression of IIB3 is also not detected in the kidney, lung, and prostate (245).

Several years ago a cDNA that reacted with a 4-kilobase phenobarbital-inducible mRNA was isolated, but this clone was not sequenced (325). More recently it was discovered that the rat IIB1 gene yields two transcripts, the typical 2-kilobase mRNA and a 4.8-kilobase mRNA that results from the use of a downstream polyadenylation site (187). It is still unclear whether a single IIB1 gene gives rise to these two transcripts or if there is a variant allele with an altered polyadenylation or 3′ processing signal that normally produces the 2-kilobase mRNA. The relationship of this 4.8-kilobase mRNA to the previously detected 4-kilobase phenobarbital-inducible transcript and its corresponding cDNA clone is unknown (325).

The complete amino acid sequence of rabbit phenobarbital-inducible P450 form 2, determined by direct protein sequencing, first revealed the presence of the IIB gene subfamily in this species (151, 334, 416). The finding of several amino acid differences between the sequences determined in two laboratories further suggested the presence of either allelic variants or more than a single gene. Several P450s in the IIB subfamily were also identified by cDNA cloning, and these exhibit considerable similarity to P450 form 2 (83, 230). At least five distinct cDNAs were isolated from a phenobarbital-induced rabbit liver library and through amino acid sequence analysis were shown to be more than 95% similar in amino acid sequence (230). This "microheterogeneity" in P450 sequence suggested the possibility of multiple rabbit IIB genes. However, it is conceivable that two or more of these cDNAs are allelic variants in an outbred strain of rabbits, since multiple allelic variants of rat IIB proteins have been identified in outbred rats (356). In any case, these data (230) support the likelihood that rabbits and rats have several closely related genes.

Chromosome mapping studies (394) and the sequence

of a partial cDNA (409) also established the existence of a IIB subfamily in the mouse. The possibility of multiple genes was further suggested by Southern blotting experiments (394). Recently two female-specific mouse cDNAs, designated pf26 and pf46, containing the full amino acid coding regions of P450s in the IIB subfamily were isolated and characterized (316). These two P450s displayed 83% amino acid similarities with each other and 82% amino acid similarity with rat IIB1. One or both of these enzymes could possess testosterone 16α-hydroxylase activity. Based on genetic evidence, using crosses of polymorphic inbred strains of mice, the pf26 P450 mRNA expression was found to be correlated with this activity. Both pf26 and pf46 are polymorphically expressed in mice; the expression of the former was inherited as an autosomal dominant trait, while the latter P450's expression was inherited as a recessive trait (316).

In humans, IIB subfamily cDNAs were isolated by screening liver libraries with rat IIB cDNA probes (276, 339). Two cDNAs were isolated that demonstrated more than 75% amino acid similarity to members of the rat IIB subfamily but less than 60% similarity to other rat P450II subfamilies (276). The locations of the few amino acid substitutions in these two human proteins corresponded to positions of variability between rat IIB1 and IIB2. In addition, an interesting variant cDNA was isolated that was lacking the sequence found in exons 5 and 6 of the gene, suggesting either the presence of a mutant gene or an anomalous splice from a normal gene (276). Others have also isolated an apparently normal and variant cDNA in the human IIB subfamily (339). This variant cDNA lacks the sequence corresponding to exon 8 and retains intron 5. Again, the nature of this defective transcript is unknown. From the available amino acid sequence of the partial cDNAs, the human IIB was found to have 78% amino acid similarity to rat IIB1 (276). The IIB subfamily was localized to human chromosome 19 (276, 377), which is consistent with the chromosome localization of the mouse IIB gene cluster (394). The enzymatic specificity of the human IIB P450s is also unknown. These studies await the isolation and expression of full-length cDNA.

5. The P450IIC gene subfamily. The members of this subfamily of P450s are, in most cases, constitutively expressed. In addition, the IIC P450s are under developmental and sex-specific regulation, and a few are also induced by phenobarbital. Rabbit cDNAs corresponding to IIC subfamily gene products were first identified by differential colony hybridization, sequenced, and found to represent a distinct P450II subfamily (252). Three clones, designated PBc1, PBc2, and PBc3, were characterized, and their deduced amino acid sequences demonstrate 72 to 88% similarity to each other and 50% similarity to rat and rabbit IIB1. The protein corresponding to the PBc1 sequence has not been purified.

Other investigators have used antibodies against the well-characterized rabbit P450 1, an active hepatic progesterone 21-hydroxylase, to isolate a cDNA clone designated p1-8 (427). A second cDNA, designated p1-88, was isolated by cross-hybridization to p1-8 and was found to share 95% of the cDNA deduced amino acid sequence of p1-8 (191). The extensive amino acid similarities between p1-8 and p1-88 and the identical electrophoretic mobilities of the proteins produced in vitro by p1-8- and p1-88-derived mRNAs suggest that either of these two P450s could contribute to progesterone 21-hydroxylase activity. Several lines of evidence indicate, however, that the p1-8 cDNA corresponds to the progesterone 21-hydroxylase or P450 1 (191). First the predominance of protein in the P450 1 preparations containing amino-terminal sequences identical to the deduced cDNA suggests that p1-8 corresponds to the progesterone 21-hydroxylase. Further evidence against the participation of the p1-88 P450 in progesterone hydroxylation was provided by the failure of a specific progesterone 21-hydroxylase-inhibitory monoclonal antibody to bind to protein synthesized in vitro from the p1-88 cDNA and the high level of binding of this antibody to p1-8-derived protein (191). Finally, in two rabbit strains polymorphic for expression of progesterone 21-hydroxylase, the levels of p1-8 mRNA correlated with enzyme activities, whereas the levels of p1-88 mRNA did not. These same types of analysis were also instrumental in establishing that PBc2 (252) encodes P450 K, a rabbit lauric acid ω-1 hydroxylase expressed in the kidney (72, 73). Other investigators have isolated the equivalent of PBc2 and a cDNA that shares 91% of the deduced amino acid sequence of p1-8 (427) from phenobarbital-treated rabbits (172), suggesting that there may be a sixth P450 in the rabbit IIC subfamily. The rabbit P450IIC genes contain nine exons with intron locations identical to those in genes of the rat IIB subfamily (114).

Several constitutively expressed P450s in the rat IIC subfamily have also been isolated and characterized, and their cDNAs have been cloned. Two members of the rat IIC family, designated P450 PB-1 (456) and P450f (368), were isolated and their amino-terminal protein sequences determined (135, 456). PB-1, also referred to as PB-C (452), is developmentally regulated in males and females (101, 452). P450f is also developmentally controlled in a manner similar to PB-1 (11, 101) and has a broad specificity and low catalytic activity toward a number of substrates (368).

Using polyclonal antibody against PB-1, investigators were able to isolate two rat cDNAs with cDNA-deduced amino-terminal sequences identical to those of PB-1 and P450f (75, 101). These cDNAs were isolated by others (213) using a cDNA probe corresponding to another IIC subfamily member, P450 M-1 (491). PB-1 and P450f demonstrate 75% amino acid similarity to each other and are 50% similar to IIB1 and IIB2 (101). The 5′ end of the PB-1 gene was isolated, sequenced, and found to

possess introns that disrupt the coding sequence in identical locations to the rat IIB1 and IIB2 genes (433). Of interest was the finding of an R.dre.1 repetitive sequence (364) in the 3′ noncoding region of the PB-1 mRNA (101). This sequence was previously thought to be expressed exclusively in rat brain RNA (412). Other R.dre.1 sequences were found in the first intron and in DNA upstream of the PB-1 gene (433).

An adult male specific P450 was purified from rats and characterized by several laboratories (29, 123, 202, 263, 291, 368, 447). This P450, designated P450h (368), metabolizes testosterone at the 16α, 2α, and 17 positions. A cDNA, designated M-1, containing the full amino acid coding region of P450h was isolated using a synthetic oligonucleotide generated from a peptide sequence (491). The amino acid sequence of M-1, as deduced from the cDNA, is 70% and 71% similar to the sequence of rat PB-1 and P450f, respectively. Others have also isolated and sequenced the male-specific P450 16α cDNA (287), which is probably the same enzyme as M-1. Expression of the M-1 mRNA is restricted to adult male livers (491); no mRNA was detected in females or in adult male lung, kidney, and testis or in ovary. The M-1 gene spans 35 kilobases of DNA and, like the IIA, IIB, and other IIC genes, contains nine exons (293).

A P450 purified from adult male rats, designated P450cc25, catalyzed both testosterone 16α-hydroxylation and vitamin D_3 25-hydroxylation (149). This enzyme was immunologically indistinguishable from M-1, and both enzymes also had identical amino-terminal sequences (149). When M-1 cDNA was expressed in yeast, however, it catalyzed only the hydroxylation of testosterone (148). These data, together with the low D_3 25-hydroxylase activity exhibited by the P450cc25 preparation, suggest that the preparation may have been contaminated with a distinct P450 vitamin D_3 25-hydroxylase. Alternatively, these data could suggest the presence of another very similar P450 gene (148). However, there is no evidence for a gene with extensive nucleotide similarity to M-1 (293, 491). It remains possible that a polymorphism in a rat M-1 allele accounts for these different activities. For instance, an amino acid difference may alter vitamin D_3 binding to the M-1 P450 active site.

The cDNA for a rat female-specific P450, designated $P450_{15\beta}$ (257), was recently cloned and sequenced (287, 495). The cDNA-deduced amino acid sequence of 15β demonstrated 68%, 68%, and 71% similarity to PB-1, P450f, and M-1, respectively, confirming that it is a member of the P450IIC subfamily. The cDNA sequence of another male-specific P450, designated P450g (135, 268, 368), also revealed that it is a member of the P450IIC family (Table 1).

Several human IIC cDNAs have also been characterized. A human cDNA, named MP-8, was isolated from a liver library (431) using specific polyclonal antibody against $P450_{MP\text{-}1}$ (389). Another cDNA, named Hp1-1, was isolated by cross-hybridization with the rabbit p1-8 cDNA (323). Other investigators have also isolated cDNAs corresponding to Hp1-1 (84, 126, 219). Hp1-1 and MP-8 demonstrate 71% and 75% cDNA-deduced amino acid sequence similarity with rat PB-1 and 77% similarity, respectively, with each other. Another cDNA, designated MP-4, isolated from the same liver library as MP-8, had only 2 base differences in its coding region from that of MP-8, yet had a distinct 3′ untranslated region (84). cDNAs were isolated from other laboratories that were almost identical to MP-4 (219, 273, 489), however, no independent clones were found that corresponded to MP-8. This clone could be derived from a third human IIC gene, or it could be an allelic variant of MP-4; a possibility that remains tentative until a second cDNA, corresponding to MP-8, is isolated.

$P450_{MP\text{-}1}$ protein, corresponding to MP-8 and MP-4, was first purified from human liver and found to have a high catalytic activity for S-mephenytoin 4-hydroxylation (130, 389), hexobarbital 3′-hydroxylation, and tolbutamide methyl hydroxylation (84). A protein corresponding to Hp1-1 was purified and found to be catalytically inactive toward mephenytoin, hexobarbital, and tolbutamide (84), and slightly active in the N-demethylation, of *d*-benzphetamine (481) and aminopyrine (251). Antibody against $P450_{MP\text{-}1}$ (that probably also cross-reacts with the Hp1-protein) inhibits S-nirvanol 4-hydroxylation, S-mephenytoin N-demethylation, and diphenylhydantoin 4-hydroxylation (389); however, the purified $P450_{MP\text{-}1}$ enzyme lacks significant activity toward these latter substrates. Hp1-1 may be the enzyme responsible for catalyzing some of these reactions, but unfortunately enzyme preparations corresponding to Hp1-1 were not analyzed for S-nirvanol 4-hydroxylation, S-mephenytoin demethylase, or diphenylhydantoin 4-hydroxylase activities. The question of substrate specificities of the IIC P450s will be resolved when the full genetic complement of human IIC cDNAs is cloned and expressed into active protein.

6. The P450IID gene subfamily. The debrisoquine 4-hydroxylase P450, named db1 (IID1), was purified from rat (103, 250) and human (56, 129) liver. This enzyme is noted for its polymorphic expression in humans (168, 260) and rats (3, 199). Antibodies and cDNA probes have been used to study the molecular basis of the human and rat drug oxidation defect.

The rat db1 was purified, and antibodies were generated and used to isolate the IID1 cDNA (103). A second cDNA, db2 (IID2), was also isolated and found to share a 78% cDNA-deduced amino acid sequence of IID1. Both IID subfamily members exhibited between 38% and 43% amino acid similarity with IIA1, IIB1, PB-1, and IIE1. In contrast, the other subfamilies, IIA, IIB, IIC, and IID, had between 45% and 55% amino acid similarity (103). These similarities revealed that the IID subfamily is a

unique lineage within the P450II family that diverged earlier than the other four subfamilies 600 million yr ago (fig. 1).

Four rat IID genes have been isolated and completely sequenced (author's laboratory, unpublished data). The calculated deduced amino acid sequence similarities of these genes ranged from 73 to 80%. Interestingly, the gene coding for IID1 was not isolated; however, a gene possessing 95% deduced amino acid sequence similarity was uncovered that had apparently diverged recently from the IID1 gene. This gene was designated IID5, and its cDNA has also been characterized (author's laboratory, unpublished data). The four rat genes each contain nine exons, span about 5 kilobases, and are arranged head to tail on a 60-kilobase segment of DNA. IID1, IID2, IID3, and IID5 are expressed in the liver and kidney, but only low level expression of the IID4 gene could be detected. This latter gene appears to be normal, suggesting that it may be expressed at a low level in liver or expressed in extrahepatic tissues. None of the rat IID genes were expressed in the lung or intestine.

By immunochemical screening, others isolated a cDNA, designated P450 UT-H, that presumably corresponds to IID1 (35). Because no sequence data are available, the relationship between this cDNA and IID1 (103) cannot be firmly established. Since the five rat IID P450s demonstrate a considerable degree of amino acid similarity, it is probable that polyclonal antibody against IID1 will cross-react with the other four proteins. In light of this possibility, the UT-H clone could correspond to any of the IID P450s expressed in the liver.

A full-length cDNA for human IID1 was isolated from a λgt11 library using anti-rat IID1 antibody (110). The human enzyme shared 46%, 44%, and 41% amino acid similarity with human IIA3 (338), MP-8 (431), and IIE1 (404), respectively. Interestingly, human IID1 was not more similar to rat IID1 than rat IID2, IID3, IID4, and IID5 (author's laboratory, unpublished data). This result is surprising, because only human and rat IID1 exhibit debrisoquine 4-hydroxylase activity. Three separate liver libraries were screened, and only cDNAs equivalent to IID1 were isolated, suggesting that no other members of the IID subfamily are expressed in human liver. Other variant RNAs derived from livers deficient in debrisoquine metabolism were isolated and sequenced (107). These results will be discussed below.

Even though only a single cDNA was found in the liver, three related IID genes were detected in the human genome (author's laboratory, unpublished data). In contrast to the rat, in which four genes demonstrated only 73 to 95% deduced amino acid similarity, humans possess three genes with 89 to 95% deduced amino acid similarity. Sequence similarities among the rat IID genes indicate the IID gene cluster was formed 90 to 120 million yr ago. Since this period was prior to the rat and human divergence about 75 million yr ago (259), both species probably originally possessed four genes. Rats recently formed another gene via a gene duplication, whereas humans may have lost a gene through mutational events. The high sequence similarities among the three human genes may have been due to gene conversion events. Evolution of the IID subfamily in humans and rats is summarized in fig. 3.

Even between rodents as similar as rats and mice which diverged 20 million yr ago (391), the IID genes can produce proteins that are enzymatically quite dissimilar. Recently, a cDNA for mouse male-specific testosterone 16α-hydroxylase (C-P-$450_{16\alpha}$) was isolated and sequenced (474), and its deduced amino acid sequence is 82%, 72%, 78%, and 70% similar to rat IID1, IID2, IID3, and IID4, respectively. Interestingly, enzyme reconstitution and antibody inhibition studies suggest that the rat IID gene products do not metabolize testosterone (author's laboratory, unpublished data). Furthermore, none of the rat IID genes are male specific (author's laboratory, unpublished data), suggesting that rat and mouse P450s have evolved distinct metabolic capacities and regulatory controls. The mouse, like the rat, may have up to five genes (474).

In summary, the IID gene subfamily has evolved differently in humans and rodents. This was probably due to differences in food consumption between species. For instance, the diet of humans may have become more

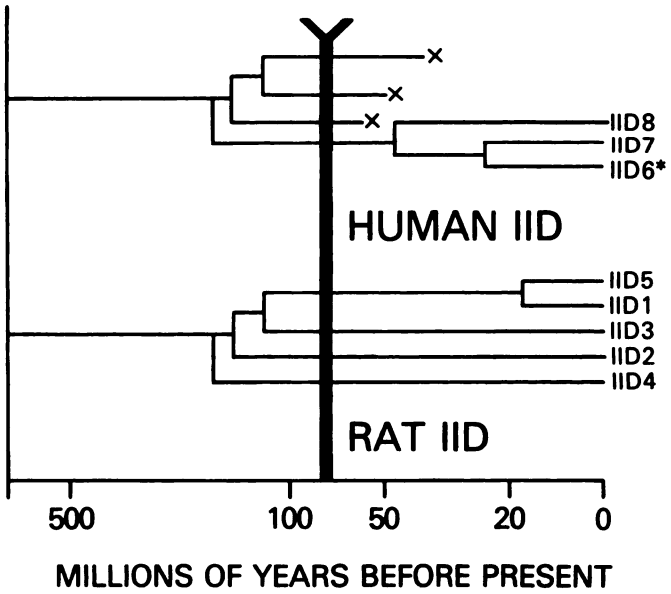

FIG. 3. Evolution of the IID subfamily in humans and rats. Based on the speciation period of rodent and human lines (about 75 million yr ago, ■) and the amino acid similarities of the rat and human P450s, it appears that four genes existed in the ancestor to humans and rats. Subsequent to the formation of these two species, three genes were lost in humans, and an additional two genes were formed more recently by gene duplication events. In rats, however, four "old genes" were maintained and one newer gene was recently formed, about 20 million yr ago. This indicates that both gene extinction and gene formation played a role in the evolution of the IID subfamily. *Referred to in the text as IID1.

restricted than rodents, resulting in lower intake of plant toxins. In the absence of selective pressure for maintenance of the detoxification enzymes, the IID genes may have started to accumulate mutations. In fact, detrimental mutations have been uncovered in all three human IID genes (author's laboratory, unpublished data).

7. The P450IIE gene subfamily. A distinct, ethanol-inducible form of P450, named IIE1, was first identified in rabbits (233, 234) and later in rats (336, 367) and humans (479, 480). This enzyme can metabolize substrates such as ethanol (234), acetone, acetoacetate, and acetol (232), diethyl ether (24), *p*-nitrophenol (231), halothane (118), benzene (190), pyridine (212a), and N-nitrosodimethylamine (162, 336, 418, 479). Its activity toward acetone and acetol oxidation suggests that IIE1 is involved in the pathway of gluconeogenesis during the fasting state (26).

The rat (404), human (404), and rabbit (212) IIE1 cDNAs have been isolated and sequenced, and their deduced amino acid sequences are about 80% similar. Only a single IIE gene has been identified in rats and humans (404, 434), whereas two highly homologous genes exist in the rabbit (210, 211). The second rabbit IIE gene, designated IIE2, was first identified through genomic cloning, and its deduced amino acid sequence is 97% similar to that of rabbit IIE1. Both genes are expressed in liver; however, only IIE1 was expressed in the kidney (211). These data indicate that the rabbit IIE1 gene recently duplicated, giving rise to two IIE subfamily members in the rabbit, is in contrast to the single IIE1 gene in humans and rats (404, 434). The two rabbit genes (211), the rat gene (434), and the human gene (432) all contain nine exons and span about 10 kilobases. The splice junctions occur at the same position in the mRNA coding region as other P450II genes sequenced to date.

8. The P450IIH gene subfamily. A cDNA for a phenobarbital- and alkylisopropylacetamide-inducible P450 was isolated from chickens (159) and designated PB15. This P450 was previously assigned to the IIC subfamily (301) based on its 57% similarity to rat IIC6. However, recent pairwise computer alignment data (312) have revealed much less similarity of PB15 to the known IIC sequences; it was therefore assigned to a new subfamily (309). Analysis of genomic DNA and genomic clones indicated the presence of two genes in the chicken IIH family.

9. The P450III gene family. A P450 that can be induced by the synthetic steroid pregnenolone 16α-carbonitrile (PCN) was identified (255), subsequently purified from rat liver, and shown to be unique from several other P450s (65, 152). Immunoinhibition and immunoquantitation studies have established that the enzymes in the P450III family, including P450p (65), P450PCN-E (123, 452), P450 3c (235), and P450 PB-2a (447), have catalytic activities for ethylmorphine N-demethylation (478), erythromycin demethylation (478), triacetyloleandomycin metabolism (478), midazolam (66), S-mephenytoin (388), and testosterone 6β-hydroxylation (108, 451, 452). As noted below, PCN-E, PCN2, and PB-2a are probably equivalent. Interestingly, in contrast to the rat (388), the human counterpart of rat P450PCN1 does not participate in the hydroxylation of S-mephenytoin (U. A. Meyer and author's laboratory, unpublished results). Antibody against a purified human P450 related to the rat enzyme also inhibits metabolism of the calcium channel-blocking drug nifedipine, demethylation of benzphetamine, 2- and 4-hydroxylation of 17β-estradiol, and aldrin epoxidation (124). Polyclonal antibody against rat P450PCN1 inhibited metabolism of the cyclic peptide immunosuppressant drug cyclosporine in human liver (237). Furthermore, the levels of a protein corresponding to rat P450PCN detected in several human liver specimens by Western blot analysis correlated with microsomal cyclosporine metabolism (237).

It is noteworthy that most of the enzyme activity data associated with rat and human P450s in the P450III family have been inferred from activity immunoinhibition data and data correlating an activity with levels of immunodetectable protein on Western blots (see above). The reason for this is that reconstitution of enzyme activity from purified preparations of P450III enzymes has not been successful. Typical reconstitution experiments rely on the use of commercial lipid preparations such as dilaurylphosphatidylcholine (DPLC) to reconstitute P450 with NADPH-P450-oxidoreductase and cytochrome b_5. Recently, it was shown that when natural lipids, extracted from microsomes, were used in the reconstitution assay, a 12-fold increase in PCN2-catalyzed propoxycoumarine O-depropylation activity was observed over the activity obtained with the DPLC lipids (485a). In another study, a mixture of lecithin, sodium cholate, and phosphatidylserine was found to stimulate reconstituted activity of PCN2 relative to DPLC-mediated reconstitution (173a). This phenomenon may be due to the presence of a lipophilic component in the reconstitution mix that is required for activity of PCN2 (173a, 485a). Other P450s, however, are not sensitive to the lipid environment, yet this finding portends caution when interpreting P450 reconstitution activity measurements.

A cDNA to rat P450PCN1 was isolated from a library constructed from polysome-immunoenriched mRNA (141). The complete amino acid sequence of full-length PCN1 was determined from this clone, establishing it as a member of a separate gene family (108). Another cDNA, apparently identical to PCN1, was isolated from a library constructed from dexamethasone-induced rats (478). A cDNA to a second distinct rat P450, designated PCN2, was isolated from an untreated adult male liver library using the PCN1 cDNA as a probe (108). PCN2 shared 90% of the nucleotide sequence and 89% of the cDNA-deduced amino acid sequence of PCN1. Evidence

for recent gene conversions was also uncovered by comparing the PCN1 and PCN2 cDNA sequence (see below). Correlation of PCN1 and PCN2 mRNA levels, measured using specific oligonucleotide probes, with protein and enzyme activity level suggests that both P450s possess testosterone 6β-hydroxylase activity (108). PCN2 has also been purified from untreated adult male rats (133) and appears to correspond to P450 2a (447) and PCN-E (123, 452).

Using a rat cDNA corresponding to PCN1, the rabbit P450 3c cDNA, a P450 constitutively expressed in liver (235), was isolated (47) and sequenced (48). The cDNA-deduced amino acid sequence of 3c was 70% and 67% similar to rat PCN1 and PCN2, respectively, and 75% and 73% similar to two human P450III cDNA-deduced sequences (15, 106, 404). The fact that the rabbit 3c is constitutively expressed argues that it may be the homologue of rat PCN2 and not rat PCN1, since the latter enzyme is not expressed in untreated animals. It should be noted, however, that the rabbit and rat lines diverged about 75 million yr ago (259) and that the rat PCN1 and PCN2 genes diverged about 22 to 41 million yr ago (108). This gene duplication may, therefore, have occurred in rats but not in rabbits. In fact, there is no evidence for a second steroid-inducible rabbit P450III gene (48).

Humans also have at least three P450III genes. $P450_{NF}$, designated NF 25 (15), and P450p (288) cDNAs were isolated from the same cDNA library and sequenced, and their deduced amino acid sequences were 98% similar. The high amino acid similarity of these two enzymes suggested that they could be allelic variants since it was recommended that P450s displaying greater than 98% amino acid sequence similarity should be considered allelic variants unless proven otherwise (301, 309). However, further sequencing of the full coding region of another cDNA corresponding to $P450_{NF}$, isolated from a library derived from a separate human liver, revealed several base changes between the 3′ noncoding regions of the P450p and $P450_{NF}$ mRNAs, including a 19-base deletion in P450p (106). Even though the amino acid coding regions of these two P450s are only 2% different, the multiple differences in their mRNA's 3′ noncoding sequence would indicate that P450p and $P450_{NF}$ are derived from separate genetic loci (106). However, it is not possible to rule out that they are allelic variants. The high degree of similarity between these genes suggests that they diverged less than 6 million yr ago and therefore would not be found in rats or rabbits. Another cDNA clone, designated NF 10, was isolated that corresponds to a second transcript of the $P450_{NF}$ gene (22a). Oligonucleotides specific to the 3′ noncoding region of NF 10 detected a 3-kilobase mRNA that results from the alternative use of a second polyadenylation signal about 800 basepairs downstream of that utilized to produce the 2-kilobase $P450_{NF}$ mRNA. This study also established that the $P450_{NF}$ mRNA was about 8-fold more abundant than that of P450p mRNA in several human liver samples (22a).

A third P450III gene product was first identified in humans by Western blot analysis of human liver microsomes with anti-rat P450PCN1 (author's laboratory, unpublished data). This protein is expressed in about 10% to 20% of the liver samples examined and has a mobility on denaturing polyacrylamide gels that is slightly lower than P450p and $P450_{NF}$. The cDNA to this enzyme, designated hPCN3, was isolated from a library constructed from a liver that only expressed the lower mobility protein. The amino acid sequence of hPCN3 deduced from the cDNA was 90% similar to P450p and $P450_{NF}$. Based upon amino-terminal protein sequence similarities, hPCN3 could correspond to the P450 expressed in fetal liver (221–223, 477) which has not yet been conclusively identified at the cDNA level.

To date, the structures of the P450III genes from rats, humans, and rabbits have not been published. The P450III gene cluster has, however, been localized to mouse chromosome 6 (395) and human chromosome 7 (25, 106).

10. The P450IV gene family. A clofibrate-inducible lauric acid ω-hydroxylase, named P450IVA1, was first purified from rats (415). This enzyme is very specific for fatty acid hydroxylations and has no detectable activity toward other substrates such as benzyphetamine, ethoxyresorufin, and testosterone. The IVA1 can also be induced by other hypolipidemic agents and is active in the oxidation of arachidonic acid (9).

Using a polyclonal antibody against purified rat IVA1, a full-length rat cDNA was isolated and sequenced (142). Since the cDNA-deduced amino acid sequence exhibited less than 35% similarity with all other P450s, it was designated as a separate gene family. The IVA1 gene is thought to have diverged early in evolution, about 1.3 billion yr ago (306, 312).

Western blot analysis using the anti-IVA1 antibody revealed two proteins with molecular weights of 51,500 and 52,000; the former represents the purified IVA1 lauric acid ω-hydroxylase. The cDNA expressed in yeast produced an enzyme that comigrated with the M_r 51,500 protein on denaturing polyacrylamide gels (142). The nature of the second immunoreactive protein is presently not known, but it may represent a second member of the P450 gene family. A second cDNA has been isolated, completely sequenced, and found to encode a P450, named IVA3, with 72% cDNA-deduced amino acid similarity with IVA1 (author's laboratory, unpublished data). It is unclear whether this protein corresponds to the M_r 52,000 protein that reacts with antibody to IVA1.

The rat IVA1 gene and a second gene, designated IVA2, were isolated, sequenced, and found to possess 13 and 12 exons, respectively (author's laboratory, unpublished data). Interestingly, this gene lacked a TATA box sequence upstream of the transcription start site. The

TATA box is found in virtually all RNA polymerase II-transcribed genes. The large number of exons in the IVA1 and IVA2 genes supports the notion that older genes possess more introns and that intron loss, not intron insertion, occurred during evolution (92). A portion of a third gene was found that is highly similar to IVA1; however, it is not known whether this gene is expressed, since its corresponding cDNA clones have not been isolated from the liver libraries.

A prostaglandin ω-hydroxylase was isolated from lungs of pregnant rabbits (466) and rabbits treated with progesterone (485). The cDNA for this enzyme was isolated and sequenced (262), and the cDNA-deduced amino acid sequence was found to exhibit 72% similarity with either rat IVA1 or IVA2. Southern blot analysis suggests that the rabbit may also have two or more P450IV genes. In fact, other fatty acid hydroxylases, apparently distinct from the lung enzyme, have been isolated from rabbit kidney (173), large colon (200), and placenta (484). P450 mRNA that hybridizes to the IVA1 or IVA2 probe is not significantly expressed in pregnant rat lung, suggesting that expression of a P450IVA prostaglandin ω-hydroxylase is specific to pregnant rabbits (author's laboratory, unpublished data). Since the lung ω-hydroxylase appears to be species specific, the physiological significance of this enzyme in lung prostaglandin metabolism remains questionable. Another enzyme unrelated to the P450IV gene family may carry out prostaglandin ω-hydroxylation in the rat lung. For instance, a member of the rabbit P450I gene family has low but significant prostaglandin hydroxylase activity (160).

11. Chromosome localization of mouse and human P450 genes. Many P450 genes diverged prior to the formation of discrete chromosomes, resulting in the dispersal of loci over several mouse and human chromosomes. Three techniques have been used to map the chromosome location of P450 genes. The most direct method is in situ localization of P450 genes on chromosome spreads. Chromosomes are karyotyped by simple histological examination and then are hybridized with radiolabeled cDNA probes. This procedure was used to map the location of the human IIA genetic locus (340). Recombinant inbred mice have also been valuable in mapping P450 genes. Using brother–sister matings over a period of more than 20 generations, chromosomal loci that contain copies of either the original mother or father gene at a particular locus are fixed. Restriction fragment length polymorphisms (RFLPs) between the two parental strains are found with the probe of interest, and this RFLP is used to screen the recombinant inbred DNAs. Tables have been constructed on data generated from mapping certain lines of recombinant inbred mice, and these are used to map new genetic loci. This procedure has been used to map the chromosome location of the mouse I (153), IIB (394), and IIC (274, 471) genes.

The most frequently used method of mapping genes is with somatic cell hybrids. Lines of mouse-hamster cells are established, and each line is vigorously karyotyped and assayed for the presence of specific mouse chromosomes. Each line, in essence, has a subset of mouse chromosomes. Probes are then used to analyze the presence or absence of specific mouse sequences in each cell line, and the results are tabulated and correlated with the presence or absence of specific chromosomes. Likewise, human-rodent cell lines have been used to determine the location of human genes.

A number of P450 genes have been mapped using the somatic cell hybrids. A list of the mouse and human genes that have been mapped to date and of their chromosome location is given in table 2. All gene families map to different chromosomes. Even within the P450II family, three subfamilies are on mouse chromosome 7, while the IIC and IID subfamily genes are located on chromosomes 19 and 15, respectively. The accumulation of large numbers of mapped loci in mice and humans has allowed the generation of tables that show the conservation of discrete regions of chromosomes. For instance, groups of genes found on chromosome 7 in mice are found on a region of chromosome 19 in humans. Human chromosome 19 is, however, composed of segments of several mouse chromosomes. This phenomenon of conservation of closely linked loci allows more refined mapping to distinct regions of the human chromosome.

The use of somatic cell hybrids containing fragments of human and mouse chromosomes also yields mapping data that more precisely localize the genes. This approach was used to localize the IA genes to the middle of mouse chromosome 9 (153), IIB genes to both human chromosome 19 in the cen-q13.3 region (276, 377) and mouse chromosome 7 (394), the IID locus to a region of human chromosome 22 distal to the *sis* oncogene (110) and to mouse chromosome 15 (103), and P450III genes

TABLE 2
Chromosome locations of P450 genes

Gene family or subfamily	Locus* symbol	Chromosome (ref.)	
		Human	Mouse
I	*CYP1A*	15 (153)	9 (154, 426)
IIA	*CYP2A*	19 (340)	7 (218)
IIB	*CYP2B*	19 (377, 276)	7 (394)
IIC	*CYP2C*	10 (323)	19 (274)
IID	*CYP2D*	22 (110)	15 (103)
IIE	*CYP2E*	10 (432)	7 (434)
III	*CYP3A*	7 (25, 106)	6 (395)
IV	*CYP4A*	1‡	4‡
XIA	*CYP11A*	15 (34)	ND§
XIB	*CYP11B*	8 (33)	ND
XVII	*CYP17A*	10 (265)	ND
XIX	*CYP19A*	15 (28)	ND
XXI	*CYP21A*	6 (460)	17 (459)

* Locus symbol was designated by Nebert *et al.* (309).
† F. Gonzalez and C. Kozak, unpublished results.
‡ F. Gonzalez and O. W. McBride, unpublished results.
§ ND, not determined.

to mouse chromosome 6 (395) and human chromosome 7 (106).

Genes can also be more precisely mapped using mouse recombinant inbred mapping and using large human pedigrees. The human P450III gene family was mapped to the 7q22 region of chromosome 7 by screening four informative CEPH (Centre d'Etude du Polymorphisme Humain) families that had been typed for several markers on chromosome 7 (25). Like the mouse recombinant inbred lines, the more gene loci that have been mapped within a given pedigree the more precise localizations can be obtained. This subchromosomal mapping can be useful from both an evolutionary standpoint and also to determine linkage or association of a P450 gene to human genetic disease.

C. Conserved Domains in P450 Proteins

1. Signal sequence and halt-transfer sequence for membrane insertion. The membrane environment is quite suitable for P450s, because many P450 substrates are hydrophobic and dissolve in the membrane lipid bilayers. The membrane also allows interactions with other P450s and the electron donor enzyme NADPH-P450-oxidoreductase (for the microsomal enzyme) or adrenodoxin and adrenodoxin reductase (for the mitochondrial enzymes). Highly specific cellular mechanisms allow the insertion of P450s and other intrinsic membrane proteins into the lipid bilayer. The microsomal P450s are inserted by the signal sequence recognition system, which involves the signal recognition particle and docking protein (reviewed in ref. 21, 370, and 463). Early studies with secretory and plasma membrane proteins established the participation of amino-terminal hydrophobic segments called "signal sequences" which direct the insertion of the proteins into the membrane or the lumen of the endoplasmic reticulum. The signal sequence is cleaved in nearly all secretory products and most membrane proteins during their translation and membrane insertion.

The P450s and other components of the microsomal mixed-function monooxygenase system were found to be synthesized primarily on membrane-bound polyribosomes (98, 99). This binding and insertion into the membrane are mediated by the hydrophobic amino-terminal sequence of these proteins. A form of P450 was one of the first intrinsic endoplasmic reticulum-bound proteins to be found inserted into membrane via a noncleavable signal sequence (12).

The amino terminus of all eucaryote P450s contains an abundance of hydrophobic residues. An acidic residue that is near the initiation methionine is followed by about 14 to 20 residues of hydrophobic amino acids, and then by several basic residues. It was suggested that the combined hydrophobic and basic segment probably serves as the "halt-transfer" signal (370). Recent studies have further established that the first amino-terminal 20 residues of P450IIB1 function as a combined insertion-halt-transfer signal; however, the following charged residues can be dispensed (289). A second halt-transfer-signal in IIB1 was located between residues 167 and 185. This segment could also serve as a membrane-anchoring domain (289). These and other studies suggest that P450s are anchored to the membrane via their amino terminii and that the bulk of the enzyme is exposed to the cytoplasmic side of the endoplasmic reticulum (71, 289, 372, 414). This orientation is also supported by immunological studies in which membrane topology was investigated using antibodies to defined peptides located throughout the IIB1 primary sequence (52). Hydropathy profile comparisons of 34 P450 sequences also allowed the identification of the transmembrane peptides within the first 66 residues of the NH_2 terminii (313).

The mitochondrial P450s are synthesized and transferred into membranes by a mechanism quite distinct from the insertion process for the microsomal enzymes. They are synthesized on membrane-free polysomes (297) and released into the cytoplasm with an amino-terminal peptide that is subsequently cleaved upon insertion into the mitochondria (378). The length of the cleavable "extrapeptide" ranges from 37 to 56 amino acids, and it was suggested that a conserved portion of this peptide, notably a periodic arginine and lysine distribution, serves to transport the P450s across the outer membrane and into the mitochondrial compartment where they are then bound to the inner mitochondrial membrane (294). Microsomal and mitochondrial P450s have, therefore, evolved distinct sequences, common to some other cellular proteins, that are required for their insertion into specific intracellular compartments.

2. Heme-binding cysteine-containing peptide. An excellent summary of the experiments leading to the conclusive identification of the fifth ligand to the heme at the active site of all P450s has been published (112). Briefly, early studies established the involvement of the thiolate group of cysteine (127, 461). Two conserved cysteine-containing regions, designated HR1 and HR2, near the N-terminal half and the C-terminal half of the P450, respectively, were identified as being highly conserved (113) when mammalian (76, 151) and bacterial (134) P450s sequences were compared. Further evidence suggesting the C-terminal cysteine as the fifth ligand bound to the heme iron was provided by comparisons of other P450 sequences, most notably those of the mitochondrial XIA1 (294) and microsomal PCN1 (105). These enzymes did not contain a cysteine equivalent to the N-terminal HR1, whereas the C-terminal cysteine remained significantly similar when all P450 sequences were compared. Finally, the crystal structure of the bacterial $P450_{cam}$ unequivocally established this latter cysteine as the heme-binding thiolate ligand (353). Conserved residues, identified by comparing the sequences derived from nine species and all gene families, are shown in fig. 4. This conserved sequence can effectively serve as a fingerprint for a P450 protein. Further analysis of this region by

		-7			-4	-2		0		+2		+4		+6		+8		+10			+13					+18
Bacteria	CIA1	F	G	H	G	S	H	L	C	L	G	Q	H	L	A	R	R	E	I	I	V	T	L	K	E	W	L
Yeast	LIA1	F	G	G	G	R	H	R	C	I	G	E	H	F	A	Y	C	Q	L	G	V	L	L	M	S	I	F
Fish	IA1	F	G	M	D	K	R	R	C	I	G	E	A	I	G	R	N	E	V	F	L	F	L	A	I	L	L
Chicken	IIH1	F	S	A	G	K	R	I	C	A	G	E	G	L	A	R	M	E	I	F	L	F	L	T	S	I	L
Cow	XIA1	F	G	W	G	V	R	Q	C	V	G	R	R	I	A	E	L	E	M	T	L	F	L	I	H	I	L
Pig	IVIIA1	F	G	A	G	P	R	S	C	V	G	E	M	L	A	R	Q	E	L	F	L	F	T	A	G	L	L
Rabbit	IIB	F	S	L	G	K	R	I	C	L	G	E	G	I	A	R	T	E	L	F	L	F	F	T	T	I	L
Rat	IIIA1	F	G	N	G	P	R	N	C	I	G	M	R	F	A	L	M	N	M	K	L	A	L	T	K	V	L
Rat	IVA1	F	S	G	G	A	R	N	C	I	G	K	Q	F	A	M	S	E	M	K	V	I	V	A	L	T	L
Mouse	IA2	F	G	L	G	K	R	R	C	I	G	E	I	P	A	K	W	E	V	F	L	F	L	A	I	L	L
Man	XXIA1	F	G	C	G	A	R	V	C	L	G	E	P	V	A	R	L	E	L	F	V	L	L	T	R	L	L
Man	XIXA1	F	G	F	G	P	R	G	C	A	G	K	Y	I	A	M	V	M	M	K	A	I	L	V	T	L	L

FIG. 4. Alignment of amino acid sequences surrounding the cysteine, designated 0, the fifth ligand to the heme iron at the enzymes' active site. Conserved residues are surrounded by *boxes*. *Shaded boxes* are totally conserved in all P450s examined to date.

site-directed mutagenesis and cDNA-directed expression is discussed below (390).

3. Other conserved regions. Other conserved regions have been identified, but their functional significance is unknown. These include an N-terminal proline-rich region, Proline Proline Glycine Proline, that may serve to join the membrane-binding N-terminus to the globular region of the P450 (20, 112). This sequence is absent in the P450IV family, however (ref. 142; author's laboratory, unpublished data). A tridecapeptide identified several years ago (151) was, in fact, found to be not well conserved when multiple P450 sequences were compared, except for the presence of two charged residues (112). Other conserved residues have been noted after comparison of multiple P450 sequences, including a glycine at position 95, a glycine/proline at position 86, and a tryptophan at position 150 (112). Functionally, the only other conserved region assumed to exist in the P450 is the NADPH-P450-oxidoreductase (or adrenodoxin) binding site. To date, however, this has not been identified.

4. P450 membrane association models and predictions. The topological association of a P450 molecule with the membrane has been predicted by comparative sequence analysis, including hydropathy plots, of multiple mammalian P450 proteins and extrapolations from the crystal structure of the soluble bacterial $P450_{CAM}$ (112, 313). Several structural and biochemical features are probably common to the mammalian microsomal P450s and are taken into consideration in the design of these models: (*a*) P450s are anchored to the lipid bilayer by a hydrophobic amino terminus; (*b*) they interact with NADPH-P450-oxidoreductase, an enzyme that is clearly exposed to the cytoplasmic face of the membrane; (*c*) they react with many hydrophobic substrates, and many of the products of P450-mediated reactions are then substrates for other microsomal enzymes such as the UDP-glucuronosyltransferases; and (*d*) the heme is either parallel or at a slight angle to the membrane surface.

D. Convergent or Divergent Evolution: The Role of Gene Conversion Events

As illustrated in fig. 5, during evolution genes can duplicate and form a gene family with, for instance, two genes. Speciation can occur to form two lines, each of which contains two genes. These genes can continue to diverge and become less and less similar to each other. This type of evolution is called divergent evolution. In addition, a member of a gene family that no longer offers a selective advantage can begin to accumulate mutations, as illustrated in fig. 5, species 1. Random base changes, deletions, and insertions can then occur so that the gene in effect disappears and is no longer recognizable, e.g., by hybridization with a probe to gene A1. Alternatively, genes can become more similar to each other through convergent evolution. In fact, evidence for the latter phenomenon has been found by comparing the same gene families in different species (163). In the case of convergent evolution, members of a multigene family in a given species are more similar to each other than to the members of the same gene family in another species, even though divergence is occurring at similar rates in both species. The primary mechanism for this type of evolution is gene conversion.

Analysis of two or more cDNA and genomic sequences of P450s within a single gene family or subfamily has revealed regions of high nucleotide similarity interspersed with regions of low similarity. The most likely mechanism for this phenomenon is gene conversion (163). A conversion event is a nonreciprocal recombination event in which a segment of one gene replaces the corresponding segment of a related gene (10). The net result of gene conversion is that two genes become more similar to each other. This is illustrated in fig. 5, in species 2 in which one of the genes donates the region around its second exon to the second gene. Evidence for gene conversion has been found in several other gene families, including gamma crystallin genes (272), human

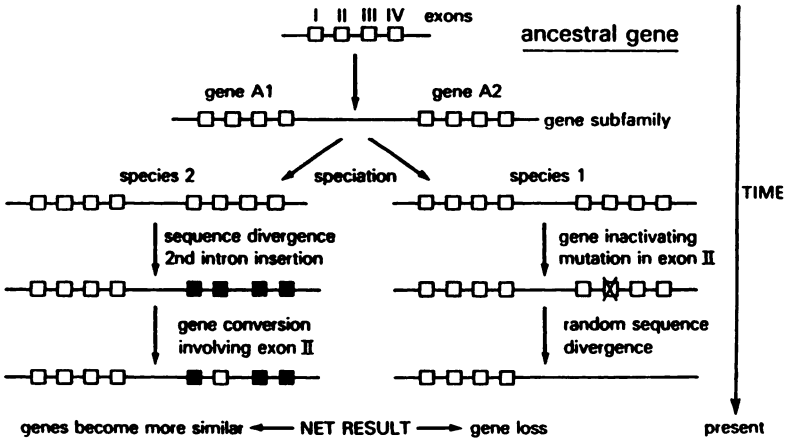

FIG. 5. Scheme depicting evolution of a P450 gene subfamily in two species. Gene conversion and gene loss are illustrated for species 1 and species 2, respectively.

T-cell antigen receptor beta-chain genes (428), hemoglobin genes (39), chorion genes (363), interferon genes (423), major histocompatibility class I genes (224, 365), and oxytocin-vasopressin (366).

Gene conversion in the P450 gene superfamily was first observed in studies of the rat P450IIB subfamily (7). As noted earlier, the IIB1 and IIB2 genes are 97% similar in nucleotide sequence; however, the distribution of dissimilar sequences is not random. Short areas of dissimilarity exist in two discrete regions of the mRNAs suggesting gene conversion (1). Evidence for gene conversion was more firmly established in the rat IIB subfamily by examining gene sequences (7). Most notably it was found that intron sequences were involved in the conversions. A segment encompassing parts of exons 7 and 8 and the entire intron 7 of the IIB1 gene and a second IIB gene were found to be unusually highly similar, suggesting that gene conversion had occurred (7). Furthermore a segment of the latter gene transferred via gene conversion to IIB2 could account for the region in exon 8 of IIB1 and IIB2 which is highly divergent. Another conversion event between the IIB1 and IIB2 genes was also postulated, encompassing the region from exons 2 through 5 and including all introns in this region (7).

Comparison of the rat PCN1 and PCN2 cDNA sequences also revealed recent gene conversion events (108). Three areas of complete nucleotide similarity were found to be interspersed with areas of dissimilarity. A region of 425 nucleotides without a single base difference was surrounded by a sequence demonstrating only 87% similarity. Despite the existence of these similar and dissimilar areas in PCN1 and PCN2, both proteins apparently possess testosterone 6β-hydroxylase activity (108).

There is evidence of gene conversion even in the P450I gene family (1). Regions in the second exons of the rat (401, 402) and mouse (215) IA1 and IA2 genes, spanning 350 and 420 base pairs, respectively, exhibit very high levels of nucleotide similarity, whereas the remaining 3′ portions of these genes are only 70% similar. It is not possible to tell whether these gene conversions occurred prior to or after the rat and mouse speciation 20 million yr ago.

Four genes in the rat IID gene cluster have been completely sequenced and are arranged head to tail on a 60-kilobase segment of DNA (author's laboratory, unpublished data). Multiple gene conversions were identified, including a segment of DNA that lies outside the coding region of the genes. Most of the conversions encompass the seventh, eighth, and ninth exons, which include the conserved cysteine-binding region. In addition, careful analysis of the genes revealed that multiple sequential gene conversions occurred among at least three of the genes.

Taken together, gene conversions seem to have played a major role in P450 gene evolution. Either recombinational conversion events occur frequently and many are fixed in the P450 genes by natural selection or the P450 families are inherently prone to recombination because of their multiplicity, regions of nucleotide similarities, or other characteristics (7). The functional significance of gene conversions in the P450 superfamily is unknown. Some of these events can serve to homogenize these genes, while others generate high divergence in limited regions of closely related proteins (7). They may afford the organism the ability to cope with the enormous numbers of substrates found in the environment.

III. P450 cDNA-directed Expression

A. The Yeast Expression Vectors

The first successful cDNA-directed expression of a P450 was performed using yeast (320). Using a well-characterized multicopy yeast expression vector containing the alcohol dehydrogenase promoter pAAH5 (5), the full length rat IA1 cDNA was expressed (320). High levels of P450 apoprotein were produced, half of which contained heme and were spectrally and enzymatically

competent. On the basis of several criteria, the mammalian P450 produced in yeast was found to be indistinguishable from the native rat enzyme (320, 373, 374).

To produce a complete mammalian electron transport system, the NADPH-P450-oxidoreductase was also expressed in yeast (295). Although the endogenous yeast oxidoreductase was functional with the rat P450, including the homologous rat enzyme, either by expression on a separate vector or by expression on the same vector as the IA1 P450, substantially enhanced P450 activity (295).

In another set of experiments, a hybrid protein, consisting of the P450 joined to the oxidoreductase, was efficiently expressed in yeast (296). The carboxy terminus of the IA1 was directly fused to amino acid residue 57 of the oxidoreductase, producing a M_r 130,000 protein. The expressed fusion protein was catalytically self-sufficient and was, in fact, 4-fold more active per molecule of hemoprotein than P450 expressed simultaneously with native oxidoreductase. Furthermore, the fused protein followed first-order enzyme kinetics instead of second-order reaction kinetics of the two-enzyme reconstituted system (296). This artificial protein is surprisingly similar to the natural M_r 119,000 phenobarbital-inducible, catalytically self-sufficient P450 (P450BM-3) from *Bacillus megaterium* (300, 458).

A recent study with rat IA2, using cDNA-directed expression in yeast in conjunction with site-directed mutagenesis, examined the role in spectral and catalytic activity of the amino acids surrounding the heme-binding cysteine (390). This study showed that the hydrophobic nature of the residues at positions −7, −5, −4, +1, and +2 (fig. 4) was important for the incorporation of heme into apoprotein. Another interesting finding was that changing the invariant Arg at −2 to a Leu resulted in a normal spectrum at 448 nm; however, the enzyme quickly denatured to 420 nm (390). These studies indicate that the heme environment of the mammalian enzyme is probably similar to that of the bacterial $P450_{cam}$ (353), as suggested by the conserved nature of the cysteine region.

In addition to the expression of full-length P450 cDNAs, the yeast system has been used to analyze the amino acids and regions of the P450 required for enzymatic activity and substrate specificity. The rat IA1 and IA2 P450s preferentially metabolize benzo(*a*)pyrene and acetanilide, respectively. Chimeric cDNAs were constructed in which the central region and carboxy terminus of IA1 were replaced with the corresponding regions of IA2 (375). The enzyme activities and substrate specificities were then assayed. From these experiments, it was concluded that the central portions of the IA1 and IA2 P450s are required for substrate binding (375). Further experiments on additional chimeric cDNAs will be required to delineate more precisely which regions of the P450 are required for substrate interactions.

In experiments designed to explore the nature of a mutant mouse IA1 gene, two amino acids critical to the maintenance of enzymatically active P450 were identified (220). Two point mutations were uncovered in a IA1 cDNA, derived from the c37 hepatoma cell line, that contains IA1 mRNA but lacks IA1 enzymatic activity; a leucine to arginine change at position 118; and an arginine to proline change at position 245. Using chimeric cDNAs expressed in yeast, the mutation at position 118 was found to have a negligible effect on activity, whereas the change of position 245 resulted in a 2- to 3-fold reduction in activity. Both amino acid changes resulted in a totally inactive protein (220).

Recent studies have uncovered the possible involvement of a threonine residue in binding of substrates to two rabbit enzymes in the P450II family (172a). The threonine-301, in two yeast cDNA-expressed P450s that carry out testosterone 16α-hydroxylase (171) and laurate ω-1 hydroxylase activities (171a), was replaced by histidine using site-directed mutagenesis (172a). The mutant P450s were devoid of hydroxylation activities even though they displayed reduced, carbon monoxide-complexed Soret bands at 450 nm and low spin type absorption spectra that were typical to their wild type-counterparts. In addition, a spectral change was not observed upon addition of lauric acid to the ferric form of the ω-1 hydroxylase in contrast to that of the expressed normal enzyme (172a). These results suggest the involvement of threonine-301 in substrate binding.

Other P450s have also been successfully expressed in yeast. These include rat IVA1 (142), rat IIA1 (298), rat M-1 (148), and mouse IA1 and IA2 (45a). The levels of expression observed for different forms of P450s have varied between laboratories and range from very high (171a, 320) to extremely low (142, 220, 298). The nature of this variability is unknown.

B. The Monkey Kidney COS Cell Transient Expression Vector

The basis of the COS cell expression system is the simian virus 40 (SV40). Monkey kidney COS cells were developed that contain a portion of the SV40 genome that codes for the T-antigen required for viral replication (93). The cDNA is expressed under the control of a strong promoter on a plasmid containing the SV40 origin of replication. This allows the propagation of multiple DNA copies in T-antigen-containing COS cells, resulting in overproduction of the cDNA-expressed protein.

The first P450 cDNA expressed in mammalian cell culture was the bovine adrenal steroid 17α-hydroxylase XVIIA1 (498). These studies firmly established that a single P450 catalyzes both 17α-hydroxylase activity and 17,20-lyase activity. Simultaneous expression of a mitochondrial and microsomal P450 allowed the full steroid biosynthetic pathway, usually only expressed in adrenal glands, to be produced in COS cells (497).

Drug- and carcinogen-metabolizing human P450s were

also successfully expressed. Expression of a human P450III cDNA (corresponding to $P450_{NF}$) in COS cells was used to demonstrate that this P450 catalyzes the oxidation of the calcium channel-blocking drug nifedipine (106). Other members of the P450III family may also metabolize nifedipine. The human debrisoquine 4-hydroxylase cDNA was also characterized by expression in COS cells (107), and human IIE1 cDNA efficiently expressed N-dimethylnitrosamine demethylase activity (432).

C. The Vaccinia Virus Vector

Although expression of cDNAs is readily achieved using the COS cell system, the level of enzyme production is, in practice, very low, requiring highly sensitive enzyme assays. In addition, expression is limited to the use of monkey kidney COS cells and their derivatives. The vaccinia virus system, on the other hand, allows the expression of large amounts of enzymes in a wide variety of mammalian cells (27, 398). The mouse IA1 and IA2 cDNAs were expressed using vaccinia virus, and both P450s displayed distinct catalytic activities that reflect the activities of the enzymes produced in whole animals (14). This system was used to establish that IA2 is an aflatoxin B_1 4-hydroxylase (67).

D. Stable Expression of P450 cDNAs in Eucaryote Cells

The COS cell and vaccinia virus systems transiently expressed P450 cDNA, since these vectors are by nature multicopy and lytic. Recently cell lines were developed that stably express the rat IIB1 cDNA (57). The endogenous hypoxanthine phosphoribosyltransferase locus in these cells is mutated at a modest 2-fold increased rate by aflatoxin B_1 when compared to the parental cells that do not express IIB1. Generation of cell lines that stably express P450s may form the basis of a new generation of toxicological test systems (57).

IV. Regulation of P450 Gene Expression

A. Inducible P450 Genes

1. 3-Methylcholanthrene/TCDD-inducible P450 genes. The most extensively studied P450 gene activation system is the 3-methylcholanthrene (MC)/2,3,7,8-tetrachlorodibenzoparadioxin (TCDD) induction response. It was recognized more than 30 yr ago that certain xenobiotic chemicals can induce their own metabolism (40). One of the first indications of this was the finding that rats fed MC have an increased capacity to metabolize certain aminoazo dyes (42). Among the enzyme activities induced by MC is the benzo(*a*)pyrene, or aryl hydrocarbon hydroxylase (AHH) activity (43), which is now known to be associated with P450IA1. Early studies using metabolic inhibitors suggested MC induction of AHH activity was due to transcription activation (86, 304).

Analysis and dissection of the mechanism of MC induction of AHH activity have been greatly aided by four fortuitous circumstances: (*a*) a polymorphism for MC induction was discovered in mice (91, 303, 420, 421); (*b*) the induction response is active in cell cultures (302, 303); (*c*) the ability to select benzo(*a*)pyrene-resistant mutant cell lines that are defective in the induction of AHH activity was developed (136, 137, 277, 278); (*d*) the potent inducing agent TCDD was discovered (117). Unfortunately, as will be discussed below, other P450 induction phenomena are more difficult to study because of the lack of an in vitro system, a suitable animal polymorphism, and a specific receptor ligand.

The mouse polymorphism was found to be due to a recessive mutation of a single gene that follows simple Mendelian inheritance (91, 421). By examining inducer-responsive and nonresponsive mice, several other enzymes were found to be under the control of this locus (named the *Ah* locus), including a form of glutathione transferase (341), a form of aldehyde dehydrogenase (59), NAD(P)H:menadione oxidoreductase (239, 361), UDP glucuronosyltransferase (333), P-glycoprotein (MDR-1) responsible for multidrug resistance (25a), and P450IA2 (109). In fact, the battery of genes regulated by the TCDD receptor may be even greater. Five unknown gene products regulated by the *Ah* locus were identified by high-resolution two-dimensional electrophoresis (393).

First evidence that the *Ah* locus was controlled by a specific receptor came with the use of the unusually potent inducing ligand TCDD (343, 344, 348, 349). The TCDD receptor binding affinity was found to segregate with inducer responsiveness in mice, suggesting that the polymorphism in the responsive and nonresponsive mice is due to a receptor defect (345). The nonresponsive mice have a variant receptor that possesses a low affinity for its ligand; therefore, their refractiveness to induction can be overcome by using higher concentrations of TCDD (344). One of the most exciting recent developments in the TCDD receptor field was the synthesis of a specific photoaffinity ligand that binds to the receptor (347). This ligand was used to demonstrate that the TCDD receptor has a molecular mass of between 95 and 126 kilodaltons depending on the organism (346). Molecular weight polymorphisms were also detected with this ligand in both rats (346) and in mice (350). The latter polymorphism was used in conjunction with recombinant inbred mice to map the receptor structural gene to mouse chromosome 12 (350). A more extensive review on the TCDD receptor has recently appeared (462). It is likely, although not yet proven, that this receptor has an endogenous ligand that plays a basic physiological or developmental role.

A 4S protein has been identified and characterized in rats that binds to polycyclic aromatic hydrocarbon inducers of IA1 such as MC (165, 166). This protein was found to interact with upstream regions of the rat IA1 gene and could play a role in its regulation (164). Recently, a substrain of Sprague-Dawley rats was found

that lacks the 4S protein (143). These rats, however, are as capable of being induced by MC and other agents as rats that possess the 4S protein. These results suggest that 4S does not play a role in the regulation of the IA1 gene (143). Although biochemical evidence for the association of the 4S protein with IA1 gene activation is suggestive, the relationship of this protein to the TCDD receptor is unclear. Direct binding of a photoaffinity ligand derivative of TCDD has identified a distinct M_r 106,000 protein in rat cytosol (346).

One of the most useful advances in the regulation of P450IA1 was the isolation of cell lines that are defective in the induction of AHH activity (136, 137, 204, 278). When benzo(*a*)pyrene is incubated with cells, it induces its own metabolism by increasing levels of the IA1 P450. This enzyme produces highly reactive metabolites of benzo(*a*)pyrene that damage DNA and protein leading to cell toxicity and death (88). This cell-killing selection was used to isolate cells resistant to benzo(*a*)pyrene, and these cells were found to be lacking IA1 enzymatic activity (136, 137, 278). The benzo(*a*)pyrene metabolites are also highly fluorescent (4, 302). This fluorescence property of benzo(*a*)pyrene metabolites has also led to the isolation of high activity variant cells through the use of a fluorescence-activated cell sorter (277, 278). These cells have increased induction of IA1 enzymatic activity.

Several lines of evidence suggest that AHH-deficient clones arise through a genetic mutation (139). The most compelling evidence is (*a*) the low spontaneous reversion frequencies which are increased by mutagen treatment (437); (*b*) the stable phenotype (136, 137); (*c*) complementation by DNA transfection (139, 290); and (*d*) direct demonstration of a gene mutation (220).

Four complementation groups of deficient cell lines that are deficient in either the TCDD receptor or the IA1 gene have been found (136, 137, 139, 204, 278). The A complementation group has a defective IA1 gene, and the B, C, and D groups have either low receptor levels (B and D groups) or are unable to translocate the inducer-receptor complex to the nucleus (C group). These latter variants may lack in a factor that promotes nuclear binding or translocation of the receptor. Alternatively a portion of the receptor protein itself or a subunit of the receptor may be mutated giving rise to the class C mutants. Using a novel positive selection procedure, involving a IA1-metabolized light-activated toxin (436), the class A mutants were rescued by transfection of rat DNA containing the IA1 gene (290).

Similarly, the class C mutants were rescued by transfection of human DNA (139). Retransfection of class C mutants with DNA isolated from the rescued cells resulted in the subsequent isolation of a unique 18-kilobase piece of human DNA that could contain the gene coding for the putative factor required for receptor nuclear translocation or binding. Rescued cells for the class B mutants that contain low receptor levels have also been obtained (139). These genetic rescue techniques should allow the isolation and sequencing of the TCDD receptor gene and other factors involved in the induction response.

Somatic cell genetics and DNA transfection have been used to identify the presence of a gene that represses IA1 benzo(*a*)pyrene hydroxylase activity (446). Transfection of DNA isolated from a dominant IA1-deficient mutant cell line into wild-type cells resulted in the recovery of a high frequency of cells that lack IA1 activity. The nature of this gene product, designated dominant negative regulator (446), has yet to be elucidated; however, it may be the putative labile negative transcriptional regulator identified by studies using cyclohexamide (104, 176). Alternatively this gene may be a mutant TCDD receptor that is incapable of forming an active transcription complex yet competes with the normal receptor (446).

The TCDD receptor mediates transcriptional activation of the IA1 gene in the intact animal (109) and in hepatoma cell cultures (177). To investigate the mechanism of this transcriptional activation, several groups have used the isolated IA1 gene in conjunction with DNA transfection and expression vector technology. The scheme for studying DNA sequence elements that control gene transcription is shown in fig. 6. Briefly, the RNA polymerase II promoter region and upstream DNA sequence are excised from the cloned gene and inserted into an expression vector. The vector most commonly used is the pSVO-*cat* (111) containing the bacterial chloramphenicol acetyl transferase (*cat*) gene minus a promoter. This enzyme is not expressed in eucaryotic cells,

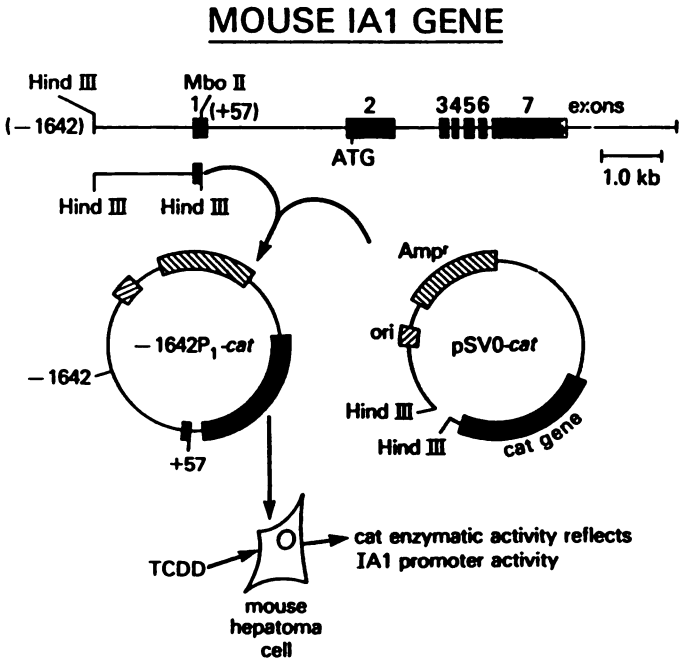

FIG. 6. Analysis of P_1450 promoter activities using the pSVO-cat (cat = chloramphenicol acetyl transferase) expression vector. The promoter region of the P_1450 gene is fused to the *cat* gene, and the construct-1642P_1-cat is transfected into hepatoma cells. *cat* enzymatic activity is then assayed.

and therefore the assay background levels are low. A P450 gene promoter inserted into the vector can drive transcription of the *cat* gene when the vector is transfected into the appropriate cell. Care must be taken to ensure translational fidelity of the *cat* mRNA by excluding the natural ATG initiator codons from the fused promoter-*cat* construct (fig. 6). In addition, splice junctions derived from the eucarotic gene should also be excluded unless a whole intron is included in the construct.

IA1 promoter-*cat* expression plasmids have been constructed from cloned mouse (104, 197), rat (78), and human (157, 182, 207) IA1 genes. All of the constructs were regulated by either TCDD or MC when transfected onto cultured cells, and deletion of certain upstream DNA abolished the induction response. These studies indicated the presence of *cis*-acting sequence elements that serve to bind receptor and/or other *trans*-acting factors that mediate induction. A *cis*-acting element is a segment of DNA directly linked to the gene, usually upstream of the transcription start site. Taking into account the altered numbering system of the IA1 promoter in one series of studies (195–197), the regulatory elements that control induction of transcription by TCDD are located at −950 and −1100 from the IA1 transcription start site. The element(s) located between −950 and −1100 is an inducible enhancer that can function regardless of orientation and distance from the transcription start site (196, 314). An enhancer is a segment of DNA, or a *cis*-acting element, that, when inserted in either orientation upstream, downstream, or within an intron of a transcription unit or gene, can increase the transcription rate of that gene. An inducible enhancer requires the presence of an inducer to increase the transcription rate. Within the −950 to −1100 region lie conserved sequence elements called drug-regulatory elements (DRE) and xenobiotic regulatory elements (XRE) that are found in multiple copies (195, 400). Sequence elements located between −500 and −800 base pairs were found to suppress inducer-independent (constitutive) expression of the IA1 promoter, suggesting the presence of an inhibitory domain (104, 157, 182, 197). A significantly high degree of nucleotide similarity was also found in this region among humans, rats, and mice (157).

The most important elements responsible for inducible enhancer activity appear to be the two 15-nucleotide XREs located between −1000 and −1100 (79). Tandem copies of the XRE increase inducible and constitutive expression when placed upstream of a heterologous SV40 promoter element minus its endogenous enhancer sequences (79). It was proposed (112) that XREs are evolutionarily related to the glucocorticoid-responsive element (GRE) and that this relationship is germane to the similarity in physicochemical properties of the TCDD receptor and the glucocorticoid receptor (46, 464). Further evidence for involvement of XREs in the induction response was found by DNA-binding assays in which DNA containing the XREs specifically bound to proteins derived from nuclear extracts (79). Furthermore, using an exonuclease protection technique on isolated nuclei (60) or a direct in vitro DNA-binding assay (53), a TCDD-dependent factor that binds to an upstream region of the mouse IA1 gene has been demonstrated. Positive gene regulation by a binding factor has been observed in other systems, including the glucocorticoid-regulated genes (438) and cadmium regulation of the metallothionein gene (384).

Other genetic evidence for the involvement of the TCDD receptor as the binding factor in regulation of IA1 was obtained with the use of the mutant cell lines discussed above. Regulation of the IA1 promoter-*cat* construct was blocked in cells that were deficient in nuclear uptake of receptor (104, 195, 400) and in cells that lacked receptor (400). Similar results were obtained with a TCDD-inducible glutathione S-transferase gene-*cat* construct (417).

Direct DNA binding of receptor to upstream IA1 DNA has not been performed because of the inability to purify the TCDD receptor to homogeneity using standard methods. However, two recent experiments suggest involvement of the TCDD receptor in binding to the XRE. Using gel retardation assays, the TCDD receptor, complexed to a specific and reversibly binding ^{125}I-labeled ligand (23), was able to bind the upstream control regions of the mouse IA1 gene (53a). Others found that incubation of the cytoplasmic fraction of untreated hepatoma cells with MC in vitro results in the appearance of a factor that binds to the XRE in the same manner as nuclear extracts from MC-treated cells (80). Definitive experiments demonstrating the role of the TCDD receptor in gene activation, however, await isolation and expression of the receptor cDNA or gene. Among the questions that can be answered using expressed receptor is whether TCDD is required for DNA binding or if TCDD modulates partitioning of the receptor between the nucleus and cytoplasm. Such issues have been explored using the glucocorticoid receptor (16, 468).

A unique and intriguing observation was made with two hepatoma class A cell lines that expressed normal levels of receptor but no AHH activity (138, 220). Interestingly, those cells contained high levels of IA1 mRNA in the absence of inducer, suggesting that the IA1 gene was constitutively activated and that this activation was the result of defective or mutant IA1 protein. In fact, these constitutively expressed mRNAs were found, by direct sequencing and expression of their corresponding cDNAs, to code for defective proteins. Based on the results of cocultivation experiments, it was further postulated that the IA1 enzyme metabolizes a diffusible endogenous substance involved in autoregulation of its own gene (138). Transfection of one of these cell lines with the IA1 promoter-*cat* vector resulted in a high level

of constitutive expression of *cat* activity (104). Deletion of DNA upstream of −398 abolished the high level of expression, indicating the involvement of a repressor factor (104). Studies with the mutant cell lines further suggest that other genes under the control of the TCDD receptor may be indirectly regulated by the activity of the IA1 gene (361).

The IA2 gene is noncoordinately regulated with the IA1 gene. However, there is controversy regarding the mechanism by which IA2 is induced in the liver. Transcriptional activation of the IA2 gene was detected in the livers of mice and rats following inducer treatment, although a substantial posttranscriptional component was suspected to play a role in the large increase in IA2 mRNA (109, 216, 399). In contrast, others did not detect increases in the rate of IA2 gene transcription (335) or detected only modest increases in transcription (392). The reasons for these discrepancies are unclear, but they may depend, to some degree, on the particular inducer used.

In general, regulation of the IA2 gene has not been as easy to study as IA1 gene regulation, because expression of this gene is not detected in established cell lines. A number of genes expressed in hepatocytes in situ become inactive when these cells are placed in primary culture or when they are transformed and become stable hepatoma cell lines (36–38). The reason for this phenomenon of "gene extinction" in cell culture is unknown; however, the mechanism apparently results in the cessation of gene transcription. It was suggested that the loss of certain gene activity in culture was due to the lack of appropriate cell-cell contact (74). Years of experimentation using different substrata to plate hepatocytes have resulted in some success in this area. Primary hepatocyte cell culture systems that permit induction of IA2 mRNA have recently been developed (335, 392). Induction of IA2 in rat hepatocyte cultures is principally due to posttranscriptional events (335, 392). These culture systems do not, however, accurately reflect the inducibility of IA1 or IA2 in the intact animal. For instance, IA1 is transcriptionally regulated in vivo but posttranscriptionally regulated in culture (392). Other investigators have developed a hepatocyte culture system that yields high levels of expression of genes that are usually turned off in primary cultures, including the induction of IIB1, IIB2, and IA2 mRNAs (380). This culture system stably expressed these genes for over 1 wk. However, induction of IA2 mRNA was not observed in this system.

To explore the lack of expression of the endogenous IA2 gene in hepatoma cells, a IA2 promoter-cat construct was employed using the system outlined in fig 6. The IA2-*cat* was not induced under the same conditions in which IA1-cat induction was observed; even constitutive expression of cat was lacking (author and D. W. Nebert, unpublished experiments). These results suggest that either a positive transcription factor required for IA2 expression is lacking or that a factor that specifically represses transcription of the IA2 gene is present. Because of the lack of an appropriate cell culture transfection system, the DNA elements involved in regulation of IA2 remain obscure. In vivo microinjection and transgenic mice experiments could possibly be used in combination with promoter-expression vectors to answer this question.

One of the features common to several genes that are not expressed in liver or hepatoma cells in culture is their tissue specificity (36–38, 74). The IA2 gene is constitutively expressed in liver and is also inducible. IA2 is not significantly expressed in extrahepatic tissues, and only high concentrations of the potent inducer TCDD cause a small increase in mRNA in the lung, kidney, and intestine (216). IA1, on the other hand, is readily inducible in virtually all tissues tested to date, even though its constitutive expression is nil (216). The increase in hepatic IA2 mRNA is due to both transcriptional activation and a large degree of mRNA stabilization, whereas control of IA1 is primarily transcriptional with only a modest posttranscriptional mRNA stabilization (216, 335, 392).

Regulation of IA1, IA2, and other genes by TCDD and related inducers is clearly very complex and will require further experimentation. Even though TCDD and other inducers are involved in the induction of these genes, other mechanisms unique to each gene come into play in their induction, constitutive expression, and tissue-specific expression. Genes such as aldehyde dehydrogenase are inducible by TCDD and yet are clearly noncoordinately regulated with the IA1 gene. To date, evidence suggests the involvement of a receptor, repressor(s), autoregulation by a small diffusible IA1 metabolite, and mRNA stability. The TCDD receptor appears to be a positive transcriptional activating element.

2. Phenobarbital-inducible P450 genes. The phenobarbital induction response was discovered more than 25 yr ago. Administration of this drug to rats resulted in a dramatic proliferation of liver endoplasmic reticulum membrane (360) and an increase in enzymes of the mixed function monooxygenase system (330–332).

The phenobarbital induction response involves increases in several mRNAs including those coding for epoxide hydratase (96, 140), NADPH-P450-oxidoreductase (97, 99, 140), UDP-glucuronosyltransferase (258), and IIB1/IIB2 (6, 99, 140). Phenobarbital-induced mRNA accumulation is due to an increase in the gene transcription rate (6, 140, 342).

The IIB1 and IIB2 genes are coordinately regulated in liver by phenobarbital. As noted earlier, these two mRNAs exhibit 97% nucleotide similarity; therefore, oligonucleotide probes specific for the IIB1 and IIB2 mRNAs were used for quantitation (326). The IIB2 gene is constitutively expressed, while IIB1 is transcriptionally inactive in the untreated rats. Phenobarbital administration results in the induction of both mRNAs; IIB1

mRNA induction was 5-fold higher than IIB2 induction (326). Significant induction of either mRNA was not detected in several other tissues examined, although IIB1 was constitutively expressed in lung and testis (324).

The expression of certain genes in the IIC family of rabbit (209, 253) and rat (13, 75) and the rat IIIA1 and IIIA2 genes (108) can also be induced by phenobarbital. Therefore, regulatory elements for phenobarbital inducibility appear to be conserved in many P450 genes.

Of interest is the inducibility of the developmentally regulated P450f gene. This gene becomes transcriptionally activated when rats reach puberty and is not significantly induced by phenobarbital when fully expressed in adult rats (101). Others isolated a rat cDNA, designated 1GC 1, that shares 98% of the deduced amino acid sequence of P450f (13). The high level of amino acid similarity between 1GC 1 and P450f suggests that these P450s may be allelic variants. This supposition is further supported by the fact that a single rat gene is detected with a P450f-specific probe (101). The P450f and 1GC 1 genes appear to be regulated differently, however. An oligonucleotide specific for 1GC 1 revealed that this gene is activated by phenobarbital in immature rats (13). In contrast only a slight increase in P450f protein was seen in 4-wk-old phenobarbital-treated rats (11). It is noteworthy that Wistar (13) and Long Evans (11) rats were used in these studies, suggesting that a polymorphism might account for this discrepancy in phenobarbital inducibility.

Phenobarbital also induces two genes in the rat P450III family (108). Both the constitutively expressed male-specific PCN2 gene and the PCN1 gene, a gene that is not expressed in either male of female untreated rats, are inducible by phenobarbital. Only PCN1 is induced by steroids, however, suggesting that these genes have overlapping regulatory controls for phenobarbital but have distinct regulatory controls for steroid inducibility and constitutive expression. Interestingly, the rabbit steroid-inducible P450 3c mRNA is not induced by phenobarbital (48).

Unfortunately data regarding the role of specific phenobarbital receptors or *cis*-acting DNA sequence elements in this induction process are lacking. Studies using radiolabeled phenobarbital failed to detect specific protein-binding complexes (422). This may be due to the low affinity of phenobarbital or its extensive metabolism by liver enzymes. However, a unique phenobarbital-like ligand, 1,4-bis[2-3,5-dichloropyridyloxy)]benzene, was discovered that is 600-fold more potent than phenobarbital as an inducer in mice but is ineffective in rats (351, 352). This compound was not significantly metabolized and, like TCDD, had a long half-life. However, binding studies have not been reported.

The lack of a suitable cell culture system has markedly hampered the ability to define *cis*-acting regulatory elements in the phenobarbital-inducible P450 genes. None of the adequately characterized tumor cell lines expresses inducible IIB1 or IIB2 genes. Therefore, transfection experiments of promoter-*cat* expression vectors have not been published. Recently, primary hepatocyte cell culture systems were developed in which IIB1 and IIB2 mRNAs are faithfully induced by phenobarbital (380, 429). Studies using the culture systems to examine the regulation of promoter-expression vectors should be forthcoming.

3. Steroid-inducible P450 genes. The rat PCN1 gene is induced by glucocorticoids such as dexamethasone and pregnenolone 16α-carbonitrile. This induction is primarily the result of an activation of PCN1 transcription (396) and an increase in mRNA (108, 141, 478). Induction of a P450III gene by dexamethasone also appears to occur in humans (288).

Of interest is the possible involvement of the glucocorticoid receptor (115, 275), which controls a variety of genes in the liver. Regulation of IIIA1, however, seems to be quite distinct from the control of classical glucocorticoid-regulated genes such as tyrosine aminotransferase (379, 381). PCN1 induction requires about a 10-fold higher level of steroid than is needed to induce tyrosine aminotransferase, and certain steroids that readily induce PCN1 fail to induce tyrosine aminotransferase (379, 381). These studies suggest that either PCN1 gene activation may depend on other factors or that differences exist between these genes in their affinity for receptor-ligand binding to *cis*-acting DNA control elements. Using promoter-expression vectors, cell culture systems (380) and cloned genes can be used to delineate the mechanisms of glucocorticoid regulation of the PCN1 gene, as described above.

One or more of the P450s in the III family are also posttranscriptionally regulated. The rabbit P450 3c mRNA is stabilized by the macrolide antibiotic triacetyloleandomycin (TAO) (47, 48). This compound does not activate transcription of the P450 3c gene. The rat PCN1 or PCN2 proteins are also stabilized by TAO (444). Dexamethasone, on the other hand, transcriptionally activates the rat PCN1 gene and simultaneously stabilizes the IIB1 and epoxide hydratase mRNAs (396).

4. Hypolipidemic drug-inducible P450 genes. The hypolipidemic drug clofibrate induces a battery of enzymes, including the peroxisomal enzymes enoyl CoA:hydratase-3-hydroxyacyl-CoA dehydrogenase, fatty acyl-CoA oxidase, 3-ketoacyl-CoA thiolase (144), D-amino acid oxidase (81), and the microsomal P450IVA1 (9, 142, 415). Many other hypolipidemic agents, including ciprofibrate, nafenopin, and the plasticizer bis(2-ethylhexyl) phthalate, also induce these enzymes. The net result of their induction is an increase in fatty acid beta oxidation. The P450IVA1 (142), enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (359), and fatty acyl-CoA oxidase (359) genes are transcriptionally activated by clofibrate administration. This activation is very rapid and occurs within 1 h of administration (142, 359).

A second P450 cDNA, designated IVA2, has recently been isolated from a clofibrate-induced liver library (author's laboratory, unpublished data). The IVA2 mRNA is markedly induced by clofibrate in liver but is constitutively expressed at high levels in the kidney. Only about a 2-fold induction of the IVA2 mRNA is observed in the kidney. IVA1 mRNA, on the other hand, is expressed at very low levels in both the liver and kidney and is highly induced in both tissues by clofibrate (142). These data suggest that the high constitutive expression of the IVA2 gene in the kidney may abrogate its inducibility IVA3 mRNA is coordinately regulated with IVA1 mRNA (author's laboratory, unpublished data).

Clofibrate-inducible IVA3 gene expression has also been detected in a rat hepatoma cell line (author's laboratory, unpublished data). The IVA1 and IVA2 genes, however, are not induced. These cells should serve as useful vehicles for the study of *cis*-acting DNA elements that are required for transcriptional activation in the IVA3 gene.

A receptor for clofibrate was purified by affinity chromatography; this molecule has a subunit molecular weight of 70,000 and exists as a dimer of 140,000 (247). Curiously, its binding affinity toward clofibrate and other ligands is about 1000-fold less than that of TCDD for the TCDD receptor (248). Availability of purified receptor should aid in identification of control elements in the IVA genes.

5. Effect of heme on P450 induction. An area that has received little attention in recent years is the effect of heme on P450 induction. In order for P450 holoenzyme to accumulate, an adequate heme pool must be available to accommodate the newly synthesized apoprotein. The rate-limiting enzyme in heme biosynthesis, 5-aminolevulinate synthase (ALV-S), is readily inducible by compounds such as phenobarbital (116) and TCDD (343). This induction is due to transcriptional activation of the ALV-S gene (267, 408). A popular hypothesis is that the effect of these inducers is to indirectly decrease the heme pool by increasing the amount of apo-P450 (266, 408). The induced apo-P450 incorporates heme from the cellular heme pool, and the resultant decreased levels of heme then derepress the ALV-S gene. This metabolic end product control could be due to heme-mediated gene repression (408). Other evidence suggests that the level of heme modulates transcription of P450 genes (18, 358). For instance, inhibition of heme synthesis or stimulation of heme degradation blocks the induction of IIB2 mRNA by phenobarbital.

B. Regulation of Constitutively Expressed P450 Genes

1. Transcriptional regulation during development. One of the most interesting regulatory phenomena is the activation of P450 gene transcription during development. At least four modes of constitutively expressed P450 gene regulation have been described: (*a*) activation of expression immediately after birth; (*b*) activation of expression at the onset of puberty; (*c*) activation of expression in males or females, referred to as a "sex imprinted" expression; and (*d*) specific suppression of gene activity in males or females at the onset of puberty.

Elevated levels of both the ethanol-inducible IIE1 gene and the IID1 and IID2 mRNAs have been detected immediately after birth in rats (103, 404, 434). Elevation of IIE1 mRNA was coincident with transcriptional activation of the IIE1 gene and begins within a few hours after birth (404, 434). mRNA levels peak at the age of 6 days (434).

The mechanism governing this regulation is unknown; however, changes in the methylation state of the IIE1 gene correlated with transcription (434). Using DNA restriction endonucleases that are sensitive to cytosine methylation, certain cytosine residues upstream of the IIE1 transcription start site were found to be demethylated within 24 h of birth (434). Further demethylation of cytosines downstream of the start site was detected at 1 wk and 10 wk of age. It is not presently known whether this demethylation is a result of transcriptional activation of the IIE1 gene or if demethylation causes gene expression.

Two genes in the rat P450IIC subfamily, PB-1 and P450f, are transcriptionally activated at the onset of puberty (101). The P450f mRNA reaches a steady-state level in adult females that is about 2-fold higher than in adult males (13, 101). P450f protein levels were also higher in adult females than males (11). The mechanism by which the PB-1 and P450f genes are activated is not understood, although serum testosterone levels do not appear to significantly affect their regulation (11, 49, 452). It has not yet been established whether pituitary hormones play a role in the regulation of these genes (452).

2. Sex imprinting and gene expression. Sex-specific P450s have been purified from rats (29, 202, 263, 292, 368, 447). Best studied are the male-specific and female-specific P450 forms which, for the purpose of this discussion, are designated P450h and P450i, respectively (368, 369), even though these enzymes have been purified by several groups. P450h is expressed only in adult male rats. This expression is dependent on androgen exposure both during the neonatal period and adulthood. Thus, castration of neonates abolishes P450h expression in adults, while castration of adults only partially decreases expression of this enzyme. This partial effect is eliminated if rats are supplemented with testosterone during adulthood, directly demonstrating a role for adult testosterone (49, 452). Similarly, testosterone injection to castrated rats during the first 3 days of life also results in significant P450h expression at adulthood, consistent with the proposal that this enzyme is one of the hepatic proteins that is subjected to neonatal "imprinting" or programming by androgen exposure (63, 128).

The cDNA and gene sequences of P450h have been

determined (287, 293, 491), and a specific oligonucleotide probe was used to quantitate P450h mRNA during development (293). These studies established that the P450h mRNA is exclusively expressed in liver of adult male rats and is absent in female liver and in several extrahepatic tissues in males and females (293). Others have also investigated levels of P450h mRNA in male and female rats and have drawn the same conclusions (287, 495). Although no direct measurements were performed, this increase probably results from the activation of gene transcription.

P450g, another male-specific rat P450, has also been purified (368). Specific anti-P450g antibodies have been used to study the regulation of this enzyme during development in various strains of rats (11, 268). P450g was undetectable in female rats from 3 to 48 wk of age (11). Interestingly, levels of P450g protein varied among individual male rats up to 20-fold and in both Long Evans (11) and Sprague-Dawley (268) rats. However, P450g protein production was minimal or absent in Fischer adult male rats (268). The reason for the variability of P450g expression is unclear, but this phenomenon may reflect the heterogenous nature of the outbred rat strains. The lack of correlation between P450g mRNA and protein levels was of interest (268). This finding suggests the production of a mRNA that yields a defective protein in the Sprague-Dawley rats. It should be noted that definitive analysis of P450g mRNA awaits isolation of the cDNA.

The rat IIA2 gene is also male specific (264). Expression of IIA2 mRNA is undetectable in females and is activated in males at the onset of puberty. The IIA1 gene, on the other hand, is expressed in adolescent males and females and is specifically suppressed only in males at puberty (264, 298). Studies of the IIA2 protein revealed that its expression in males is programmed by neonatal androgen exposure (454).

The sex-specific P450i, also designated UT-I, 2d, female, and 15β, is specific to female rats during adulthood, although it is expressed in both sexes pubertally. In female rats, expression of this P450 is only partially dependent on estrogen exposure, while in male rats exposure to androgens during the neonatal period programs for suppression of this P450 at puberty (49, 257, 452, 486). Levels of the female-specific P450i mRNA correlate with protein expression during development and after treatment of rats with various hormone-altering regimens, suggesting that the response is mediated at the transcriptional level (287, 495).

The role of pituitary hormones in the regulation of sex-specific P450s has been extensively investigated (205, 286, 291, 292, 317, 454, 486). Adult male rats have pulsatile secretions of pituitary growth hormone, while adult females have more constant levels, and these patterns are determined by both neonatal androgen exposure and adult testosterone levels (186). Hypophysectomy reduces expression of P450h, and this can be reversed by periodic injections of growth hormone (205, 287, 291, 292, 486). IIA2 expression in males, on the other hand, is not dependent on pulsatile growth hormone secretion, unlike P450h (454). Growth hormone secretion is also involved in the regulation of the female-specific P450i. Continuous infusion of growth hormone reestablishes levels of the female-specific P450i in hypophysectomized females (205, 257), and this results from a specific increase in P450i mRNA levels (287, 495). Growth hormone, in some cases, also suppresses P450 expression, as will be discussed below.

Developmental and sex-specific regulation of P450s in mice has also been investigated. P450s expressed in either male or female mice were isolated, their cDNAs were characterized, and regulation was studied (407, 474). The male-specific C-P-450$_{16\alpha}$ was found to reside in the IID gene family. Surprisingly, no sex-specific rat IID genes have been found. Since the mouse 16α cDNA hybridizes to several genes, a specific oligonucleotide probe was used to quantitate its mRNA levels in animals undergoing various hormone treatments (474). The C-P-450$_{16\alpha}$ mRNA detected with the oligonucleotide was undetectable in female livers and in livers from adult males that had been castrated at birth or as adults. The male-specific decrease in mRNA was reversed by testosterone treatment, suggesting a role for androgens in the regulation of P450 16α mRNA activity. Based on analysis of enzyme activities and mRNA levels using oligonucleotide probes specific to the C-P-450$_{16\alpha}$ mRNA, it was postulated that there are two testosterone 16α-hydroxylases that are differentially regulated; one enzyme is reversibly regulated by androgens while the other is "imprinted" for expression by castration at birth (474). The putative imprinted enzyme would not be activated by testosterone treatment of adults. This is in contrast to the regulation of rat P450h, as described above, since expression of the rat enzyme is reversed by adult testosterone treatment of neonatally gonadectomized rats (49, 452). Conclusive evidence for the existence of the irreversibly imprinted mouse gene awaits its isolation and sequencing and the study of its regulation with an oligonucleotide probe.

Another female-specific testosterone 16α-hydroxylase in the IIB subfamily has been characterized and designated I-P-450$_{16\alpha}$ (318, 319). This I-P-450$_{16\alpha}$ gene was derepressed in castrated adult BALB/cJ but not 129/J mice (318). Crosses between these two strains indicated that derepression was inherited as an autosomal dominant trait localized near to the *Coh* locus on mouse chromosome 7. Genetic crosses also established that the expression of I-P-450$_{16\alpha}$ in BALB/cJ females is regulated by a different genetic locus than that governing repression in BALB/cJ males (316), although both loci are located on the same region of chromosome 7. Treatment of castrated male BALB/cJ mice with estrogens repressed the level of expression of the I-P-450$_{16\alpha}$ gene

(318). Of interest, however, is the reason why this gene is expressed in female mice, since both males and females possess hepatic estrogen receptors. Perhaps other sex-specific factors exist that function in concert with the estrogen receptor in regulating the I-P-450$_{16\alpha}$ gene. In any case, these studies are an excellent demonstration of the power of mouse genetics in studying sex-specific developmental regulation of P-450s.

3. Sex-specific gene suppression. The sex-specific expression of certain male and female P450s results from the suppression of gene expression. For instance, the expression of PCN2 mRNA is specifically decreased in female rats when they reach puberty (108). This results in a loss of constitutively expressed testosterone 6β-hydroxylase in the female rat (108, 205, 452). Expression of IIA1 mRNA is also decreased in males at puberty (264, 298), resulting in a decline in IIA1 protein and its associated testosterone 7α-hydroxylase activity (264, 454).

Growth hormone appears to play a role in the suppression of P450 expression. Hypophysectomy resulted in an increase in PCN2 and testosterone 6β-hydroxylase activity, while high levels of growth hormone, administered by either intermittent injection or infusion, decreased levels of this enzyme in adult male rats (205, 452, 454, 488). These data suggest that the developmental decrease in PCN2 expression is the result of continuously high levels of growth hormone such as are found in adult females. Expression of the enzyme in immature males and females could be the result of low levels of growth hormone secretion. It was also suggested that suppression of PCN2 expression in females could be the result of decreased circulating corticosteroid (450). Other P450s in the IIB subfamily are also modulated by pituitary hormone secretions (487).

4. Developmental regulation of P450 induction. The induction of the P450I and P450IIB genes is also regulated as a function of development. TCDD induces expression of the IA1 gene as early as 10 days of gestational age in the mouse (169, 430). However, the IA2 gene is not activated by TCDD administration until shortly after birth. In contrast, induction of IA1 and IA2 expression in the rat by MC did not occur until 1 wk after birth (90). These results suggest a species-specific difference in developmental control of IA1 induction.

Growth hormone was also shown to have a role in regulation of rat IIE1 (467). Hypophysectomy of male and female rats resulted in 5-fold to 10-fold respective increases in IIE1 content. This increase was partially repressed when these rats were treated with growth hormone. This is another example of suppression of P450 expression by growth hormone that needs to be further examined at the gene level.

Hepatic induction of the rat IIB1 and IIB2 mRNAs by phenobarbital is first apparent 2 days prior to birth (day 22 of gestation), and the ratio of the inducible levels of these two mRNAs varied as a function of age (89, 90). Induction of other P450 genes, such as the steroid- and clofibrate-inducible genes, has not been examined in the developing rat. In general, most P450s are not expressed or induced prior to birth. The cellular mechanisms controlling inducible prenatal P450 expression are currently unknown.

5. Tissue-specific gene expression. The majority of P450 genes that have been characterized to date are expressed in hepatocytes. More specialized steroid hydroxylases, involved in the biosynthesis of aldosterone and cortisol, are found almost exclusively in steroidogenic tissues such as the adrenal gland and certain reproductive organs such as the testis and ovary. Certain forms of P450s have been detected in most tissues examined, although their levels in extrahepatic tissues are considerably lower than in liver. The TCDD-inducible IA1 is ubiquitous and is found in a wide variety of tissues only after inducer treatment.

Even though IA1 is detectable after inducer treatment in many tissues, the IA2 gene is virtually inactive except in the liver (216). In six tissues examined, IA2 mRNA was undetected after MC treatment and was expressed in very low amounts, at the limit of detectability, and only after high doses of TCDD. IA1 was easily induced in all tissues examined with low levels of TCDD and with the weaker inducer MC (216). The limited extrahepatic induction of IA2 mRNA was primarily the result of mRNA stabilization. It is unknown if IA2 protein is found in extrahepatic tissues. Substantial transcriptional activity of the IA2 gene was detected in rat kidney; however, no mature IA2 mRNA was found (335). This indicates that IA2 expression in extrahepatic tissue may be regulated at the level of RNA processing or mRNA transport. Others did not detect IA2 transcription activity in rat prostate (399).

In the P450IIA subfamily, IIA1 and IIA2 mRNAs were only detectable in the liver (264). Even after induction with MC, IIA1 mRNA was seen exclusively in the liver. In contrast, a related P450, IIA3, was expressed and inducible only in the lung, and its mRNA was undetectable in the liver, kidney, and intestine (218). These data indicate that distinctly different control elements have evolved for three genes in the IIA subfamily: (*a*) inducibility of IIA1 by MC; (*b*) induction of IIA2 in males at the onset of puberty; and (*c*) tissue-specific expression of IIA1 and IIA2 (liver) and IIA3 (lung).

The rat IIB genes are also under tissue-specific regulation. IIB1 and IIB2 exhibit 97% amino acid sequence similarity and yet are differentially expressed in extrahepatic tissues. In studies using the oligonucleotide probe to the IIB1 and IIB2 mRNAs, IIB2 mRNA was found to be constitutively expressed in liver but was undetectable in the lung, kidney, and testis (324). On the other hand, the IIB1 probe detected a mRNA that was present in the lung and testis. Since this IIB1 oligonucleotide did not

distinguish between other members of the IIB1 subfamily (7), it is not possible to conclude that the lung and testis mRNA is IIB1. This mRNA was not detectable in untreated liver or kidney, and the lung IIB mRNA was not significantly induced by phenobarbital treatment. In contrast, a mRNA reacting with the IIB1 probe was readily induced by phenobarbital in the small intestine of the rat in the absence of IIB2 induction (424). This induced expression was predominantly confined to the enterocytes. The rat IIB3 mRNA was found to be constitutively expressed in male and female liver and not inducible by phenobarbital (245). This mRNA was not detectable in the lung, kidney, and prostate.

P450s have also been detected in rat brain (203a, 441). Antibodies that react with both IIB1 and IIB2 revealed the presence of these enzymes in the Bergmann glial cells of the cerebellum and, to a lesser extent, in other regions of the brain (441). IA1 was also found in the brains of 3-methylcholanthrene-treated rats (203a). Significant levels of immunodetectable enzyme were detected in nerve fibers of the globus pallidus and, to a lesser extent, in other regions. The role of P450s in brain metabolism, however, is unknown.

IIB1, or a related protein, and its activity were detected in lung and adrenal microsomes of untreated rats but were absent in the kidney and intestine. Phenobarbital administration caused modest increases in IIB1 in these tissues, while IIB2 was induced only in the adrenal gland (32). However, inducibility varied among animals. For instance, IIB2 induction was detected in only two of six preparations of lung microsomes (32). These data are in contrast to those of the mRNA measurements described above and may reflect the sensitivity level of the assays used. For this reason it is sometimes difficult to ascertain whether a particular gene is expressed at a significant level in extrahepatic tissue. Even very low levels of expression may be of consequence in extrahepatic drug and carcinogen metabolism.

In the rabbit, three very similar IIB cDNAs were identified, and one was expressed exclusively in the lung (83). This mRNA was not induced by phenobarbital, whereas the two mRNAs in liver were readily induced. Of interest was the finding that the IIB1 mRNA in rat lung and testis did not have significant polyadenylation [poly (A)] tracts in contrast to liver IIB1 mRNA (324). Since poly (A) tracts on mRNAs are known to get shorter as mRNAs age, perhaps insignificant length poly (A) tracts may reflect an exceptional stability of the IIB1 mRNA in these tissues. Tissue-specific differences in poly (A) tracts have not previously been described for other genes. However, in contrast to these results, a rabbit constitutively expressed IIB mRNA was found to be almost totally polyadenylated (83).

Tissue-specific expression has also been detected in the IIC subfamily. The adult male-specific rat P450h mRNA was found in the liver but not in the lung, kidney, and testis (491). The rat PB-1 mRNA was not found in the kidney and lung, but substantial amounts were detected in adult female brain (213). Therefore, this enzyme may serve a physiological role in the brain, perhaps in the metabolism of steroid hormones. Members of the rabbit IIC subfamily are also regulated in a tissue-specific manner (253). PBc2 mRNA was expressed in the kidney at about 15% of the level found in the liver, while PBc1 and PBc3 mRNAs were expressed only in the liver. None of these mRNAs were expressed in the lung.

Other P450II subfamilies are under tissue-specific regulation. The rat IID genes are expressed in the liver and kidney but not in the lung and intestine (author's laboratory, unpublished data), and the rat IIE1 gene is expressed and can be induced in the liver, lung, and kidney (405). The rabbit IIE1 mRNA has been detected in the kidney and liver, and the mRNA for a second rabbit gene product, IIE2, was found only in the liver (211). The rabbit IIE1 protein has also been detected in nasopharyngeal tissues (54).

Extrahepatic expression of the P450III family has recently been investigated in rats and humans. Using the human P450p cDNA probe and a specific antibody, a high level of expression of a member of this family was detected in human intestine (445). Levels of this protein in intestinal microsomes were similar to those in liver and were correlated with erythromycin demethylase activity. Rat intestinal villus tip cells also had a high concentration of at least two P450III gene products that were noncoordinately regulated by dexamethasone and were differentially distributed between the villus tip and intestinal crypts (445). Expression of P450III members in the intestine may have profound clinical significance in drug metabolism and adsorption (445).

Expression of the P450IV genes may be ubiquitous in many tissues. P450 proteins that are fatty acid and/or prostaglandin ω-hydroxylases have been characterized from placenta (484), kidney (142, 173), colon (200), intestine (243), liver (142, 415), lung (466, 485), and polymorphonuclear leukocytes (387). cDNA sequences provide firm evidence that the rat liver and kidney (142) and rabbit lung (262) enzymes are distinct members of the P450IV family. The rat IVA1 and IVA2 genes are regulated differently in the liver and kidney. IVA1 mRNA is constitutively expressed at a low level in the kidney and liver and is also induced in both tissues by clofibrate (142). IVA2 is expressed constitutively at a high level in the kidney but is not significantly induced in this tissue by clofibrate (author's laboratory, unpublished data). This gene is coordinately regulated with IVA1 in the liver, however.

The rabbit prostaglandin ω-hydroxylase is specifically expressed at high levels in the lungs of pregnant or progesterone-treated animals (466, 485). The cDNA for this enzyme is very similar to those of the rat IVA1 and IVA2 enzymes; these enzymes exhibit 74% and 76%

amino acid similarity, respectively, with the rabbit enzyme (author's laboratory, unpublished data). This cDNA reacted with mRNA found in the liver, kidney, placenta, and induced lung. It is unclear whether more than one mRNA is being detected in these tissues, because Southern blot analysis suggests the presence of several P450IV genes in rats and rabbits (142, 262).

C. Posttranscriptional Regulation of P450

As mentioned earlier, IA2 is regulated to a large extent posttranscriptionally (216, 335, 392). After comparing transcription rates and mRNA levels, it was concluded that IA2 mRNA is markedly stabilized in liver and extrahepatic tissues by TCDD administration. IA1, on the other hand, is regulated primarily at the transcriptional level.

When rats are given dexamethasone, mRNAs for IIB1/IIB2 and the NADPH-P450 oxidoreductase are specifically stabilized in the absence of an increase in transcription (396). However, the PCN1 gene is transcriptionally activated (396), and induction of PCN1 protein is directly proportional to mRNA levels after steroid administration in rats or primary hepatocytes (444). The antibiotic TAO caused an increase in the level of a P450 that is indistinguishable from the dexamethasone-induced enzyme PCN1; however, this increase occurred in the absence of an increase in mRNA in rat hepatocytes cultured in vitro (444). In contrast, dexamethasone causes an increase in PCN1 (or PCN2) mRNA in these cultures (444). Both agents increase the mRNA(s) in the intact animal. In a new hepatocyte culture system containing "matrigel" (226), both TAO and dexamethasone increase levels of PCN1/PCN2 mRNA (380). These results suggest differences in the mechanism of action of TAO and dexamethasone. For instance, it is possible that TAO stabilizes mRNA, whereas dexamethasone increases transcription. Indeed, the latter compound has been shown to increase the rate of transcription of the rat PCN1 gene (396), while TAO was found to stabilize the P450 3c mRNA, a P450 in the III family that is expressed in untreated rabbits (48). The possibility still exists that TAO stabilizes the constitutively expressed PCN2 mRNA, and dexamethasone activates the PCN1 gene.

Induction of a P450III protein occurs in the rat as well as in primary cultures of hepatocytes (444). Protein radiolabeling experiments confirmed that TAO induction of P450p (PCN1 or PCN2) is due in part to a decrease in P450 degradation, while the degradation rates of other P450s were not affected by TAO. It has been postulated that this effect results from binding of TAO or its metabolite to the P450III protein (444). In fact, TAO binds to the enzyme in vivo, and this binding disrupts catalytic activity and inactivates the enzyme, suggesting that the long-held generalization that inactive proteins are more rapidly degraded might not apply to P450III (444). It should be noted that this study did not differentiate between the PCN1 or PCN2 as the site of action of TAO. It is reasonable to suspect in the absence of expression of the PCN1 gene in untreated rats (108) that the constitutively expressed PCN2 protein is being stabilized. Indeed, PCN2 and its mRNA are detectable at low levels in adult females, albeit at a level considerably lower than adult males (108).

One of the most profound examples of posttranscriptional regulation is seen with the IIE1 enzyme. This P450 is induced under a variety of conditions, including ethanol, acetone, and pyrazole treatment; fasting; and streptozotocin- or alloxan-induced diabetes. Administration of small organic compounds such as acetone, pyrazole, and ethanol to rats causes a rapid induction of IIE1 protein without affecting levels of IIE1 mRNA (404). Similarly, rabbit IIE1 protein is induced by imidazole without a sufficient increase in its mRNA (212). These results indicate a posttranscriptional regulation of IIE1 at either the translational level or by stabilization of the protein against degradation. Recent studies, using in vivo pulse labeling with amino acids, ruled out a translation increase and further suggested that the IIE1 protein is stabilized by pyrazole treatment (406). In the untreated rat, IIE1 was degraded biphasically with half-lives of 7 and 37 h whereas in rats pretreated with pyrazole, only the 37-h component of the degradation curve was noted. No change was noted in the turnover of total microsomal protein, suggesting that the effect was specific. The mechanism of this specific stabilization is unclear; however, it may be due to ligand binding. This is supported by studies using hepatocyte cultures (64). When rat hepatocytes were placed in culture, levels of IIE1 protein rapidly decreased. When these cells were incubated with ethanol or other inducers, the protein was stabilized against degradation. Furthermore, a correlation was found between the concentration of inducer required for stabilization and the binding affinity of the inducer for the enzyme. These results suggest that binding of inducers to IIE1 results in a protection of the enzyme against degradation (64).

In spontaneously diabetic rats or in rats made diabetic by treatment with streptozotocin or alloxan, IIE1 protein is induced to 6-fold above the level seen in untreated rats (17, 58, 68, 405). This increase is accompanied by up to a 10-fold increase in IIE1 mRNA in the absence of an increase in transcription of the IIE1 gene (405). When diabetic rats are given insulin, the effect is reversed (17, 58, 68). These data indicate that, in the diabetic rat, either the IIE1 mRNA is stabilized or that the efficiency of pre-mRNA is markedly enhanced. It was postulated, based on results correlating β-hydroxybutyrate levels with IIE1 protein levels, that ketone bodies are either directly or indirectly involved in the induction response (17). In this regard, fasting also causes an increase in IIE1 mRNA and protein (161) in addition to elevated levels of ketone bodies (279). The complex transcrip-

tional and posttranscriptional regulation of IIE1 is summarized in fig. 7.

P450IIB1/IIB2 protein and mRNA were increased in rats fed ethanol or acetone or animals that were starved (189). This study also showed a marked increase in transcription of the IIB genes after acetone treatment, whereas others found only a modest increase in IIB1/IIB2 mRNAs after treatment with methylpyrazole (404). The transcriptional activation of IIB1/IIB2 is in marked contrast to the posttranscriptional regulation of IIE1. It is quite interesting that these two P450 subfamilies are regulated differently by the same compound.

V. Genetic Heterogeneity and Polymorphisms of P450 Expression in Rodents

P450s appear to be one of the most polymorphic groups of enzymes. As discussed above, a tremendous variability of P450 expression is seen among different species of mammals. These intra- and interspecies variations in activities and enzymes suggest that many P450s are not important for day-to-day survival under steady-state conditions.

In defining a polymorphism in an activity, it is important to delineate the mechanism by which the polymorphism is generated. For instance, a loss of activity could be due to a loss of expressed protein or to a change in amino acid sequence leading to a loss of enzyme activity. In the latter case, the decrease in activity toward one substrate does not mean that the enzyme is inactive toward other substrates. This is particularly important in view of the pronounced overlapping substrate specificity observed for many forms of P450. Careful study of animal P450 polymorphisms may allow us to decipher those P450s that have crucial physiological roles, either biosynthetic or catabolic.

The mouse *Ah* locus polymorphism is due to an altered TCDD receptor having markedly different affinities for ligands such as benzo(*a*)pyrene, dibenz(*a*)anthracene, MC, and TCDD (306, 462). To date, no mouse strain lacking the receptor has been described, and various mammalian species contain receptors with markedly different affinities for their ligands. It is possible that there is an endogenous ligand that plays a key role either during development or under other circumstances. The ubiquitous nature of the IA1 gene supports a crucial role for the P450I family. Virtually every species examined has the IA1 protein or inducible benzo(*a*)pyrene hydroxylase activity.

In contrast to the IA gene family, enzymes in the P450II family are highly polymorphic. P450 polymorphisms in the IIB gene locus have been detected using two-dimensional electrophoresis (356). These studies established the existence of separate genetic loci for the IIB1 and IIB2 genes and further showed the presence of multiple allelic forms of the enzymes in various rat strains (356). Marked variability in protein levels of these enzymes among different rat strains has also been demonstrated (357, 469). For example, the Marshall 520/N strain of rat was deficient in the expression of IIB2 (357). The mechanism of this deficiency has not been established.

A defect in expression of IIB2 was found in a Japanese colony of the Sprague-Dawley rat strain Qdj:SD (145). This defect was spread throughout a colony of rats at Kyushu University and is due to an autosomal recessive mutation. The mutation has yet to be defined, but it

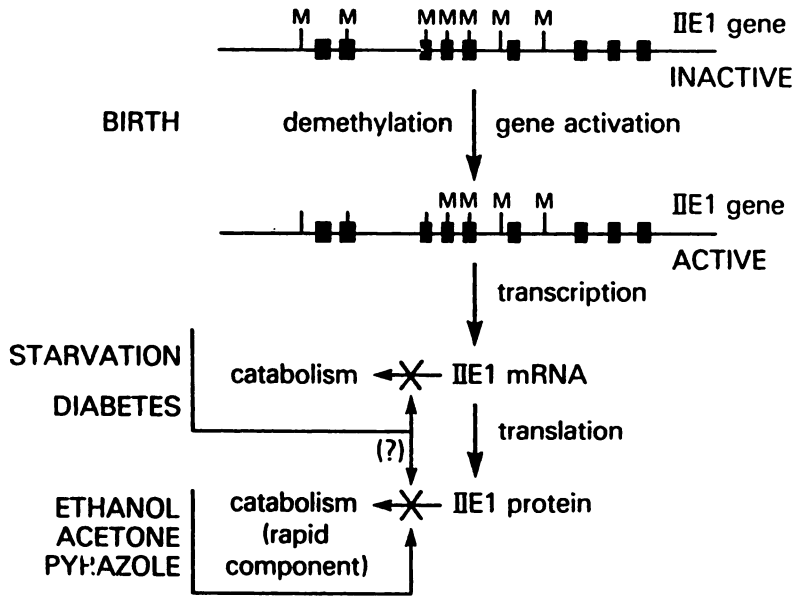

FIG. 7. Scheme depicting transcriptional and posttranscriptional regulation of IIE1 by various physiological or chemical signals. The IIE1 gene becomes transcriptionally activated after birth coincident with demethylation of certain cytosine residues (*M*) at the genes' 5′ end. In the adult, IIE1 is regulated posttranscriptionally by either protein and/or mRNA stabilization. It is unknown whether starvation or the diabetic state also stabilizes protein (indicated by ?).

could act to decrease IIB2 mRNA by several mechanisms, including: (*a*) lowering the transcription rate of the IIB2 gene; (*b*) the IIB2 pre-mRNA transcript could be inefficiently processed; or (*c*) the IIB2 mRNA could be usually unstable. No obvious nucleotide differences were found in the upstream DNA sequence of the Qdj:SD IIB2 gene when compared to a normal IIB2 gene (145).

As noted earlier, polymorphisms were detected in the expression of male-specific P450g in two rat strains (11, 268) and in the rabbit IIC5 gene (192, 427). The mechanism of the defect in rats is unclear, although it was postulated to be due to an altered mRNA (268). In rabbits, however, the polymorphic expression of P450 1 is due to differences in mRNA concentration (191, 427), suggesting the presence of mutations that either effect DNA elements controlling transcription of the P450 1 gene, pre-mRNA processing, or mRNA stability.

A rat model for the human debrisoquine 4-hydroxylase polymorphism was described in the DA rat (3). The female DA rat is deficient in metabolism of debrisoquine compared to Lewis and Sprague-Dawley rats. Controversy exists about the nature of this polymorphism. The female DA rat was shown to have lower levels of IID1 protein (250) and mRNA (35) than the Sprague-Dawley rat. Using immunoblotting techniques, others found no difference in IID proteins between male and female DA and Sprague-Dawley rats despite a 5-fold lower enzyme activity in female DA rats (103). It was recognized, however, that several P450s in the IID subfamily are expressed in the rat, and two have been purified (103). Genes and cDNAs representing five members of the IID subfamily have been isolated and sequenced, and their deduced amino acid sequences are about 73 to 95% similar to each other (author's laboratory, unpublished data). These data suggest that caution should be observed when using antibodies against IID1 for quantitation purposes because of potential cross-reactivities. In addition, the cDNAs have several areas of complete sequence identity. Using probes specific for each mRNA, however, it was further confirmed that the DA rat polymorphism is not due to a decrease in IID1 mRNA but may result in amino acid differences that alter catalytic activity of the protein (103). This result is in contrast to studies in humans in which the lack of protein was found to be associated with the poor metabolizer phenotype and the absence of IID1 enzymatic activity (107, 493). Taken together, these results suggest that rats may not be an adequate model for the human drug metabolism defects. Recent studies suggest that non-human primates might be more appropriate for pharmacogenetic investigations (179).

An interesting polymorphism was described for the expression of a mouse testosterone 16α-hydroxylase, designated I-P-450$_{16\alpha}$, a member of the IIB subfamily (316, 318, 319). Among 10 strains of mice, this enzyme and its mRNA were specifically repressed only in females of the 129/J strain. The sex-specific expression in active strains was inherited as an autosomal dominant trait from a locus on chromosome 7 (316). Interestingly, repression of I-P-450$_{16}$ in the 129/J strain was overcome by phenobarbital administration (319). These results suggest that the mutation affects only constitutive expression of an otherwise normal gene.

VI. Anomalous P450 Gene Transcripts and Pseudogenes

Pseudogenes are defective or nonfunctional genes that are prominent in many gene families. These genes arise through mutational inactivation, faulty gene duplication, or gene conversions and retroposon transpositions. To date, only one P450 pseudogene has been cloned and sequenced. A rabbit gene in the IIB subfamily was isolated and found to possess an altered 3′ splice signal in intron 2 and multiple deletions and insertions that would yield a defective protein (494).

Several anomalous P450 transcripts have recently been described. A pseudogene in the rat IIC subfamily related to PB-1 was postulated to produce a variant RNA transcript that contains a region of 159 nucleotides that appears to correspond to the presumptive entire eighth exon of the gene (213). This transcript could also be produced through an alternative splicing of a normal gene (213). The eighth exon replacement lacks the crucial heme-binding region of the P450 and would therefore be likely to produce a nonfunctional enzyme. Developmental regulation of the variant gene was similar to that of PB-1, suggesting that it possesses all of the control elements of the normal gene (213). The delineation of the mechanism by which this altered PB-1 gene is generated awaits the isolation and sequencing of its genomic clone.

In contrast to the PB-1 exon replacement gene, others have found evidence of a transcript in the mouse IID family that is lacking exon 6 (474). The putative protein produced from this variant is truncated by a premature termination codon and would be only 294 amino acids in length with a molecular weight of 33,300. A small protein that copurified with the mouse IID testosterone 16α-hydroxylase was thought to be a candidate for the truncated peptide (474). The abundance and regulation of this variant transcript were not addressed. Since multiple genes were identified in the mouse IID family, it remains likely that the variant transcript is the product of a pseudogene.

A second variant mouse transcript was found that is derived from a IIB gene (316). This transcript contains a 27-base pair insertion just prior to the region of the mRNA coding for the cysteine-containing heme-binding region, between the eighth and ninth exons. Interestingly, this transcript was found in several inbred strains of mice at approximately a 1:50 ratio of variant:normal mRNA (316). It is unclear whether this is produced from

an alternative splicing of a normal gene or from a pseudogene.

In humans, a variant RNA transcript that corresponds to the Hp1-1 cDNA has been cloned (323). It was postulated that this transcript is generated through alternative splicing in which a 3′ splice acceptor site in the intron of the Hp1-1 gene is used. Since this site is 39 base pairs upstream of the 3′ acceptor site producing the normal mRNA, a variant transcript is formed that contains a 39-nucleotide insertion. However, because of the additional 39 nucleotides, the Hp1-1-related protein would be prematurely terminated, resulting in the absence of the heme-binding region. The protein would therefore be enzymatically nonproductive. No precedent for alternative splicing exists in rodent P450s, and no clear function can be associated with this variant protein. It would seem entirely plausible that the variant transcript is the result of a mutant second allele of the Hp1-1 gene present in the liver that was analyzed. It is also noteworthy that a human genetic polymorphism in mephenytoin metabolism has been described in the IIC family (175, 241, 457); therefore, mutant genes may be common in this subfamily.

A defective transcript has also been found in the human IIB subfamily (276). This variant RNA contains intron 5, lacks exon 8, and could be the result of a pseudogene or a mutant allele of an otherwise functional gene. The former possibility is supported by Southern blot analysis showing that at least two genes are present in the human IIB subfamily (276). In any case, this subfamily is a candidate for a polymorphic human locus.

VII. Human P450 Genes

A. Characterization of Human P450s

One of the principal issues in human P450 research is whether individual variability in levels of certain P450s is associated with variable drug oxidations or susceptibility or resistance to chemically induced cancer. Indeed, recent studies have linked a P450 to a common drug metabolism defect (107, 110). P450s have not yet been clearly linked to human cancer.

Several laboratories have purified P450s from human livers; these organs were usually obtained from kidney donors or accident victims. The human IA2 was first purified as a phenacetin O-deethylase (56). This enzyme was also isolated in a catalytically inactive form by immunoaffinity chromatography using antibody against the rat ortholog of IA2, and its content was analyzed in several different liver specimens (476). IA2 probably metabolizes many of the substrates metabolized by its rodent counterparts, although this was not directly tested due to the denatured form of the purified human enzyme. Recent immunochemical evidence suggests that human IA2 demethylates theophylline (362) and is involved in O-deethylation of phenacetin (385), confirming an earlier report. Using antibodies specific for either rat IA1 or IA2, levels of IA2 were found to vary up to 10-fold among 10 liver specimens; however, only one liver contained a protein that reacted with the IA1 antibody (476). In this study no correlation of IA1 or IA2 content was found with smoking or drug treatments. In a separate study of 45 human liver specimens, IA2 was found at variable levels in all livers, whereas IA1 was undetected (385). In this study a correlation was found between levels of expression of IA2 and cigarette smoking (385). These results suggest that a marked heterogeneity exists in the expression of IA2 in human liver and that this enzyme is readily inducible in smokers. Further, IA1 may not be significantly expressed in untreated human liver; however, it has been detected in the placenta of smokers (403). These results are reminiscent of the inducibility of IA1 in extrahepatic tissues and the lack of IA1 expression in untreated rodent liver. Others found a correlation between MC-induced lymphocyte AHH activity and human IA1 mRNA expression (181). Marked intraindividual variations in maximally induced IA1 mRNA content were also evident.

Several groups have purified and studied a human P450 in the P450III family (124, 208, 223). It is likely that multiple P450III proteins are expressed in liver, as suggested by cDNA cloning (15, 106, 288). Therefore, for the purpose of this discussion, the human enzyme, referred to below as P450III, could represent one or more enzymes. Interestingly, a P450III protein is probably one of the only P450s significantly expressed in human fetal liver (223). Immunochemically detectable levels of one or more P450III proteins, varying from 5-fold to more than 10-fold, have been detected in human liver samples (106, 124, 451, 490). There is also evidence that a P450III enzyme can be induced in humans by glucocorticoids (288). The variability in P450III expression could have considerable clinical implications, since P450III metabolizes a variety of drugs, including the calcium channel-blocker nifedipine (106, 124), the contraceptive agent 17α-ethynylestradiol (121), progesterone (383, 451), the antiarrhythmic quinidine (125), the tranquilizer midazolam (238), the immunosupressant cyclosporine (237), and the antibiotic erythromycin (443). A P450III enzyme is also the major testosterone-metabolizing enzyme in human liver, carrying out hydroxylation at the 6β position (208, 451). Individual variability in hepatic P450III content could, therefore, have serious consequences in drug therapy. For instance, it was noted that cyclosporine therapy is complicated by coadministration of several other drugs (see references in 237). It is now apparent that this could result from the interaction of these drugs with a cyclosporine-metabolizing P450III (237).

The major P450 expressed in human fetal liver, P450HFLa, was purified (223) and shown to be related to $P450_{NF}$ and P450HLp in the P450III gene family (222). This enzyme was found in several fetal liver specimens that lacked detectable levels of several other P450s typ-

ically expressed in adult liver (477). HFLa catalyzes the 16α-hydroxylation of dehydroepiandrosterone 3-sulfate (222), testosterone 6β-hydroxylation, 7-ethoxycoumarin O-dethylation, and benzo(a)pyrene hydroxylation (221). The human fetal expression of a P450III gene is in contrast to rats, since PCN2 of the P450III family is not expressed until after birth (108). The function of hepatic P450HFLa during development is unknown, but it may play a major role in fetal metabolism and elimination of drugs and other substances.

Several members of the human IIC subfamily have been purified, including MP-1 (431), the S-mephenytoin 4-hydroxylase (130, 389), and P450 HLx (481). Among the drugs metabolized by MP-1 or related enzymes are the antiepileptic drug S-mephenytoin (130, 389) and the barbiturate hexobarbital (228). Other drug substrates for MP-1 include ethotoin, mephobarbital, methsuximide, and phensuximide (132). However, there is controversy regarding the relationship of MP-1 to tolbutamide metabolism. Although MP-1 carries out tolbutamide oxidation in vitro, in vivo studies suggest no correlation between mephenytoin and tolbutamide metabolism (229).

The human IIE1 was purified from liver and shown to catalyze demethylation of N-nitrosodimethylamine (480). Immunochemical studies also established a high correlation between N-nitrosodimethylamine demethylase and IIE1 protein in 16 liver specimens (490). Protein levels and activity varied up to 5-fold among liver samples, although one individual had an extremely high level of enzyme (490). Others have also documented interindividual variability in IIE1 protein expression (479). It remains to be established whether IIE1 expression is correlated with nitrosamine-associated cancer (162, 246).

B. Characterization of Defective P450 Genes Coding for Human Drug-metabolizing Enzymes—The Debrisoquine 4-Hydroxylase Polymorphism

The debrisoquine 4-hydroxylase polymorphism was discovered by urine metabolite testing of human subjects more than 10 yr ago (260; reviewed in refs. 61, 167, and 168). Two phenotypes were identified, the poor metabolizer and the extensive metabolizer. The former excretes debrisoquine, the unmetabolized parent compound, while the latter individuals excrete a large amount of the 4-hydroxy metabolite. A single P450, designated IID1, purified from human (19, 56, 129) and rat (103, 250) metabolizes the substrates associated with the in vivo characterized phenotype. The metabolism of other drugs, including sparteine (61, 62), dextromethorphan (242), bufuralol (50, 56, 129), and codeine (51), is affected in the same way by the debrisoquine 4-hydroxylase polymorphism, and hence these are metabolized by the same enzyme. Drugs such as debrisoquine and bufuralol are inactivated by IID1-mediated metabolism, while codeine is demethylated to its active component morphine (51). Polyclonal antibody against the rat enzyme effectively inhibited debrisoquine and bufuralol metabolism in human liver microsomes (107, 493). In addition, a strong correlation was found between bufuralol 1′-hydroxylase activity and IID1 protein expression in 29 human liver samples, suggesting that the debrisoquine polymorphism might result from an absence of IID1 protein (107).

Normal and mutant IID1 gene products were cloned and sequenced to study the mechanism by which the poor metabolizer phenotype is generated. The normal IID1 cDNA, isolated from an extensive metabolizer liver (110), carried out bufuralol oxidation when expressed in a COS cell expression vector (107). cDNA analysis of RNA from poor metabolizer livers revealed variant RNAs that were either longer or shorter than the normal IID1 mRNA transcript. The sequence of cDNAs isolated from the livers of poor metabolizers showed that the variant RNAs were the result of defective IID1 genes (107). These genes contained mutations that resulted in incorrectly spliced pre-mRNAs. One mutant allele produced a transcript from which the fifth intron could not be removed by splicing and another mutant allele produced a transcript from which the sixth intron could not be excised. A third variant RNA transcript was found that was generated from the incorrect use of a 3′ acceptor site in exon 6 and possibly a faulty pre-mRNA cleavage. Identification of these defects established the basis for the hypothesis that the debrisoquine hydroxylase polymorphism is a genetic mutation. By extrapolation from the frequency of poor metabolizer phenotypes of this autosomal recessive deficiency, it was estimated that mutant alleles account for 35 to 43% of all IID1 genes. Interestingly the poor metabolizer phenotype has not been found in Japanese (299).

C. Diagnostic Analysis of Mutant IID1 Genes

By studying RFLP linkage to IID1 alleles, it is possible to identify mutant alleles in individual subjects. The strategy for this type of analysis is depicted in fig. 8. The principle behind linkage of RFLPs to mutant genes is as follows. The eucaryotic genome contains a large amount of unused DNA that does not serve a coding purpose. Typically, genes flanked by this DNA can be subjected to random, nondetrimental deletions, insertions, and base changes. Introns are also subjected to multiple nondetrimental mutations, since the bulk of intron sequences carry no functional splicing signals. These changes can be detected and analyzed, in many cases, by restriction enzymes. As shown in fig. 8*A*, if a particular RFLP is linked to a mutant allele, the RFLP can be used as a convenient marker for this allele. In this hypothetical case, normal and mutant alleles of the gene exist in the population. The mutant allele is linked to an upstream *Eco*RI site (site 2) that is missing in the normal allele, resulting in two different size fragments on a Southern blot. It should be noted that this *Eco*RI site is in the upstream DNA and does not affect the activity of the gene. Therefore, the missing *Eco*RI site is "linked"

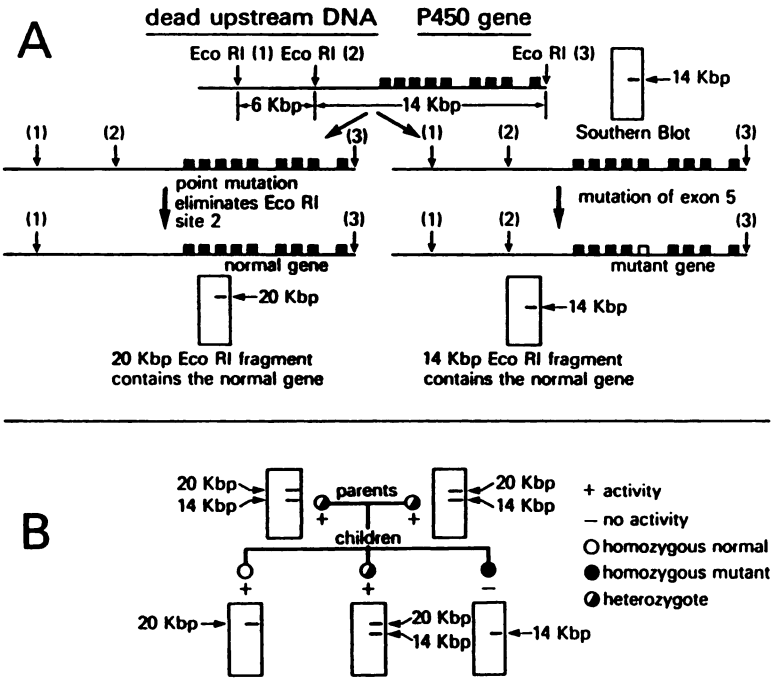

FIG. 8. Detection of mutant genes using RFLPs. *A* shows a hypothetical genome that contains a gene with nine exons. The lineage on the *left* has lost an *Eco*RI restriction enzyme site resulting in a 20-kilobase *Eco*RI fragment that anneals with a probe that detects the gene. The lineage on the *right* remains intact except for the mutation of exon 3, resulting in a defective gene. Since the *Eco*RI site 2 is still intact, a 14-kilobase fragment is produced by hybridization with a probe to the gene. Therefore, the 20-kilobase fragment is linked to a normal gene, and the 14-kilobase fragment is linked to a mutant gene. These fragments constitute an RFLP. A family analysis of this RFLP and its relationship to the activity of the gene is depicted in *Kbp*, kilobase pairs.

to the gene. This strategy was used to analyze a human population for debrisoquine metabolism. Analysis of families (Fig. 8*B*) is particularly useful in establishing the linkage between mutant alleles and RFLPs.

To determine RFLP linkage to IID1 alleles, a population of 53 unrelated individuals and 6 families were phenotyped for debrisoquine metabolism by urine test and leukocyte analysis (397). DNAs were isolated and analyzed by Southern blots using the IID1 cDNA probe. A comparison of RFLPs detected with various restriction enzymes with debrisoquine phenotypes showed the presence of two independent mutated alleles of the IID1 gene (397). These two alleles represented about 75% of all poor metabolizer phenotypes. RFLPs that correlate with the remaining 25% of poor metabolizers have not been found. These may represent various more recent gene-inactivating mutations. In any case, these studies confirm the genetic mechanism for the in vivo phenotyped debrisoquine 4-hydroxylase polymorphisms.

D. Other Defective Human P450s

At least three other polymorphic drug metabolism phenotypes have been characterized. The S-mephenytoin phenotype has been extensively studied and shown to be a result of an autosomal recessive mutation (174, 175, 241, 440, 457, 465). This drug metabolism deficiency is very prominent in oriental subjects, affecting up to 18% of the population (440). There are multiple gene products related to the S-mephenytoin hydroxylase (84, 126, 219, 431), and one of these may be responsible for the polymorphic tolbutamide metabolism (229).

A polymorphism associated with the P450III gene family (15, 106, 288) has been described for the calcium channel-blocker nifedipine (225). This polymorphism has yet to be adequately confirmed by other laboratories, and family studies have not been undertaken.

VIII. Summary and Future Prospects

Since 1980 a large body of information has accrued on the structure, evolution, and regulation of P450s, and a systematic P450 nomenclature system has been established. It has also become clear that rodent and human P450s, particularly those in the P450II family, are quite different, in terms of the numbers of P450 genes, the complexity of P450 genes, the catalytic activities of P450s, and the regulation of P450 genes. These data explain the wide differences between rodents and other mammals in drug metabolism and clearance, toxicity and susceptibility to chemically induced cancer. The dramatic difference in the evolution of P450s and their activities further suggests that the selective pressure for P450 evolution is probably environmental, including dietary habits. For instance, ingestion of plant alkaloids and toxins by different species of mammals may have governed the composition of their P450 gene milieu. It is now becoming clear that the many drug- and carcinogen-metabolizing activities that are unique to a particular species and even strain of an animal are due to qualitative

differences in P450 genes. Profound differences exist even in human populations. For instance, the Japanese population contains 18% poor metabolizers of S-mephenytoin, while Caucasians have 1 to 5% (175, 241, 440, 457). The debrisoquine 4-hydroxylase polymorphism, however, has not been seen in Oriental subjects. Genetic diversity of P450s between the major races should be examined carefully.

One of the principal reasons for the genetic diversity of P450 genes between species is the gene duplication events that have occurred during the last 5 to 50 million yr, following the divergence of many species. For instance, the human and rodent lines split about 75 million yr ago. Monkeys may be a more reasonable species than humans for studying drug and carcinogen metabolisms. This is an area that has not been explored at the biochemical level.

Because humans have many P450 genes, it is possible that other mutant alleles have escaped detection because of the lack of a specific phenotyping test. Direct isolation of human cDNAs and analysis of liver specimens may identify additional mutant human genes. Polymorphic expression of P450s might explain other drug reactions and may form the basis for individual susceptibility to chemically induced lung cancer. For instance, lung cancer patients who smoke have been found to be predominantly extensive metabolizers of debrisoquine (8). These patients very efficiently convert debrisoquine to its 4-hydroxy metabolite. Although the IID1 is not known to metabolize several common carcinogens (473), an exhaustive survey of carcinogen metabolism by this enzyme has not been carried out. This study is now feasible because we have the ability to express the IID1 cDNA in culture (107). It must also be determined whether expression of other P450s is associated with certain cancers.

The inability to correlate human and rodent drug and carcinogen metabolism further stresses the importance of thorough analysis of human P450s. Since many human P450s may be difficult to purify directly, this can be accomplished by direct cDNA cloning using cross-reacting rodent cDNA probes. The cDNAs can then be expressed into active enzymes and their substrate specificities assessed. The probes can also be used to search for mutant P450 genes and polymorphisms.

The P450 gene superfamily is probably the most complex with respect to regulatory controls. A tremendous amount of information can be gained about the molecular mechanisms of developmental gene regulation and regulation by chemical inducers and hormones. The mechanisms governing transcriptional and posttranscriptional regulation must be accurately defined. For instance, the receptors and factors associated with dexamethasone, clofibrate, and phenobarbital induction should be characterized. The hormonal controls and *cis*-acting elements associated with developmental regulation should be delineated.

Another major question is whether certain P450s play a crucial role during development or in homeostasis. For example, is the IA1 gene involved in development, and is the IIE1 gene a key enzyme in ketone body metabolism and gluconeogenesis? Are some of the drug-metabolizing liver enzymes also involved in brain steroid metabolism? Are the P450IV fatty acid-metabolizing enzymes involved in arachidonic acid, prostaglandin, and leukotriene metabolic pathways of key physiological importance?

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