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Evolution of the cytochrome P450 genes

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1. The P450 gene superfamily is presently known to contain more than 78 members, divided into 14 families.

2. The superfamily has undergone divergent evolution, and the ancestral gene is probably more than 2 billion years old.

3. The recent 'burst' in new P450 genes, particularly in the II family during the past 800 million years, appears to be the result of 'animal–plant warfare'.

4. Due to the presence or absence of a particular P450 gene in one species but not the other, it may not be correct to extrapolate toxicity or cancer data from rodent to human.

5. Increases in the P450 gene product (enzyme induction) almost always reflect an elevated rate in gene transcription, although there are several exceptions.

6. The mechanisms of P450 gene regulation (induction) by classes of inducers might become better understood through the comparison of different phyla that differ in response to a particular class of inducers.

7. Amongst several carefully selected phyla, delineation between which electron donor (presence of Fe_2S_2 protein or NADPH-P450 oxidoreductase, or both) interacts with P450 may provide valuable information about the evolution of eukaryotes from prokaryotes.

Introduction

It is now clear that what appeared 10–20 years ago to be a small number of cytochrome P450 proteins was simply a reflection of the limitations in biochemical methodology at the time. With the advent of recombinant DNA and ancillary techniques during this past decade, we have come to appreciate the magnitude and complexity of the P450 gene superfamily.

Experimental

The procedures used for nucleotide and amino acid sequencing, as well as computer analyses of these data, have been detailed elsewhere (Nebert *et al.* 1987, Nebert and Gonzalez 1987, Nelson and Strobel 1987).

Results and discussion

Evolution of the gene product

The divergence of distinct P450 gene families from a common ancestor has been estimated with the aid of computer algorithms for sequence alignment and phylogenetic tree construction. From such estimations a nomenclature system has been proposed, with Roman numerals and capital letters denoting families and subfamilies, respectively (Nebert *et al.* 1987). The nomenclature for human and

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mouse chromosomal loci (P450 genes) has also been suggested (Nebert *et al.* 1989). Thus, for example, the *CYP1A1* gene in family I encodes P450 IA1, having trivial names of mouse or human P_1450, rat P450c and rabbit form 6. The *CYP17* gene in family XVII, as another example, encodes steroid 17α-hydroxylase.

If one examines the amino acid sequences by global alignment programs, most P450 proteins are markedly different. However, there is a 26-residue region near the carboxy-terminus of all P450 proteins that exhibits a high degree of similarity (Adesnik and Atchison 1986, Black and Coon 1986, Nebert and Gonzalez 1987). These data strongly suggest that all P450 genes—ranging from bacteria to humans—have diverged from the same ancestral gene, which probably existed more than 2 billion years ago (figure 1).

In our data base we have more than 100 amino acid sequences representing 78 unique P450 genes, plus six pseudogenes (Nebert *et al.* 1989). The source of knowledge about these genes includes protein, cDNA or genomic DNA data from 11 eukaryotes and two prokaryotes. Each P450 gene produces a single protein (the enzyme). There is no evidence of alternative splicing, i.e. differential processing of the P450 transcript such that entire exons or portions of exons are removed or added in order to produce a gene product with a new catalytic function. The P450 gene superfamily comprises at least 14 families (figure 1). At present, family II has eight subfamilies, and families IV and XI each have two subfamilies.

From 3003 pairwise comparisons of the 78 available aligned P450 amino acid sequences, a P450 protein sequence from one gene family has been found to exhibit $\leqslant 40\%$ identity to that from any other family. This definition of a P450 gene family is arbitrary, but has turned out to be very useful (Nebert *et al.* 1989). Within a single family the P450 protein sequences are $>40\%$ identical with two minor exceptions: (1) the nuclear genes encoding two mitochondrial P450 proteins, scc and 11β, are included in the same family (family XI), even though they are only 37·5–38·8% identical; (2) the IID subfamily includes the most distant members of the P450 II family. Exclusion of the IID sequences results in all remaining family II sequences being $\geqslant 41{\cdot}6\%$ similar to each other; when the IID sequences are included, this value drops to $\geqslant 32{\cdot}1\%$. Mammalian sequences within the same subfamily are always $>59\%$ identical. Inclusion of more distant species within the same subfamily (e.g. chicken 17α and trout IA1) drops this value to $>47\%$. By pairwise comparisons of aligned P450 sequences (Nelson and Strobel 1987), the chicken PB15 is considered an 'outlier', necessitating assignment to its own subfamily. The trout IA1 and the chicken 17α, however, are clearly the orthologous genes of the mammalian IA1 and 17α genes, respectively, and do not justify assignment to their own unique subfamilies.

It is interesting to note that the chicken sequences (17α, IIH1 and aromatase) do not all branch from the mammalian genes at the same evolutionary distance. In fact, the human and chicken *CYP16* genes appear to have diverged more than twice as long ago as the human and chicken *CYP19* genes, yet the original gene undoubtedly existed before the predecessors of mammals and birds split. This discrepancy points out the difficulties with any computer program that attempts to define a phylogenetic tree from a combination of fossil and molecular biology data (discussed in Nebert and Gonzalez 1987, Nelson and Strobel 1987). Another consideration is the real possibility that the *CYP17* and the *CYP19* genes have not diverged at the same rate.

In those instances that have been examined thus far, genes within a defined subfamily have been found to lie within the same 'cluster' on the chromosome

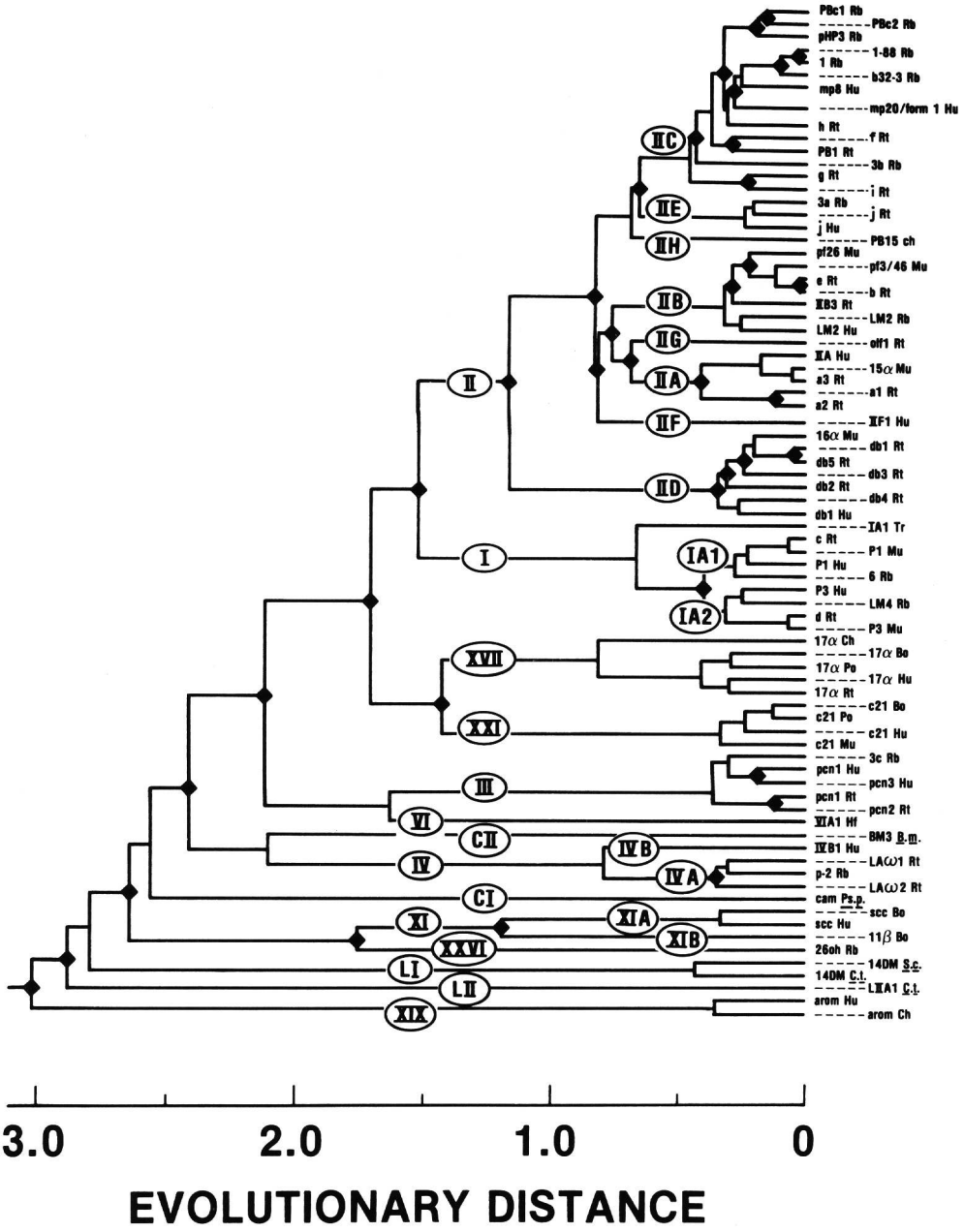

Figure 1. Unweighted-pair-group method of analysis (UPGMA) phylogenetic tree of the P450 gene superfamily based on the available sequences of 78 unique genes.

The names and sources of these genes are summarized in Nebert *et al.* 1989. The UPGMA is described by Sneath and Sokal 1973). The 39 gene duplications established to date are denoted by diamonds. The divergence between bacterial and eukaryotic genes ($d=2{\cdot}5$) has been set at 1400 million years—base pairs (Mybp), the date of the earliest evidence of eukaryotic microfossils (Vidal 1984). The estimations of time in the oldest part of the tree are subject to the largest error (discussed in Nelson and Strobel 1987).

(Nebert *et al.* 1989). Where the classification of proteins encoded by orthologous genes cannot be certain—particularly between widely diverged species and especially in subfamilies containing three or more genes—sequential numbering on a chronological basis has been recommended as the protein sequences become available in the literature (e.g. *CYP2D1*, *CYP2D2*, *CYP2D3*, etc.).

There appears to have been an 'explosion' of new P450 genes, particularly in family II, during the past 800 million years (figure 1). Family II is known to have had a minimum of 24 gene duplications. The emergence of a new gene results from a sequence of events: (1) a gene duplication event, followed by (2) divergence (or 'drift') due to mutations and, ultimately, (2) fixation of a gene having in some or most instances selective advantages during evolutionary pressures. The emergence of many new P450 genes, particularly in family II, most likely reflects 'animal–plant warfare' that has been carried on during the past 800 million years. As animals diverged from plants and began to ingest plants, the plants enhanced their own survival by synthesizing new metabolites that are increasingly toxic, as well as less palatable and/or less digestible. In return, animals responded by developing new enzymes to process the new noxious plant metabolites. Twentieth-century drugs and pesticides are often derived from plant metabolites and resemble, in chemical structure, such metabolites. Examples include digitoxin, warfarin, ergotamine, quinine and ellipticine. It is therefore not surprising that animals possess sufficiently diverse P450 enzymes to handle essentially any newly synthesized pharmaceutical agent or chemical: the P450 superfamily in animals has evolved under this kind of evolutionary pressure for at least 800 million years.

The evolutionary scheme in figure 1 might be appreciated by regulatory agencies from a different standpoint. Since the time of the human–rodent split approximately 80 million years ago, it is entirely possible that different mammalian species will have varying numbers of functional P450 genes in their corresponding subfamily. Four such examples have already been well documented (Nebert and Gonzalez 1989). (1) The rat IID subfamily has five active genes, while the human IID subfamily has only one active gene and two pseudogenes. (2) Rabbit forms 1 and 1–88 in the IIC subfamily have diverged after the rabbit–rodent separation, meaning that one of these two genes does not exist in the rat or mouse (or human). (3) Rat b and e diverged approximately 12 million years ago, whereas the rat–mouse split occurred about 17 million years ago; this means that the orthologue of rat b (or e) does not exist in the mouse (or rabbit or human). (4) The rabbit IIE subfamily has two members that arose via gene duplication after the rabbit–rodent divergence, meaning that one of these two genes does not exist in the rat or mouse (or human). These observations provide an evolutionary explanation for differences in drug metabolism between, for example, human and rat. These data might be important for regulatory agencies and pharmaceutical companies to consider: the degree of toxicity, teratogenicity or carcinogenicity of a new drug determined in the rat may not be similar to that in the human—due to the presence or absence of a particular P450 gene in one of the two species.

Evolution of P450 regulation

Many P450 genes have been shown to be under complex, and highly unique, control during development and under the influence of sex-specific signals (Nebert and Gonzalez 1987). By far the most common mechanism for increases in the P450 enzyme involves transcriptional activation of the gene (table 1). Genes of all nine

Table 1. Classification of P450 inducers.

Inducing chemicals	P450 gene family or subfamily affected
Polycyclic hydrocarbons, 2,3,7,8-tetrachlorobenzo-*p*-dioxin, benzoflavones	I, IIA
Phenobarbital, terpenoids	IIB, IIC, III, VI
Ethanol, acetone, pyrazole, imidazole	IIE†‡
Pregnenolone 16α-carbonitrile, dexamethasone	III
Rifampicin, triacetyloleandromycin, griseofulvin	III†
Clofibrate, other peroxisome proliferators	IV
ACTH (cyclic AMP)	XI, XVII, XIX, XXI
Tetradecane	LII
Camphor	CI
Phenobarbital	CII

Unless otherwise indicated, an increase in P450 protein has been shown to reflect increases in the transcriptional rate of the gene (Nebert and Gonzalez 1989).

† Post-transcriptional.

‡ Post-translational.

mammalian families are known to respond to exogenous inducers or endogenous signals in this way.

In addition, several P450 genes have been shown to be regulated post-transcriptionally (Nebert and Gonzalez 1987, 1989, Gonzalez 1988). For example, treatment of rabbits with the macrolide antibiotic triacetyloleandromycin results in an increase in 3c (P450 III) mRNA without any increase in transcriptional rate of the gene. Treatment of rats with dexamethasone leads to marked increases in b (IIB) mRNA in the absence of any increase in the transcriptional rate of the gene. Treatment of rats with ethanol, acetone or 4-methylpyrazole causes a rapid increase in the j protein (IIE1) in the absence of any increase in j mRNA, suggesting a post-translational induction process. On the other hand, rats rendered diabetic exhibit a 10-fold increase in j mRNA in the absence of an increase in transcription. Treatment of mice with TCDD leads to a combination of transcriptional and post-transcriptional effects on constitutive and inducible *CYP1A1* and *CYP1A2* gene expression (family I) (Nebert and Jones 1989), as well as striking tissue-specific differences in these effects (Kimura *et al.* 1986).

The *CYP11A1*, *CYP11B1*, *CYP17*, *CYP19* and *CYP21* genes are transcriptionally activated by certain endogenous signals (e.g. ACTH, prostaglandins, FSH, hCG, gastrin, prolactin, LH) through the action of cyclic AMP (Miller 1988, Nebert and Gonzalez 1987 and references therein). It appears that cAMP does not stimulate P450 gene transcription directly, but rather by way of an intermediate protein.

Inducible P450 enzymic activity has been detected in at least 15 (table 2 and figure 2) of the 89 phyla categorized in the Whittaker five-kingdom classification system (Margulis 1981, Field *et al.* 1988). However, a phylogenetic study of P450 gene expression and inducibility has by no means been performed in any systematic, exhaustive manner. It is possible that the induction response to a class of compounds will have evolved in particular phyla as a response to selective advantages during evolution.

It is intriguing that fish, reptiles and amphibians appear to exhibit P450 I induction by polycyclic hydrocarbons but evidently do not respond to P450 induction by phenobarbital (Schwenn and Mannering 1982). If this finding is

Table 2. Summary of phyla in which P450 activity has been detected and in which P450 induction by foreign chemicals is known to occur.

Superkingdom Phylum	P450†	References
Prokaryota		Parsons *et al.* 1983
Kingdom Monera		
Nitrogen-fixing bacteria (*Beijerinckia*)	BPh	Gibson *et al.* 1975
Pseudomonads	CAM	Unger *et al.* 1986
Aeroendospora (*Bacillus*)	PB	Wen and Fulco 1987
Actinobacteria (*Streptomyces*)	IsoF	Romesser and O'Keefe 1986 Sarlaslani and Kunz 1986
Eukaryota		
Kingdom Protoctista		
Zoomastigina (*Trypanosoma cruzi*)	2	Agosin *et al.* 1976, 1984
[?] Ciliophora (*Tetrahymena*)	none?	Iida *et al.* 1979
Kingdom Fungi		
Zygomycota (*Rhizopus*)	1, 3	Dus *et al.* 1976, Breskvar and Hudnik-Plevnik 1978, Matthews and van Etten 1983
Ascomycota (*Saccharomyces, Aspergillus, Penicillium, Cunninghamella elegans*)	1, 2	Ferris *et al.* 1973, Cerniglia and Gibson 1979, Lin and Kapoor 1979, Woods and Wiseman 1980, Bhatnagar *et al.* 1982, Dutta *et al.* 1983, King *et al.* 1983, Kappeli 1986, Kalb *et al.* 1987
Deuteromycota (*Candida*)	ALK	Sanglard *et al.* 1987
Kingdom Animalia		
Nematoda (*Caenorhabditis elegans*)	1	
Mollusca	none?	
Annelida	1	Lee *et al.* 1981
Arthropoda		
Crustacea	1, 2	Batel *et al.* 1988
Insecta	1, 2	Capdevila *et al.* 1973, Chang *et al.* 1983
Echinodermata	1	
Chordata		
SUBPHYLUM		
Vertebrata		
CLASS		
Agnatha	1	
Chondrichthyes	1	
Osteichthyes	1	
Amphibia	1	
Reptilia	1	
Aves (birds)	1, 2	
Mammalia	1, 2, 3, 4, A	
Kingdom Plantae		
Angiospermophyta	2, 4	van der Trenk and Sandermann, Jr, 1980, Salaun *et al.* 1978, 1981, Hendry and Jones 1984

The order of these major phyla and classes is taken from the modified Whittaker five-kingdom classification system (Margulis 1981). The reference list is by no means intended to be complete, particularly in **Kingdom Animalia**, for which there exist innumerable publications describing P450 activity and inducibility. We were unable to find any unequivocal study of P450 induction in **Mollusca**. It should be noted that, in addition to the inducing agents listed here, induction of plant P450 has been reported to occur by wounding, geraniol, *trans*-cinnamate, ethanol, herbicides and manganese (Benveniste *et al.* 1977, Reichhart *et al.* 1980), and *Streptomyces* P450 can be induced by soybean flour (Sarlaslani and Kunz 1986).

† Abbreviations: BPh, biphenyl induction of prokaryotic P450; CAM, camphor-inducible; PB, phenobarbital induction of prokaryotic P450; IsoF, induction by isoflavonoids; ALK, alkane-inducible; 1, induction by tetrachlorodibenzo-*p*-dioxin, benzoflavone and/or polycyclic hydrocarbons; 2, phenobarbital-inducible; 3, steroid-inducible; 4, clofibrate-inducible; A, cyclic AMP-mediated induction by ACTH.

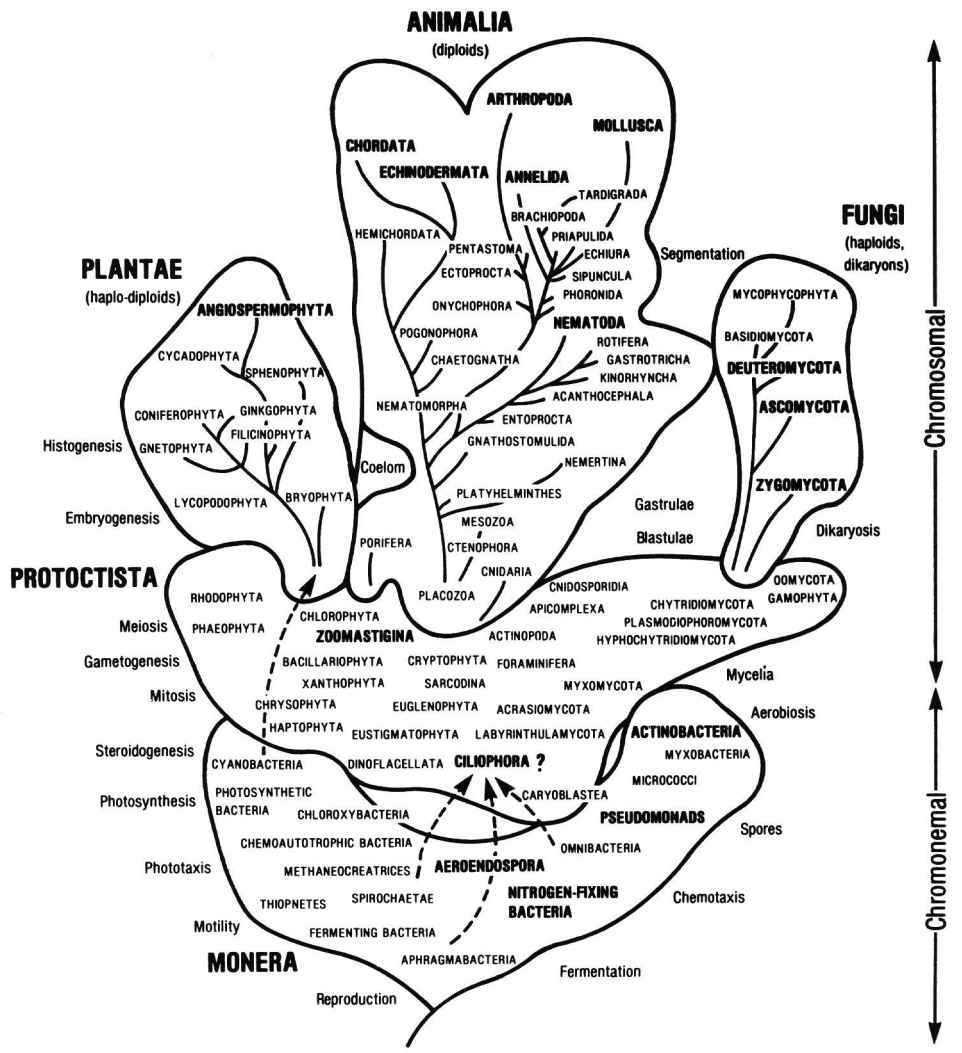

Figure 2. Illustration of the modified Whittaker five-kingdom classification system of 89 phyla. Modified from Margulis 1981. The 16 phyla *in bold*, to our knowledge, represent those in which inducible P450 gene expression (i.e. enzyme activity or spectral data in most cases) has been studied to date. 'CILIOPHORA?' indicates that no microsomal P450 enzyme activity was detected in *Tetrahymena* (Iida *et al.* 1979). (Reproduced with permission from the author and W. H. Freeman and Company, San Francisco).

correct, it is conceivable that these classes of vertebrates might have lost the phenobarbital induction system because of a survival advantage to living in water, while the land vertebrates proper retained phenobarbital inducibility. To understand why P450 genes in fish, reptiles and amphibians do not respond to phenobarbital while P450 genes in insects do, for example, may provide valuable insight into the mechanism of P450 induction by phenobarbital.

Evolution of the electron transport system

Figure 2 illustrates how few phyla have actually been proven to have P450 genes, compared with the total number of phyla that have been characterized. One possible

bridge between the original P450 ancestor and present-day P450 genes might lie in the reductase that supplies reducing equivalents to the P450 enzyme. One of the earliest systems in prokaryotes includes putidaredoxin, a ferredoxin (Fe_2S_2 protein), which shuttles electrons from NADH via putidaredoxin reductase, to P450cam and ultimately to oxygen during the metabolism of camphor (figure 3). Besides the CI family, the only other P450 families interacting with a similar electron transport pathway are families XI and XXVI; these genes encode mitochondrial P450 proteins that utilize the Fe_2S_2 protein adrenodoxin, which shuttles electrons from NADPH via adrenodoxin reductase. It is possible that mitochondria arose as bacterial endosymbionts within an ancestral eukaryotic cell—such as the α-purple bacterium (Yang *et al.* 1985)—and that mitochondrial genes 'escaped' to the nucleus on one or more occasions. The similarity between putidaredoxin and putidaredoxin reductase in pseudomonads, and mitochondrial adrenodoxin and adrenodoxin reductase in vertebrates, is consistent with this view.

How did the microsomal P450 redox system come to exist? One of the other early systems in prokaryotes is represented by the combination of NADPH-P450 oxidoreductase and P450 from family CII in the same protein molecule (Narhi and Fulco 1987, Wen and Fulco 1987). This system appears to be quite similar to present-day mammalian microsomal systems (figure 3). Interestingly, the amino acid sequences of families CII (*B. megaterium*) and IV (mammals) are more similar to one another than to any of the other 12 gene families (figure 1), and both gene products catalyze the ω-hydroxylation of fatty acids, prostaglandins and detergents (Nebert and Gonzalez 1989). One possibility, therefore, is that the first eukaryote originated from a fusion between two prokaryotes containing the CI (Fe_2S_2 protein) and the CII (NADPH-P450 oxidoreductase) electron transport systems, respectively (Nelson and Strobel 1987).

Another possibility is that the NADPH-P450 oxidoreductase is quite recent in eukaryote evolution, and was the result of a fusion between the prokaryotic FAD-containing ferredoxin reductase and the prokaryotic FMN-containing flavodoxin genes (Porter and Kasper 1986). The resultant NADPH-P450 oxidoreductase,

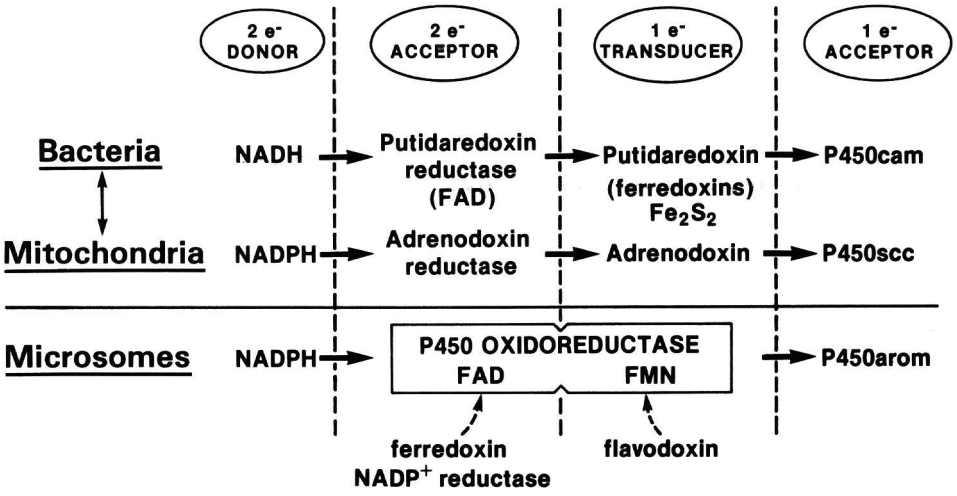

Figure 3. Summary of the prokaryotic, mitochondrial and microsomal P450 electron transport pathways.

containing both FAD and FMN, in the early eukaryote then acquired several important early functions such as participation in haem oxygenation and the cyanide-sensitive fatty acid desaturase system. With the development of nuclear chromosomal genes encoding both mitochondrial and microsomal proteins in the ancestral eukaryote, the mitochondrial P450 proteins continued to be serviced by their mitochondrial Fe_2S_2 reductase while the newly evolved microsomal P450 proteins were in need of a reductase having a satisfactory redox potential. Perhaps the NADPH-P450 oxidoreductase, because it already existed for haem oxygenation and fatty acid desaturation, was recruited for this purpose and became a successful partner in the microsomal P450 monooxygenase system.

This division between mitochondrial and microsomal enzymes might have occurred in some, but not other, phyla of the Protoctista (figure 2); it would therefore be interesting to see if some Protoctista phyla contain either the mitochondrial or microsomal P450 monooxygenase system, but not both. In accordance with this argument, it should be pointed out (table 2) that one laboratory reported no detection of microsomal P450 enzymic activity in *Tetrahymena pyriformis*, a member of the Ciliophora phylum in the Protoctista Kingdom (Iida *et al.* 1979).

In either scenario discussed above, one would view the microsomal P450 electron transport system as relatively 'modern', compared with the mitochondrial P450 electron transport pathway.

Evolution of catalytic activities

A very early role for prokaryotic P450 involved the assimilation of environmental chemicals. The induction of P450cam by camphor and P450alk by alkanes, and subsequent metabolism of these inducers as an energy source, are two examples of this role in pseudomonads (Unger *et al.* 1986) and *Candida tropicalis* (Sanglard *et al.* 1987), respectively. The induction of benzpyrene hydroxylase by benzpyrene, and subsequent metabolism of this inducer in organisms as diverse as mammals (Nebert and Gonzalez 1987) and *Cunninghamella elegans* (Cerniglia and Gibson 1979) might be considered to resemble the example in bacteria and yeast.

The other early role for P450 seems to centre on steroids. Metabolism of one or more steroids is effected by P450 enzymes in families I (e.g. *CYP1A2* encodes oestrogen 2-hydroxylase), II, III, XI, XVII, XIX, XXI, XXVI and LI. The oldest connection between P450 and cholesterol is probably the removal of the 14-methyl group from lanosterol (family LI). Cholesterol side-chain cleavage is found in plants that make cardiac glycosides (Petersen and Seitz 1985). Steroidogenesis originated with the earliest of the Protoctista (figure 2). This is not to be confused with phyla that developed later, such as Nematoda and Arthropoda, which appear to have lost squalene cyclase so that they depend upon dietary sterols; the subsequent P450 steroidogenesis pathways would be expected to have remained intact in these two phyla. Indeed, oxygenated ecdysteroids are used as hormones by nematodes and arthropods. Cholesterol biosynthesis was apparently carried out even before the divergence of prokaryotes from eukaryotes (figure 2), because there are three types of bacteria known to contain—and presumably synthesize—cholesterol or partially demethylated lanosterol derivatives (Bloch 1983). It would be of interest if some phyla in the Protoctista Kingdom have not developed steroidogenesis and do not have P450 genes associated with steroidogenesis.

Evolution of substrate reactions

The P450 genes might also be divided by means of a consideration of substrate reactions, i.e. ω-hydroxylation versus steroid metabolism. The ω-hydroxylation of alkanes (family LII) is similar to the ω-hydroxylation of fatty acids and prostaglandins (family IV); thus, one might consider a P450 ancestral gene having given rise to families IV, LII, CI and CII that appear to bear no obvious relationship to steroid substrates. All of the other families so far characterized—I, II, III, XI, XVII, XIX, XXI, XXVI and LI—encode at least one gene having steroid metabolism properties. The subsequent expansion of P450 genes, especially in family II, leading to many P450 enzymes taking part in animal–plant warfare, was discussed earlier.

A third class of catalytic activities might involve P450-mediated reactions in which atmospheric oxygen or electron transport is not required. An example of this would be thromboxane synthase (Haurand and Ullrich 1985) and prostacyclin synthase (Hecker and Ullrich 1988). This system is microsomal and appears to be so specialized (i.e. specific to mammalian platelets and megakaryocytes) that these P450 genes will probably be found to be more recently evolved, compared with those of pseudomonads and Bacillus. Unfortunately, at the present time these genes remain to be isolated and characterized.

Plant versus animal P450 genes

Other than studies in mammals, P450 induction by 'classes' of inducers has gone largely unstudied. Interestingly, P450 induction by clofibrate occurs not only in mammalian liver but in higher plants such as the Jerusalem artichoke and potato tuber (table 2). A much better understanding of the evolution of P450 regulatory mechanisms and classes of inducers is needed. It is anticipated, for example, that more comparative phylogenetic work on the P450 gene superfamily will lead to important advances in the fields of entomology and botany. Such studies should aid in the development of nutritional crops for third-world countries through the genetic engineering of successful insecticide-, pesticide- and herbicide-resistant plants.

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