The Molecular Biology of Drug-Metabolizing Enzymes

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Structure and expression of genes coding for components of the cytochrome P-450-mediated mono-oxygenase

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The cytochrome P-450-mediated mono-oxygenase system plays an important role in the synthesis of endogenous compounds such as steroids, fatty acids and prostaglandins. In effect, cytochromes P-450 contain an NADPH-dependent cytochrome P-450 reductase. Individual organisms usually possess a multiplicity of cytochromes P-450, many of which are inducible by a variety of foreign compounds.

Our group is investigating the structure and function of components of the mono-oxygenase system and the organization and expression of the corresponding genes. In particular, we are interested in the molecular mechanism by which cytochromes P-450 are induced by foreign compounds.

We have purified, from rat liver microsomal membranes, cytochrome P-450 reductase, cytochrome P-450 and several different cytochromes P-450. We have raised antibodies to, and isolated cloned cDNAs coding for each of these proteins. In this paper we will present aspects of our work on the various protein components of the mono-oxygenase.

Abbreviation used: PB P-450, the major phenobarbital-inducible form of cytochrome P-450.

The term cytochrome P-450 refers to a superfamily of haemoproteins that range in amino acid sequence homology from < 20% to > 97%. Based on amino acid sequence data a recent attempt has been made, by several workers in the field, to rationalize P-450 gene nomenclature (Neber et al., 1987).

Induction of cytochrome P-450 by phenobarbital

We are particularly interested in the induction, by the barbiturate phenobarbital, of two rat liver cytochromes P-450 known as P-450s b and c. These proteins are members of the P450IIH sub-family. As their amino acid sequences are over 97% homologous they will be referred to collectively as PB P-450. For many years it has been known that treatment of rats with phenobarbital increases the total content of cytochromes P-450 in liver microsomal membranes by 2–3-fold. However, using radioimmunoassay and enzyme-linked immunosorbent assays we have shown that the relatively small increase in total cytochromes P-450 is due to a greater than 40-fold increase of specific cytochromes P-450, namely PB P-450 (Phillips et al., 1981, 1983a). Solution hybridization experiments, using a cloned cDNA coding for a microheterogeneous variant of P-450e, revealed that the increase in the protein is mediated by a 20-fold induction in the amount of the corresponding mRNA (Phillips et al., 1983b). Nuclear run-off transcription assays demonstrated that the PB P-450 mRNA increase can be almost entirely accounted for by an increase in the transcription of genes coding for PB P-450 (Pike et al., 1985). Thus to increase PB P-450 in liver microsomal membranes phenobarbital must act, either directly or indirectly, at the level of the genome. Consequently there is little or no change in the rate of processing of the primary transcripts in the nucleus, in the rate of transport of the RNA from nucleus to cytoplasm, or in the stability of the mRNA in the cytoplasm.

Analysis of total liver mRNA by Northern blot hybridization showed that PB P-450 is encoded by a single mRNA size class of about 2100 nucleotides. The mRNA is present in the livers of control animals and is substantially induced by phenobarbital treatment. It is also induced, but to a lesser extent, by pregnenalone-16α-carbonitrile, dexamethasone and isosafrole. These three compounds are known also to induce other forms of cytochromes P-450. Although β-naphthoflavone is itself an inducer of the total content of cytochromes P-450, it actually decreases the amount of PB P-450 mRNA and protein (Shephard et al., 1982; Phillips et al., 1983a,b). Thus cytochrome P-450 induction is not a
highly specific process. For instance, an individual species of cytochrome P-450 can be induced by several structurally diverse foreign compounds, a particular chemical can induce more than one cytochrome P-450, and inducers of some cytochromes P-450 can simultaneously decrease the amounts of others.

We next addressed the question of whether all inducers of PB P-450 exert their action via the same mechanism. PB P-450 is induced about 20-fold by isosafrole and, as is the case with phenobarbital, the induction of the protein is mediated by an increase in the amount of the corresponding mRNA in the cytoplasm. But, in contrast to phenobarbital, isosafrole causes only a small increase in the rate of transcription of PB P-450 genes. Thus, isosafrole must induce PB P-450 via a different mechanism from that used by phenobarbital.

**Organization of the P450IIB gene sub-family**

We have used cloned cDNAs to investigate the genes coding for PB P-450. Southern blot hybridization experiments performed under conditions of low stringency revealed that, in a wide variety of rodents and rabbit, PB P-450 is part of a multigene family. The size of this gene family in most rodents is about 70 000 base pairs, whereas the gene family must have undergone an amplification in rats some time after their divergence from mice about 17 million years ago.

We have isolated several genomic DNA clones coding for members of the rat P450IIIB sub-family. Hybridization with unique 19-mer synthetic oligonucleotides enabled us to distinguish between genes coding for cytochromes P-450b and P-450e. Although one of the genomic clones hybridized strongly to a P-450c cDNA clone, it hybridized to neither the P-450b-specific nor the P-450e-specific oligonucleotides. DNA sequencing revealed that this particular gene coded for a cytochrome P-450 that was intermediate in sequence between P-450b and e. This previously uncharacterized gene was termed P-450b* to indicate the fact that it was more similar to P-450b than to P-450e. Analysis of the structure of the P-450b* gene showed that the number and size of its exons were the same as in genes coding for P-450b and P-450e. However, the size of some of the introns differed. For instance, in the gene coding for P-450b* the intron between exons 7 and 8 is about 150 base pairs shorter than the corresponding intron in P-450b and P-450e genes.

**Expression of individual members of the P450IIIB gene sub-family**

Synthetic 19-mer oligonucleotides specific for mRNAs coding for P-450s b, e or b* were used to investigate the expression of the corresponding genes. Northern blot hybridization experiments demonstrated that mRNAs for P-450s b and e are present in the liver of untreated rats and both are inducible by phenobarbital treatment. However, the induction of P-450b mRNA is greater than that of P-450e mRNA. mRNAs encoding P-450b and e were also present in both lung and kidney but at a lower level than in liver. P-450b* mRNA was not detected in liver, lung or kidney from control animals, nor was it inducible in any of these tissues by phenobarbital.

We have used immunohistochemical techniques to determine the regional localization of PB P-450 in various tissues. The results of these experiments indicate that in liver PB P-450 is induced by phenobarbital preferentially in the central lobular regions. The induction of members of other families of cytochromes P-450 occurs more uniformly throughout the tissue. In lung PB P-450 is located mainly in the Clara cells, which comprise only a few per cent of the cells present in this tissue. In kidney PB P-450 is present only in the proximal tubules and then only in small amounts.

The human P450II family

Individual human variations are well known in the metabolism of certain therapeutic drugs. This metabolic variability may be due, in part, to genetically determined factors relating to the structure and expression of cytochromes P-450. As a first step towards investigating this we have isolated cloned cDNAs coding for cytochromes P-450 of man. Our approach has been to use our rat P-450 cDNA clones to screen human liver cDNA libraries. Using a rat P-450c cDNA clone as a molecular hybridization probe we have isolated cloned sequences coding for several different human cytochromes P-450. One of these, pH1P450(1) (Phillips et al., 1985a), codes for a cytochrome P-450 whose amino acid sequence is 51% homologous to rat P-450e and 60% homologous to rat P-450a. At the present time it is believed that this human protein represents an as yet uncharacterized member of the P450IIA sub-family and has been designated P450IIA2.

Southern blotting experiments showed that the gene coding for this human cytochrome P-450 is a member of a small gene family of between 40000 and 50000 base pairs. Analysis of genomic DNA from representatives of different types of primates demonstrated that the size of this gene family has been conserved throughout primate evolution. A small gene family was also found in non-primate mammals such as cow, horse and cat.

Analysis of human liver RNA by Northern blot hybridization showed that pH1P450(1) hybridized to two mRNAs of 1850 and 2750 nucleotides. The lack of a suitable experimental system means that we are unable to demonstrate directly that this cytochrome P-450 is inducible by phenobarbital in man. Nevertheless, pH1P450(1) hybridizes, under relatively high stringency, to a phenobarbital-inducible marmoset liver mRNA of 1900 nucleotides.

We have used Southern blotting and hybridization in situ to map the P450IIA gene sub-family to the long arm of human chromosome 19 (Phillips et al., 1985b). The examination of stable reciprocal translocations having break points on 19q has enabled us to map the regional localization of the gene family more precisely to 19q13.1–13.2 (i.e. approximately the middle third of the long arm of chromosome 19) (Davis et al., 1986).

Another of the human cDNA clones we have isolated codes for a cytochrome P-450 whose greatest homology is to a rabbit liver cytochrome P-450 known to hydroxylate progesterone at position 21 (Tukey et al., 1985). The sequence of this human cytochrome P-450 indicates that it is a member of the P450IIIC sub-family.

We have isolated cloned cDNAs coding for several other members of the P450II family. The sequences of these are currently being determined.

**Cytochrome P-450 reductase**

In contrast to the cytochrome P-450 superfamily the other major protein components of the mono-oxygenase, namely cytochrome P-450 reductase, cytochrome b, and cytochrome b, reductase, are all present in microsomal membranes as single molecular species. We have used the antibodies that we raised to rat cytochrome P-450 reductase (Shephard et al., 1983) to isolate, from a rat liver zgl expression library, cloned cDNAs coding for this protein. These cDNAs have been used to isolate cloned sequences coding for human cytochrome P-450 reductase.

Nucleotide sequencing of the cloned cDNAs revealed that cytochrome P-450 reductase has been far more highly conserved throughout evolution than cytochromes P-450. The overall homology of the rat and human cytochrome P-450 reductases is about 95% at the amino acid level and 90% at the nucleotide level. Thus cytochrome P-450 reductase has evolved very slowly (at a rate of a 1% change in amino acid
sequence about every 17 million years), whereas cytochromes \( P-450 \) have evolved relatively rapidly (with a 1% change every 4 million years). The fact that cytochrome \( P-450 \) reductase is so highly conserved is not surprising considering that it has to bind FAD, FMN, NADPH and many different cytochromes \( P-450 \).

**Cytochrome \( b \) and cytochrome \( b \_1 \) reductase**

In addition to their involvement in the mono-oxygenase system cytochrome \( b \) and cytochrome \( b_1 \) reductase play important roles in fatty acid desaturation and in the reduction of methaemoglobin in erythrocytes. We have used antibodies to isolate cloned cDNAs coding for rat liver cytochrome \( b \) and cytochrome \( b_1 \) reductase. Comparison of the rat cytochrome \( b \) cDNA sequence with that of a human cytochrome \( b \) cDNA we have isolated showed that the homology at the amino acid level is 90%. Although high, this is not as great as that observed for cytochrome \( P-450 \) reductase. The cDNA clones have been used to isolate cloned genomic sequences coding for cytochrome \( b_1 \). The structure and organization of the gene are currently being analysed.

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**Pretranslational hormonal control of male-specific cytochrome \( P-450_{16a} \) in rat liver**

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The adult rat liver is characterized by sexual differences with respect to the metabolism of certain drugs and steroids. Furthermore, the major oxidative metabolism in the liver is catalysed by isoenzymes of cytochrome \( P-450 \) (Gustafsson et al., 1983). Two sex-specific forms of steroid-metabolizing cytochrome \( P-450 \) isoenzymes have been purified and characterized in our laboratory, the male-specific 16a-hydroxylase (\( P-450_{16a} \)) and the female-specific 15b-hydroxylase (\( P-450_{15b} \)). Polyclonal as well as monoclonal antibodies have been raised against these proteins (MacGeoch et al., 1984; Morgan et al., 1985).

The sexual differentiation of \( P-450_{16a} \) and \( P-450_{15b} \) is dependent on the action of sex hormones. Neither androgens nor oestrogens, however, exert their actions directly on the liver but via the hypothalamo–pituitary axis with growth hormone (GH) as the direct effector (Gustafsson et al., 1983). GH has been shown to regulate certain steroid-metabolizing enzymes by means of its sexually differentiated secretory pattern (Eden, 1979). The feminine type of steroid metabolism, high level of \( P-450_{15b} \) and low level of \( P-450_{16a} \), depends on the continuous presence of GH in serum and can be manifested in intact male rats or in hypophysectomized rats of both sexes by continuous administration of GH via osmotic minipumps. In the sexually mature male rat, however, GH secretion is characterized by regular bursts every 3–4 h with low or undetectable levels between peaks. This male-specific secretory pattern of GH leads to a liver which is masculine with respect to steroid-metabolizing enzymes (high \( P-450_{15b} \), low \( P-450_{16a} \)), whereas the absence of GH, as in hypophysectomized animals, results in enzyme levels that are intermediate between the normal male and female levels (MacGeoch et al., 1987).

To construct a cDNA library from normal male rat liver, total RNA was prepared using a standard guanidine/HCl technique, and poly(A)\(^+\) mRNA was purified by two cycles of oligo(dT)-cellulose chromatography. cDNA was made according to Gubler & Hoffman (1983) and the size of the cDNA obtained was 500–4000 base pairs. After ligation of linkers the cDNA was cloned into the EcoRI site of \( \lambda \)gt11. The library contained 600 000 independent recombinants and was initially screened with a \( P-450_{15b} \)-specific polyclonal antiserum with a goat anti-rabbit alkaline phosphatase conjugate as a second antibody. The sensitivity of the screening procedure allowed the detection of less than 2 ng of antigen. Re-screening of tentative positive clones with both polyclonal and monoclonal antibodies gave rise to two strongly responding clones, clone 1.1 and clone 1.3. After \( \lambda \)-phage DNA preparation the cDNA was cleaved out with EcoRI and analysed by agarose gel electrophoresis (Maniatis et al., 1982). The insert from clone 1.1 was cleaved into three different fragments of 1800, 670 and 240 base pairs respectively. To investigate whether this multiplicity of fragments was due to cloning of more than one cDNA into the same vector or if it was due to internal EcoRI sites in the cDNA all three fragments were purified and labelled with \( [\alpha-\text{\textsuperscript{125}}\text{P}]\text{dCTP} \) and used as probes in a Northern blot analysis. Twenty micrograms of total RNA from male or female rat livers were subjected to Northern blot analysis. Hybridization with the three probes revealed that only the 1800 base pair fragment specifically hybridized to male RNA of the appropriate size. The other two fragments, 670 and 240 base pairs, respectively, were not sex specific and hybridized peaks.

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