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Analysis of two benzo[a]pyrene-resistant mutants of the mouse hepatoma Hepa-1 $P_1$450 gene via cDNA expression in yeast

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Two benzo[a]pyrene-resistant mutant clones (c1 and c37) of the mouse hepatoma Hepa-1 wild-type (wt) cell line were examined for their lack of $P_1$450 [aryl hydrocarbon (benzo[a]pyrene) hydroxylase (AHH)] activity. From λgt11 cDNA libraries, the nearly full-length $P_1$450 cDNAs of wt, c1 and c37 were isolated and sequenced. The c1 cDNA was found to have a single mutation leading to premature termination of the protein after Asn-414; a rapidly migrating band corresponding to this truncated protein was found on Western immunoblots. The c37 cDNA was found to have two point mutations, leading to Leu-118→Arg-118 and Arg-245→Pro-245, but otherwise to encode the normal (524-residue) protein; the mature protein was confirmed by Western blot analysis. $P_1$450 cDNA from wt, c1 and c37 and chimeric cDNAs between wt and c37 were inserted into the expression vector pAAH5 and expressed in *Saccharomyces cerevisiae* strain 50.L4. The Leu-118→Arg-118 mutation alone was found to have negligible effect on AHH activity, while the Arg-245→Pro-245 mutation alone leads to a 2- to 3-fold decrease in enzyme activity. The two mutations together totally abrogate AHH activity. The biologic mutant c37 provides the first evidence for the importance of Arg-245, and the complementary function of Leu-118, in normal $P_1$450 enzymic function. This alteration in a single amino acid from arginine to proline might block electron flow directly, or change secondary structure of the protein, such that normal monooxygenation of benzo[a]pyrene cannot occur.

Key words: mouse $P_1$450 gene expression/somatic cell genetics/yeast expression vector pAAH5/Western immunoblots/mRNA instability/chimeric cDNAs/hybrid proteins

Introduction

The P450 gene superfamily (Nebert *et al.*, 1987) encodes proteins (cytochromes P450) important in the metabolism of endogenous substrates such as steroids, fatty acids, prostaglandins, leukotrienes, pheromones and phytoalexins (Boobis *et al.*, 1985; Ortiz de Montellano, 1986). Many of these same monooxygenases also metabolize innumerable drugs, chemical carcinogens and other environmental pollutants (Boobis *et al.*, 1985; Ortiz de Montellano, 1986).

P450I represents one of eight known mammalian families in the P450 gene superfamily (Nebert *et al.*, 1987). Within the P450I family, the two mouse genes (trivial names $P_1$450 and $P_3$450) are induced by combustion products such as TCDD (2,3,7,8-

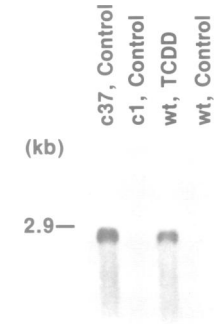

Fig. 1. Northern blot of $P_1$450 mRNA from wt, c1 and c37 cell lines. Poly(A) RNA was prepared by the guanidine thiocyanate method (Chirgwin *et al.*, 1979) followed by oligo(dT) column chromatography (Aviv and Leder, 1972). One μg of poly(A) RNA from the various samples of TCDD-treated or control cells was electrophoresed on 1% agarose gels containing 2.2 M formaldehyde and blotted to nylon filters (Nytran™; 0.45 μm pore size) (Schleicher and Schuell, Inc., Keene, NH). The filters were then probed with $P_1$450 nick-translated full-length cDNA (Kimura *et al.*, 1984). TCDD treatment (10 nM) of wt cells was carried out for 24 h before the RNA isolation. Upon longer exposure time of the autoradiogram, it has been shown that the constitutive $P_1$450 mRNA concentration of c1 cells is more than 10 times that of wt cells (Hankinson *et al.*, 1985). The c1 mutant exhibits no increase in $P_1$450 mRNA following TCDD treatment (Hankinson *et al.*, 1985), whereas the TCDD-treated c37 line shows a small (~50%) increase over constitutive $P_1$450 mRNA concentrations (data not included).

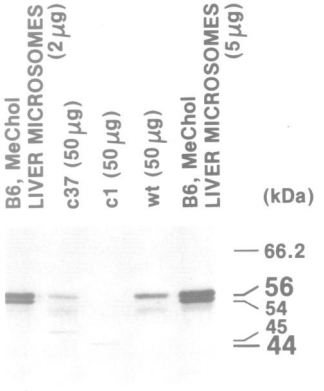

Fig. 2. Western immunoblot of $P_1$450 protein from wt, c1 and c37 cell lines. The wt cultures were treated with 10 nM TCDD for 24 h before harvesting. The sonicated cell lysate (50 μg), and 3-methylcholanthrene (MeChol)-treated B6 mouse liver microsomes as positive controls (2 μg and 5 μg), were electrophoresed on 7% $NaDodSO_4$-polyacrylamide gels. Under these experimental conditions, the immuno-crossreacting $P_3$450 in mouse liver migrates distinctly more rapidly (~2 kd) than $P_1$450. Mol. wt standards consisted of prestained protein markers (BRL, Gaithersburg, MD): the migration positions of bovine serum albumin (66.2 kd) and ovalbumin (45 kd) are shown.

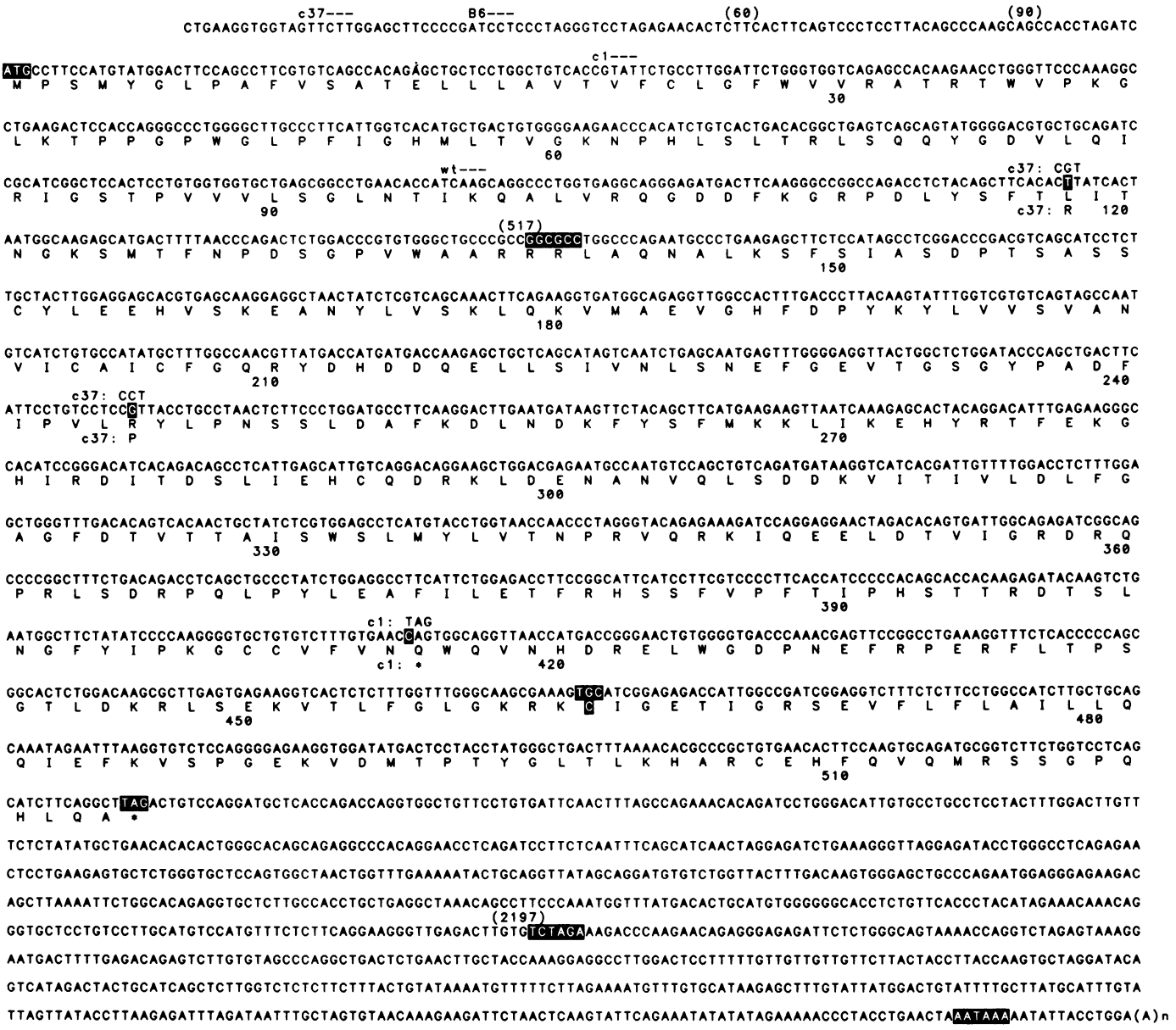

Fig. 3. Mouse P_1450 cDNA nucleotide and amino acid sequence. The numbers of several nucleotide positions are given in parentheses above the cDNA sequence, and the amino acid residues are numbered below. The 5′ ends of the c37, c1 and wt cDNA (nucleotide positions 13, 164 and 388, respectively) and the 5′ end of the B6 mouse P_1450 T4 polymerase-edited cDNA (nucleotide position 31) are shown above the nucleotide sequence. The B6 P_1450 edited cDNA was obtained by deleting the 5′ vector sequence and poly(GC) tract and a portion of the 5′ nontranslated region of pP_1450FL in the Okayama-Berg vector (Gonzalez *et al.*, 1984a) with use of the Cyclone System™ (IBI, New Haven, CT). Enclosed in blackened boxes are the initiation codon, the two mutations in the c37 P_1450 cDNA, the *Nar*I site (nucleotide position 517), the one mutation in the c1 P_1450 cDNA leading to premature termination, the cysteine codon and the cysteine residue necessary for the heme-binding site, the termination codon, the *Xba*I site (nucleotide position 2197), and the putative polyadenylation signal. A second *Xba*I (not darkened) is located at nucleotide position 2249.

tetrachlorodibenzo-*p*-dioxin) and benzo[a]pyrene; in turn, benzo[a]pyrene is an excellent substrate for the induced P_1450 [aryl hydrocarbon (benzo[a]pyrene) hydroxylase (EC1.14.14.1)] protein (Nebert and Gonzalez, 1987). Although the parent benzo[a]pyrene is not carcinogenic, the action of P_1450 metabolism leads to reactive intermediates shown in many systems to be carcinogenic, mutagenic, toxic, and teratogenic. Since the same sort of metabolic potentiation by P450I enzymes occurs with more than a dozen polycyclic hydrocarbons besides benzo[a]pyrene, it has been suggested that increased levels of these mammalian enzymes are highly correlated with environmental carcinogenesis (Conney, 1982; Pelkonen and Nebert, 1982; Jerina, 1983; Gonzalez *et al.*, 1987).

A chronologic sequence of events during the P450I induction process has been established (Nebert and Gonzalez, 1987): inducers such as TCDD or benzo[a]pyrene bind to the aromatic hydrocarbon (Ah) receptor with an apparent $k_d < 1.0$ nM (Poland *et al.*, 1976; Okey *et al.*, 1979); a temperature-dependent step is necessary before the inducer-receptor complex can bind to chromatin (Okey *et al.*, 1980); the transcriptional rate of the P_1450 and P_3450 genes and the corresponding mRNA levels increase in mouse liver (Gonzalez *et al.*, 1984b); finally, enhanced catalytic activities of both P_1450 and P_3450 proteins occur in the endoplasmic reticulum (Negishi *et al.*, 1981). A TCDD-induced rise in the transcriptional rate of the mouse P_1450 gene has also been demonstrated in cell culture (Israel and Whitlock, 1984).

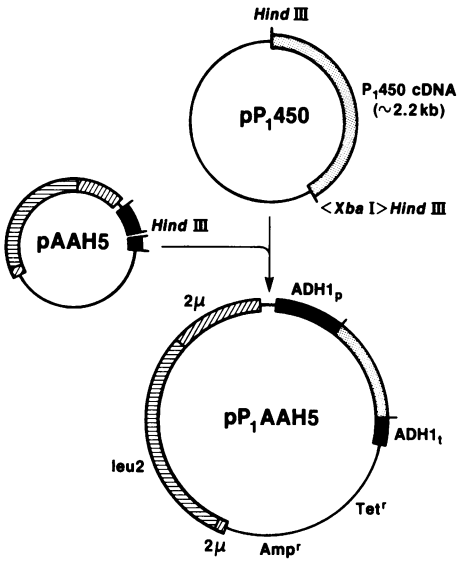

Fig. 4. Construction of P_1450 cDNAs (~2.2 kb) into the yeast expression vector pAAH5. Since the wt and c1 cDNAs did not contain the 5′ portion of the translated region (Figure 3), B6 mouse P_1450 cDNA (nucleotides 31–2622) was used as the wt cDNA and the 5′ segment of B6 mouse P_1450 cDNA (nucleotides 31–517) was ligated at the *Nar*I site to the c1 cDNA (nucleotides 518–622), in the same manner that the chimeric cDNAs were constructed. The P_1450 cDNAs thus obtained, which lack either 13 bp (c37; c37-wt) or 31 bp (wt, c1, wt-c37) of the 5′ noncoding region, were digested with *Xba*I (nucleotide position 2197), and both 5′ and 3′ ends were converted to *Hin*dIII sites for insertion into pAAH5. $ADH1_p$ and $ADH1_t$, yeast alcohol dehydrogenase-1 promoter and terminator sequences; Tet^r and Amp^r, tetracycline- and ampicillin-resistance genes; 2μ, sequences needed for yeast origin or replication; *leu2*, sequences needed for leucine biosynthesis and, hence, necessary for the selection of plasmid-containing yeast after transformation of leucine-free medium. The lengths of each region are drawn approximately to size in pP_1AAH5 (*e.g.* the *leu2* gene is ~4.5 kb).

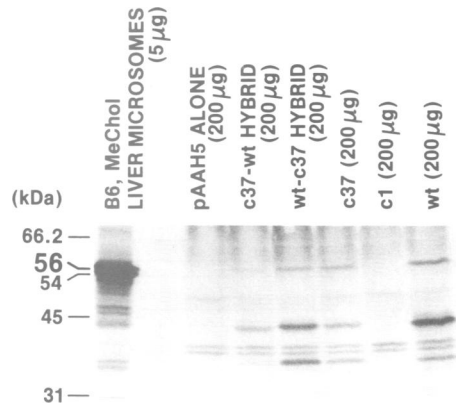

Fig. 5. Western immunoblot of P_1450 protein from several cDNA constructs expressed in yeast. pAAH5 represents yeast containing no mouse cDNA. The wt, c1 and c37 lanes represent yeast carrying the entire P_1450 cDNA translated region from the corresponding cell lines. The c37-wt hybrid represents yeast bearing a chimeric cDNA that codes for the NH_2-terminal 139 amino acids from c37 and the remaining 385 amino acids from wt; the wt-c37 hybrid represents yeast carrying the reverse combination. Yeast microsomes (200 μg), and 3-methylcholanthrene (MeChol)-treated B6 mouse liver microsomes as a positive control (5 μg), were run on 8% $NaDodSO_4$-polyacrylamide gels. The standard prestained protein markers are described in the legend of Figure 2; in this case, the location of carbonic anhydrase (31 kd) is also shown.

From mouse hepatoma Hepa-1 cell cultures treated with benzo[a]pyrene, it is possible to select benzo[a]pyrene-resistant (BP^r) mutants that exhibit neither control nor inducible P_1450 catalytic activity, because BP^r mutants do not generate the cytotoxic benzo[a]pyrene reactive intermediates (Gelboin *et al.*, 1969; Benedict *et al.*, 1972; Hankinson, 1979). Complementation studies have characterized several defects each believed to represent a distinct gene encoding a product responsible for a step necessary in the P_1450 induction process (Hankinson, 1981; 1983; Legraverend *et al.*, 1982; Hankinson *et al.*, 1985). These data have led to at least one structural gene and two regulatory gene classes of mutant lines (complementation groups A, B and C, respectively): P_1^-, lacking AHH activity but having a normal functional Ah receptor; r^-, lacking a functional Ah receptor; and nt^-, having an impaired nuclear translocation of the TCDD·receptor complex (Hankinson, 1981, 1983; Legraverend *et al.*, 1982; Hankinson *et al.*, 1985; Montisano and Hankinson, 1985; Nebert and Gonzalez, 1987). An independent laboratory has characterized the r^- and nt^- but not the P_1^- variant lines (Miller *et al.* 1983).

In the present report we examine the P_1450 structural gene of two P_1^- mutants from the standpoint of the mechanism underlying the lack of either control or inducible AHH activity. Interestingly, whereas one of the P_1^- variant cell lines displays premature termination of the P_1450 protein, the other variant line has a P_1450 protein with two amino acids differing from that of the Hepa-1 wt line. Using P_1450 cDNA from these biologic mutants in a yeast expression system, we show that one of the two amino acid changes is important in determining P_1450 catalytic activity and that both amino acid changes in concert are absolutely essential for the activity.

Results and Discussion

P_1450 mRNA and protein in wt, c1 and c37 cell lines

Hepa-1 wt cells exhibit a large increase in P_1450 mRNA concentration following TCDD treatment, as compared with virtually undetectable levels before TCDD treatment (Figure 1; lanes 3 and 4). In contrast, c1 and especially c37 exhibit elevated levels of constitutive P_1450 mRNA, which is a characteristic of the Group A, subgroup IV, mutant lines (Hankinson *et al.*, 1985). The fact that c1 has a much lower concentration of constitutive P_1450 mRNA than c37 (Figure 1; lanes 1 and 2) might be explained by the fact that c1 contains a premature termination codon while c37 does not (*vide infra*). This phenomenon of mRNA instability when the mRNA encodes a truncated mutant protein is not understood but has been described for β-globin in several of the β-thalassemias (Collins and Weissman, 1984; Orkin and Kazazian, 1984).

Western blot analysis in wt and c37 cell lysates (Figure 2) revealed P_1450 proteins of a size identical to that in mouse liver microsomes. The homologous P_3450 protein (~54 kd) was seen in mouse liver microsomes but not in the cell lysates. Although the mol. wt of the unmodified P_1450 protein is 59 229 following cDNA analysis (Kimura *et al.*, 1984), under our experimental conditions the band migrates with a relative size of ~56 kd. Interestingly, c1 exhibits a unique band of ~44 kd (Figure 2); this finding suggests that a truncated protein of approximately 410 amino acids is present in c1 rather than degraded. The remaining bands in Figure 2 are believed to be proteolytic products.

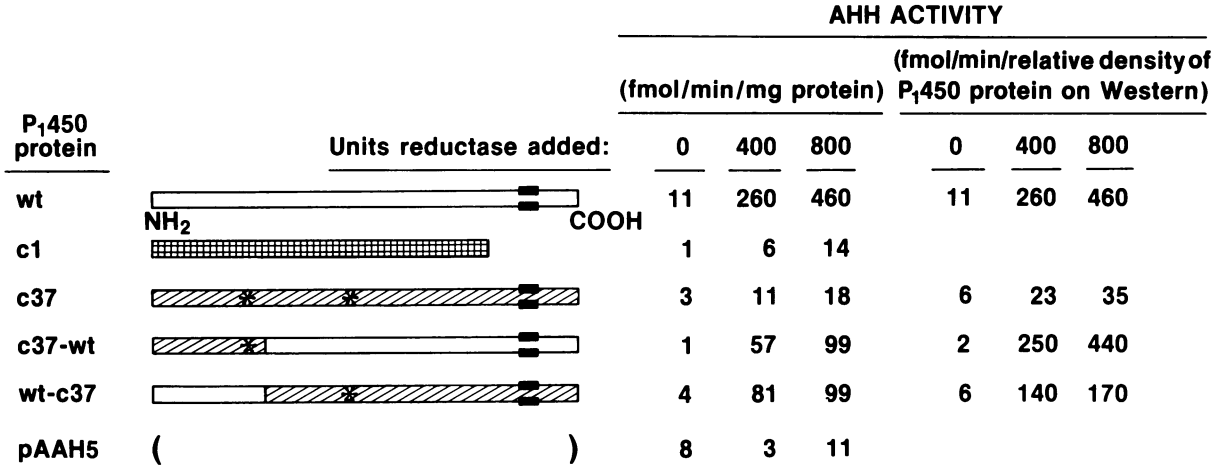

Fig. 6. Aryl hydrocarbon hydroxylase (AHH) expression of wt, c1 and c37 and hybrid P_1450 proteins in yeast microsomes. The bars represent the 524-residue P_1450 protein (NH_2-terminus at left, COOH-terminus at right), and the thickened bracket near the COOH-terminus represents the 21-residue conserved cysteinyl-containing fragment needed for heme binding in the enzyme active-site (Adesnik and Atchison, 1986; Black and Coon, 1987; Nebert and Gonzalez, 1987). The c1 contains a P_1450 414-residue truncated protein, whereas the c37 contains a 524-residue P_1450 protein with two mutations denoted by asterisks (Arg-118 and Pro-245). The enzyme activity is expressed as fmol of phenolic benzo[a]pyrene product formed per min—either per mg protein (left) or per density of the P_1450 protein band on Western blot relative to that of wt (right). Because the yeast containing c1 and the pAAH5 vector alone do not exhibit any P_1450 protein band, no values per density of the P_1450 protein can be expressed in these samples at right. A second experiment (yeast transformation, Western blot of microsomes, and AHH determinations) produced similar results. Values are shown in the absence and in the presence of two concentrations of NADPH-P450 oxidoreductase.

P_1450 cDNA sequences in the three cell lines

Sequence comparisons of P_1450 cDNAs from the CB7BL/6N inbred mouse strain (B6), the wt Hepa-1 parent line, and the c1 and c37 mutants (Figure 3) revealed: (i) complete identity of P_1450 cDNA (nucleotide positions 13−2622) between the c37 cell line and B6 mouse liver, except for the two mutations noted below; (ii) a single base difference between wt and c1, resulting in an early termination codon after Asn-414; and (iii) two point mutations between wt and c37, resulting in Leu-118→Arg-118 and Arg 245→Pro-245. It is particularly noteworthy that only these three mutations were found in the P_1450 nearly full-length cDNAs from wt, c1 and c37 cell lines—and that no other nucleotides varied (even in the nontranslated regions) among B6 mouse liver, the wt cell line, and the two mutant cell lines. If one assumes that the missing 5′ sequences of wt and c1 are identical to those of c37 and the B6 mouse, the premature termination of the c1 P_1450 is consistent with the Western immunoblot band of ~44 kd (Figure 2).

Yeast expression of P_1450 protein

About 2.2 kb of P_1450 cDNA from wt, c1 and c37 were inserted via *Hin*dIII linkers into the yeast expression vector pAAH5 (Figure 4). The *Nar*I site (nucleotide position 517 in Figure 3) conveniently divides the two mutations found in the c37 P_1450 cDNA and was used for constructing c37-wt and wt-c37 chimeric cDNAs. These chimeras were also inserted into pAAH5. The yeast bearing the various plasmids were grown in minimal medium without leucine and then transferred to YPD medium.

The 56-kd P_1450 protein band (Figure 5) is observed in the microsomes of yeast carrying the wt and c37 and the chimeric wt-c37 and c37-wt cDNA-containing plasmids. These four yeast samples also exhibit several smaller immunoreactive bands (~43 kd and ~36 kd) believed to be proteolytic products of mouse P_1450 protein because these more rapidly migrating bands are not seen in yeast microsomes carrying pAAH5 alone or the c1 cDNA-containing plasmid. Although the 44-kd protein band was observed in c1 lysate (Figure 2), the fact that this protein is not found in yeast microsomes (Figure 5) probably reflects the generally high protease activity in yeast.

Yeast expression of wt, c1 and c37 AHH activity

Significantly elevated P_1450 enzymic activity was found in yeast carrying the wt, c37-wt and wt-c37 P_1450 cDNA-containing plasmids (Figure 6), when compared with that containing pAAH5 alone or the c1 or c37 P_1450 cDNA-containing constructs. Significant AHH activity absolutely required the addition of the flavoprotein NADPH-P450 oxidoreductase *in vitro*. This finding is not consistent with that reported by Oeda and coworkers (1985).

The reaction curve of the oxidoreductase added exhibits first-order kinetics for wt and c37-wt, in contrast to saturation kinetics for wt-c37. The latter observation suggests that NADPH-P450 oxidoreductase may not control the reaction rate in wt-c37 and that the reaction is directly affected by other steps such as electron flow within the P_1450 molecule or between the P_1450 protein and the substrate.

The c37-wt and wt-c37 data are not strikingly different when the AHH activity was measured in fmol/min/mg microsomal protein. However, when the intensity of the P_1450 protein band on the Western immunoblot (Figure 5) is taken into account, the c37-wt AHH specific activity of 440 is approximately the same as the wt AHH specific activity of 460, whilst the wt-c37 AHH activity is about one-half to one-third as high (Figure 6 at *right*). AHH-specific activities in the range of 10 to 20 (fmol/min/mg protein) are believed to represent the yeast endogenous P450 activity (Callen *et al.*, 1980; Woods and Wiseman, 1980; Kärgel *et al.*, 1984; Käppeli, 1986).

Several conclusions can be drawn from this study. The c1 P_1450 has no AHH activity because it lacks the heme-binding peptide in the enzyme active-site. The c37 P_1450 has two mutations (residues 118 and 245) and has negligible AHH activity. Although the Leu-118→Arg-118 mutation alone has little effect on AHH activity (Figure 6), the Arg-245→Pro-245 mutation alone causes a 2- to 3-fold decrease in functional enzymic activity, and the two mutations in concert totally block functional

AHH activity. This study illustrates that the use of biologic mutants is a very powerful tool for gaining insight into important amino acids, or regions of the protein, responsible for catalytic activity.

Materials and methods

Materials

The Hepa-1 wt (Benedict *et al.*, 1973; Bernhard *et al.*, 1973) and the c1 variant (Hankinson *et al.*, 1985) cell lines have been characterized. Like c1, the c37 line is one of many Group A, subgroup IV, mutant lines having negligible AHH activity and elevated $P_1$450 mRNA in control and TCDD-treated cells (Hankinson *et al.*, 1985). The yeast expression vector pAAH5, carrying the *leu2* gene and the yeast ADH1 promoter and terminator constructed from the plasmid YEp13 (Ammerer, 1983) was kindly provided by Benjamin D.Hall (University of Washington, Seattle). The *Saccharomyces cerevisiae* strain 50.L4 (*MATα trp1-1 leu2-3 leu2-112 gal 1 ura3-50 his*$^-$ *cup1*^{s}) was a gift of Dennis Thiele (National Cancer Institute, Bethesda, MD). NADPH-P450 oxidoreductase, purified from the liver of phenobarbital-treated rats, and anti-(rat)P450c antibody were generous gifts of Kiyoshi Nagata (Keio University, Tokyo). The conditions for the culturing and harvesting of the yeast cells (Oeda *et al.*, 1985) and of the wt, c1 and c37 cell lines (Legraverend *et al.*, 1982; Hankinson, 1983; Hankinson *et al.*, 1985) have been described in detail.

Western blot analysis

Microsomes from yeast cells were isolated by the zymolase method (Oeda *et al.*, 1985). Protein concentrations were determined by the BCA method (Pierce Chemical Co., Rockford, IL) with bovine serum albumin as standard. Western blotting was performed as detailed (Towbin *et al.*, 1979). The blots were treated with rabbit anti-(rat)P450c serum, diluted 200-fold prior to use, and then with goat anti-(rabbit)IgG conjugated with alkaline phosphatase (KPL Laboratories, Gaithersburg, MD). Color development was carried out with 5-bromo-4-chloro--3-indolyl phosphate and nitroblue tetrazolium (BCIP/NBT) as substrates (KPL Laboratories).

cDNA isolation and sequencing

cDNA libraries from the wt, c1 and c37 cell lines were constructed in λgt11 (Young and Davis, 1983) and screened with the mouse $P_1$450 full-length cDNA (Kimura *et al.*, 1984). The longest clone obtained from each library was then sequenced. Sequencing was carried out by the M13 shotgun cloning protocols (Deininger, 1983) and the dideoxy method (Sanger *et al.*, 1977). When necessary, restriction fragments were also isolated, cloned into M13 and sequenced. Each nucleotide was sequenced at least once on both strands and usually three to seven times.

Enzyme assay

$P_1$450 catalytic activity (AHH activity) in yeast microsomes was performed as detailed (Nebert, 1978). Spec. act. denotes femtomoles of phenolic benzo[a]pyrene produced per min per mg protein during a 30-min incubation of the reaction mixture at 37°C. The amount of hydroxylated benzo[a]pyrene product formed was also compared with the relative intensity of $P_1$450 protein bands on Western immunoblots scanned with a Beckman DU-8 spectrophotometer.

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