Annu. Rev. Pharmacol. Toxicol. 1995. 35:369–90

CYTOCHROMES P450 EXPRESSION SYSTEMS

Frank J. Gonzalez and Kenneth R. Korzekwa
Laboratory of Molecular Carcinogenesis, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

KEY WORDS: cDNA expression, NADPH-P450 oxidoreductase, cytochrome b5, yeast, COS cells, vaccinia virus, baculovirus, retrovirus, B lymphoblastoid cells

ABSTRACT

Catalytically active cytochrome P450 enzymes have been successfully expressed in bacterial, yeast, and mammalian cells. A variety of expression vectors have been used, resulting in both transient and stable expression. The system of choice depends on the goals of a particular project. Factors such as expense, ease of use, and yields required should govern the decision whether to use bacterial, yeast, insect, or mammalian cDNA expression. High-level expression of mammalian P450s in bacteria usually requires modifications of the amino-terminal region of the enzyme. The *Escherichia coli* P450-OR fusion proteins may also come of age for use in fermentation-production processes for the chemical industry. Many cytochromes P450 have been expressed in yeast, with variable levels of expression. Baculovirus, albeit somewhat tedious in having to individualize expression conditions, can produce high levels of enzyme. The standard mammalian cell expression systems, both transient and stable, have been of tremendous value to drug metabolism and carcinogenesis research and will continue to play a role in these areas.

INTRODUCTION

The production of proteins and active enzymes has been critical to the advancement of several areas of molecular biology, including biotechnology, gene regulation, and enzyme structure and function. Knowing the cDNA of a particular protein means having a virtually unlimited source of that protein. Biotechnology drugs can be efficiently mass produced and purified for human

therapy. Transcription factors and growth factors present in minuscule amounts in cells can be generated in sufficient quantity to allow direct physical measurements of protein-protein and protein-DNA interactions. Enzymes can be generated at amount and purity levels suitable for production of crystals for X-ray diffraction. Amino acid changes can be readily introduced into enzymes for structure function studies. With these capabilities, cDNA expression systems have propelled a number of fields of investigations forward.

Several factors should be considered when deciding upon the proper cDNA expression system to use for a particular protein or enzyme. Yields and expense are of primary concern, thus bacterial systems are usually the systems of choice. However, proteins can undergo posttranslational processing, which includes proteolytic cleavage, glycosylation, and phosphorylation. When processing is required for full enzymatic and biologic activities, certain expression systems may not be suitable. An obvious example would be the use of *Escherichia coli* to express a protein that requires precise processing and/or glycosylation for its biological activity.

Mammalian P450s represent a special challenge for cDNA expression (1). They are intrinsic membrane-based proteins that require the presence of a noncovalently bound heme (protoporphyrin IX). Certain steroidogenic and cholesterol-metabolizing P450s are found in mitochondria and require processing for insertion into the inner membrane. The majority of P450s, including the xenobiotic-metabolizing enzymes, are found in the endoplasmic reticulum network of the cell; the bulk of the enzyme faces the cytoplasmic surface of the lipid bilayers as opposed to the lumen, where secreted proteins and certain transferases are localized. The lipid composition of the membrane could possibly effect activities of specific P450 forms (2). With the exception of aromatase (CYP19) (3–5), there is no convincing evidence that P450s are glycosylated, and although phosphorylation of P450s has been documented, this modification may play no role in modulation of catalytic activity (6, 7). Thus, to express the functional activity of a P450, a cell must have adequate heme biosynthetic capabilities and ample intracellular membranes. P450s also require other enzymatic components for full activity, including the flavoprotein NADPH-P450 oxidoreductase (OR) and, in some cases, cytochrome b_5 (1). The OR must interact directly with the P450 to transfer the required two electrons from NADPH. Cytochrome b_5 is necessary for increasing electron transfer for certain P450 forms and specific substrates.

There are two ways to determine catalytic activities of cytochromes P450. First, they can be assayed in cell lysates or subcellular fractions of membranes having the P450 and other components required for activity. Second, P450s can be reconstituted with membranes, OR, and cytochrome b_5 into lipid vesicles, and full catalytic activities can be accomplished. However, the lipid composition and reconstitution conditions must sometimes be customized for

each P450 form. Thus, an expressed P450 can be characterized in situ or can be purified and reconstituted. Both prokaryote and eukaryote systems have been used to produce catalytically active P450s. *E. coli* is the most commonly used host organism for expression and has been widely used for inexpensive production of recombinant proteins; it is increasingly used in the expression of P450s. Yeast, baculovirus, and several mammalian cell systems have been exploited for production of P450s. The various properties and successes of each system are discussed in the sections below.

P450 EXPRESSION IN BACTERIA

High-level expression of mammalian P450s in bacteria is difficult to achieve without modifications of the amino-terminal region of the enzyme. A native rabbit CYP2E1 was produced in a catalytically active form (8). Levels of expression were reported to be 0.3% of total *E. coli* protein. Precise determinations of the amount of active enzyme produced per liter of culture were not reported, but spectral determinations of the carbon monoxide–bound reduced membrane fractions are suggestive of low levels of holoenzyme (Figure 3A in Ref. 8). Removal of amino acid residues 3 through 29 did not improve levels of expression (8), but the truncated enzyme was catalytically active (9). Surprisingly, a modified form with a segment of the amino-terminal sequence removed was also tightly bound to the bacterial lipid bilayers, suggesting that the hydrophobic amino terminus is not absolutely required for anchoring the protein to the membrane.

The cholesterol 7α-hydroxylase CYP7A1 was expressed in *E. coli* after slight modification of the amino-terminal sequence (10). The sequence *Met Met Ala* was changed to *Met Ala* at the initiator methionine. An alanine codon was placed at codon 2 because it is the preferred codon for high-level expression of T7 phage proteins in *E. coli* (11, 12). Rabbit CYP2E1 naturally contains *Ala* at position 2, which may account for the successful expression of this unmodified P450 form, albeit at a low level. The slightly modified CYP7A1 was expressed at about 0.2% of total protein, similar to the rabbit CYP2E1. The activity and spectral properties of the expressed enzyme were not determined. When amino acids 2 through 24 of CYP7A1 were deleted, expression increased 10-fold, and the enzyme was preferentially found in the soluble portion of the cellular extract, in contrast to the full-length P450, which was found in the membrane fraction (10). Expressed CYP7A1 could be purified to a specific content of 2.72 nmol mg^{-1}. Theoretically specific contents should approach 20, suggesting that only a portion of the protein exist as a holoenzyme. The P450 was active, indicating that the N-terminal region is not required for catalytic activity. This is similar to the findings with expressed

rabbit CYP2E1. However, in contrast to CYP2E1, reconstitution required no phospholipid and an excess of OR (20:1).

A modified form of bovine CYP17A1 was produced in *E. coli* (13). In this case, the second codon was changed to a GCT-encoding alanine, and codons 4 and 5 were modified to TTA in order to make the 5′ end of the mRNA AT rich, similar to other *E. coli* mRNAs (14). The last nucleotides of codons 6 and 7 were also changed to A and T, respectively, to minimize the possibility of secondary structure. In the absence of these modifications, apoprotein was not found in the cellular extracts. The modified version was produced as holoenzyme and could be reconstituted with OR to generate the proper catalytic activities. Levels of expression were up to 16 mg P450 per liter of culture.

Because the NH_2-terminal modification of CYP17A1 was found to markedly increase expression in *E. coli*, it was used to replace the NH_2 regions of other mammalian P450s as a means to enhance expression. This modification would not be expected to alter catalytic activities, since, as noted above, one can dispense with the hydrophobic region and maintain activities (9, 10, 13). A list of P450s that have been expressed in *E. coli* is shown in Table 1. In most cases, the modified CYP17A1 5′ end was used to replace the natural 5′ end of the cDNAs. With this change, up to 700 nmol L^{-1} of medium of active enzyme was produced, but the levels varied with the P450 form; values as low as 20 nmol L^{-1} were reported for CYP2C9 (23). Expression of human CYP1A1 was accomplished by replacing the second codon *Leu* for *Ala* and by making the 5′ region AT rich using silent substitution, which resulted in yields of up to 25 nmol L^{-1}. The house fly CYP6A1 was also expressed by changing only the second codon to an *Ala* (27, 28).

A rapid purification procedure has been developed for isolation of P450s from bacterial membrane extracts (22). In the presence of high detergent concentrations, most bacterial proteins were found to bind DEAE-Sephacel while P450s pass through in the void volume. Removal of detergent with hydroxyapatite resulted in P450 preparations that had specific contents of up to 15 nmol mg^{-1} (21). Further chromatography on CM-Sepharose resulted in almost pure preparations with specific contents of up to 23 nmol mg^{-1} (21). High detergent concentration was found to destabilize the P450, resulting in P420, but this problem could be overcome by addition of α-naphthoflavone—a specific ligand and substrate for CYP1A1 and CYP1A2—and 4-methylpyrazole, which binds to CYP2E1. Thus, the high yields, inexpensive expression conditions, and ease of purification make *E. coli* a suitable host for P450 preparation. However, the enzyme must be reconstituted with OR and cytochrome b_5 in order to measure catalytic activities. Ideally, it would be desirable to coexpress the OR and cytochrome b_5 with the P450 in *E. coli* to generate an intact metabolic system. In situ metabolism of steroids was obtained with the bovine CYP17A1, indicating that bacteria have an electron transport chain

Table 1 Expression of P450s in *Escheria Coli*

P450	Modification	Vector	Cells	Yield	Reference
rbCYP2E1	None	pKKHC[a]	MV1304[b]	0.3%[c]	8
rbCYP2E1	NH_2 deletion 3-29	pKKHC	MV1304	0.1%[c]	8
raCYP7A1	NH_2 deletion 3-24	pKK233-1	XL1-Blue	2.0%[c]	10
bCYP17A1	ATGTGGCTGCTCCTGGCTGTC GCT TTATTA A T	pCWOrit[d]	JM1-0	16mg/L	13
CYP2C3	Modified CYP17A1 replacement	pCWOrit	JM109	400 nmol/L	15
hCYP1A2	Modified CYP17A1 replacement	pCWOrit	JM109	700 nmol/L	16
CYP4A11	Modified CYP17A1 replacement	pCWOrit	JM109	40 nmol/L	17
bCYP4A1-OR	Modified CYP17A1 replacement	pCWOrit	NR[e]	700 nmol/L	18
CYP3A4-OR	Modified CYP17A1 replacement	pCWOrit	DH5α	200 nmol/L	19
CYP17A1-OR	Modified CYP17A1 replacement	pCWOrit	DH5α	700 nmol/L	20
CYP3A4	NH_2 deletion 3-12	pCWOrit	DH5α	370 nmol/L	21
hCYP1A2	Modified CYP17A1 replacement	pCWOrit	DH5α	245 nmol/L	22
CYP2C9	Modified CYP17A1 replacement	pCWOrit	DH5α	20 nmol/L	23
hCYP2E1	Ala 2nd codon, NH_2 deletion 3-21	pCWOrit	DH5α	40 nmol/L	24
hCYP1A1	Ala 2nd codon	pCWOrit	DH5α	25 nmol/L	25
CYP6A1	Ala 2nd codon	pCWOrit	DH5α	20 mg/L[f]	26

[a] pKKHC was derived from pKK233-1 (Pharmacia LKG Biotechnology, Inc.) by replacing the pBR322 origin of replication with that from a pUC-derived plasmid. This vector has an IPTG-inducible *trc* promoter.
[b] MV1304 is a recA$^-$ derivative of JM105 (US Biochemical Corp.).
[c] This value is probably based on apoprotein determinations and does not reflect the level of expression of holoenzyme.
[d] pCWOrit is a derivative of pHSe5 and contains two *tac* promoter cassettes (Pharmacia) that are inducible by IPTG.
[e] Not reported.
[f] The yields were quoted for P450 in the membrane fraction per liter of culture and therefore may be an underestimation of actual yields.

that could function with this P450 form (13). The nature of the *E. coli* enzymes responsible for electron transfer to the mammalian enzyme is unknown.

In an effort to produce a catalytically sufficient P450, a CYP4A1-OR fusion protein was constructed using the same strategy described for a rat CYP1A1-OR fusion produced in yeast (29–31). Amino acid 509 of CYP4A1 was fused to amino acid 57 of rat OR via a *Ser Thr* hinge, and the critically modified N-terminal segment of bovine CYP17A1 was used at the 5′ end of CYP4A1 by fusing it to codon 23 (18). Similar strategies were used to prepare fusion proteins for CYP3A4 (19) and CYP17A1 (18, 20). The CYP4A1-OR fusion was functionally active toward lauric acid, albeit the ratio of $\omega/\omega - 1$ was much higher than expected for the purified enzyme. Addition of purified OR to the fusion protein resulted in higher levels of the minor metabolite in addition to an overall increase in rate of metabolism. These reactions occurred in the absence of added phospholipid (18).

Studies of the CYP17A1-OR fusion protein also revealed catalytic activities similar to those of the nonfusion P450, and surprisingly, the turnover rate for

progesterone or pregnenolone was considerably higher for the purified membrane-free form. Adding phospholipids inhibited activity, and in contrast to the results with CYP4A1, additional OR did not increase the rate of progesterone 17-hydroxylation (20). The rate of formation of dehydroepiandrosterone from 17α-hydroxy pregnenolone, due to the inherent lyase activity of the enzyme, was very low and could be stimulated by cytochrome b_5. This cytochrome had no stimulatory effect on the 17-hydroxylation reaction. The rate of metabolism of progesterone in the intact *E. coli* cells is considerably lower than that obtained with purified enzyme, indicating that the intracellular lipid environment is inhibitory or that the reducing equivalents in the cells are not sufficient (20). In contrast, intact yeast cells expressing the fusion protein show high rates of metabolism (31).

The CYP3A4-OR fusion is catalytically sufficient in the presence of an unsaturated lipid, detergent, and cytochrome b_5 for hydroxylation of steroids and the N-oxidation of nifedipine. The unsaturated lipid and small amount of detergent is also required for reconstitution of the native enzyme and is unique to the CYP3A P450s (32). However, in its purified state the fusion protein can metabolize erythromycin and benzphetamine. The ability of intact cells to metabolize CYP3A4 substrates was not determined.

At this time, no crystal structure of a membrane-bound P450 has been determined. However, three non-membrane-bound bacterial P450s were crystallized, and their structures were elucidated (33–35). These structures may provide a very important link between the primary sequence of the mammalian enzymes and their predicted tertiary structures. Although these enzymes were originally purified from their original bacterial sources, they have since been cloned and expressed at high levels in *E. coli*. Expression has provided native proteins (36), mutants of $P450_{cam}$ (37, 38), and the individual domains of $P450_{BM\text{-}3}$ (39, 40), which is a catalytically self-sufficient P450 containing both P450 and reductase domains. Generally, several hundred nanomoles per L culture can be obtained, an amount similar to that obtained for modified mammalian P450s. In conclusion, the bacterial system of P450 production will have tremendous utility in the inexpensive production of antibodies, crystallization, physicochemical analyses, and possible bioreactors for production of metabolites.

P450 EXPRESSION IN YEAST

The first system used to successfully express a mammalian P450 was yeast. Rat CYP1A1 was produced in *Saccharomyces cerevisiae* using the vector pAAH5, which has an alcohol dehydrogenase promoter (41, 42). Almost 1% of total yeast protein was represented by P450 and several milligrams of active

Table 2 Expression of P450s in yeast

P450	Vector	Cells	Yield[a]	Reference
CYP1A1	p31GAPFL	YHE2	156 pmol/mg	49
CYP1A1	p31RIT	YHE2		
CYP1A1-OR	p31GAPFL	YHE2		51
CYP1A1-OR				
CYP1A2	p31GAPFL	YHE2		52
CYP1A2-OR	p31GAPFL	YNW64[b]		53
CYP1A2-OR	pye DP	W303.1B		54
CYP2C8	pAAH5	334	250 pmol/mg	55
CYP2C9	YEp24	MT8-1	200 pmol/mg	56
CYP2C9	pAAH5	AH22		57
CYP2C9	pAAH5	D12	1 nmol/L	58
CYP2C9	pAAH5	334	250 pmol/mg	55
CYP2C18	pAAH5	334	60 pmol/mg	55
CYP2C19	pAAH5	334	17 pmol/mg	55
CYP2D6	pMA91	AH22	98 pmol/mg	59
CYP2D6	pYeDP	Y1153	50 pmol/mg	60
CYP2A4(NF-25)[c]	pYeDP	Y1153	50 pmol/mg	61
CYP3A4(PCN1)[c]	pAAH5	W303.1B	8×10^5 molecules per cell	62
CYP3A4	pYeDP	AH22,D12	3 nmol/L	63

[a] Microsomal protein.
[b] This strain contains a disrupted endogenous OR gene.
[c] The NF-25 and PCN1 variants of CYP3A4 differ by two amino acids.

protein could easily be purified. Many P450s have been produced in yeast since this initial report (43–48). Table 2 lists some of the xenobiotic-metabolizing P450s that have been expressed in yeast. A variety of vectors were used in these studies, in addition to the constitutively expressed pAAH5, and variable levels of expression were reported. Yields range from subnanomolar to hundreds of nanomoles per liter. Differences in levels of expression could be due to stability differences between individual P450 forms or to inadequate attention to proper expression vector design. For example, nontranslated regions of cDNAs could inhibit proper transcription and translation (64). The vector pYeDP1 and its derivatives have been used to produce many P450s, some at quite high levels (48). This vector uses the constitutive phosphoglycerate kinase promoter. The acid phosphatase promoter was used to express rat CYP1A2 (65) and human CYP1A1 (49).

The inducible YEp13 vector containing a metalothionine CUP1 gene promoter was used to produce rabbit CYP2E1 and CYP2E2 (50, 66, 67). The alkaline phosphatase promoter in the pHE11 vector can be stimulated by

shifting cells to a low-phosphate buffer (49). In most cases continuous growth in low phosphate can result in P450 expression. Similarly, the pYeDP-based vectors can be modulated by changing glucose and galactose concentrations in the medium. However, these manipulations can be rather cumbersome with large cultures. The use of an inducible promoter is advantageous when the protein product is lethal, resulting in loss of the foreign DNA from the yeast cell and preferential overgrowth of wild-type cells. Certain P450 forms known to be expressed at low levels might benefit from an inducible promoter. However, direct comparison of an inducible and constitutive promoter revealed no significant difference in levels of expression of human CYP1A1 (49).

The most commonly used strain of *S. cerevisiae* for P450 expression is AH22 (41). Other yeast strains, including YHE2 (49), 50.L4 (66), W303.1B (64), and Y1153 (60) have also yielded active P450s. These strains differ primarily on the basis of various selectable markers and mutations. It is unclear why certain *S. cerevisiae* yeast strains such as AH22 can be used to express active P450s while others, having similar genotypes, produce no P450 under identical conditions. Yeast can be catalytically self-sufficient, since the endogenous OR can support electron transport of mammalian P450. However, the yeast OR may not have adequate amounts or activity to fully support catalytic activities, in which case the addition of exogenous OR to microsomal preparations is required. Mammalian OR has been coexpressed with P450 in yeast, resulting in increased catalytic activities (68). Other investigators have increased levels of expression of the endogenous OR up to 30-fold by placing the cloned OR under control of the strong galactose-inducible promoter and by using homologous recombination to replace the natural yeast OR (43).

The endogenous yeast cytochrome b_5 was found to be an inefficient electron donor for mammalian P450s; therefore, the human counterpart was also placed under control of the galactose-inducible promoter, resulting in high-level expression in the presence of galactose (43). Crossing yeast strains that contained high human OR and cytochrome b_5 resulted in a strain that had all the necessary electron-transport components for fully active CYP1A1 and CYP3A4 monooxygenase activity (54, 69). However, differences in the abilities of individual human P450 forms to interact with OR and cytochrome b_5 were observed; CYP3A4 required more OR than CYP1A1. CYP1A1 turnover for ethoxyresorufin *O*-deethylation was increased fourfold with OR; this increase was similar to that seen by other investigators when human OR was coexpressed with the P450 (45). Also, CYP3A4 activity increased over 60-fold, an effect that was attributed to the requirement of CYP3A4 for cytochrome b_5 for maximal activities. Substitution of human OR for the yeast enzyme resulted in increased P450 activation, which demonstrated that the human OR is more efficient than yeast OR (45).

Yeast was the first system used to produce a functional P450-OR fusion with

rat CYP1A1 and OR (29, 30). Shibata et al also produced a CYP17A1-OR fusion (31). The success of this construction was not totally surprising given that the *Bacillus megaterium* $P450_{BM-3}$ consist of a catalytically self-sufficient natural fused enzyme of 120 kDa that contains a C-terminal domain flavoprotein (36).

Many P450s have been expressed in yeast (see reviews 44 and 48 for references). Table 2 lists only those human P450 forms that were produced using the yeast system. Yeast appears to be the expression system of choice for site-directed mutagenesis studies (e.g. 70–72). Analyzing the effects of mutations is simpler when the enzymes can be purified and reconstituted, because the amounts of lipid, cytochrome b_5, and OR can be controlled. Under ideal conditions, with carefully constructed vectors, the yeast system has proven to be quite versatile in the expression of microsomal P450s; yields are usually around 400 nmol L^{-1}. However, to date, the expression of mitochondrial P450s has not been reported.

The preparation of yeast hosts with high human OR and human cytochrome b_5 levels renders an intracellular membrane environment suitable for optimal P450 expression (45). Full metabolic pathways involving expressed phase II enzymes, such as epoxide hydratase, can be generated in a yeast cell (45). Yeast thus represents a system easily accessible to many laboratories for P450 production.

P450 EXPRESSION IN MAMMALIAN CELLS

Transient Expression Systems

COS CELLS The African green monkey kidney–derived CV1 cells were the first mammalian cell–derived system for expression of a mammalian P450. SV40-transformed COS-1 cells support expression of vectors containing the SV40 origin of replication. Because the virus used to transform these cells has a defective origin of replication, SV40 does not replicate even though the T antigen is expressed. To produce an active P450 in COS cells, the cDNA must be cloned into an expression vector and the construct DNA transfected into COS-1 or COS-7 cells. The DNA replicates in the cells, and after two to three days the cells can be harvested for activity determinations. The dual catalytic activities of 17α-hydroxylation of progesterone followed by 17,20-lyase activity to produce dehydroepiandrosterone was demonstrated using the vector pCD with COS-1 cells (73). The COS cell system is among the most widely used because of its simplicity and the fact that cDNAs do not have to be modified before they are inserted into the vectors. Activities can be determined within just a few days after cloning the cDNAs into the vectors (74, 75). These cells have adequate OR and cytochrome b_5 levels to support P450 activities. P450s have been expressed using a variety of vectors, including p91023(B), pCD, pSVL, and pCMV, which differ primarily at the promoter used for

transcription. The pCMV vector contains a powerful human cytomegalovirus promoter that yields levels of expressed P450 of up to 10-fold higher than other vector systems (74).

The COS cell system has been used in structure function studies to determine the effects of amino acid substitution on catalytic activities (75–78). These studies have been very informative when site-directed mutation results in an enzyme with altered ratios of metabolites. However, due to the low levels of expression, difference spectroscopy cannot easily and accurately be used to determine absolute P450 holoenzyme contents. Therefore, whenever a particular mutant is catalytically inactive, it is impossible to determine whether the P450 did not fold properly and incorporate heme or whether the amino acid alteration affected the enzyme's active site. A list of human P450s expressed in COS cells is displayed in Table 3. The COS cell system can be recommended as a screening tool for P450 catalytic activities.

VACCINIA VIRUS Vaccinia virus has been used to express a number of rat and human P450s, albeit by a single laboratory (84, 85). The limited use of vaccinia in the P450 community is due in part to the availability of other systems, the safety concerns in using the attenuated derivative of a cow pox virus, and the numerous steps required to generate a recombinant virus (84). Vaccinia has a large genome of almost 200 kbp that necessitates the use of in situ recombination for generation of P450-cDNA-containing viruses. The cDNA is first inserted into a plasmid, and the plasmid is introduced into a mammalian cell line with wild-type virus. By virtue of homology between the virus and plasmid DNA, recombination occurs. The recombinants are then selected by an X-gal plaque identification procedure (84). Once a virus containing a P450 cDNA is obtained, it can be used to infect any mammalian cell line to produce active P450. In contrast to several other systems, the vaccinia expression system does not require modification of the cDNA, since 5′ and 3′ untranslated regions do

Table 3 Expression of human P450s in COS cells

P450	Vector	Reference
CYP1A1	pSVL	79
CYP1A2	pSVL	79
CYP2A6	pSVL	80
CYP2C8	p90123(B)	77
CYP2C9	p90123(B)	77
cyp2C18	pCMV-4	81
CYP3A4	pCMV-2	82
CYP3A5	pCMV-2	82
CYP4A11	pCMV-4	83

not interfere with expression levels. Similar levels of expression ranging from 15 to 25 pmol mg^{-1} total cellular protein can be obtained for different P450s. HepG2 cells are used as hosts for expression because they have ample endoplasmic reticulum, OR, and cytochrome b_5. The vaccinia virus is a lytic virus that kills the cells; thus the cells must be harvested after one to three days of infection.

Recombinant vaccinia virus have been used for site-directed mutagenesis studies (86, 87) and for expression of a number of human P450 forms, OR, and cytochrome b_5 (Table 4). This system has been particularly useful in drug metabolism studies. When the recombinant vaccinia viruses are used to infect HepG2 cells, maximal P450 catalytic activities obtained are usually equal to or greater than the optimized reconstituted systems. These cells also have microsomal epoxide hydrolase in quantities similar to those of liver microsomes (100). For metabolites produced by a single liver P450 form, the expression system can reproduce the K_m values observed in liver microsomes (91). Because the P450 contents can be quantitated and only NADPH addition is required, the vaccinia virus expression system is ideal for screening P450s for the potential metabolism of new drugs. These enzymes have been used to assess mutagen activation in the Ames test (88, 101, 102).

P450 EXPRESSION IN INSECT CELLS

Baculovirus

Baculovirus shares many similarities with the vaccinia virus; it contains a large genome, it has a lytic life cycle, and construction of recombinant viruses

Table 4 Expression of human P450s, OR, and cytochrome b_5 in vaccinia virus

Enzyme	Reference
CYP1A1	88
CYP2A6	89
CYP2B6	90
CYP2C8	91
CYP2C9	91
$CYP2C9_{R144C}$	92
CYP2D6	93
CYP2E1	94
CYP2F1	95
CYP3A4	96
CYP3A5	96
CYP4B1	97
OR	98
Cytochrome b_5	99

requires in situ recombination (103, 104). However, the baculovirus expression is restricted to insect cells. High levels of expression can be obtained with many recombinant proteins. P450 can be expressed at levels of 300–1000 pmol mg^{-1} total cell lysate, but only when hemin is added to the culture medium during the course of the infection cycle (105, 106). Only a small number of P450s have been expressed with this system even though it is commercially available through a number of vendors (83, 105, 107–112). The percentage of apoproteins that have heme ranges from ~50% (105) to >90% (112). The reason for this variability is unknown.

CYP4A11 (83), CYP3A4 (112), and CYP19A1 (111) are the only human P450s that have been expressed in baculovirus. All were characterized and found to exhibit the proper catalyzing activities after reconstitution with OR. CYP19A1 has also been shown to be glycosylated in insect cells (5) as it is in human placenta (3). Baculovirus host cells are deficient in all electron-transport components required for P450 expression. These must be added directly to cell extracts for P450 activity assays. The P450 can also be purified using a few column steps (112). Another alternative is to coexpress the OR and P450 in the same cell. This coexpression technique has yielded turnover numbers for catalytic activities that approximate activities expected of the purified reconstituted enzymes (113). Vectors are now available from the Invitrogen Corporation of San Diego, California, for production of recombinant baculoviruses containing two and three independently transcribed cassettes. These new developments may allow simultaneous expression of P450 OR and cytochrome b_5 to reconstitute activities in situ.

STABLE EXPRESSION SYSTEMS

B Lymphoblastoid Cells

B lymphoblastoid cells have proven to be extremely useful for expressing human P450s (114–117). The B lymphoblastoid cells, designated AHH-1 TK+/–, are anchorage independent and support replication of extrachromosomal vectors containing the origin of replication of Epstein-Barr virus (118). These cells contain single copies of the thymidine kinase gene and hypoxanthine phosphoribosyltransferase gene and thus are ideally suited for the study of genetic toxicology end points. The AHH-1 cells contain adequate OR and cytochrome b_5 to support P450 catalytic activities.

P450 cDNAs are inserted into plasmid vectors containing selectable markers that either confer resistance to hygromycin B or allow growth in 1-histidinol in the absence of histidine. The hygromycin B and 1-histidinol selection produce episomal copy numbers of 5 and 40 per cell, respectively (116). The cDNAs are under control of the Herpes Virus thymidine kinase promoter that was shown to be more efficiently expressed in AHH-1 cells than the CMV

Table 5 Expression of P450s in B lymphoblastoid cells

P450	Vector	Yield[a]	Reference
CYP1A1	pHSR	25	119
CYP1A2	pEBVHistk	40	119
CYP2A6	pMF6	55	118
CYP2B6	pHyHo	60	117
CYP2D6	pEBVHistk	160	120
CYP2E1	pHyHo	40	117
CYP3A4	pHyHo	20	117

[a] pmol mg^{-1} of microsomal protein.

promoter. Individual cDNAs yielded variable levels of expression of P450 enzymes, and this was due in part to the deleterious effects of 5′ and 3′ untranslated regions. Even upon removal or alteration of these sequences, expression can still vary, possibly owing to different stabilities of the expressed P450 forms. Expression levels can be elevated by increasing the copy number of the cDNA and in some cases by coexpressing OR. By various manipulations, the expression of CYP3A4 was increased almost two orders of magnitude from the original established cell line (117). Maximal activities of CYP3A4 were achieved by increasing OR content; this P450 requires higher levels of OR, as noted for other systems (69, 112). The human P450s produced in B lymphoblastoid cells are listed in Table 5. In some cases the expression levels of newly developed cell lines have increased considerably from those achieved in earlier published reports (121–123).

Multiple P450s can be simultaneously expressed in a single cell using two separate vectors, each containing several cDNA expression cassettes (124). A cell line designated, MCL-5, contains CYP1A1, CYP1A2, CYP2E1, CYP3A4, and microsomal epoxide hydratase and responds to different classes of chemical carcinogens, including the polycyclic aromatic hydrocarbons, aromatic amines, low molecular weight nitrosamines, and aflatoxins. MCL-5 cells are prototypes for cells that could be used in prospective determinations of potential mutagens and carcinogens.

In conclusion, development of the B lymphoblastoid cell lines requires painstaking trial and error in order to achieve maximal expression. Once developed, the cell lines are stable in culture for several months and can be stored and recultured at will. Metabolism studies can be performed using either microsomes or by incubating substrates with the cells in situ.

Retrovirus

A limited number of P450s have been expressed using retroviruses (125–129). In contrast to the B lymphoblastoid stable expression system, the retrovirus

system produces stably integrated cDNAs. To produce a cell line expressing a P450, the cDNA is placed into a plasmid under control of the retrovirus long terminal repeat (LTR). The plasmid also contains a packaging signal and a selectable marker, usually the bacterial phosphoribosyltransferase II gene, that confers resistance to the antibiotic G418 in eukaryote cells. The construct is transfected into packaging cells where the DNA is packaged and viral particles are secreted. Since the original DNA cannot be replicated, these viral particles can only infect and integrate into a cell's genome. The recombinant virus can be used to infect most mammalian cell lines. Individual G418-resistant clones are selected and analyzed for P450 production. In most cases a single copy of the cDNA is integrated into the chromosome and its integration site probably determines the level of P450 expression in individual clones. The amount of expression is low compared to B lymphoblastoid cells; values of 10 pmol mg^{-1} microsomal protein have been reported (127).

Retroviral expression has the advantage of stable integration in a variety of different cell lines. The HepG2 cells with OR and cytochrome b_5 have been used to express human CYP2E1 (127). Human CYP2A6 has also been expressed (128, 129). The retrovirus system has not, however, gained widespread use because of the complexity and length of time required for isolating an expressing clone.

Other Stable Expression Systems

V79 cells have been developed to stably express P450s (130, 131). These cells were chosen because of their widespread use in genetic toxicology and eukaryote mutagenesis assays. The cells are transformed using a standard SV40-derived expression vector containing a selectable marker. Spontaneous integration occurs, and expressing clones are selected. Several rat P450s have been produced in V79 cells in addition to human CYP1A1 (132), CYP1A2 (133), and CYP2D6 (134, 135). The V79 cells that express P450s have been used for metabolism studies and in cytotoxicity assays.

By using a similar strategy, human CYP1A2 was expressed in human bronchial epithelial cells (136). These cells were immortalized by the SV40 T antigen, yet they retain characteristics of the primary cells. They also express OR and other phase II enzymes. Both retroviral and standard transfection were used to produce stable recombinants containing integrated cDNA expression cassettes driven by the CMV promoter. These cells can be used in cytotoxicity and mutation assays.

The human CYP1A2 was also expressed together with human *N*-acetyltransferase 2 (NAT2) (137). The CYP1A2, NAT2, and OR cDNAs were simultaneously expressed in Chinese hamster CHL cells, and the resultant cells were found to be sensitive to the cytotoxic effects of heterocyclic amines 2-amino-3-methylimidazo[4,5-f] quinoline (IQ) and 2-amino-3,8-dimethyl-

imidazo[4,5-f] quinoxaline (MeIQx). Cells expressing only CYP1A2 and OR were not sensitive to the heterocyclic arylamines, thus establishing a role for NAT2 in the activation process in situ.

Aromatase has been expressed in Chinese hamster ovary cells using a pH β Apr-1-neo expression vector with an aromatase expression that is under the control of a β-actin promoter (138). The copy number in Chinese hamster ovary (CHO) cells was estimated to be 15. Although the amount of aromatase expressed is too small for quantitation by difference spectra, the sensitivity of the aromatase assay makes this a useful expression system. Several aromatase mutants have been expressed and used in structure-function studies (4).

CONCLUSIONS

Several systems have been used to produce catalytically active P450s (139). The system of choice depends on the goals of a particular project. Factors including expense, ease of use, and required yields should govern the decision of whether bacterial, yeast, insect, or mammalian cDNA expression systems should be used. As knowledge continues to accumulate on the optimum N-terminal amino acid sequences required for mammalian P450 production in *E. coli,* this system may be ideal for making large amounts of P450s for physiochemical and, possibly, crystallization studies. The yeast system should also continue to be of use in generating ample amounts of enzyme. Expression in yeast may result in intact metabolic factories that can be used in large-scale metabolite production. The *E. coli* P450-OR fusion proteins may also come of age for use in fermentation production processes in the chemical industry. Baculovirus, albeit somewhat tedious in its required individualized expression conditions, will also be of value in P450 research requiring high levels of enzyme. The standard mammalian cell systems, both transient and stable, have been tremendously valuable to drug and carcinogenesis research and will continue to play a role in these areas. In particular, eukaryote mutagen-testing systems that rely on human P450s will be invaluable in the pharmaceutical and chemical industries. The future holds much promise for the continued use of P450 cDNA expression systems.

Literature Cited

1. Schenkman JB, Greim H, eds. 1993. *Handbook of Experimental Pharmacology.* Berlin: Springer-Verlag. 737 pp.

2. Imaoka S, Imai Y, Shimada T, Funae Y. 1992. Role of phospholipids in reconstituted cytochrome P450 3A form and mechanism of their activation of

catalytic activity. *Biochemistry* 31: 6063–69

3. Sethumadhavan K, Bellino FL, Thotakura NR. 1991. Estrogen synthetase (aromatase). The cytochrome P-450 component of the human placental enzyme is a glycoprotein. *Mol. Cell. Endocrinol.* 78:25–32
4. Chen S, Zhou D, Swiderek KM, Kadohama N, Osawa Y, Hall PF. 1993. Structure-function studies of human aromatase. *J. Steroid Biochem. Mol. Biol.* 44:347–56
5. Shimozawa O, Sakaguchi M, Ogawa H, Harada N, Mihara K, Omura T. 1993. Core glycosylation of cytochrome P-450 (arom). Evidence for localization of N terminus of microsomal cytochrome P-450 in the lumen. *J. Biol. Chem.* 268: 21,399–21,402
6. Koch JA, Waxman DJ. 1991. P450 Phosphorylation in isolated hepatocytes and in vivo. *Methods Enzymol.* 206:305–15
7. Mendez JF, Machu TK, Sony BJ, Browning MD, Deitrich RA. 1993. Phosphorylation of cytochrome P4502E1 (CYP2E1) by calmodulin-dependent protein kinase, protein kinase C, and cAMP dependent protein kinase. *Alcohol* 28:445–51
8. Larson JR, Coon MJ, Porter TD. 1991. Alcohol-inducible cytochrome P450IIE1 lacking the hydrophobic NH_2-terminal segment retains catalytic activity and is membrane-bound when expressed in *Escherichia coli*. *J. Biol. Chem.* 266:7321–24
9. Larson JR, Coon MJ, Porter TD. 1991. Purification and properties of a shortened form of cytochrome P-450 2E1: deletion of the NH_2-terminal membrane insertion signal peptide does not alter the catalytic activities. *Proc. Natl. Acad. Sci. USA* 88:9141–45
10. Li YC, Chaing JY. 1991. The expresssion of catalytically active cholesterol 7α-hydroxylase in *Echerichia coli*. *J. Biol. Chem.* 266:19186–91
11. Dunn JJ, Studier FW. 1983. Complete nucleotide sequence of bacteriophage T7 DNA and the locations of T7 genetic elements. *J. Mol. Biol.* 166:477–535
12. Looman AC, Bodlaender J, Comstock LJ, Eaton D, Jhurani P, et al. 1987. Influence of the codon following the AUG initiation codon on the expression of a modified *lacZ* gene in *Escherichia coli*. *EMBO.J.* 6:2489–92
13. Barnes HJ, Arlotto MP, Waterman MR. 1991. Expression and enzymatic activity of recombinant cytochrome P450 17-hydroxylase in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 88:5597–5601
14. Stormo GD, Schneider TD, Gold LM. 1982. Characterization of translational initiation sites in *E. coli*. *Nucleic Acids Res.* 10:2971–96
15. Richardson TH, Hsu M-H, Kronbach T, Barnes HJ, Chan G, et al. 1993. Purification and characterization of recombinant-expressed cytochrome P450 2C3 from *Escherichia coli*: 2C3 encodes the 6β-hydroxylase deficient form of P450 3b. *Arch. Biochem. Biophys.* 300:510–16
16. Fisher CW, Caudle DL, Martin-Wixtrom C, Quattrochi LC, Tukey RH, et al. 1992. High-level expression of functional human cytochrome P450 1A2 in *Escherichia coli*. *FASEB.J.* 6:759–64
17. Palmer CNA, Richardson TH, Griffin KJ, Hsu M-H, Muerhoff AS, et al. 1993. Characterization of a cDNA encoding a human kidney, cytochrome *P*-450 4A fatty acid omega-hydroxylase and the cognate enzyme expressed in *Escherichia coli*. *Biochim. Biophys. Acta Gene. Struct. Expression* 1172:161–66
18. Fisher CW, Shet MS, Caudle DL, Martin-Wixtrom CA, Estabrook RW. 1992. High-level expression in *Escherichia coli* of enzymatically active fusion proteins containing the domains of mammalian cytochromes P450 and NADPH-P450 reductase flavoprotein. *Proc. Natl. Acad. Sci. USA* 89:10,817–21
19. Shet MS, Fisher CW, Holmans PL, Estabrook RW. 1993. Human cytochrome P450 3A4: enzymatic properties of a purified recombinant fusion protein containing NADPH-P450 reductase. *Proc. Natl. Acad. Sci. USA* 90:11748–52
20. Shet MS, Fisher CW, Arlotto MP, Shackleton CHL, Holmans PL, et al. 1994. Purification and enzymatic properties of a recombinant fusion protein expressed in *Escherichia coli* containing the domains of bovine P450 17A and rat NADPH-P450 reductase. *Arch. Biochem. Biophys.* 311:402–17
21. Gillam EMJ, Baba T, Kim B-R, Ohmori S, Guengerich FP. 1993. Expression of modified human cytochrome P450 3A4 in *Escherichia coli* and purification and reconstitution of the enzyme. *Arch. Biochem. Biophys.* 305:123–31
22. Sandhu P, Guo Z, Baba T, Martin MV, Tukey RH, Guengerich FP. 1994. Expression of modified human cytochrome P450 1A2 in *Escherichia coli*: stabilization, purification, spectral characterization, and catalytic activities of the enzyme. *Arch. Biochem. Biophys.* 309: 168–77
23. Sandhu P, Baba T, Guengerich FP. 1993. Expression of modified cytochrome P450 2C10 (2C9) in *Escherichia coli*,

purification, and reconstitution of catalytic activity. *Arch. Biochem. Biophys.* 306:443–50

24. Gillam EMJ, Guo Z, Guengerich FP. 1994. Expression of modified human cytochrome P450 2E1 in *Escherichia coli*, purification and spectral and catalytic properties. *Arch. Biochem. Biophys.* 312:59–66
25. Guo Z, Gillam EMJ, Ohmori S, Tukey RH, Guengerich FP. 1994. Expression of modified human cytochrome P450 1A1 in *Escherichia coli* effects of 5′ substitution, stabilization, purification, spectral characterization and catalytic properties. *Arch. Biochem. Biophys.* 312: 436–46
26. Andersen JF, Utermohlen JG, Feyereisen R. 1994. Expression of house fly CYP6A1 and NADPH-cytochrome P450 reductase in *Escherichia coli* and reconstitution of an insecticide-metabolizing P450 system. *Biochemistry* 33:2171–77
27. Zysset T, Wietholtz H. 1991. Pharmacokinetics of caffeine in patients with decompensated Type I and Type II diabetes mellitus. *Eur. J. Clin. Pharmacol.* 41:449–52
28. Korzekwa K, Nagata K, Trager WF, Gillette JR. 1989. Metabolism of the B-ring of testosterone by the rat cytochrome P-450 system. *Drug Metab. Rev.* 20:535–39
29. Murakami H, Yabusaki Y, Sakaki T, Shibata M, Ohkawa H. 1987. A genetically engineered P450 monooxygenase: construction of the functional fused enzyme between rat cytochrome P450s and NADPH-cytochrome P450 reductase. *DNA Cell Biol.* 6:189–97
30. Yabusaki Y, Murakami H, Sakaki T, Shibata M, Ohkawa H. 1988. Genetically engineered modification of P450 monooxygenases: functional analysis of the amino-terminal hydrophobic-region and hinge region of the P450/reductase fused enzyme. *DNA Cell Biol.* 7:701–11
31. Shibata M, Sakaki T, Yabusaki Y, Murakami H, Ohkawa H. 1990. Genetically engineered P450 monooxygenases: construction of bovine P450c17/yeast reductase fused enzymes. *DNA. Cell Biol.* 9:27–36
32. Eberhart DC, Parkinson A. 1991. Cytochrome P450 IIIA1 (P450p) requires cytochrome b_5 and phospholipid with unsaturated fatty acids. *Arch. Biochem. Biophys.* 291:231–40
33. Poulos TL. 1991. Modeling of mammalian P450s on basis of P450cam X-ray structure. *Methods Enzymol.* 206:11–30
34. Ravichandran KG, Boddupalli SS, Hasemann CA, Peterson JA, Deisenhofer J. 1993. Crystal structure of hemoprotein domain of P450BM-3, a prototype for microsomal P450s. *Science* 261: 731–36
35. Hasemann CA, Ravichandran KG, Peterson JA, Deisenhofer J. 1994. Crystal structure and refinement of cytochrome $P450_{terp}$ at 2.3 Å resolution. *J. Mol. Biol.* 236:1169–85
36. Narhi LO, Wen LP, Fulco AJ. 1988. Characterization of the protein expressed in *Escherichia coli* by a recombinant plasmid containing the Bacillus megaterium cytochrome P-450BM-3 gene. *Mol. Cell. Biochem.* 79:63–71
37. Atkins WA, Sligar SG. 1988. The roles of active site hydrogen bonding in cytochrome P-450cam as revealed by site-directed mutagenesis. *J. Biol. Chem.* 263:18842–49
38. Tuck SF, Graham-Lorence S, Peterson JA, Ortiz de Montellano PR. 1993. Active sites of the cytochrome $p450_{cam}$ (CYP101) F87W and F87A mutants. Evidence for significant structural reorganization without alteration of catalytic regiospecificity. *J. Biol. Chem.* 268: 269–75
39. Oster T, Boddupalli SS, Peterson JA. 1991. Expression, purification, and properties of the flavoprotein domain of cytochrome P-450BM-3. Evidence for the importance of the amino-terminal region for FMN binding. *J. Biol. Chem.* 266:22718–25
40. Li H, Darwish K, Poulos TL. 1991. Characterization of recombinant Bacillis megaterium cytochrome P-450BM-3 and its two functional domains. *J. Biol. Chem.* 266:11909–14
41. Oeda K, Sakaki T, Ohkawa H. 1985. Expression of rat liver cytochrome P-450MC cDNA in *Saccharomyces cerevisiae. DNA Cell Biol.* 4:167–75
42. Sakaki T, Oeda K, Miyoshi M, Ohkawa H. 1985. Characterization of rat cytochrome $P-450_{mc}$ synthesized in *Saccharomyces cerevisiae. J. Biochem.* 98: 167–75
43. Urban P, Cullin C, Pompon D. 1990. Maximizing the expression of mammalian cytochrome P-450 monooxygenase activities in yeast cells. *Biochimie* 72: 463–72
44. Guengerich FP, Brian WR, Sari M-A, Ross JT. 1991. Expression of mammalian cytochrome P450 enzymes using yeast-based vectors. *Methods Enzymol.* 206:130–45
45. Urban P, Truan G, Gautier J-C, Pompon D. 1993. Xenobiotic metabolism in humanized yeast: engineered yeast cells producing human NADPH-cytochrome

P-450 reductase, cytochrome *b*5, epoxide hydrolase and *P*-450s. *Biochem. Soc. Trans.* 21:1028–34
46. Eugster H-P, Sengstag C. 1993. *Saccharomyces cerevisiae*: an alternative source for human microsomal liver enzymes and its use in drug interaction studies. *Toxicology* 82:61–73
47. Fulco AJ. 1991. P450BM-3 and other inducible bacterial P450 cytochromes: biochemistry and regulation. *Annu. Rev. Pharmacol. Toxicol.* 31:177–203
48. Urban P, Truan G, Bellamine A, Laine R, Gautier J-C, Pompon D. 1994. Engineered yeast simulating P450-dependent metabolism: tricks, myths, and reality. See Ref. 139, In press
49. Eugster H-P, Sengstag C. 1990. Constitutive and inducible expression of human cytochrome P450IA1 in yeast *Saccharomyces cerevisiae*: an alternative enzyme source for in vitro studies. *Biochem. Biophys. Res. Commun.* 172:737–44
50. Ching MS, Lennard MS, Tucker GT, Woods HF, Kelly DE, Kelly SL. 1991. Expression of alcohol-inducible rabbit liver cytochrome P450 3a in *Saccharomyces cerevisiae* with the copper-inducible promoter. *Biochem. Pharmacol.* 42:753–58
51. Eugster H-P, Bärtsch S, Würgler FE, Sengstag C. 1992. Functional co-expression of human oxidoreductase and cytochrome P450 1A1 in *Saccharomyces cerevisiae* results in increased EROD activity. *Biochem. Biophys. Res. Commun.* 185:641–47
52. Eugster H-P, Probst M, Würgler FE, Sengstag C. 1993. Caffeine, estradiol, and progesterone interact with human CYP1A1 and CYP1A2: evidence from cDNA-directed expression in *Saccharomyces cerevisiae*. *Drug Metab. Dispos.* 21:43–49
53. Sengstag C, Eugster H-P, Würgler FE. 1994. High promutagen activating capacity of yeast microsomes containing human cytochrome P-450 1A and human NADPH-cytochrome P-450 reductase. *Carcinogenesis* 15:837–44
54. Truan G, Cullin C, Reisdorf P, Urban P, Pompon D. 1993. Enhanced in vivo monooxygenase activities of mammalian P450s in engineered yeast cells producing high levels of NADPH-P450 reductase and human cytochrome b_5. *Gene* 125:49–55
55. Goldstein JA, Faletto MB, Romkes-Sparks M, Sullivan T, Kitareewan S, et al. 1994. Evidence that CYP2C19 is the major (*S*)-mephenytoin 4′-hydroxylase in humans. *Biochemistry* 33:1743–52
56. Yasumori T, Murayama N, Yamazoe Y, Abe A, Nogi Y, et al. 1989. Expression of a human P-450IIC gene in yeast cells using galactose-inducible expression system. *Mol. Pharmacol.* 35:443–49
57. Ohgiya S, Komori M, Fujitani T, Miura T, Shinriki N, Kamataki T. 1989. Cloning of human cytochrome P-450 cDNA and its expression in *Saccharomyces cerevisiae*. *Biochem. Int.* 18:429–38
58. Brian WR, Srivastava PK, Umbenhauer DR, Lloyds RS, Guengerich FP. 1989. Expression of a human cytochrome P-450 protein with tolbutamide hydroxylase activity in *Saccharomyces cerevisiae*. *Biochemistry* 28:4993–99
59. Ellis SW, Ching MS, Watson PF, Henderson CJ, Simula AP, et al. 1992. Catalytic activities of human debrisoquine 4-hydroxylase cytochrome P450 (CYP2D6) expressed in yeast. *Biochem. Pharmacol.* 44:617–20
60. Krynetsky EY, Drutsa VL, Kovaleva IE, Luzikov VN, Uvarov VY. 1993. Effects of amino terminus truncation in human cytochrome P450IID6 on its insertion into the endoplasmic reticulum membrane of *Saccharomyces cerevisiae*. *FEBS. Lett.* 336:87–89
61. Renaud JP, Cullin C, Pompon D, Beaune P, Mansuy D. 1990. Expression of human liver cytochrome P450 IIIA4 in yeast. A functional model for the hepatic enzyme. *Eur. J. Biochem.* 194: 889–96
62. Brian WR, Sari M, Iwasaki M, Shimada T, Kaminsky LS, Guengerich FP. 1990. Catalytic activities of human liver cytochrome P-450 IIIA4 expressed in *Saccharomycescerevisiae*. *Biochemistry* 29:11280–92
63. Peyronneau M-A, Renaud J-P, Jaouen M, Urban P, Cullin C, et al. 1993. Expression in yeast of three allelic cDNAs coding for human liver *P*-450 3A4: different stabilities, binding properties and catalytic activities of the yeast-produced enzymes. *Eur. J. Biochem.* 218:355–61
64. Pompon D. 1988. cDNA cloning and functional expression in yeast *Saccharomyces cerevisiae* of naphthoflavone-induced rabbit liver P450 LM4 and LM6. *Eur. J. Biochem.* 177: 285–93
65. Shimizu T, Sogawa K, Fujii-Kuriyama Y, Takahashi M, Ogoma Y, Hatano M. 1986. Expression of rat cytochrome P450d in *Saccharomyces cerevisiae*. *FEBS. Lett.* 207:217–21
66. Fujita VS, Thiele DJ, Coon MJ. 1990. Expression of alcohol-inducible rabbit liver cytochrome P450 3a in *Saccharomyces cerevisiae* with the copper-in-

ducible promoter. *DNA. Cell Biol.* 9: 111–18

67. Pernecky SJ, Porter TD, Coon MJ. 1990. Expression of rabbit cytochrome P-450IIE2 in yeast and stabilization of the enzyme by 4-methylpyrazole. *Biochem. Biophys. Res. Commun.* 172:1331–37
68. Murakami H, Yabusaki Y, Ohkawa H. 1986. Expression of rat NADPH-cytochrome P-450 reductase in *Saccharomyces cerevisiae*. *DNA Cell Biol.* 5: 1–10
69. Peyronneau M-A, Renaud J-P, Truan G, Urban P, Pompon D, Mansuy D. 1992. Optimization of yeast-expressed human liver cytochrome P450 3A4 catalytic activities by coexpressing NADPH-cytochrome P450 reductase and cytochrome b_5. *Eur. J. Biochem.* 207:109–16
70. Uno T, Imai Y. 1989. Identification of regions functioning in substrate interaction of rabbit liver cytochrome P-450 (laurate (omega-1)-hydroxylase). *J. Biochem.* 106:569–74
71. Shimizu T, Murakami Y, Hatano M. 1994. Glu^{318} and Thr^{319} mutations of cytochrome P450 1A2 remarkably enhance homolytic O-O cleavage of alkyl hydroperoxides. An optical absorption spectral study. *J. Biol. Chem.* 269: 13,296–13,304
72. Iwasaki M, Darden TA, Parker CE, Tomer KB, Pedersen LG, Negishi M. 1994. Inherent versatility of P-450 oxygenase. Conferring dehydroepiandrosterone hydroxylase activity to P-450 2a-4 by a single amino acid mutation at position 117. *J. Biol. Chem.* 269:9079–83
73. Zuber MX, Simpson ER, Waterman MR. 1986. Expression of bovine 17α-hydroxylase cytochrome P-450 cDNA in nonsteroidogenic (COS1) cells. *Science* 234:1258–61
74. Clark BJ, Waterman MR. 1991. Heterologous expression of mammalian P450 in COS cells. *Methods Enzymol.* 206: 100–8
75. Higashi Y, Fujii-Kuriyama Y. 1991. Functional analysis of mutant P450(C21) genes in COS cell expression system. *Methods Enzymol.* 206:166–73
76. Kronbach T, Larabee TM, Johnson EF. 1989. Hybrid cytochromes P-450 identify a substrate binding domain in P-450IIC5 and P-450IIC4. *Proc. Natl. Acad. Sci. USA* 86:8262–65
77. Veronese ME, Doecke CJ, Mackenzie PI, McManus ME, Miners JO, et al. 1993. Site-directed mutation studies of human liver cytochrome *P*-450 isoenzymes in the CYP2C subfamily. *Biochem. J.* 289:533–38
78. Luo Z, He Y, Halpert JR. 1994. Role of residues 363 and 206 in conversion of cytochrome P450 2B1 from a steroid 16-hydroxylase to a 15α-hydroxylase. *Arch. Biochem. Biophys.* 309:52–57
79. McManus ME, Burgess WM, Veronese ME, Huggett A, Quattrochi LC, Tukey RH. 1990. Metabolism of 2-acetylaminofluorene and benzo(a)pyrene and activation of food-derived heterocyclic amine mutagens by human cytochromes P450. *Cancer Res.* 50:3367–76
80. Miles JS, McLaren AW, Forrester LM, Glancey MJ, Lang MA, Wolf CR. 1990. Identification of the human liver cytochrome P-450 responsible for coumarin 7-hydroxylase activity. *Biochem. J.* 267: 365–71
81. Furuya H, Meyer UA, Gelboin HV, Gonzalez FJ. 1991. Polymerase chain reaction-directed identification, cloning, and quantification of human CYP2C18 mRNA. *Mol. Pharmacol.* 40:375–82
82. Ball SE, Maurer G, Zollinger M, Ladona M, Vickers AE. 1992. Characterization of the cytochrome P-450 gene family responsible for the N-dealkylation of the ergot alkaloid CQA 206–291 in humans. *Drug Metab. Dispos. Biol. Fate Chem.* 20:56–63
83. Imaoka S, Ogawa H, Kimura S, Gonzalez FJ. 1993. Complete cDNA sequence and cDNA-directed expression of CYP4A11, a fatty acid omega-hydroxylase expressed in human kidney. *DNA Cell Biol.* 12:893–99
84. Gonzalez FJ, Aoyama T, Gelboin HV. 1991. Expression of mammalian cytochrome P450 using vaccinia virus. *Methods Enzymol.* 206:85–92
85. Gonzalez FJ, 1993. Molecular biology of human xenobiotic-metabolizing cytochromes P450: role of vaccinia virus cDNA expression in evaluating catalytic function. *Toxicology* 82:77–88
86. Hanioka N, Gonzalez FJ, Lindberg NA, Liu G, Gelboin HV, Korzekwa KR. 1992. Site-directed mutagenesis of cytochrome P450s CYP2A1 and CYP2A2: influence of the distal helix on the kinetics of testosterone hydroxylation. *Biochemistry* 31:3364–70
87. Aoyama T, Korzekwa K, Nagata K, Adesnik M, Reiss A, et al. 1989. Sequence requirements for cytochrome P-450IIB1 catalytic activity. Alteration of the stereospecificity and regioselectivity of steroid hydroxylation by a simultaneous change of two hydrophobic amino acid residues to phenylalanine. *J. Biol. Chem.* 264:21,327–33
88. Aoyama T, Gonzalez FJ, Gelboin HV. 1989. Human cDNA-expressed cyto-

chrome P450 IA2: mutagen activation and substrate specificity. *Mol. Carcinog.* 2:192–98

89. Yamano S, Tatsuno J, Gonzalez FJ. 1990. The CYP2A3 gene product catalyzes coumarin 7-hydroxylation in human liver microsomes. *Biochemistry* 29: 1322–29
90. Yamano S, Nhamburo PT, Aoyama T, Meyer UA, Inaba T, et al. 1989. cDNA cloning and sequence and cDNA-directed expression of human P450 IIB1: identification of a normal and two variant cDNAs derived from the CYP2B locus on chromosome 19 and differential expression of the IIB mRNAs in human liver. *Biochemistry* 28:7340–48
91. Rettie AE, Korzekwa KR, Kunze KL, Lawrence RF, Eddy AC, et al. 1992. Hydroxylation of warfarin by human cDNA-expressed cytochrome P-450: a role for P-4502C9 in the etiology of (*S*)-warfarin-drug interactions. *Chem. Res. Toxicol.* 5:54–59
92. Rettie AE, Wienkers LC, Gonzalez FJ, Trager WF, Korzekwa KR. 1994. Impaired (*S*)-warfarin metabolism catalyzed by the R144C allelic variant of CYP2C9. *Pharmacogenetics* 4:39–42
93. Tyndale R, Aoyama T, Broly F, Matsunaga T, Inaba T, et al. 1991. Identification of a new variant CYP2D6 allele lacking the codon encoding Lys-281: possible association with the poor metabolizer phenotype. *Pharmacogenetics* 1:26–32
94. Patten CJ, Ishizaki H, Aoyama T, Lee M, Ning SM, et al. 1992. Catalytic properties of the human cytochrome P450 2E1 produced by cDNA expression in mammalian cells. *Arch. Biochem. Biophys.* 299:163–71
95. Nhamburo PT, Kimura S, McBride OW, Kozak CA, Gelboin HV, Gonzalez FJ. 1990. The human CYP2F gene subfamily: identification of a cDNA encoding a new cytochrome P450, cDNA-directed expression, and chromosome mapping. *Biochemistry* 29:5491–99
96. Aoyama T, Yamano S, Waxman DJ, Lapenson DP, Meyer UA, et al. 1989. Cytochrome P-450 hPCN3, a novel cytochrome P450IIIA gene product that is differentially expressed in adult human liver. *J. Biol. Chem.* 264:10,388–95
97. Nhamburo PT, Gonzalez FJ, McBride OW, Gelboin HV, Kimura S. 1989. Identification of a new P450 expressed in human lung: complete cDNA sequence, cDNA-directed expression, and chromosome mapping. *Biochemistry* 28:8060–66
98. Yamano S, Aoyama T, McBride OW, Hardwick JP, Gelboin HV, Gonzalez FJ. 1989. Human NADPH-P450 oxidoreductase: complementary DNA cloning, sequence and vaccinia virus–mediated expression and localization of the CYPOR gene to chromosome 7. *Mol. Pharmacol.* 36:83–88
99. Aoyama T, Nagata K, Yamazoe Y, Kato R, Matsunaga E, et al. 1990. Cytochrome b5 potentiation of cytochrome P-450 catalytic activity demonstrated by a vaccinia virus–mediated in situ reconstitution system. *Proc. Natl. Acad. Sci. USA* 87:5425–29
100. Shou M, Korzekwa KR, Crespi CL, Gonzalez FJ, Gelboin HV. 1994. The role of twelve cDNA expressed human, rodent, and rabbit cytochromes P450 in the metabolism of benzo[a]pyrene and benzo[a]pyrene *trans*-7,8-dihydrodiol. *Mol. Carcinog.* 10:159–68
101. Aoyama T, Yamano S, Guzelian PS, Gelboin HV, Gonzalez FJ. 1990. Five of 12 forms of vaccinia virus–expressed human hepatic cytochrome P450 metabolically activate aflatoxin B1. *Proc. Natl. Acad. Sci. USA* 87:4790–93
102. Aoyama T, Gelboin HV, Gonzalez FJ. 1990. Mutagenic activation of 2-amino-3-methylimidazo[4,5-f]quinoline by complementary DNA-expressed human liver P-450. *Cancer Res.* 50:2060–63
103. Smith GE, Fraser MJ, Summers MD. 1983. Molecular engineering of the *Autographa California* nuclear polyhidrosis genome: deletion mutations within the polyhedron gene. *J. Virol.* 46:584–93
104. Luckow VA, Summers MD. 1988. Trends in the development of baculovirus expression vectors. *BioTechnology* 6:47–55
105. Asseffa A, Smith SJ, Nagata K, Gillette J, Gelboin HV, Gonzalez FJ. 1989. Novel exogenous heme-dependent expression of mammalian cytochrome P450 using baculovirus. *Arch. Biochem. Biophys.* 274:481–90
106. Gonzalez FJ, Kimura S, Tamura S, Gelboin HV. 1991. Expression of mammalian cytochrome P450 using baculovirus. *Methods Enzymol.* 206:93–99
107. Ohta D, Matsu-ura Y, Sato R. 1991. Expression and characterization of a rabbit liver cytochrome P450 belonging to the P450IIB subfamily with the aid of the baculovirus expression vector system. *Biochem. Biophys. Res. Commun.* 175:394–99
108. Lähde M, Raunio H, Pelkonen O, Karp M, Oker-Blom C. 1993. Expression of human placental cytochrome P450 aromatase (CYP19) cDNA in insect cells

using a luciferase based baculovirus vector. *Biochem. Biophys. Res. Commun.* 197:1511–17

109. Imaoka S, Wedlund PJ, Ogawa H, Kimura S, Gonzalez FJ, Kim H-Y. 1993. Identification of CYP2C23 expressed in rat kidney as an arachidonic acid epoxygenase. *J. Pharmacol. Exp. Ther.* 267: 1012–16
110. Ma R, Cohen MB, Brenbaum MR, Schuler MA. 1994. Black swallowtail (*Papilio polyxenes*) alleles encode cytochrome P450s that selectively metabolize linear furanocourmarins. *Arch. Biochem. Biophys.* 310:322–40
111. Sigle RO, Titus MA, Harada N, Nelson SD. 1994. Baculovirus-mediated high-level expression of human placental aromatase (CYP19A1). *Biochem. Biophys. Res. Commun.* 201:694–700
112. Buters JTM, Korzekwa KR, Kunze KL, Omata Y, Hardwick JP, Gonzalez FJ. 1994. cDNA-directed expression of human cytochrome P450 CYP3A4 using baculovirus. *Drug Metab. Dispos. Biol. Fate. Chem.* 22:688–92
113. Tamura S, Korzekwa KR, Kimura S, Gelboin HV, Gonzalez FJ. 1992. Baculovirus-mediated expression and functional characterization of human NADPH-P450 oxidoreductase. *Arch. Biochem. Biophys.* 293:219–23
114. Crespi CL, Langenbach R, Penman BW. 1990. The development of a panel of human cell lines expressing specific human cytochrome P450 cDNAs. In *Mutation and the Environment,* ed. ML Mendelsohn, RL Albertini, pp. 97–106. New York: WileyLiss
115. Gonzalez FJ, Crespi CL, Gelboin HV. 1991. DNA-expressed human cytochrome P450s: a new age of molecular toxicology and human risk assessment. *Mutat. Res.* 247:113–27
116. Langenbach R, Smith PB, Crespi C. 1992. Recombinant DNA approaches for the development of metabolic systems used in in vitro toxicology. *Mutat. Res.* 277:251–75
117. Crespi CL, Langenbach R, Gelboin HV, Gonzalez FJ, Penman BW. Human cells as an expression system for cytochromes P450. See Ref. 139, In press
118. Sugden B, Marsh K, Yates J. 1985. A vector that replicates as a plasmid and can be efficiently selected in B-lymphoblasts transformed by Epstein-Barr virus. *Mol. Cell. Biol.* 5:410–13
119. Penman BW, Chen L, Gelboin HV, Gonzalez FJ, Crespi CL, 1994. Development of a human lymphoblastoid cell line constitutively expressing human CYP1A1: substrate specificity with model substrates and promutagens. *Carcinogenesis* In press
120. Penman BW, Reece J, Smith T, Yang CS, Gelboin HV, et al. 1993. Characterization of a human cell line expressing high levels of cDNA-derived CYP2D6. *Pharmacogenetics* 3:28–39
121. Crespi CL, Steimel DT, Aoyama T, Gelboin HV, Gonzalez FJ. 1990. Stable expression of human cytochrome P450IA2 cDNA in a human lymphoblastoid cell line: role of the enzyme in the metabolic activation of aflatoxin B1. *Mol. Carcinog.* 3:5–8
122. Crespi CL, Penman BW, Steimel DT, Gelboin HV, Gonzalez FJ. 1991. The development of a human cell line stably expressing human CYP3A4: role in the metabolic activation of aflatoxin B1 and comparison to CYP1A2 and CYP2A3. *Carcinogenesis* 12:355–59
123. Crespi CL, Penman BW, Gelboin HV, Gonzalez FJ. 1991. A tobacco smoke–derived nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, is activated by multiple human cytochrome P450s including the polymorphic human cytochrome P4502D6. *Carcinogenesis* 12:1197–1201
124. Crespi CL, Gonzalez FJ, Steimel DT, Turner TR, Gelboin HV, et al. 1991. A metabolically competent cell line expressing five cDNAs encoding procarcinogen-activating enzymes: application to mutagenicity testing. *Chem. Res. Toxicol.* 4:566–72
125. Battula N, 1989. Transduction of cytochrome P3-450 by retroviruses: constitutive expression of enzymatically active microsomal hemoprotein in animal cells. *J. Biol. Chem.* 264:2991–96
126. Nouso K, Thorgeirsson SS, Battula N. 1992. Stable expression of human cytochrome P450IIE1 in mammalian cells: metabolic activation of nitrosodimethylamine and formation of adducts with cellular DNA. *Cancer Res.* 52: 1796–1800
127. Dai Y, Rashba-Step J, Cederbaum AI. 1993. Stable expression of human cytochrome P4502E1 in HepG2 cells: characterization of catalytic activities and production of reactive oxygen intermediates. *Biochemistry* 32:6928–37
128. Tiano HF, Hosokawa M, Chulada PC, Smith PB, Wang R-L, et al. 1993. Retroviral mediated expression of human cytochrome P450 2A6 in C3H/10T1/2 cells confers transformability by 4-(methylnitrosamino)- 1-(3-pyridyl)- 1-butanone (NNK). *Carcinogenesis* 14:1421–27
129. Salonpää P, Hakkola J, Pasanen M, Pelkonen O, Vähäkangas K, et al. 1993.

Retrovirus-mediated stable expression of human CYP2A6 in mammalian cells. *Eur. J. Pharmacol. Environ. Toxicol. Pharmacol.* 248:95–102
130. Doehmer J, Oesch F. 1991. V79 Chinese hamster cells genetically engineered for stable expression of cytochromes P450. *Methods Enzymol.* 206:117–23
131. Doehmer J, 1993. V79 Chinese hamster cells genetically engineered for cytochrome P450 and their use in mutagenicity and metabolism studies. *Toxicology* 82:105–18
132. Schmalix WA, Mäser H, Kiefer F, Reen R, Wiebel FJ, et al. 1993. Stable expression of human cytochrome P450 1A1 cDNA in V79 Chinese hamster cells and metabolic activation of benzo[a]pyrene. *Eur. J. Pharmacol. Environ. Toxicol. Pharmacol.* 248:251–61
133. Wolfel C, Heinrich-Hirsch B, Schulz-Schalge T, Seidel A, Frank H, et al. 1992. Genetically engineered V79 Chinese hamster cells for stable expression of human cytochrome P450IA2. *Eur. J. Pharmacol.* 228:95–102
134. Fischer V, Vogels B, Maurer G, Tynes RE. 1992. The antipsychotic clozapine is metabolized by the polymorphic human microsomal and recombinant cytochrome P450 2D6. *J. Pharmacol. Exp. Ther.* 260:1355–60
135. Fischer V, Vickers AEM, Heitz F, Mahadevan S, Baldeck J-P, et al. 1994. The polymorphic cytochrome P-4502D6 is involved in the metabolism of both 5-hydroxytryptamine antagonists, tropisetron and ondansetron. *Drug Metab. Dispos.* 22:269–74
136. Mace K, Gonzalez FJ, McConnell IR, Garner RC, Avanti O, et al. 1994. Increased activation of promutagens in a human bronchial epithelial cell line stably expressing the human CYP1A2. *Mol. Carcinog.* In press
137. Yanagawa Y, Sawada M, Deguchi T, Gonzalez FJ, Kamataki T. 1994. Stable expression of human CYP1A2 and *N*-acetyltransferases in Chinese hamster CHL cells: mutagenic activation of 2-amino-3-methylimidazo[4,5-*f*]quinoline and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline. *Cancer Res.* 54:3422–27
138. Zhou D, Pompon D, Chen S. 1990. Stable expression of human aromatase complementary DNA in mammalian cells: a useful system for aromatase inhibitor screening. *Cancer Res.* 50:6949–54
139. Waterman M, Hildebrand M, eds. 1994. *Assessment of the Use of Single Cytochrome P450 Enzymes in Drug Research, Proceedings of the Ernst Schering Research Foundation Workshop.* Berlin: Springer-Verlag. In press