0026-895X/89/040443-07$02.00/0

MOLECULAR PHARMACOLOGY, 35:443-449

Expression of a Human P-450IIC Gene in Yeast Cells Using Galactose-Inducible Expression System

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Received August 15, 1988; Accepted January 3, 1989

SUMMARY

A cDNA of a human liver cytochrome P-450, corresponding to P-450 human-2, was expressed in *Saccharomyces cerevisiae* cells by the use of a galactose-inducible expression vector containing the *GAL7* promoter and terminator. In Western blots using anti-P-450 human-2 IgG, a single band, which exhibited mobility identical to that of authentic P-450 human-2 purified from human liver, was detected in microsomes of the yeast cells. The amount synthesized in yeast was estimated to be approximately 1% of the total cell protein, and approximately 25% of the cytochrome existed in the holoenzyme state. Microsomes from the P-450 human-2-producing yeast showed a catalytic activity towards benzo(*a*)pyrene, and the activity was significantly enhanced by the addition of purified NADPH-cytochrome P-450 reductase. The yeast microsomes also catalyzed (*S*)-mephenytoin 4-hydroxylation but not the demethylation. The present results indicate that the yeast cells containing P-450 human-2 cDNA synthesize a functionally active form of the enzyme, the chemical and catalytic properties of which are identical to those of the human liver preparation.

Cytochrome P-450 comprises a superfamily of enzymes that play an important role in the oxidative and reductive biotransformations of endogenous and exogenous chemicals including drugs and carcinogens. Recent studies involving protein chemistry and molecular cloning technology revealed that multiple forms of cytochrome P-450 exist in livers of experimental animals and they have been classified into families on the basis of similarities in their structures or response to drug inducers (1). Recently, several cytochrome P-450s have been purified from human livers, and the catalytic activities of these enzymes were determined in reconstituted systems. However, these results vary with the purity of the enzyme. In addition, high amino acid similarities and even identical amino-terminal protein sequences are observed in cytochrome P-450s in human livers. Especially in the case of hepatic (*S*)-mephenytoin 4-hydroxylase, multiple forms with identical amino-terminal protein sequences were purified from human livers, although the apparent molecular weights of these forms are different from each other (2, 3). Recently, we also purified two cytochrome P-450s, P-450 human-1 and P-450 human-2, from human livers (4). The latter showed high catalytic activities towards various drugs including benzo(*a*)pyrene and ethylmorphine, suggesting its substantial role in xenobiotic and carcinogen metabolisms in human livers (4). Interestingly, P-450 human-2 also has an amino-terminal protein sequence identical to those of the reported (*S*)-mephenytoin 4-hydroxylases (2, 3). We then isolated a cDNA clone from a λgt11 cDNA library, using anti-P-450 human-2 serum (5), and showed the presence of a close similarity to a cDNA MP-8, presumably encoding one of the (*S*)-mephenytoin 4-hydroxylases (7).[1] However, obvious similarity was observed neither in their 3′ noncoding sequences nor in the length of the whole sequence. In addition, several genes related to human (*S*)-mephenytoin 4-hydroxylase exist in human livers (8). To further assess the genetic polymorphism of (*S*)-mephenytoin 4-hydroxylase and to better understand the characteristics of this form of cytochrome P-450, P-450 human-2, we used a recombinant DNA technique and expressed its cDNA in yeast cells using a galactose-inducible expression vector (9).

Materials and Methods

Enzymes and chemicals. Restriction endonucleases and other modifying enzymes were purchased from Takara Shuzo Co. (Kyoto,

This work was supported in part by Grant-in-Aids for Encouragement of Young Scientists from the Ministry of Education, Science and Culture, for New Drug Development from the Ministry of Health and Welfare of Japan, and a grant from the Japan Health Science Foundation.

[1] The related cDNA sequences with high similarity have been listed as P-450IIC8 or IIC9 in the nomenclature proposed by Nebert *et al.* (6).

ABBREVIATIONS: mephenytoin, 3-methyl-5-phenyl-5-ethylhydantoin; bp, base pairs; *ADH*, yeast alcohol dehydrogenase; *GAL7*, α-D-galactose-1-phosphate uridyltransferase; HPLC, high performance liquid chromatography; IgG, immunoglobin G fraction; *PHO*, yeast acid phosphatase; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; nirvanol, 5-phenyl-5-ethylhydantoin; [ ], plasmid-carrier state.

Japan) and Toyobo Co. (Osaka, Japan). Zymolyase 100T was from Seikagaku Kogyo Co. (Tokyo, Japan). [α-^{32}P]dCTP (3000 Ci/mmol) was obtained from Amersham International (Buckinghamshire, UK). Random primed DNA labeling kit was obtained from Boehringer Mannheim (Mannheim, FRG). Nitrocellulose filters for immunoblot analysis were from Schleicher and Schuell (Dassel, FRG), and nylon membrane filters for RNA blot analysis were from Pall Ultra Filtration Corp. (Glen Cove, NY). Mephenytoin was kindly provided by Drs. D. Römer and H. Stähelin (Sandoz, Ltd., Basel, Switzerland). Authentic standards of its *S*-enantiomer and metabolites, 4-hydroxy-(*S*)-mephenytoin and (*S*)-nirvanol (the demethylated product), were kindly provided by Prof. F. P. Guengerich (Vanderbilt University, Nashville, TN). All other reagents were of the highest quality commercially available.

Media. Bacterial media were LB (1% trypton, 0.5% yeast extract, 1% NaCl) supplemented with 0.1 mg/ml ampicillin when necessary. Enriched minimal media for the growth of yeasts contained 0.67% yeast nitrogen base without amino acids, 0.5% casamino acids (Difco Laboratories, Detroit, MI), 0.002% L-tryptophan, 0.004% adenine sulfate, and 2% each of the following carbon sources for the indicated medium: glucose for SGlu; galactose for SGal; and glycerol and sodium lactate for SGlyLac.

Construction of expression vector pAA7 containing the *GAL7* promoter and terminator. Plasmid pAA7 (Fig. 1) consisted of pNI2 (a 7.5-kbp fragment between *Bam*HI and *Sal*I), a derivative of YEp24 (10), the promoter region of the yeast *GAL7* gene (11) (a 274-bp fragment between *Bam*HI and *Bgl*II), and its 3′ region (a 2.2-kbp fragment between *Bgl*II and *Eco*RI) covering a part of the coding region together with the transcription terminator (12). Plasmid pNI2 was constructed by eliminating two *Eco*RI sites flanking 2 μm of DNA in YEp24 by filling-in with Klenow fragment followed by blunt-end ligation. The junction between *Sal*I of pNI2 and *Eco*RI of the distal end of *GAL7* was ligated after both sites were rendered blunt with mung bean nuclease followed by ligation.

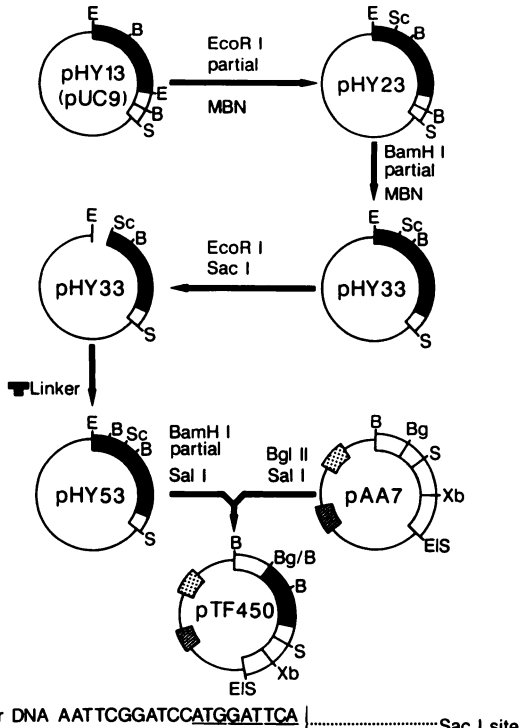

Fig. 1. Construction of plasmids for the expression of P-450 human-2 cDNA. The DNA linker includes the coding sequence (the *underlined* sequence) from the initiation codon to the *Sac*I site. The restriction sites in the plasmids and other abbreviations are as follows: *B*, *Bam*HI; *Bg*, *Bgl*II; *E*, *Eco*RI; *S*, *Sal*I; *Sc*, *Sac*I; *Xb*, *Xba*I; *MBN*, mung bean nuclease; *partial*, partial digestion. ■, P-450 human-2 cDNA; □, the yeast *URA3* gene; ▨, 2 μm circle DNA; □, in pAA7, the yeast *GAL7* gene containing the promoter and terminator; and the *single line*, in pAA7, pBR322 DNA. *E/S* indicates the blunt end ligated position to which *Eco*RI and *Sal*I sites are converted.

Plasmid preparation and transformation. The plasmid DNA was prepared from an *Escherichia coli* strain by the alkaline extraction method (13). *E. coli* transformation was carried out by the conventional method (14) and yeast transformation by the lithium ion method (15). Transformants were selected on LB agar with ampicillin for *E. coli* and SGlu agar lacking uracil for yeasts.

Preparation of total RNA from yeast and RNA blot analysis. The plasmid pTF450 was introduced to *Saccharomyces cerevisiae* MT8-1 (a *ade his3 leu2 trpl ura3*) (9) and transformant yeasts were grown in the noninducing medium SGlyLac. When the cell density reached 10^7/ml, galactose was added to a final concentration of 2%. Samples (5 ml) were withdrawn from a culture at the indicated periods, and total RNA was extracted by the procedures described by Elder *et al.* (16). RNA blot analysis was carried out according to the reported protocols (15), using P-450 human-2 cDNA from pHY13 (5) as the probe. Prehybridization, hybridization, and washing processes were performed as described (17).

Preparation of crude extracts for P-450 human-2 protein analysis. A yeast strain MT8-1 harboring either pAA7 or pTF450 was grown in the noninducing medium SGlyLac, and aliquots (10 ml) were taken at the indicated periods after the addition of galactose, as described above. Subsequent procedures were performed as described by Handa *et al.* (18). SDS-polyacrylamide gel electrophoresis was conducted according to the method of Laemmli (19) with an acrylamide concentration of 7.5%. Immunoblot analysis was performed as described by Towbin *et al* (20), using anti-P-450 human-2 IgG (4). The amount of immunoreactive protein was quantitated by densitometric readings in proportion to the densities of the purified proteins on the same blot.

Preparation of yeast microsomes from large scale culture. *S. cerevisiae* MT8-1 cells carrying the plasmid pAA7 or pTF450 were grown to the stationary phase and then an aliquot (30 ml) was introduced into SGal medium (2.0 liters, pH 7.2, adjusted with 0.2 M K_2HPO_4). The cells were collected at the logarithmic phase of the growth by centrifugation, and microsomes were prepared by the method described by Oeda *et al.* (21) with minor modifications. The cells were washed with distilled water and resuspended to less than 0.5 g of cells/ml in buffer I (0.1 M Tris-sulfate, pH 9.4, 10 mM dithiothreitol). The suspension was incubated with gentle shaking for 15 min at 25°. The cells were washed twice with buffer II (10 mM Tris-chloride, pH 7.5, 0.65 M sorbitol, 0.1 mM dithiothreitol, 0.1 mM EDTA) and suspended to less than 0.15 g of cells/ml in buffer III (10 mM Tris-chloride, pH 7.5, 2.0 M sorbitol, 0.1 mM dithiothreitol, 0.1 mM EDTA) containing 0.1 mg/ml Zymolyase 100T. The suspension was incubated with gentle shaking for 1 hr at 30°, washed twice with buffer III, and then resuspended in buffer II containing 0.1 mM PMSF. The resulting spheroplasts were disrupted by sonication (10 W, 1 min, 5 times) and homogenized in a Dounce tissue grinder. The lysate was centrifuged at 3000 × *g* for 15 min to precipitate cell debris, then the supernatant was centrifuged at 10,000 × *g* for 20 min to recover crude mitochondrial fraction. The supernatant obtained was ultracentrifuged at 125,000 × *g* for 90 min to separate the microsomal fraction from the soluble fraction. The sediment recovered as the microsomal pellet was resuspended in buffer II containing 0.1 mM PMSF.

Assay of benzo(*a*)pyrene and (*S*)-mephenytoin metabolism. The incubation mixture for the assay of benzo(*a*)pyrene hydroxylation consisted of 4 mg of yeast microsomal protein, a variable amount of purified rat NADPH-cytochrome P-450 reductase, anti-P-450 human-2 IgG (when necessary), 100 mM potassium phosphate buffer (pH 7.4), 0.1 mM EDTA, an NADPH-generating system (6 mM $MgCl_2$, 0.8 mM NADP, 8 mM glucose 6-phosphate, and 1 unit of glucose 6-phosphate dehydrogenase), and 0.1 mM substrate in a final volume of 1 ml. All

determinations were performed in duplicate. Reactions were initiated by the addition of the NADPH-generating system and were carried out at 37° for 1 hr. Activity of benzo(*a*)pyrene hydroxylase was quantitated as described by Nebert and Gelboin (22). Assay of (*S*)-mephenytoin metabolism was performed as described by Meier *et al.* (23) with some modifications. The incubation mixture contained 4 mg of yeast or 300 μg of human liver microsomal protein; the reductase and the antibody (when necessary) described above were used in a final volume of 250 μl. The microsomal suspension was preincubated for 5 min at 37° with 25 μl of the NADPH-generating system and buffer system described above. The reaction was started by the additon of 25 μl of 200 μM (*S*)-mephenytoin dissolved in 2.5% propylene glycol. The incubation was terminated after 1 hr with 100 μl of a 2% sodium azide solution containing 2.5 μM sodium phenobarbital (internal standard). The mixture was extracted with 5 ml of dichloromethane, and then the extract was evaporated under a gentle stream of nitrogen. The residue was dissolved in 100 μl of water and a 50-μl aliquot was subjected to HPLC analysis. The HPLC system consisted of a JASCO TRI-Rotor-V pump, an UVIDEC-100-III UV spectrophotometer set at 204 nm, and a Nucleosil 7 C_{18} column (4.6 × 300 mm), and the metabolites were eluted with a mixture of 6% acetonitrile/25% methanol/69% 20 mM $NaClO_4$ (pH 2.5, adjusted with perchloric acid) at a flow rate of 1 ml/min. The amount formed was determined by the peak height of the metabolite using a standard curve generated by the authentic standard.

Other methods. The content of cytochrome P-450 in the microsomes was determined according to the method of Omura and Sato (24), except for the inclusion of 0.2% Emulgen 913 in solubilization buffer. Protein content was determined by the method of Lowry *et al.* (25), using bovine serum albumin as the standard.

Results

Construction of plasmids for expression of P-450 human-2. The recombinant plasmid pTF450, which directed synthesis of P-450 human-2 protein in *S. cerevisiae* cells, was constructed as shown in Fig. 1. The cDNA, which has been previously cloned in the plasmid pHY13 , consisted of a 1464-bp protein-coding sequence and a 383-bp segment of the 3′ noncoding region (5). Starting from this plasmid, an *Eco*RI site located at the downstream of P-450 human-2 cDNA was cleaved, and its cohesive ends were blunted by mung bean nuclease to form the plasmid pHY23. A similar procedure was carried out at the neighboring *Bam*HI site above the *Eco*RI site to form the plasmid pHY33. Plasmid pHY33 was digested with *Eco*RI and *Sac*I, and a small *Eco*RI-*Sac*I fragment was replaced by a synthetic DNA linker (Fig. 1) to construct pHY53, containing the entire coding region of P-450 human-2 cDNA. The synthetic DNA linker was designed to include the 9-bp sequence of the 5′ terminus of the coding sequence for P-450 human-2, which was lacking in a previously isolated cDNA (5). The 9-bp sequence was predicted by the amino-terminal of the purified protein and also by the 5′ terminal nucleotide sequence of the cDNA for rat P-450 (M-1) (26), the entire sequence of which was highly homologous to that of P-450 human-2 cDNA. Then P-450 human-2 cDNA, excised from pHY53 by *Bam*HI partial and *Sal*I complete digestion, was inserted between the *GAL7* promoter and terminator of the expression vector pAA7.

Transcription and expression of P-450 human-2 cDNA in yeast. The plasmid pTF450 or pAA7 was introduced into MT8-1. Whole-cell extracts prepared from both MT8-1[pTF450] and MT8-1[pAA7] were analyzed by immunoblotting using anti-P-450 human-2 IgG. As shown in Fig. 2, a protein reacting with anti-P-450 human-2 IgG was observed as a major band in extracts from MT8-1[pTF450] that migrated at a position identical to that of authentic P-450 human-2 (M_r 56,000). The P-450 human-2 protein was clearly detectable 60 min after the addition of galactose to the culture, and its amount in yeast increased with the incubation period up to 180 min. On the other hand, the extracts from MT8-1[pAA7] did not show any immunodetectable protein. Total RNA isolated from MT8-1[pTF450] was analyzed by RNA blotting using the ^{32}P-labeled P-450 human-2 cDNA from pHY13 as the probe. As shown in Fig. 3, a distinct band of P-450 human-2-specific mRNA was detected only 15 min after the addition of galactose, and its amount reached a plateau level by 90 min. Judging from the relative position of 18S and 28S ribosomal RNA markers, the size of P-450 human-2 mRNA synthesized in yeast was estimated to be approximately 2.0 kb, which was consistent with the size predicted from the construction of the plasmid if transcription of the P-450 cDNA begins and ends at the respective points at which *GAL7* normally starts and terminates.

Quantitation of P-450 human-2 hemoprotein in yeast. Subcellular localization of P-450 human-2 in yeast was examined by immunoblotting using anti-P-450 human-2 IgG. P-450 human-2 was mainly localized in microsomes (60%). The protein was also detectable in the mitochondria (13%) and the cell debris (27%) but not in the soluble fraction (data not shown). The reduced CO-difference spectra were obtained with the microsomes prepared from MT8-1[pTF450] and MT8-1[pAA7] (Fig. 4). Microsomal fraction of MT8-1[pTF450] showed a prominent absorption peak at 450 nm, although no appreciable peak was detected around 450 nm in the spectrum of MT8-1[pAA7] microsomes. These results, together with the immunological data, indicate that P-450 human-2 protein represents approximately 1% of the total cell protein, and 25% of this P-450 exists as the holoenzyme state. The specific content of P-450 human-2 in MT8-1[pTF450] microsomes ranged from 0.14 to 0.2 nmol/mg of protein. Unless the pH of a culture was kept neutral by the addition of K_2HPO_4, the holoenzyme content decreased to half of that in a culture containing K_2HPO_4, in which a 5-fold increase of the total amount of P-450 protein was obtained.

Monooxygenase activity of P-450 human-2 expressed in yeast. Monooxygenase activities of the yeast microsomes towards benzo(*a*)pyrene and (*S*)-mephenytoin are shown in Table 1. MT8-1[pTF450] microsomes showed approximately 25-fold higher activity than those of MT8-1[pAA7] in benzo(*a*)pyrene hydroxylation. The specific activity was considerably lower (approximately 1%) than that of purified P-450 human-2 in the reconstituted system (4) but significantly increased with the amount of purified rat NADPH-cytochrome P-450 reductase. To confirm that benzo(*a*)pyrene hydroxylation in the yeast is catalyzed by P-450 human-2, the effect of anti-P-450 human-2 IgG on that activity was examined. The addition of 0.096 mg of anti-P-450 human-2 IgG to a reaction mixture containing 1 mg of the microsomal protein inhibited the activity by 95%. However, anti-P-450 human-2 IgG did not inhibit the activity of MT8-1[pAA7] microsomes (see Fig. 6). The titration curve and the rate of inhibition were almost the same as those previously reported for liver microsomes (4). MT8-1[pTF450] microsomes also catalyzed (*S*)-mephenytoin 4-hydroxylation but not the demethylation. Typical chromatograms of (*S*)-mephenytoin metabolites are shown in Fig. 5. Similar to benzo(*a*)pyrene hydroxylation, the activity was enhanced by the addition of the reductase, whereas no such

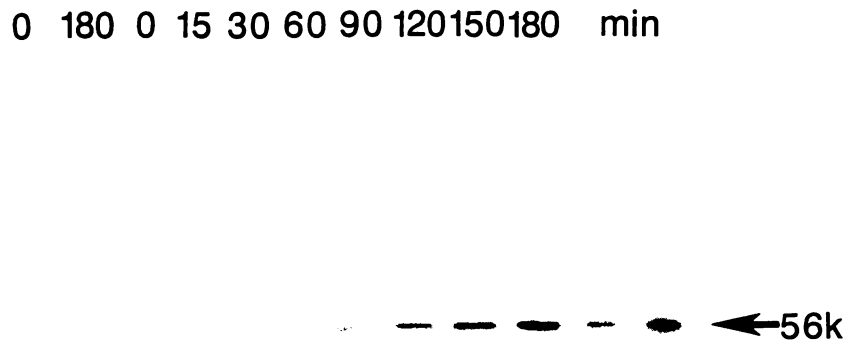

Fig. 2. Immunoblot analysis of P-450 human-2 protein synthesized in yeast using anti-P-450 human-2 IgG. Each *lane* contained 5 μg of the extract prepared from MT8-1[pAA7] or MT8-1[pTF450], which was harvested at indicated periods after galactose addition. Different amounts (A, 0.375 pmol; B, 0.75 pmol) of purified P-450 human-2 were used as the authentic standard.

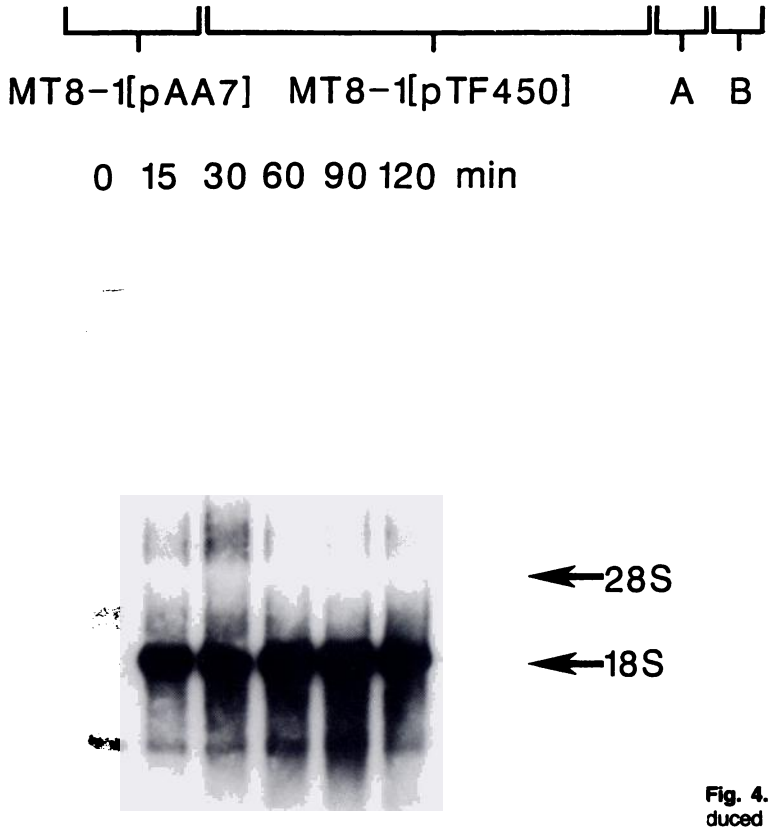

Fig. 3. RNA blot analysis of P-450 human-2 mRNA in yeast. Each *lane* contained 5 μg of total RNA isolated from MT8-1[pTF450]. *Numbers at top* indicate periods after galactose addition. Yeast ribosomal RNA markers are indicated at 18S and 28S.

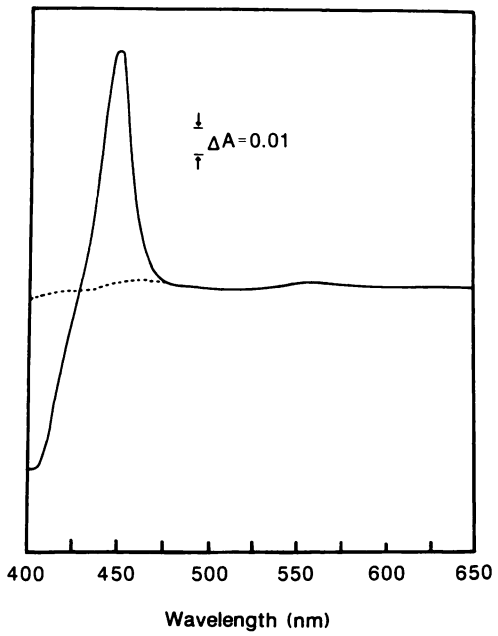

Fig. 4. Reduced CO-difference spectra of yeast microsomes. The reduced CO-difference spectra of cytochrome P-450 were obtained according to the method of Omura and Sato (24). Spectra were recorded with 25 mg/ml microsomal protein.—— MT8-1[pTF450]; – – –, MT8-1[pAA7].

enhancement was detected with those of MT8-1[pAA7]. The addition of 0.096 mg of anti-P-450 human-2 IgG to 1 mg of the microsomal protein inhibited its activity almost completely (Fig. 6).

Correlation between catalytic activities towards (*S*)-mephenytoin and immunochemically determined P-450 human-2 content in human liver. Activities of (*S*)-mephenytoin 4-hydroxylation and demethylation in human liver microsomes were examined to discern the relationship with P-450 human-2 content in human liver microsomes. A good correlation (r = 0.709) was observed between the activity of (*S*)-mephenytoin 4-hydroxylation and the content of P-450 human-2 in human livers; however, no significant correlation between its demethylation and the P-450 human-2 content was observed (Fig. 7). Although microheterogeneities are known in the related genes of P-450 human-2 (7, 8), these results indicate that P-450 human-2 exists as a functionally active form of (*S*)-mephenytoin 4-hydroxylase in human liver microsomes.

Discussion

Many forms of cytochrome P-450 have been isolated from experimental animal and human tissue, and the cDNAs of some

TABLE 1

Benzo(*a*)pyrene hydroxylase and (*S*)-mephenytoin 4-hydroxylase activities of microsomal fractions prepared from MT8-1[pAA7] and MT8-1[pTF450]

Reaction mixture: Microsomes	Reaction mixture: Rat f_{PT}[c]	Benzo(*a*)pyrene hydroxylase activity[a]	(*S*)-Mephenytoin 4-hydroxylase activity
	units	*pmol/hr/nmol of P-450 (pmol/hr/mg of microsomal protein)*	*pmol/hr/nmol of P-450*
MT8-1[pAA7]	0	(0.276)	ND[d]
MT8-1[pAA7]	1.0	(0.493)	ND
MT8-1[pTF450]	0	118 (4.16)	218
MT8-1[pTF450]	0.25	175 (6.18)	302
MT8-1[pTF450]	0.5	234 (8.28)	459
MT8-1[pTF450]	1.0	355 (12.55)	724
MT8-1[pTF450]	1.5	409 (14.46)	843

[a] The activity of purified P-450 human-2 from liver microsomes in the reconstituted system was 0.214 nmol/min/nmol of P-450 (4).
[b] The detection limit of the metabolite was 7.5 pmol/hr/nmol of P-450.
[c] NADPH-cytochrome P-450 reductase.
[d] ND, not detected.

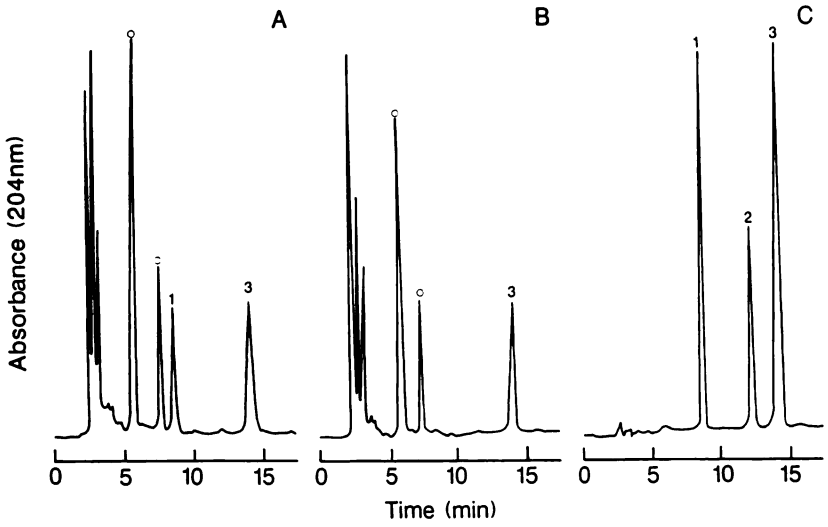

Fig. 5. Typical chromatograms of (*S*)-mephenytoin metabolites. Extracts (50 μl) of the incubated mixture containing microsomes from MT8-1[pTF450] (A) and MT8-1[pAA7] (B) were injected for HPLC. Separations of the authentic standards (50 μl) containing 1 μmol each of 4-hydroxy-(*S*)-mephenytoin (*peak 1*), (*S*)-nirvanol (*peak 2*), and phenobarbital (*peak 3*, internal standard) are shown in C. Other peaks (○) were also detected at the chromatogram of the extracted incubation mixture without substrate.

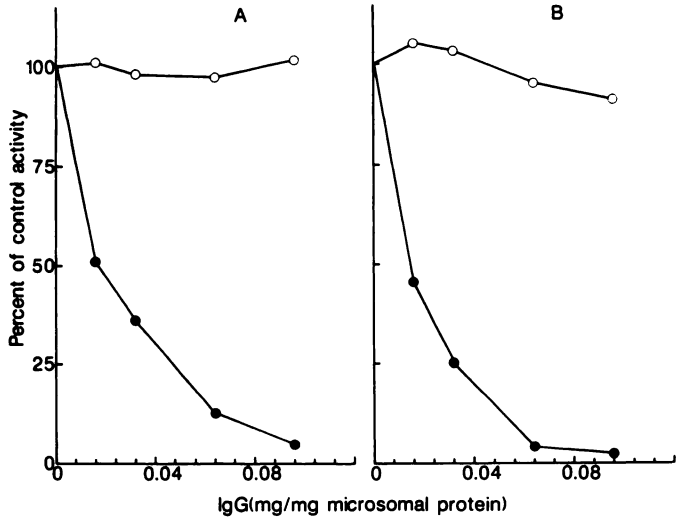

Fig. 6. Inhibition of benzo(*a*)pyrene hydroxylase (A) and (*S*)-mephenytoin 4-hydroxylase (B) activities in MT8-1[pTF450] microsomes by anti-P-450 human-2 IgG. The specific content of P-450 human-2 in the microsomes was 0.14 nmol/mg of protein. Incubations were carried out with MT8-1[pTF450] microsomes and 1 unit of purified rat NADPH-cytochrome P-450 reductase. ●, Anti-P-450 human-2 IgG; ○, nonimmune IgG.

P-450s were cloned and expressed in yeast or mammalian cells (21, 27–34). Although expression in yeast of P-450 cDNAs derived from human livers has not yet been reported, we have successfully expressed a cDNA cloned from λgt11 cDNA library of human origin and produced a functionally active form of a human cytochrome P-450 in yeast. Microsomes from the yeast bearing P-450 human-2 cDNA catalyzed benzo(*a*)pyrene hydroxylation, which was observed with the purified P-450 in the reconstituted system (4). Furthermore, the rate of inhibition of the hydroxylation in the yeast microsomes by anti-P-450 human-2 IgG (Fig. 6) was almost identical to that in human livers. The expressed protein also catalyzed (*S*)-mephenytoin 4-hydroxylation. Although several human cDNAs have been isolated in relation to (*S*)-mephenytoin 4-hydroxylase, none of these genes has been shown conclusively to encode this enzyme. We have demonstrated that P-450 human-2 cDNA encodes a functionally active (*S*)-mephenytoin 4-hydroxylase. P-450 human-2 cDNA showed a high sequence similarity to a cDNA MP-8, presumably encoding one of the human (*S*)-mephenytoin 4-hydroxylases (7). There were only four nucleotide differences in their coding sequences. However, no obvious similarity was observed in their 3′ noncoding sequences, except for the first 75 bases, and in the length of the complete sequence. Although a genomic DNA blot analysis using nonoverlapping 5′ and 3′ portions of MP-8 suggested that approximately seven

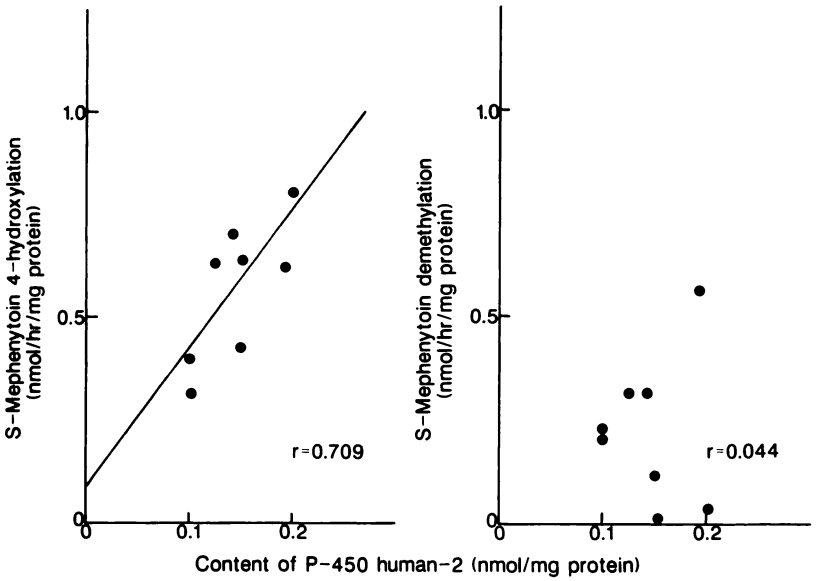

Fig. 7. Correlation between (*S*)-mephenytoin 4-hydroxylase activity of human liver microsomes and immunochemically determined P-450 human-2 content in human liver. The *line* was drawn according to the results of a linear regression analysis. Each *point* represents a different individual liver of a Japanese subject.

related sequences were present in this gene family (8), it is unknown whether P-450 human-2 cDNA is an allelic variant of MP-8 or its duplicated gene formed by alternative splicing. In order to better understand the genetic polymorphism of (*S*)-mephenytoin hydroxylation, it will be necessary to ascertain that the related genes including MP-8 encode functionally active (*S*)-mephenytoin 4-hydroxylases. Recently Meier *et al.* (35) reported that genetic polymorphism of mephenytoin hydroxylation was due to the presence of a functionally altered P-450 and not to be a decreased amount of intact enzyme protein. However, a good correlation was observed between microsomal (*S*)-mephenytoin 4-hydroxylation and the content of P-450 human-2 in human livers (Fig. 7). These results indicate that P-450 human-2 catalyzing benzo(*a*)pyrene hydroxylation is a form of (*S*)-mephenytoin 4-hydroxylase in human livers.

The production of cytochrome P-450 in yeast was previously performed by the use of the *ADH1* (21, 27–30) or *PHO5* (34) promoters. In the present study, we have demonstrated that the *GAL7* promoter is also a powerful tool for the synthesis of cytochrome P-450 in yeast. The yield of P-450 human-2 achieved by the present expression system, approximately 1% of the total protein, appears comparable to those of the P-450s in the previous reports. The yield may be improved severalfold by amplifying the positive regulatory gene *GAL4* with a multicopy plasmid (12). One problem that remains to be overcome, how can the proportion of holoenzyme in the total P-450 produced in yeast be increased? In this work, we used a high pH medium and observed an increase in the proportion of holoenzyme, compared with that in the conventional medium, but at the expense of normal yeast growth. In order to synthesize a functionally active P-450 in yeast with a better yield, a systematic search for optimum growth conditions, as well as for genetic methods, is underway in our laboratories.

Acknowledgments

We are indebted to Dr. M. Niwa (Fujisawa Pharmaceutical Co., Osaka, Japan) for the synthesis of the linker DNA and to K. Suzuki and K. Yamauchi for their skillful assistance.

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