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THE EXPRESSION OF HUMAN CYTOCHROME P450IA1 IN THE YEAST *SACCHAROMYCES CEREVISIAE**

MICHAEL S. CHING,†‡ MARTIN S. LENNARD,† GEOFFREY T. TUCKER,†
H. FRANK WOODS,† DIANE E. KELLY†§ and STEVEN L. KELLY†§

† University Department of Medicine and Pharmacology, Royal Hallamshire Hospital, Sheffield S10 2JF; and § Krebs Institute for Biomolecular Research, Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield S10 2TN, U.K.

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Abstract—Data from animal studies suggest that cytochrome P450IA1 catalyses the metabolic activation of several procarcinogenic compounds. In the present study, we have expressed human cytochrome P450IA1 in yeast cells. A 1.70 kb *BclI/Bam*HI fragment containing a full-length human cytochrome P450IA1 cDNA was inserted into the *Bgl*II expression site of the yeast expression plasmid pMA91 thereby allowing the ATG initiation codon to be located adjacent to the PGK (phosphoglycerate kinase) promoter. The resulting recombinant plasmid, pCK-1, was introduced into *Saccharomyces cerevisiae* strains ATCC 44773 and AH22. Microsomes prepared from yeast transformants of strain ATCC 44773 contained undetectable levels of cytochrome P450. In contrast, microsomes from strain AH22 contained cytochrome P450 with a specific content of 33.3 ± 10.8 pmol/mg of microsomal protein and showed a reduced carbon monoxide difference spectrum with a peak at 448 nm. Control yeast cells transformed with pMA91 showed no cytochrome P450. Western blots were carried out using an antibody that reacts against rat cytochrome P450IA1 and an antibody that reacts against a synthetic peptide representing a short sequence of human cytochrome P450IA1. A band with a molecular weight of 54 kD was observed in microsomes of yeast transformed with pCK-1, but not with pMA91. When microsomes from yeast transformed with pCK-1 were incubated with benzo(*a*)pyrene (10 min, 10–160 μM), an estimated K_m value of 7 μM was obtained. The availability of yeast cells with functionally active human cytochrome P450IA1 will facilitate molecular structure–activity studies of procarcinogen and drug metabolism by this enzyme in man.

The cytochrome P450I gene family comprises two members, IA1 and IA2, which are ubiquitous in mammals. Although cytochrome P450IA1 has been detected immunologically at very low levels in rabbit lung [1], it is usually detectable only after treatment with inducing agents such as 3-methylcholanthrene. The enzyme appears to have an important role in the carcinogenic activation of polycyclic aromatic hydrocarbons including benzo(*a*)pyrene [2, 3]. Considerable data are available regarding rat and mouse cytochrome P450IA1 [4, 5] but the role of the human isoenzyme remains poorly defined. This isoenzyme has not been isolated or purified from human tissues [3, 4] and it is not constitutively expressed in human liver [4, 6, 7]. Data from animal studies of cytochrome P450IA1 cannot be extrapolated directly to humans. For example, whereas mouse [8] cytochrome P450IA1 has virtually no capacity to activate the carcinogen 2-acetylaminofluorene, the rat and human isoenzymes are capable of N-hydroxylating this compound [9–11].

Recent studies highlight the contribution of cytochrome P450IA1-mediated metabolism to chemical carcinogenesis in man. McLemore *et al.* [12] examined cytochrome P450IA1 gene expression in normal lung tissue and in lung tissue from patients with various primary pulmonary carcinomas. In normal lung tissue from active smokers, 89% expressed cytochrome P450IA1 mRNA whereas expression was absent in normal tissue from non-smokers. A strong positive correlation was also observed between P450IA1 gene expression and ethoxyresorufin O-deethylation in lung microsomes. Cytochrome P450IA1 gene expression was also found in lung cancer patients, even in the absence of smoking and other environmental inducers, suggesting alterations in cytochrome P450IA1 gene regulation in lung cancer. Another study has shown a correlation between enhanced lung cancer risk and an *Msp*I restriction fragment length polymorphism of the human cytochrome P450IA1 gene [13].

Central to the problem of defining the activity of this enzyme is the availability of suitable methods of isolating and characterizing single isoenzymes. In previous studies, human [10, 11] and mouse [14] cytochrome P450IA1 cDNA has been expressed in human cell lines and Cos-1 cells as functionally active protein. Rat [15, 16] and mouse [17, 18] cytochrome P450IA1 have also been expressed as functionally active protein in yeast. The aim of the present study was to express human cytochrome P450IA1 in the yeast *Saccharomyces cerevisiae* using the constitutive PGK (phosphoglycerate kinase) promoter. Eugster *et al.* [19] reported recently the expression of human cytochrome P450IA1 in yeast, although in that study a high background of endogenous yeast cytochrome P450 was observed which is known to possess a

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‡ To whom correspondence should be addressed.

metabolic capacity in carcinogen activation. In our expression system there was no detectable contribution from the endogenous enzyme.

MATERIALS AND METHODS

Enzymes and chemicals. Restriction endonucleases and other modifying enzymes were purchased from Gibco BRL (Paisley, U.K.) and culture media were obtained from Difco (Michigan, U.S.A.). Nitrocellulose filters for Western blotting were purchased from Schleider and Schuell (Dassel, Germany) and alkaline phosphatase-conjugated goat (anti-rabbit and anti-mouse) IgG and benzo(*a*)pyrene were obtained from the Sigma Chemical Co. (Poole, U.K.). All other reagents were of the highest available grade.

Microbial strains and plasmids. The *Escherichia coli* strains used in this study were DH5α (F^-, *recA*1 *endA*1 *gyrA*96 *thi*-1 *hsdR*17 *relA*1 *suppE*44 $\lambda -$ Δ*lacU*169 θ80*lacZ* Δ M15) obtained from Gibco BRL and GM242 (F^-*dam*$^-$ *recA*1 *suppE*44 *sin*-2 *tw*-1 *leuB*6 *proA*2 *his*4 *metB*1 *lacY*1 *galK*2 *ara*-14 *tsx*-33 *phi*-1 *deoB*6 *rpsL*260) obtained from Prof. J. R. Guest (Department of Microbiology, University of Sheffield). Yeast strain ATCC 44773 (a *leu*2-3*leu*2-112 *his*3-Δ 1 *ura*3-52 *trp*1-289) was obtained from the American Type Culture Collection and yeast strain AH22 (ρ^o a *leu*2-3 *leu*2-112 *can*1 *cir*$^+$ *his*4-519) was obtained from Dr H. F. J. Bligh (Department of Molecular Biology, University of Edinburgh). The recombinant plasmid which carries the full-length cDNA encoding human cytochrome P450IA1 was a gift from Dr D. W. Nebert (National Institutes of Health, Maryland, U.S.A.) and the yeast expression plasmid pMA91 carrying the PGK promoter and terminator [20] was a gift from Dr S. Kingsman (Department of Biochemistry, University of Oxford).

Media. *E. coli* was cultivated in L-broth containing 1% (w/v) bactotryptone, 0.5% (w/v) yeast extract and 1% (w/v) sodium chloride supplemented with 0.1 mg/mL of ampicillin where necessary. Minimal medium YM containing 1.34% (w/v) yeast nitrogen base without amino acids, 3% (w/v) glucose, 40 μg/mL *l*-histidine, 40 μg/mL uracil and 40 μg/mL *l*-tryptophan was used to cultivate yeast strain ATCC 44773. YM medium containing 40 μg/mL *l*-histidine was used to cultivate yeast strain AH22.

The construction of expression plasmid pCK-2 containing the PGK *promoter*. Plasmid pCK-1, which directed the synthesis of cytochrome P450IA1 in yeast strains ATCC 44773 and AH22, was constructed as shown in Fig. 1. All restriction endonuclease digestions, ligations and dephosphorylations were carried out according to standard techniques [21]. The 1.70 kb *Bcl*I/*Bam*HI fragment containing the full-length cytochrome P450IA1 cDNA was ligated into the *Bgl*II expression site of pMA91, thereby allowing the ATG start codon to be located adjacent to the PGK promoter. Restriction map analysis of the recombinant plasmid pCK-1 was used to confirm that the fragment was inserted in the correct orientation.

Cultivation of yeast cells. Yeast transformations were carried out by the lithium ion method [22]. ATCC 44773 and AH22 yeast cells transformed with either pMA91 or pCK-1 were grown to late logarithmic growth phase in YM media and then harvested.

Preparation of yeast microsomes. Harvested yeast cells were resuspended in 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM EDTA and 0.65 M sorbitol (buffer A) and then broken in a glass bead mill-type cell homogenizer (Braun, model 853022, Germany) at 4000 rpm for 1–2 min with carbon dioxide cooling. The homogenate was centrifuged at 2000 *g* for 5 min to remove residual whole cells and the supernatant was then centrifuged at 15,000 *g* for 20 min to remove cell debris and mitochondria. After centrifuging the supernatant at 100,000 *g* for 90 min at 4° (Beckman L-80 ultracentrifuge, 70 Ti rotor), the microsomal pellet was resuspended in buffer A at a protein concentration of 10–15 mg/mL.

Western blotting. SDS–polyacrylamide gel electrophoresis was carried out by the method of Laemmli [23] using an acrylamide concentration of 10% (w/v). After electrophoresis, the proteins were transferred by electroblotting (Biorad Trans Blot apparatus, Watford, U.K.) to a nitrocellulose filter and probing was carried out according to the method of Burnette [24]. The two primary antibodies used in this study were gifts from Dr A. R. Boobis (Department of Clinical Pharmacology, Royal Postgraduate Medical School, London). One was a monoclonal antibody raised in mice against rat cytochrome P450IA1 (antibody 3/4/2) [25]. This antibody cross-reacts with human cytochrome P450IA2 and is believed to cross-react with human cytochrome P450IA1, since it was predicted that the epitope with which this antibody reacts should be present on both cytochrome P450IA1 and IA2 in man [25]. The other primary antibody was a polyclonal antibody raised in rabbits against a synthetic peptide corresponding to amino acid sequence position 290–297 (Gln-Glu-Lys-Gln-Leu-Asp-Glu-Asn) which appears to be unique to human cytochrome P450IA1 (R. J. Edwards, personal communication). Liver microsomes prepared from rats induced with β-naphthoflavone were used as controls in the Western blotting.

Assays. Protein concentration was measured by the method of Lowry *et al.* [26] using bovine serum albumin as a standard. The cytochrome P450 content of microsomes was measured by reduced carbon monoxide difference spectroscopy [27] using a model PU8800 split beam scanning spectrophotometer (Phillips, Cambridge, U.K.). An extinction coefficient of 91 $mM^{-1}\,cm^{-1}$ was used for reduced carbon monoxide bound cytochrome P450 between 448 and 490 nm. Benzo(*a*)pyrene hydroxylase activity over the concentration range of 10 to 160 μM was measured in triplicate according to the method of Nebert [28]. Each 1 mL of incubation mixture contained yeast microsomes (at a final microsomal protein concentration of around 2.5 mg/mL), $MgCl_2$ (0.61 mg), NADPH (0.4 μmol), bovine serum albumin (5 mg), benzo(*a*)pyrene (using the required amount of a concentrated stock 2 mg/mL in acetone) and phosphate buffer, pH 7.4 (0.1 M). The mixture was incubated in a shaking water bath at 37° for

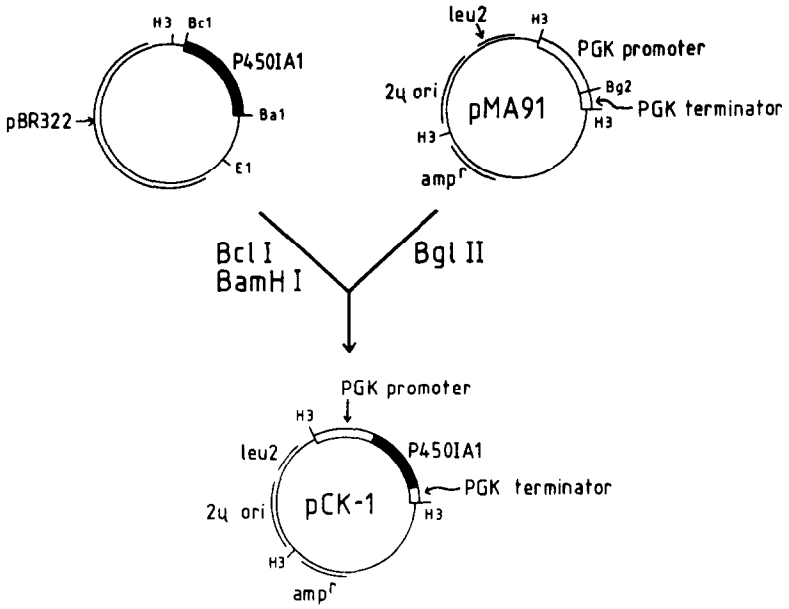

Fig. 1. Construction of plasmid pCK-1. The dark-boxed region indicates the protein coding sequence and the open-boxed region indicates the promoter and terminator sequences. The restriction sites are Ba1, *Bam*HI; Bc1, *Bcl*1; Bg2, *Bgl*II; E1, *Eco*R1; H3, *Hin*dIII. The restriction map of each plasmid is drawn to scale.

10 min and a preliminary experiment showed that the reaction rate was linear during incubation.

RESULTS

The spectrum of yeast microsomes prepared from AH22 cells transformed with pCK-1 showed a Soret peak at 448 nm which was not evident in the microsomal fraction prepared from AH22::pMA91 cells (Fig. 2). In contrast, no evidence of cytochrome P450 expression was observed in strain ATCC 44773. The content of human cytochrome P450IA1 haemoprotein in AH22::pCK-1 yeast microsomes was 33.3 ± 10.8 pmol/mg of microsomal protein (range 19.1–46.5 pmol/mg, N = 7 preparations). In the Western blot, using antibody 3/4/2, a band with molecular weight 56 kD was present in β-naphthoflavone induced rat liver microsomes, and a band with molecular weight 54 kD was present in AH22::pCK-1 microsomes but not in AH22::pMA91 microsomes (Fig. 3). Using the human cytochrome P450IA1 anti-peptide antibody, a band with molecular weight of 54 kD was present in AH22::pCK-1 microsomes only (data not shown). At a substrate concentration of 80 μM, the microsomal fraction prepared from AH22::pCK-1 cells catalysed benzo(*a*)pyrene metabolism (1.39 ± 0.42 fluorescence units/mg protein/min, range 1.00–1.99, N = 4 preparations) whereas no activity was observed in AH22::pMA91 microsomes (<0.014 fluorescence units/mg protein/min, N = 3 preparations). A plot of velocity of reaction against substrate concentration is shown in Fig. 4. Transformation of these data according to Lineweaver–Burk gave an estimated K_m value of 7 μM.

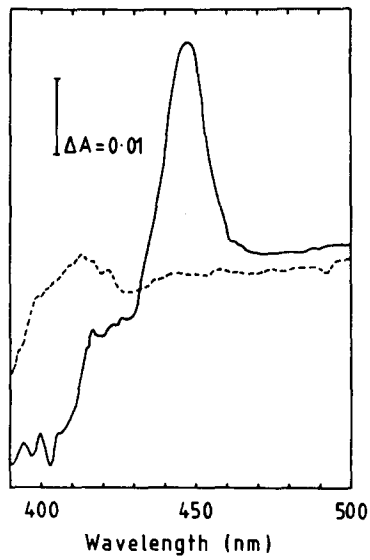

Fig. 2. The reduced carbon monoxide difference spectrum of the microsomal fraction from strain AH22 yeast cells transformed with expression plasmid pCK-1 (——) (protein concentration = 10 mg/mL) and the control plasmid pMA91 (– – –) (protein concentration = 9 mg/mL).

DISCUSSION

The characterization of individual cytochrome P450 isoenzymes using conventional biochemical methods is difficult because of the presence of multiple forms of P450s in tissues, the similar physical

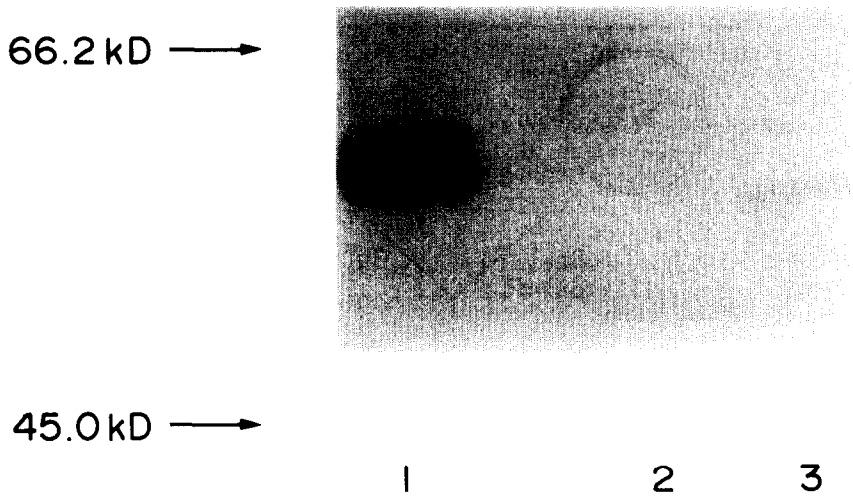

Fig. 3. Western blot using primary antibody 3/4/2. Lane 1; positive control of β-naphthoflavone induced rat liver microsomes (protein loading = 3.3 μg, specific content of P450 = 1.2 nmol/mg). Lane 2; microsomes from yeast transformed with pMA91 (protein loading = 0.45 mg). Lane 3; microsomes from yeast transformed with pCK-1 (protein loading = 0.51 mg, specific content of P450 = 29 pmol/mg).

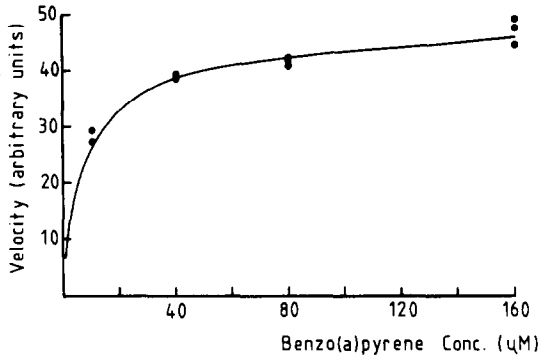

Fig. 4. Plot of velocity of benzo(*a*)pyrene metabolite production vs substrate concentration in microsomes from AH22 yeast transformed with expression plasmid pCK-1.

properties of these membrane bound proteins and, in the case of human isoenzymes, the limited availability of human tissue [2]. In contrast, cytochrome P450 cDNA-directed expression in yeast offers several advantages. The life-cycle, physiology and genetics of yeast are well understood, they are easy to grow in batch or chemostat culture and the cloned P450s are in a normal physiological environment, bound to smooth endoplasmic reticulum and coupled to cytochrome P450 reductase [29]. The lower cost and potentially higher P450 yield in *S. cerevisiae* is an advantage in comparison to mammalian cell expression systems.

The level of cytochrome P450IA1 expression achieved with the PGK promoter in AH22 cells was similar to that obtained for other mammalian P450s in yeast. These include rat cytochrome P450IIE1 (30–60 pmol/mg) [30], rat P450IIB1 (68 pmol/mg) [31], mouse P450IA1 (approximately 100 pmol/mg) [18] and a human P450IIC (approximately 40 pmol/mg) [32]. In contrast, using the ADH promoter in AH22 yeast cells, microsomal protein contents of up to 400–500 pmol/mg have been reported for rat cytochrome P450IA1 [33] and a chimeric rat IA1/IA2 protein [34]. In the present study, AH22 :: pCK-1 microsomes exhibited aryl hydrocarbon hydroxylase activity. In addition, the Michaelis constant for the expressed human enzyme (K_m = 7 μM) was an order of magnitude lower than that reported for endogenous yeast cytochrome P450LI (K_m = 111 μM) [35].

During this work, we examined initially expression using pCK-1 in the strain ATCC 44773 but were unable to observe any cytochrome P450 in the microsomal fraction using reduced carbon monoxide difference analysis. Use of the *GAL*1 inducible promoter for expression was also investigated but only a low level (≤5 pmol/mg) of cytochrome P450IA1 was produced. With both the PGK and *GAL*1 promoters, the intervening sequence between the yeast promoter and the ATG start codon of the cDNA was at a minimum and, therefore, theoretically ideal. These results indicate the importance of yeast strain and promoter in achieving optimal expression. The difference between expression from pCK-1 in strains AH22 and ATCC 44773 appears not to be related to the ability of the strain to produce endogenous cytochome P450LI as both strains fall into the ascribed category of non-producer as defined by King *et al.* [36].

After completion of our work, Eugster *et al.* [19] reported the expression of human cytochrome P450IA1 in *S. cerevisiae* under the control of the constitutive *GAPFL* promoter and the inducible *PHO5* promoter. However, expression of the human enzyme was achieved in the presence of a significant

background of endogenous yeast cytochrome P450LI and it was necessary to use ketoconazole to prevent carbon monoxide binding to the yeast enzyme, so that CO difference spectra could be used to measure the human enzyme content of microsomes. The background level of yeast cytochrome P450LI would also complicate analysis of the activity and contribution of human cytochrome P450IA1 metabolism, as the activation of a variety of procarcinogens by the yeast enzyme is well established [37]. Using the culture conditions reported here with strain AH22, yeast cytochrome P450 and benzo(*a*)pyrene hydroxylase activity in control AH22::pMA91 microsomes could not be detected.

In conclusion, we have successfully expressed human cytochrome P450IA1 using the PGK promoter in AH22 yeast cells. The availability of yeast cells with functionally active human cytochrome P450IA1 will facilitate molecular structure–activity studies of procarcinogen and drug metabolism by this enzyme in man.

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