Expression of cytochrome P-450$_d$ by *Saccharomyces cerevisiae*

Toru Shimizu, Kazuhiro Sogawa*, Yoshiaki Fujii-Kuriyama*, Masae Takahashi, Yoshiro Ogoma and Masahiro Hatano

Chemical Research Institute of Non-Aqueous Solutions, Tohoku University, Sendai 980 and **Department of Biochemistry, Cancer Institute, Japanese Foundation for Cancer Research, Toshima-ku, Tokyo 170, Japan*

Received 15 August 1986

Rat liver microsomal cytochrome P-450$_d$ was abundantly expressed in the yeast *Saccharomyces cerevisiae* by using a yeast-*Escherichia coli* shuttle vector consisting of rat liver P-450$_d$ cDNA and yeast acid phosphatase promoter. The expressed cytochrome P-450$_d$ was immunologically crossed with rat liver P-450$_d$. The hydroxylase activity of estra-1,3,5(10)-triene-3,17β-diol was 11 nmol/min per nmol P-450$_d$, which is comparable to that reported previously for rat liver P-450$_d$. The expressed P-450$_d$ content was nearly 1% of total yeast protein as estimated from immunoblotting, hydroxylase activity and optical absorption of the reduced CO form.

Cytochrome P-450 (Yeast) Expression cDNA

1. INTRODUCTION

A family of heme-containing monooxygenases, collectively called cytochrome P-450 (P-450), is widespread throughout nature and plays an important role in a variety of metabolic hydroxylations [1]. Isosafrole induces predominantly a unique P-450, cytochrome P-450$_d$ (P-450$_d$), in rat liver microsomes [2]. P-450$_d$ has an efficient catalytic activity toward the 2-hydroxylation of 17β-estradiol. This activity is in contrast with those of other forms of rat liver P-450, P-450$_a$, P-450$_b$, which are predominantly induced by phenobarbital, and P-450$_c$, which is predominantly induced by 3-methylcholanthrene [3]. Kawajiri et al. [4] determined the coding nucleotide sequence of mRNA for P-450$_d$ by sequence analysis of cloned cDNAs. As the first step toward elucidation of a structure-function relationship in the P-450$_d$ molecule, we constructed a direct expression plasmid of P-450$_d$ in the yeast by using cDNA of P-450$_d$ and a yeast-*E. coli* shuttle vector, pAM82 [5], which carries a yeast acid phosphatase promoter. The constructed expression plasmid of P-450$_d$ allowed high expression of P-450$_d$ in the yeast up to 1% of the total yeast protein. The expressed P-450$_d$ of the yeast was identical to rat liver P-450$_d$ in terms of immunocrossing, hydroxylase activity of the 2-position of 17β-estradiol and optical absorption spectra.

Abbreviations: P-450, cytochrome P-450; P-450$_d$, cytochrome P-450$_d$; 17β-estradiol, estra-1,3,5(10)-triene-3,17β-diol; isosafrole, 5-(1-propenyl)-1,3-benzodioxole; phenobarbital, 5-ethyl-5-phenyl-2,4,6(1H,3H,5H)-pyrimidinetrione; 3-methylcholanthrene, 1,2-dihydro-3-methylbenz(j)aceanthrylene; ampicillin, 6-[D-(2-amino-2-phenylacetoamido)]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo(3,2,0)heptane-2-carboxylic acid

2. MATERIALS AND METHODS

Yeast strain AH22 (*a leu2 his4 can1 cir*$^+$) was kindly supplied by Dr K. Matsubara of Osaka University.

P-450$_d$ cDNA was obtained as described [4]. A yeast-*E. coli* shuttle vector, named pAM82, consisting of yeast acid phosphatase was kindly sup-

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

plied by Dr K. Matsubara of Osaka University. This vector carries marker *ars1, 2-μori, leu2* and an ampicillin-resistant marker of *E. coli* plasmid pBR322 [5].

Restriction endonucleases, other nucleic acid modifying enzymes and phosphorylated *Xho*I linker were purchased from Takara Shuzo (Kyoto). Leupeptin and pepstatin A were purchased from Peptide Institute (Osaka). Other reagents used were of the highest guaranteed grade and were used without further purification.

Transformation was carried out as in [5]. Leu^+ transformants were selected on Burkholder minimal medium containing 2% agar. The cultivation and the induction of yeast cells were performed as described in [5]. Microsomes were prepared according to Aoyama et al. [6]. To minimize decomposition of $P\text{-}450_d$ in the yeast, cells were lysed by a French press and spun down [6]. $P\text{-}450_d$ was purified from the microsomes according to Hashimoto and Imai [7]. Hydroxylase activity of $P\text{-}450_d$ was measured by the method by Ryan et al. [2,3].

3. RESULTS AND DISCUSSION

3.1. *Construction of expression vector*

cDNA of $P\text{-}450_d$ incorporated into pBR322 was doubly digested by exonucleases *Rsa*I and *Acc*I (fig.1). After the sticky end of the *Acc*I site was converted to the flush end by the Klenow fragment of *E. coli* DNA polymerase in the presence of deoxyribonucleoside 5'-triphosphates, the phosphorylated *Xho*I linker was ligated to both sides of the $P\text{-}450_d$ cDNA. The $P\text{-}450_d$ cDNA with *Xho*I linkers on both ends was digested with *Xho*I to

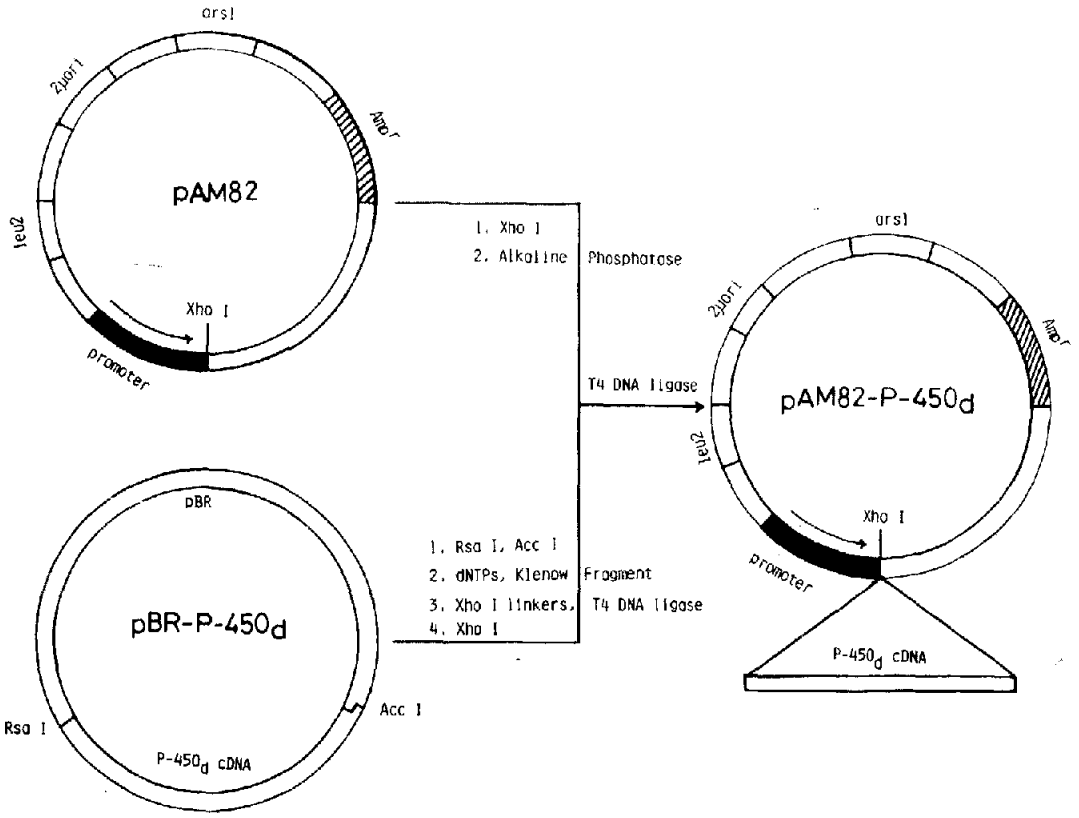

Fig.1. Construction procedure of the expression vector, pAM82-$P\text{-}450_d$. $P\text{-}450_d$ cDNA in pBR322 was digested by *Acc*I and *Rsa*I. After the sticky end of the *Acc*I site was filled with dNTPs by the Klenow fragment, phosphorylated *Xho*I linker was ligated to both ends of $P\text{-}450_d$ cDNA by T_4 DNA ligase. The $P\text{-}450_d$ cDNA was cleaved by *Xho*I and then inserted into the *Xho*I-digested pAM82. The expression vector pAM82-$P\text{-}450_d$ was thus constructed.

create the *Xho*I sticky ends on both ends of the P-450$_d$ cDNA. The yeast-*E. coli* shuttle vector, pAM82, was digested by *Xho*I, the site of which is located just after the yeast acid phosphatase promoter (fig.1). The P-450$_d$ cDNA having *Xho*I sites was inserted into the *Xho*I-digested pAM82 by T_4 ligase. The P-450$_d$ expression vector constructed as mentioned was transferred into *E. coli*, HB101. Purified P-450$_d$ expression vector was then transferred into the yeast, *S. cerevisiae*.

3.2. *Expression of P-450$_d$ in terms of Western blotting*

Expression of P-450$_d$ was studied by immunoblotting (Western blotting) using anti-P-450$_d$ IgG and peroxidase-conjugated goat anti-rabbit IgG (fig.2). The SDS-solubilized fraction of the whole yeast cells containing the P-450$_d$ expression vector, pAM82-P-450$_d$, showed a clear band (lane 2, fig.2), which reacts with anti-P-450$_d$ IgG, at an M_r identical to rat liver P-450$_d$ (lane 1, fig.2). Expression of P-450$_d$ was estimated at nearly 1% of the total yeast protein from immunoprecipitations of authentic rat liver P-450$_d$. Probably partially decomposed forms of P-450$_d$ were detected in the lower M_r regions. The whole yeast cell without the expression vector, pAM82-P-450$_d$, or with the vector pAM82 did not show any band in Western blotting. To reduce the content of the partially decomposed P-450$_d$, hemin was added to the induction medium. As can be seen in lanes 2 and 3 of fig.2, the content of the decomposed P-450$_d$ did not decrease considerably. We changed the phosphate concentration in the first culture for cell

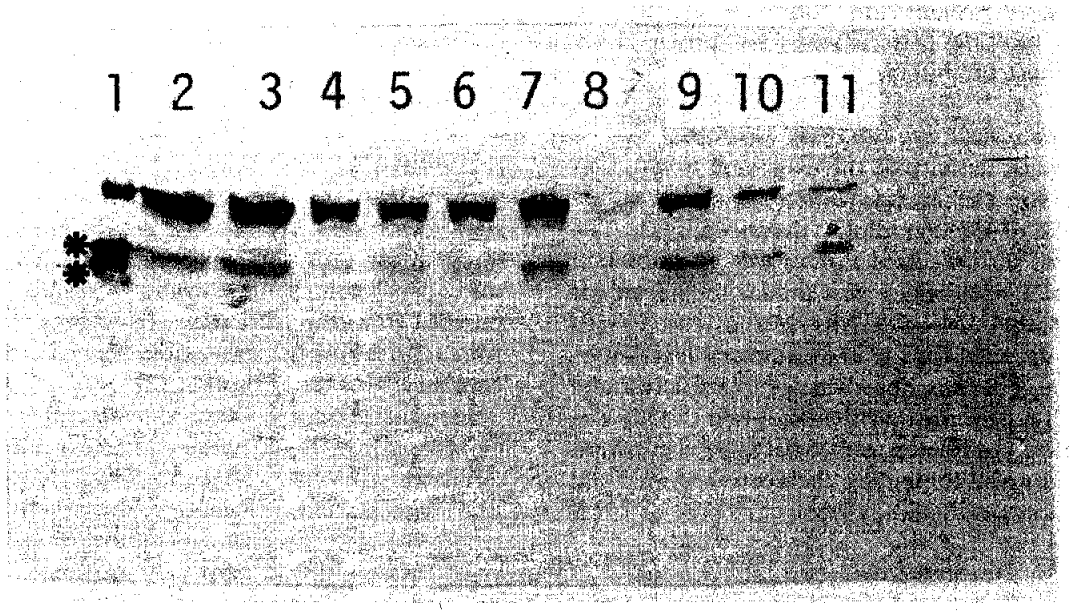

Fig.2. Western blotting of rat liver P-450$_d$ (lane 1) and P-450$_d$ expressed in the yeast AH22 (lanes 2–11). Lanes: 1, rat liver P-450$_d$, 0.3 μg protein; 2, yeast cultured in 1 l low-phosphate minimal medium after the first cultivation in 1 l high-phosphate (1.5 g KH_2PO_4/l) Burkholder minimal medium in the presence of 50 mg hemin; 3, yeast cultured as for lane 2 in the absence of hemin; 4, yeast cultured in 1 l low-phosphate minimal medium after the first cultivation in a high-phosphate (37.5 g KH_2PO_4/l) minimal medium. 250 ml of the first cultivation was used for the induction in the low-phosphate medium; 5, yeast cultured as in lane 4, but the phosphate content of the first medium was 7.5 g/l; 6, yeast cultured in the same way as lane 4, but the phosphate content of the first medium was 1.5 g/l; 7, yeast cultured first in low-phosphate minimal medium. We did not change the content of phosphate; 8, yeast cultured in 1 l low-phosphate minimal medium after first cultivation in 1 l high-phosphate (1.5 g KH_2PO_4/l) minimal medium; 9, yeast cultured as in lane 8. But 250 ml of the first culture solution was used for the second cultivation; 10, yeast cultured as in lane 8. However, 63 ml of the first culture solution was used for the second cultivation; 11, yeast cultured as for lane 8. But 16 ml of the first culture solution was used for the second cultivation. Protein contents of the yeast applied for electrophoresis were nearly 10 μg. Asterisks for lane 1 indicate degraded forms of the native rat P-450$_d$.

growth (lanes 4–7, fig.2). Expression of P-450$_d$ did not seem to be strongly dependent on the phosphate concentration in the first culture. The content of the decomposed P-450$_d$ was also not changed by varying the phosphate concentration in the first culture. The ratios of the culture volume for cell growth to that for induction appeared to be important for protection against degradation of the product. Therefore, we changed these ratios by reducing the first culture volume to improve the expression efficiency and to reduce the content of the decomposed P-450$_d$ (lanes 8–11, fig.2). An optimum ratio of the culture volume for cell growth to that for induction was 1/4 so that we could obtain the highest expression of P-450$_d$ with minimal decomposition.

To minimize the decomposition of P-450$_d$ in the yeast, we added the inhibitors, leupeptin and pepstatin A. Significant improvement in the Western blotting patterns was not observed by adding these inhibitors.

3.3. *Hydroxylase activity of 17β-estradiol*

Enzymatic activity of the expressed P-450$_d$ was studied for the hydroxylation reaction at the 2-position of 17β-estradiol as described in [2]. The activity of the expressed P-450$_d$ was 11 nmol/min per nmol P-450$_d$ which was similar to that of rat liver P-450$_d$ [2]. The activity of the yeast P-450$_d$ was not detected for the mitochondrial fraction of the yeast, but was observed for the microsomal fraction, suggesting that the expressed P-450$_d$ was localized in the microsomes. Microsomes prepared from the yeast containing the vector pAM82 did not show any hydroxylation activity of 17β-estradiol.

3.4. *Optical absorption of the expressed P-450$_d$*

When whole yeast cells transfected with the P-450$_d$ expression vector were treated with CO gas and sodium dithionite, an absorption maximum at 448 nm was observed (fig.3), suggesting that P-450$_d$ is produced in the yeast to a considerable extent. On the other hand, the yeast harboring the vector pAM82 did not give rise to the absorption maximum around 450 nm. From the absorption magnitude of the reduced CO form of P-450$_d$ in the yeast, the amount of the expressed P-450$_d$ is estimated to be 1% of the total yeast protein, which is consistent with the estimation of expressed P-450$_d$ from Western blotting. Microsome preparations from the yeast containing the expression vector also gave rise to an absorption maximum at 448 nm, while mitochondria did not show the absorption band around 450 nm. P-450$_d$ purified from the yeast was a ferric high-spin type with a Soret absorption maximum at 392 nm in accordance with that reported [2].

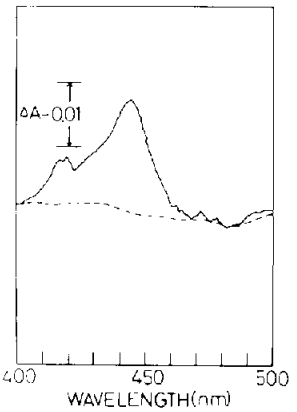

Fig.3. Difference absorption spectrum of the reduced CO form of P-450$_d$ which was expressed in the yeast harboring the expression vector pAM82-P-450$_d$ (——). The spectrum was taken for 1 ml yeast solution which was suspended with distilled water after the yeast cells in the 50 ml cultivated solution were harvested. The yeast cells harboring the vector pAM82 did not show a band around 450 nm (– – –).

In conclusion, we have demonstrated that a cloned rat liver P-450$_d$ gene can be expressed efficiently in yeast cells under the control of the yeast acid phosphatase promoter. Oeda et al. [8] reported a high expression of rat liver P-450$_c$ in the yeast under the control of a yeast alcohol dehydrogenase promoter. The efficiency of the expression of P-450$_d$ in the yeast of our system is as high as that of P-450$_c$ by using the alcohol dehydrogenase promoter [8]. We attempted to express P-450$_d$ by using the yeast alcohol dehydrogenase promoter, but did not succeed in the high expression of P-450$_d$ for unknown reasons.

ACKNOWLEDGEMENTS

We are deeply indebted to Dr K. Kawajiri for giving us anti-P-450$_d$ IgG, to Dr J. Nikawa and Dr

T. Kuga for instruction on the transformation of the yeast and to Dr H. Ohkawa for valuable discussions. We gratefully acknowledge Dr K. Matsubara of Osaka University for giving us access to the yeast strain AH22 and the expression vector, pAM82. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan.

REFERENCES

[1] Sato, R. and Omura, T. (1978) Cytochrome P-450, Kodansha, Tokyo.
[2] Ryan, D.E., Thomas, P.E. and Levin, W. (1980) J. Biol. Chem. 255, 7941–7955.
[3] Ryan, D.E., Thomas, P.E., Korzeniowski, D. and Levin, W. (1979) J. Biol. Chem. 254, 1365–1374.
[4] Kawajiri, K., Goto, O., Sogawa, K., Tagashira, Y., Muramatsu, M. and Fujii-Kuriyama, Y. (1984) Proc. Natl. Acad. Sci. USA 81, 1649–1653.
[5] Miyanohara, A., Toh-e, A., Nozaki, C., Hamada, F., Ohtomo, N. and Matsubara, K. (1983) Proc. Natl. Acad. Sci. USA 80, 1–5.
[6] Aoyama, Y., Yoshida, Y. and Sato, R. (1984) J. Biol. Chem. 259, 1661–1666.
[7] Hashimoto, C. and Imai, Y. (1976) Biochem. Biophys. Res. Commun. 68, 821–827.
[8] Oeda, K., Sakaki, T. and Ohkawa, H. (1985) DNA 4, 203–210.