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ISOLATION OF THE ALKANE INDUCIBLE CYTOCHROME P450 (P450alk) GENE FROM THE YEAST *CANDIDA TROPICALIS*

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Summary: The gene for the alkane-inducible cytochrome P450, P450alk, has been isolated from the yeast *Candida tropicalis* by immunoscreening a λgt11 library. Isolation of the gene has been identified on the basis of its inducibility and partial DNA sequence. Transcripts of this gene were induced by alkane to levels 500 to 1000 fold over those detected in glucose-grown cells. The nucleotide sequence of the 3' portion of this gene revealed a coding sequence for the heme binding segment characteristic of the P450 gene family.

Cytochromes P450 are widely distributed hemoproteins which catalyze the monooxygenation of a large variety of compounds using molecular oxygen and reducing equivalents provided by a flavoprotein (1,2). The yeast *Candida tropicalis* has been reported to contain two cytochrome P450 types: one catalyzing the 14α-demethylation of lanosterol (P450lan) and the other an alkane inducible form (P450alk) which hydroxylates n-alkanes at the terminal position (3). As the first enzyme in the pathway for alkane assimilation, P450alk is essential for the growth of *C. tropicalis* on this carbon source (4). Cellular levels for both of these two distinct P450s are regulated according to the carbon source utilized and also are affected by exogenous factors such as oxygen partial pressure (3,4). We are interested in the isolation and characterization of these P450 genes in order to elucidate the molecular basis of their regulation and to enable structure-function comparisons with P450s from higher eukaryotes and from *Sacchromyces cerevisiae* (5). Here we report the isolation from *C. tropicalis* of the gene for P450alk, or P450LIB1 according to the nomenclature of Nebert et al (6).

Material and methods

The yeast used was *C. tropicalis* ATCC 750. Two libraries were constructed with *Sau*3A partially digested genomic DNA, one of 8 to 10 kb fragments inserted in the *Bam*HI site of YRp vector pAB107 (7) and one of smaller fragments linked with

Abbreviations: IPTG: Isopropyl β-thiogalactopyranoside, aa: amino acid.

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*Eco*RI linkers in the λgt11 expression system. The latter library was constructed according to Huynh *et al.* (8). *E. coli* Y1090 was used as the host for the library and *E. coli* Y1089 as a lysogenic strain. Immunoscreening of the expression library was performed according to Huynh et al. (8) using the BLOTTO procedure (9) with polyvalent rabbit antibody prepared with P450alk protein isolated from *C. tropicalis* (10). Positive signals were detected with ^{125}I-protein A. Affinity purification of the P450alk antibody with β-galactosidase fusion protein from recombinant phage clones was carried out as described by Johnson *et al.* (11). Polyacrylamide electrophoresis was performed according to Lämmeli (12). For the isolation of RNA, cells were grown at 30°C on a rotary shaker (400 rpm) to mid-log phase on complete medium containing 2% glucose or 1% tetradecane (10). Following the addition of 0.05 mg/ml Actinomycin D, the cultures were continued for two minutes and then cooled on ice. Cells were pelleted and washed with TE (10 mM Tris-HCl, pH 7.2, 1 mM EDTA). Following disruption of cells with glass beads, total RNA was isolated according to Chirgwin *et al.* using isothiocyanate buffer (13). The mRNA fraction was isolated by affinity chromatography on oligo-dT cellulose. RNA was glyoxylated prior to electrophoresis in 1% agarose with 10mM NaHPO4 (pH 7.0) as running buffer. RNA was blotted on Genescreenplus as described by the supplier (DuPont). Radiolabelled DNA was prepared by random primer labelling (14). DNA sequencing was performed according to Ahmed (15) with quasi-end labelling as described by Duncan (16).

Results and Discussion

P450alk gene isolation

Immunoscreening of the λgt11 library yielded 7 positive clones, identified as isolates 4,5,11,13,14,19,24. These were then analyzed for the production of IPTG-inducible proteins in *E. coli* Y1089 lysogenic strains. Immunoblots of cell extracts revealed 4 classes of P450 immunoreactive proteins with apparent molecular weights of 162, 145, 132 and 120 kDa, (Fig. 1). The same protein bands were also detected by β-galactosidase antibody (data not shown), demonstrating that these proteins were β-galactosidase fusion proteins. Thus, each of these phage clones contained a DNA insert oriented in phase with the transcriptional reading frame of the *lac*Z gene segment in λgt11. To confirm the P450alk gene identity of these clones, fusion protein coded by clone 11 in *E. coli* Y1090 was used to obtain affinity-purified P450alk antibody. This antibody was then immunoblotted with partially purified P450alk. As shown in Fig. 2, affinity-purified P450alk antibody was able to detect a single band of 54 kDa corresponding to the molecular weight of the silver stained P450alk protein. When we used the DNA insert from clone 11 to probe the six other clones in a Southern blot, only clones 5, 19 and 24 cross-hybridized (data not shown), thus eliminating the three other isolated clones as gene candidates. Presumably, the isolation of these three clones occurred due to the presence of P450alk non-specific antibodies in our initial preparation. Fig. 3 shows DNA insert sizes from each confirmed clone and their corresponding P450 coding sizes. Since for each clone the coding size of the yeast portion of the fusion protein is shorter than the total insert size, it is predicted that these inserts contain the termination codon originating from the 3' end of the P450alk gene itself (indicated in Fig. 3 by the vertical bar on each clone).

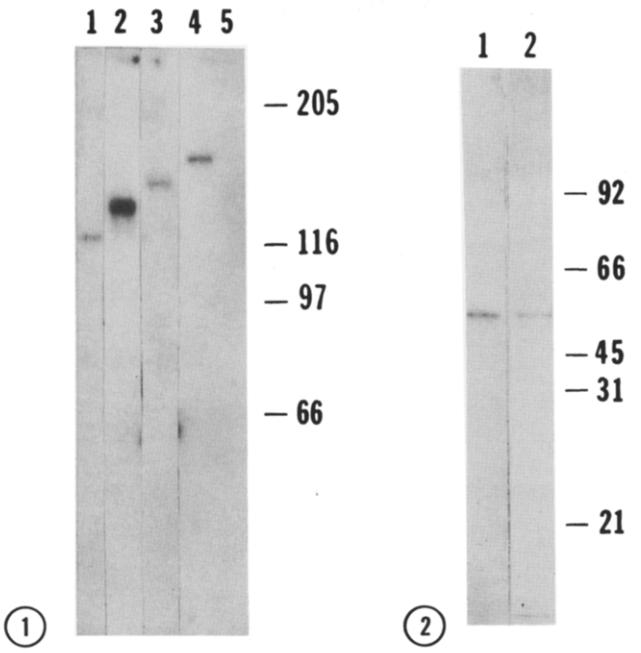

Fig. 1. Immunoblot of *E. coli* Y1089 lysates (after lytic induction at 42°C and IPTG induction). Lane 1, clone 11; lane 2, clone 5; lane 3, clone 13; lane 4, clone 4; lane 5, λgt11. Western blot on nitrocellulose was probed using P450alk antibody, which had been previously cross-absorbed with *E. coli* antigens. Signals were detected by ^{125}I-protein A labelling. Numbers on the right side of the Fig. show the position of molecular weight standards in kDa.

Fig. 2. Detection of P450alk using affinity purified antibody. Lane 1: Immunoblot of partially purified P450alk using affinity purified P450alk antibody. Antisera was affinity purified using lysates of IPTG induced *E. coli* Y1090 obtained with clone 11. The positive band was detected by ^{125}I protein A labelling. Lane 2: silver stain of partially purified P450alk in 10% SDS-PAGE. The prominent band in both lanes had an apparent molecular weight of 54 kDa.

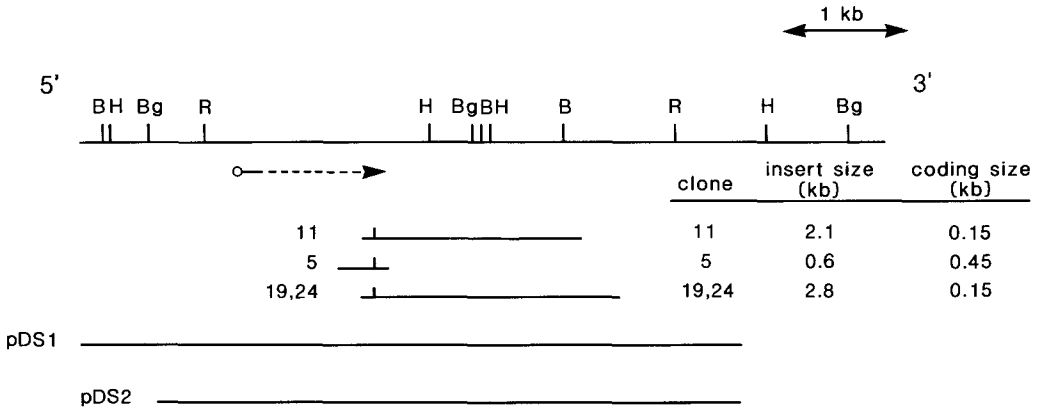

Fig. 3. Genomic restriction map of the P450alk gene. Radiolabelled inserts of clones 11 and 5 were used as probes for the P450alk gene in *C. tropicalis* DNA digested with: B, *Bam*HI; H, *Hind*III; Bg, *Bgl*II and R, *Eco*RI. Insert from clone 11 was aligned based upon digestion with *Bgl*II, which gave 2 fragments of 0.9 and 1.2 kb. The dashed arrow indicates the orientation and location of the P450alk gene based upon the predicted size of this gene and the calculated position of the 3' end coding segment for inserts of clones 11, 5, 19 and 24.

Genomic restriction map of the P450alk gene

A restriction map of the P450alk gene was constructed using EcoRI inserts from clones 5,11,19 and 24 as probes in Southern blots of genomic digests of *C. tropicalis* (Fig. 3). The orientation (5' ---> 3') of the chromosomal gene was determined by analyzing hybridization characteristics of inserts from clones 11 and 5. The insert from the latter clone, which contains a coding sequence larger than that accounted for in clone 11, hybridized with the same 2.3 kb *Hind*III/*Eco*RI fragment detected by the insert from clone 11 but did not hybridize with genomic fragments detected by the 3' end of clone 11 insert (Fig. 3). This indicated that this 2.3 kb *Hind*III/*Eco*RI fragment contained the P450alk gene, considering that the required coding sequence for a 54 kDa protein should be approximately 1.5 kb. Screening of the pAB107 library with clone 11 as a probe yielded plasmids pDS1 and pDS2. Restriction analysis of both plasmids revealed that they contain inserts large enough to contain the entire P450alk gene (Fig. 3).

Northern blot analysis of mRNA from glucose- and tetradecane-grown cells

As tetradecane and other related alkanes were reported to induce the biosynthesis of the P450alk protein (4, 17), we should be able to detect an increase of the P450alk gene transcripts in cells growing with tetradecane. The 2.3 kb

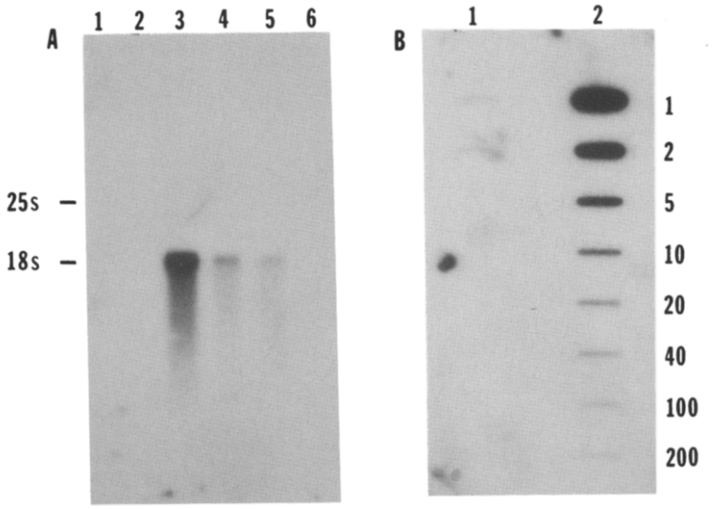

Fig. 4. mRNA analysis. A) Northern blot of mRNA from tetradecane- (t) and glucose-grown (g) cells from *C. tropicalis* probed with the 2.3 kb *Hind*III/*Eco*RI fragment containing the P450alk gene. Lanes 1 and 2: 10 µg and 5 µg mRNA (g). Lanes 3,4,5,6: 5, 1, 0.5, 0.1 µg mRNA (t). The apparent molecular size of the major band obtained in lanes 3-6 was 1.75 kb based upon the position of the 25 and 18S ribosomal RNA indicated in the left of the Fig. As a control for the presence of mRNA in lanes 1 and 2, ADE2 gene from *C. albicans* was used as a probe of this same stripped Northern blot and showed bands of approximately the same intensity in lanes 2 and 3 (data not shown). B) Slot blots of 1) mRNA (g), 5 µg and 2) mRNA (t), 1 µg. Dilution coefficients are shown on the right side of the Fig. X-ray film was preflashed and exposed for 20 hrs. Relative intensity of signals determined by densitometry indicated that mRNA (t) is 500 to 1000 fold higher than mRNA (g).

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GTT GTT ATG TAC ACG ATT CTT GCT ACT CAC AGA GAT AAA GAC ATT TAT GGT GAA GAT GCT
Val Val Met Tyr Thr Ile Leu Ala Thr His Arg Asp Lys Asp Ile Tyr Gly Glu Asp Ala
 .   :   .   :   :   .   .   .   .   .   .   .   :   .   .   .
Thr Glu Val Tyr Pro Ile Leu Ser Ser Ala Leu His Asp Pro Gln Tyr Phe Asp His Pro
                390                                     400

TAT GTT TTC AGG CCA GAA AGA TGG TTT GAA CCT GAA --- ACC AGA AAA TTG GGC TGG GCA
Tyr Val Phe Arg Pro Glu Arg Trp Phe Glu Pro Glu --- Thr Arg Lys Leu Gly Trp Ala
 :   .   :   :   .   .   .   .   .   .   .   X   .   .   :
Asp Ser Phe Asn Pro Glu His Phe Leu Asp Ala Asn Gly Ala Leu Lys Lys Ser Glu Ala
                410                                     420

                                        +++
TAT GTT CCA|TTC AAT GGC GGT CCA AGA ATT TGT TTG GGT CAA CAG TTT GCT TTA ACT GAA
Tyr Val Pro|Phe Asn Gly Gly Pro Arg Ile Cys Leu Gly Gln Gln Phe Ala Leu Thr Glu
 .   .   :  |:   .   .   :   :   :   :   :   .   .   :   .   :
Phe Met Pro|Phe Ser Thr Gly Lys Arg Ile Cys Leu Gly Glu Gly Ile Ala Arg Asn Glu
           |    430                 +++             440

GCA TCA TAT GTC|ACT GTT AGA TTG CTT CAA GAA TTT GGT AAC TTG AAA CAA GAT CCA AAT
Ala Ser Tyr Val|Thr Val Arg Leu Leu Gln Glu Phe Gly Asn Leu Lys Gln Asp Pro Asn
               |  .   .   .   :   :   .   X   .
Leu Phe Leu Phe|Phe Thr Thr Ile Leu Gln Asn Phe Ser Val Ser Ser His Leu Ala Pro
               |450                                     460

ACT GAA TAT CCA CCA AAA TTA CAA AAC ACA TTG ACT TTG TCT CTT TTT GAA GGT GCT GAA
Thr Glu Tyr Pro Pro Lys Leu Gln Asn Thr Leu Thr Leu Ser Leu Phe Glu Gly Ala Glu

Lys Asp Ile Asp Leu Thr Pro Lys Glu Ser Gly Ile Gly Lys Ile Pro Pro Thr Ile Gln
                470                                     480

GTA CAA ATG TAT TTA ATT TTA TAA GTT ATT CAA AAA CGT GTT TTT GGA ATT GCG TTC ACG

Val Gln Met Tyr Leu Ile Leu ---

Ile Cys Phe Ser Ala Arg ---
                490
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Fig. 5. Nucleotide sequence of the 3' position of the P450alk gene from *C. tropicalis*. The derived aa sequence is shown immediately below the nucleotide sequence. The aa sequence of the P450b gene is shown in italics (19). This sequence has been aligned with the yeast sequence by the FASTP algorithm on BIONET (18), with the invariant cysteine marked (+++). This algorithm marks the region of highest homology at the beginning and end with an X and indicates identical aa (:) and conservative replacements (.). Numbers below the aa rat liver sequence indicate their respective position from the first aa. Two vertical lines delimit the 21 aa HR2 region common for P450 primary structures.

*Hind*III/*Eco*RI fragment isolated from pDS1 was used to probe Northern blots of mRNA fractions from glucose- and tetradecane-grown cells. As shown in Fig. 4A, this 2.3 kb fragment does contain a coding sequence inducible by tetradecane. The size of the inducible mRNA transcript, 1.75 kb, is appropriate for an mRNA encoding the P450alk protein. This procedure did not detect this mRNA from glucose-grown cells. However, slot blot analysis of mRNA from glucose-grown and tetradecane-grown cells shows that a 500 to 1000 fold elevation of P450alk mRNA occurred in cells grown on tetradecane (Fig. 4B). Comparable results were obtained by Wiedmann et al., for the levels of P450 transcripts in *C. maltosa* grown on glucose or on alkanes (17). They showed that induction on alkane resulted in a 500 fold increase in P450 transcripts.

Partial nucleotide sequence of the P450alk gene

From pDS1, we subcloned the 4.1 kb *Bam*HI fragment containing the whole structural P450alk gene into pAA3.7x, a plasmid which generates transposon-promoted deletions used for plasmid sequencing (15). We sequenced the 3' segment of the cloned fragment, as the region predicted to contain the 3' end of the P450alk gene. A sequence of approximately 300 bp revealed an open reading frame with a termination

codon. A search (18) for similarities in the National Biomedical Research Foundation Database showed that this translated sequence was clearly related to the P450 gene family. Our amino acid sequence was most similar to the C-terminal sequence of the phenobarbital-inducible P450 gene from rat liver, P450b (19). Included in this region is a pattern of invariant and variant amino acids highly conserved among P450 families, termed HR2 (20), which makes up a part of the heme binding site and contains a cysteine residue as the fifth ligand of the iron molecule. The DNA sequence we have determined and the characteristic HR2 segment it encodes are shown in Fig. 5.

In conclusion, we have shown here that we have isolated a cytochrome P450 gene from *C. tropicalis* and that this gene is induced by growth on tetradecane. Sequencing of the complete P450alk gene is in progress. Experiments are planned to examine the control of its transcription in *C. tropicalis*. Moreover, since *S. cerevisiae* is unable to grow on alkanes and is likely to lack this particular gene, we will test the expression of the P450alk gene and the function of its product in this organism.

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