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Cytochrome P450 oxidoreductase gene and its differentially terminated cDNAs from the white rot fungus *Phanerochaete chrysosporium*

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Abstract The white rot fungus *Phanerochaete chrysosporium* metabolizes a range of xenobiotics via P450 mono-oxygenation, particularly under peroxidase-suppressing culture conditions. Here we report the cloning and analysis of the gene from this fungus for the cytochrome P450 oxidoreductase (CPR) and its differentially terminated cDNAs. Using a PCR-based approach with degenerate primers, a 285-bp genomic fragment was isolated from the two widely studied strains BKM-F 1767 and ME 446, and was identified as a *CPR* gene segment based on sequence comparison with the database. A clone containing the full-length *CPR* gene was isolated from a BKM-F 1767 genomic library using the PCR-generated segment as a probe, and the 3937-bp insert was sequenced by gene walking. Based on the detection of conserved CPR motifs, a coding region of 2381 bp was identified with a 991-bp segment 5′ to the putative ATG start codon. Two cDNAs with differentially terminated transcripts were isolated and sequenced. Comparison of the gene and the cDNA sequences confirmed the presence of three introns (62 bp, 50 bp, and 58 bp). Sequence identity and a phylogenetic comparison of the deduced protein (736 aa) with other CPRs in the database suggested that *P. chrysosporium* CPR is the largest CPR known and is more closely related to animal (36–38%) and yeast (37–38%) CPRs than to plant CPRs (33–35%). The availability of this gene will facilitate further studies on understanding the potent xenobiotic mono-oxygenation systems in this model white rot fungus.

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Introduction

Phanerochaete chrysosporium is the most extensively studied white rot fungus for ligninolysis and xenobiotic metabolism and thus has served as the model organism for wood rotting basidiomycete fungi. This organism is capable of the biotransformation of a range of environmental chemicals including substituted or unsubstituted monocyclic and polycyclic aromatics, alicyclics, and aliphatics (reviewed in Bumpus and Aust 1987; Pszczynski and Crawford 1995). Since the discovery of the extracellular peroxidases in this fungus in 1983, the role of these enzymes in catalyzing an initial free radical-based oxidation has been elucidated for several recalcitrant xenobiotics, including PAHs, dioxins, chlorophenols and nitoaromatics (Hammel 1995; Valli et al. 1992).

However, subsequent independent studies showed that extracellular peroxidases are not always involved or else are not critical in the initial oxidation of several pollutant chemicals. Results from metabolite identification and from the use of a peroxidase-negative mutant or P450 inhibitors suggested P450 mono-oxygenation as an alternative oxidizing system in this fungus for the biotransformation of different xenobiotics (Sutherland et al. 1991; Yadav and Reddy 1992, 1993; Kullman and Matsumura 1996). Recent studies gave spectral evidence for the P450 protein in cell extracts (Masaphy et al. 1996; Yadav and Loper 1997) and demonstrated benzo(a)pyrene hydroxylase activity in this fungus (Masaphy et al. 1996). However, attempts at isolation of *P. chrysosporium* P450 system components have met with only limited success (Yadav and Loper 1997; Kullman and Matsumura 1997; Yadav and Loper 1998).

A typical microsomal P450 system in eukaryotes contains a mono-oxygenase and a reductase compo-

nent. The latter, NADPH-dependent cytochrome P450 oxidoreductase (EC 1.6.2.4; CPR), is usually a common electron donor to multiple mono-oxygenases, although multiple *CPRs* have been reported in certain plants (Lesot et al. 1995; Urban et al. 1997). The electron transfer proceeds from NADPH to FAD, to FMN in the CPR, to the P450 heme. This flavoprotein is also involved in other physiological functions, acting as an electron donor to cytochrome b5 (Enoch and Strittmatter 1979), heme oxygenase (Schacter et al. 1972), squalene epoxidase (Ono et al. 1977), and the fatty acid elongation system (Ilan et al. 1981). CPR has also been shown to initiate lipid peroxidation by one-electron reduction of molecular oxygen (Sevanian et al. 1990). Interestingly, it has been hypothesized that lipid peroxidation in intact *P. chrysosporium* cells is involved in the degradation of polycyclic aromatic hydrocarbons that are not the substrates for lignin peroxidases due to their high (>7.55 eV) ionization potential (Moen and Hammel 1994; Bogan and Lamar 1995).

The CPR gene has been isolated from selected animal species (Porter and Kasper 1985; Katagiri et al. 1986; Yamano et al. 1989; Ohgiya et al. 1994), insects (Hovemann et al. 1997), plants (Shet et al. 1993; Durst and Nelson 1995; Urban et al. 1997), and yeasts (Yabusaki et al. 1988; Sutter and Loper 1989; Sutter et al. 1990; Miles 1992; Kargel et al. 1996); however, among filamentous fungi, the gene has only been reported from the ascomycete fungus *Aspergillus niger* (van den Brink et al. 1995). The importance of coregulation of the CPR component and the mono-oxygenase component of the P450 system has been indicated in different studies (Sutter et al. 1990; Ohkuma et al. 1995), with balanced expression of both P450 mono-oxygenase and reductase shown to be critical in achieving optimized P450 activity in *A. niger* (van den Brink et al. 1996). It is apparent that characterizing the P450 oxidoreductase will be important in the analysis of P450-catalyzed metabolism in *P. chrysosporium*.

This report on the isolation and characterization of the *CPR* gene and its cDNAs provides the first complete sequence of a P450-system component from *P. chrysosporium*. Availability of this gene will facilitate the understanding and manipulation of P450 catalysis in this model organism and may eventually help in the development of an in vitro reconstituted P450 enzyme system for applications in xenobiotic de-toxification and stereospecific chemical biotransformation.

Materials and methods

Strains and culture conditions

P. chrysosporium strains BKM-F 1767 (ATCC 24725) and ME 446 (ATCC 34541) were cultured at 37 °C in malt extract broth (Difco Laboratories) as previously described (Yadav and Reddy 1993). Fungal mycelia, harvested by vacuum filtration and washed with sterile water, were frozen at −80 °C for subsequent use in DNA isolation.

PCR amplification of the *CPR* sequence from genomic DNA

Total genomic DNA was isolated from the frozen mycelial mass using the method of Lee et al. (1988). Degenerate primers were designed based on relatively conserved sequences in the FMN-binding region of known CPRs (see Fig. 1). Multiple alignment of the representative CPR amino-acid sequences from taxonomically diverse species helped identify the consensus sequence for the two regions FMN-1 and FMN-2 (Fig. 1). The PCR reaction mixture (50 µl) consisted of 100–400 ng of genomic DNA template, 10 mM Tris HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 200 µM of each of the four dNTPs (Pharmacia, LKB Biotechnology), 2.5 U of *Taq* DNA polymerase (Stoffel fragment) as recommended in the GeneAmp kit (Perkin-Elmer), and 100 ng each of forward and reverse degenerate primers. The PCR conditions included an initial template denaturation at 94 °C for 3 min followed by 35 cycles of amplification by repeating denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min. An additional elongation cycle consisting of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 10 min was used before cooling to 4 °C. A PCR product corresponding to a distinct band of about 300-bp size was detected for both the BKM-F 1767 and ME 446 strains. Direct sequencing of the amplicon, gel-purified using a GeneClean II kit (BIO 101, Inc.), yielded a distinct sequence of 285-bp size in both cases. BLAST homology screening (Altschul et al. 1990) demonstrated that the sequence was related to *CPR* genes.

Isolation of the full-length gene

A λ DASH genomic library of *P. chrysosporium* BKM-F 1767 obtained from Dr. Ming Tien of the Pennsylvania State University was plated on NZCYM plates as phage plaques and blotted onto Magnagraph nylon membranes (MSI Technologies) per standard procedures (Sambrook et al. 1989; Ausubel et al. 1994–1998). The library was screened for the *CPR* gene using the amplicon as a probe following the procedures described earlier (Yadav and Loper 1999). Hybridization was performed at 56 °C overnight using a ^{32}P-radiolabeled probe (final radioactivity level of 1×10^6 dpm/ml of hybridization fluid). Hybridized membranes were washed in 2× SSC, 0.1% SDS twice for 20 min at room temperature and once for 10 min at 56 °C, and then exposed at room temperature to a Phosphor Image Screen to develop the hybridization signal. Three

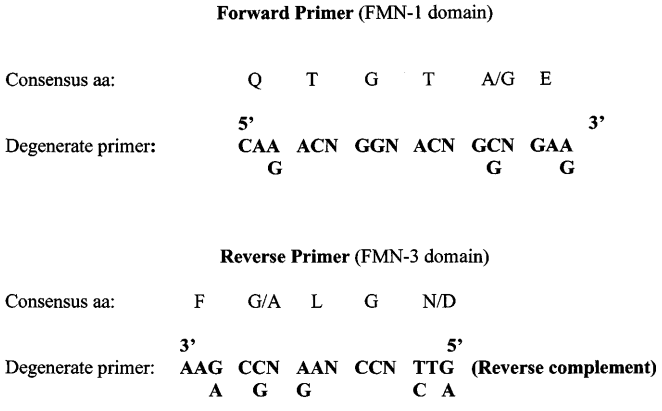


Fig. 1 Degenerate primers used for the PCR-amplification of *CPR* gene segments from *P. chrysosporium* strains BKM-F 1767 and ME 446. The forward primer was based on the conserved FMN-1 binding region (QTGTA/GE) and the reverse primer was based on the conserved FMN-3 binding region (FG/ALGN/D) of *CPR* genes from taxonomically diverse species including human, plant (*Arabidopsis thaliana*), trout, *Coenorhabditis elegans*, *Drosophila melanogaster*, *Aspergillus niger*, and yeasts (*Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Candida tropicalis*, *Candida maltosa*)

putative clones from the first screening were purified by re-hybridization in a secondary and tertiary (wherever required) screening under similar conditions and their DNA was isolated using a Qiagen kit (Qiagen, Inc.). End-sequencing of the insert DNA (using the flanking T7 and T3 primers located on the vector ends) led to the isolation of a genomic clone carrying the full-length *CPR* gene.

Isolation of cDNA

A *P. chrysosporium* BKM-F 1767 cDNA library, prepared in the λYES vector from cultures grown under high nitrogen conditions, was obtained from Dr. Ming Tien (Zapanta et al. 1998). The stock was screened as a phage library using the 285-bp amplicon as a probe, to isolate the *CPR* cDNA clones per standard procedures (Sambrook et al. 1989). Three putative clones were re-grown as phagemid clones (Zapanta et al. 1998) to isolate enough DNA for sequencing. The inserts were end-sequenced using vector-based primers to confirm their identity.

Sequence determination and analysis

DNA sequencing was performed by the University's DNA Core facility using an automatic DNA sequencer system (Applied Biosystems). DNA inserts or PCR products were sequenced by "gene walking". Sequencing primers based on the predetermined amplicon sequence were used to initiate gene walking on the genomic insert. The cDNA clones were sequenced by gene walking beginning at the vector ends. The sequences were analyzed by computer-based methods described previously (Yadav and Loper 1999). Sequences of the *CPR* gene and the two cDNAs are available in Genbank under the accession numbers AF193060 (*CPR* gene), AF193061 (cDNA-1), and AF193062 (cDNA-2).

Results

Gene isolation

The 285-bp amplicons from BKM-F 1767 and ME 446 (see Materials and Methods) showed absolute nt identity in their overlapping 251-bp sequence, excluding the 33-bp end-sequences generated by the degenerate primers (data not shown). Further work involved only the BKM-F 1767 strain, and the λ DASH genomic library was screened using as a probe the 285-bp amplicon from that strain in order to isolate the full-length gene. Of the three putative genomic clones isolated, one carried the full-length gene as determined by end-sequencing of the insert followed by BLAST homology searches. A 3937-bp sequence was generated by sequencing in both directions (Fig. 2).

cDNAs

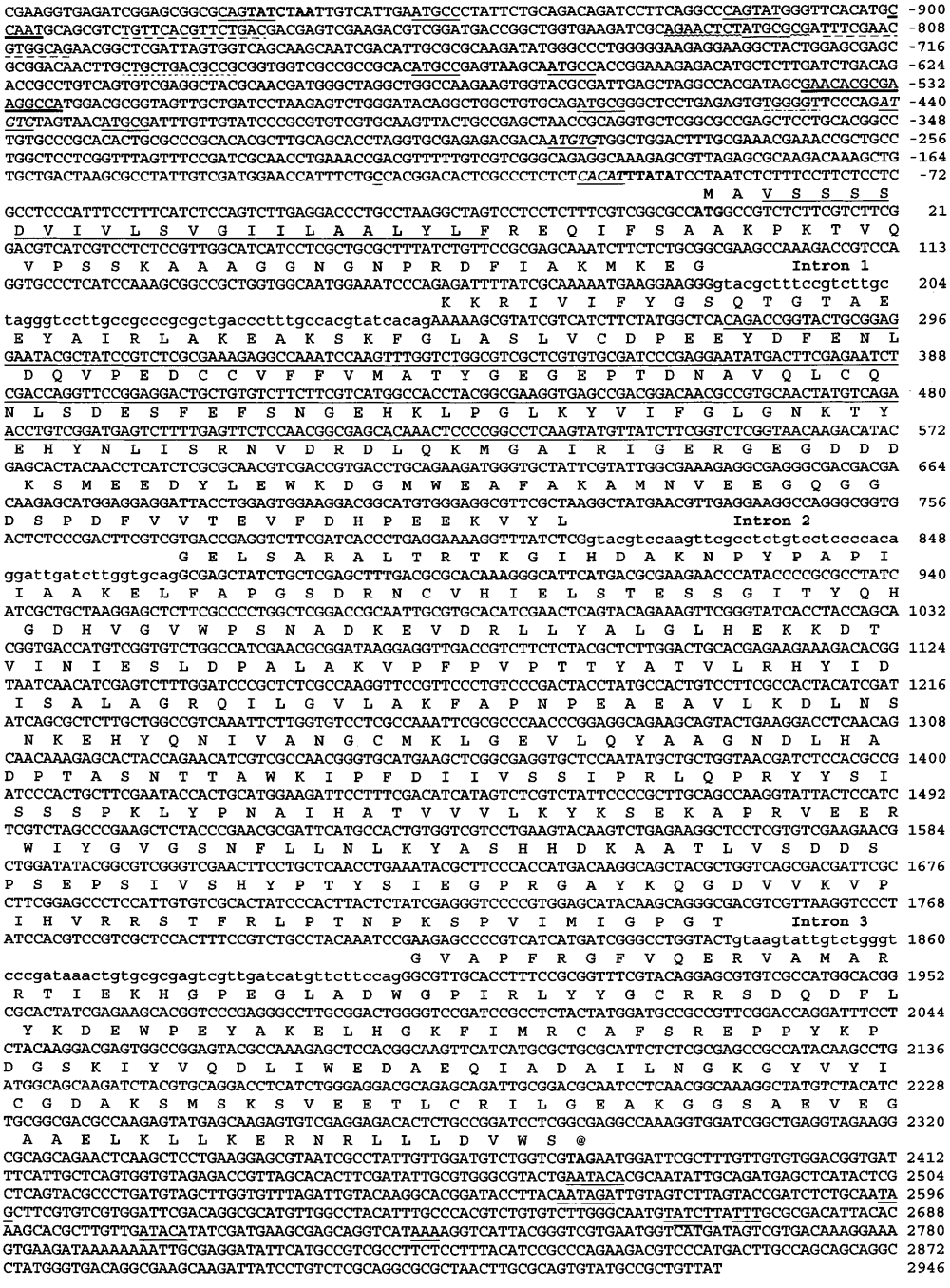
Screening a cDNA library using the 285-bp amplicon as a probe yielded two putative cDNA clones. Sequencing of these gave two cDNA species, cDNA-1, 2441 bp, and cDNA-2, 2201 bp. These cDNAs are of identical overlapping sequence but differ in their ends. At the 3′ end this is a result of differential termination or the recognition of different polyadenylation sites: the larger species has a 200-bp 3′ noncoding region attached to a 21-bp-long poly A tail, whereas cDNA-2 has a 29-bp-long 3′ noncoding region with a 97-bp-long poly A tail (Fig. 3).

The two cDNAs also differ at their 5′ ends but this is apparently due to degradation of the transcript or incomplete first-strand synthesis of cDNA-2. In cDNA-1, there are two putative in-frame ATG codons (identifiable in the genomic sequence at 992 and 1167 bp downstream from the 5′ end; see Fig. 2), whereas cDNA-2 has only the downstream ATG. We assigned the initiation methionine to the upstream ATG codon (in cDNA-1) since it allows for a hydrophobic transmembrane segment between the initial methionine and the putative FMN-binding domain (see Figs. 2 and 4), typical of other CPR genes. On this basis cDNA-1 allows for a 2208-bp coding region (736 deduced aa for a protein with a calculated molecular weight of 81,637 Da) with a 9-bp upstream 5′ non-coding region. It has an in-frame termination codon (TAG) at about 22 bases upstream (see Fig. 2) as is typical of expressible genes. The hydrophobic transmembrane region predicted by TMpred analysis is 22-aa long (present between amino acids 3 to 24), similar in size to that reported for *Drosophila* CPR (Hovemann et al. 1997) but longer than that for *Candida* species (18–19 aa).

Gene sequence analysis

Based on the assigned start codon, the genomic sequence contained the entire coding region of 2381 bp with a flanking 991-bp upstream region and a 556-bp downstream region (Fig. 2). The 2381-bp coding region has unique sites for the restriction enzymes *Not*1 (130 bp from the 5′ end), *Pst*1 (612 bp), *Xho*1 (879 bp), *Eco*RV (1214 bp), *Hae*II (1220 bp), *Sac*II (1916 bp) and *Bgl*II (2146 bp), and is not cleavable by the common restriction enzymes *Eco*R1, *Hin*dIII, *Kpn*1, *Spe*1 and *Xba*1. A frame shift in the coding region and gaps in the alignment between this and the known CPR sequences in the database indicated the presence of introns. A comparison of the genomic and cDNA sequence of the gene led to the identification of three introns including a 62-bp class-0 intron, a 50-bp class-1 intron, and a 58-bp class-0 intron (Fig. 2). These introns conform in size (49–78 bp) to those reported for peroxidase genes from this organism (see Gold and Alic 1993). The coding region (excluding introns) is relatively GC-rich with a base composition of A (22.57%), T (21.35%), G (26.96%), and C (29.13%).

The region surrounding the assigned start codon contains a purine (G) at the −3 position, as observed for several highly expressed genes in yeast (Hamilton et al. 1987), and conforms to the eukaryotic consensus GNNATGG. This sequence (GCCATGG) has one base variation from the consensus sequence (GACATGG) observed for the lignin peroxidase genes of this organism (see Gold and Alic 1993). NNPP analysis for eukaryotic promoter prediction indicated a putative transcription



start site at the −125 nt position on the (−) strand upstream of the assigned translation start codon. Two TATA boxes, a cellular and viral type TATA beginning at the −95 nt position on the (−) strand and an avian C-type TATA beginning at the −960 nt position on the (+) strand, are present. A CCAAT box beginning at the −896 nt position was also detected (Fig. 2). These features are in contrast to those reported for the mammalian CPR which, like house-keeping genes, lacks the classical TATA and CCAAT boxes (O'Leary et al. 1994). The presence of other possible genetic elements and repeat sequences are indicated in Fig. 2. Among these, a pentanucleotide sequence ATGTG (or its complement CACAT) reported for the n-alkane-inducible *CPR* gene of *Candida maltosa* (Ohkuma et al. 1995) and for P450alk genes of alkane-assimilating yeasts (Yadav and Loper 1999), occurs thrice in this promoter region. Also present are the consensus sequences for the glucocorticoid response element, aryl hydrocarbon receptor/aryl hydrocarbon nuclear translocator (ARNT) heterodimers, ARNT homodimers, and a cAMP-responsive element-binding protein (CRE) sequence AATTCAAT (see Fig. 2). Consensus sequences for the steroid hormone-responsive element (TGACCT) and for the xenobiotic-responsive element, XRE (TTGCGTG), implicated in binding the xenobiotic-Ah receptor com-

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Fig. 2 Nucleotide and deduced amino-acid sequence of the *CPR* gene from strain BKM-F 1767. Numbering for the sequence begins with the putative initial ATG codon. Amino-acid residues are indicated as a *single letter notation* above the second base for each codon. The sequence coinciding with the 285-bp amplicon is *underlined* in the coding region. The start codon, the stop codon, the predicted transcription initiation site base (*underlined*) and the TATA boxes are shown in bold letters. Other genetic elements in the 5′ non-coding region are marked as follows: CCAAT box (*underlined, thick*), repeat element sequence ATGTG or its complementary sequence CACAT (*italicized and underlined*), other repeat sequences cited under the Results section (*underlined*), cAMP responsive element (*underlined, dotted*), glucocorticoid responsive element (*underlined, wave*), AhR/ARNT heterodimer sequence (*underlined, thick*), ARNT homodimer sequence (*underlined, dash*), and alcohol dehydrogenase gene regulator-1 sequence (*underlined, dot-dash*). Bases shared by the overlapping genetic elements in the 5′ region are *double underlined*. In the 3′ non-coding region, potential tripartite signals and poly A-addition signal(s) are *underlined*

plex, have been detected in the promoter region of the rat liver CPR gene (O'Leary et al. 1994). XRE and CRE elements have also been reported for the promoter regions of the lignin peroxidase genes in this fungus (see Gold and Alic 1993). Promoter-analysis studies directed at these elements will help to resolve their specific role in transcriptional regulation of the flanking metabolic genes in *P. chrysosporium*.

The 3′ flanking region of the *CPR* gene contains two variants of the polyA signal (AATAAA-3′) beginning at nt positions 2471 and 2562, and a variant of the tripartite signal [5′-TAG—TA(T/A)GT—TTT] beginning at nt position 2595. Another tripartite signal is present on the (−) strand between nt positions 2666 and 2733. These sequences (underlined in Fig. 2) are typical of eukaryotic genes and are believed to play a role in transcription termination and polyadenylation. However, the isolated cDNA species in fact polyadenylated at base positions 2240 and 2411.

Deduced protein

The deduced amino-acid (aa) sequence of the *P. chrysosporium* CPR was aligned with known CPRs from taxonomically diverse species. Coupled with available information on the functional domains involved in the binding of the cofactors FMN, FAD and NADPH, and in the P450 protein binding for CPRs and other flavoproteins (Porter and Kasper 1985, 1986; Sutter et al. 1990; van den Brink et al. 1995), this permitted the identification of these domains in the *P. chrysosporium* CPR (Fig. 4). By this comparison, 100 invariant residues (out of a total of 736), largely concentrated over these functional domains, were identified in the *P. chrysosporium* CPR.

A sequence identity comparison of the deduced protein with other CPRs using Alignplus indicates that the *P. chrysosporium* CPR is most related to that of the fungus *Aspergillus niger* (43%), followed by yeasts and animal CPRs (36–38%), and is most distant from the CPR of plants (33–35%).

Discussion

Using a PCR approach with degenerate primers based on the relatively conserved FMN-binding domain of the known CPRs, we isolated a genomic amplicon of the expected size (285 bp) from the two widely used strains of *P. chrysosporium*, BKM-F 1767 and ME 446. The amplicon was identified as a *CPR* sequence by its homology to known *CPRs* in the database. The two strains had an identical sequence for this segment and therefore only the gene from BKM-F 1767 was pursued. Screening its genomic library using the 285-bp product as a probe, we obtained a 3937-bp sequence containing the entire coding region (2381 bp) interrupted by three small introns (62 bp, 50 bp, and 58 bp) and the flanking 5′ (991 bp) and 3′ (556 bp) regions.

The two cDNAs with their different polyadenylation sites were isolated from a cDNA library prepared from nitrogen-sufficient (non-ligninolytic) cultures. As the xenobiotic catabolic activities in this organism vary between the nutrient-deficient (ligninolytic) and nutrient-sufficient (non-ligninolytic) cultures, it will be interesting to study expression of the two transcript types under

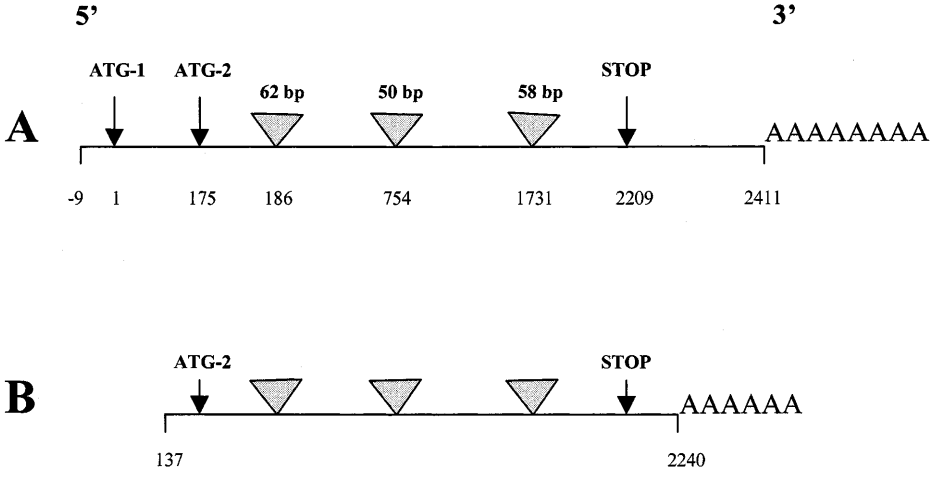

Fig. 3 Comparison of cDNA-1 (**A**) and cDNA-2 (**B**). The two in-frame ATG codons and the stop codon are shown by the *arrows*. The *inverted triangles* represent the spliced introns with their size (bp) indicated on the top. Base numbers 137 and 2240 in **B** conform to their location in **A** (cDNA-1)

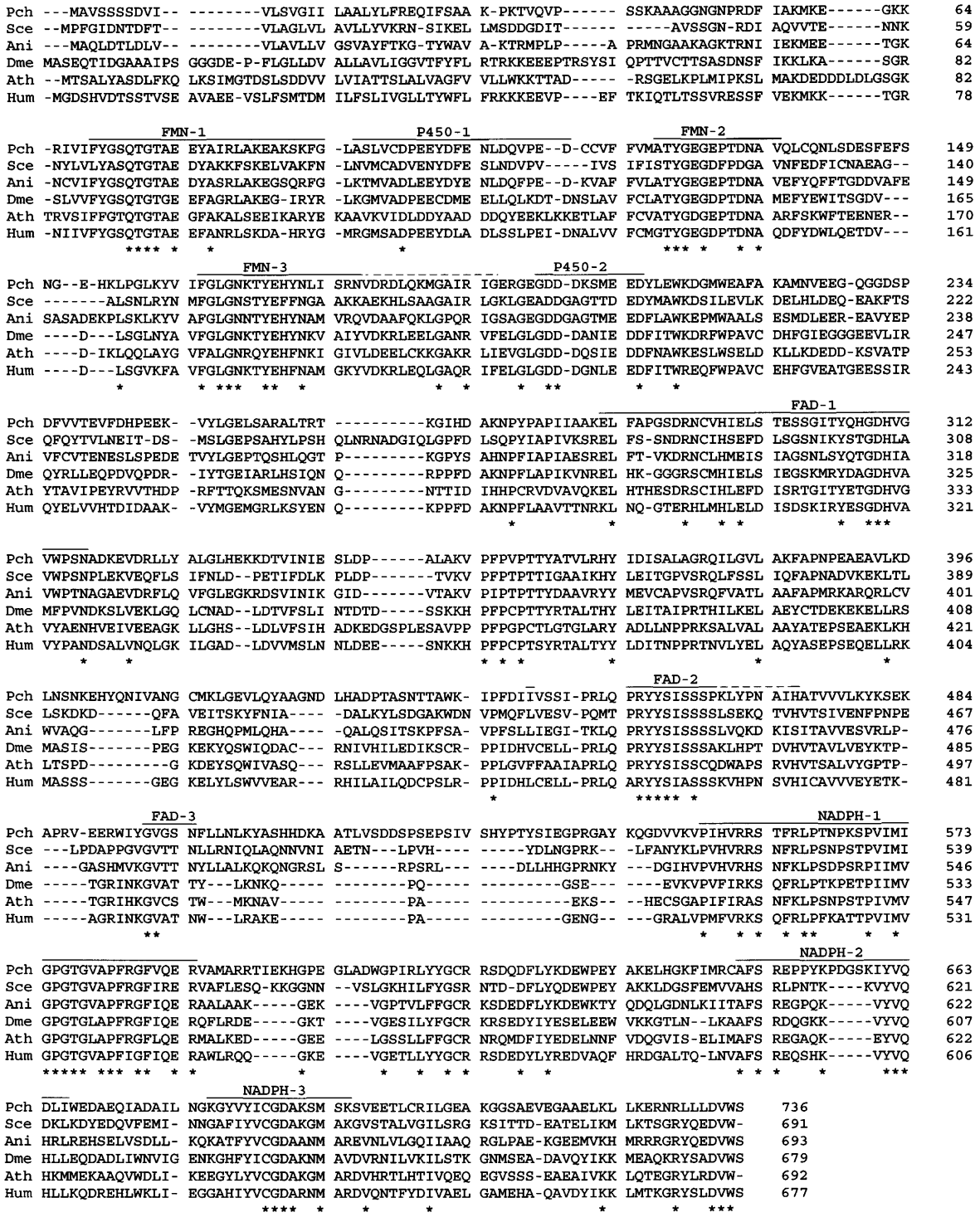

Fig. 4 Alignment of the deduced amino-acid sequences of the cloned *CPR* and representative CPRs from taxonomically diverse species. The CLUSTAL W 1.7 program was employed. Position of the proposed function domains is represented by the *overlines*. Invariant amino-acid residues are indicated by the *underlying asterisks*. Abbreviations: *Pch* (*Phanerochaete chrysosporium*), *Sce* (*Saccharomyces cerevisiae*), *Ani* (*Aspergillus niger*), *Dme* (*Drosophila melanogaster*), *Ath* (*Arabidopsis thaliana*), *Hum* (Human)

varied nutrient conditions as an indication of their relative physiological significance.

The *P. chrysosporium* deduced CPR has an amino-terminal hydrophobic region consisting of a 22-aa-long transmembrane segment typical of known CPRs. This domain is not sequence-conserved among species (Fig. 4) but is known to anchor the CPR protein to the endoplasmic reticulum membrane in the cell, a function essential for correct interaction between the CPR and

the P450 mono-oxygenase components for P450 catalysis. In the *P. chrysosporium* CPR, putative trypsin cleavable sites as predicted by Gene Runner analysis are present downstream from this transmembrane segment (the first trypsin site being at amino-acid position 25 from the N-terminal end). Such trypsin recognition sites are characteristic of higher eukaryotic CPRs, and are known to be responsible for trypsin-mediated release of a catalytically inactive soluble CPR protein from its membrane-anchoring domain (Black and Coon 1982).

Functional domains in the deduced CPR

Despite the low sequence identity of the *P. chrysosporium* CPR with other known CPRs (33–43%), the binding domains for FMN-, FAD-, and NADPH-binding are all highly conserved. These domains are believed to have been derived from two ancestral flavoproteins, the FMN-containing bacterial flavodoxin and the FAD-containing ferredoxin NADP+ reductase (FNR), by fusion of the encoding genes (Porter and Kasper 1985, 1986; Sutter et al. 1990; Smith et al. 1994).

FMN-binding domain

In this domain, three regions (designated as FMN-1, FMN-2 and FMN-3 in Fig. 4) were identified showing homology to the two originally proposed FMN-binding sequences (FMN-1 and FMN-3) for rat CPR (Porter and Kasper 1985) and the third FMN-binding region (FMN-2) as suggested for the Aspergillus CPR (van den Brink et al. 1995) based on the multiple alignment of known CPRs and comparison with the flavodoxin sequence (Karplus et al. 1991; Watt et al. 1991). The identified FMN-1, FMN-2, and FMN-3 domains in *P. chrysosporium* CPR contain the consensus sequences S/T-x-T-G-T (aa residues 72–76), A/S-T-Y-G-D/E (aa residues 123–127), and G-N-x-x-Y-E-x-x-N (aa residues 166–174), respectively. In the FMN-1 consensus region, the first aa residue is serine in *P. chrysosporium* as in other fungal and animal CPRs in contrast to a threonine in plant CPRs.

FAD-binding domain

Three FAD-binding domains (FAD-1, FAD-2 and FAD-3) identified for the *A. niger* CPR (van den Brink et al. 1995) were detected in the *P. chrysosporium* CPR (Fig. 4). The core consensus sequences believed to be responsible for binding of FAD in the three domains are G-D-H (aa residues 308–310) for FAD-1, P/A-R-Y-Y-S-I-S/A-S-S/C-x (aa residues 455–464) for FAD-2, and G-V-x-T/S-N/T (aa residues 495–499) for the FAD-3 region.

NADPH-binding domain

Three NADPH-binding regions (NADPH-1, NADPH-2 and NADPH-3) with their distinctive structural features and their individual putative roles in NADPH-binding described for CPRs (van den Brink et al. 1995) are detectable for the *P. chrysosporium* CPR (Fig. 4). The NADPH-1 region (aa residues 552–589) is a glycine-rich segment with 20 invariant residues. It includes a consensus sequence G-T-G-x-A-P (aa residues 576–581), that can be originally localized to the G-T-G-x-x-P segment of FNR, responsible for binding to the NADPH ribose group. The NADPH-2 region in CPRs (aa residues 646–666) contains three conserved amino acids (Ser648, Arg649 and Lys654) that are characteristic of NADP-binding proteins but not of NADH-binding proteins and are involved in NADP phosphate binding. Among these, Arg649 is believed to be critical in discriminating between NADPH and NADH (Sem and Kasper 1993). The NADPH-3 region (aa residues 681–695) of all CPRs has an invariant cysteine residue (position 687) which is believed to interact with the NADP ribose group.

P450 protein-binding domain

Acidic aa residues, present in the vicinity of FMN-binding domains, are important for the interaction of CPR with the cytochrome P450 protein and cytochrome c as studied in the rat CPR by site-directed mutagenesis (Shen and Kasper 1995). Based on the presence of these residues, two putative cytochrome P450-binding regions (P450-1 and P450-2) have been tentatively assigned in different CPRs including those of fungi (Sutter et al. 1990; van den Brink et al. 1995). The corresponding regions in the *P. chrysosporium* CPR were characterized by the presence of acidic amino acids (aspartic acid at positions 100, 105, 110, 115, 199–201, 207; glutamic acid at positions 102, 103, 107, 114, 205, 206). In the P450-1 region (aa residues 95–115), an aspartic acid residue (Asp100) was conserved among all CPRs. In the P450-2 region (aa residues 199–207), three aspartic acid residues (aa positions 199, 200 and 207) are invariant in all known CPRs.

The 3′ terminus sequence in the coding region of all CPRs contains a conserved consensus G/K/N-R-Y-x-x-D-V/T-W; among all fungal CPRs, including yeast, this sequence is G-R-Y-Q-E-D-V-W. However, the *P. chrysosporium* CPR has only a part of this region (D-V-W) conserved. There is an additional Ser residue at the 3′ coding region of CPR in some species. Interestingly, this serine residue is present among animal CPRs as well as in *A. niger* and *P. chrysosporium*, but is absent in yeast and plant CPRs.

Phylogenetic analysis

A phylogenetic comparison with the available CPR protein sequences in the database by the neighbor joining method showed that the *P. chrysosporium* CPR represents a distinct homolog occupying a separate

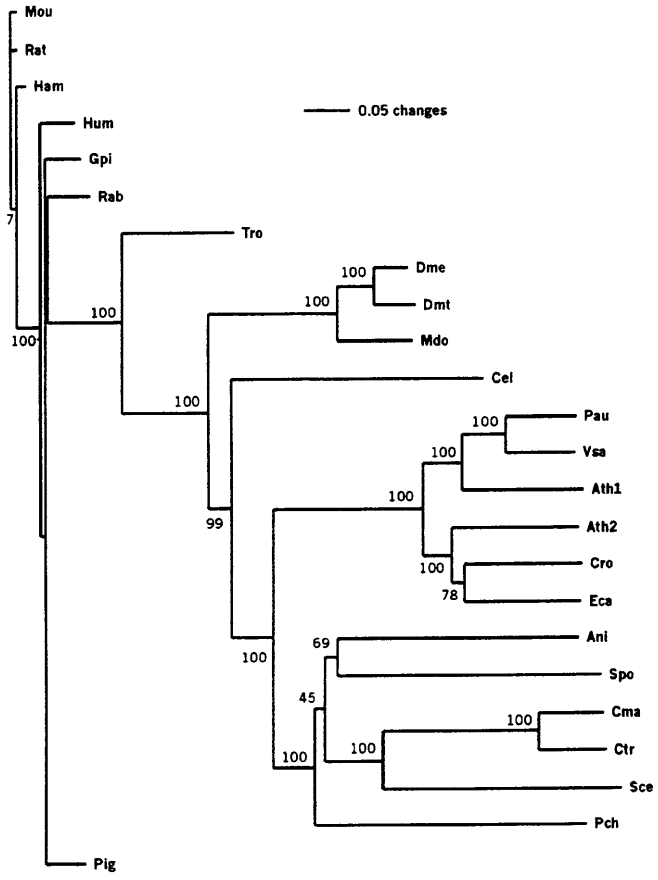

Fig. 5 Phylogenetic analysis of cytochrome P450 oxidoreductases. *P. chrysosporium* CPR (this data) and all others from the genetic database were used. Distance analysis and tree construction was done by the neighbor-joining method. The *numbers* indicate percent support for specific nodes after 1000 replications of bootstrap analysis. The *bar* represents a 5% estimated sequence difference. Abbreviations: *Pch* (*Phanerochaete chrysosporium*), *Sce* (*Saccharomyces cerevisiae*), *Ctr* (*Candida tropicalis*), *Cma* (*Candida maltosa*), *Spo* (*Schizosaccharomyces pombe*), *Ani* (*Aspergillus niger*), *Ath* (*Arabidopsis thaliana*), *Eca* (*Eschscholtzia californica*), *Cro* (*Catharanthus roseus*), *Vsa* (*Vicia sativa*), *Pau* (*Phaseolus aureus*), *Cel* (*Caenorhabditis elegans*), *Mdo* (*Musca domestica*), *Dmt* (*Drosophila mettleri*), *Dme* (*Drosophila melanogaster*), *Tro* (Trout), *Rab* (Rabbit), *Gpi* (Guinea pig), *Pig* (Pig), *Hum* (Human), *Ham* (Hamster), *Rat* (Rat), *Mou* (Mouse)

branch in the fungal cluster on the phylogenetic tree, and is closer to animal than to plant CPRs (see Fig. 5).

This work describes the first report on a *CPR* gene from Basidiomycetes and is an important step in the molecular characterization of catabolic P450 systems in *P. chrysosporium*. In addition to expanding the *CPR* gene diversity among fungi, the availability of the *CPR* gene from this model fungus will facilitate further studies to understand P450 mono-oxygenation mechanisms and metabolic manipulations in white rot fungi.

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