

Methods To Investigate the Expression of Lignin Peroxidase Genes by the White Rot Fungus *Phanerochaete chrysosporium*

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Two methods allowing the analysis of expression of specific lignin peroxidase (LPO) genes from white rot fungi are presented. In the first method, degenerate oligonucleotide primers derived from amino acid sequence motifs held in common among all members of the LPO gene family are used to prime the polymerase chain reaction (PCR) amplification of LPO-related nucleotide sequences from cDNA prepared by using RNA from ligninolytic cultures. The PCR products are cloned and analyzed by restriction cleavage and DNA sequencing. This method was applied to the analysis of transcripts from carbon-limited cultures of *Phanerochaete chrysosporium* BKM-F-1767, revealing two major classes of PCR products. One class showed DNA sequences with a high degree of similarity to the previously described CLG4 cDNA sequence (H. A. De Boer, Y. Zhang, C. Collins, and C. A. Reddy, *Gene* 60:93–102, 1987), whereas the other harbored DNA sequences with similarities to the L18 cDNA sequence previously described for *P. chrysosporium* OGC101 (T. G. Ritch, Jr., V. J. Nipper, L. Akileswaran, A. J. Smith, D. G. Pribnow, and M. H. Gold, *Gene* 107:119–126, 1991). The second method is based on nuclease protection assays involving isoenzyme-specific RNA probes. By using this method, the L18-related gene of *P. chrysosporium* BKM-F-1767 was found to be expressed under conditions of carbon and of nitrogen limitation, although the transcript levels were found to be higher in carbon-limited cultures. Furthermore, it was found that omission of veratryl alcohol addition to the culture did not affect the levels of the L18-related transcripts in carbon-limited cultures.

The biodegradation of lignin by white rot fungi is a complex reaction which appears to be achieved by the concerted action of a number of enzymes (reviewed in references 32 and 55). The first lignin-degrading enzyme, ligninase (lignin peroxidase [LPO]) from the white rot basidiomycete *Phanerochaete chrysosporium* was described in 1983 (20, 61). LPOs have since been isolated from the culture supernatants of other white rot basidiomycetes including *Trametes versicolor* (13, 28), *Phlebia radiata* (42), *Coriolorsis occidentalis* (41), and *Bjerkandera adusta* (29). In *P. chrysosporium* these enzymes are present as multiple isozymes (31, 48), and isoelectric focusing gels revealed up to 15 different peroxidases including LPOs and Mn(II)-dependent peroxidase (MnP) (34, 35), whereas the high-pressure liquid chromatography analysis by Kirk et al. (31) indicated the presence of 10 extracellular heme proteins. Cross-reaction of polyclonal antibodies directed against single isozymes with all other isozymes, but not with MnP, have indicated that LPOs are structurally related (31, 34). This homology was verified by amino acid composition comparisons (34), by N-terminal sequence analysis (21, 43, 66), and by peptide mapping of the different isozymes (15, 34). All these experiments indicated early on that LPOs are similar and that they constitute an isozyme family.

With the goal of more clearly defining the origin(s) of the numerous LPO isozymes which were initially thought to be posttranslational variants of the same gene product, the isolation and characterization of LPO genes and cDNA copies have been undertaken by several groups (reviewed in references 1 and 47). A total of five different LPO cDNA

sequences from *P. chrysosporium* BKM-F-1767 have been described in the literature (Table 1), and a number of genomic LPO sequences and allelic variants thereof from the *P. chrysosporium* BKM-F-1767 strain have also appeared (Table 1). LPO cDNA and genomic sequences from *P. chrysosporium* ME446 and OGC101 (3, 5, 49, 50) and from other white rot fungi including *B. adusta* (30), *P. radiata* (51), and *T. versicolor* (4) have recently also been analyzed. Taken together, the results indicate that the diversity of LPO isozymes must be due, in part, to the genomic multiplicity of the LPO sequences.

The regulation of LPO gene expression has been investigated by using Northern (RNA) blots (2, 25, 27) and, more recently, by using competitive polymerase chain reaction (PCR) (58). The data obtained indicate that LPO-related transcripts appear under conditions of carbon or nitrogen limitation and that the levels of specific transcripts can be affected by the culture conditions.

In this paper we describe alternative approaches to the analysis of the expression of specific LPO genes and to the identification of LPO transcripts from hitherto unknown LPO genes. We also present the nucleotide sequence of a new LPO cDNA from *P. chrysosporium* BKM-F-1767.

MATERIALS AND METHODS

Chemicals, radiochemicals, and enzymes. All chemicals were commercial preparations of reagent or sequencing grade whenever possible. [α -thio- 35 S]dATP (1,000 Ci mmol $^{-1}$), [α - 32 P]dCTP (3,000 Ci mmol $^{-1}$), and [α - 32 P]UTP (800 Ci mmol $^{-1}$) were from Amersham.

Strains, media, and growth conditions. The bacterial strains used were *Escherichia coli* DH5 α (Bethesda Re-

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TABLE 1. Cloned lignin peroxidase genes from *P. chrysosporium* BKM-F-1767

Sequence	Characteristics	Reference(s)
LPOA	Genomic clone	57, 66
LIPA	Allelic form of LPOA	19
ML-1	cDNA clone corresponding to LPOA	62
ML-4	cDNA and genomic clones corresponding to LIPA	2
LPOB	Genomic clone, linked to LPOA	26
LIPB	Allelic form of LPOB	19
ML-5	cDNA clone	2
CLG4	cDNA clone	11
CLG5	cDNA clone	11
GLG5	Genomic clone corresponding to CLG5	19
GLG2	Genomic clone related to CLG5	68
GLG6	Genomic clone	40
0282	Genomic clone	54
V4	Genomic clone	54
LPO811	cDNA clone	This work

search Laboratories), Y1090 (67), BB4 (56), and XL1-Blue (6). They were cultivated in LB or 2× YT medium (52). *P. chrysosporium* BKM-F-1767 (ATCC 24725) (7) was used and cultures were prepared as described by Leisola et al. (34, 35).

RNA isolation. Total-cell RNA was isolated by grinding frozen cells into a fine powder with a mortar and pestle under liquid nitrogen. The frozen powder was suspended in 1 ml of 4 M guanidine isothiocyanate buffer per g of wet cells (52), and total-cell RNA was isolated as described previously (8, 60). The poly(A)-containing RNA fraction was purified by binding to and elution from oligo(dT)-cellulose (New England BioLabs) (52).

cDNA library construction and screening. cDNA synthesis was carried out with 2.5 µg of poly(A) RNA and 40 U of Moloney murine leukemia virus reverse transcriptase (Pharmacia) as described by Haymerle et al. (22). The cDNA was provided with *EcoRI* adaptors (New England BioLabs), ligated with *EcoRI*-cut λZAP DNA (Stratagene) (56), and packaged in vitro by using Gigapack II Plus packaging extracts (Stratagene). Plaque screening with cDNA or oligonucleotide probes was done as described by Davis et al. (9).

PCR. Double-stranded cDNA was prepared as described above. For PCR amplification, 10 µg of each primer, 20 ng of cDNA, 10 µl of a 2 mM deoxynucleoside triphosphate solution, 10 µl of 10× *Taq* polymerase buffer (100 mM Tris-HCl [pH 9.0], 500 mM KCl, 15 mM MgCl₂, 1% Triton X-100, 0.1% gelatin [Anawa, Wangen, Switzerland]), and 2.5 U of *Taq* polymerase (Anawa) were combined and the volume was adjusted to 100 µl. Denaturation, annealing, and polymerization were carried out for 1.5 min at 94°C, 2 min at 50°C, and 2 min at 72°C, respectively. The cycle was repeated 30 times. After a 10-min incubation at 72°C, 10 µg of carrier glycogen (Boehringer) were added and the reaction was extracted with 100 µl of chloroform. The DNA was purified by adsorption to Prep-A-Gene (Bio-Rad) and subsequently digested with *EcoRI* and *PstI* and fractionated on a

low-melting-temperature agarose gel (Pharmacia). The DNA band of the predicted size was cut out and ligated (18) with *EcoRI*- and *PstI*-digested pBluescript SK- vector DNA (Stratagene) (56). Plasmid harboring inserts were identified by colony screening (17). The agarose gel band harboring the PCR product was radiolabeled by the oligolabeling procedure of Feinberg and Vogelstein (16) and used as a hybridization probe.

DNA sequencing. Plasmid DNA was obtained from λZAP clones by coinfecting cells with lambda and the helper phage R408 (56). DNA sequencing reactions were performed by the method of Sanger et al. (53) on double-stranded plasmid DNA as the template (24) with either T7 polymerase (Pharmacia) or Sequenase (United States Biochemicals) (59) as specified in the step-by-step protocol from United States Biochemicals. Deletion subclones were created by using restriction enzymes. Computer analysis of the sequence data was performed with the programs of Queen and Korn (45) and Devereux et al. (12).

RNA mapping of RNA. [³²P]RNA probes were prepared by using T3 or T7 RNA polymerase and [³²P]UTP essentially as described by Sambrook et al. (52) and Krieg (33), except that the final UTP concentration in the reaction was 10 µM. Unlabeled RNA was synthesized by using 500 µM UTP in the reaction. Hybridizations were carried overnight at 52°C with 5 to 10 µg of total RNA in 30 µl of 80% FAHB (52). RNase ONE (Promega) (37, 38) was used for digestion at 30°C for 60 min. Five units of enzyme in 300 µl of RNase digestion buffer (10 mM Tris-HCl [pH 7.5], 5 mM EDTA [pH 8.0], 200 mM sodium acetate [pH 8.0]) was used. To stop the reaction, 20 µl of a mixture containing 2% sodium dodecyl sulfate and 1 µg of *E. coli* tRNA was added and the samples were precipitated with ethanol. The protected RNA hybrids were analyzed on a 6.5% polyacrylamide-8 M urea gel.

Nucleotide sequence accession number. The LPO811 nucleotide sequence reported in this paper has been entered in the GenBank Data Library and assigned accession no. L08963.

RESULTS AND DISCUSSION

PCR-based strategy to investigate the expression of specific LPO genes. One of the characteristics of peroxidases is that they contain two histidine residues that are essential for activity. The so-called proximal histidine is the axial ligand of the heme, and the other one, the so-called distal histidine, is believed to be involved in charge stabilization during reaction of the heme prosthetic group with H₂O₂ (reviewed in reference 10). These residues have also been identified in LPOs (23, 62). The comparison of the proximal and distal His residues and surrounding regions present in the various LPO sequences from *P. chrysosporium* BKM-F-1767 revealed two conserved amino acid motifs (Fig. 1A). The corresponding nucleotide sequences (Fig. 1B) allowed the design of two sets of degenerate oligonucleotides (Fig. 1C) encoding all of the hitherto known LPO gene sequences. Set D, encoding the distal His plus surrounding regions, was 32-fold degenerated, whereas set P, encoding the proximal His residue plus flanking regions, was 144-fold degenerated (Fig. 1C). The two oligonucleotide mixtures were used as the opposing primers in the PCR. Double-stranded cDNA prepared from poly(A) RNA of 6-day-old carbon-limited cultures was used as the template for the reaction. On the basis of the distance of the target amino acid sequences, the predicted size of the amplified PCR product was roughly 430 bp. After completion of the PCR reaction, the product was digested with *EcoRI* and *PstI* and analyzed by agarose gel

(A) Protein sequences

Distal His region	Proximal His region
F H D S I A I A	A H S I A A V V A

(B) Nucleotide sequences

Distal His region	Proximal His region
LPOB TTC CAC GAT GCT ATT GCC ATC	GCC CAC TCC GTT GCT GCA GTC
LPOA TTC CAC GAC TCC ATC GCA ATT	GCG CAC TCC GTC GCA GCG GTG
0282 TTC CAC GAC TCC ATC GCA ATT	GCG CAC TCC GTC GCG GCA GTC
V4 TTC CAC GAC GCC ATC GCG ATC	GCG CAT TCT GTC GCT GCC GCC
CLG4 TTC CAC GAC TCC ATC GCT ATC	GCC CAC TCC ATC GCG GCT GCG
CLG5 TTC CAC GAC GCC ATC GCT ATC	GCA CAC TCC GTC GCG GCT GCC
ML-5 TTC CAC GAC TCG ATC GCC ATC	GCC CAC TCC GTT GCT GCA GTC
GLG6 TTC CAC GAC GCC ATC GCT ATC	GCT CAC TCC GTC GCT GCG GCC
Consensus TTC CAC GAT ^T GCC ^T ATC ^T GCN ATC	GCN CAC TCC GTC GCT ^T GCN ^G GTC
C T G	A

(C) PCR primers

Oligo D

5' GGAA TTC CAC GAT GCN ATC GC
 C T T

Oligo P

5' AACTGCA GC ^TAGC ^GAAT ^GAGA ^GATG ^CTGC
 C G

FIG. 1. PCR strategy to analyze the expression of LPO genes based on degenerate primers encoding the proximal and distal His residues plus surrounding regions. (A) Amino acid sequences in the vicinities of the proximal and distal His residues in LPOs from *P. chrysosporium* BKM-F-1767. The conserved His residues are shown in boldface type. (B) Corresponding nucleotide sequences. (C) PCR primers used. The underlined nucleotides were added to create *EcoRI* (Oligo D) and *PstI* (Oligo P) cleavage sites.

electrophoresis (Fig. 2A). A major band of the predicted size was observed (Fig. 2A, lane 1), cut out of the gel, and then ligated to pBluescript SK- plasmid DNA previously cut with *EcoRI* and *PstI*. Recombinant plasmids were analyzed by restriction cleavage, and two different classes of inserts were apparent (Fig. 2B). One class was readily cleaved with the enzyme *SalI* (Fig. 2B, lane 3) but not with *RsaI* (Fig. 2B, lane 1), whereas the other class was cleaved with *RsaI* (Fig. 2B, lane 2) but not with *SalI* (Fig. 2B, lane 4). Of the 24 PCR clones investigated, 13 showed the pattern seen in lanes 1 and 3 and the rest revealed the pattern seen in lanes 2 and 4. To identify the origin of the cloned PCR products, several members of the two groups of clones described above were subjected to a partial DNA sequence analysis in the vicinity of the region encoding the proximal His (Fig. 2C and D). This analysis revealed the presence of two kinds of cDNA sequences, one of them showing a high degree of similarity to the previously described CLG4 cDNA sequence (11) (Fig. 2D) and the other one being similar to the L18 cDNA sequence (Fig. 2C) previously found in *P. chrysosporium* OGC101 (49). The analysis also showed that the two kinds of sequences were about equal in abundance and, conse-

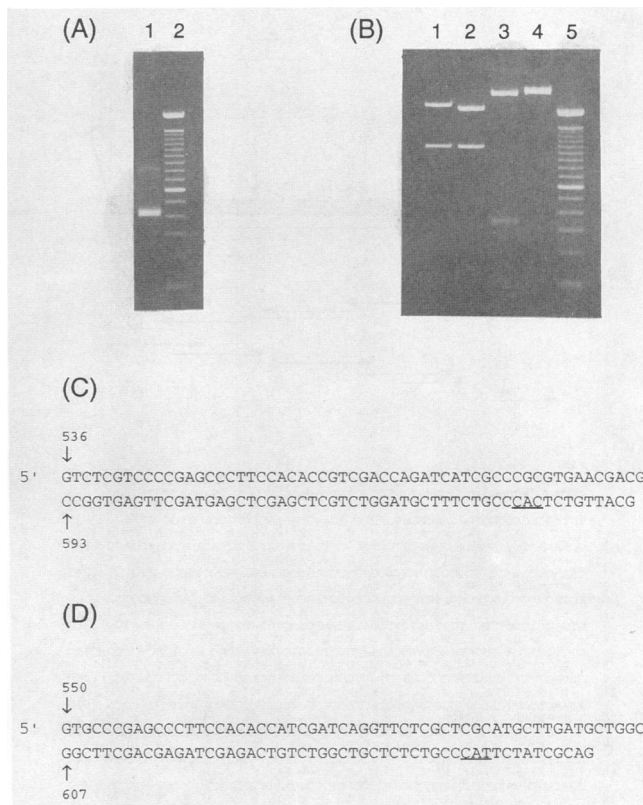


FIG. 2. Analysis of PCR products. (A) Analysis of PCR products by agarose gel electrophoresis. Lanes: 1, PCR product; 2, 100-bp ladder (Bethesda Research Laboratories). (B) Restriction analysis of cloned PCR products. Lanes: 1 and 2, *RsaI* cleavage; 3 and 4, *SalI* cleavage; 5, 100-bp ladder. (C and D) Partial DNA sequences of PCR products encoding the proximal His plus flanking regions. The codons encoding the proximal His residues are underlined. The sequence shown in panel C, corresponding to the insert of the plasmid shown in panel B, lanes 1 and 3, was numbered by the method of Ritch et al. (50), whereas the sequence shown in panel D, corresponding to the insert of the plasmid shown in panel B, lanes 2 and 4, was numbered by the method of De Boer et al. (11).

quently, that the two sequences represent the major transcripts present in C-limited cultures. This assumption is supported by the results obtained from the screening of the cDNA library described below.

Isolation and sequence analysis of a novel LPO cDNA from *P. chrysosporium* BKM-F-1767. To isolate a full-length copy of the novel L18-like cDNA from BKM-F-1767, a bacteriophage λZAP-based cDNA library was constructed with RNA from 6-day-old carbon-limited *P. chrysosporium* cultures and LPO cDNAs were identified by plaque screening with a previously isolated partial LPO cDNA clone (66) and subsequent DNA sequencing. Of the five clones investigated, two appeared to be similar to the partial DNA sequence shown in Fig. 2D and two others were similar to the partial DNA sequence of the PCR product shown in Fig. 2C and with the *P. chrysosporium* OGC101 L18 cDNA sequence (49, 50). One of the L18-like clones was analyzed in more detail and will be referred to here as LPO811. The restriction map of the corresponding insert is shown in Fig. 3A, and the nucleotide sequence of the mRNA-like strand and the deduced amino acid sequence are presented in Fig.

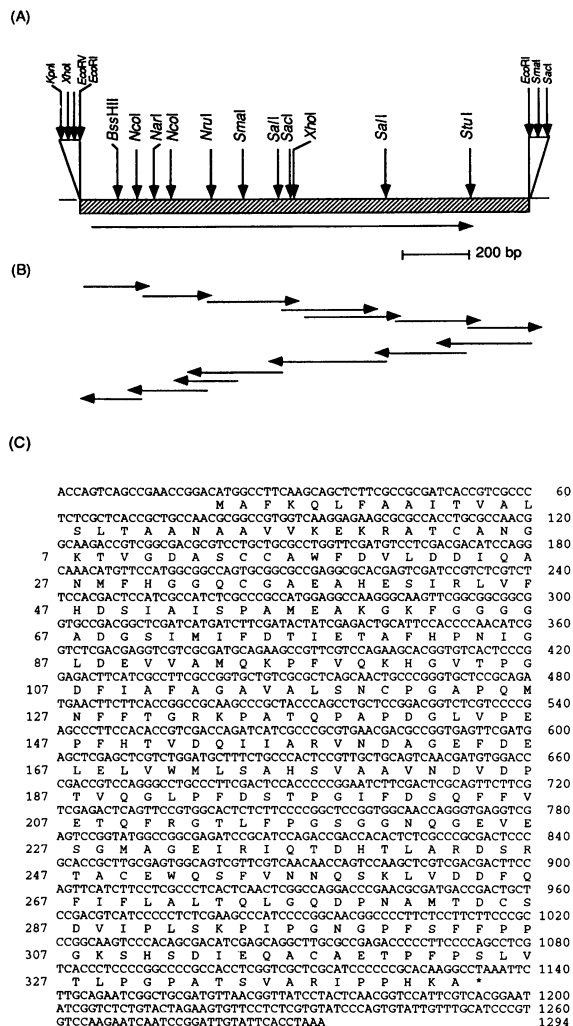


FIG. 3. Molecular analysis of the LPO811 cDNA. (A) Restriction map. The two triangles represent the polylinker regions of the pBluescript SK+ vector (56). The arrow indicates the protein-coding region. (B) Sequencing strategy. (C) Nucleotide sequence and deduced amino acid sequence. The numbers on the left refer to amino acids of the mature protein, and the numbers on the right refer to nucleotides.

3C. The LPO811 cDNA is 1,291 bp long, the protein-coding region being flanked by 20 bp at the 5' end and 155 bp at the 3' end. The mature protein of 343 amino acids is preceded by a leader sequence of 28 predominantly hydrophobic amino acids, ending with the dibasic residues Lys-Arg. One potential N-glycosylation site can be identified at amino acids 257 to 259. In addition, there are 45 potential O-glycosylation sites. The codon usage is extremely biased in favor of codons ending with C and/or G residues, the third base being preferentially a C residue. A comparison of the nucleotide sequences encoded by the LPO811 and L18 clones revealed differences at 11 positions. Nine of these differences do not affect the amino acid sequence, but two G-to-A changes at positions 870 and 871 lead to a glycine-to-asparagine change affecting amino acid 256 (Fig. 3C). Also, a T-to-C change at position 893 created a second *SalI* site within the LPO811 sequence. These changes may be due to strain differences or to different allelic forms.

The juxtaposition of the deduced amino acid sequence encoded by the LPO811 cDNA with the N-terminal amino acid sequences of several LPO isozymes (21, 43, 66) made it possible to correlate the LPO811 encoded protein. From the protein sequence shown in Fig. 3C, it can be concluded that the LPO811-encoded protein corresponds to the pI 4.15 isozyme (21) as far as the N-terminal sequences are concerned. The N-terminal amino acid sequence of the pI 4.2 isozyme isolated from *P. chrysosporium* INA-12 (43) also appears to correspond to the isozyme encoded by the LPO811 cDNA.

Analysis of specific LPO transcripts by nuclease protection assays. Techniques that allow the detection of single-base changes in both cloned and genomic DNA fragments have previously been worked out by Myers et al. (39). In this approach, mismatches are revealed after cleavage with RNase A of RNA-DNA duplexes consisting of a uniformly labeled single-stranded RNA probe made by runoff transcription in vitro and a DNA sequence. Using the same general strategy, we have worked out a sensitive procedure capable of differentiating transcripts derived from different LPO genes. For this purpose a labeled RNA probe was made extending from the *StuI* site at positions 1129 to 1134 (Fig. 3A) up to the very 3' end of the transcript derived from the LPO811 gene. The sequence of this region varies quite substantially among all LPO genes, and thus it is possible to differentiate the transcripts from different LPO genes. RNA made in vitro by using the cloned CLG4-related cDNA (65), a partial LPOA genomic sequence (66), or the cloned LPO811 cDNA as the template were used to assess the specificity of the system (Fig. 4A, lanes 2 to 4). In parallel, the three plasmids were used as controls in the nuclease protection assay (lanes 5 to 7). It is evident from Fig. 4A that the assay conditions used are well suited to discriminate among different LPO nucleotide sequences and that the protected fragments observed agree with the sizes of the expected hybrids. Therefore the protection method is specific for LPO811 transcripts. In Fig. 4B, lanes 2 to 7, the appearance of LPO811 transcripts in nitrogen-limited and carbon-limited cultures of *P. chrysosporium* BKM-F-1767 is presented. Such transcripts were found under conditions of nitrogen limitation and of carbon limitation, although the transcripts in N-limited cultures appeared earlier and the transcript levels were higher in C-limited cultures. It has previously been shown (14, 36, 63, 64) that veratryl alcohol (3,4-dimethoxybenzyl alcohol) added as a supplement to cultures of *P. chrysosporium* enhances LPO activity through an induction type of mechanism and/or by protecting the enzyme against inactivation by hydrogen peroxide. The results presented in Fig. 4B, lanes 8 and 9, show that the LPO811 transcript levels were not affected by the presence or absence of veratryl alcohol in the cultures, even though the LPO activity in the veratryl alcohol-containing culture was about 25 times higher (0.26 versus 0.01 U ml⁻¹), indicating that veratryl alcohol does not exert its effect at the RNA level as far as the LPO811 gene is concerned.

Concluding remarks. The PCR-based strategy will be useful for identifying transcripts from hitherto unidentified LPO genes from *P. chrysosporium* and from other white rot fungi grown under various physiological conditions. The strategy differs in this respect from other methods (2, 25, 27, 58) which have been used in the past to investigate the transcription of LPO genes. These methods all rely on gene probes from previously identified LPO genes, and therefore sequences different from the already known ones will generally not be detected. In fact, LPO811 transcripts have so

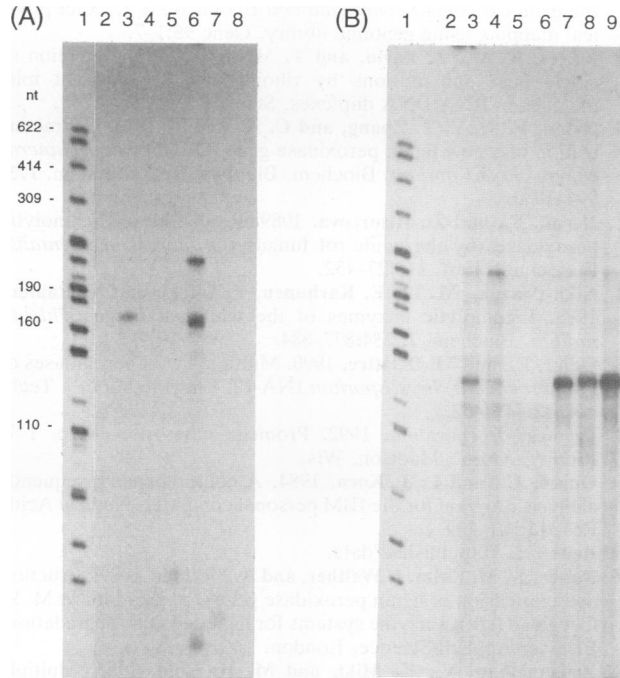


FIG. 4. Analysis of LPO811 gene-related transcripts by the RNase ONE protection assay. (A) RNase mapping with synthetic RNAs and DNA. Lanes: 1, marker fragments; 2, in vitro-synthesized CLG4-like RNA (100 ng) (a plasmid harboring a full-length CLG4-like cDNA in pBluescript KS+ [65] previously cut with *BscI* had been used as a template); 3, in vitro-synthesized LPO811 RNA (100 ng) (pBluescript SK+/LPO811 DNA previously cut with *Bam*HI had been used as a template); 4, in vitro-synthesized LPOA RNA (100 ng) (pBluescript KS+ carrying a partial LPOA sequence lacking about 1 kb of the protein-coding region at the 5' end [66] previously cut with *Eco*RV had been used as a template); 5, pBluescript KS+ harboring a full-length CLG4-like cDNA previously cut with *BscI* (50 ng); 6, pBluescript SK+/LPO811 DNA (50 ng) previously cut with *Bam*HI; 7, pBluescript KS+ carrying a partial LPOA sequence as described above (50 ng) previously cut with *Eco*RV; 8, *E. coli* tRNA (10 μ g). (B) RNA from *P. chrysosporium* BKM-F-1767. Total RNA (5 μ g) from fungal pellets grown under carbon- or nitrogen-limited conditions was analyzed. Lanes: 1, marker fragments; 2 to 4, RNA from 2-, 4-, or 6-day nitrogen-limited cultures, respectively; 5 to 7, RNA from 2-, 4-, and 6-day carbon-limited cultures, respectively; 8, RNA from a veratryl alcohol-containing, 5-day carbon-limited culture; 9, RNA from a 5-day carbon-limited culture not containing veratryl alcohol. Marker fragments used were end-labeled pBR322 *Msp*I fragments. The numbers on the left refer to the sizes of the fragments in nucleotides (nt).

far not been detected in *P. chrysosporium* BKM-F-1767. One potential drawback of the method presented here is that minor transcripts will be detected only if a large number of cloned PCR fragments are being analyzed. The technique is not limited to double-stranded cDNA, since single-stranded DNA templates could easily be prepared from total-cell RNA by reverse transcription with either oligo(dT) or LPO-specific oligonucleotides. By using cellular DNA as a template in the PCR reaction, the same strategy should also be useful to rapidly analyze LPO or MnP gene families in white rot fungi and to identify individual members. The nuclease strategy, on the other hand, complements existing techniques aimed at investigating individual transcripts from gene families. Similar results were obtained by using a mixture of RNase A and RNase T₁ (46). RNase A in

combination with RNase T₁ has previously been used to detect single-base-pair mismatches in RNA-DNA hybrids (39), and RNase ONE has been reported to be equally discriminatory (44). Therefore the technique should be specific enough to discriminate between the transcripts derived from allelic gene copies.

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