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Cloning and molecular analysis of a cDNA and the *Cs-mnp1* gene encoding a manganese peroxidase isoenzyme from the lignin-degrading basidiomycete *Ceriporiopsis subvermispora*

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Abstract

A cDNA (MnP13-1) and the *Cs-mnp1* gene encoding for an isoenzyme of manganese peroxidase (MnP) from *C. subvermispora* were isolated separately and sequenced. The cDNA, identified in a library constructed in the vector Lambda ZIPLOX, contains 1285 nucleotides, excluding the poly(A) tail, and has a 63% G+C content. The deduced protein sequence shows a high degree of identity with MnPs from other fungi. The mature protein contains 364 amino acids, which are preceded by a 24-amino-acid leader sequence. Consistent with the peroxidase mechanism of MnP, the proximal histidine, the distal histidine and the distal arginine are conserved, although the aromatic binding site (L/V/I–P–X–P) is less hydrophilic than those of other peroxidases. A gene coding for the same protein (*Cs-mnp1*) was isolated from a genomic library constructed in Lambda GEM-11 vector using the cDNA MnP13-1 as a probe. A subcloned *Sac*I fragment of 2.5 kb contained the complete sequence of the *Cs-mnp1* gene, including 162 bp and 770 bp of the upstream and downstream regions, respectively. The *Cs-mnp1* gene possesses seven short intervening sequences. The intron splice junction sequences as well as the putative internal lariat formation sites adhere to the GT–AG and CTRAY rules, respectively. To examine the structure of the regulatory region of the *Cs-mnp1* gene further, a fragment of 1.9 kb was amplified using inverse PCR. A putative TATAA element was identified 5′ of the translational start codon. Also, an inverted CCAAT element, SP-1 and AP-2 sites and several putative heat-shock and metal response elements were identified.

Keywords: Lignocellulose biodegradation; Wood-rotting fungi; cDNA and genomic clones; Introns; Heat-schock element; Metal-response element

1. Introduction

Ceriporiopsis subvermispora is a white rot basidiomycete that efficiently degrades wood (Otjen et al., 1987) as well as grass lignocelluloses (Akin et al., 1995). Its ligninolytic system is composed of manganese peroxidase (MnP) and laccase (Rüttimann et al., 1992). The former is a heme-containing enzyme that oxidizes Mn(II) to Mn(III) (Glenn et al., 1986), which in turn oxidizes phenolic substrates in vitro and presumably lignin itself in natural environments. Laccase is a copper-containing phenoloxidase, although in the presence of appropriate substrates, it is able to attack non-phenolic compounds indirectly (Bourbannais and Paice, 1992). *C. subvermispora* does not produce lignin peroxidase (LiP), another heme protein possessing the unique ability to attack non-phenolic residues generating cation radicals that eventually decay to smaller compounds (Kirk et al., 1986). Recent reports indicate that in cultures of this fungus, MnP itself mediates the oxidation of non-phenolic lignin structures through a novel mechanism involving peroxidation of unsaturated lipids (Jensen et al., 1996).

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Abbreviations: bp, base pairs; cDNA, DNA complementary to RNA; HSE, heat shock element; LiP, lignin peroxidase; MnP, manganese peroxidase; *mnp*, gene encoding for manganese peroxidase; MRE, metal response element; PCR, polymerase chain reaction; pI, isoelectric point.

MnP seems to be the most ubiquitous ligninolytic enzyme among white-rot fungi (Orth et al., 1993; Hatakka, 1994). In cultures of *C. subvermispora*, the titers of MnP correlate positively with mineralization of synthetic lignin (Rüttimann-Johnson et al., 1993). Analysis by isoelectrofocusing of samples withdrawn from the cultures reveals the presence of up to 11 MnPs, with an isoenzyme pattern that varies according to the conditions of growth (Lobos et al., 1994). The isoenzymes differ with respect to their substrate specificity and the concentration of Mn(II) required for optimal activity (Urzúa et al., 1995). N-terminal sequences of several of these MnP isoenzymes are clearly distinct, suggesting the existence of more than one gene (Lobos et al., 1994). The production of MnP as an enzyme family is not unique to *C. subvermispora* (Leisola et al., 1987; Johansson and Nyman, 1993; Rüttimann-Johnson et al., 1994; Périé et al., 1996), although the physiological significance of this multiplicity is not well understood.

Genetic studies constitute a suitable approach to gain insight into the latter phenomenon. Three genes for MnP have been identified in the fungus *Phanerochaete chrysosporium* (Godfrey et al., 1990; Mayfield et al., 1994; Alic et al., 1997), which are regulated in response to nutrient limitation, heat shock, concentration of Mn(II) and hydrogen peroxide (Pribnow et al., 1989; Brown et al., 1991, 1993; Mayfield et al., 1994; Li et al., 1995). In *Trametes versicolor*, N-terminal sequencing suggests the presence of three genes (Johansson et al., 1993), one of which has been recently sequenced at the nucleotide level (Johansson and Nyman, 1996). Two MnP cDNAs from the fungus IZU-154 (Matsubara et al., 1996) and a *mnp* gene from *Pleurotus ostreatus* (Asada et al., 1995) have also been sequenced.

The aim of our studies is to verify whether there is a gene family coding for the MnPs in *C. subvermispora* and to determine the conditions governing expression of selective isoenzymes. For this purpose, we have constructed both cDNA and genomic libraries of the fungus. This work reports the independent isolation and sequencing of a cDNA from one of the MnP isoenzymes and a *Cs-mnp* gene encoding the same protein.

2. Materials and methods

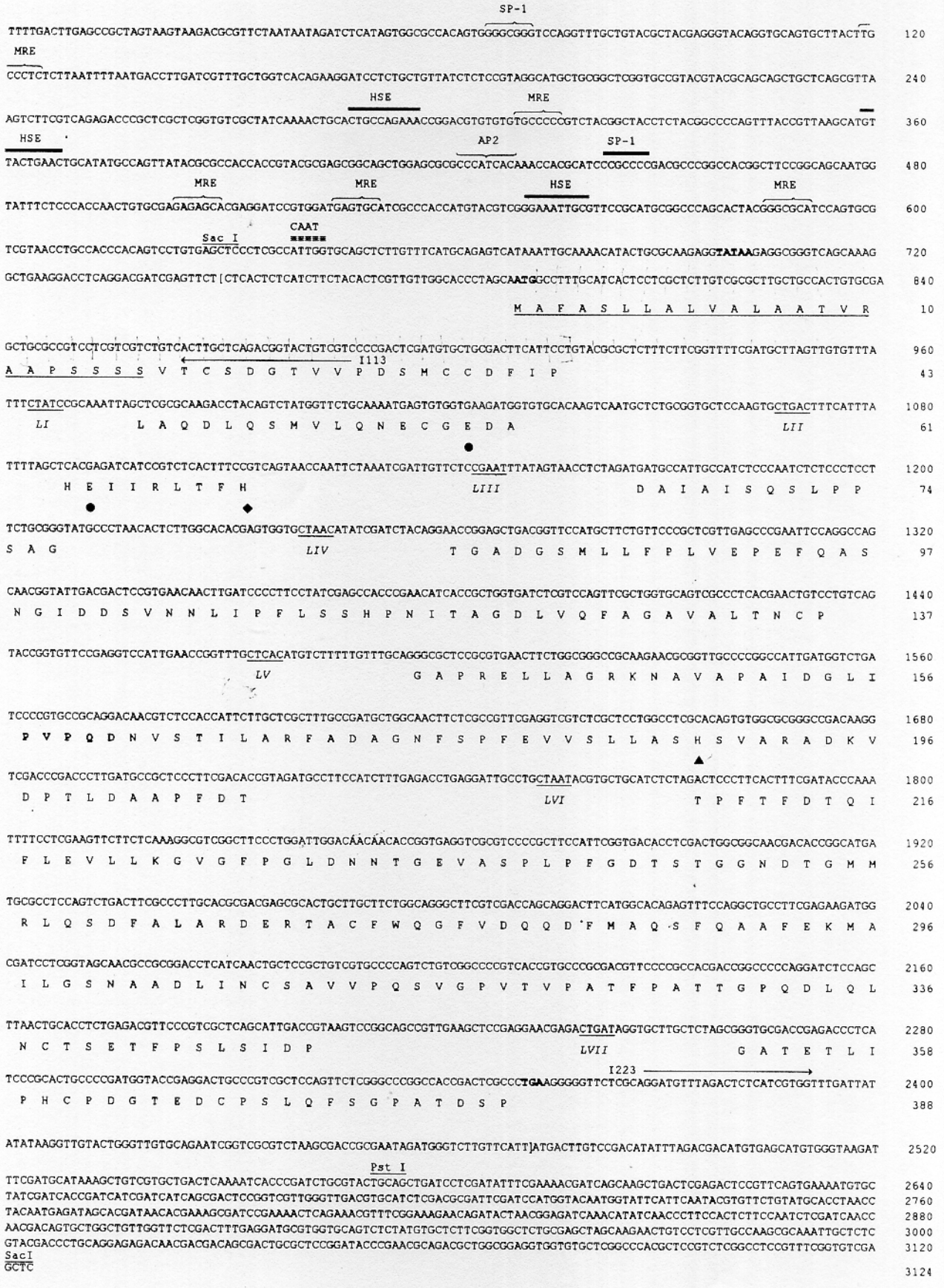
2.1. Construction of a cDNA library in Lambda ZIPLOX

Nitrogen-frozen mycelium of *C. subvermispora* (strain FP105757, Forest Products Laboratory, Madison, WI) was blended under dry ice, and the frozen powder was added to 12.5 ml of homogenization solution (0.1% DEPC, 0.2 M Tris–HCl pH 8.5, 50 mM EGTA, 0.25 M NaCl, 0.6% *p*-aminosalysilic acid and 1% triisopropylnaphthalenesulfonic acid) and 12.5 ml of water-saturated phenol. One gram of sterile glass beads (106 mm) (Glass Beads, Sigma) was added, and the mixture was incubated on ice for 60 min with occasional stirring. After centrifugation at $12\,000 \times g$ for 15 min at 4°C, the supernatant was extracted twice with phenol: chlorophorm (1:1). Poly(A)-containing RNAs were purified by oligo(dT)-cellulose column chromatography (GIBCO BRL) from 1.74 mg of total RNA obtained by ethanol precipitation of the aqueous phase. Then, synthesis of cDNA and size fractionation of the products were conducted using the Superscript Lambda System (GIBCO BRL) according to the manufacturer's instructions. cDNA fragments were cloned in Lambda ZIPLOX (GIBCO BRL) and packaged in vitro using the Packagene Lambda DNA System (Promega). Automatic subcloning in pZL1, after screening the library with a heterologous probe (see text), was performed according to D'Alessio et al. (1992). The cDNA was directly sequenced by the dideoxy chain termination method (Sanger et al., 1977) using a Sequenase Version 2.0 kit from US Biochemicals.

2.2. Construction of a genomic DNA library in Lambda Gem-11

C. subvermispora FP105757 was grown for 10 days in YMPG medium (Rüttimann et al., 1992). The mycelium was harvested by filtration through miracloth, immediately frozen in liquid nitrogen and stored at −80°C until use. The frozen mycelium (8 g) was ground in a coffee grinder with small chunks of dry ice until powdered. After sublimation of the ice, the powder was added to 25 ml of TE buffer (10 mM Tris–HCl, pH 8.0; 1 mM EDTA) and 5 ml of 5% SDS and incubated at room temperature for 15 min. Nucleic acids were extracted with one volume of phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous phase was treated with 1 mg of proteinase K at 50°C for 30 min and extracted again as above. The nucleic acids were precipitated with ethanol, resuspended in TE buffer and incubated with 1 mg of RNase at 37°C for 30 min. The proteinase K treament, organic extraction and ethanol precipitation steps were repeated, and the final DNA

Fig. 1. The nucleotide and predicted amino-acid sequence of the *C. subvermispora Cs-mnp1* gene (Genbank accession no. AF013257). The start codon is followed by a 24-amino-acid signal peptide (underlined). The symbols '◆' and '▲' denote the distal and proximal histidine, respectively, whereas the symbol '·' denotes residues Glu35 and Glu39. Square brackets indicate the 5′ and polyadenylation site in the cDNA, respectively. The putative polyadenylation signal is shown in parentheses. TATA box is in bold and underlined. An inverted CCAAT element (ATTGG) is shown as well as putative metal response elements (MRE) and heat shock elements (HSE). SP1 and AP2 recognitions sites are also indicated (see text for more details). Internal lariat formation sites are overlined and numbered from *LI* to *LVII*. *Sac*I as *Pst*I recognition sites are also shown. The sequence and orientation of the oligonucleotides I113 and I223 are marked.


SP-1
MRE
HSE
MRE
HSE
AP2
SP-1
MRE
MRE
HSE
MRE
CAAT
Sac I
I113
I223
Pst I
SacI

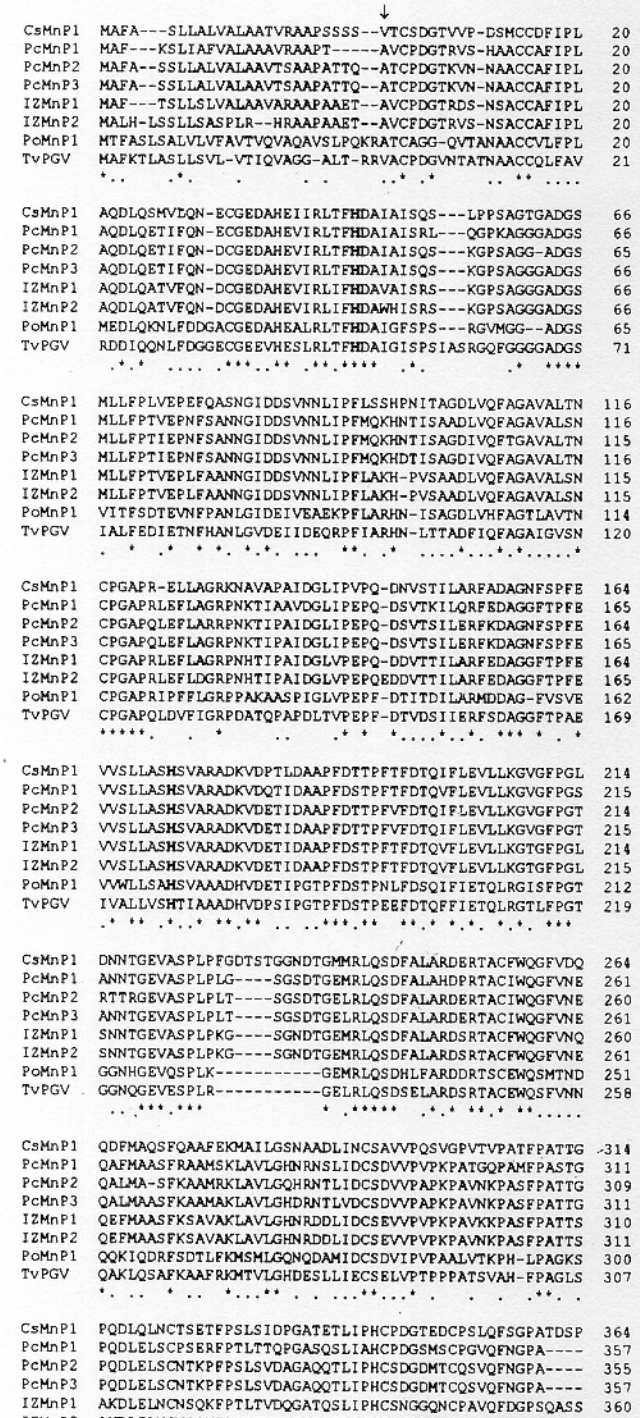


Fig. 2. Alignment of the primary structures of MnP from *C. subvermispora* and MnPs from different fungi. Sequence of CsMnP1 from *C. subvermispora* was deduced from the cDNA (this work); PcMnP1 (Pribnow et al., 1989), PcMnP2 (Mayfield et al., 1994) and PcMnP3 (Alic et al., 1997) are from *P. chrysosporium*; PoMnP (Asada et al., 1995) is from *P. ostreatus* and TvMPGI (Johansson and Nyman, 1996) is from *T. versicolor*. Identical and conserved residues are shown by asterisks and dots, respectively. The arrow marks the N-terminal of the mature proteins. Distal and proximal His residues are in bold type. The multiple alignment was carried out using the CLUSTAL PCGENE program from Intelligenetics

preparation was resuspended in TE buffer. Five hundred micrograms of DNA were partially digested with *Sau*3AI, size-fractionated and cloned in *Bam* HI-linearized vector Lambda GEM-11 (Promega Biotec, Madison, WI) following the supplier's instructions. In-vitro packaging was as described above for the cDNA library. Propagation and amplification of the genomic library were performed by infection of *E. coli* LE392. Plaque lifts were probed with *C. subvermispora* MnP13-1 cDNA under high stringency conditions. The sequencing of both strands of the *Sac* I fragment (see text) was as indicated previously for the cDNA.

2.3. Amplification of regulatory sequences by inverse PCR

Genomic DNA (4 μg) from *C. subvermispora* was digested with *Pst*I (30 U) for 3 h at 37°C in a final reaction volume of 40 μl. The enzyme was inactivated by heating at 65°C for 15 min. DNA was then extracted with one volume of phenol:chloroform:isoamyl alcohol (25:24:1), one volume of chloroform:isoamyl alcohol (24:1) and finally precipitated with ethanol. The final reaction product was resuspended in water to a final concentration of 40 ng/ml. For ligation, 30 μl of digested DNA (1.2 μg) were incubated at 12°C for 16 h with 1 U of T4 DNA ligase in a final volume of 50 μl. PCR reactions were carried out using *Pfu* DNA polymerase (Stratagene, La Jolla, CA) using 5 μl of the ligation reaction (120 ng of DNA) in a final volume of 50 μl. PCR products were cloned into *Eco*RV-linearized Bluescript KSII vector. The authenticity of the product was confirmed by Southern blotting and DNA sequencing.

3. Results and discussion

3.1. Cloning and sequencing of cDNA MnP13-1

A cDNA library was constructed in Lambda ZIPLOX using RNA isolated from *C. subvermispora* grown for 10 days in liquid cultures of defined composition (Rüttimann et al., 1992). The cDNA MnP13-1 clone was isolated by probing the library with a cDNA corresponding to MnP isoenzyme H3 from *P. chrysosporium* (Orth et al., 1994) as a probe. Subcloning and sequencing of the fragments yielded the complete sequence of this cDNA (Genbank accession no. U60413). Its characteristics are implicit in Fig. 1, which depicts the sequence of the corresponding MnP gene (see later). The cDNA clone consists of 1285 bp, excluding the poly(A) tail, and possesses 51 bp in the 5′ non-coding region. The nucleotide sequence flanking the first ATG codon, GCAATGG, follows the proposed eukaryotic initiation sequence of (A/G)NNATGG (Kozak,

1981). Translation starting at this ATG codon predicts a preprotein of 388 amino acids that contains, based on previously determined N-terminal sequences of several MnPs (Lobos et al., 1994), a leader sequence of 24 amino acids and a deduced molecular mass of 40.4 kDa. The leader sequence is predominantly hydrophobic, ending in four serine residues. It differs from leader sequences described in MnPs from other white-rot fungi, which, in general, exhibit a highly variable C-terminal region (Tien and Tu, 1987; Pribnow et al., 1989; Jönsson and Nyman, 1994; Orth et al., 1994; Asada et al., 1995; Matsubara et al., 1996). Variability at the cleavage sites is not surprising, since it has been reported that leader sequences and cleavage sites are highly variable among prokaryotes and eukaryotes (von Heijne, 1985). Cleavage of this leader peptide yields a mature protein of 38.1 kDa, which is 58% of the experimentally determined molecular mass for the isoenzymes of pI between 3.2 and 3.53 (65 kDa) (Lobos et al., 1994). This difference could be assigned to glycosylation of the protein (Farrel et al., 1989). Like other MnP proteins (Tien and Tu, 1987; Pribnow et al., 1989; Orth et al., 1994), the MnP13-1 deduced amino-acid sequence contains six potential sites for *N*-glycosylation with the general sequence N-Xaa-T/S. Moreover, 16% of the total amino acids correspond to either T or S, which constitute potential sites for O-glycosilation. Surprisingly, although the RNA had been prepared from *C. subvermispora* grown in liquid cultures, the N-terminal amino-acid sequence deduced from the MnP13-1 cDNA clone is more similar to the MnPs isolated from cultures on wood (Lobos et al., 1994).

As with MnPs from other basidiomycetes, the presence of the consensus polyadenylation site (AATAAA) (Proudfoot and Brownlee, 1976) was not apparent in this clone. Other groups have shown that polyadenylation signals in filamentous fungi and yeast do not always match the canonical sequence exactly (Ballance, 1986; Peterson and Myers, 1993; Heidmann et al., 1994). However, the sequence TATATA located 68 bp upstream of the poly(A) tail (Fig. 1) is similar to the so-called upstream element in *S. cerevisiae* (Russo et al., 1991; Heidmann et al., 1994), and therefore, it might constitute a putative polyadenylation site.

3.2. *Characterization of deduced protein and alignments. Amino-acid residues involved in catalysis*

A comparison of the amino-acid sequence of MnP13-1 with MnPs from other basidiomycetes revealed a high degree of identity (Fig. 2), the main differences arising from the presence of T instead of a highly conserved G at residue 61 and an insertion of four aa residues after G^{233}. The amino-acid composition of MnP13-1 exhibits a high content of D+E relative to K+R, which is consistent with the acidic pI determined for the MnPs secreted by the fungus when growing on pine wood chips (Lobos et al., 1994). It shares with other MnPs the manganese binding sites E^{35} and E^{39} (Sundaramoorty et al., 1994), and, as expected for a peroxidase, the distal histidine H^{46}, proximal histidine H^{172} and the distal arginine R^{42} are all conserved (Fig. 3A). Interestingly, a four-amino-acid motif, L/V/I–P–Xaa–P, assigned as an aromatic binding site (Veitch and Williams, 1990), also shares an identity with other peroxidases, although it is less hydrophylic due to the presence of V^{143} and P^{144} (Fig. 3B). Should this feature be shared with other MnP isoenzymes from *C. subvermispora*, it may provide an explanation for their ability to oxidize aromatic compounds in the absence of Mn(II) (Urzúa et al., 1995).

3.3. *Isolation and sequencing of* Cs-mnp1 *gene*

A genomic library of *C. subvermispora* was prepared in Lambda GEM-11 and screened using cDNA MnP13-1 as a probe under high stringency conditions. Characterization by Southern blotting of one genomic clone yielded a *Sac*I fragment of 2.5 kb. This fragment contained the entire open reading frame of the *Cs-mnp1* gene plus 162-bp in the 5′ non-coding region and 770-bp of 3′ non-coding sequence. The sequence of *Cs-mnp1* is shown in Fig. 1.

Coding region and flanking untranslated regions of the genomic clone are identical to cDNA MnP13-1 (Fig. 1). Alignment of the cDNA and *Cs-mnp1* allowed the localization of seven short intervening sequences with sizes ranging between 52 and 60 bp (Fig. 4). The final intron splits a codon for proline, as it occurs in genes *mnp1* and *mnp2* of *P. chrysosporium* (Godfrey et al., 1990; Mayfield et al., 1994). Sequences at the intron splicing junctions adhere to the GT–AG rule. In turn, three putative internal lariat formation sites match the consensus sequence CTRAY (Padgett et al., 1989), whereas other three differ from it in only one nucleotide. Comparison of the structure of the *Cs-mnp1* gene of *C. subvermispora* and five *mnp* genes from other basidiomycetes reveals an almost perfect alignment between *Cs-mnp1* and *mnp2* of *P. chrysosporium* (Mayfield et al., 1994) (Fig. 4). Both genes have an additional intron splitting exon 3 of *mnp1* and *mnp3* of *P. chrysosporium* at the codon for the distal histidine H^{46} (Gold and Alic, 1993).

3.4. *Regulatory sequences of* Cs-mnp1 *gene*

To examine further the regulatory sequences of the *Cs-mnp1* gene, the 5′ and 3′ non-coding flanking regions were isolated by inverse PCR (Triglia et al., 1988). For this purpose, two primers were designed, based on the

A

Peroxidase	aa	distal His	proximal His	aa
Turnip	35	SILRLFFHDCF	----RDMVALSGAHTIG	177
HRP	35	SILRLHTHDCF	----SDLVALSGGHTFG	173
CCP	45	VLVRLAWHTSG	----REVVALMGAHALG	177
PcLiP(H8)	40	ESIRLVRHDSI	----LELVWMLSAHSVA	179
TVLiP1	48	ESLRLTFHDAI	----ILTVWLLTAHTVA	183
PcMnP(H3)	37	EVIRLQFHDAI	----FEVVSLLASHSVA	175
IZMnP1	39	EVIRLIFHDAV	----FEVVSLLASHSVA	175
PoMnP	41	EALRLTFHDAI	----VEVVWLLSAHSVA	163
TvPGV	41	ESLRLTFHDAI	----AEIVALLVSHTIA	169
CsMnP1	39	EIIRLTFHDAI	----FEVVSLLASHSVA	175

B

Peroxidase								
TOPA		I	P	S	P	F	E	
HRP		L	P	A	P	F	F	
PcMnP1	141	I	P	E	P	Q	D	146
IZMnP1	140	V	P	E	P	Q	D	145
PoMnP	141	V	P	E	P	F	D	146
TvPGV	146	V	P	E	P	F	D	152
PcLiP(H8)		V	P	E	P	F	H	
CiP		I	P	G	P	G	N	
CsMnP1	140	I	P	V	P	Q	D	145

Fig. 3. Comparison of CsMnP with other peroxidases at regions near the proximal and distal His (**A**) and the aromatic binding site (**B**). The aa are aligned assuming a similar location of the His residues. Identical amino acids are in bold type. The amino-acid sequences are from turnip and horseradish peroxidase (HRP) (Welinder and Mazza, 1977), cytochrome-c peroxidase (CCP; Kaput et al., 1982), *P. chrysosporium* LiP isoenzyme H8 (Tien and Tu, 1987), *T. versicolor* LiP (Black and Reddy, 1991), *P. chrysosporium* MnP isoenzyme H3 (Orth et al., 1994); fungus IZU-154 MnP isoenzyme 1 (Matsubara et al., 1996), *P. ostreatus* MnP (Asada et al., 1995); *T. versicolor* MnP (Johansson and Nyman, 1996); TOPA (Diaz-De-Leon et al., 1993); CCP (Kaput et al., 1982) and MnP13-1 (this work).

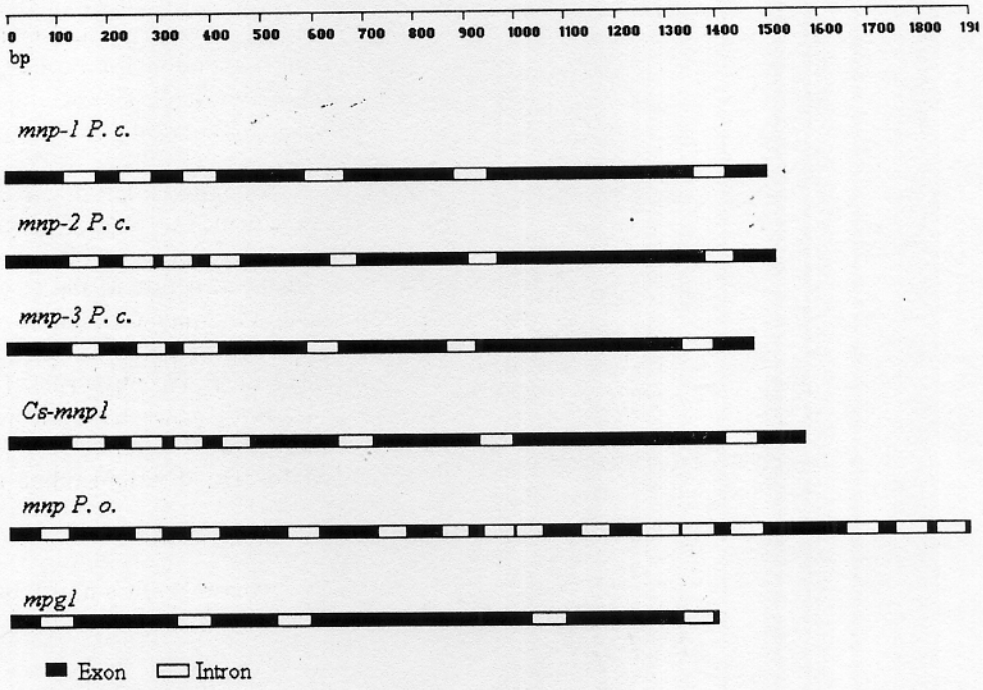


Fig. 4. Intron/exon structure of MnP genes *mnp1*, *mnp2* and *mnp3* from *P. chrysosporium*, *Cs-mnp1* from *C.subvermispora*, *mnp* from *P. ostreatus* and *mpg1* from *T. versicolor*. The exons are indicated by solid black lines, whereas the open boxes correspond to the introns.

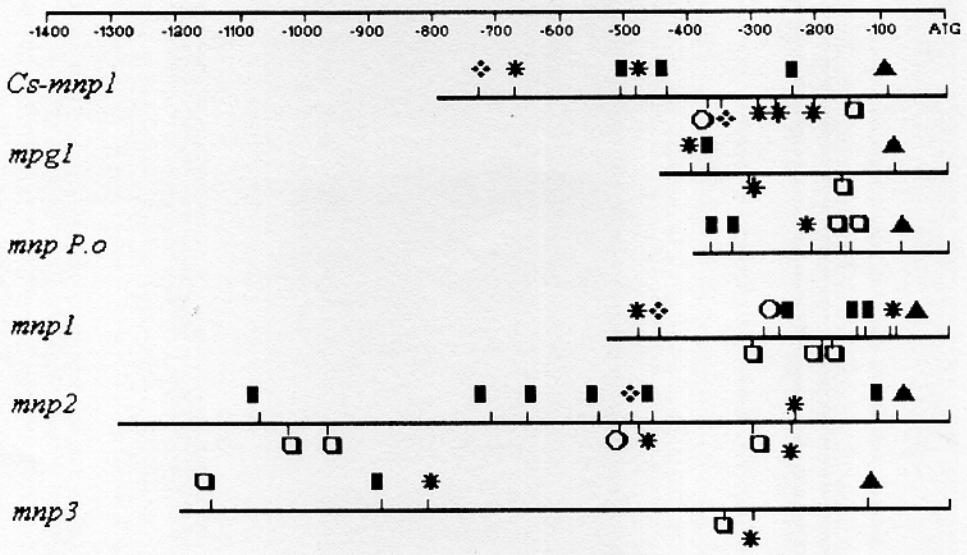


Fig. 5. Comparison of promoter elements of MnP genes *mnp1*, *mnp2* and *mnp3* from *P. chrysosporium*, *Cs-mnp1* from *C. subvermispora*, *mnp* from *P. ostreatus* and *mpg1* from *T. versicolor*. Several putatative elements as TATA box (▲), CCAAT box (□), MRE (*), HSE (■), Sp1 (❖) and AP-2 (○) recognition sites are indicated. Besides the TATA box, no relevant site conservation is observed for these elements among the various genes.

sequence of the cDNA MnP13-1. These were primer I113 (5′..ACGACAGTACCGTCTGAGCAAGT..3′), the 3′ end of which was located 75 bp downstream from codon for the first methionine and the primer I223 (5′..GGATGTTTAGACTCTCATCGTGG..3′), with its 3′ end located 37 bp downstream of the termination codon. Since Southern blotting analysis had shown that a *Pst*I fragment of 3.8 kb contains the entire *Cs-mnp1* gene, genomic DNA was digested with this enzyme and the fragments obtained were recircularized and PCR-amplified with the primers I113 and I223. A product of 1900 bp was cloned in Bluescript KSII vector linearized with *Eco*RV. The sequence of this DNA product showed that it contained approximately 1700 bp of the upstream regulatory region of *Cs-mnp1* and only 208 bp of the 3′ non-coding end. The *Pst*I site corresponding to the point of circularization of the DNA fragments was found to be located 100 bp downstream of the polyadenylation site (Fig. 1).

Sequencing of 789 bp in the 5′ flanking region of *Cs-mnp1* showed a TATAA element 91 bp upstream of the ATG codon (Fig. 1). In addition, this segment includes an inverted CCAAT element (ATTGG) (Dynan and Tjian, 1985) at position −150 and two CAAT boxes (Ballance, 1986) at −315 and −116, the latter in an inverted fashion. In turn, SP-1 transcription factor recognition sites (GGGCGG) (Dynan and Tjian, 1985) were found at −347 (inverted) and −727, whereas a AP-2 transcription binding site (CCCATCAC) (Faisst and Meyer, 1992) occurs at position −367. A putative heat-shock element (HSE) at −238 matches the consensus sequence 5′-NGAANNTTCN-3′ (Bonner et al., 1994) in nine out of ten positions, whereas two other putative HSE elements at positions −431 and −502 match the consensus 5′-NTTCNNGAAN-3′ (Bonner et al., 1994). Additional studies of the 5′ region of the *Cs-mnp1* gene revealed the presence of several metal response element (MRE) (consensus sequence TGCRCNC, Thiele, 1992). Two direct MRE sequences matching six out of seven positions are located at −479 and −671, and two inverted MRE sequences are found at positions −205 and −264. Another inverted MRE element differing in only one nucleotide from the consensus sequence is located at position −286.

Although the regulatory elements of *Cs-mnp1* are similar to those described for *mnp* genes in other fungi, they differ from them in number, position and orientation (Fig. 5). The significance of multiple putative HSE and MRE boxes present in *mnp* genes is still unknown, and sequences required for binding of the heat shock transcription factor in filamentous fungi remain to be defined. Transcription factors responding to the concentration of manganese, the connection of this metal with the MRE and examination of possible relationships between regulation by this metal and heat shock constitute matters of future studies. In *C. subvermispora* cultures, MnP titres respond to MnII concentrations (Rüttimann et al., 1992), although the effects of heat shock on MnP levels are unknown.

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