REGULATION OF MANGANESE PEROXIDASE GENE EXPRESSION IN THE BASIDIOMYCETE *PHANEROCHAETE CHRYSOSPORIUM*

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ABSTRACT

The Regulation of Manganese Peroxidase Gene Expression
in the Basidiomycete *Phanerochaete chrysosporium*

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The expression of manganese peroxidase (MnP) in nitrogen-limited cultures of *Phanerochaete chrysosporium* is regulated by Mn ion, heat shock (HS), and H_2O_2 at the level of gene transcription. We have constructed a homologous gene reporter system to further examine the regulation of two *mnp* genes, *mnp1* and *mnp2*, encoding individual MnP isozymes. Internal deletions of 234 and 359 bp were made within the coding regions of the *mnp1* and *mnp2* genes, respectively. The truncated *mnp* genes were subcloned into the shuttle vector pOGI18, which includes the *Schizophyllum commune ade5* gene as a selectable marker and transformed into a *P. chrysosporium* Ade 1 auxotrophic mutant. Northern blot analysis of purified Ade$^+$ transformants demonstrated that both of the truncated *mnp* genes were regulated in a manner similar to the endogenous *mnp* genes with respect to nitrogen limitation and induction by Mn, HS, and H_2O_2.

Reverse transcriptase polymerase chain reaction (RT-PCR) has allowed detailed analysis of differential expression between and within families of genes involved in lignocellulose degradation. RT-PCR was used here to further our investigation of *mnp* gene expression in a more detailed manner than previous northern blot analysis. Competitive RT-PCR allowed the comparison of *mnp1*, *mnp2*, and *mnp3* expression patterns. We demonstrated that there is a basal level of *mnp* when cells are grown in nitrogen sufficient cultures, as well as nitrogen limited cultures, in the absence of Mn. Furthermore, we present evidence for differential

expression within the *mnp* gene family under limiting nitrogen in the presence of Mn. Additionally we show that RT-PCR can be used to quantitate the expression of M2ClaI, a truncated reporter gene previously used to examine the regulation of *mnp2*.

CHAPTER 1
INTRODUCTION

Lignin, cellulose, and hemicellulose are the essential components of plant cell walls, occurring not only in wood but in all true vascular plants, ferns, and club mosses. After cellulose, lignin is the second most abundant renewable carbon-containing substance on Earth. It constitutes 15–30% of the plant cell wall, forming a matrix that surrounds cellulose (Kirk & Farrell, 1987). Lignin is a naturally occurring recalcitrant compound, unable to be degraded by most organisms. However, mineralization of lignin is important both environmentally and economically. By facilitating the utilization of cellulose, lignin degradation becomes an essential biochemical step in the recycling of carbon (Crawford, 1981). In addition, the presence of lignin in wood and woody plants prevents the efficient utilization of cellulose as a source of fuel, food, and fiber (Crawford, 1981; Eriksson, 1990).

The basidiomycetous fungi causing white-rot decay in wood are the only known organisms which are capable of degrading lignin extensively to carbon dioxide and water in pure culture (Kirk & Farrell, 1987; Gold et al., 1989). For the past twenty years, research centered on the biochemistry, molecular biology and the genetics of the lignin-degrading fungus *Phanerochaete chrysosporium* (Buswell & Odier, 1987; Kirk & Farrell, 1987; Gold et al., 1989; Gold & Alic, 1993; Broda et al., 1996) has made it the best studied and understood of lignin-degrading organisms. During secondary metabolism, the onset of which is triggered by limiting nutrients such as nitrogen, *P. chrysosporium* secretes two families of unique heme peroxidases, manganese peroxidase (MnP) and lignin peroxidase (LiP), and a H_2O_2-generating system which are the major components of its extracellular lignin degradative system

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(Kirk & Farrell, 1987; Gold & Alic, 1993). Both LiP and MnP are glycoproteins of $M_r \sim 40,000-46,000$, containing one mole of heme per mole of enzyme.

The oxidative reaction mechanisms for LiP and MnP differ. LiP catalyses the one electron oxidation of aromatic rings of lignin and lignin model compounds to generate substrate aryl cation radicals (Renganathan et al., 1986; Kirk & Farrell, 1987; Marquez et al., 1988; Schoemaker, 1990). MnP oxidizes Mn^{2+} to Mn^{3+}. Chelated with organic acids such as oxalate, Mn^{3+} can diffuse from the enzyme surface and oxidize the insoluble terminal substrate, lignin (Glenn & Gold, 1985; Glenn et al., 1986; Wariishi et al., 1988, 1992; Gold et al., 1989). The cation radicals generated by LiP and the Mn^{3+} from MnP are considered to be effective in the oxidation of lignin, resulting in depolymerization.

LiP and MnP both occur as series of isozymes encoded by several genes. At least six LiPs (H1, H2, H6, H8, H9, H10) and four MnPs (H3, H4, H5, H7) can be resolved from *P. chrysosporium* strain BKM-F-1767 by high performance liquid chromatography (Broda et al., 1996). The sequences of *lip* cDNAs and genomic clones for isozymes H2, H8, H6, H10 have been determined (Gold & Alic, 1993). The sequences of *mnp* cDNAs (Pease et al., 1989; Pribnow et al., 1989; Mayfield et al., 1994a; Orth et al., 1994) and genomic clones (*mnp1*, *mnp2*, and *mnp3*) encoding alleles of three MnP isozymes from *P. chrysosporium* have been reported (Godfrey et al., 1990; Mayfield et al., 1994a; Alic et al., 1997).

1.1   Metal Ions and Their Regulation of Gene Expression

1.1.1 Metal ions in biological systems

Metal ion homeostasis is essential in living cells, and many cellular proteins require metal ions such as Cu, Zn, and Fe as cofactors and as structural components. However, some of these same metals can be toxic at higher concentrations. For example, low concentrations of Fe can result in the loss of biological function of many DNA-binding proteins. High concentrations of Fe can cause cell damage by generating free radicals from metabolites such as H_2O_2 and O_2 via Fenton reactions. Heavy metals (Cd, Pb, Hg) are important environmental pollutants, owing to their

toxicity, prevalence, and persistence. A high-affinity metal such as Pd^{2+} has a very similar electronic configuration to a low-affinity metal such as Ca^{2+}, and, as a result, Pb^{2+} can replace Ca^{2+} as a biological ligand. Neurotoxicity can be caused by the activation of protein kinase C by low concentrations of Pb^{2+} that substitute for Ca^{2+} in second messenger metabolism (Goldstein, 1993). Because metal ions can be both essential as well as toxic to living systems, elucidation of their cellular roles and regulation of metal homeostasis is an important area of research. Such research has shown that metal ions have a direct role in the regulation of genes that are responsible for their uptake, homeostasis, and detoxification (O'Halloran, 1989; Ralston & O'Halloran, 1990; Thiele, 1992; Zhu & Thiele, 1996).

1.1.2 Metal ion regulation of gene expression

Metal-regulated gene transcription is well documented (O'Halloran, 1989, 1993; Kapoor et al., 1990; Neilands, 1990; Ralston & O'Halloran, 1990; Thiele, 1992; Zhu & Thiele, 1996). The interdisciplinary study of metal ions in biological systems has resulted in dramatic strides toward understanding the interactions of metal ions with nucleic acid and proteins. Iron, mercury, and copper regulating systems illustrate three different mechanisms for metal regulation of gene transcription. The ferric uptake regulation (Fur) protein acts as an Fe-responsive repressor in the prokaryote *Escherichia coli* (O'Halloran, 1989, 1993). The transcriptional regulator, Mer, is responsible for mercury detoxification in bacterial systems, playing a role as activator and repressor of essential structural genes (O'Halloran, 1989, 1993). In eukaryotes, metallothionein (MT) biosynthesis is controlled by a soluble protein which, after binding Cu or Zn, activates gene transcription by binding to *cis*-acting sequences in the promoter of the MT gene (Thiele, 1992; Zhu & Thiele, 1996). In yeast, the product of the MT gene (CUP1) is responsible for Cu homeostasis (Thiele, 1992). Mouse MTI and MTII were initially characterized for their ability to sequester and protect cells against heavy metals. The functional roles for MT of higher eukaryotes are now known to include zinc homeostasis and protection from oxidative stress (Thiele, 1992; Tamai et al., 1993; Dalton et al., 1994, 1996).

1.1.3 Metal-regulated transcription in eukaryotes

All higher eukaryotic MT promoters from *Drosophila* to humans contain multiple copies of a semiconserved sequence that is responsible for induction by metals, referred to as a metal-responsive element (MRE) (Imbert et al., 1990). Using site-directed mutagenesis experiments, heterologous promoters, and sequence comparisons, the MREs were defined as a 12–15-base pair (bp) sequence consisting of a highly conserved core, TGC(A/G)CNC, having less conserved flanking nucleotides (Culotta and Hamer, 1989; Imbert et al., 1990; Thiele, 1992).

It is known that the sensor and the MRE-binding protein are one and the same in both baker's yeast, *Saccharomyces cerevisiae*, and pathogenic yeast, *Candida glabrata* (Thiele, 1992; Zhu & Thiele, 1996). In mammals, the precise mechanisms by which metalloregulation occurs via MREs are complex and poorly understood. Unlike the yeast MT systems which respond only to Cu and Ag, the mammalian metallothioneins are expressed in response to a wide variety of metals such as Zn, Cd, Cu and Pb. Detailed biochemical experiments including *in vivo* transcriptional experiments and footprinting indicate that a metal responsive factor binds to the MT promoter (Zhu & Thiele, 1996). Similar to the model for *S. cerevisiae*, there is evidence that the transcription factors involved in this process are pre-existing, because transcription in mammalian cells occurs in the presence of protein synthesis inhibitors (Karin et al., 1980). There is recent evidence that the mouse MT-1 transcription factor may be sequestered by the MTF-1 inhibitor in the absence of metal (Palmiter, 1994).

Several *trans*-acting metal transcription factors (MTFs) which bind to repeated MREs have been cloned and characterized (Radtke et al., 1993; Brugnera et al., 1994). The MTF-1 isolated from mouse is the most thoroughly understood in terms of its structure and mechanism of action. Consistent with MTF-1 functioning as a transcription factor, it contains TF-IIIA-like Zn fingers (Cys2–His2), an acidic region, a proline-rich region, and a serine–threonine-rich region (Radtke et al., 1993). Similar to the studies in yeast, the transfection of MTF –/– mouse stem cells with MTF cDNA clearly demonstrates that MTF-1 is required for the metal activation of mammalian MT genes (Zhu & Thiele, 1996) in a dose-dependent manner.

1.1.4 Manganese

Manganese, like iron, copper and zinc, is an essential trace element for cells and is a required cofactor for a variety of redox and non-redox enzymes. Several energy-dependent Mn^{2+}-specific transport systems have been demonstrated in bacteria and eukayotes such as filamentous fungi and yeast (Parkin & Ross, 1985; Hockertz et al., 1987; Gadd & Laurence, 1996). Mn is required for the synthesis of several secondary metabolites (Weinberg, 1982; Scott et al., 1986), and there are reports of Mn-inducible enzymes (Brown et al., 1990; Kapoor et al., 1990; Périé & Gold, 1991). Moreover, like Fe and Ca, evidence for Mn^{3+} as an important regulatory switch has been obtained both in fungi (Williams, 1982; Garraway & Evans, 1984) and in bacteria (Auling, 1983; Archibald & Duong, 1986).

1.2   The Regulation of Gene Expression by Heat Shock and Oxidative Stress

1.2.1 Stress response in biological systems

When living systems are exposed to adverse environmental conditions, they display a rapid molecular response to maintain cellular homeostasis. This stress response can be a result of temperature shifts, exposure to toxic metals, metabolic inhibitors, and chemicals that produce oxidative stress such as menadione, hydrogen peroxide (H_2O_2), and tert-butylhydroquinone (tBHQ). The primary research focus has been on molecular events occurring upon a shift in temperature: the heat shock (HS) response.

HS elicits the synthesis of proteins termed heat shock protein (HSPs). HSPs are ubiquitous. Several HSP families can be distinguished and are grouped based on their average molecular weights. Other proteins. such as mouse heme oxygenase-1 (HO-1) (Alam & Smith, 1994), mouse MT (Dalton et al., 1996), *Neurospora crassa* peroxidase (Kapoor et al., 1990), *S. cerevisiae* ubiquitin (Mager & De Kruijff, 1995) and *P. chrysosporium* MnP (Gold & Alic, 1993), are also strongly enhanced upon exposure of cells to stress. These proteins are also considered HSPs and sometimes referred to as stress proteins (Patrusky, 1990). Many of the proteins termed HSPs

are expressed under non-stress growth conditions, indicating that HSPs also play roles in normal cellular processes.

1.2.2 HS and oxidative stress regulation of gene expression

Eukaryotic HSPs and HS-responsive proteins are regulated at the level of gene transcription by *trans*-acting heat shock factors (HSFs) which bind to repeated heat shock elements (HSEs) in the promoter regions of HS genes (Lindquist, 1986; Morimoto, 1993). Several HSFs have been cloned and characterized (Sorger & Pelham, 1988; Wiederrecht et al., 1988; Clos et al., 1990; Schuetz et al., 1991; Gallo et al., 1993; Sheldon & Kingston, 1993). Work on the *Drosophila HSP70* gene identified a 14-bp consensus sequence (5'CnnGAAnnTTCnnG3') (Pelham, 1982). More recently a revision of the HSE was proposed based on sequence comparisons of genetically defined HS regulatory regions of *D. melanogaster* and site-directed mutagenesis experiments (Xiao et al., 1991). The identified DNA sequences are composed of contiguous arrays of inverted 5-bp units, nGAAn.

1.3 Regulation of *mnp* Gene Transcription by Mn, HS, and Oxidative Stress

1.3.1 MnP gene sequences

MnP occurs as a series of isozymes encoded by a family of genes and the sequences of cDNA (Pease et al., 1989; Pribnow et al., 1989; Mayfield et al., 1994a; Orth et al., 1994), and the genomic clones (Godfrey et al., 1990; Mayfield et al., 1994a; Alic et al., 1997) encoding alleles of three MnP isozymes from *P. chrysosporium* have been determined. The structures of the proteins are very similar with respect to the size of the signal peptide, the size of the mature protein, and the amino acids involved in peroxidase function. Those forming the Mn-binding site and those amino acids involved in disulfide bond formation are conserved in the *mnp* sequences (Gold & Alic, 1993; Alic et al., 1997). The coding regions of the *mnp* genes exhibit at least 66% identity at the nucleotide level and at least 85% identity at the amino acid level.

The structures of the genes encoding the three MnPs is also similar (Fig. 1.1). The *mnp2* gene has seven introns, whereas the *mnp1* and *mnp3* genes have six introns. The positions of five of the introns are common to all three *mnp* genes. It has been suggested that *P. chrysosporium mnp* genes can be divided into three subfamilies on the basis of intron–exon structure (Alic et al., 1997). The promoter regions of the *mnp1*, *mnp2*, and *mnp3* genes contain a TATAAA element and CCAAT elements (i.e., ATTGG). *mnp1* and *mnp2* contain a putative SP-1 site (GGGCGG). All three genes contain multiple putative MREs, identical to the consensus MREs (TGC(A/G)CNC) found in mouse and other metallothionein genes (Imbert et al., 1990). Additionally, the promoter regions of the *mnp* genes contain multiple HSEs similar to NTTCNNGAAN (Gold & Alic, 1993). The HSEs match the consensus in six or seven of the eight positions. Little is known about whether these putative regulatory domains possess a physiological function.

1.3.2 Mn regulation

The accumulation of *mnp* transcript and protein are dependent on the presence of manganese (Mn) (Bonnarme & Jeffries, 1990; Brown et al., 1990, 1991; Mayfield et al., 1994a). This Mn-dependent induction of *mnp* occurs only under conditions of nitrogen starvation, and Mn is the only metal which has been found to mediate this effect (Brown et al., 1991). Immunoblots confirm that MnP protein is expressed only in the presence of Mn, indicating that Mn is required for MnP synthesis (Brown et al., 1990). In addition, *mnp* mRNA is detectable on northern blots of RNA isolated from nitrogen-limited cultures grown in the presence but not in the absence of Mn (Brown et al., 1991). *mnp* transcript can be induced by the addition of Mn to the five-day-old Mn-deficient cultures within 40 min. The amount of mRNA obtained is a function of the amount of Mn added, reaching a maximum at 180 μM in the final concentration. In contrast to its effect on *mnp* gene transcription, Mn has no significant effect on growth as indicated by the mycelial dry weight and on rates of nitrogen consumption (Brown et al., 1990). The RNA synthesis inhibitor actinomycin D prevents the accumulation of *mnp* mRNA (Brown et al., 1991), which is additional
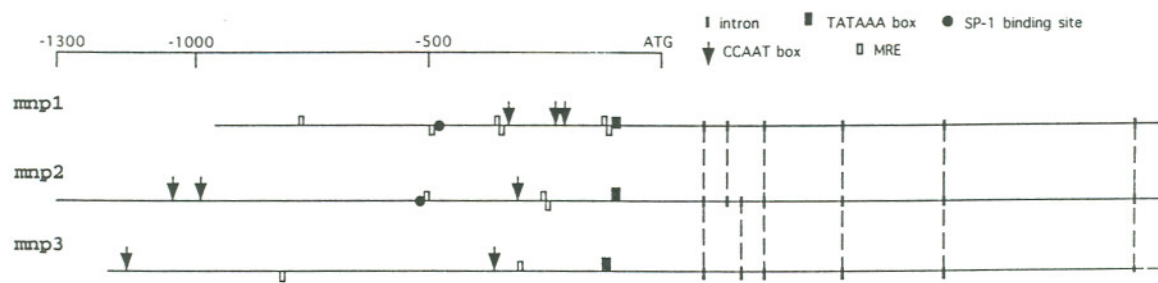
Fig. 1.1 Comparison of promoter elements and intron positions in three *P. chrysosporium mnp* genes (Alic et al., 1997).

evidence that Mn acts at the level of gene transcription. Also, the *mnp1*-ODase reporter, which is a fusion of the promoter region of the *mnp1* gene with the coding sequence for the *S. commune ura1* gene, is inducible by Mn (Godfrey et al., 1994), indicating that the control mechanism is contained within the *mnp1* promoter region.

As mentioned above, several regions of the *mnp* promoters contain sequences identical to the metallothionein MRE consensus sequence (Brown et al., 1991; Gold & Alic, 1993; Alic et al., 1997). In the yeast metallothionein system, an intracellular metalloregulatory protein functions as both a metal receptor and a *trans*-acting transcription factor, binding to the *cis*-acting MREs to increase gene expression dramatically (Thiele, 1992). Since metallothionein genes are known not to be inducible by Mn, *mnp* could represent a related yet distinct metal-responsive system. To date, this is the only Mn regulation of gene transcription to be studied at the molecular level, and it cannot be assumed that conservation of the nucleotide sequence means conservation of function. In fact, although the mouse heme oxygenase-1 (HO-1) gene is activated by heavy metals, deletion analysis of the putative MRE within the HO-1 promoter indicates that this sequence is not responsive to Cd^{2+} or Zn^{2+} (Alam & Smith, 1994).

The conserved minimal MRE sequence is small and thus would be expected to occur frequently in the genome just by chance, as in the case of HO-1. The fact that the three *mnp* promoters contain multiple copies of the MRE leads to the speculation that the conservation here is significant. The theme of multiple copies of relatively small functional sequences appears to be common among eukaryotic promoters and enhancers (Stuart et al., 1985). As shown in Fig. 1.1, the positions and numbers of the MREs within the *mnp* promoters are different. The MREs also differ in orientation and specific sequence. The two proximal pairs of MREs in the *mnp1* gene promoter overlap to form a 4-bp palindrome (TGCA). The proximal MRE pair in the *mnp2* gene contains a 6-bp palindrome (TGCGCA). The MREs of mammalian MT genes are often present in two clusters, wherein each cluster contains several tandem duplications of the consensus sequence (Stuart et al., 1985). The fact that two pairs of MREs in the *mnp1* promoter and one pair of MREs in the *mnp2* promoter contain

palindromes, suggests that the overlap may have physiological significance and could be analogous to the cluster arrangement. The promoters of *mnp1, mnp2,* and *mnp3* also contain single putative MREs. The transcription of *mnp* genes may be differentially regulated by Mn as a result of the location and specific sequence of Mn response elements.

1.3.3 HS and H_2O_2 regulation

HS and oxidative stress also mediate the accumulation of *mnp* transcript under conditions of nitrogen starvation. HS induction is a far more rapid response than Mn induction. *mnp* mRNA is detectable within 15 min on northern blots of RNA isolated from five-day-old nitrogen-limited Mn-deficient cultures grown at 37°C and transferred to 45°C (Brown et al., 1993). In contrast, Mn-induced mRNA is detectable only after 45 min (Brown et al., 1991). Under the same culture conditions, a range of 0.25–1.0 mM H_2O_2 will induce *mnp* transcription. In contrast to Mn-dependent induction of *mnp*, immunoblots confirm that MnP protein is not expressed in the presence of HS or H_2O_2 alone, emphasizing the requirement on Mn in MnP synthesis (Brown et al., 1991; Li et al., 1995). Additionally, if HS is conducted in the presence of Mn, higher levels of MnP are produced, suggesting that the two effects are additive and that Mn may be required for a post-transcriptional step in MnP production under HS.

In addition to thermal stress, a wide variety of chemical agents are known to induce HSPs (Morimoto, 1993; Choi & Alam, 1996) in both prokaryotes and eukaryotes. Since lignin degradation is an oxidative process during which the fungus produces H_2O_2 as part of the extracellular lignin-degrading system, it is not surprising to find induction of *mnp* gene transcription by H_2O_2. It is possible that in *P. chrysosporium* oxidative stress acts through the same mechanism as HS. However, it has recently been reported that oxidative stress activates MT through the metal-responsive transcription factor (Dalton et al., 1996), so it is possible that here oxidative response could be acting through the metal-responsive system. As mentioned above, all *mnp* genes contain putative HSEs, but, as in the case of MREs

found in heme oxygenase, sequence identity does not confirm function. Although components of HS systems in yeast and mammals have been isolated and cloned and the minimal HSE required for induction defined (Young & Craig, 1993; Kroeger & Morimoto, 1994), very little is known about such systems in filamentous fungi.

1.3.4 Reporter constructs for studying the regulation of mnp gene expression

To determine the role of these promoter sequences, a fusion of the *mnp1* promoter to the *Schizophyllum commune ura1* gene encoding orotidylate decarboxylase (Odase) was constructed. The promoter–reporter gene was subcloned into the shuttle vector pOGI18, which includes the *S. commune ade5* gene as a selectable marker, and transformed into a *P. chrysosporium* Ade1Ura3 auxotrophic mutant. ODase assays of purified Ade^+Ura^+ transformants demonstrated that ODase activity is detected only during secondary metabolic growth, and the pattern of ODase expression is similar to that of endogenous MnP expression (Godfrey et al., 1994). This suggests that 1,500-bp of the *mnp1* promoter is sufficient to regulate a heterologous gene in a manner analogous to the regulation of endogenous *mnp* genes with respect to Mn, nutrient levels, and metabolic growth phase.

1.4 RT-PCR as a Technique to Study the Influence of Nitrogen and Carbon on Differential Gene Regulation within *mnp* and *lip* Gene Families

1.4.1 Nitrogen and carbon regulation of ligninolysis

It is not surprising that the degradation of lignin by *P. chrysosporium* is expressed in response to nitrogen limitation, because the nitrogen level in wood is low (C:N ratio is 350–500:1) (Cowling & Merrill, 1966). It has been shown that, as nitrogen becomes limiting, primary exponential growth discontinues, and this is why lignin degradation is considered to be a secondary metabolic event (Jeffries et al., 1981). Lignin degradation also occurs during depletion of carbon and sulfur (Jeffries et al., 1981). Carbon repression of lignin degradation is not related to carbon (glucose) catabolite repression, since ligninolytic activity appears at the same time in

cultures grown in either repressive (glucose) or nonrepressive (glycerol) carbon sources (Fenn & Kirk, 1981). In contrast, the addition of ammonium nitrate and many amino acids, such as glutamate, glutamine and histidine, repress lignin degradation (Fenn & Kirk, 1981; Kirk & Farrell, 1987). Nitrogen depletion has been correlated with the production of the secondary metabolite veratryl alcohol (Fenn & Kirk, 1981; Kirk & Farrell, 1987), secondary metabolic extracellular polysaccharide production (Leisola et al., 1986), and cAMP levels (MacDonald et al., 1984; Boominathan & Reddy, 1992). cAMP is known to be a catabolite regulator. For these reasons, the repression of lignin degradation is believed to occur via a nitrogen regulatory mechanism.

Lignin alone will not support growth of *P. chrysosporium* or the synthesis of the ligninolytic system (Kirk & Farrell, 1987). *P. chrysosporium* requires an additional carbon source in the form of hemicellulose, cellulose, or glucose. Most studies on the lignin degradative system of *P. chrysosporium* have been done under conditions of limiting nutrient nitrogen in glucose-supplemented cultures. There is evidence that nitrogen regulation may be strain dependent (Buswell & Odier, 1987) and that it may be affected by the available carbon source (Gold & Alic, 1993).

1.4.2 *RT-PCR analysis of differential gene expression within mnp and lip gene families*

Early studies on extracellular isozyme profiles (Pease et al., 1989; Pribnow et al., 1989; Boominathan et al., 1990; Tien & Myer, 1990; Datta et al., 1991; Pease & Tien, 1992), enzymes assays (Pease & Tien, 1992), and probing of transcripts with gene-specific probes (Boominathan et al., 1993) indicated that members of the MnP and LiP gene families are differentially regulated. However, the protein products are difficult and tedious to resolve and assay independently. Transcript levels that are low may not be detectable by northern blotting techniques, quantitation of probed transcript can lack sensitivity, and cross-hybridization of the probe to similar transcripts can influence the specificity of the analysis. Reverse transcriptase polymerase chain reaction (RT-PCR) has shown great promise as a method to study the differential level of gene expression within families of genes involved in

lignocellulose degradation in culture (Covert et al., 1992; Stewart et al., 1992; Brooks et al., 1993; Reiser et al., 1993; Tempelaars et al., 1994; Broda et al., 1995) and in as pollution-contaminated soil (Lamar et al., 1995; Bogan et al., 1996a,b). The advantages of using RT-PCR instead of northern blotting are increased sensitivity, decreased analysis time, and the avoidance of radioactive probes.

The quantitative RT-PCR technique being used to study *lip* and *mnp* gene expression is based on a modification of the protocol of Gilliland et al. (1990). To quantitate the initial transcript levels, several dilutions of competitive genomic templates are added to individual PCR reactions and co-amplified with target cDNA. The initial concentration of specific cDNAs is determined by estimating the dilution points at which the target cDNA and competitive template are equivalent. A cDNA with a new restriction site is ideal for the competitive template. Restriction enzyme digest following PCR will distinguish the competitive DNA from the target cDNA. However, genomic plasmid DNA can be used as a competitive template provided the PCR target spans an intron. The amplified competitive template can then be distinguished from the target cDNA by size.

RT-PCR of total RNA is particularly well suited for analysis of gene families such as *lip* and *mnp*, because a single primer can be used to synthesize the first-strand cDNA. First-strand cDNAs can then be PCR amplified with gene-specific upstream primers. The targets span at least one intron allowing the differentiation of genomic DNA and cDNA. A disadvantage in using genomic DNA is the possibility that it may not be amplified as efficiently as target cDNA, either because of size or increased melting temperature (Gilliland et al., 1990). However, among the *lip* genes and *mnp* genes, transcripts and genomic structures are highly similar with respect to overall length, intron position, intron length, and nucleotide sequence (Stewart et al., 1992; Gold & Alic, 1993; Alic et al., 1997). Thus, relative comparisons of gene expression among these genes can be considered valid when upstream primers are located approximately equidistant from the conserved downstream primer.

RT-PCR was used to determine the relative transcript levels of *lip* genes in both carbon- and nitrogen-limited cultures (Stewart et al., 1992), as well as in nitrogen-limited cultures utilizing different carbon sources (Broda et al., 1995). The

amounts of LiPA, LiPB, and 0282 gene transcripts were similar in both nitrogen- and carbon-limited culture conditions. In contrast, GLG5, V4 and GLG4 gene transcripts were dramatically altered by the nitrogen- and carbon-limited culture conditions. In low carbon, the transcript for GLG4 was ~1000-fold more abundant than under high carbon conditions. When Avicel, a model compound for cellulose, was used as the sole carbon source, LIG1, LIG2, LIG3, and LIG5 transcripts were detected. In ball-milled straw, a model compound for lignocellulose, LIG1 and LIG5 transcripts were detected. LIG5 was the only transcript detected in low glucose. Both studies confirm that within the *lip* family there is differential gene expression.

Quantitation of three *mnp* mRNAs during a 2.5-week time course of *P. chrysosporium*-colonized soil revealed coordinate regulation of the gene family (Bogan et al., 1996b). Other studies have found differences based on culturing conditions. For example, the major MnP isozymes isolated during aspen wood degradation (Datta et al., 1991) differed from those isolated from liquid cultures (Pribnow et al., 1989). Culture conditions, including the nature of both the carbon source and the nitrogen source, the ratio of C:N, solid versus liquid, agitating versus stationary, buffer, O_2 and differences in strain, are all important parameters that may explain the variations in results obtained in separate studies.

1.5   Thesis Overview

As reviewed above, the expression of manganese peroxidase (MnP) in nitrogen-limited cultures of *P. chrysosporium* is regulated by Mn ion, HS, and H_2O_2. This thesis describes two methods to explore the regulation of gene expression for individual members of the *mnp* gene family: the truncated-gene reporter system and RT-PCR. These techniques should facilitate studies on the mechanism of regulation of *mnp* gene expression.

CHAPTER 2
TRUNCATED-GENE REPORTER SYSTEM FOR STUDYING THE REGULATION OF MANGANESE PEROXIDASE EXPRESSION

2.1   Introduction

The white-rot basidiomycete fungus *Phanerochaete chrysosporium* has been studied extensively for its ability to degrade lignin (Buswell & Odier, 1987; Gold & Alic, 1993) and a wide variety of aromatic pollutants (Bumpus & Aust, 1987; Hammel et al., 1989; Valli et al., 1992; Joshi & Gold, 1993). Two families of peroxidases, lignin peroxidase (LiP) and manganese peroxidase (MnP), and an H_2O_2-generating system are the major components of this organism's extracellular lignin-degrading system (Gold et al., 1989; Gold & Alic, 1993; Joshi & Gold, 1993). MnP is a well-characterized H_2O_2-requiring heme glycoprotein of $M_r \sim 46,000$ (Glenn & Gold, 1985; Wariishi et al., 1988; Gold et al., 1989; Gold & Alic, 1993). The enzyme oxidizes Mn^{2+} to Mn^{3+}; the latter, complexed with an organic acid chelator such as oxalate which is secreted by the fungus, oxidizes the terminal phenolic substrate (Glenn & Gold, 1985; Wariishi et al., 1992; Kuan & Tien, 1993b; Kishi et al., 1994). X-Ray crystallographic (Sundaramoorthy et al., 1994) and site-directed mutagenesis (Kusters-van-Someren et al., 1995; Kishi et al., 1996) studies have defined the Mn-binding site of MnP. MnP occurs as a series of isozymes encoded by a family of genes, and the sequences of cDNA (Pease et al., 1989; Pribnow et al., 1989; Mayfield et al., 1994a; Orth et al., 1994) and genomic clones (Godfrey et al., 1990; Mayfield et al., 1994a), encoding alleles of three MnP isozymes, have been determined.

MnP expression is regulated at the level of gene transcription by the depletion of nutrient nitrogen (Pribnow et al., 1989). In addition, MnP activity is dependent on

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the presence of Mn^{2+} in the culture medium (Bonnarme & Jeffries, 1990; Brown et al., 1990) and *mnp* gene transcription is regulated by Mn^{2+} (Brown et al., 1990; Godfrey et al., 1994), as well as by heat shock (HS) (Godfrey et al., 1990; Brown et al., 1993; Gold & Alic, 1993), H_2O_2, and other chemical stresses (Li et al., 1995). To date, this is the only example of Mn regulation of gene transcription to be studied at the molecular level.

Our previous northern blot analysis of *mnp* transcription did not distinguish between individual *mnp* gene products (Brown et al., 1990, 1991, 1993; Li et al., 1995). In this report, we demonstrate that differentially truncated genes can be used to examine the regulation of closely related MnP isozymes by factors such as nutrient nitrogen, Mn, HS, and H_2O_2.

2.2   Materials and Methods

2.2.1 Organisms

P. chrysosporium wild-type strain OGC101, a derivative of BKM-F-1767, and the auxotrophic strain OGC107-1 (Ade1) were maintained as described (Gold et al., 1982). *Escherichia coli* XL1-Blue (Stratagene, La Jolla, CA) and DH5αF′ (BRL, Gaithersberg, MD) were used for subcloning plasmids.

2.2.2 Construction of pAM1Bal31

A 2.8-kb *NcoI–MluI* fragment of the *P. chrysosporium mnp1* gene, including 936 bp of sequence 5′ to the ATG translation start codon and 204 bp 3′ to the poly(A) site (Gold et al., 1982), was subcloned into *NcoI–MluI*-digested pET-11d (Novagen, Bagsvaerd, Denmark). The plasmid containing the insert was cut with *Dra*III at a unique site within the sixth *mnp1* exon (Gold et al., 1982) and used as a substrate for the exonuclease *Bal*31 (New England Biolabs, Beverly, MA). The progress of the reaction was monitored by agarose gel electrophoresis. Following religation, the truncated insert was sequenced and found to contain a 234-bp deletion encompassing nucleotides 1014–1248 of the *mnp1* gene (Fig. 2.1A). The insert was
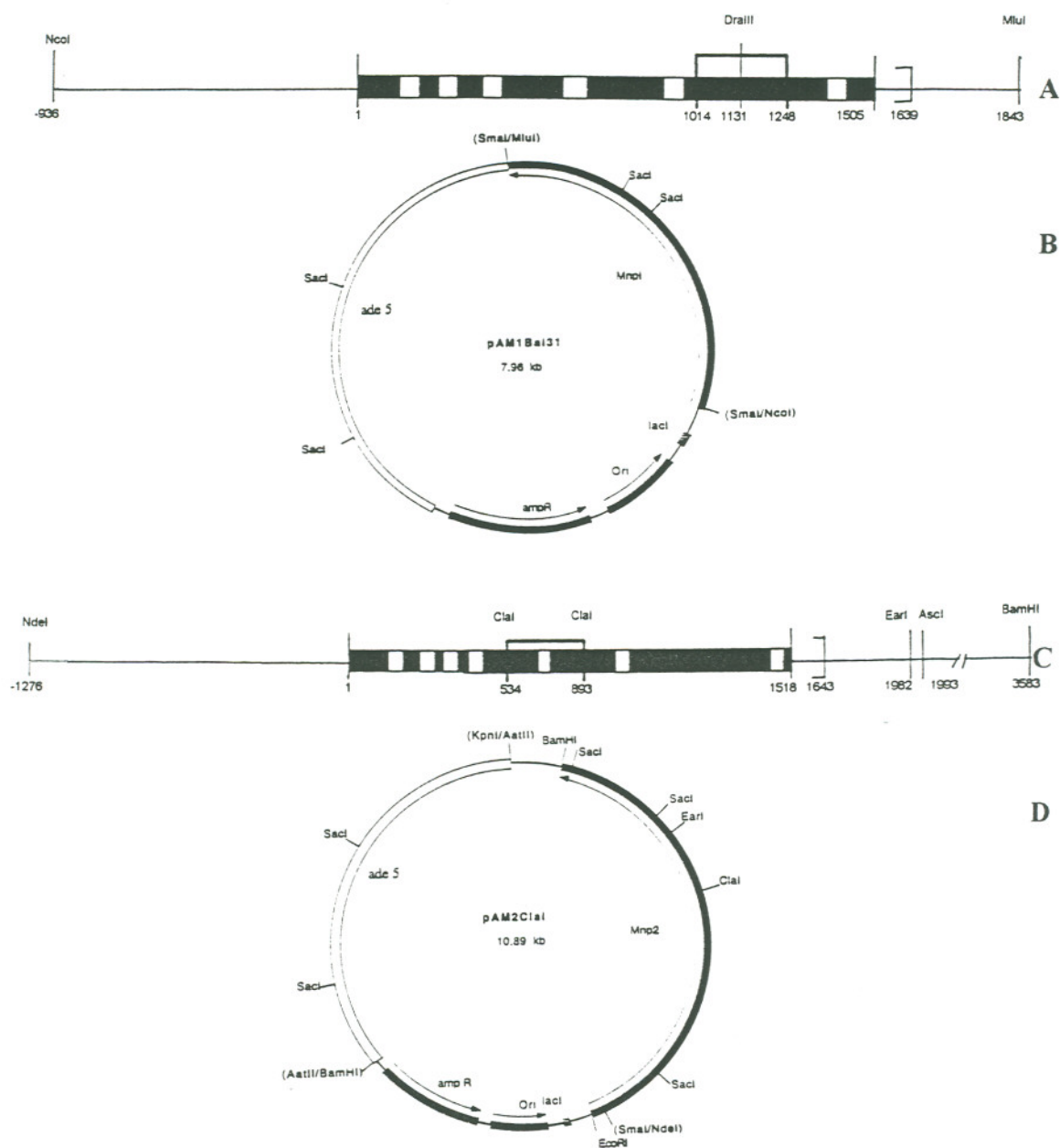
Fig. 2.1 Construction of pAM1Bal31 and pAM2ClaI. Genomic maps of *mnp1* (**A**) and *mnp2* (**C**) indicating the deleted regions: Closed boxes represent exons, open boxes represent introns. The polyadenylation sites are indicated by]. Plasmids pAM1Bal31 (**B**) and pAM2ClaI (**D**): The *S. commune ade5* gene, pUC18 sequences from pOGI18 (Godfrey et al. 1994), and the truncated *mnp* gene inserts are indicated. Restriction sites in parentheses were eliminated during subcloning.

cut out with *Nco*I and *Mlu*I, blunt-ended by a fill-in reaction with T4 polymerase, and ligated into *Sma*I-digested pOGI18 (Godfrey et al., 1994) (Fig. 2.1B). The structure of the construct pAM1Bal31 was confirmed by restriction mapping.

2.2.3 Construction of pAM2ClaI

A 3.3-kb *Nde*I–*Asc*I fragment of the *P. chrysosporium mnp2* gene, including 1.3 kb of the sequence 5' to the translation start codon and 350 bp 3' to the poly(A) site (Mayfield et al., 1994a), was blunt-ended with T4 polymerase and subcloned into *Sma*I-digested pUC18. This plasmid was digested with *Cla*I to create an internal deletion of 359 bp, encompassing nucleotides 534–893 of the *mnp2* coding region. To create pAM2ClaI, an *Eco*RI–*Ear*I fragment encompassing the truncated *mnp2* gene and an *Ear*I–*Bam*HI fragment encompassing an additional 1.6 kb downstream of the *mnp2* gene (Fig. 2.1C) were ligated in a three-way ligation to *Eco*RI–*Bam*HI-digested pOGI18 (Godfrey et al., 1994) (Fig. 2.1D). The structure of this construct was confirmed by restriction mapping.

2.2.4 Fungal transformations

Protoplasts of *P. chrysosporium* Ade1 basidiospores were transformed with *Eco*RI-linearized pAM1Bal31 (1.7 μg), pAM2ClaI (1.0 μg), or pOGI18 (1.0 μg) as described (Alic et al., 1989, 1990). Prototrophic transformants were transferred to minimal medium, screened by northern blotting for expression of the truncated genes, and purified by isolation of single basidiospores (Alic et al., 1987).

2.2.5 Culture conditions

P. chrysosporium strains were grown at 37°C from a conidial inoculum in 20-ml stationary cultures in 250-ml Erlenmeyer flasks as described (Kirk et al., 1978; Brown et al., 1991), with 2% glucose as the carbon source, 1.2 mM (limiting nitrogen) or 12 mM ammonium tartrate (sufficient nitrogen), and 20 mM sodium-2,2-dimethyl succinate (pH 4.5) as the buffer, without added Mn. Cultures were purged with 100% O_2 on day 3. After five days, cultures were induced with $MnSO_4$ (180

μM final concentration) for 2 h or with a 45°C HS or H_2O_2 (1.0 mM final concentration) for 1 h, as described (Brown et al., 1991, 1993; Li et al., 1995).

2.2.6 RNA preparation and northern blot hybridization

RNA isolation, northern blotting, and hybridization were performed as previously described (Brown et al., 1993, Li et al., 1995). Electrophoresis was conducted in a 1.5% agarose gel containing 1.6 M formaldehyde. The *mnp1* cDNA (Pribnow et al., 1989) and the *P. chrysosporium* gene encoding glyceraldehyde-3-phosphate dehydrogenase (*gpd*) (Mayfield et al., 1994b) were used for random primed synthesis of the ^{32}P-dCTP-labeled (Amersham, Arlington IL) probes.

2.2.7 Enzyme activity and protein analysis

MnP activity assays were performed as previously described (Li et al., 1995). Intracellular and extracellular protein preparations, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and western blot (immunoblot) analysis were as described (Brown et al., 1990).

2.3   Results and Discussion

2.3.1 Truncated mnp reporter genes

The lignin degradative system of *P. chrysosporium* is expressed during secondary metabolic growth, the onset of which is triggered by the depletion of nutrient nitrogen (Buswell & Odier, 1987; Kirk & Farrell, 1987; Gold & Alic, 1993). Likewise, LiP and MnP activities are detectable only during the secondary metabolic growth phase (Kirk & Farrell, 1987; Gold & Alic, 1993), and northern blot analysis has shown that the expression of *lip* and *mnp* genes is controlled at the level of transcription by nutrient nitrogen (Tien & Tu, 1987; Li et al., 1994). MnP also is regulated at the level of gene transcription by Mn^{2+}, the substrate for the enzyme (Brown et al., 1991; Godfrey et al., 1994; Li et al., 1995), and by HS (Godfrey et al., 1990; Brown et al., 1993) and oxidative stress (Li et al., 1995). Induction with Mn and HS or oxidative stress appears to be additive (Brown et al., 1993; Li et al.,

1995). Putative MREs and HSEs in the *mnp1* and *mnp2* promoters (Mayfield et al., 1994a; Li et al., 1995) may be involved in regulation by these factors. Thus, the regulation of *mnp* genes apparently involves a hierarchy of environmental signals.

Since *mnp1* and *mnp2* are closely related sequences encoding individual isozymes of MnP (Godfrey et al., 1990; Mayfield et al., 1994a), our previous studies on *mnp* gene regulation did not distinguish between the two genes (Brown et al., 1991, 1993; Li et al., 1995). The *mnp1* cDNA used as a probe for northern blot analysis, as well as the polyclonal antibody to MnP1, cross-reacts with other members of the *mnp* gene family. Therefore, we have developed a reporter gene system utilizing truncated *mnp1* and *mnp2* genes to monitor the induction of individual members of the *mnp* gene family. Because of the difficulties encountered in expressing foreign reporter genes in homobasidiomycetes, truncated constructs also were used to examine expression of the *Sc4* and *gpd* genes from *Schizophyllum commune* (Schuren et al., 1993).

Internal deletions were made within the coding regions of the *mnp1* and *mnp2* genes (Fig. 2.1). pAM1Bal31 contains a 234-bp deletion of *mnp1*, coding for a predicted mRNA of ∼ 1080 nt as compared with 1314 nt for the *mnp1* cDNA (Pribnow et al., 1989) (Fig. 2.1A). pAM1Bal31 includes 204 bp downstream of the poly(A) site. pAM2ClaI contains a 359-bp deletion of *mnp2* that encompasses a 53-bp intron and predicts an mRNA of ∼ 1002 nt as compared with 1308 nt for the *mnp2* cDNA (Mayfield et al., 1994a) (Fig. 2.1C). *mnp2* deletion plasmids were constructed with either 350 bp or 1940 bp downstream of the poly(A) site to determine whether the additional 3′ sequence affected expression of the construct. No difference was observed in expression of the two constructs (data not shown), and pAM2ClaI, containing the additional 3′ sequence, was used in all further experiments.

Seven of the 33 Ade[+] transformants obtained with pAM1Bal31 were screened by northern blot analysis for Mn induction of the truncated *mnp1* message. Four transformants expressed the truncated message under these conditions. Eight of the 30 Ade[+] transformants obtained with pAM2ClaI were screened for Mn induction of the truncated *mnp2* message. Three transformants produced the truncated message

under these conditions. These results are similar to those obtained with *mnp1* expression under control of the *P. chrysosporium gpd* promoter (Mayfield et al., 1994b) and expression of the *S. commune ura1* gene under control of the *mnp1* promoter (Godfrey et al., 1994). Since multiple copies of transforming DNA integrate ectopically in *P. chrysosporium*, expression of introduced genes is likely to be affected by the copy number and integration sites (Alic et al., 1990, 1991; Akileswaran et al., 1993). These results indicate that the deletions in the *mnp1* and *mnp2* genes do not prevent the production and processing of stable mRNA. Transformants which expressed the truncated *mnp1* and *mnp2* messages at levels similar to endogenous *mnp* were purified by isolation of single basidiospores (Alic et al., 1991) and used for further analysis.

2.3.2 Mn induction of truncated mnp genes

Nitrogen-limited cultures of wild-type *P. chrysosporium* and pOGI18, pAMIBal31, and pAM2ClaI transformants were grown at 37°C in the absence of Mn. On day 5, Mn was added to a final concentration of 180 μM, and the cells were harvested after 2 h. As shown in Fig. 2.2, the accumulation of both endogenous and truncated *mnp* mRNA was readily detectable in cultures induced with Mn. Only trace amounts of *mnp* mRNAs were detected from uninduced cultures in the absence of Mn (see Fig. 2.4). Reprobing of the blot with the *P. chrysosporium gpd* gene demonstrated that each lane contained approximately equal amounts of mRNA (Fig. 2.2). These results indicate that the truncated and endogenous *mnp* genes are regulated by Mn in a similar manner and confirm our earlier results indicating that both the *mnp1* and *mnp2* genes are regulated by Mn (Brown et al., 1991; Mayfield et al., 1994a). Comparison with an RNA ladder confirms that the endogenous and truncated mRNAs are of the expected size (Fig. 2.2).

Extracellular MnP activity and the production of MnP protein in wild-type *P. chrysosporium* and pOGI18, pAMIBal31, and pAM2ClaI transformants were examined in the presence and absence of Mn. Mn-deficient nitrogen-limited cultures were induced with Mn as above, and MnP activity in the extracellular medium was assayed after 24 h. MnP activity was detected only in the extracellular medium of

Fig. 2.2 Induction of *mnp* gene transcription by Mn. Five-day-old nitrogen-limited cultures of wild type (lane 1), pOGI18 transformant (lane 2), pAM1Bal31 transformant (lane 3), and pAM2ClaI transformant (lane 4) were induced with $MnSO_4$ (180 μM final concentration). Cells were harvested and total RNA was extracted and probed as described in the text. The upper blot was probed with the *mnp1* cDNA and the lower blot with the *P. chrysosporium gpd* gene.
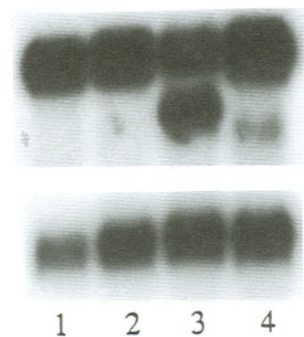
Fig. 2.3 Induction of *mnp* gene transcription by HS. Five-day-old nitrogen-limited, Mn-deficient cultures of wild type (lane 1), pOGI18 transformant (lane 2), pAM1Bal31 transformant (lane 3), and pAM2ClaI transformant (lane 4) were transferred to a 45°C water bath for 1 h. Cells were harvested and total RNA was extracted and probed as described in the text. The upper blot was probed with the *mnp1* cDNA and the lower blot with the *P. chrysosporium gpd* gene.
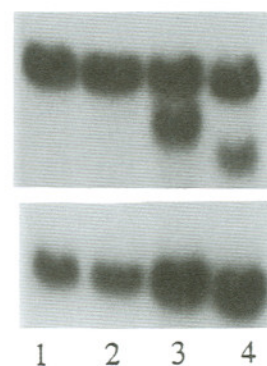
Fig. 2.4 Induction of *mnp* gene transcription by H_2O_2. Five-day-old nitrogen-limited, Mn-deficient cultures were harvested (lanes 1–3) or induced with H_2O_2 for 1 h before harvesting (lanes 4–7), and total RNA was extracted and probed as described in the text: pOGI18 transformant (lanes 1 and 5), pAM1Bal31 transformant (lanes 2 and 6), pAM2ClaI transformant (lanes 3 and 7), and wild type (lane 4). The upper blot was probed with the *mnp1* cDNA and the lower blot with the *P. chrysosporium gpd* gene.
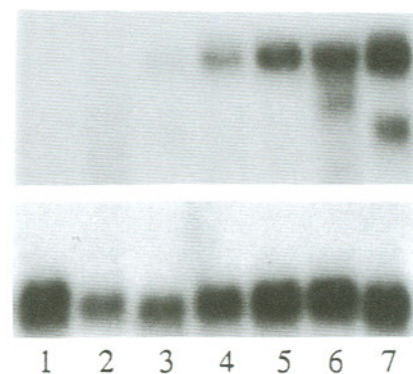
cultures induced with Mn (data not shown). The truncation in the *mnp1* gene was predicted to result in a reading frame shift, changing the amino acid sequence downstream of the truncation and leading to premature termination of the protein. In contrast, the truncation in the *mnp2* gene was predicted to result in the elimination of amino acids 85–186 of the mature MnP protein. Thus, any protein produced from the truncated *mnp* genes would be easily distinguishable from endogenous MnP on the basis of molecular weight. Western blot analysis was performed on intracellular and extracellular protein extracted from Mn-deficient, nitrogen-limited cultures induced with Mn as above. Although native MnP protein was detected under these conditions in all cultures, no truncated MnP protein was evident (data not shown). This suggests that the deletions in the *mnp1* and *mnp2* genes were sufficient to preclude translation, processing, and/or folding of MnP protein.

2.3.3 Nitrogen regulation of truncated mnp genes

Neither endogenous nor truncated *mnp* mRNAs were detected following Mn induction of nitrogen-sufficient cultures (data not shown), confirming our previous results (Pribnow et al., 1989; Brown et al., 1991) and indicating that the truncated reporter genes are regulated similarly to the endogenous *mnp1* and *mnp2* genes with regard to nitrogen.

2.3.4 HS induction of truncated mnp genes

Nitrogen-limited, Mn-deficient cultures of pOGI18, pAMIBal31, and pAM2ClaI transformants, as well as wild-type *P. chrysosporium*, were grown at 37°C for five days and then treated with a 45°C HS for 1 h prior to harvesting. As seen in Fig. 2.3, both endogenous and truncated *mnp* mRNAs were detected from heat-shocked cells, indicating that the truncated *mnp* genes are regulated by HS in a manner analogous to the endogenous *mnp* genes.

2.3.5 H₂O₂ induction of truncated mnp genes

Mn-deficient nitrogen-limited cultures of wild-type *P. chrysosporium* and pOGI18, pAMIBal31, and pAM2ClaI transformants were grown at 37°C for five days

and then treated with H_2O_2 (final concentration 1.0 mM) for 1 h. As seen in Fig. 2.4, the accumulation of both endogenous and truncated *mnp* mRNA was detected in all cells exposed to H_2O_2. Only trace amounts of *mnp* mRNAs were detected from uninduced cultures in the absence of Mn (Fig. 2.4), indicating that the endogenous and truncated *mnp* genes are induced in a similar manner by Mn, HS, and H_2O_2.

2.3.6 Conclusions

Our results demonstrate that truncated reporter genes can be used to examine the expression of individual members of a gene family. The experiments described here indicate that both the truncated *mnp1* and *mnp2* genes are regulated by the same factors which regulate the endogenous *mnp* genes and imply that our previous results using the *mnp1* cDNA as a probe for northern blots are applicable to both *mnp1* and *mnp2*. Our results also indicate that 936 bp of the *mnp1* promoter and 1.3 kb of the *mnp2* promoter are sufficient for regulation of *mnp* gene expression by nitrogen limitation, Mn, HS, and H_2O_2, confirming and extending our previous results with the *mnp1* promoter (Godfrey et al., 1994).

The truncated reporter system described here has several advantages in addition to distinguishing between individual members of a gene family. In these experiments, endogenous *mnp* expression serves as an internal control for expression of introduced *mnp* reporter genes, making this a particularly attractive system for studying mutations in the *mnp* promoters. In addition, this system can be used for studying *mnp* gene expression in the absence of Mn, a condition under which no MnP protein is produced (Brown et al., 1993; Li et al., 1995). The truncated reporter genes described here will be used for analyzing the expression of mutated *mnp* promoters in order to identify *cis*-acting sequences that are involved in the regulation of *mnp* gene expression by Mn and other factors. Similar truncations of additional *mnp* and *lip* genes also will be utilized as reporters.

CHAPTER 3
RT-PCR ANALYSIS OF THE TRANSCRIPTIONAL REGULATION OF THE
MANGANESE PEROXIDASE GENE FAMILY

3.1   Introduction

The white-rot basidiomycete fungus *Phanerochaete chrysosporium* has been
studied extensively for its ability to degrade lignin (Buswell & Odier, 1987; Kirk &
Farrell, 1987; Gold & Alic, 1993) and a wide variety of aromatic pollutants (Bumpus
& Aust, 1987; Hammel et al., 1989; Valli et al., 1992; Joshi & Gold, 1993). Two
families of peroxidases, lignin peroxidase (LiP) and manganese peroxidase (MnP) and
an H_2O_2-generating system are the major components of this organism's extracellular
lignin-degrading system (Kirk & Farrell, 1987; Gold et al., 1989; Gold & Alic,
1993). The best-studied MnP isozyme from *P. chrysosporium* is a well-characterized
H_2O_2-requiring heme glycoprotein of M_r ~46,000 (Glenn & Gold, 1985; Wariishi et
al., 1988, 1989; Gold et al., 1989; Gold & Alic, 1993). The enzyme oxidizes Mn^{2+}
to Mn^{3+}; the latter, complexed with an organic acid chelator such as oxalate which is
secreted by the fungus, oxidizes the terminal phenolic substrate (Glenn & Gold, 1985;
Wariishi et al., 1992; Kuan & Tien, 1993a; Kishi et al., 1994) and possibly
nonphenolic substituents via a radical mediator (Wariishi et al., 1989; Bao et al.,
1994). X-Ray crystallographic (Sundaramoorthy et al., 1994) and site-directed
mutagenesis (Kusters-van-Someren et al., 1995; Kishi et al., 1996) studies have
defined the Mn-binding site of MnP.

MnP occurs as a series of isozymes encoded by a family of genes, and the
sequences of cDNA (Pease et al., 1989; Pribnow et al., 1989; Mayfield et al., 1994a;
Orth et al., 1994) and genomic clones (Godfrey et al., 1990; Mayfield et al., 1994a;
Alic et al., 1997) encoding alleles of three MnP isozymes from *P. chrysosporium*

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have been determined. MnP expression is regulated at the level of gene transcription by the depletion of nutrient nitrogen (Pribnow et al., 1989). In addition, MnP activity is dependent on the presence of Mn^{2+} in the culture medium (Bonnarme & Jeffries, 1990; Brown et al., 1990), and *mnp* gene transcription is regulated by Mn^{2+} (Brown et al., 1990, 1991; Godfrey et al., 1994). To date, this is the only case of Mn regulation of gene transcription to be studied at the molecular level. MnP also is regulated at the level of gene transcription by heat shock (HS) (Brown et al., 1993), H_2O_2, and other chemical stresses (Li et al., 1995).

Reverse transcriptase polymerase chain reaction (RT-PCR) has allowed detailed analysis of differential expression between and within families of genes involved in lignocellulose degradation in liquid cultures (Covert et al., 1992; Stewart et al., 1992; Brooks et al., 1993; Reiser et al., 1994; Tempelaars et al., 1994; Broda et al., 1995, 1996) as well as pollutant-contaminated soil (Lamar et al., 1995; Bogan & Lamar, 1996; Bogan et al., 1996b). In addition to the ability to distinguish among very similar RNAs, RT-PCR has been shown to be 1,000–10,000-fold more sensitive than northern blotting techniques (Byrne et al., 1988; Wang et al., 1989; Sooknanan et al., 1993). RT-PCR takes hours to perform instead of days and avoids the use of ^{32}P-labeled probes.

Herein, RT-PCR is used to further our investigation of *mnp* gene expression in a more detailed manner than our previous northern blot analysis. Competitive RT-PCR is used to compare the expression patterns of genes encoding three MnP isozymes, *mnp1, mnp2*, and *mnp3*. Here, we demonstrate that there is a basal level of the three *mnp* gene transcripts when the cells are grown without Mn in nitrogen-sufficient cultures as well as nitrogen-limited cultures. Furthermore, we present evidence for the differential expression within the *mnp* gene family under nitrogen-limiting conditions in the presence of Mn, with or without agitation. Our results indicate that RT-PCR can be used to quantitate the expression of M2ClaI, a truncated reporter gene previously used to examine the regulation of *mnp2* (Gettemy et al., 1997).

3.2   Materials and Methods

3.2.1 Organisms

P. chrysosporium wild-type strain OGC101, a derivative of BKM-F-1767, and pAM2ClaI (Gettemy et al., 1997) transformants were maintained as described previously (Gold et al., 1982).

3.2.2 Culture conditions

P. chrysosporium strains were grown at 37°C from conidial inocula in 20-ml stationary cultures in 250-ml Erlenmeyer flasks as described previously (Kirk et al., 1978; Brown et al., 1991). The medium contained mineral salts with trace elements (without Mn) (Kirk et al., 1978), 2% glucose as the carbon source, and 1.2 mM (limiting nitrogen) or 12 mM ammonium tartrate. Cultures were grown with and without added Mn (180 μM). Cultures were incubated under air for two days; on day 3 they were purged with 100% O_2 for 10 min. For the induction studies, five-day-old cultures were treated with Mn (180 μM final concentration) for 4 h or with 45°C HS for 1.5 h, prior to harvesting as described (Brown et al., 1993; Li et al., 1995). For the temporal expression experiment, the cultures were incubated under air for two days, after which they were purged daily with 100% O_2 for 10 min.

OGC101 was also grown at 28°C from a mycelial fragment inocula in 1-liter agitated cultures in 2-liter Erlenmeyer flasks as described previously (Glenn & Gold, 1985). The medium contained 2% glucose, 1.2 mM ammonium tartrate, 20 mM 2,2-dimethylsuccinate (pH 4.5) as buffer, and minimal salts with trace elements (6$\times$) (Kirk et al., 1978; Gold et al., 1984). The cultures were grown for five days before harvesting. They were incubated under air for two days, after which they were purged daily with 100% O_2.

3.2.3 RNA extraction

Cells were filtered through Miracloth (Calbiochem, La Jolla, CA), pressed firmly between layers of paper towel, frozen rapidly in liquid nitrogen, and stored at −80°C. The frozen cells were disrupted and the RNA isolated by homogenizing with

acid-washed glass beads in a mini-bead beater in the presence of 4 M guanidinium thiocyanate (Janssen, 1987). The supernatants were extracted with chloroform, and the RNA was precipitated with isopropanol. An overnight 4 M LiCl wash of the isopropanol precipitated RNA aided in the removal of carbohydrates, genomic DNA, and large ribosomal RNA. The RNA was then washed with 75% ethanol and solubilized in diethyl pyrocarbonate-treated water (DEPC). For each sample, the absorbance of the solution was measured at 260 and 280 nm in order to quantitate and determine the purity of the RNA.

3.2.4 *Reverse transcription polymerase chain reaction*

All RT-PCRs were performed with a DNA thermal cycler (ERICOMP, San Diego, CA). The reverse transcription (RT) reaction (25 μl) contained 2 μg of total RNA, 15 U of Moloney murine leukemia virus RT (GIBCO BRL, Gaitherburg, MD), 40 U RNasin (Promega Biotech Inc., Madison, WI) and 3 pmol of the appropriate 3' primer (ORPRC, Beaverton OR) (Table 3.1), RT buffer, and 10 mM dNTPs (USB, Cleveland, OH) (Frohman et al., 1988). The RT reactions contained an annealing step, 5 min at 70°C with slow cooling to 30°C, followed by extension, 60 min at 42°C and 30 min at 52°C. Following the RT reaction, the volume was increased to 100 μl with DEPC-treated water. PCRs (50 μl) contained 1 U of deep Vent polymerase (NEB, Inc., Beverly, MA) and 30 pmol each of the upstream and downstream primers (Table 3.1), buffer (NEB, Beverly, MA), and 20 mM dNTPs. The PCR temperature program was 94°C for 6 min, 54°C for 2 min, and 72°C for 40 min for 1 cycle, followed by 94°C for 1 min, 54°C for 2 min, and 72°C for 5 min for 35 cycles, and a final 15-min extension at 72°C (Stewart et al., 1992).

3.2.5 *Competitive RT-PCR*

Competitive RT-PCR reactions were conducted as described above, except that known amounts of plasmids containing the full-length genomic sequences (gDNA) for each gene (Godfrey et al., 1990; Mayfield et al., 1994a, 1994b; Alic et al., 1997) were added as competitive template (Gilliland et al., 1990). The competitive DNAs were added in a series of dilutions of known concentration. Introns within the

TABLE 3.1 Gene-specific PCR primers and probes

Gene[a]	5′ Primer[b]	3′ Primer[b]	cDNA (bp)[c]	gDNA (bp)[c]	Probes[b]
mnp1	ccggtcaacggcttggtattc	cgttgttgg(c/g/a)(g/a)agaagttgg	336	512	cgctgttcgtgctgcccccactgcggt
mnp2	agctctcaaggacatccgcac	cgttgttgg(c/g/a)(g/a)agaagttgg	346	565	cataactcgcgccgccccgactgcgga
mnp3	ccgacggtaccaaggtcaac[d]	cgttgttgg(c/g/a)(g/a)agaagttgg	229	399	catttcgcaaagcaagggaccgagcg
mnp2ClaI	agctctcaaggacatccgcac	taccgagtcgaagggcgc	322/681	950	cataactcgcgccgccccgactgcgga
gpd	cgtatcgtcctccgtaatgc	actcgttgtcgtaccaggag	905	1106	

[a] *P. chrysosporium* genes encoding manganese peroxidases (*mnp1, mnp2, mnp3*), truncated reporter manganese peroxidase (*mnp2ClaI*), and glyceraldehyde phosphate dehydrogenase (*gpd*).
[b] Primer/probe design based on published sequences; *mnp1* (Godfrey et al., 1990), *mnp2* (Mayfield et al., 1994), *mnp3* (Alic et al., 1997), *gpd* (Mayfield 1994b).
[c] gDNA, size predicted for PCR amplification of genomic sequence; cDNA, size predicted for PCR amplification of cDNA sequence.
[d] Primer designed by Bogan et al., 1996.

competitive template allowed the target cDNAs and the genomic products to be separated on agarose gels.

The ratio of plasmid size (competitive template) to PCR target size can be used to adjust for the relative molar concentrations of *mnp* and glyceraldehyde-3-phosphate dehydrogenase (*gpd*) sequences. This is an important conversion when comparing the quantities of the individual *mnp* transcripts. The *mnp1* genomic template is a plasmid of 8835 bp, and the PCR target size is 512 bp, yielding a plasmid-to-gDNA target ratio of 17.25. The *mnp2* genomic template is a plasmid of 7680 bp, and the PCR target size is 565 bp, yielding a plasmid-to-target ratio of 13.59. The *mnp3* genomic template is a plasmid of 6114 bp, and the PCR target size is 399 bp, yielding a plasmid-to-target ratio of 15.3. The *mnp2* plasmid was also used as the competitive template for M2ClaI with the gDNA PCR target size of 950 bp, yielding a plasmid-to-target ratio of 5.51.

mRNA transcripts corresponding to *gpd* were examined as a control. Competitive genomic template was used as described above in order to quantitate the message. The *gpd* genomic template is the plasmid of 6257 bp, and the PCR target size is 905 bp, yielding a plasmid-to-target ratio of 6.9.

Following amplification, 5 μl of the PCR products were analyzed by electrophoresis on 1.5% agarose gels and stained with ethidium bromide. Gels were visualized with UV light and photographed. The Polaroid photos of the ethidium-stained gels were scanned using Adobe Photoshop 2.5 software. Figs. 3.2, 3.3, and 3.6 were arranged using Canvas 3.0. To quantitate the amount of PCR product, a densitometer was used on the scan of ethidium-stained gels (IP lab gel software).

3.2.6 Southern blot analysis

Southern blots of *mnp* PCR products were analyzed with gene-specific oligonucleotide probes (Table 3.1) to ensure the specificity of each PCR primer pair for its target sequence. 500 pg of each genomic plasmid were digested with *Eco*RI; 5 μl of *mnp* PCR product derived from genomic DNA, and 5 μl of *mnp* RT-PCR product derived from total RNA extracted from 5-day-old limiting nitrogen cultures

were separated on an agarose gel and stained with ethidium. The DNAs were blotted to nylon membranes (Micron Separations Inc., Westboro, MA) and UV cross-linked (UV Stratalinker; Statagene Inc., La Jolla, CA). Blots were prehybridized for 1 h in $5 \times$ SSC, 1% SDS, $5 \times$ Denhardt's solution, 25 mM NaPO$_4$ (pH 7.0), and 100 μg/ml herring sperm DNA. Probes (10 pmol) were end labeled with α^{32}-P-dATP using T4 polynucleotide kinase (NEB, Inc., Beverly, MA). The unincorporated label was not removed. Hybridizations were carried out overnight at 55°C. Blots were washed with $6 \times$ SSC and 1% SDS at room temperature for 5 min three times and at 50°C for 2 min two times. Blots were exposed to Kodak XAR film overnight.

3.3   Results

3.3.1 Detection of mnp transcripts using RT-PCR

Three specific *mnp* transcripts were detected using RT-PCR. A conserved *mnp* downstream primer (Table 3.1) was used to synthesize the first-strand cDNAs from *P. chrysosporium* total RNA. The individual *mnp* cDNAs were then amplified using PCR with the conserved *mnp* downstream primer and *mnp*-specific upstream primers (Table 3.1). The target sites for PCR included introns to distinguish between RNA and any contaminating genomic DNA PCR products. Fig. 3.1 shows the amplified segments of the individual *mnp* genes and *gpd*. The predicted sizes are indicated in Table 3.1. As shown in Fig. 3.2, the identity of the ethidium-stained PCR products was confirmed by Southern blot hybridization with gene-specific oligonucleotide probes. In contrast to earlier reports (Brooks et al., 1993; Broda et al., 1995), there was no genomic DNA contamination detectable in ethidium-stained gels.

Initially RT-PCR was conducted on RNA samples derived from cultures of wild-type strain OGC101. Nitrogen-sufficient and nitrogen-limited cultures were grown at 37°C in the absence of Mn and in the presence of 180 μM Mn. On day 5, the individual cultures were harvested and RNA was extracted. All three *mnp* transcripts were amplified from nitrogen-limited day-5 cultures grown at 37°C in the presence of 180 μM Mn as well as nitrogen-limited day-5 cultures grown at 37°C in
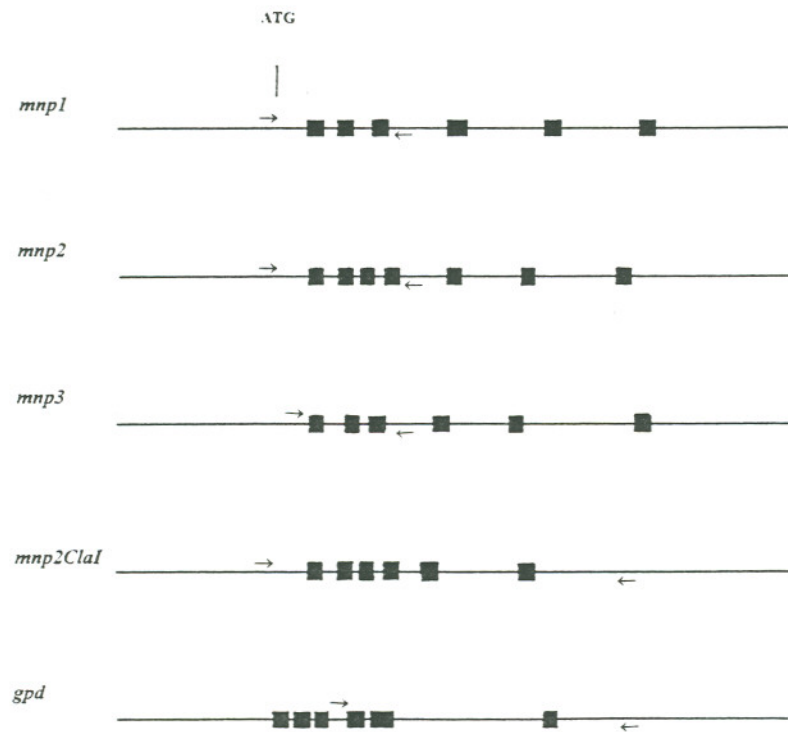
Fig. 3.1 Schematic representation of three closely related *mnp* genes, the truncated-*mnp2* reporter gene, *gpd*, and the strategy for PCR amplification. Coding regions are shown as solid lines, and introns are shown as solid boxes. Positions of the downstream primers (←), upstream primers (→), and the specific oligo nucleotide probes (—) are indicated.
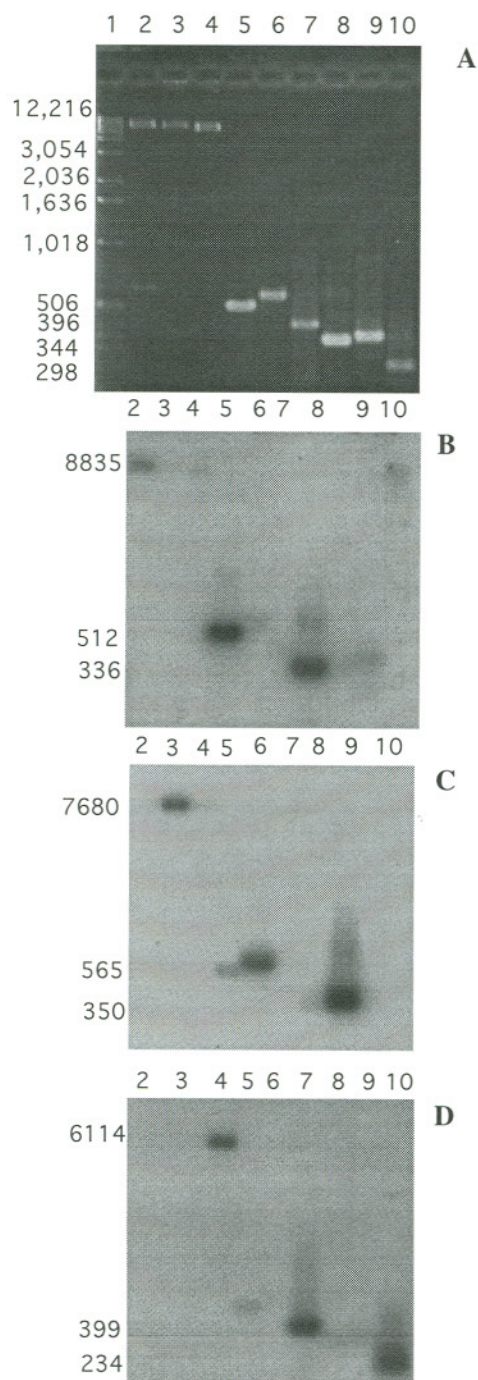
Fig. 3.2 Specificity of *mnp* PCR products. **(A)** Ethidium-stained gel. Lanes 2, 3, and 4: *Eco*RI-cut plasmids; lanes 5, 6, 7: products derived by PCR amplification of *P. chrysosporium* genomic DNA; lanes 8, 9, 10 were generated by RT-PCR of RNA extracted from day 5 nitrogen-limited *P. chrysosporium* cultures. Lanes 2, 5, and 8 contain *mnp1*; lanes 3, 6, and 9 contain *mnp2*; lanes 4, 7, and 10 contain *mnp3*. The leftmost lane contains 1-kb molecular size markers, and the sizes of the bands (base pairs) are on the left. **(B)** Southern blot of gel in **A** probed with the *mnp1*-specific probe. **(C)** Southern blot of gel in **A** probed with the *mnp2*-specific probe. **(D)** Southern blot of gel in **A** probed with the *mnp3*-specific probe.

the absence of Mn and induced on day 5 with 180 μM Mn for 4 h or HS for 1.5 h. All three *mnp* transcripts were also detected by RT-PCR from day-5 nitrogen-sufficient cultures grown at 37°C in the presence of 180 μM Mn, as well as in the absence of Mn, and day-5 nitrogen-limited cultures grown without Mn.

3.3.2 *Competitive PCR to quantitate individual mnp messages*

Competitive PCR was used to quantitate the three *mnp* transcripts derived from cultures grown under each condition. The RT reaction was repeated as before except that, in addition to the conserved *mnp* primer, a 3' *gpd* primer (Table 3.1) was added as a control. For each cDNA, eight identical PCR reactions were spiked with serial dilutions of the appropriate competitive genomic template. A ninth control reaction was performed containing all components of the PCR reaction except RNA template. Following PCR, the products generated by the competitive DNAs and the cDNAs were analyzed by gel electrophoresis and ethidium staining. The initial concentration of specific cDNAs was determined by estimating the dilution points at which a set of cDNA and competitive template PCR products were equivalent. This is illustrated in Fig. 3.3A, which shows the competitive RT-PCR quantitation of *mnp2* mRNA. The cDNA levels as determined for the three *mnp* and *gpd* genes are shown graphically in Fig. 3.3B and C. Adjusting for the relative molar concentrations of the *mnp* and *gpd* genes, the transcript concentrations ranged from 10^{-3} to 4 pg/20 ng total RNA.

As shown in Fig. 3.3, nitrogen-sufficient and nitrogen-limited cultures of OGC101 were grown at 37°C in the absence of Mn and in the presence of 180 μM Mn. On day 5, the individual cultures were harvested and RNA was extracted. Competitive RT-PCR on the RNA from these cultures demonstrated that under conditions of sufficient nitrogen the concentration of *mnp* transcript was unaffected by the presence of Mn. The basal levels for *mnp1*, *mnp2*, and *mnp3* were ~0.002 pg/20 ng total RNA in the presence and absence of Mn. In nitrogen-limited cultures with 180 μM Mn, the *mnp* transcripts increased ~100-fold for *mnp1* (~0.3 pg/20 ng), ~1600-fold for *mnp2* (~4 pg/20 ng), and <2-fold for *mnp3* (0.003 pg/20 ng) (Fig. 3.3B and C). The *gpd* transcript was ~1 pg/20 ng under nitrogen-limiting
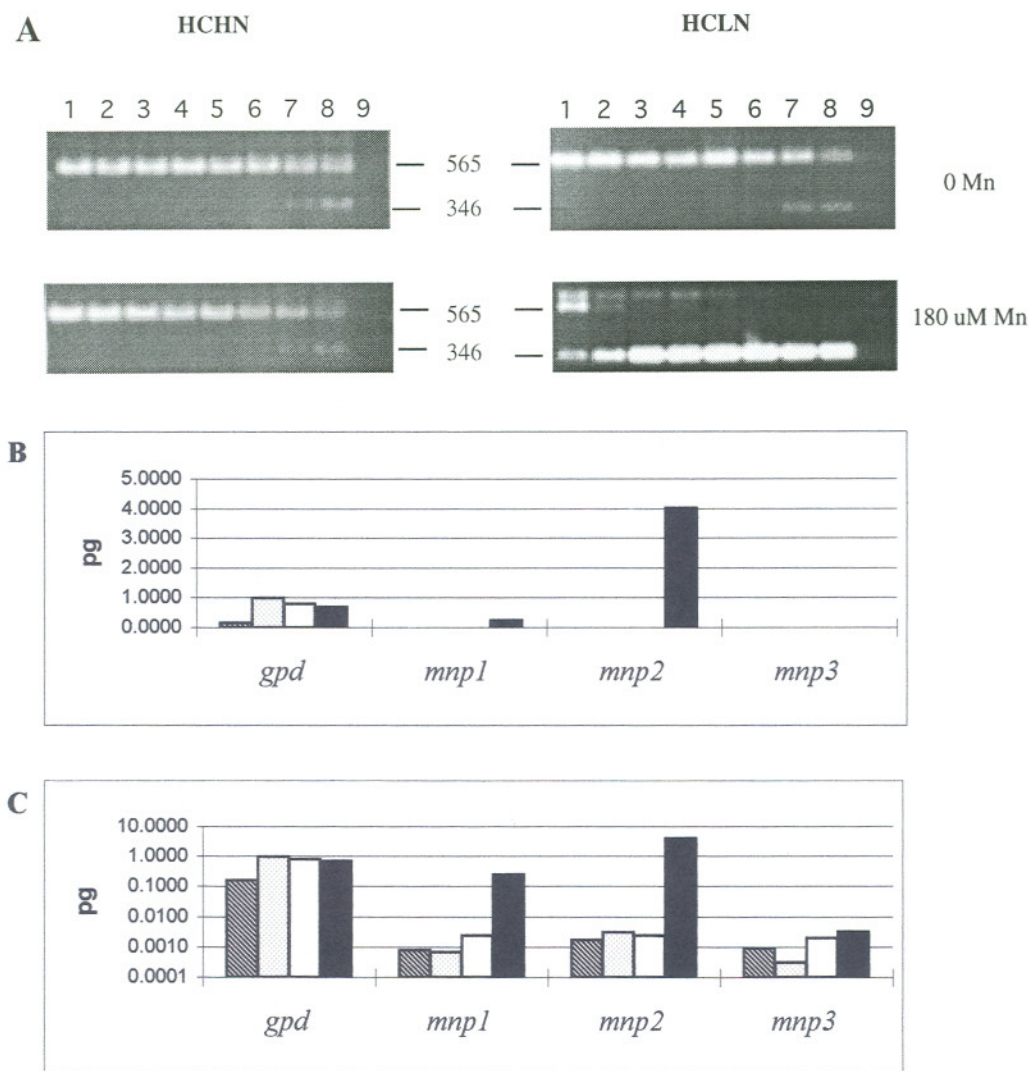
Fig. 3.3 mRNA levels of *mnp1*, *mnp2*, *mnp3*, and *gpd*. (A) Competitive RT-PCR quantitation of *mnp2* mRNA. Each PCR reaction contained cDNA derived from total RNA isolated from five-day-old cultures with sufficient nitrogen (HN) or limited nitrogen (LN), with or without 180 μM Mn. Lanes 1–8 contained 100, 50, 10, 5, 1, 0.5, 0.1, and 0.05 pg, respectively, of competitive genomic *mnp2* DNA. Lane 9 lacked template. **(B)** Bar graph of mRNA levels for *mnp1*, *mnp2*, *mnp3*, and *gpd*, determined as shown in **A**. HN = /, HN 180 μM Mn = ·, LN = **white**, LN 180 μM Mn = **black**. **(C)** Log scale graph of mRNA levels in **B**.

conditions. In the day-5 nitrogen-sufficient cultures grown in the presence of Mn, the *gpd* transcript was ~1 pg/20 ng; however, in the absence of Mn, there was a 10-fold decrease in *gpd* transcript (Fig. 3.3B and C).

3.3.3 Mn induction

To determine the effect on *mnp* expression upon the addition of Mn to uninduced cultures, nitrogen-limited cultures of OGC101 were grown at 37°C in the absence of Mn. On day 5, Mn was added to a final concentration of 180 μM, and the cells were harvested after 4 h. The concentration of *mnp1* transcripts increased ~2-fold over that from uninduced cultures. The concentration of *mnp2* transcript increased ~70-fold over that from uninduced cultures. The *mnp3* transcript increased <2-fold over that from uninduced cultures (data not shown).

3.3.4 HS induction

To compare *mnp* transcript induction by HS with the induction by Mn, nitrogen-limited Mn-deficient cultures of OGC101 were grown at 37°C for five days and then treated with a 45°C HS for 1.5 h prior to harvesting. The concentration of *mnp1* transcripts increased ~2-fold over that from uninduced cultures. The concentration of *mnp2* transcript increased ~70-fold over that from uninduced cultures. The *mnp3* transcript increased <2-fold over that from uninduced cultures (data not shown).

3.3.5 Agitated cultures

Agitated cultures were grown to compare *mnp* transcript levels isolated under these conditions to transcript levels from stationary cultures. Nitrogen-limited cultures of OGC101 were grown at 28°C in the presence of 6× trace elements. On day 5, the individual cultures were harvested and RNA was extracted. All three *mnp* transcripts were detected. The level of *mnp1*, *mnp2*, and *mnp3* transcripts were ~0.7 pg/20 ng, ~0.1 pg/20 ng, and ~0.002 pg/20 ng, respectively, as shown graphically in Fig. 3.4.

A

B
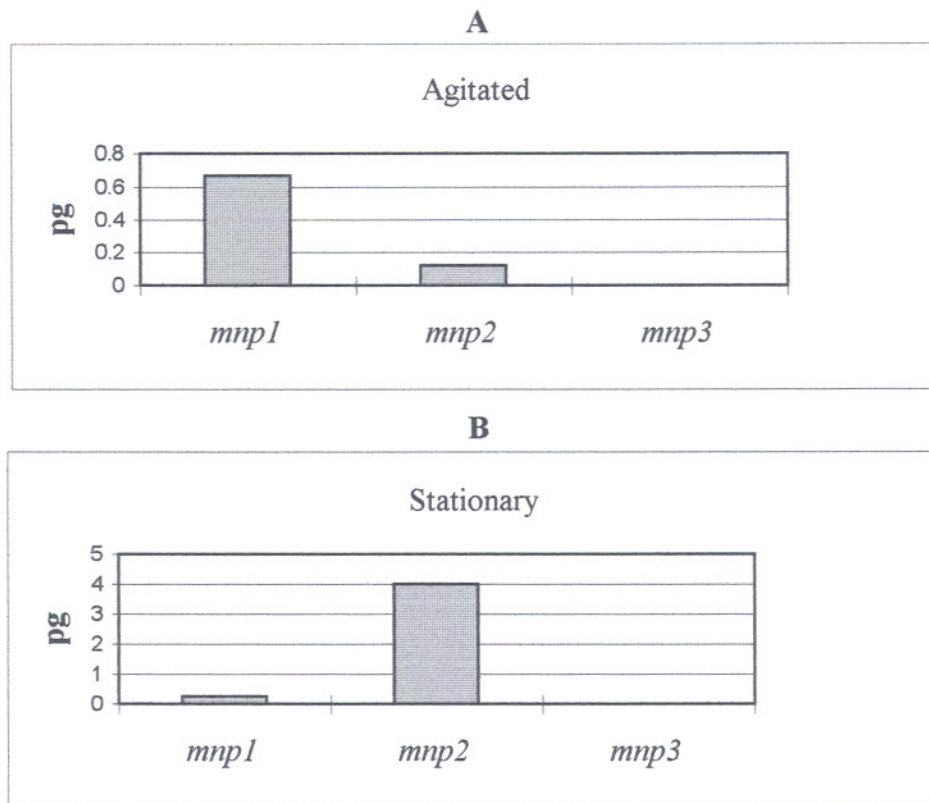
Fig. 3.4 mRNA levels of *mnp1, mnp2,* and *mnp3* in agitated (**A**) or stationary (**B**) cultures.
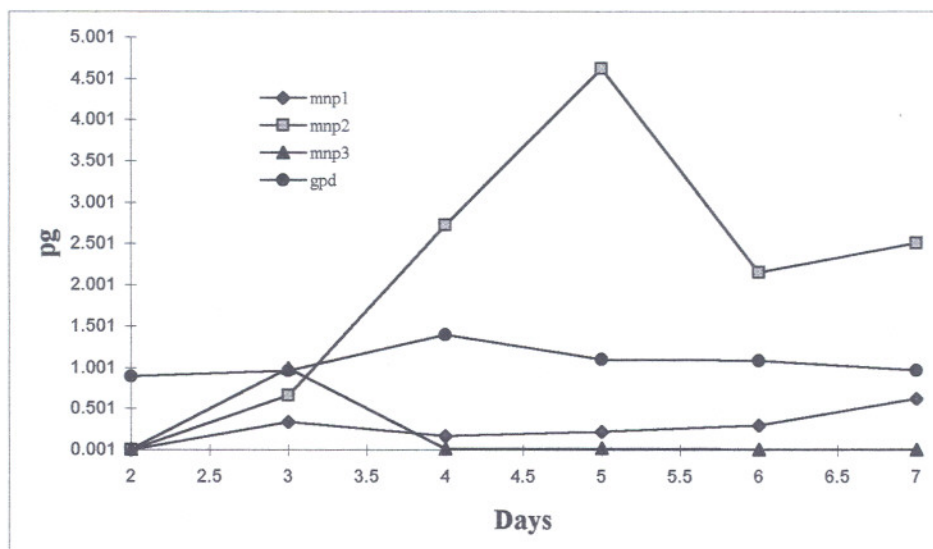
Fig. 3-5. Temporal variation in the amount of *mnp* and *gpd* transcripts in mRNA extracted from *P. chrysosporium* nitrogen limited cultures containing 180 μM Mn.

3.3.6 Temporal production of mnp gene transcripts monitored by competitive RT-PCR

To determine the expression profiles of *mnp1*, *mnp2*, and *mnp3* under nitrogen limitation over time, cultures of OGC101 were grown at 37°C in the presence of 180 μM Mn. cDNA was prepared from RNA isolated from each of two flasks on days 2–7 after inoculation. Competitive RT-PCRs were performed as before, except only four quantitative PCR reactions were run for each cDNA (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} ng). Fig. 3.5 shows the temporal variations in the levels of *mnp1*, *mnp2*, and *mnp3* mRNA under these culture conditions. All three transcripts were present on day 2. *mnp1* increased on day 3 by ~ 300-fold over day 2 and remained relatively constant through day 7. *mnp2* gradually increased on days 2–4, peaking on day 5 at ~ 1000-fold over the level on day 2, and then decreased gradually on days 6 and 7. *mnp3* peaked on day 3 with an ~ 100-fold increase over day 2 and returned to low levels on days 4–7. The levels for *gpd* remained relatively constant on days 2–7.

3.3.7 Competitive RT-PCR to quantitate the expression of the truncated mnp2 message

We recently constructed a truncated gene reporter system to examine the regulation of two genes, *mnp1* and *mnp2* (Gettemy et al., 1997). Competitive RT-PCR was used to quantitate the expression of the endogenous and truncated *mnp2* messages in the pAM2ClaI transformant strain. Both messages were detected using RT-PCR. A unique *mnp2* downstream primer was used to synthesize the first-strand cDNA. The messages were amplified using PCR with *mnp2*-specific downstream and upstream primers (Table 3.1). The target sites for the PCR included introns and the deleted region of the truncated reporter gene to distinguish between the truncated message, the endogenous *mnp2* mRNA, and *mnp2* genomic DNA.

Nitrogen-limited cultures of the pAM2ClaI transformant were grown at 37°C in the absence of Mn. On day 5, Mn was added to a final concentration of 180 μM, and the cells were harvested after 4 h. As shown in Fig. 3.6 the induction of both the
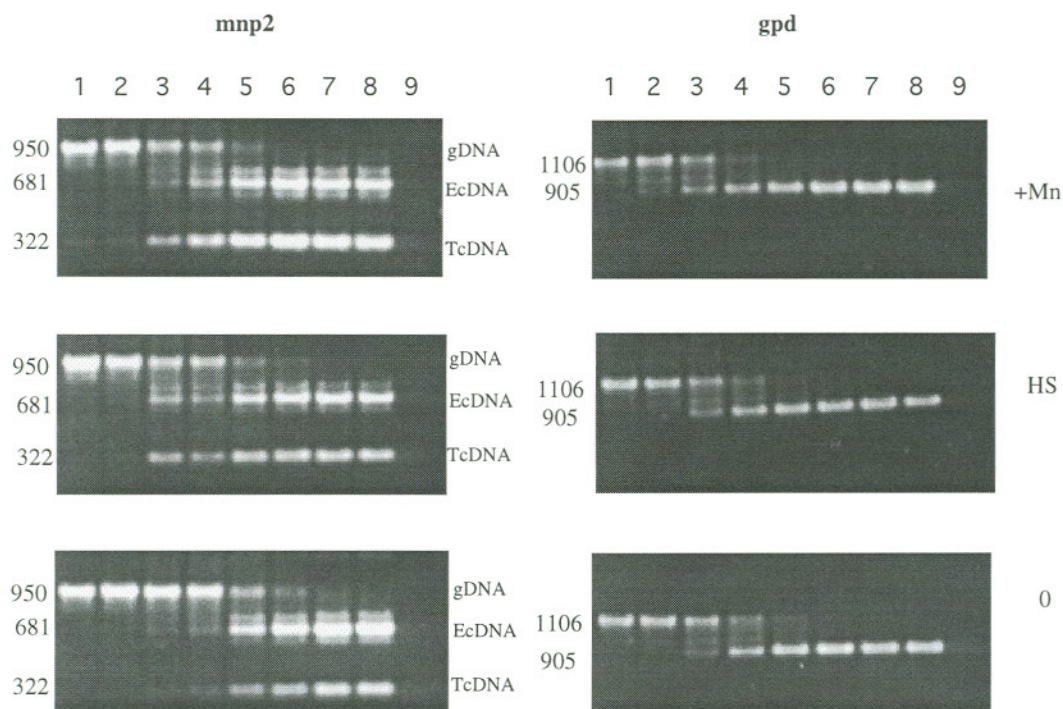
Fig. 3.6 Induction of truncated *mnp2* gene transcription by Mn and HS. Five-day-old nitrogen-limited cultures of a pAM2ClaI transformant were grown without Mn or induced with $MnSO_4$ (180 μM final concentration) or HS induced by transfer to a 45°C water bath for 90 min. Cells were harvested and the RNA was extracted. Competitive RT-PCR of the endogenous *mnp2* and the truncated *mnp2* and *gpd* mRNAs were performed as described in the text. Lanes 1–8: 100, 50, 10, 5, 1, 0.5, 0.1, and 0.05 pg, respectively, of competitive template. Lane 9 is a 0 control, containing all PCR components except template. The sizes of of the genomic DNA and the cDNA products are indicated at the left margin in base pairs.

endogenous and the truncated *mnp2*s was readily detectable in the Mn-induced cultures.

The predicted sizes of the endogenous *mnp2* PCR product, the truncated *mnp2* PCR product, and the genomic PCR product were 681, 322, and 950 bp, respectively. Comparison with a 1-kb DNA ladder (Promega Biotech Inc., Madison, WI) showed that the PCR products of both the endogenous and truncated *mnp2* messages were of the expected size, and the identities of the ethidium-stained PCR products were confirmed by hybridization with a gene-specific oligonucleotide probe (data not shown).

Nitrogen-limited Mn-deficient cultures of a pAM2ClaI transformant were grown at 37°C in the absence of Mn for five days and then treated with 45°C HS for 1.5 h prior to harvesting. As shown in Fig. 3.6, the induction of both the endogenous and the truncated *mnp2* transcripts was readily detectable in the HS-induced cultures.

3.4   Discussion

The lignin degradative system of *P. chrysosporium* is expressed during secondary metabolic (idiophasic) growth, the onset of which is triggered by limiting nutrient nitrogen (Buswell & Odier, 1987; Kirk & Farrell, 1987; Gold & Alic, 1993). Likewise, LiP and MnP activities are detectable in the extracellular medium only during the secondary metabolic phase of growth (Kirk & Farrell, 1987), and northern blot analysis has demonstrated that LiP and MnP expression is controlled at the level of gene transcription by nutrient nitrogen (Tien & Tu, 1987; Pribnow et al., 1989; Li et al., 1994). There is additional evidence that various isozymes of LiP and MnP may be differentially regulated by carbon and nitrogen (Holzbaur et al., 1988; Pease et al., 1989; Stewart et al., 1992; Broda et al., 1995, 1996).

Our previous results demonstrated that MnP is regulated by Mn^{2+}, the substrate for the enzyme, at the level of gene transcription (Brown et al., 1990, 1991). The *mnp1, mnp2,* and *mnp3* promoter regions contain multiple putative consensus metal response elements (MREs) (Brown et al., 1991; Mayfield et al.,

1994a; Alic et al., 1997) that are identical to the *cis*-acting sequences responsible for heavy metal induction of mouse and other metallothionein genes (Thiele, 1992). The *mnp1, mnp2, and mnp3* promoters also contain putative heat shock elements (HSEs) (Godfrey et al., 1990; Mayfield et al., 1994a; Alic et al., 1997). HS, as well as other chemicals that are known to induce the HS response, also induce *mnp* gene transcription, suggesting that the HSEs are physiologically functional (Brown et al., 1993; Li et al., 1995). However, the induction of mouse metallothionein-1 gene transcription by oxidative stress is mediated in part by MREs in the proximal promoter region (Dalton et al., 1996), and it is possible that the induction of *mnp* by H_2O_2 is also regulated by MREs. Neither MREs nor HSEs have been found in the promoter regions of sequenced *lip* genes.

Competitive RT-PCR has been established as a sensitive, rapid, and accurate method to study the transcription of lignocellulolytic genes from *P. chrysosporium* (Covert et al., 1992; Stewart et al., 1992; Reiser et al., 1993; Tempelaars et al., 1994; Broda et al., 1995, 1996; Lamar et al., 1995; Bogan & Lamar, 1996; Bogan et al., 1996b). The use of a single downstream primer in the RT reaction is similar to a technique employed earlier to study *lip* gene expression (Stewart et al., 1992; Lamar et al., 1995) as well as *mnp* gene expression (Bogan et al., 1996b). The method was used here to investigate *mnp* gene expression in more detail than our previous northern blot analyses. In particular, we wanted to use competitive RT-PCR to investigate the expression of three specific *mnp* genes under various culture conditions. Our results indicate that *mnp* genes are constitutively expressed at a very low level under nitrogen-sufficient conditions and are differentially regulated in response to Mn, culture age, and agitation.

RT-PCR was conducted on total RNA derived from cultures of OGC101, the strain used in our earlier studies. Preliminary RT-PCR analyses were carried out to establish the culture conditions for detection of *mnp* transcripts. In our earlier studies, *mnp* transcript and MnP protein were not detected in nitrogen-sufficient and nitrogen-limited cultures without Mn. By RT-PCR, the transcripts of the three *mnp* genes were detected in nitrogen-limited day-5 cultures grown in the presence of Mn as

well as day-5 Mn-deficient nitrogen-limited cultures that were induced with Mn or HS. However, transcripts of all three of the *mnp* genes were also detected in RNA isolated from day-5 nitrogen-sufficient cultures grown in the presence or the absence of Mn, as well as nitrogen-limited day-5 cultures grown in the absence of Mn. This discrepancy probably is due to the increased sensitivity of the PCR technique, which amplifies transcripts that are below the level of detection by northern blot analysis.

The expected PCR products for each *mnp* transcript were found under all culture conditions analyzed. RT-PCR can easily distinguish between spliced and unspliced transcripts; however, differential splicing in regions not amplified by the PCR primers or slight variations in transcriptional start sites would not be detectable in the experiments presented here. Although the *mnp* transcripts were readily detectable from heat-shocked cells in the absence of Mn, neither MnP activity nor MnP protein is detectable from cells that have been subjected to HS in the absence of Mn (Brown et al., 1993). The presence of active MnP protein from cells that have been heat shocked in the presence of Mn indicates that elevated temperature is not a barrier to translation of *mnp* mRNA. It is possible that *mnp* HS transcripts differ in some way from Mn-induced transcripts and are not translated, at least in the absence of Mn.

Quantitation of the three *mnp* transcripts under various culture conditions establishes that there is a very low constitutive level of *mnp* transcription (~ 0.002 pg/20ng total RNA). The presence of 180 μM Mn in nitrogen-sufficient cultures has no effect on the basal level of expression, as illustrated for *mnp2* in Fig. 3.3. Using RT-PCR, *lip* (LIG5) expression also was detected under high-nitrogen conditions at a low basal level (Brooks et al., 1993). Taken together, these results suggest that the expression of *lip* and *mnp* is not completely repressed by excess nitrogen.

The *mnp* genes are differentially regulated in 5-day-old nitrogen-limited cultures. The presence of 180 μM Mn in day-5 nitrogen-limited cultures results in an increase in *mnp1* and *mnp2* transcript levels, but no increase in *mnp3* transcript. Induction by Mn (180 μM) or HS (45°C) of day-5 Mn-deficient nitrogen-limited cultures results in an increase in *mnp1* and *mnp2* expression and no increase in *mnp3*

expression. The levels of *mnp2* are consistently higher than *mnp1* or *mnp3* under these conditions. These results indicate that transcriptional regulation is unique for each *mnp* gene and may be the result of the number and the position of *cis*-acting sequences in the individual *mnp* gene promoters.

As shown in Fig. 3.7, the positions, orientations and sequences of the MREs within the *mnp* promoters differ. The two proximal pairs of MREs in the *mnp1* gene promoter overlap to form a 4-bp palindrome (TGCA). The proximal MRE pair in the *mnp2* gene contains a 6-bp palindrome (TGCGCA). The MREs of mammalian MT genes are often present in two clusters, wherein each cluster contains several tandem duplications of the consensus sequence. The fact that two pairs of MREs in the *mnp1* promoter and one pair of MREs in the *mnp2* promoter contain palindromes suggests that the overlap may have physiological significance and could be analogous to the cluster arrangement. The promoters of *mnp1* and *mnp2* also contain single putative MREs. The promoter of *mnp3* contains only single putative MREs. In all six MREs found in the *mnp1* promoter, the R of the consensus (TGCRCNC) is A. In the three MREs found in *mnp2*, the R is always G, and the two MREs found in *mnp3* contains G or A at the R position. There are also differences in the nucleotide found at the variable position of the core (TGCRCNC). The distance from the MREs to the TATA box in each promoter, as well as the positions of the MREs in relation to each other, also varies. Variations such as these have been found to contribute to differential metal-induced promoter activity in both the mouse metallothionein and the trout metallothionein promoters (Searle et al., 1987; Samson & Gedamu, 1995).

Under agitating conditions, the primary protein isolated from *P. chrysosporium* is the MnP1 isozyme (Glenn & Gold, 1985); therefore, we were surprised to find that under stationary conditions the *mnp2* transcript levels were ∼ 16-fold higher than *mnp1* levels. To determine if the protein levels correspond to the transcript levels in agitating cultures, RT-PCR was conducted on RNA extracted under these culture conditions. Under agitating conditions, the *mnp1* transcript levels are 7-fold higher than the *mnp2* transcript levels, as seen in Fig. 3.4.

A

mnp1

```
-104    g  c  g  t  g  T  G  C  A  C  G  c  a  g  -89c
        c  g  c  A  C  A  C  G  T  g  c  g  t  c

-328    c  a  a  g  c  g  T  G  C  A  C  A  C  c  -313
        g  t  t  c  G  C  A  C  G  T  g  t  g  g

-491    g  g  c  T  G  C  A  C  T  C  g  c  c  c  -479
        c  c  g  a  c  g  t  g  a  g  c  g  g  g

-733  t  g  g  c  g  g  g  t  g  c  a  a  g  c-746
      a  c  c  g  C  C  C  A  C  G  T  t  c  g
```

mnp2

```
-247    g  a  t  g  T  G  C  G  C  A  C  g  t  t  -232
        c  t  a  C  A  C  G  C  G  T  g  c  a  a

-488    c  g  c  g  g  g  c  g  c  a  c  a  a  a  -473
        g  c  g  C  C  C  G  C  G  T  g  t  t  t
```

mnp3

```
-308    c  t  g  t  g  g  g  c  g  c  a  c  a  a  -294
        g  a  c  a  C  C  C  G  C  G  T  g  t  t

-813    c  c  a  T  G  C  A  C  T  C  t  t  c  t  -798
        g  g  t  a  c  g  t  g  a  g  a  a  g  a
```

CONSENSUS   **TGC(A/G)CNC**

B

Fig. 3.7 **(A)** Positions and orientations of the putative MREs within the promoter regions of *mnp1* (Godfrey et al., 1990), *mnp2* (Mayfield et al., 1994), and *mnp3* (Alic et al., 1997). The upper and lower lines correspond to the coding strand of the *mnp* genes and the complementary strand, respectively. Numbers refer to the nucleotide positions with respect to the translation start codon. The core MRE consensus sequence (Stuart et al., 1985) for MT is presented for comparison. **(B)** Comparison of promoter elements and intron positions in three *P. chrysosporium mnp* genes (Alic et al., 1997).

To determine the expression profiles of *mnp1*, *mnp2*, and *mnp3* under conditions of nitrogen limitation, cDNA was prepared from RNAs isolated from duplicate cultures on each of days 2–7 following inoculation. Quantitation of these members of the *mnp* gene family via competitive RT-PCR reveals that *mnp1*, *mnp2*, and *mnp3* have distinct temporal transcription patterns under these culture conditions (Fig. 3.5). This data is consistent with previous data on the expression of MnP under liquid culture conditions (Pease et al., 1992). In contrast, RT-PCR of *mnp* transcripts isolated from *P. chrysosporium* grown in soil indicates these *mnp* genes are coordinately regulated (Bogan et al., 1996b). In order to determine if the transcript peak observed for *mnp3* (Fig. 3.5) is related to Mn levels, a temporal expression analysis of nitrogen-limited cultures without Mn will need to be carried out. There is sufficient variability among MRE sequences in the *mnp* promoters (Fig. 3.7), both within the core and the flanking sequences, to allow several levels of MRE-dependent transcription. Additionally, the MREs in cooperation with other non-MRE elements could result in differential metal regulation.

The temporal expression of *mnp1* and *mnp2* confirm our earlier northern blot analysis of *mnp* gene expression (Brown et al., 1991, 1993). However, earlier northern blot analyses did not detect *mnp* message on day 3, as seen here using RT-PCR of *mnp3* (Fig. 3.5) (Brown et al., 1991). The *mnp1* cDNA was used as the probe for these earlier northern blots. It is possible that hybridization of a *mnp1* cDNA probe to *mnp3* RNA is not as strong as the hybridization of *mnp1* cDNA with *mnp1* and *mnp2* RNA. This may explain why the peak of *mnp3* was not detected previously (Brown et al., 1991). The *mnp1* cDNA does not probe the *mnp2* cDNA as strongly as the *mnp1* cDNA (data not shown).

The *gpd* transcript was analyzed in conjunction with the *mnp* transcripts as an internal control. Under limiting nitrogen conditions, *gpd* levels are quite constant. However, under the high nitrogen culture conditions in the absence of Mn, the *gpd* transcript is ~ 10-fold lower. It has been reported that mRNA levels of β-actin and dihydrofolate reductase also are not always constant and may vary with culture conditions (Elder et al., 1988; Schmidt & Merrill, 1991).

We also have demonstrated that competitive RT-PCR can be used to monitor expression of the *mnp2* truncated reporter and the endogenous *mnp2* gene in the pAM2ClaI transformants. Induction by Mn or a 45°C HS of 5-day-old Mn-deficient nitrogen-limited cultures results in an increase in both the endogenous *mnp2* transcript and the truncated *mnp2* transcript (Fig. 3.6). These results confirm our earlier northern blot analysis using the truncated *mnp* gene reporter system (Gettemy et al., 1997) which demonstrated that a truncated *mnp2* reporter is regulated in the same fashion as the endogenous *mnp* genes and, in particular, the endogenous *mnp2* gene.

Competitive RT-PCR as an assay of the reporter gene RNA provides a more accurate method for determining the activity of putative regulatory sequences as compared with northern blot analysis. In addition, competitive PCR measures the transcription product directly, in contrast to standard reporter systems that measure transcription by protein assay, relying on the synthesis and/or secretion of a reporter protein.

The similarities between the Mn regulation of *mnp* gene transcription and other metalloregulatory systems suggest that a Mn-binding transacting transcription factor may be involved. The truncated promoter in conjunction with RT-PCR may now be used to examine the possible roles of the putative MREs in Mn regulation of *mnp* gene transcription with eventual isolation of the transcription factor(s) involved.

CHAPTER 4
CONCLUDING REMARKS

4.1 Summary of Research

The goal of this work was to increase our understanding of the regulation of manganese peroxidase gene expression in the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Specifically, we have developed an homologous truncated gene reporter system to further examine the regulation of two *mnp* genes, *mnp1* and *mnp2*. With this system we have demonstrated that both of the truncated *mnp* genes are regulated in a manner similar to the full-length endogenous *mnp* genes with respect to nitrogen limitation and regulation by Mn, HS, and H_2O_2. Furthermore, we have shown that quantitative RT-PCR is a sensitive and rapid technique for analyzing specific *mnp* genes. Using RT-PCR we have demonstrated that three *mnp* genes are differentially expressed in relationship to factors such as nutrient nitrogen and Mn. Additionally, we have shown that competitive RT-PCR can be used to quantitate the expression of the three MnP genes and the expression of the truncated gene reporter, *M2ClaI*.

4.2 Future Directions

The truncated reporter system, in conjunction with RT-PCR, can now be used to define the specific *cis*-acting sequences required for regulation of *mnp1* and *mnp2* transcription. Once the *cis*-acting sequences are defined, the *trans*-acting factors can be isolated. These components will contribute to our the understanding of *mnp* regulation. Mn regulation of these *mnp* genes, for example, can be explained by either positive or negative regulation. In a negative regulatory model, the repressors

would limit the expression of *mnp* under high nitrogen and low Mn conditions; thus, a shift to low nitrogen and high Mn might dislodge these repressors from the promoter. In a positive regulatory model, high Mn in the presence of low nitrogen might trigger positive-acting transcription factors to bind to the *mnp* promoters and stimulate transcription. The molecular mechanism regulating *mnp* could possibly require a combination of both positive and negative factors.

In order to determine the precise promoter sequences necessary for regulation of *mnp* by nitrogen, Mn, and HS, a deletion series of the promoters of the truncated genes could be constructed. This is similar to the studies conducted on the mouse MT-1 promoter (Stuart et al., 1985). These progressive 5′ to 3′ deletions could be made with the unidirectional exonuclease III method. In addition, 3′ to 5′ promoter deletions could be constructed using exonuclease III from a restriction site just upstream of the *mnp* TATAA element. This deletion series would be sequenced and cloned into the shuttle vector pOGI18 and used to transform *P. chrysosporium*. Truncated gene reporter expression could then be compared to that of the wild type promoter–reporter fusion under various conditions of high and low nitrogen, the absence and presence of Mn, and HS.

The putative MREs and HSEs of *mnp1* and *mnp2* could also be eliminated by deletion or mutation directly by polymerase chain reaction. We are interested in the contribution of the MRE sequence to transcription in the context of native *mnp* promoter sequences, orientation, and distance from the TATAA box and other response elements. The *mnp1* promoter contains two overlapping putative MRE pairs and two single putative MREs, and the *mnp2* promoter contains only one overlapping pair and one single putative MRE. Because of this apparent simplicity, the *mnp2* promoter may be more amenable to mutational analysis. Recently, site-directed mutagenesis with PCR was used to replace the putative MREs in the *mnp2* promoter with a non-related *Spe*I site of the same length, maintaining the proper spacing within the promoter. The mutations have been confirmed by sequencing. Constructs consisting of these mutant promoters and the truncated *mnp2* gene have been cloned into pOGI18 and transformed into the Ade1 strain. Prototrophic transformants have

been screened for expression of the truncated gene following induction with both Mn and HS. Purified transformants will be examined for expression of the truncated *mnp2* gene under various conditions using quantitative RT-PCR.

To determine if the *cis*-acting sequences identified in the above experiments are essential for the regulation of transcription of *mnp* genes, it would be useful to transfer these sequences to a non-inducible promoter and show that this transfer renders the recipient gene inducible. Previous studies of synthetic MREs fused to a minimal promoter were used when investigating the requirement for metal induction of MT transcription in mammalian cells (Searle et al., 1987; Culotta & Hamer, 1989). This experiment could be achieved with the *mnp* promoter elements by linking the Mn-responsive *cis*-acting sequence to the LiP promoter, another secondary metabolic gene, which is not inducible by Mn. If this rendered the LiP gene Mn-responsive following its transformation into *P. chrysosporium*, the role of the sequence could be confirmed, and biochemical methods then could be used to isolate and characterize the relevant *trans*-acting factors.

4.3   Significance and Conclusions

Progress in the above objectives will provide some of the information required for a comprehensive understanding of the detailed mechanisms of *mnp* transcriptional regulation. Elucidation of Mn-regulated gene transcription in *P. chrysosporium* will not only increase our knowledge of metal regulation but also of secondary metabolism. This work also could aid in understanding the relationship between metal ion and HS regulation of genes. Finally, research is needed to study the ability of *P. chrysosporium* to decompose environmental pollutants in soils and effluents. *P. chrysosporium* is exceptionally versatile at degrading pollutants, because it produces a variety of non-specific peroxidases such as MnP which oxidizes a wide variety of toxic aromatics. The understanding of the regulation of genes implicated in pollution degradation would greatly enhance the potential for utilizing this organism in bioremediation applications.

LITERATURE CITED

Akileswaran, L., Alic, M., Clark, E. K., Hornick, J. L., and Gold, M. H. (1993) Isolation and transformation of uracil auxotrophs of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *Curr. Genet.* 23:351–356.

Alam, J., Cai, J., and Smith, A. (1994) Isolation and characterization of the mouse heme oxygenase-1 gene. Distal 5′ sequences are required for induction by heme or heavy metals. *J. Biol. Chem.* 269:1001–1009.

Alic, M., Letzring, C., and Gold, M. H. (1987) Mating system and basidiospore formation in the lignin-degrading basidiomycete *Phaerochaete chrysosporium*. *Appl. Environ. Microbiol.* 53:1464–1469.

Alic, M., Kornegay, J. R., Pribnow, D., and Gold, M. H. (1989) Transformation by complementation of an adenine auxotroph of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 55:406–411.

Alic, M., Clark, E. K., Korngay, J. R., and Gold, M. H. (1990) Transformation of *Phanerochaete chrysosporium* and *Neurospora crassa* with adenine biosynthetic genes from *Schizophyllum commune*. *Curr. Genet.* 17:305–311.

Alic, M., Mayfield, M. B., Akileswaran, L., and Gold, M. H. (1991) Homologous transformation of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *Curr. Genet.* 19:491–494.

Alic, M., Akileswaran, L., and Gold, M. H. (1997) Characterization of the gene encoding manganese peroxidase isozyme 3 from *Phanerochaete chrysosporium*. *Biochim. Biophys. Acta* 1338:1–7.

Archibald, F. S. and Duong, M. N. (1986) Superoxide dismutase and oxygen toxicity defenses in the genus *Neisseria*. *Infect. Immunol.* 51:631–641.

Auling, G. (1983) The effect of manganese limitation on DNA precursor biosynthesis during nucleotide fermentation with *Brevibacterium ammoniagenes* and *Micrococcus luteus*. *Eur. J. Appl. Microbiol. Biotechnol.* 18:229–235.

Bao, W., Fukushima, Y., Jensen, K. A., Jr., Moen, M. A., and Hammel, K. E. (1994) Oxidative degradation of non-phenolic lignin during lipid peroxidation by fungal manganese peroxidase. *FEBS Lett.* 354:297–300.

Bogan, B. W. and Lamar, R. T. (1996) Polycyclic aromatic hydrocarbon-degrading capabilities of *Phanerochaete laevis* HHB-1625 and its extracellular ligninolytic enzymes. *Appl. Environ. Microbiol.* 62:1597–1603.

Bogan, B. W., Schoenike, B., Lamar, R. T., and Cullen, D. (1996a) Expression of *lip* genes during growth in soil and oxidaion of anthracene by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 62:3697–3703.

Bogan, B. W., Schoenike, B., Lamar, R. T., and Cullen, D. (1996b) Manganese peroxidase mRNA and enzyme activity levels during bioremediation of polycyclic aromatic hydrocarbon-contaminated soil with *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 62:2381–2386.

Bonnarme, P. and Jeffries, T. W. (1990) Mn(II) regulation of lignin peroxidases and manganese-dependent peroxidases from lignin-degrading white-rot fungi. *Appl. Environ. Microbiol.* 56:210–217.

Boominathan, K. and Reddy, C. A. (1992) cAMP-mediated differential regulation of lignin peroxidase and manganese-dependent peroxidase production in the white-rot basidiomycete *Phanerochaete chrysosporium*. *Proc. Natl. Acad. Sci. U.S.A.* 89:5586–5590.

Boominathan, K., Dass, S. B., Randall, T. A., and Reddy, C. A. (1990) Nitrogen-deregulated mutants of *Phanerochaete chrysosporium*—a lignin-degrading basidiomycete. *Arch. Microbiol.* 153:521–527.

Boominathan, K., D'Souza, T. M., Naidu, P. S., Dosoretz, C., and Reddy, C. A. (1993) Temporal expression of the major lignin peroxidase genes of *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 59:3946–3950.

Broda, P., Birch, P. R., Brooks, P. R., and Sims, P. F. (1995) PCR-mediated analysis of lignocellulolytic gene transcription by *Phanerochaete chrysosporium*: substrate-dependent differential expression within gene families. *Appl. Environ. Microbiol.* 61:2358–2364.

Broda, P., Birch, P. R., Brooks, P. R., and Sims, P. F. (1996) Lignocellulose degradation by *Phanerochaete chrysosporium*: gene families and gene expression for a complex process. *Mol. Microbiol.* 19:923–932.

Brooks, P., Sims, P., and Broda, P. (1993) Isozyme specific polymerase chain reaction analysis of differential gene expression: a general method applied to lignin peroxidase genes of *Phanerochaete chrysosporium*. *Biotechnology N. Y.* 11:830–834.

Brown, J. A., Glenn, J. K., and Gold, M. H. (1990) Manganese regulates expression of manganese peroxidase by *Phanerochaete chrysosporium*. *J. Bacteriol.* 172:3125–3130.

Brown, J. A., Alic, M., and Gold, M. H. (1991) Manganese peroxidase gene transcription in *Phanerochaete chrysosporium*: activation by manganese. *J. Bacteriol.* 173:4101–4106.

Brown, J. A., Li, D., Alic, M., and Gold, M. H. (1993) Heat shock induction of manganese peroxidase gene transcription in *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 59:4295–4299.

Brugnera, E., Georgiev, O., Radtke, F., Heuchel, R., Baker, E., Sutherland, G. R., and Schaffner, W. (1994) Cloning, chromosomal mapping and characterization of the human metal-regulatory transcription factor MTF-1. *Nucleic Acids Res.* 22:3167–3173.

Bumpus, J. A. and Aust, S. D. (1987) Biodegradation of DDT [1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane] by the white rot fungus *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 53:2001–2008.

Buswell, J. A. and Odier, E. (1987) Lignin degradation. *Crit. Rev. Biotechnol.* 6:1–60.

Byrne, B. C., Li, J. J., Sninsky, J., and Poiesz, B. J. (1988) Detection of HIV-1 RNA sequences by *in vitro* DNA amplification. *Nucleic Acids Res.* 16:4165.

Choi, A. M. and Alam, J. (1996) Heme oxygenase-1: function, regulation, and implication of a novel stress-inducible protein in oxidant-induced lung injury. *Am. J. Respir. Cell Mol. Biol.* 15:9–19.

Clos, J., Westwood, J. T., Becker, P. B., Wilson, S., Lambert, K., and Wu, C. (1990) Molecular cloning and expression of a hexameric *Drosophila* heat shock factor subject to negative regulation. *Cell* 63:1085–1097.

Covert, S. F., Vanden Wymelenberg, A., and Cullen, D. (1992) Structure, organization, and transcription of a cellobiohydrolase gene cluster from *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 58:2168–2175.

Cowling, E. B. and Merrill, W. (1966) Nitrogen in wood and its role in wood deterioration. *Can. J. Bot.* 44:1539–1554.

Crawford, R. L. (1981) *Lignin Biodegradation and Transformation,* Wiley-Interscience, New York.

Culotta, V. C. and Hamer, D. H. (1989) Fine mapping of a mouse metallothionein gene metal response element. *Mol. Cell. Biol.* 9:1376–1380.

Dalton, T., Palmiter, R. D., and Andrews, G. K. (1994) Transcriptional induction of the mouse metallothionein-I gene in hydrogen peroxide-treated Hepa cells involves a composite major late transcription factor/antioxidant response element and metal response promoter elements. *Nucleic Acids Res.* 22:5016–5023.

Dalton, T. P., Li, Q., Bittel, D., Liang, L., and Andrews, G. K. (1996) Oxidative stress activates metal-responsive transcription factor-1 binding activity. Occupancy *in vivo* of metal response elements in the metallothionein-I gene promoter. *J. Biol. Chem.* 271:26233–26241.

Datta, A., Bettermann, A., and Kirk, T. K. (1991) Identification of a specific manganese peroxidase among ligninolytic enzymes secreted by *Phanerochaete chrysosporium* during wood decay. *Appl. Environ. Microbiol.* 57:1453–1460.

Elder, P. K., French, C. L., Subramaniam, M., Schmidt, L. J., and Getz, M. J. (1988) Evidence that the functional β-actin gene is single copy in most mice and is associated with 5′ sequences capable of conferring serum- and cycloheximide-dependent regulation. *Mol. Cell. Biol.* 8:480–485.

Eriksson, K. E. L. (1990) Biotechnology in the pulp and paper industry. *Wood Sci. Technol.* 24:79–101.

Fenn, P. and Kirk, T. K. (1981) Relationship of nitrogen to the onset and suppression of ligninolytic activity and secondary metabolism in *Phanerochaete chrysosporium.* *Arch. Microbiol.* 130:59–65.

Frohman, M. A., Dush, M. K., and Martin, G. R. (1988) Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. U.S.A.* 85:8998–9002.

Gadd, G. M. and Laurence, O. S. (1996) Demonstration of high-affinity Mn^{2+} uptake in *Saccharomyces cerevisiae*: specificity and kinetics. *Microbiology* 142:1159–1167.

Gallo, G. J., Prentice, H., and Kingston, R. E. (1993) Heat shock factor is required for growth at normal temperatures in the fission yeast *Schizosaccharomyces pombe.* *Mol. Cell. Biol.* 13:749–761.

Garraway, M. O. and Evans, R. C. (1984) *Fungal Nutrition and Physiology*, Wiley, New York.

Gettemy, J. M., Li, D., Alic, M., and Gold, M. H. (1997) Truncated-gene reporter system for studying the regulation of manganese peroxidase expression. *Curr. Genet.* 31:519–524.

Gilliland, G., Perrin, S., and Bunn, H. F. (1990) *Competitive PCR for Quantitation of mRNA*, Academic Press, San Diego.

Glenn, J. K. and Gold, M. H. (1985) Purification and characterization of an extracellular Mn(II)-dependent peroxidase from the lignin-degrading basidiomycete, *Phanerochaete chrysosporium. Arch. Biochem. Biophys.* 242:329–341.

Glenn, J. K., Akileswaran, L., and Gold, M. H. (1986) Mn(II) oxidation is the principal function of the extracellular Mn-peroxidase from *Phanerochaete chrysosporium. Arch. Biochem. Biophys.* 251:688–696.

Godfrey, B. J., Mayfield, M. B., Brown, J. A., and Gold, M. H. (1990) Characterization of a gene encoding a manganese peroxidase from *Phanerochaete chrysosporium. Gene* 93:119–124.

Godfrey, B. J., Akileswaran, L., and Gold, M. H. (1994) A reporter gene construct for studying the regulation of manganese peroxidase gene expression. *Appl. Environ. Microbiol.* 60:1353–1358.

Gold, M. H. and Alic, M. (1993) Molecular biology of the lignin-degrading basidiomycete *Phanerochaete chrysosporium. Microbiol. Rev.* 57:605–622.

Gold, M. H., Cheng, T. M., and Mayfield, M. B. (1982) Isolation of complementation studies of auxotrophic mutants of the lignin-degrading basidiomycete *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* 44:996–1000.

Gold, M. H., Kuwahara, M., Chiu, A. A., and Glenn, J. K. (1984) Purification and characterization of an extracellular H_2O_2-requiring diarylpropane oxygenase from the white rot basidiomycete, *Phanerochaete chrysosporium. Arch. Biochem. Biophys.* 234:353–362.

Gold, M. H., Wariishi, H., and Valli, K. (1989) Extracellular peroxidases involved in lignin degradation by the white rot basidiomycete *Phanerochaete chrysosporium. ACS Symp. Ser.* 389:127–140.

Goldstein, G. W. (1993) Evidence that lead acts as a calcium substitute in second messenger metabolism. *Neurotoxicology* 14:97–101.

Hammel, K. E., Tardone, P. J., Moen, M. A., and Price, L. A. (1989) Biomimetic oxidation of nonphenolic lignin models by Mn(III): new observations on the

oxidizability of guaiacyl and syringyl substructures. *Arch. Biochem. Biophys.* 270:404–409.

Hockertz, S., Schmid, J., and Auling, G. (1987) A specific transport system for manganese in the filamentous fungus *Aspergillus niger. J. Gen. Microbiol.* 133:3513–3519.

Holzbaur, E. L., Andrawis, A., and Tien, M. (1988) Structure and regulation of a lignin peroxidase gene from *Phanerochaete chrysosporium. Biochem. Biophys. Res. Commun.* 155:626–633. (Erratum 157:417)

Imbert, J., Culotta, V., Furst, P., Gedamu, L., and Hamer, D. (1990) Regulation of metallothionein gene transcription by metals. In *Metal-Ion Induced Regulation of Gene Expression* (Eichhorn, G. L. and Marzilli, L. G., ed.), Vol. 3, pp. 139–164, Elsevier, New York.

Janssen, K. (1987) Single-step RNA isolation from cultured cells or tissues. In *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. ed.), pp. 4.2.4–4.2.6. Vol. 2, Greene Publishing Assoc., Brooklyn, NY, and John Wiley, Media, PA.

Jeffries, T. W., Choi, S., and Kirk, T. K. (1981) Nutritional regulation of lignin degradation by *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* 42:290–296.

Joshi, D. K. and Gold, M. H. (1993) Degradation of 2,4,5-trichlorophenol by the lignin-degrading basidiomycete *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* 59:1779–1785.

Kapoor, M., Sreenivasan, G. M., Goel, N., and Lewis, J. (1990) Development of thermotolerance in *Neurospora crassa* by heat shock and other stresses eliciting peroxidase induction. *J. Bacteriol.* 172:2798–2801.

Karin, M., Andersen, R. D., Slater, E., Smith, K., and Herschman, H. R. (1980) Metallothionein mRNA induction in HeLa cells in response to zinc or dexamethasone is a primary induction response. *Nature* 286:295–297.

Kirk, T. K. and Farrell, R. L. (1987) Enzymatic "combustion": the microbial degradation of lignin. *Annu. Rev. Microbiol.* 41:465–505.

Kirk, T. K., Schultz, E., Connors, W. J., Lorenz, L. F., and Zeikus, J. G. (1978) Influence of culture parameters on lignin metabolism by *Phanerochaete chrysosporium. Arch. Microbiol.* 117:277–285.

Kishi, K., Wariishi, H., Marquez, L., Dunford, H. B., and Gold, M. H. (1994) Mechanism of manganese peroxidase compound II reduction. Effect of organic acid chelators and pH. *Biochemistry* 33:8694–8701.

Kishi, K., Kusters-van Someren, M., Mayfield, M. B., Sun, J., Loehr, T. M., and Gold, M. H. (1996) Characterization of manganese(II) binding site mutants of manganese peroxidase. *Biochemistry* 35:8986–8994.

Kroeger, P. E. and Morimoto, R. I. (1994) Selection of new HSF1 and HSF2 DNA-binding sites reveals difference in trimer cooperativity. *Mol. Cell. Biol.* 14:7592–7603.

Kuan, I. C. and Tien, M. (1993a) Glyoxylate-supported reactions catalyzed by Mn peroxidase of *Phanerochaete chrysosporium*: activity in the absence of added hydrogen peroxide. *Arch. Biochem. Biophys.* 302:447–454.

Kuan, I. C. and Tien, M. (1993b) Stimulation of Mn peroxidase activity: a possible role for oxalate in lignin biodegradation. *Proc. Natl. Acad. Sci. U.S.A.* 90:1242–1246.

Kusters-van Someren, M., Kishi, K., Lundell, T., and Gold, M. H. (1995) The manganese binding site of manganese peroxidase: characterization of an Asp179Asn site-directed mutant protein. *Biochemistry* 34:10620–10627.

Lamar, R. T., Schoenike, B., Vanden Wymelenberg, A., Stewart, P., Dietrich, D. M., and Cullen, D. (1995) Quantitation of fungal mRNAs in complex substrates by reverse transcription PCR and its application to *Phanerochaete chrysosporium*-colonized soil. *Appl. Environ. Microbiol.* 61:2122–2126.

Leisola, M. S., Schmidt, B., and Fiechter, A. (1986) Enzymatic determination of veratryl alcohol. *Anal. Biochem.* 155:108–111.

Li, D., Alic, M., and Gold, M. H. (1994) Nitrogen regulation of lignin peroxidase gene transcription. *Appl. Environ. Microbiol.* 60:3447–3449.

Li, D., Alic, M., Brown, J. A., and Gold, M. H. (1995) Regulation of manganese peroxidase gene transcription by hydrogen peroxide, chemical stress, and molecular oxygen. *Appl. Environ. Microbiol.* 61:341–345.

Lindquist, S. (1986) The heat-shock response. *Annu. Rev. Biochem.* 55:1151–1191.

MacDonald, M. J., Paterson, A., and Broda, P. (1984) Possible relationship between cyclic AMP and idiophasic metabolism in the white rot fungus *Phanerochaete chrysosporium*. *J. Bacteriol.* 160:470–472.

Mager, W. H. and De Kruijff, A. J. (1995) Stress-induced transcriptional activation. *Microbiol. Rev.* 59:506–531.

Marquez, L., Wariishi, H., Dunford, H. B., and Gold, M. H. (1988) Spectroscopic and kinetic properties of the oxidized intermediates of lignin peroxidase from *Phanerochaete chrysosporium. J. Biol. Chem.* 263:10549–10552.

Mayfield, M. B., Godfrey, B. J., and Gold, M. H. (1994a) Characterization of the *mnp2* gene encoding manganese peroxidase isozyme 2 from the basidiomycete *Phanerochaete chrysosporium. Gene* 142:231–235.

Mayfield, M. B., Kishi, K., Alic, M., and Gold, M. H. (1994b) Homologous expression of recombinant manganese peroxidase in *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* 60:4303–4309.

Morimoto, R. I. (1993) Cells in stress: transcriptional activation of heat shock genes. *Science* 259:1409–1410.

Neilands, J. B. (1990) Molecular aspects of regulation of high affinity iron absorption in microorganisms. *Adv. Inorg. Biochem.* 8:63–90.

O'Halloran, T. V. (1989) Metalloregulatory proteins: metal-responsive molecular switches governing gene expression. In *Metal Ions in Biological Systems* (Sigel, H. ed.), Vol. 25, pp. 105–146, Marcel Dekker, New York.

O'Halloran, T. V. (1993) Transition metals in control of gene expression. *Science* 261:715–725.

Orth, A. B., Rzhetskaya, M., Cullen, D., and Tien, M. (1994) Characterization of a cDNA encoding a manganese peroxidase from *Phanerochaete chrysosporium*: genomic organization of lignin and manganese peroxidase-encoding genes. *Gene* 148:161–165.

Palmiter, R. D. (1994) Regulation of metallothionein genes by heavy metals appears to be mediated by a zinc-sensitive inhibitor that interacts with a constitutively active transcription factor, MTF-1. *Proc. Natl. Acad. Sci. U.S.A.* 91:1219–1223.

Parkin, M. J. and Ross, I. S. (1986) The specific uptake of manganese in the yeast *Candida utilis. J. Gen. Microbiol.* 132:2155–2160.

Patrusky, B. (1990) A biological imperative. *Natl. Sci. Found. Mosaic* 3:2–11.

Pease, E. A. and Tien, M. (1992) Heterogeneity and regulation of manganese peroxidases from *Phanerochaete chrysosporium. J. Bacteriol.* 174:3532–3540.

Pease, E. A., Andrawis, A., Tien, M. (1989) Manganese-dependent peroxidase from *Phanerochaete chrysosporium*. Primary structure deduced from cDNA sequence. *J. Biol. Chem.* 264:13531–13535.

Pelham, H. R. (1982) A regulatory upstream promoter element in the Drosophila hsp 70 heat-shock gene. *Cell* 30:517–528.

Périé, F. H. and Gold, M. H. (1991) Manganese regulation of manganese peroxidase expression and lignin degradation by the white rot fungus *Dichomitus squalens*. *Appl. Environ. Microbiol.* 57:2240–2245.

Pribnow, D. G, Mayfield, M. B., Nipper, V. J., Brown, J. A., Gold, M. H. (1989) Characterization of a cDNA encoding a manganese peroxidase, from the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *J. Biol. Chem.* 264:5036–5040.

Radtke, F., Heuchel, R., Georgiev, O., Hergersberg, M., Gariglio, M., Dembic, Z., and Schaffner, W. (1993) Cloned transcription factor MTF-1 activates the mouse metallothionein I promoter. *EMBO J.* 12:1355–1362.

Ralston, D. M. and O'Halloran, T. V. (1990) Metalloregulatory proteins and molecular mechanisms of heavy metal signal transduction. *Adv. Inorg. Biochem.* 8:1–31.

Reiser, J., Walther, I. S., Fraefel, C., and Fiechter, A. (1993) Methods to investigate the expression of lignin peroxidase genes by the white rot fungus *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 59:2897–2903.

Reiser, J., Muheim, A., Hardegger, M., Frank, G., and Fiechter, A. (1994) Aryl-alcohol dehydrogenase from the white-rot fungus *Phanerochaete chrysosporium*. Gene cloning, sequence analysis, expression, and purification of the recombinant enzyme. *J. Biol. Chem.* 269:28152–28159.

Renganathan, V., Miki, K., and Gold, M. H. (1986) Role of molecular oxygen in lignin peroxidase reactions. *Arch. Biochem. Biophys.* 246:155–161.

Samson, S. L. and Gedamu, L. (1995) Metal-responsive elements of the rainbow trout metallothionein-B gene function for basal and metal-induced activity. *J. Biol. Chem.* 270:6864–6871.

Schmidt, E. E. and Merrill, G. F. (1991) Changes in dihydrofolate reductase (DHFR) mRNA levels can account fully for changes in DHFR synthesis rates during terminal differentiation in a highly amplified myogenic cell line. *Mol. Cell. Biol.* 11:3726–3734.

Schoemaker, H. E. (1990) On the chemistry of lignin biodegradation. *Recl. Trav. Chim. Pays-Bas* 109:255–272.

Schuetz, T. J., Gallo, G. J., Sheldon, L., Tempst, P., and Kingston, R. E. (1991) Isolation of a cDNA for HSF2: evidence for two heat shock factor genes in humans. *Proc. Natl. Acad. Sci. U.S.A.* 88:6911–6915.

Schuren, F. H. J., Harmsen, M. C., and Wessels, J. G. H. (1993) A homologous gene-reporter system for the basidiomycete *Schizophyllum commune* based on internally deleted homologous genes. *Mol. Gen. Genet.* 238:91–96.

Scott, R. E., Jones, A., and Gaucher, G. M. (1986) Manganese and antibiotic biosynthesis. III. The site of manganese control of patulin production in *Penicillium urticae. Can. J. Microbiol.* 32:273–279.

Searle, P. F., Stuart, G. W., and Palmiter, R. D. (1987) Metal regulatory elements of the mouse metallothionein-I gene. *EXS* 52:407–414.

Sheldon, L. A. and Kingston, R. E. (1993) Hydrophobic coiled-coil domains regulate the subcellular localization of human heat shock factor 2. *Genes Dev.* 7:1549–1558.

Sooknanan, R., Malek, L., Wang, X. H., Siebert, T., and Keating, A. (1993) Detection and direct sequence identification of BCR-ABL mRNA in Ph[+] chronic myeloid leukemia. *Exp. Hematol.* 21:1719–1724.

Sorger, P. K. and Pelham, H. R. (1988) Yeast heat shock factor is an essential DNA-binding protein that exhibits temperature-dependent phosphorylation. *Cell* 54:855–864.

Stewart, P., Kersten, P., Vanden Wymelenberg, A., Gaskell, J., and Cullen, D. (1992) Lignin peroxidase gene family of *Phanerochaete chrysosporium*: complex regulation by carbon and nitrogen limitation and identification of a second dimorphic chromosome. *J. Bacteriol.* 174:5036–5042.

Stuart, G. W., Searle, P. F., and Palmiter, R. D. (1985) Identification of multiple metal regulatory elements in mouse metallothionein-I promoter by assaying synthetic sequences. *Nature* 317:828–831.

Sundaramoorthy, M., Kishi, K., Gold, M. H., and Poulos, T. L. (1994) The crystal structure of manganese peroxidase from *Phanerochaete chrysosporium* at 2.06-Å resolution. *J. Biol. Chem.* 269:32759–32767.

Tamai, K. T., Gralla, E. B., Ellerby, L. M., Valentine, J. S., and Thiele, D. J. (1993) Yeast and mammalian metallothioneins functionally substitute for yeast copper-zinc superoxide dismutase. *Proc. Natl. Acad. Sci. U.S.A.* 90:8013–8017.

Tempelaars, C. A., Birch, P. R., Sims, P. F., and Broda, P. (1994) Isolation, characterization, and analysis of the expression of the *cbhII* gene of *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 60:4387–4393.

Thiele, D. J. (1992) Metal-regulated transcription in eukaryotes. *Nucleic Acids Res.* 20:1183–1191.

Tien, M. and Myer, S. B. (1990) Selection and characterization of mutants of *Phanerochaete chrysosporium* exhibiting ligninolytic activity under nutrient-rich conditions. *Appl. Environ. Microbiol.* 56:2540–2544.

Tien, M. and Tu, C.-P. D. (1987) Cloning and sequencing of a cDNA for a ligninase from *Phanerochaete chrysosporium*. *Nature (London)* 326:520–523.

Valli, K., Wariishi, H., and Gold, M. H. (1992) Degradation of 2,7-dichlorodibenzo-*p*-dioxin by the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *J. Bacteriol.* 174:2131–2137.

Wang, A. M., Doyle, M. V., and Mark, D. F. (1989) Quantitation of mRNA by the polymerase chain reaction. *Proc. Natl. Acad. Sci. U.S.A.* 86:9717–9721.

Wariishi, H., Akileswaran, L., and Gold, M. H. (1988) Manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*: spectral characterization of the oxidized states and the catalytic cycle. *Biochemistry* 27:5365–5370.

Wariishi, H., Dunford, H. B., MacDonald, I. D., and Gold, M. H. (1989) Manganese peroxidase from the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Transient-state kinetics and reaction mechanism. *J. Biol. Chem.* 264:3335–3340.

Wariishi, H., Valli, K., and Gold, M. H. (1992) Manganese(II) oxidation by manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*: kinetic mechanism and role of chelators. *J. Biol. Chem.* 267:23688–23695.

Weinberg, E. D. (1982) Biosynthesis of microbial metabolites—regulation by mineral elements and temperature. In *Overproduction of Microbial Products* (Krumphanzl, V., Sikyt, B., and Vanck, Z., eds.), pp. 181–194, Academic Press, London.

Wiederrecht, G., Seto, D., and Parker, C. S. (1988) Isolation of the gene encoding the *S. cerevisiae* heat shock transcription factor. *Cell* 54:841–853.

Williams, R. J. P. (1982) Free manganese (II) and iron (II) cations can act as intracellular cell controls. *FEBS Lett.* 140:3–10.

Xiao, H., Perisic, O., and Lis, J. T. (1991) Cooperative binding of Drosophila heat shock factor to arrays of a conserved 5 bp unit. *Cell* 64:585–593.

Young, M. R. and Craig, E. A. (1993) *Saccharomyces cerevisiae* HSP70 heat shock elements are functionally distinct. *Mol. Cell. Biol.* 13:5637–5646.

Zhu, Z. and Thiele, D. J. (1996) Toxic metal-responsive gene transcription. *EXS* 77:307–320.

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The author's publication list includes the following:

Kishi, K. Hildenbrand, D. P., Kuster-van-Someren, M., Gettemy, J., Mauk, A. G., and Gold, M. H. (1997) Site-directed mutations at phenylalanine-190 of manganese peroxidase: effects on stability, function, and coordination. *Biochemistry* **36,** 4268–4277.

Gettemy, J. M., Li, D., Alic, M., and Gold, M. H. (1997) Truncated-gene reporter system for studying the regulation of manganese peroxidase expression. Submitted to *Curr. Genet.*