A Promoter-Reporter Construct for Studying the Regulation of Manganese Peroxidase Gene Expression in *Phanerochaete chrysosporium*

Bruce John Godfrey
B.S., University of Oregon, 1983

A dissertation submitted to the faculty of
the Oregon Graduate Institute of Science & Technology
in partial fulfillment of the
requirements for the degree
Doctor of Philosophy
in
Molecular Biology

July, 1996

The dissertation "A Promoter-Reporter Construct for Studying the Regulation of Manganese Peroxidase Gene Regulation in *Phanerochaete chrysosporium*" by Bruce J. Godfrey has been examined and approved by the following Examination Committee:

Michael H. Gold, Thesis Advisor
Professor

James M. Gregg
Professor

Matthew S. Sachs
Assistant Professor

David R. Boone
Professor

DEDICATION

This thesis is dedicated to the pursuit of imagination in learning and science, both of which are becoming increasingly difficult in this time of budget cuts and practical justification in our society's scientific and educational institutions, and to those who persevere in this course. May they succeed beyond our wildest dreams.

ACKNOWLEDGMENTS

My advisor, Dr. Michael H. Gold, made this research and degree possible, with his confidence in me and his encouragement. His attention to detail in writing and oral presentations set a good example for all of his students.

I would like to acknowledge my colleagues in the Gold lab, especially Mary Mayfield and Lakshmi Akileswaran, whose contributions of labor and experience were instrumental in carrying this research to completion. I would also like to acknowledge the rest of the members of the Gold lab during this time—Dave Pribnow, Margaret Alic, Julie Brown, Khadar Valli, Tom Ritch, Valerie Nipper, Barry Brock, Dinesh Joshi, Urs Tuor, Hiroyuki Wariishi, Li Dan, T. C. Sheng, and Katsu Kishi. I received useful advice and ideas from all of these people. I also obtained some wonderful ethnic recipes from some of them. Julie Brown and Dave Pribnow, in particular, laid the foundations for my work in their own projects. I must also acknowledge my departmental committee members, Drs. Matthew Sachs and James Cregg, for all their valuable advice, teaching, and the occasional use of their equipment.

Computers played an important role in carrying out this research. I started in graduate school largely computer illiterate, and I owe many thanks to Tom Ritch for getting me started as a knowledgeable computer user and to Claude Errera for teaching me more about their use and much about their internal workings and maintenance. I also owe many thanks to Doug Davis, the OGI machinist, who taught me a great deal about machining and constructing equipment.

Finally, I must thank those closest to me for their support and encouragement through graduate school—my parents and sister for encouraging my interests while I was young and then helping me our whenever necessary while I was in school. Finally, I owe much to my wife, Laurie, who I met at OGI, for her proofreading and innumerable other forms of support and encouragement during this tremendously busy time.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

A PROMOTER-REPORTER CONSTRUCT FOR STUDYING THE REGULATION OF MANGANESE PEROXIDASE GENE EXPRESSION IN *PHANEROCHAETE CHRYSOSPORIUM*

Bruce J. Godfrey, Ph.D.

Supervising Professor: Michael H. Gold

Three major factors control the expression of the *Phanerochaete chrysosporium* manganese peroxidase (*mnp*) gene. Nutrient nitrogen availability controls the onset of idiophasic growth. Once idiophase is established, the other two factors, Mn^{II} and heat shock, act as inducers of *mnp* transcription. In many other metal-regulated genes, the transcriptional response to metal induction has been traced to DNA sequence elements in the respective promoters using promoter-reporter gene fusion experiments.

To determine whether Mn^{II}-responsive *cis*-acting sequence elements are present in the *mnp* promoter, genomic clones encoding MnP isozymes 1 and 2 were isolated and sequenced. Consensus metal response elements (MREs) were found in the 5′ upstream regions of both the *mnp1* and *mnp2* genes. The putative MRE sequences present in the *mnp* genes are identical to the consensus MRE sequences found in mammalian metallothionein genes.

A 1500-bp fragment of the *mnp1* promoter was fused to the coding region of a reporter gene [orotidylate decarboxylase (ODase)] in plasmid pAMO. This promoter-reporter fusion construct was used to transform a *P. chrysosporium* mutant lacking endogenous ODase activity. ODase activity was detected in transformants only during idiophasic growth, and the pattern of ODase expression was similar to that of

endogenous MnP. ODase activity and MnP activity were induced synchronously by addition of Mn^{II} to nitrogen-limited, Mn-deficient cultures. Growth in high-nitrogen medium suppressed Mn^{II} induction of both the ODase and endogenous MnP genes.

A 750-bp fragment of the *mnp1* promoter, fused to the coding region of the ODase gene, pAMO-*Eag*, was also regulated by Mn^{II} and nutrient nitrogen in the same manner as both the endogenous *mnp1* gene and the construct containing 1500-bp of *mnp1* promoter sequence. Transformants containing pAMO-*Eag* constructs with either point mutations or linker insertion mutations in the MRE sequences were examined for Mn^{II} induction of reporter gene expression. Mn^{II}-dependent expression of the reporter was reduced in transformants of pAMO-*Eag* containing either type of mutation. These results demonstrate a role of the MRE sequences in mediating the transcriptional effect of Mn^{II} on *mnp* gene expression in *P. chrysosporium*.

CHAPTER 1
INTRODUCTION

1.1 Lignin Degradation by White-Rot Fungi

White-rot basidiomycete fungi are the only organisms known to be capable of extensively mineralizing lignin in pure culture. The white-rot basidiomycete *Phanerochaete chrysosporium* is the most intensively studied wood-rotting fungus. *P. chrysosporium* has served as the laboratory model white-rot basidiomycete due to its high rate of lignin degradation (105) and to its ability to grow at higher temperatures than most basidiomycetes. *P. chrysosporium* is capable of degrading all of the polymers in wood (92, 106). Most laboratory studies of biological lignin degradation have made use of one of two strains of *P. chrysosporium*, BKM-F-1767 and BKM derivative OGC101, and ME446.

The biochemistry of lignin degradation by *P. chrysosporium* has been reviewed elsewhere (37, 74, 105). Briefly, the lignin degradative system of this fungus consists primarily of two families of extracellular peroxidases, each containing several isozyme forms, and a hydrogen peroxide-generating system (71, 105, 111, 189). The peroxidases, lignin peroxidase (LiP) and manganese peroxidase (MnP), each oxidize a different group of substructures in lignin in one-electron steps, forming cation radicals and phenoxy radicals respectively. Ensuing reactions leading to the mineralization of lignin include nonenzymatic free radical reactions with water, protons, and oxygen, and enzyme-catalyzed steps involving further oxidations and reductions of reaction intermediates (80, 105, 194, 199, 206). The hydrogen peroxide-generating enzymes include the extracellular glyoxal oxidase (103) and may also include glucose-2-oxidase and methanol oxidase (105).

1.2 Physiology and Regulation of Lignin Degradation

The expression of the lignin degradative system of *P. chrysosporium* occurs in response to nitrogen starvation (98, 104, 106, 161). Primary microbial growth ceases when an essential macronutrient becomes depleted. Thus, lignin degradation by *P. chrysosporium* is thought to occur during secondary metabolic, or idiophasic, growth. It has been shown that lignin degradation can also be induced by limitation of carbon or sulfur, but not by phosphorous (98). Lignin alone cannot serve as a growth substrate; a cosubstrate is required (112, 197). The rate of lignin degradation by *P. chrysosporium* under conditions of carbon limitation is relatively slow, presumably due to the shortage of energy-producing cosubstrates (54).

The addition of excess nitrogen to ligninolytic cultures represses lignin degradation and restores primary growth (56, 105). The suppression of ligninolytic activity by addition of ammonium or glutamate occurs in cultures grown on either glucose or glycerol (56). Ligninolytic activity appears in cultures grown with glucose or glycerol as carbon sources, though ligninolytic activity is higher in cultures grown on glucose than on other carbon sources (56). The addition of glucose to ligninolytic cultures grown on glycerol does not suppress ligninolytic activity (56). Thus, lignin degradation appears to be under the control of a nitrogen-dependent regulatory mechanism, and carbon catabolite repression does not appear to be involved. However, there is some evidence that nitrogen regulation may be strain-dependent (36) and that it may depend on the available carbon source (166, 193).

The mechanism through which nitrogen levels affect the transition to idiophase and the induction of LiPs and MnPs has been sought in biochemical studies focused on the ubiquitous transducer molecule, cAMP. MacDonald et al. monitored the changes in adenylate cyclase activity, intracellular cAMP concentration, and extracellular cAMP in *P. chrysosporium* cultures entering idiophase (120). Intracellular cAMP was observed to increase as the cultures became ligninolytic. The activity of adenylate cyclase was found to increase about 4.5-fold in the cytoplasmic fraction at the same time as the intracellular cAMP level was observed to rise (119). No phosphodiesterase activity was detected, but extracellular cAMP accumulated

logarithmically during primary growth and remained at a constant low level during idiophase (119). Thus, it appears that intracellular cAMP levels are regulated by a combination of synthesis by adenylate cyclase and secretion, and that a rise in the intracellular concentration is correlated with the onset of ligninolytic activity.

In addition to nitrogen regulation, Mn^{II} is known to regulate the expression of the MnPs (32, 98, 147). The effect of Mn^{II} has been shown to be at the level of transcription (30). Oxygen is also known to affect the titres of ligninolytic enzymes in *P. chrysosporium* cultures (15, 54, 115). Heat shock (31) and various forms of chemical stress (115) have also been shown to induce MnP transcription. The addition of veratryl alcohol (3,4-dimethoxybenzyl alcohol), a secondary metabolite of *P. chrysosporium* and many other white-rot fungi, or benzyl alcohol to nitrogen-limited cultures results in an increased LiP titre in the culture fluid (54). Veratryl alcohol appears not to act as an inducer of the lignin-degrading system (39), as it has been shown to protect LiP from inactivation by excess hydrogen peroxide (199), so it could be stabilizing LiP protein.

1.3 Peroxidase Expression Studies

The Mn^{II} requirement of MnP and the differences in substrate specificity between MnP and LiP make it possible to distinguish between the two peroxidases in enzyme assays. Nitrogen-deregulated mutants of *P. chrysosporium* have been obtained using polymeric dyes, which act as substrates for the lignin-degrading system, as the screen (69, 78). Nitrogen-deregulated strains, which can degrade lignin on nitrogen-rich media, include one that is LiP^-, MnP^+ (27). This indicates that the regulation of these two enzymes with respect to nitrogen can be uncoupled.

The cloning of cDNAs and genomic sequences encoding several LiPs and two MnPs has allowed the primary sequences of these enzymes to be deduced and has made possible the studies of their expression at the molecular level (reviewed in ref. 74). The effects of various inducers have now been observed on the transcription of the peroxidase genes using cDNA probes of RNA blots. The promoters of these genes can now be analyzed and cis-acting sequences involved in their responses to

activators like Mn^{II} can be identified. Ultimately sequence-specific DNA-binding proteins, which mediate the effect of the inducers on transcription, can be identified.

1.3.1 Lignin peroxidases

The LiP isozymes of *P. chrysosporium* are encoded by a family of closely related genes which all cross-hybridize to some extent on Southern blots. The first cDNA was cloned from BKM-F-1767, encoding the highly expressed isozyme H8 (211, 213). Since then, three cDNAs encoding isozyme H8 (9, 165, 191) and about 10 genomic clones of H8 (9, 65, 93, 94, 136, 164, 170, 180, 201) and related genes have been sequenced. Genomic and cDNA sequences are also available for isozymes H2 (25, 138), H10 (25, 65, 212), H6 (9), and one uncharacterized isozyme (137). The *lip* genes are grouped into clusters in the *P. chrysosporium* genome. In BKM-F-1767, five *lip* genes have been mapped to one dimorphic chromosome with a sixth gene encoding isozyme H2 on the second dimorphic chromosome (65). In ME446, the *lip* genes have been localized to two unlinked clusters, one of which contains the *H2* gene (156).

The *lip* genes encode mature proteins of 343–345 amino acids which are preceded by 27- or 28-amino acid leader sequences (9, 25, 137, 138, 191). The leader sequence of H8 from OGC101 has been shown to contain a 21-residue signal peptide and a seven-residue propeptide which is cleaved *in vitro* by pancreatic microsomes (165). The seven-amino acid propeptide is followed by a Lys-Arg KEX2-type cleavage site. All the sequenced *lip* genes contain eight or nine introns ranging in length from 49 to 78 bp. The *lip* genes have a strong codon bias in favor of G+C-rich codons (164). The coding regions contain 60–65% G+C residues, whereas the 3′ noncoding regions contain 44–49% G+C. The overall genome of *P. chrysosporium* contains 59% G+C (154). The *lip* genes contain a TATA box 66 to 81 bp upstream of the translation initiation codon and a CAAT sequence positioned between −107 and −228 (74). The transcription start site has been mapped downstream of these sequences in two genes (93, 212).

Sequence elements similar or identical to those which bind cAMP response element binding protein (CREB) and the cAMP-responsive activating protein AP-2 have been found in the promoter regions of many but not all *lip* genes (47, 74, 164). The identification of the putative binding sites similar to those for the mammalian transcriptional regulatory protein AP-2 in several *P. chrysosporium* peroxidase genes suggests that cAMP may be involved directly in the regulation of the lignin-degrading system in this fungus (47).

In vitro translation and northern blot analysis of poly(A) RNA using cDNA probes have shown that expression of LiP in strain BKM-F-1767 and BKM-derivative OGC101 is controlled at the level of transcription by nutrient nitrogen availability (116, 191). There is also evidence that the various isozymes of LiP in this strain are differentially expressed in response to nitrogen and carbon limitation. Nitrogen-deregulated mutants of *P. chrysosporium* apparently express a different complement of isozymes, based on their chromatographic elution profile, than do wild-type strains (27, 110, 190). It has been shown that in *P. chrysosporium* strain BKM-F-1767, although LiP isozymes H2 and H8 are both produced under nitrogen limitation, the *H2*-encoding transcript is more than 1000-fold more abundant under carbon than under nitrogen limitation (182).

In a recent study of the effects of various cAMP-affecting drugs on LiP and MnP expression, it was shown that addition of atropine (an adenylate cyclase inhibitor), theophylline (an inhibitor of adenylate cyclase and cAMP-dependent phosphodiesterase), or histamine (a cAMP-dependent phosphodiesterase stimulant) to ligninolytic cultures results in suppression of LiP activity (28). MnP activity was also suppressed, but higher doses of inhibitors were needed to achieve the same degree of suppression.

1.3.2 Manganese peroxidases

The various isozymes of MnP are produced only in nitrogen-limited cultures which contain micromolar concentrations of Mn^{II}. Once a cDNA clone encoding a MnP protein was obtained, it was shown that Mn^{II} acted at the level of transcription to induce the expression of MnP. The first *mnp* cDNA sequence, encoding the MnP1

protein, was reported from this laboratory by Pribnow et al. in 1988 (152). A cDNA encoding H4 from BKM-F-1767 was sequenced in 1989 (146), and in 1990 the first *mnp* gene was cloned and sequenced in our laboratory (73). The regulation of MnP expression by nitrogen and Mn^{II} was being studied concurrently with the cloning and sequencing efforts (32). The work on the transcriptional regulation led to the discovery of heat-shock induction of the *mnp1* gene (31, 73), and consensus heat-shock elements were found in the promoter sequence of the gene (73). Subsequently, cDNAs for two H4-encoding alleles (MnP2a and MnP2b) from O101 and a genomic clone of MnP2 have been sequenced (125).

The *mnp1* gene from O101 encodes a mature protein of 357 amino acids preceded by a 21-amino acid leader sequence (152). The H4-encoding sequences from BKM-F-1767 and O101 predict a 358-amino acid mature protein and a 24-amino acid leader sequence (125, 146). The two MnP isozymes share about 70% nucleotide sequence identity, 88% amino acid sequence identity, and cross-hybridize readily. The *mnp* and *lip* cDNAs share 50–65% sequence identity at the amino acid level and about 60% identity at the nucleotide level (74). The coding regions of the *mnp* genes also have a high G+C content, but their codon bias indices are lower than those of the *lip* genes (164). The MnP leader sequences lack an obvious propeptide sequence as found in the *lip* genes. The *mnp1* gene contains six introns of from 57–72 bp, and the *mnp2* gene contains seven introns of from 50 to 55 bp. The positions of six of the introns are identical in the two *mnp* genes, but the additional intron in *mnp2* splits the distal His codon of exon 3 in the *mnp1* gene. There is little similarity in intron positions between the *lip* and *mnp* genes (74).

The 5′ untranslated regions of the *mnp* genes contain a TATAA element 81 bp upstream from the translation initiation codon. They also contain three inverted CCAAT elements (ATTGG on the coding strands) and several other eukaryotic consensus regulatory sequences. The *mnp* promoter regions contain putative recognition sites for the general transcription factor SP-1, and the *mnp1* and *mnp2* genes contain possible AP-2 sites (73, 125). Both *mnp* promoter regions also contain eukaryotic consensus heat-shock elements (HSEs) (118) and metal regulatory elements

(MREs) identical to the MREs found in the mouse metallothionein *(mMT)* genes (45). The *mnp1* gene (73) contains four HSEs within 400 bp of the translation initiation codon and the *mnp2* gene (125) contains six HSEs within 1100 bp of its translation initiation codon. These HSEs match the consensus sequence in six or seven of the eight conserved positions and are interspersed with the inverted CCAAT elements in an arrangement similar to that seen in the mammalian *Hsp70* genes (81). Four of the six putative MREs of the *mnp1* promoter occur in pairs with a 4 bp palindromic overlap, as do two of the three MREs in the *mnp2* promoter. This arrangement is not found in the mammalian *MT* genes.

The accumulation of MnP activity in the extracellular medium depends on the presence of the substrate Mn^{II}. Western blots of intracellular and extracellular *P. chrysosporium* O101 proteins probed with polyclonal MnP antibodies have demonstrated that MnP protein is present only under nitrogen-limited conditions (1.2 mM ammonium tartrate as the sole nitrogen source) and only in the presence of Mn^{II} (32). *mnp* mRNA can only be detected in nitrogen-limited cultures grown in the presence of Mn^{II}. Likewise, *in vitro* translation of poly(A) RNA from nitrogen-limited cultures yields MnP protein only if Mn^{II} is present in the culture (30). Thus, Mn^{II} does not act to regulate secretion of pre-existing protein or to activate pre-existing protein; rather, it acts to regulate the synthesis of *mnp* mRNA.

Addition of Mn^{II} to 5-day-old nitrogen-limited cultures results in the appearance of *mnp* mRNA in about 40 minutes. The amount of mRNA detected is a function of the amount of Mn^{II} added up to 180 μM. The addition of 180 μM Mn^{II} to 5-day-old high nitrogen cultures (12 mM ammonium tartrate) does not result in the appearance of *mnp* message or activity, thus nitrogen regulation overrides Mn^{II} regulation. No metal other than Mn^{II} has been found to induce MnP transcription or the appearance of MnP activity.

The presence or absence of 0.1 mM concentrations of Mn^{II} has no significant effect on growth of *P. chrysosporium* strains O101 or BKM-F-1767 in culture as measured by mycelial dry weight or rates of carbon or nitrogen consumption. It also does not affect the total amount of extracellular protein produced (32). Several

protein bands in addition to MnP appear on western blots of *in vitro* translated mRNA from O101 cultures grown in the presence of Mn^{II}, which suggests that there are other Mn^{II}-regulated genes in *P. chrysosporium* (74).

mnp gene transcription has been found to be induced by a one-hour 45°C heat shock in the *P. chrysosporium* strain O101 (31, 73). Like Mn^{II}, heat shock only induces MnP transcription in nitrogen-limited cultures (31). The effects of heat shock and Mn^{II} on MnP transcription appear to be additive on northern blots. The heat shock-induced message is no longer detectable on northern blots one hour after the cultures are returned to 37°C, and no MnP protein is produced from the heat-shock message in the absence of Mn^{II}. However, in the presence of Mn^{II}, MnP protein is detectable on western blots and as MnP activity in the culture fluid of heat-shocked cells (31). These results suggest that Mn^{II} may be required for a post-transcriptional step in MnP expression, such as processing or transport of the primary transcript, mRNA stabilization, or translation.

Both *mnp* genes sequenced to date contain consensus HSEs. HSEs are cis-acting DNA sequence elements to which a heat-shock transcription factor has been shown to bind and cause transcriptional activation (118). These sequences are not found in any reported *lip* genes. HSEs are among the most conserved transcription factor binding sites known in the eukaryotes, being essentially the same in organisms from yeast to insects to man (118). Thus, it is likely that these sequences in the *mnp* promoter mediate the heat-shock induction seen in the *mnp* genes. The heat-shock transcription factor has been found to mediate transcriptional activation in response to influences other than heat, such as heavy metals, oxidative stress, and toxic substances like ethanol and arsenite. The physiological role of heat-shock induction of *mnp* genes in *P. chrysosporium* is not understood, but it may be related to the H_2O_2 produced during lignin degradation and/or the thermophilic nature of this fungus.

Experiments testing the ability of several factors known to induce many other heat-shock genes have been carried out in *P. chrysosporium* using *mnp* mRNA accumulation to indicate induction (115). As in the case of heat shock, addition of

0.25–1.5 mM H_2O_2 to nitrogen-deficient cultures lacking Mn^{II} results in *mnp* mRNA accumulation. No *mnp* mRNA is detected when H_2O_2 is added to nitrogen sufficient cultures. More MnP activity is produced by 5-day-old nitrogen-deficient cultures treated with Mn^{II} and H_2O_2 than when treated with either inducer alone. Addition of ethanol, peracetic acid, sodium arsenite, 2,4-dichlorophenol, or dimethyl formamide to 5-day-old nitrogen-deficient cultures also causes *mnp* mRNA accumulation (115).

1.3.3 Perspectives on the regulation of ligninolytic peroxidase expression in *P. chrysosporium*

Three major factors affecting the expression of LiP and MnP emerge from the foregoing discussions. Nutrient nitrogen availability acts as the overriding regulator, controlling the transition from primary growth to idiophasic growth. The second messenger cAMP also may be involved in the onset of idiophase and in the expression of both LiP and MnP. Mn^{II} availability controls the expression of MnP once nitrogen limitation occurs. Heat shock affects the expression of the *mnp* genes and is also dependent on pre-existing nitrogen limitation. Translation of the heat shock-induced *mnp* message appears to depend on Mn^{II} availability, suggesting that Mn^{II} availability is key to the expression of MnP under nitrogen-limited conditions.

Carbon limitation has an effect similar to that of nitrogen limitation on at least some of the *lip* and perhaps *mnp* genes. Less work has been done on carbon regulation, which is not expected to occur during growth of the fungus on wood, its natural substrate. A carbon cosubstrate, which presumably provides an energy source, is required for lignin degradation, suggesting that slow lignin degradation would be likely to occur under carbon-limited conditions.

1.4 Regulation of Metallothionein Gene Expression by Metals

1.4.1 Metallothioneins

Metallothioneins (MTs) are small heavy metal-binding proteins of about 60 amino acids which contain a high molar percentage of cysteines. MT was first discovered as a cadmium and zinc metalloprotein in horse kidney. Similar small

proteins were subsequently discovered in a wide range of eukaryotic organisms in many different tissues and cell types. MTs bind heavy metals by means of clusters of cysteine residues through thiolate bonds. Synthesis of MT is homeostatically regulated in cells and organisms by their level of exposure to heavy metals (85).

The function of MTs has been debated since their discovery. A role in metal detoxification is clear in lower eukaryotes such as yeast (38, 187), but there are other possible roles in metal metabolism which are particularly likely in mammals (101). These other possible roles include intracellular trace metal homeostasis, control of the intracellular redox potential, activated oxygen detoxification, and sulfur metabolism (59). In some mammalian cells, MT transcription is also induced by glucocorticoids, interferon, and stress conditions (85). The possibility that MTs are involved in the transfer of metal ions to apometalloenzymes has also been studied. Mammalian zinc-MT is able to reactivate various zinc-dependent enzymes, such as carbonic anhydrase, aldolase, thermolysin, and alkaline phosphatase, with rates comparable to those observed with the inorganic salts (117, 196). Transfer of copper to the binuclear copper proteins tyrosinase and hemocyanin has been studied with *Neurospora crassa* copper-MT (19).

In eukaryotes, the MTs are among the most intensively studied examples of metal-regulated transcriptional activation. The rapid and powerful activation of *MT* gene transcription in response to various transition metals such as zinc, copper, cadmium, and lead is considered to be part of the metal detoxification function of these genes. Work in the laboratories of Richard Palmiter (183) and Dean Hamer (40) with the mouse *MT* gene provided the first experimental evidence for the existence of promoter sequences responsible for metal-activated transcription of MT. The yeast *Saccharomyces cerevisiae* contains a copper-specific MT which was identified as a dominant Mendelian locus, *CUP1*, conferring copper resistance on yeast cells (38). Because of the facile genetic manipulations possible in yeast, this copper resistance determinant is now the best understood eukaryotic metal-responsive transcription system (186). The first information about the structure and action mechanism of a eukaryotic metal-responsive transcription factor was obtained from research on the yeast CUP1 system (63).

1.4.2 Mammalian metallothioneins

As reviewed by Hamer in 1986 (85), mammalian MTs are 61- or 62-amino acid proteins, containing 20 cysteines, 6–8 lysines, 7–10 serines, and no aromatics or histidines. The majority of the cysteine residues are present in cys–cys and cys-x-cys clusters. All vertebrates examined contain two or more isoforms of MT which can be grouped into two classes, MT-I and MT-II. The metal content of MT purified from mammalian tissues is highly variable and depends on organism, tissue, and metal exposure history. MT isolated from human liver autopsy samples contains almost exclusively zinc, whereas MT from kidneys contains substantial amounts of cadmium and copper. MT isolated from organisms that have been intentionally exposed to heavy metals contain predominantly the administered metal.

1.4.2.1 *Metal binding properties*

The mammalian MT proteins form two domains when folded around metals, with nine of the Cys residues in the N-terminal β-domain and 11 in the C-terminal α-domain. The two-domain structure has been confirmed by X-ray crystallography of the rat Cd-Zn MT-II. The metals interact with the protein exclusively through Cys thiolate bonds with all the Cys residues. The α-domain contains the A metal cluster and binds four atoms of zinc or cadmium, or five to six atoms of copper. The β-domain contains the B metal cluster and binds four atoms of zinc or cadmium, or six atoms of copper. All metal ions are tetrahedrally coordinated by combinations of bridging and terminal thiolate ligands. The copper MT isolated from *N. crassa* contains only 25 amino acids, but the seven cysteines can be precisely aligned with those in the amino terminal copper-binding domain of mammalian MT (114). The *N. crassa* protein binds six copper atoms *in vivo*, or three atoms of zinc, cadmium, mercury, cobalt, or nickel *in vitro* (20).

1.4.2.2 *Regulation of expression*

Expression of *MT* genes is induced by heavy metal ions and in mammalian cells by circulating factors such as hormones and interferon. Mammalian MT

synthesis is accompanied by increases in translatable *MT* mRNA and is blocked by actinomycin D, indicating that heavy metal ions must act at the level of mRNA synthesis, processing, or degradation (85). Nuclear "run-off" experiments demonstrated that synthesis of *MT* RNA increased 17–25-fold within an hour in cadmium-exposed nuclei, whereas maximal mRNA accumulation occurs 4–6 hours after cadmium exposure in whole tissue. These results, together with the observed inducibility of *MT* promoter/reporter gene constructs in transfected cells indicate that heavy metal regulation occurs largely or exclusively at the level of transcription initiation (50).

The introduction of cloned *MT* genes and constructs, containing promoter sequences derived from cloned genes, back into cells from which they were derived has been used to study the cis-acting control sequences. Many of the experiments using reporter genes under the control of the cloned *MT* promoter sequences provide a simple assay system for promoter activation (85). This reporter gene method has been instrumental in the mapping of the cis-acting control elements of the mouse *MT* promoter (45).

Several different types of gene transfer systems are available for use with mammalian cells. These include stable transfection, transient transfection, transient viral infection, and DNA tumor virus (i.e., bovine papilloma virus) infection/transformation. Stable transfection results in introduced, or transfected, DNA being integrated into the chromosome. The reintroduced gene typically is present in 1–50 tandemly arranged copies integrated into a single, but random, chromosomal site. A stably transfected cell line can be studied over a long period of time, but there is a high degree of clone-to-clone variability in copy number, expression level, and inducibility. Many of these limitations are overcome by the use of infection or acute transfection. It should be noted that, in filamentous fungi, the only DNA transformation methods generally available produce stable transformants analogous to the stable transfectants just described.

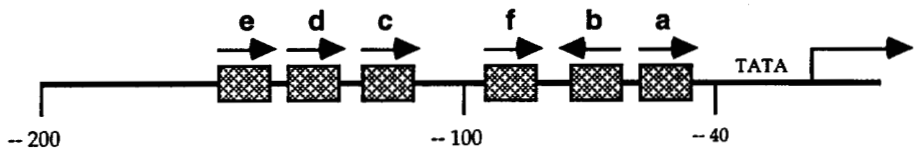
Mammalian cells can also be transfected with a plasmid DNA gene fusion construct or infected with a construct cloned into a viral genome such as SV40. Infected cells are assayed 12–72 hours later, before the viral DNA integrates into the

genome or kills the cells (40). Cells transfected with naked plasmid DNA, introduced into the cells in the same way used to make stable transfectants, can be used in a similar manner (175). During the first 12–72 hours, the DNA will not yet have integrated into the chromosome and the transfectants can be treated as if the plasmid was present in the nucleus as episomal DNA. This is referred to as acute or transient transfection. The advantage of these acute methods is the simplicity and reproducibility, as the transformants are all comparable. However, the copy number can be nonphysiologically high, and long-term experiments are not possible (85).

1.4.2.3 *The mouse metallothionein, mMT-I, promoter*

The cDNA and gene encoding the mouse metallothionein I (mMT-I) were cloned in the Palmiter laboratory (51). These clones provided the first direct evidence for the metal-activated transcription of *MT* genes and the experimental tools for the analysis of a *MT* promoter. The *mMT-I* promoter was used in promoter/reporter gene fusion experiments where different lengths of the promoter were deleted or linkers were inserted in various sites, and the effects on expression levels and metal inducibility were observed. These experiments, referred to as deletion and linker-scanning mutagenesis, led to the conclusion that there are several sequence elements in the promoter which contribute to the overall magnitude of the metal-responsive transcription. These cis-acting sequence elements were named metal responsive elements (MREs), and are present in all *MT* promoters that have been examined. Fine mapping studies, utilizing point mutations, have defined the MRE sequences. Those results, combined with sequence comparison studies, have established the MRE core consensus sequence which is necessary for efficient metal-inducible transcription. These results are diagrammed in Fig. 1.1 (40, 45, 132, 183).

The MREs have been found to function as modular transcriptional control elements. Each of the MREs from the *mMT-I* gene, when placed in front of the herpes virus thymidine kinase promoter, shows a different level of metal-activated transcriptional potency. Dimers of MREd produced a 20-fold induction in response to zinc (184). A single synthetic MREd is transcriptionally responsive to the same metals as an intact *mMT-I* promoter (45). It has also been demonstrated that a



MRE consensus CTCTG**TGCRCNC**GGCCC

Fig. 1.1 Organization of MREs in the mouse metallothionein I promoter. The location and orientation of MREs a through f are indicated relative to the binding site for transcription factor IID (TATA binding factor) and numbered from the start of transcription (arrow). This summarizes data from several functional analyses and *in vivo* DNA binding studies. (Redrawn from ref. 85)

multimerized MREd could act as a metal-inducible enhancer in that it could activate the rabbit *β-globin* gene promoter from a long distance, in either orientation, and from upstream or downstream of the *β-globin* transcript initiation site (208). The *mMT-I*, the human *MT-IIA*, and several other *MT*s have MREs interdigitated with other regulatory elements, which suggests that a number of different and possibly important protein–protein interactions may contribute to the regulatory mechanisms in mammalian *MT* promoters (186).

1.4.2.4 Trans-*acting factors*

Since MREs are transcriptionally responsive to multiple metals, models of how transcription is modulated by these elements must presume that there is either one pre-existing factor in responsive cells that binds to or is otherwise activated by multiple metals, or that there are multiple factors that are each activated by one or a small number of metals. The first evidence for the existence of a positive-acting metal-responsive transcription factor came from a simple competition experiment. A *mMT-I/GalK* fusion gene was used as an indicator, and wild-type or mutated MREs on *mMT-I* promoter fragments were used as competitors to detect the presence of a titratable MRE binding factor in cultured monkey kidney cells (177). Cadmium-dependent DNA–protein interactions have also been observed in *in vivo* methylation protection assays at all five MREs in the rat *MT-I* promoter (8). Results of a range of *in vitro* experiments have led to the conclusion that the simple model in which the transcription factor is activated directly by metal binding is likely to be correct.

Wold's laboratory carried out a detailed study of protein–DNA interactions in the *mMT-I* promoter using both DMS and DNAseI footprinting and testing responses to both cadmium and zinc (132). These studies also showed that MREd, the most potent of the MREs in isolation, was occupied in cells not exposed to exogenous zinc. The pattern of occupation was not consistent with binding of SP-1, whose site overlaps MREd. This site may be of such high affinity that it is occupied at even the low level of zinc found in untreated cells, or it is a target site for other DNA-binding proteins *in vivo*. A third possibility is that the two overlapping proteins bind

cooperatively, thus reducing the amount of zinc necessary to cause the protein–DNA interaction.

The formation of a cadmium-inducible DNA–protein complex of low affinity was observed by Andersen and colleagues in rat cells (7). The binding of this factor required at least two MREs and was too weak to be detected by standard footprinting procedures. Complex formation occurred only with cadmium and no other metals. Cross-linking experiments were used to estimate the molecular mass of a protein from this complex at about 39 kDa.

The existence of a protein in rat liver nuclear extracts which binds in a zinc-dependent manner to the mMT-I MREa in electrophoretic mobility shift assays has been demonstrated (174). This activity has been termed zinc activated protein (ZAP). Mouse MREa is capable of metal regulation *in vivo* without any apparent overlapping regulatory sites. ZAP binding also depends on a transcriptionally competent MRE core element and binding *in vitro* is maximally activated by 60–100 μM $ZnSO_4$, the same concentration range known to efficiently induce mMT-I transcription in cell cultures. A similar zinc-inducible mMT-I MREd binding activity has been found in HeLa cells (a human cell line) (208).

1.4.2.5 *Purification and cloning of metal-responsive MRE-binding proteins*

Most investigators trying to purify an MRE-binding factor have focused on detecting zinc responsive proteins. This approach can be complicated by the fact that some MREs, such as mMREd, overlap binding sites for zinc finger-type DNA-binding proteins like SP-1. For this reason, Imbert et al. (97) used an MRE from trout *MT*-B *(tMT-B)* gene in an affinity chromatography protocol to purify a mouse protein. There was no experimental evidence for overlapping regulatory elements in the *tMT* promoter, and a *tMT-B/CAT* reporter fusion containing only the tMREa element was inducible by cadmium and zinc in mouse L cells. These investigators purified a mouse factor to near homogeneity which they designated metal response element binding factor I (MBF-I). It has a molecular mass of about 74 kDa and binds an oligonucleotide containing a functional MRE, but not an MRE with point mutations in critical core residues. However, no experimental evidence that this factor binds to

functional *mMTI* MREs *in vitro* has been presented, and MBF-I only stimulates transcription from a tMRE template in response to zinc. Recently, sequence information from MBF-I was used to isolate a cDNA clone which turned out to be the RP-A factor, a zinc finger protein which functions in DNA replication. The relationship of this clone to MBF-I activity has not yet been clarified.

Seguin and coworkers have purified a protein which binds to mMT-I MREd in the presence of zinc (MEP-1) (176). The MRE binding activity of this factor in *in vivo* footprinting assays could be inactivated by 1,10-phenanthroline and restored by addition of zinc. Competition studies *in vitro* with other transcriptionally functional MREs, but not those with nonfunctional point mutations, indicate that only functional MREs compete for binding to MREd. MEP-1 has been purified to homogeneity by a protocol utilizing a zinc chelating sepharose column.

Radtke and colleagues (153) cloned a factor which was previously characterized on the basis of its ability to bind to the MREs of the *mMT-I* promoter (208). The factor was termed MTF-1 and was shown to bind most strongly to MREd of the *mMT-I* promoter. As MREd overlaped an SP-1 binding site, an MRE was designed which had the same high affinity for MTF-1 as MREd but without the SP-I site. This synthetic MRE was used to screen a cDNA expression library for MTF-1. A cDNA clone was isolated which encodes a protein of 72.5 kDa that contains six zinc fingers and a transcriptional activation domain containing an acidic region and a proline-rich region. The recombinant MTF-1 protein displayed binding to MREs *in vitro* that is indistinguishable from that of the natural MTF-1. Binding of both proteins to MREs was abolished by EDTA or *o*-phenanthroline. It appears that the MTF-1 factor may contain either a zinc-responsive domain in addition to the zinc fingers, or it may contain one or more zinc fingers that bind zinc in a lower than normal affinity, with a binding constant near the physiologically tolerated zinc concentration.

The most complete purification of a zinc-responsive MRE-binding factor reported to date was recently published by Otsuka et al. (142). These researchers achieved a 16,000-fold purification of a zinc-responsive factor from HeLa cells,

which bind to the MREs of the human *MT-IIA* gene. The purification strategy used a biotinylated MREa oligonucleotide to form a complex with the MRE-binding protein, allowing the complex to be separated from the crude mixture by avidin affinity chromatography. This protein was efficiently separated from the zinc-responsive factor, ZRF, by this procedure. Amino acid sequencing of peptide fragments of ZRF revealed that it is homologous to the mMTF-1 cDNA isolated by Radtke et al. from mouse cells. ZRF complexed with authentic and mutated MRE sequences in band shift assays behaved as expected for a protein whose sequence-specific binding activity depends on the presence of zinc. Chelators easily disrupted the complexes and mutations in important MRE core sequence bases also reduced binding activity substantially. It appears that ZRF is the human homolog of mMTF-1.

1.4.2.6 *Relationships between the various MRE-binding proteins identified to date*

There are two general models for how MREs mediate the transcriptional activation of *MT* genes (186). The presence of a pre-existing metal sensing factor is implicit in both models, a view which is supported by the lack of requirement for *de novo* protein synthesis for transcriptional activation. The simplest model for the mechanism of action of a higher eukaryotic metal responsive transcription factor (MRTF) has been referred to as the "flexible protein" model. In this model, a single MRTF is proposed to exist which has a metal-binding domain which can fold to accommodate all the heavy metals which activate transcription of *MT* genes, and has an MRE-binding domain which is "uncovered" or "formed" by the binding of a metal. The second general model, known as the *MRTF* gene family model, proposes the existence of a family of related transcription factors, each member of which specializes in binding one or a small subset of the metals which activate *MT* gene transcription. Again, binding of the metal by the MRTF protein would activate the MRE-binding function in some way. Other less direct mechanisms are also possible, involving various accessory "adaptor" proteins to accomplish the MRE binding in response to a wide range of metals, or even involving a cascade of protein kinases as

in prokaryotes, but the evidence so far is consistent with the existence of one or more direct MRE-binding MRTFs.

The experimental evidence to date suggests that there are multiple metal-dependent MRE-binding factors. It appears that, since these MRE-binding activities only respond to a subset of the metals known to activate transcription from *MT* promoters, there must be a family of MRTFs with different metal specificities. The differences in preferred coordination geometry of metals such as zinc (tetrahedral) and copper (trigonal) are consistent with this idea. However, cadmium can also be stably coordinated in a tetrahedral ligand environment much like zinc, but MEP-1, MTF-1, and ZAP do not respond to cadmium, so additional parameters must be involved in determining the metal specificities of these proteins.

1.4.3 Yeast copper metallothionein

The *CUP1* gene of *S. cerevisiae* encodes a 61-amino acid protein that shares the cys-x-cys and cys–cys motifs with the mammalian MTs, but the overall arrangement of cys residues is only partially conserved. It contains 12 cysteines which constitute 20% of the residues, whereas cysteines constitute 32% of the human MT. The yeast copper-MT, or cuprothionein, also contains 10% glutamate which gives the protein a net negative charge. It binds eight copper atoms per polypeptide in trigonal arrays via cysteine thiolates as a Cu-S polynuclear cluster. Cuprothionein protects yeast cells from copper toxicity by binding and sequestering intracellular copper. In contrast to the wide array of metals which induce mammalian MTs, only copper and, more weakly, silver induce *CUP1* transcription.

The cloning of the *CUP1* gene in 1984 (38) allowed promoter/reporter fusion gene experiments to be conducted. Analysis of the reporter fusion results led to the identification of the promoter region responsible for copper induction. A 431-bp DNA fragment spanning from 33 bp upstream of the *CUP1* ATG translation initiation codon to position -464 was ligated upstream of the translation start codon of the *Escherichia coli galK* gene in a yeast/*E. coli* shuttle plasmid. Studying constructs with deletions and point mutations in the promoter of the *galK* reporter fusion demonstrated the existence of multiple sequences important for copper inducibility of

the *CUP1* promoter between -105 and -230. This region was designated the CUP1 upstream activating sequence (UAS). An element of the CUP1 UAS located between positions -108 and -139 confers copper activation when inserted as a tandem repeat in a heterologous promoter. These results suggested the existence of a specific positively acting MRTF.

Such a copper regulated transcription factor was sought by genetic means, by mutagenizing a copper-resistant yeast strain and screening for mutants which failed to activate CUP1 in response to copper. Strains bearing a recessive mutation with that phenotype were found in two laboratories and called *ace1-1* (185) and *cup2* (33). These were later demonstrated to be alleles of the same gene. The wild-type ACE1 was cloned by transforming an *ace1-1* mutant strain with a yeast genomic library and selecting for the colonies which had recovered copper resistance. The deduced amino acid sequence of the *ace1* gene product revealed a polypeptide with a cysteine-rich N-terminal half containing numerous positively charged residues and an acidic C-terminal region (63). A fusion of this protein with *E. coli* β-galactosidase was found to concentrate in the nucleus, as would be expected of a DNA-binding transcription factor (185).

ACE1 expressed in *E. coli* was used to demonstrate direct copper-dependent binding of ACE1 to CUP1 UAS sequences by DNaseI footprinting (53). These results showed three regions of DNaseI protection between -110 and -220 in the CUP1 promoter. Those results are consistent with earlier promoter mutation results and allowed a consensus copper-activating sequence to be proposed. Experiments with a truncated version of the ACE1 protein that includes only the N-terminal domain were used to demonstrate that this domain contains the DNA-binding activity and the copper-binding activity (63). The DNA-binding ability of this protein was found to be dependent on the presence of copper in gel mobility shift assays using a wild-type CUP1 UAS oligonucleotide as the probe DNA.

Comparison of the amino acid sequence of ACE1 with MT reveals four stretches of sequence in the N-terminal domain which cannot be accommodated in a MT-like structure. These segments contain many basic residues and are predicted to have strong β-turns that could project outward from the copper core. This feature led

to speculation that these segments could be DNA-binding loops, as they are similar in size to loops predicted for zinc-finger proteins (63). A conformational effect of copper binding can be seen in the difference in protease susceptibility of the apo- and copper-containing proteins, similar to the results with MTs. MTs are considered to be random coil structures until they bind a metal. Copper can be removed from the ACE1 protein by KCN, which is a good chelator of Cu^{I}, but if the Cu-ACEI complex is pre-incubated with CUP1 UAS, the copper cannot be removed by chelation. This suggests that DNA binding stabilizes the copper-containing ACE1 structure.

The ACE 1 protein has an N-terminal domain that is composed of a MT-like structure where the external loops have been enlarged and contain positively charged residues which form sequence-specific DNA-binding structures. The C-terminal domain shares a concentration of acidic residues with other known transcriptional activation domains. Like a MT, the N-terminal domain does not fold into a definable active structure unless it binds copper (33, 63). This structure is further reinforced when it binds to the CUP1 UAS sequences. This view of ACE1 is supported by work on the original mutant used to clone the gene *ace1-1*. In the *ace1-1* mutant, one cysteine residue is changed to a tyrosine. Whereas ACE1 binds six atoms of copper, *ace1-1* only binds five atoms. This protein, defective in copper binding and the ability to induce CUP1 *in vivo*, has been shown to bind to only a subportion of the CUP1 UAS promoter region and displays very weak transcriptional activation (33).

1.5 Gene Expression in Filamentous Fungi

1.5.1 Background

Filamentous fungi have been part of man's food technology for all of recorded history. More recently, filamentous fungi have been used in controlled fermentation processes for the production of metabolites, including pharmaceuticals and industrial enzymes. Development of these industrial processes has resulted in highly productive fungal strains. Most strain improvement to date has been accomplished by mutagenesis and screening for desired properties, an approach which does not require knowledge of the fundamentals of gene expression in these organisms. However, the

application of recombinant DNA technology is beginning to make a contribution to strain development, allowing modifications not possible by genetics, including the introduction of biosynthetic capacities not normally found within a particular genus (12).

A DNA transformation system is one of the primary requirements for the use of recombinant DNA techniques in any organism. The bulk of the work on the development of fungal transformation systems has been done with members of the industrially important Ascomycete genera, mainly *Aspergillus* and *Penicillium*, and with the scientifically important *N. crassa*. One common type of transformant selection uses complementation of a nutritional auxotrophic mutant strain by a gene which compensates for the biosynthetic deficiency. The complementing gene is usually from the same species or another related species to ensure that it can be expressed in the target cells. Auxotroph complementation is often the easiest type of selection scheme to develop as it does not require complicated recombinant DNA constructions. For this reason, auxotroph complementation is often developed first for a given fungus, and then that transformation system is used to develop a selection scheme that is usable in wild-type cells, such as antibiotic resistance (57).

A number of positive selection systems based on antibiotic resistance have been developed for filamentous fungi. In those cases where the antibiotic resistance gene is of prokaryotic origin, such as the kanamycin-, G418- (neomycin), and hygromycin-resistance genes, it has been necessary to use a fungal promoter to direct transcription of the gene (13, 34). Heterologous protein expression, which includes expression of all the commonly used reporter genes, has been more difficult to achieve in basidiomycetes than in ascomycetes. This, and the lack of industrial interest in this class of fungi, has left the current understanding of gene expression in basidiomycetes in a more primitive state.

1.5.2 Filamentous fungal gene structure

In general, filamentous fungal genes are more similar to the genes of higher eukaryotes than to yeast or prokaryotes. Most filamentous fungal genes contain introns with conserved splice sequences (12, 83). The genomes of *Aspergillus*

nidulans and *N. crassa* contain very little repetitive DNA (108, 192). The genomes of some filamentous fungi are unusually G+C rich. Both of the basidiomycetes *Schizophyllum commune* and *P. chrysosporium* (154) contain an average of over 55% G+C in their genomes, with the coding regions of some genes exceeding 60% G+C (152). A large number of genes of the euascomycetes *N. crassa* and *A. nidulans* have been cloned and sequenced, and the general characteristics of other filamentous fungal genes appear to be similar to these (12, 83).

There are several known cases where functionally related genes are clustered (83). In one such case, the *Arom* locus of *A. nidulans*, a single 5.3-kb message has been found that appears to be the result of the fusion of five independent prokaryotic cistrons into a single large polyfunctional eukaryotic cistron (42, 89). The *trp-1/trpC* genes also encode trifunctional proteins in most fungi, a feature common in microorganisms (83). The basidiomycetes *S. commune* and *P. chrysosporium* also may contain genes in the adenine and tryptophan biosynthetic pathways that encode multifunctional proteins (3, 179). Clustering occurs among the genes involved in sporulation in *A. nidulans*, and there is evidence of cluster-specific positional effects on the expression of some of these genes (129). At least some of the *cellulase* and *lip* genes in *P. chrysosporium* have also been found to occur in clusters (44, 65).

1.5.3 Basidiomycete gene expression systems

1.5.3.1 *Background*

Comprehensive studies of gene expression and protein secretion have not been undertaken in basidiomycetous fungi. However, results of many attempts to express prokaryotic and eukaryotic genes as selectable transformation markers and as reporter genes are available. The majority of transformation markers that have been used are genes which complement an auxotrophic mutant strain of the fungus to be transformed. These biosynthetic pathway genes are often from a different but related organism, so they give some indication of the range of species which have compatible gene expression parameters. The use of antibiotic resistance genes as markers is desirable since mutant strains are not necessary as hosts. However, it has been

difficult to create transformation vectors using antibiotic resistance markers for filamentous basidiomycetes because of the generally more limited range of acceptable heterologous genes as compared to ascomycetes.

1.5.3.2 *Transformation selection markers*

The first published report of the transformation of a basidiomycete fungus was for the hemibasidiomycete yeast *Ustilago maydis*, which was transformed with a plasmid based on the yeast 2-micron circle in 1983 (14). The first report of the transformation of a filamentous homobasidiomycete was the transformation of *S. commune* in 1986 (135). This transformation made use of a tryptophan biosynthetic gene, *trp1*, which was cloned from *S. commune*. Since then, *Coprinus cinereus* (22) and *P. chrysosporium* (4) have also been transformed, either by complementation of auxotrophic strains with a biosynthetic gene from the same organism or from another homobasidiomycete.

Three forms of antibiotic resistance have also been used as selection markers in homobasidiomycetes. The kanamycin resistance determinant from Tn903, which had been found to function in other fungi without the addition of eukaryotic control sequences, was used to construct a transformation vector for *P. chrysosporium* (158). Hygromycin B resistance has also been expressed in *S. commune* using the *S. commune trp1* promoter and terminator to control transcription. However, the transformation frequency was low (131). The plasmid pAN7-1, which contains the *A. nidulans gpd* promoter controlling the expression of the *E. coli hygB* gene with termination provided by the *A. nidulans trpC* terminator, has been used to transform several basidiomycetes to hygromycin resistance. These include *Laccaria lacata* (16), *Pleurotus ostreatus* (148), and *Hebeloma cylindrosporum* (123). In these cases, pAN7-1, which uses ascomycete transcriptional control sequences and a prokaryotic transformation selection marker, does not function as well in basidiomycetes as it does in ascomycetes. The reduced level of expression may be due to relatively poor function of the ascomycete promoter or to methylation of the DNA, as has been observed in *S. commune* (131), *H. cylindrosporum* (123), and *P. ostreatus* (148).

Resistance to the antibiotic phleomycin has been expressed in *S. commune*, using the homologous *gpd* promoter and terminator (172), and in *P. chrysosporium*, using a homologous histone *H4* promoter and an *A. nidulans* terminator (66). Phleomycin resistance in *P. chrysosporium* is only an effective selection during the protoplast regeneration stage of the transformation process. Regenerated mycelium do not take up enough phleomycin to inhibit their growth. However, in *S. commune*, phleomycin resistance provides efficient selection for transformants at all growth stages. Hygromycin also has relatively little effect on *P. chrysosporium* mycelium, and it was not expressed efficiently in *S. commune*.

Kanamycin is the only antibiotic which has been reported to provide selection on *P. chrysosporium* mycelium. The only vectors described which have used kanamycin resistance in *P. chrysosporium* fortuitously became extrachromosomally maintained plasmids (158–160). This was thought to have occurred through a recombination event with a postulated endogenous low copy number plasmid. Even though the kanamycin resistance gene in these plasmids was shown to be methylated, it still expresses efficiently enough to provide kanamycin resistance at low copy number. The only apparent drawback to the best kanamycin selection plasmid that has been described so far is its low transformation efficiency, in the range of 20 transformants per microgram of plasmid DNA. This plasmid does not integrate into the host chromosome, so it cannot be used for purposes requiring targeted integration of transforming DNA such as gene disruption experiments.

1.5.3.3 *Studies of heterologous gene expression in basidiomycetes*

A series of experiments intended to investigate the range of heterologous genes that could be expressed in the homobasidiomycete *C. cinereus* has been reported (41). These experiments made use of a tryptophan auxotrophic mutant of *C. cinereus* and cloned *trp* genes from several other organisms. Cotransformation experiments were performed in which the *C. cinereus trp2.1* mutant was transformed with the *A. nidulans trpC*, or isocitrate lyase gene *(acuD)*, or with a hygromycin resistance construct designed for *U. maydis*. The cotransformants were tested for the expression

and integration of unselected heterologous genes. The cross-species functionality of the *trp* genes was used to isolate the corresponding *trp* genes of all the fungi used in this study as well as those of *Cochliobolus heterostrophus* (195), *Penicillium chrysogenum* (168), and *Phycomyces blakesleeanus* (162).

The *trp* genes of the two basidiomycetes *S. commune* and *P. chrysosporium*, and of *C. cinereus* itself, were able to transform the *C. cinereus trp-2.1* mutant to prototrophy. No Trp^+ transformants of the *C. cinereus trp-2.1* strain were obtained using the *A. nidulans trpC* gene. In the cotransformation experiments, a *C. cinereus trp-1/trp-2* double mutant strain was used as the host. Transformed oidia (spores) were allowed to regenerate and form colonies on indole-containing media to select for Trp^+ transformants. Selection for Trp^+ allows regeneration without requiring activity from the *A. nidulans* gene. The cotransformants were then screened for expression of the *A. nidulans trpC* gene. The *A. nidulans* gene was found to successfully integrate into the genome of *C. cinereus*, but no evidence of expression was found, either during vegetative growth or during spore regeneration. In the same experiment using both *C. cinereus trp-1* and *trp-2* genes as cotransforming DNAs, 60% of cotransformants expressed the nonselected *trp2* gene, so the failure of the *A. nidulans* gene to express was due to an inherent incompatibility with *C. cinereus*.

Cotransformation was also used to test the expression of the *A. nidulans acuD* in *C. cinereus*. The equivalent gene from *C. cinereus*, *acu-7*, had been expressed in an *A. nidulans acuD* mutant. The *A. nidulans acuD* gene failed to express in *C. cinereus* even though 42% of the cotransformants contained the acuD DNA. Finally, a hygromycin resistance construct that had been developed to transform the hemibasidiomycete yeast *U. maydis* was tested in *C. cinereus*. This plasmid, pHL1, contained the *U. maydis hsp70* promoter and terminator controlling the transcription of the bacterial hygromycin phosphotransferase gene, *hygB* (82). Cotransformation of pHL1 into a *C. cinereus trp-1* mutant strain did not give any hygromycin-resistant transformants when 20 out of 26 transformants contained the *U. maydis hygB* construct.

The heterologous *trp* gene transformation results confirm that gene transfer between different homobasidiomycete species is possible. The *trp* gene cotransformation results suggest that ascomycete genes will often not express in basidiomycetes. Thus, differences in transcriptional regulatory mechanisms may explain the lack of expression of the ascomycete genes in *C. cinereus*, though other sources of incompatibility are not excluded. All three of the homobasidiomycete species tested here are members of the same major class subdivision, the hymenomycetes. The failure of the *U. maydis* hygromycin resistance construct to express in *C. cinereus* could be due to incompatibility of transcriptional controls, but could also be due to other differences in the prokaryotic hygromycin phosphotransferase gene itself.

The first transformation system reported for *P. chrysosporium* used an adenine biosynthetic gene, *ade2* (61), from the related basidiomycete *S. commune* to complement the adenine-requiring *P. chrysosporium* auxotroph, *ade2* (4). The heterologous *ade2* gene gave a transformation frequency of 100 or more transformants per μg of DNA. Since then, three other *P. chrysosporium* auxotrophic strains have been transformed with heterologous genes from other fungi. The *ade1* strain of *P. chrysosporium* was transformed with the *ade5* gene cloned from *S. commune* (61) at an efficiency exceeding 200 transformants per μg of DNA. The enzyme encoded by the *ade5* gene was identified by using it to transform biochemically characterized *N. crassa* adenine auxotrophs (3). The *N. crassa ade2* strain was successfully transformed, thus identifying the enzyme encoded by *ade2* as phosphoribosylaminoimidazole synthetase.

The other two *P. chrysosporium* auxotrophs that have been transformed are the uracil-requiring mutants *ura2* and *ura11* (1). The *ura2* strain of *P. chrysosporium*, and an *ade2ura2* double mutant, were transformed by the *ura5* gene from the ascomycete *Podospora anserina* (1). The transformation frequency was much lower than for the other heterologous transformations of *P. chrysosporium*, about 10 transformants per μg of DNA. However, the transformation frequency was also very

low for this plasmid in *P. anserina* (17). The *P. anserina ura5* gene encodes the enzyme orotidylate pyrophosphorylase.

The *P. chrysosporium ura11* strain was transformed with the *ura3* gene from *S. commune*, which encodes orotidylate decarboxylase (ODase) (62). The *S. commune* plasmid, pEF1, transformed *P. chrysosporium ura11* at an efficiency of ~600 transformants per μg, comparable to that for the heterologous ade transformations described above. The *P. chrysosporium* ODase gene was cloned and found to give a transformation frequency of about 200 transformants per μg in the *ura11* strain. Homologous transformation of *P. chrysosporium ade1* by the *P. chrysosporium* pADE1 plasmid also gave over 100 transformants per μg, not much different from the efficiency of the corresponding heterologous transformation using the *S. commune* gene (6). However, the transformants developed sooner than those produced by the *S. commune ade5* gene. It appears that the differences in efficiency of gene expression between *P. chrysosporium* and *S. commune* genes in *P. chrysosporium* strains is small.

All the *P. chrysosporium* transformations discussed above, including those which used homologous DNA, resulted in ectopic integration of one or more copies of the transforming DNA. Since homologous integration of introduced genes is apparently rare in *P. chrysosporium* (1, 3, 6), an efficient selection scheme is necessary to isolate transformants from targeted integration events. Gene replacement has been achieved in *P. chrysosporium* by using sequences homologous to the *P. chrysosporium ura3* (ODase) gene to direct the integration of the *S. commune ade2* gene (2). Transformants, in which the *ura3* gene was interrupted by integration of the ura/ade construct, were selected by their resistance to 5-fluoroorotate. The ability to target transforming DNA to specific sites in the genome will make reverse genetic experiments possible. It will also facilitate studies of gene regulation using promoter/reporter gene fusions.

Overall, these findings lead to the general conclusion that only promoters from homobasidiomycete genes will function efficiently enough in these species to direct expression of antibiotic resistance markers for transformation or heterologous protein

production. The fact that the *C. cinereus acu-7* gene can be expressed in *A. nidulans*, but the reverse is not possible, suggests that the protein coding sequences are compatible between these fungi. The expression of the *P. anserina ura5* gene in *P. chrysosporium* is the only reported case of an ascomycete gene functioning in a basidiomycete, whereas the reverse has been reported several times.

The strategy of using a promoter from a gene which is expressed constitutively at high levels and whose structure and sequence is highly conserved among related organisms, such as the gene encoding glyceraldehyde-3-phosphate dehydrogenase, has worked well in the construction of transformation vectors for ascomycetes. The plasmid pAN7-1 is a good example of this. The *gpd/hygB* construct it contains has been expressed in over 20 filamentous fungi, mostly ascomycetes. These successes, and the relative ease of cloning this gene in most organisms using heterologous DNA probes, makes it a good choice for many gene expression purposes.

Work towards the goal of duplicating this strategy in basidiomycetes was published in 1992 (88). These researchers cloned and sequenced genomic clones of *gpd* genes from three basidiomycete fungi, *S. commune*, *P. chrysosporium*, and *Agaricus bisporus*. These highly conserved gene sequences were used to make a phylogenetic sequence comparison with other organisms. The results of the comparison suggested that filamentous fungi in general may be more closely related to the metazoa than to the plants and that the ascomycetous yeasts are even more distant from the filamentous fungi than are the plants. Overall, these observations are consistent with trends in gene compatibility between fungi and other organisms discussed above.

The cloned *S. commune* glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene has been used to develop a reporter gene system and a selectable transformation marker based on antibiotic resistance. Schuren et al. (171) made internal deletions in the coding regions of the *gpd* gene and the *Sc4* gene, which encodes a protein found in the basidiospore wall, and transformed these truncated gene constructs into *S. commune*. In this type of reporter experiment the expression of the native gene and the internally deleted version are assessed at the same time on northern blots. The

Sc4 construct contained 1140 bp upstream of the initiation codon, and the GPD constructs contained 1300 bp of upstream sequence. Both genes expressed as expected in some of the respective transformants, so it can be assumed that they both contained enough of the regulatory sequences necessary for normal gene expression. No correlation was found between copy number of integrated plasmid and expression level. There were clear differences in expression among the transformants, presumably due to the sites of integration (171).

In 1994 Schuren and Wessels described a highly efficient transformation of *S. commune* using the *gpd* promoter and terminator controlling the expression of a gene encoding bleomycin resistance (172). This was the first report of the transformation of a basidiomycete to antibiotic resistance that gave high transformation frequency and did not involve an autonomously replicating plasmid. The bleomycin resistance gene from *Streptoalloteichus hindustanus*, *Sh ble*, encodes a small, 124 amino acid protein, which binds with strong affinity to bleomycin and related antibiotics. The *Sh ble* gene is fairly G+C rich having a G+C content of 70%, similar to the genomic G+C content of *S. commune* and *P. chrysosporium*.

Since the *Sh ble* gene inserted into the *gpd* deletion construct previously used as a reporter was transcribed, a translational fusion was made between *ble* and the *gpd* promoter sequences to create a transformation vector. This vector contained 1300 bp of *gpd* 5′ sequences and expressed the *ble* gene efficiently enough to yield 5,000–10,000 transformants per μg DNA under optimized conditions. The ability of the *ble* gene to produce phleomycin resistant transformants was also used as an assay for promoter function in 5′ deletion experiments. A set of deletions into the *gpd* promoter, leaving from 750–50 bp of 5′ untranslated region, was made. These experiments showed that a 130-bp fragment contains sequences sufficient to produce the maximal number of phleomycin-resistant transformants. All the constructs described above contained the *S. commune gpd* terminator. A construct was also tested in which this terminator was not present and few transformants were obtained (172).

The plasmid pAN8-1, which contains the *A. nidulans gpd* promoter controlling expression of the *Sh ble* gene, was also tested and gave very few transformants in *S. commune* (172). This result is consistent with those from several other attempts to express genes in a basidiomycete using ascomycete control sequences. A translational fusion between the *A. bisporus gpd* promoter and *Sh ble* gene was also used to transform *S. commune* (172). This construct gave 50–100 transformant/μg, confirming that heterologous basidiomycete promoter sequences are more compatible with the transcriptional machinery of *S. commune* than are ascomycete promoters.

1.5.3.4 *Conclusions—parameters affecting gene expression in basidiomycetes*

Several lower basidiomycetes have been transformed successfully by constructs in which ascomycete promoter and terminator sequences were used to control the expression of a heterologous selectable marker gene. However, the homobasidiomycete fungi do not appear to accept these foreign sequences as readily. In the cases where transformation has been achieved with such constructs, the transformation efficiency has been fairly low, and there have been reports of rearrangements of the integrated plasmid, methylation of the integrated DNA, and spontaneous reversion of the transformant phenotype. Some or all of these problems have been successfully avoided when homologous promoter and/or terminator sequences have been used. However, significantly different results are obtained depending on which transformation marker gene has been used in the transformation of a homobasidiomycete.

For example, *hygB* sequences express, and are largely protected from methylation, in *S. commune* by using homologous transcriptional control sequences (131). However, the transformation frequency of 50–100 transformants/μg is still very low compared to that obtained with the phleomycin resistance marker (172). The only obvious differences between the *ble* gene and the *hygB* gene are their overall G+C content and their lengths. The *ble* gene has a G+C content of 70%, close to the 60% in the *S. commune gpd* gene, and 58% overall in its genomic DNA. The *Sh ble* gene is also fairly short at only 372 bp. In contrast, the *hygB* gene has a G+C

content of about 40% and a length of N bases. If the G+C content were actually an important factor in determining whether or not a heterologous gene will be transcribed and translated in a basidiomycete, it would argue that these fungi had a way of recognizing foreign DNA based either on sequence composition or on the basis of some discreet sequence element(s). If the latter were the case, then this sequence is apparently either found, or lacking, more often in DNA of low G+C content.

1.5.4 DNA Methylation—Possible effects on fungal transgene expression

1.5.4.1 *Occurrence of DNA methylation*

One of the most widely reported modifications of DNA introduced into fungal cells by transformation is methylation. The preferential methylation of foreign sequences seems to imply that at least some fungi have a system that recognizes foreign sequences and responds by methylating them. Some filamentous fungi are known to be able to recognize and methylate repeated sequences. In *N. crassa* the methylation leads to C-to-T transition mutations which accumulate to the point of gene inactivation. This phenomenon is known as repeat induced point (RIP) mutation (178). A similar phenomenon has been reported in *Ascobolus immersus*, which was termed methylation induced pre-meiotically (MIP). MIP does not result in point mutations, only in the methylation of cytosine bases in repeated sequences (167).

DNA methylation systems are widespread in nature, and they all include methylating enzymes which methylate specific sequences (48). In prokaryotes, such sequence-specific methylases are accompanied by a restriction enzyme which cuts the sequence if it is not methylated. These methylation/restriction systems serve to prevent foreign DNA, which may be unmethylated or methylated in a different pattern, from functioning in the host organism (29). Both deoxyadenosine and deoxycytosine are methylated in prokaryotes.

1.5.4.2 *DNA methylation in eukaryotes*

Eukaryotes methylate DNA almost exclusively on deoxycytidine (dC) residues in the 5 position. These 5-mdC bases occur in mammalian and fungal cells predominantly in CpG dinucleotide sequences. Chromosomal methylation patterns in

mammals are inherited and are copied from the parent strand, which is hemimethylated initially during DNA replication. This process has been termed maintenance methylation. A methyltransferase exists which may be involved in maintenance methylation. The enzyme recognizes a CpG dinucleotide methylated on only one strand and symmetrically methylates the other strand. It is not known whether *de novo* methylation of sequences introduced into mammalian cells involves the same enzyme as maintenance methylation does.

DNA methylation appears to be a means of modulating gene expression in eukaryotes (23, 48). The importance of methylation in mammals has been demonstrated by creating transgenic mice which are homozygous for the disruption of the DNA methyltransferase. Even though approximately 30% of the methyltransferase activity remains in the homozygotes, mutant embryos are stunted and die at mid-gestation. Maintenance methylation does appear to be reversible as there are several cases in which a gene in a tissue where it is expressed is found to be un- or under-methylated and the same gene in tissues where it is not expressed is found to be highly methylated. These results point to an important role for DNA methylation in cell differentiation and development in mammals. This idea and the possible evolution of methylation systems and enzymes from prokaryotes to assume these functions in higher eukaryotes has been reviewed recently by Bestor (21). On the other hand, there are many eukaryotes which do not appear to contain methylated DNA, including the lower eukaryotes widely used for genetic research, yeast, *Caenorhabditis elegans*, and *Drosophila menagaster* (121).

There are known to be at least two types of methylcytosine-binding proteins (MeCP) in mammals which bind to 5m-CpG dinucleotides and are probably involved in mediating the effects of 5m-CpG. Work on these 5m-CpG binding proteins was reviewed recently (23). One of these proteins, MeCP-1, can bind to individual 5m-CpG dinucleotides and the other, MeCP-2, binds *in vitro* only to sequences containing at least 12 symmetrically methylated CpG pairs. MeCP-1 appears to be involved in a general repression mechanism of promoters containing CpG sequences. Several studies have shown that transcription is sensitive to any methylation near the promoter, not just to methylation at specific sites. The strength of repression depends

on the density of 5m-CpGs near the promoter. The role of MeCP-2 is less clear. It is found chromosomally bound and is concentrated in heterochromatic regions known to be rich in 5m-CpG. This localization is consistent with a role in long-term gene repression, which might be required for some developmentally regulated genes.

1.5.4.3 *Occurrence of DNA methylation in fungi*

Methylcytosine has been detected to varying degrees in the genomes, or in DNA introduced into the genome by transformation, in many but not all filamentous fungi. These include *Physarum polycephalum, P. blakesleeanus, N. crassa, A. immersus, Sporotrichum dimorphosphorum, Candida albicans, Sclerotium rolfsii, Phymatotrichum omnivorum*, *C. cinereus*, *S. commune*, *P. chrysosporium*, and *P. ostreatus*. The occurrence and possible role in differentiation of DNA methylation in fungi has been reviewed (121). Methylated sequences in fungi are not randomly dispersed through the genomes but are found in clusters. The repeated sequences of ribosomal DNA genes have been found to be methylated in many fungi. Methylation patterns in DNA near centromeres in *C. cinereus* were shown to be inherited both mitotically and meiotically. Methylation of rDNA genes in *S. commune* and *N. crassa* have been shown to be differentially methylated depending on developmental state. A general increase in DNA methylation is seen in the dormant sclerotia of *P. omnivorum* as compared to the metabolically active mycelium of the fungus. The morphogenetic change from the yeast form to the mycelial form in *C. albicans*, which is thought to be related to the acquisition of pathogenicity, correlates with an increase in the level of 5-mdC. In general, it appears that DNA methylation may play a similar role in developmental gene regulation in filamentous fungi and mammals.

1.5.4.4 *DNA methylation in basidiomycetes*

C. cinereus is the only basidiomycete in which methylation of repeated sequences has been studied directly. Freedman and Pukilla studied methylation effects on the endogenous *trp1* gene in *C. cinereus* strains constructed with 1, 2, or 3 copies of the gene in either tandem arrays or as ectopic integrants (60). Methylation

of the *trp* gene was only observed when more than one copy was present. Among the strains carrying duplications and triplications, no significant difference in the extent of methylation was found between tandem and ectopic integrations. Methylation was found only at the CpG dinucleotide, and no C-to-T transitions were observed in the progeny of crosses between *trp1* duplication strains. However, methylation did lead to gene inactivation and the appearance of a Trp^- phenotype.

The extent of methylation of genomic DNA can be assessed at CCGG sites using a pair of restriction endonuclease isoschizomers where one isoschizomer, *Msp*I, is sensitive to methylation of dC in the internal CpG only, and the other, *Hpa*II, is sensitive to methylation of either dC base. This type of analysis has been used to demonstrate methylation of CCGG sequences in DNA introduced into a basidiomycete in several instances. The loss of some *EcoR*I, *Pst*I and *Sca*I sites in plasmid pPO-1, a plasmid derived from pAN7-1 by spontaneous recombination in *P. ostreatus*, led the investigators to test for DNA methylation. Both dC bases in the majority of CCGG sites were found to be methylated. This was proposed to be the reason for the lower than expected transformation frequency for the replicative plasmid pPO-1 (148).

The extent of methylation and expression of *E. coli hygB* constructs containing either heterologous or homologous promoters cotransformed into *S. commune* were compared by Mooibroeck et al. (131). In transformants of the constructs which used an *A. nidulans* promoter sequence (pAN7-1), *Hpa*II/*Msp*I isoschizomer analysis showed heavy methylation of the *E. coli hygB* and *A. nidulans gpd* sequences. The inhibition of *Hpa*II in particular indicated a preference for methylation of CG pairs over CC pairs, consistent with the general pattern of DNA methylation in eukaryotes. No methylation was observed in the cotransformed pAM1 plasmid, which contained the *S. commune trp* selection marker. A plasmid containing only a 200-bp fragment of the *S. commune Sc4* gene as the homologous sequence showed little or no methylation in a cotransformation experiment.

An *hygB* fusion with the homologous *trp1* promoter was also tested for HPT expression and methylation in cotransformants. Methylation was slight or absent in all transformants tested. All *hygB*-containing cotransformants appeared to express

hygromycin resistance, with half of them growing at 25 μg/ml hygromycin. One possible explanation proposed by the authors is that plasmids containing exclusively heterologous sequences are preferentially integrated into the genome of *S. commune* in areas which are not actively transcribed and are highly methylated. Conversely, plasmids containing homologous sequences would preferentially integrate in areas which are very lightly methylated and transcriptionally active.

In a recent attempt to create a practical dominant selectable transformation marker for use in *P. chrysosporium*, Gessner and Raeder (66) used a cloned homologous histone *H4* gene as a source of a promoter to drive the expression of the SH *ble* gene. One of their constructs fortuitously contained an entire homologous *H3* gene as well. The transformants from plasmids which contained the *H3* gene along with the *H4/ble* fusion did not express the *ble* gene and were found to be extensively methylated, whereas the transformants from plasmids containing only the *H4/ble* fusion expressed the *ble* gene well and showed little evidence of methylation. Since multiple copies of both plasmids were present in the respective transformants, something other than repeats of a sequence must have been responsible for the methylation. The authors suggested that overexpression of the *H3* gene, which would result in deleterious effects of histone excess, probably created a selection for inactivation of the *H3* gene.

The filamentous fungi are among the most primitive eukaryotes in which developmental changes and cellular differentiation can be observed. Of the filamentous fungi, the homobasidiomycetes display the greatest variety of differentiated cell types. It is interesting that methylation of DNA introduced by transformation has been reported in all the basidiomycetes that have been assayed for methylation. The available evidence seems to suggest that in many filamentous fungi, including basidiomycetes, methylation may prevent foreign sequences from expressing or prevent repeated sequences from over-expressing. Methylation of foreign, usually repeated, DNA sequences may be contributing to the generally observed lack of correlation between copy number and expression level of transformed genes. This same phenomenon is widely reported in transgenic plants (58).

CHAPTER 2

CHARACTERIZATION OF A GENE ENCODING A MANGANESE PEROXIDASE FROM *PHANEROCHAETE CHRYSOSPORIUM*

2.1 Introduction

During secondary metabolic growth, the lignin-degrading basidiomycete *Phanerochaete chrysosporium* secretes two extracellular heme peroxidases, manganese peroxidase (MnP) and lignin peroxidase (LiP), which, along with an H_2O_2-generating system, are apparently the major components of its lignin degradative system (80, 105). The structure and mechanism of LiP have been studied extensively (80, 105, 124, 188), and cDNA (25, 191) and genomic sequences (10, 180, 201) encoding several LiP isozymes have been reported.

The second ligninolytic enzyme, MnP, has also been identified (111), purified and characterized (68, 70). This peroxidase is an H_2O_2-dependent heme glycoprotein of M_r ~46,000 with an iron protoporphyrin IX prosthetic group. Like LiP, MnP exists as a series of isozymes (80, 145). MnP oxidizes Mn^{II} to Mn^{III} which, in turn, oxidizes a variety of phenols and phenolic lignin model compounds (68, 80, 202). The catalytic activity of MnP is stimulated by various organic acids such as lactate and malonate (68, 70, 203) which act as Mn^{III} chelators.

cDNA sequences for two MnP isozymes have been reported (146, 152), and the deduced amino acid (aa) sequence identity with the various LiP proteins is approximately 50%. The aim of the present study was to determine the nucleotide (nt) sequence of an *mnp* genomic clone and study the regulation of its transcription by heat shock (HS). This clone corresponds to the *mnp1* cDNA reported earlier (152).

2.2 Results and Discussion

2.2.1 Sequence of the *mnp1* gene

Characterization by Southern blotting of one genomic clone *(mnp1)* suggested that it encodes the *mnp1* gene. A set of overlapping restriction fragments of the *mnp1* genomic clone was sequenced by the strategy shown in Fig. 2.1. The 3′-terminal 900 bp were sequenced on only one strand using two different templates by three different methods: PolIk with standard nt, PolIk with deaza dGTP, and *Taq* polymerase with deaza dGTP. No discrepancies were found. A synthetic oligo primer was required to obtain the sequence of the 3′-terminal 300 bp.

2.2.2 Introns and exons

The sequences of the coding region and the 3′- and 5′-untranslated regions of the *mnp1* genomic clone match the *mnp1* cDNA exactly (152) (Fig. 2.2). This allowed the ready deduction of intron positions. The six introns in the *mnp1* gene vary in size from 57 to 72 bp, and their positions are shown in Fig. 2.3 in comparison with the positions of the eight introns in the *lip*-H8 gene (10, 170, 180, 201). Despite the strong homology in the coding regions between the *mnp1* gene (152) and several *lip* genes (25, 180, 191), there is little similarity in the locations of introns between *mnp1* and the *lip* genes. Codons for the distal His and distal Arg, aa residues thought to be involved in the heterolytic cleavage of H_2O_2 during the formation of the oxidized enzyme compound I (150, 152, 203), are together in exon 3 in the *mnp1* gene but are split by intron 2 in the *lip* genes (Fig. 2.3). In both proteins, the distal His and distal Arg are separated by 3 aa, and presumably are part of a functional domain. The codon for the proximal His (His^{173}) (130, 152) is located in exon 5 in the *mnp1* gene.

MnP appears to lack the putative 7-aa propeptide found between the signal peptide and the N terminus of the mature LiP protein (170), and the sequences encoding the MnP-1 signal peptide and N terminus of the mature protein are on the same exon. In the *lip* gene, the sequence encoding the signal peptide is separated

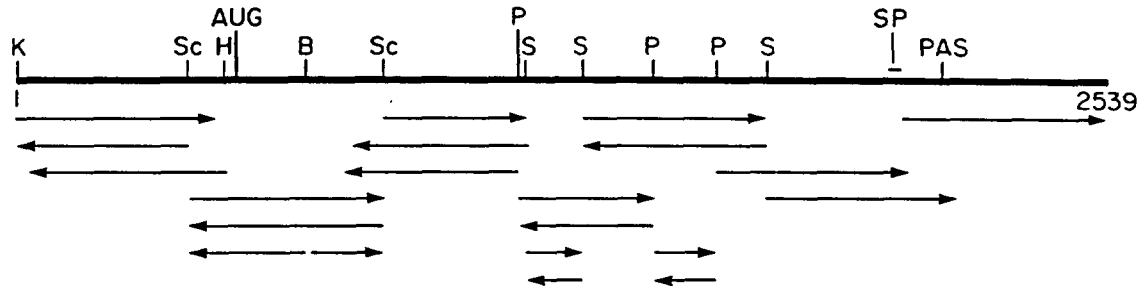


Fig. 2.1 Strategy for sequencing the *mnp1* gene. Arrows indicate the strand sequenced and the extent of the sequence determined from various restriction sites. Restriction endonucleases: B: *Bst*YI, H: *Hin*cII, K: *Kpn*I, P: *Pst*I, S: *Sal*I; Sc: *Sac*I. SP indicates the position at which a 17-bp synthetic oligo primer was annealed to extend the sequence in the 3' direction on the coding strand. AUG: start codon; PAS: polyadenylation site. Organisms: *P. chrysosporium* strain OGC101 (5) was used throughout this study. *Escherichia coli* strain DH5αF' (BRL) was used for the growth of all plasmid and phage M13 subclones. *E. coli* strain LE392 was used as the host for λEMBL3. Genomic library: genomic DNA was isolated from high nitrogen (12 mM ammonium tartrate) 2-day old shaking cultures (79) as described (64). A partial digest of the genomic DNA was carried out with *Mbo*I and fragments of between 10 and 20 kb were isolated from a sucrose gradient and ligated into λEMBL3 arms (122). Ligation products were packaged using a Gigapack kit (Stratagene) and amplified as described (122). Placque lifts were probed with a *mnp1* cDNA (55, 152). One λEMBL3 genomic clone yielded a 6.5-kb *Bam*HI fragment that encompassed all of the *Sal*I fragments which previously hybridized with the *mnp1* cDNA probe on Southern blots. This 6.5-kb fragment was subcloned in pUC18 and used as the source for all sequencing template subclones.

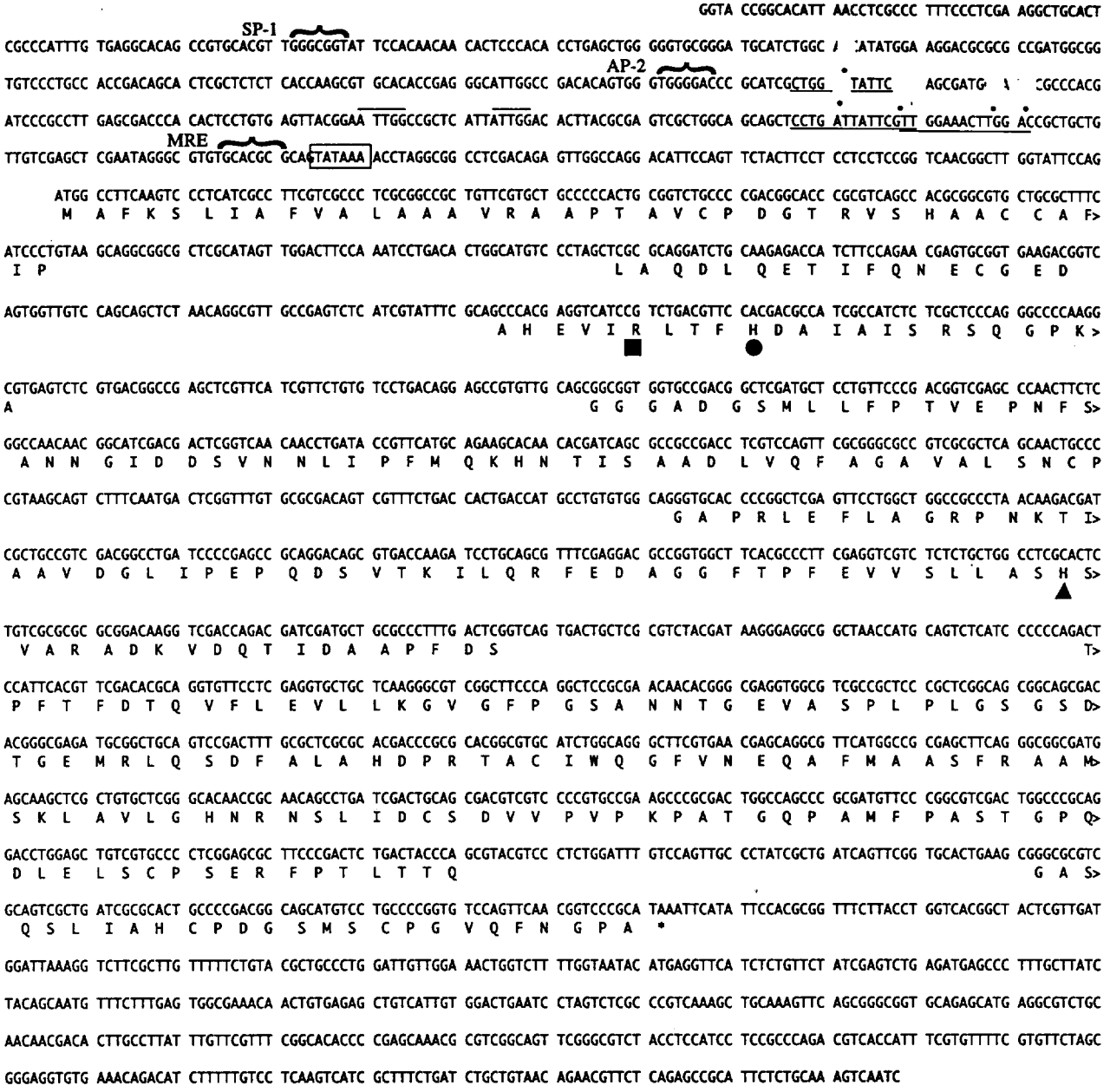


Fig. 2.2 The nucleotide sequence for the *mnp1* gene and the deduced amino acid sequence. The six introns are indicated as interruptions in the aa sequence. The three putative HSE sequences are underlined. Nucleotide residues in the HSEs, which vary from the consensus C—GAA—TTC—G, are indicated with dots. The three inverted CCAAT elements (ATTGG) are overlined. The putative MREs and the putative SP-1 and AP-2 recognition sites are indicated. The TATAAA sequence is boxed. The symbol [indicates the 5′ end of the corresponding cDNA.

The start codon is followed by an apparent 21-aa signal peptide which is underlined. ● and ■ denote the distal His and Arg, respectively. ▲ denotes the proximal His, and the symbol] indicates the site of polyadenylation in the cDNA. Sequencing templates were generated by subcloning restriction fragments into M13mp18/19 from which single-stranded templates were prepared by standard methods (127). Sequence analysis: PolIk sequencing kits were obtained from New England Biolabs and Boehringer-Mannheim and the *Taq* polymerase kit was obtained from U.S. Biochemicals. Chain extension products for dideoxy sequence analysis (169) were labeled with ^{35}S. Gels were prepared and run as described (140).

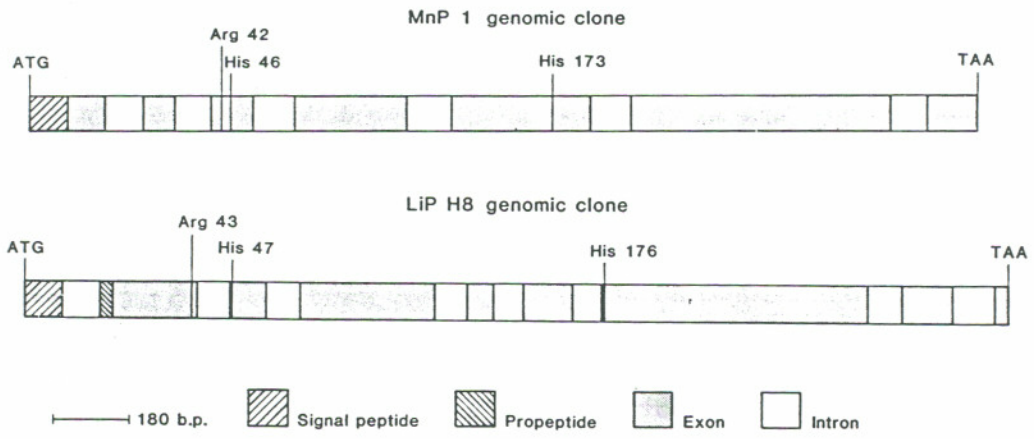


Fig. 2.3 Comparison of the structures of the *mnp1* and *lip*-H8 genes from the start to stop codons. The positions of the distal $His^{46/47}$, $Arg^{42/43}$, and proximal $His^{173/176}$ for *mnp1* and *lip*-H8, respectively, are indicated.

from that encoding the propeptide by an intron (Fig. 2.3) (170). MnP also lacks the short C-terminal proline-rich sequence which is separated from the remainder of the coding region by an intron in all *lip* genes (Figs. 2.2 and 2.3) (10, 180, 201).

The intron splice junction sequences for the *mnp1* gene are compared with consensus sequences for other filamentous fungi (11) in Table 2.1. Intron splice junction sequences all adhere to the GT—AG rule. All of the putative internal lariat formation sites (143) conform to the consensus CTRAY except intron 2 which has a C in place of the R.

The 59% G+C content for the 5′-upstream region of the *mnp1* gene is identical to that for the total *P. chrysosporium* genomic DNA (154). The total G+C content of the introns is 56%, and for the 3′-untranslated and downstream region it is 41%. In contrast, the *mnp1* coding region has an extreme (68%) G+C bias (152).

2.2.3 Regulatory sequences

The 5′-upstream region of the *mnp1* gene (Fig. 2.2) contains a TATAAA element 81 bp upstream from the start codon. In addition, three inverted CCAAT elements (ATTGG) are found at positions -181, -195, and -304 with respect to the start codon (52, 81). Three sequences resembling the consensus eukaryotic HSE (147a), C—GAA—TTC—G, occur at positions -129, -141, and -162 upstream of the start codon (underlined in Fig. 2.2). These three putative heat shock element (HSE) sequences match the consensus in 5, 6, and 7 of the 8 positions, respectively. The two most proximal putative HSEs of *mnp1* at positions -129 and -141 overlap but not in the standard fixed spatial arrangement (18); therefore, they probably do not constitute a doublet. These putative HSEs are interspersed with the inverted CCAAT elements in an arrangement similar to that reported for the human and rodent HS protein *hsp70* promoters (81). We have failed to identify sequences closely resembling a HSE in any of the *lip* genomic sequences which have been reported (10, 180, 201).

The multiplicity of putative HSEs found in the *mnp1* gene conforms to a common motif in HS genes (18, 118). However, many HS genes have long 5′-

Table 2.1 Intron-Exon Junction Sequences in the *mnp1* Gene[a]

Intron No.	5' Splice site	Distance (bp)	Internal site (lariat formation)	Distance (bp)	3' Splice site
1	GTAAGC	-30-	TCCTGAC	-12-	CTAG
2	GTCAGT	-33-	GTCTCAT	-12-	GCAG
3	GTGAGT	-32-	TCCTGAC	-13-	GCAG
4	GTAAGC	-36-	TTCTGAC	-19-	GCAG
5	GTCAGT	-28-	GGCTAAC	-17-	CCAG
6	GTACGT	-27-	CGCTGAT	-14-	GAAG
mnp-1 consensus	GTRAGY C	-27-36-	CTRAY	-12-19	GYAG C
lip- H8 [b] consensus	GTRNRY	-27-34-	CTRA	-6-11	YAG
Ascomycete [c] consensus	GTAYGT A		TGCTAAC AA G		ACAG CT

[a] Y = C or T; R = A or G.

[b] The *lip*-H8 junction sequences (10, 180, 201) are summarized as shown.

[c] The ascomycete consensus sequences sequences are from ref. 11.

untranslated leader sequences and most lack introns (118). Even though the *mnp1* gene lacks the latter two characteristics of HS genes, the expression of *mnp1* does appear to display some HS characteristics. Normal *mnp1* gene transcription is induced under nitrogen-limiting conditions only when the cells are grown in the presence of Mn^{II} (32). As shown in Fig. 2.4, northern blot analysis indicates that when *P. chrysosporium* is cultured under low nitrogen conditions, either the presence of Mn or a 45°C HS for 1 h results in accumulation of MnP1 mRNA, suggesting that HS as well as Mn can induce *mnp1* gene transcription. These results suggest that the putative HSEs found in the promoter region of the *mnp1* gene function in a physiological manner.

Many non-HS stress factors, including respiratory poisons and oxidizing agents, induce the expression of HS proteins (139). For example, H_2O_2 induces HS proteins as well as superoxide dismutase and catalase in prokaryotes (43, 163). Lignin degradation by *P. chrysosporium* is an oxidative process (80, 105). In addition, H_2O_2 is produced by *P. chrysosporium* under ligninolytic conditions (80, 105). Therefore, H_2O_2, oxygen, or carbon-centered radicals generated during lignin degradation (37, 80, 105, 188, 206) may affect *mnp1* gene expression via mechanisms involving these putative HSEs. Inspection of the 5′ upstream region of the *mnp1* gene (Fig. 2.2) also revealed two putative MREs at positions -91 and -481 with respect to the start codon. The MRE at position -481 conforms exactly to the consensus sequence TGCRCYCG (where R and Y are a purine and a pyrimidine, respectively) found in mammalian metallothionein genes (144). The MRE at position -91 varies from the consensus at only one position. Since MnP production is not regulated by heavy metals (32), it is conceivable that these putative MREs mediate the effect of Mn on *mnp1* gene transcription as previously described (32). In addition, the promoter region of the *mnp1* gene contains a putative SP-1 recognition site (GGGCG) with the preferred flanking bases (52) at position -446 from the start codon and a putative AP-2 recognition site (TGGGGA) (209) at position -285. SP-1 and AP-2 are general transcription factors initially identified in the SV40 enhancer and found in many eukaryotic genes (99).

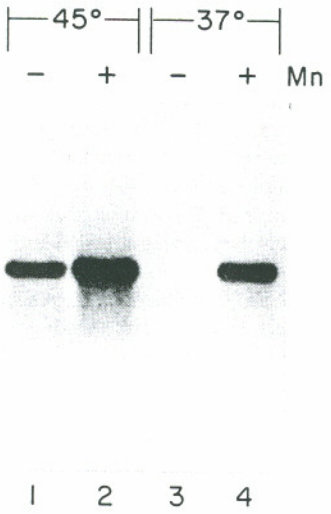


Fig. 2.4 Northern blot analysis of *mnp1* gene transcription. Cultures were grown under limiting nitrogen conditions at 37°C in the absence of Mn (32). On day 4, cultures were induced either by adding Mn to a final concentration of 180 μM (lanes **2** and **4**) or by heat shocking at 45°C (lanes **1** and **2**). After a 1-h induction period, the cells were filtered and frozen. RNA was extracted, electrophoresed in 1.0% agarose containing 0.7 M formaldehyde, transferred to Biotrace RP membranes, and analyzed as previously described (32).

2.3 Acknowledgments

We are grateful to D. Pribnow for help with construction of the genomic DNA library and to M. Alic for valuable discussions. This work was supported by grants from the U.S. Department of Energy (DE-FG07-87ER13715), the U.S. Department of Agriculture (86-FSTY-9-0207), and the National Science Foundation (DMB-8904358).

CHAPTER 3

CHARACTERIZATION OF THE *mnp2* GENE ENCODING MANGANESE PEROXIDASE ISOZYME 2 FROM *PHANEROCHAETE CHRYSOSPORIUM*

3.1 Introduction

White-rot basidiomycetes are primarily responsible for the initiation of lignin decomposition in wood (80, 105). During secondary metabolic (idiophasic) growth, the lignin-degrading basidiomycete *Phanerochaete chrysosporium* secretes two families of extracellular peroxidases, lignin peroxidase (LiP) and manganese peroxidase (MnP), which, along with an H_2O_2-generating system, are apparently the major components of its lignin degradative system (87, 204).

MnP has been purified and characterized (70, 80, 204). It is an H_2O_2-dependent 46-kDa heme glycoprotein which, like LiP, exists as a family of isozymes (113). MnP oxidizes Mn^{2+} to Mn^{3+} which, in turn, oxidizes a variety of phenols including lignin model dimers and polymeric lignin (70, 80, 204).

cDNA sequences encoding two different isozymes of MnP have been reported (146, 152). Using a *mnp* cDNA probe, we have demonstrated that MnP expression is regulated at the level of gene transcription by Mn ions and heat shock (30–32, 73). Recently, we also characterized a genomic clone, *mnp1*, encoding MnP isozyme 1 (73). Here, the nucleotide (nt) sequence of a genomic clone encoding MnP isozyme 2 (*mnp2*) was determined and compared with previously reported nt sequences encoding MnPs.

3.2 Experimental and Discussion

3.2.1 Sequence of the *mnp2* gene

A set of overlapping restriction fragments of two *mnp2* cDNAs subcloned into M13mp18 or M13mp19 were sequenced as described (152). Characterization by Southern blotting of one genomic clone suggested that it encoded the *mnp2* gene. A set of nested deletions generated by exonuclease III digestion was used for sequencing this genomic clone. The *mnp2* sequence and the predicted translation product are shown in Fig. 3.1. The experimentally determined N-terminal sequence of the first 20 amino acids (aa) of mature *mnp2*, beginning with Ala^{1} of mature MnP (Ala^{25} in Fig. 3.1), matches the predicted translation product, indicating this gene encodes *mnp2*. It also matches the first 20 aa of the predicted translation product of the cDNA λMP-1 (146). The coding region sequences and the 5′ and 3′ untranslated regions of the *mnp2* gene differ from our *mnp2a* cDNA at only two individual nt positions and from our *mnp2b* cDNA at seven positions. These minor differences suggest that our two cDNAs and one genomic sequence are derived from allelic genes. The *mnp2* coding region described here also is similar to that of the λMP-1 cDNA sequence reported earlier (146), differing at only nine nt positions. The coding region of the *mnp2* and *mnp1* genes exhibit 81% identity at the nt level and 85% identity at the aa level.

3.2.2 Introns and exons

The near identity between the two cDNAs and the genomic sequences described here allowed the ready deduction of intron positions. The seven introns in the *mnp2* gene vary in size from 50 to 55 bp. The positions of the seven introns in *mnp2* are compared with the positions of the six introns in *mnp1* (Fig. 3.2) (73). All of the introns in *mnp1* align with introns in *mnp2*. The additional intron in *mnp2* is located in a position which splits exon 3 of *mnp1* at the codon for the catalytic distal His^{46} (74, 150). The intron splice junction sequences all adhere to the GT—AG rule, and all of the putative internal lariat formation sites (143) conform to the consensus

Fig. 3.1 The nucleotide sequence of the *mnp2* gene and the deduced aa sequence. cDNA library construction, plaque screening using a polyclonal antibody, and cDNA sequencing were as described (152). Restriction mapping of replicative forms was used to differentiate various *mnp1* and *mnp2* cDNA clones. Restriction fragments of *mnp2* cDNA clones were subcloned directly from gels into M13mp18 or M13mp19 from which single-stranded templates were prepared (127). Fragments were sequenced in both directions by the dideoxy method (169) using [α-^{35}S]dATP (NEN-Dupont). Genomic DNA was isolated, digested, and ligated into λEMBL3 arms as described (122). Ligation products were packaged and amplified as described (73). Plaque lifts were probed with a *mnp2* cDNA (55). One λEMBL3 clone yielded a 4.8-kb *Hin*dIII-*Sph*I fragment which hybridized with the *mnp2* cDNA on Southern blots, and this was subcloned into pUC18. Subsequently, *Hin*dIII + *Bam*HI were used to release the 4.8-kb fragment for subcloning into Bluescript (Stratagene, La Jolla, CA). Exonuclease III was used to generate overlapping deletion subclones (91). Organisms: *P. chrysosporium* strain OGC101 (5) was used throughout this study. *Escherichia coli* strain DH5αF′ or XL-1 Blue was used as described (73, 152). Purification of MnP2 protein was as described (70) except that fast protein liquid chromatography using a Mono-Q column was used to separate several MnP

isozymes. Sequencing of the N terminus of the mature MnP2 protein was as described (152). The seven introns are indicated as interruptions in the aa sequence. The six putative HSE sequences are underlined. The three inverted CCAAT elements (ATTGG) are overlined. The TATAA box is boxed. The putative AP-2 site is double-overlined. The putative MRE and the putative SP-1 binding site are indicated. The start codon is followed by an apparent 24-aa signal peptide which is underlined. The symbol [indicates the 5′ end, and the symbol] indicates the site of polyadenylation of the corresponding cDNA. The braced { } 249 nt sequence encompassing the translation start codon was used to probe the northern blot in Fig. 3.3.

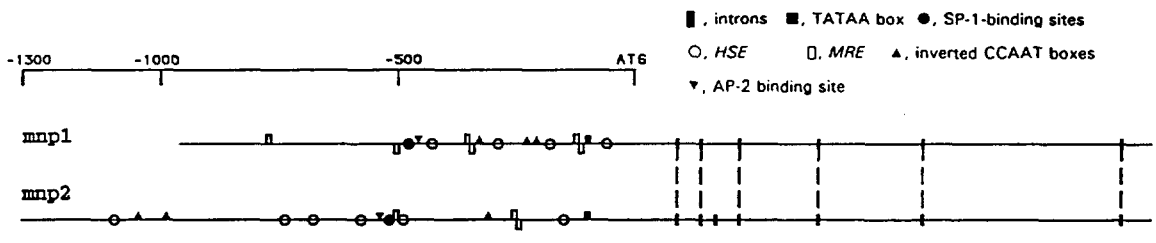


Fig. 3.2 Comparison of the structure of the *mnp1* and *mnp2* genes.

CTRAY except for the site in intron 4 which has a C instead of an R. The pattern of intron number and position has been used to classify the large family of *lip* genes from *P. chrysosporium* into four subfamilies (74, 164). Our results suggest that *mnp1* and *mnp2* represent two gene subfamilies.

3.2.3 Signal peptide (SP)

Deduced aa sequences from the nt sequences encoding *mnp2* described here and previously (146) suggest a mature 358-aa protein preceded by a 24-aa leader sequence characteristic of a SP (90). In contrast, the sequence of *mnp1* suggests a SP of 21 aa (152). Both the *mnp1* and *mnp2* deduced aa sequences lack the 6–7-aa propeptide found between the SP and the mature LiP protein in all *lip* sequences (165). The sequences encoding the SP and N terminus of the mature protein are on the same exon in the *mnp* genes. In all *lip* genes but one, the sequence encoding the SP is separated from that encoding the propeptide by an intron (165, 170).

3.2.4 Regulatory sequences

Fig. 3.1 shows the sequence of 1,287 nt of the 5′ upstream region of the *mnp2* gene. This region contains a TATAAA element 81 bp upstream of the start codon. In addition, three inverted CCAAT elements (ATTGG) (52) are found at nt positions -301, -966, -1,026 with respect to the start codon. Six sequences resembling the consensus eukaryotic HS elements (HSE) (118), C-GAA-TTC-G, occur at nt -110, -459, -542, -654, -711, and -1,071 with respect to the start codon. These six HSEs each match the consensus in six or seven of the eight positions. The 5′ upstream region of the *mnp2* gene also reveals three putative metal response elements (MRE). The putative MRE at nt -237 and the reverse complements at nt -238 and -479 with respect to the start codon conform to the consensus sequence TGCRCNC found in mouse metallothionein (MT) genes (96). The pair of MREs at nt -237/238 overlap and form a 4-bp palindrome (TGCA). The identical overlap occurs in the two proximal pairs of MRE in the *mnp1* gene (73) (Fig. 3.2). The *mnp2* gene contains a putative SP-1-recognition site (5′-GGGCGG) (52) at nt -492. The *mnp2* gene

contains one putative inverted AP-2 recognition site (5′-CCCACGGC) at nt -508 which exactly matches a recently postulated AP-2 consensus sequence for *lip* genes (47). The *mnp1* gene also contains a putative inverted AP-2 recognition site (5′-CCCACACC) at nt -425, exactly matching this consensus sequence. In contrast, neither of the two *mnp1* promoter sequences recently suggested to be AP-2 recognition sites (47) exactly match this proposed AP-2 consensus sequence. Furthermore, other sequences within the *mnp1* and *mnp2* gene promoter match AP-2 consensus sequences which have been experimentally verified in mammalian cells (95).

The positions of the regulatory elements in the 5′ upstream region of *mnp2* are compared with those in the promoter of *mnp1* in Fig. 3.2. The 5′ upstream regions of both genes contain a TATAA box, multiple inverted CCAAT boxes, and multiple putative HSE and MRE. Although the positions of the TATAA box, one of the CCAAT boxes and one of the MRE, relative to the ATG are conserved in the two genes, the positions and/or numbers of the HSE and other MRE, CAAT boxes and AP-2 sites differ between the two genes. In particular, the 5′ upstream region of *mnp1* contains four HSE and six MRE, while the 5′ upstream region of *mnp2* contains six HSE and three MRE. The number, position, and orientation of HSE and MRE varies within the promoters for various HS proteins and mammalian MT (96, 118, 186). Using the *mnp1* cDNA as a probe of northern blots, we have shown that Mn ion regulation of MnP expression occurs at the level of gene transcription (30). These results suggest that the putative MRE observed in the *mnp1* gene may be involved in Mn ion regulation of MnP expression. A specific probe to *mnp2* using a fragment from the 5′-noncoding region of the *mnp2* gene (Fig. 3.1) was used to determine whether Mn ion regulates the expression of this gene. Southern blots of the purified *mnp1* and *mnp2* genes demonstrate that the *mnp2* specific probe hybridizes only with *mnp2* (data not shown). As shown in Fig. 3.3, northern blot analysis indicates that when *P. chrysosporium* is grown at 37°C under low nitrogen conditions, the addition of 180 μM $MnSO_4$ results in the specific accumulation of *mnp2* RNA, suggesting that *mnp2* transcription is regulated by Mn ion.

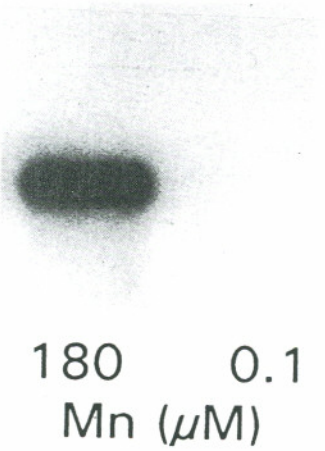


Fig. 3.3 Northern blot analysis of *mnp2* gene transcription. Cultures were grown under limiting nitrogen conditions in the presence of 0.1 or 180 μM $MnSO_4$ as described (32). On day 5, the cells were filtered and frozen in liquid nitrogen. RNA was extracted from 40 mg of frozen cells as described (30) except that after chloroform extraction the aqueous phase was precipitated with 2 M LiCl before the final 70% EtOH precipitation. The RNA was electrophoresed in 1.0% agarose containing 0.7 M formaldehyde, transferred to Biotrace HP membranes (Gelman, Ann Arbor, MI), and analyzed as described (30). The 249-nt fragment shown in Fig. 3.1 was used as a template for randomly primed synthesis of a labeled specific probe (55).

The best-studied system for metal regulation of transcription in eukaryotes is MT biosynthesis (186). MT in yeast, encoded by the *cup1* gene, is activated in the presence of Cu^{2+} by binding of the Ace1 protein to *cis*-acting sequences in the promoter region of *cup1*. Multiple copies of *cis*-acting elements, found in both orientations, are responsible for heavy metal induction of mammalian MT genes. The similarities between the Mn ion regulation of *mnp* genes and metal regulation of MT genes, including the presence of multiple putative MRE in both sequenced *mnp* gene promoters, suggest that a Mn-binding transcription factor may be involved. We are using the DNA transformation system that we developed for *P. chrysosporium* (74) to examine the role of these putative MRE and HSE in the regulation of *mnp* gene transcription.

3.2.5 Conclusions

(1) The gene encoding MnP2 was isolated and sequenced. The coding region of the *mnp2* gene is interrupted by seven small introns. The promoter region contains a putative TATAA box, HSE, MRE, inverted CCAAT boxes, and an SP-1-binding site. All of these putative consensus sequences are also found in the promoter region of the *mnp1* gene.

(2) A northern blot experiment suggests that *mnp2* gene transcription is regulated by Mn ion.

3.3 Acknowledgments

This work was supported by grants DMB-9104610 and MCB-9207997 from the National Science Foundation and DE-FG06-92ER20093 from the U.S. Department of Energy, Division of Energy Biosciences, Office of Basic Energy Sciences. The authors thank Dr. Margaret Alic for valuable discussions.

CHAPTER 4
A REPORTER GENE CONSTRUCT FOR STUDYING THE REGULATION OF MANGANESE PEROXIDASE GENE EXPRESSION

4.1 Introduction

The white-rot basidiomycete *Phanerochaete chrysosporium* has been the focus of numerous studies on the degradation of lignin (37, 80, 105) and aromatic pollutants (35, 86, 100, 198). Two families of peroxidases, lignin peroxidase (LiP) and manganese peroxidase (MnP), along with an H_2O_2 generating system, are the major components of this organism's extracellular lignin degrading system (74, 80, 105). MnP has been purified and extensively characterized (70, 74, 202, 203). MnP is an H_2O_2- and Mn^{II}-dependent, heme-containing glycoprotein of M_r ~46,000 (74, 80). This enzyme oxidizes Mn^{II} to Mn^{III}; the latter, complexed with an organic acid chelator such as oxalate or malonate, secreted by *P. chrysosporium*, oxidizes the terminal phenolic substrate (70, 109, 205). MnP occurs as a family of isozymes encoded by a series of genes, and the sequences of cDNA (146, 152) and genomic clones (73, 74, 125) encoding two MnP isozymes have been determined. As an idiophasic protein, the expression of MnP is activated at the level of transcription by the depletion of nutrient nitrogen (74, 80, 105, 152), and there is evidence that the MnP isozymes may be differentially regulated by carbon and nitrogen (147). MnP expression also is dependent on the presence of Mn^{II} in the culture medium (26, 32), and *mnp* gene transcription is regulated by Mn^{II} (30, 32). In addition, we demonstrated that *mnp* gene transcription is regulated by heat shock (31, 73, 74).

The promoter regions of the two sequenced *mnp* genes (73, 74, 125) contain putative heat shock elements (HSEs) (118) and metal response elements (MREs) (96, 186). To further examine the putative *cis*-acting sequences involved in the regulation

of *mnp* gene expression by nitrogen, Mn^{II}, and heat shock, we have constructed a promoter-reporter system in which the promoter region of the *mnp1* gene is fused to the coding region of the orotidylate decarboxylase (ODase) gene (*ura1*) from *Schizophyllum commune* acting as a reporter. This exogenous promoter-reporter was used to examine regulation of the *mnp* system.

4.2 Materials and Methods

4.2.1 Organisms

P. chrysosporium OGC101, *ade1* and *ura11* mutants, and the *ade1ura11* double mutant were as described (1, 5). *Escherichia coli* strains XL1-Blue and DH5α were used for subcloning plasmids.

4.2.2 Enzymes and chemicals

Orotate phosphoribosyl transferase, orotidylate decarboxylase, and sodium orotate were obtained from Sigma. ^{14}COOH-orotic acid was obtained from DuPont-New England Nuclear. Restriction enzymes, T4 DNA ligase, and T4 DNA polymerase were obtained from New England Biolabs or Promega. Reaction conditions were as recommended by suppliers; otherwise, they were standard (122).

4.2.3 Construction of pOGI 18

A 3.8-kb *Bam*HI-*Kpn*I fragment containing the *S. commune ade5* gene from plasmid pAde5-2g (3) and *Aat*II-digested pUC18 were treated in separate reactions with T4 polymerase in the presence of all four deoxynucleoside triphosphates to create blunt-ended fragments. These fragments were ligated and the resulting plasmid was transformed into DH5α cells. The cells were plated onto nitrocellulose filters on Luria broth-ampicillin (LBamp) plates. Filters were probed with the ^{32}P-labeled *Bam*HI-*Kpn*I fragment to identify recombinant colonies (122). Recombinants were streaked onto LBamp plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside), and several blue

colonies were picked for plasmid preparations and subsequent restriction mapping. One colony which gave the expected restriction pattern was selected as the *P. chrysosporium ade1* transformation vector. A map of this plasmid, pOGI 18, is shown in Fig. 4.1a.

4.2.4 Construction of pAMO

A 6.5-kb λEMBL3 *Bam*HI fragment containing the *mnp1* gene (73) was subcloned into pUC18, after which a 2.1-kb *Sal*I fragment containing the 5′ end of the coding region and 1.4 kb of 5′ untranslated sequence was subcloned into pUC18. A *Sal*I-*Hin*cII subfragment which spans from 1.4 kb upstream of the coding region to 15 bp downstream of the TATAAA sequence was isolated from this subclone by digesting with *Sal*I and then with *Hin*cII. The *S. commune ura1* gene was obtained from R. Ullrich as the plasmid pEF1 (62). The translation initiation codon of this gene resides within a unique *Bsp*HI site and the coding region of the gene was isolated as a *Bsp*HI-*Bam*HI fragment. A synthetic linker was used to replace the *mnp1* 5′ sequences between the *Hin*cII site and initiation codon and to adapt the *mnp1* promoter fragment to the *Bsp*HI overhang on the *ura1* gene (Fig. 4.1b). The promoter-reporter fusion was initially assembled in pUC18 as pMO (data not shown). The three fragments were combined in an equimolar ratio with *Sal*I-*Bam*HI-digested pUC18, ligated, and transformed into XL1-Blue cells. To construct pAMO (Fig. 4.1b and c), pMO was cut at the unique *Sph*I site and treated with T4 polymerase to create blunt ends. The complete fusion construct was then released from pUC18 by digestion with *Eco*RI and subcloned into *Sma*I-*Eco*RI-digested pOGI 18. The construction was confirmed by restriction mapping and by sequencing an *Eag*I-*Sal*I fragment spanning the fusion junction which had been subcloned into pBluescriptIISK+ to provide single-stranded DNA.

4.2.5 Fungal transformations

The double mutant *ade1ura11* was transformed as described elsewhere (3, 4). One microgram of the plasmid pAMO, linearized at the unique *Eco*RI site (Fig.

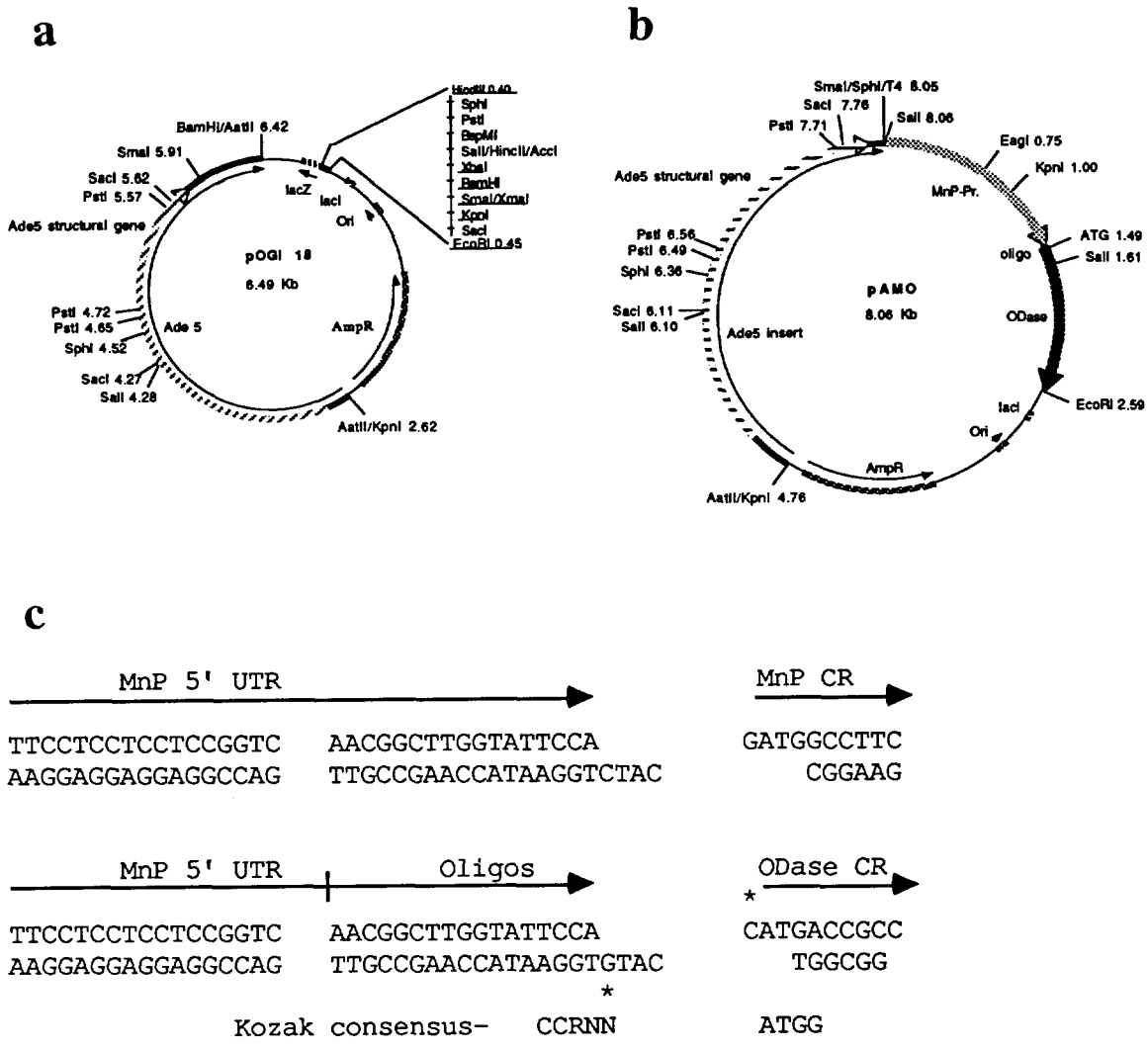


Fig. 4.1 Restriction map of pOGI 18. (a) The light slashed line indicates the approximate location of the *ade5* gene, and the dark line indicates *ade5* flanking sequence. The light line indicates pUC18 sequence. (b) Restriction map of pAMO, containing the *mnp* promoter-*ura1* reporter in pOGI 18. The positions and orientation of the *mnp* promoter and *ura1* reporter are indicated. The *Sma*I site used for insertion of the *mnp-ura1* fragment is in a section of the *ade5* insert not required for function. (c) Fusion junction of the *mnp* promoter-*ura1* reporter construct. The single-base-pair change in the *mnp1* sequence still conforms to the eukaryotic translation initiation consensus sequence (107). UTR, untranslated region; CR, coding region.

4.1b), and 2 × 10^6 protoplasts were used for each transformation and selected on medium containing uracil. Approximately 30 Ade^+ transformants were obtained per μg of plasmid DNA. Twenty Ade^+ transformant colonies were transferred to Vogel medium containing 1% glucose, 0.01% uracil, and 1.5% agar (GV-ura) and GV slants (5, 76). Those transformants which grew only on GV-ura but not on GV were transferred to rich slants, containing Vogel medium, 3% malt extract, 0.5% tryptone, 0.5% yeast extract, 1.5% agar, 0.01% uracil, and 0.001% adenine (76) for maintenance. Transformants were purified by fruiting and plating basidiospores as described previously (5). The modified fruiting media (5, 75) consisted of 4.5% Walseth cellulose, 0.01% uracil, and 0.001% adenine, in 1/6-strength Vogel medium.

4.2.6 Culture conditions

Mutant and transformant cultures were maintained on rich slants as described above, containing adenine and uracil. The basal medium (HCLN) (30, 106) contained 2% glucose and 1.2 mM ammonium tartrate and a trace element solution containing no $MnSO_4$. $HCLN_{AUY}$ is HCLN supplemented with 0.001% adenine, 0.01% uracil, 0.01% yeast extract (Difco), and either 0.25 or 180 μM $MnSO_4$. HCHN basal medium contained 2% glucose and 24 mM ammonium tartrate.

Initially, single basidiospore isolates of each transformant were tested for expression of the reporter gene. Isolates were grown at 37°C from a conidial inoculum in 20-ml stationary cultures of $HCLN_{AUY}$ medium, containing 0.25 μM $MnSO_4$ in 250-ml Erlenmeyer flasks. Cultures were incubated under air for 5 days and then purged with 100% O_2 on day 5, and $MnSO_4$ (180 μM) was added on day 6. Cells were harvested by filtration through Miracloth 36 h after Mn^{II} induction, frozen in liquid N_2, and stored at -80°C.

Subsequent experiments were conducted with isolate A141. This isolate was initially grown from a conidial inoculum in $HCLN_{AUY}$ medium containing either 0.25 or 180 μM $MnSO_4$. Cultures were incubated under air for 4 days and subsequently purged with O_2 at subsequent 48-h intervals. Extracellular MnP activity was assayed and cells were harvested as described.

For the Mn induction experiments, cultures of isolate A141 were grown for 5 days under air in $HCLN_{AUY}$ or $HCHN_{AUY}$ medium containing 0.25 μM $MnSO_4$ and then purged with O_2. On day 6, 180 μM $MnSO_4$ was added after which extracellular MnP activity was assayed as indicated and cells were harvested and stored at -80°C.

4.2.7 Intracellular enzyme extracts

Frozen cells (100 mg) were first crushed and then shaken for 15 s with cold, dry glass beads on a mini-bead beater (Biospec Products, Bartlesville, OK). An 800-μl volume of ice-cold 50 mM Na-phosphate (pH 7.0) was added, and the tube was shaken on the bead beater for an additional 60 s. Another 800 μl of buffer was added and the mixture was shaken for 3 min on the bead beater. The broken cell preparation was centrifuged for 10 min at 12,000 rpm at 4°C in an Eppendorf microcentrifuge. Supernatants were assayed for ODase activity either immediately or after storage for up to 1 week at -20°C. Protein concentration was determined by the bicinchoninic acid protein assay.

4.2.8 ODase assay

The ODase assay, measuring the evolution of $^{14}CO_2$ from ^{14}COOH-orotidyl monophosphate, is a modification of that described previously (151). ^{14}C-labeled orotic acid, phosphoribosyl pyrophosphate (PRPP), and orotidine-5′-phosphate pyrophosphorylase (OPPase) were used to generate ^{14}COOH-orotidyl monophosphate in the coupled reaction shown in Fig. 4.2. The reaction was carried out in a 15-ml Falcon centrifuge tube stoppered with an Aldrich Suba-Seal stopper. The $^{14}CO_2$ released was absorbed onto a piece of Whatman 3MM paper (1 × 1.5 cm) presaturated with 50 μl of 1 M NaOH and suspended from the Suba-Seal stopper. The 1-ml reaction mixture contained 500 μM orotate (specific activity 0.113 μCi/μM), 5 mM $MgCl_2$, 1 mM PRPP, and 2 mM dithiothreitol in 50 mM Na phosphate, pH 7.0. The reaction was started by the addition of extract and 1 U of OPPase. Incubation was at 37°C with gentle agitation for 1 h. The reaction was stopped by the addition, with a needle, of 200 μl of 1 M H_2SO_4. The tubes were left in the water bath for an additional hour to complete the adsorption of the $^{14}CO_2$ onto

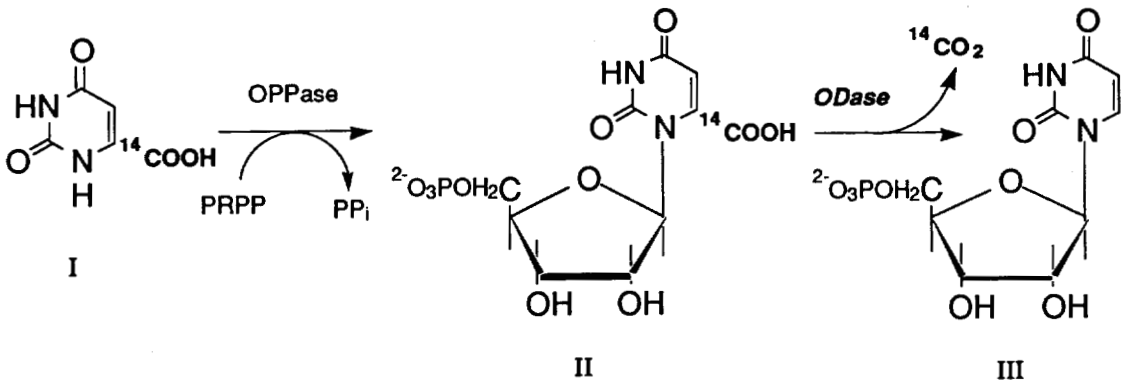


Fig. 4.2 Reactions of the two-step coupled ODase assay. **I**, ^{14}C-orotic acid; **II**, ^{14}COOH-orotidyl monophosphate; **III**, uracil monophosphate; PRPP, phosphoribosyl pyrophosphate; PPi, inorganic pyrophosphate; OPPase, orotidylate pyrophosphorylase.

the paper. The papers were dried, and counts were performed in a scintillation counter.

MnP activity was measured by monitoring the formation of Mn^{III} malonate at 270 nm as described elsewhere (205). Reaction mixtures (1 ml) contained 0.2 mM $MnSO_4$, 0.2 mM H_2O_2, and 50 μl extracellular medium in 50 mM Na malonate, pH 4.5. MnP and ODase activity were measured in triplicate cultures.

4.3 Results

4.3.1 Construction of vectors

The *E. coli-P. chrysosporium* shuttle vector, pOGI 18, contains the *S. commune ade1* gene (61) encoding phosphoribosyl aminoimidazole synthetase (3) on a 6.5-kb *Bam*HI/*Kpn*I fragment ligated into the unique *Aat*II site of pUC18. This plasmid, pOGI 18 (Fig. 4.1a), provided blue-white selection for DNA inserted in the multiple cloning site of pUC18 and complementation of the *P. chrysosporium ade1* strain as previously reported for the pAde5-2g plasmid (3).

4.3.2 *mnp* promoter-ODase reporter fusion, pAMO

The *mnp* promoter-ODase reporter construct contained a 1.4-kb *Sal*I-*Hin*cII fragment of the *mnp1* gene, which spans from 1,400 bases 5′ of the cDNA start (152) to 15 bases downstream of the TATAAA box; a synthetic oligonucleotide linker which recreates the *mnp1* 5′ untranslated region (UTR) from the *Hin*cII site to the translation initiation codon; and the coding region of the *S. commune ura1* gene from the initiation codon to the end of the pEF1 insert (1.65 kb) (62). The single base change before the ATG, as shown in Fig. 4.1c, conforms to the CCRNNATGG eukaryotic initiation consensus sequence (107). However, the *mnp1* gene differs from the consensus in several positions (152). The complete *mnp-ura* construct was subcloned into pOGI 18 as an *Sph*I/*Eco*RI fragment to create pAMO (Fig. 4.1b).

4.3.3 Transformation and characterization of transformants

An *ade1ura11* mutant lacking any endogenous ODase activity was used as the host strain for transformations. This double mutant, when transformed with pOGI 18 and grown with uracil supplementation, produces MnP and LiP under idiophasic growth conditions but grows more slowly than the *ade1* and OGC101 strains.

Of the 20 Ade^+ transformants examined, induction of ODase in response to exogenous Mn^{II} varied from 0- to 25-fold with an average induction of 7.7-fold. The transformant (A141) with the highest Mn^{II} induction of the reporter gene was selected for further study. From a Southern blot, we estimated that one to two copies of the plasmid had integrated into the genome in this transformant (data not shown).

4.3.4 Effect of Mn on expression of ODase and *mnp1* in cultures of the transformant A141

Time courses for the appearance of extracellular MnP activity and intracellular ODase activity are shown in Fig. 4.3. Cultures grown in the presence of 0.25 μM Mn^{II} had no detectable MnP activity through day 8 and had minimal ODase activity during the same period, whereas in cultures containing 180 μM Mn^{II}, both MnP and ODase activity first appeared on day 6 and reached a maximum on day 8. With this transformant, MnP activity appeared approximately 48 h later than in the wild-type strain, presumably owing to the mutant strain's slower growth.

The induction of MnP and ODase activity by Mn^{II} is shown in Fig. 4.4. The transformant was grown for 5 days in the presence of 0.25 μM Mn^{II}, after which the cultures were purged with O_2. On day 6, 180 μM Mn^{II} was added to the medium and the cultures were reincubated. No MnP activity was detected before the addition of 180 μM Mn^{II}. MnP activity reached a maximum 30 h after the addition of 180 μM Mn^{II} and declined thereafter. This is similar to our previous results with the wild-type strain (32). In parallel with the appearance of MnP activity, ODase activity steadily increased for 30 h after the addition of Mn^{II}, reaching a maximum induction of $\sim$25-fold. Beyond 30 h, ODase activity leveled off. In contrast to the induction of activity in HCLN cultures, neither MnP nor ODase activity was seen for the first 48 h after 180 μM Mn^{II} was added to cultures grown for 6 days in HCHN medium containing 0.25 μM Mn^{II} (Fig. 4.4).

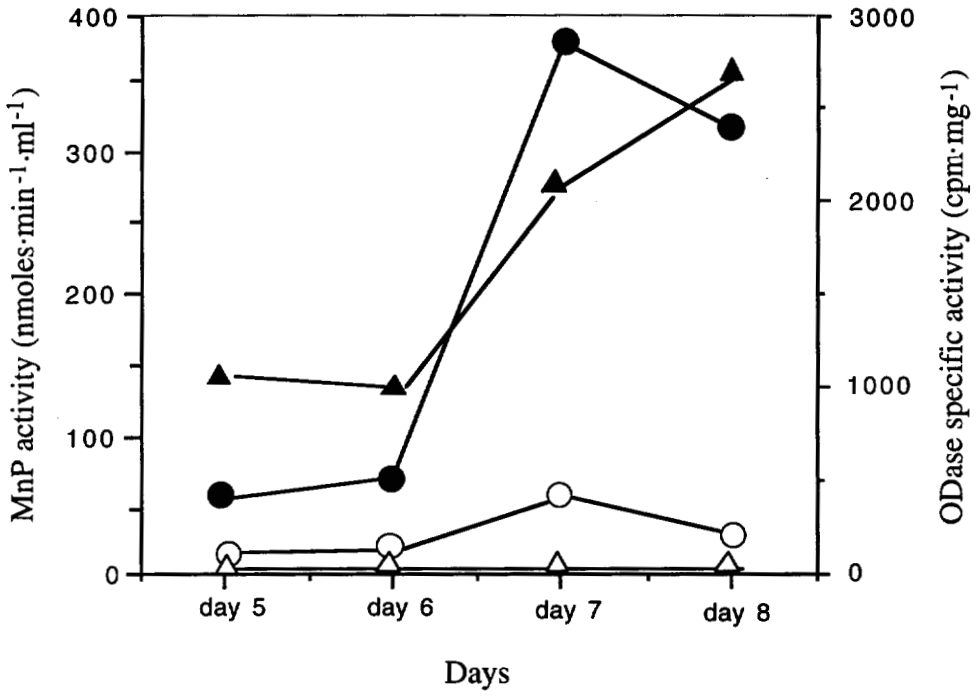


Fig. 4.3 Effect of Mn supplementation on the appearance of extracellular MnP and intracellular ODase activity. Nitrogen-limited cultures of the transformant A141 were grown in the presence of 180 or 0.25 μM $MnSO_4$ from a conidial inoculum as described in the text. MnP activity from triplicate cultures grown in the presence of 180 (▲) or 0.25 (△) μM Mn and ODase activity from triplicate cultures grown in the presence of 180 (●) or 0.25 (○) μM Mn were assayed as described in the text.

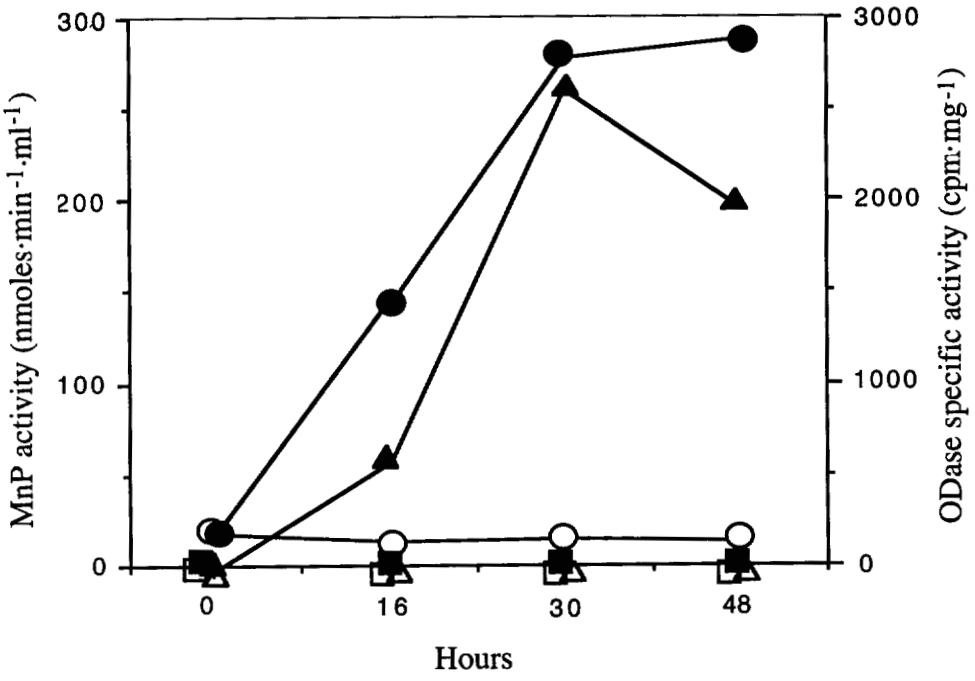


Fig. 4.4 Induction of extracellular MnP and intracellular ODase activity by Mn. Nitrogen-limited or nitrogen-sufficient, Mn-deficient cultures of the transformant A141 were grown for 6 days, after which 180 μM $MnSO_4$ was added to the experimental flasks. ODase activity in nitrogen-limited cultures induced with Mn (●) or not induced (○), and in nitrogen-sufficient cultures induced with Mn (■) was assayed as described in the text. MnP activity in nitrogen-limited cultures induced with Mn (▲) or not induced (△), and in nitrogen-sufficient cultures induced with Mn (□) was assayed as described in the text.

4.4 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 (37, 80, 105). Likewise, MnP and LiP activities are detectable in the extracellular medium only during the secondary metabolic phase of growth (74, 105). Northern (RNA) blot analysis has demonstrated that expression of both MnP and LiP is controlled at the level of gene transcription by nutrient nitrogen (152, 191).

Previously we demonstrated that MnP expression also is regulated by Mn^{II}, the substrate for the enzyme, at the level of gene transcription (30, 32) and that the addition of Mn^{II} to nitrogen-limited, Mn-deficient cultures on days 4, 5, or 6 results in detectable *mnp* mRNA within 40 min (30). In the white-rot basidiomycete *Dichomitus squalens*, MnP expression and lignin degradation also have been shown to be dependent on the presence of Mn^{II} (149). Examination of the promoter regions of the *mnp1* and *mnp2* genes (73, 74, 125) revealed the presence of putative MREs within 800 bp of the translation initiation codon. These sequences are identical to *cis*-acting MRE sequences responsible for heavy-metal induction of mammalian metallothionein genes (96, 186). Interestingly, examination of the *mnp1* promoter region also revealed the presence putative HSEs within 400 bp upstream of the *mnp1* translation initiation codon (73, 74). Likewise, six HSEs are found within 1,100 bp of the translation initiation codon of the *mnp2* gene (74, 125). Previously, we reported on the heat shock induction of *mnp* gene transcription, even in the absence of Mn^{II}, suggesting that the HSEs are physiologically functional (31, 74, 125). Since neither MREs nor HSEs are found in *lip* gene promoters (74), these sequences may be involved in specific aspects of *mnp* gene regulation, in contrast to the shared mechanisms regulating these peroxidases by factors such as nutrient nitrogen depletion (74, 105).

Several metal-ion-regulated gene transcription systems have been studied in detail (96, 157, 186). Most of these are single-component systems, wherein a single

intracellular metalloregulatory protein functions as both the metal receptor and the *trans*-acting transcription factor. Although the possibility that Mn^{II} is acting through a multicomponent signal transduction system has not been ruled out, the existence of an Mn^{II}-binding transcription factor similar to the Cu-binding Ace1 protein that activates transcription of the yeast metallothionein gene (186) is an attractive model for the *mnp* system. Although Mn^{II} is involved in the synthesis of some secondary metabolites in other fungi (173, 207), this is, to our knowledge, the first instance of Mn^{II} regulation of gene transcription to be studied at the molecular level.

We chose the *S. commune ura1* gene as a reporter because this gene complements *ura11* mutants of *P. chrysosporium* (1), ensuring that *ura1* is expressed in this organism. The availability of an *ade1ura11* double mutant enabled us to transform with the shuttle vector and select for Ade^{+} transformants. It also allowed for measurement of ODase activity encoded by the exogenous *ura1* gene without interference by endogenous ODase activity. ODase has no known substrates other than OMP, eliminating interference with the assay by other components of crude cell extracts. Furthermore, ODase activity in crude cell extracts is stable for at least 1 week when the extracts are stored at -20°C.

Transformation of *P. chrysosporium* with heterologous plasmid DNA results in incorporation of the DNA into multiple ectopic locations (3, 4). pOGI vector derivatives containing, for the most part, heterologous DNA also presumably integrate at ectopic locations. Thus, in these experiments, the site of integration has not been controlled. However, the expression of the endogenous *mnp* gene can be used as an internal control, and direct comparison of the expression of MnP with that of ODase has been made.

The results in Fig. 4.3 show that the time course of ODase expression in the transformant parallels that of MnP expression. The expression of both enzymes occurs during secondary metabolic (idiophasic) growth and is dependent on the presence of high levels of Mn^{II} in the culture medium. Both ODase and MnP activities in the transformant are expressed on days 7 and 8, with little or no activity in cells grown in 0.25 μM Mn^{II}.

The role of Mn^{II} in regulating the expression of the reporter gene can be examined most directly with induction experiments, wherein the effect of Mn^{II} is independent of other variables such as nutrient nitrogen and atmospheric O_2 levels in the flasks. The results in Fig. 4.4 show that the addition of 180 μM Mn^{II} to cultures grown for 6 days in the presence of 0.25 μM Mn^{II} leads to the simultaneous induction of both endogenous MnP and ODase reporter activity. In this experiment, the reporter activity is induced ~25-fold over the baseline level. Both MnP activity and ODase activity reach a maximum 30 h after the addition of Mn^{II}. Subsequently, MnP activity decreases while the ODase activity levels off, suggesting that ODase protein or message has a longer half-life than MnP protein or message *in vivo*. Finally, addition of 180 μM Mn^{II} to cells grown in HCHN medium does not lead to induction of either the reporter activity or endogenous MnP activity.

We conclude from these results that 1,500 bp of sequence immediately upstream of the *mnp* translation start site is sufficient to regulate the *ura1* reporter in a manner analogous to the regulation of the endogenous *mnp* genes with respect to Mn^{II}, nutrient nitrogen levels, and metabolic phase of growth. These results suggest that this first promoter-reporter construct for *P. chrysosporium* should prove useful for studying the roles of the putative MREs and other *cis*-acting sequences in the regulation of *mnp* gene transcription by Mn^{II}, heat shock, and nutrient nitrogen levels. We are constructing deletion mutants of the *mnp* promoter and site-directed mutants altered in the MRE sequences to delineate the role of the MREs in the regulation of *mnp* by Mn^{II} and possibly other factors.

4.5 Acknowledgments

This work was supported by grants DMB 9104610 and MCB 9207997 from the National Science Foundation. We thank Robert Ullrich, University of Vermont, for supplying plasmid PEF1 containing the *S. commune ura1* gene and Margaret Alic for valuable discussions.

CHAPTER 5
MUTATIONAL ANALYSIS OF METAL RESPONSE ELEMENTS IN THE MANGANESE PEROXIDASE GENE PROMOTER FROM *PHANEROCHAETE CHRYSOSPORIUM*

5.1 Introduction

The white-rot basidiomycete *Phanerochaete chrysosporium* has been the focus of numerous studies on the degradation of lignin (37, 80, 105) and aromatic pollutants (35, 84, 100, 198). 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 (74, 80, 105). MnP has been purified and extensively characterized (70, 80, 202, 203) as an H_2O_2-requiring, heme-containing glycoprotein of M_r ~46,000 (74, 80). The enzyme oxidizes Mn^{II} to Mn^{III}; the latter, complexed with an organic acid chelator such as oxalate or malonate, secreted by *P. chrysosporium*, oxidizes the terminal phenolic substrate (70, 109, 205). MnP occurs as a series of isozymes encoded by a family of genes, and the sequences of cDNA (125, 141, 146, 152) and genomic clones (73, 74, 125) encoding several MnP isozymes have been determined.

As an idiophasic protein, the expression of MnP is regulated at the level of transcription by the depletion of nutrient nitrogen (74, 105, 152). MnP expression also is dependent upon the presence of Mn^{II} in the culture medium (26, 32), and *mnp* gene transcription is regulated by Mn^{II} (30, 32). In addition, *mnp* gene transcription is regulated by heat shock (31, 73, 74) and by H_2O_2 and other chemical stresses (115).

The promoter regions of the two sequenced *mnp* genes (73, 74, 125) contain putative heat shock elements (HSEs) (118) and metal response elements (MREs) (96,

186). The MRE consensus sequence was originally defined in mammalian metallothionein (MT) genes as a positive *cis*-acting transcriptional control element (96, 183). To examine the function of these putative *cis*-acting sequences in the *mnp1* gene we developed a promoter-reporter system in which the *mnp1* gene promoter was fused to the coding region of the orotidylate decarboxylase (ODase) gene, *ura1*, of *Schizophyllum commune*, as the reporter (72). Herein, we examine the Mn inducibility of a series of promoter-reporter fusion constructs containing point or linker-insertion mutations in the putative MRE pairs of the *mnp 1* promoter.

5.2 Materials and Methods

5.2.1 Organisms

P. chrysosporium OGC101, and the *ade1ura11* double mutant strains were as described previously (1, 5) *Escherichia coli* XL1-Blue (Stratagene, La Jolla, CA), DH5α (BRL, Gaithersberg, MD), and GM119 were used as plasmid hosts for genetic constructions.

5.2.2 Chemicals and enzymes

Orotate phosphoribosyl transferase, orotidylate decarboxylase, and sodium orotate were obtained from Sigma. ^{14}COOH orotic acid and ^{32}P-dCTP were obtained from Du Pont-New England Nuclear. Restriction enzymes, T4 DNA ligase, and T4 DNA polymerase were obtained from New England Biolabs or Promega. Reaction conditions were as recommended by suppliers, otherwise they were standard (122). Random hexamer primed ^{32}P-labelled DNA probes were prepared using an Amersham MultiPrime kit. Dideoxy DNA sequencing was conducted using the Sequenase 2.0 kit from U.S. Biochemicals. Primers were 17mer oligonucleotides synthesized at the Biotechnology Core Facility at Oregon State University.

5.2.3 Construction of pAMO-*Eag*

To construct pAMO-*Eag* (Fig. 5.1), pMO (72) was cut at the unique *Eag*I site followed by treatment with T4 polymerase to create blunt ends. This truncated *mnp* promoter-ODase fusion construct then was released from pUC18 by digestion with *Eco*RI and subcloned into *Sma*I-*Eco*RI-cut pOGI 18 (72). The *Eag*I site was recreated upon ligating the T4 polymerase-filled end into the *Sma*I site. The construction was confirmed by restriction mapping the complete plasmid and by sequencing a subcloned *Eag*I-*Sal*I fragment spanning the fusion junction. The sequencing construct, pMOES, contained the *Eag*I-*Sal*I fragment from pAMO-*Eag* subcloned into pBluescriptII SK+.

5.2.4 Construction of pAMO-*Eag* plasmids containing mutant MREs

The *Eag*I-*Sal*I fragment subcloned into pBluescriptII, pMOES, was used as the source of single-stranded DNA for oligonucleotide-directed mutagenesis using the T7-GEN *in vitro* mutagenesis kit from U.S. Biochemical. The 2-bp changes in the proximal and distal MRE pairs (Fig. 5.2) were created using a 20-bp synthetic oligonucleotide containing the desired sequence, according to the instructions. The mutations introduced a unique *Hpa*I restriction site, which was used to screen for the mutated sequences among the reaction products, and for subsequent creation of the linker insertion mutants. The plasmids bearing the 2-bp point mutations in the proximal MRE pair, and in both the proximal and distal MRE pairs, were pMOESdM1 and pMOESdM1dM2, respectively (Fig. 5.3a).

To construct the 3 linker insertion mutants, pMOESdM1dM2 was cut with *Kpn*I-*Sac*I to isolate a fragment of the promoter containing only the distal mutant MRE. This fragment then was exchanged with the same fragment from wild type pMOES to create pMOESdM2. A blunt-ended 10-bp synthetic oligo duplex containing a *Bcl*I site was ligated into the *Hpa*I site of both pMOESdM1 and pMOESdM2. The initial products of these ligations contained multiple copies of the linker. DNA preparations were made of each plasmid product in *E. coli* GM119 cells (Dam^-) to avoid methylation of the *Bcl*I sites. The unmethylated DNA was cut with

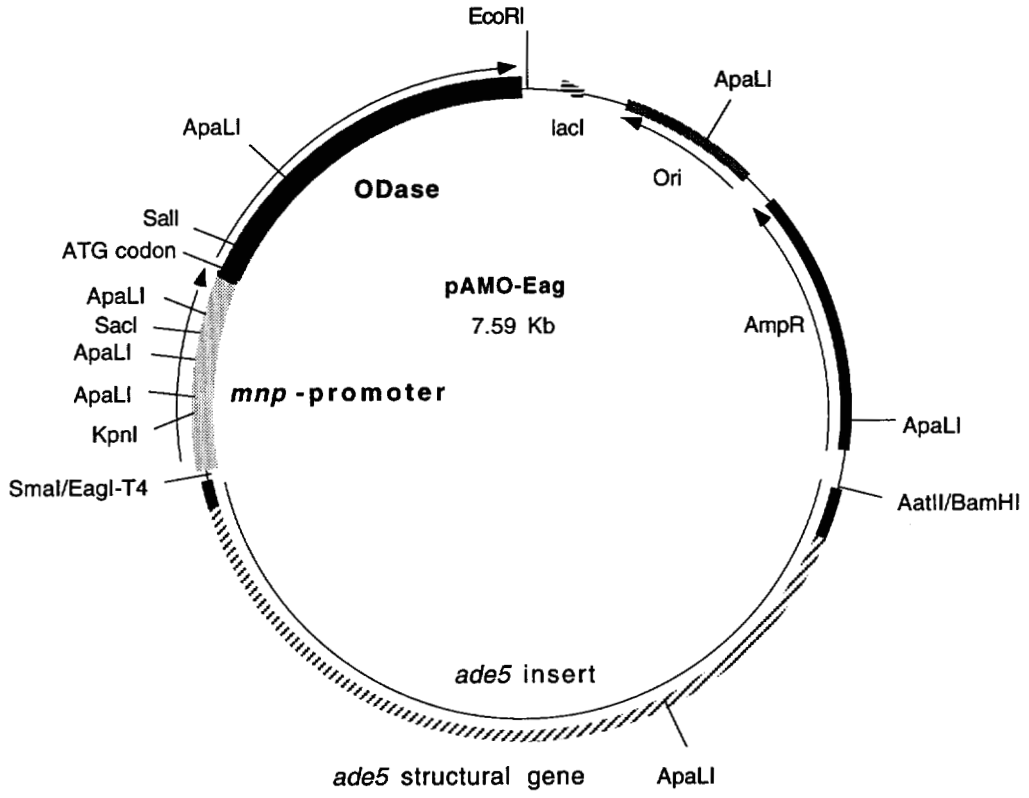


Fig. 5.1 Restriction map of plasmid pAMO-*Eag*. The light slashed line indicates the approximate location of the *S. commune ade5* gene; the adjacent dark solid line indicates the *ade5*-containing insert. The positions and orientations of the *mnp* promoter are as indicated. The remainder of the sequence is from pUC18.

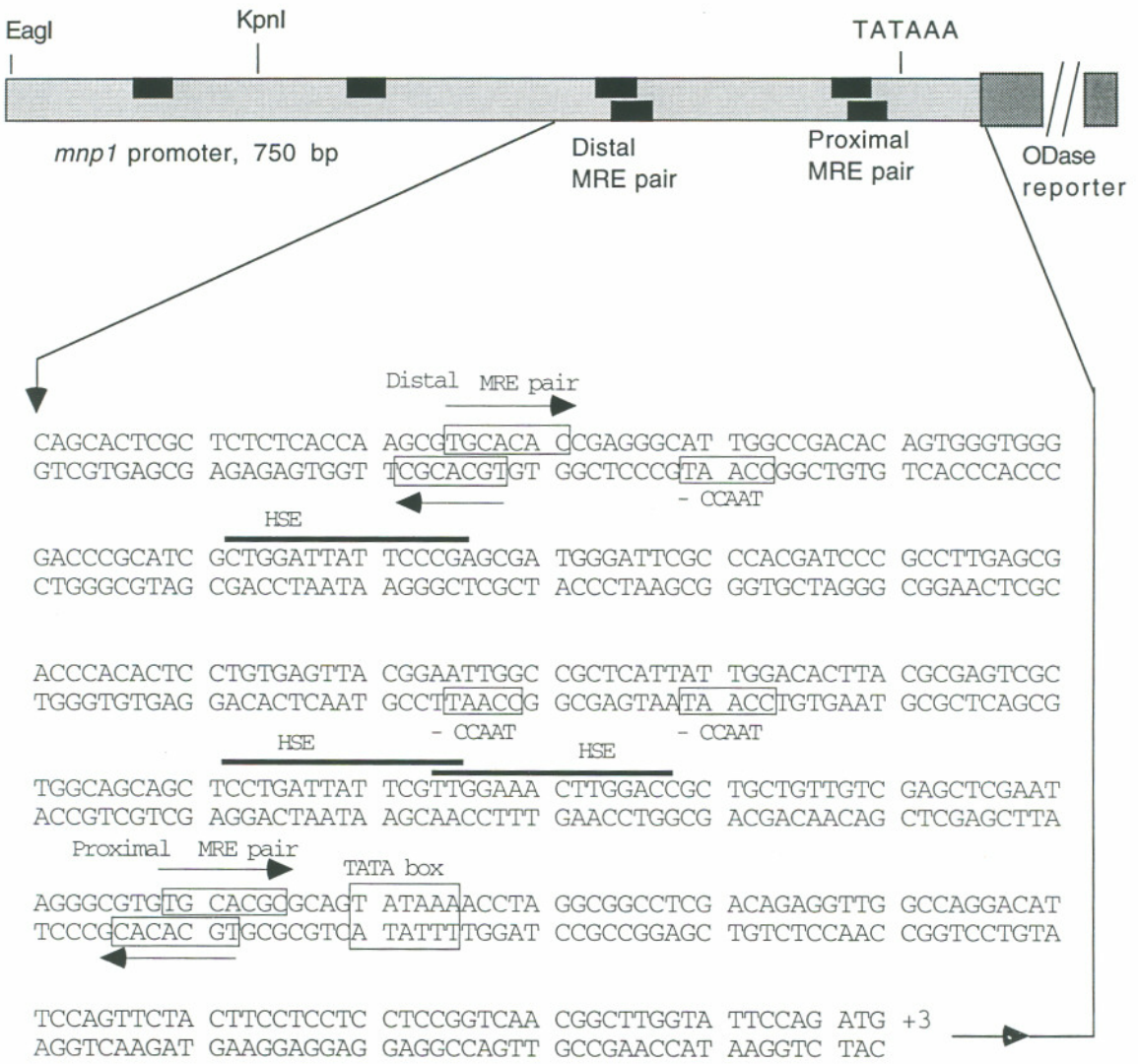


Fig. 5.2 Putative metal response sequences and other cis-acting elements in the *mnp1* promoter in pAMO-*Eag*. MRE = metal response element; HSE = heat shock element; ■ = MRE.

a

Consensus MRE:	TGCRCNC
Proximal MRE Pair:	GTGTGCACGC CACACGTGCG
Distal MRE Pair:	GCGTGCACGC CGCACGTGCG
Point Mutant (P-1) Version,	GTGTTAACGC CACAATTGCG
Point Mutant (P-2) Version,	GCGTTAACGC CGCAATTGCG
10 bp. Linker (L-1):	GCTGATCACG CGACTAGTGC
Linker inserted in HpaI site:	GTGTT-GCTGATCACG-AACGC CACAA-CGACTAGTGC-TTGCG

b

Construct designation	Proximal MRE pair	Distal MRE pair
wild type	wild type	wild type
P-1	point mutant	wild type
P-1/P-2	point mutant	point mutant
L-1	linker insertion	wild type
L-2	wild type	linker insertion
L-1/L-2	linker insertion	linker insertion

Fig. 5.3 Construction of the MRE mutants. (a) Sequence changes used to make the mutant MRE constructs. (b) The mutant MRE constructs are designated as point mutants (P) or linker insertion mutants (L). The proximal MRE pair is assigned the number 1, and the distal pair is assigned the number 2.

*Bcl*I, the plasmid bands were isolated from agarose gels to eliminate excess copies of the linker, and the linear plasmids were circularized to create pMOES B1 and pMOES B2. The double linker insertion mutant, pMOES B1B2, was created by isolating the B2 insertion on a *Kpn*I-*Sac*I fragment and exchanging this fragment with that from pMOES B1. A version of pMO, pMO-*Eag*, was created which lacks the *Sal*I and *Kpn*I sites in the adjacent polylinker to facilitate subcloning of the mutant promoter constructs into pOGI. pMO-*Eag* contains the *Eag*I-EcoRI fragment from pMO subcloned into a modified pBluescriptII SK+ vector, pBluescriptII ΔSK. The vector pBluescriptII ΔSK was prepared by restricting pBluescriptII SK+ with *Sal*I and *Asp*718I, treating with T4 polymerase in the presence of dG and dC only, and religating. pBluescriptII ΔSK therefore lacks the *Sal*I, *Xho*I, *Apa*I, and *Kpn*I sites, and retains functional lacα-complementation selection for inserts. The *Eag*I-*Sal*I fragments from the various mutant versions of pMOES were all subcloned into pMO-*Eag* by exchanging the mutant *Eag*I-*Sal*I fragments for the original *Eag*I-*Sal*I fragment of pMO-*Eag*, after verifying their sequences. The pAMO-*Eag* mutant constructs (Fig. 5.3b) were made by subcloning the *Eag*I-*Eco*RI fragments from the mutant pMO-*Eags* into pOGI 18 as described above for pAMO-*Eag*.

5.2.5 *P. chrysosporium* transformation and purification of transformants

The double mutant *ade1ura11* was transformed as described (3, 4), with pAMO. The plasmids were linearized at the unique *Eco*RI site and 0.5 to 1 μg of DNA was used to transform 2×10^6 protoplasted basidiospores. 15–25 transformants were transferred to slants of Vogel's medium containing 1% glucose, 0.01% uracil, and 1.5% agar (GV/ura) and to GV slants (5, 76). Those transformants which grew on GV/ura but not on GV were transferred for maintenance to slants containing Vogel's medium, 3% malt extract, 0.5% tryptone, 0.5% yeast extract, 1.5% agar, 0.01% uracil, and 0.001% adenine (rich medium) (76). Transformants were purified by fruiting, plating basidiospores at high dilution, and picking single basidiospore-derived colonies, as described (5). The modified fruiting media consisted of 4.5%

Walseths cellulose, 1/6× Vogel's salts (5, 75), 0.01% uracil, and 0.001% adenine, pH 4.8.

5.2.6 Media and growth conditions

Cultures were maintained on rich slants, as described above. High-carbon low-nitrogen (HCLN) minus Mn medium containing 2% glucose and 1.2 mM ammonium tartrate was prepared as described (30, 106), using a trace element solution containing no added $MnSO_4$. $HCLN_{AUY}$ 0.25 μM Mn^{II} was HCLN supplemented with 0.001% adenine, 0.01% uracil, 0.01% yeast extract (Difco), and 0.25 μM $MnSO_4$, and was used for the minus Mn media in the induction experiments (72).

5.2.7 Screening transformants for Mn-inducible reporter expression

Single basidiospore isolates of each transformant were tested for expression of the reporter gene by growing in 20-ml stationary liquid cultures in $HCLN_{AUY}$ 0.25 μM $MnSO_4$ for 5 days under air. Five flasks of each isolate were flushed with O_2 on day 5. On day 6, $MnSO_4$ (180 μM final concentration) was added to three of the flasks to induce expression of the reporter, and all flasks were again purged with O_2. Cells were harvested by filtration through Miracloth (Calbiochem) 40 h after Mn induction, frozen in liquid N_2 and stored at -80°C as described (72). The Mn^{II}-induction time course for the wild-type (pAMO-*Eag*) construct was performed as described (72).

5.2.8 Enzyme assays

Cell extracts were prepared and assayed for orotidylate decarboxylase activity as described previously (72). MnP assays (205) were performed immediately before harvesting, after which the cells were frozen in liquid nitrogen and stored at -80°C.

5.2.9 Preparation and analysis of genomic DNAs

Genomic DNA was prepared from shake cultures grown in MYV supplemented with 0.01% uracil and 0.001% adenine, as described (4). The cells were harvested by filtration through Miracloth, pressed dry between paper towels,

weighed, frozen in liquid N_2, and stored at -80°C. Genomic DNA was prepared by powdering 200 mg of frozen cells in a liquid N_2-chilled mortar and pestle, scraping the powder into disposable sterile 15-ml tubes, containing 750 μl of lysis buffer (155) and 650 μl of TE equilibrated phenol, and then vortexing gently by hand. After 5 min at RT 150 μl of $CHCl_3$:isoamyl alcohol (iAA), 24:1, was added; the tubes were hand vortexed, and centrifuged at 16,000 $\times$ *g* for 20 min. The aqueous supernatants were treated with 40 μg/ml RNaseA at 37°C for 1 h, extracted once each with phenol/CHCl3:iAA 1:1, and $CHCl_3$:iAA, and centrifuged for 15 min at 16,000 $\times$ *g*. Genomic DNA was precipitated from the aqueous phase by adding 0.5 volumes of 40% PEG 8000, 30 mM $MgCl_2$, mixing, and incubating at RT for 20 min. The DNA was pelleted by centrifuging at 16,000 $\times$ *g* for 20 min, washed twice with 70% EtOH, and dried *in vacuo*.

The pellets were resuspended in 400 μl of 10 mM Tris, 1 mM EDTA (TE), pH 8.0. Contaminating carbohydrates were removed by bringing the DNA samples to 0.25 M NaCl, adding 0.35 volumes of ethanol (128), and centrifuging for 15 min in a microcentrifuge. The supernatants were transferred to fresh tubes, and 1 ml of ethanol was added to precipitate the DNA. The precipitate was washed with 70% ethanol and dried, the resulting pellets were resuspended in 50 μl of TE, pH 8.0 and incubated overnight at 4°C. The 260 nm/280 nm ratios were about 2.0, as described (67).

Genomic DNA samples, 1.0 μg each, were digested with *Apa*LI and electrophoresed on a 15-cm 0.7% agarose gel. Four lanes of 1.0 μg each of wild-type genomic DNA spiked with 1.0, 3.0, 5.0, and 10.0 copy equivalents of the pAMO-*Eag* plasmid cut with *Apa*LI were included on each gel as quantitation standards. DNA was transferred to Magna NT nylon membranes (3). The blots were probed with ^{32}P-dCTP labeled random hexamer-primed linear pAMO-*Eag* plasmid, washed in 2 $\times$ SSC, 1% SDS followed by 0.2% SSC, 0.1% SDS at 68°C, and imaged on a Molecular Dynamics Phosphorimager to quantitate the bands. Band intensities were determined and the number of integrated copies estimated using IP LabGel software.

5.3 Results

5.3.1 Construction of the 750-bp *mnp* promoter-ODase construct pAMO-*Eag* and mutant constructs

The wild-type plasmid used in this study, pAMO-*Eag*, was a variant of pAMO (72) wherein the *mnp* promoter was truncated at an *Eag*I site 768 bp 5′ of the translation initiation codon of the reporter gene. A restriction map of pAMO-*Eag* is shown in Fig. 5.1. The *mnp1* promoter is depicted in Fig. 5.2, with the MREs and several other putative *cis* elements indicated, along with the sequence of the region containing the mutations. Five mutant versions of pAMO-*Eag* also were constructed. Two of these mutant promoters, pAMO-*Eag* P-1 and pAMO-*Eag* P-1/P-2, contained 2-bp point mutations in the proximal, and in both the distal and proximal overlapping MRE pairs, respectively (Fig. 5.3a). Alterations of either of these two internal bases of the consensus MRE sequence in mammalian MT result in large decreases in metal inducibility of reporter constructs (45).

More profound disruptions of the MRE sequences were made by ligating 10-bp synthetic oligonucleotide linkers, containing *Bcl*I sites, into the *Hpa*I sites resulting from construction of the point mutations, as shown in Fig. 5.3a. These linkers had the same G+C/A+T ratio of 0.6 as the entire *mnp1* promoter sequence (73). The 10-bp linker insertions may have changed the spacing between other active promoter sequences, but would not alter the face of the DNA strand to which factors would bind, relative to each other. The new sequences in the *mnp* promoter context had no sequence identity to other *cis*-acting elements in the transcription factor database supplied with MacVector software (IBI).

5.3.2 Transformation and characterization of the Mn-inducibility of the transformants

The pAMO-*Eag* plasmids, containing the *S. commune ade5* gene as the selectable transformation marker, were transformed into the $Ade^{-}Ura^{-}$ double auxotrophic strain of *P. chrysosporium*, *ade1ura11*, lacking endogenous ODase

activity. Of the 15 randomly-picked primary transformants of each plasmid construct, between 11 and 14 yielded ade$^+$ strains. These single basidiospore isolates were screened for Mn^{II}-inducible expression of the reporter gene as described above.

As shown in Fig. 5.4, a time course of Mn-induction demonstrated that the wild-type promoter-reporter construct containing 768 bp of promoter sequence was regulated in the same manner as described previously for a construct containing 1500 bp of native *mnp1* promoter sequence (72). The pAMO-*Eag* transformant was grown for 5 days in HCLN cultures in the presence of 0.25 μM Mn, after which the cultures were purged with O_2. On day 6, 180 μM Mn was added and the cultures were reincubated. MnP activity reached a maximum 30 h after the addition of 180 μM Mn. In parallel with the appearance of MnP activity, ODase reporter activity steadily increased for 30 h after the addition of 180 μM Mn, reaching a maximum induction of ~33-fold. No significant MnP or ODase activity was detected in uninduced cultures. In contrast to the induction of activity in HCLN cultures, neither MnP nor ODase activity was observed after 180 μM Mn was added to cultures grown in HCHN medium containing 0.25 μM Mn (Fig. 5.4).

A wide variation was seen in ODase reporter expression levels within each class of mutant transformant, as would be expected from the ectopic integration of a variable number of plasmids. About 20% of the transformants from each group gave little or no ODase activity. For this analysis we disregarded any transformants with Mn^{II} induced ODase expression levels that were less than 10% those of the highest in that group, as well as any transformants that constitutively expressed ODase in the absence of Mn^{II}.

The mean Mn^{II}-induced ODase expression for each group of transformants is shown in Table 5.1, along with the maximum and minimum values measured in each group. The Mn^{II} induction ratio (the quotient of Mn^{II} induced over uninduced ODase expression) for each group of transformants also is given in Table 5.1 as the mean for the group. None of the mutations completely abolished Mn^{II} induction. The 7-fold mean induction observed for of the linker insertion constructs was the lowest induction ratio measured for any group of transformants. The point mutations and the linker insertion mutations had a similar effect on the average level of Mn^{II}-induced

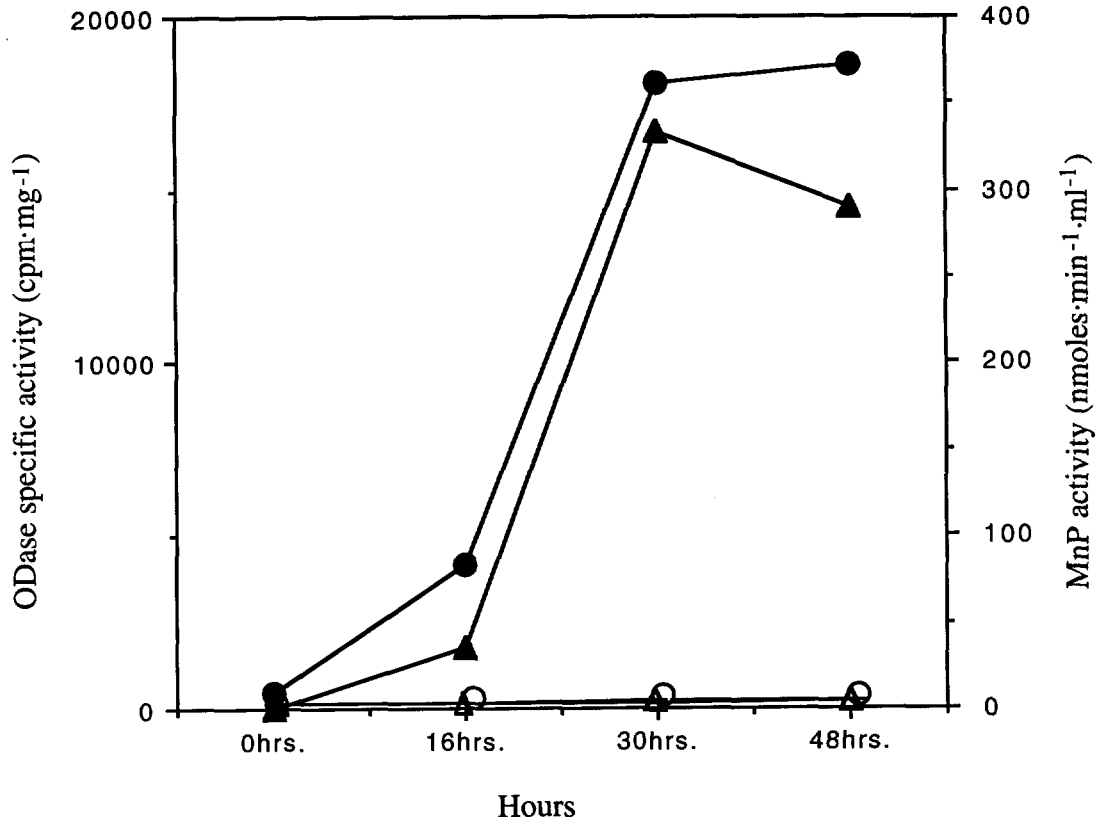


Fig. 5.4 Induction of extracellular MnP and intracellular ODase activities by Mn^{II}. Nitrogen-limited or nitrogen-sufficient, Mn-deficient cultures of a pAMO-*Eag* transformant were grown for 6 days, after which 180 μM $MnSO_4$ was added to the experimental flasks. ODase activity in nitrogen-limited cultures induced with Mn^{II} (●), and nitrogen-sufficient cultures induced with Mn^{II} (○). MnP activity in nitrogen-limited, Mn^{II}-induced cultures (▲), and in nitrogen-sufficient, Mn^{II}-induced cultures (△). MnP and ODase activities were assayed as described in Materials and Methods.

Table 5-1 Reporter Expression from *mnp* Promoter Constructs with Mutations in MREs

MRE Construct (number of transformants analyzed)	Average Mn-induced ODase specific activity	Max./Min. ODase specific activity values	Average induction ratio, (range)	Average copy number	Max./Min. copy numbers
wild type (8)	13,000	23,300 3,300	28.6 (67-1.8)	4	9/1
P-1 (11)	4,600	10,000 960	14.4 (35-3.7)	11	31/1
P-1/P-2 (14)	3,200	7,300 770	9.8 (24-0.4)	8	27/3
L-1 (8)	5,000	17,500 700	7.6 (12.5-2.5)	16	38/4
L-2 (6)	2,900	6,900 670	7.6 (22.8-2.8)	11	22/3
L-1/L-2 (5)	4,900	8,300 2,000	6.4 (11-2.3)	9	17/5

ODase reporter specific activity values as cpm/mg. Induction ratio is the ratio of reporter expression in a Mn-treated culture to that in a minus Mn culture. ODase expression was measured as decribed in Materials and Methods. The number of copies of each plasmid integrated was estimated from Southern blots, as described in Materials and Methods.

reporter activity, resulting in 30–45% of the mean wild type construct expression levels (2900 to 5000 cpm/mg versus the 12,900 cpm/mg for the wild-type promoter constructs).

The approximate number of plasmid copies integrated into the genomes of the transformants was estimated from Southern blots (data not shown). Transformants with the wild-type promoter contained on average fewer than four copies of the plasmid, whereas transformants with the mutant promoters contained on average more than eight copies (Table 5.1). Since the analyzed transformants were chosen for Mn^{II}-regulated ODase expression, this observation was consistent with the relative weakness of Mn^{II}-induction among the mutant MRE constructs. Even high gene dosages did not overcome the effects of the mutations.

5.3.3 Statistical analysis of the reporter expression data

The reporter gene expression data were analyzed to determine the significance and standard errors of the mean induction ratios and mean reporter expression levels for each group of constructs. Fig. 5.5a shows the mean Mn^{II}-induced reporter expression level for each group of mutant constructs. All of the mutant construct expression levels fell below the mean expression level of the wild-type construct. Fig. 5.5b shows a similar pattern for the induction ratio data.

An analysis of variance (181) was performed on the induced and uninduced ODase expression of the transformants. An unpaired t-test applied to the induction ratio data (181) detected significant differences in the mean induction ratios ($P<0.05$), compared to the wild type transformants, for P-1/P-2 and L-1, the two groups having a sufficient sample size. To determine whether the changes in induction ratio were due primarily to changes in the Mn^{II}-induced ODase expression level or to the uninduced baseline expression level, a paired t-test was performed on the induced and uninduced expression data separately. No significant differences were detected in the uninduced expression of the reporter in any of the constructs. A difference at the $P<0.05$ significance level, relative to the wild type, was found for the induced ODase expression of all the mutant constructs. Therefore, the changes in induction ratio caused by the MRE mutations appear to be due to changes in the level of Mn^{II}-induced reporter gene expression.

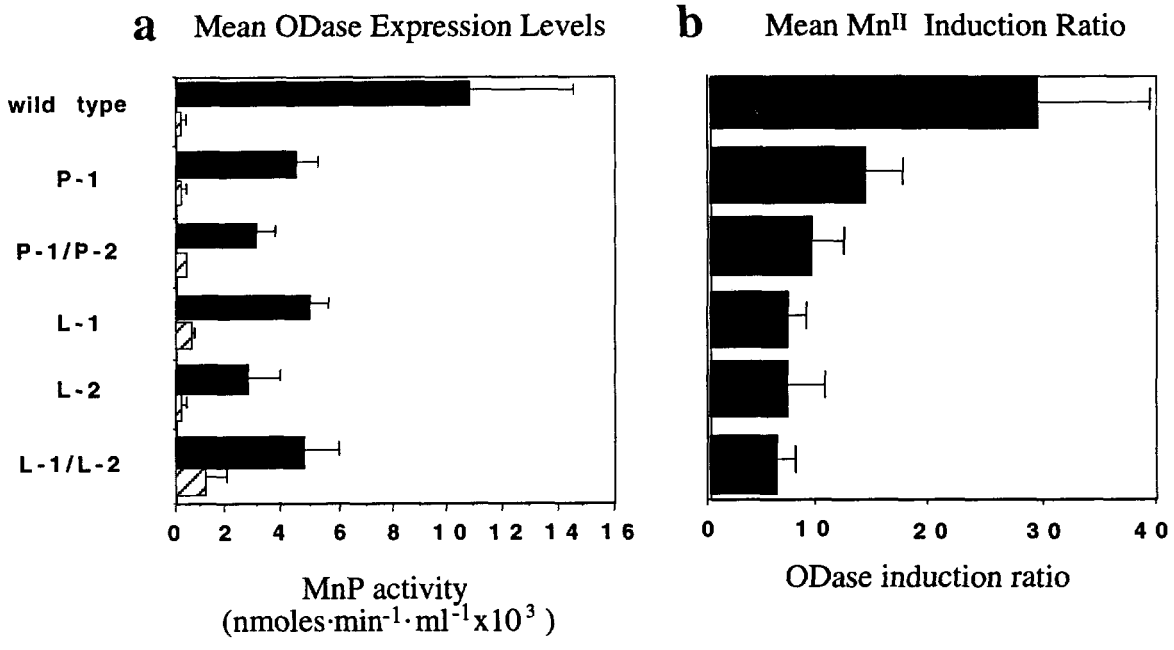


Fig. 5.5 Comparison of the mean ODase expression levels and induction ratio of the wild-type and mutant promoter-reporter constucts. (a) Mean Mn^{II}-induced (■) and uninduced (▨) expression of ODase reporter activity for each construct. (b) Mean induction ratios in response to Mn^{II} for each construct. Error bars indicate standard error of the mean.

5.4 Discussion

The lignin degrading system of the white-rot basidiomycete *P. chrysosporium* is expressed during idiophasic growth, the onset of which is triggered by limitation of nutrient nitrogen (74, 105). Accordingly, the activities of the ligninolytic peroxidases, LiP and MnP, are detectable in the extracellular medium during idiophasic growth, and northern blot analysis has demonstrated that LiP and MnP expression are controlled at the level of gene transcription by nutrient nitrogen limitation (74, 116, 152).

We have shown that MnP expression is regulated by Mn^{II}, but not by other metals, at the level of transcription (30). We also have shown that *mnp* gene transcription is induced by heat shock (31, 73) and a variety of chemical and oxidative stresses, including H_2O_2 (115). Chemical stresses including heavy metals, organic compounds and oxidizing agents are known to induce the expression of heat shock proteins (118). Mn^{II}, heat shock, and chemical stresses induce *mnp* gene transcription only under nitrogen limitation, suggesting that MnP expression is regulated in a hierarchical manner with nutrient nitrogen regulation overriding other transcriptional regulators.

Both the *P. chrysosporium mnp1* and *mnp2* genes (73, 74, 125) contain short promoter sequences matching exactly the consensus MREs (TGCRCXC), as defined for mammalian MT genes (183, 186). Transcription of mammalian MT genes is mediated by *cis*-acting promoter elements, MREs, in response to the presence of heavy metals (186). Similar Cys-rich proteins have been identified in yeasts and fungi (38, 114, 133, 134). Transcription of the copper MT gene in yeast is controlled by a metal-activated transcription factor which binds to the *cis*-acting elements in the MT gene promoters that are distinct from the MREs defined above (186). In this work we have used promoter-reporter constructs to determine the role of consensus MRE sequences in the Mn^{II} regulation of *mnp* gene expression.

The effect of manganese on the expression of the reporter gene in *P. chrysosporium* can be observed directly in an induction experiment, wherein the effect

of manganese is distinct from the effects of other nutritional regulators, such as nitrogen and oxygen availability. Some variability in Mn-inducibility of the reporter gene results from the ectopic integration of the introduced DNA (3, 4), since only 50–75% of the screened transformants expressed the reporter gene at levels ≥ 10% of that of the most strongly expressing transformants. This suggests that the expression levels of the reporter constructs are a function both of the structure of the construct and the site of genomic integration. Interestingly, the wild-type promoter construct transformants contained on average fewer than four copies of the plasmid, whereas the mutant transformants contained on average greater than eight copies (Table 5.1), suggesting that there may be selective pressure against overexpression of ODase.

The results in Fig. 5.4 show that the time course of ODase expression in the wild-type transformant parallels that of MnP expression. This demonstrates that 750 bp of sequence 5′ of the translation start site of the *mnp1* gene are sufficient to confer wild-type regulation of the ODase reporter with respect to nutrient nitrogen limitation and Mn availability. Mutations in the *mnp* promoter consensus MRE sequences that would cause a decrease in the transcriptional activation of mammalian MT genes (45) also decrease the expression of the ODase reporter gene in *P. chrysosporium* (Table 5.1, Fig. 5.5). Point mutations in both of the MRE pairs, as well as the linker insertion constructs, resulted in at least a 50% decrease in the average level of reporter gene expression (Fig. 5.5). The double point and linker insertion mutations cause a particularly strong decrease of the induction ratio (Fig. 5.5). The two sets of mutant transformants with a sufficiently large sample size, P-1/P-2 and L-1, showed a statistically significant difference in induction ratio as compared to the wild-type. These two groups have similar mean induction ratios of 1/3 to 1/4 of the wild type induction ratio. The other groups of linker insertion mutants caused mean induction ratio decreases which were similar to those of the P-1/P-2 and L-1 groups.

Analysis of the reporter gene expression data revealed a significant difference ($P<0.05$) in the level of Mn-induced ODase expression between the mutant constructs and the wild type. The differences in uninduced expression are not significant. These results indicate that the decrease in induction ratios among the

mutant constructs is due to the reduction in Mn-induced expression of the reporter, rather than to changes in the uninduced expression level. The residual 7-fold Mn^{II} induction observed in constructs with both MRE pairs disrupted may be due to the two single unaltered MREs upstream of the two pairs (Fig. 5.2). The apparent residual induction also may, in part, be a result of the slow accumulation of the ODase activity in the cells. We found that the *S. commune ura1* gene encodes a stable intracellular enzyme in *P. chrysosporium* (72), whereas MnP, a secreted protein, is slowly inactivated in the culture fluid (32, 74).

In mammalian MT genes the MREs often are present in clusters, each containing several tandem duplications of the consensus sequence (186) and the MRE sequences often overlap with other consensus elements such as SP-1 sites (40, 85, 186, 208). This cluster arrangement may be reflected in the overlapping paired MREs found in the *P. chrysosporium mnp* promoters (73, 74, 125). Disruption of either cluster of MREs in a mammalian MT promoter results in a substantial decrease in heavy metal inducibility of the gene (40). Similarly, disruption of either pair of MREs results in a substantial decrease in Mn^{II} inducibility of the *mnp* gene promoter-reporter fusion (Fig. 5.5). Both sequenced *mnp* genes contain one overlapping MRE pair near the TATA sequence and a single MRE (*mnp2*) or another overlapping pair (*mnp1*) 200–240 bp upstream. In both *mnp* genes the spacing between one MRE and the next is 200–240 bp. The *mnp1* gene also contains two single upstream MREs (Fig. 5.2) (73, 74).

Point mutations in the consensus *mnp* MRE sequences that cause a significant decrease in metal inducibility of mammalian MT (45) likewise decrease *mnp* gene expression, suggesting that the DNA binding domain of the putative transcription factor in *P. chrysosporium* may be similar to the MRE binding domain of the metal-activated factor(s) in mammals.

We conclude that the consensus MRE sequences present in the *mnp* promoter are involved in the regulation of the *mnp 1* gene by Mn. Elucidation of the exact mechanism of Mn regulation of *mnp* gene transcription awaits further experimentation.

5.5 Acknowledgments

We thank Margaret Alic for valuable discussions. This work was supported by grants MCB-9405978 and MCB-9506338 from the National Science Foundation and DE-FG06-92ER20093 from the U.S. Department of Energy, Division of Energy Biosciences.

CHAPTER 6
CONCLUSIONS AND FUTURE DIRECTIONS

The development of molecular genetic techniques for *Phanerochaete chrysosporium* began with the selection of auxotroph mutants (6). The subsequent development of a DNA transformation system, using complementation of an auxotrophic mutant by a gene from another basidiomycete (4, 6), has opened many new avenues for fruitful research. These include studies of the roles of individual ligninolytic enzymes in lignin degradation, studies of the mechanisms of these enzymes, and the development of a reporter gene system for studying gene regulation in *P. chrysosporium* (72, 74). Understanding gene regulation might contribute to the development of practical applications for *P. chrysosporium* in bioremediation, renewable resource utilization, and fungal protein expression.

The experimental work described in the preceding chapters includes the isolation and sequencing of two genes encoding isozymes of the ligninolytic enzyme manganese peroxidase (MnP). Analysis of these genes revealed the presence of promoter sequences identical to metal-responsive sequences found in mammalian metallothionein genes. A biosynthetic gene from a related fungus, *Schizophyllum commune*, was used with a double auxotrophic *P. chrysosporium* mutant to develop a reporter gene system. The final chapter of this thesis describes the use of a reporter system to demonstrate that the putative metal regulatory elements in the promoters of the MnP genes are apparently involved in the regulation of these genes by manganese II (Mn^{II}) ions. Proof that these putative promoter elements are the sequences which mediate the transcriptional response to manganese will come from experiments in which these putative manganese responsive sequences (MREs) are shown to confer manganese inducibility upon a gene not normally regulated by a metal.

There are many questions to be answered about the mechanism of transcriptional regulation by Mn^{II}. These include the following:

(1) Is the effect a positive activation of transcription or the relief of transcriptional repression by Mn^{II}?

(2) Are the MRE sequences themselves necessary and sufficient to provide manganese-responsive transcription, or are other unidentified sequences required before Mn^{II} induction can occur?

(3) Does the transcriptional effect of manganese require the presence of at least one overlapping pair of MRE sequences, or will individual MREs also provide Mn^{II} inducibility?

(4) Are the order and spacing of the MRE sequences important to their apparent function as manganese-dependant transcriptional control sequences? As shown in Fig. 5.2, both MnP genes have an overlapping pair of MREs most proximal to the TATA element and one or more single MREs and/or pairs upstream from this proximal pair. The spacing of the putative MREs relative to each other is also similar in both promoters, with 200-240 bp between consecutive MREs.

(5) Can the Mn^{II} induction ratio be increased further in a synthetic promoter that contains more paired MREs or a different configuration of MREs?

(6) Can a transcription factor which binds to the MRE sequence be isolated for study?

(7) Is that putative transcription factor related to those which activate metallothionein genes in mammals, or is the only similarity the DNA sequence to which it binds?

Many of these questions can be addressed using ODase or possibly another reporter gene. Obtaining answers to some of these questions will require the creation of further genetic constructions, and some may require screening mutant libraries using a biochemical selection scheme. The next logical step to take towards answering these questions is to confirm that the MRE sequences in the MnP promoters are in fact the primary sequences which mediate the transcriptional response to manganese. The most convincing demonstration of this would be to put

the MRE sequences into the promoter of another gene and assay for manganese induction of that gene.

An experiment is needed to prove that the MRE sequences are necessary and sufficient to confer manganese inducibility on a gene in *P. chrysosporium*. One could take advantage of the work described here with the *S. commune* ODase gene. ODase is a biosynthetic gene needed by the cell at all times, a so-called "housekeeping gene," so it is expected to have a constitutive promoter and expression pattern. The promoter is likely to be fairly simple, probably providing basal expression with only a few hundred base pairs of sequence upstream of the TATA element. Inserting one or a few copies of the MRE pair sequences upstream of the TATA box in the *S. commune* or *P. chrysosporium ura1* genes might cause Mn^{II}-activated expression in addition to the basal level of ODase in some of the constructs if the MRE alone is sufficient. Several versions of this construct, with MRE pairs inserted in different positions, should be made. The insertions may, in some cases, interfere with the basal promoter function.

If the MRE pairs act as transcriptional activators, then ODase expression should increase beyond the normal basal level when Mn^{II} is added to the cultures. The increase in ODase activity should be easily detectable in the assay described in Chapter 3, above. If the MRE pairs function as repressors, then even the basal expression of ODase should be inhibited until Mn^{II} is added to the cultures. If the latter turned out to be the case, the first evidence would probably be that transformants containing this construct only grow on minimal media that is supplemented with Mn^{II} or uracil. ODase activity would be detectable in transformants grown on uracil-supplemented media only if Mn^{II} were added to the culture. Even if the manganese-responsive transcription factor (MRTF) is only produced during idiophase, this type of construct should work since the ODase gene is efficiently expressed during both primary and secondary metabolism.

If the ODase gene does become regulated by manganese in the constructs described above, further experiments would be possible. The importance of the spatial relationship between the MREs, could be explored. The relative strengths of single versus paired MREs could be assayed. Ultimately, a very strongly inducible

artificial promoter or enhancer could be made. A strong and inducible basidiomycete promoter would be very useful for fermentative protein production in this class of fungi. In addition to being a useful laboratory tool for peroxidase protein engineering experiments, this could lead to biotechnological applications.

Ultimately a reporter gene that can be used in wild-type *P. chrysosporium* strains will need to be found. The ODase-deficient mutant strain provides another unique research opportunity in addition to being the host for ODase reporter experiments. Using a Ura^- mutant, one could devise a positive selection scheme to isolate a *P. chrysosporium* strain lacking MRTF activity. An MRTF mutant could provide valuable evidence of the identity of any putative MRTF clone that might be isolated in the future. It may be possible to make a plasmid construct that contains a version of the *P. chrysosporium* or *S. commune* ODase gene in which the promoter is stripped down to just the TATA element, with synthetic DNA containing MRE sequences inserted in place of the original basal promoter elements, as was done in yeast for cuprothionien (38).

ura11 transformants of the resulting construct would grow on unsupplemented media only in the presence of manganese. Such transformed strains could be used to select for mutations in the manganese-responsive transcription factor by using the suicide substrate 5-fluoro-orotate to kill all transformants with an active ODase gene (24). Conidia from the chosen strain would be mutangenized by X-ray or UV treatment and then plated on colony-forming media (1, 2, 76, 77) containing FOA. Only those conidia in which the MRTF was not expressed in an active form would grow. Even if the action of Mn^{II} is indirect, i.e. the MRTF is activated by phosphorylation instead of Mn^{II} binding, the trans-acting factor which activates the MnP promoter in response to the presence of manganese might still be mutated by this strategy.

The final step would be to separate those strains in which the ODase gene itself was damaged from those in which the MRTF was damaged. Crossing the strains with an ODase-minus mutant strain would give the result that those which had a damaged MRTF would be able to survive on minimal media supplemented with manganese. Those with damaged ODase genes would remain unable to grow on

minimal media with or without manganese. Molecular analysis provides an alternative approach to analyzing the mutant strains. The presence of the ODase gene could be checked by Southern blotting of the genomic DNA from mutagenized strains. X-ray mutagenesis usually results in large deletions, so this approach would probably distinguish between the two possibilities. Alternatively, polymerase chain reactions could be used to amplify the ODase gene for limited sequencing.

In order to answer specific questions about the nature of the transcription factor which mediates manganese induction, the gene or cDNA encoding the factor will have to be cloned. An MRTF mutant strain could be used to clone the MRTF gene by complementation with a genomic library prepared from wild-type *P. chrysosporium* DNA. There are several more efficient and commonly used strategies for cloning transcription factors, including screening an expression library with DNA containing the sequence to which the factor is known to bind (49), screening a cDNA library with degenerate oligos whose sequences are based on known sequence motifs present in other related transcription factors, and complementation in yeast.

With the active MRE sequence well defined as a result of experiments described above, it may be possible to use that sequence to isolate a transcription factor clone from an expression library. A secondary metabolic cDNA expression library has already been made from OGC101 (152). Briefly, one would make a complementary pair of synthetic oligonucleotides containing several copies of the active MRE sequence. This double-stranded synthetic DNA would be radioactively labeled and used as a probe to screen the λgt11 expression library in *Escherichia coli* (49, 200). A positive clone would be confirmed by expressing it in *E. coli* and showing that it specifically binds to the MRE DNA sequence in gel shift assays.

Yeast genetics provides powerful tools for cross-species cloning. An MRE-binding transcription factor from mouse cells has already been cloned by genetic complementation in yeast (210). The author was not able to conclusively demonstrate that the mouse factor cloned in yeast acted as a metal-dependant transcription factor in mouse cells. Nonetheless, the work demonstrates the usefulness of the yeast complementation strategy. Such unclear results are less likely to result from attempting to isolate an MRE-binding factor from *P. chrysosporium* in yeast

cells, because regulatory complexities such as tissue-specific and hormone-activated regulation and their required ancillary transcription factors are not likely to occur in *P. chrysosporium*.

The strategy for cloning an MRE-binding transcription-activating factor by genetic complementation in yeast is as follows (210). A selectable yeast gene such as Galk is stripped of all promoter elements, but the TATA box and engineered DNA containing the MRE sequences previously shown to respond to Mn^{II} in *P. chrysosporium* is inserted in place of the original promoter. This construct is transformed into a yeast strain which can still provide selection for another transformation marker. A *P. chrysosporium* cDNA library is created in a yeast expression vector. This library is transformed into the yeast strain described above. The transformants are plated on medium containing galactose as the carbon source. Only colonies which are able to activate expression of Galk by virtue of the introduction of a *P. chrysosporium*-derived MRE-binding transcription factor will grow on the glactose medium.

Once a clone of an MRE-binding transcription factor is isolated by some means, its identity must be proved. Expressing the protein in *E. coli* and using the recombinant protein in a gel shift assay would show that it has the correct sequence specificity in binding. The correct protein should bind only to the MRE sequence and not to unrelated competitor DNA. To prove that the protein actually serves as the MRTF in *P. chrysosporium* it should be demonstrated that the clone can rescue an Mn^{II}-activationless mutant *P. chrysosporium* strain by transformation. Ultimately, the amino acid sequence of the MRTF protein would be derived from the sequence of the gene. Comparison of the MRTF sequence with that from other metal-activated transcription factors may reveal why the DNA sequence to which it binds is the same as that found in mammalian metallothionein genes. It could also suggest the mechanism of interaction with manganese.

REFERENCES

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

2. Alic, M., L. Akileswaran, and M. H. Gold. 1993. Gene replacement in the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *Gene* **136:**307-311.

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

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

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

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

7. Andersen, R. D., S. J. Taplitz, A. M. Oberbauer, K. L. Calame, and H. R. Herschman. 1990. Metal-dependant binding of a nuclear factor to the rat metallothionein-I promoter. *Nucleic Acids Res.* **18:**6049-6055.

8. Andersen, R. D., S. J. Taplitz, S. Wong, G. Bristol, B. L. Larkin, and H. R. Herschman. 1987. Metal-dependant binding of a factor *in vivo* to the metal-responsive elements of the metallothionein 1 gene promoter. *Mol. Cell. Biol.* **7:**3574-3581.

9. Andrawis, A., E. A. Pease, I. Kuan, E. Holzbaur, and M. Tien. 1989. Characterization of two lignin peroxidase clones from *Phanerochaete chrysosporium*. *Biochem. Biophys. Res. Commun.* **162:**673-680.

10. Asada, Y., Y. Kimura, M. Kuwahara, A. Tsukamoto, K. Koide, A. Oka, and M. Takanami. 1988. Cloning and sequencing of a ligninase gene from a lignin-degrading basidiomycete, *Phanerochaete chrysosporium*. *Appl. Microbiol. Biotechnol.* **29:**469-473.

11. Ballance, D. J. 1986. Sequences important for gene expression in filamentous fungi. *Yeast* **2:**229-236.

12. Ballance, D. J. 1991. Transformation systems for filamentous fungi and an overview of fungal gene structure, p. 1-29. *In* S. A. Leong and R. M. Berka (ed.), *Molecular Industrial Mycology: Systems and Applications for Filamentous Fungi*. Marcel Dekker, New York.

13. Ballance, D. J., F. P. Buxton, and G. Turner. 1983. Transformation of *Aspergillus nidulans* by the orotidine-5′-phosphate decarboxylase gene of *Neurospora crassa*. *Biochem. Biophys. Res. Commun.* **112:**284-289.

14. Banks, G. R. 1983. Transformation of *Ustilago maydis* by a plasmid containing yeast 2μ DNA. *Curr. Genet.* **7:**73-77.

15. Bar-Lev, S. S., and T. K. Kirk. 1981. Effects of molecular oxygen on lignin degradation by *Phanerochaete chrysosporium*. *Biochem. Biophys. Res. Commun.* **99:**373-378.

16. Barrett, V., R. K. Dixon, and P. A. Lemke. 1990. Genetic transformation of a mycorrhizal fungus. *Appl. Microbiol. Biotechnol.* **33:**313-316.

17. Bégueret, J., V. Razanamparany, M. Perrot, and C. Barreau. 1984. Cloning gene *Ura5* for the orotidylic acid pyrophosphorylase of the filamentous fungus *Podospora anserina*: transformation of protoplasts. *Gene* **32:**487-492.

18. Bienz, M., and H. R. B. Pelham. 1987. Mechanism of heat shock gene activation in higher eukaryotes. *Adv. Genet.* **24:**31-72.

19. Beltramini, M., and K. Lerch. 1982. Copper transfer between *Neurospora* copper metallothionein and type 3 copper apoproteins. *FEBS Lett.* **142:**219-222.

20. Beltramini, M., K. Lerch, and M. Vasak. 1984. Metal substitution of *Neurospora* copper metallothionein. *Biochemistry* **23:**3422-3427.

21. Bestor, T. H. 1990. DNA methylation: evolution of a bacterial immune function into a regulator of gene expression and genome structure in higher eukaryotes. *Philos. Trans. R. Soc. London Ser. B* **326:**179-187.

22. Binninger, D. M., C. Skrzynia, P. J. Pukkila, and L. A. Casselton. 1987. DNA-mediated transformation of the basidiomycete *Coprinus cinereus. EMBO J.* **6:**835-840.

23. Bird, A. P. 1992. The essentials of DNA methylation. *Cell* **70:**5-8.

24. Boeke, J. D., F. LaCroute, and G. R. Fink. 1984. A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. *Mol. Gen. Genet.* **197:**345-346.

25. de Boer, H. A., Y. Z. Zhang, C. Collins, and C. A. Reddy. 1987. Analysis of nucleotide sequences of two ligninase cDNAs from a white-rot filamentous fungus, *Phanerochaete chrysosporium. Gene* **60:**93-102.

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

27. Boominathan, K., S. Balachandra-Dass, T. A. Randall, R. L. Kelley, and C. A. Reddy. 1990. Lignin peroxidase-negative mutant of the white-rot basidiomycete *Phanerochaete chrysosporium. J. Bacteriol.* **172:**260-265.

28. Boominathan, K., and C. A. Reddy. 1992. cAMP-mediated differential regulation of lignin peroxidase and manganese-dependant peroxidase production in the white-rot basidiomycete *Phanerochaete chrysosporium. Proc. Natl. Acad. Sci. USA* **89:**5586-5590.

29. Boyer, H. W. 1971. DNA restriction and modification mechanisms in bacteria. *Annu. Rev. Microbiol.* **25:**153-176.

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

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

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

33. Buchman, C., P. Skroch, J. Welch, S. Fogel, and M. Karin. 1989. The CUP2 gene product, regulator of yeast metallothionein expression, is a copper-activated DNA-binding protein. *Mol. Cell. Biol.* **9**:4091-4095.

34. Bull, J. H., and J. C. Wootton. 1984. Heavily methylated amplified DNA in transformants of *Neurospora crassa*. *Nature* **310**:701-704.

35. Bumpus, J. A., and S. D. Aust. 1987. Biodegradation of environmental pollutants by the white rot fungus *Phanerochaete chrysosporium*: involvement of the lignin-degrading system. *Bioessays* **6**:166-170.

36. Buswell, J. A., B. Mollet, and E. Odier. 1984. Ligninolytic enzyme production by *Phanerochaete chrysosporium* under conditions of nitrogen sufficiency. *FEMS Microbiol. Lett.* **25**:295-299.

37. Buswell, J. A., and E. Odier. 1987. Lignin biodegradation. *Crit. Rev. Biotechnol.* **6**:1-60.

38. Butt, T. R., E. J. Sternberg, J. A. Gorman, P. Clark, D. Hamer, M. Rosenberg, and S. T. Crooke. 1984. Copper metallothionein of yeast, structure of the gene, and regulation of expression. *Proc. Natl. Acad. Sci. USA* **81**:3332-3336.

39. Cancel, A. M., A. B. Orth, and M. Tien. 1993. Lignin and veratryl alcohol are not inducers of the ligninolytic system of *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **59**:2909-2913.

40. Carter, A. D., B. K. Felber, M. J. Walling, M.-F. Jubier, C. J. Schmidt, and D. H. Hamer. 1984. Duplicated heavy metal control sequences of the mouse metallothionein-I gene. *Proc. Natl. Acad. Sci. USA* **81**:7392-7396.

41. Casselton, L. A., and A. de la Fuente Herce. 1989. Heterologous gene expression in the basidiomycete fungus *Coprinus cinereus*. *Curr. Genet.* **16**:35-40.

42. Charles, I. G., J. W. Keyte, W. J. Brammar, M. Smith, and A. R. Hawkins. 1986. The isolation and nucleotide sequence of the complex AROM locus of *Aspergillus nidulans*. *Nucleic Acids Res.* **14**:2201-2213.

43. Christman, M. F., R. W. Morgan, F. S. Jacobson, and B. N. Ames. 1985. Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. *Cell* **41**:753-762.

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

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

46. Davies, R. W. 1991. Molecular biology of high-level recombinant protein production system in *Aspergillus*, p. 31-81. *In* S. A. Leong and R. M. Berka (ed.), *Molecular Industrial Mycology: Systems and Applications for Filamentous Fungi*. Marcel Dekker, New York.

47. Dhawale, S. S. 1993. Is an activator protein-2-like transcription factor involved in regulating gene expression during nitrogen limitation in fungi? *Appl. Environ. Microbiol.* **59:**2335-2338.

48. Doerfler, W. 1983. DNA methylation and gene activity. *Annu. Rev. Biochem.* **52:**93-124.

49. Jin, W. D., A.-L. Boutillier, M. J. Gluckman, S. R. Salton, J.-P. Loeffler, and J. L. Roberts. 1994. Characterization of a corticotropin-releasing hormone-responsive element in the rat proopiomelanocortin gene promoter and molecular cloning of its binding protein. *Mol. Endocrinol.* **8:**1377-1388.

50. Durnam, D. M., and R. D. Palmiter. 1981. Transcriptional regulation of the mouse metallothionein-1 gene by heavy metals. *J. Biol. Chem.* **256:**5712-5716.

51. Durnam, D. M., F. Perrin, F. Gannon, and R. D. Palmiter. 1980. Isolation and characterization of the mouse metallothionein-I gene. *Proc. Natl. Acad. Sci. USA* **77:**6511-6515.

52. Dynan, W. S., and R. Tijian. 1985. Control of eukaryotic messenger RNA synthesis by sequence-specific DNA-binding proteins. *Nature (London)* **316:**774-778.

53. Evans, C. F., D. R. Engelke, and D. J. Thiele. 1990. ACE1 transcription factor produced in *Escherichia coli* binds multiple regions within the yeast metallothionein upstream activation sequences. *Mol. Cell. Biol.* **10:**426-429.

54. Faison, B. D., and T. K. Kirk. 1985. Factors involved in the regulation of a ligninase activity in *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **49:**299-304.

55. Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132:**6-13.

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

57. Fincham, J. R. S. 1989. Transformation in fungi. *Microbiol. Rev.* **53:**148-170.

58. Finnegan, J., and D. McElroy. 1994. Transgene inactivation: plants fight back! *Bio/Technology* **12:**883-888.

59. Foulkes, E. C. (ed.). 1982. *Biological Roles of Metallothionein.* Elsevier/North-Holland, New York.

60. Freedman, T., and P. J. Pukkila. 1993. *De novo* methylation of repeated sequences in *Coprinus cinereus*. *Genetics* **135:**357-366.

61. Froeliger, E. H., A. M. Muñoz-Rivas, C. A. Specht, R. C. Ullrich, and C. P. Novotny. 1987. The isolation of specific genes from the basidiomycete *Schizophyllum commune*. *Curr. Genet.* **12:**547-554.

62. Froeliger, E. H., R. C. Ullrich, and C. P. Novotny. 1989. Sequence analysis of the *ura1* gene encoding orotidine-5'-monophosphate decarboxylase of *Schizophyllum commune*. *Gene* **83:**387-393.

63. Furst, P., S. Hu, R. Hackett, and D. Hamer. 1988. Copper activates metallothionein gene transcription by altering the conformation of a specific DNA binding protein. *Cell* **55:**705-717.

64. Garber, R. C., and O. C. Yoder. 1983. Isolation of DNA from filamentous fungi and separation of nuclear, mitochondrial, ribosomal, and plasmid components. *Anal. Biochem.* **135:**416-422.

65. Gaskell, J., E. Dieperink, and D. Cullen. 1991. Genomic organization of lignin peroxidase genes of *Phanerochaete chrysosporium*. *Nucleic Acids Res.* **19:**599-603.

66. Gessner, M., and U. Raeder. 1994. A histone H4 promoter for expression of a phleomycin-resistance gene in *Phanerochaete chrysosporium*. *Gene* **142:**237-241.

67. Glasel, J. A. 1995. Validity of nucleic acid purities monitored by 260nm/280nm absorbance ratios. *Biotechniques* **18:**62-63.

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

69. Glenn, J. K., and M. H. Gold. 1983. Decolorization of several polymeric dyes by the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **45**:1741-1747.

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

71. Glenn, J. K., M. A. Morgan, M. B. Mayfield, M. Kuwahara, and M. H. Gold. 1983. An extracellular H_2O_2-requiring enzyme preparation involved in lignin biodegradation by the white rot basidiomycete *Phanerochaete chrysosporium*. *Biochem. Biophys. Res. Commun.* **114**:1077-1083.

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

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

74. Gold, M. H., and M. Alic. 1993. Molecular biology of lignin degradation by *Phanerochaete chrysosporium*. *Microbiol. Rev.* **57**:605-622.

75. Gold, M. H., and T. M. Cheng. 1979. Conditions for fruit body formation in the white rot basidiomycete *Phanerochaete chrysosporium*. *Arch. Microbiol.* **121**:37-41.

76. Gold, M. H., and T. M. Cheng. 1978. Induction of colonial growth and replica plating of the white rot basidiomycete *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **35**:1223-1225.

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

78. Gold, M. H., J. K. Glenn, and M. A. Alic. 1988. Use of polymeric dyes in lignin biodegradation assays. *Methods Enzymol.* **161**:74-78.

79. Gold, M. H., M. Kuwahara, A. A. Chiu, and J. K. Glenn. 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.

80. Gold, M. H., H. Wariishi, and K. Valli. 1989. Extracellular peroxidases involved in lignin degradation by the white rot basidiomycete *Phanerochaete chrysosporium*, p. 127-140. *In* J. R. Whitaker and P. E. Sonnet (ed.), *Biocatalysis in Agricultural Biotechnology*, Vol. 389. American Chemical Society, Washington, D.C.

81. Greene, J. M., Z. Larin, I. C. A. Taylor, H. Prentice, K. A. Gwinn, and R. E. Kingston. 1987. Multiple basal elements of a human hsp70 promoter function differently in human and rodent cell lines. *Mol. Cell. Biol.* **7**:3646-3655.

82. Gritz, L., and J. Davies. 1983. Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*. *Gene* **25**:179-188.

83. Gurr, S. J., S. E. Unkles, and J. R. Kinghorn. 1987. The structure and organization of nuclear genes of filamentous fungi, p. 93-139. *In* J. R. Kinghorn (ed.), *Gene Structure in Eukaryotic Microbes*. IRL Press, Oxford.

84. Hammel, K. E. 1989. Organopollutant degradation by ligninolytic fungi. *Enzyme Microb. Technol.* **11**:776-777.

85. Hamer, D. H. 1986. Metallothionein. *Annu. Rev. Biochem.* **55**:913-951.

86. Hammel, K. E. 1989. Organopollutant degradation by ligninolytic fungi. *Enzyme Microb. Technol.* **11**:776-777.

87. Hammel, K. E., and M. A. Moen. 1991. Depolymerization of a synthetic lignin *in vitro* by lignin peroxidase. *Enzyme Microb. Technol.* **13**:15-18.

88. Harmsen, M. C., F. H. J. Schuren, S. M. Moukha, C. M. van Zuilen, P. J. Punt, and J. G. H. Wessels. 1992. Sequence analysis of the glyceraldehyde-3-phosphate dehydrogenase genes from the basidiomycetes *Schizophyllum commune*, *Phanerochaete chrysosporium*, and *Agaricus bisporus*. *Curr. Genet.* **22**:447-454.

89. Hawkins, A. R. 1987. The complex *Arom* locus of *Aspergillus nidulans*. Evidence for multiple gene fusions and convergent evolution. *Curr. Genet.* **11**:491-498.

90. von Heijne, G. 1985. Signal sequences: the limits of variation. *J. Mol. Biol.* **184:**99-105.

91. Henikoff, S. 1987. Unidirectional digestion with exonuclease III in DNA sequence analysis. *Methods Enzymol.* **155:**156-165.

92. Higuchi, T. 1990. Lignin biochemistry: biosynthesis and biodegradation. *Wood Sci. Technol.* **24:**23-63.

93. Holzbaur, E. L. F., and M. Tien. 1988. Structure and regulation of a lignin peroxidase gene from *Phanerochaete chrysosporium. Biochem. Biophys. Res. Commun.* **55:**626-633.

94. Huoponen, K., P. Ollikka, M. Kälin, I. Walther, P. Mäntsälä, and J. Reiser. 1990. Characterization of lignin peroxidase-encoding genes from lignin-degrading basidiomycetes. *Gene* **89:**145-150.

95. Imagawa, M., R. Chiu, and M. Karin. 1987. Transcription factor AP-2 mediates induction by two different signal-transduction pathways: protein kinase C and cyclic AMP. *Cell* **51:**251-260.

96. Imbert, J., V. Culotta, P. Furst, L. Gedamu, and D. Hamer. 1990. Regulation of metallothionein gene transcription by metals, p. 139-164. *In* G. L. Eichorn and L. G. Marzilli (ed.). *Metal-Ion Induced Regulation of Gene Expression*, Vol. 8. Elsevier Science Publishing, Inc., New York.

97. Imbert, J., M. Zafarullah, V. C. Culotta, L. Gedamu, and D. Hamer. 1989. Transcription factor MBF-I interacts with metal regulatory elements of higher eucaryotic metallothionein genes. *Mol. Cell. Biol.* **9:**5315-5323.

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

99. Jones, N. C., P. W. J. Rigby, and E. B. Ziff. 1988. Trans-acting protein factors and regulation of eukaryotic transcription: lessons from studies on DNA tumor viruses. *Genes Dev.* **2:**267-281.

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

101. Karin, M. 1985. Metallothioneins: proteins in search of a function. *Cell* **41:**9-10.

102. Kelly, J. M., and M. J. Hynes. 1987. Multiple copies of the *amdS* gene of *Aspergillus nidulans* cause titration of trans-acting regulatory proteins. *Curr. Genet.* **12:**21-31.

103. Kersten, P. J., and D. Cullen. 1993. Cloning and characterization of a cDNA encoding glyoxal oxidase, a H_2O_2-producing enzyme from the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *Proc. Natl. Acad. Sci. USA* **90:**7411-7413.

104. Keyser, P., T. K. Kirk, and J. G. Zeikus. 1978. Ligninolytic enzyme system of *Phanerochaete chrysosporium*: synthesized in the absence of lignin in response to nitrogen starvation. *J. Bacteriol.* **135:**790-797.

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

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

107. Kozak, M. 1983. Comparison of initiation of protein synthesis in procaryotes, eucaryotes, and organelles. *Microbiol. Rev.* **47:**1-45.

108. Krumlauf, R., and G. A. Marzluf. 1980. Genome organization and characterization of the repetitive and inverted repeat DNA sequences in *Neurospora crassa*. *J. Biol. Chem.* **225:**1138-1145.

109. Kuan, I.-C., K. A. Johnson, and M. Tien. 1993. Kinetic analysis of manganese peroxidase. *J. Biol. Chem.* **268:**20064-20070.

110. Kuwahara, M., and Y. Asada. 1987. Production of ligninases, peroxidases and alcohol oxidase by *Phanerochaete chrysosporium* and its mutant, p. 171-176. *In* E. Odier (ed.), *Lignin Enzymatic and Microbial Degradation*. Institut National de la Recherche Agronomique, Paris.

111. Kuwahara, M., J. K. Glenn, M. A. Morgan, and M. H. Gold. 1984. Separation and characterization of two extracellular H_2O_2-dependant oxidases from ligninolytic cultures of *Phanerochaete chrysosporium*. *FEBS Lett.* **169:**247-250.

112. Leatham, G. F. 1986. The ligninolytic activities of *Lentinus edodes* and *Phanerochaete chrysosporium*. *Appl. Microbiol. Biotechnol.* **24:**51-58.

113. Leisola, M. S. A., B. Kozulic, F. Meussdoerffer, and A. Fiechter. 1987. Homology among multiple extracellular peroxidases from *Phanerochaete chrysosporium*. *J. Biol. Chem.* **262:**419-424.

114. Lerch, K. 1980. Copper metallothionein, a copper-binding protein from *Neurospora crassa*. *Nature* **284:**368-370.

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

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

117. Li, T.-Y., A. J. Kraker, C. F. Shaw III, and D. H. Petering. 1980. Ligand substitution reactions of metallothioneins with EDTA and apo-carbonic anhydrase. *Proc. Natl. Acad. Sci. USA* 77:6334-6338.

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

119. MacDonald, M. J., R. Ambler, and P. Broda. 1985. Regulation of intracellular cyclic AMP levels in the white-rot fungus *Phanerochaete chrysosporium* during the onset of idiophasic metabolism. *Arch. Microbiol.* **142:**152-156.

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

121. Magill, J. M., and C. W. Magill. 1989. DNA methylation in fungi. *Dev. Genet.* **10:**63-69.

122. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

123. Marmeisse, R., G. Gay, J.-C. Debaud, and L. A. Casselton. 1992. Genetic transformation of the symbiotic basidiomycete fungus *Hebeloma cylindrosporum*. *Curr. Genet.* **22:**41-45.

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

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

126. Mellon, F. M., and L. A. Casselton. 1988. Transformation as a method of increasing gene copy number and gene expression in the basidiomycete fungus *Coprinus cinereus*. *Curr. Genet.* **14:**451-456.

127. Messing, J. 1983. New M13 vectors for cloning. *Methods Enzymol.* **101:**20-78.

128. Michaels, S. D., M. C. John, and R. M. Amasino. 1994. Removal of polysaccharides from plant DNA by ethanol precipitation. *Biotechniques* **17:**274,276.

129. Miller, B. L., K. Y. Miller, K. A. Roberti, and W. E. Timberlake. 1987. Position-dependent and -independent mechanisms regulate cell-specific expression of the SpoC1 gene cluster of *Aspergillus nidulans*. *Mol. Cell. Biol.* **7:**427-434.

130. Mino, Y., H. Wariishi, N. J. Blackburn, T. M. Loehr, and M. H. Gold. 1988. Spectral characterization of manganese peroxidase, an extracellular heme enzyme from the lignin-degrading basidiomycete, *Phanerochaete chrysosporium*. *J. Biol. Chem.* **263:**7029-7036.

131. Mooibroek, H., A. G. J. Kuipers, J. H. Sietsma, P. J. Punt, and J. G. H. Wessels. 1990. Introduction of hygromycin B resistance into *Schizophyllum commune*: preferential methylation of donor DNA. *Mol. Gen. Genet.* **222:**41-48.

132. Mueller, P. R., S. J. Salser, and B. Wold. 1988. Constitutive and metal-inducible protein:DNA interactions at the mouse metallothionein I promoter examined by *in vivo* and *in vitro* footprinting. *Genes Dev.* **2:**412-427.

133. Munger, K., U. A. Germann, and K. Lerch. 1987. The *Neurospora crassa* metallothionein gene—regulation of expression and chromosomal location. *J. Biol. Chem.* **262:**7363-7367.

134. Munger, K., and K. Lerch. 1985. Copper metallothionein from the fungus *Agaricus bisporus*: chemical and spectroscopic properties. *Biochemistry* **24:**6751-6756.

135. Muñoz-Rivas, A. M., C. A. Specht, B. J. Drummond, E. Froeliger, C. P. Novotny, and R. C. Ullrich. 1986. Transformation of the basidiomycete, *Schizophyllum commune*. *Mol. Gen. Genet.* **205:**103-106.

136. Naidu, P. S., and C. A. Reddy. 1990. Nucleotide sequence of a new lignin peroxidase gene GLG3 from the white-rot fungus, *Phanerochaete chrysosporium*. *Nucleic Acids Res.* **18:**7173.

137. Naidu, P. S., Y. Z. Zhang, and C. A. Reddy. 1990. Characterization of a new lignin peroxidase gene (GLG6) from *Phanerochaete chrysosporium*. *Biochem. Biophys. Res. Commun.* **173:**994-1000.

138. Naidu, P. S., Y. Z. Zhang, and C. A. Reddy. 1989. PhaLip 2 Accession No. X15599. GenBank.

139. Nover, L. 1984. Inducers of heat shock protein synthesis, p. 7-12. *In* L. Nover (ed.), *Heat Shock Response of Eukaryotic Cells*. Springer-Verlag, Berlin.

140. Ornstein, D. L., and M. A. Kashdan. 1985. Sequencing DNA using sulfur-35 labelling: a troubleshooting guide. *BioTechniques* **3:**476-483.

141. Orth, A. B., M. Rzhetskaya, D. Cullen, and M. Tien. 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.

142. Otsuka, F., A. Iwamatsu, K. Suzuki, M. Ohsawa, and D. H. Hamer. 1994. Purification and characterization of a protein that binds to metal responsive elements of the human metallothionein II_A gene. *J. Biol. Chem.* **269:**23700-23707.

143. Padget, R. A., M. M. Konarska, P. J. Grabowski, S. F. Hardy, and P. A. Sharp. 1989. Lariat mRNAs as intermediates and products in the splicing of messenger RNA precursors. *Science* **225:**898-903.

144. Palmiter, R. D., G. W. Stuart, and P. F. Searle. 1985. Metal regulatory elements in metallothionein gene promoters, p. 144-149. *In* Y. Gluzman (ed.), *Current Communication in Molecular Biology: Eukaryotic Transcription: The Role of cis- and trans-acting Elements in Initiation*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

145. Paszczynski, A., V.-B. Huynh, and R. L. Crawford. 1986. Comparison of ligninase-I and peroxidase-M2 from the white-rot fungus *Phanerochaete chrysosporium*. *Arch. Biochem. Biophys.* **244:**750-765.

146. Pease, E. A., A. Andrawis, and M. Tien. 1989. Manganese-dependant peroxidase from *Phanerochaete chrysosporium*: primary structure deduced from cDNA sequence. *J. Biol. Chem.* **264:**13531-13535.

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

147a. Pelham, H. R. B. 1982. A regulatory upstream promoter element in the *Drosophila melanogaster* Hsp70 heat-shock gene. *Cell* **30:**517-528.

148. Peng, M., N. K. Singh, and P. A. Lemke. 1992. Recovery of recombinant plasmids from *Pleurotus ostreatus* transformants. *Curr. Genet.* **22:**53-59.

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

150. Poulos, T. L., and J. Kraut. 1980. The stereochemistry of peroxidase catalysis. *J. Biol. Chem.* **255:**8199-8205.

151. Prabhakararao, K., and M. E. Jones. 1975. Radioassay of orotic acid phosphoribosyl transferase and orotidylate decarboxylase utilizing a high-voltage paper electrophoresis technique or an improved $^{14}CO_2$ release method. *Anal. Biochem.* **69:**451-457.

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

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

154. Raeder, U., and P. Broda. 1984. Comparison of the lignin-degrading white rot fungi *Phanerochaete chrysosporium* and *Sporotrichum pulverulentum* at the DNA level. *Curr. Genet.* **8:**499-506.

155. Raeder, U., and P. Broda. 1985. Rapid preparation of DNA from filamentous fungi. *Lett. Appl. Microbiol.* **1:**17-20.

156. Raeder, U., W. Thompson, and P. Broda. 1989. RFLP-based genetic map of *Phanerochaete chrysosporium* ME446: lignin peroxidase genes occur in clusters. *Mol. Microbiol.* **3:**911-918.

157. Ralston, D. M., and T. V. O'Halloran. 1990. Metalloregulatory proteins and molecular mechanisms of heavy metal signal transduction, p. 1-31. *In* G. L. Eichorn and L. G. Marzilli (ed.), *Metal-ion Induced Regulation of Gene Expression*, Vol. 8. Elsevier Science Publishing, Inc., New York.

158. Randall, T., T. R. Rao, and C. A. Reddy. 1989. Use of a shuttle vector for the transformation of the white rot basidiomycete, *Phanerochaete chrysosporium*. *Biochem. Biophys. Res. Commun.* **161:**720-725.

159. Randall, T., and C. A. Reddy. 1991. An improved transformation vector for the lignin-degrading white rot basidiomycete *Phanerochaete chrysosporium*. *Gene* **103:**125-130.

160. Randall, T., C. A. Reddy, and K. Boominathan. 1991. A novel extrachromosomally maintained transformation vector for the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *J. Bacteriol.* **173:**776-782.

161. Reid, I. D. 1983. Effects of nitrogen sources on cellulose and synthetic lignin degradation by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **45:**838-842.

162. Revuelta, J. L., and M. Jayaram. 1987. *Phycomyces blakesleeanus* TRP1 gene: organization and functional complementation in *Escherichia coli* and *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **7:**2664-2670.

163. Richter, H. E., and P. C. Loewen. 1981. Induction of catalase in *Escherichia coli* by ascorbic acid involves hydrogen peroxidase. *Biochem. Biophys. Res. Commun.* **100:**1039-1046.

164. Ritch, T. G., Jr., and M. H. Gold. 1992. Characterization of a highly expressed lignin peroxidase-encoding gene from the basidiomycete *Phanerochaete chrysosporium*. *Gene* **118:**73-80.

165. Ritch, T. G., Jr., V. J. Nipper, L. Akileswaran, A. J. Smith, D. G. Pribnow, and M. H. Gold. 1991. Lignin peroxidase from the basidiomycete *Phanerochaete chrysosporium* is synthesized as a preproenzyme. *Gene* **107:**119-126.

166. Roch, P., J. A. Buswell, R. B. Cain, and E. Odier. 1989. Lignin peroxidase production by strains of *Phanerochaete chrysosporium* grown on glycerol. *Appl. Microbiol. Biotechnol.* **31:**587-591.

167. Rossignol, J. L., and M. Picard. 1991. *Ascobolus immersus* and *Podospora anserina*: sex , recombination, silencing and death, p. 267-290. *In* J. W. Bennet and L. L. Lasure (ed.), *More Gene Manipulations in Fungi*. Academic Press, San Diego.

168. Sánchez, F., A. Tourino, S. Traseira, A. Pérez-Aranda, V. Rubio, and M. A. Penalva. 1986. Molecular cloning and characterization of the trpC gene from *Penicillium chrysogenum*. *Mol. Gen. Genet.* **205:**248-252.

169. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463-5467.

170. Schalch, H., J. Gaskell, T. L. Smith, and D. Cullen. 1989. Molecular cloning and sequences of lignin peroxidase genes of *Phanerochaete chrysosporium*. *Mol. Cell. Biol.* **9**:2743-2747.

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

172. Schuren, F. H. J., and J. G. H. Wessels. 1994. Highly-efficient transformation of homobasidiomycete *Schizophyllum commune* to phleomycin resistance. *Curr. Genet.* **26**:179-183.

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

174. Searle, P. F. 1990. Zinc dependant binding of a liver nuclear factor to a metal response element MRE-α of the mouse metallothionein-I gene and variant sequences. *Nucleic Acids Res.* **18**:4683-4690.

175. Searle, P. F., G. W. Stuart, and R. D. Palmiter. 1985. Building a metal-responsive promoter with synthetic regulatory elements. *Mol. Cell. Biol.* **5**:1480-1489.

176. Séguin, C. 1991. A nuclear factor requires Zn^{2+} to bind to a regulatory MRE element of the mouse gene encoding metallothionein-1. *Gene* **97**:295-300.

177. Séguin, C., B. K. Felber, A. D. Carter, and D. H. Hamer. 1984. Competition for cellular factors that activate metallothionein gene transcription. *Nature* **312**:781-785.

178. Selker, E. U. 1991. Repeat-induced point mutation and DNA methylation, p. 258-265. *In* J. W. Bennet and L. L. Lasure (ed.), *More Gene Manipulations in Fungi*. Academic Press, San Diego.

179. Sims, P., A. Brown, C. James, U. Raeder, A. Schrank, and P. Broda. 1988. Molecular genetics of lignocellulose degradation in *Phanerochaete chrysosporium*, p. 365-377. *In* J.-P. Aubert, P. Beguin, and J. Millet (ed.), *Biochemistry and Genetics of Cellulose Degradation*. Academic Press, London.

180. Smith, T. L., H. Schalch, J. Gaskell, S. Covert, and D. Cullen. 1988. Nucleotide sequence of a ligninase gene from *Phanerochaete chrysosporium*. *Nucleic Acids Res.* **16:**1219.

181. Snedecor, G. W., and W. G. Cochran. 1980. *Statistical Methods*, 7th ed. Iowa State University Press, Ames Iowa.

182. Stewart, P., P. Kersten, A. Vanden Wymelenberg, J. Gaskell, and D. Cullen. 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.

183. Stuart, G. W., P. F. Searle, H. Y. Chen, R. L. Brinster, and R. D. Palmiter. 1984. A 12-base-pair DNA motif that is repeated several times in metallothionein gene promoters confers metal regulation to a heterologous gene. *Proc. Natl. Acad. Sci. USA* **81:**7318-7322.

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

185. Szczypka, M. S., and D. J. Thiele. 1989. A cysteine-rich nuclear protein activates yeast metallothionein gene transcription. *Mol. Cell. Biol.* **9:**421-429.

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

187. Thiele, D. J., M. J. Walling, and D. Hamer. 1986. Mammalian metallothionein is functional in yeast. *Science* **231:**854-856.

188. Tien, M. 1987. Properties of ligninase from *Phanerochaete chrysosporium* and their possible applications. *Crit. Rev. Microbiol.* **15:**141-168.

189. Tien, M., and T. K. Kirk. 1983. Lignin-degrading enzyme from the hymenomycete *Phanerochaete chrysosporium* burds. *Science* **221:**661-663.

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

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

192. Timberlake, W. E. 1978. Low repetitive DNA content in *Aspergillus nidulans*. *Science* **202:**973-975.

193. Tonon, F., C. Prior de Castro, and E. Odier. 1990. Nitrogen and carbon regulation of lignin peroxidase and enzymes of nitrogen metabolism in *Phanerochaete chrysosporium*. *Exp. Mycol.* **14:**243-254.

194. Tuor, U., H. Wariishi, H. E. Schoemaker, and M. H. Gold. 1992. Oxidation of phenolic arylglycerol-β-aryl ether lignin model compounds by manganese peroxidase from *Phanerochaete chrysosporium*: oxidative cleavage of an α-carbonyl model compound. *Biochemistry* **31:**4986-4995.

195. Turgeon, B. G., W. D. MacRae, R. C. Garber, G. R. Fink, and O. C. Yoder. 1986. A cloned tryptophan-synthesis gene from the Ascomycete *Cochliobolus heterostrophus* functions in *Escherichia coli*, yeast and *Aspergillus nidulans*. *Gene* **42:**79-88.

196. Udom, A. O., and F. O. Brady. 1980. Reactivation *in vitro* of zinc-requiring apo-enzymes by rat liver zinc-thionein. *Biochem. J.* **187:**329-335.

197. Ulmer, D. C., M. S. A. Leisola, B. H. Schmidt, and A. Fiechter. 1983. Rapid degradation of isolated lignins by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **45:**1795-1801.

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

199. Valli, K., H. Wariishi, and M. H. Gold. 1990. Oxidation of monomethoxylated aromatic compounds by lignin peroxidase: role of veratryl alcohol in lignin biodegradation. *Biochemistry* **29:**8535-8539.

200. Vinson, C. R., K. L. LaMarco, P. F. Johnson, W. H. Landschulz, and S. L. McKnight. 1988. *In situ* detection of sequence-specific DNA binding activity specified by a recombinant bacteriophage. *Genes Dev.* **2:**801-806.

201. Walther, I., M. Kalin, J. Reiser, F. Suter, B. Fritsche, M. Saloheimo, M. Leisola, T. Teeri, J. K. C. Knowles, and A. Fiechter. 1988. Molecular analysis of a *Phanerochaete chrysosporium* lignin peroxidase gene. *Gene* **70:**127-137.

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

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

204. Wariishi, H., K. Valli, and M. H. Gold. 1991. *In vitro* depolymerization of lignin by manganese peroxidase of *Phanerochaete chrysosporium*. *Biochem. Biophys. Res. Commun.* **176:**269-275.

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

206. Wariishi, H., K. Valli, and M. H. Gold. 1989. Oxidative cleavage of a phenolic diarylpropane lignin model dimer by manganese peroxidase from *Phanerochaete chrysosporium*. *Biochemistry* **28:**6017-6023.

207. Weinberg, E. D. 1982. Biosynthesis of microbial metabolites—regulation by mineral elements and temperature, p. 181-194. *In* V. Krumphanzel, B. Sikyta, and S. Vanek (ed.), *Overproduction of Microbial Products*. Academic Press, Ltd., London.

208. Westin, G., and W. Schaffner. 1988. A zinc-responsive factor interacts with a metal-regulated enhancer element (MRE) of the mouse metallothionein-I gene. *EMBO J.* **7:**3763-3770.

209. Wingender, E. 1988. Compilation of transcription regulating proteins. *Nucleic Acids Res.* **16:**1879-1902.

210. Xu, C. 1993. cDNA cloning of a mouse factor that activates transcription from a metal response element of the mouse metallothionein-I gene in yeast. *DNA Cell Biol.* **12:**517-525.

211. Zhang, Y. Z., and C. A. Reddy. 1988. Use of synthetic oligonucleotide probes for identifying ligninase cDNA clones. *Methods Enzymol.* **161:**228-237.

212. Zhang, Y. Z., C. A. Reddy, and A. Rasooly. 1991. Cloning of several lignin peroxidase (LiP)-encoding genes: sequence analysis of the *LIP6* gene from the white rot basidiomycete *Phanerochaete chrysosporium*. *Gene* **97:**191-198.

213. Zhang, Y. Z., G. J. Zylstra, R. H. Olsen, and C. A. Reddy. 1986. Identification of cDNA clones for ligninase from *Phanerochaete chrysosporium* using synthetic oligonucleotide probes. *Biochem. Biophys. Res. Commun.* **137:**649-656.

BIOGRAPHICAL SKETCH

Bruce Godfrey was born in Portland, Oregon, in 1960. He attended public primary and secondary schools in Portland. During his youth, Bruce developed an interest in science, especially chemistry, which he has pursued continuously since. In 1979 he enrolled at the University of Oregon in Eugene as a chemistry major. During college his interests broadened to include molecular biology and biotechnology, and he received credit for undergraduate research in molecular biology in the laboratory of Dr. Edward Herbert. He earned a B.S. degree in Chemistry from the University of Oregon in 1983.

Following graduation, he took a job at Molecular Probes, Inc., as an organic chemist. He was hired in 1985 by Dr. Herbert in the Chemistry Department at the University of Oregon as a research assistant doing molecular biology. When the Herbert lab moved to the Vollum Institute for Advanced Biomedical Research in Portland a year later, he continued employment part-time as a research assistant in the Herbert lab and part-time running the oligonucleotide synthesis facility for the Institute.

He learned of the research on the fungus *Phanerochaete chrysosporium* in Michael Gold's lab at the Oregon Graduate Institute from a seminar given at the Oregon Health Sciences University by Dave Pribnow. The work Dr. Pribnow described fit several of Bruce's interests, including chemistry, molecular biology, and biomass utilization. In 1987 Bruce enrolled as a graduate student in the department of Chemistry, Biochemistry, and Molecular Biology, to work in Dr. Gold's lab. His thesis research involved developing some basic tools for molecular genetic manipulations in *P. chrysosporium*. He defended his thesis at OGI in August 1995 and took a postdoctoral position at the Beckman Institute at the University of Illinois

in September. At the Beckman Institute he will be working on projects involving biophysics, structural biology, and molecular biology with Dr. Steven Sligar.

List of Publications

Grimes, M., A. Iacangelo, L. Eiden, B. Godfrey, and E. Herbert (1987) Chromogranin A: the primary structure deduced from cDNA clones reveals the presence of pairs of basic amino acids. *Ann. N.Y. Acad. Sci.* **493,** 351-378.

Bond, C. T., G. Nilaver, B. Godfrey, E. A. Zimmerman, and J. P. Adelman (1988) Characterization of complementary deoxyribonucleic acid for the precursor of porcine motilin. *Mol. Endocrinol.* **2,** 175-180.

Beinfeld, M. C., C. T. Bond, G. Nilaver, D. Daihk, B. Godfrey, and J. P. Adelman (1988) Heterogeneity of motilin immunoreactivity in mammalian tissue. *Synapse* **2,** 266-275.

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

Gold, M. H., J. A. Brown, B. J. Godfrey, M. B. Mayfield, H. Wariishi, and K. Valli (1991) Structure and regulation of a Mn peroxidase gene from *Phanerochaete chrysosporium*, p. 188-199. In G. F. Leatham and M. E. Himmel (ed.), *Enzymes in Biomass Conversion.* American Chemical Society, Washington, D.C.

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

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