A Reporter Gene Construct for Studying the Regulation of Manganese Peroxidase Gene Expression

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The orotidylate decarboxylase (ODase) gene (ura1) from Schizopyllum commune was utilized as a reporter for studying Mn regulation of the manganese peroxidase (MnP) gene (mnp) from the lignin-degrading basidiomycete Phanerochaete chrysosporium. A 1,500-bp fragment of the mnp1 promoter was fused upstream of the coding region of the ODase gene in a plasmid (pMAMO) containing the S. commune ade5 gene as a selectable marker. pMAMO was used to transform a P. chrysosporium adel ura11 mutant lacking endogenous ODase activity. When the P. chrysosporium transformant was grown in nitrogen-limited, Mn(II)-sufficient cultures, ODase activity was detected only during secondary metabolic growth and the pattern of ODase expression was similar to that of endogenous MnP. When Mn was added to 6-day-old nitrogen-limited, Mn-deficient cultures, both ODase activity and MnP activity were induced synchronously with maximal activity at 30 h. Growth in high-nitrogen-concentration medium suppressed the induction of both the ODase and endogenous MnP. These results indicate that this promoter-reporter construct can be used to study the regulation of the mnp gene.

The white rot basidiomycete Phanerochaete chrysosporium has been the focus of numerous studies of the degradation of lignin (10, 18, 22) and aromatic pollutants (9, 19, 21, 38). Two families of peroxidases, lignin peroxidase (LiP) and manganese peroxidase (MnP), along with an H2O2-generating system, are the major components of this organism's extracellular lignin-degrading system (15, 18, 22). MnP has been purified and extensively characterized (13, 15, 39, 40). MnP is an H2O2- and Mn(II)-dependent, heme-containing glycoprotein of M, ~46,000 (15, 18). This enzyme oxidizes Mn(II) to Mn(III); the latter, complexed with an organic acid chelator such as oxalate or malonate, is secreted by P. chrysosporium, oxidizes a terminal phenolic substrate (13, 25, 41). MnP occurs as a family of isozymes encoded by a series of genes, and the sequences of cDNA (29, 33) and genomic clones (14, 15, 28) encoding two MnP isozymes have been determined. As an idiopathic protein, the expression of MnP is activated at the level of transcription by the depletion of nutrient nitrogen (15, 18, 22, 33), and there is evidence that the MnP isozymes may be differentially regulated by carbon and nitrogen (30). MnP expression also is dependent on the presence of Mn(II) in the culture medium (5, 7), and mnp gene transcription is regulated by MnS (6, 7). In addition, we demonstrated that mnp gene transcription is regulated by heat shock (8, 14, 15).

The promoter regions of the two sequenced mnp genes (14, 15, 28) contain putative heat shock elements (HSEs) (26) and metal response elements (MREs) (20, 36). To further examine the putative cis-acting sequences involved in the regulation of mnp gene expression by nitrogen, Mn, 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 Schizopyllum commune acting as a reporter. This exogenous promoter-reporter was used to examine regulation of the mnp system.

MATERIALS AND METHODS

Organisms. P. chrysosporium OGC101, adel and ura11 mutants, and the adel ura11 double mutant were as described previously (1, 4). Escherichia coli XL1-Blue and DH5α were used for subcloning plasmids.

Enzymes and chemicals. Orotate phosphoribosyl transferase, orotidylate decarboxylase, and sodium orotate were obtained from Sigma. 14COOH-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 (27).

Construction of pOGI 18. A 3.8-kb BamHI-Kpn1 fragment containing the S. commune ade5 gene from plasmid pAde5-2g (2) and AdelII-digested pHCl8 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 32P-labeled BamH1-Kpn1 fragment to identify recombinant colonies (27). 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 adel transformation vector. A map of this plasmid, pOGI 18, is shown in Fig. 1a.

Construction of pAMO. A 6.5-kb λEMBL3 BamH1 fragment containing the mnp1 gene (14) was subcloned into pUC18, after which a 2.1-kb SaI fragment containing the S' end of the coding region and 1.4 kb of S' untranslated sequence was subcloned into pUC18. A SaI-HincII subfrag-
ment 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 Sall and then with HindII. The S. commune ura1 gene was obtained from R. Ullrich as the plasmid pEF1 (12). The translation initiation codon of this gene resides within a unique BspHI site, and the coding region of the gene was isolated as a BspHI-BamHI fragment. A synthetic linker was used to replace the mnpl 5' sequences between the HindII site and the initiation codon and to adapt the mnpl promoter fragment to the BspHI overhang on the ura1 gene (Fig. 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 Sall-BamHI-digested pUC18, ligated, and transformed into XL1-Blue cells. To construct pAMO (Fig. 1b and c), pMO was cut at the unique SphI site and treated with T4 polymerase to create blunt ends. The complete fusion construct was then released from pUC18 by digestion with EcoRI and subcloned into SmaI-EcoRI-digested pOGI 18. The construction was confirmed by restriction mapping and by sequencing an Eagl-Sall fragment spanning the fusion junction which had been subcloned into pBluescriptISK+ to provide single-stranded DNA.

**Fungal transformations.** The double mutant adel ura11 was transformed as described elsewhere (2, 3). One microgram of the plasmid pAMO, linearized at the unique EcoRI site (Fig. 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 (4, 16). Those transformants which grew 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 (16) for maintenance. Transformants were purified by fruiting and plating basidiospores as described previously (4). The modified fruiting media (4, 17) consisted of 4.5% Walseth
cellulose, 0.01% uracil, and 0.001% adenine in 1/6-strength Vogel medium.

Culture conditions. Mutant and transformant cultures were maintained on rich slants as described above, containing adenine and uracil. The basal medium (HCLN) (6, 23) contained 2% glucose and 1.2 mM ammonium tartrate and a trace element solution containing no MnSO₄. HCLN(AUY) is HCLN supplemented with 0.001% adenine, 0.01% uracil, 0.01% yeast extract (Difco), and either 0.25 or 180 μM MnSO₄, 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 MnSO₄ in 250-ml Erlenmeyer flasks. Cultures were incubated under air for 5 days and then purged with 100% O₂ on day 5, and MnSO₄ (180 μM) was added on day 6. Cells were harvested by filtration through Miracloth 36 h after Mn induction, frozen in liquid N₂, 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 MnSO₄. Cultures were incubated under air for 4 days and subsequently purged with O₂ at 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 MnSO₄ and then purged with O₂. On day 6, 180 μM MnSO₄ was added, after which extracellular MnP activity was periodically assayed as indicated and cells were harvested and stored at −80°C.

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, Okla.). 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.

ODase assay. The ODase assay, measuring the evolution of 14CO₂ from 14C-oxalic acid, was a modification of that described previously (32). 14C-labeled oxalic acid, phosphoribosyl pyrophosphate, and orotidine-5'-phosphate pyrophosphorylase were used in the coupled reaction shown in Fig. 2. The reaction was carried out in a 15-ml Falcon centrifuge tube stoppered with an Aldrich Suba-Seal stopper. The 14CO₂ released was absorbed onto a piece of Whatman 3MM paper (1 by 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 MgCl₂, 1 mM phosphoribosyl pyrophosphate, and 2 mM dithiothreitol in 50 mM Na phosphate, pH 7.0. The reaction was started by the addition of extract and 1 μl of orotidine-5'-phosphate pyrophosphorylase. Incubation was at 37°C with gentle agitation for 1 h. The reaction was stopped by the addition, of a needle, of 200 μl of 1 M H₂SO₄. The tubes were left in the water bath for an additional hour to complete the adsorption of the 14CO₂ onto 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 (41). Reaction mixtures (1 ml) contained 0.2 mM MnSO₄, 0.2 mM H₂O₂, and 50 μl of extracellular medium in 50 mM Na malonate, pH 4.5. MnP and ODase activities were measured in triplicate cultures.

RESULTS

Construction of vectors. The E. coli P. chrysosporium shuttle vector, pOGI 18, contains the S. commune ade1 gene (11) encoding phosphoribosyl amoinoimidazole synthetase (2) on a 6.5-kb BamHI-KpnI fragment ligated into the unique AarII site of pUC18. This plasmid, pOGI 18 (Fig. 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 pAdes5-2g plasmid (2).

mpn promoter-ODase reporter fusion, pAMO. The mpn promoter-ODase reporter construct contained a 1.4-kb SalI-HincII fragment of the mpnl gene, which spans from 1,400 bases 5' of the cDNA start (33) to 15 bases downstream of the TATAAA box; a synthetic oligonucleotide linker which recreates the mpnl 5' untranslated region from the HincII site to the translation initiation codon; and the coding region of the S. commune ural1 gene from the initiation codon to the end of the pEF1 insert (1.65 kb) (12). The single base change before the ATG, as shown in Fig. 1c, conforms to the CCRNNATGG eukaryotic initiation consensus sequence (24). However, the mpnl gene differs from the consensus in several positions (33). The complete mpnl-ural1 construct was subcloned into pOGI 18 as an SphI-EcoRI fragment to create pAMO (Fig. 1b).

Transformation and characterization of transformants. An ade1 ural1 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 idiosyncratic 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 varied from 0- to 25-fold with an average induction of 7.7-fold. The transformant (A141) with the highest Mn 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).

Effect of Mn on expression of ODase and mpnl in cultures of the transformant A141. Time courses for the appearance of extracellular MnP activity and intracellular ODase activity are shown in Fig. 3. Cultures grown in the presence of 0.25 μM Mn had no detectable MnP activity through day 8 and had minimal ODase activity during the same period, whereas in cultures
containing 180 μM Mn, both MnP and ODase activities 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 activities by Mn is shown in Fig. 4. The transformant was grown for 5 days in the presence of 0.25 μM Mn, after which the cultures were purged with O2. On day 6, 180 μM Mn was added to the medium and the cultures were reincubated. No MnP activity was detected before the addition of 180 μM Mn. MnP activity reached a maximum 30 h after the addition of 180 μM Mn and declined thereafter. This is similar to our previous results with the wild-type strain (7). In parallel with the appearance of MnP activity, ODase activity steadily increased for 30 h after the addition of Mn, reaching a maximum induction of ~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 was added to cultures grown for 6 days in HCHN medium containing 0.25 μM Mn (Fig. 4).

**DISCUSSION**

The lignin-degradative system of *P. chrysosporium* is expressed during secondary metabolic (idiophase) growth, the onset of which is triggered by limiting nutrient (10, 18, 22). Likewise, MnP and LiP activities are detectable in the extracellular medium only during the secondary metabolic phase of growth (15, 22). Northern (RNA) blot analysis has demonstrated that expression of both MnP and LiP is controlled at the level of gene transcription by nutrient nitrogen (33, 37).

Previously we demonstrated that MnP expression also is regulated by Mn(II), the substrate for the enzyme, at the level of gene transcription (6, 7) and that the addition of Mn to nitrogen-limited, Mn-deficient cultures on day 4, 5, or 6 results in detectable *mnp* mRNA within 40 min (6). In the white rot basidiomycete *Dichomitus squalens*, MnP expression and lignin degradation also have been shown to be dependent on the presence of Mn (31). Examination of the promoter regions of the *mnp1* and *mnp2* genes (14, 15, 28) 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 (20, 36). Interestingly, examination of the *mnp1* promoter region also revealed the presence of putative HSEs within 400 bp upstream of the *mnp1* translation initiation codon (14, 15). Likewise, six HSEs are found within 1,100 bp of the translation initiation codon of the *mnp2* gene (15, 28). Previously, we reported on the heat shock induction of *mnp* gene transcription, even in the absence of Mn, suggesting that the HSEs are physiologically functional (8, 15, 28). Since neither MREs nor HSEs are found in *lip* gene promoters (15), 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 (15, 22).

Several metal-ion-regulated gene transcription systems have been studied in detail (20, 34, 36). 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 is acting through a multicomponent signal transduction system has not been ruled out, the existence of an Mn-binding transcription factor similar to the Cu-binding Ace1 protein that activates transcription of the yeast metallothionein gene (36) is an attractive model for the *mnp* system. Although Mn is involved in the synthesis of some secondary metabolites in other fungi (35, 42), this is, to our knowledge, the first instance of Mn 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 *ura1* mutants of *P. chrysosporium* (1), ensuring that *ura1* is expressed in this organism. The availability of an *adel ura1* 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 *ura*1 gene without interference by endogenous ODase activity. ODase has no known substrates other than orotidyl monophosphate, 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 (2, 3). pOG1 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 *mpn* 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. 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 (idiopathic) growth and is dependent on the presence of high levels of Mn 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.

The role of Mn in regulating the expression of the reporter gene can be examined most directly with induction experiments, wherein the effect of Mn is independent of other variables such as nutrient nitrogen and atmospheric O2 levels in the flasks. The results in Fig. 4 show that the addition of 180 μM Mn to cultures grown for 6 days in the presence of 0.25 μM Mn 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. 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 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 *mpn* translation start site is sufficient to regulate the *ura*1 reporter in a manner analogous to the regulation of the endogenous *mpn* genes with respect to Mn, 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 *mpn* gene transcription by Mn, heat shock, and nutrient nitrogen levels. We are constructing deletion mutants of the *mpn* promoter and site-directed mutants altered in the MRE sequences to delineate the role of the MREs in the regulation of *mpn* by Mn and possibly other factors.

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