

Metal-responsive elements in *Pleurotus ostreatus* laccase gene promoters

Vincenza Faraco,¹ Paola Giardina² and Giovanni Sannia¹

Correspondence
Giovanni Sannia
sannia@unina.it

¹Dipartimento di Chimica Organica e Biochimica, Università di Napoli Federico II, Complesso Universitario Monte S. Angelo, Via Cinthia, I-80126 Napoli, Italy

²Centro Regionale di Competenza Applicazioni Tecnologico-Industriali Di Biomolecole E Biosistemi, Regione Campania, Italy

Fungal laccase gene transcription is strongly induced by copper ions; notably, some laccase promoters contain multiple putative metal-responsive elements (MREs). Previously, it has been demonstrated that the *Pleurotus ostreatus* laccase genes *poxc* and *poxa1b* are transcriptionally induced by copper, and several putative MREs were found in the promoter regions of these genes, which extend for about 400 nt upstream of the start codon (ATG). Identification of MRE sequences, which are protected by protein binding in the *poxc* and *poxa1b* promoter regions, has been achieved by footprinting analyses. Electromobility shift assays led to the evaluation of the ability of the identified MREs to bind protein(s), and the role of specific nucleotides of these elements in complex formation has also been analysed. The formation of complexes between analysed MREs and fungal proteins requires the absence of metal ions. Proteins extracted from fungus grown in copper-depleted medium are able to form complexes with MREs, whilst proteins extracted from fungus grown in copper-containing medium are able to form complexes only in the presence of a metal chelator. Moreover, copper-depleted proteins are unable to form complexes when copper or zinc ions are added. UV-cross-linking analyses led to the determination of the molecular masses of the MRE-binding proteins. In the *poxa1b* promoter, a GC-rich region, homologous to the core binding site for transcription factor Sp1, decreases the binding affinity of the adjacent MRE, affecting its interactions with fungal protein factors.

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INTRODUCTION

White rot fungi are the most active micro-organisms degrading lignin, a complex aromatic biopolymer that is extremely recalcitrant to degradation (Kirk & Farrell, 1987). These fungi produce different oxidative enzymes, with broad substrate specificity, which can also be used to degrade a vast range of toxic aromatic pollutants (Hammel, 1995; Rodriguez *et al.*, 1999).

Fungal laccases (benzenediol: oxygen oxidoreductases; EC 1.10.3.2) are ligninolytic enzymes that belong to the class of blue copper oxidases, which catalyse the one-electron oxidation of many aromatic substrates with the simultaneous reduction of molecular oxygen to water (Thurston, 1994). The existence of multiple genes encoding different laccase isoenzymes has been demonstrated in several fungi (Mansur *et al.*, 1997; Smith *et al.*, 1998; Yaver & Golightly,

1996; Giardina *et al.*, 1999). Moreover, laccase gene expression depends on cultural conditions, and differentially regulated systems to control laccase production have been reported (Collins & Dobson, 1997; Mansur *et al.* 1998; Muñoz *et al.*; 1997; Yaver *et al.*, 1996). Among the various inducers tested, copper ions greatly increase laccase gene transcription in several fungi (Collins & Dobson, 1997; Karahanian *et al.*, 1998; Soden & Dobson, 2001; Galhaup *et al.*, 2002; Palmieri *et al.*, 2000).

Pleurotus ostreatus is a basidiomycete that secretes several laccase isoenzymes, four of which, named POXC (Giardina *et al.*, 1996), POXA1w (Palmieri *et al.*, 1997), POXA3 and POXA1b (Giardina *et al.*, 1999), have been purified and characterized. Four different *P. ostreatus* laccase genes and their corresponding cDNAs have been cloned and sequenced: *poxc* (Giardina *et al.*, 1996) (previously named *pox2*), *poxa1b* (Giardina *et al.*, 1999), *poxa3* (Gene Bank accession no. AJ344434) and *pox1* (Giardina *et al.*, 1995) (encoding an as yet unidentified laccase isoenzyme). The addition of copper sulphate to *P. ostreatus* growth medium causes a marked increase of total laccase activity and a transcription induction of *poxc* and, mostly, *poxa1b* genes (Palmieri *et al.*, 2000).

Abbreviations: BCS, bathocuproinedisulfonic acid; EMSA, electromobility shift assay; MRE, metal-responsive element.

The GenBank accession numbers for the *poxc* (previously named *pox2*) and *poxa1b* gene sequences reported in this article are Z49075 and AJ005017, respectively.

Nucleotide sequences of the *poxc* and *poxalb* promoter regions, extending about 400 nt upstream of the start codon (ATG), have been analysed, and multiple putative regulatory sites such as metal-responsive elements (MREs), xenobiotic-responsive elements and heat-shock elements have been identified in them. The sequences of all MREs are similar to the core MRE consensus sequence (5'-TGCRNC-3') identified in metallothionein (*mt*) gene promoters (Thiele, 1992). Other laccase promoters have been reported to contain multiple putative MRE sites (Karahanian *et al.*, 1998; Mansur *et al.*, 1998; Galhaup *et al.*, 2002; Klonowska *et al.*, 2001).

Metal-regulated gene transcription systems play important roles in metal homeostasis and detoxification (Kägi & Schäffer, 1988), and are widespread in eukaryotic organisms (Hill *et al.*, 1991; Hagen *et al.*, 1988; Greco *et al.*, 1990). The best-characterized example of a metal-regulated transcription system is that of the *mt* genes. In *mt* promoters from higher eukaryotes, multiple copies of MREs constitute the *cis*-acting sequences responsible for heavy-metal induction of *mt* gene expression (Culotta & Hamer, 1989). The role of metallothioneins in protection from metal toxicity correlates with the ability of several metal ions, including zinc, copper, cadmium and others, to activate *mt* gene transcription (Hamer, 1986). Mechanisms of metal regulation have so far been elucidated for *mt* gene transcription systems (Zhou & Thiele 1991; Andersen *et al.* 1987, 1990; Mueller *et al.*, 1988) and for some other metal-responsive transcription systems (Carri *et al.*, 1991; Merchant *et al.*, 1991; Williams & Morimoto, 1990; Jin & Ringertz, 1990). Regulation of *mt* genes occurs via a metal-regulatory protein which functions both as a metal receptor and a transcription factor.

To shed light on the mechanism of copper regulation of *poxc* and *poxalb* transcription, we identified the MREs involved in protein binding in the *poxc* and *poxalb* promoter regions by footprinting analyses. Furthermore, the ability of each element to bind protein(s) has been evaluated and the role of specific nucleotides in the identified elements has been analysed.

METHODS

Organism and culture conditions. White rot fungus *P. ostreatus* ATCC MYA-2306 was maintained by periodic transfer at 4 °C on potato/dextrose agar plates (Difco) in the presence of 0.5% yeast extract (Difco). The mycelium was grown both in liquid basal medium (PDY: 24 g potato/dextrose broth l⁻¹, 5 g yeast extract l⁻¹) and in the same medium supplemented with 150 µM CuSO₄ at the time of inoculation. Mycelium was also grown in copper-depleted PDY medium, obtained by adding 33 µM bathocuproinedisulfonic acid (BCS), as Cu(I) chelator, in combination with 1 mM ascorbic acid, used to reduce Cu(II) to Cu(I) (Averbeck *et al.*, 2001). The mycelium was grown for 48 h after inoculation.

Preparation of mycelium crude extract. A total protein extract from *P. ostreatus* mycelium was prepared as follows. Lyophilized cells were ground in a mortar with a pestle. The ground material was resuspended in cold extraction buffer [200 mM Tris/HCl pH 8.0, 400 mM (NH₄)₂SO₄, 10 mM MgCl₂, 1 mM EDTA, 10% (v/v) glycerol, 1 mM PMSF, 7 mM β-mercaptoethanol] and then

centrifuged at 4 °C for 1 h at 15 000 g. (NH₄)₂SO₄ up to 80% saturation was added to the supernatant and material was centrifuged for 1 h at 15 000 g. The pellet was resuspended in a minimal volume of protein buffer (20 mM HEPES pH 8.0, 7 mM β-mercaptoethanol, 1 mM PMSF, 20% glycerol) and dialysed extensively against the same buffer. EDTA-treated proteins were obtained by incubating proteins from the mycelium grown in the basal medium with 10 mM EDTA, then the chelating agent was removed by dilution of the treated proteins in protein buffer followed by ultrafiltration (Centricon Ultrafree Max 5 kDa; Millipore). Protein concentration was determined using the Bio-Rad Protein Assay with bovine serum albumin as the standard.

Footprinting analyses. Probes for footprinting analyses were prepared by PCR amplification. Plasmids containing the *poxc* and *poxalb* promoters, extending about 1400 nt upstream of the start codon (ATG), were used as templates for PCRs. Two different fragments, pc1 and pc2, covering the +11 to -220 and -32 to -276 regions of the *poxc* gene, respectively, were generated by PCR using the oligonucleotide primers pc-1-f (-220 to -203) and pc-1-r (+12 to -7), respectively, for the amplification of fragment pc1 and the oligonucleotide primers pc-2-f (-276 to -259) and pc-2-r (-32 to -49) for the amplification of fragment pc2 (Fig. 1). Only one fragment, palb, consisting of the -23 to -232 region of *poxalb* was generated by PCR using the oligonucleotide primers designated palb-f (-233 to -216) and palb-r (-23 to -39) (Fig. 1). One of the PCR primers was end-labelled using T4 Polynucleotide kinase (Roche) and [γ-³²P]ATP (Amersham); it was separated from free [γ-³²P]ATP using the QIAquick Nucleotide Removal kit (Qiagen). Labelled fragments were purified on a 7% native polyacrylamide gel, eluting overnight at 37 °C in diffusion buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, pH 8, 0.1% SDS). The mixture was phenol-extracted and ethanol-precipitated. The binding reaction was performed as described in the 'Electromobility shift assays' section of Methods, except for using at least fivefold amounts of extract and purified probe (250 000–500 000 c.p.m.). After incubation for 20 min, MgCl₂ at a final concentration of 15 mM and 0.3 U DNAase I (Roche) were added to the mixture. Digestion was carried out for 1 min at room temperature and the reaction was stopped by adding EDTA to a final concentration of 35 mM. Samples were immediately loaded onto a 5% native polyacrylamide gel (29:1) in 0.5 × TBE (45 mM Tris/HCl pH 8.0, 45 mM boric acid, 1 mM EDTA). After electrophoretic separation, regions of the gel corresponding to the probe and to DNA-protein complexes were excised and eluted overnight at 37 °C in diffusion buffer. The mixtures were phenol-extracted, ethanol-precipitated, dissolved in sequencing load mix [100 mM NaOH/formamide (1:2), 0.1% bromophenol blue, 0.1% xylene cyanol] and loaded onto a 7% polyacrylamide/urea DNA sequencing gel. A Maxam-Gilbert A+G reaction was carried out on the same probes in order to localize protected sequences.

Electromobility shift assays (EMSAs). Synthetic oligomers, corresponding to the identified MRE sequences and indicated in Table 1, were annealed and ³²P-end-labelled with T4 Polynucleotide kinase (Roche). EMSAs were performed in a reaction volume of 20 µl, in binding buffer (20 mM HEPES pH 7.6, 100 mM NaCl, 0.7 mM β-mercaptoethanol, 0.1 mM PMSF, 10% glycerol) using 1.0–10 µg of poly(dI-dC)•poly(dI-dC) (Roche) as a non-specific competitor for the binding reaction. Unless specified otherwise, the reaction mixture contained 2 mM EDTA. Total protein extract (10 µg) was incubated with the ³²P-end-labelled DNA oligomer (3 ng, 50 000–100 000 c.p.m.) in binding buffer in the presence of poly(dI-dC)•poly(dI-dC) for 20 min at room temperature. Mixtures were then analysed by electrophoresis on a 7% native polyacrylamide gel (29:1 cross-linking ratio) in 0.5 × TBE. Electrophoreses were performed at room temperature at 200 V (20 V cm⁻¹). The gels were dried and analysed by autoradiography.

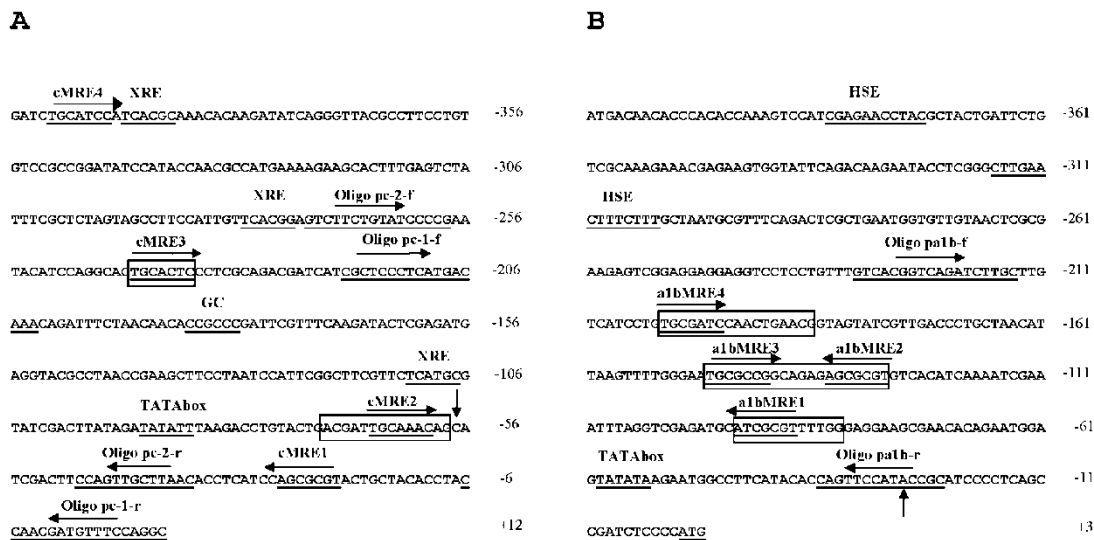


Fig. 1. Nucleotide sequences of the *P. ostreatus poxc* (A) and *poxa1b* (B) promoter regions, extending about 400 nt upstream of the start codon (ATG). Transcription-initiation sites are indicated by vertical arrows. The putative TATA box, GC box, MREs, heat-shock elements (HSEs) and xenobiotic-responsive elements (XREs) are underlined. *poxc* MREs are named cMREs, whilst *poxa1b* MREs are named a1bMREs. In each promoter, the MRE sites are numbered according to their proximity to the start codon. The orientation of MREs is indicated by arrows. Oligonucleotides used for probe amplifications are also indicated. MRE sequences identified by footprinting analyses are boxed.

In competition experiments, incubations were performed after the addition of eight- to 240-fold molar excesses of unlabelled competitor DNA to the reaction mixture, containing proteins and poly(dI-dC)•poly(dI-dC). For chelation inactivation experiments, specified amounts of EDTA (0.1–20 mM) or Cyclam (1,4,8,11-tetraazacyclotetradecane; 0.1–5 mM) were added to protein extracts and incubation was then performed at room temperature for 20 min. Radiolabelled DNA was then added, and the mixture was incubated for another 20 min at room temperature. Metal treatment was performed by pre-incubating EDTA-treated proteins or BCS-proteins with 0.1–10 mM CuSO₄, ZnSO₄ or MgCl₂ for 20 min before the addition of the probe.

UV cross-linking. The binding reaction between radiolabelled MRE oligonucleotides and protein extracts was performed as described in the 'Electromobility shift assays' section of Methods, except for using threefold amounts of protein extract and probe. After incubation at room temperature for 20 min, the reaction mixture was exposed to UV light (300 nm) for 20 min. Samples were then separated by SDS-PAGE (12% acrylamide, 0.1% SDS), as described by Laemmli (1970). The apparent molecular mass of the proteins bound to radiolabelled oligonucleotides was determined by calibration of the gel with a Pre-stained Protein Molecular Weight Marker (Fermentas MBI), containing β -galactosidase (118.0 kDa), bovine serum albumin (79.0 kDa), ovalbumin (47.0 kDa), carbonic anhydrase (33.0 kDa), β -lactoglobulin (25.0 kDa) and lysozyme (19.5 kDa). The gels were dried and analysed by autoradiography.

RESULTS

Fine-mapping of transcription factor binding sites in the *poxc* and *poxa1b* promoters

Putative regulatory sites, such as MREs, xenobiotic responsive elements and heat-shock elements, identified in the

poxc and *poxa1b* promoter regions, extending about 400 nt upstream of the start codon (ATG), are shown in Fig. 1.

DNase I protection of putative MREs was analysed by footprinting assays, performed on two *poxc* (pc1 and pc2) promoter fragments and one *poxa1b* (pa1b) promoter fragment, incubating each of them with cellular proteins extracted from fungus grown in the presence of CuSO₄. Fig. 2 shows the results of the footprinting assays performed using the pc2 fragment. Bound proteins protect the –58 to –71 region, including the cMRE2 site (–60 to –66) and a small region (–242 to –236) corresponding to cMRE3 (exactly matching the core MRE consensus sequence). Footprinting analyses on the pa1b probe led to the identification of the protected region –186 to –202, including the a1bMRE4 site (–83 to –94) containing the a1bMRE1 site, and –128 to –147, including the a1bMRE2 and a1bMRE3 sites (data not shown). It is worth noting that two MREs of the *poxa1b* promoter, a1bMRE2 and a1bMRE3, are located in a single large protected region. Footprinting analyses on the pc1 probe (data not shown) revealed that cMRE1, located downstream of the *poxc* transcription-initiation site, is not protected. Sequences of the protected regions are boxed in Fig. 1.

EMSAs of identified MRE sequences

Oligomers corresponding to the identified MRE sequences (Table 1a) were synthesized and used in EMSAs in order to analyse interactions with proteins extracted from *P. ostreatus* grown in medium in the absence or in the presence of added copper (basal- or +Cu-proteins,

Table 1. Nucleotide sequences of the oligomers used in the EMSAs

Underlined sequences indicate MRE core sequences. Bold letters indicate mutated nucleotides. (a) Oligomers corresponding to the MRE sequences; (b) mutated oligomers differing from a1bMRE2 by a single substitution in one of the variable positions of the core sequence; (c) oligomers used in the analyses of the adjacent MREs.

Oligomer	Sequence
(a)	
cMRE1	GATC C ATCCAGCGCGTAC
cMRE2	GATC A CGATTGCAAACAG
cMRE3	GATCGCACTGCACTCCCT
a1bMRE1	GATCATGCATCGCGT T TTGG
a1bMRE2	GCAGAGAGCGCGTCTAG
a1bMRE3	GATC T GCGCCGGCAGAG
a1bMRE4	GATC T GCGATCCAACGAACG
(b)	
a1bMRE2	GCAGAGAGCGCGTCTAG
Mut 4	GCAGAGAGCACGTCTAG
Mut 5	GCAGAGAGAGCGTCTAG
Mut 6C	GCAGAGAGCGCGTCTAG
Mut 6T	GCAGAGATCGCGTCTAG
Mut 6A	GCAGAGAAACGCGTCTAG
Mut 7C	GCAGAGCGCGCGTCTAG
Mut 7G	GCAGAGGGCGCGTCTAG
(c)	
a1bMRE2-3	GATC T GCGCCGGCAGAGAGCGCGT
a1bMRE2-cMRE1	GATCAGCGCGTGCAGAGAGCGCGT
cGC	GATCAACACCGCCCGATT

repectively). The mobility of all MRE oligomers tested was retarded, except for cMRE1, a putative MRE, which was not protected in footprinting experiments. It is worth noting that complexes with the same mobility are formed in the presence of both +Cu- and basal-proteins. Fig. 3 shows the results obtained using the cMRE3 oligomer incubated with +Cu-proteins. The specificity of the binding was demonstrated by competition EMSAs, using increasing molar excesses (8-, 40- and 80-fold) of unlabelled specific and a-specific competitors. To compare the binding capacity of the different MREs, cross-competition analyses were performed by incubating each labelled MRE with +Cu-proteins in the presence of increasing molar excesses (40-, 80- and 120-fold) of the various unlabelled oligomers (data not shown). Results showed that a1bMRE2 is able to compete, at the lowest molar excess, with all the other oligomers, indicating that this element binds the protein factor(s) with the highest affinity. Therefore, cross-competition analyses were performed using radiolabelled a1bMRE2 and increasing amounts of each of the MREs as competitors (Fig. 4); an affinity scale of *poxc* and *poxa1b* MRE towards the factor(s) was then determined: a1bMRE2 > a1bMRE4/cMRE3 > a1bMRE1/cMRE2 > a1bMRE3.

Analysis of the core sequences in the *poxc* and *poxa1b* MRE

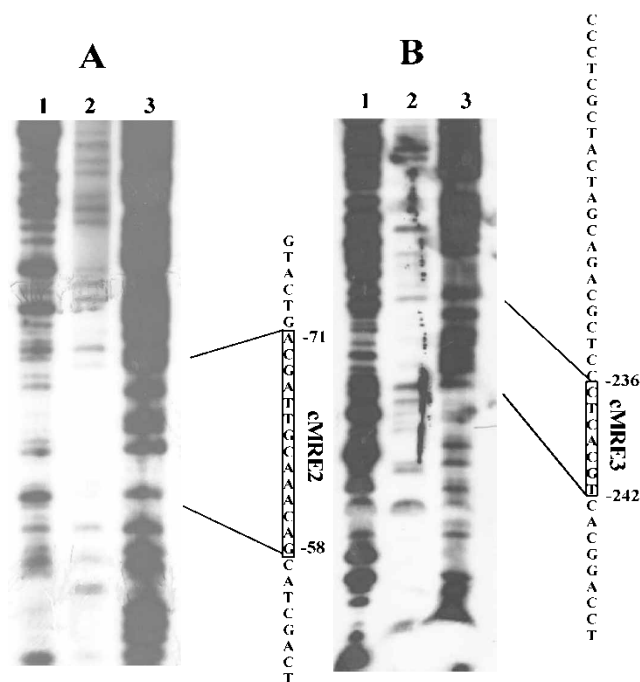


Fig. 2. DNA footprinting of the pc2 fragment obtained by amplification with radiolabelled oligonucleotides pc2r (A) and pc2f (B). Lanes: 1, DNase I digestion of probe; 2, DNase I digestion of probe after incubation with +Cu-proteins; 3, Maxam-Gilbert reaction products.

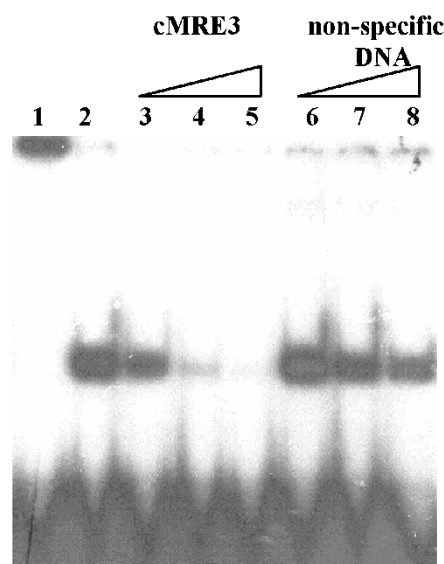


Fig. 3. EMSA of cMRE3 incubated with +Cu-proteins. Lanes: 1, probe; 2, probe incubated with +Cu-proteins; 3, 4 and 5, competition with 8-, 40- and 80-fold molar excesses of unlabelled cMRE3, respectively; 6, 7 and 8, competition with 8-, 40- and 80-fold molar excesses of non-specific oligonucleotide, respectively.

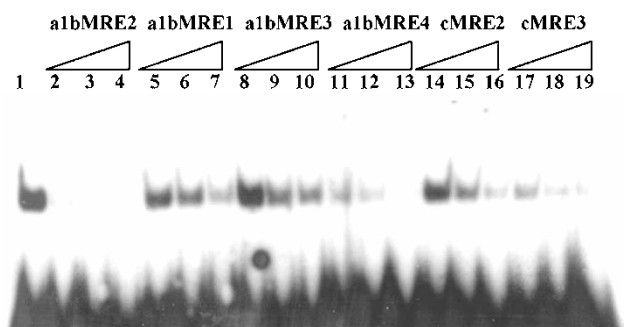


Fig. 4. Cross-competition of ^{32}P -labelled a1bMRE2 electrophoretic mobility shift (lane 1) by 40- (lanes 2, 5, 8, 11, 14 and 17), 80- (lanes 3, 6, 9, 12, 15 and 18) and 120-fold (lanes 4, 7, 10, 13, 16 and 19) molar excesses of each of the other unlabelled MRE oligonucleotides.

oligomers indicates variability in the last four positions of these sequences. The role of each base in complex formation was then investigated using mutated oligomers differing from a1bMRE2 by a single substitution in one of the variable positions of the core sequence (Table 1b). Fig. 5 shows the cross-competition EMSA performed using these oligomers as competitors of a1bMRE2 in complex formation. Changing G to A in the fourth position, or G to C or T in the sixth position, decreased the ability of the oligomers to compete in the complex formation as compared to that of a1bMRE2 itself. However, changing C to A in the fifth position or G to A in the sixth position did not significantly affect the binding affinity. As a consequence, an optimal MRE sequence was deduced: TGCG^C/_AG^G/_{AA}.

All the above-described experiments have been performed

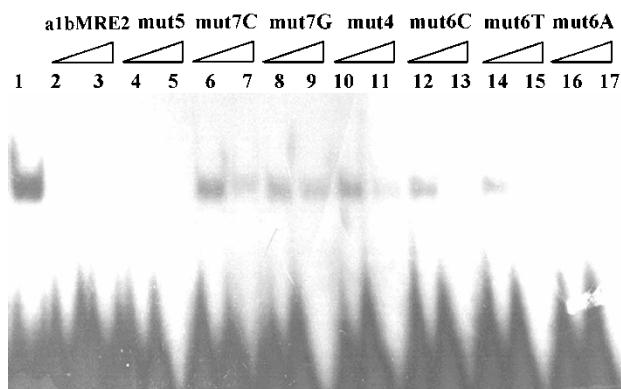


Fig. 5. Cross-competition of ^{32}P -labelled a1bMRE2 electrophoretic mobility shift (lane 1) by the mutated oligomers indicated in Table 1 [120-fold (even-numbered lanes) and 240-fold (odd-numbered lanes) molar excesses].

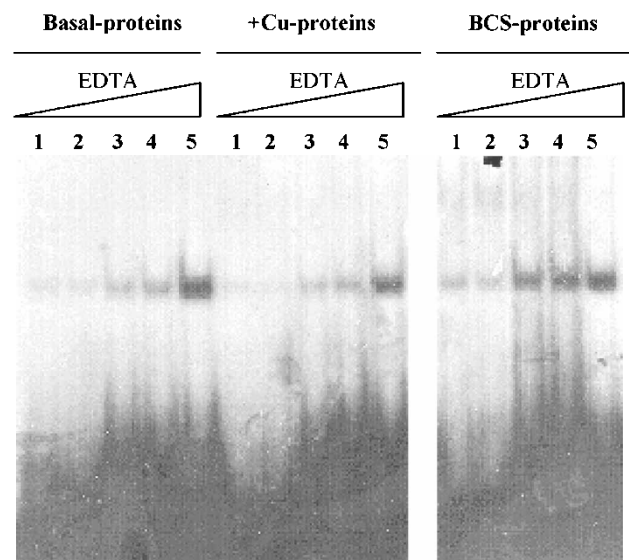


Fig. 6. Effect of EDTA on the EMSA of a1bMRE2 incubated with basal-, +Cu- or BCS-proteins. Lanes 1, 2, 3, 4 and 5, band shift assay with proteins pre-incubated with 0, 0.1, 0.5, 2.0 and 10.0 mM EDTA, respectively.

in the presence of EDTA in the reaction mixture. To investigate the role of metal ions in complex formation, EMSAs were performed incubating radiolabelled a1bMRE2 with +Cu- or basal-proteins in the absence or in the presence of increasing amounts of EDTA (Fig. 6) or Cyclam (0.1–20 mM EDTA, 0.1–5 mM Cyclam). Results indicated that complex formation is dependent on the presence of chelating agent, and the intensity of the band corresponding to the complex increased with chelator concentration. Lower concentrations of Cyclam (1 mM) than EDTA (10 mM) are needed to obtain the maximum band intensity. EMSAs were performed with proteins extracted from mycelium grown in copper-depleted medium containing the Cu(I) chelator BCS (BCS-proteins). As shown in Fig. 6, the band corresponding to the complex formed with BCS-proteins was observable even in the absence of EDTA or Cyclam, and a lower EDTA concentration (0.5 mM compared to 10 mM) was needed to reach the maximum band intensity.

Furthermore, when EMSAs were performed using EDTA-treated proteins (basal-proteins incubated in the presence of 10 mM EDTA and dialysed extensively), a clear signal due to the complex was detected even in the absence of added EDTA (Fig. 7, lane 1). To investigate the effect of metal addition, EDTA-treated proteins were incubated with increasing amounts of CuSO_4 , ZnSO_4 or MgCl_2 . As shown in Fig. 7, by adding 1 mM CuSO_4 to the reaction mixture, the band corresponding to the complex disappeared, whilst 1 mM ZnSO_4 reduced its intensity and MgCl_2 had a slighter effect.

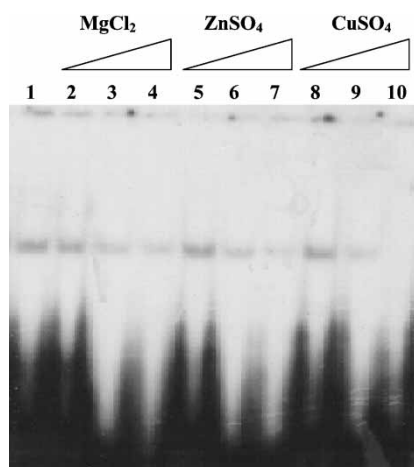


Fig. 7. Effect of metal addition on the EMSA of a1bMRE2 incubated with EDTA-treated proteins (lane 1); lanes 2, 3 and 4, addition of 0.1, 0.5 and 1 mM MgCl_2 , respectively; lanes 5, 6 and 7, addition of 0.1, 0.5 and 1 mM ZnSO_4 , respectively; lanes 8, 9 and 10, addition of 0.1, 0.5 and 1 mM CuSO_4 , respectively.

Analysis of adjacent MREs

As described above, a single extended region (−128 to −147) containing the a1bMRE2 and a1bMRE3 sites is protected in the *poxa1b* promoter. An oligonucleotide corresponding to the sequence −128 to −147 (a1bMRE2-3; Table 1c) was used as competitor in an EMSA of radiolabelled a1bMRE2. The results of this experiment, shown in Fig. 8, demonstrated that a1bMRE2-3 competed with a1bMRE2 at a higher molar excess than the specific competitor. Therefore, the binding capacity of a1bMRE2 is reduced by the adjacent a1bMRE3. A new oligomer

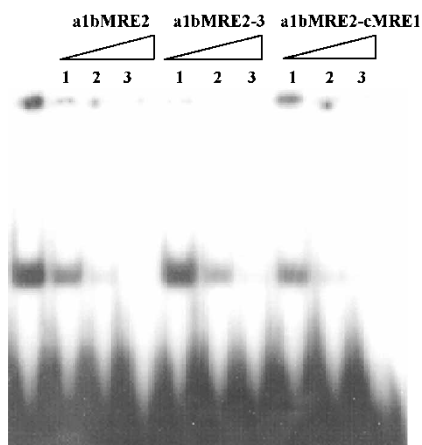


Fig. 8. Cross-competition of ^{32}P -labelled a1bMRE2 electrophoretic mobility shift by 8- (lane 1), 40- (lane 2) and 80-fold (lane 3) molar excesses of unlabelled a1bMRE2, a1bMRE2-3 or a1bMRE2-cMRE1 oligonucleotide, respectively.

(a1bMRE2-cMRE1; Table 1c) containing a1bMRE2 and cMRE1 (which does not bind any factor) was used as a control; it was a competitor as effective as a1bMRE2 alone (Fig. 8), thus demonstrating that the behaviour of a1bMRE2-3 is independent of its length.

UV-cross-linking analyses

Results of UV-cross-linking analyses performed using radiolabelled MRE oligonucleotides are shown in Fig. 9. All oligonucleotides bind a 25 kDa protein factor; a1bMRE2 binds an additional 30 kDa protein, whereas a1bMRE2-3 binds a 15 kDa factor instead of the 30 kDa protein. The binding specificity of a1bMRE2 was demonstrated by specific and a-specific competition experiments. Due to the similarity of a1bMRE3 to GC (Sp1 binding) sequences, an oligonucleotide corresponding to the putative GC sequence of the *poxc* promoter (cGC; Table 1c) was used and proved to bind a 15 kDa factor. Hence, a1bMRE2 binds a 25 kDa and a 30 kDa protein factor, whilst a1bMRE2-3 is able to bind a 25 kDa and a 15 kDa factor, the latter having the same apparent molecular mass as the GC binding protein.

DISCUSSION

Multiple putative MREs were identified in the *P. ostreatus* lactase *poxc* and *poxa1b* gene promoter regions, which extended about 400 nt upstream of the start codon (ATG). These elements show nucleotide sequences similar to the core MRE consensus sequence TGCPuCXC which is known to be involved in the metal response of metallothionein (*mt*) genes in higher eukaryotes. Multiple copies of these *cis*-acting DNA elements are required for metal-induced transcription activation of mammalian *mt* genes (Culotta & Hamer, 1989). MREs in both orientations are present in the *poxc* and *poxa1b* promoters; this feature is a common characteristic of MRE sites in *mt* genes (Thiele, 1992).

All putative MREs in the *poxc* and *poxa1b* promoters are recognized by fungal proteins, except for cMRE1, which is located downstream of the transcription-initiation site. Footprinting analyses of the *poxa1b* promoter showed the occurrence of a large protected region including a1bMRE2 and a1bMRE3 sites with opposite orientations. It is noteworthy that cMRE2, located downstream of the putative TATA box, is protected in the *poxc* promoter; to the best of our knowledge, no functional MRE site with this location has been reported in metal-responsive promoters characterized so far (Koizumi *et al.*, 1999).

Proteins extracted from fungus grown in medium with (+ Cu-proteins) or without (basal-proteins) copper addition, or in copper-depleted medium (BCS-proteins), were used in EMSAs of MRE oligomers, corresponding to the identified MRE sequences. DNA–protein complexes with the same electrophoretic mobility were formed by BCS-proteins in the absence of EDTA, whilst basal- or +Cu-proteins needed addition of EDTA to the reaction mixture to produce complexes. In any case, an increase in the EDTA

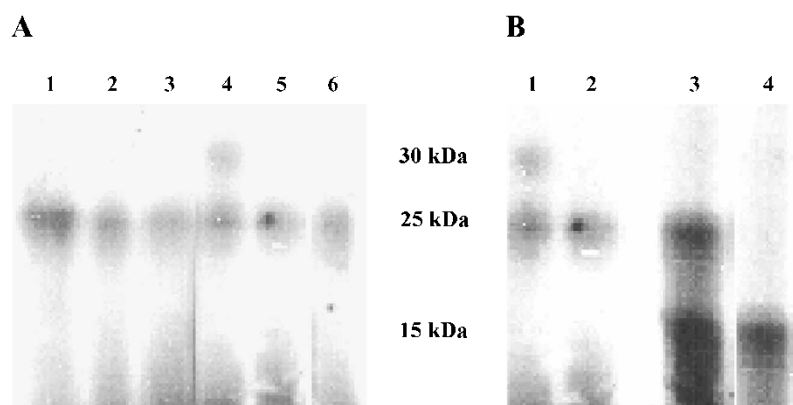


Fig. 9. UV-cross-linking analysis of +Cu-proteins incubated with (A) cMRE2 (lane 1), cMRE3 (lane 2), a1bMRE1 (lane 3), a1bMRE2 (lane 4), a1bMRE3 (lane 5) and a1bMRE4 (lane 6), or (B) incubated with a1bMRE2 (lane 1), a1bMRE3 (lane 2), a1bMRE2-3 (lane 3) and cGC (lane 4).

concentration enhanced the intensity of the band due to the complex formation. However, the addition of copper or zinc ions to copper-depleted proteins made them unable to bind to MREs. These results strongly suggest that a specific factor is able to bind these DNA sequences only when metals are absent from the reaction mixture. Even the low levels of copper found in the basal medium (1–2 μ M) make this protein factor(s) unable to form complex, probably through a copper-induced conformational change of this factor(s), inhibiting its ability to bind DNA.

Cross-competition analyses among wild-type or mutated oligomers allowed us to determine an affinity scale of the tested MREs and to define the optimal binding sequence whose differences with the reported higher eukaryotes MRE consensus sequence are essentially in the fifth and seventh positions (Thiele, 1992). a1bMRE2 proved to bind protein factor(s) with higher affinity than the other MRE oligomers. Results of UV-cross-linking analyses indicated that a1bMRE2 behaves differently from all the other MREs, binding not only the 25 kDa factor, recognized by all MRE oligomers tested, but also a 30 kDa factor. This behaviour does not occur when the adjacent a1bMRE3 site, a GC-rich region, is present. This site decreases the a1bMRE2 binding affinity and causes the binding of a 15 kDa protein instead of the 30 kDa factor. A GC-rich region, homologous to the core binding site for mammalian transcription factor Sp1, has been found adjacent to the MRE core sequence in some *mt* promoters (Culotta & Hamer, 1989), and a regulatory role of Sp1, binding to this GC-rich region, in MRE-mediated transcriptional activation has been demonstrated (Ogra *et al.*, 2001). As a matter of fact, a 15 kDa protein binds the putative GC sequence of the *poxc* promoter. However, further investigations are needed to verify that, in *P. ostreatus*, (i) the response to metals involves the binding of a negative-acting regulatory factor to the MRE heptanucleotide core and (ii) the flanking sequences can either influence this binding or affect interactions with other factors.

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