Binding of nuclear proteins to the promoter region of the laccase gene *Cs-lcs1* from the basidiomycete *Ceriporiopsis subvermispora*

Rubén Polanco[a], Sergio Lobos[b], and Rafael Vicuña[a,*]

[a]*Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile and Millenium Institute for Fundamental and Applied Biology, Santiago, Chile*

[b]*Laboratorio de Bioquímica General, Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Santiago, Chile*

Abstract

The white rot basidiomycete *Ceriporiopsis subvermispora* secretes the ligninolytic enzymes manganese-dependent peroxidase (MnP) and laccase to the extracellular medium. The promoter region of the laccase gene (*Cs-lcs1*) possesses several putative metal responsive elements (MRE), as well as a putative target site responding to copper termed ACE, similar to the one found in yeast. In this work, we show by electrophoretic mobility-shift assays that the migration of DNA probes containing either MRE sites or the ACE element are retarded in their mobility after incubation with nuclear extracts from *C. subvermispora*. Competition experiments suggested the presence of defined binding proteins recognizing these elements.

1. Introduction

C. subvermispora is a white-rot fungus that shows high selectivity towards lignin when growing on wood [1]. Its ligninolytic system is composed of manganese-dependent peroxidase (MnP) and laccase [2]. Both activities are secreted as families of isoenzymes, with isoelectrofocusing patterns that vary according to the composition of the growth medium [3]. The MnP isoenzymes differ with respect to their substrate specificity and the concentration of Mn(II) required for optimal activity [4]. MnP activity is not detected in liquid cultures lacking manganese [1], whereas laccase titers are negligible in copper-limited cultures [5,6].

To date, we have identified, cloned and sequenced four genes coding for MnP: *Cs-mnp1*, *Cs-mnp2A*, *Cs-mnp2B* and *Cs-mnp3* [7,8]. *Cs-mnp2A* and *Cs-mnp2B* correspond to alleles of the same gene. Analysis of homokaryonic strains by PCR amplification followed by DNA sequencing has revealed the presence of alleles for the genes *Cs-mnp1* and *Cs-mnp3* as well [9]. In contrast, present evidence points to the presence of a single gene coding for laccase, namely *Cs-lcs1* [6].

The upstream regions of *Cs-mnp* genes contain a TATA box, AP-1 and AP-2 sites, as well as putative sites for transcription regulation by metals (MRE), cAMP (CRE), xenobiotics (XRE) and heat shock (HSE) [8]. MREs have been identified in animals and plants as the target site of transcription factors responding to toxic concentrations of cadmium, copper and zinc [10,11]. Found widespread in the upstream region of metallothionein (MT) genes, putative MREs have the consensus sequence TGCRCNC [10]. We have previously reported that transcription of genes *Cs-mnp2A* and *Cs-mnp2B*, but not that of *Cs-mnp3*, is activated by manganese.

In turn, the promoter region of *Cs-lcs1* contains a TATA box, two CAAT sites, five putative MREs and an ACE-like element [8]. The latter was originally identified in the promoter of the two MT [12] and the zinc superoxide dismutase [13] genes in *Saccharomyces cerevisiae* as a recognition site for the transcription factor ACE1 which responds to Cu(I) and Ag(I), but not to Zn(II). In agreement with this evidence, transcription of *Cs-lcs1* is activated by copper and silver, but not by Zn(II) [6]. The MT genes of *Candida glabrata* possess a similar element that is the target site for the transcription factor termed AMT1 [14].

We are interested in identifying proteins from *C. subvermispora* that bind specifically to the MRE and the ACE sites, since it is conceivable that such proteins may regulate the expression of genes coding for MnP and laccase in response to metals. As a first approach to identify proteins with this role, we are using electrophoretic mobility-shift assays (EMSA). The results described below suggest that

* Corresponding author. Tel.: +56-2-686-2663; fax: +56-2-222-5515.
E-mail address: rvicuna@genes.bio.puc.cl (R. Vicuña).

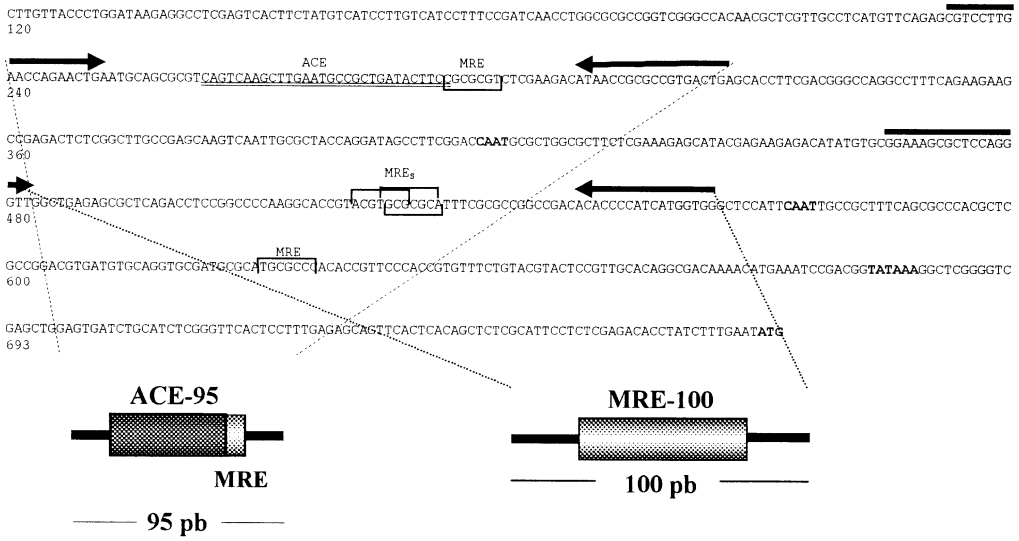

Fig. 1. Design of DNA probes. Partial sequence of the promoter region of the *Cs-lcs1* gene upstream of the initiation codon. The arrows encompass the nucleotides comprising the primers employed for the generation of the two DNA probes by PCR amplification.

nuclear proteins from *C. subvermispora* bind specifically to DNA probes containing either MRE or ACE elements.

2. Materials and methods

2.1. Strain

C. subvermispora strain FP-105752 was obtained from the Center for Forest Mycology Research (Forest Products Laboratory, Madison, WI). Culture conditions were as previously described [2].

2.2. Preparation of nuclear extracts

The procedure developed for the preparation of extracts enriched in nuclear proteins was based in those published for the isolation of nuclei from *Aspergillus nidulans* [15] and of nuclear extracts from *Arabidopsis thaliana* [16]. The details of the final procedure will be published elsewhere. The final protein concentration in the solution of nuclear extracts (0.15 ml) varied between 2 and 4 mg/ml, as measured by the Bradford method using the Bio-Rad protein assay kit.

2.3. Preparation of the labeled probes

The probes containing the ACE-like element with an adjacent MRE box (ACE-95) and another one containing three overlapping putative MRE boxes (LMRE-100), were obtained by PCR amplification of specific segments of the upstream region of the *Cs-lcs1* gene (Fig. 1). They were labeled by either of the following protocols: a) prior to the PCR reaction, the primers were phosphorylated with polynucleotide kinase and [γ-^{32}P]ATP, or b) the PCR reaction was conducted in the presence of [α-^{32}P]dCTP. Radioactive labeled probes were purified by polyacrylamide gel electrophoresis [17].

2.4. EMSA

The conditions were based on those previously reported by Remondelli et al. [18]. Reaction mixtures (30 μl) contained: 20 mM Hepes/KOH buffer pH 7.5, 6.6 mM $ZnSO_4$, 0.1 mM $CuSO_4 \times 5H_2O$, 40 mM KCl, 2 mM EDTA, 4 mM DTT, 2% PEG 4K, 12% glycerol, 6 μg of BSA, 30 ng of poly dI-dC, 2.5 μg of plasmid Bluescript KSII, 2–15 μg of nuclear extract and 100 ng (20,000−40,000 cpm) of the labeled DNA probe. After incubation for 30−40 min on ice, samples were separated by electrophoresis on 4% native polyacrylamide gels in TBE buffer (22.5 mM Tris Base, pH 8.4, 22.5 mM H_3BO_4 and 0.63 mM EDTA) and run at 100V for 4 h at room temperature using TBE as runing buffer.

3. Results and discussion

Previous work in our laboratory had shown that the highest titers of MnP and laccase in liquid cultures of defined composition are detected at day 10 and between days 12 and 14, respectively [2,5]. Accordingly, we prepared a time course experiment with mycelium harvested at days 8, 10, 12 and 14. DNA-protein complexes were observed by EMSA with both DNA probes. In both cases, formation of the complex appeared to be optimum with protein extracts prepared with cultures harvested at day 10, with an abrupt disappearance of the bands corresponding to the DNA-protein complexes in the incubations with day 12 extracts (data not shown).

A key aspect in EMSA assays is the specificity with

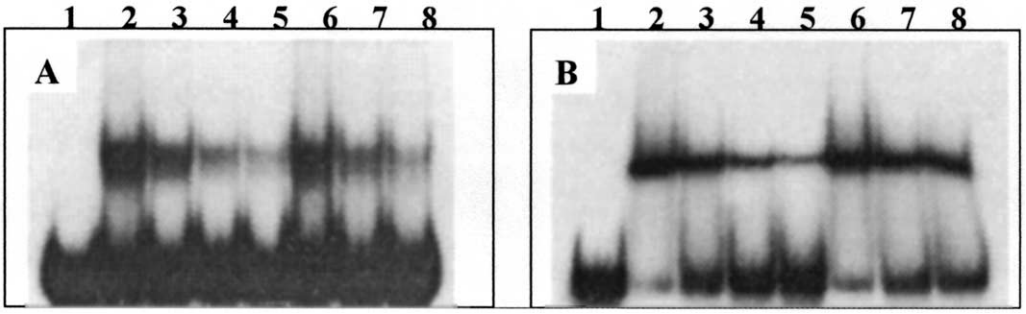

Fig. 2. Competition analysis of the DNA binding assays. Binding assays were conducted with the ACE probe (**A**) or the MRE probe (**B**), with protein extracts prepared with mycelium grown in standard medium and harvested at day 10. Lanes contain: (1) free probe; (2) standard binding reaction without competitor (see Methods); (3–5) binding assays conducted in the presence of a 10-, 30- and 50-fold excess of unlabeled specific DNA probe; (6–8) binding assays conducted in the presence of a 10-, 30- and 50-fold excess of plasmid Bluescript KSII as a non-specific competitor.

which proteins bind to DNA. Competition experiments constitute a traditional approach to inspect into this critical feature. Therefore, incubations were conducted in the presence of an excess of either unlabeled probe or a non-related DNA molecule (plasmid Bluescript KSII). Although as observed in Fig. 2 there is some reduction in the intensity of the retarded band when competing with non-related DNA, this effect is much more pronounced with the corresponding homologous probes, especially in the case of the MRE-100 probe. These results provide additional evidence that the complexes observed with the ACE and the MRE probes arise as a result of the binding of defined proteins to each of these probes.

It is conceivable that the putative MRE sites found in the promoter region of genes coding for MnP and laccase in *C. subvermispora* may play a similar role as they do in plants and animals. To our knowledge, activation of transcription of MnP genes by metals such as cadmium, copper and zinc in basidiomycetes has not been reported. On the other hand, it has been suggested that MREs present in the promoters of genes coding for MnP could be the target site of transcription factors responding to manganese [19]. However, this metal has been found to affect the levels of mRNA from MnP genes lacking MREs [20]. Therefore, both the role of the putative MREs in basidiomycetes, if any, as well as the mechanism of action of manganese in these microorganisms, remains to be established. In contrast, it is likely that the ACE-like box found in the laccase gene promoter in *C. subvermispora* is equivalent to the ACE recognition elements described in yeast [12,13] and in the basidiomycete PM1 [21], since transcription of this gene is activated by copper and silver, but not by zinc [6].

An indication that the complexes observed in this work might arise due to the binding of specific proteins to the probes is that they are observed only with extracts from day 8 and day 10 cultures. The time course experiment also indicates that the binding of nuclear proteins to the probes precedes the peaks of enzymatic activity, as expected. A higher displacement in both cases with the unlabeled homologous probes as compared to unrelated DNA (Fig. 2) constitutes an additional indication of specific protein binding to DNA. It should be noted that these differences between the two complexes are observed in spite of the presence of a MRE box adjacent to the ACE sequence in the ACE-95 probe, indicating that protein binding to the ACE site predominates in this probe.

We are now in the process of purifying the proteins that bind to each DNA probes by affinity chromatography.

Acknowledgments

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