The copper-dependent ACE1 transcription factor activates the transcription of the *mco1* gene from the basidiomycete *Phanerochaete chrysosporium*

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We have previously identified and functionally characterized the transcription factor ACE1 (Pc-ACE1) from *Phanerochaete chrysosporium*. In *Saccharomyces cerevisiae*, ACE1 activates the copper-dependent transcription of target genes through a DNA sequence element named ACE. However, the possible target gene(s) of Pc-ACE1 were unknown. An *in silico* search led to the identification of putative ACE elements in the promoter region of *mco1*, one of the four clustered genes encoding multicopper oxidases (MCOs) in *P. chrysosporium*. Since copper exerts an effect at the transcriptional level in MCOs from several organisms, in this work we analysed the effect of copper on transcript levels of the clustered MCO genes from *P. chrysosporium*, with the exception of the transcriptionally inactive *mco3*. Copper supplementation of cultures produced an increment in transcripts from genes *mco1* and *mco2*, but not from *mco4*. Electrophoretic mobility-shift assays revealed that Pc-ACE1 binds specifically to a probe containing one of the putative ACE elements found in the promoter of *mco1*. In addition, using a cell-free transcription system, we showed that in the presence of cuprous ion, Pc-ACE1 induces activation of the promoter of *mco1*, but not that of *mco2*.

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INTRODUCTION

The lignin-degrading basidiomycete *Phanerochaete chrysosporium* has an impressive array of extracellular enzymes involved in lignocellulose degradation. Among them, the extensively characterized lignin and manganese peroxidases (LiP and MnP, respectively) have been described as true ligninases because of their high redox potential (Martinez *et al.*, 2005; Kersten & Cullen, 2007). A third kind of enzyme also involved in lignin degradation, although not produced by *P. chrysosporium*, is the phenol oxidase termed laccase (Larrondo *et al.*, 2003; Baldrian, 2006). Laccases belong to the family of multicopper oxidases (MCOs), a large group of copper-containing proteins that include mammalian ceruloplasmin, plant ascorbate oxidases and fungal Fet3 ferroxidases (Solomon *et al.*, 1996). Spectroscopic and X-ray crystallographic studies have revealed that MCOs contain at least one blue copper or

type 1 site (T1) and a type 2/type 3 (T2/T3) trinuclear copper cluster as the minimal functional unit. These copper centres, located in the active site of these enzymes, play a key role in catalysis (Solomon *et al.*, 1996; Baldrian, 2006).

In addition to the structural and catalytic roles played by copper, some reports have shown that this metal also affects the expression of some MCOs. For instance, copper increases mammalian ceruloplasmin mRNA levels in hepatoma cells through copper-induced promoter activation (Martin *et al.*, 2005). Copper has also been shown to activate the transcription of *CmA04*, a gene encoding ascorbate oxidase in the plant *Cucumis melo* (Sanmartin *et al.*, 2007), as well as to increase mRNA levels of *fet3*, a gene encoding a ferroxidase involved in iron uptake in *Saccharomyces cerevisiae* (Gross *et al.*, 2000). Laccases are not an exception in this respect. Thus, copper regulates laccase mRNA levels in the fungi *Trametes versicolor* (Collins & Dobson, 1997), *Ceriporiopsis subvermispora* (Karahanian *et al.*, 1998), *Pleurotus ostreatus* (Palmieri *et al.*, 2000), *Pleurotus sajor-caju* (Soden & Dobson, 2001, 2003) and *Trametes pubescens* (Galhaup *et al.*, 2002).

Analysis of the promoter region of some of the MCO-encoding genes has shown the presence of a copper-dependent responsive element named ACE (activation of

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Abbreviations: EMSA, electrophoretic mobility-shift assay; MCO, multicopper oxidase; qRT-PCR, real-time quantitative RT-PCR.

A supplementary figure is available with the online version of this paper.

cup1 expression), that was previously described in yeast as the recognition site for the ACE1 transcription factor (Evans *et al.*, 1990). ACE1 is a member of a group of fungal transcription factors that contain a copper-fist cysteine-rich DNA-binding domain located in the N-terminal region (Hu *et al.*, 1990). This *trans*-acting regulatory protein activates the transcription of the *S. cerevisiae* metallothionein-encoding genes *cup1* (Thiele, 1988; Buchman *et al.*, 1989; Culotta *et al.*, 1989; Evans *et al.*, 1990; Thorvaldsen *et al.*, 1993) and *crs5* (Culotta *et al.*, 1994) and the superoxide dismutase gene (*sod1*) (Gralla *et al.*, 1991; Carri *et al.*, 1991) in response to copper. This metal is essential for the DNA-binding activity of ACE1 (Winge, 1998). Interestingly, we have recently identified the gene encoding ACE1 in *P. chrysosporium* (*Pc-ace1*), the first basidiomycetal orthologue of the yeast ACE1 transcription factor (Polanco *et al.*, 2006). However, until this work, the possible target gene(s) of *Pc-ACE1* remained unknown.

Previously, we have shown that the genome database of *P. chrysosporium* (<http://genome.jgi-psf.org/Phchr1/Phchr1.home.html>) lacks laccase-encoding sequences. Instead, we identified four clustered MCO genes (designated *mco1* to *mco4*) distantly related to laccases (Larrondo *et al.*, 2003, 2004). Heterologous expression of *mco1*-cDNA in *Aspergillus nidulans* showed that the substrate specificity of the recombinant MCO1 differs from that of laccases. For example, MCO1 has strong ferroxidase activity, with a K_m value similar to Fet3 protein from *S. cerevisiae* (Larrondo *et al.*, 2003). Although extracellular MCO1 shows biochemical and structural similarities with the membrane-bound Fet3 from yeast, we have recently identified and characterized *Pc-fet3*, the gene encoding the canonical ferroxidase involved in iron uptake in *P. chrysosporium* (Larrondo *et al.*, 2007a). The presence of *Pc-fet3* and the lack of a C-terminal transmembrane domain anchor in MCO1, which is distinctive of Fet3 proteins, supports the assertion that MCO1 and Fet3 play different roles in the cell (Larrondo *et al.*, 2003, 2007a). A recent and detailed phylogenetic analysis of more than 350 MCOs, including those from *P. chrysosporium*, supports this statement (Hoegger *et al.*, 2006). To date, the physiological function of MCO1 remains obscure.

In an effort to gain insight into the role of MCO1, we decided to analyse the transcriptional regulation of *mco1* and the molecular mechanisms involved in it. The fact that copper affects the transcription of various MCO-encoding genes in different organisms, plus the presence of putative ACE elements in the promoter of *mco1* (Polanco *et al.*, 2006), led us to analyse the effect of copper on the expression of this novel group of multicopper oxidases recently discovered in *P. chrysosporium* (Larrondo *et al.*, 2003). In this work we show that *mco1* is a target gene of the recently described ACE1 transcription factor in *P. chrysosporium*.

METHODS

Strain and culture conditions. *P. chrysosporium* homokaryotic strain RP-78 was obtained from the Center for Mycology Research,

Forest Products Laboratory, Madison, WI, USA. For solid-state incubations, *P. chrysosporium* spores were inoculated on potato-dextrose agar plates and grown for 1 week at 39 °C. Spores were collected by flooding the agar plates with 5 ml sterile water, and 2×10^6 spores were inoculated in 20 ml carbon-limited stationary liquid cultures as previously described (Brown *et al.*, 1993). Cultures were harvested after 4 days of incubation. When indicated, they were supplemented with CuSO_4 to a final concentration of 0.25 mM for the indicated times.

Analysis of the promoter regions of *mco* genes. Using the reported cDNA sequences of *mco1*, *mco2*, *mco3* and *mco4* (GenBank accession numbers AY225437, AY532139, AY532142 and AY532149, respectively) and by means of BLAST, *mco* genes were localized in the *P. chrysosporium* genome database (<http://genome.jgi-psf.org/Phchr1/Phchr1.home.html>). Taking into consideration the presence of several gene models, each promoter was manually obtained and arbitrarily defined as the corresponding intergenic region. This procedure led to uncovering of promoter regions of 1407, 827 and 537 bp for *mco1*, *mco2* and *mco3*, respectively. Since the closest upstream gene model for *mco4* is located 5.2 kbp away, the promoter region was arbitrarily defined as the sequence 1500 bp upstream from the *mco4* translation start site. These sequences were examined for the presence of putative ACE elements using the MatInspector software (<http://www.genomatix.de/products/MatInspector/>). The analysis was restricted to fungal transcription binding sites.

RNA extraction. After 4 days of growth, the mycelia obtained from five independent flasks (processed as a batch) were separated from the culture fluid by filtration through Miracloth (Calbiochem) and frozen in liquid nitrogen. The frozen mycelium was ground to a powder in a mortar containing liquid nitrogen. The powder was homogenized in the same mortar by the addition of 4 ml Tris/HCl buffer (0.2 M Tris/HCl pH 7.5, 0.5 M NaCl, 0.01 M EDTA, 1% SDS, 50 mM β -mercaptoethanol) and 4 ml phenol/chloroform/isoamyl alcohol (25:24:1, by vol.). Each sample was transferred to a 50 ml RNase-free tube and vortex-homogenized. In the same tube and using a sterile syringe and needle, the fluid was pipetted up and down several times to shear genomic DNA (gDNA). Thereafter, the fluid was transferred to four 2 ml Eppendorf tubes. After centrifugation (15 min at 14 000 r.p.m.), the aqueous phase was phenol-extracted in a clean tube and the RNA was obtained as described by Manubens *et al.* (2003). Ten micrograms of total RNA was fractionated by electrophoresis in a formaldehyde-agarose gel (1.2% w/v) and RNA integrity was verified by ethidium-bromide staining. Poly(A) mRNA was obtained from 100 μ g total RNA using the mRNA DIRECT kit (Dynal), according to manufacturer's instructions. To ensure the absence of gDNA, the poly(A) mRNA obtained was incubated at 37 °C for 30 min using RQ1 RNase-free DNase (Promega), according to the manufacturer's instructions. For each sample, 2 μ l treated mRNA was collected before proceeding with the retrotranscription step. To check for potential gDNA contamination, RT-minus reactions were carried out in real time using SYBR Green detection chemistry. No cycle threshold (C_t) values were obtained, at least under the cycle conditions used (see below). Only those mRNA samples that did not show gDNA contamination were reverse transcribed using the MMLV reverse transcriptase (Invitrogen) for 45 min at 42 °C, according to manufacturer's instructions. The cDNA obtained was diluted twofold.

Real-time quantitative RT-PCR (qRT-PCR). Relative quantification of *mco* transcript levels was performed in real time using the Brilliant SYBR Green QPCR Master Reagent kit and the Mx3000P detection system (Stratagene). Primer sequences and predicted T_m values, as well as amplicon length, are shown in Table 1. Levels of mRNA from the glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene were used for normalization. The qRT-PCR mixture (25 μ l)

Table 1. Oligonucleotides employed for qRT-PCR

The T_m values were calculated using the nearest-neighbour method (Breslauer *et al.*, 1986).

Primer	Sequence (5'–3')	Predicted T_m (°C)	Amplicon length (bp)
<i>mco1</i> Fw	CATAACCGAGTGCAGGAATACC	58.6	101
<i>mco1</i> Rv	GCGTGTCTAATGCGAATGC	59.3	
<i>mco2</i> Fw	CTCCGGTCAAATTCCTAGCT	58.9	107
<i>mco2</i> Rv	ACCCAGGGCGAAAGGTA	59.5	
<i>mco4</i> Fw	TGCGATAAACGGTATTGGTCAGT	59.9	91
<i>mco4</i> Rv	TATAAGACGCAGGCGGTAGGT	58.1	
<i>gapdh</i> Fw	TGCTGATGCCCCCATGTT	60.0	81
<i>gapdh</i> Rv	CAGGACGCGTTCGAGATG	58.0	

contained 2.0 μ l cDNA template and 140 nM of each primer. The qRT-PCR was performed under the following conditions: 10 min at 95 °C followed by 40 cycles of 30 s at 94 °C, 30 s at 56 °C and 30 s at 72 °C, followed by a melting cycle from 55 °C to 95 °C to check for amplification specificity. C_t values were obtained during the elongation period of the qRT-PCR. A previous standard quantification curve with several serial dilutions of RT-PCR products was constructed for each gene to calculate the amplification efficiency (E) according to the equation $E = [10^{(-1/\text{slope})}] - 1$. The E values obtained for *mco1*, *mco2*, *mco4* and *gapdh* were 96 %, 99 %, 98 % and 94 %, respectively. These values were used to obtain a more accurate ratio between the gene of interest (GOI) and the expression of the housekeeping (HK) gene, using the following equation: $[(1 + E_{\text{GOI}})^{-[\Delta\text{GOI} - \Delta\text{GOI control}]}] / [(1 + E_{\text{HK}})^{-[\Delta\text{HK} - \Delta\text{HK control}]}]$. Values are referred to the culture not supplemented with copper (control). All experiments were performed in two biological and three technical replicates.

In vitro transcription–translation of the *P. chrysosporium* ACE1 transcription factor (Pc-ACE1). The previously cloned Pc-ACE1 transcription factor (Polanco *et al.*, 2006) was subcloned into the *NdeI* and *BamHI* sites of the bacterial expression vector pET21a (Novagen). Primers to obtain the *S. cerevisiae* ACE1 (Sc-ACE1) coding sequence (5'-CATATGGTCGTAATTAACGGGGTCA-3', direct, and 5'-GGATCCCCTTGTAATGTGAGTTA-3', reverse) were designed in accordance with the reported sequence (GenBank accession number AY557820). Because of the absence of introns, the Sc-ACE1 coding sequence was obtained by PCR using gDNA. The Sc-ACE1 transcription factor was also cloned into the *NdeI* and *BamHI* sites of the pET21a vector. Nucleotide sequences were determined with the ABI Prism Big Dye terminator cycle sequencing kit on ABI automated sequencers (Applied Biosystems). Bacterial heterologous expression was unsuccessful (data not shown). Therefore, taking a different approach, both plasmids were linearized at the *BamHI* site and used for *in vitro* synthesis of their corresponding mRNA using the Riboprobe *in vitro* Transcription System (Promega). mRNA integrity and size specificity was verified by ethidium-bromide staining (see supplementary Fig. S1, available with the online version of this paper). One microgram of each mRNA was further incubated for 90 min at 30 °C with the Flexi Rabbit Reticulocyte Lysate System (Promega) in the presence of [35 S]methionine. Thereafter, 1 μ l of each translation product was separated by SDS-PAGE using standard procedures. The SDS-PAGE gel was fixed, dried and exposed on scientific autoradiographic imaging films (Kodak), thus allowing the confirmation of the predicted molecular mass of each protein (see Fig. S1).

Preparation of labelled probes. A probe containing two previously described ACE elements from the *S. cerevisiae* *cup1* gene (Culotta *et al.*, 1989) was PCR-amplified using primers 5'-GATTCTTTTGCT-GGCATTTC-3' (direct) and 5'-GACAATCCATATTGCGTTG-3'

(reverse). A specific PCR product was obtained, cloned into pGEM-T vector (Promega) and sequenced. The probe was PCR-amplified in the presence of [α - 32 P]dCTP using the obtained plasmid as a template. In addition, 32 P-end-labelled double-stranded oligomers containing one of the ACE elements identified in the promoter region of *mco1* were used as probe. Radioactive labelled probes were purified by PAGE.

Electrophoretic mobility-shift assays (EMSAs). Standard binding reaction mixtures (30 μ l) contained: 30 mM HEPES/KOH buffer pH 7.5, 2.0 mM EDTA, 0.1 mM ZnSO₄, 0.12 mM AgNO₃, 40 mM KCl, 7.5 mM DTT, 12 % glycerol, 3 % PEG 4000, 30 ng poly(dI-dC) and 300 ng Bluescript KSII plasmid. The concentrations of CuSO₄ and ascorbic acid (included to reduce Cu²⁺ to Cu⁺) were 0.15 mM, unless otherwise specified. To each binding reaction, 2 μ l rabbit reticulocyte lysate containing the synthesized Sc-ACE1 or Pc-ACE1 transcription factor was added, as well as 50 ng (20 000–40 000 c.p.m.) labelled DNA probe. After incubation for 15 min at room temperature, samples were separated by electrophoresis as described previously (Polanco *et al.*, 2002). No DNA–protein interaction was observed when binding assays were carried out with 2 μ l rabbit reticulocyte lysate in which the translation reaction was omitted (data not shown). Binding assays shown in Fig. 3(D) were carried out in the absence of AgNO₃. For competition experiments, labelled probe was premixed with the unlabelled probe or the non-specific competitor.

Pc-ACE1-mediated *in vitro* transcription. Promoter activation mediated by Pc-ACE1 was studied using an *in vitro* transcription system. First, a 500 bp DNA fragment corresponding to the upstream sequence from the *mco1* translation start site containing one ACE element was PCR amplified using primers 5'-ATTAATCTAGAGGCGCTCCAGACC-3' (direct) and 5'-AAGCTTGCGATAATGCAAAGTGAAGGATG-3' (reverse). After this procedure, the 589 bp CMV promoter of the mammalian expression vector pEGFP-N1 (Clontech) was removed using *Asel* and *HindIII* restriction endonucleases and replaced with the aforementioned 500 bp *mco1* promoter fragment. Similarly, the complete *mco2* promoter (as defined above, 'Analysis of the promoter regions of *mco* genes'; 827 bp) was PCR-amplified using primers 5'-ATTAATGGCGAGGGGTGCCGTTGCT-3' (direct) and 5'-AAGCTTTGCGAGCAGAGGCGGTGG-3' (reverse) and cloned into the pEGFP-N1 vector as mentioned. In each case, a plasmid containing the respective *mco* promoter region was linked to an EGFP reporter gene. *In vitro* transcription mixtures (25 μ l) contained 3.3 μ l HeLa nuclear extract (Promega), 0.4 mM of each ribonucleotide, 100 ng linearized plasmid DNA template, 40 units RNase OUT and 3 μ l rabbit reticulocyte lysate containing the *in vitro*-synthesized Pc-ACE1 transcription factor in 20 mM HEPES pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 3 mM MgCl₂, 20 % (v/v) glycerol. When indicated, reaction mixtures were also supplemented with 25 μ M CuSO₄, 25 μ M

ascorbate and 25 μ M KCN. Control reactions were carried out with 3 μ l rabbit reticulocyte lysate in which the translation reaction was omitted. Following transcription, reporter gene mRNA levels were measured by qRT-PCR using primers 5'-GAGAAGCGCGATC-ACATGGT-3' (direct) and 5'-TTACTTGTACAGCTCGTCCATGC-3' (reverse), as described above.

RESULTS

Effect of copper on transcript levels of *mco* genes

The effect that copper exerts at the transcriptional level of *mco* genes in various organisms (see above), and the previous detection of putative ACE elements in the promoter of *mco1* from *P. chrysosporium* (Polanco *et al.*, 2006), prompted us to examine whether this metal regulates the expression of *mco1*, *mco2* and *mco4*. As described previously, the *mco3* gene is transcriptionally inactive due to the insertion of an 8.14 kb gypsy-class retroelement within the 12th intron (Larrondo *et al.*, 2007b). Therefore, it was not analysed in this study. As shown in Fig. 1, the addition of copper to the culture medium led to a 4-fold increase in *mco1*-transcript levels after 30 min, while *mco2* transcripts were increased 2.5-fold. In the case of *mco4*, no major change was observed during the period analysed.

Analysis of *mco* promoter regions

These results prompted us to examine the promoters of *mco* genes to look for the presence of ACE elements. The

Fig. 1. Effect of Cu^{2+} on transcript levels from *mco* genes. *P. chrysosporium* was grown for 4 days in C-starved medium and then harvested (C, Control) or exposed to CuSO_4 to a final concentration of 250 μ M for the indicated times. After each period of time, the cultures were harvested, mRNA was isolated and qRT-PCR was performed as described in Methods. The *mco3* gene was not analysed since it is transcriptionally inactive (Larrondo *et al.*, 2007b).

four clustered *mco* genes (*mco1* to *mco4*) are located on Scaffold 9 of the assembly version 2.0 of the *P. chrysosporium* genome database (Larrondo *et al.*, 2004). Their respective promoter regions were defined and searched for the presence of regulatory elements as described in Methods. As expected, two putative ACE elements were identified in the promoter of *mco1* (Polanco *et al.*, 2006) (Fig. 2A). Both adhere to the consensus sequence of the previously described ACE elements in the promoter regions of the *S. cerevisiae* *cup1* and *sod1* genes (Fig. 2B). The ACE sequences in the promoter of *mco1* are centred between positions –306 to –292 and –849 to –835 from the *mco1* translation start site (Fig. 2C). On the other hand, although *mco2*-transcript levels showed a slight increase (Fig. 1), no putative ACE elements were identified in its promoter. Likewise, no ACE-like sequences were found in the upstream regulatory region of *mco4*.

DNA-binding activity of Pc-ACE1

It seemed highly likely that the higher levels of *mco1* transcripts observed upon incubation of cultures with copper are mediated by Pc-ACE1. In order to test this hypothesis, as a first approach we characterized the DNA-binding activity of the *in vitro*-synthesized Pc-ACE1 protein to the two previously described ACE elements from the *S. cerevisiae* *cup1* gene (Culotta *et al.*, 1989). The EMSAs shown in Fig. 3 illustrate the specific binding of Pc-ACE1 to a DNA probe containing these sequences. In this experiment, two DNA–protein complexes were observed (Fig. 3A, lane 2). The lower-mobility complex appears as a slight band signal that shows some level of competition with the unlabelled probe. On the other hand, a stronger band corresponding to a higher-mobility DNA–protein complex was readily displaced when binding assays were carried out in the presence of a molar excess of unlabelled DNA probe (Fig. 3A, lanes 3–5). Displacement was much more limited when assays were conducted in the presence of non-specific DNA competitor (Fig. 3A, lanes 6–8). Similar results were obtained using the same DNA probe with the *in vitro*-synthesized ACE1 protein from *S. cerevisiae*, used as a positive control (see supplementary Fig. S1, available with the online version of this paper).

In order to determine whether Pc-ACE1 can specifically interact with a probe containing one of the ACE sequences identified in the promoter of *mco1*, a probe containing the element located between positions –306 and –292 was synthesized (Fig. 3B). As shown in Fig. 3(C), this ^{32}P -labelled 40 bp oligomer forms an easily observed DNA–protein complex with Pc-ACE1. An equivalent result was also observed using a 100 bp DNA probe (data not shown). To confirm the specificity of this association, competition experiments were carried out using double-stranded unlabelled 40 bp DNA oligomers containing either the same ACE element or a mutant version of this ACE with five point mutations (Fig. 3B). Formation of the DNA–protein complex was completely inhibited by incubation

Fig. 2. Representation of the four clustered *mco* genes from *P. chrysosporium*. (A) The schematic representation (to scale) shows the four *mco* genes and some neighbouring gene models (*pe*, phosphoesterase; *dd*, dihydrolipoamide dehydrogenase) located on Scaffold 9, between coordinates 12 862 and 48 804. To simplify the figure, two gene models between the dihydrolipoamide dehydrogenase gene model and *mco4* were omitted. Genes and gene models are represented in black boxes showing transcriptional orientation, whereas promoters and intergenic regions are shown in white spaces. The 8.14 kbp *pcr1* retroelement (grey box) interrupts the *mco3* gene (Larrondo *et al.*, 2007b). *mco1* promoter, 1407 bp; *mco2* promoter, 827 bp; *mco3* promoter, 537 bp; *mco4* promoter, 1500 bp. (B) Alignment of the ACE elements from the *S. cerevisiae* *cup1* and *sod1* genes (*Sc-cup1* and *Sc-sod1*) and the two ACE elements identified in the promoter of *mco1*. All ACE elements adhere to the consensus sequence H(T)HNNGCTGD (H, not G, N, any nucleotide, D, not C). Residues shown in bold type have been assigned a key role involved in DNA–protein interaction (Gralla *et al.*, 1991; Beaudoin *et al.*, 2003). (C) The *mco1* promoter region, as defined in Methods. ACE elements (grey boxes) are denoted with their respective positions (superscript numbers) relative to the *mco1* translation start site (ATG).

with excess wild-type oligomer, whereas the mutant competitor was less effective (Fig. 3C). Although this result indicates that the mutated sequence is important for the observed protein–DNA interaction, some additional DNA sequences may also be involved in complex stabilization.

A further control of binding specificity was conducted by measuring the effect of copper on complex formation. As indicated in Methods, standard binding assays were conducted in the presence of this metal. No DNA–protein complex was observed in the absence of copper (Fig. 3D, lane 7). Addition of KCN, a specific chelator of Cu^+ , inhibited the DNA–protein complex formation in a concentration-dependent manner (Fig. 3D, lanes 3–5). To avoid copper reduction, a binding assay containing 25 μM CuSO_4 was also carried out, in the absence of ascorbic acid. As shown in Fig. 3(D), lane 6, a weak DNA–protein complex signal was observed under these conditions. These results indicate that Pc-ACE1, similar to Sc-ACE1 (Buchman *et al.*, 1989), requires Cu^+ for binding to the DNA probe.

Pc-ACE1 mediates the activation of ACE-containing promoters

Three different lines of evidence strongly suggest that Pc-ACE1 mediates *mco1* promoter activation: (a) the copper-dependent increase of *mco1*-mRNA; (b) the presence of two ACE elements in the promoter region of this gene and (c) the copper-dependent DNA-binding of Pc-ACE1 to one of these two ACE elements. In order to confirm that Pc-ACE1 is involved in the copper-dependent transcription

induction of the *mco1* promoter, a cell-free transcription system was employed. This consisted of a linearized plasmid containing the *mco1* promoter region linked to an EGFP reporter gene, a HeLa nuclear extract providing RNA polymerase plus general transcription factors and the Pc-ACE1 protein (see Methods). The basal level of transcription was greatly increased by adding Pc-ACE1 in the presence of Cu^+ (Fig. 4, lanes 1 and 2). In contrast, no transcriptional activation was observed when ascorbate was omitted, to prevent reduction of Cu^{2+} to Cu^+ (Fig. 4, lane 3) or when Cu^+ was chelated with KCN (Fig. 4, lane 4). Finally, the transcriptional activity of the *mco2* promoter was also tested. In this case, no transcriptional activation was observed (Fig. 4, lanes 5 and 6).

DISCUSSION

In this work, we report that copper increases mRNA levels of *mco1* and *mco2*, but not of *mco4*. Among these genes, only the promoter of *mco1* showed the presence of two copper-dependent *cis*-acting regulatory elements known as ACE. These had been originally identified in the promoter regions of the *S. cerevisiae* copper metallothioneins *cup1* and *crs5*, as well as in the promoter region of the superoxide dismutase gene *sod1* (Culotta *et al.*, 1989, 1994; Gralla *et al.*, 1991). This element is the recognition site for the ACE1 transcription factor, which activates the transcription of target genes in response to copper. Prior to the present work, we had reported the cloning and sequencing of *Pc-ace1*, the gene encoding an ACE1 transcription factor in *P. chrysosporium*. However, until

Fig. 3. Competition analysis by EMSAs. (A) Top, schematic representation of the *S. cerevisiae cup1* gene promoter and its related 100 bp DNA probe containing two ACE elements (white boxes); the position of each element (superscript numbers) is indicated with respect to the translation start site (ATG). Bottom, binding assays conducted with this probe (ACE_{cup1}) and *in vitro*-synthesized Pc-ACE1 protein. Lanes contain: (1) free probe; (2) standard binding reaction without competitor; (3, 4, 5) binding assays in the presence of 10-, 25- or 50-fold excess of unlabelled specific DNA probe, respectively; (6, 7, 8) binding assays in the presence of 10-, 25- or 50-fold excess, respectively, of a 101 bp unrelated DNA as a non-specific competitor. (B) Synthetic oligonucleotides used as double-stranded competitors in the experiment shown in (C). Boxes denote the wild-type ACE element (WT) and the corresponding region within the mutant (MUT) competitor. Wild-type and substituted residues within the ACE consensus sequence are indicated in bold type. (C) Binding assays conducted with a 40 bp DNA probe containing the ACE element centred between positions -306 and -292 from the *mco1* translation start site (Fig. 2C) and the *in vitro*-synthesized Pc-ACE1 transcription factor. Lanes contain: (1) free probe; (2) standard binding reaction without competitor; (3, 4, 5) binding assays in the presence of 10-, 25- or 50-fold excess unlabelled specific WT DNA probe, respectively; (6, 7, 8) binding assays in the presence of 10-, 25- or 50-fold excess unlabelled MUT competitor, respectively. (D) Binding assays conducted with the same DNA probe and Pc-ACE1 transcription factor. Lanes contain: (1) free probe; (2) standard binding reaction; (3, 4, 5) binding reactions in the presence of 25, 50 or 100 mM KCN, respectively, as Cu⁺-specific chelator; (6) binding reaction in the presence of 150 µM Cu²⁺ or (7) in the absence of copper.

Fig. 4. Pc-ACE1-dependent promoter activation of an ACE-containing promoter. *In vitro* transcription of constructions *mco1*-promoter:EGFP (lanes 1 to 4) and *mco2*-promoter:EGFP (lanes 5 and 6) as template DNAs. Reaction mixtures were supplemented with Cu⁺ (added as 25 μ M CuSO₄-ascorbate, see Methods) and 3 μ l of either control rabbit reticulocyte lysate translation mixture (ACE-) or an identical mixture containing the *in vitro*-translated Pc-ACE1 transcription factor (ACE+). As a control, a reaction supplemented with 25 μ M ascorbate (Asc, lane 3) was carried out. KCN, a Cu⁺ specific chelator, was also used (Cu⁺-KCN, lane 4). Transcripts were quantified by qRT-PCR ($n=3$). Values are referred to the *in vitro* transcription assay carried out in the absence of Pc-ACE1 (lane 1).

now, the possible target gene(s) of Pc-ACE1 remained unknown.

The *Pc-ace1* cDNA complements *in vivo* a mutant yeast strain lacking a functional ACE1 transcription factor. In this ACE1 Δ yeast strain, Pc-ACE1 was strictly required for growth in the presence of a high concentration of copper. Notably, Pc-ACE1 restored the copper inducibility of the yeast *cup1* gene (Polanco *et al.*, 2006). In order to characterize the DNA-binding activity of Pc-ACE1, EMSAs were carried out using the *in vitro*-synthesized Pc-ACE1 transcription factor. This protein was tested with DNA probes containing ACE elements either from yeast or from *P. chrysosporium*. As a positive control, we also synthesized the *S. cerevisiae* ACE1 (Sc-ACE1). EMSAs using a DNA probe containing the two previously described ACE elements from the promoter region of the *cup1* gene from yeast (Culotta *et al.*, 1989) showed specific DNA-protein interaction of both Sc-ACE1 (Fig. S1) and Pc-ACE1 (Fig. 3).

With each transcription factor, two DNA-protein complexes were obtained. Cysteine amino acids located within the copper-fist DNA-binding domain of Sc-ACE1 have been described as key residues that participate in DNA-binding activity and also as being involved in DNA target site specificity (Buchman *et al.*, 1990). Since these cysteine

residues are conserved in Pc-ACE1 (Polanco *et al.*, 2006), a similar binding pattern of the two transcription factors was not unexpected. Two DNA-protein complexes had also been observed with Sc-ACE1 and a similar DNA probe (Hu *et al.*, 1990). In that work, the authors observed that the lower-mobility complex was obtained only at high protein concentrations, whereas the higher-mobility complex was observed at both low and high protein concentrations. When the *cup1* ACE elements are placed in separate probes, Sc-ACE1 binds efficiently, forming only one DNA-protein complex (Hu *et al.*, 1990). Consequently, it has been proposed that when both elements are located within the same DNA molecule, the higher-mobility complex corresponds to the occupancy of one DNA-binding site, whereas the DNA-protein complex showing a lower migration in the gel results from the occupancy of both ACE elements (Hu *et al.*, 1990). This result clearly indicates that Pc-ACE1, when interacting with and recognizing canonical ACE elements, behaves like Sc-ACE1. It also explains, at a molecular level, the reported copper-dependent Pc-ACE1-mediated induction of the yeast *cup1* gene in a ACE1 Δ background (Polanco *et al.*, 2006).

The presence of two ACE elements in the promoter of *mco1* led us to examine the binding of Pc-ACE1 to at least one of them. The chosen element was centred between positions -306 and -292 from the *mco1* translation start site, similar to one of the ACE elements of the yeast *cup1* gene (see Fig. 3). Fig. 3(C) illustrates that Pc-ACE1 does interact with this ACE element. The specificity of this interaction was tested in competition experiments with the same unlabelled probe and with an unlabelled probe including five point mutations. Four of these five mutations were located within the invariant 5'-GCTG-3' ACE core (Fig. 3B) (Gralla *et al.*, 1991). Although some displacement of the complex was observed with the mutant probe, the complex was completely displaced with a 50-fold molar excess of the unlabelled probe possessing the wild-type ACE element. This result indicates that the complex was formed by sequence-specific interactions, as has also been demonstrated for Cuf1, a *Schizosaccharomyces pombe* transcription factor that has a DNA sequence binding specificity similar to the ACE1 protein from *Saccharomyces cerevisiae* (Beaudoin *et al.*, 2003). The requirement for Cu⁺ in the incubation mixture is also indicative of the specificity of this binding (Fig. 3D). It has been described that this metal, when binding to the copper regulatory domain within Sc-ACE1, stabilizes a specific tertiary fold forming a tetracopper thiolate cluster (Winge, 1998).

In order to demonstrate that Pc-ACE1 can stimulate the transcriptional activity of an ACE-containing promoter, an *in vitro* transcription assay was developed. As shown in Fig. 4, Pc-ACE1 enhances the transcription of a reporter gene linked to a DNA fragment encompassing 500 bp upstream of the *mco1* translation start site. This promoter fragment contains the same ACE element first studied by EMSA. Interestingly, no transcriptional activation was observed in the absence of Cu⁺, or when Cu⁺ was

chelated with KCN. These results may account for the higher *mco1*-mRNA levels observed *in vivo* in cultures of *P. chrysosporium* supplemented with copper.

We also observed higher *mco2*-mRNA levels when cultures of *P. chrysosporium* were supplemented with copper. The absence of ACE elements in the promoter of *mco2* made this result difficult to interpret. We then decided to test whether Pc-ACE1 can stimulate the transcription of a reporter gene linked to the promoter of *mco2*. As described in Results, this stimulation does not take place, suggesting that *mco2* may not be a direct target of Pc-ACE1. In this regard, through hierarchical clustering of microarray data, a group of six differentially expressed genes that are highly induced in response to copper have been observed in *S. cerevisiae*. Among these, only *cup1* and *crs5*, but not the other four genes, contain ACE elements in their respective promoter regions (van Bakel *et al.*, 2005). It is conceivable that copper may induce gene expression by a mechanism different to that mediated by ACE1 transcription factor, such as, for example, the generation of reactive oxygen species. Clearly, further work is necessary to clarify the molecular mechanisms underlying the increment in *mco2*-mRNA levels in response to copper.

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