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The PcACE1 transcription factor from *Phanerochaete chrysosporium* contains a Cys- and Ser-rich transactivation domain

Feiruz Barham, Patricia Roman and *Paulina Bull

Departamento de Genética Molecular y Microbiología. Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile.

ABSTRACT

Transcription factor Ace1 from *Saccharomyces cerevisiae* regulates the expression of target genes when the copper concentration reaches 200 μM levels. We are studying the ortholog of Ace1 from fungus *Phanerochaete chrysosporium* PcACE1, isolated by complementation in yeast. In this report we show the localization of the transactivation region of PcACE1. Different PcACE1 fragments were ligated in frame to the GAL4 DNA-binding domain by site-directed mutagenesis in a suitable yeast expression vector. Transformation of an appropriate *Saccharomyces cerevisiae* strain was used as host. This strain contains the fusion *GAL1:lacZ* in its genome under the control of promoter sequences recognized by GAL4. Finally, we measured β-galactosidase activity in each yeast clone. The activation of the reporter gene is proportional to the transactivation capacity of the transcription factor PcACE1.
The results obtained indicate that PcACE1 transactivation domain is located in the carboxy terminal half and contains an array of cysteines in the form of Cys-X-Cys and Cys-X_2-Cys and a 60% of Ser. Therefore, these results show that this type of Cys motif can function as transcription activating domain not only in transcription factors that respond to minimal copper concentrations but also in those that respond to high copper concentrations. This is the first transactivation domain reported in a basidiomycete fungus.

INTRODUCTION

Copper is a key element in living organisms. It plays a vital role as a cofactor of some metalloenzymes, such as superoxide dismutase, cytochrome C oxidase and copper radical oxidases (Rutherford and Bird, 2004). However, high copper levels are toxic, because they can produce free radicals that damage nucleic acids, proteins and lipids. Several mechanisms regulate copper concentration very closely (Balamurugan and Schaffner, 2006). For instance, extracellular copper as Cu^{+2} has to be reduced to Cu^{+1} by Fre1 reductase before entering cells through specific transporters, ctr1 and ctr3. As well, chaperone Atx1 binds Cu^{+1} in the cytoplasm, so that this metal is never left free. A third regulatory mechanism is found in the nucleus; six transcription factors sense the amount of copper; some of them are activated when copper concentration is high (Ace1, Amt1 and Crf1), whereas the others are activated when copper concentration is low (Mac1, GRISEA and Cuf1) (Rutherford and Bird, 2004).

Ace1 (activator of Cup expression) from *Saccharomyces cerevisiae,* activates a number of genes: cup1, *crs*5 and superoxide dismutase (Thiele, 1988). Amt1 is the functional ortholog of ACE1 in *Candida glabrata* (Zhou and Thiele 1991), and Crf1, the ortholog of *Yarrowia lipolytica* (García et al., 2002). These three transcription factors confer resistance to high copper concentrations. They share three conserved Cys motifs. The first, with the sequence **C-**X_2-**C-**X_8-**C-**X-**H,** (X: any other amino acid), is located between amino acids 11-25, corresponding to a Zinc DNA binding domain (Farrell et al., 1996, Turner et al., 1998). The second motif, **C-**X_2-**C-**$X_{12\text{-}14}$**C-**X_2-**C,** corresponding to amino acids 43-63, captures Cu^{+1}, producing cuprous-thiolate clusters (Brown et al., 2002). A third Cys motif, **C-**X-**C-**X_5-**C-**X-**C**, is located about 20 amino acids further downstream. These three highly conserved motifs are important to sense copper concentrations. In fact, Ace1 DNA binding activity depends on the presence of Cu^{+1} (Zhou and Thiele, 1993).

Mac1 (**m**etal binding **ac**tivator) from *Saccharomyces cerevisiae* is active at low copper concentrations, and induces the expression of the high affinity copper transport system, *ctr*1, *ctr*3 and reductase *fre*1 (Jungmann et al., 1993, Georgatsou et al., 1997). Cuf1, from *Schizosaccharomyces pombe*, stimulates copper transport from the vacuole to the cytoplasm through *ctr*6 (Beaudoin and Labbé, 2001). GRISEA, the ortholog of Mac1 in *Podospora anserina*, activates the expression of the genes *PaSOD*2 and *PaCtr*3, encoding a mitochondrial manganese superoxide dismutase and a copper transporter, respectively (Borghouts et al., 2002). Mac1 contains a motif similar to the first Cys DNA-binding motif present in transcription factors Ace1, Amt1 and Crf1. However, it lacks second and third motifs. On the other hand, it contains two Cys-rich motifs at the carboxy terminal region designated as C1 **(C-**X-**C-**X_4-**C-**X-**C-**X_2-**C-**X_2-**H)**, at amino acid position 264**,** and C2 (**C-**X-**C-**X_4-**C-**X-**C-**X_4-**C-**X-**C-**X_2-**C-**X_2-**H),** at position 315 (Graden and Winge, 1997). Each motif binds four Cu^{+1} (Brown et al., 2002). GRISEA and Cuf1 also have similar Cys-rich motifs at the carboxy terminal region (Rutherford and Bird, 2006).

Transcription factor PcACE1 from basidiomycete fungus *Phanerochaete chrysosporium* is another member of the family of transcription factors that regulates copper concentration. It was cloned by Polanco et al. (2006) and is activated when copper concentration is high. Functionally, it is the ortholog of Ace1 from *Saccharomyces cerevisiae*. This was demonstrated by complementation assays using a *S. cerevisiae* ace1Δ strain, in which the capacity of this mutant to grow at high copper concentration was restored when PcACE1 cDNA was introduced (Polanco et al., 2006). Moreover, *in vitro* transcribed and translated PcACE1 protein was able to bind in a gel-shift

* Corresponding author: Dra. Paulina Bull, Pontificia Universidad Católica de Chile, Facultad de Ciencias Biológicas, Dpto. Genética Molecular y Microbiología. Alameda 340, Santiago, Chile. Phone 56-2-3542663 - Fax 56-2-3542185. e-mail: pbull@bio.puc.cl

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assay to the promoter of the yeast metallothionein in the presence of copper, but not in its absence (Canessa et al., 2008). The PcACE1 amino acid sequence contains the same three Cys motifs present at the amino terminus of Ace1, Amt1 and Crf1, and additional three. Table 1 shows the amino acid sequence of the six Cys motifs present in the amino acid sequence of PcACE1. The fourth Cys motif, **C**-X-**C**-X_3-**C**-X-**C**-X_2-**C**-X_2-**H,** is highly similar to C1 from Mac1, and at the carboxy terminus of GRISEA and Cuf1, transcription factors that are active at low copper concentration (Rutherford and Bird, 2004). Another unique finding is that PcACE1 contains 68% serines within the amino acid sequence $(S)_5$-H-$(S)_3$-H-R-S between amino acid residues 546 and 564, an unusually high number.

In this report we present evidence that the fifth and sixth Cys-rich motifs from PcACE1 and the Ser-rich motif located further downstream are involved in transcription transactivation.

METHODS

Strains and culture media

S. cerevisiae YM335::RY171 (*a gal4-536 ura3-52 ade2-101 lys2-801 His3-200met*) is a derivative from YM335 having a GAL1-lacZ fusion gene integrated at the *URA3* locus and has been previously described (Ma and Ptashne, 1987). Yeast cells were grown in YPD (10 g yeast extract, 10 g bactopeptone and 2% dextrose or glucose for 1 lt) or synthetic defined media without His (SC-His) (Johnston, 1994). SC-His medium was prepared as follows: **Mix 1**: To 500 ml of water, 6.7 g yeast nitrogen base (without amino acids), 10 mg adenine, 40 mg uracil and 50 mg Tyr, were added. **Mix 2**: To 100 ml of water, 200 mg Arg, 600 mg Ile, 600 mg Leu, 400 mg Lys, 100 mg Met, 600 mg Phe, 1g Thr and 400 mg Trp, were added. **Mix 3**: 435 ml of sterile water was added to Mix 1 plus 10 ml of Mix 2 and 5 ml of 1M Na_2HPO_4 (pH 7).The carbon sources for the defined media, where appropriate, were glucose, glycerol, ethanol and/or galactose at 2% final concentration (Johnston, 1994).

Plasmid constructions

The method designed by Ma and Ptashne (1987) was used. Several groups have been able to localize the transcriptional activation domain in different transcription factors, for instance Ace1 (Hu et al., 1990), Mac1 (Graden and Winge, 1997), and proto-oncogenes c-*rel* (Bull et al., 1990) and c-*rel*B (Ryseck et al., 1992), among others. Vector pMA424 (Ma and Ptashne, 1987) contains the ADH promoter followed by the DNA binding domain of the yeast transcription factor GAL4 (amino acids 1-147) and the ADH termination domain. The last two are separated by a BamH1 site. The plasmid also contains a HIS3 gene for selection of the transformed yeast (Ma and Ptashne, 1987).

PcACE1 cDNA cloned in pET21a (+) between Nde1 (5′) and BamH1 (3′) sites was kindly provided by Dr. Rafael Vicuña. Oligonucleotides containing BamH1 sites were designed for site-directed mutagenesis in different places of PcACE1 cDNA. Care was taken to keep the coding sequence in frame with the GAL4 DNA-binding domain. Oligonucleotides and position are shown in Table 2. PCR amplification of PcACE1 cDNA was performed with Taq Platinum DNA polymerase in 25 µl solution. PcACE1 fragments were ligated into the unique BamH1 site of pMA424. Recombinant plasmids containing fragments of PcACE1 cDNA were named pFB1 to pFB10. Figure 1 shows a schematic representation of the 10 different PcACE1 fragments obtained. In this figure, the position of the Cys motifs are highlighted in black and the position of the Ser motif in gray (amino acids 546-564).

Yeast transformation

Yeast cells auxotrophic for His and carrying the promoter of GAL1 in the genome, followed by the reporter gene β-galactosidase, were transformed with the previously obtained recombinant plasmids. Later, the reporter activity was analyzed in each yeast transformant (Ma and Ptashne, 1987).

One isolated colony *of S. cerevisiae* YM335::RY171 was grown in YPD culture medium for 16 hr at 30ºC. 100 ml of YPD medium was inoculated with 1 ml of saturated culture that was obtained the night before. The culture was grown until Abs_{600}

TABLE 1
Localization and sequence of the Cys motifs in the amino acid sequence of PcACE1

	Position (aa)	Amino acid sequence
1	11-25	**C**-E-T-**C**-I-K-G-H-R-S-S-N-**C**-K-**H**
2	43-61	**C**-D-H-**C**-R-E-L-R-K-T-K-Q-V-H-V-K-**C**-V-**C**
3	126-136	**C**-T-**C**-K-T-T-G-I-**C**-N-**C**
4	298-312	**C**-D-**C**-G-P-N-**C**-A-**C**-P-G-**C**-V-I-**H**
5	430-439	**C**-K-**C**-P-H-R-V-**C**-A-**C**
6	450-457	**C**-T-**C**-P-S-**C**-N-**H**

The positions of the amino acid residues (aa) are shown, together with the respective amino acid sequence. Cys and His residue pairs are in bold.

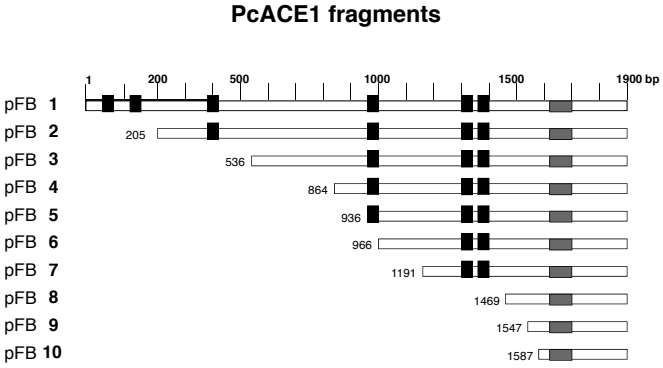

Figure 1. A diagram of PcACE1 cDNA fragments obtained by PCR and cloned into the BamHI site of pMA424 is shown. The names of clones obtained (pFB1 to pFB10) are shown to the left; numbers on the top of the figure correspond to length in base pairs of complete PcACE1 cDNA. All fragments share 3′ end of cDNA and differ in the 5′ end. Numbers immediately before each fragment correspond to the nucleotide at which the fragment begins. The Cys motifs are in black, and the position of the Ser motif in gray (amino acids 546-564). The length of each box is not to scale.

0.4-0.6, divided in 4 sterile tubes and incubated on ice, after which they were centrifuged for 7.5 min at 4,400 rpm in Sorvall SS34 rotor, followed by a centrifugation in a microfuge for 1 min at 14,000 rpm. The supernatant was discarded and the cells were resuspended in 1 ml sterile water. This was done twice. Then, they were suspended in 1 ml of 0.1 M lithium acetate. After another centrifugation, the cells were resuspended in 0.4 ml of 0.1 M lithium acetate. Aliquots of 100 µl were placed in new tubes, and the following reagents were added: 240 µl 50% PEG 3,500; 36 µl 1 M lithium acetate; 25 µl denatured salmon sperm DNA (10 mg/ml) and 90 ng of supercoiled pFB plasmid. After one min vortexing, tubes were incubated for 30 min at 28°C, followed by incubation at 42°C for 15 min with gentle agitation. Afterwards, tubes were centrifuged at 14,000 rpm for 1 min; the supernatant was discarded and the pellet was washed twice with 1 ml of sterile water. Part of the supernatant was discarded. The remaining yeast cells were plated on petri dishes containing SC-His and 2% glucose. Colonies were visualized after incubation at 28°C for 3 days.

Induction of transformed yeast cells and determination of b-galactosidase activity

Transformed yeast cells were cultured in liquid SC–HIS and 2% glucose medium for 18 hrs. The medium was then changed to the induction medium (SC-His, glycerol, ethanol and galactose) and incubated for 4 hr at 30°C.The induced cells were concentrated to 1 ml by centrifugation and the medium was changed to 1 ml Buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ and 2.7 ml b-mercapto-ethanol). Yeast cells were lysed by addition of 70 ml of 0.1% SDS and 100 ml chloroform, and vortexed for 10 sec. Then, 200 µl of 4 mg/ml o-nitrophenyl-β-D-galactopyranose (substrate) were added, incubated for 5 min at 30ºC. The reaction was stopped with 0.5 ml 1 M sodium carbonate. After centrifugation for 3 min, the product (o-nitrophenol) was quantified by absorbance at 420 nm as Yocum et al. (1984). The determination of each sample was repeated 3 to 6 times. Enzyme activity was determined using the following formula:

β-galactosidase specific activity (units) = Abs $_{420}$ x 1000/ time (min) x vol (ml) x ABS $_{600}$.

The standard errors were within 10%.

All DNA manipulations were performed with standard procedures (Sambrook et al., 1989). The plasmid constructs were verified by restriction enzyme mapping and confirmed by double-stranded sequencing (Macrogen, Korea).

RESULTS AND DISCUSSION

Any transcription factor contains at least two domains; the DNA binding domain and the transcription transactivation domain (Ptashne, 1988). Both domains are key to a functional transcription factor. Interestingly, Ma and Ptashne (1987) demonstrated that these modules can be interchanged between two different transcription factors. Each DNA binding fragment retains its properties and binds to the same nucleotide sequence recognized by the original transcription factor in the promoter of the target gene. This finding allowed us to determine the localization of transactivation domains of several transcription factors, using the DNA binding domain of a well-known transcription factor such as GAL4.

The amino acid sequence of transcription activation domains can be highly variable; for instance, transcription factor Sp1 has a transactivation domain rich in glutamines, and CTF1 is rich in prolines. VP16 of Herpes simplex virus contains hydrophobic and aromatic residues. On the other hand, GCN4 contains acidic residues in the transactivation domain. NF-kB p65 needs to be phosphorylated in order to be active (Schmitz et al., 1995). The transcription activation domain of Mac1 maps to the C1 Cys-rich domain (Graden and Winge, 1987).

TABLE 2
Oligonucleotides utilized for site-directed Mutagenesis

Name	Nucleotide sequence	Position (bp)
RV172	5′GCCGTCAGGGATCCCTCCAGGCTTCGAGT	942
RV173	5′GACTACAGCCAGCGGATCCGAGCCCCTATTGCA	1471
RV174	5′TTCTGCTGCGACAGGGATCCCTCGCGTGTCACTCA	1593
RV176	5′GGATCCTTAGAATATCCGTGGACTGCCATCGTCA	1902-1869
RV177	5′TCGCCGGAATTCCGGGGATCC	pMA424
RV178	5′AGGATCCTAGTCGGTGAGAA	1
RV179	5′TCCGGCGCAGGGATCCCCTCGTCG	202
RV180	5′AAACAGACGGATCCTTCCACGT	537
RV181	5′CCTCCCACCGGGATCCCTGCTGCGA	865
RV183	5′ATGTGGCAGTCCTGGATCCTCTGGCCCAAT	1191
RV184	5′TCCGTTAGGGATCCGGGGCGTCTT	1548
RV203	5′AACGAATGAACGGGATCCCTTCCACT	1061

The BamH1 site in each oligonucleotide is underlined. All oligonucleotides are FORWARD, except RV176, which is REVERSE.

We decided to map the transactivation domain of the transcription factor PcACE1 using the method described by Ma and Ptashne (1987). Full-length PcAce1 or partial sequences of it were fused to the GAL4 DNA binding domain and assayed for transcription activation. Figure 2 shows the percentage of b-galactosidase activity obtained with each yeast transformant. 100% was defined as activity of the full-length GAL4 yeast transcription factor (positive control). pFB1, pFB2 and pFB3, the largest open reading frames, showed no reporter activity. However, when the first 850 bp of the 5′ terminal sequences of PcACE1 were deleted (clones pFB4, pFB5, pFB6 and pFB7), transactivation activity was present, suggesting that an inhibitory region had been eliminated. The C terminal half of the protein functions as a transcription activator.

The highest reporter activity was obtained with clones pFB5 and pFB6, yielding about 80% of the positive control. In this region, the PcACE1 fragments contain the Cys motifs 5 and 6 and the Ser-rich motif. Surprisingly, Mac1, which is active only at very low copper concentrations, contains a similar transcription activating domain at the Cys-rich region (Graden and Winge, 1997). Therefore, these results indicate that this type of Cys motif can function as a transcription activating domain not only in transcription factors that respond to minimal copper, but also in some that respond to high copper concentrations, as in this case.

pFB8, pFB9 and pFB10 have about 30% transactivation activity. Interestingly, this region only has the Ser-rich motif and lacks Cys motifs. These results suggest that there could be two transactivating regions in PcACE1, one containing the Cys motifs and a second containing the Ser-rich motif. It would be interesting to look for phosphorylation in Ser. There are examples of activation by phosphorylation, such as Mac1 (Heredia et al., 2001) or C-terminal domain of the largest subunit from yeast RNA polymerase II (Kim et al., 2009).

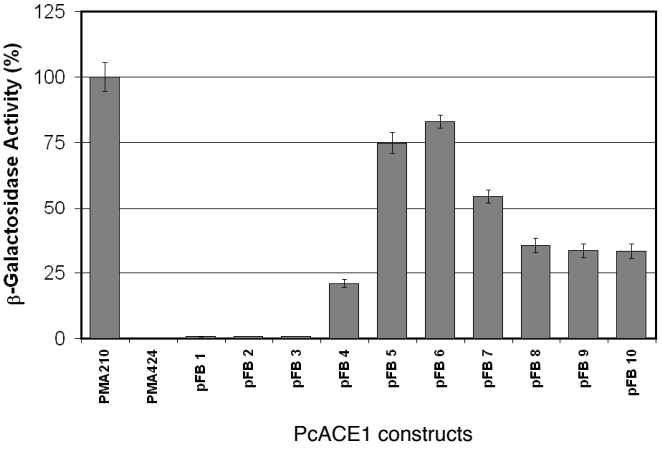

Figure 2. β-galactosidase activity of yeast transformed with clones pFB1 to pFB10. Plasmids expressing the GAL4-PcACE1 chimeras (pFB1 to pFB10), wild type GAL4 (pMA210) or GAL4 DNA binding domain (pMA424) were introduced into a yeast strain lacking a functional GAL4 gene but bearing a Gal80 gene and an integrated GAL1::lacZ fusion gene (YM335::RY171). β-galactosidase activities were measured from cells grown in the presence of galactose, glycerol and ethanol. Each experiment was done 3 to 7 times except pFB6 and pFB7, which is the mean of three different clones.

Why are the pFB1, pFB2 and pFB3 clones inactive, in spite of the presence of all Cys and Ser-rich motifs? There are other examples in which the full-length transcription factor is inactive, in spite of the presence of the transactivation domain, for instance the proteins c-REL (Bull et al., 1990) and c-RELB (Ryseck et al., 1992). There are a few possible explanations for this result. First, Ace1 from *S. cerevisiae* is active only in the presence of copper (Furst and Hamer, 1989). Copper may cause a conformation change in PcACE1 by forming a cuprous thiolate, similar to Ace1 (Brown et al., 2002) and Mac1 (Jensen and Winge, 1998). It could also be that the addition of the DNA binding domain of GAL4 (147 amino acids), produces a steric hindrance that prevents the correct folding of the protein. On the other hand, pFB1 contains two different DNA binding sequences, one from GAL4, and the other from PcACE1. Each would try to bind to its target promoter, and the binding would be not productive. In the case of pFB2 and pFB3, there could be a competition for other necessary regulators. Site-directed mutagenesis of crucial amino acids will be able to determine the important amino acids.

Taken together, in this work, we show the transactivation domain of PcACE1 maps to the carboxy terminus, where two conserved Cys- and Ser-rich motifs are localized. Future experiments will help to better understand the transcriptional mechanisms involved in fungal copper homeostasis.

These data contribute to the understanding of how the copper levels are regulated in a basidiomycete fungus.

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