6896–6907 *Nucleic Acids Research, 2011, Vol. 39, No. 16* *Published online 17 May 2011*
doi:10.1093/nar/gkr228

Identification of a DNA-binding site for the transcription factor Haa1, required for *Saccharomyces cerevisiae* response to acetic acid stress

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Received December 14, 2010; Revised March 29, 2011; Accepted March 30, 2011

ABSTRACT

The transcription factor Haa1 is the main player in reprogramming yeast genomic expression in response to acetic acid stress. Mapping of the promoter region of one of the Haa1-activated genes, *TPO3*, allowed the identification of an acetic acid responsive element (ACRE) to which Haa1 binds *in vivo*. The *in silico* analysis of the promoter regions of the genes of the Haa1-regulon led to the identification of an Haa1-responsive element (HRE) 5′-GNN(G/C)(A/C)(A/G)G(A/G/C)G-3′. Using surface plasmon resonance experiments and electrophoretic mobility shift assays it is demonstrated that Haa1 interacts with high affinity (K_D of 2 nM) with the HRE motif present in the ACRE region of *TPO3* promoter. No significant interaction was found between Haa1 and HRE motifs having adenine nucleotides at positions 6 and 8 (K_D of 396 and 6780 nM, respectively) suggesting that Haa1p does not recognize these motifs *in vivo*. A lower affinity of Haa1 toward HRE motifs having mutations in the guanine nucleotides at position 7 and 9 (K_D of 21 and 119 nM, respectively) was also observed. Altogether, the results obtained indicate that the minimal functional binding site of Haa1 is 5′-(G/C)(A/C)GG(G/C)G-3′. The Haa1-dependent transcriptional regulatory network active in yeast response to acetic acid stress is proposed.

INTRODUCTION

The *Saccharomyces cerevisiae* transcriptional activator Haa1 was first included into a family of fungal copper-regulated transcription factors, based on the identification of a putative copper-regulatory domain within its DNA-binding domain (DBD; 1). Besides Haa1, this family also includes the *S. cerevisiae* Ace1 and Mac1 transcription factors, *Candida glabrata* Amt1 and *Schizosaccharomyces pombe* Cuf1 (2). Unlike its homologs, the function of Haa1 is independent of the copper-status of the cell (1) suggesting that its physiological function is not related to copper homeostasis. A biological role for Haa1 in yeast tolerance to acetic and propionic acids was established in a previous study (3). The expression of the *HAA1* gene was shown to reduce the duration of the adaptation period of a yeast cell population suddenly exposed to toxic concentrations of these two short chain carboxylic acids, by decreasing the weak acid-induced loss of cell viability (3). More recently, the role of Haa1 in tolerance to lactic acid was also demonstrated (4). Acetic, propionic and lactic acids are widely used by food and beverage industries as preservative agents. However, the activity of spoilage yeasts and molds resistant to these weak acids seriously limits their usefulness, also bringing major economic losses (5). Acetic acid is also a byproduct of *S. cerevisiae* alcoholic fermentation and together with high concentrations of ethanol and other toxic metabolites, acetic acid may contribute to fermentation arrest and reduced ethanol productivity (5). This weak acid is also present in lignocellulosic hydrolysates, a highly interesting non-feedstock substrate in industrial biotechnology (6).

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The molecular mechanisms underlying response and resistance to acetic acid and to other weak acids have been studied in *S. cerevisiae* to guide the design of more efficient preservation strategies and the engineering of more robust industrial strains to be used in processes in which yeast is explored as a cell factory and tolerance to acetic acid is required (5,7).

The transcriptional activation of 80% of the acetic acid-responsive genes is Haa1 dependent (8). This high percentage of direct or indirect Haa1 target genes points out this transcription factor as a key player in the control of yeast genomic expression program in response to acetic acid stress (8). The expression of a number of genes of the Haa1-regulon was found to confer yeast protection against acetic acid (8). Those having the more prominent effect were (i) *TPO2* and *TPO3*, encoding two plasma membrane transporters of the Major Facilitator Superfamily proposed to mediate the efflux of acetate from the cell interior during cultivation in the presence of acetic acid (3); (ii) *SAP30*, whose gene product is a subunit of the histone deacetylase Rpd3 complex, recently demonstrated to be involved in the regulation of yeast transcriptional response to environmental stress (9); and (iii) *HRK1*, which encodes a kinase belonging to a family of kinases involved in the post-translational regulation of plasma membrane transporters (10). The internal levels of acetic acid accumulated during growth of Δ*haa1* cells in the presence of the acid were higher than those registered in the parental strain (3), this being attributed to the reduced transcriptional activation of Haa1-target genes required for the reduction of the internal concentration of acetate (3,8). The objective of this work was the identification of the DNA motif used by Haa1 to activate the expression of acetic acid-responsive genes. The functional binding site of Haa1 is here described and the Haa1-dependent transcriptional regulatory network active in yeast response to acetic acid stress is proposed.

MATERIALS AND METHODS

Strains and growth media

Saccharomyces cerevisiae BY4741 (*MATa*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*), the deletion mutant BY4741_*Δhaa1* (*MATa*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*, *YPR008w::kanMX4*) and the *HAA1*-TAP strain (*MATa ade2 arg4 leu2-3,112 trp-289 ura3-52*) were all obtained from the Euroscarf collection. These yeast strains were batch-cultured, at 30°C, with orbital agitation (250 r.p.m.), in YPD growth medium (2% glucose, 2% bactopeptone and 1% yeast extract) or in minimal growth medium MM4 [contains, per liter, 1.7 g yeast nitrogen base without amino acids or NH_4^+, 20 g glucose, 2.65 g $(NH_4)_2SO_4$, 20 mg methionine, 20 mg histidine, 60 mg leucine and 20 mg uracil]. *Escherichia coli* BL21-CondonPlus(DE3)-RIL cells [genotype B F^- *ompT hsdS*(rB- mB-) *dcm*$^+$ *Tet*^{r} *gal* λ(DE3) *endA* Hte (*argU ileY leuW* Cam^r)] (Stratagene) were used for the over-expression of Haa1(DBD)-His_6 fusion protein.

Plasmids

A list of plasmids used in this study is available in Table 1. The construction of the *lacZ* fusion plasmid p*TPO3*::*lacZ* in the cloning vector pAJ152 was described before (3). Plasmids p*TPO3(-790)*::*lacZ*, p*TPO3(-590)*::*lacZ* and p*TPO3(-400)*::*lacZ* were constructed by cloning the 790, 590 and 400 bp DNA region upstream of *TPO3* start codon in the BamHI/HindIII sites of pAJ152 vector. p*ACRE-CYC1*::*lacZ* plasmid was constructed by cloning the acetic acid responsive element (ACRE) found in *TPO3* promoter (located between positions −790 to −590 upstream of its start codon) into the XhoI/XbaI sites of the pNB404 vector (11). Plasmid pACRE*-*CYC1*::*lacZ* was obtained by site-directed mutagenesis of the Haa1 responsive element (HRE) present in ACRE, using pACRE-*CYC1*::*lacZ* as template. Plasmid

Table 1. List of plasmids used in this study

Plasmid name	Description	Reference
p*TPO3*::*lacZ*	Expression fusion plasmid in which 1000 bp *TPO3* promoter were fused with *lacZ* coding sequence in pAJ152 basal vector	(3)
p*TPO3(-790)*::*lacZ*	Expression fusion plasmid in which 790 bp *TPO3* promoter were fused with *lacZ* coding sequence in pAJ152 basal vector	This study
p*TPO3(-590)*::*lacZ*	Expression fusion plasmid in which 590 bp *TPO3* promoter were fused with *lacZ* coding sequence in pAJ152 basal vector	This study
p*TPO3(-400)*::*lacZ*	Expression fusion plasmid in which 400 bp *TPO3* promoter were fused with *lacZ* coding sequence in pAJ152 basal vector	This study
pNB404	UAS-less vector containing the minimal promoter *CYC1* fused to *lacZ* coding sequence	(11)
pACRE-*CYC1*::*lacZ*	pNB404-derived vector that contains the ACRE region from *TPO3* promoter cloned upstream of minimal promoter *CYC1*;	This study
pACRE*-*CYC1*::*lacZ*	Vector derived from pACRE-*CYC1*::*lacZ* containing the HRE motif mutated	This study
pACRE (-740/-590) *CYC1*::*lacZ*	pNB404-based vector that contains the last 150 bp of the ACRE region found in *TPO3* promoter (between nucleotides −740 and −590) cloned upstream of minimal promoter *CYC1* and of *lacZ* coding sequence	This study
pACRE (-790/-690) *CYC1*::*lacZ*	pNB404-based vector that contains the first 100 bp of the ACRE region found in *TPO3* promoter (between nucleotides −790 and −690) cloned upstream of minimal promoter *CYC1* and of *lacZ* coding sequence	This study
pET23a(+)-*HAA1*	IPTG-inducible plasmid that drives the expression of Haa1 DNA-binding domain (N-terminal 123 residues) fused to a six histidine C-terminal tag	This study

pHaa1(DBD)::His_6 was obtained by cloning the DBD of Haa1 mapped to the N-terminal 123 residues of the protein (1), in the XhoI/BamHI sites of pET23a(+) expression vector (Novagen).

Determination of *TPO3* expression levels using *lacZ* fusion plasmids

The expression of the *lacZ* reporter gene produced in yeast transformants harboring the *lacZ* fusions with truncated regions of *TPO3* promoter was determined based on the quantification of β-galactosidase activity. Cells harboring the p*TPO3*::*lacZ*, p*TPO3(-790)*::*lacZ,* p*TPO3(-590)*::*lacZ* and p*TPO3(-400)*::*lacZ* plasmids were cultivated until mid-exponential phase (OD_{600nm} = 0.8 ± 0.05) in MM4 growth medium (at pH 4.0, using HCl as the acidulant) lacking uracil for plasmid maintenance. These cells were re-inoculated, at an OD_{600nm} of 0.2 ± 0.05, into this same basal medium either or not supplemented with acetic acid (60 mM). A stock solution of 5 M acetic acid (prepared in water and adjusted to pH 4.0 with NaOH) was used to supplement the growth medium. After 8 h of incubation in the presence or absence of acetic acid, cells were harvested by filtration, washed two times with water and the filters were kept at −20°C until further use. The determination of β-galactosidase activity produced from plasmids p*TPO3*::*lacZ*, p*TPO3(-790)*::*lacZ,* p*TPO3(-590)*::*lacZ* and p*TPO3(-400)*::*lacZ* in these cells was performed as described before (12). The assessment of the expression of *lacZ* gene (through β-galactosidase activity) in yeast cells harboring p*ACRE-CYC1*::*lacZ* or p*ACRE***-CYC1*::*lacZ* plasmids in the presence or absence of acetic acid stress (40 mM) was performed using the same protocol. Since the *CYC1* promoter is very weak and produces low levels of background expression of *lacZ* reporter gene (11), a lower concentration of acetic acid having a milder toxic effect on cell viability had to be used to assess β-galactosidase activity in yeast cells transformed with pNB404-based plasmids.

In vivo binding of Haa1 to *TPO3* promoter region in the presence or absence of acetic acid stress

In vivo binding of Haa1 to the promoter region of *TPO3* gene was assessed by chromatin immunoprecipitation (ChIP). For this, *HAA1*-TAP cells, which express Haa1 fused to a C-terminal tandem affinity purification (TAP) tag, were cultivated in YPD growth medium (adjusted at pH 4.0 with HCl) until mid-exponential phase (OD_{600nm} = 0.8 ± 0.05) and then re-inoculated in fresh YPD growth medium (at pH 4.0; used as control culture) or in this same basal medium supplemented with acetic acid (60 mM). The volume of inoculum used was calculated to obtain cell suspensions with an OD_{600nm} of 0.2 ± 0.05. After 30 min of cultivation in the presence or absence of acetic acid, 1% formaldehyde was added to both cultures and cells were left to grow for another 30 min. After that time, 125 mM glycine were added to both cellular suspensions for quenching and incubation proceeded for another 5 min. Cells were then harvested by centrifugation (8000 r.p.m., 5 min, 4°C, rotor JA20 Beckman), washed with Tris buffer (at pH 8.0) and frozen at −80°C until further use. DNA extraction and subsequent immunoprecipitation was performed as described before (13). Reversed cross-linked input and output DNAs were purified with polymerase chain reaction (PCR) purification columns (Qiagen). Two complementary oligonucleotides (I_1 5′-TTCTCTGTGCTTGG CGAGGGGTTTACTGGAGCCCAATC-3′ and I_2 3′-A AGAGACACGAACCGCTCCCCAAATGACCTCGG GTTAG-5′) were selected for the amplification of the ACRE region found in *TPO3* promoter using as templates the purified input and output DNAs collected. A DNA fragment within the *QDR3* promoter was used as a negative control since this gene is not regulated by Haa1 (our unpublished data).

Expression and purification of Haa1(DBD)-His_6 peptide in *E. coli*

Codon-optimized *E. coli* BL21 RIL cells (DE3, pLysS) (Stratagene) were transformed with plasmid p*HAA1*(*DBD*)::His_6 to yield expression of Haa1(DBD)-His_6 peptide. Transformants were cultivated at 37°C with orbital agitation (250 r.p.m.) in LB growth medium, supplemented with chloramphenicol (30 μg/ml) and ampicillin (150 μg/ml), until exponential phase (OD_{640nm} = 0.6 ± 0.05). At that point, 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) were added to the culture to induce the expression of the Haa1(DBD)-His_6 fusion peptide and cells were left to grow for another 3 h. Cells were harvested by centrifugation (8000 r.p.m., 8 min, 4°C, rotor JA20 Beckman), washed with ice-cold distilled water and frozen at −80°C until further use. Crude protein extracts were obtained by sonication and subsequent centrifugation at 18 000*g* for 1 h at 4°C. The clarified supernatant was loaded into a His-trap column (GE Healthcare) previously equilibrated with 10 ml of ice-cold washing buffer [10 mM phosphate buffer (pH 7.4), 150 mM NaCl, 10 mM imidazole). Column washing was started with 5 ml of washing buffer and elution of the Haa1(DBD)-His_6 peptide was performed using a stepwise increasing gradient of imidazole concentration (in the range of 20–500 mM) in the wash buffer. The fractions containing the purified peptide were recovered, concentrated using Amicon ultracel filters (molecular weight cutoff of 10 kDa) (Millipore) and applied in a 75 Sephadex HR10-300 GL3-kDa–70-kDa column (GE Healthcare) for subsequent fast protein liquid chromatography (FPLC) purification. The fractions containing the purified protein were collected, concentrated and the protein concentration in each fraction was determined using Protein Assay Reagent (BioRad). Confirmation that the collected protein corresponded to the the Haa1(DBD)-His_6 peptide was performed by western-blot using an antibody against the histidine tag (acquired from Santa Cruz Biotech).

Electrophoretic mobility shift assays

The *in vitro* interaction of Haa1(DBD)-His_6 peptide with the HRE found in *TPO3* promoter was carried out by electrophoretic mobility shift assays (EMSAs). For this, 10 pmol of a 20-bp sequence of *TPO3* promoter that

spans the HRE motif (specifically from −770 to −790 bp of *TPO3* promoter) was radiolabeled at their 5′-terminus with [γ-^{32}P]-ATP using T4 phage kinase (Invitrogen). After labeling, 11 pmol of the complementary oligonucleotide were added to the suspension and the mixture was left at room temperature for 2 h to promote the annealing of both oligonucleotides. The oligoduplex obtained was purified with ProbeQuantG columns (GE Healthcare). The *in vitro* interaction assays were carried out in 20 µl of final volume using 10 fmol of the radiolabeled oligoduplex and a range of 0–25 ng of the purified Haa1(DBD)-His$_6$ peptide. The binding buffer used was 65 mM KCl, 10% glycerol, 10 mM Tris (pH 8.0), 0.025% Nonidet P40 and 20% bovine serum albumin. Binding reactions were left for 20 min at 30°C and then loaded on a 8-cm × 7-cm 6% acrylamide gels (pre-run at 100 V for 1 h). The gels were run at 11°C and 150 V until the bromophenol blue dye had migrated at least two-thirds of the way down the gel. The temperature of the electrophoresis was carried out at 11°C to prevent an eventual complex denaturation during the course of the electrophoresis as the result of the heating that could be generated during running of the samples in the acrylamide gel, a procedure that is been used before with success in the study of the interactions established between yeast transcription factors and their target genes (14). In other trials, the electrophoresis was run without temperature control, but this did not lead to an alteration of the results obtained. The signal obtained in the gels was acquired by laser-based imaging system using the Typhoon Trio equipment.

Measurement of dissociation constants of Haa1 HRE complexes by surface plasmon resonance

The DBD of Haa1, over-expressed and purified in *E. coli* using the protocol described above, was immobilized by the amine coupling procedure in flow cell 2 of the CM5 sensor chip (GE Healthcare) on a Biacore 2000 system (GE Healthcare), according to the manufacturer's instructions. Briefly, the surface was activated with a 1:1 mixture of 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS), injected during 5 min using a flow rate of 10 µl/min. Then, 5 µg/ml of Haa1 DBD were injected during 5 min at the same flow rate. After the injection of the ligand, ethanolamine was injected over the surface to deactivate it. The immobilization of the protein originated a response of 900 RU. On flow cell 1, no protein was immobilized and this cell was used as the control blank cell. Biosensor assays were run at 25°C in the same buffer used to carry out the EMSA assays. The sequences of the 10 DNA oligomers used in the analysis are provided in Supplementary Table 1. Nine of them correspond to the HRE motif and respective mutations and the last one is a random sequence, which was used as a negative control. The DNA oligomers were injected over flow cells 2–1 for 5 min at concentrations of 10, 25, 50, 100 and 200 nM using a flow rate of 20 µl/min. The dissociation was allowed to occur during 4 min in the running buffer. All experiments included triple injections of each oligomer concentration to determine the reproducibility of the signal. Bound oligomers were removed after each cycle with a 30-s wash with NaOH 50 mM. The effect of these washes with NaOH in Haa1 activity was assessed by performing five independent injections of one of the DNA oligos interspersed by a washing step. The replicas performed were almost coincident indicating that the binding partners were stable throughout the time required for the experiment and that the regeneration conditions were appropriate. After each cycle, the signal was stabilized during 1 min before a new oligomer injection. Data from flow cell 1 was used to correct for refractive index changes and nonspecific binding. Rate constants were calculated using the BIAEVALUATION 3.0 software package by fitting the sensograms obtained for each DNA oligomer to a 1:1 Langmuir binding model. A chi-square test was performed to estimate the reliability of the fitting and values below 10, considered by the manufacturer to be the maximum acceptable value for proper data fitting, were obtained for all the sensograms analyzed. K_D value was calculated as the ratio between k_d and k_a. Each DNA-oligomer assay was run at least three times each to determine the robustness of the data obtained. The values presented in Table 2 are the average of these three independent assays and the error presented corresponds to the associated standard deviation between these three experiments. To further confirm the results obtained, the interaction between Haa1 and the HRE motif present in *TPO3* promoter (considered the wild-type HRE) was performed by immobilizing the DNA oligomer from *TPO3* promoter that spans the HRE motif biotinylated at its 5′terminus in a streptavidin-coated chip (Biacore SA; General Healthcare). The Haa1 DBD peptide was then injected over it. On flow cell 1 no substrate was added so this could be used as the control blank cell and on flow cell 2 we immobilized the biotinylated DNA oligomer. The target substrate was captured on flow cell 2 by manually injecting 20 µl of a 500-nM solution of the substrate in reaction buffer at a 20 µl/min flow rate. The immobilization of the DNA sequence originated a response of 300 RU. The assays were run at 25°C in the buffer described above. The Haa1 DBD peptide was injected over flow cells 1 and 2 for 5 min at concentrations of 20, 25, 30, 35, 40 and 45 nM using a flow rate of 20 µl/min. The dissociation was allowed to occur for 4 min in the running buffer. All experiments included triple injections of each protein concentration to determine the reproducibility of the signal. Bound protein was removed with a 30-s wash with 2 M NaCl. After each cycle, the signal was stabilized during 1 min before a new protein injection. Data from flow cell 1 was used to correct for refractive index changes and nonspecific binding. The values of k_a, k_d and K_D were estimated using the same methodology as described above.

RESULTS

TPO3 promoter contains an upstream activating sequence necessary for Haa1 dependent *TPO3* transcriptional activation in response to acetic acid stress

The promoter region of *TPO3* gene was used to map the possible existence of one (or more) *cis*-acting regulatory

Table 2. Definition of the minimal functional Haa1-binding site

HRE motif	k_a (1/Ms) ($\times 10^4$)	k_d (1/s) ($\times 10^{-4}$)	K_D (nM)	Relative K_D
GGCGAGGGG	4.5 ± 0.3	1.1 ± 0.1	2.0 ± 0.2	1.0
AGCGAGGGG	7.6 ± 0.5	1.5 ± 0.2	1.9 ± 0.3	1.0
GGCCAGGGG	11.0 ± 1.0	4.9 ± 0.7	4.3 ± 0.3	2.2
GGCGCGGGG	7.1 ± 0.3	9.7 ± 0.5	13.6 ± 0.9	6.8
GGCGAAGGG	0.39 ± 0.02	13.0 ± 2.0	396.0 ± 51.1	198.0
GGCGAGAGG	3.9 ± 0.6	11.0 ± 2.0	21.4 ± 6.1	10.7
GGCGAGGCG	5.2 ± 0.3	21.0 ± 2.0	38.5 ± 4.5	19.3
GGCGAGGAG	0.33 ± 0.06	320.0 ± 30.0	6780.0 ± 865	3390.0
GGCGAGGGA	4.2 ± 0.2	54.0 ± 3.0	119.0 ± 3.6	59.5
Negative control	0.01 ± 0.002	130.0 ± 10.0	>10 000	>5000

The affinity of Haa1 to HRE motifs predicted from the degenerate consensus sequence obtained by *in silico* analysis of the promoter region of genes of the Haa1 regulon (shown in Figure 3) was compared by surface plasmon resonance. The association and dissociation rates (k_a and k_d) of complexes established between Haa1 and 37-bp DNA sequences harboring these motifs were obtained by fitting the sensograms to a Langmuir 1:1 model (Supplementary Figures S1A and S2), as detailed in 'Materials and Methods' section. The K_D values were calculated as the ratio between the dissociation and association constants. The error associated to this value is the standard deviation of three independent experiments that were carried out to study the interaction of Haa1 DBD peptide with each oligonucleotide. Relative K_D values were calculated relative to the value obtained for the HRE motif present in *TPO3* promoter (GGCGAGGGG) to which Haa1 was found to bind *in vivo* (Figure 1B). A random DNA sequence was used as a negative control.

element(s) that could be used by Haa1 as binding site(s). *TPO3* was selected with this objective among other genes regulated by Haa1 because it is a proposed direct Haa1 target gene (1) and it is induced in response to acetic acid in a Haa1-dependent manner (3,8). Moreover, a functional *TPO3*::*lacZ* fusion plasmid (p*TPO3*::*lacZ*), suitable for easy assessment of *TPO3* expression, was available (3). The acetic acid-induced transcriptional activation of *TPO3* was compared in cells carrying the p*TPO3*::*lacZ* plasmid (which contains the full-length *TPO3* promoter fused to *lacZ*) and in cells harboring plasmids p*TPO3(-790)*::*lacZ*, p*TPO3(-590)*::*lacZ* and p*TPO3(-400)*::*lacZ*, in which progressive 5′-truncations, of ~200 bp each, of *TPO3* promoter were performed (Figure 1A). In wild-type cells transformed with the p*TPO3*::*lacZ* plasmid the levels of *TPO3* expression where ~5-fold higher in acetic acid-stressed cells than in control cells (Figure 1A). However, in Δ*haa1* cells no significant increase in *TPO3* expression was found upon acetic acid challenge (Figure 1A) consistent with the described involvement of Haa1 in the activation of *TPO3* transcription under acetic acid stress (3,8). Elimination of the −790 to −590-bp portion of *TPO3* promoter abolished the Haa1-dependent transcriptional activation of this gene induced by acetic acid (Figure 1A), suggesting that this region contains an acetic acid responsive element (ACRE). ChIP analysis of the ACRE region within *TPO3* promoter (–590 to −790) confirms that Haa1 binds *in vivo* to this region under acetic acid stress as well as in unstressed yeast cells (Figure 1B).

ACRE confers an acetic-acid-responsiveness to the minimal promoter *CYC1*

To examine whether ACRE could regulate a heterologous reporter gene in an acetic acid-dependent-manner, this DNA region was cloned, in its natural orientation, in the UAS-less vector pNB404::*lacZ* (Figure 2). pNB404 is a UAS-less vector that contains only the minimal transcriptional and translational initiation sites from the *CYC1* gene, leading to a very low background expression of the reporter gene *lacZ* (11). Fusion of the ACRE sequence to *CYC1* promoter was sufficient to confer an acetic-acid-dependent regulation to *lacZ* expression, under the dependence of *HAA1* expression (Figure 2).

ACRE contains a DNA motif enriched in the promoter region of Haa1-regulated genes that is a functional binding site for Haa1

The results support the conclusion that the ACRE sequence found in *TPO3* promoter includes a functional binding site for Haa1, here designated HRE. To narrow down the ACRE to a binding site, truncated versions of this DNA element were cloned in the pNB404 vector and the acetic-acid-responsiveness of these constructs was compared with the one provided by a full-length ACRE sequence (Figure 2). The results obtained clearly show that the putative HRE is located between nucleotides −690 and −590 of *TPO3* promoter (Figure 2). To identify the HRE motif within this 100-bp region, the promoter regions (considered to be the 1000-bp upstream of start codon) of the 85 genes activated in response to acetic acid stress in a Haa1 dependent manner (8) were searched for enriched DNA motifs, using the AlignACE DNA motif finder (Aligns Nucleic Acid Conserved Elements; http://atlas.med.harvard.edu/) (15), using a strategy that is detailed in ref. 16. The algorithm identified a number of over-represented DNA motifs in the data set but only one of these motifs, 5′-GNN(G/C)(A/C)(A/G)G(G/C/A)G-3′, was within the 100-bp region of ACRE present in *TPO3* promoter presumed to harbor the HRE (Figure 3A). Reinforcing the idea that this motif is the functional binding site for Haa1, its mutation in *TPO3* promoter abrogated the Haa1-dependent acetic-acid-responsiveness of the pACRE-*CYC1*::*lacZ* construct (Figure 3B). To confirm that Haa1 interacts with the HRE motif identified in *TPO3* promoter an EMSA was carried out. For this, the Haa1 DBD was produced in *E. coli* and purified by immunoaffinity chromatography and FPLC. The

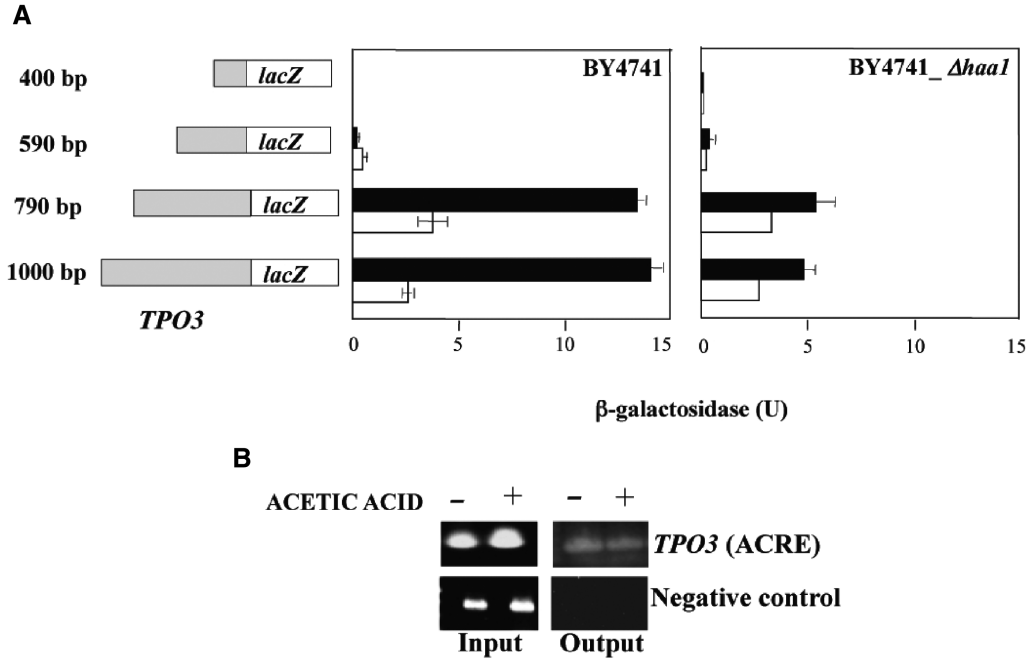
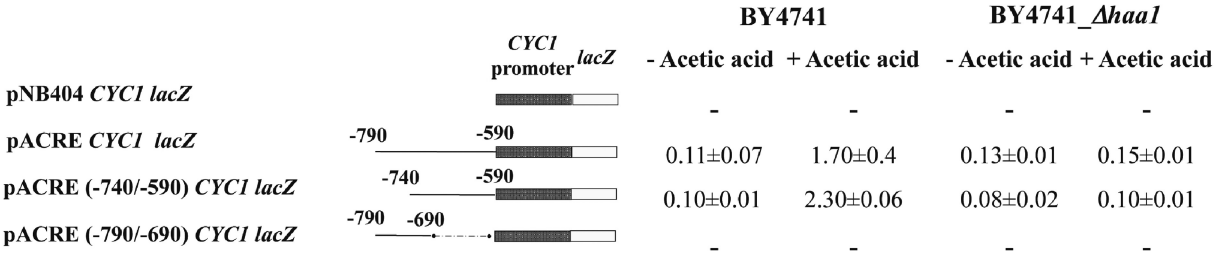


Figure 1. (**A**) Mapping of an upstream activating sequence underlying the Haa1 dependent activation of *TPO3* transcription under acetic acid stress. Progressive 5′ deletions, of ~200 bp each, of *TPO3* promoter were performed, according with the schematic representation shown, and the levels of β-galactosidase (U) produced from these truncated constructs were compared with those produced by a construct containing the full-length *TPO3* promoter (containing 1000 bp) in the absence (white bars) or presence of 60 mM acetic acid, at pH 4.0 (dark bars). Values are means of, at least, three independent experiments. (**B**) *In vivo* binding of Haa1 to ACRE region of *TPO3* promoter. Yeast cells expressing a Haa1–TAP fusion protein were cultivated for 30 min in the absence (–) or presence (+) of acetic acid and total DNA was extracted from these cells, immunoprecipitated with an anti-TAP antibody and purified (output). An aliquot of DNA not subjected to the immunoprecipitation step was used as a control (input). Oligonucleotides spanning the ACRE region of *TPO3* promoter were used to amplify the DNA signal in both extracts.



	BY4741		BY4741_*Δhaa1*	
	- Acetic acid	+ Acetic acid	- Acetic acid	+ Acetic acid
pNB404 *CYC1 lacZ*	-	-	-	-
pACRE *CYC1 lacZ*	0.11±0.07	1.70±0.4	0.13±0.01	0.15±0.01
pACRE (-740/-590) *CYC1 lacZ*	0.10±0.01	2.30±0.06	0.08±0.02	0.10±0.01
pACRE (-790/-690) *CYC1 lacZ*	-	-	-	-

Figure 2. To narrow down the ACRE sequence into a smaller DNA fragment, overlapping fragments of this DNA sequence, depicted by horizontal dark bars, were cloned in front of *CYC1* minimal promoter and upstream of *lacZ* reporter gene. The levels of β-galactosidase produced by these constructs were compared with the level produced by a construct containing the full ACRE sequence in the presence or absence of acetic acid stress. The different plasmids were named according to the number of the initial nucleotide of *TPO3* promoter included in the constructs, as described in Table 1. β-Galactosidase activities represent the average of three independent experiments and (–) stands for a non-detectable activity.

interaction of this peptide with ACRE was tested using an oligonucleotide from *TPO3* promoter that spans the candidate motif. EMSA experiments confirmed the existence of a strong interaction between the Haa1(DBD)–His_6 peptide and the HRE motif (Figure 4). A similar result was obtained using a full-length Haa1 (data not shown).

Definition of HRE motifs that serve as functional binding sites for Haa1

The results described above point out the degenerate DNA motif 5′-GNN(G/C)(A/C)(A/G)G(G/C/A)G-3′ as the functional binding site of Haa1. Surface plasmon resonance (SPR) was used to estimate the kinetic constants (k_a, association rate constant; k_d, dissociation rate constant; and K_D, dissociation constant) of the formation of complexes established between Haa1 and those DNA motifs predicted by the degenerate HRE sequence. This information is considered essential, as it will allow the identification of those HRE motifs that serve as functional Haa1 binding sites, also providing an important insight into the identification of bases required for an efficient Haa1–DNA complex formation. The mutations of the HRE motif were created by site-directed mutagenesis of the motif present in *TPO3* promoter, 5′-GGCGAGGGG

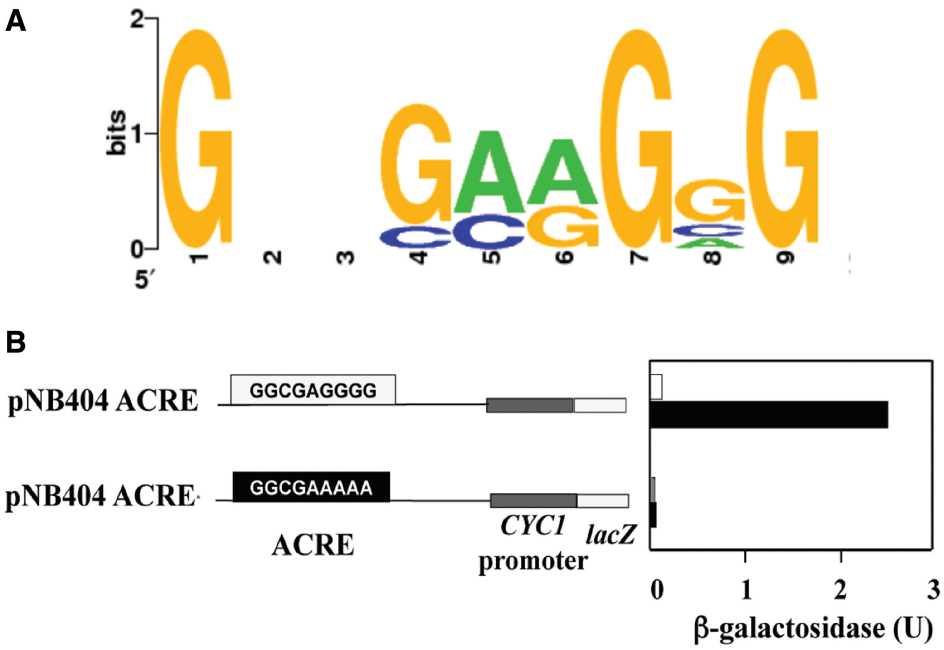


Figure 3. (**A**) Schematic representation of the DNA motif identified by the AlignAce algorithm as being enriched in the promoter region of the 85 genes activated by Haa1 in response to acetic acid stress (8). (**B**) Effect of the DNA motif identified by the referred bioinformatic analysis in the Haa1 dependent transcriptional activation of *TPO3* gene induced by acetic acid.

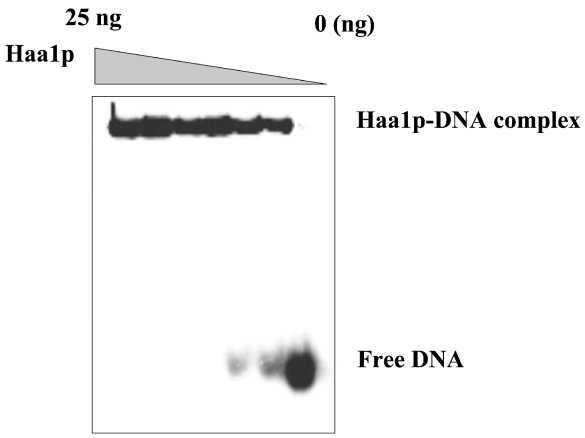


Figure 4. *In vitro* interaction of Haa1 with the HRE motif found in *TPO3* promoter sequence. Different amounts of the Haa1 DNA-binding domain, expressed in *E. coli* and purified by affinity chromatography and FPLC, were incubated for 20 min at 30°C with a ^{32}P-radiolabeled oligonucleotide from *TPO3* promoter containing the candidate Haa1 binding motif (GGCGAGGGG). The protein–DNA complexes were separated from free DNA probe by polyacrylamide gel electrophoresis and a representative result of the gels obtained is shown.

-3′ (considered to be the 'wild-type' HRE motif), since Haa1 was found in our study to establish a strong *in vitro* interaction with this DNA sequence (Figure 4). The sensograms obtained used to estimate the kinetic association and dissociation constants are shown in Supplementary Figures S1 and S2. Remarkably, among the HRE motifs tested the motif present in *TPO3* promoter was the one having the highest affinity to Haa1 (corresponding to the lowest K_D value obtained, 2 nM). By analyzing the k_a and k_d values we were also able to see that the Haa1–HRE complex associates rapidly and dissociates with a low velocity which is indicative of the formation of a stable complex (Table 2). To further confirm these results, the Haa1–HRE interaction was monitored using a reverse experimental strategy, that is, immobilizing the biotinylated oligo that contains the HRE motif in a streptavidin-coated chip and letting the peptide flow (Supplementary Figure S2). Expectedly, the values of the kinetic constants obtained, k_a $1.5 \times 10^5\,M^{-1}\,s^{-1}$ and k_d of $6.3 \times 10^{-4}\,s^{-1}$, are different but the resulting K_D value (4.3 nM) is close to the one that had been obtained using the protein has the immobilized ligand (which was 2.0 nM). A random DNA oligonucleotide was used as a negative control to test the specificity of the Haa1–HRE interaction. As expected, Haa1 had a highly impaired affinity for this DNA sequence, based on the low association rates (k_a of $1.1 \times 10^4\,M^{-1}\,s^{-1}$) and the high dissociation rates of the complex formed (k_d $1.3 \times 10^{-4}\,s^{-1}$) (Table 2). The affinity of Haa1 toward an HRE motif that harbors an adenine nucleotide at position 1 instead of a guanine was not affected (K_D of 1.9 nM, compared to 2 nM). Taking into account that k_a and k_d values are similar to the ones obtained for the 'wild-type' HRE motif, we may infer that the stability of the complex was not altered. This observation suggests that the capacity of Haa1 to recognize the HRE sequence is independent of the type of purine present at position 1. In fact, the first three nucleotides of the degenerate HRE motif should be dispensable

for Haa1 binding since no nucleotide conservation is found at positions 2 and 3 of this motif (Figure 3A). The replacement of the other conserved guanine nucleotides of the HRE motif at positions 7 and 9 for adenines had little effect on the Haa1–HRE association rate; however, these Haa1–'mutant HRE' complexes were less stable than a Haa1–'wild-type HRE', based on the higher complex dissociation rates obtained (Table 2). In particular, an almost 50-fold increase in dissociation rate was observed for the complex established between Haa1 and the GGCGAGGGA motif, suggesting that guanine nucleotide at position 9 is essential for efficient Haa1 binding. Consequently, HRE motifs with mutations in this position are unlikely to serve as functional Haa1 binding sites. Regarding the nucleotide degeneracy predicted at positions 4, 5 and 8 the results obtained demonstrate that although Haa1 has a lower affinity toward HRE motifs that differ from the one present in *TPO3* promoter, the values of the different kinetic parameters determined do not exclude the majority of the motifs tested as being functional binding sites (Table 2). The exceptions are the motifs having adenine nucleotide at position 8 (GGCGAGGAG) or at position 6 (GGCGA AGGG) which bind poorly to Haa1 (K_D of 6780 and 396 compared to 2.2 nM) (Table 2). Altogether, the results obtained with the SPR analysis indicate that the minimal functional Haa1 binding site is 5′-(G/C)(A/C)GG(G/C)G-3′.

Transcriptional regulatory network mediated by Haa1 in response to acetic acid stress

To reveal the transcriptional regulatory network governed by Haa1 under acetic acid stress we have searched the promoter region of the 85 genes whose expression is activated by Haa1 in response to acetic acid stress (8) for the HRE motif 5′-(G/C)(A/C)GG(G/C)G-3′. Approximately 55% of these Haa1-regulated genes harbor in their promoter region one or more copies of the minimal HRE motif suggesting that these are direct Haa1 targets (Figure 5A). Among these genes are *TPO3*, *TPO2, YPR157w* and *PHM8* (Figure 5A) which have been previously proposed to be directly regulated by Haa1 (1). In line with this, we have found that under acetic acid stress Haa1 binds *in vivo* to the promoter region of *TPO3* and *TPO2* genes (Figure 1B and our unpublished data). The effect of Haa1 in the transcriptional activation of genes that do not have a HRE motif is likely to be indirect and mediated by other transcription factors, whose expression is presumably regulated, directly or indirectly, by Haa1. Among the Haa1-activated genes under acetic acid stress that have a HRE motif in their promoter regions are *MSN4*, *FKH2* and *MCM1*, which encode three transcriptional activators (8). The genes presumed to be indirectly regulated by Haa1 were clustered with Msn4, Fkh2 and Mcm1, based on the existence of previous documented regulatory associations with these transcription factors, using the YEASTRACT database (17) (Figure 5B). Seven of the indirect Haa1 targets have documented regulatory associations with Msn4p, two with Fkh2p and two with Mcm1p (Figure 5B). However, for 19 Haa1 indirect targets it was not possible to find any association with these transcription factors (Figure 5B). Although the possibility exists that the description of Msn2-, Fkh2- and Mcm1-regulons is incomplete, this observation suggests the existence of additional transcription factor(s) contributing to the Haa1-dependent transcriptional regulatory network active under acetic acid stress (Figure 5B). Although not explicitly considered in the model, the combinatorial regulation of acetic-acid-responsive genes that are, directly or indirectly, regulated by Haa1, cannot of course be ignored.

DISCUSSION

In this study, it is demonstrated that the *S. cerevisae* transcription factor Haa1 requires the DNA sequence 5′-(G/C)(A/C)GG(G/C)G-3′ in the promoter region of acetic-acid-responsive genes to activate their expression. This DNA sequence, designated HRE, is the first functional binding site described for Haa1. Consistent with this result, Haa1 was found to interact with sequences harboring this HRE motif in a large-scale study carried out to identify the interactions occurring among a large number of yeast transcription factors and oligonucleotides containing evolutionary conserved sequences presumed to contain functional binding sites (14). Since the Haa1 DBD is highly homologous to Ace1 DBD it was thought that the two transcription factors could have similar DNA-binding sites (18), as found to occur with the Haa1 and Ace1 homologs Cuf1 and Amt1 (19,20). However, the Haa1-binding site here described does not resemble the Ace1 DNA target sequence. Other evidences also indicate that, *in vivo*, Haa1 and Ace1 bind to different DNA sequences, in specific, the over-expression of Haa1 in a mutant devoid of *ACE1* expression does not restore the expression of Ace1p target genes [(1) and our unpublished data] and no induction of Haa1-target genes is registered upon Ace1 overexpression in a Δ*haa1* background (our unpublished data). Consistent with these observations, no Ace1 binding site is found enriched in the promoter of Haa1-regulated genes and vice versa. Moreover, the sets of genes documented as being regulated by Ace1 and Haa1 do not overlap (17). It is likely that the Haa1 and Ace1 DBDs have a different structure, despite the homology of the amino acid sequence of these two peptides. The presence of a unique segment between residues 81 and 91 in Haa1 DBD could prevent the formation of a two-lobe structure similar to that of Ace1 (21). To bind DNA Ace1 requires the formation of a polycopper cluster within the DBD for stabilization of a functional conformer of the protein (18,21,22), thus activating Ace1p under copper-induced stress and maintaining an unstructured inactive form in the absence of copper stress (21,23). However, the activity of Haa1 is independent of the copper-status of the cell [(1) and our unpublished data] and the mutation of cysteine residues mediating copper binding in Ace1p does not reduce the ability of Haa1 to activate gene transcription (our unpublished data). These observations clearly indicate that the role played by copper in structuring Haa1 and Ace1 is

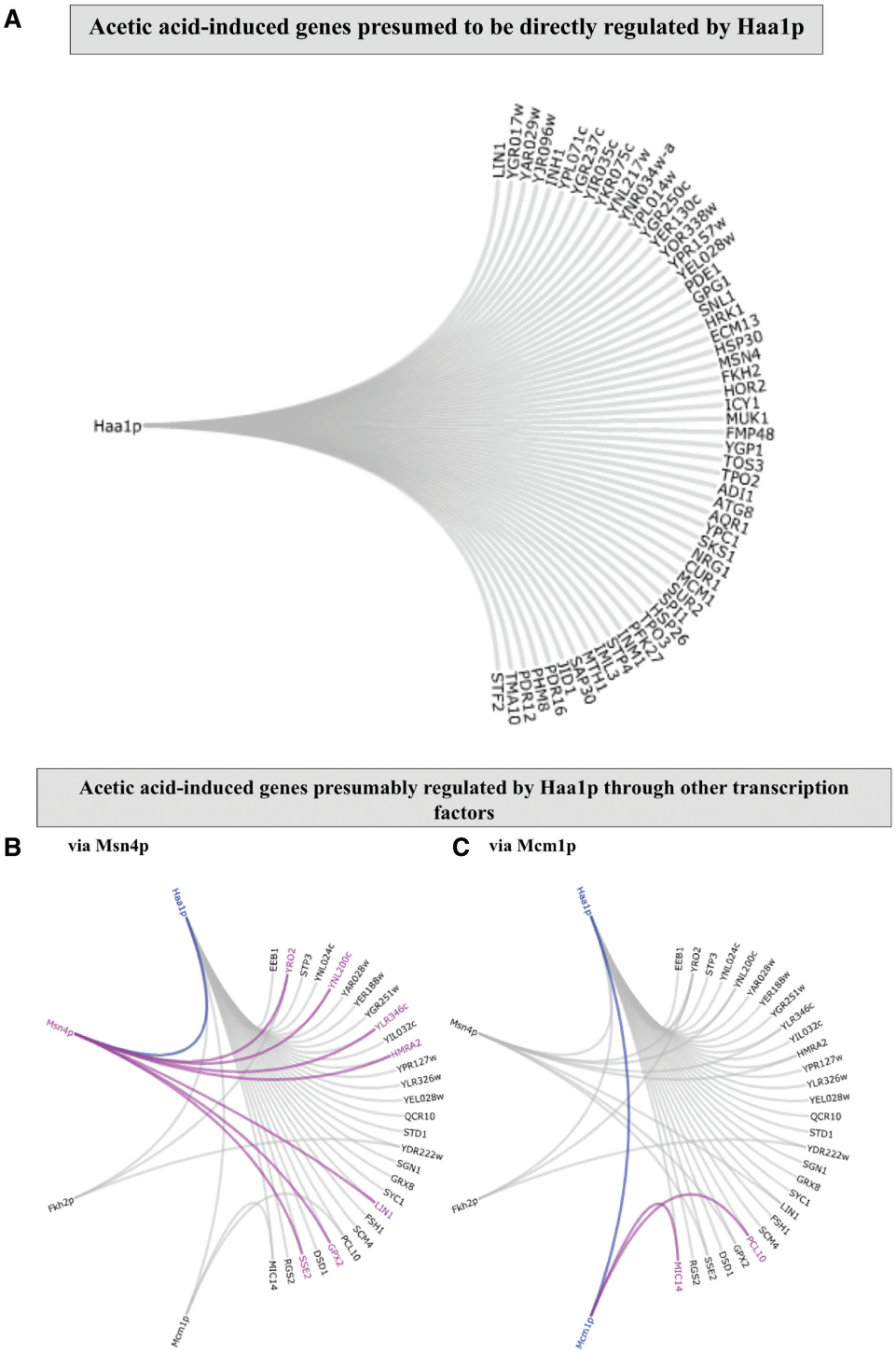


Figure 5. Putative Haa1 dependent transcriptional regulatory network active under acetic acid stress. The promoter region of the genes whose transcription was found to be activated by Haa1 in acetic acid-challenged cells was searched for the Haa1-binding motif (5′-(G/C)(A/C)GG(G/C)G-3′) using the YEASTRACT database. The genes that have at least one HRE motif in their promoter region, presumably the direct Haa1 target genes, are shown in (**A**). The documented targets of the Msn4p, Mcm1p or Fkh2p transcription factors were searched among the Haa1-activated genes that do not have a HRE motif in their promoter region (**B–D**). The non-direct Haa1-target genes which are also not documented targets of Msn4p, Fkh2p or Mcm1p were clustered under the putative regulation of an unidentified factor(s), X (**E**). All the transcriptional regulatory networks shown were built using the tools available in the YEASTRACT database (17).

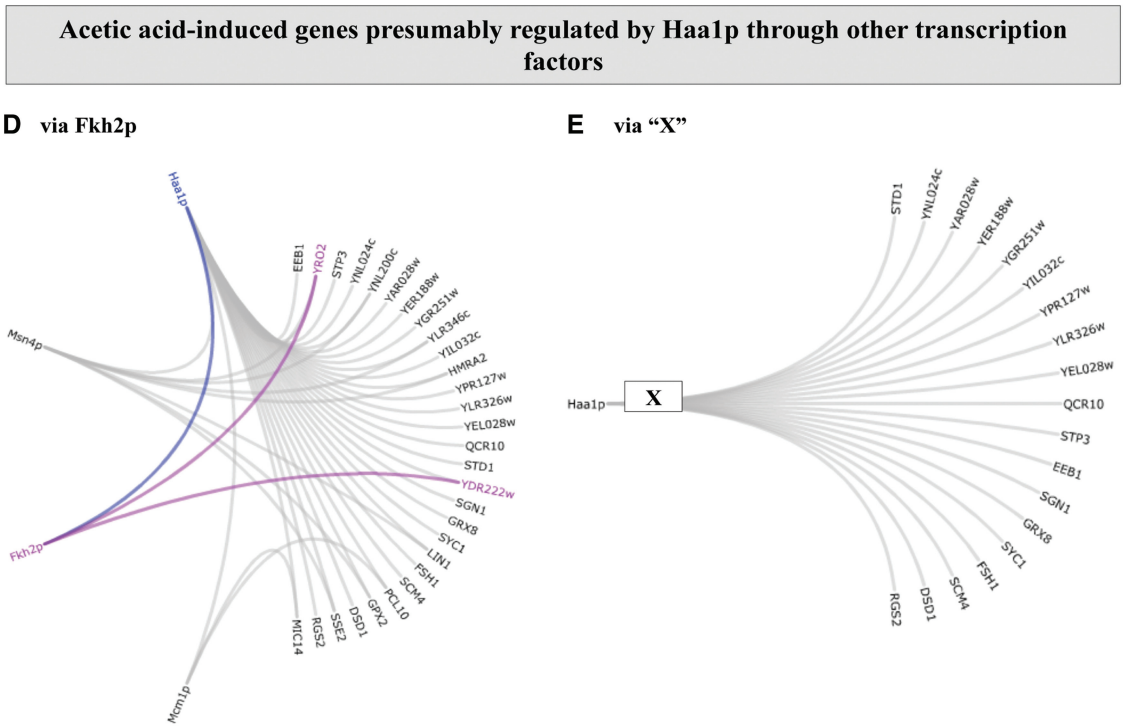


Figure 5. Continued.

different, although they do not exclude the possible stabilization of Haa1 DBD through the formation of a polycopper cluster.

One of the main differences between the Haa1 and Ace1 binding sites (5′-THNNGCTG-3′) is the absence in the HRE motif of a 5′-(A/T)-rich region which is essential for Ace1 binding and for binding of Cuf1 and Amt1 to their target DNA sequences (19–21,24). Ace1 was found to establish minor groove DNA contacts with this (A/T)-hook, through a RGRP motif (G = glycine; R = arginine; P = proline) present in its DBD (21,24,25), and strong major groove interactions with the guanine nucleotides of the GCTG core (18). A closer inspection of the promoter region of the Haa1 regulated genes harboring an HRE motif also shows that in 77% of the cases this motif is preceded by at least two adenine and/or thymine nucleotides. It is thus hypothesized that Haa1 may use these adenine or thymine nucleotides to promote minor groove contacts with HRE. Consistently, the RGRP motif is also present in Haa1 DBD between positions 36 and 39 and the mutation of arginine 38 abolishes the ability of Haa1 to induce gene expression and to bind the HRE motif (our unpublished data). Since the replacement of the conserved guanine nucleotides at positions 3 and 6 of the HRE motif (considered positions 6 and 9 in the degenerate HRE sequence) significantly destabilizes the Haa1–HRE complex, as assessed by the SPR analysis carried out, it is presumed that these nucleotides could mediate major groove contacts between Haa1 and the HRE motif.

It is interesting to note that no correlation was found between the number of HRE motifs having higher affinity for Haa1 in the promoter region of the Haa1-dependent genes and these genes' transcription. For example, the motif with the highest affinity to Haa1, 5′GAGGGG3′, is present in the promoter region of *TPO3*, *TPO2*, *STF2* and *AQR1* genes, whose transcriptional activation in response to acetic acid stress mediated by Haa1 range from 1.5-fold (for *AQR1*) to 18.7-fold (for *TPO2*) (8). This result suggests that other factors, besides DNA-binding affinity, are involved in the Haa1-dependent control of gene expression under acetic acid stress. Haa1 was found to bind to the promoter region of the *TPO3* gene independently of the presence of acetic acid in the growth medium (Figure 1B) and to be constitutively localized in the nucleus (our unpublished data). In the absence of acetic acid, no significant effect of *HAA1* expression in the yeast transcriptome was found, not even in the expression of genes activated in response to acetic acid stress in a Haa1-dependent manner, including *TPO3* (8). These observations suggest that Haa1 is apparently non-functional in unstressed yeast cells independently of its possible binding to the promoter region of target genes. The same behavior was described for War1 transcription factor, required for yeast adaptation and resistance to propionic and sorbic acids (26). War1 also binds to the promoter region of the *PDR12* gene but in the absence of these weak acids this transcription factor does not induce *PDR12* transcription (27,28). It is possible that the transactivation potential of Haa1 is enhanced under acetic acid stress leading to the increased recruitment of the transcriptional machinery to the promoter region of its target genes and, consequently, to their up-regulation, as demonstrated for Yap1 under benomyl stress (29) or

for War1 under propionic acid- and sorbic acid- induced stress (27). War1 activation was found to occur upon binding of sorbate and propionate anions (27), a mechanism also described for other xenobiotic compounds or cell metabolites and yeast transcription factors in particular for Pdr1p upon binding of ketoconazole (30), Leu3 upon binding of the intermediate of leucine biosynthesis α-isopropyl malate (31), Ppr1, upon binding of the pyrimidine biosynthesis intermediate dihydroorotic acid (32) and Lys14 upon binding of α-amino adipate semialdehyde (33). The possible interaction of Haa1 with acetate is an interesting possibility to explain the hypothesized activation mechanism of Haa1 under acetic acid stress.

A putative transcriptional regulatory network active under acetic acid stress and controlled by Haa1 is proposed in this work (Figure 5). Almost 55% of the genes whose transcription is upregulated under acetic acid stress in a Haa1-dependent manner are presumed to be primary targets of Haa1 as they harbour in their promoter at least one copy of the HRE motif. The effect of Haa1 in the expression of genes that do not have a HRE motif in their promoter is proposed to be mediated by Fkh2, Msn4 or Mcm1 transcription factors, whose transcription was found to be activated under acetic-acid-stress in a Haa1-dependent manner (Figure 5) (34). Msn4p, together with its close homolog Msn2p, mediates yeast transcriptional response to environmental stress (35), consistent with the classification of five (*YRO2*, *YNL200c*, *LIN1*, *GPX2* and *SSE2*) of the eight Haa1 indirect targets putatively co-regulated by Msn4 as stress-responsive genes, specifically (35). The transcription factor Fkh2p is involved in the regulation of genes related with the transition of G2/M phase and Mcm1 is involved in the regulation of genes related with cell mating type and pheromone response. Since a number of the putative indirect Haa1-target genes are not documented targets of Msn4, Fkh2 or Mcm1, the possible participation of an unidentified transcription factor (designated 'X') in the Haa1-mediated transcriptional regulatory network is anticipated, although no Haa1-dependent transcriptional activation of a gene encoding a transcription factor was detected (8). Interestingly, 10 out of the 19 genes clustered in the hypothetical 'X'-regulon are documented targets of Sfp1, a transcription factor involved in ribosome biogenesis in response to nutrient starvation and other stresses (36). Acetic acid stress was shown to induce a rapid degradation of ribosomal RNA (37) and a large set of genes involved in ribosome biogenesis were identified as determinants of resistance to acetic acid (34). However, the possible participation of Sfp1 in the control of the expression of the Haa1-regulated genes and/or the identification of other transcription factors that cooperate with Haa1 in the control of yeast genomic expression under acetic acid stress have to be proved. Nevertheless, results of the present study are an important contribution to the better understanding of the complexity of yeast transcriptional regulatory networks active under stress, in particular the one mediated by Haa1 in acetic-acid-challenged cells.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

FUNDING

FEDER, Fundação para a Ciência e a Tecnologia (FCT) and PTDC Program (contracts PTDC/AGR-ALI/102608/2008, PTDC/EBB-EBI/108517/200 and ERA-IB/0002/2010, in the framework of the FP6 EraNet Programme on Industrial Biotechnology INTACT (Integral Engineering for Acetic Acid Tolerance in yeast); a post-doctoral grant to N.P.M. (SFRH/BPD/46982/2008) and a PhD grant to R.G.M. (SFRH/BD/36024/2007); and a grant from the National Institute of Health (contract ES03817 to D.R.W.). Funding for open access charge: Fundação Ciência e Tecnologia-PTDC program.

Conflict of interest statement. None declared.

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