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Chromatin-mediated transcriptional regulation by the yeast architectural factors NHP6A and NHP6B

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The *Saccharomyces cerevisiae* NHP6A and NHP6B proteins are chromatin architectural factors, functionally and structurally related to the mammalian high mobility group (HMG)-1 and -2 proteins, a family of non-sequence-specific DNA binding proteins. *nhp6a nhp6b* mutants have various morphological defects and are defective in the induced expression of several RNA polymerase II-transcribed genes. We found that NHP6A/B proteins are also required for full induction of the yeast *CHA1* gene. Importantly, *CHA1* basal level expression is increased 10-fold in an *nhp6a nhp6b* double deletion mutant. Micrococcal nuclease and DNase I analysis of the *CHA1* gene in this strain showed an open promoter structure, characteristic of the activated state of this promoter, even under non-inducing conditions. To address the possible function of the NHP6A/B proteins in chromatin-mediated gene regulation, we performed whole-genome transcriptional profiling of a Δ*nhp6a* Δ*nhp6b* yeast strain. Our results suggest that NHP6A/B proteins play an important regulatory role, repressing as well as potentiating expression of genes involved in several cellular processes, and that NHP6A/B control is exerted at the level of the individual gene.

Keywords: *CHA1*/chromatin/HMG-like proteins/*Saccharomyces cerevisiae*/transcriptional regulation

Introduction

The bulk of eukaryotic DNA is found assembled into chromatin. Although one important function of chromatin is to compact DNA, it has become apparent that chromatin structure also plays an important role in transcriptional regulation. Nucleosomes, the structural subunits of chromatin, provide a means to control the accessibility of regulatory factors to cognate sites and potentiate interactions between distant regulatory elements (Grunstein, 1992; Felsenfeld *et al*., 1996; Wolffe *et al*., 1997). Sequence-specific regulatory factors might function, at least in part, by counteracting or enhancing chromatin-mediated repression. To date, studies on chromatin structure have focused primarily on histone–DNA interactions. Non-histone chromatin proteins provide an extra layer of possible interactions, adding to the functional and structural complexity of the chromatin fiber (Bustin and Reeves, 1996; Bustin, 1999; Bianchi and Beltrame, 2000).

The *Saccharomyces cerevisiae CHA1* gene encodes the catabolic L-serine (L-threonine) dehydratase, which is responsible for biodegradation of serine and threonine. We have shown that expression of the *CHA1* gene is transcriptionally induced by serine and threonine, and that two promoter elements, $UAS1_{CHA}$ and $UAS2_{CHA}$, are required and sufficient to confer serine/threonine inducibility to yeast genes (Bornæs *et al*., 1993). Furthermore, the two *cis*-acting sequences are bound by Cha4p, a gene-specific transcriptional activator (Holmberg and Schjerling, 1996). In previous studies we used accessibility to modifying nucleases, such as micrococcal nuclease (MNase) and DNase I, to determine the *in vivo* chromatin structure of the *CHA1* chromosomal locus, both in the non-induced state and upon induction. Thus, we reported that upon activation, a precisely positioned nucleosome (nuc-1) occluding the TATA box and the transcription start site at *CHA1* is removed, and that this structural transition is independent of the SWI/SNF or ADA complex (Moreira and Holmberg, 1998).

Recent studies have shown that transcriptional regulation by some eukaryotic enhancers requires the assembly of a specific nucleoprotein complex likely to involve architectural factors (Paull *et al*., 1993; Tjian and Maniatis, 1994; Yie *et al*., 1999). To further our understanding of the molecular mechanisms behind the structural transitions at the *CHA1* promoter and their relationship to the process of gene activation, we investigated the role of architectural factors in this process.

Two non-sequence-specific DNA-binding proteins, non-histone proteins 6A and 6B (NHP6A/B), have been identified in *S.cerevisiae* in a search for proteins that could functionally replace HU in Hin-mediated DNA inversion (Paull and Johnson, 1995). Two highly related genes, *NHP6A* and *NHP6B*, encode the NHP6A/B proteins and deletion of both genes is required for any observable phenotype (Costigan *et al*., 1994). NHP6A/B are members of the high mobility group (HMG)-1 and -2 family proteins, and are required for activated gene expression, both *in vitro* and *in vivo*, of a subset of RNA polymerase II-transcribed genes (Paull *et al*., 1996). HMG-1 and -2 are very abundant chromatin-binding proteins, shown to bind substrates with a wide minor groove, such as four-way junctions, severely undertwisted DNA, *cis*-platinated DNA, and DNA at the entry and exit points of nucleosomes (Wright and Dixon, 1988; Bianchi *et al*., 1992; Pil and Lippard, 1992; Bustin, 1999; Bianchi and Beltrame, 2000). Although the exact cellular function of HMG-1/-2 proteins remains unknown, stimulatory (Stoute and Marzluff, 1982; Tremethick and Molloy, 1988; Singh

and Dixon, 1990; Shykind *et al*., 1995) and inhibitory (Ge and Roeder, 1994; Stelzer *et al*., 1994) effects on transcription, as well as on *in vitro* nucleosome assembly and chromatin organization, DNA repair and recombination, have been described (Bonne-Andrea *et al*., 1984; Nightingale *et al*., 1996; van Gent *et al*., 1997; Melvin and Edwards, 1999).

In this study we report on the effect of deleting the yeast genes encoding HMG-1/-2-like proteins (NHP6A/B) on *CHA1* chromatin structure and transcriptional activity, as well as on global gene expression.

Results

Basal and activated CHA1 gene expression in a Δnhp6a Δnhp6b double mutant

The yeast NHP6A/B proteins are members of the HMG-1/2 family of proteins, a group of proteins reportedly involved in both the repression and mediation of *in vitro* nucleosome assembly (reviewed in Bustin *et al*., 1990). The conflicting reports from various *in vitro* experimental approaches have long obscured the biological function(s) played by HMG-1/-2 proteins. It is widely accepted, nonetheless, that these proteins play a structural role in the regulation of chromatin (Bustin, 1999).

Expression of the yeast *CHA1* gene is induced ~100-fold by the presence of serine in the growth medium (Ramos and Wiame, 1982; Petersen *et al*., 1988). Northern blot analysis of total RNA isolated from wild type (wt) and a Δ*nhp6a* Δ*nhp6b* (Δ) strain showed that *CHA1* transcript levels in cells grown under inducing conditions were reduced by ~70% in the mutant strain compared with the isogenic wild type (Figure 1, upper panel, Δ +Ser and wt +Ser, respectively). We also analyzed transcript levels from the *CHA1* gene in cells grown under non-inducing conditions, and detected no difference between the wild-type and mutant cells (Figure 1, upper panel, wt –Ser and Δ –Ser, respectively). Thus, activated expression of *CHA1* depends on the presence of NHP6A and/or NHP6B proteins.

Any moderate effect of the NHP6A/B proteins on *CHA1* basal level transcription would not be noticeable in our northern blot analysis since *CHA1* basal transcription is very low. To address this point we examined the expression pattern of the *CHA1* gene in the Δ*nhp6a* Δ*nhp6b* strain using a reporter construct. We transformed the mutant and its isogenic wild type with a centromeric reporter plasmid, pTK120 (a *CHA1::lacZ* translational fusion described in Bornæs *et al*., 1993). Levels of β-galactosidase activity were measured with or without the addition of serine to the growth medium (Figure 1, lower panel). Consistent with the results for *CHA1* mRNA levels, activation of the *CHA1–lacZ* construct was reduced by ~50% in *nhp6b nhp6b* cells compared with its wild-type counterpart (Figure 1, lower panel, compare standard deviation of Δ +Ser with wt +Ser). Interestingly, the basal level expression of the *CHA1–lacZ* fusion construct was increased 10-fold in the mutant strain (Figure 1, lower panel, compare standard deviations of Δ with wt). These results show that NHP6A/B proteins play a dual role in the regulation of the *CHA1* gene. They potentiate *CHA1* transcriptional activation in the presence of serine and repress *CHA1* basal expression under non-inducing growth conditions.

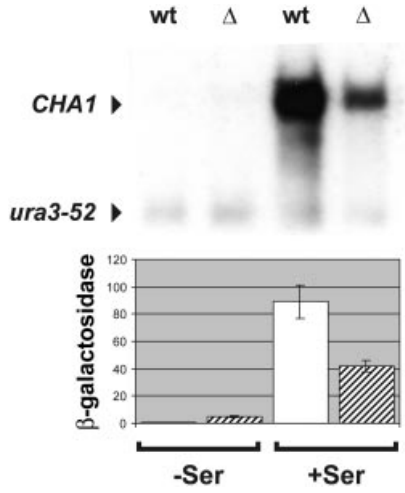

Fig. 1. Transcriptional activity of the *CHA1* gene in cells lacking the NHP6A and NHP6B proteins. Upper panel: northern blot analysis of a Δ*nhp6A* Δ*nhp6B* mutant strain (RJY6012; Δ) and an isogenic wild-type strain (SEY6210; wt). Ten micrograms of total RNA isolated from cells grown in the absence (–Ser) or presence (+Ser) of serine were electrophoresed in a 1.5% formaldehyde–agarose gel, blotted and hybridized with ^{32}P-labeled probes for the *CHA1* and *URA3* genes. Lower panel: β-galactosidase assay of a reporter plasmid, pTK120, containing a *CHA1::lacZ* translational fusion. Cells grown in the absence (–Ser) or presence (+Ser) of serine were harvested and β-galactosidase activity determined.

Chromatin structure of the CHA1 gene in a Δnhp6a Δnhp6b strain

Induction of *CHA1* expression in the presence of serine in the growth medium is mediated by the activator Cha4p through two *cis*-acting elements, $UAS1_{CHA}$ and $UAS2_{CHA}$, present in the promoter region (Holmberg and Schjerling, 1996). A striking chromatin transition takes place at the *CHA1* gene upon induction (Moreira and Holmberg, 1998), with the remodeling of a nucleosome (nuc-1) positioned over the putative TATA box (positions –132 and –82) and transcription start site (position –20), and disarrangement of the nucleosomes arrayed over the coding sequence (schematized in Figure 2). The observed chromatin remodeling in this promoter is independent of the SWI/SNF and ADA/GCN5 complexes (Moreira and Holmberg, 1998), two multimeric complexes implicated in counteracting chromatin-mediated repression (reviewed in Winston and Carlson, 1992; Pazin and Kadonaga, 1997a, b; Struhl, 1998).

To investigate whether the decrease in activated expression of *CHA1* in the *nhp6a nhp6b* mutant reflects a defective activation-dependent chromatin remodeling of the *CHA1* promoter, MNase and DNase I digests of RJY6012 (Δ*nhp6a* Δ*nhp6b*) and SEY6210 (wild type) cells were carried out (Figure 3, Δ and wt, respectively). We obtained the previously observed band pattern in the wild type in the absence (Figure 3, wt –Ser) or presence (Figure 3, wt +Ser) of serine, namely a strong hypersensitive site in the 5′ flank and an ordered nucleosomal array covering both the coding region and the TATA box in uninduced cells. Upon induction, the nucleosome covering

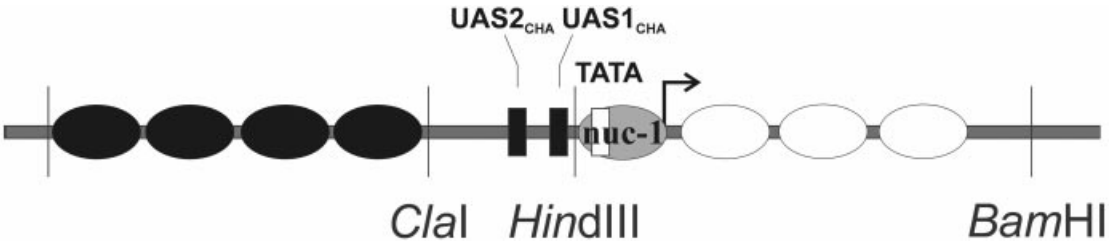

Fig. 2. Chromatin organization of the *CHA1* gene. Nucleosomes are depicted as filled ellipses, or as open ellipses for nucleosomes rearranged upon induction, and the nucleosome covering the TATA box, displaced upon induction, is marked nuc-1. Relevant restriction sites and *cis*-acting sequences are shown. $UAS1_{CHA}$ and $UAS2_{CHA}$ are represented by filled rectangles, and the TATA element by an open rectangle.

the TATA region is remodeled and the pattern of the coding region becomes diffuse. The mutant strain, however, showed a drastic effect of the NHP6 proteins on *CHA1* chromatin. MNase and DNase I digests of mutant nuclei (Figure 3, Δ) yielded patterns different from those of the isogenic wild type (Figure 3, compare Δ with wt). Mutant cells grown under non-inducing as well as inducing conditions show an expanded hypersensitive region with absence of nuc-1 (Figure 3, Δ) and display a disarrayed nucleosomal assembly over the coding sequence. The observed chromatin structure at the *CHA1* promoter in cells grown under non-inducing conditions correlates well with the 10-fold increase in basal expression in the *nhp6a nhp6b* double mutant. The fact that nuc-1 is remodeled in *nhp6a nhp6b* cells grown under non-inducing conditions could explain the measured increase in basal level expression. We have shown previously that in the *CHA1* promoter the process of nucleosome disruption precedes that of transcriptional initiation (Moreira and Holmberg, 1998). Our observation that the *CHA1* promoter is remodeled under non-inducing conditions in a strain lacking NHP6A and NHP6B suggests that these proteins participate in control of the stability of the TATA-occluding nucleosome in the *CHA1* promoter.

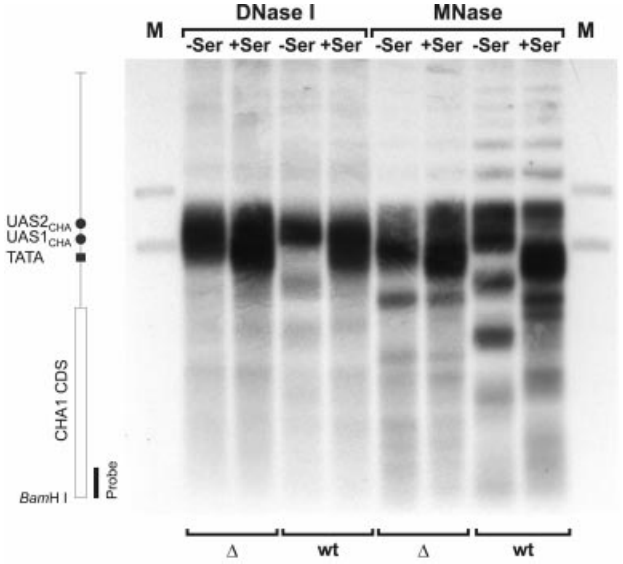

Fig. 3. Chromatin analysis of the *CHA1* gene in the Δ*nhp6a* Δ*nhp6b* double mutant (RJY6012; Δ) and the isogenic wild-type strain (SEY6210; wt). MNase- and DNase I-based mapping of nucleosome organization was carried out as described previously (Moreira and Holmberg, 1998). Cells were grown in the absence (–Ser) or presence (+Ser) of serine, nuclei purified and digested for 10 min with 20 U/ml DNase I or 100 U/ml MNase. DNA was isolated, digested with *Bam*HI, separated on a 1% agarose gel, blotted and hybridized with a ^{32}P-labeled *CHA1*-specific PCR amplificate. Lanes M contain restriction enzyme double digests of genomic DNA with *Bam*HI and *Cla*I or *Bam*HI and *Hin*dIII, to generate position marker fragments. The vertical map indicates the relative positions of the various *cis*-acting sequences and the *CHA1* coding sequence (CDS).

Chromatin structure of the PHO5 gene in a Δnhp6a Δnhp6b strain

The yeast *PHO5* gene is a well studied case of a promoter possessing a nucleosome placed over the TATA box, which is involved in gene regulation (reviewed in Svaren and Hörz, 1997). Gene-specific activator binding and nucleosome disruption are two separate events in the *CHA1* promoter, with the former preceding the latter (Moreira and Holmberg, 1998). In the remodeling of the *PHO5* promoter by the activator Pho4p, however, these two functions seem to be linked (Svaren and Hörz, 1997). Paull *et al*. (1996) demonstrated that induced expression of the *PHO5* gene is marginally decreased (80% of wild type) and that basal level expression is very slightly increased (~2.5-fold) in an *nhp6a nhp6b* strain. To discover whether the increase in basal transcription of the *PHO5* gene correlated with remodeling of the nucleosomal structure of its promoter, we examined the structure of the *PHO5* gene in *nhp6a nhp6b* cells grown under non-inducing conditions by MNase analysis. In spite of the slight increase in basal transcription in the *nhp6a nhp6b* strain (Paull *et al*., 1996; data not shown) we could not observe any difference between the *PHO5* promoter structure in this strain and its wild-type counterpart (data not shown). Since the effect that the deletion of *NHP6A/B* has on *PHO5* basal transcription is quite small (~2.5-fold), it is possible that only a fraction of cells have increased expression and chromatin remodeling of the *PHO5* gene. Our analysis could then conceivably reflect a mixture of two structures, with a minor remodeled form being obscured by a predominant assembled form. To investigate this possibility, we used accessibility of the *PHO5* promoter to the restriction endonuclease *Cla*I, an assay shown previously to reflect the extent of disruption of nucleosome –2 at the *PHO5* promoter (Almer *et al*., 1986). In both the *nhp6a nhp6b* strain (Δ) and its wild-type counterpart (wt), accessibility to *Cla*I was <10% (Figure 4), showing that the *PHO5* promoter is not remodeled in the mutant strain.

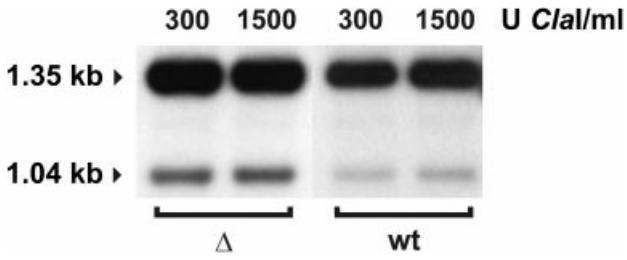

Fig. 4. Chromatin accessibility analysis of the *PHO5* promoter in cells lacking NHP6A and NHP6B. Restriction enzyme-based mapping of nucleosome organization was carried out essentially as described in the legend for Figure 3, except that isolated nuclei were digested for 30 min with *Cla*I. DNA was isolated, digested with *Apa*I, separated on a 1% agarose gel, blotted, and hybridized with a ^{32}P-labeled *PHO5*-specific PCR amplificate. A 1.35 kb *Apa*I fragment is generated in the absence of cleavage by *Cla*I, and a 1.04 kb fragment is generated if the *Cla*I site is accessible.

Thus, the alteration in chromatin structure observed at the *CHA1* promoter in *nhp6a nhp6b* cells is not due to a defect in a general chromatin component.

Genome-wide expression patterns in cells lacking the NHP6A/B proteins

The completion of the sequencing project for the *S.cerevisiae* genome made it possible to quantitate mRNA levels for all identified open reading frames (ORFs) (DeRisi *et al*., 1997; Wodicka *et al*., 1997). We used commercially available high-density arrays containing a total of 6144 yeast ORFs spotted on two nylon membrane microarrays (filters I and II) to quantitate global mRNA levels in yeast cells devoid of NHP6A/B proteins. If one considers only those genes whose expression changed >3-fold in two independent experiments, 197 genes corresponding to 3.2% of the yeast genome are affected in the *nhp6a nhp6b* double mutant compared with the isogenic wild-type strain (Table I). Of these, 114 (1.9%) are up-regulated and 83 (1.4%) are down-regulated in the *nhp6a nhp6b* double mutant. Analysis of the affected ORFs revealed some interesting patterns of expression. The ORFs that show the greatest differences in expression (>50-fold) are mostly **a**-specific genes, genes involved in mating-type determination that are normally expressed at very low levels or not at all in α cells (e.g. *ASG7* or *MFA1*). One field from a microarray membrane is shown in Figure 5, for both RJY6012 (*nhp6a nhp6b*; Δ) and SEY6210 (wild type; wt), with differentially expressed mating genes highlighted for comparison. In total, 21 genes involved in the pheromone response pathway show differential expression in the *nhp6a nhp6b* mutant (Table I, shown in red, bold typeface). Moreover, nine pheromone-regulated genes required for yeast mating differentiation (Erdman *et al*., 1998) are up-regulated in the mutant strain (Table I, boxed genes). Interestingly, both sets of genes show only transcriptional derepression in *nhp6a nhp6b*. A third group of genes identified in our analysis was that of genes involved in methionine biosynthesis (Table I, shown in blue, bold typeface), with both up- and down-regulated genes. We could not discern any further functional grouping in the remaining genes, which could reveal an effect of NHP6A/B proteins on any additional cellular process. Thus, NHP6A/B proteins affect the transcription of several genes, positively as well as negatively, involved in a variety of cellular processes, but particularly those implicated in the mating response and methionine biosynthesis, and pheromone-regulated genes.

Northern blot analysis of selected loci in nhp6a nhp6b double mutants

To assess the validity of the results obtained through our genome-wide analysis, we examined the expression of some of the deregulated loci using northern blot analysis. One locus, *ILV1*, which had shown no difference in expression in our microarray analysis, was used as hybridization control. We isolated total RNA from strains RJY6012 (*MAT*α Δ*nhp6a* Δ*nhp6b*; Δ *MAT*α) and SEY6210 (isogenic wild-type; wt *MAT*α) and investigated the transcript levels of various genes by northern blotting. Additionally, since we had observed a considerable effect on **a**-specific genes, we included a *MAT***a** Δ*nhp6a* Δ*nhp6b* mutant strain (DY2382; Δ *MAT***a**) and its isogenic wild-type strain (DY150; wt *MAT***a**) in this analysis. Northern blot analysis of these strains (Figure 6) reiterates the effects we had observed with the high-density array analysis (Table I). Thus, **a**-specific genes such as *ASG7*, *STE2*, *BAR1*, *AGA2* and *MFA1* display very low or undetectable levels of transcription in SEY6210 (*MAT*α wt), but robust expression in RJY6012 (*MAT*α Δ*nhp6a* Δ*nhp6b*), resulting in very large ratios of induction, similar to those observed in our microarray assay. Interestingly, with the exception of *ASG7*, which shows a slight increase (3-fold), these genes show no difference (*STE2*, *AGA2* and *FIG2*) or even a slight decrease (2-fold for *BAR1* and *MFA1*) in expression in the **a** mating-type strain (Figure 6; cf. DY150 and DY2382). Other loci, such as *FUS3* and *PCL2*, show ratios of induction in *MAT*α *nhp6a nhp6b* of 4- and 6-fold, respectively, and somewhat lower levels of derepression (2- and 3-fold, respectively) in *MAT***a** *nhp6a nhp6b*. As expected from the microarray analysis, *MET14* expression is decreased in *MAT*α *nhp6a nhp6b* (12-fold). Also, in the case of *MET14*, transcript levels are unaltered in the *MAT***a** *nhp6a nhp6b* mutant. We concluded that the effects we had observed in our whole genome analysis correctly reflect the patterns of gene expression in yeast cells lacking NHP6 proteins. Furthermore, for some genes the deregulation is mating-type dependent.

Pheromone production in Δnhp6a Δnhp6b double mutants

Our genome-wide analysis of the Δ*nhp6a* Δ*nhp6b* mutant strain revealed substantial differences in the expression of genes involved in the pheromone response pathway. To determine whether the observed transcriptional effects had a physiological effect, we used a previously described plate assay for pheromone production (Sprague, 1991). Briefly, the strains to be tested are spotted onto a lawn of cells from an **a**-factor tester strain (*MAT*α *sst2*) and an α-factor tester strain (*MAT***a** *bar1*). These tester strains are hypersensitive to pheromone and will not grow in the immediate vicinity of a pheromone-producing strain of the opposite mating type, thus resulting in a clear zone or halo around the spotted cells.

We spotted strains RJY6012 (*MAT*α Δ*nhp6a* Δ*nhp6b*; Δ *MAT*α), SEY6210 (*MAT*α wild-type; wt *MAT*α), DY2382 (*MAT***a** Δ*nhp6a* Δ*nhp6b*; Δ *MAT***a**) and DY150 (*MAT***a**

Table I. Genes that display differential expression in a Δ*nhp6A* Δ*nhp6B* mutant strain

Genes with increased expression in RJY6012 (Δ*nhp6A* Δ*nhp6B*)			Genes with decreased expression in RJY6012 (Δ*nhp6A* Δ*nhp6B*)			Fold difference
AGA2 T	ASG7 S,T	BAR1 T				>50
KAR4 S,T	MFA1 T	MFA2 T				
STE2 T	YML047c	YNL279w S,T				
AGA1 S	FIG2 S,T	FUS1 S,T	MET10	MET14		20-50
FUS2 T						
HEM2	NHP6B	RBK1	CYC1	HXT2	INO1 S	9-20
SAG1 S	YFL027c	YGL052w	MEF2	MET3 T	SAM2	
YGL053w	YGL090w S	YHL045w	STP4 S	URP2	YER069w	
YIL080w T	YIL082w T	YIR039c	YER156c	YHR095w	YML058w	
YJR150c T	YMR107w T	YOL091w				
YPL156c						
AFR1 T	LEE1	NRG1	SAR1	YDR134c S	YGR053c	6-7
PCL2 T	SOM1	STE3 S	YNL179c			
YAR031w	YDL085w	YER106w				
YH028w	YHR180w	YIL117c T				
YLL055w T	YPL272c					
CYB2	PGM2	SLT2	ACB1	CYS4	SAM1	5
SUL1 S,	TEC1 S	YAR053w	YBR077c	YDL039c	YDL060w	
YDR125c T	YIL011w T	YKL161c T	YGL054c	YGR260w	YHR133c	
YMR065w	YMR206w T	YNL042w	YJL159w	YMR145c	YNL141w S	
YNR004w	YPR015c					
AMD2	BOI2	FAR1 S,T	AGP1 S	COX8	CPH1	4
FET3 S,T	GCV1 S	GPA1 S	CYH2	HIS1	MDH1	
LEU1	MDH2	PDE1	PIR3 T	PMP2	RNR2 T	
PRY2	PTP2	STE12	SUI2	SUR4	UBI4	
YBR066c	YGL051w	YGP1 S,T	YBR106w	YDR083w	YDR100w	
YHL046c T	YIL165c S	YJL105w	YDR101c	YHR148w	YLR074c	
YKR091w	YLL047w	YLR042c S	YNL174w	YPL158c S	YPR028w	
YMR096w	YMR097c	YNR068c				
YOR382w T	YPL033c					
HMLα1 S	ARG3 S	CDC13	ATP16	CHO1	COX17	3
CUP9	FIG1 S,T	FUS3 S,T	FUN30	GLN4	GNS1	
MET28	MFα2 S	PAU2 T	HHF2	HXK1 S	IPP1	
PAU5 T	POS5	RPI1 S	MRPL31	PBI2 S	PMI40	
SLI15	YBL062w	YBR032w	POL30	RPA12	RPL5A	
YCR041w S	YDL239c	YDR124w	SLF1	SSA1	SSA2	
YDR249c	YDR453c	YDR542w T	SSE1	STI1	USS1	
YGL261c T	YGR294w T	YIL176c T	YBR025c	YBR089w	YCR016w	
YIR035c	YLL064c T	YLR205c	YGR142w	YHR104w	YIL158w	
YLR231c	YML117w	YMR041c	YKL195w	YLR301w	YML093w	
YOR080w			YOR286w	YPR126c		

[S]Genes with differential expression in *swi/snf* mutants (Sudarsanam *et al*., 2000).
[T]Genes with deregulated expression in cells lacking Tup1p (DeRisi *et al*., 1997).

wild type; wt *MAT***a**) onto a lawn of either MT503 (*MAT*α *sst2*; **a**-factor tester strain) or XP635-10C (*MAT***a** *bar1*; α-factor tester strain) cells (Figure 7). Strains RJY6012 and SEY6210 show a halo in the α-factor tester strain but not in the **a**-factor tester strain, corresponding to production of α- but not **a**-pheromone by these strains. This is not surprising in the case of SEY6210 since this is an α strain; however, in the case of RJY6012 we had observed transcription of **a**-specific genes including *MFA1* and *MFA2* (Table I), the genes encoding **a**-pheromone, and would thus expect this strain to produce **a**-pheromone as well. Seemingly, expression of *MFA1* and *MFA2* is not sufficient to allow RJY6012 to secrete biologically active **a**-pheromone. Additionally, the halo produced by RJY6012 is substantially smaller (~2- to 3-fold) than that of its isogenic counterpart. Even though this plate-based assay is of a qualitative rather than quantitative nature, the greatly reduced size of the halo implies a considerable reduction in α-pheromone secretion, which is probably due to the increased expression of *BAR1* (the

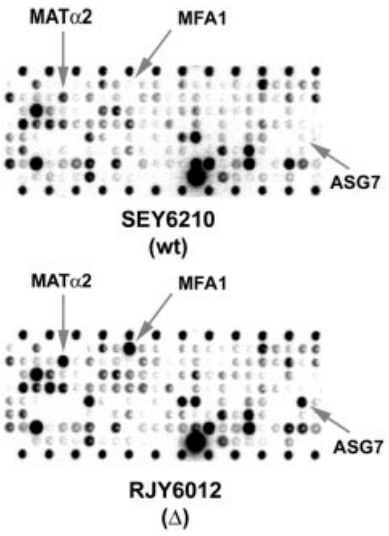

Fig. 5. Transcriptional analysis by microarray hybridization. Membranes containing 6144 yeast ORFs were hybridized with labeled RNA from strains RJY6012 (*MAT*α Δ*nhp6a* Δ*nhp6b*) or SEY6210 (*MAT*α wt). One field from such a membrane is shown, with genes involved in the mating response and found to be deregulated in the mutant strain indicated by gray arrows.

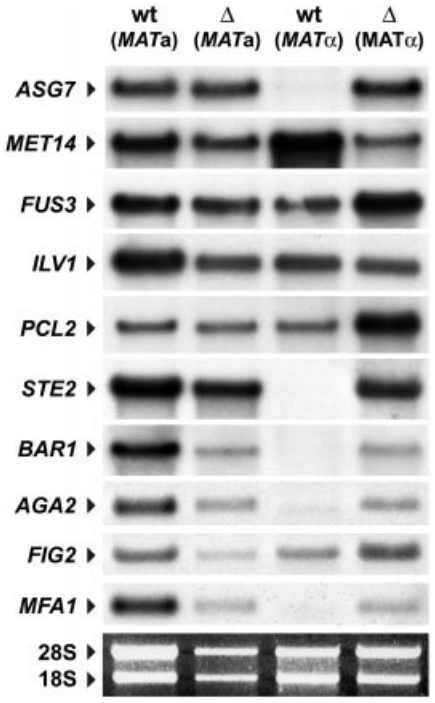

Fig. 6. Expression of various loci in cells lacking NHP6A and NHP6B. Northern blot analysis of a *MAT*α Δ*nhp6a* Δ*nhp6b* mutant strain [Δ (*MAT*α); RJY6012] and its isogenic wild-type counterpart [wt (*MAT*α); SEY6210] plus a *MAT***a** Δ*nhp6a* Δ*nhp6b* mutant strain [Δ (*MAT***a**); DY150] and its isogenic wild-type counterpart [wt (*MAT***a**); DY2382]. Ten micrograms of total RNA isolated from cells grown in minimal medium were electrophoresed in a 1.5% formaldehyde–agarose gel, blotted and hybridized with ^{32}P-labeled probes for the different genes. An ethidium bromide staining of the gel is shown as loading control.

barrier protease that degrades α factor) in *nhp6a nhp6b*. Conversely, DY150 and DY2382 show a halo in the **a**-factor tester strain but not in the α-tester strain, corresponding to production of **a**- but not α-pheromone by these strains. Again, we observed a reduction (~50%) in the size of the halo produced by the *MAT***a** *nhp6a nhp6b* mutant strain (DY2382) compared with the isogenic wild type (DY150), consistent with the down-regulation of *MFA1* in this strain. These results lead us to conclude that even though expression of **a**-specific genes, including those coding for **a**-pheromone, is greatly up-regulated in *MAT*α *nhp6a nhp6b* cells, the strain maintains its apparent mating type and secretes only α-pheromone, albeit to a lesser extent. Similarly, the *MAT***a** *nhp6a nhp6b* mutant maintains its apparent mating type and secretes only **a**-pheromone.

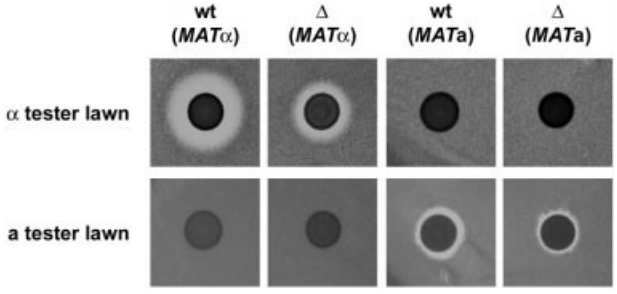

Fig. 7. Halo assay for production of mating pheromone. Ten microliters of a cell suspension (~1 × 10^{10} cells/ml) from strains RJY6012 (*MAT*α Δ*nhp6a* Δ*nhp6b*), SEY6210 (*MAT*α wt), DY2382 (*MAT***a** Δ*nhp6a* Δ*nhp6b*) and DY150 (*MAT***a** wt) were spotted onto a plate, grown overnight at 30°C and replica plated onto a lawn of 1–5 × 10^7 cells of either MT503 (**a**-factor tester strain) or XP635-10C (α-factor tester strain) cells. Plates were then incubated at room temperature for 2–4 days.

Discussion

In this article we present data showing that NHP6A and/or NHP6B proteins are required not only for full activation but also for repression of basal expression of the *CHA1* gene, and for nucleosome positioning under non-inducing conditions, suggesting a role for these proteins in chromatin organization *in vivo*. Furthermore, a genome-wide analysis of cells lacking NHP6A/B identified various genes with deregulated levels of expression, with some discrete subsets of genes being affected. The pattern of gene expression in *nhp6a nhp6b* cells, with 114 up-regulated genes and 83 down-regulated genes, shows that these proteins are generally required for both activation and repression of target genes. These data suggest an important role of NHP6A/B in chromatin-mediated gene regulation, both positive and negative, in several yeast cellular processes.

Regulation of CHA1 gene expression and chromatin structure by NHP6A/B

The stimulatory function of NHP6A and/or NHP6B on *CHA1*-activated transcription, determined by northern blot analysis and β-galactosidase measurements (Figure 1, cf. wt +Ser and Δ +Ser), is in accord with the previously reported observation that expression of a number of RNA polymerase II-transcribed genes is impaired in an *nhp6a nhp6b* mutant. This effect was proposed to be due to the

lack of formation at the TATA box of a TBP–TFIIA–NHP6A/B complex with increased affinity for TFIIB (Paull *et al*., 1996), a process probably similar to that observed with HMG-1/2 and the *in vitro* activated transcription of the adenovirus major late promoter (Shykind *et al*., 1995). Activated transcription of the *CHA1* gene is dependent on the presence of a functional TATA element (at position –82), and activation-impaired TBP mutants, defective in interactions with TFIIA, TFIIB and the TATA box, abrogate activated *CHA1* expression (Moreira and Holmberg, 1998). Thus, the drastic decrease in induced *CHA1* expression observed in the *nhp6a nhp6b* mutant is readily explained, assuming a similar mechanism of activation for this gene to that already suggested, with NHP6A/B proteins participating in a TBP–TFIIA–NHP6A/B ternary complex with increased affinity for TFIIB. But what of the difference in *CHA1* basal transcription and chromatin structure in Δ*nhp6a* Δ*nhp6b* cells?

The increase in *CHA1* basal expression in Δ*nhp6a* Δ*nhp6b* cells under non-inducing growth conditions (Figure 1, cf. wt –Ser and Δ –Ser) suggests the NHP6A/B proteins somehow to be involved in the repression of this gene. HMG-1/-2 proteins have previously been shown to act as repressors of class II transcription, either by forming an HMG1–TBP–DNA complex capable of inhibiting pol II transcription *in vitro* (Ge and Roeder, 1994) or by HMG-2-mediated repression after assembly of a TBP–TFIIA–promoter complex (Stelzer *et al*., 1994). However, these two mechanisms are unlikely to explain the observed loss of repression at *CHA1*. The *CHA1* gene has a precisely positioned nucleosome (nuc-1; see Figure 2) that occludes the TATA box under non-inducing growth conditions (Figure 3, wt –Ser), thus reducing basal transcription of this gene to undetectable levels. Therefore, formation of a repressive complex including NHP6A/B, and TBP–TFIIA–DNA or TBP–DNA cannot be viewed as a possible mechanism of action at the *CHA1* gene, since formation of such a complex is incompatible with the observed presence of nuc-1 at the *CHA1* promoter in a non-induced, wild-type strain (Figure 3, wt –Ser). A more likely function for NHP6A/B at the *CHA1* promoter is that these proteins are required either for assembly of a repressive nucleoprotein complex (e.g. nucleosome) or function as auxiliary co-repressors, facilitating the recruitment/binding of a repressive factor(s) working through chromatin (e.g. histone deacetylase activity).

Alternatively, the expression of some general chromatin factor might be impaired in the *nhp6a nhp6b* double deletant, which would also result in an altered chromatin structure at the *CHA1* promoter and elevated levels of basal expression due to increased accessibility of the TATA box. If this were the case, then we would expect to see a similar situation at other loci, also normally associated with nucleosomes under non-inducing conditions. We chose to examine the *PHO5* gene, where a nucleosome positioned over the TATA element is involved in gene regulation (reviewed in Svaren and Hörz, 1997). We compared the structure of the *PHO5* gene in the *nhp6a nhp6b* double deletion mutant with an isogenic wild type grown under non-inducing conditions. A restriction enzyme accessibility assay (Figure 4) showed no difference in Δ*nhp6a* Δ*nhp6b PHO5* promoter accessibility relative to its wild-type counterpart. In conclusion, although a slight derepression of basal transcription occurs in the *PHO5* gene in Δ*nhp6a* Δ*nhp6b* (Paull *et al*., 1996; data not shown), in contrast to *CHA1* no structural change could be detected in the *PHO5* promoter region. These results argue that the effect of NHP6A/B on the *CHA1* promoter is specific rather than generalized, as one would expect should the levels of some general chromatin component, such as histones, be impaired in the *nhp6a nhp6b* deletion mutant.

Another possible explanation for the observed up-regulation of *CHA1* transcript levels is that expression of some factor directly involved in modulating *CHA1* chromatin structure is down-regulated in Δ*nhp6a* Δ*nhp6b*. We have shown previously that *CHA1* expression is up-regulated and chromatin structure altered in a *sir4* strain (Moreira and Holmberg, 1998). Furthermore, we have also shown that RSC, an essential ATP-driven chromatin-remodeling complex, is required for chromatin-mediated transcriptional repression of the yeast *CHA1* (Moreira and Holmberg, 1999). Should expression of any of these genes be defective in Δ*nhp6a* Δ*nhp6b*, the outcome would be the exact phenotype we observed, namely showing increased basal expression and altered chromatin structure at the *CHA1* gene.

A genomic view of NHP6A/B cell function

To determine whether the effects we observed at the *CHA1* gene in *nhp6a nhp6b* were due to impaired expression of some repressive factor, and to gain some insight into the cellular function(s) of NHP6A/B proteins, we performed transcriptional whole-genome analysis in an *nhp6a nhp6b* double deletion mutant. We could identify 197 genes, corresponding to 3.2% of the yeast genome, differentially expressed in *nhp6a nhp6b* (Table I). Of these, 114 (1.9%) are up-regulated and 83 (1.4%) are down-regulated, suggesting that NHP6A/B are involved in transcriptional repression at other loci than *CHA1*. Some interesting patterns of expression emerged in this analysis, with various genes involved in the mating response (Table I, shown in red, bold typeface), pheromone-regulated genes required for yeast mating differentiation (Table I, boxed genes), and genes involved in methionine biosynthesis (Table I, shown in blue, bold typeface) deregulated in *nhp6a nhp6b*. However, we did not observe any difference in the expression of *SIR4*, RSC components, or of any other factor that could otherwise explain the deregulation at *CHA1*, leading us to conclude that NHP6A and B have a function in chromatin-mediated gene regulation.

HMG-1/-2 proteins have been shown to be involved in nucleosome assembly *in vitro* and chromatin organization (Bonne-Andrea *et al*., 1984; Nightingale *et al*., 1996). Additionally, a recent report has shown that expression of the *S.cerevisiae HO* gene, where chromatin structure plays an important regulatory role, requires NHP6A/B. Moreover, a *gcn5 nhp6a nhp6b* triple mutant has severe growth defects, suggesting that the SAGA histone acetyltransferase complex and NHP6A/B proteins function in parallel pathways, and indicating a possible role for NHP6A/B proteins in chromatin-mediated gene regulation (Yu *et al*., 2000).

The patterns of gene expression we observed in Δ*nhp6a* Δ*nhp6b* cells raise an interesting question as to the global

in vivo function(s) of these proteins. The large number of genes affected in a common cellular process is striking (Table I, genes that are red bold typeface, boxed, or blue bold typeface). These effects were confirmed by northern blot analysis of the expression of several genes (Figure 6), and therefore can be construed as real and not an artifact of the microarray analysis. Furthermore, the biological assay for pheromone production (Figure 7) showed a quantifiable physiological difference between Δ*nhp6a* Δ*nhp6b* and isogenic wild-type cells, clearly reflecting the differences we detected at the transcriptional level. Thus, even considering the possibility that only one gene in a particular pathway is an actual target for NHP6A/B proteins, with the remaining genes being indirect effects, the profile of gene expression in *nhp6a nhp6b* is provocative, providing a wealth of information and suggesting a possible mechanism of action for these proteins.

Analysis of the affected genes in *nhp6a nhp6b* (Table I) reveals no clustering in the positions of these genes, but rather that they are spread throughout the genome. These data show that the control of gene expression by NHP6A/B proteins is gene specific rather than affecting larger chromosomal domains. Furthermore, to determine whether some common sequence could be associated with dependence on NHP6A/B, we used two sequence analysis programs, MEME (http://atlas.med.harvard.edu; Bailey and Gribskov, 1998) and AlignACE (http://meme.sdsc.edu; Hughes *et al*., 2000), to analyze the promoter regions of the affected genes (up to 800 bp 5′ of the ATG codon), but were unable to identify any common motifs that could serve as cognate sites for NHP6A/B. This negative result suggests that NHP6A/B may be targeted to specific promoters by gene-specific transcription factors.

We also compared the set of genes identified in the genome-wide expression analysis we performed in an *nhp6a nhp6b* double mutant with data sets for mutants involved in chromatin-mediated gene regulation available in the literature. Interestingly, the published datasets for *swi/snf* (Sudarsanam *et al*., 2000) and *tup1* mutants (DeRisi *et al*., 1997) show a high degree of overlap with our dataset. In fact, 40 out of the 197 genes (20%) affected in the *nhp6a nhp6b* mutant are also affected in a strain lacking *tup1*, with 32 of the 197 (16%) affected in *swi–snf* mutants (Table I, genes labeled with superscript S and T for *swi–snf* and *tup1*, respectively). If one considers only those genes that are up-regulated in the *nhp6a nhp6b* mutant, then the relative proportion of common affected genes increases to 35/114 (30%) and 25/114 (21%) for the *tup1* and *swi–snf* mutants, respectively.

Overexpression of NHP6A/B suppresses defects in the Slt2/Mpk1 MAP kinase (MAPK) pathway, and genetic and phenotypic analysis of *nhp6a nhp6b* mutants suggests that NHP6A/B function downstream of Slt2 (Costigan *et al*., 1994). Interestingly, we find that *SLT2* expression is up-regulated 5-fold in *nhp6a nhp6b* (Table I). Moreover, the SBF transcription activator complex (composed of Swi4p and Swi6p) is itself a target of the Slt2/Mpk1 MAP kinase (MAPK) pathway (Madden *et al*., 1997), and a high copy suppressor screen for proteins required for full activity of the SBF complex identified NHP6A (Sidorova and Breeden, 1999). The SBF complex binds to cognate elements in target genes such as *HO* and *CLN1*, the expression of which requires NHP6A/B (Sidorova and Breeden, 1999; Yu *et al*., 2000). No direct interaction between NHP6A and the SBF complex could be demonstrated (Sidorova and Breeden, 1999). However, the experiment was not carried out in the context of chromatin, and it is conceivable that the effect of NHP6A on Swi6-regulated promoters is through chromatin modulation.

We propose that NHP6A/B proteins function *in vivo* as co-regulatory factors, directly involved in recruiting or in stabilizing interactions of *trans*-acting factors with cognate sequences. NHP6A/B proteins could be responsible for modulating chromatin structure at target promoters, allowing additional regulatory factors to be recruited. This function might be particularly important in cases where factors have no DNA-sequence specificity, such as for Ssn6–Tup1, or weak sequence specificity, such as for the SWI/SNF complex. This model would also explain the effect we observed at the *CHA1* gene. We have previously reported that a SWI/SNF homologous complex termed RSC is required for repression of *CHA1* basal expression (Moreira and Holmberg, 1999). Should NHP6A/B be involved in recruitment of the RSC complex to the *CHA1* promoter, one would expect that in the absence of NHP6A/B proteins, targeting would be impaired and deregulation of the *CHA1* gene would occur, as indeed we have observed.

Materials and methods

Strains

Strains RJY6012 (*MATα his3-Δ200 leu2-3,112 ura3-52 trp1-Δ201 lys2-801 suc2-Δ9 gal3 nhp6A::ura⁻ nhp6B::LEU2*) and SEY6210 (*MATα his3-Δ200 leu2-3,112 ura3-52 trp1-Δ201 lys2-801 suc2-Δ9 gal3*) were provided by Reid C.Johnson. Strains MT503 (*MATα sst2-1 leu2-3,112 his3 can1*) and XP635–10C (*MAT***a** *bar1 gal2 leu2-3,112*) were provided by Vivian Mckay. Strains DY150 (*MAT***a** *ade2 can1 his3 leu2 ura3 trp1*) and DY2382 (*MAT***a** *ade2 can1 his3 leu2 ura3 trp1 nhp6A::URA3 nhp6B::HIS3*) were provided by David J.Stillman.

Cells were grown in minimal medium (0.67% bacto yeast nitrogen base without amino acids, 2% glucose, buffered with 10 g succinic acid and 6 g NaOH per liter) supplemented with the required amino acids at appropriate concentrations, in the absence (–Ser) or presence (+Ser) of serine as inducer (1 g/l).

Restriction endonucleases and DNA-modifying enzymes were purchased from Boehringer Mannheim (Mannheim, Germany), *Taq* polymerase was from Pharmacia (Amersham Pharmacia Biotech) and radiolabeled nucleotides were from ICN Pharmaceuticals, Inc. (Costa Mesa, CA).

Indirect end-labeling chromatin analysis

Isolation of nuclei and nuclease digestion were carried out as described (Moreira and Holmberg, 1998). Restriction endonuclease cleavage was performed under similar conditions as for MNase and DNase I, except that nuclei were treated for 1 h at 37°C. Subsequently, samples were treated with proteinase K and genomic DNA isolated. After secondary digestion with the appropriate restriction enzyme, the treated DNA samples were electrophoresed in 1% agarose gels in 1× TBE, transferred onto Positive™ nylon membranes (Oncor, Gaithersburg, MD) and hybridized following standard protocols (Sambrook *et al*., 1989).

Northern blot analysis

Total RNA was isolated from untreated nuclei using the Qiagen RNeasy Total RNA Kit (Qiagen, Germany) according to the manufacturer's instructions. Ten micrograms of RNA per sample were loaded onto a 1.4% agarose–formaldehyde gel and electrophoresed in 1× MOPS, transferred onto Positive™ nylon membranes and hybridized following standard protocols (Sambrook *et al*., 1989). Transcript levels were quantified using a Cyclone Storage Phosphor System (Packard Instrument Company, Meriden, CT).

Radiolabeling of probes

Labeling was carried out as described (Espelund *et al*., 1990). A ^{32}P-labeled PCR fragment covering positions +300 to +554 relative to the translational initiation codon was used for chromatin and northern blot analysis of the *CHA1* gene. A ^{32}P-labeled PCR fragment covering positions –1300 to –1086 relative to the translational initiation codon was used as probe for chromatin and restriction enzyme analysis of the *PHO5* gene. The *URA3* messenger was detected with a ^{32}P-labeled PCR amplificate covering positions –23 to +151. All other transcripts were detected with a ^{32}P-labeled PCR amplificate encompassing the entire ORF, from position +1 to the last nucleotide in the stop codon.

β-galactosidase activity

β-galactosidase measurements were performed as described previously (Remacle and Holmberg, 1992), with all values shown in Miller units (Miller, 1972).

Genome-wide expression analysis using high-density microarrays

Wild-type yeast cells (SEY6210) and cells bearing a double Δ*nhp6A* Δ*nhp6B* deletion (RJY6012) were grown in parallel in minimal medium at 30°C. Cultures were harvested at OD_{600} = 1. Total RNA was isolated as previously described. One microgram of total RNA per sample was labeled and hybridized to Yeast GeneFilters® Microarrays filters I and II (Research Genetics, Huntsville, AL), according to the manufacturer's instructions. Quantitation was performed on a Cyclone Storage Phosphor System (Packard Instrument Company, Meriden, CT). Overall hybridization results from the two strains were normalized by setting the hybridization intensities of the control spots present in a given filter to be identical in both samples. Two independent hybridizations were performed for each sample. The results from the two hybridizations were averaged and only those genes that showed a similar effect in both hybridizations were considered in our analysis.

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