The EMBO Journal (2004) 23, 333–342 | © 2004 European Molecular Biology Organization | All Rights Reserved 0261-4189/04
www.embojournal.org

THE EMBO JOURNAL

Nhp6 facilitates Aft1 binding and Ssn6 recruitment, both essential for *FRE2* transcriptional activation

George S Fragiadakis[1,2], Dimitris Tzamarias[1,3] and Despina Alexandraki[1,2,*]

[1]Institute of Molecular Biology and Biotechnology-FORTH, University of Crete, Vassilika Vouton, Heraklion, Crete, Greece, [2]Department of Biology, University of Crete, Greece and [3]School of Science and Technology, Hellenic Open University, Greece

We found Nhp6a/b yeast HMG-box chromatin-associated architectural factors and Ssn6 (Cyc8) corepressor to be crucial transcriptional coactivators of *FRE2* gene. *FRE2* encoding a plasma membrane ferric reductase is induced by the iron-responsive, DNA-binding, transcriptional activator Aft1. We have shown that Nhp6 interacts directly with the Aft1 N-half, including the DNA-binding region, to facilitate Aft1 binding at *FRE2* UAS. Ssn6 also interacts directly with the Aft1 N-half and is recruited on *FRE2* promoter only in the presence of both Aft1 and Nhp6. This Nhp6/Ssn6 role in Aft1-mediated transcription is *FRE2* promoter context specific, and both regulators are required for activation-dependent chromatin remodeling. Our results provide the first *in vivo* biochemical evidence for nonsequence-specific HMG-box protein-facilitated recruitment of a yeast gene-specific transactivator to its DNA target site and for Nhp6-mediated Ssn6 promoter recruitment. Ssn6 has an explicitly coactivating role on *FRE2* promoter only upon induction. Therefore, transcriptional activation in response to iron availability involves multiple protein interactions between the Aft1 iron-responsive DNA-binding factor and global regulators such as Nhp6 and Ssn6.

The EMBO Journal (2004) **23,** 333–342. doi:10.1038/sj.emboj.7600043; Published online 22 January 2004
Subject Categories: chromatin & transcription; signal transduction
Keywords: Aft1; iron regulation; Nhp6; Ssn6; transcriptional activation

Introduction

Gene expression involves dynamic and highly regulated processes of interactions between protein and DNA components of the chromatin. Transcription of most eucaryotic genes involves several DNA-binding activators that recognize distinct sequences on a single promoter. Pathway-specific transactivators, responding to diverse cellular signals, cooperate with global activators or repressors to exert their specific function.

*Corresponding author. Institute of Molecular Biology and Biotechnology-FORTH, University of Crete, Vassilika Vouton, PO Box 1527, Heraklion, Crete GR-71110, Greece. Tel.: +30 281 039 1161; Fax: +30 281 039 1101; E-mail: alexandr@imbb.forth.gr

Received: 22 August 2003; accepted: 25 November 2003; Published online: 22 January 2004

Iron uptake and homeostasis is a complex process and, in *Saccharomyces cerevisiae*, involves many proteins, including iron reductases (Fre1–6), oxidases (Fet3, Fet5), permeases (Ftr1, Fth1, Fet4), siderophore transporters (Arn1–4) and cell wall mannoproteins (Fit1–3) (Kosman, 2003). Transcription of the corresponding genes is regulated by the availability of iron, being induced under low-iron conditions. Iron-dependent regulation is mediated by Aft1 (Yamaguchi-Iwai *et al*, 1995) and/or the recently identified Aft2 (Blaiseau *et al*, 2001; Rutherford *et al*, 2003) DNA-binding transcription factors. Aft1 binds to the FeRE consensus sequence PyPuCACCCPu, present on various promoters, in an iron-dependent manner (Yamaguchi-Iwai *et al*, 1996). It contains a basic putative DNA-binding domain within residues 140–280 of its N-terminal region and a strong transactivation domain within residues 413–572 of its glutamine-rich C-terminus (Yamaguchi-Iwai *et al*, 2002). Aft1 responds to the iron concentration of the cell, remaining in the cytoplasm when iron is replete (Yamaguchi-Iwai *et al*, 2002). Its subcellular localization is regulated by an N-terminal nuclear export signal sequence. The mechanism by which Aft1 senses iron is still unexplored and may involve direct iron binding to the protein. A putative iron binding CysXCys motif, located in the middle of the molecule, affects Aft1 transactivation potential since a Cys291Phe substitution causes constitutive transcriptional activation (Yamaguchi-Iwai *et al*, 1995). Two histidine-rich domains, located N- and C-terminally, respectively, may bind iron leading to an intramolecular interaction, as was shown for other metal-related activators (Jensen and Winge, 1998).

FRE1 (Dancis *et al*, 1992) and *FRE2* (Georgatsou and Alexandraki, 1994) are the most extensively studied iron reductase genes. *FRE2* transcription solely depends on Aft1 whereas *FRE1* is also induced by Aft2 (Rutherford *et al*, 2003) and, in the absence of extracellular copper, by the copper-regulated DNA-binding transcriptional activator Mac1 (Yamaguchi-Iwai *et al*, 1997). The highly inducible and simply regulated *FRE2* gene is a suitable model for transcriptional regulation studies. Interestingly, in a genetic screen seeking positive regulators of *FRE2* expression, we identified *NHP6A* gene encoding the Nhp6a architectural protein. On the other hand, a previous report suggested that Nhp6b protein physically interacts with Tup1 (Laser *et al*, 2000) while Ssn6 and Tup1 transcriptional corepressors were shown to affect reductive iron uptake in yeast (Lesuisse *et al*, 2001). On this basis, we have examined the functional interplay of these general cofactors in *FRE2* transcription in relation to Aft1 function.

Nhp6a and its counterpart Nhp6b (both termed Nhp6) are small, abundant, chromatin-associated, non-histone yeast proteins. They belong to the HMG-box family of high-mobility-group proteins, being structurally and functionally homologous to the ubiquitous mammalian HMGB1/2 proteins (Kolodrubetz and Burgum, 1990). HMGB1/2 appear to act

as architectural facilitators to overcome the rigidity barrier of DNA in the assembly of nucleoprotein complexes, in a variety of DNA-related processes such as transcription, replication, recombination and repair (Bustin, 1999; Thomas and Travers, 2001). Nhp6 consists of a single HMG box that functions as a sequence-independent DNA-binding domain and a short basic N-terminal tail essential for high-affinity DNA binding (Yen *et al*, 1998). It preferentially binds distorted (e.g. micro-circular or cisplatinated) DNA structures and induces a large bend to linear DNA *in vitro* (Paull and Johnson, 1995; Yen *et al*, 1998; Wong *et al*, 2002).

Either *nhp6a*Δ or *nhp6b*Δ mutant exhibits no observable phenotype, reflecting a functional redundancy of the two proteins (Costigan *et al*, 1994). Notably, Nhp6b, less abundant than Nhp6a, is increased in *nhp6a*Δ cells to compensate for the absence of its counterpart (Kolodrubetz *et al*, 2001). On the other hand, *nhp6*ΔΔ double mutant exhibits several phenotypes, including slow growth at 30°C, cessation of growth at 38°C in the absence of osmotic support (1 M sorbitol) along with cellular and cytoskeletal morphology defects (Costigan *et al*, 1994). This phenotypic variety is consistent with the affected transcription of several RNA polymerase II-transcribed genes in the *nhp6*ΔΔ strain (Paull *et al*, 1996; Sidorova and Breeden, 1999; Moreira and Holmberg, 2000; Yu *et al*, 2000, 2003) and the Nhp6-stimulated TFIIIC-dependent transcription of *SNR6* gene by RNA polymerase III (Kruppa *et al*, 2001; Lopez *et al*, 2001; Martin *et al*, 2001). Furthermore, accumulating evidence shows that Nhp6 is directly involved in the modulation of chromatin state (Moreira and Holmberg, 2000; Brewster *et al*, 2001; Formosa *et al*, 2001; Lopez *et al*, 2001; Szerlong *et al*, 2003).

Ssn6 is a major transcriptional coregulator in yeast. It was identified as the corepressor required, in a complex with Tup1, for the transcriptional inhibition of various genes involved in diverse physiological pathways. This complex is recruited to different promoters via interactions with specific DNA-binding regulatory proteins and inhibits transcription, with the Tup1 subunit being the repressor, by affecting both chromatin structure and the basic transcription machinery (Smith and Johnson, 2000). Experiments using artificial reporter genes have shown that Ssn6–Tup1 can also act as a transcriptional coactivator, with this function predominantly mediated by Ssn6 (Conlan *et al*, 1999). Genetic and biochemical analyses have revealed a number of natural genes as targets of Ssn6–Tup1 positive action (Zhang and Guarente, 1994; Conlan *et al*, 1999; Papamichos-Chronakis *et al*, 2002; Proft and Struhl, 2002).

In this work, we show that induced *FRE2* transcription relies on the collaborative function of the two general transcriptional coregulators, Nhp6 and Ssn6, necessary for Aft1 function. We demonstrate a distinct relation of Nhp6 and Ssn6 with Aft1 on *FRE2* promoter, providing new roles for both coregulators and showing that specific protein interactions modulate Aft1 functionality in response to iron availability.

Results

SSN6 and NHP6A/B are necessary for induced FRE2 transcription

We isolated *NHP6A* gene as a high-copy suppressor of a genomic mutation that abolishes *FRE2*, but not *FRE1*, transcriptional induction (genetic screen in Materials and methods). Preliminary transcriptional analysis of a *FRE2-LacZ* reporter gene in *NHP6A*, *SSN6* and *TUP1* mutants revealed a strong positive effect of each of the three genes on induced transcription, with the effect of *TUP1* being less prominent (data not shown). On this basis, we examined the accumulation of *FRE2* mRNA, as well as of *FRE1* for comparison, in *ssn6*Δ, *nhp6*ΔΔ and *ssn6*Δ *nhp6*ΔΔ mutants, compared to a wild-type strain, under basal and inducing conditions. We used an *nhp6*ΔΔ double mutation since disruption of both *NHP6* genes is required to obtain observable phenotypes (Costigan *et al*, 1994). Transcriptional activation of *FRE2* in the absence of iron was completely eliminated in all mutant strains (Figure 1A), revealing a dramatic effect of the *SSN6* and *NHP6A/B* deletions on induced *FRE2* transcription. Basal mRNA levels (in iron-replete medium) were hardly detected in wild-type and mutant strains, and therefore we could not draw any safe conclusion concerning the effect of the above deletions on *FRE2* basal transcription. Under copper-depleted conditions, *FRE2* transcription was at basal levels in all strains examined, in agreement with previously reported results (Georgatsou *et al*, 1997). This was an important control since the Fe(II) chelator used in our experiments also chelates Cu(II) (see Materials and methods).

The results for *FRE1* transcription revealed a more complex regulation (Figure 1B) consistent with the known action of multiple DNA-binding transactivators on *FRE1* promoter. Basal transcription of *FRE1* in the *ssn6*Δ strain was at wild-type levels while transcriptional induction was significantly lower, more prominently in the absence of copper, indicating that the *SSN6* deletion affected induced *FRE1* transcription. In *nhp6*ΔΔ cells, basal transcription was drastically reduced and transcriptional induction was also reduced (more prominently in the absence of copper), but to a lesser extent, suggesting that the *NHP6A/B* deletion affected predominantly noninduced *FRE1* transcription. The *ssn6*Δ *nhp6*ΔΔ triple mutation produced results similar to those of *ssn6*Δ and *nhp6*ΔΔ alone, under inducing conditions, whereas it revealed an epistatic effect of *nhp6*ΔΔ on *ssn6*Δ in *FRE1* basal transcription.

Our data so far revealed a new regulatory role of *SSN6* and *NHP6A/B* in metal-regulated transcription. Since these genes were essential for induced *FRE2* transcription, the question that arose next was whether this role directly associated with the action of Aft1 transactivator. Unlike *FRE1* whose expression is not solely affected by Aft1, *FRE2* expression, under iron-depletion conditions, is completely eliminated by *AFT1* deletion (Yamaguchi-Iwai *et al*, 1995; our observations).

SSN6 and NHP6A/B promote Aft1-mediated transcription

In order to test whether *SSN6* and *NHP6A/B* are involved in Aft1-mediated *FRE2* transcription, we examined transcriptional induction in wild-type and mutant cells grown in iron-depleted medium, using a $FRE2_{UAS}$-*HIS3-LacZ* reporter gene. This gene comprises a region from *FRE2* promoter that contains the Aft1 binding consensus element and a TATA region from *HIS3* promoter known to be unaffected by *NHP6A/B* (Paull *et al*, 1996) and *SSN6* (data not shown), inserted upstream of the *LacZ* gene. As shown in Figure 2A, β-galactosidase activity was drastically and similarly reduced in both *ssn6*Δ and *nhp6*ΔΔ, compared to wild-type strain,

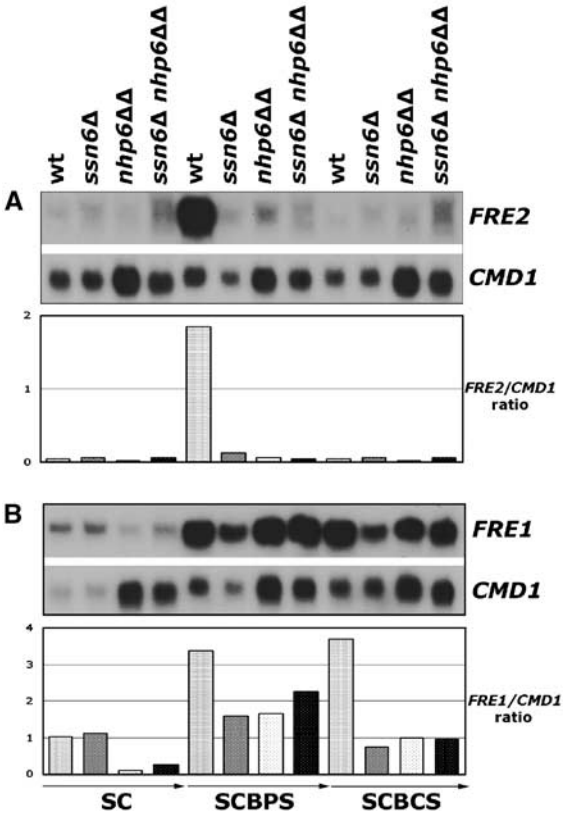

Figure 1 Effect of *SSN6* and *NHP6A/B* on metal-regulated transcription. (**A**) Northern analysis of total RNA extracted from the indicated strains grown in metal-replete (SC), iron-depleted (SCBPS) or copper-depleted (SCBCS) medium using radiolabeled *FRE2* and *CMD1* (internal control) probes. (**B**) The same blot using *FRE1* and *CMD1* probes. Bands were quantified using the PhosphorImager and ImageQuant software, and bars represent the indicated intensity ratios (normalized mRNA levels).

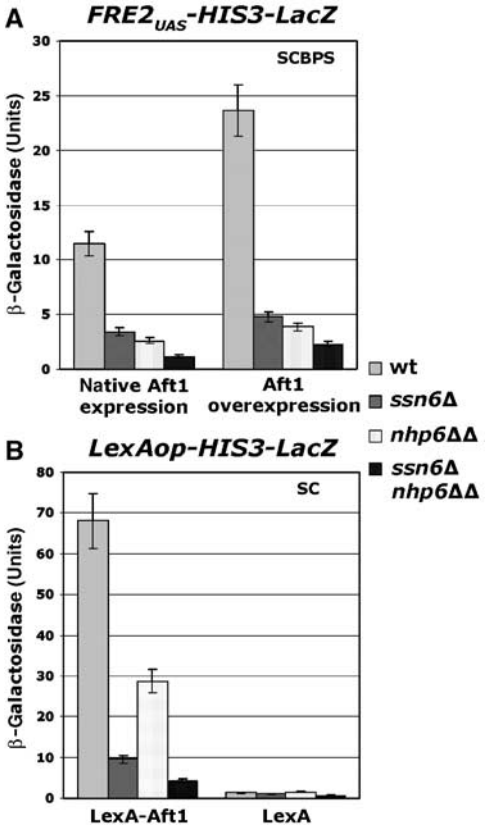

Figure 2 Effect of *SSN6* and *NHP6A/B* on Aft1-mediated transcription. (**A**) β-Galactosidase activity units obtained from the indicated strains cotransformed with $FRE2_{UAS}$-*HIS3-LacZ* and pYX142 or pYX142-*AFT1* (*AFT1* overexpression) grown in SCBPS. (**B**) β-Galactosidase activity units obtained from the indicated strains cotransformed with *LexAop-HIS3-LacZ* and LexA-Aft1 or LexA expression plasmid grown in SC medium. (The activity in SCBPS exceeds measurable levels.) Values in (A) and (B) represent the average obtained from three independent transformants.

indicating a considerable effect of *SSN6* and *NHP6A/B* on Aft1-mediated transcription. Interestingly, it was further reduced in the triply deleted *ssn6*Δ *nhp6*ΔΔ strain, implying a cooperative role for these genes. Furthermore, concomitant overexpression of *AFT1*, although increasing β-galactosidase activity in wild-type strain, did not rescue the activity of mutant strains (Figure 2A), confirming that the observed effect on transcription was mediated through Aft1 transactivator. We further investigated the effect of *SSN6* and *NHP6A/B* on the transactivation function of Aft1 by artificially tethering a LexA-Aft1 protein on another reporter gene containing a LexA binding site upstream of *HIS3-LacZ*. β-galactosidase activity was decreased by 85% in *ssn6*Δ, compared to wild-type cells, indicating that *SSN6* is mainly responsible for full transactivation by LexA-Aft1 (Figure 2B). On the other hand, nearly half of the wild-type activity was retained in *nhp6*ΔΔ strain, suggesting that the effect of *NHP6A/B* is exerted partly on the transactivation potential and mainly on the DNA-binding function of Aft1. These observations indicate that *SSN6* and *NHP6A/B* promote the Aft1 activation potential from *FRE2* promoter in a distinct manner.

SSN6 and NHP6A/B deletion effects are promoter context specific

In order to examine whether the effects of *SSN6* and *NHP6A/B* are common to all Aft1-responsive genes, we analyzed the RNA accumulation patterns of four additional iron-regulated genes. These were selected according to the iron regulation literature (Rutherford *et al*, 2003): *ARN2* (*TAF1*) as the only other gene, in addition to *FRE2*, that is considerably, and probably solely, upregulated by Aft1, *FRE3* as the most homologous to *FRE2* and the most prominently regulated by Aft1 of all *FRE* genes, and *FIT2* as well as *FET3* as highly induced by Aft1 and less by Aft2. Of these genes, only the expression of *ARN2* was drastically reduced in either *ssn6*Δ or *nhp6*ΔΔ background and practically abolished in the double mutant, reproducing the results obtained for the expression of *FRE2* gene (Figure 3). *ARN2*, like *FRE2*, is probably regulated only by Aft1 using one FeRE element. *FRE2* contains an additional but less functional FeRE element (GS Fragiadakis, data not shown). *FET3*, *FIT2* and possibly *FRE3* promoters are regulated by both Aft1 and Aft2 and, moreover, contain multiple FeRE elements. These findings imply that *SSN6* and *NHP6A/B* deletion effects are promoter context specific.

SSN6 and NHP6A/B are required for the activation-dependent chromatin remodeling of FRE2 promoter

To investigate whether specific chromatin organization is involved in the transcriptional activation of *FRE2* gene and

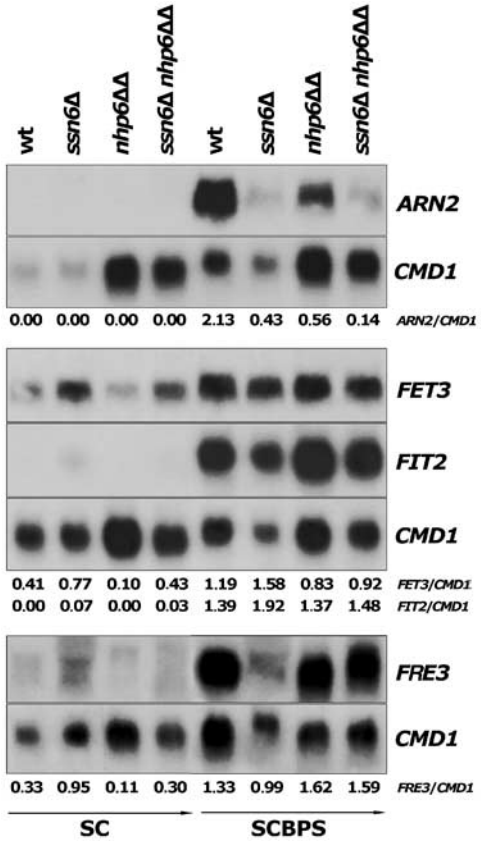

Figure 3 Effect of *SSN6* and *NHP6A/B* on Aft1-regulated genes. Same blots and analysis for SC and SCBPS RNA samples as described for Figure 1, using *ARN2*, *FET3*, *FIT2*, *FRE3* and *CMD1* probes.

affected by *SSN6* and *NHP6A/B* deletions, we first subjected wild-type cells grown under iron-replete or iron-depleted conditions to micrococcal nuclease digestion followed by indirect end-labeling analysis (Figure 4, lanes 2 and 3). Two different DNA band patterns were obtained, corresponding to the repressed or activated state indicating an activation-dependent chromatin change on *FRE2* promoter. More specifically, nucleosomes were positioned on repressed *FRE2* promoter. A region including the UAS_{Aft1} was protected in the repressed state while it became nuclease hypersensitive in the activated state, suggesting that remodeling of a UAS_{Aft1}-occluding nucleosome (nuc-2, upstream of TATA) took place upon induction. The band patterns obtained from similarly analyzed *nhp6*ΔΔ or *ssn6*Δ cells under induction conditions (Figure 4, lanes 4 and 5) resembled that of repressed wild-type cells, indicating that the absence of Nhp6 or Ssn6 resulted in defective activation-dependent chromatin remodeling of *FRE2* promoter. This is in accordance with the severe decrease in induced *FRE2* transcription observed in *nhp6*ΔΔ and *ssn6*Δ strains. Therefore, chromatin remodeling of *FRE2* promoter takes place under induction conditions and strictly depends on the presence of Nhp6 and Ssn6 proteins.

Aft1 recruitment on FRE2 promoter requires the presence of Nhp6

To further understand the activation process on *FRE2* promoter, we tested its occupancy by the Aft1 and Nhp6 factors *in vivo*, performing chromatin immunoprecipitation (ChIP)

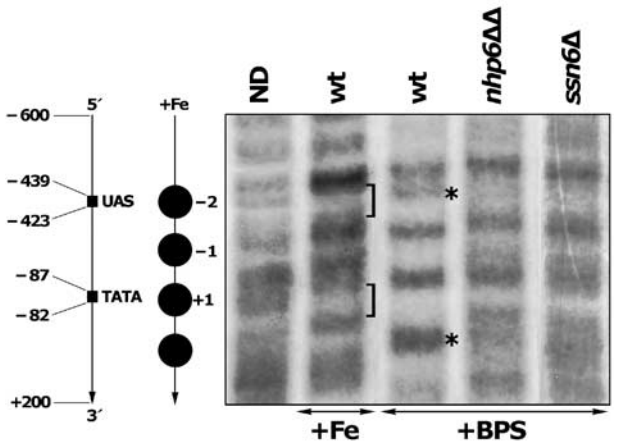

Figure 4 Low-resolution chromatin analysis of the *FRE2* promoter. Wild-type cells under repression (+Fe, SC supplemented with 200 μM $FeCl_3$ 30 min prior to cell collection) and wild type, *nhp6*ΔΔ and *ssn6*Δ under induction (+BPS) conditions were subjected to MNase digestion followed by indirect end-labeling. On the left, nucleosomes seen as protected areas on the repressed *FRE2* promoter are depicted as circles. The adjacent map indicates the relative positions of the UAS_{Aft1} and the presumptive TATA box. Brackets indicate the corresponding protected regions and asterisks indicate induction-specific MNase sensitivity sites. Lane ND contains a naked genomic DNA digest.

assays in cells grown under conditions of iron depletion. As shown in Figure 5A, the occupancy of *FRE2* promoter by the endogenous Aft1 protein, tagged with 9 Myc epitopes, depended quantitatively on the presence of Nhp6. In contrast, Aft1-9Myc recruitment on *FRE1* promoter (analyzed in parallel) was similar in wild-type and *nhp6*ΔΔ cells. Similar results were obtained using an HA-tagged Aft1 (data not shown). Given that Aft1 bound to both promoters, this finding indicated that Nhp6 intervenes in Aft1 binding on *FRE2* and not on *FRE1*, acting in a promoter context-dependent manner. Indeed, we found Nhp6a present on both *FRE1* and *FRE2* promoters, as well as on several other promoters and coding regions, at similarly high levels (data not shown). Nhp6, as a nonsequence-specific DNA-binding protein whose interaction with chromatin is more stable and permanent than that of other transiently binding regulatory factors (Bustin, 1999), was expected to crosslink efficiently in many chromosomal positions. Our experiments demonstrated that, specifically in the *FRE2* promoter context, Nhp6 facilitated quantitative Aft1 DNA binding.

Ssn6 is recruited on FRE2 promoter and this recruitment is dependent upon the presence of both Aft1 and Nhp6

To address whether Ssn6 protein physically associates with *FRE2* promoter, we used an HA-tagged Ssn6 derivative expressed in *ssn6*Δ to avoid interference by endogenous Ssn6. As assayed by ChIP performed in cells grown in iron depletion (Figure 5B), HA-Ssn6 was specifically recruited on *FRE2* and *FRE1* promoter (analyzed in parallel), whereas it occupied at background levels an *ACT1* coding sequence. *AFT1* gene deletion almost eliminated HA-Ssn6 recruitment on *FRE2* while, in contrast, did not significantly affect recruitment on *FRE1* promoter. Deletion of *NHP6A/B* genes considerably reduced HA-Ssn6 recruitment on *FRE2* promoter while recruitment on *FRE1* was not significantly affected (Figure 5C). We conclude that Ssn6 associates with *FRE2*

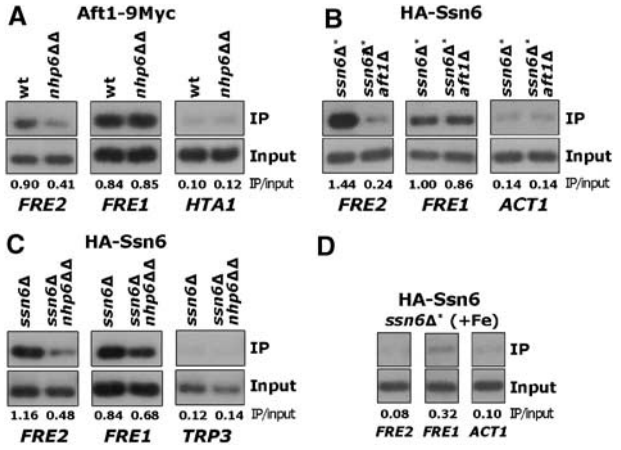

Figure 5 Association of Aft1 and Ssn6 with *FRE1* and *FRE2* promoters. (**A**) Wild-type and *nhp6*ΔΔ cells, carrying chromosomal *AFT1*-9Myc, grown in SCBPS, were subjected to ChIP with anti-Myc, followed by PCR analysis of the immunoprecipitated (IP) and input DNA using primers specific for *FRE1*, *FRE2* and *HTA1* promoters. (**B**) *ssn6*Δ* and *ssn6*Δ* *aft1*Δ cells, transformed with an HA-Ssn6 expressing plasmid, were grown and analyzed as above using anti-HA and primers specific for *FRE1* and *FRE2* promoters and *ACT1* coding region. (**C**) *ssn6*Δ and *ssn6*Δ *nhp6*ΔΔ cells were transformed, grown and analyzed as above using anti-HA and primers specific for *FRE1*, *FRE2* and *TRP3* promoters. (**D**) *ssn6*Δ* cells transformed with an HA-Ssn6 expressing plasmid and grown under high-iron conditions (SC supplemented with 200 μM $FeCl_3$ 30 min prior to cell collection) were analyzed as above using anti-HA and primers specific for *FRE1* and *FRE2* promoters and *ACT1* coding region. Bands in (A–D) were quantified using the PhosphorImager and ImageQuant software, and numbers express the indicated ratios. The specific recruitment of HA-Ssn6 on *FRE1* and *FRE2* promoters in *ssn6*Δ cells of two different genetic backgrounds was comparable.

promoter and its recruitment is quantitatively dependent on the presence of Aft1 and Nhp6. In fact, under high-iron conditions, when Aft1 is cytoplasmic, we observed no recruitment of Ssn6 on *FRE2* promoter (Figure 5D), consistent with its dependency on the presence of Aft1. On the other hand, Ssn6 is also present at *FRE1* promoter, but independently of Aft1 and Nhp6. Under high-iron conditions, recruitment of Ssn6 on *FRE1* promoter was observed at low levels (Figure 3D), consistent with the Aft1-independent low *FRE1* expression in these conditions (data not shown, but similar to SC conditions shown in Figure 1).

Ssn6 and Nhp6a associate with Aft1 in vivo but not with one another

A preliminary indication for an Aft1–Ssn6 interaction *in vivo* was obtained by two-hybrid analysis (data not shown). A similar analysis of potential Aft1–Nhp6 interaction was not feasible because hybrids of Nhp6a with an activation or DNA-binding domain, expressed from strong promoters on multicopy plasmids, probably caused a dramatic decrease in growth rate, being toxic for cells. On this basis, we performed copurification assays *in vivo* in *aft1*Δ cells expressing HA-Aft1 and 6xHis-Ssn6 or 6xHis-Nhp6a proteins in order to verify the interdependence of Aft1 with Ssn6 and Nhp6 on *FRE2* promoter. These assays were carried out under iron-depletion conditions to ensure quantitative Aft1 colocalization with Ssn6 and Nhp6 in the nucleus. The 6xHis-tagged proteins bound on Ni-NTA beads are shown in Figure 6A. HA-Aft1 specifically copurified with 6xHis-Ssn6 as well as with 6xHis-Nhp6a (Figure 6B, left), indicating an association with each of the two proteins. Interestingly, in a copurification assay carried out under iron-replete conditions, we did not observe Aft1–Nhp6a association (data not shown). The endogenous Tup1, used as a positive control of the assay, also copurified with 6xHis-Ssn6 as expected (Figure 6B, right), but did not copurify with 6xHis-Nhp6a under either iron-depletion (Figure 6B, right) or iron-replete conditions (data not shown).

Since Aft1 was found to associate with each of Ssn6 and Nhp6a, we next examined whether Ssn6 and Nhp6a associate with one another as well. The *in vivo* copurification assay was performed in *nhp6*ΔΔ cells expressing HA-Nhp6a and 6xHis-Ssn6 proteins. As shown in Figure 6C, HA-Nhp6a did not copurify with 6xHis-Ssn6 (left panel) while Tup1 did (right panel), indicating that Nhp6a did not associate with Ssn6 under the conditions employed. We used iron-depletion conditions as Ssn6 and Nhp6 may require the presence of Aft1 in order to interact. However, in this experiment Aft1 was expressed from the native *AFT1* gene at levels significantly lower than those of the other two proteins. The 'reverse' copurification experiment, under the same conditions, using HA-Ssn6 and 6xHis-Nhp6a proteins was not informative due to high nonspecific retention of HA-Ssn6 on the Ni-NTA agarose beads (data not shown).

The above data revealed that Aft1 associates with Ssn6 and Nhp6a *in vivo*, consistent with its interdependence with each of these proteins on *FRE2* promoter. They also showed that Ssn6 and Nhp6a may not interact directly *in vivo*, although they are linked through Aft1. Furthermore, this protein complex is most likely formed on DNA, since interaction between Aft1 and Ssn6 was abolished in cells lacking Nhp6 as shown by *in vivo* copurification of HA-Aft1 with the 6xHis-Ssn6 in wild-type and *nhp6*ΔΔ cells (Figure 6D).

Ssn6 and Nhp6a interact directly with the N-terminal half of Aft1 in vitro

We subsequently examined whether the *in vivo* detected associations reflected physical contacts between the involved proteins. For this, we tested whether bacterially produced GST-fused N-Ssn6, or GST-fused Nhp6a, could associate *in vitro*, in the absence of additional yeast proteins, with bacterially produced 6xHis-tagged Aft1 and the N-half or C-half Aft1 derivatives (Figure 7A). The entire Ssn6 protein could not be produced in bacteria. However, the N-Ssn6 derivative tested in this assay, containing the entire TPR domain of Ssn6 (10 tandem repeats) known to be involved in distinct protein–protein interactions (Tzamarias and Struhl, 1995), could restore *FRE2* gene induction to wild-type levels, when introduced in an *ssn6*Δ strain (data not shown).

As shown in Figure 7B, GST-N-Ssn6 as well as GST-Nhp6a, but not GST alone, interacted with 6xHis-Aft1 (7B-1), 6xHis-N-Aft1 (7B-2), but not with 6xHis-C-Aft1 (7B-3), indicating a direct contact between residues of the N-half of Aft1 and the TPR domain of Ssn6 or some portion of the Nhp6a protein. The fact that Nhp6a interacted directly with the DNA-binding region of Aft1 further supported our finding, described above, for Nhp6-facilitated Aft1 binding on *FRE2* promoter. Finally, GST-N-Ssn6 interacted with 6xHis-N-Tup1 as expected, while GST-Nhp6a did not (7B-4), in accordance with what we observed *in vivo*.

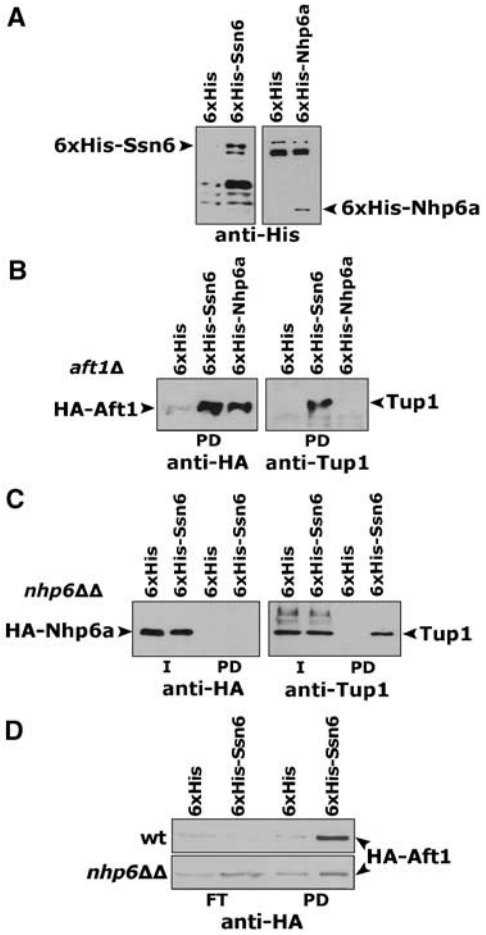

Figure 6 Association of Ssn6 and Nhp6a with Aft1 *in vivo*. (**A**) Protein extracts from *aft1*Δ cells grown in SCBPS and expressing 6xHis or 6xHis-Ssn6 or 6xHis-Nhp6a along with HA-Aft1 were incubated with Ni-NTA beads. Bound proteins were analyzed by SDS–PAGE and immunoblotting using anti-His to detect 6xHis-Ssn6 and 6xHis-Nhp6a. (**B**) Pulled-down (PD) HA-Aft1 was detected by anti-HA (left) and pulled-down endogenous Tup1 by anti-Tup1 (right). (**C**) Extracts from *nhp6*ΔΔ cells grown in SCBPS and expressing 6xHis or 6xHis-Ssn6 along with HA-Nhp6a were incubated with Ni-NTA beads. Pulled-down (PD) and input (I) proteins were analyzed by SDS–PAGE and immunoblotting using anti-HA to detect HA-Nhp6a (left) and anti-Tup1 to detect endogenous Tup1 (right). (**D**) Extracts from wild-type and *nhp6*ΔΔ cells grown in SCBPS and expressing 6xHis or 6xHis-Ssn6 along with HA-Aft1 were incubated with Ni-NTA beads. Pulled-down (PD) and flow-through (FT) proteins were analyzed by SDS–PAGE and immunoblotting using anti-HA to detect HA-Aft1.

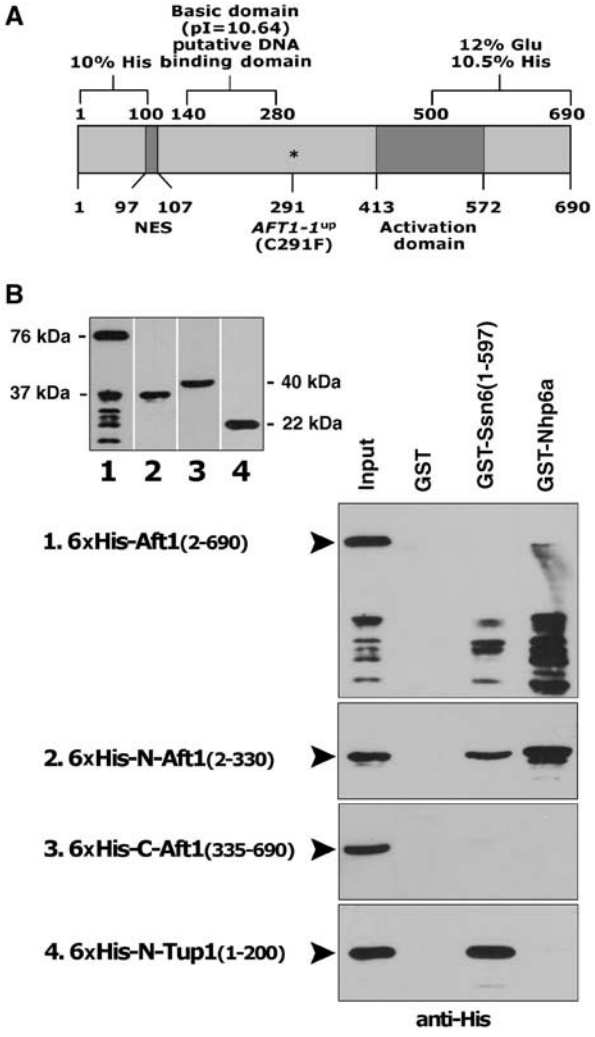

Figure 7 Interaction of Aft1 with Ssn6 and Nhp6a *in vitro*. (**A**) Diagrammatic representation of the Aft1 protein sequence. (**B**) GST or GST-Ssn6(1-597) or GST-Nhp6a bound on glutathione agarose beads were incubated with purified 6xHis-tagged Aft1 derivatives or N-Tup1 (positive control) and analyzed by SDS–PAGE and immunoblotting using anti-His antibody. The input lane contains 20% of the total amount of each 6xHis-tagged protein incubated with the beads. Multiple bands observed in B-1 correspond to Aft1 degradation products. Top left inset: electrophoretic pattern of the 6xHis-tagged proteins (input amounts).

Taken together, our data indicate the following in iron starvation: (a) Aft1 binding on *FRE2* promoter largely depends on its direct interaction with Nhp6a. The possibility of DNA bending playing a major role cannot be excluded. (b) Ssn6 is recruited on *FRE2* promoter via direct interaction with Aft1. (c) Each of Nhp6 and Ssn6 is required for activation-specific chromatin organization. Based on these indications, we propose a model of concerted action of Nhp6 and Ssn6 proteins on *FRE2* promoter, required for Aft1-induced transcription (see Discussion).

Discussion

Accumulating data concerning the specific roles of Nhp6 and Ssn6 commonly used factors on various regulated promoters in yeast reveal new functions and molecular interplays. *FRE2* gene is induced upon iron limitation by the iron-responsive, DNA-binding, transcriptional activator Aft1. Although cellular iron concentration regulates the subcellular localization of Aft1, we found that Nhp6 and Ssn6 global coregulators are necessary to potentiate its activation function acting together at UAS_{Aft1} on the simply regulated *FRE2* promoter.

Nhp6 acts at FRE2 UAS regulating predominantly Aft1 DNA binding

Nhp6 regulates the transcription of a number of RNA polymerase II-transcribed genes. *In vivo* experiments with chimeric promoter constructions have suggested that Nhp6 acts, in most cases, at the core promoters of RNA polymerase II-transcribed genes, whereas *in vitro* binding experiments have demonstrated that it promotes the formation of a complex

with TBP and TFIIA, at the TATA box, with enhanced affinity for TFIIB (Paull *et al*, 1996). Recent genetic analysis has shown that Nhp6 stimulates TBP binding to *HO* promoter, leading to initiation of transcription (Yu *et al*, 2003). Here, we show that *FRE2* is another RNA polymerase II-transcribed gene whose activated transcription depends on Nhp6 protein. Nhp6 is predominantly required at *FRE2* UAS rather than at the core promoter since transcriptional activation of a $FRE2_{UAS}$-*HIS3-LacZ* gene, in which the *FRE2* core promoter has been replaced by the Nhp6-independent respective region from *HIS3*, was reduced by 80% in *nhp6*ΔΔ strain. Thus, Nhp6 seems to intervene in the function of *FRE2* gene's DNA-binding transactivator, Aft1. Interestingly, LexA-Aft1-mediated activation of a *LexAop-HIS3-LacZ* gene was reduced by only 50% in the same strain, revealing that, in the *FRE2* promoter context, Nhp6 predominantly affects the DNA-binding function of Aft1. This was confirmed by showing that recruitment of Aft1 on *FRE2* promoter is quantitatively dependent upon the presence of Nhp6. We further demonstrated *in vivo* association of Nhp6 with Aft1 and *in vitro* direct interaction of Nhp6 with the N-half of Aft1 correlated with its DNA-binding domain. Therefore, we reason that, *in vivo*, Nhp6 enhances the binding of Aft1 to its cognate DNA site at *FRE2* promoter. Consistent with this, under induction conditions, when Aft1 is nuclear, we observed nucleosomal remodeling at the region of UAS_{Aft1}, possibly to improve its accessibility, and this was dependent on the presence of Nhp6. In addition to its effect on Aft1 DNA binding, Nhp6 may also affect directly Aft1 activation function since we observed a residual Nhp6 effect on LexA-Aft1-mediated activation (Figure 2B). These effects could be mediated by chromatin structure-modulating activities known to associate genetically and/or physically with Nhp6 (Yu *et al*, 2000, 2003; Brewster *et al*, 2001; Formosa *et al*, 2001; Szerlong *et al*, 2003). A possible role of SAGA on *FRE2* promoter was implied by the significant transcriptional reduction of *FRE2* mRNA accumulation in a *gcn5*Δ strain (unpublished observations).

An indication that Nhp6 mediates its biological functions by interacting with different proteins at various promoters was previously reported using engineered mutations affecting its RNA polymerase II- or III-related functions but not its DNA-binding and -bending properties (Kruppa and Kolodrubetz, 2001). It is also known that Nhp6 is required for *GAL1* UAS activity (Paull *et al*, 1996), and a direct interaction between the activation domain of Gal4 transactivator and Nhp6b has been demonstrated (Laser *et al*, 2000). However, how this interaction assists Gal4 in its function remains unknown. On the other hand, the mammalian HMGB1/2 proteins enhance the *in vitro* DNA-binding ability of several sequence-specific transcription factors by directly interacting with them *in vitro* and this has been correlated with their role in activation or repression of transcription *in vivo* (Thomas and Travers, 2001; Agresti and Bianchi, 2003). Therefore, a chaperone role has been proposed for nonsequence-specific HMGB proteins in facilitation and stabilization of transcription factor binding, allowing additional proteins or protein complexes to be recruited. Our results provide *in vivo* biochemical evidence for a similar mode of action of the yeast Nhp6 HMGB protein in facilitating the binding of Aft1 transactivator to its target site on *FRE2* promoter.

Ssn6 corepressor is recruited under inducing conditions on FRE2 promoter and mediates transcriptional activation

Our data point to a crucial coactivating role of Ssn6 in Aft1-mediated *FRE2* transcriptional activation. Ssn6 exerts its action at *FRE2* UAS by dramatically affecting Aft1 transactivation function, as revealed by the $FRE2_{UAS}$-*HIS3-LacZ* and *LexAop-HIS3-LacZ* transcriptional activation in an *ssn6*Δ strain. Moreover, similarly to Nhp6, Ssn6 is required for the observed chromatin remodeling of the activated *FRE2* promoter. Since we showed that Ssn6 is recruited on *FRE2* promoter, we assume that Ssn6 may recruit nucleosome modifying and/or chromatin remodeling activities in this particular promoter context. A positive role for the Ssn6–Tup1 complex in transcription has been reported for *CIT2* gene encoding a citrate synthase, regulated by Rtg3, a bHLH/L-Zip DNA-binding transcriptional activator, upon mitochondrial dysfunction (Conlan *et al*, 1999). The authors proposed that specific metabolic signals may convert the Ssn6–Tup1 transcriptional corepressor to a coactivator on certain promoters. *GAL1* and a set of Sko1-regulated osmotic stress-inducible genes have also been identified as targets of Ssn6–Tup1 positive action (Papamichos-Chronakis *et al*, 2002; Proft and Struhl, 2002). The corepressor complex is continuously tethered on these promoters under both repressing and inducing conditions. Upon induction, instead of being released, it facilitates recruitment of SAGA and/or SWI/SNF coactivators that alleviate Ssn6–Tup1-mediated repression. We showed that on *FRE2* promoter Ssn6 is recruited only under inducing conditions, and this depends quantitatively on the presence of Aft1. Moreover, *ssn6*Δ analysis did not reveal any repressive role of Ssn6 under iron-replete conditions. Therefore, we reason that the Ssn6–Tup1 complex is tethered at *FRE2* promoter upon induction and that, thereon, it has an explicitly coactivating role. We assume a Tup1 effect similar to that of Ssn6 since we noticed considerable reduction of induced *FRE2* transcription in *tup1*Δ cells (unpublished observations). The activation function of Ssn6–Tup1 is in agreement with the previously reported decrease in the rate of iron reductive uptake (ferric citrate) in *ssn6*Δ or *tup1*Δ strains (Lesuisse *et al*, 2001). However, the iron reductive uptake results reflect the overall effect of *FRE1* and *FRE2* reductase genes as well as of Aft1-dependent oxidase and permease genes.

Formation of an Aft1–Nhp6–DNA ternary complex on FRE2 promoter allows Ssn6 recruitment, a crucial step for activation

We showed that Ssn6 recruitment, in the particular *FRE2* promoter context, is induced by Nhp6. By analogy, Nhp6 could be involved in tethering Ssn6–Tup1 to various loci. A previously reported Nhp6b–Tup1 interaction (Laser *et al*, 2000) is in support of this notion. However, we were not able to detect interaction between Nhp6a and Tup1 in both our *in vivo* and *in vitro* experiments nor association between Ssn6 and Nhp6 proteins. Therefore, Ssn6 appears to be recruited on *FRE2* promoter via direct interaction with an Aft1–Nhp6–DNA ternary complex. In fact, its recruitment depends on the presence of both Aft1 and Nhp6, while its *in vivo* copurification with Aft1 depends on the presence of Nhp6. Upon recruitment, Ssn6 may further stabilize Aft1 DNA binding, since we found *in vitro* direct interaction

of Ssn6 with the N-half of Aft1 (DNA-binding domain) rather than the C-half (transactivation domain) of Aft1. Consistent with this, Aft1 recruitment on *FRE2* promoter was found to be slightly reduced in an *ssn6*Δ strain (unpublished observations).

Based on the above results taken together, we propose a simple model illustrating the sequence of events leading to the formation of an Ssn6–Aft1–Nhp6–DNA quaternary complex on *FRE2* promoter, responsible for the observed full transcriptional activity (Figure 8). In the absence of either Ssn6 or Nhp6, activity is severely reduced, while in the absence of both it is almost abolished (Figure 2A), revealing an Ssn6–Nhp6 functional cooperativity on *FRE2* promoter.

On the other hand, *FRE1* promoter provides a context where Nhp6 and Ssn6 act independently. Although both factors affect *FRE1* gene expression, each of Ssn6 and Nhp6, as well as Aft1, are independently recruited on *FRE1* promoter. This may be due to the fact that *FRE1* transcription depends on different DNA-binding activators besides Aft1. Considering also our presented RNA analysis of other iron-regulated genes, we reason that promoter context-determined protein interactions decide where, when and how global regulators will exert their function.

Materials and methods

Yeast strains and media

*ssn6*Δ (*cyc8*-Δ*9*::*HIS3*) is an FT5 (*MATa ura3-52 trp1*-Δ*63 his3*-Δ*200 leu2*::*PET56*) derivative (Tzamarias and Struhl, 1994). *nhp6a*Δ n*hp6b*Δ (*nhp6*ΔΔ) and *ssn6*Δ *nhp6*ΔΔ were generated by replacement of the *Bst*EII-*Nde*I fragment of *NHP6A* with *KanMX4* and of the *Sph*I-*Msc*I fragment of *NHP6B* with the *URA3* gene and recovery of uracil auxotrophy on 5-FOA-containing plates (Ausubel *et al*, 1987–2003). FT5(*AFT1-9Myc*) and *nhp6*ΔΔ(*AFT1-9Myc*) strains were generated by C-terminal tagging the genomic *AFT1* (Knop *et al*, 1999). *aft1*Δ is S288C derived (Georgatsou *et al*, 1997). The S288C-derived *ssn6*Δ* was generated by replacing the *Pst*I fragment of *SSN6* by *KanMX4*.

Synthetic complete (SC) medium (0.67% yeast nitrogen base, 20 amino acids, uracil, adenine, 2% glucose, 1.23 μM $FeCl_3$ and 0.25 μM $CuSO_4$; Difco) was supplemented with 100 μM bathophenanthroline disulfonic acid-Na_2 salt (BPS) as Fe(II) and Cu(II) chelator (in effect chelator of reduced iron) or with 100 μM bathocuproine disulfonic acid-Na_2 salt (BCS) as Cu(I) chelator.

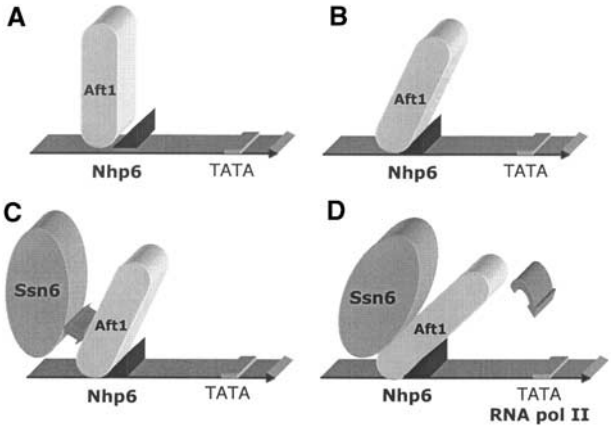

Figure 8 Model depicting how Nhp6a and Ssn6 distinctly and cooperatively potentiate Aft1-mediated transcription on *FRE2* promoter. (**A**) Upon induction under iron depletion, Nhp6 is in proximity with chromatin and Aft1 interacts with DNA and Nhp6. (**B**) Structural alterations are induced, resulting in enhanced Aft1 binding to UAS. (**C**) Ssn6 interaction with Aft1 is favored. (**D**) The presence of Ssn6 on the promoter further stabilizes Aft1 DNA binding and ensures access to the RNA polymerase II transcription machinery components, resulting in full transcriptional activation.

Plasmids

FRE2$_{UAS}$*-HIS3-LacZ* contains *FRE2* promoter (−655 to −419), upstream of the *HIS3* T_R element of the VS11, *URA3*-marked, low-copy plasmid (Georgakopoulos and Thireos, 1992). *LexAop-HIS3-LacZ* contains a single LexA operator upstream of the *HIS3* T_R. LexA-Aft1 plasmid contains the *AFT1* coding sequence between the *Sma*I and *Kpn*I sites of YCp91 (Tzamarias and Struhl, 1994). HA-Aft1 plasmid contains an *EcoR*I-*Nco*I/blunt fragment from LexA-Aft1 plasmid between the *EcoR*I and *Sac*I/blunt sites of pYX142 (Novagen). HA-Ssn6 plasmid contains an *EcoR*I-*Sac*I fragment from LexA-Ssn6 plasmid (Tzamarias and Struhl, 1995) between the *EcoR*I and *Sac*I sites of pYX142. HA-Nhp6a plasmid was constructed by replacing the *Sma*I-*Nhe*I fragment of HA-Ssn6 plasmid with the *NHP6A* coding sequence cloned bluntly between *Sma*I and *Nhe*I/blunt sites. 6xHis-Ssn6 plasmid contains a *Sma*I-*Nco*I fragment from HA-Ssn6 plasmid between the *Nhe*I/blunt and *Nco*I sites of pVTU260 (EUROSCARF). 6xHis-Nhp6a plasmid contains a *Bam*HI fragment of *NHP6A* inserted between the *Nhe*I/blunt and *Bam*HI sites of pVTU260. Fusion proteins are functional as judged by phenotypic complementation of the corresponding mutants, and protein levels are not significantly affected in mutant strains as confirmed by Western blot analysis. GST-N-Ssn6(1-597) plasmid contains a *Sma*I-*Pvu*II fragment from HA-Ssn6 plasmid into the *Sma*I site of pGEX-T2 (Pharmacia). GST-Nhp6a plasmid contains the *NHP6A* coding sequence into the *Sma*I site of pGEX-T2. *AFT1* coding sequence was cloned into the *Pvu*II site of pRSET-C (Invitrogen) to yield the 6xHis-Aft1(2-690) plasmid. 6xHis-N-Aft1(2-330) plasmid was generated by removal of the *Nsi*I-*Nco*I fragment from 6xHis-Aft1(2-690). 6xHis-C-Aft1(335-690) plasmid contains a PCR-synthesized *AFT1* fragment (+1001 to +2070) into the *Pvu*II site of pRSET-C. 6xHis-N-Tup1(1-200) plasmid derived from pRSET-A.

Oligonucleotide primers were purchased from the Microchemistry Lab at FORTH and MWG-Biotech. All PCR primer sequences are available on request. The polymerases used for PCR were Vent (New England Biolabs) or *Taq* (MINOTECH Biotechnology).

Genetic screen

In a *leu2-1 ura3-52* S288C-derived strain, *HIS3* gene was replaced with a *FRE2-HIS3* hybrid containing the *FRE2* regulatory region (−816 to −1) fused to the *HIS3* coding region, leading to iron-dependent histidine auxotrophy. In this selection scheme, we sought mutants affecting putative regulators of *FRE2* but not *FRE1* transcription. A *FRE2*-affecting *trans* semidominant mutation that caused very poor growth on low-iron plates lacking histidine, compared to a similarly engineered wild-type auxotroph, was isolated. We did not identify the mutated gene (it was not an *AFT1* allele), but instead sought suppressors of this mutation. A YEp13-based high-copy yeast genomic library (Kim Nasmyth), containing inserts of 5–10 kb, was introduced in the mutant strain, and transformants, initially selected for leucine prototrophy, were screened for growth on plates containing iron-depleted (100 μM BPS) minimal medium lacking histidine, in the presence of 1 mM of 3-aminotriazole, a competitive inhibitor of His3 protein, to repress basal *HIS3* expression. Five plasmids carried inserts from chromosome XVI, with *NHP6A* gene being responsible for suppression. *NHP6A* did not complement the chromosomal mutation since the *NHP6A* allele from the mutant strain could also suppress the *FRE2* mutant phenotype.

RNA analysis

Total RNA was extracted by the acid phenol method (Ausubel *et al*, 1987–2003) from cultures grown to an OD_{550} of 0.6–1.0 in SC medium or initially in SC and then in SCBPS for 7 h or SCBCS for 3 h.

β-Galactosidase and one-hybrid assays

In all, 5 ml of yeast cultures were grown in SC to an OD_{550} of 1.5, diluted and regrown for 7 h in SC or SCBPS, and β-galactosidase activity was measured (Ausubel *et al*, 1987–2003).

In vivo nucleosome-remodeling assay

Chromatin analysis of *FRE2* promoter was performed by micrococcal nuclease (MNase) digestion using nystatin-permeabilized spheroplasts (Venditti and Camilloni, 1994) followed by indirect end-labeling. Secondary digestion was with *Xho*I at position +901, and a ^{32}P-labeled fragment from position +441 to +864 was used as a probe.

Chromatin immunoprecipitation assays

In all, 50 ml of yeast cultures grown to an OD_{550} of 0.6–1.0 initially in SC and then in SCBPS for 7 h were used for ChIP assays (Kuo and Allis, 1999) with polyclonal anti-HA and anti-Myc antibodies (Santa Cruz). Immunoprecipitated (3/100 µl) and total input DNA (3/1000 µl) were analyzed by PCR (4 min at 94°C; 30 s at 94°C, 30 s at 52°C, 55 s at 72°C (27 cycles); 5 min at 72°C). PCR products electrophoresed on 1.7% agarose gels were quantified using the PhosphorImager and ImageQuant software (Molecular Dynamics). The pairs of primers used were specific for promoter or ORF regions: *FRE2*(−596/−297), *FRE1*(−353/−1), *HTA1*(−665/−373), *TRP3*(−146/+143) and *ACT1*(+415/+724).

6xHis pull-down assays in yeast cells

Cells cotransformed with plasmids expressing 6xHis- and HA-tagged proteins were grown to an OD_{550} of 0.8, initially in SC and then in SCBPS for 7 h. Proteins were extracted from lysed cells in 50 mM HEPES-KOH, pH 7.5, 5 mM magnesium acetate, 75 mM potassium acetate, 0.1% NP-40, 0.5 mg/ml BSA, 5 mM imidazole, 10% glycerol and protease inhibitors. The supernatant was incubated with Ni-NTA beads for 1 h at 4°C. Beads were washed in the same buffer without BSA. Retained proteins were eluted in gel loading buffer and analyzed by SDS–PAGE and immunoblotting (Ausubel *et al*, 1987–2003) using polyclonal anti-His, anti-HA (Santa Cruz) and anti-Tup1 antibodies. Secondary antibodies were visualized by an ECL Western blotting detection kit (Pierce).

In vitro interaction assays

GST-tagged proteins were expressed in *E. coli* ER2566 (Tzamarias and Struhl, 1995). 6xHis-tagged proteins expressed in ER2566, purified by Ni-NTA chromatography (Qiagen), were eluted in 75 mM KCl, 20 mM Tris, pH 8.00, 0.01% NP-40, 0.25% BSA, 250 mM imidazole and protease inhibitors and incubated with glutathione agarose bead-bound GST or GST hybrid protein for 8–12 h, at 4°C, in 200 µl of the above buffer without imidazole. Beads were washed in the same buffer without BSA and retained proteins were eluted in gel loading buffer and analyzed by SDS–PAGE and immunoblotting using a polyclonal anti-His antibody (Santa Cruz).

Acknowledgements

We thank A Voutsina, E Georgatsou, Y Papanikolau, I Topalidou, G Thireos and I Talianidis for helpful discussions, M Papamichos-Chronakis for the 6xHis-N-Tup1 plasmid, Tup1 antibody and advice, N Gounalaki for the *nhp6*ΔΔ, *ssn6*Δ *nhp6*ΔΔ FT5-derived strains, G Thireos for *LexAop-HIS3-LacZ* plasmid, A Tartas for constructing the LexA-Aft1 plasmid and I Kagiampakis for computational assistance. This work was supported by the Greek Ministry of Development-GSRT (IMBB funding and PENED grant).

References

Agresti A, Bianchi ME (2003) HMGB proteins and gene expression. *Curr Opin Genet Dev* **13:** 170–178

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1987–2003) *Current Protocols in Molecular Biology*. New York: Greene Publishing Associates and Wiley-Interscience

Blaiseau PL, Lesuisse E, Camadro JM (2001) Aft2p, a novel iron-regulated transcription activator that modulates, with Aft1p, intracellular iron use and resistance to oxidative stress in yeast. *J Biol Chem* **276:** 34221–34226

Brewster NK, Johnston GC, Singer RA (2001) A bipartite yeast SSRP1 analog comprised of Pob3 and Nhp6 proteins modulates transcription. *Mol Cell Biol* **21:** 3491–3502

Bustin M (1999) Regulation of DNA-dependent activities by the functional motifs of the high-mobility-group chromosomal proteins. *Mol Cell Biol* **19:** 5237–5246

Conlan RS, Gounalaki N, Hatzis P, Tzamarias D (1999) The Tup1–Cyc8 protein complex can shift from a transcriptional co-repressor to a transcriptional co-activator. *J Biol Chem* **274:** 205–210

Costigan C, Kolodrubetz D, Snyder M (1994) NHP6A and NHP6B, which encode HMG1-like proteins, are candidates for downstream components of the yeast SLT2 mitogen-activated protein kinase pathway. *Mol Cell Biol* **14:** 2391–2403

Dancis A, Roman DG, Anderson GJ, Hinnebusch AG, Klausner RD (1992) Ferric reductase of *Saccharomyces cerevisiae*: molecular characterization, role in iron uptake, and transcriptional control by iron. *Proc Natl Acad Sci USA* **89:** 3869–3873

Formosa T, Eriksson P, Wittmeyer J, Ginn J, Yu Y, Stillman DJ (2001) Spt16-Pob3 and the HMG protein Nhp6 combine to form the nucleosome-binding factor SPN. *EMBO J* **20:** 3506–3517

Georgakopoulos T, Thireos G (1992) Two distinct yeast transcriptional activators require the function of the GCN5 protein to promote normal levels of transcription. *EMBO J* **11:** 4145–4152

Georgatsou E, Alexandraki D (1994) Two distinctly regulated genes are required for ferric reduction, the first step of iron uptake in *Saccharomyces cerevisiae*. *Mol Cell Biol* **14:** 3065–3073

Georgatsou E, Mavrogiannis LA, Fragiadakis GS, Alexandraki D (1997) The yeast Fre1p/Fre2p cupric reductases facilitate copper uptake and are regulated by the copper-modulated Mac1p activator. *J Biol Chem* **272:** 13786–13792

Jensen LT, Winge DR (1998) Identification of a copper-induced intramolecular interaction in the transcription factor Mac1 from *Saccharomyces cerevisiae*. *EMBO J* **17:** 5400–5408

Knop M, Siegers K, Pereira G, Zachariae W, Winsor B, Nasmyth K, Schiebel E (1999) Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. *Yeast* **15:** 963–972

Kolodrubetz D, Burgum A (1990) Duplicated NHP6 genes of *Saccharomyces cerevisiae* encode proteins homologous to bovine high mobility group protein 1. *J Biol Chem* **265:** 3234–3239

Kolodrubetz D, Kruppa M, Burgum A (2001) Gene dosage affects the expression of the duplicated NHP6 genes of *Saccharomyces cerevisiae*. *Gene* **272:** 93–101

Kosman DJ (2003) Molecular mechanisms of iron uptake in fungi. *Mol Microbiol* **47:** 1185–1197

Kruppa M, Kolodrubetz D (2001) Mutations in the yeast Nhp6 protein can differentially affect its *in vivo* functions. *Biochem Biophys Res Commun* **280:** 1292–1299

Kruppa M, Moir RD, Kolodrubetz D, Willis IM (2001) Nhp6, an HMG1 protein, functions in SNR6 transcription by RNA polymerase III in *S. cerevisiae*. *Mol Cell* **7:** 309–318

Kuo MH, Allis CD (1999) *In vivo* cross-linking and immunoprecipitation for studying dynamic protein:DNA associations in a chromatin environment. *Methods* **19:** 425–433

Laser H, Bongards C, Schuller J, Heck S, Johnsson N, Lehming N (2000) A new screen for protein interactions reveals that the *Saccharomyces cerevisiae* high mobility group proteins Nhp6A/B are involved in the regulation of the GAL1 promoter. *Proc Natl Acad Sci USA* **97:** 13732–13737

Lesuisse E, Blaiseau PL, Dancis A, Camadro JM (2001) Siderophore uptake and use by the yeast *Saccharomyces cerevisiae*. *Microbiology* **147:** 289–298

Lopez S, Livingstone-Zatchej M, Jourdain S, Thoma F, Sentenac A, Marsolier MC (2001) High-mobility-group proteins NHP6A and NHP6B participate in activation of the RNA polymerase III SNR6 gene. *Mol Cell Biol* **21:** 3096–3104

Martin MP, Gerlach VL, Brow DA (2001) A novel upstream RNA polymerase III promoter element becomes essential when the chromatin structure of the yeast U6 RNA gene is altered. *Mol Cell Biol* **21:** 6429–6439

Moreira JM, Holmberg S (2000) Chromatin-mediated transcriptional regulation by the yeast architectural factors NHP6A and NHP6B. *EMBO J* **19:** 6804–6813

Papamichos-Chronakis M, Petrakis T, Ktistaki E, Topalidou I, Tzamarias D (2002) Cti6, a PHD domain protein, bridges the

Cyc8–Tup1 corepressor and the SAGA coactivator to overcome repression at GAL1. *Mol Cell* **9:** 1297–1305

Paull TT, Carey M, Johnson RC (1996) Yeast HMG proteins NHP6A/B potentiate promoter-specific transcriptional activation *in vivo* and assembly of preinitiation complexes *in vitro*. *Genes Dev* **10:** 2769–2781

Paull TT, Johnson RC (1995) DNA looping by *Saccharomyces cerevisiae* high mobility group proteins NHP6A/B. Consequences for nucleoprotein complex assembly and chromatin condensation. *J Biol Chem* **270:** 8744–8754

Proft M, Struhl K (2002) Hog1 kinase converts the Sko1–Cyc8–Tup1 repressor complex into an activator that recruits SAGA and SWI/SNF in response to osmotic stress. *Mol Cell* **9:** 1307–1317

Rutherford JC, Jaron S, Winge DR (2003) Aft1p and Aft2p mediate iron-responsive gene expression in yeast through related promoter elements. *J Biol Chem*

Sidorova J, Breeden L (1999) The MSN1 and NHP6A genes suppress SWI6 defects in *Saccharomyces cerevisiae*. *Genetics* **151:** 45–55

Smith RL, Johnson AD (2000) Turning genes off by Ssn6–Tup1: a conserved system of transcriptional repression in eukaryotes. *Trends Biochem Sci* **25:** 325–330

Szerlong H, Saha A, Cairns BR (2003) The nuclear actin-related proteins Arp7 and Arp9: a dimeric module that cooperates with architectural proteins for chromatin remodeling. *EMBO J* **22:** 3175–3187

Thomas JO, Travers AA (2001) HMG1 and 2, and related 'architectural' DNA-binding proteins. *Trends Biochem Sci* **26:** 167–174

Tzamarias D, Struhl K (1994) Functional dissection of the yeast Cyc8–Tup1 transcriptional co-repressor complex. *Nature* **369:** 758–761

Tzamarias D, Struhl K (1995) Distinct TPR motifs of Cyc8 are involved in recruiting the Cyc8–Tup1 corepressor complex to differentially regulated promoters. *Genes Dev* **9:** 821–831

Venditti S, Camilloni G (1994) *In vivo* analysis of chromatin following nystatin-mediated import of active enzymes into *Saccharomyces cerevisiae*. *Mol Gen Genet* **242:** 100–104

Wong B, Masse JE, Yen YM, Giannikopoulos P, Feigon J, Johnson RC, Giannikoupolous P (2002) Binding to cisplatin-modified DNA by the *Saccharomyces cerevisiae* HMGB protein Nhp6A. *Biochemistry* **41:** 5404–5414

Yamaguchi-Iwai Y, Dancis A, Klausner RD (1995) AFT1: a mediator of iron regulated transcriptional control in *Saccharomyces cerevisiae*. *EMBO J* **14:** 1231–1239

Yamaguchi-Iwai Y, Serpe M, Haile D, Yang W, Kosman DJ, Klausner RD, Dancis A (1997) Homeostatic regulation of copper uptake in yeast via direct binding of MAC1 protein to upstream regulatory sequences of FRE1 and CTR1. *J Biol Chem* **272:** 17711–17718

Yamaguchi-Iwai Y, Stearman R, Dancis A, Klausner RD (1996) Iron-regulated DNA binding by the AFT1 protein controls the iron regulation in yeast. *EMBO J* **15:** 3377–3384

Yamaguchi-Iwai Y, Ueta R, Fukunaka A, Sasaki R (2002) Subcellular localization of Aft1 transcription factor responds to iron status in *Saccharomyces cerevisiae*. *J Biol Chem* **277:** 18914–18918

Yen YM, Wong B, Johnson RC (1998) Determinants of DNA binding and bending by the *Saccharomyces cerevisiae* high mobility group protein NHP6A that are important for its biological activities. Role of the unique N terminus and putative intercalating methionine. *J Biol Chem* **273:** 4424–4435

Yu Y, Eriksson P, Bhoite LT, Stillman DJ (2003) Regulation of TATA-binding protein binding by the SAGA complex and the Nhp6 high-mobility group protein. *Mol Cell Biol* **23:** 1910–1921

Yu Y, Eriksson P, Stillman DJ (2000) Architectural transcription factors and the SAGA complex function in parallel pathways to activate transcription. *Mol Cell Biol* **20:** 2350–2357

Zhang L, Guarente L (1994) Evidence that TUP1/SSN6 has a positive effect on the activity of the yeast activator HAP1. *Genetics* **136:** 813–817