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

George S Fragiadakis1,2, Dimitris Tzamaris1,3 and Despina Alexandraki1,2,*

1Institute of Molecular Biology and Biotechnology-FORTH, University of Crete, Vassilika Vouton, Heraklion, Crete, Greece, 2Department of Biology, University of Crete, Greece and 3School 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.

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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.

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 (Paul and Johnson, 1995; Yen et al., 1998; Wong et al., 2002).

Either nhp6Δ or nhp6βΔ mutant exhibits no observable phenotype, reflecting a functional redundancy of the two proteins (Costigan et al., 1994). Notably, Nhp6β, less abundant than Nhp6α, is increased in nhp6Δ cells to compensate for the absence of its counterpart (Kolodrubetz et al., 2001). On the other hand, nhp6Δ 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 nhp6Δ strain (Paul et al., 1996; Siderova 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 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 ssn6Δ, nhp6ΔΔ and ssn6Δ nhp6ΔΔ mutants, compared to a wild-type strain, under basal and inducing conditions. We used an nhp6ΔΔ 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 ssn6Δ 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 nhp6ΔΔ 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 ssn6Δ nhp6ΔΔ triple mutation produced results similar to those of ssn6Δ and nhp6ΔΔ alone, under inducing conditions, whereas it revealed an epistatic effect of nhp6ΔΔ on ssn6Δ 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-LacZ-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 (Paul 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 ssn6Δ and nhp6ΔΔ, compared to wild-type strain,
indicating a considerable effect of SSN6 and NHP6A/B on Aft1-mediated transcription. Interestingly, it was further reduced in the triply deleted ssn6Δ nhp6ΔΔ 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 ssn6Δ, 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 nhp6ΔΔ 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 ssn6Δ or nhp6ΔΔ 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
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 nhp6Δ/Δ or ssn6Δ/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 nhp6Δ/Δ and ssn6Δ/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) 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 nhp6Δ/Δ 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 Nhp6Δ 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 ssn6Δ 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
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 Sn6 on FRE2 promoter (Figure 5D), consistent with its dependency on the presence of Aft1. On the other hand, Sn6 is also present at FRE1 promoter, but independently of Aft1 and Nhp6. Under high-iron conditions, recruitment of Sn6 on FRE1 promoter was observed at low levels (Figure 3D), consistent with the FRE1-independent low level of Aft1 expression in these conditions (data not shown, but similar to SC conditions shown in Figure 1).

Sn6 and Nhp6a associate with Aft1 in vivo but not with one another

A preliminary indication for an Aft1–Sn6 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 multicycoplasms, probably caused a dramatic decrease in growth rate, being toxic for cells. On this basis, we performed copurification assays in vivo in aft1Δ cells expressing HA-Aft1 and 6xHis-Sn6 or 6xHis-Nhp6a proteins in order to verify the interdependence of Aft1 with Sn6 and Nhp6 on FRE2 promoter. These assays were carried out under iron-depletion conditions to ensure quantitative Aft1 colocalization with Sn6 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-Sn6 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-Sn6 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 Sn6 and Nhp6a, we next examined whether Sn6 and Nhp6a associate with one another as well. The in vivo copurification assay was performed in nhp6ΔΔ cells expressing HA-Nhp6a and 6xHis-Sn6 proteins. As shown in Figure 6C, HA-Nhp6a did not copurify with 6xHis-Sn6 (left panel) while Tup1 did (right panel), indicating that Nhp6a did not associate with Sn6 under the conditions employed. We used iron-depletion conditions as Sn6 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-Sn6 and 6xHis-Nhp6a proteins was not informative due to high nonspecific retention of HA-Sn6 on the Ni-NTA agarose beads (data not shown).

The above data revealed that Aft1 associates with Sn6 and Nhp6a in vivo, consistent with its interdependence with each of these proteins on FRE2 promoter. They also showed that Sn6 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 Sn6 was abolished in cells lacking Nhp6 as shown by in vivo copurification of HA-Aft1 with the 6xHis-Sn6 in wild-type and nhp6ΔΔ cells (Figure 6D).

Sn6 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-Sn6, 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 Sn6 protein could not be produced in bacteria. However, the N-Sn6 derivative tested in this assay, containing the entire TPR domain of Sn6 (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, introduced in an sn6Δ strain (data not shown).

As shown in Figure 7B, GST-N-Sn6 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 Sn6 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 Nhp6a-facilitated Aft1 binding on FRE2 promoter. Finally, GST-N-Sn6 interacted with 6xHis-N-Tup1 as expected, while GST-Nhp6a did not (7B-4), in accordance with what we observed in vivo.

Figure 5 Association of Aft1 and Sn6 with FRE1 and FRE2 promoters. (A) Wild-type and nhp6ΔΔ 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) sn6Δ and sn6Δ aft1Δ cells, transformed with an HA-Sn6 expressing plasmid, were grown and analyzed as above using anti-HA and primers specific for FRE1 and FRE2 promoters and ACT1 coding region. (C) sn6Δa and sn6Δa nhp6ΔΔ cells were transformed, grown and analyzed as above using anti-HA and primers specific for FRE1, FRE2 and TRP3 promoters. (D) sn6Δa cells transformed with an HA-Sn6 expressing plasmid and grown under high-iron conditions (SC supplemented with 200 μM FeCl3, 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-Sn6 on FRE1 and FRE2 promoters in sn6Δa cells of two different genetic backgrounds was comparable.

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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\textsubscript{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 TFIIH (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\textsubscript{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\textsubscript{D}A 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 gcn5A 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\textsubscript{UAS}/His3-LacZ and LexAop-His3-LacZ transcriptional activation in an ssn6\textsubscript{D}A 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; Profi 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\textsubscript{D}A 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, therefore, 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\textsubscript{A} 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\textsubscript{D}A or tup1\textsubscript{A} 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 Nhp6–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 ssn6Δ 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

ssn6Δ (cys2-809::HIS3) is an FTS (MATa ura3-52 trp1-Δ63 his3-Δ200 leu2-2::PET56) derivative (Tzamarias and Struhl, 1994). nhp6Δ nhp6ΔΔ and ssn6Δ nhp6ΔΔ were generated by replacement of the BstEl-Idel fragment of NHp6A with KanMX4 and of the Spht-Mclsl fragment of NHp6Δ with the URA3 gene and recovery of uracil auxotrophy on 5-FOA-containing plates (Ausubel et al., 1987–2003). FT5(AFT1-9Myc) and nhp6ΔΔ(AFT1-9Myc) strains were generated by C-terminal tagging the genomic AFT1 (Knope et al., 1999). aft1Δ is S288C derived (Georgatsou et al., 1997). The S288C-derived ssn6Δ* was generated by replacing the PstI fragment of Ssn6 with KanMX4.

Synthetic complete (SC) medium (0.67% yeast nitrogen base, 20 amino acids, uracil, adenine, 2% glucose, 1.23 μM FeCl3, and 0.25 μM CuSO4·Difco) was supplemented with 100 μM bathophenanthroline disulfoxonic acid-Na2 salt (BPS) as Fe(II) and Cu(II) chelator (in effect chelator of reduced iron) or with 100 μM bathocuproine disulfoxonic acid-Na2 salt (BCS) as Cu(II) chelator.

Plasmids

FRE2ΔEAS-HIS3-LacZ contains FRE2 promoter (−655 to −419), upstream of the HIS3 TR element of the VS1, URA3-marked, low-copy plasmid (Georgakopoulos and Theireos, 1992). LexA-NHP6-HIS3-LacZ contains a single LexA operator upstream of the HIS3 TR. LexA-Aft1 plasmid contains the AFT1 coding sequence between the SmaI and KpnI sites of Ycpl91 (Tzamarias and Struhl, 1994). HA-Aft1 plasmid contains an EcoRI-Ncol/blunt fragment from LexA-Aft1 plasmid between the EcoRI and Saci/blunt sites of pY142 (Novagen). HA-Ssn6 plasmid contains an EcoRI-SacI fragment from LexA-Ssn6 plasmid (Tzamarias and Struhl, 1995) between the EcoRI and SacI sites of pY142. HA-Nhp6a plasmid was constructed by replacing the SmaI-Nhel fragment of HA-Ssn6 plasmid with the NHp6A coding sequence cloned bluntly between Smal and Nhel/blunt sites. 6xHis-Ssn6 plasmid contains a Sncol-Nhel fragment from HA-Ssn6 plasmid between the Nhel/blunt and Ncol sites of pVTU260 (EUROSCARF). 6xHis-Nhp6a plasmid contains a BamHI fragment of NHp6A inserted between the Nhel/blunt and BamHI 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 SmaI-PstI fragment from HA-Ssn6 plasmid into the Smal site of pgEX-T2 (Pharmacia). GST-Nhp6a plasmid contains the NHp6A coding sequence cloned into the SmaI site of pgEX-T2. AFT1 coding sequence was cloned into the PvuII 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 Ncol-Nhel fragment from 6xHis-Aft1(2-690). 6xHis-N-Aft1(335-690) plasmid contains a PCR-synthesized AFT1 fragment (+100 to +2070) into the PvuII 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-amino triazolone, 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 OD550 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 OD550 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 (MNaše) digestion using nystatin-permeabilized spheroplasts (Venditti and Camilli, 1994) followed by indirect end-labeling. Secondary digestion was with Hhp1 at position +901, and a 32P-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 OD550 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 were 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.

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