Evidence That *TUP1/SSN6* Has a Positive Effect on the Activity of the Yeast Activator HAP1

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ABSTRACT

The activity of the yeast transcriptional activator HAP1 is controlled by heme and the heme effect is mediated through the heme domain of HAP1. In this report, we show that HAP1 activity is significantly reduced in strains deleted of *TUP1* or *SSN6*, and addition of a heme analog does not allow HAP1 to regain its full activity. Deletion of the heme domain alleviates the requirement for *TUP1/SSN6*. The results suggest that TUP1/SSN6 have a positive effect on the activity of HAP1 and this effect is mediated through the heme domain. Although TUP1/SSN6 generally repress transcription of many genes, our data indicate they may have positive effect on the expression of certain genes.

HAP1 is a transcription factor that controls the expression of many mitochondrial enzymes involved in the oxidative phosphorylation-electron transport pathway. It is a zinc finger DNA binding protein and its activity is modulated by the availability of oxygen (PFEIFER *et al.* 1989). The presence of oxygen allows synthesis of heme which serves as the inducer of HAP1 activity *in vivo*. HAP1 is a 1483 residue protein containing a zinc finger DNA binding domain and dimerization domain between residues 1–148, a heme responsive domain between residues 244 and 444, a highly acidic activation domain between residues 1309 and 1483, and a 800-residue internal region of unknown function. The zinc finger of HAP1 is homologous to a family of yeast transcription factors including GAL4, LAC9 and MAL63 (JOHNSTON 1987).

HAP1 forms dimers and binds to different sequence elements, such as the UAS1 of *CYC1* (iso-1-cytochrome *c*), the UAS of *CYC7* (iso-2-cytochrome *c*), and the UAS of *CYT1* (cytochrome c_1). *In vitro*, HAP1 will not bind to DNA unless heme is included in the reaction. *In vivo*, HAP1 will only activate the promoters when the level of heme is sufficiently high (GUARENTE *et al.* 1984; PFEIFER *et al.* 1987, 1989). HAP1 activity in wild-type cells can be activated further by the addition of the heme analog, deuteroporphyrin IX (dpIX), to the media (GUARENTE *et al.* 1984; PFEIFER *et al.* 1989). Many lines of evidence suggest that heme regulates HAP1 activity by binding directly to the heme domain of HAP1 (PFEIFER *et al.* 1989; ZHANG *et al.* 1993). Binding of heme allows HAP1 to dimerize and bind to DNA. Further, recent experiments in this (L. ZHANG and L. GUARENTE, manuscript submitted) and the Guiard laboratory (FYTLOVICH *et al.* 1993) show that one or more cellular factors interacts with the heme domain of HAP1 and represses its DNA binding and transcriptional activity in the absence of heme. That is, heme initiates a series of events leading to HAP1 activation, including the disassembling of the multiple component, HAP1 repression complex, and dimeriztion of HAP1 (L. ZHANG and L. GUARENTE, manuscript submitted).

TUP1/SSN6 form a high molecular weight complex that represses transcription of many promoters (WILLIAMS *et al.* 1991; KELEHER *et al.* 1992). Repression of transcription by α2 and MIG1 also requires TUP1/SSN6 (reviewed in TRUMBLY 1992). Further, positioning TUP1/SSN6 at a lexA site via fusion to lexA also elicits repression, suggesting that the TUP1/SSN6 complex is the active agent in mediating repression by α2 and MIG1 (KELEHER *et al.* 1992; TRUMBLY 1992). Deletion or mutations of *TUP1* or *SSN6* increases transcription from many yeast promoters, including *SUC2* and *ANB1* (SCHULTZ and CARLSON 1987; ZHANG *et al.* 1991). This could occur, for example, if the TUP1/SSN6 complex bound to the transcriptional activators in these systems to repress their activity. Thus, we explored the possibility that the general repressor, TUP1/SSN6, was the cellular factor that bound HAP1 in the absence of heme. However, our experiments show that, in contrast to our expectation, TUP1/SSN6 have a positive role in the heme induction of HAP1 activity. We discuss this finding in light of a model for heme regulation of oxygen-induced genes in yeast.

MATERIALS AND METHODS

Yeast strains and methods: *Saccharomyces cerevisiae* strains used were BWG 1-7a (**a**, *leu2-2, 112, his4-519, ade1-100, ura3-52*) (GUARENTE *et al.* 1984), LPY22 (**a**, *leu2-2, 112, his4-519, ade1-100, ura3-52, hap1Δ::LEU2*) (TURCOTTE and GUARENTE 1992), 1-7aΔ*tup1*, and LPY22Δ*tup1*. Deletion of *TUP1* was constructed using plasmid pFW36 as described (WILLIAMS and TRUMBLY 1990). 1-7aΔ*ssn6* was generated using plasmid pDSB as described (TRUMBLY 1988). The deletions were confirmed by Southern analysis or complementation. Cells were grown in YPD or synthetic complete media (ROSE *et al.* 1988).

TABLE 1

Effect of deleting *TUP1 or SSN6* on the β-galactosidase activities of various reporters

Reporter	WT	Δ*ssn6*	Δ*tup1*
ANB1	<1	400	150
UAS2up1/*CYC1*	48	33	30
His 66 (*HIS4*)	89	56	67
SD5 (*GAL*)	1163	1203	1706
UAS1/*CYC1*	220	10	14

β-Galactosidase activities of different reporters were assayed in 1–7a, 1–7aΔ*tup1*, and 1–7aΔ*ssn6* cells. Cells were grown in 2% glucose dropout medium or 2% galactose dropout medium for assaying SD5 reporter activity. All the reporters were constructed using the basal *CYC1* promoter lacking any UASs. The UAS2up1/*CYC1* reporter and the UAS1/*CYC1* reporter contain the *CYC1* promoter sequences up to −265, and −229 to −312, respectively (GUARENTE *et al.* 1984). SD5 contains the UAS of *GAL 1–10* promoter (GUARENTE *et al.* 1982). His 66 contains the 89bp *HIS4* promoter fragment containing BAS1, BAS2, and GCN4 binding sites (ARNDT *et al.* 1987). The *ANB1* reporter contains sequences upstream to −312 of pLG669-Z (GUARENTE *et al.* 1984). WT = wild type.

β-Galactosidase assays: Cells carrying plasmids were generally grown in selective synthetic complete media containing 2% glucose (ROSE *et al.* 1988). Cells carrying HAP1 expression plasmids under the control of UAS*GAL* (TURCOTTE and GUARENTE 1992; *SD5HAP1*) were grown in 2% raffinose and then induced with 2% galactose for 8 hr before assaying for β-galactosidase activity. Strains deleted of *TUP1* or *SSN6* were maintained at room temperature instead of 30°. EDTA was added to the cells prior to β-galactosidase assays as described (TRUMBLY 1986). At least three independent transformants were assayed and average values were listed here.

Yeast extracts and DNA-binding assays: Cells were grown as described above. 200 ml of cells were harvested by centrifugation and washed in buffer A [200 mM Tris-HCl, pH 8.0, 400 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 7 mM 2-mercaptoethanol, 1 μg/ml leupeptin, 1 μg/ml pepstatin], and resuspended in buffer B [20 mM Hepes, pH 8.0, 5 mM EDTA, 20% glycerol, 1 mM PMSF, 7 mM 2-mercaptoethanol, 1 μg/ml leupeptin, 1 μg/ml pepstatin]. Then, cells were permeabilized by agitation with 0.75 volume of glass beads as described by PFEIFER *et al.* (1987). Extracts were collected after centrifugation of the permeabilized cells. The radiolabeled UAS1/*CYC1* was prepared as described (TURCOTTE and GUARENTE 1992; ZHANG *et al.* 1993). The binding reactions were carried out exactly as described (ZHANG *et al.* 1993). The reaction mixtures were loaded onto 4% polyacrylamide gels in 1/2TBE and electrophoresis was carried out at 4°.

RESULTS

TUP1/SSN6 affects HAP1 transcriptional activity: HAP1 associates with a high molecular complex in the absence of heme to repress its activity (FYTLOVICH *et al.* 1993; L. ZHANG and L. GUARENTE, manuscript submitted). We wished to investigate whether TUP1/SSN6 comprised this repression complex. If so, activation by HAP1 may no longer require heme in a *tup1* or *ssn6* deletion mutant. Table 1 shows the effects of deleting *TUP1* or *SSN6* on the activities of various reporters. Deletions of *SSN6* or *TUP1* had no significant effect on transcription by the *HIS4* (His-66) promoter (ARNDT *et al.* 1987), the *GAL1-10* (SD5) promoter (GUARENTE *et al.* 1982), or the HAP2/3/4-responsive UAS2up1 element (GUARENTE *et al.* 1984). Also, as expected, the *ANB1* promoter was greatly stimulated in the Δ*ssn6* or Δ*tup1* strains (ZHANG *et al.* 1991). However, contrary to expectation, the level of UAS1 was greatly decreased in the Δ*ssn6* or Δ*tup1* strains. This is the first instance in which SSN6 and TUP1 have been shown to be positive regulators. This unusual finding prompted us to abandon the model that TUP1/SSN6 repressed HAP1 and to investigate further their requirement to activate HAP1.

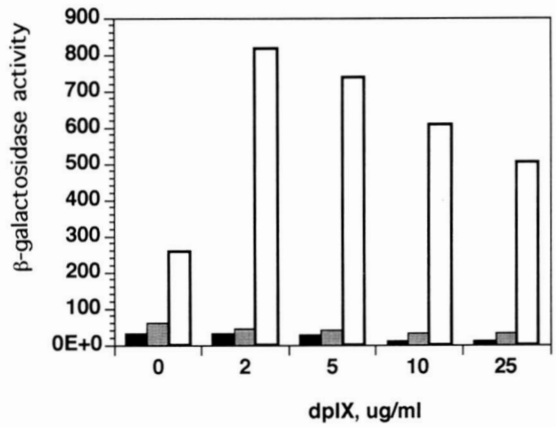

FIGURE 1.—The effect of the heme analogue, dpIX, on the HAP1 activity in cells deleted of *TUP1* or *SSN6*. β-Galactosidase activity of UAS1/*CYC1* reporter (pLGΔ312ΔAX, GUARENTE *et al.* 1984) was assayed in 1-7a, 1-7aΔ*tup1*, and 1-7aΔ*ssn6* cells in the presence of various concentrations of dpIX. The unfilled, filled, and black bars represent the β-galactosidase activity in 1-7a, 1-7aΔ*tup1*, and 1-7aΔ*ssn6* cells, respectively.

TUP1/SSN6 affects HAP1 activity through the heme domain: To determine if the effect of *TUP1/SSN6* deletion on HAP1 activity was related to heme synthesis, we tested how HAP1 activity is affected by the addition of the heme analog, deuteroporphyrin IX (dpIX). Addition of dpIX to heme deficient cells allows HAP1 to gain full activity, while addition of dpIX to wild type cells further enhance HAP1 activity above its normal level (GUARENTE *et al.* 1984; PFEIFER *et al.* 1989). If the lower activity of HAP1 in Δ*tup1* or Δ*ssn6* cells are due to deficiency in heme synthesis, addition of dpIX should complement that defect and allows HAP1 to gain full activity. β-galactosidase assays show that dpIX increased activity of a *TUP1* strain above the normally activated levels as expected, but did not affect the low levels in the Δ*tup1* strain (Figure 1). This result suggests that the reduction of HAP1 activity in the absence of TUP1 or SSN6 gene product is not due to the lack of heme synthesis, but may indicate a more direct involvement of these factors in activation by HAP1.

To map which domain of HAP1 was responsible for mediating the effects of TUP1/SSN6, we inserted plasmids expressing HAP1 or deletion derivatives into

HAP1 Constructs	Δ*hap1TUP1*	Δ*hap1Δtup1*
HAL	1861	1444
HAP1	1058	294
ΔBe-B	1862	1443
ΔBe-K	945	1380
ΔBg-K	1585	307
ΔB-K	1577	360
ΔKpn	2.6	3.4
SD5HAP1	539	164

FIGURE 2.—The heme domain mediates the effect of TUP1 on the HAP1 activity. β-Galactosidase activity of UAS1/*CYC1* reporter (pLGΔ312ΔAX-*HIS*, TURCOTTE and GUARENTE 1992) was assayed in LPY22 cells (Δ*hap1TUP1*) or cells deleted of *TUP1* (*hap1* Δ*tup1*) in the presence of various HAP1 deletion constructs (derivatives of pHAP1, PFEIFER *et al.* 1989), which were expressed from the HAP1 promoter except for *SD5HAP1*(expressed from the *GAL1-10* promoter). The HAP1 DNA binding domain (DNA), the heme domain (HEME), the activation domain (ACT), and relevant restriction sites are shown. HAL is a fusion protein containing the HAP1 DNA binding domain (1–244) and the GAL4 activation domain (753–881). ΔKpn is a HAP1 derivative deleted of the activation domain and does not activate transcription (PFEIFER *et al.* 1989).

strains deleted for *HAP1* or both *HAP1* and *TUP1*. The activity of HAP1 was significantly reduced in the absence of TUP1 whether the protein was expressed from its own promoter (HAP1) or the GAL promoter (*SD5HAP1*, Figure 2). The level of activation was significantly higher in this experiment than that of Table 1, probably because higher levels of HAP1 were expressed from the high copy plasmids. This elevation reduces the fold-effect of deleting *TUP1*, although a significant effect remains. Because the reduction in UAS1 activity in the Δ*tup1* strain was observed when HAP1 was expressed from the GAL promoter, the requirement for TUP1 is not related to transcription of the HAP1 gene (see Table 1).

Deletions outside of the heme domain (444–1307) did not alleviate the requirement for TUP1 (see row ΔBg-K or row ΔB-K, Figure 2). However, deletion of the heme domain abolished the requirement (see row ΔBe-K and row ΔBe-B, Figure 2). Similar experiments in Δ*ssn6* cells also show this effect of deleting the heme

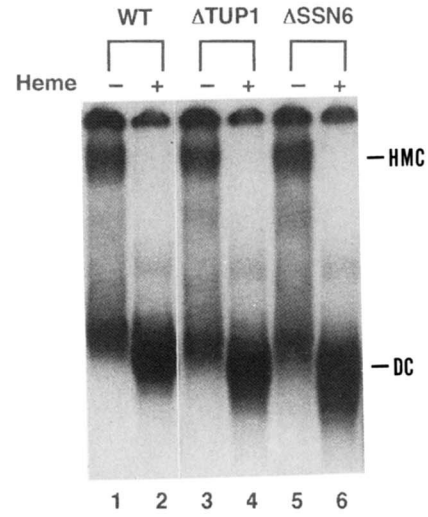

FIGURE 3.—TUP1 and SSN6 have no effect on DNA-binding of both HMC and DC. DNA-binding reactions were carried out using extracts prepared from 1-7a, 1-7aΔ*tup1*, and 1-7aΔ*ssn6* cells overexpressing HAP1. A final concentration of 20 ng/μl heme was included in the DNA binding reaction mixtures loaded in lanes 2, 4 and 6. To reveal the high molecular weight complex (HMC), the gel was run for an extended time period at 4° and the free probe was out of the gel.

domain (data not shown). These data show that TUP1/SSN6 are required as positive regulators, only when the heme domain of HAP1 is present.

Previously, we (L. ZHANG and L. GUARENTE, manuscript submitted) and FYTLOVICH *et al.* (1993) observed that one or more cellular factors interacts with the heme domain of HAP1 in the absence of heme. Addition of heme disrupts this high molecular weight, multiple component, HAP1 complex and allows HAP1 to dimerize, leading to HAP1 DNA binding and transcriptional activation (ZHANG *et al.* 1993; L. ZHANG and L. GUARENTE, manuscript submitted). Since the TUP1/SSN6 requirement for HAP1 activity appeared to function through the heme domain of HAP1, it seemed possible that TUP1/SSN6 might assist the conversion of the high molecular weight complexes (HMC) to dimeric complexes (DC) upon heme addition. However, we found that deletion of *TUP1/SSN6* has no effect on the formation of the high molecular weight complex or dimeric complexes (Figure 3). TUP1/SSN6 are therefore likely involved in the activation process after the high molecular weight complexes are converted to dimeric complexes by heme.

DISCUSSION

Previously, it has been shown that TUP1/SSN6 are required for repression of promoters such as *SUC2* and are required for repression by α2, a1/α2 and MIG1 (KELEHER *et al.* 1992; TRUMBLY 1992). TUP1 and SSN6 are associated together in a high molecular weight complex and both contain tandem amino acid repeats that

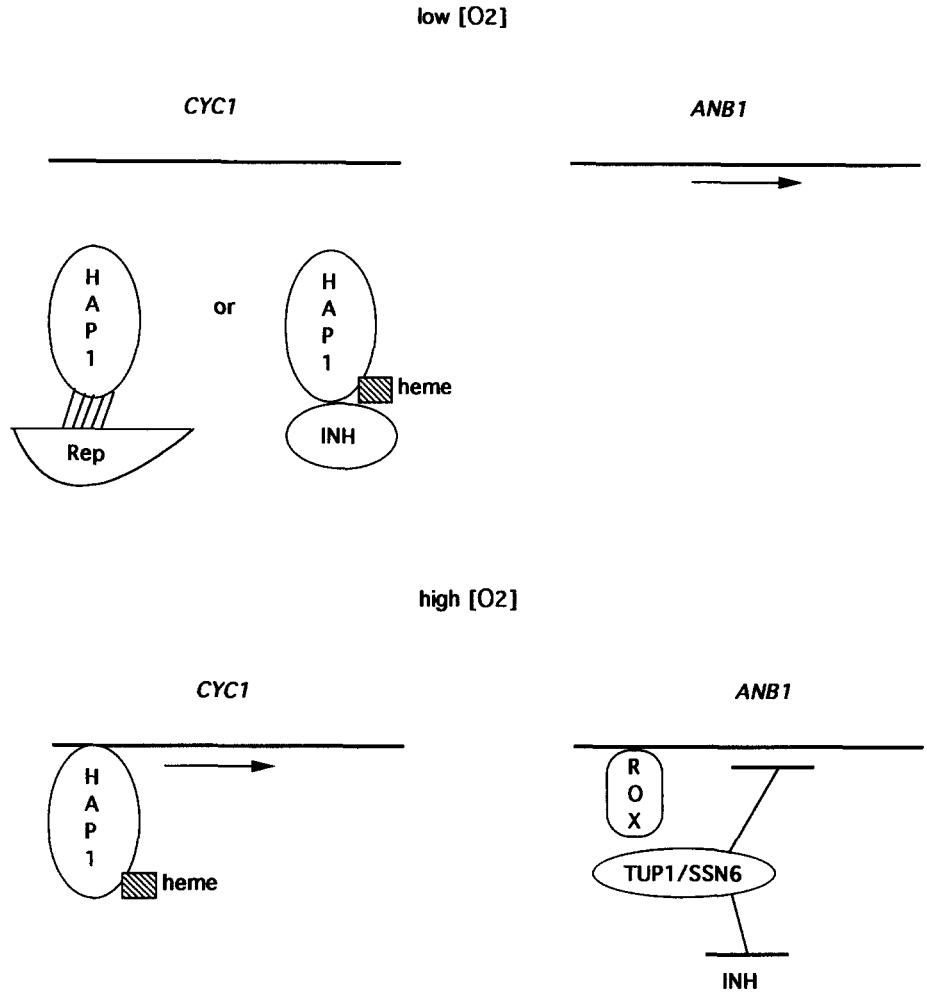

FIGURE 4.—A model illustrating the effect of TUP1/SSN6 on the HAP1 controlled *CYC1* promoter and the *ANB1* promoter. Under low oxygen condition, HAP1 activity is repressed by the repressor (Rep) or the inhibitor (INH) while the *ANB1* promoter is active. Under high oxygen condition, HAP1 is active and the *CYC1* promoter is transcribed but the INH and the *ANB1* promoter are repressed by TUP1/SSN6. ROX is a repressor of the *ANB1* promoter (LOWRY and ZITOMER 1988).

are believed to form coiled-coil structures (SHULTZ *et al.* 1990; WILLIAMS *et al.* 1991; WILLIAMS and TRUMBLY 1990). These coiled-coil structures could allow TUP1/SSN6 to interact with other proteins, thereby regulating their activities.

In this report, we show that TUP1/SSN6 plays a positive role in mediating the heme induction of HAP1 transcriptional activity. When *TUP1* or *SSN6* is deleted from the chromosome, HAP1 activity is reduced and addition of the heme analogue, deuteroporphyrin IX, does not allow HAP1 activity to be recovered. Deletion of the heme domain of HAP1 alleviates the requirement for TUP1/SSN6. However, TUP1/SSN6 are not present in a higher molecular weight HAP1 complex formed in the absence of heme and are not required for conversion of high molecular weight repression complexes to dimeric complexes in response to heme.

What do TUP1/SSN6 do to activate HAP1? One model is that they bind to the heme domain to increase the ability of HAP1 to activate transcription. However, this seems unlikely given that TUP1/SSN6 can repress the *CYC1* promoter when bound nearby via fusion to lexA (KELEHER *et al.* 1992). A second model is that TUP1/SSN6 repress a gene encoding an inhibitor of HAP1 activity (*INH*) that functions through the heme domain when heme is bound to the protein. We imagine that such an inhibitor might function under the conditions of low concentrations of intracellular heme to maintain tight repression of HAP1 activity. Thus, under these conditions, HAP1 uncomplexed with heme would be repressed by the previously identified putative repressor (Rep; L. ZHANG and L. GUARENTE, manuscript submitted; FYTLOVICH *et al.*, 1993) and HAP1 complexed with heme would be repressed by the INH (Figure 4). Upon induction, Rep would be neutralized by conversion of all of HAP1 to the heme-bound form, and, we imagine, synthesis of INH may be down regulated by a mechanism that uses TUP1/SSN6. By this view, the heme domain of HAP1 provides a surface that can be contacted by several regulatory proteins in the cell. The interaction between INH and HAP1 could be weak and transient, since we did not detect any stable INH-HAP1 complexes in DNA mobility shift assays (not shown).

TUP1/SSN6 have been shown to be involved in the transcriptional regulation of various genes such as glucose repression of *SUC2* and repression of a specific genes by $\alpha 2$ and $\mathbf{a1}/\alpha 2$ (SCHULTZ and CARLSON 1987; KELEHER *et al.* 1992). In this report, we show that TUP1/SSN6 has a positive role in HAP1 activity. HAP1 activity is high when the level of oxygen (or heme) is high and

it is inactive in the absence of oxygen. HAP1 activates the expression of many enzymes such as cytochrome *c* and c_1 under aerobic condition. Since TUP1/SSN6 is also involved in the repression of *ANB1*, a gene which is expressed under anaerobic conditions but repressed under aerobic conditions (ZHANG *et al.* 1991), it appears that TUP1/SSN6 play a global role in the transcriptional regulation of genes whose expression is under the control of the oxygen level in the environment (see Figure 4). However, since HAP1 is not required for growth on nonfermentable carbon sources, the effect of TUP1/SSN6 on HAP1 cannot account for the poor growth of Δ*tup1* or Δ*ssn6* strains on nonfermentable carbon sources. We believe the TUP1/SSN6 effect on HAP1 activity is more related to cellular response to oxygen than carbon source.

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