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Regulation of the *HAP1* gene involves positive actions of histone deacetylases

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Abstract

The yeast transcriptional regulator Hap1 promotes both transcriptional activation and repression. Previous studies have shown that Hap1 binds to the promoter of its own gene and represses its transcription. In this report, we identified the DNA site that allows Hap1-binding with high affinity. This Hap1-binding site contains only one CGG triplet and is distinct from the typical Hap1-binding upstream activation sequences (UASs) mediating transcriptional activation. Furthermore, at the *HAP1* promoter, Ssa is bound to DNA with Hap1, whereas Hsp90 is not bound. Intriguingly, we found that histone deacetylases, including Rpd3, Hda1, Sin3 and Hos1, are not required for the repression of the *HAP1* gene by Hap1. Rather, they are required for transcriptional activation of the *HAP1* promoter, and this requirement is dependent on the *HAP1* basal promoter. These results reveal a complex mechanism of transcriptional regulation at the *HAP1* promoter, involving multiple DNA elements and regulatory proteins.

Keywords: Transcriptional repression; Hap1; Histone deacetylase; DNA-binding; CGG triplet; Ssa; Hsp90; Molecular chaperone

The yeast heme activator protein Hap1 is an important regulator mediating oxygen and heme regulation [1–4]. Previous ChIP–chip studies have identified >200 genes, to which Hap1 can bind and regulate [5,6]. This points to the potential of the Hap1 protein in controlling transcription of diverse genes. Hap1 not only activates transcription of many genes involved in respiration and in controlling oxidative damage, in response to heme or oxygen, but it also represses certain genes, such as its own gene [4,7]. Interestingly, Hap1 activates transcription in a heme-dependent manner, whereas it represses the *HAP1* gene in a heme-independent manner [4,7,8]. The mechanism of heme-dependent transcriptional activation by Hap1 has been well studied [4,9–11]. Previous studies have shown that heme regulation of Hap1 activity requires molecular chaperones Hsp90, Hsp70 and its cochaperones Ydj1 and Sro9 [12–16]. Hsp70 and its cochaperones Ydj1 and Sro9 confer Hap1 repression at low heme levels, whereas Hsp90 promotes heme activation of Hap1 [12–15,17].

The mode of Hap1-binding to its activated genes is well understood [18–21]. The promoters of many Hap1-activated genes generally contain a direct repeat of two CGG triplets separated by a six nucleotide spacer (optimal site: CGGnnnTAnCGG). In contrast, the promoter of the *HAP1* gene does not contain such a site, although it binds to Hap1 with high affinity [7]. Our previous studies showed that besides the DNA-binding domain, none of the Hap1 domains plays a dominant role in repression [7]. The function of Ssa (Hsp70), but not Hsp90, is required for transcriptional repression by Hap1 [7]. Transcriptional regulators that can both activate and repress transcription are found in both yeast and higher organisms. Examples include the yeast Rap1 protein and the mammalian proteins, such as YY1 and Myc [22]. Generally, such regulators mediate activation and repression by interacting with

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different protein partners. A common mechanism of transcriptional repression is through chromatin remodeling complexes, such as the histone deacetylase Rpd3 complex and the Swi/Snf complex [23–25].

Here, we sought to elucidate the molecular mechanism by which Hap1 represses the *HAP1* gene. We first mapped the high affinity Hap1-binding DNA site by using biochemical methods. We then asked whether Ssa binds to DNA together with Hap1. Finally, we determined if several histone deacetylases, including Rpd3, Hda1, Sin3, Hos1 and Hos3, are involved in Hap1-mediated transcriptional repression. Our results suggest that the regulation of the *HAP1* gene is mediated by a novel and complex mechanism.

Materials and methods

Yeast strains and reporters. Yeast strains used were JEL1 (*MATα leu2 trp1 ura3-52 nprb1-1122 pep4-3 ΔHis3::pGAL10-GAL1*) [26], BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0), BY4741Δsnf2::Mxkan4, BY4741Δsin3::Mxkan4, BY4741Δ*rpd3*::Mxkan4, BY4741Δ*hda1*::Mxkan4, BY4741Δ*hos1*::Mxkan4, BY4741Δ*hos2*::Mxkan4, BY4741Δ*hos3*::Mxkan4. The BY4741 strains [27] were purchased from Open Biosystems.

Preparation of yeast cell extracts. Yeast JEL1 (*MATα leu2 trp1 ura3-52 nprb1-1122 pep4-3 ΔHis3::pGAL10-GAL1*) [26] cells were transformed with the Hap1 expression plasmid under the control of the *GAL1* promoter or its own promoter [26]. Cell extracts were prepared as described previously [26]. Protein concentrations were determined by the BCA (bicinchoninic acid) protein assay kit (Pierce).

Electrophoretic mobility shift assays (EMSAs) and DNA pull down. DNA-binding reactions were carried out exactly as described [17,26,28]. Radioactivity of the interested bands was visualized and quantified by using the PhosphoImage™ system (Molecular Dynamics). DNA pull-down was performed as described previously [13,15]. Pulled down proteins were analyzed by SDS–PAGE, followed by Western blotting analysis.

β-Galactosidase assays. The β-galactosidase levels from the UAS1/*CYC1*–TATA–*lacZ* or *HAP1–lacZ* or *HAP1–CYC1–lacZ* (with the *HAP1* promoter sequence −1 to −150 replaced by the *CYC1* promoter sequence −1 to −178) reporter gene [7] were measured in BY4741 cells, grown in synthetic complete medium containing 2% glucose or 2% raffinose and 2% galactose. To examine the effect of Hap1 on reporter activities, the Hap1 expression plasmid or the empty vector was transformed into the cells along with the reporter gene.

Results

The Hap1 protein binds specifically to the HAP1 promoter sequence −341 to −380

To identify the Hap1-binding site in the *HAP1* promoter, we performed electrophoretic mobility shift assays by using radiolabeled DNA containing various regions of the promoter (Fig. 1). As shown in Fig. 1, the DNA fragment containing the whole promoter sequence −1 to −461 ("−" sign is omitted in the Fig. 1) was shifted upwards in extracts prepared from cells expressing Hap1 (lane 1, Fig. 1), compared to extracts from cells without Hap1 (lane 2, Fig. 1). This suggests that Hap1 can bind to the whole promoter sequence, although the complexes were too large to be distinctively identified. In contrast, on the DNA fragment containing the promoter sequence −1 to −300 (lanes 3 and 4) or −1 to −150 (lanes 5 and 6), the complexes formed in the presence (lanes 3 and 5) or absence (lanes 4 and 6) of Hap1 were largely the same, suggesting that Hap1 did not bind to DNA fragments containing these sequences. Subsequently, we examined Hap1 complexes formed on DNA fragments containing progressively shorter promoter sequence in the −300 to −461 region. The DNA fragment containing the promoter sequence −341 to −380 exhibited strong Hap1-dependent complex formation (lanes 21 and 22, Fig. 1). The DNA fragment containing −321 to −360 overlaps with the −341 to −380 DNA fragment and exhibited strong complex formation (lanes 19 and 20), although the percentage of bound vs. free was higher for the −341 to −380 DNA fragment. All longer DNA fragments containing the −341 to −380 region formed Hap1-dependent complexes (see lanes 7–14). The DNA fragment containing the −380 to −461 region also formed a Hap1-dependent complex (lanes 15 and 16), but it was much weaker, compared to that formed on the −341 to −380 DNA fragment.

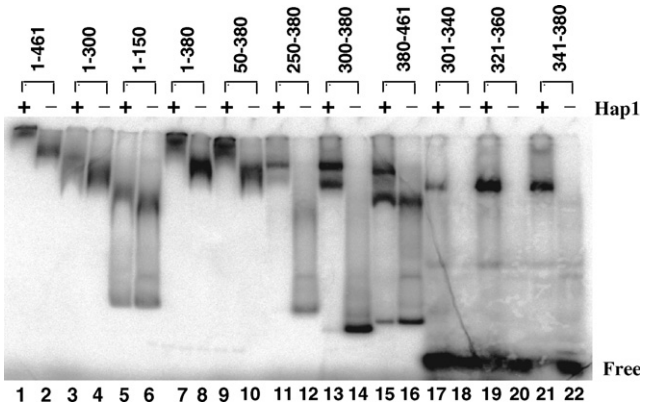

Fig. 1. The DNA–proteins complexes formed on various regions of the *HAP1* promoter. Note that the "−" sign in the nucleotide number is omitted in the figure. Extracts were prepared from cells bearing a Hap1 expression vector (+ Hap1) or an empty vector (− Hap1). Extracts were incubated with radiolabeled DNA fragments containing various regions of the *HAP1* promoter.

To verify the specificity of the Hap1 complexes formed on the *HAP1* promoter, we performed a competition experiment (Fig. 2). As expected, the UAS/*CYC7* Hap1-binding site had no effect on complex formation on the DNA fragment containing the promoter sequence −1 to −300 (lanes 1–3) or −1 to −150 (lanes 5–7, Fig. 2). This shows that complex formation on these DNA fragments was not dependent on Hap1. In contrast, the UAS/*CYC7* Hap1-binding site competed off two Hap1 complexes formed on the −300 to −380 DNA fragment (lanes 10 and 11, Fig. 2). This DNA fragment contains two Hap1-binding sites: one in −301 to −340 (lanes 17 and 18, Fig. 1) and one in −341 to −380 (lanes 21 and 22, Fig. 1). Likewise, on the −380 to −461 DNA fragment (lanes 13–15), the Hap1-dependent upper complex, but not the lower complex, was competed off by the UAS/*CYC7* site. The Hap1-dependent complex formed on the

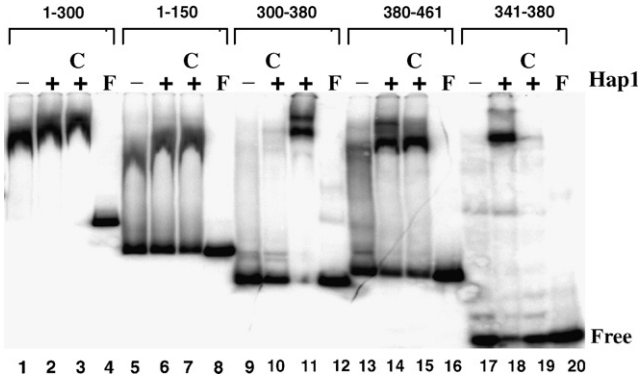

Fig. 2. The effects of unlabeled competitor Hap1-binding site on the formation of DNA–protein complexes formed on various regions of the *HAP1* promoter. Extracts were prepared from cells bearing a Hap1 expression vector (+ Hap1) or an empty vector (− Hap1). In lanes 4, 8, 12, 16 and 20, no extract (marked as F) was included. In lanes 3, 7, 10, 15 and 19 (marked as C), unlabeled DNA containing a high affinity Hap1-binding UAS site was included as competitor DNA.

−341 to −380 DNA fragment was completely competed off (lanes 17–19, Fig. 2). These results strongly suggest that the complex formed on the sequence −341 to −380 is dependent on Hap1, because an independent Hap1-binding site competed it off completely, but did not affect non-Hap1–DNA complexes formed on other sequences.

To further verify the specific association of Hap1 with the promoter sequences, we used Hap1 antibody to perform supershift or disruption of the Hap1–DNA complexes. As shown in Fig. 3, for the Hap1-independent complexes formed on the −1 to −300 (lanes 5–8, Fig. 3) or −1 to −150 (lanes 9–12, Fig. 3) DNA fragment, addition of the Hap1 antibody or preimmune serum had no effect on the complex formation, as expected. For the Hap1-dependent complexes formed on the −1 to −461 (lanes 1–4, Fig. 3) or −1 to −380 (lanes 13–16, Fig. 3) DNA fragment, addition of the Hap1 antibody (lanes 3 and 15), but not

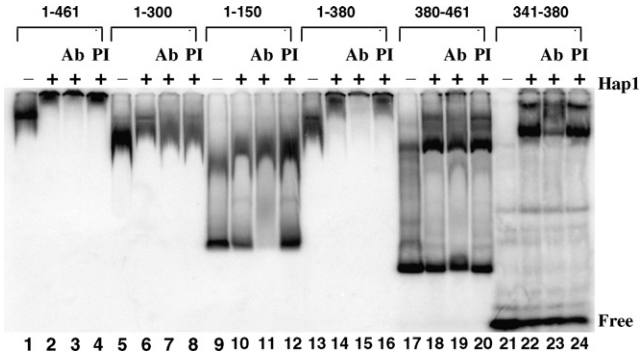

Fig. 3. The effects of Hap1 antibodies on the formation of the DNA–protein complexes formed on various regions of the *HAP1* promoter. Extracts were prepared from cells bearing a Hap1 expression vector (+ Hap1) or an empty vector (− Hap1). Extracts were incubated with radiolabeled DNA fragments containing various regions of the *HAP1* promoter. In lanes 3, 7, 11, 15, 19 and 23 (+ Ab), antiserum against Hap1 was included in the reaction mixtures; in lanes 4, 8, 12, 16, 20 and 24, preimmune serum was included in the reactions mixtures.

preimmune serum (lanes 4 and 16), supershifted or disrupted the complex. For the complexes formed on the −380 to −461 fragment (lanes 17–20, Fig. 3), the upper Hap1-dependent complex was supershifted (lane 19), while the lower, Hap1-independent one was not affected (see also Fig. 2 above). For the complex formed on the sequence −341 to −380 (lanes 21–24, Fig. 3), the lower Hap1-dependent complex was supershifted. The upper complex appeared to be disrupted. Evidently, these complexes were selectively supershifted or disrupted by Hap1 antibodies. These results show that the complexes formed on the −341 to −380 DNA fragment contained Hap1.

To unequivocally demonstrate that Hap1 is indeed bound to the −341 to −380 sequence, we performed DNA pull-down. As shown in Fig. 4A, the DNA fragment containing the −1 to −150 (lane 1) or −1 to −300 (lane 2) sequence did not pull down any Hap1, indicating that Hap1 cannot bind to these sequences, as expected [12]. For controls, we show that a mutant Hap1-binding site did not pull down any Hap1 in every experiment (see lanes 9, 11, 19 and 20, Fig. 4A). The lower bands below the highest Hap1 band (Fig. 4A) were Hap1 degradation products formed during the relatively long procedure of DNA pull-down. All other promoter DNA fragments containing the −341 to −380 fragment pulled down high levels of Hap1 (lanes 3–8, 12–14, and 17, Fig. 4A). The DNA fragment containing a shorter −345 to −373 sequence pulled down a high level of Hap1 (lane 21, Fig. 4A). Together, these results in Figs. 1–4A demonstrate that Hap1 binds specifically and strongly to the *HAP1* promoter DNA sequence −341 to −380. This site contains only one CGG triplets, and is thus distinct from the UASs that mediate transcriptional activation by Hap1 (Fig. 4B) [20].

Ssa, but not Hsp90, is bound with Hap1 to the HAP1 promoter −341 to −380 sequence

Previous biochemical studies showed that at the conventional UASs, Ssa is continuously bound to Hap1 [12]. Addition of heme significantly enhances the association of Hsp90 with Hap1 [12,14]. Thus, we determined whether Ssa and Hsp90 are bound to Hap1 at the *HAP1* promoter and whether heme affects their association with Hap1. We detected the protein levels of Hap1, Ssa and Hsp90 pulled down by the *HAP1* promoter element −341 to −380 (lanes 5 and 6, Fig. 4C) or −345 to −373 (lanes 3 and 4, Fig. 4C), in the absence (lanes 3 and 5) or presence of heme (lanes 4 and 6). For controls, we examined the proteins pulled down by a mutant Hap1-binding site (lanes 1 and 2). We found that Ssa was continuously associated with Hap1 at the *HAP1* promoter element, regardless of heme levels (Fig. 4C). In contrast, Hsp90 was not associated with Hap1, even in the presence of heme. This is contrary to the heme-enhanced association of Hsp90 with Hap1 at UASs. These results show that on the *HAP1* promoter, Hap1 was associated only with Ssa, not with Hsp90, even at a high heme level.

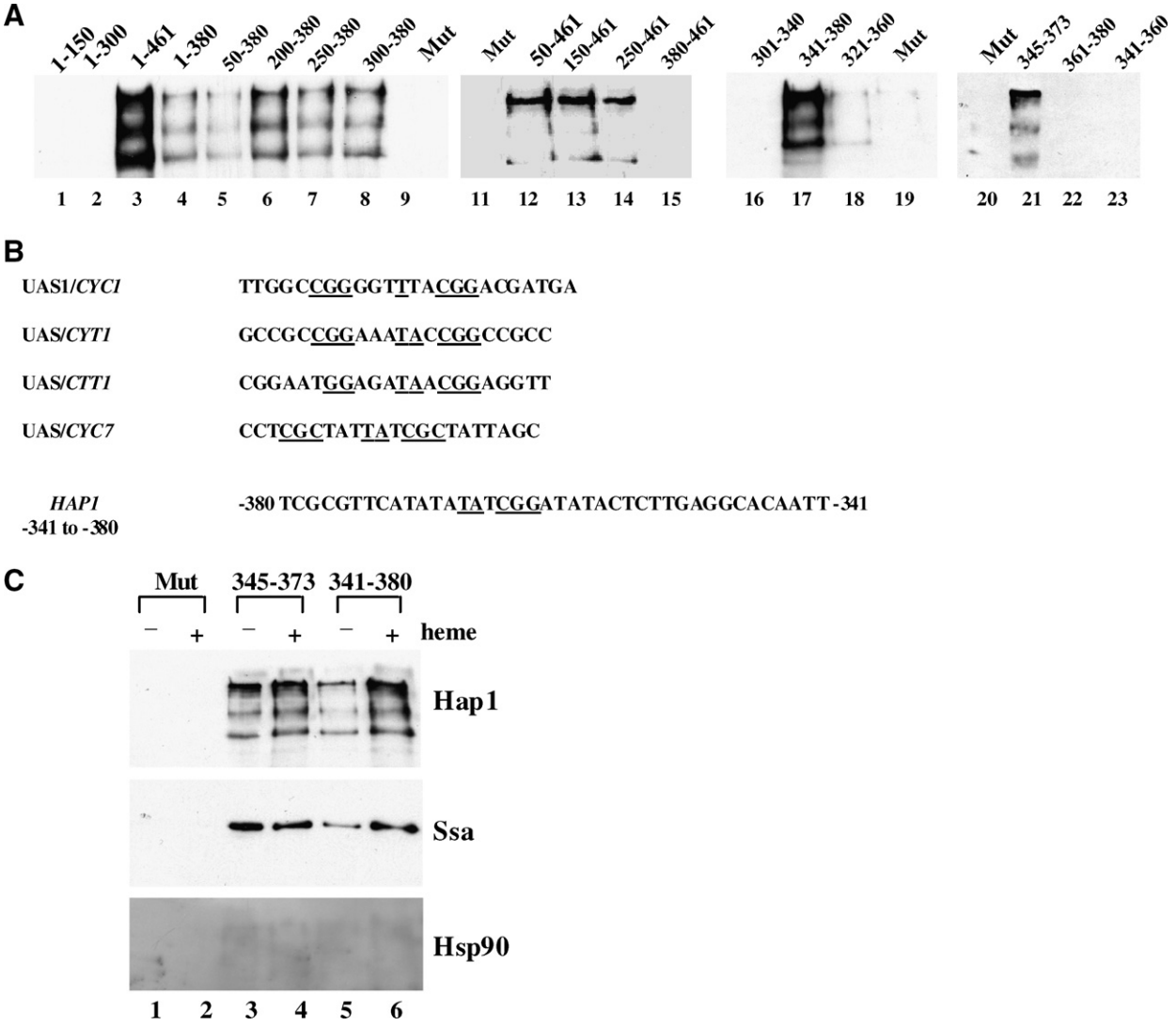

Fig. 4. (A) The levels of Hap1 proteins pulled down by DNA containing various *HAP1* promoter regions. Extracts prepared from cells expressing Hap1 were used to perform DNA pull-downs with DNA containing various regions of the *HAP1* promoter or a mutant Hap1-binding site (Mut, CGGACTCATCCG). (B) Sequence comparison of typical UAS sites mediating Hap1 transcriptional activation and the identified Hap1-binding site in the *HAP1* promoter. Shown here are the UASs of the *CYC1*, *CYT1*, *CTT1*, and *CYC7* promoters, and the −341 to −380 sequence of the *HAP1* promoter. (C) Ssa, but not Hsp90, is associated with Hap1 on the *HAP1* promoter. DNA pull-downs were performed with DNA containing the −341 to −380 or −345 to −373 sequence of the *HAP1* promoter or a mutant Hap1-binding site (Mut, CGGACTCATCCG). In lanes 2, 4 and 6 (+ heme), 2 μg/ml heme was included in the reaction mixtures.

Histone deacetylases, including Rpd3, Hda1, Sin3 and Hos1, positively regulate the HAP1 gene

Histone deacetylases often play a general role in suppressing the transcription of various genes [23–25]. Thus, we asked whether these histone deacetylases also play a role in suppressing the *HAP1* gene by Hap1. We examined the promoter activities of the *HAP1* gene (Table 1) in wild-type and mutant strains with one of the histone deacetylase genes deleted. For controls and comparisons, we measured the *CYC1* promoter activities in all strains. In addition, we measured the activities of a *HAP1–CYC1–lacZ* fusion promoter [7] (Table 1), whose activity is not affected by Hap1.

The activities of the *CYC1–lacZ* and *HAP1–lacZ* reporters in wild type and mutant cells were as expected from previous studies [7] (Table 1). Intriguingly, deletion of each of the histone deacetylase genes, including *RPD3*, *HDA1*, *HOS1*, *SIN3* and *HOS3*, significantly reduced the *HAP1–lacZ* reporter activity (Table 1), whether or not Hap1 was expressed. For comparisons, we found that deletion of each of the histone deacetylase genes did not significantly affect, or somewhat enhanced in the case of Δ*sin3*, the *CYC1–lacZ* or *HAP1–CYC1–lacZ* fusion reporter activity (Table 1). We also found that deletion of *SNF2* significantly reduced the *HAP1–lacZ* reporter activity, but did not considerably affect the *CYC1–lacZ* or *HAP1–CYC1–lacZ* fusion reporter activity (Table 1). These results show

Table 1
The effects of histone deacetylases on the promoter activities of the *HAP1* and *CYC1* genes[a]

Strains	Reporter (Exp. Vec.)				
	HAP1–lacZ (*GAL1–HAP1*)	*HAP1–lacZ* (vector)	*HAP1–CYC1–lacZ*	*CYC1–lacZ* (*GAL1–HAP1*)	*CYC1–lacZ* (vector)
BY4741	90 ± 4	227 ± 31	107 ± 33	247 ± 32	5 ± 1
Δrpd3	14 ± 3	21 ± 5	55 ± 18	231 ± 50	5 ± 1
Δhda1	10 ± 0.7	31 ± 4	58 ± 10	191 ± 8	22 ± 2
Δhos1	13 ± 0.5	29 ± 5	71 ± 3	245 ± 80	5 ± 1
Δhos3	15 ± 1	54 ± 8	77 ± 6	198 ± 26	8 ± 1
Δsin3	14 ± 4	15 ± 3	268 ± 39	433 ± 30	13 ± 2
Δsnf2	11 ± 0.6	17 ± 2	298 ± 28	180 ± 10	14 ± 2

[a] Yeast strains were transformed with the indicated *lacZ* reporter gene plasmid and the expression vector (Exp. Vec.) for Hap1 (*GAL1–HAP1*) or the empty vector. β-Galactosidase activities were measured and calculated as Miller units.

that histone deacetylases do not help suppress the *HAP1* promoter, but selectively activate the promoter.

Discussion

In this report, we used electrophoretic mobility shift assays and DNA pull-downs to identify and confirm a high affinity Hap1-binding site on the *HAP1* gene promoter. The high-affinity Hap1-binding site is mapped within the promoter sequence −341 to −380 (Fig. 4). This sequence differs from the previously identified UASs in that it contains only one single CGG triplet (Fig. 4B). Nonetheless, it contains part of the optimal sequence TAnCGG (Fig. 4B). This site likely represents a new class of Hap1-binding sites different from typical UASs. Hap1 appears to bind to this site along with Ssa. However, Hsp90 does not seem to bind to this site even in the presence of heme. This is again different from Hap1-binding at UASs. Previous studies showed that heme enhances the binding of Hsp90 to Hap1 at UASs [12]. The difference in Hsp90–Hap1 association at the *HAP1* promoter and UASs suggests that Hap1–chaperone interactions are affected by DNA elements.

Histone deacetylases are often global transcriptional repressors [23–25]. Interestingly, here we found that histone deacetylases Rpd3, Sin3, Hda1, Hos1 and Hos3 all play a positive role in transcription driven by the *HAP1* promoter. Deletion of any one of the genes significantly and selectively reduced the *HAP1* promoter activity (Table 1). This positive role of histone deacetylases is not dependent on Hap1, because deletion of their genes reduced the *HAP1* promoter activity, whether or not Hap1 was expressed (Table 1). However, the positive role of histone deacetylases is promoter specific and is dependent on the basal *HAP1* promoter region −1 to −150. When this region was replaced by the basal promoter region of the *CYC1* gene in the *HAP1–CYC1–lacZ* fusion promoter, deletion of the histone deacetylase genes no longer considerably affect the promoter activity (Table 1).

The histone deacetylase Rpd3 has previously been shown to activate transcription of genes involved in heat shock and osmotic stress [23–25]. Studies in yeast show that it activates transcription of *GAL* genes, DNA damage inducible genes, and anaerobic *DAN* genes [29,30]. Here we found that the positive role of histone deacetylases in the regulation of the *HAP1* gene is not dependent on Hap1. Rather, it is dependent on the basal promoter −1 to −150 region. The positive role of Rpd3 and other histone deacetylases in the expression of the *HAP1* gene may reflect a general function of histone deacetylases in the transcription of many genes.

Acknowledgments

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