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Long-Range Interactions at the *HO* Promoter

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The *SWI5* gene encodes a zinc finger DNA-binding protein required for the transcriptional activation of the yeast *HO* gene. There are two Swi5p binding sites in the *HO* promoter, site A at −1800 and site B at −1300. Swi5p binding at site B has been investigated in some detail, and we have shown that Swi5p binds site B in a mutually cooperative fashion with Pho2p, a homeodomain protein. In this report, we demonstrate that Swi5p and Pho2p bind cooperatively to both sites A and B but that there are differences in binding to these two promoter sites. It has been shown previously that point mutations in either Swi5p binding site only modestly reduce *HO* expression in a *PHO2* strain. We show that these mutant promoters are completely inactive in a *pho2* mutant. We have created stronger point mutations at the two Swi5p binding sites within the *HO* promoter, and we show that the two binding sites, separated by 500 bp, are both absolutely required for *HO* expression, independent of *PHO2*. These results create an apparent dilemma, as the strong mutations at the Swi5p binding sites show that both binding sites are required for *HO* expression, but the earlier binding site mutations allow Swi5p to activate *HO*, but only in the presence of Pho2p. To explain these results, a model is proposed in which physical interaction between Swi5p proteins bound to these two sites separated by 500 bp is required for activation of the *HO* promoter. Experimental evidence is presented that supports the model. In addition, through deletion analysis we have identified a region near the amino terminus of Swi5p that is required for *PHO2*-independent activation of *HO*, suggesting that this region mediates the long-range interactions between Swi5p molecules bound at the distant sites.

The *Saccharomyces cerevisiae HO* gene encodes an endonuclease which is responsible for initiating mating type switching in yeast (for reviews, see references 8 and 20). When yeast cells divide, they do so asymmetrically, producing a large mother cell and a small daughter cell. Only the mother cell is able to switch its mating type, and the ability to switch mating type requires transcription of the *HO* gene. Daughter cells do not express *HO* and are therefore not capable of switching their mating type. The *SWI5* gene was identified originally as a positive transcriptional regulator of *HO* transcription, and *SWI5* has been implicated in the asymmetric expression of *HO* (19, 21, 27). Recent work has shown that the *ASH1* gene product is required to repress *HO* transcription in daughter cells (2, 25).

SWI5 encodes a 709-amino-acid protein which contains three zinc finger DNA-binding domains near its carboxy terminus (26). Swi5p binds DNA in vitro via these zinc finger motifs to a site in the *HO* promoter located approximately 1,300 nucleotides (nt) upstream of the *HO* ATG start codon (16, 26). Recently, a second Swi5p binding site within the *HO* promoter has been identified that is also required for proper transcriptional regulation of *HO* in vivo (27).

The new Swi5p binding site is located approximately 1,800 nt upstream of the *HO* ATG start codon and has been named site A, while the original site at −1300 has been named site B. The interaction of Swi5p with site B has been examined extensively in vitro, and these studies identified an additional protein, Pho2p (also known as Bas2p or Grf10p), that binds cooperatively with Swi5p to site B (4, 5). Cooperative binding of Swi5p and Pho2p to site B is required for transcriptional activation of both an *HO-lacZ* reporter gene and a heterologous reporter plasmid containing only the site B (Swi5p-Pho2p) upstream activation sequence (5).

In this paper, we show that Swi5p and Pho2p can both bind to site A individually and there is cooperativity in the binding by Swi5p and Pho2p. To examine the role of *PHO2* in the transcriptional activation of the *HO* promoter through sites A and B, we examined the expression of *HO* with mutations in the promoter at either site A or B, in the presence or absence of *PHO2*. These experiments show that *PHO2* is required for *HO* expression from promoters containing these mutant Swi5p binding sites. We also show that a severe mutation at either the site A or B Swi5p binding site is sufficient to eliminate *HO* transcription in a *PHO2* strain, demonstrating that both sites are essential. Our results suggest that long-distance interactions at the *HO* promoter between these widely spaced Swi5p binding sites are important in transcriptional activation of *HO*.

MATERIALS AND METHODS

Strains. The strains used in this study (shown in Table 1) are isogenic in the K765 background (18). Strains DY1665 and DY3621 were made by restoring the *HO* gene at its chromosomal locus in the *HO*::*SUP4*-o strain K765 as previously described (18). Strains DY2174 and DY2170 contain the a1 and b1 point mutations, respectively, at sites A and B of the *HO* promoter, and strain DY2180 contains both point mutations (27). The *PHO2* genes in strains DY1665, DY2174, DY2170, DY2180, and DY3621 were replaced by a *pho2*::*LEU2* allele (5), thus creating strains DY2519, DY2406, DY2407, DY2408, and DY3912, respectively. Strains DY3895, DY3936, DY3982, and DY3986, containing the *SWI5*Δ3 allele, were constructed by standard genetic methods (24). Strains DY4178 and DY4180, containing *HO* promoter mutations, were constructed by gene replacement using plasmids M3163 and M3164, respectively. All gene replacements were confirmed by Southern blot analysis.

Plasmids. Plasmid M1402 was constructed by inserting a 46-nt double-stranded oligonucleotide containing the Swi5p and Pho2p binding sites (4) from the site B region into plasmid pIC20R (14). Plasmid M1679 was constructed by

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TABLE 1. Yeast strains

Strain	Genotype[a]
DY1665	*HO*
DY2170	*HO* (b1 promoter mutation)
DY2174	*HO* (a1 promoter mutation)
DY2180	*HO* (a1 and b1 promoter mutations)
DY2406	*HO* (a1 promoter mutation) *pho2*::*LEU2*
DY2407	*HO* (b1 promoter mutation) *pho2*::*LEU2*
DY2408	*HO* (a1 and b1 promoter mutations) *pho2*::*LEU2*
DY2519	*HO pho2*::*LEU2*
DY3621	*HO*
DY3895	*HO pho2*::*LEU2 SWI5*Δ3
DY3912	*HO pho2*::*LEU2*
DY3936	*HO SWI5*Δ3
DY3982	*HO* (a1 promoter mutation) *SWI5*Δ3
DY3986	*HO* (b1 promoter mutation) *SWI5*Δ3
DY4178	*HO* (a3 promoter mutation)
DY4180	*HO* (b3 promoter mutation)

[a] All strains are isogenic in the K765 background (18) and have the genotype *MAT***a** *HML***a** *HMR***a** *ade2-1 ade6 can1-100 his3-11,15 leu2-3,112 met trp1-1 ura3.*

cloning an *Nsi*I-*Xho*I (from the *Xho*I-229 linker insertion; see reference 18) fragment from the *HO* promoter (−1902 to −929 from the ATG) into *Pst*I-*Xho*I-digested pBSKS+ (Stratagene). Plasmids M2808, M2810, M3266, and M3267 contain *HO* promoter fragments with a1, b1, a3, and b3, respectively, cloned into Bluescript. The a3 and b3 mutations (see Fig. 7) were made by site-directed mutagenesis with the Altered Sites mutagenesis kit (Promega). The template for the *HO* riboprobe, plasmid M558, contains an *Xho*I (from the *Xho*I-207 linker insertion; see reference 18)-*Hin*PI fragment (−425 to +85 from the ATG) cloned into pBSKS+ (Stratagene), with the orientation such that T7 RNA polymerase can be used to synthesize a riboprobe from a *Hin*dIII-digested template. The template for the actin riboprobe, plasmid M2312, contains the *Eco*RI-*Kpn*I fragment (−479 to +1213 from the ATG) cloned into pSP65 (Promega), with the orientation such that SP6 RNA polymerase can be used to synthesize a riboprobe from a *Bst*BI-digested template.

In vitro binding analyses. Protein purification, probe labeling, gel retardation, DNase I footprinting, and measurement of disassociation rates were performed as previously described (4, 5). The binding reaction mixtures contained 100 to 125 μg of poly(dI · dC) per ml. Probes for gel retardation assays were prepared as follows. The site A probe containing 139 nt of *HO* sequences (−1902 to −1763, where +1 is ATG) was prepared from M2222, and equivalent probes containing the a1 and a3 mutations were prepared from M2808 and M3266, respectively. For some experiments, a site B probe was prepared from M1402 containing 46 nt of *HO* sequences (−1328 to −1282). Site B probes containing the wild-type, b1, or b3 sequence containing 134 nt of *HO* sequences (−1350 to −1216) were prepared from M2808, M2810, and M3267, respectively. For DNase I protection experiments, a site A probe containing *HO* sequences from −1902 to −1544 (from the ATG) was prepared from plasmid M1679.

Quantitation of *HO* RNA levels. The *HO* and actin probes for RNase protection were prepared essentially as previously described (1) by using *Hin*dIII-cleaved M558 DNA and *Bst*BI-cleaved M2312 templates and the appropriate RNA polymerase, except that 0.625 mM unlabeled CTP was included in the actin riboprobe synthesis reaction mixture. The unlabeled CTP was added to decrease the specific activity of the actin riboprobe, and this allows the *HO* and actin signals to be visible on the same autoradiographic exposure. The probes were purified by electrophoresis on a denaturing gel. For the RNase protection experiments, total cellular RNA was purified as previously described (17) and 20 μg of RNA was lyophilized in a Speed-Vac and resuspended in 30 μl of hybridization solution [80% formamide, 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.4), 400 mM NaCl, 1 mM EDTA, 5×10^5 cpm of the *HO* riboprobe, 2×10^5 cpm of the actin riboprobe]. The hybridization reaction mixtures were incubated at 85°C for 5 min and transferred immediately to a 48°C bath for overnight incubation. The samples were treated with 5.3 μg of RNase A per ml for 60 min at 30°C, digested with 125 μg of proteinase K per ml in 0.5% sodium dodecyl sulfate for 45 min at 37°C, extracted with phenol-chloroform-isoamyl alcohol (25:24:1), ethanol precipitated, and electrophoresed on a sequencing gel. After electrophoresis, the gel was dried and autoradiographed with an intensifying screen and preflashed Cronex film (Dupont) at −70°C. Quantitative analysis was performed with a Molecular Imager using Phosphor Analyst Software (Bio-Rad). Radioactivity in each band was measured, the background level from the corresponding position of the no-RNA lane was subtracted, and the *HO* value was normalized by dividing by the value for the actin internal control. S1 nuclease protection assays were performed essentially as previously described (10), with oligonucleotides specific for *HO* (5′ GCCCTGTGTGACA TTTATGACGCGGGCAGCGGAGCCATCTGCGCACATAACGTAAGAGT TAGCCCACCGC 3′) and actin (5′ GGAAGAGTACAAGGACAAAACGGC TTGGATGGAAACGTAGAAGGCATTCCA 3′).

RESULTS

The Swi5p protein binds to the *HO* promoter at a site 1,300 nt upstream of the *HO* ATG start codon (16, 26). Tebb et al. (27) demonstrated that Swi5p binds to the *HO* promoter at an additional site approximately 1,800 nt upstream of the *HO* start codon. The −1800 and −1300 Swi5p binding sites have been named sites A and B, respectively. Swi5p binds to site B cooperatively with an additional protein, Pho2p, and this cooperative binding is required for proper transcriptional activation of *HO*. We therefore decided to investigate whether Pho2p is required for transcriptional activation of *HO* through the site A Swi5p binding site.

Binding of Swi5p and Pho2p to site A in vitro. To investigate the characteristics of Swi5p and Pho2p in vitro DNA binding to site A, histidine-tagged Swi5p and Pho2p fusion proteins were expressed in *Escherichia coli* and purified to near homogeneity by using nickel column chromatography as described previously (4, 5). These purified fusion proteins were used in a gel retardation assay to characterize the binding of Swi5p and Pho2p to sites A and B.

Swi5p and Pho2p have been shown previously to bind cooperatively to site B (5). Figure 1B demonstrates this cooperative DNA binding by Swi5p and Pho2p to radiolabeled site B. As increasing amounts of Swi5p protein are added to the binding reaction mixtures containing a set amount of Pho2p and a site B probe, a slower-migrating ternary complex is formed (compare lanes 2 through 8 to lanes 10 through 16 in Fig. 1B). This slower-migrating complex contains both Swi5p and Pho2p proteins (4). The cooperative nature of this binding is revealed by comparison of the amount of protein-DNA complex produced when the same amount of Swi5p is incubated without Pho2p (Fig. 1B, lane 4) or with Pho2p (Fig. 1B, lane 12).

A radiolabeled DNA probe containing site A was used in a gel retardation experiment to determine whether Swi5p and Pho2p can bind to this region of the *HO* promoter. As seen in Fig. 1A (lanes 2 to 8), Swi5p bound to the site A probe. Moreover, Swi5p has a higher affinity for site A than for site B. This can be seen by comparing lanes 2 to 8 of Fig. 1A to the same lanes in Fig. 1B, where site B requires a substantially higher concentration of Swi5p protein to produce an equivalent amount of the protein-DNA complex. Pho2p also binds to both sites A and B (Fig. 1A and B, lanes 9). Gel retardation experiments were also conducted with site A and B probes with increasing amounts of Pho2p, and it appears that addition of Pho2p produces more protein-DNA complexes with site A than with site B (Fig. 2). A gel retardation assay, however, measures binding to any and all possible binding sites. The presence of multiple shifted species with the site A probe suggests that there are several low-affinity binding sites within this probe, and they could all contribute to the primary shifted complex. DNase I footprinting, in contrast, allows measurement of binding to a specific DNA sequence within a probe. As shown in Fig. 3, Swi5p binding to the site A probe is centered on the GCGTGGCAG sequence, in agreement with the methylation interference data (27). We attempted to identify the Pho2p binding site at site A by DNase I footprinting, but no region of clear protection was visible when Pho2p alone was incubated with DNA (data not shown). Although Pho2p alone is unable to footprint the site A region, a Pho2p footprint can be seen when Swi5p is included in the binding reaction mixture (see below). These results contrast with the experiments with site B, in which a clear DNase I footprint of Pho2p was ob-

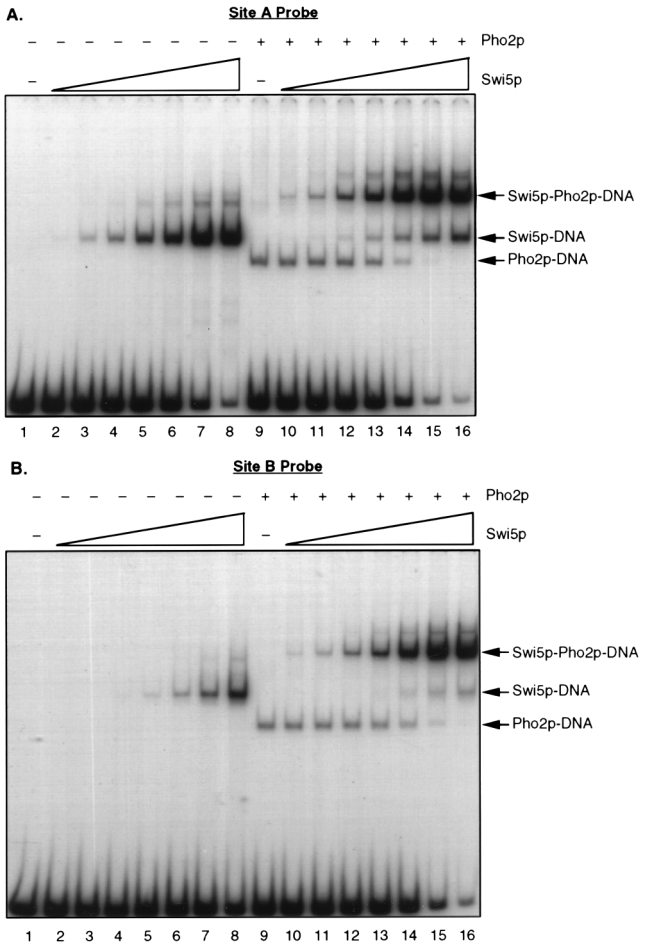


FIG. 1. Binding of Swi5p and Pho2p to sites A and B of the *HO* promoter. (A) Site A probe. (B) Site B probe. Lanes: 1, no protein added; 9 to 13, 250 ng of Pho2p; 2 and 10, 0.86 ng of Swi5p; 3 and 11, 2.5 ng of Swi5p; lanes 4 and 12, 7.8 ng of Swi5p; 5 and 13, 23 ng of Swi5p; 6 and 14, 70 ng of Swi5p; 7 and 15, 210 ng of Swi5p; 8 and 16, 630 ng of Swi5p.

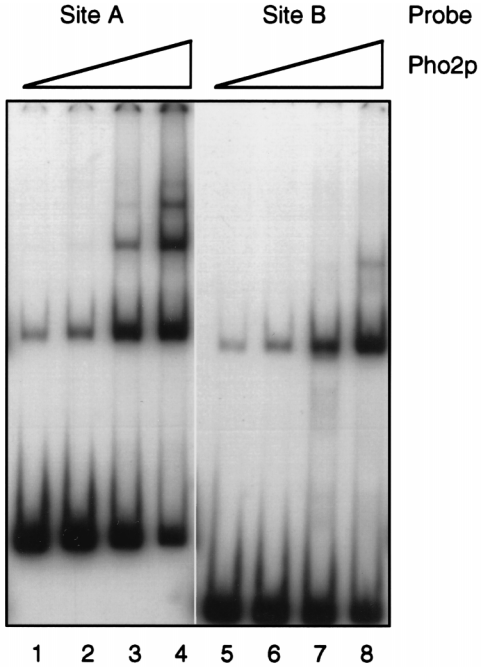


FIG. 2. Binding of Pho2p to sites A and B of the *HO* promoter. (A) Site A probe. (B) Site B probe. Lanes: 1 and 5, 30 ng of Pho2p; 2 and 6, 100 ng of Pho2p; 3 and 7, 330 ng of Pho2p; 4 and 8, 1 μg of Pho2p.

tained (5), and suggest that Pho2p binds with much lower affinity to site A than to site B.

Cooperative binding at site A. Because Swi5p and Pho2p both bind to site A, we wanted to determine if these two proteins bind cooperatively to site A, as was observed for site B (4, 5). In the gel retardation experiment (Fig. 1), Pho2p does not appear to stimulate the binding of Swi5p to site A, since, at any particular concentration of Swi5 protein, similar amounts of ternary complex and binary Swi5p-DNA complex are formed (compare lanes 2 through 8 to lanes 10 through 16 in Fig. 1A). However, the DNase I footprint experiment (Fig. 3A) provides some evidence of cooperativity. There is only partial protection from DNase I with the amount of Swi5p protein used in this experiment (lane 2). However, when Pho2p is included in a binding reaction with Swi5p, the Swi5p binding site is completely protected (compare lanes 3 through 5 to lane 2), suggesting that Pho2p can, at least to some degree, promote Swi5p binding to site A. Moreover, a weak Pho2p footprint is visible when Pho2p is incubated with Swi5p (lanes 3 to 5); little protection in this region by Pho2p is seen in the absence of Swi5p (data not shown). Part of the DNA sequence within the Pho2p footprint is AATT (Fig. 3B), which conforms to the consensus homeodomain binding site. The distance between the Pho2p and Swi5p binding sites at site A is 19 nt greater than at site B, roughly two turns of the DNA helix. We have shown previously that precise spacing between the Pho2p and Swi5p binding sites is not critical for cooperative binding (3). We conclude that Swi5p binding promotes Pho2p binding to this site A region of the *HO* promoter.

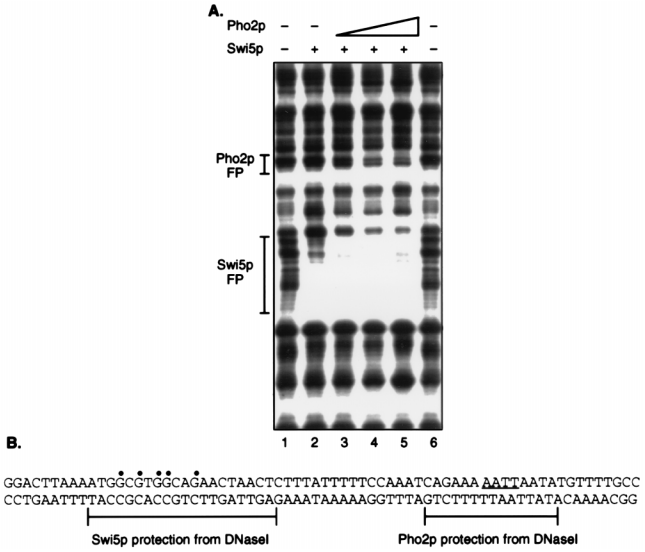


FIG. 3. DNase I footprint (FP) at site A of the *HO* promoter. (A) DNase I footprinting was performed with the indicated proteins. The following amounts of protein were used: Swi5p, 210 ng (lanes 2 to 5); Pho2p, 50 ng (lane 3), 100 ng (lane 4), and 200 ng (lane 5). The regions of protection are indicated. (B) Sequence of the site A region. The guanosine residues whose methylation interferes with Swi5p binding (27) are indicated by dots. The homeodomain consensus sequence is underlined.

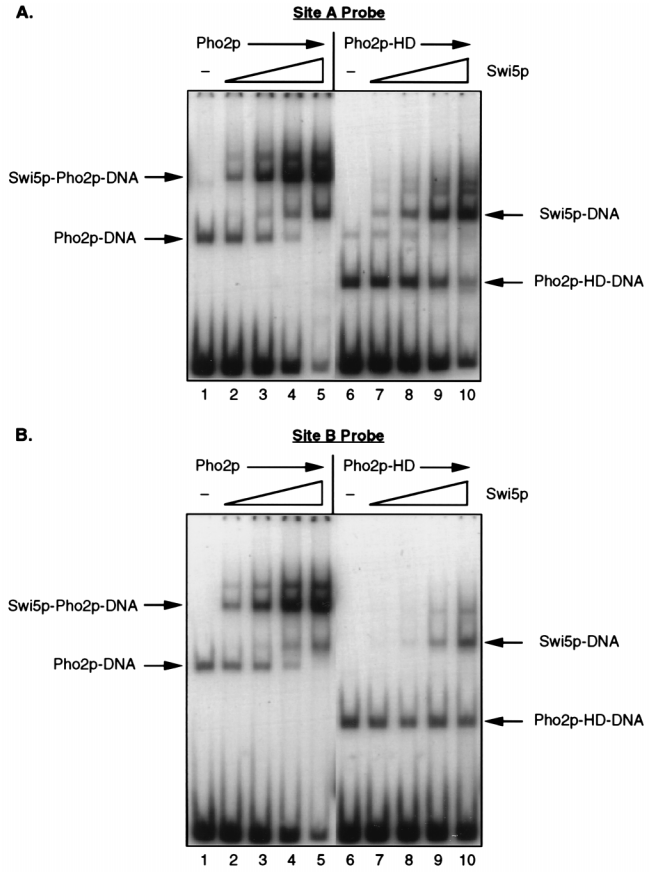


FIG. 4. The homeodomain of Pho2p is not sufficient for cooperative binding at site A or B. (A) Site A probe. (B) Site B probe. Lanes: 1 to 5, Pho2p (amino acids 35 to 528); 6 to 10, Pho2p-HD homeodomain (amino acids 4 to 170); 1 and 6, no Swi5p; 2 and 7, 2.3 ng of Swi5p; 3 and 8, 7 ng of Swi5p; 4 and 9, 21 ng of Swi5p; 5 and 10, 63 ng of Swi5p.

Although the gel retardation assay results in Fig. 1 did not provide strong evidence of mutually cooperative binding at site A, there is additional evidence of Swi5p-Pho2p interactions at site A. A ternary complex was seen when Swi5p and Pho2p were both incubated with the site A probe (Fig. 1A, lanes 10 through 16). The experiment shown in Fig. 4 demonstrates that formation of this ternary complex requires specific interactions between Swi5p and Pho2p. In this experiment, binding of Swi5p to site A was examined in the absence and presence of two Pho2p proteins, nearly full-length Pho2p and Pho2p-HD, which is a derivative of Pho2p containing only the homeodomain DNA-binding motif. We have shown that this Pho2p-HD version can bind to site B but cannot bind cooperatively with Swi5p (3). Both the Pho2p and Pho2p-HD proteins bind to site A and B probes in a gel retardation experiment (Fig. 4A and B, lanes 1 and 6). Slower-migrating ternary complexes containing both Swi5p and Pho2p are formed when both Swi5p and Pho2p are incubated in the presence of a site A or B probe (Fig. 4A and B, lanes 2 through 5). However, a slower-migrating ternary complex was not seen when Pho2p-HD was incubated in the presence of Swi5p with either a site A or B probe, even though Pho2p-HD binds to site A and B probes alone (Fig. 4A and B, lanes 6 through 10). These results suggest that there are protein-protein interactions between Swi5p and Pho2p that occur at both sites A and B and that the Pho2p-HD protein does not contain protein sequences required for interaction with Swi5p.

TABLE 2. Disassociation half-lives of protein-DNA complexes[a]

Protein(s)	Disassociation half-life (min)	
	Site A	Site B
Pho2p	$\ll$0.25	$\ll$0.25
Swi5p	16	4
Pho2p, Swi5p	20	20

[a] Disassociation half-lives of protein-DNA complexes were determined as previously described (5). Protein-DNA complexes were preformed by incubation at 25°C for 60 min before addition of excess unlabeled specific competitor DNA. The amount of specific competitor added is sufficient to block DNA binding to the labeled probe when the unlabeled competitor is added at the start of the binding reaction. After addition of the unlabeled competitor DNA, the incubations were continued for various times before gel loading. Protein-DNA complexes were quantitated with a PhosphorImager.

In summary, binding of Swi5p and Pho2p is cooperative with both the site A and B probes derived from the *HO* promoter. However, there are important differences in binding to the two probes. At site B, binding is mutually cooperative, with each protein promoting binding by the other protein (5). At site A, where Pho2p binding is very weak, Pho2p binding is strongly promoted by Swi5p but Swi5p binding is only modestly stimulated by Pho2p.

Stability of protein-DNA complexes. The cooperative binding of Swi5p and Pho2p to site B is due, at least in part, to the increased stability of the Swi5p-Pho2p-site B ternary complex compared to the Swi5p-site B or Pho2p-site B binary complex (5). Disassociation rates of the binary and ternary complexes were determined by allowing a protein(s) to form equilibrium complexes with a labeled DNA probe containing the binding site and then adding a large excess of unlabeled binding site DNA. At various time points after the addition of unlabeled DNA, the binding reactions were assayed with a gel retardation assay and the radiolabeled protein-DNA complexes were visualized by autoradiography. If the protein(s) disassociates from the probe during the incubation with unlabeled binding site DNA, it will likely bind to the excess unlabeled DNA and there will be a decrease in the amount of the specific radiolabeled protein-DNA complex detected.

Table 2 shows the results of the disassociation rate experiment performed with the Pho2p-DNA, Swi5p-DNA, and Pho2p-Swi5p-DNA complexes with either a site A or a site B probe. Pho2p disassociation from both the site A and B probes was extremely rapid. The half-life of each Pho2p-DNA complex was less than 15 s, and there was no detectable difference between the disassociation rates of the Pho2p-site A and Pho2p-site B complexes. The Swi5p-DNA complexes were much more stable. Swi5p disassociated from site B with a half-life of approximately 4 min in this experiment, but the half-life of the Swi5p-site A complex was approximately 16 min. Thus, the Swi5p-site A complex was about four times more stable than the Swi5p-site B complex. The Swi5p-Pho2p-DNA complexes had very similar disassociation half-lives. The disassociation half-lives of the Pho2p-Swi5p-site B and Pho2p-Swi5p-site A complexes were approximately 20 min, which is in agreement with the previous measurement of Pho2p-Swi5p-site B complex stability (5).

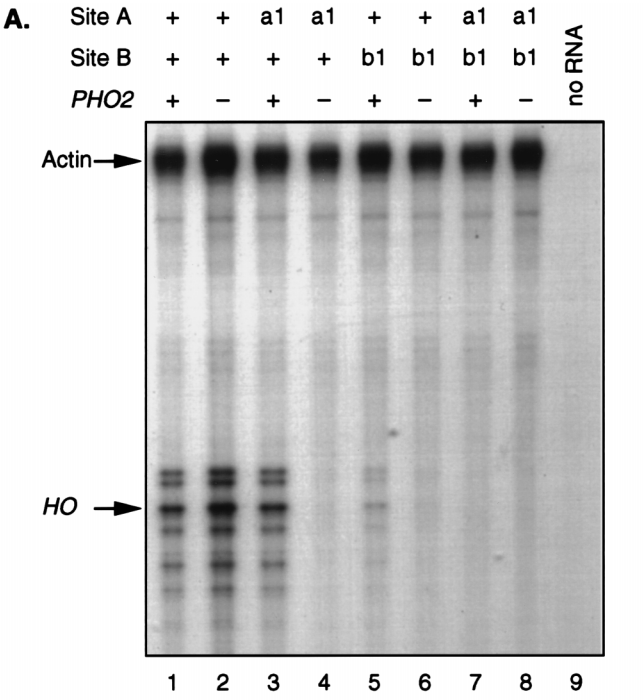
At site B, the ternary complex disassociates between 5 and 10 times slower (see reference 5) than the Swi5p-site B complex and at least 80 times slower than the Pho2p-site B complex. At site A, the disassociation half-life of the Pho2p-Swi5p-site A ternary complex was approximately 20 min, which is about 1.25 times as long as that of the Swi5p-site A complex and at least 80 times as long as that of the Pho2p-site A

complex. Thus, addition of Pho2p to the Swi5p-site A complex increased the stability of the ternary complex only slightly compared to that of the Swi5p-site A binary complex, which supports the conclusion from the gel retardation experiments that Pho2p only modestly stimulates binding of Swi5p to site A. In addition, since the disassociation half-life of the ternary complex is at least 80 times as long as that of the Pho2p-site A binary complex, it appears that Swi5p greatly enhances the binding of Pho2p to site A.

In vivo role of *PHO2* in *HO* transcription. Tebb et al. (27) created a set of strains which had mutations at the site A and B Swi5p binding sites within the *HO* promoter and determined the effects of these mutations on *HO* expression. To analyze the role of *PHO2* in *HO* expression at these sites, we disrupted the *PHO2* gene in these strains bearing the single site A or B mutation (called a1 or b1, respectively) or the site A and B double mutation (a1 and b1) and measured *HO* RNA levels with an RNase protection assay. RNA levels were quantitated with a PhosphorImager and are presented as the ratio of *HO* RNA to the actin internal control RNA. As shown in Fig. 5, in agreement with Tebb et al. (27), the individual a1 and b1 mutations reduce but do not eliminate *HO* transcription in a *PHO2 SWI5* strain, while the double mutation (a1 and b1) has essentially no *HO* expression.

The *pho2* null mutation had no effect on the expression of the wild-type *HO* gene (Fig. 5). This result differs from that in which a *pho2* mutation reduced the expression of a *HO*::*lacZ* reporter by a factor of 4 (5). Although this difference may be due to differences in strain background, the use of the *HO*::*lacZ* reporter is more likely the cause. The *pho2* null mutation had a much more striking effect on the strains containing *HO* promoter mutations (Fig. 5). In a *PHO2* wild-type strain, the b1 promoter mutation reduces expression to 22%, but in a *pho2* mutant strain, expression is reduced to 4%. The results obtained with the a1 promoter mutation are even more striking. The *HO* promoter with the a1 mutation is expressed at 97% in a wild-type strain, but its expression is reduced to 1% in the *pho2* mutant. Thus, *HO* expression is independent of *PHO2* when both Swi5p binding sites are intact. However, *PHO2* is required for *HO* expression when binding of Swi5p to one of the two binding sites is compromised.

The site A and B Swi5p binding sites are somewhat different, and there may be some flexibility in DNA site recognition by zinc finger proteins (27). The a1 and b1 mutations generated at sites A and B, respectively, each contain substitutions of only 2 nt (Fig. 6). Thus, these mutations may substantially reduce Swi5p binding without completely eliminating the affinity of Swi5p for these mutant sites. Due to the cooperative DNA binding by Swi5p and Pho2p, it is possible that Pho2p stimulates binding of Swi5p to the mutant sites and thus promotes transcription in vivo. To test this hypothesis, DNA probes were prepared from the a1 and b1 mutant binding sites and used for the in vitro binding studies shown in Fig. 7. First, Swi5p does bind to the a1 mutant binding site (Fig. 7A, lane 12). However, Swi5p binding to this mutant site is drastically reduced compared to that of wild-type site A (compare lanes 10 to 12 to lanes 2 to 4 in Fig. 7A). Relatively little protein-DNA complex is seen when Swi5p alone is incubated with the a1 mutant site (Fig. 7A, lanes 10 to 12), while addition of Pho2p to the same amount of Swi5p markedly stimulates binding (Fig. 7A, lanes 13 to 15). Similar results are obtained with the b1 mutant binding site (Fig. 7B): Swi5p binds very poorly to the mutant site, and the cooperative interactions allow Pho2p to substantially increase formation of protein-DNA complexes containing Swi5p.



		W.T.	*pho2*⁻
+	+	100	107
a1	+	97	1
+	b1	22	4
a1	b1	2	4

FIG. 5. Effect of *pho2* mutation and promoter mutations on *HO* expression. (A) RNase protection assays using probes specific for *HO* and actin (internal control). Yeast strains were constructed in which either or both Swi5p binding sites were mutated and that contained either the *PHO2* or the *pho2*::*LEU2* allele. RNA was prepared from the following strains: lane 1, DY1665; lane 2, DY2519; lane 3, DY2174; lane 4, DY2406; lane 5, DY2170; lane 6, DY2407; lane 7, DY2180; lane 8, DY2408. Lane 9 contained no added RNA. (B) Summary of transcription data. The data in A were subjected to PhosphorImager analysis. *HO* expression, normalized to the actin internal control, is given as a percentage of the wild-type (W.T.) level.

Sites A and B are not redundant. What would be the effect of "null" mutations at site A or B that totally disrupt binding of Swi5p, such that binding cannot be rescued by Pho2p? We expected *HO* expression to be eliminated, even in a *PHO2* strain. The a1 and b1 mutations, with substitutions in 2 nt of the Swi5p binding site, reduce but do not eliminate Swi5p binding. We used site-directed mutagenesis to create new mutations at sites A and B, the a3 and b3 mutations, which contain substitutions at either four or five positions in the Swi5p binding sites (Fig. 6). Based upon a consensus Swi5p binding site, and from in vitro methylation and hydroxyl radical interference studies (4), we expected these changes to eliminate binding.

We determined the in vivo effects of these mutations at the Swi5p binding sites on *HO* gene transcription. Yeast strains were constructed with the site A Swi5p binding site replaced by the a3 mutant site and site B replaced with the b3 mutant site,

Wild Type Site A	CTGCCACGC GACGGTGCG
a1 Mutation	CTGCgACGt GACGcTGCa
a3 Mutation	aTGtaAtcC tACatTagG
Wild Type Site B	ACCAGCATGC TGGTCGTACG
b1 Mutation	ACtAGtATGC TGaTCaTAGC
b3 Mutation	cttActATGC gaaTgaTACG

FIG. 6. DNA sequences of wild-type and mutant Swi5p binding sites. The nucleotide sequences from sites A and B are indicated, along with the nucleotide substitutions for the a1, a3, b1, and b3 mutations. The orientation of the site A sequence is inverted with respect to that in Fig. 3. The guanosine residues whose methylation interferes with Swi5p binding (4, 27) are underlined.

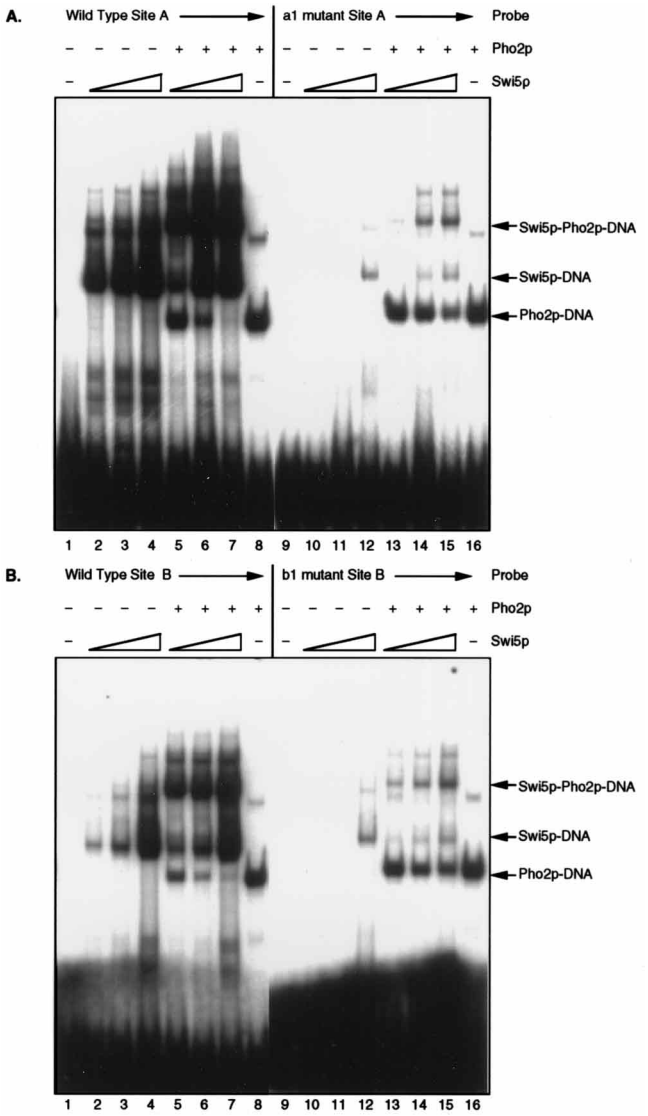


FIG. 7. Stimulation by Pho2p of Swi5p binding to mutant binding sites. (A) Lanes: 1 to 8, wild-type site A probe; 9 to 16, a1 mutant site A probe. (B) Lanes: 1 to 8, wild-type site B probe; 9 to 16, b1 mutant site B probe. 1 and 9, no protein added; 5 to 8 and 14 to 16, 6 ng of Pho2p; 2, 5, 10, and 13, 56 ng of Swi5p; 3, 6, 11, and 14, 167 ng of Swi5p; 4, 7, 12, and 15, 500 ng of Swi5p. The preparations of Swi5p and Pho2p purified from an *E. coli* expression system are different from those used in Fig. 1 to 5.

and an S1 nuclease protection assay was used to measure *HO* RNA levels. As shown in Fig. 8A, the a1 mutation does not reduce *HO* expression at all and the b1 mutation reduces *HO* expression to 22% of that of the wild type. In contrast, the a3 and b3 mutations completely eliminate *HO* expression. Once again, the residual expression from the a1 and b1 mutant promoters is largely *PHO2* dependent. This demonstrates that sites A and B are not functionally redundant but that a strong mutation in either Swi5p binding site is sufficient to block *HO* expression.

In vitro DNA-binding studies were performed to examine the binding of Swi5p to the mutant binding sites. As shown in Fig. 8B, Swi5p does not bind to the a3 mutant site (lanes 10 to 12) and addition of Pho2p is largely unable to stimulate binding (lanes 13 to 16). This binding is significantly different from that seen with the a1 mutant site, where Swi5p binds weakly and Pho2p stimulates binding (Fig. 8B, lanes 2 to 4 and 5 to 7). This result demonstrates that the a3 mutation, which eliminates Swi5p binding to site A, is sufficient to block *HO* expression.

Different results were obtained with the b3 promoter mutation at site B. In vitro DNA-binding studies show that Swi5p binds with similar affinities to the b1 and b3 mutant sites and that Pho2p stimulates binding to both to equivalent degrees (Fig. 8C). Dutnall et al. (7) have observed th Swi5p binds weakly in vitro to a sequence adjacent to site B. This weak binding may explain the similar in vitro affinities of Swi5p for the b1 and b3 mutant probes; however, the b3 mutation causes a greater reduction in *HO* expression in vivo.

A specific region of Swi5p is required for *PHO2*-independent activation. An extensive deletion analysis of Swi5p has been conducted (27). Small deletions in the zinc finger DNA-binding domain, or in the region just N terminal, eliminate the ability of Swi5p to activate *HO* expression in vivo (Fig. 9A). This essential region N terminal to the zinc fingers contains specific structural elements important for DNA binding of Swi5p (7) and also overlaps the Pho2p interaction domain (3). Remarkably, small deletions (40 to 150 amino acids deleted) throughout the protein extending from amino acids 8 to 465 have no effect on Swi5p activity in vivo, although larger deletions produce inactive proteins (27).

We examined these Swi5p deletions to see if any of them require Pho2p for activation of *HO*. Strains containing the various Swi5p deletion mutants and either the wild-type *PHO2* gene or a *pho2* gene disruption were constructed, and *HO* expression was analyzed on Northern blots (data not shown). The results summarized in Fig. 9A show that most of these Swi5p deletion mutants are independent of *PHO2*. However, one mutant, *SWI5*Δ3 (lacking amino acids 24 to 100), is com-

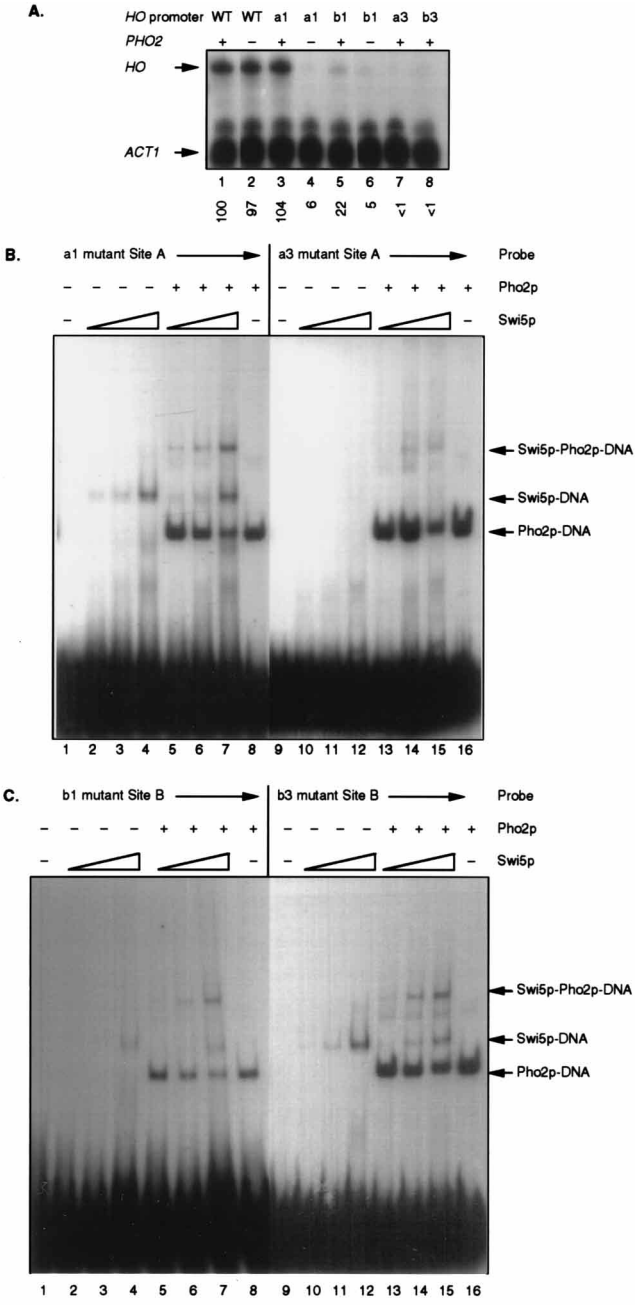


FIG. 8. Ability of Swi5p binding site mutations to eliminate *HO* expression. (A) S1 nuclease protection assays using probes specific for *HO* and actin (internal control). Yeast strains were constructed which had the a1, b1, a3, or b3 promoter mutation and contained either the *PHO2* or *pho2*::*LEU2* allele. RNAs from the following strains were prepared: lane 1, DY3621; lane 2, DY3912; lane 3, DY2174; lane 4, DY2406; lane 5, DY2170; lane 6, DY2407; lane 7, DY4178; lane 8, DY4180. WT, wild type. (B) Gel retardation assay. Lanes: 1 to 8, a1 mutant site A probe; 9 to 16, a3 mutant site A probe; 1 and 9, no protein added; 5 to 8 and 14 to 16, 6 ng of Pho2p; 2, 5, 10, and 13, 56 ng of Swi5p; 3, 6, 11, and 14, 167 ng of Swi5p; 4, 7, 12, and 15, 500 ng of Swi5p. The preparations of Swi5p and Pho2p purified from an *E. coli* expression system are different from those used in Fig. 1 to 5. (C) Gel retardation assay. Lanes: 1 to 8, b1 mutant site B probe; 9 to 16, b3 mutant site B probe. Protein samples were added as in panel B.

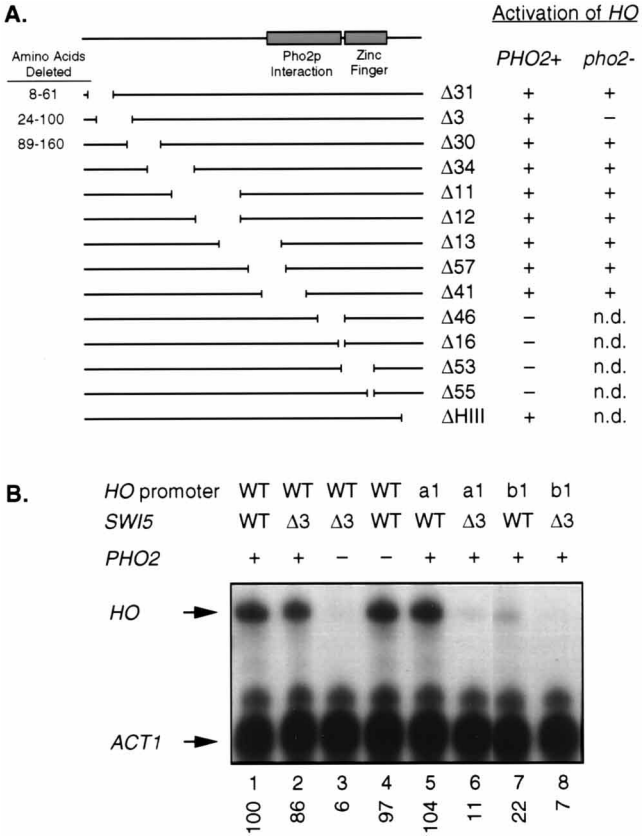


FIG. 9. Requirement of a specific region of Swi5p for *PHO2*-independent activation of *HO*. (A) Effects of internal deletions in Swi5p on *HO* expression in *PHO2* and *pho2* strains. The diagram shows the locations of the zinc finger region and a region required for in vitro cooperative binding with Pho2p (3). The plus and minus symbols summarize *HO* expression as determined by Northern blot analysis. n.d., not done. (B) S1 nuclease protection assay using probes specific for *HO* and actin (internal control). Yeast strains were constructed which differed at the *SWI5* locus (wild type [WT] or *SWI5*Δ3 deletion allele), at the *PHO2* locus (*PHO2* or *pho2*::*LEU2* null allele) and at the *HO* locus (wild type or the a1, b1, a3, or b3 promoter mutation). RNAs from the following strains were prepared: lane 1, DY3621; lane 2, DY3936; lane 3, DY3895; lane 4, DY3912; lane 5, DY2174; lane 6, DY3982; lane 7, DY2170; lane 8, DY3986.

pletely unable to activate *HO* in a *pho2* mutant. Swi5p mutants Δ31 and Δ30, lacking residues 8 to 61 and 89 to 160, respectively, are completely *PHO2* independent. These results suggest that the region required for *PHO2*-independent activation includes amino acids 24 to 100, and the critical region might be as small as amino acids 61 to 89.

The *PHO2* dependence of the *SWI5*Δ3 deletion mutant can be seen in the results of the S1 nuclease protection measurements of *HO* expression shown in Fig. 9B. The *SWI5*Δ3 mutation reduces *HO* expression to 86% (lane 2), while a *pho2* null mutation reduces *HO* expression to 97% (lane 4). However, *HO* expression is practically eliminated in the *SWI5*Δ3 *pho2* double mutant (lane 3). Thus, the *SWI5*Δ3 or *pho2* mutation, when present singly, has essentially no effect on *HO* expression, while there is a synergistic effect of combining the two mutations. Figures 5 and 8 show that a single mutation either of *pho2* or of a Swi5p binding site at the *HO* promoter had little effect on transcription, but the double mutant sharply reduced *HO* expression. We were therefore interested in determining whether the *SWI5*Δ3 mutation acts synergistically with the Swi5p binding site mutations. Crosses were performed to isolate strains in which the *SWI5*Δ3 allele was present with either the a1 or the b1 promoter mutation, and *HO* expression was compared to that of a strain with the wild-type SWI5 allele or the wild-type *HO* promoter. The data in Fig. 9B (lanes 1, 2, and

5 to 8) show that combining the *SWI5*Δ3 and the promoter mutations also reduced *HO* expression synergistically.

DISCUSSION

The *SWI5* gene encodes a DNA-binding factor required for transcriptional activation of the yeast *HO* gene (26). A *swi5* mutation leads to a 100-fold decrease in *HO* expression. The *HO* promoter contains two binding sites for Swi5p, site A at −1800 (upstream of the ATG codon) and site B at −1300. The a1 and b1 mutations in these binding sites lead to a partial drop in promoter activity, and mutation of both binding sites leads to a 100-fold drop in *HO* expression, equivalent to the *swi5* null mutation (27). Based on these results, it was suggested that the two Swi5p binding sites are partially redundant. However, we now show that these a1 and b1 mutations do not completely eliminate Swi5p binding and that Pho2p stimulates Swi5p binding to these mutant sites. Strains were constructed with more severe mutations in the Swi5p binding sites at the *HO* promoter, and either of these mutations, a3 or b3, is sufficient to eliminate *HO* expression. These results demonstrate that these two Swi5p binding sites, separated by 500 bp, are both essential for *HO* transcription.

We have shown previously that Swi5p binds cooperatively to the site B region of the *HO* promoter with Pho2p, a homeodomain protein (4, 5). We now show that Swi5p and Pho2p both bind to site A, but their interaction with site A differs substantially from their interaction with site B. First, Swi5p appears to bind with higher affinity to site A than to site B. Second, Pho2p binds to both sites A and B with extremely low affinity. Finally, cooperative interactions between Swi5p and Pho2p are different at sites A and B. At site B, Swi5p and Pho2p bind DNA in a mutually cooperative fashion since each protein increases the apparent affinity of the other for DNA. At site A, in contrast, Swi5p significantly increases the affinity of Pho2p for DNA but Pho2p only marginally increases the affinity of Swi5p for DNA. The conclusion that the cooperative binding of Swi5p and Pho2p is different at sites A and B is supported by both gel retardation experiments and measurements of the disassociation half-lives of protein-DNA complexes.

We determined the effects of a *pho2* null mutation on *HO* expression by using strains with the wild-type *HO* promoter or strains with mutations in the site A and B Swi5p binding sites (Fig. 5). The *pho2* mutation has little effect on the native *HO* promoter containing intact Swi5p binding sites. Mutation of either of the Swi5p binding sites leads to a reduction in *HO* expression in a *PHO2* strain, but disruption of the *PHO2* gene essentially eliminates expression from these mutant promoters. This is particularly striking with the a1 mutation at site A in the promoter, which has essentially wild-type promoter activity when *PHO2* is present, but a *pho2* mutation reduces the activity of this promoter to 1% of that of the wild type.

Based on the in vitro cooperative DNA binding of Swi5p and Pho2p and the effects of the *pho2* mutation on activation of site A or B mutant *HO* promoters, we propose the following model for *HO* activation. Two molecules of Swi5p, one bound at site A and one bound at site B, physically interact, thus creating a DNA loop (Fig. 10, line a). Swi5p must bind to these two sites, separated by 500 bp, and then interact to produce the C complex, which is stable. We postulate that the C complex, or the DNA loop it generates, is required to activate *HO* transcription. Pho2p is not needed for this interaction, since *HO* expression from the wild-type promoter is not affected by the *pho2* mutation (Fig. 10, line b). Although a mutation at one of the Swi5p binding sites may prevent Swi5p from binding alone, we propose that cooperative interactions with Pho2p promote Swi5p binding, despite the promoter mutation (Fig. 10, line c). Although binding to mutant site A may be reduced so that complex A (Fig. 10, line c) may exist only transiently, any interaction between the two sites would lead to formation of the C complex, which is still stable. However, when Swi5p binding is compromised by mutation at site A, the A complex does not form in a *pho2* mutant (Fig. 10, line d), and neither does the C complex, which is required for transcriptional activation. We have examined one prediction of this model by testing in vitro whether the affinity of Swi5p to site A is increased by the presence of site B on the same DNA molecule. The results were negative, in that there was no difference in affinity for the DNA templates with one or two binding sites (data not shown). This negative result does not necessarily contradict the model, for an additional factor may be required either to help bend the DNA or to mediate Swi5p interactions, and this factor was absent from the in vitro experiment.

The model suggests that an interaction between the two Swi5p binding sites is required for *HO* promoter activation. The model leads to two predictions. First, it suggests that the a1 and b1 mutations in sites A and B do not completely block Swi5p binding but that there will be residual Swi5p binding activity and that this binding activity will be stimulated by cooperative interactions with Pho2p. This prediction is supported by the data in Fig. 7. The a1 and b1 promoter mutations have only 2 nt substitutions in the Swi5p binding site, and thus it is reasonable that Swi5p binding is reduced but not eliminated. The second prediction is that a more substantial promoter mutation that completely blocks Swi5p binding will completely kill the promoter, even in a *PHO2* strain. The a3 mutation at site A of the *HO* promoter fulfills these requirements: Swi5p does not bind to this mutant site, and this mutation eliminates *HO* expression in a *PHO2* strain (Fig. 10, line e). In contrast, the b3 mutation at site B prevents *HO* expression without completely eliminating the binding of Swi5p to the mutant site in vitro. Although Swi5p appears to bind to the b1 and b3 mutant sites similarly in vitro, the two mutations have different characteristics in vivo. The b1 mutation reduces *HO* expression to 22% of that of the wild type, while *HO* expression in the b3 mutant is reduced to <1% (Fig. 10, line f). We suggest that the in vitro binding assay may lack the sensitivity to detect subtle differences in Swi5p binding to the b1 and b3 sites.

Our analysis of deletion mutants suggests that a specific region of Swi5p is required for this long-range interaction in *HO* activation (Fig. 9). Deletion of amino acids 24 to 100 of Swi5p results in an allele which is totally *PHO2* dependent. The data suggest that the critical region of Swi5p for *PHO2*-independent activation of *HO* may be as small as residues 61 to 89. We suggest that this region of Swi5p (shown as the stippled region of Swi5p in Fig. 10) may interact with a second Swi5p molecule, allowing *HO* expression in a *pho2* mutant (Fig. 10, line b). This interaction may be direct, or it could require an additional protein factor. Although the Swi5Δ3p allele can activate *HO* in a wild-type strain (Fig. 10, line g), either a *pho2* (line h) or a *HO* promoter mutation (line i) prevents activation by Swi5Δ3p. How does the Swi5Δ3p deletion protein achieve this long-range interaction in a *PHO2* strain? One possibility is shown in Fig. 10, line g, where the Swi5Δ3p protein bound at site A interacts with the Pho2p molecule bound at site B and the Swi5Δ3p protein at site B interacts with Pho2p at site A. In this tetramer model, each Swi5Δ3p molecule interacts with two Pho2p molecules, one bound at the adjacent DNA and one bound adjacent to the other Swi5Δ3p. Thus, Swi5Δ3p is unable to activate in a *pho2* mutant because neither Pho2p nor the specific region of Swi5p is able to promote loop formation (Fig.

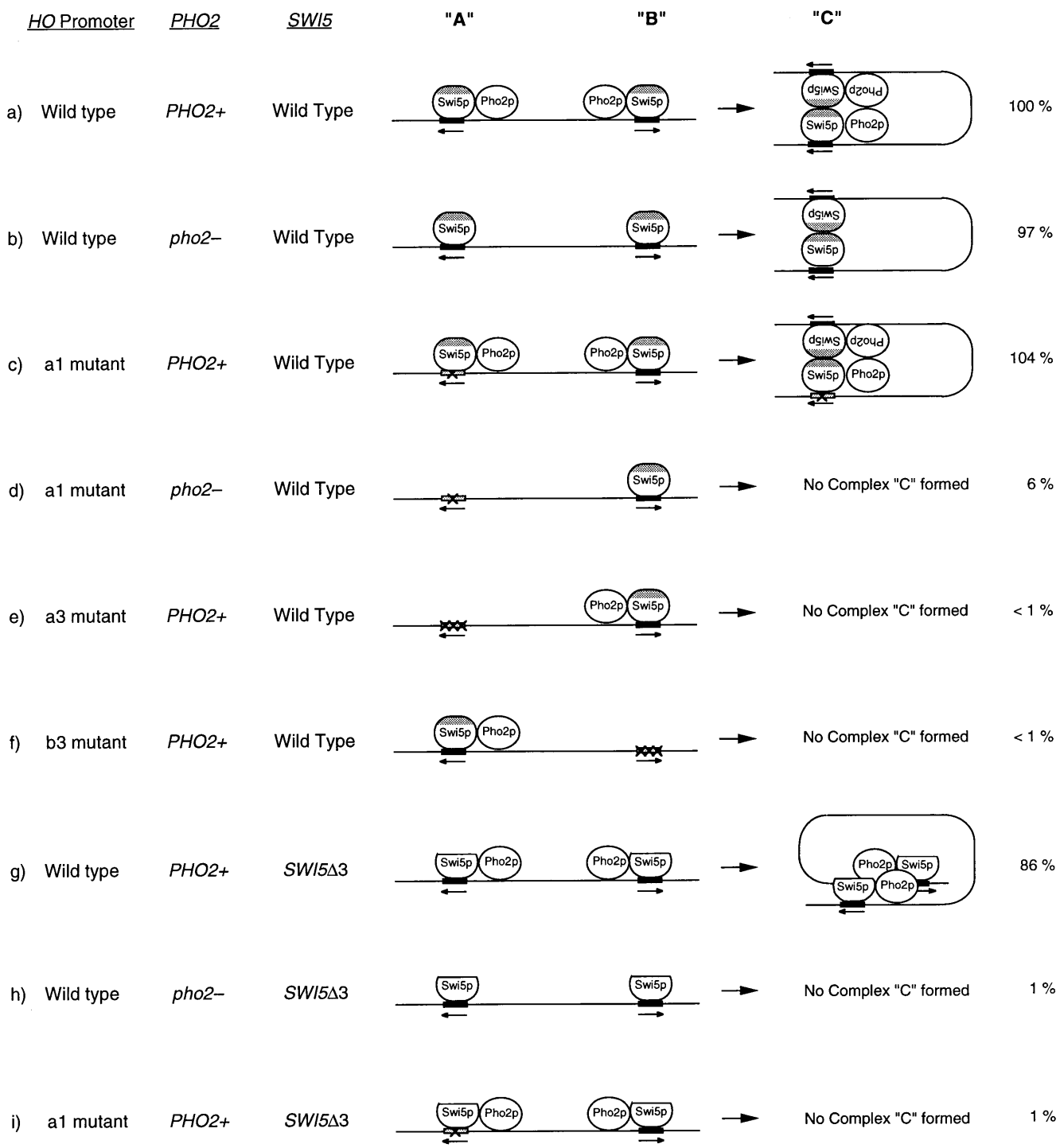


FIG. 10. Model for Swi5 activation of *HO* expression. Swi5p molecules bound at sites A and B interact to form productive complex C. For the wild-type promoter, this interaction is independent of Pho2p. The Swi5Δ3p deletion allele is unable to activate *HO* transcription in a *pho2* mutant or in a strain with the a1 promoter mutation. Correspondingly, the a1 promoter mutant is also dependent on *PHO2* for *HO* activation. In contrast, the a3 and b3 mutations inactivate the *HO* promoter, even in a *PHO2* strain. The percent *HO* expression values are taken from Fig. 5 and 9. See the text for a discussion.

10, line h). Studies of cooperative binding by Swi5p and Pho2p when the spacing between the binding sites is altered suggest that there is flexibility in the Swi5p-Pho2p interaction (3). Alternatively, interactions between the two DNA-bound Pho2p molecules may promote long-range interactions between the two Swi5Δ3p molecules. Other models are also possible. Pho2p participates in the transcriptional activation of the *PHO5* gene, where it collaborates with Pho4p (22, 28). Recent data suggest that Pho2p activates *PHO5* by increasing the accessibility of the Pho4p activation domain (23), and thus,

Pho2p could alter the structure of Swi5p. Finally, we suggest that Swi5Δ3p is unable to activate the a1 mutant promoter (Fig. 10, line i) because any A complex that does form in the absence of the specific region of Swi5p is so short lived that the C complex does not form efficiently.

Why would a loop between Swi5p binding sites at sites A and B be so important for activation of *HO* transcription? The transcriptional regulation of *HO* is extremely complex. *HO* transcription requires multiple events at distinct points in the cell cycle, with Swi5p required at the first step in *HO* promoter activation (8, 20). Swi5p levels are not detectable at the time *HO* is expressed in late G1, suggesting that either Swi5p performs some function in early G_1 which changes the activation state of the promoter or Swi5p persists in a stable complex at the *HO* promoter (27). In this discussion, we have emphasized a potential cooperative interaction between two Swi5p molecules bound to these sites as a mechanism for Swi5p function. However, formation of a loop structure may not be important just for stabilization of Swi5p binding, but the loop structure itself may promote activation, possibly by affecting the structure of chromatin. Other models besides DNA looping are also possible, such as binding by Swi5p and Pho2p at sites A and B acting to disrupt one or more nucleosomes present at the *HO* promoter.

Genetic analysis of transcriptional regulators of *HO* supports the hypothesis that chromatin structure is important for *HO* expression. First, the *SWI*/*SNF* genes (*SWI1*, *SWI2* [*SNF2*], *SWI3*, *SNF5*, and *SNF6*) are required for *HO* activation, and it is believed that the *SWI*/*SNF* gene products are required for the remodeling of chromatin (6, 29). Strains with a *swi2*/*snf2* mutation show defects in displacing nucleosomes from the *SUC2* (9, 15) and *HIS4* promoters (12). Moreover, mutations in *SIN1* (*SPT2*), *SIN2*, or *SIN4* allow *HO* to be expressed in a *swi5* mutant and analysis of these mutations suggests that chromatin structure plays an important role in *HO* regulation. Specifically, the *SIN1* gene encodes an HMG-like protein (13), the *sin2* mutation is due to a dominant mutation in histone H3 (8), and the *sin4* mutation affects the structure of chromatin (11). These results suggest that changes in chromatin structure can bypass the normal Swi5p requirement for *HO* activation. Although the genetic analyses strongly suggest that chromatin structure is important for *HO* gene regulation, additional work is needed to establish the role of chromatin remodeling in activation of *HO* expression.

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