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Genetics

Internal deletions in the yeast transcriptional activator HAP1 have opposite effects at two sequence elements

(transcriptional activation/DNA-binding/multisequence recognition/zinc finger)

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ABSTRACT **In this report we study the effects of internal deletions of the yeast transcriptional activator HAP1 (CYP1) on activity at two dissimilar DNA binding sites, upstream activation sequence 1 (UAS1) of *CYC1* (iso-1-cytochrome *c*) and *CYC7* (iso-2-cytochrome *c*). These deletions remove up to 1061 amino acids of the 1483-residue protein and bring the carboxyl-terminal acidic activation domain closer to the amino-terminal DNA-binding domain. Surprisingly, the deletions have opposite effects at the two sites; activity at UAS1 increases with deletion size, while activity at *CYC7* decreases. The mutant with the largest deletion, mini-HAP1, has no measurable activity at *CYC7* but binds normally to the site *in vitro*. In contrast, a protein with the DNA-binding domain of HAP1 fused to the acidic activation domain of GAL4 is active at both UAS1 and *CYC7*. These findings are discussed in the context of two models that suggest how the DNA sequence can alter the activity of the bound HAP1. In a separate experiment, we generate a mutation in the DNA-binding domain of HAP1 that requires the addition of zinc for binding to either UAS1 or *CYC7 in vitro*. This finding shows that a zinc finger anchors DNA binding to both types of HAP1 sites.**

Eukaryotic transcriptional activators are endowed with several distinct activities, including DNA-binding, transcriptional activation, and ability to respond to regulatory signals (1, 2). In many cases, these activities appear to lie in separate parts of the proteins. A case in point is the yeast activator HAP1 (CYP1; Fig. 1). The ability of HAP1 to bind specifically to its sites lies in the first 148 residues of the 1483 amino acid protein (3). To activate the transcriptional machinery, HAP1 requires an acidic region between residues 1308 and 1483 (4). Finally, the ability of the protein to respond to the effector, heme, lies in a region adjacent to the DNA-binding domain (4).

The DNA-binding domain of HAP1 contains six cysteine residues and is similar to a set of fungal activators, including GAL4 (1). Indeed, mutation of Cys-64 or Cys-81 of the protein knocks out its ability to bind to DNA (3). This single domain of the protein directs binding to two dissimilar classes of sequence elements. One class of element is found in the upstream activation sequences (UASs) of *CYC1* (iso-1-cytochrome *c*) (4), *CYT1* (J. Schneider and L.G., unpublished data), and *CTT1* (5). The UAS1 of *CYC1* actually contains two HAP1 binding sites, UAS1A (previously thought to bind a distinct factor termed RAF; J. Schneider and L.G., unpublished data) and UAS1B. These sites all contain regions of obvious sequence similarity. The other class of element is exemplified by a single site in the *CYC7* (iso-2-cytochrome *c*) UAS (6). This site bears no discernible similarity to any of the sites of the first class. All of these binding sites respond to HAP1 *in vivo*.

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FIG. 1. Functional domains of HAP1. The DNA-binding (DNA) domain and acidic activation region (ACT) were shown to lie between residues 1–148 and 1308–1483, respectively (3, 4). The region between residues 247 and 444 (HEME) was found to be necessary to repress the DNA-binding of HAP1 in the absence of the inducer, heme (3). The large internal region of HAP1, between residues 444 and 1308, regulates the activity of HAP1 and is a central subject of this paper.

The simple picture of functionally distinct domains within the activator has been blurred by the finding that a single mutation in the DNA-binding domain of HAP1, HAP1-18 (CYP1-18) (7, 8), greatly increases its activity at the *CYC7* site without altering its affinity for the site (9). The change Ser-63 → Arg at the base of the proposed zinc finger evidently increases the interaction between HAP1 and the transcriptional machinery. We have suggested, therefore, that the DNA-binding and acidic domains are both directly involved in contacting the machinery (9). Similar inferences were drawn from an analysis of the effects of mutations in the DNA-binding domain of the glucocorticoid receptor (10).

One interesting corollary to this picture of activation is that the DNA sequence bound could directly influence the ability of the bound protein to activate, much like an allosteric effector influences an enzyme. The multisequence recognition of HAP1 may thus create another layer of control in this fashion. Does HAP1 behave differently at the two sequence motifs? *In vivo*, there are two salient differences in the activity of UAS1B versus *CYC7*. The former site responds well to HAP1 and gives rise to glucose-repressed transcription that is higher in lactate medium than in glucose medium (11), while the latter site responds poorly and gives rise to a level of transcription that is actually lower in lactate than in glucose (12). The HAP1 response to carbon at *CYC7* is counterbalanced by a second element in the *CYC7* promoter that is HAP1-independent and gives rise to induction in lactate (12). It is not possible to deduce whether the differences in the behavior of HAP1 at UAS1 versus *CYC7* are intrinsic properties of the bound activator or are due to other proteins that bind to one or the other of these sites *in vivo*.

An approach to begin to examine whether HAP1 behaves differently at the two sites is to determine whether the effects of mutations in HAP1 that lie outside of the DNA-binding domain are coordinate at UAS1 and *CYC7*. In this report, we test a series of internal deletions in HAP1 for activation at

Abbreviation: UAS, upstream activation sequence.

UAS1 and *CYC7*. This analysis shows a striking disparity in the behavior of these mutants at the two sites. In a separate experiment, we obtain additional evidence that the zinc finger of HAP1 is essential for binding to both UAS1 and *CYC7* by studying the effects of mutations in the loop of the finger. We present a model for the differential activity of HAP1 mutants at the two sites that rests on the notion of DNA sequence-induced changes in the transcriptional activator.

MATERIALS AND METHODS

Strains and Media. All strains are derivatives of BWG1-7A (4). Integrant strains bearing a *CYC1-lacZ* reporter driven by UAS1 or a *CYC7-lacZ* reporter driven by the *CYC7* promoter have been described (4, 12). The strain with UAS1B was constructed by inserting a 23-base oligonucleotide encoding UAS1B into the *Xho* I site of pLG-178, removing the 2-μm origin of replication and integrating the resulting plasmid into the yeast genome. The *CYC7* mutant H strain bore a 2-μm plasmid marked with *LEU2* and with a *CYC7-lacZ* fusion extending out to −273 in *CYC7* DNA. Cells were grown in minimal medium containing yeast nitrogen base without amino acids with 2% glucose.

Plasmid Constructions. The *HAP1* deletion set has been described (3). The SP65 mini-*HAP1*-containing plasmid was constructed by digesting pSP65HAP1 (3) with *Bst*EII and *Kpn* I and religating in the presence of *Bam*HI linkers. Mutagenesis of codon 79 was as described for codon 63 mutagenesis (9, 13) and is detailed in the legend to Fig. 3. pHAP1-GAL4 was constructed by digesting pHAP1 (3) with *Bst*EII (which cleaves at codon 245 of *HAP1*) and *Bgl* II (which cleaves at codon 749 of *HAP1*). The *Bst*EII end was rendered flush with DNA polymerase Klenow fragment. A *Pvu* II–*Bam*HI fragment (bearing the 3′ 129 codons of *GAL4*) from pRB1027 (14) was inserted to create the *HAP1-GAL4* fusion.

Gel Shift. *In vitro* transcriptions and translations were as described (9). Protein was synthesized unlabeled and, in parallel, labeled with [^{35}S]methionine to check that synthesis of the correctly sized protein occurred. Unlabeled protein was bound to probes described below in the presence of 250 ng of salmon sperm DNA. The UAS1 probe was prepared by cleaving pLG-312-229 with *Xho* I, filling in with [α-^{32}P]dNTPs, and cleaving with *Sma* I. The *CYC7* probe was prepared by cleaving pTPCYC7 at 5′ position −273 and 3′ position −195 or pTPCYC7 at 5′ position −273 and 3′ position −130 (12) with *Xho* I and *Bgl* II and filling in with [α-^{32}P]dNTPs. The *lexA* probe was prepared by cleaving the pUC18 derivative bearing the 22-base-pair *lexA* operator (legend to Fig. 5) with *Eco*RI and *Hin*dIII and filling in with [α-^{32}P]nucleotides. Gel shift was carried out as described (9).

RESULTS

Mutations in the Loop of the Zinc Finger. We wished to obtain further evidence that the zinc finger of HAP1 was required for binding to both UAS1 and *CYC7*. Johnston found that changing a proline residue to leucine in the loop of the zinc finger of GAL4 decreased the affinity of the protein for zinc, resulting in a zinc-dependent phenotype (15). HAP1 also has a proline at the corresponding position of the loop, residue 79 of the protein. Thus, we mutagenized this codon *in vitro* (Fig. 2 legend and *Materials and Methods*). Changes of Pro-79 to serine and valine were generated, but the alteration to leucine was not recovered. The DNA-binding domains of the mutants (residues 1–245) were synthesized *in vitro* and bound to UAS1 or *CYC7* probes in the presence of increasing concentrations of zinc (Fig. 2). While the Val-79 mutant was totally inactive at all zinc concentrations, the Ser-79 mutant displayed zinc-dependent binding. The optimal concentration of zinc (5 μM) was much lower than the concentration used to rescue the GAL4 mutant, which was 2 mM zinc (15). At the highest concentration of zinc, for reasons that are not clear, no binding to either probe was observed. Germane to the issue of multisequence recognition, the requirement for zinc was identical at both UAS1 and *CYC7*. We presume that the wild-type DNA binding domain binds zinc with such a high affinity that dependency could not be demonstrated without some zinc depletion step.

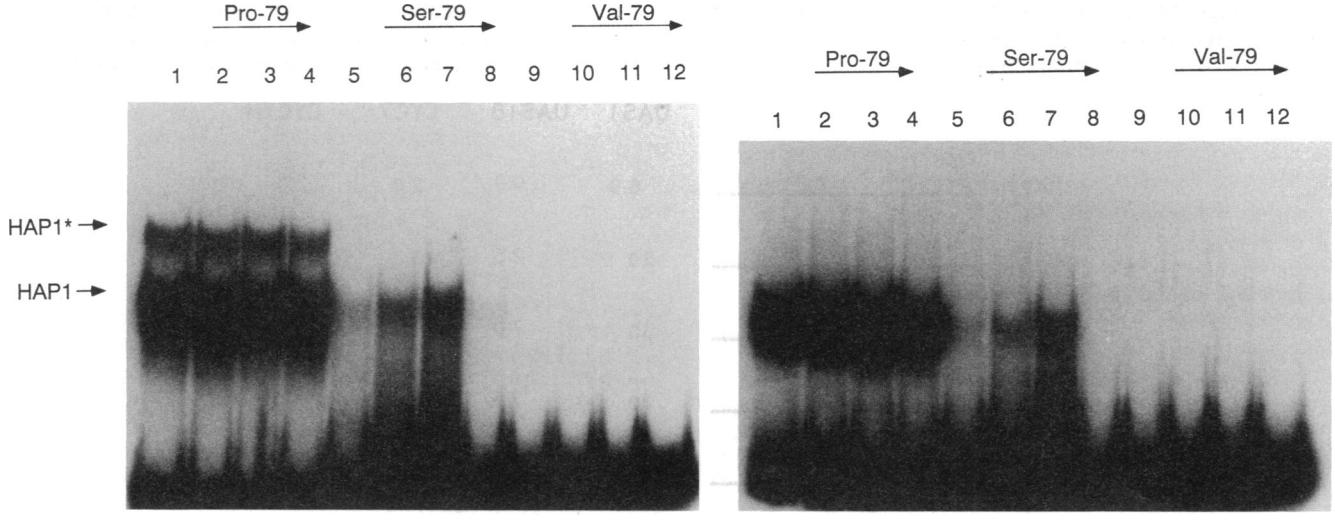

FIG. 2. Binding of HAP1 mutants of Pro-79. Pro-79 was replaced by valine or serine by oligonucleotide-directed mutagenesis. An oligonucleotide, 5′-TGGCAGTGT(A or G)(A or C)TCTGAGTTT-3′, was synthesized and annealed to the coding strand of an M13 mp18 subclone of pSP65HAP1 bearing the *Sma* I–*Hin*dIII region of *HAP1* and extended (9, 13). After sequencing candidate phages, clones that bore alterations of the proline codon (CCA) to serine (TCA) or valine (GTA) were identified. Double-stranded DNA was prepared, and the *Sma* I–*Hin*dIII fragment encoding the DNA-binding domain was inserted into pSP65, also digested with *Sma* I/*Hin*dIII. The resulting plasmids were cleaved with *Bst*EII and used for *in vitro* transcription and translation in a wheat-germ extract. Translation extract was mixed with ^{32}P-labeled UAS1 (−312 to −229) (*Left*) or *CYC7* (−273 to −130) (*Right*) probes in the presence of 250 ng of salmon sperm DNA. Lanes: 1, 5, and 9, 0.0 μM $ZnCl_2$; 2, 6, and 10, 1.5 μM $ZnCl_2$; 3, 7, and 11, 5.0 μM $ZnCl_2$; 4, 8, and 12, 15.0 μM $ZnCl_2$. HAP1 indicates binding to the UAS1B site, and HAP1* indicate binding to UAS1A and UAS1B.

The codon 79 mutants were recombined into an expression plasmid, and their ability to activate UAS1 or *CYC7* was checked *in vivo* in the presence of increasing zinc concentrations in the medium (data not shown). While the Val-79 mutant was inactive at all concentrations of zinc, the Ser-79 mutant was about as active as wild type in the absence of any zinc supplement. The relatively high affinity of this mutant for zinc compared with the GAL4 mutant could explain why a zinc dependence could not be demonstrated *in vivo*. A failure to observe inhibition of activity of the mutant at high concentrations of zinc in the medium could reflect an inability to achieve high concentrations in the cell. Again, these properties of the mutants applied equally to UAS1 and *CYC7*. The above findings support earlier genetic findings indicating the importance of Cys-64 and Cys-81 (3). We conclude that the cysteine-rich region in the DNA-binding domain of HAP1 is, in fact, a zinc finger and that zinc is required equally for binding to UAS1 and *CYC7*.

Internal Deletions in HAP1. There exists a large internal region in HAP1 (residues 247–1308) that spaces the DNA-binding and acidic domains (3). This region is not required for activation of UAS1 but is involved in regulation of HAP1 activity (3). Removal of residues 247–445 results in heme-independent activity *in vivo* and heme-independent DNA binding *in vitro*. Further, deletions carboxyl-terminal to this region cause an additional elevation in activity at UAS1. Given the role of the large spacer in modulating HAP1 activity, we reasoned that this region might be a target for mutations that would show differential effects at the two binding sites. Therefore, we determined the effects of deletions in HAP1 on a *CYC1-lacZ* reporter driven by either UAS1 or the *CYC7* promoter *in vivo*. The reporters were integrated into the genome of a *hap1* mutant strain, and *HAP1* and its deleted derivatives were transformed into the strains on 2-μm plasmids.

Fig. 3 displays our findings. At UAS1, all of the deletion mutants gave rise to increased expression compared with wild type, in part because the heme regulatory domain was missing. In the extreme case, the 247–1308 deletion, mini-HAP1, had a level of activity about 10 times that of the wild type. In striking contrast, the activity at the *CYC7* promoter, although higher than that of wild type in the mutant with the shortest deletion removing the heme domain, decreased with deletion size in all mutants with larger deletions. In the extreme, mini-HAP1 was totally inactive, giving a level of expression equal to the background observed in a mutant deleted of the acidic activation domain.

UAS1 and the *CYC7* promoter are complex; the former contains two HAP1 binding sites, and the latter contains one HAP1 site and a site for a different factor that induces the promoter in lactate (12). To examine the effects of the HAP1 deletions on UASs with single binding sites for the activator, we used deleted versions of UAS1 and the *CYC7* promoter that removed all upstream DNA except for a single HAP1 binding site in each case. The UAS1 derivative contains only UAS1B, and the *CYC7* derivative *CYC7* mutant H is missing the L site but retains the HAP1 site. Because the levels of activity of *CYC7* H were low, it was necessary to transfer the construct to a 2-μm plasmid to bring activity into an assayable range. The pattern of expression seen at UAS1B and *CYC7* H was identical to that of the intact promoters, although the levels were lower (Fig. 3). Thus, these experiments show that internal deletions in HAP1 exhibit opposite effects at the two sequence motifs: activity at UAS1 increases, and activity at *CYC7* decreases.

Previous work has shown that residues 1–148 of HAP1 are sufficient for specific binding to UAS1 or *CYC7* (3). Further, the Δ247-444 deletion mutation described above is fully capable of *CYC7* activation *in vivo*. Nevertheless, it was important to demonstrate directly that the inability of mini-HAP1 to activate transcription was not due to an inability of that particular mutant protein to bind to *CYC7*. Thus, we synthesized mini-HAP1 *in vitro* and, in parallel, the DNA-binding domain of HAP1 as a control, and we analyzed binding to UAS1 and *CYC7* DNA probes (Fig. 4). Binding of mini-HAP1 (as well as the DNA-binding domain alone) to both UAS1 and *CYC7* probes was comparable. Binding to a DNA probe that does not respond to HAP1, the *lexA* operator, was not observed. These results indicate that the mini-HAP1 retains the ability to bind specifically to the *CYC7* site.

HAP1 Fusion to the GAL4 Activation Domain. The above results suggest that the HAP1 acidic domain cannot activate transcription at *CYC7* when fused directly to the HAP1 DNA-binding domain. We wished to determine whether this property was specific for the HAP1 acidic domain or would apply more generally to acidic activation domains. Accordingly, the acidic activation domain of GAL4 (16) was fused to

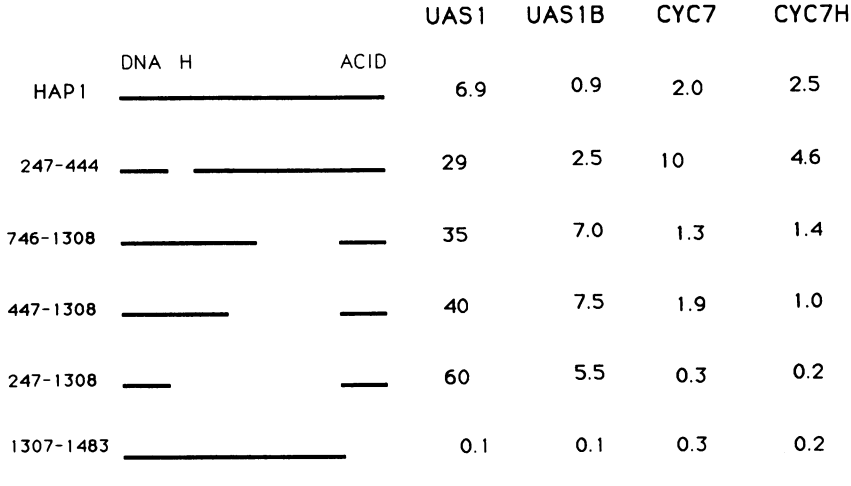

FIG. 3. Internal deletions in *HAP1*. Deletions of the indicated residues of *HAP1* were constructed as described in plasmids bearing *HAP1*. Constructs were transformed into strains bearing an integrated *CYC1-lacZ* reporter driven by UAS1 (−312 to −229) or the *CYC7* promoter extending out to −354. The UAS1B integrant bears a synthetic site inserted into the UAS cloning region of pLG-312. The 2-μm sequences were removed to facilitate integration. The *CYC7* mutant H promoter bears a 5′ deletion ending at −273, leaving intact the HAP1 binding site. The other active site in the *CYC7* promoter, the L site, has been removed. The *CYC7* H construct was studied on a 2-μm plasmid. β-Galactosidase assays were performed in triplicate with an error of <10%. The DNA-binding (DNA), acidic (ACID), and heme (H) domains of HAP1 are indicated.

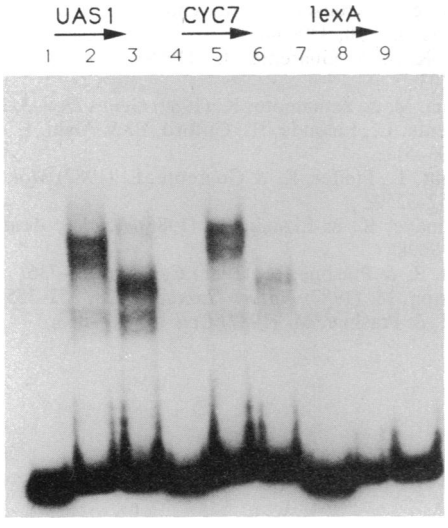

FIG. 4. Binding of mini-HAP1 to UAS1 and *CYC7*. A plasmid containing mini-*HAP1* fused to the SP6 promoter was constructed as described. This plasmid was cleaved with *Xho* I, which cleaves past the 3′ end of the mini-*HAP1* gene, or with *Bam*HI which cleaves at codon 245 between the DNA-binding and acidic domains. The DNAs were transcribed, and RNAs were translated as described. Translations were carried out with unlabeled amino acids or with [^{35}S]methionine in parallel. The ^{35}S-labeled reactions were monitored on SDS/polyacrylamide gels for synthesis of proteins. Probes used contained UAS1 (−312 to −229), *CYC7* (−273 to −195), or the *lexA* operator as a control. The *lexA* site, a 26-base *Xho* I fragment, was subcloned from pRB1155 into the *Sal* I site of pUC18 and was excised with *Eco*RI and *Hin*dIII. In all binding reactions, the unlabeled *in vitro* translation products were bound to probes labeled with ^{32}P (indicated above the lanes) in the presence of 250 ng of salmon sperm DNA. Lanes: 1, 4, and 7, probes alone; 2, 5, and 8, mini-HAP1; 3, 6, and 9, DNA-binding domain of HAP1.

the DNA-binding domain of HAP1 (residues 1–247) and the ability of this fusion to activate at UAS1 and *CYC7* was determined. HAP1-GAL4 could activate at both UAS1 and *CYC7* (Fig. 5). This finding demonstrates several key points. First, the HAP1 DNA-binding domain (residues 1-247) is capable of binding to the *CYC7* site *in vivo*. Moreover, the inability of mini-HAP1 to activate transcription is specific for the HAP1 acidic domain. In other words, the GAL4 acidic domain overrides the defect in mini-HAP1. The possible nature of this specificity will be considered below.

DISCUSSION

The experiments presented above show that the DNA-binding domain of HAP1 is a zinc finger and that this structure is needed for binding to both the UAS1 and *CYC7* classes of sequence elements. Previous studies indicated that the amino acids involved in binding to UAS1 and *CYC7* were not identical. Saturation mutagenesis of codon 63 showed that arginine, methionine, or isoleucine at that position bound much better to *CYC7* than to UAS1 (9). The wild-type protein

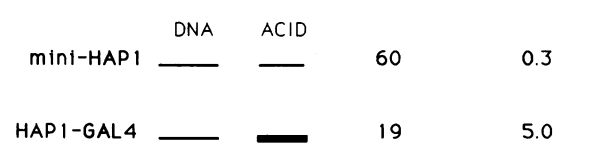

FIG. 5. Comparison of HAP1-GAL and mini-HAP1. The UAS1 and *CYC7* strains used and the β-galactosidase assays are as in Fig. 4. The thick bar represents the acidic domain of GAL4 (residues 753–881) fused to residues 1–247 of HAP1 as described.

binds with equal affinity to the sites. Thus, our picture of this domain of the protein is that of a surface that recognizes both sequence elements, probably by making different protein–DNA contacts.

Earlier studies provided the hint that the bound activator might have different properties when bound at the two sites. In this work, we investigated this question by selecting a series of internally deleted mutants of HAP1 and examining their activities at UAS1 and *CYC7 in vivo*. Strikingly, the deletions had opposite effects at the two sites. The activity of UAS1 increased with the deletion size and the activity of *CYC7* decreased. In the extreme, mini-HAP1, a fusion of the carboxyl-terminal acidic domain to the amino-terminal DNA-binding domain, had high activity at UAS1 and no activity at *CYC7*. This derivative bound equally well to both sites *in vitro*.

How do the mutants display differential effects? Previous work suggested that, in addition to the acidic domain, the DNA-binding domain contacted the transcriptional machinery (9). This inference was based on the fact that the 1–18 mutation greatly increased the activity at *CYC7* without affecting binding to the site. Thus, we proposed that the 1–18 mutation increased transcription by strengthening the contact between the *CYC7*-bound activator and the machinery (9). This cooperation between the acidic and DNA-binding domains in the activation process could explain the effects of the deletions. In one model we imagine that both the DNA-binding and acidic domains of HAP1 contact two distinct protein surfaces in the transcriptional machinery. We further speculate that the conformation of the DNA-binding domain differs when HAP1 is bound at UAS1 versus *CYC7*. These two conformers of the DNA-binding domain could contact the machinery differently, conceivably in distinct regions. Thus, the geometry of the contacts between the two HAP1 domains and the machinery could differ when the protein is bound at UAS1 versus *CYC7*. Given such a difference, internally deleted derivatives that reduce the spacing and alter the geometry between the DNA-binding and acidic domains of HAP1 could prevent the simultaneous contacts with the machinery when bound at *CYC7* but not UAS1.

That activity at *CYC7* is restored in a fusion of residues 1–247 to the acidic domain of GAL4 is consistent with this model. This restoration could occur if the geometry between the acidic domain of GAL4 and the DNA-binding domain of HAP1 in this fusion protein again allowed simultaneous contacts with the machinery.

An alternative hypothesis that accounts for the differential effects of the HAP1 deletions at the two sites is that the *CYC7* conformer of the DNA-binding domain masks the acidic domain by an intramolecular interaction. Masking may be favored in HAP1 deletion derivatives in which the two domains are brought closer together. If the masking required a specific interaction between the two domains of HAP1, this model would also explain the properties of HAP1-GAL4. A model that we do not favor is that the acidic domain of mini-HAP1 can mask a portion of the DNA-binding domain, thereby preventing binding to *CYC7* but not UAS1. This seems unlikely because *in vitro* synthesized mini-HAP1 binds equally well to both sites.

One interesting feature of either of the above models is the direct role of the DNA binding site in setting the level of activity of a transcriptional activator. Such a role would create an additional layer of control and give added specificity to DNA sequences in the activation process. It remains to be determined biochemically whether DNA sequence in fact could alter the conformation of a protein, and whether such an alteration might be signaled to the transcriptional machinery.

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1. Guarente, L. (1987) *Annu. Rev. Genet.* **21,** 425–452.
2. Struhl, K. (1989) *Annu. Rev. Biochem.* **58,** 1051–1077.
3. Pfeifer, K., Kim, K. S., Kogan, S. & Guarente, L. (1989) *Cell* **56,** 291–301.
4. Pfeifer, K., Arcangioli, B. & Guarente, L. (1987) *Cell* **49,** 9–18.
5. Winkler, H., Adam, G., Mattes, E., Schanz, M., Hartig, A. & Ruis, H. (1988) *EMBO J.* **7,** 1799–1804.
6. Pfeifer, K., Prezant, T. & Guarente, L. (1987) *Cell* **49,** 19–27.
7. Clavilier, L., Auber, G., Somlo, M. & Slonimski, P. (1976) *Biochemie* **58,** 155–172.
8. Verdiere, J., Creusot, F., Guarente, L. & Slonimski, P. (1986) *Curr. Genet.* **10,** 339–342.
9. Kim, K. S. & Guarente, L. (1989) *Nature (London)* **342,** 200–203.
10. Schena, M. & Yamamoto, K. (1989) *Genes Dev.* **3,** 1590–1601.
11. Guarente, L., Lalonde, B., Gifford, P. & Alani, E. (1984) *Cell* **36,** 503–511.
12. Prezant, T., Pfeifer, K. & Guarente, L. (1987) *Mol. Cell. Biol.* **7,** 3252–3259.
13. Nakamaye, K. & Ekstein, F. (1986) *Nucleic Acids Res.* **14,** 9679–9698.
14. Brent, R. & Ptashne, M. (1985) *Cell* **43,** 729–736.
15. Johnston, M. (1987) *Nature (London)* **328,** 352–355.
16. Ma, J. & Ptashne, M. (1987) *Cell* **48,** 847–853.