HAP1 positive control mutants specific for one of two binding sites

Bernard Turcotte and Leonard Guarente

Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 USA

The expression of the yeast *CYC1* and *CYC7* genes is controlled by the HAP1 activator. A GAL4-like zinc finger (residues 1–148) specifies binding to the dissimilar sites UAS1 (of *CYC1*) and CYC7, and an acidic domain (residues 1307–1483) is essential for activation of transcription. To analyze how HAP1 binds to UAS1 and CYC7, we performed saturation mutagenesis of the DNA-binding domain and recovered mutants with altered activity. Class 1 mutants had a reduced activity at both UAS1 and CYC7, and class 2 mutants selectively eliminated activity at CYC7. Surprisingly, several mutants of both classes exhibited wild-type DNA binding, indicating that they were specifically defective in activation. These positive control (PC) mutants alter residues that bracket the zinc finger. We explain these mutants in a model involving cofactor proteins that bind UAS1 and CYC7 along with HAP1. The existence of PC mutants that only affect activity at CYC7 raises the possibility that different cofactors may exist for UAS1 and CYC7.

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Eukaryotic enhancers are sites found at locations distant from transcription initiation sites, which augment transcription (Banerji et al. 1981; Moreau et al. 1981). The activity of enhancers depends on transcriptional activator proteins that bind to the sites. Activators contain two functional domains: one that dictates site-specific DNA binding to the enhancer, and one dedicated to the activation of the transcriptional machinery (Brent and Ptashne 1985). Activation domains fall into several classes, the most prevalent of which contains a high concentration of acidic amino acids (Hope and Struhl 1986; Ma and Ptashne 1987). These acidic domains are functional in eukaryotes that range from yeast to mammals, an indication of striking conservation (for review, see Guarente and Bermingham-McDonogh 1992).

The enhancer and the initiation site–proximal promoter element, the TATA box, can be separated by hundreds of nucleotides, creating the capacity for complex arrays of enhancers and activators at a single promoter. In addition to the complexity of a multitude of activators acting at a single promoter, the existence of additional factors, or cofactors, can influence the potency of enhancers. For example, binding of the yeast mating-type regulators $\alpha 1$ and $\alpha 2$ to their target sites is aided by the binding of MCM1 to adjacent DNA sequences (Keleher et al. 1988; Jarvis et al. 1989; Passmore et al. 1989). In the yeast GAL system, the product of *GAL11* potentiates full activity of the GAL4 activator (Suzuki et al. 1988) and shows specificity for the DNA-binding domain of GAL4 (Himmelfarb et al. 1990). This GAL4 domain contains a zinc finger of 6 cysteine residues that is very similar to DNA-binding domains of a number of yeast activators.

The activity of one of these activators, PPR1, is reduced in a *gal11* mutant, suggesting that GAL11 may be a cofactor for this family of yeast activators (Himmelfarb et al. 1990).

Another yeast activator with similarity to GAL4 throughout the zinc finger domain is HAP1 (Pfeifer et al. 1989). When compared with the other members of this family, HAP1 contains a longer amino-terminal extension adjacent to the finger. For example, the first cysteine residue of GAL4 is at residue 11, whereas in HAP1 the corresponding residue is at position 64 (Pfeifer et al. 1989). HAP1 activates transcription of the two genes encoding cytochrome *c*, *CYC1* and *CYC7* (Guarente et al. 1984; Prezant et al. 1987; Zitomer et al. 1987), as well as genes encoding other heme-related proteins (Winkler et al. 1988; Schneider and Guarente 1991). The DNA-binding activity of HAP1 itself is stimulated by heme, which, presumably, binds to an internal sequence in the protein that otherwise blocks DNA binding. The structure of HAP1 contains discrete domains. The DNA-binding domain lies between residues 1 and 148, the heme regulatory domain is between residues 245 and 444, and the acidic activation domain is between residues 1307 and 1483 (Pfeifer et al. 1989).

Strikingly, while HAP1-binding sites at UAS1 (upstream activating sequence 1) of *CYC1*, *CYT1*, and *CTT1* are similar, the CYC7 site shows no discernible similarity. Several findings suggest that the same HAP1 DNA-binding domain binds to the dissimilar sequences, UAS1 and CYC7. First, the sites cross-compete for HAP1 binding with an equal affinity (Pfeifer et al. 1987b). Second, mutations in the DNA-binding domain, including mu-

tations in the Cys residues, knock out binding to both sites (Pfeifer et al. 1989). Gel-shift complexes of HAP1 or truncated derivatives to UAS1 and CYC7 show identical mobilities, suggesting that the same species of the protein binds to the sites. In addition, when the first 148 amino acids of HAP1 were translated in vitro, the polypeptide bound to both UAS1 and CYC7 (Pfeifer et al. 1989).

What biological role might be served by multisequence recognition? Perhaps the most striking feature of the dissimilar HAP1 sites is that transcriptional activation by UAS1 and CYC7 differs quantitatively and qualitatively in vivo. Thus, when synthetic UAS1 and CYC7 oligonucleotides are inserted into the same promoter construct, the level of activation driven by HAP1 is 5- to 10-fold higher for UAS1 (Kim and Guarente 1989). Furthermore, while the activity of HAP1 at UAS1 increases in cells shifted from glucose to lactate, the activity of HAP1 at CYC7 decreases (L. Guarente, unpubl.).

An additional clue regarding the differential activity of UAS1 and CYC7 comes from the *HAP1-18* mutation (Creusot et al. 1988; Pfeifer et al. 1989). This mutation was originally identified because it increases the activity of HAP1 at CYC7 10- to 100-fold (Clavilier et al. 1976). We found that the *HAP1-18* mutation also increased the activity of HAP1 dramatically at the synthetic CYC7 oligonucleotide in front of a minimal *CYC1* promoter (Kim and Guarente 1989). The mutation changes Ser-63 to Arg, immediately adjacent to the Cys cluster in the DNA-binding domain of the protein. A secondary effect of the change is that the mutant does not bind to UAS1. When Ser-63 is systematically changed to 15 other residues, only Arg-63 shows elevated activation of CYC7, although several other changes, like *HAP1-18*, selectively knock out binding to UAS1 (Kim and Guarente 1989).

Several models are consistent with these findings. We have suggested that the DNA sequence itself may alter

the conformation of the DNA-binding domain to allow that part of HAP1 to contact the transcriptional machinery differently when bound at the two sites (Kim and Guarente 1989). To begin to address the question of how HAP1 function might differ at UAS1 versus CYC7, we have performed a systematic genetic analysis of the DNA-binding domain of HAP1 described herein. In so doing, we have isolated mutations that affect the activities of UAS1 and CYC7 differentially. A novel class of mutants retains the ability to bind to both sites but has lost the ability to activate specifically at one. From these results, we suggest that HAP1 activity in vivo is due partly to cofactor proteins that bind to CYC7 and UAS1. Our results raise the possibility that a single activator can interact with two different cofactors when bound at different sequence elements.

Results

Genetic strategy

We wished to carry out a saturation mutagenesis of the DNA-binding domain of HAP1 and then screen for mutations that differentially affected UAS1 (of CYC1) and the CYC7 element. To facilitate the mutagenesis, we constructed a plasmid that bore the HAP1 DNA-binding domain on a cassette so that it could be transferred from the substrate for mutagenesis to a yeast expression plasmid. The DNA-binding domain of HAP1 was transferred to a plasmid with an M13 origin, and single-stranded DNA was prepared. Saturation mutagenesis of codons 48–130 was carried out using nine overlapping oligonucleotides (Fig. 1), which were synthesized such that they contained, on average, one mutation per oligonucleotide. Each synthetic oligonucleotide was extended using the single-stranded substrate, and resulting plasmids were transformed into *Escherichia coli*. Greater than 10^4 in-

Figure 1. Nucleotide and amino acid sequence of the DNA-binding domain of HAP1. Amino acid numbering is given at left. Circled amino acids are cysteine residues of the zinc finger involved in binding to zinc. The rectangle shows the position of the *HAP1-18* mutation. Arrows indicate the sequence of the spiked oligonucleotides that were used for saturation mutagenesis (see Materials and methods).

dependent colonies were pooled, and the cassette was transferred to a yeast expression plasmid under control of the UAS_{GAL} .

To screen for mutations that affected UAS1 and CYC7 differentially, the yeast strain LPY22 ($\Delta hap1$) was transformed with mutagenized HAP1 plasmid. The strain also contained a *lacZ* reporter driven by the *CYC1* TATA box–mRNA initiation region and either UAS1 or CYC7 UAS. Both UAS1 and CYC7 contained two HAP1-binding sites that direct activation via the acidic activation domain of HAP1. Deletion of this domain in the HAP1 Δ Kp construct (or HAP1-18 Δ Kp) reduced activity to background levels (Table 1, column one). Transformants were transferred on filters to X-gal plates with galactose to induce expression of HAP1. Colonies with a reduced or an increased level of β -galactosidase were chosen for analysis. The HAP1 plasmids from these candidates were recovered in bacteria and retransformed into yeast strains bearing each of the reporters.

Classes of mutants recovered

The levels of β -galactosidase in yeast cells retransformed with mutant candidates were assayed (Table 1). Mutants fell into two main categories: Class 1 showed a reduced

level of activity at both UAS1 and CYC7 (Table 1, column one); class 2 mutants were fully active at UAS1 but totally inactive at CYC7 (Table 1, column one). The reciprocal class, which would reduce activity at UAS1 but not CYC7, was not observed (with the exception of the HAP1-18 mutation, see below). Mutations of both classes were sequenced. Many class 1 mutations resulted in chain termination and are not shown in Table 1. The class 1 and class 2 mutants described below all bore missense mutations.

We wished to test the possibility that class 1 mutants had a reduced binding affinity for both UAS1 and CYC7, whereas class 2 mutants had lost the ability to bind to CYC7 but not UAS1. For this purpose, gel-shift analysis was carried out in yeast extracts from cells that were partially induced for HAP1 synthesis in 1% galactose plus 1% glucose (Fig. 2). HAP1 and HAP1-18 binding were also measured as controls.

Many mutants did display a reduction in DNA binding, which could account for their loss in activation ability [R57H, K76T (Fig. 2); K71N, K76R, C81Y, K86I, and FS91 (data not shown)]. Unexpectedly, some mutants in both class 1 and class 2 displayed a level of DNA binding approximately equal to or greater than the wild-type protein ($\geq 90\%$ of the wild-type-binding activity). The quantitation of the binding data by the PhosphorImager is

Table 1. *In vivo* activation and *in vitro* binding of wild-type and mutant HAP1

Plasmid	Mutation	In vivo activation (β -gal units)		In vitro binding (% wild type)	
		<i>CYC1</i>	<i>CYC7</i>	<i>CYC1</i>	<i>CYC7</i>
HAP1	Wild type	68	21	100	100
HAP1 Δ Kp	Δ 1307–1483	5.0	0.2	—	—
HAP1-18	Ser-63–Arg	2.3	264	3	50
HAP1-18 Δ Kp	Δ 1307–1483	2.2	0.5	—	—
Class 1					
R57H	Arg-57–His	10	0.7	40	15
PC4	Pro-61–Gln	18	0.6	150	95
K76T	Lys-76–Thr	12	0.5	80	30
P79R	Pro-79–Arg	17	0.3	—	—
PC5	His-94–Asp	18	0.4	75	90
PC6	Thr-127–Ile Leu-128–Arg	40	0.9	60	105
PC7	Ser-63–Gly	3.7	0.5	<1	170
K71N	Lys-71–Asn	5.3	0.6	<1	<1
K76R	Lys-76–Arg	5.2	0.5	<1	<1
C81Y	Cys-81–Tyr	5.8	0.7	<1	<1
K86I	Lys-86–Ile	2.0	1.2	<1	<1
Class 2					
PC1	Lys-54–Glu	55	0.4	290	270
PC2	Arg-55–Gly	257	0.8	415	385
DM1	Pro-61–Ala Ser-63–Cys	84	0.2	60	40
I66S	Ile-66–Ser	108	0.3	20	10
PC3	Gln-98–Leu Ala-101–Glu	69	0.6	105	90

Mutations or deletions of HAP1 mutants are given in the two left columns. *In vivo* activity was determined by measuring β -galactosidase levels produced from reporters pLG Δ 312 Δ AX–*HIS* (*CYC1*) or pCYC7-5–*HIS4* (*CYC7*) as described in Material and methods. *In vitro* binding was measured from the gel shown in Fig. 2 using a PhosphorImager (Molecular Dynamics). All data were obtained under moderate levels of HAP1 using a 1% glucose–1% galactose mix. Deletion of amino acids 1307–1483 removes the acidic activation domain.

Figure 2. In vitro binding of wild-type and HAP1 mutants. (*Middle*) Extracts from cells expressing wild-type and HAP1 mutants were prepared as described in Materials and methods and used for gel-shift assay using a probe corresponding to the UAS1 of *CYC1* (1) or the UAS from *CYC7* (7). With the *CYC1* probe, a nonspecific signal with a faster mobility than HAP1 was also detected. This nonspecific signal was similar in all lanes, indicating that no general protein degradation occurred during extract preparation and that similar amounts of protein were loaded in each lane. Shown are histograms giving the in vivo activity of the mutants using the *CYC1* (*top*) or the *CYC7* reporter (*bottom*). Histograms are derived from data presented in Table 1.

shown in Table 1. Mutants that can still bind to their DNA sites but are unable to activate transcription were identified in the λ repressor (Guarente et al. 1982b; Hochschild et al. 1983) and in the glucocorticoid receptor (Skena et al. 1989) and termed PC mutants. In addition, mutations in Oct-1 were identified that prevented interaction with VP16 but did not prevent DNA binding (Stern and Herr 1991). It is inferred that PC mutants have lost the ability to make a protein contact with the transcriptional machinery that is critical to the activation process.

Some positive-control mutants displayed the PC property at both UAS1 and *CYC7*, that is, they were class 1 mutants (*PC4*, *PC5*, and *PC6*). One other mutant, *PC7*, displayed the PC property at *CYC7* and was unable to bind to UAS1. This change is of particular interest because it occurs at the 1–18 position altering Ser-63 to Gly. Strikingly, *PC1*, *PC2*, and *PC3* behaved like wild-type HAP1 at UAS1 in terms of binding and activation but were PC mutants at *CYC7*. These PC mutants provide insight into the mechanism of HAP1 activation of transcription, as we discuss below.

A final class of mutant had elevated activity at *CYC7* (the *HAP1-18* phenotype). DNA sequence analysis of three independent mutants of this class revealed three different nucleotide alterations to change Ser-63 to Arg (data not shown). Evidently, the only change in the pro-

tein that gives a level of expression at *CYC7* that is elevated over wild type is Ser-63 to Arg. This is consistent with earlier findings that two different *HAP1-18*-type mutations changed Ser-63 to Arg (Creusot et al. 1988; Pfeifer et al. 1989).

The location of the PC mutations in HAP1 is striking. They all lie in the regions immediately to the amino or carboxyl side of the cysteine cluster of HAP1 (Fig. 3). Mutations in positions within the cysteine cluster invariably affect DNA binding to both UAS1 and *CYC7*. Position 63 appears to be unique in differentially affecting binding to one site (UAS1) but not to the other (*CYC7*). Previous work indicated that serine, threonine, or cysteine residues were required at position 63 for binding to UAS1, whereas these and many other residues allowed binding at *CYC7* (Kim and Guarente 1989). This work furthers our understanding of the importance of position 63 in activation at *CYC7*; Ser-63 gives a moderate level of transcriptional activation at *CYC7*, Arg-63 displays an accentuated level of activation, and Gly-63 does not activate.

HAP1 activity in a *gal11* deletion strain

GAL11 is thought to aid activity of GAL4 and PPR1 by interacting in some way with the zinc finger (Himmel-

Figure 3. Positions and amino acid changes of HAP1 mutants that bind to UAS1 and CYC7 but show a reduced or no transcriptional activity (PC mutants). Circles represent amino acids; numbers represent their respective positions. Arrows indicate the mutations. The HAP1 zinc finger was drawn assuming that it has a structure similar to GAL4 (Pan and Coleman 1990). Cysteine residues involved in zinc binding are shown in bold-face type.

farb et al. 1990). Because the zinc fingers of GAL4 and HAP1 are very similar (43% identical), it was possible that GAL11 also modulated the activity of HAP1. The PC mutants could be defective in such an interaction. To test the effect of GAL11 on HAP1 activity, we deleted *GAL11* from LPY22 (Δ hap1). The chromosomal deletion removes the promoter and most of the coding region of *GAL11*. We then introduced into the *GAL11* or *gal11* strains plasmids expressing HAP1, HAP1-PC3, or HAP1-18 under the control of the *HAP1* promoter along with a reporter plasmid, with the *lacZ* gene driven by UAS1 or CYC7. In control experiments, we found that the level of a GAL4-driven reporter, pLGS5 (Guarente et al. 1982a), was reduced 80-fold in the *gal11* mutant, whereas the activity of a reporter driven by LexA-GCN4 (Hope and Struhl 1986) was unaffected in the *gal11* strain (Table 2). HAP1 activity at UAS1 was reduced only about threefold by the *gal11* deletion, and activity at CYC7 was reduced less than twofold. Activity of PC3 at UAS1 was affected to a similar extent as wild-type HAP1. From these results, we conclude that GAL11 plays at best a minor role in modulating HAP1 activity at both UAS1 and CYC7. Similarly, HAP1-18 activity at CYC7 was reduced only twofold in the *gal11* mutant (Table 2). This finding indicates that the increase in CYC7 expression caused by the HAP1-18 mutation is not due to a favored interaction with GAL11.

Discussion

Multisequence recognition: DNA binding

Previous work showed that HAP1 bound primarily to one face of the DNA helix at both UAS1 and CYC7 and

that major groove contacts were dispersed over three turns of the helix at both sites (Pfeifer et al. 1987b). Furthermore, at both sites, the major groove contacts were spread over a 17-nucleotide region and HAP1 made a single contact on the reverse face of the helix at one end of each site. Recent experiments indicate that HAP1 binds to these sites as a dimer (L. Zhang and L. Guarente, unpubl.).

The saturation mutagenesis of the HAP1 DNA-binding domain could potentially provide insight by identifying mutants that selectively bound to UAS1 or CYC7. However, the only changes that significantly affected binding selectively were at Ser-63, a position already known to be critical for binding to UAS1 but not CYC7. This suggests that the same set of amino acid residues of HAP1, except Ser-63, are important for binding to both sites. Ser-63 is uniquely important for binding to UAS1. In this regard, one primary difference in major groove contacts at UAS1 and CYC7 is two additional contacts at UAS1, at G262, and on the opposite strand at G252 (Pfeifer et al. 1987b). These residues are 10 bases apart and are therefore on the same face of the helix. We speculate that Ser-63 of two monomers of HAP1 directly contacts G262 and G252 in UAS1.

Multisequence recognition: site-specific cofactors

In studying what biological role may be served by evolution of two HAP1-binding sites of different sequence, we have reported differences in the levels of activation and extent of regulation between UAS1 and CYC7 (Kim and Guarente 1989; L. Guarente, unpubl.). Furthermore, past work showed that certain derivatives of HAP1 bear-

Table 2. Effect of *GAL11* deletion on HAP1 activity at CYC1 (reporter pLG4312AAX-HIS and CYC7 (reporter pCYC7-5-HIS4)

Reporter	Activator	Strain	
		wild type	<i>gal11</i> ⁻
CYC1	HAP1	3,045	930
	HAP1-18	25	60
	HAP1-PC3	4,200	1410
	—	20	40
CYC7	HAP1	740	475
	HAP1-18	6,720	3210
	HAP1-PC3	15	NT
	—	35	20
pLGS5	GAL4	25,000	320
LexA (RB115)	LexA-GCN4	3,845	4350

HAP1 expression vectors are under the control of their own promoter (see Material and methods), and YEP352 (Hill et al. 1986) was used in the -HAP control. In vivo activity was determined by measuring β -galactosidase activity, as described in Material and methods, using a wild-type (LPY22) or a *gal11*⁻ (LPgal11⁻) strain. β -Galactosidase units are in nmoles/min per mg of protein. (NT) Not tested.

ing internal deletions bound normally to both sites but were specifically defective in activation at CYC7 (Kim et al. 1990). These findings suggested that there was some difference in activation by HAP1 at UAS1 versus CYC7.

What might this difference be? A very interesting type of mutation that emerged from our screen bound normally to both UAS1 and CYC7 but were defective in activation of transcription. One class of these PC mutants was defective in activation at both UAS1 and CYC7, whereas a second class activated like wild type at UAS1 but was inactive at CYC7. All of these PC mutations were in residues that flank the 6 cysteine residues of the zinc finger (Fig. 3). These PC mutations lie >1200 residues away from the carboxyl acidic activation domain of HAP1.

From these findings we surmise that the DNA-binding domain of HAP1 contains specificity to interact with another protein factor, or cofactor, most probably by cobinding with that factor at HAP1 sites *in vivo*. Recently, the three-dimensional structure of the DNA-binding domain of GAL4 bound to DNA has been determined (Marmorstein et al. 1992). GAL4 binds as a dimer to its recognition sequence with a twofold symmetry. Interestingly, contacts with DNA are located at both ends of the UAS leaving a long stretch of DNA at the center accessible for binding of another protein in the major groove. Assuming that HAP1 has a structure similar to GAL4, the residues altered by *PC1*, *PC2*, *PC3*, *PC4*, *PC5*, and *PC7* mutations could be in a position to interact with a cofactor bound to the center of the site (Fig. 4, model 1). Protein-protein interactions of this type have been observed in the case of the yeast repressor

$\alpha 2$ and the factor MCM1 (Keleher et al. 1988). The *PC6* double mutant lies in more distal residues that are important in dimerization (L. Zhang and L. Guarente, unpubl.) and may alter the conformation of the DNA-binding domain of HAP1, resulting in a loss of interaction with a cofactor when bound at CYC7. We note that the PC mutations do not alter the complex between HAP1 in yeast extracts and its binding sites, suggesting that the cofactor is too unstable or its affinity for the HAP1-DNA complex is too low for detection by gel shift.

Perhaps the most intriguing class of mutations, exemplified by *PC1*, *PC2*, and *PC3*, behave like the wild-type protein at UAS1 and are PC mutants at CYC7. How can we explain these effects? One reasonable model is that HAP1 associates with two different cofactors that are specific for UAS1 and CYC7 (Fig. 4, model 2). This specificity could be determined by the difference in sequences of the two sites. Different cofactors might also explain why UAS1 activity is higher than that of CYC7 even though HAP1 binds to the sites with the same affinity (Kim and Guarente 1989). The *PC1*, *PC2*, and *PC3* changes would selectively knock out the interaction with the CYC7 cofactor by this model. However, this class of mutants do not eliminate model 1, if we assume that the cofactor-DNA interaction at UAS1 is stronger than at CYC7 and can compensate for the loss of interaction energy caused by *PC1*, *PC2*, and *PC3*. It will be necessary to identify the gene encoding the CYC7 cofactor to clearly discriminate between the two cofactor models.

We tested the possibility that GAL11 was a HAP1 cofactor because it affects the activity of GAL4 and shows specificity for the DNA-binding domain of GAL4, which is similar to that of HAP1. However, removing GAL11 from cells by gene deletion exerted a minor reduction in the activity of UAS1 (threefold) and had a slight effect at CYC7 (less than twofold), indicating that GAL11 is not an important cofactor for HAP1.

One HAP1 mutant that is striking in its effects is *HAP1-18*, changing Ser-63 to Arg, which elevates HAP1 activity at CYC7 10-fold or more. The mutation does not increase the steady-state level of HAP1 nor the affinity of HAP1 for CYC7 (Kim and Guarente 1989). We have speculated that *HAP1-18* increases the affinity of HAP1 for some component of the transcriptional machinery, which we now envision as a cofactor at CYC7. The change of Ser-63 to Gly (*PC7*) has the opposite effect of *HAP1-18*. The mutant *PC7* is inactive at CYC7, although it still binds to DNA. We expect this change to disrupt interaction with a CYC7 cofactor. In a previous study (Kim and Guarente 1989), we found that the change from Ser-63 to Gly displayed somewhat reduced binding when synthesized *in vitro* and partial activation potential *in vivo* at CYC7 (Kim and Guarente 1989). In that study, the CYC7 reporter contained the entire CYC7 promoter and was integrated into the yeast genome. Apparently, the CYC7 reporter used here bearing only the HAP1-binding site oligonucleotide provides a more stringent assay for HAP1 activation.

This study (Table 1) and previous experiments have

Figure 4. Models for interaction between HAP1 and cofactor(s). HAP1 is shown bound to UAS1 or CYC7 as a dimer. In model 1, only one cofactor interacts with the DNA-binding domain of HAP1 and a UAS. Thick stripes represent a strong interaction with the HAP1-DNA complex at UAS1; thin stripes represent a weaker interaction at CYC7. In model 2, two different cofactors, each specific for a given UAS, are responsible for the differential activity at UAS1 and CYC7.

shown that the acidic domain at the carboxy-terminal part of HAP1 is necessary for activation while the PC mutants indicate that the activation domain is not sufficient for activation of transcription. One possibility to account for these observations is that the cofactor acts in synergy with the acidic domain to stabilize an interaction with a target protein of the transcriptional machinery. Alternatively, the cofactor could help to stabilize occupancy of the UAS by HAP1 *in vivo*.

In summary, our mutational analysis of the DNA-binding domain of HAP1 has provided insights into DNA binding and transcriptional activation at dissimilar UASs. Most novel is the surmise that HAP1 uses a cofactor at CYC7 and, potentially, a different cofactor at UAS1. The difference in sequences of the two sites could specify which cofactor is bound. The genetic approach illustrated here may be an effective route to begin to identify cofactors for other activators.

Materials and methods

Strains and media

Strains are derivatives of BWG1-7A (Guarente and Mason 1983). LPY22 contains a *LEU2* deletional insertion in *HAP1* between the *HindIII* and the *KpnI* sites of *HAP1*. This totally removes HAP1 functional sequences. LPY22 was rendered *trp1*[−] by integrating the *URA3-hisG* cassette (Alani et al. 1987) into the *TRP1* gene and selecting for *URA*⁺ and subsequently *gal11*[−] (Himmelfarb et al. 1990). This disruption replaces the *GAL11* sequences from 123 bp upstream of the ATG to nucleotide +2341 by the *TRP1* gene. Southern blotting confirmed proper integration. Rich (YPD), minimal (SD), and synthetic complete (SC) media were prepared as described (Sherman et al. 1986).

Plasmids

Plasmid pEMBL-HAP1 was constructed by subcloning the 1.3-kb *BamHI* fragment (encoding the amino-terminal part of HAP1) from SD5-HAP1 (Kim and Guarente 1989) into the *BamHI* site of pEMBL18+ (Dente et al. 1985). An *XhoI* site was then introduced by site-directed mutagenesis at nucleotide 111 relative to the ATG using the oligonucleotide 5' GCCAA-CACCTCGAGTAATCT-3' to give the plasmid pEMBL-HAP1-CAS. Introduction of the *XhoI* site did not change the HAP1 amino acid sequence. SD5-HAP1-CAS was constructed in two steps. The unique *XhoI* site of SD5-HAP1 in the *CYC1* promoter was destroyed by cutting the plasmid with *XhoI*, filling in with Klenow, and ligating. The resulting plasmid was then used to replace its *BamHI* fragment by the one from pEMBL-HAP1-CAS, thus giving a plasmid with an *XhoI*-*BstEII* (nucleotide +739 relative to the ATG) cassette fragment.

Deletion mutants SD5-HAP1ΔKp and SD5-HAP1-18ΔKp were constructed by linearizing the plasmids SD5-HAP1 and SD5-HAP1-18 (Kim and Guarente 1989) by *KpnI*, filling in with Klenow, and inserting an *XbaI* linker (New England Biolabs) containing nonsense codons in the three reading frames. HAP1 expression vector under the control of its own promoter was constructed in two steps. The *AatII*-*NcoI* fragment (containing a 2μ origin of replication) was isolated from plasmid YEP352 (Hill et al. 1986) and subcloned into the *AatII*-*NcoI* backbone of plasmid pRS306 (Sikorski and Hieter 1989), giving p306-2μ containing a unique *PstI* and *XhoI* sites in the polylinker region. The 5.3-kb *PstI*-*XhoI* fragment containing the *HAP1* gene (from

−227 to +5013 bp relative to the AUG) was isolated from plasmids pHAP1 (Pfeifer et al. 1987b) and subcloned into the *XhoI*-*PstI* backbone of p306-2μ to give pHAP1-S. *HAP1* mutations in plasmids pHAP1-18 (Pfeifer et al. 1989) and PC3 (Table 1) were transferred to the expression vector pHAP1-S by replacing their corresponding *EcoRI* fragments (+176 to +602 relative to the ATG) to give pHAP1-18-S and pHAP1-PC3, respectively (Table 2).

The *CYC1* reporter, pLGΔ312ΔAX-HIS, was constructed by removing the *URA3* marker of plasmid pLGΔ312ΔAX (Guarente et al. 1984) by digesting with *StuI* and inserting a blunt-ended *SacI*-*BstEII* genomic fragment from the *HIS4* gene (Donahue et al. 1982). The *CYC7* reporter, *CYC7*-5-HIS4, was constructed by removing the *URA3* marker from plasmid *CYC7*-5, containing two copies of the HAP1-binding site at *CYC7* (Kim and Guarente 1989), by digesting with *StuI*, adding *NotI* linkers, and inserting a blunt-ended *SacI*-*BstEII* fragment of the *HIS4* gene (Donahue et al. 1982) flanked by *NotI* linkers.

Saturation mutagenesis of the DNA-binding domain of HAP1

Single-stranded DNA from plasmid pEMBL-HAP1-CAS was prepared (Sambrook et al. 1989) and used for saturation mutagenesis with spiked oligonucleotides (level of contamination, 2.5% for each of three other nucleotides) (see Fig. 1). Mutagenesis was performed using the phosphorothionate-modified DNA method, according to Nakamaye and Eckstein (1986), with a kit from Amersham following the manufacturer's protocol. *In vitro*-mutated DNA was then transformed into *E. coli* (TG1). Greater than 10⁴ independent colonies were then scraped off the plates and grown for plasmid isolation by cesium chloride banding (Sambrook et al. 1989). Mutated fragments were then excised from pEMBL-HAP1-CAS by cutting with *XhoI* (at position +111 relative to the ATG) and *BstEII* (+739) for subcloning into the *XhoI*-*BstEII* backbone of the yeast expression vector SD5-HAP1-CAS. The latter contained the *URA3* marker and a 2μ origin of replication. The ligation mix was then transformed into *E. coli* MC1061, and the colonies were pooled for plasmid isolation. The DNA was then used to transform the yeast strain LPY22 bearing a *HIS4*-marked reporter (see above) on minimal plates. Colonies were then transferred with a nitrocellulose filter onto a minimal X-gal plate containing 2% galactose. Colonies with altered β-galactosidase activity relative to wild type were then grown for plasmid isolation and transformation into *E. coli*. Mutations were identified by sequencing the double-stranded DNA (Sanger et al. 1977). Mutant phenotypes were confirmed by transforming them back into the LPY22 strain with either the *CYC1* or the *CYC7* reporters.

β-Galactosidase assays

HAP1 expression vectors under the control of UAS_{GAL} Cells were grown to saturation in minimal media containing 2% raffinose; they were then diluted 20 times in SD media containing 1% glucose and 1% galactose and grown for ~18 hr before assaying for β-galactosidase (Table 1; Guarente and Mason 1983).

gal11[−] strain Cells were grown to saturation in YPD; they were then spun, washed in water, and diluted 2000 times (LPY22 strain) or 50 times (LPgal11[−] strain) in SC media with 2% glucose lacking histidine (or 2% galactose plus histidine for the reporter pLGS5). Cells were then grown for ~20 hr before assaying for β-galactosidase activity (Table 2; Rose and Botstein 1983).

Preparation of extracts and DNA-binding assays

Cells were grown exactly as described for HAP1 expression vec-

tors under the control of UAS_{GAL}. Extracts were then prepared, and the DNA-binding assay was carried out as described (Pfeifer et al. 1987a) using complementary oligonucleotides with *Xho*I ends filled in with the Klenow fragment and [α -³²P]dATP and [α -³²P]dGTP. The sequence of the *CYC1* probe is 5'-TC-GAGTGGCCGGGTTTACGGACGATGA-3', and that of the *CYC7* probe is 5'-TCGACCCTCGCTATTATCGCTATTAGC-3'. Quantitative data were obtained by measuring the amount of radioactivity present in the retarded bands using a PhosphorImager (Molecular Dynamics).

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HAP1 positive control mutants specific for one of two binding sites.

B Turcotte and L Guarente

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