Identification and characterization of HAP4: a third component of the CCAAT-bound HAP2/HAP3 heteromer

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The *CYC1* gene of *Saccharomyces cerevisiae* is positively regulated by the HAP2 and HAP3 proteins, which form a heteromeric complex that binds to a CCAAT box in the upstream activation site, UAS2, and which activate transcription in a nonfermentable carbon source. We carried out a genetic analysis to identify additional *trans*-acting regulatory factors exerting their effects through UAS2. We present the identification and characterization of a new locus, *HAP4*, which is shown to encode a subunit of the DNA-binding complex at UAS2. In the *hap4* mutant, the binding of HAP2 and HAP3 (HAP2/3) is not observed in vitro. The *HAP4* gene is regulated transcriptionally by a carbon source, suggesting that it encodes a regulatory subunit of the bound complex. The sequence of HAP4 shows a highly acidic region, which innactivated the protein when deleted. Replacement of this region with the activation domain of *GAL4* restored activity, suggesting that it provides the principal activation domain to the bound HAP2/3/4 complex.

[*Key Words*: HAP4; CCAAT-binding factor; transcription; *Saccharomyces cerevisiae*]

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Activation of eukaryotic gene expression is mediated by the binding of distinct regulatory factors to specific upstream DNA sequence elements (for reviev, see Guarente 1988; Ptashne 1988). In recent years, it has become apparent that the cell often makes use of a flexible and economical system of combinatorial control. By exploiting different combinations of regulatory elements and proteins, the cell can modulate precisely the expression of a given gene, as well as maximize its use of a given activator. Numerous enhancers in yeast and higher cells work on this principle (Yamamoto 1985; McKnight and Tjian 1986; Jones et al. 1988). One means of achieving such control consists of an enhancer made up of discrete neighboring binding sites for different regulatory factors. The varied combinations of the cognate factors at adjacent elements can provide expression in response to a distinct set of signals. Another means of mixing and matching is provided by regulatory complexes consisting of heterologous protein subunits. Thus, both the DNA specificity of a given activator and its specificity for interaction with other proteins allow the cell to tailor its gene expression precisely.

There are several examples of such combinations of proteins in higher cells. The herpes simplex virus product VP16 forms a complex with cellular DNA-binding proteins, including Oct-1, and increases their ability to activate the transcriptional machinery (McKnight et al. 1987; Gerster and Roeder 1988; Preston et al. 1988; Triezenberg et al. 1988b). For this purpose, VP16 bears an acidic region comparable to the activation domains of the yeast activators GCN4 and GAL4 (Hope and Struhl 1986; Giniger and Ptashne 1987; Ma and Ptashne 1987; Triezenberg et al. 1988a). In a variation on this theme, the cellular factors Fos and AP-1 form a heteromeric complex with a higher affinity for the AP1-binding site than AP1 alone (Halazonetis et al. 1988; Kouzarides and Ziff 1988; Nakabeppu et al. 1988). AP-1 is a DNA-binding factor related to the avian oncogene *jun* and the yeast GCN4 (Bohmann et al. 1987; Vogt et al. 1987; Angel et al. 1988); however, although it has been demonstrated to be an activator when fused to the DNA binding domain of *lexA*, Fos has no DNA-binding activity by itself (Chiu et al. 1988; Lech et al. 1988). Both AP-1 and OCT-1 can bind DNA without contribution from the additional factor. In yet another variation, however, the HeLa cell CCAAT-binding factor CP1 has been shown to require both of two separate chromatographic fractions to bind the adenovirus major late promoter CCAAT box in vitro (Chodosh et al. 1988a).

The yeast mating type control system provides an additional example of proteins working in combination (Bender and Sprague 1987; Goutte and Johnson 1988; Keleher et al. 1988; Tan et al. 1988). In this case, MATα1 and a second factor called pheromone/receptor transcription factor (PRTF) bind together to regulate α-specific genes positively. MATα2, again cooperating with PRTF, binds to repress **a**-specific genes. However, in the diploid, MATα2 and MAT**a**1 act together at a new regulatory site to repress haploid-specific genes. In different combinations at the DNA, the same proteins can act in different ways.

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It was demonstrated previously that the positive regulators HAP2 and HAP3 bind as a heteromeric complex (HAP2/3) to the upstream activation site UAS2 of the yeast *CYC1* gene (Olesen et al. 1987; Hahn and Guarente 1988). Mutations in *HAP2* or *HAP3* affect expression of several other cytochrome genes as well as *CYC1*, and also affect the *HEM1* gene (Guarente et al. 1984; Keng and Guarente 1987; Trueblood et al. 1988; Schneider 1989). Several of these genes are known to be induced when the cells are shifted from glucose to a nonfermentable carbon source such as lactate. Under these conditions, the cells require cytochromes for respiratory growth. Thus, the HAP2/3-system activates genes globally encoding cytochromes and related proteins when cells undergo the shift to a nonfermentable carbon source. For the wild-type UAS2, the induction from glucose to lactate is some 50-fold (Guarente et al. 1984). The HAP2/3-binding site in UAS2 and the UASs of other genes under its control contains a CCAAT box (in region 1 of the UAS; see Fig. 1). Linker substitutions or deletions within the UAS2 CCAAT box itself or within sequences 20 bases upstream of the box abolished HAP2/3 binding, as well as the activity of the site in vivo (Forsburg and Guarente 1988). A base substitution in UAS2 (UP1) that generates a perfect CCAAT sequence from the wild-type UAS2 sequence CCAAC increased activity in all carbon sources in vivo and increased the affinity of the site for HAP2/3 binding in vitro (Guarente et al. 1984; Olesen et al. 1987). A factor independent of HAP2/3 binds adjacent to the heteromer in the downstream region 2 of the UAS; although this region provides a very low level of activity by itself, its presence augments the activity of region 1 by some fivefold (Olesen et al. 1987; Forsburg and Guarente 1988).

In this paper we present the isolation, cloning, and characterization of an additional positive regulator of region 1 of UAS2. Like HAP2 and HAP3, HAP4 is required for growth on a nonfermentable carbon source. It regulates the same range of UAS elements that respond to HAP2 and HAP3 and is regulated transcriptionally by a shift to a nonfermentable carbon source. Thus, regulation of *HAP4* may account for the regulation of UAS2. Preliminary biochemical characterization of the *HAP4* gene product demonstrated that HAP4 is required for binding to UAS2 by HAP2/3 in vitro and that it binds with HAP2 and HAP3 at UAS2. Also, we present evidence suggesting that HAP4 contains an activation domain.

Results

Isolation of hap4-1 and hap4-2

We carried out a genetic analysis to determine whether any additional *trans*-acting factors, besides HAP2 and HAP3, are involved in UAS2 regulation. To identify such factors, we used a mutant screen similar to that used to isolate mutations in *HAP2* and *HAP3*. We began with cells carrying a high-copy *CYC1–lacZ* fusion plasmid, the expression of which was driven by UAS2UP1. The plasmid produced ~100 units (Miller 1972) of β-galactosidase. These cells were mutagenized with ethylethase sulfonate (EMS) and plated directly on X-Gal glucose plates. Colonies were screened for a loss of β-galactosidase activity, indicated by a change in color from dark blue to light blue (see Materials and methods). From two pools of 20,000 cells each, 30 candidates with <25 units of β-galactosidase activity were identified. By complementation, in which we assayed β-galactosidase activity from the UAS2 fusion in the diploid, we identified two of these as alleles of *HAP2*, and two as alleles of *HAP3*. Candidate strains, including both *hap2*$^-$ strains and one *hap3*$^-$ strain as positive controls, were cured of the fusion-bearing plasmid and transformed with additional fusion plasmids in which *CYC1–lacZ* expression was driven by other UAS elements, such as UAS1 or UAS–*HIS4*. Five UAS2-specific mutants were identified. Two were the previously identified *hap2*$^-$ alleles, and a third was the *hap3*$^-$ allele. The remaining two mutants defined a new complementation group, which we termed *HAP4* (Table 1).

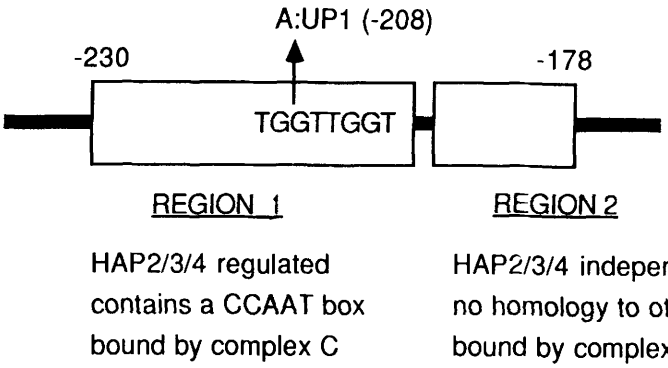

Figure 1. Summary of UAS2 organization. UAS2 consists of two regions. Region 1 is bound by complex C, known to contain HAP2 and HAP3 (Olesen et al. 1987; Forsburg and Guarente 1988; Hahn and Guarente 1988). Region 1 extends from −230 to −200, relative to the upstream-most RNA start site. It contains the sequence TGGTTGGT, which contains a G→A transition at −208 in UAS2UP1, increasing the homology to the HAP2 and HAP3 consensus TNATTGGT (Guarente et al. 1984; Forsburg and Guarente 1988). This sequence encodes a CCAAT box on the opposite strand. Region 2, from −192 to −178, has little activity by itself but substantially enhances the activity of region 1 (Forsburg and Guarente 1988). Region 2 is bound by a HAP2/3-independent factor (Olesen et al. 1987; Forsburg and Guarente 1988).

Table 1. *UAS specificity of* HAP4

UAS element	Wild-type (units β-gal)	*hap4-2* (units β-gal)
UAS2UP1	90	8
UAS1	69	66
UAS–*HIS4*	35	53
Region 1 (UP1) only	12	1
Region 2 only	0.5	0.6
UAS–*GAL* (in glucose)	0.1	0.1
UAS–*GAL* (in galactose)	500	600

Each value indicates the average β-galactosidase activity obtained from at least two transformants of each plasmid. Each vector contains a *CYC1–lacZ* fusion driven by the indicated UAS. Assays were carried out as described in Materials and methods. Unless indicated otherwise, all assays were carried out on cells grown in glucose media. Duplicate assays varied by <30%. The sources of these UAS elements are as follows: UAS2UP1 (pLGΔ-265UP1) and UAS1 (pLGΔ-229-178), Guarente et al. (1984); UAS–*HIS4* [pHYC3(169)], Hinnebusch et al. (1985). Region 1 of UAS2UP1 (containing the UP1 mutation; pSLFΔ188-194UPK) and region 2 of UAS2 (pSLFΔ203K), Forsburg and Guarente (1988). UAS–*GAL* (pLGSD5), Guarente et al. (1982).

These new mutants, which were isolated independently (one from each of the starting pools), had the same phenotypes. As was the case for *hap2* and *hap3* mutants, *hap4* mutants were petite and failed to grow on nonfermentable carbon sources such as lactate. We presume that this phenotype is a result of the failure to produce sufficient levels of cytochromes for respiratory growth. Both *hap4* mutant strains produced 8 units of β-galactosidase activity (compared to the wild-type level of ~100 units) from a UAS2UP1–*lacZ* fusion. As is the case for *hap2* and *hap3*, the new isolates specifically affected the activity of the CCAAT box region (region 1) of UAS2 (Table 1). That is, when transformed with plasmids carrying deletion constructions of UAS2, the new mutants only affected the activity of the region 1 fusion. When crossed to wild type and sporulated, the petite phenotype and the failure to activate UAS2UP1 cosegregated as a single nuclear locus, and when appropriately marked spores were crossed together, they failed to complement for lactate growth (see Materials and methods). Finally, using *lacZ* fusions to UASs from the *COX4*, *CYT1*, and *HEM1* genes, we determined that the new locus regulates other genes known to be affected by mutations in *HAP2* or *HAP3* (data not shown). Because these phenotypes were similar to those observed in *hap2* or *hap3* cells, we named the new locus *HAP4*.

Cloning and characterization of HAP4

The petite phenotype of *hap4-1* and *hap4-2* provided a convenient selection by which to isolate the wild-type gene. We transformed *hap4-2* cells with a single-copy yeast library and selected for growth on lactate. We isolated two clones, characterized the restriction map of the inserts, and determined that they overlapped by ~8 kb. We reduced the insert to a 3.5-kb *Bgl*II insert that complemented both *hap4-1* and *hap4-2* strains in either single copy or in high copy (pSLF402; see Fig. 2). This fragment also directed integration of the plasmid to the *hap4* locus. Additional subcloning in a high-copy vector allowed us to reduce this insert still further, to a 2-kb *Cla*I–*Bgl*II fragment (pSLF405; see Fig. 2).

A disruption of the *HAP4* locus was constructed by replacing the 800-bp *Cla* fragment with the *LEU2* gene (Fig. 2). Deletion of the *Cla* fragment abolished complementation by the clone. The phenotype of this disruption was indistinguishable from that of a disruption of *HAP2* or *HAP3* (Pinkham and Guarente 1985; Hahn et al. 1988). In all cases, there were a basal 2 units of activity from the UAS2UP1–*lacZ* fusion. Thus, the original isolates of *hap4* were somewhat leaky with respect to UAS2 activity.

Regulation of HAP4

Northern analysis of *HAP4* RNA levels indicated that unlike *HAP2* and *HAP3* (Pinkham and Guarente 1985; Hahn et al. 1988), this gene is induced strongly by a shift in carbon source from glucose to lactate (Fig. 3). Cells grown in lactate produced four- to fivefold more *HAP4* transcript (by densitometry) than cells grown in glucose. However, mutations in *HAP2*, *HAP3*, or *HAP4* had no effect on the expression of *HAP4* in glucose-grown cells (data not shown), suggesting that there is no autoregulation of this gene. Therefore, regulation of *HAP4* may account for at least some of the carbon-source regulation seen at UAS2 and at the UAS elements of other genes also regulated by *HAP2*, *HAP3*, and *HAP4*. The apparent size of the *HAP4* RNA was ~2.5 kb.

Sequence of HAP4

The complete nucleotide sequence and the predicted amino acid sequence of the *HAP4* gene are shown in Figure 4. The original smallest complementing subclone (pSLF405; see Fig. 2) was shown by this analysis to truncate a large open reading frame (ORF) at the *Bgl*II site indicated in Figure 4. Truncated and full-length *HAP4* were indistinguishable in terms of complementation for growth on lactate and UAS2UP1 activity (data not shown). The ORF continued for an additional 200 bp beyond this site.

This ORF predicted a protein of 554 amino acid residues, giving an estimated molecular mass of 62 kD. The sequence showed no apparent homology to any other protein in a database search. It contains a very short basic region between residues 54 and 80, followed by an asparagine-rich tract that ends with 7 Asn residues in a row. Most notable, however, is the carboxyl terminus, which is extremely acidic overall, with two particularly acidic blocks. Between residues 519 and 549 and between residues 424 and 471, the protein is 30% acidic, indicated in Figure 4. The *Bgl*II site, which marked the

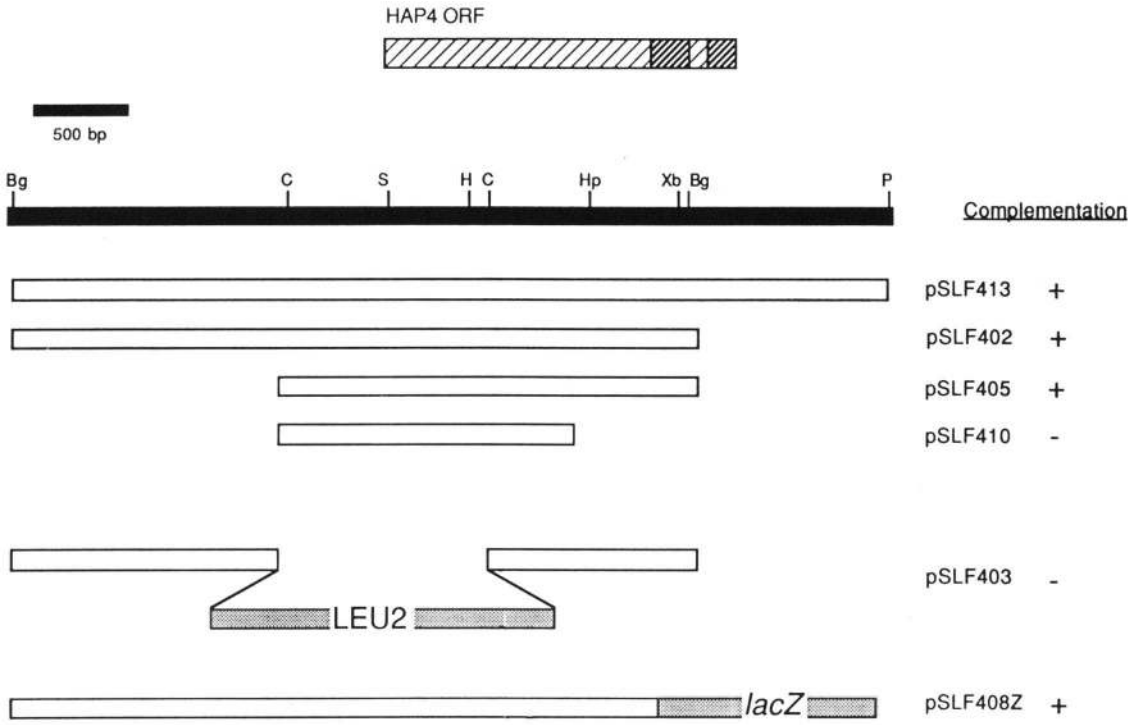

Figure 2. Subclones of *HAP4*. All clones were constructed in a high-copy vector. Details of constructions are described in Materials and methods. Complementation of *hap4-2* by all *HAP4* clones was assessed both by streaking on YEP lactate places and by β-galactosidase assays on vector pSLF265UPLEU. All positive clones were indistinguishable from one another in terms of growth and activity. The bar above the restriction map indicates the HAP4 ORF. The shading indicates the regions of most acidity. (Bg) *Bgl*II; (S) *Stu*I; (H) *Hin*dIII; (C) *Cla*I; (Xb) *Xba*I; (P) *Pst*I.

end of the complementing truncation of *HAP4* (pSLF405; see Figs. 2 and 4), cut off one, but not both, of these acidic domains at residue 476. However, a further truncation at the unique *Hpa*I site (pSLF410; see Figs. 2 and 4), at residue 327, cut off both acidic regions and failed to complement for growth on lactate. Therefore, assuming this further truncation is a stable protein, the acidic region is essential to HAP4 function, as will be discussed further below.

The RNA start site was determined using primer extension (Fig. 5). The primer was synthesized to hybridize to the sequence just downstream of the predicted initiator ATG. There are three start sites. The two principal start sites provide a leader of ~280 nucleotides in length; the third start is ~50 nucleotides farther upstream. This long leader includes two upstream ATGs, initiating ORFs of nine and three residues, respectively. Thus, *HAP4* joins a handful of other yeast genes with known upstream ATGs (Cigan and Donahue 1987). It is interesting to speculate that these upstream ORFs may provide translational regulation of *HAP4*, as is the case for *GCN4* (Hinnebusch, 1984; Thireos et al. 1984). Because UAS2 is regulated by heme as well as by carbon source, it is possible that an additional, specific level of regulation of *HAP4* would occur in response to heme levels. Indeed, the yeast catalase T protein has been shown to be regulated translationally by heme (Hamilton et al. 1982).

Construction of a HAP4–lacZ fusion

We constructed a bifunctional fusion between *HAP4* and β-galactosidase (Fig. 2). This construction, pSLF408Z, fused the *lacZ* gene to codon 465 of *HAP4* (Materials and methods; see Figs. 2 and 4). The construction preserved the major complementing portion of the *HAP4* gene and nearly 2 kb of upstream sequences. This fusion complemented a *hap4*$^-$ strain for growth on lactate, although the growth rate was slightly slower than wild type. The fusion was regulated in the same way as the *HAP4* mRNA; i.e., activity was induced four- to fivefold when cells were shifted from glucose to a nonfermentable carbon source (data not shown).

HAP4 *is required for binding of* HAP2/3

Because HAP4 exerts its effect through the same region of UAS2, as does HAP2 and HAP3, we assumed that HAP4 function is mediated in some way via these previously isolated positive factors. Mutations in *HAP4* have no effect on the expression of a *HAP2–lacZ* fusion (data not shown). Thus, it is possible to make at least two models as to the function of HAP4 in regulation of UAS2, given the fact that HAP2 and HAP3 are part of a DNA-binding complex. HAP4 could regulate directly the complex formed by HAP2 and HAP3, for example,

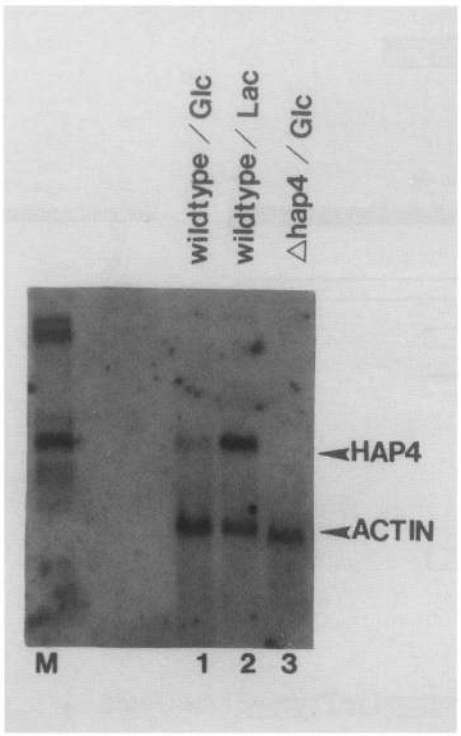

Figure 3. Northern blot of *HAP4*. (M) Marker, containing λ DNA cut with *Pst*I; (lane *1*) wild-type cells, glucose media; (lane *2*) wild-type cells, lactate media; (lane *3*) *hap4* :: *LEU2* cells, glucose media. Ten micrograms of total RNA was loaded in each lane. The probes were the 800-bp *Cla*I fragment from pSLF401 (see Fig. 2), the actin gene on an sp65 vector, and λ cut with *Pst*I.

by physically modifying the proteins. Phosphorylation is one means of covalent modification that has been implicated in control of the yeast regulator ADR1 (Cherry et al. 1989), or HAP4 could interact with the complex. We first attempted to determine whether HAP4 is required for in vitro binding of the HAP2/3 complex to UAS2UP1, using DNA mobility shift gel assays and crude yeast extracts.

Under normal conditions, the HAP2/3 complex can be visualized only in extracts from cells grown in the fully inducing carbon source lactate (Olesen et al. 1987), but *hap4*$^-$ cells cannot be grown in lactate because they are petite. To obviate this difficulty, a DNA mobility shift gel assay was carried out on extracts from galactose-grown cells that carried a plasmid placing expression of *HAP2* under control of UAS_{GAL} (Fig. 6). Expression of this UAS is unaffected by mutations in *HAP4* (Table 1). HAP2 overproduction from this plasmid allows visualization of the HAP2/3 complex when the cells are grown in galactose (Olesen et al. 1987). When bound to a DNA probe containing UAS2UP1, two complexes were detected in extracts from glucose-grown cells, called A and B (Fig. 6, lane 1), and a third complex in galactose, called C (Fig. 6, lane 3). Earlier work (Olesen et al. 1987) demonstrated that complexes A and B are HAP2- and HAP3-independent and are unaffected by changes in carbon source. Complex A was localized to region 2 of UAS2, activity of which is unaffected by mutations in *HAP2*, *HAP3*, or *HAP4* in vivo (Olesen et al. 1987; Forsburg and Guarente 1988). Complex B cannot be localized to any portion of the UAS, and its appearance varies unpredictably (Olesen et al. 1987; Forsburg and Guarente 1988). Complex C, which bound specifically to region 1 of UAS2, was shown to contain both HAP2 and HAP3 (Olesen et al. 1987).

We transformed the HAP2 overproducer plasmid into *hap4*$^-$ cells and compared extracts from *hap4*$^-$ cells grown in glucose (Fig. 6, lane 2) and in galactose (Fig. 6, lane 4) to wild-type extracts. Complex C alone was not resolved in *hap4*$^-$, galactose-grown cells. Therefore, within the limits of this assay system, we conclude that HAP4 is required for formation of the HAP2/3-containing complex C.

HAP4 is a component of the HAP2/3 complex at UAS2

Both HAP2 and HAP3 were shown to be present in complex C by constructing size variants and demonstrating that the mobility of complex C altered with the size of the proteins (Olesen et al. 1987). This approach requires that the size variants be fully functional, as they must provide complementing activity in the lactate conditions required for the visualization of complex C. We employed this method to determine whether HAP4 also is contained in complex C. Using wild-type HAP4 and two size variants, we carried out DNA-mobility shift gel assays, where these variants were the only source of HAP4 activity. Besides the wild-type protein, we used the functional truncation pSLF405, which removed the carboxyl terminus at codon 478 (the downstream *Bgl*II site in Fig. 2) and the bifunctional *HAP4–lacZ* fusion (pSLF408Z; Fig. 2). Extracts from lactate-grown cells bearing these variants were prepared and used for binding. The results are shown in Figure 7. The mobility of complex C clearly shifted in response to the change in the size of HAP4. The truncated HAP4 migrated more quickly, and the mobility of the HAP4–β-galactosidase fusion was retarded, relative to the migration of the wild-type complex. This demonstrates that HAP4 also is a part of complex C at UAS2, a complex thus consisting of at least three proteins.

HAP4 is an activator

The acidic carboxyl terminus of HAP4 is similar to that found in numerous other activators. Extensive work on *GCN4* and *GAL4* has demonstrated that an 'acid blob' provides an activation domain, which can be fused to a variety of DNA-binding domains and still function (Brent and Ptashne 1985; Hope and Struhl 1986; Giniger and Ptashne 1987; Ma and Ptashne 1987). Furthermore, the acid blob works in a variety of eukaryotic cells (Fischer et al. 1988; Kakidani and Ptashne 1985; Webster et al. 1988). The strikingly acidic regions found in HAP4 therefore suggested that the carboxyl terminus

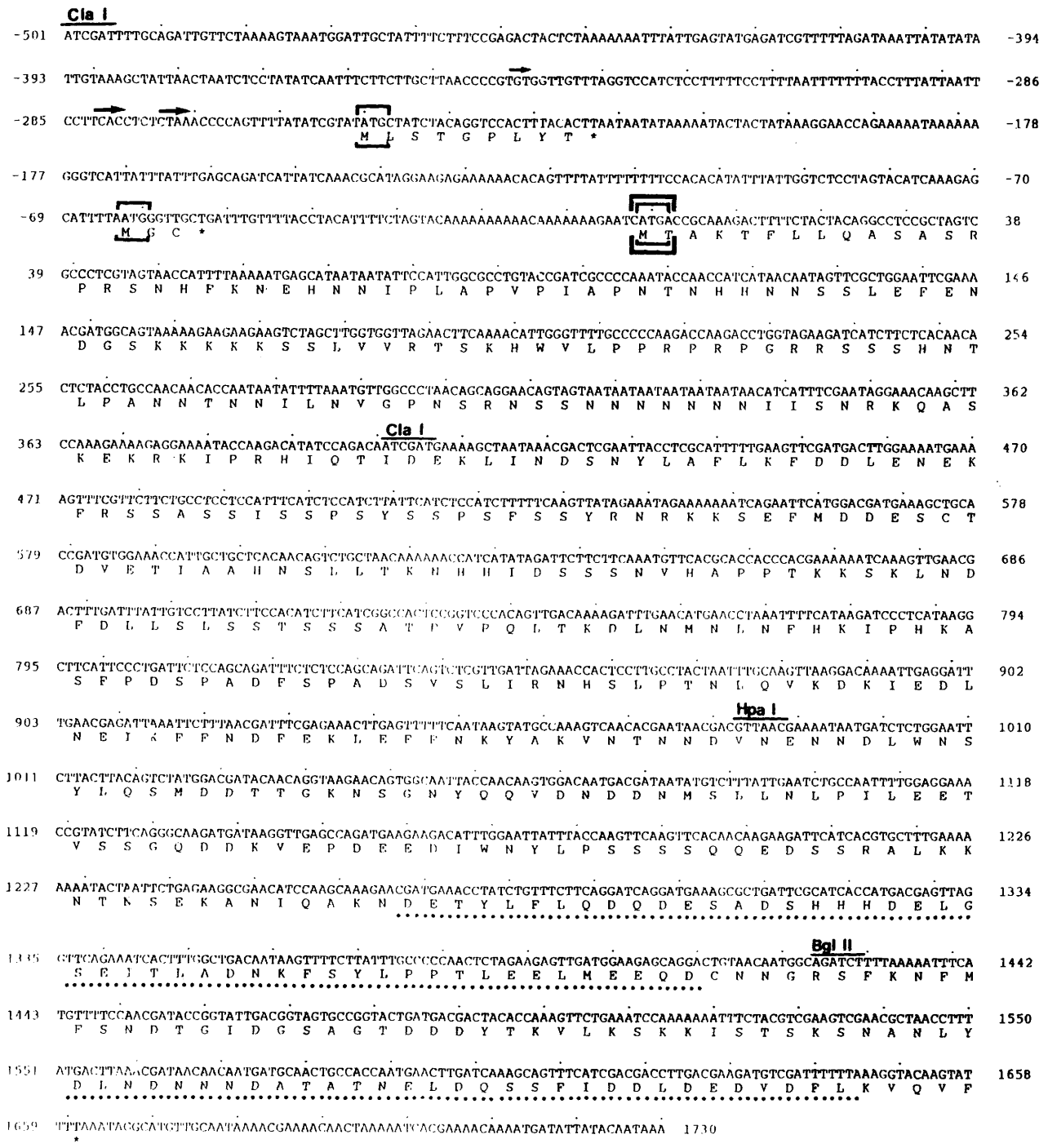

Figure 4. Sequence of the *HAP4* gene and predicted amino acid sequence. Sequencing was carried out using the Sequenase/dideoxy chain termination method on M13 clones carrying restriction fragments of *HAP4*, so that both strands and all junctions were sequenced. Each clone was sequenced at least in duplicate. The three ATGs are bracketed; the presumed initiator ATG is bracketed in double lines. The RNA start sites are indicated by arrows over the sequence. The *ClaI* sites, the *BglII* site, and the *HpaI* site are indicated. A dotted underline marks the acidic domains.

of HAP4 may provide the activation domain for the UAS2 complex. There are only very short regions of acidity in the HAP2 or HAP3 proteins (Pinkham et al. 1987; Hahn et al. 1988). In addition, it suggested that HAP4 may contain two domains, a carboxyl terminal activation domain and an amino terminus required to interact with the HAP2 and HAP3 proteins. Activators such as GCN4 and GAL4 contain two domains also, the second of which recognizes and binds the cognate activation sequence.

The above model suggested that it might be possible to restore function to a noncomplementing truncated

Figure 5. Primer extension. RNA was isolated from wild-type cells carrying the high-copy plasmid pSLF401. A primer of the sequence 5′-GAGGGCGACTAGCGGAGG-3′ was synthesized and end-labeled with [γ-^{32}P]dATP, and extension was carried out as described in Materials and methods. Forty micrograms of RNA from lactate-grown cells was used. The marker lane (lane *2*) contains the 'C' sequencing reaction carried out on an M13 clone carrying the left-hand portion of HAP4, using the same primer. (Lane *1*) RNA; (lane *2*) C reaction.

derivative of HAP4, missing both acidic regions, by addition of an activation domain from a different yeast activator. (This assumes that the nonfunctional truncation protein is indeed synthesized and stable.) We constructed a fusion of the amino terminus of HAP4 to the acidic region of GAL4. The truncated HAP4 domain

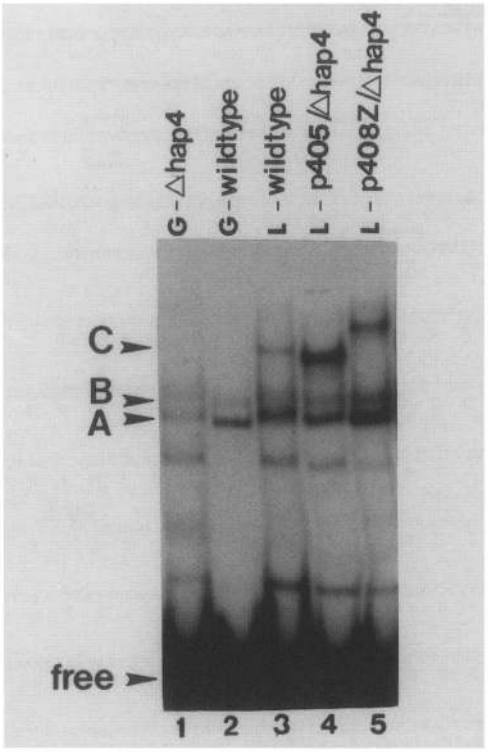

Figure 6. HAP4 is required for binding of HAP2/3 complex. A binding gel was run using labeled UAS2UP1 probe and crude cell extracts. All extracts are from cells transformed with plasmid pJO71, which places HAP2 under control of UAS–*GAL*. (Lane *1*) Wild-type cells, pJO71, glucose media; (lane *2*) *hap4* cells, pJO71, glucose media; (lane *3*) wild-type cells, pJO71, galactose media; (lane *4*) *hap4* cells, pJO71, galactose media. The typical UAS2–protein complexes A, B, and C are indicated.

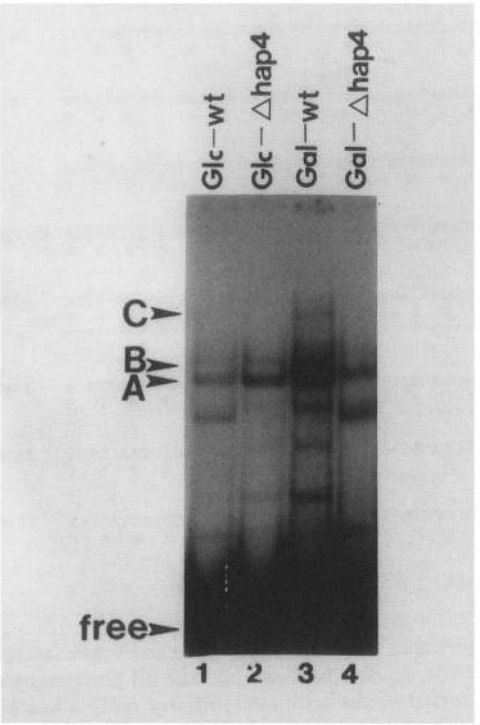

Figure 7.. HAP4 is part of the complex at UAS2. A binding reaction was carried out using UAS2UP1 probe and crude extracts containing size variants of HAP4. (Lane *1*) *hap4* :: *LEU2* cells grown in glucose; (lane *2*) wild-type cells grown in glucose; (lane *3*) wild-type cells grown in lactate; (lane *4*) *hap4* :: *LEU2* cells, containing the truncation pSLF405, grown in lactate; (lane *5*) *hap4* :: *LEU2* cells, containing the fusion pSLF408Z, grown in lactate. The construction of the plasmids is described in Materials and methods. The UAS2 complexes are indicated.

from pSLF410 (Figs. 2 and 8), containing residues 1–327, lacked both acidic regions and failed to complement for growth on lactate. It was fused to the activating domain, residues 752–881 from the carboxyl terminus of GAL4 (Brent and Ptashne 1985). This *HAP4–GAL4* fusion was transformed into cells carrying the *hap4* disruption and scored for growth on lactate. The fusion allowed normal growth on lactate and full activity from UAS2UP1 (Fig. 8). This result is consistent with the model that the acidic domain of HAP4 (residues 424–554) is an activation domain. If this is so, residues 1–327 of HAP4 must encode all the information necessary to anchor HAP4 to HAP2/3.

Discussion

In this paper we present the isolation and characterization of a third positive regulatory factor required for activation of UAS2 of the yeast *CYC1* gene. *HAP4* encodes a protein of 554 amino acids with a highly acidic region near its carboxyl terminus. Using size variants of *HAP4*, we show that complex C, containing UAS2, HAP2, and HAP3, also contains HAP4. Thus, the complex bound at

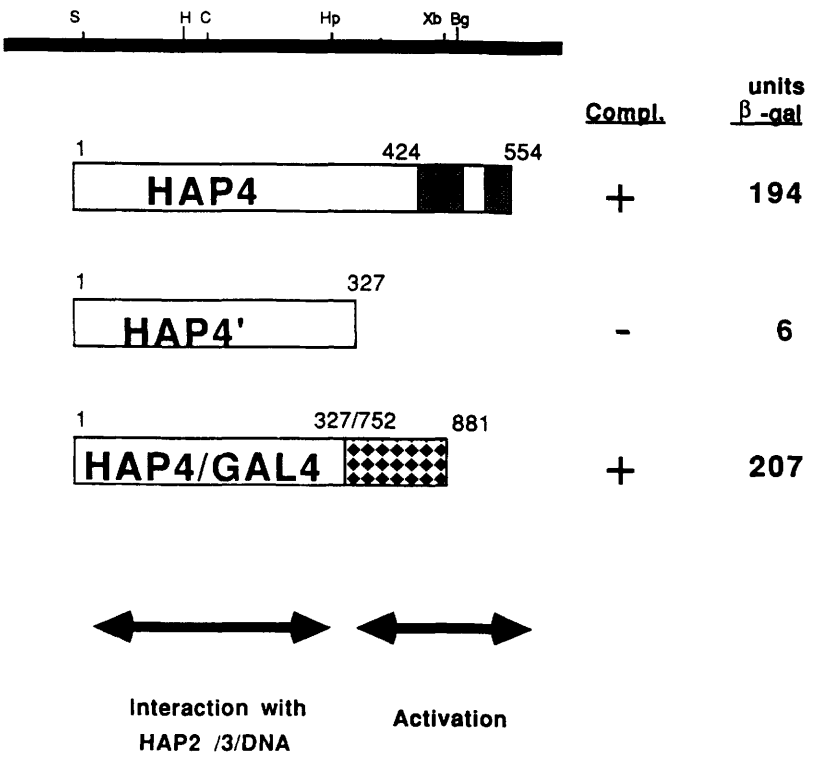

Figure 8. HAP4 provides a possible activation domain. Three constructions are described. The shaded areas indicate the acidic regions (and presumed activating domains). HAP4 wild-type protein provided by pSLF406 complements for growth on lactate. pSLF410, which truncates HAP4 at the *HpaI* site at residue 327, fails to complement. HAP4–GAL4, provided by pSLF414, complements fully. It consists of the truncation at the *HpaI* site of HAP4, fused to a *PvuII–BamHI* fragment containing the carboxyl terminus of GAL4. Complementation for lactate growth was assessed in both the *hap4* :: *LEU2* strain, and *hap4-2*. Activity was measured by cotransforming *hap4-2* with the UAS2UP1 vector pSLF265UPLEU and the *HAP4* vectors pSLF406, pSLF410, or pSLF414. Liquid assays were carried out on duplicate transformants in galactose. The average value of duplicate assays is presented.

the CCAAT box of UAS2 is a heteromeric trimer, at least.

It is very likely that HAP4 is a part of the HAP2/3 complex apart from the DNA as well. The formation of complex C is abolished by mutations in any one of these three *HAP* genes. It was shown previously that a UAS2-binding complex containing HAP2 and HAP3 could be purified intact over four successive chromatographic steps (Hahn and Guarente 1988). We reason that HAP4 must also be a part of that purified HAP complex (1) because UAS2-binding activity was recovered, and (2) because the electrophoretic mobility of the protein–DNA complex did not change during the purification.

Transcriptional activators are endowed with three basic functions: site-specific DNA binding, transcriptional activation, and ability to respond to regulatory signals. In transcriptional activators that contain a single protein, all the functions must be accommodated in one polypeptide. In the case of the yeast activators GCN4 and GAL4, there are distinct and separable domains within the protein for DNA-binding and transcriptional activation (Hope and Struhl 1986; Keegan et al. 1986; Ma and Ptashne 1987). In contrast, a heteromeric complex has the potential to separate functions between subunits. For example, the herpes viral protein VP16 augments transcription of particular genes by complexing with specific DNA-binding proteins and providing an acidic activation domain (Gerster and Roeder 1988; Preston et al. 1988; Triezenberg et al. 1988a,b). Mechanisms by which activators respond to regulatory signals are diverse and include sites in the protein that bind effectors (e.g., glucocorticoid receptor; Rasconi and Yamamoto 1987), sites in the protein that are modified covalently (e.g, ADR1; Cherry et al. 1988), regulation in the synthesis of the activator (e.g., GCN4; Hinnebusch 1984; Thireos et al. 1984), and regulation by direct interaction with another protein (e.g., GAL4–GAL80; Lue et al. 1987). Any component in the regulatory complex at the DNA may be targeted for regulation by these means.

Two properties of *HAP4* are relevant to how these basic functions are distributed among components of the HAP2/3/4 regulatory system. First, levels of *HAP4* RNA are regulated substantially by carbon catabolite repression, whereas levels of *HAP2* or *HAP3* RNA are not (Pinkham and Guarente 1985; Hahn et al. 1988). Because induction in a nonfermentable carbon source is the major regulatory response of genes controlled by HAP2/3/4, thus *HAP4* provides a regulated subunit to the complex formed at UAS2. We do not know yet whether regulation of *HAP4* transcription is the only way in which the carbon source signal is transduced to this complex. Another regulatory system in which the synthesis of the activator is regulated is that of general control of amino acid biosynthesis in yeast; in this case, translation of the mRNA encoding the activator GCN4 is regulated by the availability of amino acids (Hinnebusch 1984; Thireos et al. 1984). In contrast, other systems subject to catabolite repression, such as the *GAL* genes, are activated by a protein, GAL4, that is constitutively synthesized (Matsumoto et al. 1978; Perlman and Hopper 1979). In these cases, post-translational modification may regulate the activity of the activator. We do not yet know whether additional systems regulate the HAP2/3/4 activation complex.

A second property of HAP4 relevant to functional do-

mains of the activation complex is that it contains a very acidic region that can be replaced by the acidic transcriptional activation domain of GAL4. This finding suggests that the activation function of the complex is provided by HAP4. More recently, we found that a *lexA*–HAP4 bifunctional fusion is a potent transcriptional activator at the *lexA* operator, even in the absence of HAP2 and HAP3 (J. Olesen and L. Guarente, unpubl.). Thus, like the herpes virus VP16, HAP4 could be imagined to have two functional domains: a carboxyl acidic activation region and an amino-terminal region anchoring it to the complex. In this sense, HAP4 is a cellular counterpart of the viral protein. Unlike VP16, HAP4 is required for DNA-binding activity to be observed. It is not known whether HAP4 holds HAP2 and HAP3 in a conformation to bind to UAS2 and related sites or whether HAP4 makes contacts with DNA. The HAP4 sequence is devoid of the DNA-binding motifs seen in most other DNA-binding proteins such as helix-turn-helix (Sauer et al. 1982), zinc fingers (Berg 1986; Green and Chambon 1987; Evans and Hollenberg 1988), or leucine zippers (Landschulz et al. 1988).

Why UAS2 requires three proteins for its activation is still an intriguing puzzle. One obvious model suggests that the yeast cell uses HAP2, HAP3, and HAP4 products in different combinations with other, not yet identified, factors to gain complexity in gene control from an otherwise limited repertoire of regulators. However, we have not yet identified any gene regulated by HAP2/3 that is not regulated by HAP4 also.

Implications for CCAAT-binding factors from higher cells

Earlier studies showed that human cells contain a set of distinct CCAAT box-binding factors that bind to a specific subset of CCAAT boxes, differing in their flanking sequences (Chodosh et al. 1988a; Dorn et al. 1988; Santoro et al. 1988). Several of these factors appear to be heteromers, and one, CP1, contains functional homologs of HAP2 and HAP3 (Chodosh et al. 1988b). The two subunits from HeLa cells, CP1A and CP1B, were separated by phosphocellulose column chromatography. Experiments by Chodosh et al. (1988b) demonstrated that HAP2 is a functional homolog of CP1B and HAP3 is a functional homology of CP1A, using in vitro DNA-binding experiments. Chodosh et al. (1988b) concluded that over evolution, both the ability of individual subunits of the complex to interact with each other and the ability of the complex to bind to a specific CCAAT box have been conserved.

How does the discovery of a third component of the DNA-binding complex affect these earlier conclusions? Given the remarkable conservation of interactions described above, it is likely that some counterpart of HAP4 exists in one of the two phosphocellulose column fractions containing CP1A or CP1B. Interestingly, although the size of CP1B estimated from glycerol gradient analysis corresponded with the deduced molecular weight of HAP2, the size of CP1A was much larger than the deduced molecular weight of HAP3. It is possible that the human equivalents of HAP3 and HAP4 are in a complex in the absence of CP1B. In the extreme case, the two functions might reside on a single polypeptide in humans and perhaps in other higher eukaryotes as well.

Although HAP2/3/4 and CP1 share structural features involved in subunit assembly and DNA recognition, they serve different functions in their respective hosts. HAP2/3/4 is a mediator of carbon catabolite control, whereas CP1, which binds to CCAAT boxes from a diverse set of genes, is apparently a constitutive transcription factor. Likewise, GCN4 in yeast is regulated by amino acid availability, while AP-1 is a member of a family of related proteins that responds to serum growth factors with the aid of Fos (Hinnebusch 1984; Thireos et al. 1984; Setoyama et al. 1986; Angel et al. 1988; Halazonetis et al. 1988). One surmises that basic rules governing protein–protein interactions and protein–DNA recognition in heteromeric binding complexes such as HAP2/3/4 were put in place early in the lineage of eukaryotes and remained invariant. In contrast, the evolutionary process has moderated the way key transcription factors interface with signal transduction pathways that govern cell growth and development, so that the factors and their cognate sequences in the different systems now provide regulation in response to very different conditions.

Materials and methods

Strains

Yeast strain BWG1-7A (*MATα ura3-52 leu2-3,112 his4-519 ade1-100*; Guarente and Mason 1983) and its *hap2-1* (Guarente et al. 1984) and *hap3-1* (Pinkham et al. 1986) derivatives were used in this study. The genetic analysis employed PSY142 (*MATα ura3-52 leu2-3,112 lys2-801*; D. Botstein lab), BWG9-A1 (*Matα ade6 his4-519 ura3-52*; Guarente and Mason 1983), JG283-2D (*MATα ura3-52 lys2-801 leu2-3,112 hap2 :: LEU2*; J. Greene), and JP2-10D (*MATα leu2-3,112 ura3-52 his4-519 hap3-1*; J. Pinkham). Strains SLF401 and SLF402 were *hap4 :: LEU2* disruptions of BWG1-7A and PSY142, respectively. *Escherichia coli* strain YMC9 was used in the isolation of the clone, HB101 for constructions, and TG1 for M13 analysis.

Media

Rich media and synthetic (minimal) media were supplemented with 2% glucose or 2% lactate, as described by Sherman et al. (1986).

Genetic analysis

Genetic techniques were carried out as described in Sherman et al. (1986).

Mutagenesis

Strain BWG1-7A was transformed with plasmid pLGΔ-265UP1 and treated with EMS to ~50% survival (Sherman et al. 1986). Cells were plated, at a concentration of ~400 colonies per plate, directly on minimal glucose plates that were supplemented with required nutrients and contained the β-galactosidase chro-

mogenic substrate X-Gal (XG). Candidate strains with a decrease in color when scanned by eye were restreaked on XG plates; those that maintained their phenotype were assayed in liquid culture, as described by Miller (1972).

hap4-1 and *hap4-2* were shown to affect single nuclear loci by crossing each with wild-type strain PSY142 and scoring segregation patterns. Seven tetrads in each cross were scored; in all cases, the petite phenotype segregated 2 : 2. For *hap4-1*, of the two tetrads screened, loss of β-galactosidase activity from UAS2UP1–*lacZ* cosegregated with the petite phenotype. For *hap4-2*, all seven tetrads were screened for UAS2UP1–*lacZ* activity, and in all cases loss of activity cosegregated with petiteness.

Library and cloning

The single-copy genomic library, a gift of Karl Pfeifer, contained a partial *Sau3AI* partial digest of 1-7A DNA in the *Bam*HI site of the vector YCp50. The library was transformed into SLF250 (*hap4-2*) cells. Transformants were selected on minimal glucose plate, scraped down, and replated on rich lactate. Positive (growing) clones were isolated at a frequency of ~1 : 1000.

Yeast DNA (teeny) prep

The putative plasmid clones of *HAP4* were isolated from yeast, as described by Osborne and Guarente (1988).

Plasmids and plasmid constructions

The original *HAP4* insert in the single-copy YCp50 vector was 16 kb. The localization of the gene on the insert was carried out in YCp50. The 3.5-kb *Bgl*II *HAP4* fragment isolated in this analysis was recloned into YCp50 (pSLF400) and also was placed in the high-copy YEp352 vector (Hill et al. 1986) at the *Bam*HI site of the polylinker (pSLF402). All other constructions described in Figure 2, with the exception of the *lacZ* fusion, were carried out in YEp352. In all cases, the *HAP4* derivative in the YEp352 background is essentially a cassette within the polylinker region of this vector.

The fusion to *lacZ* was constructed by placing a 10-bp *Bam*HI linker at the *Xba*I site in pSLF402, to allow an in-frame fusion to the *Bam*HI site at the 5′ end of the *CYC1–lacZ* fusion in pSLFΔ178K (Forsburg and Guarente 1988). The *HAP4* moiety was removed from this intermediate with a *Kpn*I–*Bam*HI digest, where *Kpn*I cuts in the polylinker upstream of the *HAP4* sequences. This fragment was ligated into the *Kpn*- to *Bam*-cut backbone from pSLFΔ178K.

The HAP4–GAL4 fusion (Fig. 8) was constructed by isolating the 1.5-kb *Kpn*I–*Hpa*I fragment containing the amino terminus of *HAP4* from pSLF405, the 1.2-kb *Pvu*II–*Bam*HI fragment containing the amino terminus of *GAL4* from pRB1027 (Brent and Ptashne 1985), and the CIP-treated *Kpn*I–*Bam*HI backbone of YEp352 (Hill et al. 1986) and ligating them together. This three-part construct was verified by restriction analysis.

The high-copy UAS2UP1–*lacZ* fusion vector pLGΔ-256UP1 has been described (Guarente et al. 1984). Its activity is ~100 units in glucose, and 600 units in lactate. Ability of the *HAP4* subclones to activate UAS2UP1 was assessed using a plasmid pSLF265UPLEU, which replaces the *URA3* marker in pLGΔ265UP1 with *LEU2* as follows: The *LEU2* gene on a *Hpa*I–*Sal*I 1.5-kb fragment from plasmid pAA101 (Andreadis et al. 1982) was isolated. A *Sal*I linker was placed at the *Sma*I site of pLGΔ-265UP1, and a *Stu*I–*Sal*I backbone fragment was isolated and ligated to the *LEU2* fragment. The *Stu*I site in the 2μ portion of the plasmid pLGΔ-265UP1 was fused to the *Hpa*I site of *LEU2*; 2μ function was maintained. The activity of this plasmid was ~150 units in glucose and 400 units in lactate.

The test plasmid pLGΔ-229-178 (UAS1 only) is described in Guarente et al. (1984). The UAS_{HIS4} fusion plasmid pHYC3(169) is described in Hinnebusch et al. (1985). The UAS2 deletion plasmids pSLFΔ178-192UPK and pSLFΔ203K are described in Forsburg and Guarente (1988). The *COX4–lacZ* fusion and *CYT1–lacZ* fusions were provided by Carrie Schneider (Schneider 1989). The *HEM1–lacZ* fusion vectors pTK1011 and pTK1012 were provided by Teresa Keng (Keng and Guarente 1987). The UAS_{GAL} fusion pLG-SD5 has been described (Guarente et al. 1982). The plasmid pJO71 (Olesen et al. 1987), which places expression of *HAP2* under the control of UAS_{GAL}, was provided by Jim Olesen, as was the *HAP2–lacZ* fusion, pJP258 (Pinkham et al. 1987).

Molecular analysis

Techniques used in general DNA isolation and manipulation were as described in Maniatis et al. (1982).

Disruption

The *hap4* :: *LEU2* disruption was constructed by replacing the 800-bp *Cla* fragment of *HAP4* with a 1.5-kb *Hpa–Sal* fragment containing *LEU2* isolated from the plasmid pAA101 (Andreadis et al. 1982), following a reaction with Klenow fragment to blunt the overhanging ends. A *Sal–Pst* fragment (the sites flank *HAP4* in the polylinker) containing the disrupted *HAP4* was isolated and used to disrupt the genomic copy of *HAP4* by the method of Rothstein (1983). Both strains BWG1-7A and PSY142 were disrupted for *HAP4*; the phenotype in both cases was identical.

Verification of clone

A construction containing the same insert as pSLF402 was placed in the YIp352 vector (Hill et al. 1986). This plasmid was cut with *Cla* to target integration into the *hap4-2* allele. After transformation with the purified gapped plasmid backbone, the resulting strain retained its petite phenotype. It was crossed against wild type and the cosegregation of the petite phenotype with the *URA3* marker verified.

Sequencing

Sequencing was carried out using dideoxy chain termination (Sanger et al. 1977) and the Sequenase system (U.S. Biochemicals).

RNA preparation

RNA was prepared as described in Osborne and Guarente (1988). For the Northern blot, RNA was isolated from wild-type cells grown in glucose and lactate and from *hap4* :: *LEU2* cells grown in glucose. For the primer extension, RNA was isolated from wild-type cells transformed with the plasmid pSLF402 and grown in lactate.

Probes (Northern)

The *HAP4* probe for the Northern blot was prepared by digesting pSLF401 with *Cla*I and purifying the 800-bp *Cla*I fragment. The probe for actin was an SP65 actin clone obtained

from T. Keng, which was linearized with *Bam*HI and labeled. Labeling was carried out by the random hexamer priming method.

Northern analysis

Total RNA (in a sample buffer of 6% formaldehyde, 50% deionized formamide, 10 μg RNA, 1× MOPS and one-tenth volume gel dye, denatured at 60° for 5 min and transferred immediately to ice) was electrophoresed on a formaldehyde agarose gel [1.5% formaldehyde (4% of a 37% formaldehyde solution), 1% agarose, 1× MOPS] at 100 V for 3 hr. The gel was rinsed 30 min in 10× SSC and transferred to GeneScreen Plus (Dupont) in 10× SSC. The filter was baked under vacuum at 80°C for 2 hr and placed in prehybridization buffer (0.5% SDS, 10× Denhardt's solution, 100 μ/ml calf thymus DNA, 4× SSC, boiled 10 min and cooled 10 min) for 5 hr at 60°C. The prehybridization solution was replaced with a hybridization solution [10× Denhardt's, 4× SSC, 10 mM Tris (pH 7.4), 100 μ/ml of calf thymus DNA, 0.1% SDS, boiled and cooled] and incubated overnight at 60°C. The filter was washed twice in 2× SSC, at room temperature for 5 min, twice in 2× SSC and 1% SDS at 60° for 15 min, and twice in 0.1× SSC at room temperature for 15 min, air-dried, and autoradiographed for 3 days.

Primer extension

Primer extensions were carried out as described in Hahn et al. (1985). The primer, of the sequence 5′-GAGGGCGACTAGCGGAGG-3′, hybridized just downstream of the *HAP4* ATG. RNA (40 μg) was hybridized with the labeled primer for the extension reaction.

Probe (DNA binding)

The probe for gel-binding assays was an 85-bp *Sma*I–*Xho*I fragment from pLGΔ-265UP1, containing all of UAS2UP1. It was end-labeled with [α-^{32}P]dATP by fill-in reaction with Klenow fragment and purified by acrylamide gel electrophoresis and electroelution.

Extract preparation

Yeast cell extracts were prepared as described by Pfeifer et al. (1987). Cells were grown to an OD_{600} of 1.0, harvested by centrifugation, resuspended in extraction buffer [200 mM Tris-HCl (pH 8.0), 400 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 7 mM 2-mercaptoethanol], and disrupted by agitation with glass beads. The extracts were centrifuged for 1 hr at 10,000*g*; the supernatant was collected and precipitated with saturated $(NH_4)_2SO_4$ added to a final concentration of 50%. The protein was resuspended in protein buffer [20 mM HEPES (pH 8.0), 5 mM EDTA, 1 mM PMSF, 20% glycerol, and 7 mM 2-mercaptoethanol].

Gel electrophoresis DNA-binding assays

Protein–DNA complexes were resolved on polyacrylamide gels as has been described (Pfeifer et al. 1987). The 20-ml binding reaction contained 1 mM dithiotreitol, 4% glycerol, 4 mM Tris (pH 8.0), 40 mM KCl, 2 mM $MgCl_2$, 100 μ/ml of BSA, and 10 mg of total protein from crude extracts. This mixture was incubated at room temperature for 10 min and loaded onto a 4% polyacrylamide gel in TBE (90 mM Tris-HCl, 90 mM H_3BO_3, 2.5 mM EDTA). The gels were run at 25 mA until the bromphenol blue ran to the base of the gel and then were dried and autoradiographed.

Computer analysis

Sequence comparison was carried out at the Massachusetts Institute of Technology Whitaker College VAX, using the NBRF and EMBL nucleotide sequence banks and the NBRF protein sequence bank.

Acknowledgments

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