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Mutations in yeast HAP2/HAP3 define a hybrid CCAAT box binding domain

Yanyan Xing, John D.Fikes and Leonard Guarente

Department of Biology, MIT, Cambridge, MA 02139, USA

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We describe a detailed genetic analysis of the DNA-binding regions in the HAP2/HAP3 CCAAT-binding heteromeric complex. The DNA-binding domain of HAP2 is shown to be a 21 residue region containing three critical histidines and three critical arginines. Mutation of an arginine at position 199 to leucine alters the DNA-binding specificity of the complex to favor CCAAC over CCAAT. Residues in HAP3 that are critical for DNA-binding comprise a short, seven amino acid region. Three different mutations in the HAP2 DNA-binding domain are suppressed by a mutation in the HAP3 DNA-binding domain. This HAP3 mutation also suppresses mutations in a different region of HAP2 which promotes subunit assembly of the complex. These findings suggest that short regions of HAP2 and HAP3 comprise a hybrid DNA-binding domain and that this domain can help hold the two subunits together in the CCAAT-binding complex.

Key words: CCAAT box/DNA binding/HAP2–HAP3/transcription complex

Introduction

Many transcription factors in eukaryotes are heteromers, i.e. oligomers containing non-identical subunits (Olesen *et al.*, 1987; Chodosh *et al.*, 1988a; Curran and Franza, 1988; Landshultz *et al.*, 1988; Murre *et al.*, 1989b). Large families of heteromeric transcription factors have been described in higher eukaryotes. Members of the leucine zipper family (Landshultz *et al.*, 1988) can dimerize via coiled-coil structures containing the leucine heptad repeats. Among these proteins are Jun and Fos, involved in promoting cell growth, as well as other members of the AP1 family (Curran and Franza, 1988). Members of the helix–loop–helix family also form heterodimers and include myoD and E12, involved in the differentiation of muscle cells (Murre *et al.*, 1989b).

The yeast CCAATT box binding factor was shown to be a heteromer containing the HAP2 and HAP3 proteins (Olesen *et al.*, 1987; Hahn and Guarente, 1988; Hahn *et al.*, 1988). *HAP2* and *HAP3* were identified because mutation in either gene abolished the activity of the CCAAT box *in vivo* thus blocking expression of nuclear genes encoding mitochondrial proteins and preventing growth on lactate medium (Pinkham and Guarente, 1985; Pinkham *et al.*, 1987; Hahn *et al.*, 1988). These mutations also eliminated a protein–DNA complex observed by gel shift analysis in extracts from wild type cells. By using size variants of HAP2 or HAP3, both proteins were shown to be present in the CCAAT-bound complex (Olesen *et al.*, 1987) and to remain stably associated with each other in the absence of DNA (Hahn and Guarente, 1988). CCAAT sequences are recognized by several classes of transcription factors in mammalian cells (Chodosh *et al.*, 1988a; Benoist and Mathis, 1990). The basis for recognition depends upon sequences that flank CCAAT. HAP2 and HAP3 were shown to be functionally conserved over evolution, since the proteins could be exchanged with fractions containing subunits of the mammalian CCAAT-binding factor, CP1, to restore DNA binding at the CCAAT box (Chodosh *et al.*, 1988b).

A third subunit of the complex exists in *Saccharomyces cerevisiae*, HAP4 (Forsburg and Guarente, 1989), which contains an acidic domain that strongly activates transcription when fused to the DNA-binding domain of lexA (Olesen and Guarente, 1990). In a strain with a deletion in the *HAP4* gene, the CCAAT box is not activated. However, this defect can be overcome by fusing the activation domain of GAL4 to HAP2 (Olesen and Guarente, 1990), showing that HAP4 is not essential for the binding of HAP2/HAP3 to CCAAT. HAP4 counterparts have not been identified from any other organism.

Mutational analysis of HAP2 delineated the functional region as a 60 amino acid core, that lay toward the carboxyl-terminal of the 265 residue protein (Olesen and Guarente, 1990; Olesen *et al.*, 1991). Further dissection of the HAP2 core divided it into two domains. The first is an ~20 amino acid region required for binding to CCAAT (DNA-binding domain). Removal of this region prevents activity at CCAAT, but does not prevent lexA–HAP2 from recruiting HAP3 and HAP4 to a lexA-binding site (Olesen and Guarente, 1990; Olesen *et al.*, 1991). The second domain of HAP2 is a region of ~25 amino acids which when mutated prevents activity at CCAAT and also recruitment of HAP3 and HAP4 at the lexA site. This region of HAP2, the subunit association domain, is therefore essential for protein–protein interaction between HAP2 and other subunits of the complex. Within the HAP2 core, the subunit assembly domain lies at the amino-terminus followed by a spacer region and the DNA-binding domain (Olesen and Guarente, 1990; Olesen *et al.*, 1991).

Clones of HAP2 counterparts have been isolated from *Schizosaccharomyces pombe* (Olesen *et al.*, 1991), mouse (Hooft van Huijsduijnen *et al.*, 1990), rat (Maity *et al.*, 1990; Vuorio *et al.*, 1990) and human (Becker *et al.*, 1991). These clones show a very strong conservation within the 60 amino acid core, and complete divergence elsewhere in the protein (see Li *et al.*, 1992). Within the core, the subunit assembly and DNA-binding domains are highly conserved, and the spacer has diverged. Unlike what has been found in leucine zipper proteins, the spacing between the subunit assembly and DNA-binding domains of HAP2 does not have to be exact, since the spacer in the *S.pombe* protein is one residue longer than in the others (Olesen *et al.*, 1991).

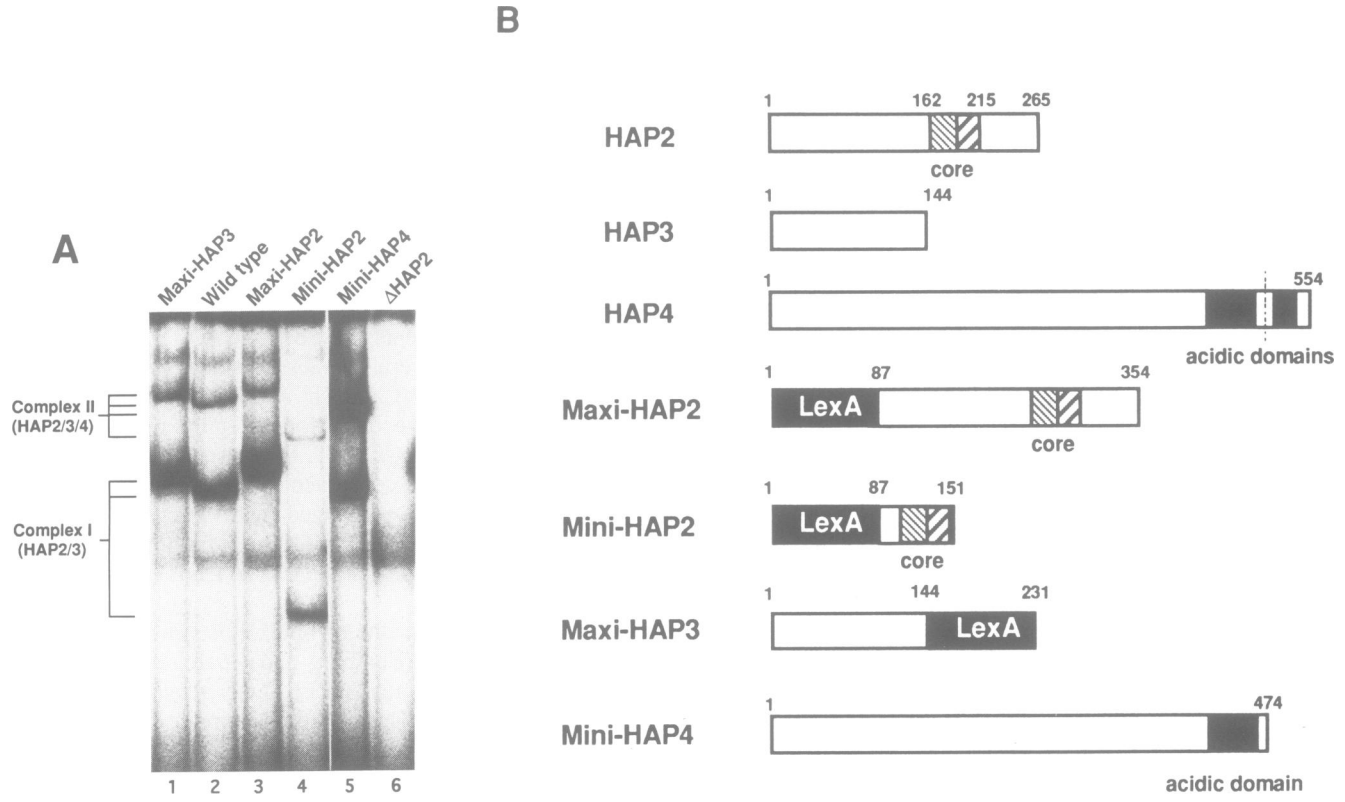

Fig. 1. Two HAP complexes are formed with the CCAAT DNA probe. (**A**) Gel shift assay of HAP complex formation. Each HAP size variant was transformed into the corresponding yeast *hap* null strain. For lanes 1–5, all the extracts were prepared from yeast cells grown in lactate-rich medium. Lane 1, Maxi-HAP3 plus HAP2 and HAP4; lane 2, wild type HAP2, HAP3 and HAP4; lane 3, Maxi-HAP2 plus HAP3 and HAP4; lane 4, Mini-HAP2 plus HAP3 and HAP4; lane 5, Mini-HAP4 plus HAP2 and HAP3; lane 6, extract prepared from JO1-1a (Δhap2) grown in glucose-rich medium. (**B**) Construction of the size variants of HAP proteins used in gel shift analysis. Hatched region in HAP2 protein indicates the subunit association and DNA-binding domains in the essential core (Olesen and Guarente, 1990); filled boxes in the HAP4 protein are the acidic activation domains (Ma and Ptashne, 1987; Forsburg and Guarente, 1989). The length of the HAP proteins (in amino acid residues) is indicated at the carboxyl-termini of the proteins.

The DNA-binding and subunit association domains of HAP2 are not similar to any motifs that have been described in other transcription factors (Landschulz *et al.*, 1988; Murre *et al.*, 1989a; for review, see Harrison, 1991). In this report, we begin a detailed study of the structural determinants of the DNA-binding domain of the HAP complex. Our findings suggest that HAP2 and HAP3 interact via DNA-binding domains of each subunit to create a hybrid CCAAT box binding structure.

Results

Biochemical assay for HAP2/HAP3 DNA-binding

Previous experiments showed that the HAP2/HAP3/HAP4 CCAAT box complex could be visualized by gel shift analysis of extracts of yeast cells grown on the carbon source lactate (Olesen *et al.*, 1987; Forsburg and Guarente, 1989). We modified that assay by using a short CCAAT box oligonucleotide instead of a yeast chromosomal fragment and by running the gel in the cold (see Materials and methods). This assay displayed two predominant complexes, I and II, shown in Figure 1A. In order to ascertain the identity of these two complexes, we constructed size variants of HAP2, HAP3 and HAP4, as depicted in Figure 1B. Maxi-HAP2 (larger than HAP2) contains a fusion of residues 1–87 of lexA to the amino-terminus of HAP2 (Pinkham *et al.*, 1987), and mini-HAP2 (shorter than HAP2) contains a fusion of this lexA moiety to the HAP2 core (Olesen and Guarente, 1990). The mobilities of both complexes I and II were altered by the HAP2 size variants (Figure 1A, compare lanes 2, 3 and 4), indicating that both contain HAP2. As expected,

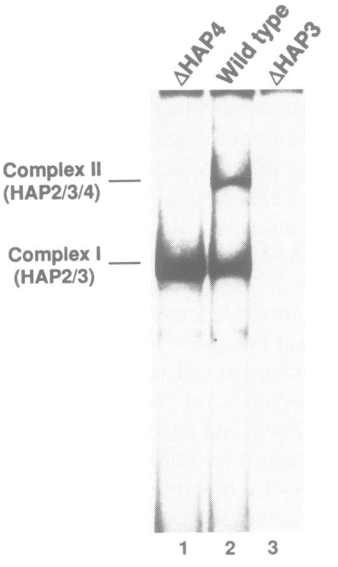

Fig. 2. Gel shift assay of HAP complex formation with and without HAP4 protein. First lane, extract prepared from a *hap4* null strain SLF401 (*hap4*::*LEU2*) grown in glucose-rich medium; lane 2, wild type cells, lactate medium; lane 3, *hap3* null strain SHY40 (*hap3*::*HIS4*), glucose-rich medium.

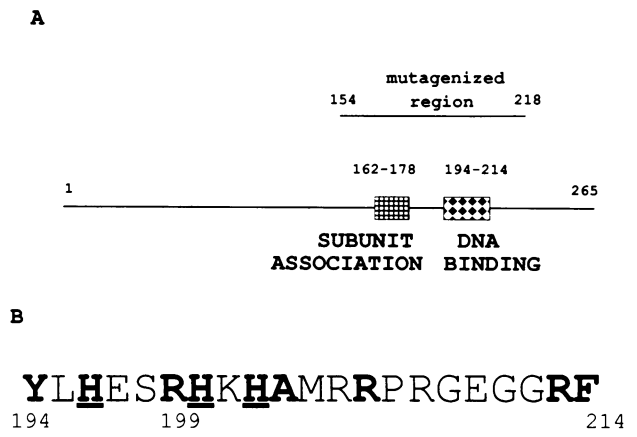

Fig. 3. Diagram of HAP2. (**A**) Domains in HAP2. The region of HAP2 subjected to oligonucleotide-directed mutagenesis is shown as well as the regions of mutations recovered in the subunit association and DNA-binding domains. (**B**) Sequence of the HAP2 DNA-binding domain. Residues which are important in DNA binding as determined by the mutational analysis are shown in bold. Arginine 199 is indicated and important histidine residues are underlined.

deletion of HAP2 abolished both complexes (see Figure 1A, lane 6). Likewise, maxi-HAP3, containing a fusion of lexA to the carboxyl-terminus of HAP3, altered the mobility of both complexes, indicating that HAP3 is also present in both complexes I and II (Figure 1A, compare lanes 1 and 2). Mini-HAP4, however, deleting a portion of the acidic activation domain of HAP4, affected the mobility of complex II but not that of complex I. This finding indicated that HAP4 was present in complex II but not in complex I (see Figure 1A, lane 5). These data show that complex I contains HAP2 and HAP3 but not HAP4, and that complex II contains all three HAP subunits.

To investigate whether complex I, containing HAP2 and HAP3, required HAP4 for its formation, we prepared extracts from a strain bearing a deletion of HAP4. As shown in Figure 2, this extract gave rise to complex I but not complex II (compare Figure 2, lanes 1 and 2). The control extract from a HAP3 deletion strain did not give rise to either complex (Figure 2, lane 3). Thus, as predicted from earlier genetic studies, HAP2 and HAP3 can associate with each other and bind to the CCAAT box in the absence of HAP4 (Hahn and Guarente, 1988; Olesen and Guarente, 1990).

Mutagenesis of the HAP2 DNA-binding domain

The entire region previously defined as the DNA-binding domain of HAP2 (Olesen and Guarente, 1990) was mutagenized by a random oligonucleotide approach, as described in Materials and methods. A chimera containing lexA1−87 fused to the HAP2 core was employed as substrate for mutagenesis. This mutagenesis strategy should produce a complete set of random mutations throughout the DNA-binding region. Mutagenized pools each spanned 11 adjacent amino acids and together covered HAP2 sequences from residue 154 to residue 218 (Figure 3). Each pool was transformed into a Δ*hap2* yeast strain with a *lacZ* reporter under the control of UAS2UP1 (Guarente *et al.*, 1984), which bears the sequence CCAAT (see Materials and methods). White or pale blue colonies were picked and the HAP2 plasmids recovered in *Escherichia coli*. Sequences were determined of those clones that yielded the mutant phenotype when retransformed into yeast. Also, β-galactosidase (β-gal)

Table I. Site-directed mutagenesis of the protein−DNA association domain of HAP2

lexA−HAP2 mutants	Growth on lactate plate	β-galactosidase activity (units)	
		UAS2UP1 (CCAAT)	lexA operator
Y194C	−	5.1	27.5
Y194D	±	6.4	35.7
H196R	−	4.7	31.7
H196D	±	4.8	42.5
H196N	+	54.8	37.8
R199G	−	3.9	54.0
H200Y	−	5.7	36.4
H200Q	±	4.8	28.9
H202Q,R208C	−	2.6	45.0
H202R	±	4.6	37.0
H202N	+	45.5	24.5
A203V	−	4.4	33.6
R206I	−	2.9	44.0
P207A	++	89.9	27.0
G209V	+	34.7	17.9
G212V	−	44.0	34.5
R213M	±	4.8	25.2
F214L	−	2.5	42.6
L215 Stop	++	106	23.3
lexA−HAP2	++	110	30

HAP2 mutations are listed in the left column. The control wild type HAP2 is at the bottom of the column. *In vivo* activity was determined by measuring β-gal levels from reporters pLGΔ265UP1 (CCAAT) and pRB115 (lexA operator) as described in Materials and methods. All cells carrying mutant and wild type HAP2 were tested in lactate-rich medium. β-gal levels were obtained from cells grown in synthetic medium supplemented with 2% glucose, as described in Materials and methods. Growth in lactate medium: ++, full growth, wild type; +, intermediate growth; ± poor growth; −, no growth.

assays were carried out to quantify the effects of the mutations on expression of the reporter (Table I).

Mutations giving rise to stop codons were found throughout the mutagenized region. Mutations were recovered between residues 162 and 178 which inactivated the subunit association domain of HAP2 and will be described in detail elsewhere. Mutations between residues 178 and 194 were not recovered (except for chain-terminating mutations), presumably because this region of HAP2 serves as a spacer and does not contain essential residues. Most importantly, mutations in the DNA-binding region of HAP2 spanned Y194−F214 (Figure 3A).

We confirmed that these mutant proteins were stable *in vivo* by showing that they gave rise to a normal level of assembly of the HAP2/HAP3/HAP4 complex at the lexA site (Brent and Ptashne, 1985) (Table I). A stop codon at position 215 did not affect activity, defining the carboxyl-terminus of the functional DNA-binding domain of F214. Several features emerge from inspection of residues altered in the mutants (Figure 3B). First, three His residues, H196, H200 and H202, are of key importance in DNA binding. The involvement of such a His cluster in DNA binding of the HAP complex raises the possibility that a metal cofactor may be a structural component of the functional HAP complex (Hooft van Huijsduijnen *et al.*, 1987, 1990). Second, three Arg residues, R199, R206 and R213, are also critical in DNA binding. Third, the domain is bounded by aromatic amino acids, Y194 and F214, which are also key residues in DNA binding. The only other strong mutation recovered was A203 →V.

Table II. CCAAT box specificity of the HAP2 protein–DNA association domain

	Growth on lactate plate	β-galactosidase activity (Units)	
		UAS2UP1 (CCAAT)	UAS2wt (CCAAC)
Wild type	++	110	3.8
R199L	+	9.4	30

Wild type HAP2 and the R199L mutant were transformed into a *hap2* null strain (JO1-1a) respectively, with reporter plasmids pLGΔ265UP1 (CCAAT) or pLGΔ265wt (CCAAC). The β-gal levels were determined in cells grown in synthetic medium supplemented with 2% glucose, as described in Materials and methods. Growth in lactate medium: ++, full growth, wild type; +, intermediate growth.

Does this HAP2 domain actually contact the CCAAT sequence? One way to demonstrate this point would be to isolate altered specificity mutants in this domain which change the DNA sequence specificity of the HAP complex. One natural site in yeast for the HAP complex is the UAS2 of CYC1 (Guarente *et al.*, 1984), which contains CCAAC instead of the canonical sequence. The alteration of UAS2 to CCAAT by the UP1 mutation increases transactivation by the HAP complex 10-fold (Forsburg and Guarente, 1988). An indicator strain bearing UAS2 instead of UAS2UP1 forms pale blue colonies on X-Gal glucose plates in response to the HAP complex. We introduced the HAP2 mutagenized pools into this strain and isolated clones that gave rise to dark blue colonies. HAP2 plasmids were recovered in *E.coli*, as above, and shown to retransform yeast to give the novel phenotype. In β-gal assays, the activity of the CCAAC-driven reporter was increased 6-fold in the mutant (Table II). Strikingly, when this mutant was introduced into a strain bearing a CCAAT-driven reporter, activity was 12-fold lower than in a strain with wild type HAP2. Only one mutation was repeatedly isolated with these properties, R199L. This mutation alters the specificity of HAP2 to increase activity at CCAAC and to decrease activity at CCAAT. As shown above, the R119G mutation abolished activity at CCAAT.

In order to show conclusively that the R199L change really altered the specificity of the HAP complex, we made extracts from the mutant strain, and performed gel shift assays using probes that carried CCAAC or CCAAT binding sites (Figure 4). The wild type HAP2 extract showed strong binding to CCAAT and no detectable binding to CCAAC (Figure 4, compare lanes 1 and 2). In contrast, the extract containing the HAP2 R199L mutant did not bind to CCAAT, but gave clear binding to CCAAC (Figure 4, compare lanes 3 and 4). These findings indicate that R199 of HAP2 is involved in discriminating the fifth base position of the CCAAT box, and demonstrates that the DNA-binding domain of HAP2 directly contacts at least part of the DNA site.

HAP3 also contains a DNA-binding domain

Because HAP3 is required for the DNA-binding activity of the complex, we surmised that, like HAP2, it contained a DNA-binding domain. To begin to delineate functional domains in HAP3, we constucted amino-terminal and carboxyl-terminal deletions in the gene. We also cloned the *S.pombe* homolog by screening a cDNA library (Fikes *et al.*, 1990) for complementation of a *hap3* mutant strain for growth on lactate (see Materials and methods). A comparsion of the sequences from *S.cerevisiae* and *S.pombe* (Figure 5A)

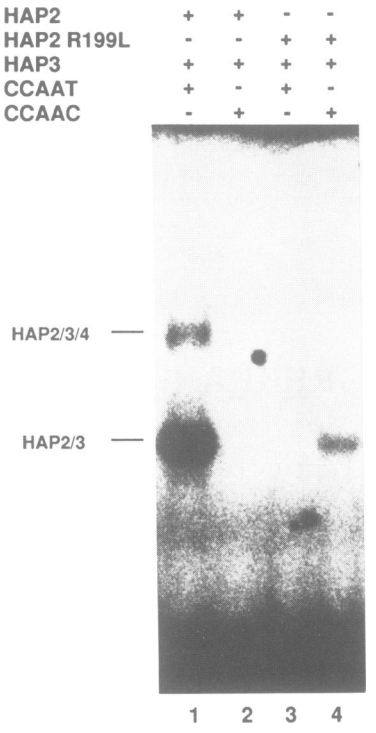

Fig. 4. Gel shift of the altered specificity mutant of HAP2. HAP2 proteins are lexA–HAP2 core fusions expressed in *hap2* null cells (JO1-1a). Extracts were prepared from growing cells in lactate-rich medium. Lanes 1 and 3, CCAAT probe added; lanes 2 and 4, CCAAC probe added; lanes 1 and 2, lexA–HAP2 core; lanes 3 and 4, lexA–HAP2 core R199L.

suggests that the amino-terminal 41 residues (missing in the *S.pombe* gene) and the carboxyl-terminal 15 residues (divergent in the *S.pombe* clone) are not essential.

This suggestion was confirmed because truncation of the amino-terminal 35 residues or the carboxyl-terminal 18 residues of the *S.cerevisiae* HAP3 did not affect function *in vivo* (Figure 6). Further, truncated forms of HAP3 gave the HAP2/HAP3 DNA gel shift complex I at the same levels as the wild type (Figure 7).

Mutagenesis was directed across the essential region of HAP3 (amino acids 35–128) as described in Materials and methods and mutants isolated after transforming a Δ*hap3* indicator strain (containing wild type HAP2 and HAP4) with the CCAAT-driven reporter (Table III). Mutants that did not activate CCAAT were isolated and tested further. The first class of mutations were not defective in activation at a lexA site in a strain expressing lexA–HAP2, which is a measure of the ability of recruit HAP2 and HAP3. These mutants actually increased activity at the lexA site relative to wild type HAP3. These mutants are therefore defective in binding to CCAAT, but still function in complex assembly. The increase in the activity at the lexA reporter is probably because CCAAT sites in yeast DNA no longer compete for the lexA–HAP complex, thereby increasing binding to the lexA site (Table I; also see Olesen and Guarente, 1990). These DNA-binding mutations are all in one region of HAP3, residues 42–48 (possibly extending to residue 52). Note that this region is highly conserved between HAP3 and its homologs from rat (Maity and de Crombrugghe, 1992), mouse (Hooft van Huijsduijnen *et al.*, 1990) and *S.pombe* (Figure 5B; also see Li *et al.*, 1992). We infer from these mutants that both HAP2 and HAP3

A

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Sp   1   MSADGLDYTNLLPIANVARIMKSALPENAKISKEAKDCVQDCVS 44
Sc  25 SSGSLQQISTLREQDRWLPINNVARLMKNTLPPSAKVSKDAKECMQECVS 74
Sp  45 EFISFVTGEASEQCTQEKRKTITGEDVLLALNTLGFENYAEVLKISLTKY 94
Sc  75 ELISFVTSEASDRCAADKRKTINGEDILISLHALGFENYAEVLKIYLAKY 124
Sp  95 REQQARSASMKETKQSRSEEPQ 116
Sc 125 RQQQALKNQLM.YEQDDEEVP 144
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                45                    68                    88
Sc      REQDRWLPINNVARLMKNTLPPSAKVSKDAKECMQECVSELISFVTSEASDRCAADKRKTINGEDILISLHALGFENYAEVLKIYLAKYRQQQA
Sp           LPIANVARIMKSALPENAKISKEAKDCVQDCVSEFISFVTGEASEQCTQEKRKTITGEDVLLALNTLGFENYAEVLKISLTKYREQQA
Mouse   REQDIYLPIANVARIMKNAIPQTGKIAKDAKECVQECVSEFISFITSEASERCHQEKRKTINGEDILFAMSTLGFDSYVEPLKLYLQKFRE
Rat     REQDIYLPIANVARIMKNAIPQTGKIAKDAKECVQECVSEFISFITSEASERCHQEKRKTINGEDILFAMSTLGFDSYVEPLKLYLQKFRE
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Fig. 5. (**A**) Amino acid sequence alignment of *S.cerevisiae* HAP3 (*Sc*) residues 25–144 (bottom) and *S.pombe* homolog (*Sp*) residues from 1 to 116 (top). Vertical bars indicate identical residues; (:) functionally conserved residues and (.) less conserved residues. (**B**) Amino acid sequence alignment of *S.cerevisiae* HAP3 residues with those of *S.pombe*, mouse and rat homologs. The residues in bold type are conserved among species.

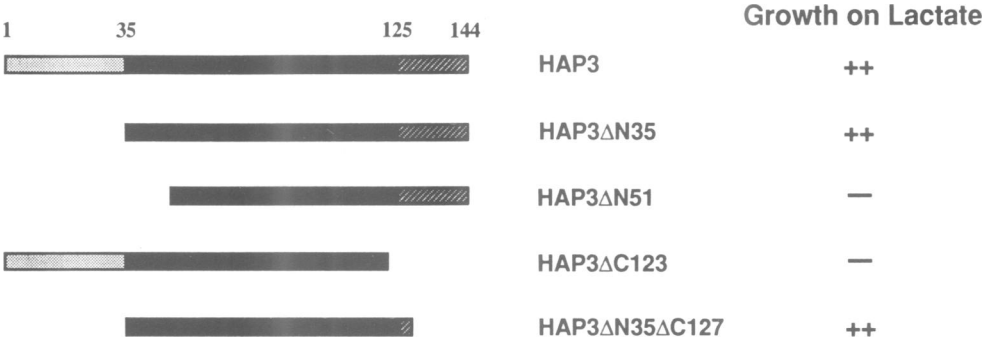

Fig. 6. Diagram of HAP3 amino- and carboxyl-terminal truncations. All the truncations were tested for their ability to support growth in lactate medium in a Δ*hap3* strain.

make contacts with DNA at the CCAAT box. Alternatively, it is possible that HAP3 does not directly contact DNA, but holds HAP2 in a proper conformation to bind to the site.

A second class of mutants was defective in the lexA assay, but was rescued by fusion of the acidic activation domain of GAL4 to HAP2. The HAP2–GAL4 assay is a measure of the ability of HAP3 derivatives to associate with HAP2 and direct binding of the complex to the CCAAT box (Olesen and Guarente, 1990). The failure of these mutants to function in a strain with wild type HAP2 and HAP4 must therefore be due to a failure to recruit HAP4 to the complex. These mutations lie in the carboxyl-terminal region of HAP3, between residues 81 (or possibly 82) and 113 (see Table III). The third class of mutants did not function either in the lexA assay for HAP2/HAP4 recruitment or in the HAP2–GAL4 assay for HAP2 association. These mutants, which fall between residues 69 and 80, could have a specific defect in interaction with HAP2 (Table III). However, because these mutants were nonfunctional in all of our assays, we cannot rule out the possibility that they are defective by virtue of instability, or some other global defect.

It has been proposed that a hybrid metal-binding domain could exist comprising residues of HAP2 and HAP3 (Hooft van Huijsduijnen *et al.*, 1990). In this regard, two cysteines of HAP3 at positions 68 and 72 would interact with the essential histidines of HAP2 to form a hybrid metal-binding domain. Mutations at these positions were not recovered from the mutagenized pools of HAP3 DNA. To investigate the possible importance of these residues further, we mutated C68 and C72 to serine by site-directed mutagenesis

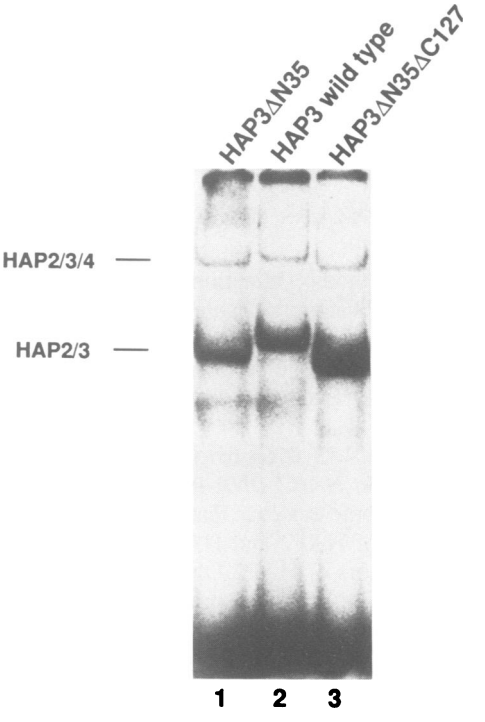

Fig. 7. Gel shift assay of HAP3 core in the HAP2/HAP3 DNA complex. Lane 1, amino-terminal truncation of HAP3; lane 2, wild type HAP3 and lane 3, amino- and carboxyl-terminal doubly truncated HAP3. All three extracts were prepared from cells grown in lactate-rich medium.

Table III. Dissection of the functional domains of the HAP3 essential core by site-directed mutagenesis

	HAP3 mutants	In *Δhap3* strain[a]	In *Δhap2Δhap3* strain[b] +lexA−HAP2		In *Δhap2Δhap3* strain[b] +HAP2−GAL4	
		Growth on lactate plate	lexA-operator β-gal (units)	Growth on lactate plate	UAS2UP1 (CCAAT) β-gal (units)	Growth on lactate plate
HAP3−DNA	E37R, L42R	−	225	−	4.2	−
	L42R	−	226	−	10.8	−
	P43R	−	256	−	6.8	−
	N46D	−	194.6	−	7.9	−
	N46Y	−	222.5	−	4.2	−
	A48P, K52R	−	232	−	5.6	−
	C68S	++	37.2	++	118	++
	M69R	−	2.8	−	7.1	−
	Q70E, E75V	−	2.6	−	7.9	−
	C72S	++	39.0	++	127	++
	C68S, C72S	++	34.6	++	115	++
	C72S, E75R	−	2.3	−	11.3	−
	L76P	−	4.3	−	8.4	−
	I77W, E83V	−	1.6	−	8.1	−
	F79L, V80E	−	1.8	−	7.2	−
HAP3−HAP4	T811, C88W	−	1.6	−	16.2	±
	S82R	−	1.2	−	58.0	+
	A84P	−	2.5	−	15.8	±
	S82R, S85R	−	2.3	−	49.3	+
	S82R, C88R	−	1.1	−	18.8	±
	C88H	++	35.3	++	132	++
	C88R	++	28.8	++	124	++
	T95A, I96R	−	1.5	−	18.6	±
	L108 Stop	−	2.5	−	38.7	+
	E111G, Y113G	−	2.4	−	40.2	+
	Y113S, V116G	−	2.2	−	39.5	+
	F110L, V116E	−	1.4	−	36.6	+
	K123 Stop	−	1.7	−	43.3	+
	HAP3 wild type	++	42.0	++	160	++

HAP3 mutations are listed in the left column. The HAP3 mutants were tested with the lexA−HAP2 plasmid (with the *HIS4* marker) in *hap2 hap3* null cells for their ability to recruit HAP2 and HAP4. The HAP3 mutations were further tested with the HAP2−GAL4 fusion (with the *HIS4* marker) in *hap2 hap3* null cells for their ability to associate with HAP2. Quantitative levels of activity were determined by measuring β-gal levels from reporters pLGΔ265UP1 (CCAAT) and pRB1155 (lexA operator) as described in Materials and methods. Growth in lactate media: ++, full growth, wild type; +, intermediate growth; ±, poor growth; −, no growth.

[a]*Δhap3* strain SHY40.

[b]*Δhap2Δhap3* strain YX-2A (see Materials and methods).

(Materials and methods). Mutation of C68 or C72 or both did not affect HAP3 activity (Table III). This finding would appear to rule out the possibility of a critical zinc finger or other metal-binding domain between these cysteines and the important histidine residues in HAP2.

Mutation in the HAP3 DNA-binding domain suppresses certain HAP2 DNA-binding mutants

In order to obtain more evidence that the two proposed DNA-binding domains of HAP2 and HAP3 interact, we sought to isolate suppressors of HAP2 DNA-binding mutants that would lie in HAP3. For this purpose, the *lexA−HAP2* mutant genes encoding Y194C, H196R, H200Y, R206I, A203V, G212V, R213S and F214C were transferred to a URA3 plasmid and transformed into a *Δhap2 Δhap3* strain (see Materials and methods). The resulting strains were transformed with the HAP3 mutagenized pools (on a LEU2 plasmid). Transformants bearing the wild type HAP3 could not grow on lactate plates because of the defects in the HAP2 DNA-binding partners. Suppressors in HAP3 that could compensate for the HAP2 defect would restore binding to CCAAT, and growth on lactate plates. Colonies that grew on lactate plates were thus isolated. HAP2 mutants and the HAP3 plasmids were recovered in *E.coli*. HAP3 candidates that would retransform the mutant HAP2 strain to allow growth on lactate plates were sequenced. Suppressors for three HAP2 mutants, Y194C, H200Y and R206I, were found in the same HAP3 mutagenized pool (see Table IV), and all bore the N45H mutation. In some cases, the N45H change was in combination with other HAP3 mutations (data not shown). To test whether the N45H change was responsible for suppression in each case, we transformed HAP3 N45H into each of the three HAP2 mutants and found that all three grew on lactate plates (Table IV). We conclude that the N45H is a suppressor of all three DNA-binding mutants of HAP2.

We next tested whether the N45H suppressor displayed allele specificity with other mutations in the DNA-binding domain of HAP2. Initially we tested suppression of R199G, a mutation in the HAP2 DNA-binding domain believed to be specifically defective in contacting CCAAT. The HAP3 suppressor did not suppress this mutation (Table IV).

Table IV. Suppression of HAP2 mutations by HAP3N45H

Region mutated	HAP2 mutations	Growth on lactate medium	
		JO2-1 (*Δhap2Δhap3*) with HAP3N45H	JO2-1 (*Δhap2Δhap3*) with wild type HAP3
Protein–DNA domain	Y194C	+	–
	R206I	+	–
	H200Y	+	–
	H196R	–	–
	R199G	–	–
	A203V	–	–
	G212V	–	–
	R213S	±	–
	F214C	–	–
Subunit association domain	N164I	+	–
	Q167L	+	–
	R175G	+	–
	HAP2 (wt)	++	++

Wild type HAP3 and suppressor HAP3N45H were transformed into JO2-1 (*Δhap2Δhap3*) expressing wild type lexA–HAP2 mutants bearing lesions in the protein–DNA and subunit association domains of HAP2. The ability of these transformants to grow in lactate medium was tested. Growth in lactate medium: ++, full growth, wild type; +, intermediate growth; ±, poor growth; –, no growth.

Further, the suppressor failed when tested with the other HAP2 DNA-binding mutants, H196R, A203V, G212V, R213S and F214C. This allele-specific suppression of HAP2 DNA-binding mutants by the N45H mutation in the DNA-binding domain of HAP3 is consistent with the hypothesis that DNA-binding domains of HAP2 and HAP3 interact to form an active structure comprising residues of both subunits. Those residues of HAP2 that are not suppressed by HAP3 N45H could directly contact the DNA, such as R199G, or alter the structure of HAP2 so that it cannot be suppressed by the HAP3 mutation. Alternatively, the allele-specificity of suppression may not bespeak protein–protein interactions between HAP2 and HAP3 in these regions, but may derive from an increase in DNA-binding affinity conferred by the N45H mutation. Those mutations in HAP2 that are not suppressed may exert a reduction in the DNA-binding affinity of the complex that is so drastic that binding is not rescued by the suppressor.

The HAP3 mutation N45H also suppresses mutations in the HAP2 subunit association domain

In a final test of the specificity of the N45H suppressor in HAP3 for mutations in HAP2, we tested several mutations in the HAP2 subunit association domain. The isolation and characterization of mutations in the HAP2 subunit assembly domain will be described elsewhere. The N164I, Q167L and R175G mutations all lie in the subunit assembly domain (Olesen and Guarente, 1990) and inactivate lexA–HAP2 at UAS2UP1 or at the lexA site (Table IV and data not shown). Surprisingly, these mutations were all suppressed by the HAP3 N45H mutation (Table IV). This finding is a strong indication that the N45H suppressor functions by increasing the avidity of association between HAP2 and HAP3. We present a model for this suppression in the Discussion.

Discussion

In this report, we have investigated DNA binding in the HAP2/HAP3/HAP4 heteromeric complex, which binds to the CCAAT box (Olesen *et al.*, 1987; Hahn and Guarente, 1988; Forsburg and Guarente, 1989). Our findings indicate that HAP2 and HAP3 bind to CCAAT in the absence of HAP4 and can be visualized as a gel shift complex. A more slowly migrating complex containing HAP4 is also evident by gel shift assay (Figure 1A). The region of HAP2 that is required to contact the CCAAT box consists of 21 residues and is bounded by the aromatic residues Tyr and Phe at either end. Within the domain are three critical His residues and three critical Arg residues (see Table I). The three key His residues suggest that a metal cofactor may be involved in the binding of the HAP2/HAP3 complex to CCAAT (Hooft van Huijsduijnen *et al.*, 1987, 1990), although we have not yet obtained clear evidence to that effect. All of the residues found critical for DNA binding by this mutagenesis study are also absolutely conserved in HAP2 counterparts from *S.pombe* (Olesen *et al.*, 1991), mouse (Hooft van Huijsduijnen *et al.*, 1990), rat (Maity *et al.*, 1990) and human (Becker *et al.*, 1991). We demonstrate that this domain directly contacts CCAAT by isolating an altered specificity mutant in one of the critical arginines, R199L (Table II). This mutation increases binding to CCAAC and virtually abolishes binding to CCAAT (Figure 4).

A short region of HAP3 is also required for binding to the CCAAT box. This region is also rigidly conserved over evolution (Li *et al.*, 1992) with the exception of N45 (see Figure 5B). The short length of this region, seven residues, makes it difficult to discern any important motifs. Of interest, however, is a suppressor mutation in this HAP3 region which restores activity to several HAP2 DNA-binding mutants. This suppressor mutation changes the non-conserved asparagine at position 45 to histidine. This suppressor does not suppress all of the HAP2 DNA-binding mutants. Notably, it fails to suppress mutation in the DNA-contacting residue, R199G (Table IV). This allele specificity could suggest that the DNA-binding domains of HAP2 and HAP3 interact directly to form a hybrid structure. Alternatively, the suppressor may increase the affinity of the HAP2/HAP3 complex for the CCAAT box and give rise to suppression of all but the strongest mutations in HAP2.

A surprising finding discriminates between these two models for suppression by the HAP2 mutation, namely, that

the mutation also suppresses lesions in the subunit association domain of HAP2 (Tabel IV). The logical inference from this is that HAP3 N45H increases the affinity between the DNA-binding regions of HAP2 and HAP3. In so doing, the mutation can suppress lesions in the HAP2 DNA-binding domain that weaken association and also mutations in the HAP2 subunit association domain.

A model for the association between HAP2 and HAP3 is shown in Figure 8. In this model we propose two regions of association between the wild type HAP2 and HAP3: strong association between the subunit association domains and weak association between the DNA-binding domains. We imagine that the interaction between the subunit association domains must be strong because a truncated HAP2 derivative missing the DNA-binding domain is still able to recruit HAP3 and HAP4 to a lexA site (see Table I, and Olesen and Guarente, 1990). Once HAP2 and HAP3 are brought together by the subunit association domains, the interaction between the DNA-binding domains would create the CCAAT-binding structure. The subunit association domain of HAP3 has not been clearly delineated, but may lie in the region between residues 69 and 80, because mutations in that region are globally defective in function, perhaps due to a defect in binding to HAP2.

The figure also presents a model for suppression of the subunit association domain mutations in HAP2 by N45H in the DNA-binding domain of HAP3. We imagine that the subunit association domain mutations all weaken the interaction between HAP2 and HAP3. By increasing the affinity in a second region of interaction, namely the DNA-binding domains of the proteins, the N45H mutation can compensate for a weakened interaction between the subunit association domains of HAP2 and HAP3. Implicit in this model are two regions of contact between HAP2 and HAP3. Thus, our model for subunit interactions in the complex differs from what has been observed in leucine zipper (Landschulz *et al.*, 1988) or helix−loop−helix proteins (Murre *et al.*, 1989a,b). In these latter cases, the DNA-binding domains in the dimer interact separately with two half sites in the DNA and not with each other. In the HAP2/HAP3 complex, we imagine that the CCAAT-binding domain involves a hybrid stucture between the subunits.

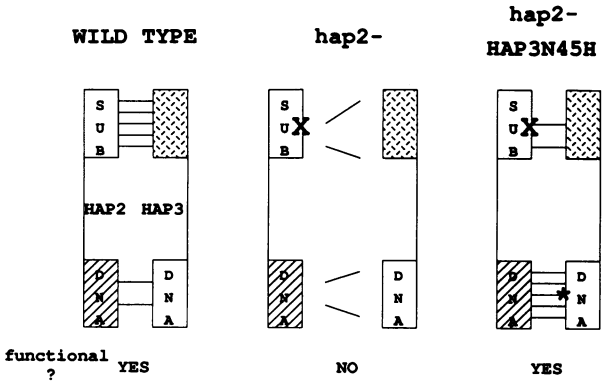

Fig. 8. Model of interactions in HAP2−HAP3 complex. In the case of the wild-type proteins, a strong interaction between the subunit association domains (SuB) of HAP2 and HAP3 (indicated by five lines) holds the complex together while a weak interaction between the DNA-binding domains (DNA) of the subunits (indicated by two lines) forms the hybrid DNA-binding structure. Mutations in the HAP2 subunit association domain (indicated by the X) prevent association of HAP2 and HAP3. Association can be re-established by the HAP3N45H mutation (indicated by the asterisk) which creates a strong interaction between the DNA-binding domains of the subunits (indicated by five lines).

Materials and methods

Strains and media

The *S.cerevisiae* strain BWG 1-7a (*MATaleu2*-3,112*his4*-519 *ade1*-100 *ura3*-52; Guarente and Mason, 1983) or isogenic derivatives were used in all experiments described. HAP2 complementation was assayed using strain JO1-1a (Δ*hap2*; Olesen and Guarente, 1990). HAP3 complementation was assayed using strain SHY40 (*hap3*::*HIS4*; Hahn *et al.*, 1988). The strain JO2-1 is an isogenic Δ*hap2*, *hap3*::*HIS4* double null strain (Olesen and Guarente, 1990). The strain YX-2A was derived from JO2-1 by deleting *HIS4* and inserting *hisG* (Alani *et al.*, 1987). The *hap4* null strain SLF401 (*hap4*::*LEU2*, Forsburg and Guarente, 1989) was used for preparation of extracts.

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

Plasmids

Vectors pRB1155 (Brent and Ptashne, 1985), pJP300 (Pinkham *et al.*, 1987), pJO323 (lexA-HAP2-core, Olesen and Guarente, 1990), pSLF405 (Forsburg and Guarente, 1989), HAP2−GAL4 (Olesen and Guarente, 1990), pLGΔ265wt (CCAAC, Guarente *et al.*, 1984) and pLGΔ265UP1 (CCAAT, Guarente *et al.*, 1984) have been described.

A *LEU2*$^+$, 2 μm plasmid for HAP2 and HAP3 expression and mutagenesis was constructed. Plasmid YEP351 (Hill *et al.*, 1986) was digested with *Pvu*II and an *Sph*I linker was ligated into the *Pvu*II site to form YEP451. An M13 phage replication origin was obtained from pBluescript KS+ digested with *Ssp*I and *Pvu*II and inserted into YEP451's unique *Hpa*I site to form YEP451S. An *Sph*I−*Sph*I fragment (765 bp) containing the ADH promoter and terminator obtained from DB20 (Becker *et al.*, 1991) was then transferred into the unique *Sph*I site of YEP451S, resulting in pDAS5.

To make the HAP2 plasmid with the *LEU2* marker for mutagenesis, pJO323 (Olesen and Guarente, 1990) was digested with *Sph*I, the 1.5 kb *Sph*I−*Sph*I fragment (ADH promoter-lexA-HAP2-core-ADH terminator) was released and ligated into YEP451S to form pYX3236. The HAP2 plasmid with the *HIS4* marker was constructed by ligating a 3.1 kb *Bam*HI−*Bam*HI fragment from pJP300 to a unique *Bam*HI site in pLGΔ312ΔAX-*HIS4* vector (Turcotte and Guarente, 1992). The plasmids with the *URA3* marker of HAP2 and its mutations were constructed by ligating a 1.5 kb *Sph*I−*Sph*I fragments of pYX3236 into the *Sph*I site of plasmid YEP352 (Hill *et al.*, 1986). To make the HAP2−GAL4 fusion with the *HIS4* marker (pHAP2-GAL4-*HIS4*), the pLGΔ312ΔAX-*HIS4* vector was digested with *Bam*HI and *Sma*I, blunted with Klenow and the vector fragment was ligated with a 2.3 kb fragment obtained from plasmid HAP2−GAL4 (Olesen and Guarente, 1990) digested with *Pvu*II and *Hinc*II.

To make the HAP3 plasmid with the *LEU2* marker for mutagenesis, pSH95 (Hahn *et al.*, 1988) was digested with *Xho*I and *Ssp*I and a 570 bp *Xho*I−*Ssp*I fragment containing a HAP3 ORF was ligated with a *Hin*dIII linker, further digested with *Hin*dIII and then ligated into pBluescript KS+ vector digested with *Sal*I and *Hin*dIII to form plasmid Y395. A *Xho*I−*Pst*I fragment released from Y395 was ligated into pUC19 digested with *Sal*I and *Pst*I to form plasmid Y395U. After digestion of Y395U with *Hin*dIII, a *Hin*dIII−*Hin*dIII fragment was obtained and ligated into pDAS5 to obtain pYX315. The Maxi-HAP3 plasmid was constructed by digestion of Y395U with *Ban*I, blunted with Klenow and ligated with the *lexA* gene *Hinc*II−*Xmn*I (blunted) fragment to form pLH. pLH was further digested with *Hin*dIII and the *Hin*dIII fragment was ligated into pDAS5 to form pYL315 (Maxi-HAP3).

β-Galactosidase assays

The appropriate isogenic yeast strain was transformed with the indicated *lacZ* reporters (2 μm, *URA3*$^+$) and lexA−HAP2 fusion plasmids (2 μm, *Leu2*$^+$ por *HIS4*$^+$) or HAP2−GAL4 plasmid (2 μm, *LEU2*$^+$ or *HIS4*$^+$) or HAP3 plasmids (2 μm, *LEU2*$^+$) by sequential transformation, using the lithium acetate protocol (Sherman *et al.*, 1986). Transformants were grown selectively in minimal medium and assayed by β-galactosidase activity as described previously (Guarente and Mason, 1983). Values reported were normalized to a control and represent the average of not less than three independent determinations, with a variation of less than 20%.

Cloning of S.pombe HAP3

SHY40 was transformed with an *S.pombe* cDNA library (Fikes *et al.*, 1990) and plated on minimal glucose selective medium (Olesen *et al.*, 1991).

Transformants were scraped, resuspended in 1 × SD (Sherman *et al.*, 1986) and plated on a rich lactate medium for selection of *HAP3*$^+$. Lactate-positive clones were obtained at a frequency of 1 in 10^5 from the library. The clones were recovered by yeast DNA preparation and transformed into an *E.coli* strain XL-1. The plasmids were recovered and retransformed into SHY40 to confirm the lactate phenotype.

HAP3 deletion constructions

HAP3ΔN35 was constructed by site-directed mutagenesis (Bio-Rad kit) introducing two *Xba*I sites into pXY315 at the nucleotide sequence of HAP3 corresponding to amino acid residues 2 and 35. After digestion with *Xba*I, the plasmid was self-ligated and checked by sequencing. The deletion did not disrupt the HAP3 open reading frame and kept the original initiation codon of HAP3. HAP3ΔN51 was constructed in a manner similar to HAP3ΔN35 but two *Xba*I sites were introduced into pYX315 corresponding to amino acid residues 1 and 49 of HAP3. After digestion of *Xba*I and self-ligation, the original initiation codon of HAP3 and the first 48 amino acid residues were removed, and the methionine at position 51 was then taken as an initiation codon. HAP3ΔC123 was constructed by introduction of a stop codon at position K123 of pYX315 and HAP3ΔC127 was constructed by introduction of a stop codon at position Q127 of HAP3ΔN35, by site-directed mutagenesis.

Saturated mutagenesis of HAP2 and HAP3

Single-stranded DNA from plasmids pYX3236 and pYX315 were prepared according to Sambrook *et al.* (1989) from *E.coli* strain CJ236 (*dut*$^-$*ung*$^-$) respectively and used for saturated mutagenesis with random oligonucleotides (the level of contamination was 2.5% for each of the three other nucleotides). Mutagenesis was performed with a kit from Bio-Rad.

Saturation mutagenesis on the HAP2 core region expanded from amino acid residues 154–218 was conducted by using seven overlapping oligonucleotides, each of 30 bases (which were synthesized such that they contained, on average, one random mutation per oligonucleotide); Saturation mutagenesis on the HAP3 core region expanded from amino acid residue 35 to residue 128 was carried out by using nine overlapping oligonucleotides, each of 33 bases (as described above). *In vitro* mutated DNA directed by each of the oligonucleotides was then transformed into *E.coli* strain DH5α. More than 10^4 colonies were obtained from each transformation and amplified in LB broth. The plasmid DNAs were purified by cesium chloride centrifugation. The purified plasmid pool of HAP2 or HAP3 mutations was then directly transformed into a yeast *hap2* or *hap3* null strain harboring pLGΔ265UP1 (CCAAT) and pLGΔ265wt (CCAAC) reporters on minimal plates, respectively. Yeast colonies were then transferred with a nitrocellulose filter on to a minimal X-Gal plate with 2% glucose. Colonies with different β-gal activity from wild type were then grown on lactate-rich plates to test *hap* mutations and in liquid glucose minimal medium for plasmid preparation. The recovered plasmids were transferred into DH5α. Mutations were identified by sequencing double-stranded DNA. The mutated plasmids were transformed back into the original *hap* null strains with pLGΔ265UP1 (CCAAT) or pLGΔ265wt to measure their β-gal activity. The phenotype of the transformants was further confirmed on lactate plates.

Both cysteine amino acid residues at positions 68 and 72 of HAP3 were changed into serine by site-directed mutagenesis individually or simultaneously. The resulting mutations were (C68S), (C72S) and (C68S, C72S).

Preparation of extracts and DNA-binding assays

Yeast cell extracts were prepared as described previously (Olesen *et al.*, 1987). The UAS2UP1 probe (CCAAT) was designed as described by Hahn and Guarente (1988) and the UAS2wt probe (CCAAC) was one nucleotide different from UAS2UP1 in the CCAAT box (T → C). The DNA probes were end-labeled by Klenow fragment with [α-^{32}P]dATP. Protein–DNA binding conditions were as described previously (Chodosh *et al.*, 1988b; Olesen and Guarente, 1990). Protein–DNA complexes were resolved by gel electrophoresis on 5% polyacrylamide gels in 0.5 × TBE at 4°C. After gel electrophoresis, the gels were dried and autoradiographed with X-ray film or a PhosphorImager (FUJIX BAS2000).

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The EMBL databank accession number for *Schizosaccharomyces pombe* PHP3 mRNA is X75072.