Assembly of the Hap2p/Hap3p/Hap4p/Hap5p-DNA Complex in *Saccharomyces cerevisiae*

David S. McNabb* and Inés Pinto

Department of Biological Sciences, University of Arkansas, Fayetteville, Arkansas 72701

Received 30 July 2005/Accepted 25 August 2005

The CCAAT-binding factor (CBF) is an evolutionarily conserved multimeric transcriptional activator in eukaryotes. In *Saccharomyces cerevisiae*, the CCAAT-binding factor is composed of four subunits, termed Hap2p, Hap3p, Hap4p, and Hap5p. The Hap2p/Hap3p/Hap5p heterotrimer is the DNA-binding component of the complex that binds to the consensus 5'-CCAAT-3' sequence in the promoter of target genes. The Hap4p subunit contains the transcriptional activation domain necessary for stimulating transcription after interacting with Hap2p/Hap3p/Hap5p. In this report, we demonstrate that Hap2p, Hap3p, and Hap5p assemble via a one-step pathway requiring all three subunits simultaneously, as opposed to the mammalian CCAAT-binding factor which has been shown to assemble via a two-step pathway with CBF-A (Hap3p homolog) and CBF-C (Hap5p homolog) forming a stable dimer before CBF-B (Hap2p homolog) can interact. We have also found that the interaction of Hap4p with Hap2p/Hap3p/Hap5p requires DNA binding as a prerequisite. To further understand the protein-protein and protein-DNA interactions of this transcription factor, we identified the minimal domain of Hap4p necessary for interaction with the Hap2p/Hap3p/Hap5p-DNA complex, and we demonstrate that this domain is sufficient to complement the respiratory deficiency of a hap4Δ mutant and activate transcription when fused with the VP16 activation domain. These studies provide a further understanding of the assembly of the yeast CCAAT-binding factor at target promoters and raise a number of questions concerning the protein-protein and protein-DNA interactions of this multisubunit transcription factor.

*Saccharomyces cerevisiae* is a respirofermentative yeast that represses respiratory metabolism when growing in medium containing glucose as the sole carbon source, even in an oxygenated environment (14, 53). Following glucose depletion, cells undergo a major reprogramming of gene expression, known as the diauxic shift, to activate the genes that encode proteins needed for respiration and gluconeogenesis (12, 16, 30, 45). Thus, the organism can utilize the ethanol that was generated during the fermentative metabolism. The CCAAT-binding factor (CBF; the Hap2p/Hap3p/Hap4p/Hap5p complex) is one of the transcriptional activators responsible for the activation of many of the genes involved in respiratory metabolism (12, 16, 45, 57), as well as other genes needed for other metabolic functions, such as ammonia assimilation (10, 11, 41).

The CCAAT-binding factor is a multisubunit transcriptional activator that binds to the 5’-CCAAT-3’ consensus elements within promoters (6, 36). This activator is unique among DNA-binding proteins in that it requires three heterologous subunits, termed Hap2p, Hap3p, and Hap5p, for DNA-binding activity (35, 38). The Hap2p/Hap3p/Hap5p trimer has been shown to be sufficient for CCAAT-specific binding at target promoters (38); however, this complex lacks the ability to activate transcription. A fourth subunit of the complex, termed Hap4p, is necessary for transcriptional activation (15). *HAP4* is subject to glucose repression (12, 15), with its expression repressed in the presence of glucose and activated in its absence, while the expression of *HAP2, HAP3*, and *HAP5* is constitutive (12). Thus, the synthesis and interaction of Hap4p with Hap2p/Hap3p/Hap5p modulate the activity of target genes. Mutations that abolish the function of any of the four Hap subunits result in the inability of yeast to grow on nonfermentable carbon sources (15, 24, 38, 40), emphasizing the importance of this protein complex as a global regulator of respiration.

Each of the DNA-binding subunits of the CCAAT-binding factor contains an essential core region that is highly conserved evolutionarily from yeast to humans (35), with the Hap3p and Hap5p core elements displaying amino acid sequence similarities to the histone fold motifs of histones H2B and H2A, respectively (2, 35). In yeast, these core regions have been shown to be essential for the assembly and DNA-binding activity of the heterotrimeric complex (37). Moreover, in vivo expressions of the conserved regions of Hap2p and Hap3p are sufficient for functional complementation of their respective null mutants (39, 55, 56). In addition to the highly conserved core region, Hap5p contains a 32-amino-acid domain that is lacking in the homologous proteins of higher eukaryotes but present in other yeasts and fungi (37). This small domain is required for the association of Hap4p with Hap2p/Hap3p/Hap5p; hence, this region was termed the Hap4p recruitment domain (37). Thus, the Hap4p recruitment domain, together with the histone fold region of Hap5p, is sufficient for functional complementation of a hap5Δ mutant (37).

For several years, Hap4p homologs were not found in other yeast or fungi; however, recent studies have revealed that homologs exist in *Kluyveromyces lactis* (4) and *Hansenula polymorpha* (51). These Hap4p homologs were shown to functionally complement an *S. cerevisiae* hap4Δ mutant in spite of the fact that the homology between these proteins was limited to a...
16-amino-acid domain (4, 51). Nevertheless, once this conserved region was identified, it became apparent that Hap4p-like proteins exist in many different yeasts and fungi (51). Moreover, the Hap4p recruitment domain of Hap5p is also conserved in these organisms (unpublished observations), suggesting that these domains may be important for Hap4p-Hap5p interactions. However, biochemical studies that directly assess the domain(s) of Hap4p needed for association with Hap2p/Hap3p/Hap5p have not been performed.

Hap4p is composed of 554 amino acid residues with a C-terminal activation domain that is between amino acid residues 330 and 554 (15). Additional studies of the Hap4p activation domain have revealed two regions capable of stimulating transcription when fused to the LexA DNA-binding domain. One region was identified, it became apparent that Hap4p-like proteins exist in many different yeasts and fungi (51). Moreover, the Hap4p recruitment domain of Hap5p is also conserved in these organisms (unpublished observations), suggesting that these domains may be important for Hap4p-Hap5p interactions. However, biochemical studies that directly assess the domain(s) of Hap4p needed for association with Hap2p/Hap3p/Hap5p have not been performed.

Hap4p is composed of 554 amino acid residues with a C-terminal activation domain that is between amino acid residues 330 and 554 (15). Additional studies of the Hap4p activation domain have revealed two regions capable of stimulating transcription when fused to the LexA DNA-binding domain. One region was identified, it became apparent that Hap4p-like proteins exist in many different yeasts and fungi (51). Moreover, the Hap4p recruitment domain of Hap5p is also conserved in these organisms (unpublished observations), suggesting that these domains may be important for Hap4p-Hap5p interactions. However, biochemical studies that directly assess the domain(s) of Hap4p needed for association with Hap2p/Hap3p/Hap5p have not been performed.

Hap4p is composed of 554 amino acid residues with a C-terminal activation domain that is between amino acid residues 330 and 554 (15). Additional studies of the Hap4p activation domain have revealed two regions capable of stimulating transcription when fused to the LexA DNA-binding domain. One region was identified, it became apparent that Hap4p-like proteins exist in many different yeasts and fungi (51). Moreover, the Hap4p recruitment domain of Hap5p is also conserved in these organisms (unpublished observations), suggesting that these domains may be important for Hap4p-Hap5p interactions. However, biochemical studies that directly assess the domain(s) of Hap4p needed for association with Hap2p/Hap3p/Hap5p have not been performed.

Hap4p is composed of 554 amino acid residues with a C-terminal activation domain that is between amino acid residues 330 and 554 (15). Additional studies of the Hap4p activation domain have revealed two regions capable of stimulating transcription when fused to the LexA DNA-binding domain. One region was identified, it became apparent that Hap4p-like proteins exist in many different yeasts and fungi (51). Moreover, the Hap4p recruitment domain of Hap5p is also conserved in these organisms (unpublished observations), suggesting that these domains may be important for Hap4p-Hap5p interactions. However, biochemical studies that directly assess the domain(s) of Hap4p needed for association with Hap2p/Hap3p/Hap5p have not been performed.

Hap4p is composed of 554 amino acid residues with a C-terminal activation domain that is between amino acid residues 330 and 554 (15). Additional studies of the Hap4p activation domain have revealed two regions capable of stimulating transcription when fused to the LexA DNA-binding domain. One region was identified, it became apparent that Hap4p-like proteins exist in many different yeasts and fungi (51). Moreover, the Hap4p recruitment domain of Hap5p is also conserved in these organisms (unpublished observations), suggesting that these domains may be important for Hap4p-Hap5p interactions. However, biochemical studies that directly assess the domain(s) of Hap4p needed for association with Hap2p/Hap3p/Hap5p have not been performed.

Hap4p is composed of 554 amino acid residues with a C-terminal activation domain that is between amino acid residues 330 and 554 (15). Additional studies of the Hap4p activation domain have revealed two regions capable of stimulating transcription when fused to the LexA DNA-binding domain. One region was identified, it became apparent that Hap4p-like proteins exist in many different yeasts and fungi (51). Moreover, the Hap4p recruitment domain of Hap5p is also conserved in these organisms (unpublished observations), suggesting that these domains may be important for Hap4p-Hap5p interactions. However, biochemical studies that directly assess the domain(s) of Hap4p needed for association with Hap2p/Hap3p/Hap5p have not been performed.
The construction of the plasmid required multiple steps. Initially, the
moter, and the 5’ and 3’ ends, respectively, along with introducing a stop codon after amino acid residue 210. The PCR product and pCITE2c were digested with BamHI/NotI restriction sites. The DNA was cloned into pET-28a at the 5’ and 3’ ends, respectively, and a termination codon after amino acid residue 180. The PCR product and pCITE2c were digested with BamHI/NotI and ligated.

For the mobility shift studies, the core domains of Hap2p and Hap3p were produced as six-His-tagged fusion proteins in E. coli BL21(DE3). The plasmid encoding the Hap2p core domain was generated by PCR amplification of HAP2 (amino acid residues 15 to 214) from the template plasmid pJP103 (40) with primers oDM0108 and oDM0141 that incorporated unique BamHI and NotI restriction sites on the 5’ and 3’ ends, respectively. The DNA was ligated into the NdeI/EcoRI sites of YCplac33 (18). Next, the DNA was digested with EcoRI/XbaI and cloned into the same sites of pJR3. The DNA was cloned into the EcoRI/XbaI sites of YCplac33 containing the HAP4 UTR (amino acid residues 1225 to 1262). The plasmid was amplified by PCR from the template plasmid pSLF413 (15) with primers oDM0108 and oDM0141 that incorporated unique PstI and EcoRI sites on the 5’ and 3’ ends, respectively. The DNA was cloned into pET-28a at the 5’ and 3’ ends, respectively, and a termination codon after amino acid residue 180. The PCR product and pCITE2c were digested with BamHI/NotI and ligated.

For preparation of six-His–Hap2p and six-His–Hap3p, the DNA was digested with XbaI/PstI and cloned into YCplac33 containing the HAP4 UTR (amino acid residues 15 to 214) coding sequence, this plasmid was designated pDM471. To add the Hap4p activation domain was amplified by PCR from the template plasmid pJR3 (52) with primers oDM0108 and oDM0141 that incorporated unique BamHI and HindIII sites on the 5’ and 3’ ends, respectively. The DNA was cloned into pET-28a at the 5’ and 3’ ends, respectively, and a termination codon after amino acid residue 180. The PCR product and pCITE2c were digested with BamHI/NotI and ligated.

The DNA was cloned into pET-28a at the 5’ and 3’ ends, respectively, and a termination codon after amino acid residue 180. The PCR product and pCITE2c were digested with BamHI/NotI and ligated.

Purification of recombinant proteins. The glutathione S-transferase fusion proteins were expressed in E. coli BL26 and purified from bacterial lysates with glutathione-Sepharose beads (Amersham Biosciences) as previously described (38). For preparation of six-His–Hap2p and six-His–Hap3p, E. coli BL21(DE3)
containing the appropriate expression plasmid was inoculated to TB medium (43) containing 40 μg of kanamycin/ml and then grown at 37°C to an optical density at 600 nm (OD<sub>600</sub>) of ~0.7. Expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactoside (IPTG), and the culture was incubated an additional 3 h at 37°C. The cells were harvested by centrifugation at 5,000 × g for 10 min, resuspended in denaturing lysis buffer (20 mM Tris [pH 7.9], 500 mM NaCl, 6 M guanidine hydrochloride, 5 mM imidazole, 1 mM phenylmethylsulfonyl fluoride, and 5 mM benzamidine) and incubated for 1 h at room temperature with agitation. The lysate was sonicated to reduce the viscosity and subsequently centrifuged at 12,000 × g for 15 min. at 4°C. The supernatant was collected and passed over a Ni-NTA agarose column (Qiagen) that had been pre-equilibrated with lysis buffer. The column was washed with lysis buffer until the absorbance at 280 nm (A<sub>280</sub>) was <0.01 and then with wash buffer (lysis buffer containing 20 mM imidazole) until A<sub>280</sub> was <0.01. The proteins were eluted with lysis buffer containing 300 mM imidazole, and 0.5 M fractions were collected. Fractions containing the recombinant proteins were identified by A<sub>280</sub> values. Small aliquots of each fraction were precipitated with trichloroacetic acid (25%), the purity of the fractions was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were visualized by Coomassie blue staining. Fractions containing the recombinant proteins were pooled and dialyzed overnight in a renaturation buffer (25 mM Tris [pH 7.9], 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 5% glycerol, and 5 mM β-mercaptoethanol). The proteins were then quantified by the bichinchoninic acid protein assay (Pierce) with bovine serum albumin as the standard, and the activity of the recombinant proteins was verified by mobility shift assays.

### In vitro transcription/translation

To express each of the Hap subunits, in vitro transcription and translation were performed using the Novagen single-tube protein system for coupled transcription/translation according to the manufacturer’s protocol. For GST pulldown experiments, the proteins were radiolabeled with [35S]methionine (DuPont-NEN) during translation. For mobility shift studies, the proteins were not radiolabeled, but a [35S]methionine-labeled parallel reaction was performed to confirm the protein size and expression by SDS–12% PAGE, followed by autoradiography. In vitro protein-protein interaction assays. For the GST pulldown assays, [35S]methionine-labeled Hap subunits were incubated with GST-GST-Hap2p, GST-Hap3p, GST-Hap5p, or a combination of recombinant proteins in a binding buffer containing 50 mM HEPEs (pH 7.9), 100 mM KCl, 1 mM EDTA, 0.1% Tween-20, 5 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride in a final volume of 200 μl. After incubation at room temperature for 30 min, 50 μl of 50% (vol/vol) glutathione-Sepharose resin (Amersham Biosciences), pre-equilibrated in binding buffer, was added to each reaction mixture, and the incubation was continued for 30 min at room temperature. The mixture was centrifuged, and the resin was washed four times with 200 μl of binding buffer. The resin was then boiled in SDS-PAGE loading buffer (25) and centrifuged, and the supernatant was run on SDS–12% PAGE gels that were subsequently fixed, dried, and exposed to a PhosphorImager screen. The efficiency of the pulldown experiment was carried out on a Molecular Dynamics PhosphorImager. For reactions requiring DNA, the CCAAT box probe was a 37-μp long double-stranded oligonucleotide with the sequence derived from CYC1 UAS2UP1 as described previously (23), and the mutated probe was identical to UAS2UP1, except the CCAAT sequence was changed to GGAAG.

DNA-binding assays and gel electrophoresis. The UAS2UP probe derived from the sequence of the CYC1 gene and containing the CCAAT box was described previously (23). The DNA probe was end labeled with [γ-32P]ATP with the Klenow fragment. All DNA-binding reactions were performed in 1× DNA-binding buffer (20 mM HEPES-NaOH [pH 7.9], 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 20% glycerol) containing 20 ng of each recombinant Hap subunit, 5 μl of the appropriately programmed in vitro transcription/translation extract, and 3 μg of poly(dI-dC) nonspecific competitor DNA. When an in vitro transcription/translation extract was not included in the DNA-binding reaction, the poly(dI-dC) concentration was reduced to 200 ng. Reactions were incubated at room temperature for 30 to 45 min, and the protein-DNA complexes were resolved by gel electrophoresis as previously described (38). The gels were subsequently fixed, and the protein-DNA complexes were visualized by autoradiography and with a PhosphorImager (Molecular Dynamics).

**β-Galactosidase assays.** β-Galactosidase assays were performed on cells grown in synthetic omission medium (SC-Ura-Leu) containing 2% glucose, raffinose, or lactate as the carbon source to an OD<sub>600</sub> of ~1.0. The cells were harvested by centrifugation, and total RNA was prepared by the glass bead-acid phenol method as previously described (1). Approximately 30 μg of each total RNA sample was loaded, separated by formaldehyde–1% agarose gel electrophoresis, and transferred to GeneScreen Plus membranes (DuPont-NEN Research Products) according to the manufacturer’s protocol. The membranes were hybridized and washed under standard high-stringency conditions (43). The HAP1, CYC1, and SNR20 probes were obtained by PCR amplification of S. cerevisiae FY250 genomic DNA using oligonucleotide primer pairs oDM169/oDM187, oDM125/oDM124, and oDM206/oDM207, respectively. The PCR products were purified by agarose gel electrophoresis and the Geneclean kit (Qiogene, Inc.). The probes were radiolabeled with [α-32P]dCTP (Amersham Biosciences) by use of a random primer labeling kit (U.S. Biochemicals) according to the manufacturer’s protocol. The transcript levels were quantified on a Molecular Dynamics PhosphorImager. For quantifying the levels of Hap6p and TBP, X-ray films were scanned and analyzed by densitometry after exposure to the ECL reagent.

### RESULTS

The assembly pathway of the Hap2p/Hap3p/Hap5p heterotrimer. The mammalian CCAAT-binding factor has been shown to follow a two-step ordered pathway of assembly, with CBF-A (Hap3p homolog) and CBF-C (Hap5p homolog) initially forming a stable dimer, and CBF-B (Hap2p homolog) subsequently binding to form the trimer (48). Two lines of evidence suggested that this two-step pathway may not exist for the S. cerevisiae CCAAT-binding factor. First, we had not observed a stable Hap3p/Hap5p dimer during our in vitro studies with purified recombinant Hap proteins (data not shown); second, it was previously shown that size variants of Hap3p and Hap5p could be interchanged with their respective counterpart parts of the CCAAT-binding factor from yeast cell extracts (38). This differed from the stability of the CBF-A/CFB-C dimer, which required denaturing conditions to separate the two subunits (34). Given these contradictions, we investigated the assembly of the Hap2p/Hap3p/Hap5p heterotrimer.

To examine the assembly of Hap2p/Hap3p/Hap5p, full-length [35S]methionine-labeled Hap2p, Hap3p, and Hap5p were synthesized by in vitro transcription/translation and the radiolabeled subunits were subsequently used for in vitro protein-protein interaction studies with purified recombinant GST, GST-Hap2p, GST-Hap3p, and GST-Hap5p. The reactions were precipitated with glutathione-Sepharose resin and the proteins associated with the GST-Hap subunits analyzed by SDS-PAGE (Fig. 1). In this assay, no binary interactions between the subunits were observed, suggesting that Hap3p and Hap5p do not form a stable dimer. In addition, none of the Hap subunits formed homodimers, as indicated by the lack of

---

**Eukaryot. Cell**

1832 McNABB AND PINTO

**EUKARYOT. CELL**
self-association, consistent with previous studies with the mammalian counterparts (48). The radiolabeled Hap proteins were precipitated only when a combination of the other two GST-Hap subunits were added to the reaction mixture (Fig. 1). From several independent experiments, the efficiency of the precipitation reaction with radiolabeled Hap3p was always lower than those containing radiolabeled Hap2p or Hap5p; however, we do not have an explanation for this observation.

To evaluate whether the presence of DNA may stabilize a Hap3p/Hap5p intermediate, the same protein-protein interaction studies were repeated in the presence of a 37-bp double-stranded CCAAT site oligonucleotide derived from the sequence of the CYC1 UAS2UP1 (23), a known Hap2p/Hap3p/Hap5p-binding site; again, we observed no stable binary interactions (data not shown). On the basis of these data, we concluded that the yeast CCAAT-binding factor does not follow a two-step assembly pathway like its mammalian homologs but requires the presence of the three Hap subunits for stable complex formation.

Sequence-specific DNA binding by Hap2p/Hap3p/Hap5p is a prerequisite for Hap4p interaction. To further dissect the assembly of the yeast CCAAT-binding factor, we examined the requirement for Hap4p interaction. This was particularly important, since the CCAAT-binding factor in higher eukaryotes lacks a Hap4p homolog and the requirements for it to bind with Hap2p/Hap3p/Hap5p have not been investigated. As mentioned previously, the Hap4p recruitment domain of Hap5p is necessary for the Hap4p-Hap2p/Hap3p/Hap5p interaction (37); however, we wanted to determine whether the other subunits of the complex were also necessary. For these studies, we used a truncated allele of HAP4, encoding amino acid residues 1 through 330, since it has been previously shown to interact with Hap2p/Hap3p/Hap5p in mobility shift assays (37). Thus, [35S]methionine-labeled Hap4p (1-330) was synthesized by in vitro transcription/translation and incubated with GST alone or GST-Hap2p/Hap3p/Hap5p in the presence or absence of a double-stranded DNA oligonucleotide containing a CCAAT box or a mutated oligonucleotide (GGAAG). The first lane represents 10% of the radiolabeled protein in each reaction mixture (10% input). The percentage of the total radiolabeled protein precipitated in each reaction is shown below panels A and B.

FIG. 1. The Hap2p/Hap3p/Hap5p heterotrimer assembles via a one-step mechanism. Protein-protein interaction studies were performed as described in Materials and Methods using [35S]methionine-labeled Hap2p, Hap3p, and Hap5p synthesized by in vitro transcription/translation in a rabbit reticulocyte lysate. The GST pulldown reaction mixtures contained the radiolabeled Hap subunit as indicated on the left of each panel along with GST, GST-Hap2p, GST-Hap3, GST-Hap5p, or a combination of GST-Hap subunits as indicated. The first lane of each panel contains 10% of the in vitro-synthesized protein added to each GST pulldown reaction mixture (10% input). The percentage of the total radiolabeled protein precipitated in each reaction is shown below the individual panels.

FIG. 2. DNA binding by Hap2p/Hap3p/Hap5p is a prerequisite for Hap4p association. (A) Protein-protein interaction studies were performed as described in Materials and Methods using [35S]methionine-labeled Hap4p (1-330) synthesized by in vitro transcription/translation. Each GST pulldown reaction mixture contained the radiolabeled Hap4p (1-330) incubated with GST, GST-Hap2p, GST-Hap3p, GST-Hap5p, or the indicated combinations. The first lane represents 10% of the radiolabeled Hap4p added to each pulldown reaction mixture (10% input). (B) Protein-protein interaction studies were performed with [35S]methionine-labeled Hap4p (1-330) incubated with GST alone or GST-Hap2p/Hap3p/Hap5p in the presence or absence of a double-stranded DNA oligonucleotide containing a CCAAT box or a mutated oligonucleotide (GGAAG). The first lane represents 10% of the radiolabeled protein in each reaction mixture (10% input). The percentage of the total radiolabeled protein precipitated in each reaction is shown below panels A and B.
the presence of a 37-bp double-stranded DNA oligonucleotide containing the sequence of the CCAAT site from CYC1 UAS2UP or an identical control DNA in which the CCAAT site was mutated to GGAAG to abolish binding by Hap2p/Hap3p/Hap5p (Fig. 2B). These data clearly demonstrated that Hap4p (1-330) could interact with Hap2p/Hap3p/Hap5p in the presence of the double-stranded CCAAT-containing oligonucleotide but not when the mutated oligonucleotide was present, suggesting that Hap2p/Hap3p/Hap5p must first bind DNA in a sequence-specific manner as a prerequisite for Hap4p interaction.

The domain of Hap4p required for interaction with Hap2p/Hap3p/Hap5p. Since the association of Hap4p with Hap2p/Hap3p/Hap5p required the heterotrimer to be bound to DNA, we investigated the region within Hap4p that was essential for the interaction(s). It has been shown that the Hap4p homologs in *S. cerevisiae*, *K. lactis*, and *H. polymorpha* share a 16-amino-acid region that is highly conserved (4, 51); however, it seemed unreasonable to assume that this domain alone would be sufficient to form a stable interaction with Hap2p/Hap3p/Hap5p. To investigate the minimal region of Hap4p sufficient for the stable binding with Hap2p/Hap3p/Hap5p, deletion analysis was performed on HAP4. For these studies, the Hap2p and Hap3p core domains and the Hap5p core plus the Hap4p recruitment domain (termed p92) (37, 38) were expressed in *E. coli* and purified as outlined in Materials and Methods. Following purification, the affinity tags were removed from each protein by thrombin cleavage. The various HAP4 truncations were synthesized via in vitro transcription/translation using two parallel reactions in which the proteins were synthesized in the presence of \[^{35}S\]methionine for SDS-PAGE analysis to concomitantly reactions in which the proteins were synthesized in the

To further explore the interactions between Hap4p and to other Hap4p homologs. Since the amino acid sequence of Hap4p has diverged dramatically in non-*Saccharomyces* yeasts (4, 51), we compared the sequence of *S. cerevisiae* Hap4p (23-180) with that of the closely related *Saccharomycyes* species *Saccharomyces bayanus* (7), and a more evolutionarily diverged species, *Saccharomyces castellii* (7), to identify any conserved domains (Fig. 3B). Besides the Hap2p/Hap3p/Hap5p interaction domain, which is conserved in all Hap4p homologs (4, 51), the only remarkable conservation was a 20-amino-acid segment at the C terminus of Hap4p (23-180) between amino acid residues 161 and 180. This region was serine rich and conserved in the most divergent species, *S. castellii*. Furthermore, Hap4p (1-165) removes most of this region, causing the loss of binding to Hap2p/Hap3p/Hap5p (Fig. 3A), indicating that it is functionally important for assembly of the Hap2p/Hap3p/Hap4p/Hap5p complex.

The stoichiometry of Hap4p in the yeast CCAAT-binding factor. To further explore the interactions between Hap4p and
Hap2p/Hap3p/Hap4p/Hap5p, we needed to determine how many copies of Hap4p are associated with each DNA-bound CCAAT-binding complex. Prior studies have demonstrated that each of the DNA-binding subunits of the mammalian CCAAT-binding factor are present in one copy per complex (29, 47), and we have performed mobility shift studies with different size variants of Hap2p, Hap3p, and Hap5p to confirm these data with yeast (data not shown). However, the number of Hap4p molecules per CCAAT-binding complex has not been established. To determine the stoichiometry of Hap4p in the DNA-bound CCAAT complex, mobility shift studies were performed using the in vitro-translated Hap4p (1-330) and Hap4p (23-180) (Fig. 4). Because the protein-DNA complexes contained identical Hap2p, Hap3p, and Hap5p subunits, any difference in mobility must be attributed to the size of the Hap4p protein. When Hap4p (1-330) and Hap4p (23-180) were both present in the DNA-binding assay, two distinct Hap2p/Hap3p/Hap4p/Hap5p complexes were observed, and their mobility corresponded to that of the Hap complexes containing either Hap4p (1-330) or Hap4p (23-180) alone (Fig. 4). If two or more molecules of Hap4p were present in each DNA-bound CCAAT complex, additional DNA-protein complexes of intermediate mobility would have been observed as a result of having a mixture of the two size variants. Thus, these data indicate that Hap4p, like Hap2p, Hap3p, and Hap5p, is present in one copy per DNA-bound CCAAT-binding complex.

In vivo complementation of a hap4Δ mutant with Hap4p (23-180) fused to the VP16 activation domain. The studies described above have shown that amino acid residues 23 through 180 are sufficient for Hap4p to associate with the Hap2p/Hap3p/Hap5p-DNA complex in vitro. To investigate whether this region was sufficient in vivo, we determined whether Hap4p (23-180) could functionally complement a hap4Δ mutant of S. cerevisiae. To mimic the normal levels of Hap4p expression in vivo, we generated an autonomously replicating sequence-centromeric (ARS/CEN) plasmid in which Hap4p (23-180) was expressed from the HAP4 promoter with its native 5′ and 3′ untranslated sequences. Two plasmids were generated for these studies. One plasmid contained HAP4 (23-180) lacking an activation domain and the other contained HAP4 (23-180) fused with the VP16 activation domain.

FIG. 4. One Hap4p molecule is present in the Hap2p/Hap3p/Hap4p/Hap5p-DNA complex. DNA mobility shift assays were performed with DNA-binding reaction mixtures containing a radiolabeled CCAAT box probe incubated with purified recombinant Hap2p core, Hap3p core, and Hap5p (p92). Rabbit reticulocyte lysates containing Hap4p (1-330), Hap4p (23-180), or an unprogrammed reticulocyte lysate were added to the binding reaction mixtures as indicated. The positions of the Hap2p/Hap3p/Hap5p heterotrimer and the Hap2p/Hap3p/Hap4p/Hap5p heterotetramers are indicated on the right. The free probe is at the bottom of the gel.

FIG. 5. In vivo complementation of a hap4Δ mutant with HAP4 (23-180)-VP16. (A) Schematic diagram of the various HAP4 alleles used to test complementation of the hap4Δ mutant. (B) Yeast strains FY250 (wt) or DMY144 (hap4Δ) were transformed with the indicated plasmids and subsequently grown on SC-Ura medium containing glucose for 3 days at 30°C. (C) Same as in panel B except the strains were grown on SC-Ura medium containing lactate as the sole carbon source. The plates were incubated for 3 days at 30°C.
VP16 activation domain (Fig. 5A). The VP16 activation domain was chosen to avoid any additional Hap4p sequences within the protein. These plasmids were introduced into strain DMY144 (hap4Δ) and the transformants were tested for their ability to grow on medium containing either glucose (Fig. 5B) or lactate (Fig. 5C) as the sole carbon source. As controls, the wild-type strains FY250 and DMY144 containing an ARS/CEN vector only and DMY144 containing an ARS/CEN plasmid with the full-length HAP4 were included. All of the strains grew normally on glucose-containing medium, demonstrating their viability; however, the strain containing Hap4p (23-180) lacking an activation domain failed to complement the respiratory defect of the hap4Δ mutant. In contrast, Hap4p (23-180) fused to the VP16 activation domain complemented the defect with the strain growing normally on lactate medium compared to the wild-type strain or the hap4Δ strain containing the full-length HAP4.

To ascertain the ability of Hap4p (23-180) to stimulate transcription, the strains containing the different HAP4 alleles were transformed with an LEU2 plasmid containing a CYC1-lacZ reporter (15). The transformants were grown in SC-Ura-Leu synthetic omission medium containing glucose, raffinose, or lactate as the sole carbon source and were subsequently assayed for ß-galactosidase activity (Table 2). The Hap4p (23-180) alone did not stimulate ß-galactosidase activity beyond that of the hap4Δ control strain; however, addition of the VP16 activation domain to the truncated protein resulted in ß-galactosidase activity that was similar to that of the strains containing the wild-type HAP4 gene. Thus, we concluded that Hap4p (23-180) is sufficient for association with Hap2p/Hap3p/Hap5p in vivo and is competent to activate transcription when fused to a heterologous activation domain.

Hap4p protein expression is induced on nonfermentable carbon source. To understand the association of Hap4p with carbon source. To understand the association of Hap4p with Hap2p/Hap3p/Hap5p in vivo and how it regulates transcription, it was important to examine the expression of Hap4p protein. It has been shown that HAP4 mRNA levels are repressed when yeasts are grown in glucose-containing medium and induced three- to fourfold in medium containing lactate as the carbon source (15). The HAP4 gene is interesting structurally because it has an unusually long 5' UTR of 270, 280, and 330 nucleotides (due to three mRNA initiation sites) that contain two short open reading frames, 27 and 9 nucleotides long, before the authentic AUG of the Hap4p open reading frame (15, 28).

![FIG. 6. Hap4p protein levels correspond to the mRNA induction.](image)

(A) Northern blot analysis was performed with total RNA isolated from yeast strain BY4733 (HAP4) that was grown in rich medium containing glucose, raffinose, or lactate as indicated. The membrane was hybridized with radiolabeled probes specific for HAP4, CYC1, or SNR20. SNR20 was used to normalize RNA loading. B. Total protein extracts were prepared from yeast strains BY4733 (untagged HAP4) and DMY187 (3xHA-tagged HAP4) that was grown in rich medium containing glucose, raffinose, or lactate as indicated. The protein extracts were separated by SDS-PAGE using a 4 to 15% gradient gel, transferred to an Immobilon-P membrane, and probed with the anti-HA monoclonal antibody 12CA5 and anti-TBP antisera. The TBP protein was used as a loading control.
HAP4 mRNA levels were induced threefold in response to growth in YPRaffinose and YPLactate, consistent with the published data (15). As a control, we examined the induction of a Hap2p/Hap3p/Hap4p/Hap5p-regulated gene, CYC1, whose mRNA levels were induced 10 to 12 fold. To evaluate the induction of Hap4p protein synthesis, the untagged yeast strain BY4733 (HAP4) and epitope-tagged strain DMY187 (HAP4-3xHA) were grown under the conditions described above, and total protein extracts were prepared and separated by SDS-PAGE. The level of Hap4p was evaluated by Western blotting with an anti-HA monoclonal antibody (Fig. 6B). As a control for protein loading, the blot was reprobed with anti-TBP antiserum. The Hap4p protein levels were found to be induced approximately threefold during the growth in YPRaffinose and YPLactate, consistent with the mRNA levels. Interestingly, there was a detectable level of HAP4 mRNA and Hap4p even under glucose-repressing conditions (Fig. 6), which raises a question as to whether other posttranslational regulatory mechanisms are involved in controlling the association of Hap4p with the Hap2p/Hap3p/Hap5p-DNA complex. It seemed surprising that a threefold increase in Hap4p protein levels would yield a 10- to 12-fold activation of the target gene CYC1. Moreover, mobility shift assays performed with extracts prepared from yeast grown on glucose do not show any detectable Hap2p/Hap3p/Hap4p/Hap5p heterotetramer bound to DNA, yet the Hap2p/Hap3p/Hap4p/Hap5p complex is observed in extracts prepared from cells grown in lactate-containing medium (see Fig. 6 of reference 38). In summary, our data show that the inducibility of Hap4p protein levels correlates with the mRNA but suggests that the association of Hap4p with Hap2p/Hap3p/Hap5p may be posttranslationally regulated.

**DISCUSSION**

In this report, we have made several novel findings related to the assembly of the CCAAT-binding factor in *S. cerevisiae*. First, the assembly of the Hap2p/Hap3p/Hap5p heterotrimer does not follow a two-step assembly pathway analogous to its mammalian counterpart (48) but assembles via a one-step mechanism requiring the presence of all three subunits for stable complex formation. Second, the interaction of Hap4p with Hap2p/Hap3p/Hap5p requires the heterotrimeric complex to be bound to DNA at a CCAAT site. Third, we have identified the minimal region of Hap4p that is necessary for interaction with the Hap2p/Hap3p/Hap5p-DNA complex both in vivo and in vitro and have shown that Hap4p is present in one copy per DNA-bound Hap complex. Fourth, we have shown that the level of Hap4p protein expression under repressing conditions correlates with the induction of HAP4 mRNA, suggesting that protein levels are not likely to be controlled via a translational mechanism that involves the short open reading frames in the 5′ UTR of the mRNA.

We suspected that the assembly of the Hap2p/Hap3p/Hap5p heterotrimer may differ from its mammalian counterparts for the reasons already enumerated. Sinha et al. (47) and Kim et al. (29) have performed detailed mutational studies of CBF-A (Hap3p homolog) and CBF-C (Hap5p homolog), respectively, to delimit the regions of each protein that are necessary for heterodimer formation. A comparison of the amino acid sequences for the yeast and mammalian homologs within these regions revealed several nonconserved amino acid residues that could explain the different stabilities of the heterodimers; however, the functional relevance remains unclear. One possibility may relate to the fact that some organisms, such as plants (13, 21) and fungi (51; our unpublished observations) have multiple genetic loci that encode different variants of each Hap subunit. For example, *Candida albicans* has two distinct genetic loci encoding Hap3p homologs (unpublished observations), while *Arabidopsis thaliana* encodes six different nuclear factors YA (NF-YAs), nine NF-YBs, and eight NF-YCs (21), the homologs of Hap2p, Hap3p, and Hap5p, respectively. In contrast, mammalian organisms appear to have a single gene encoding each CCAAT-binding factor subunit (32, 49). Thus, the organisms that encode multiple variants of each subunit may have evolved with a less stable heterodimer to facilitate the interchange of subunits for different roles in gene regulation. Although *S. cerevisiae* does not have multiple loci encoding each Hap subunit, it may have evolved a similar pattern of stability.

The requirement for Hap2p/Hap3p/Hap5p to be bound to DNA as a prerequisite for Hap4p association raised a number of questions about the protein-protein and/or protein-DNA contacts within the complex. We have previously shown that the Hap4p recruitment domain of Hap5p is necessary for Hap4p to stably associate with Hap2p/Hap3p/Hap5p (37); however, the data in this report show that DNA binding is also important. We have previously reported that the Hap4p recruitment domain of Hap5p fused to the LexA DNA-binding domain is not sufficient to recruit Hap4p to a promoter containing lexA-binding sites (37). Thus, DNA and the Hap4p recruitment domain of Hap5p alone are not sufficient to bring Hap4p to a promoter, implying that Hap2p and Hap3p are also needed. In fact, previous studies have suggested that Hap4p may interact with Hap5p (55); however, the nature of this interaction is not understood. We envision two models for the interaction of Hap4p with the DNA-protein complex which would not be mutually exclusive. The first model involves a conformational change in Hap2p/Hap3p/Hap5p after binding to its cognate CCAAT site that allows the stable association of Hap4p. The alternative model involves Hap4p directly contacting both DNA and Hap2p/Hap3p/Hap5p for stable assembly. By this model, the Hap4p-DNA contact does not have to be sequence specific, since the DNA-bound Hap2p/Hap3p/Hap5p would provide sequence specificity. These hypotheses will be resolved by additional studies using fluorescence spectroscopy and DNA-protein cross-linking experiments.

The minimal functional region of Hap4p required for interaction with the Hap2p/Hap3p/Hap5p-DNA complex lies between amino acid residues 23 and 180. The evolutionarily conserved domain of Hap4p (4, 51) is within this domain, and we presume that it is required for contact with the Hap4p recruitment domain on Hap5p, although this interaction has not yet been established. Interestingly, Stebbins and Triezenberg (50) have shown that Hap4p contains two transcriptional activation domains. By fusing various segments of Hap4p to the LexA DNA-binding domain and assaying their activation potential with a *lexA-lacZ* reporter, these investigators identified two distinct activation domains. The first activation domain was mapped between amino acid residues 124 and 329, and a sec-
ond mapped between amino acid residues 359 and 476. They generated point mutations within the first activation domain, namely, a triple point mutant (F148S, L149S, and F151S) that nearly abolished transcriptional activation. Our data raise a question about whether this region is competent to stimulate transcription in the context of the native CCAAT-binding factor, since Hap4p (23-180) failed to activate transcription unless fused to the VP16 activation domain (Table 2). However, we cannot rule out the possibility that additional C-terminal residues between amino acids 180 and 329 may contribute to activated transcription.

Why is the Hap4p subunit absent from the CCAAT-binding complexes of higher eukaryotes? One the basis of studies performed with the mammalian CCAAT-binding factor, this DNA-binding complex functions as a proximal promoter factor that works synergistically with other highly regulated activators to control gene expression (36). The mammalian homologs of Hap2p and Hap5p contain glutamine-rich regions, like that of another proximal promoter factor, Sp1 (8), that function as activation domains (9). Thus, the mammalian CCAAT-binding factor appears to have evolved to serve a more general function in transcription at numerous target genes (36), while the fungal CCAAT-binding factors appear to act as gene-specific transcriptional regulators. Thus, controlling the expression of Hap4p is pivotal in the regulation of downstream target genes.

On the basis of our results, we propose a model for the assembly of Hap2p/Hap3p/Hap4p/Hap5p in S. cerevisiae. Initially, Hap2p, Hap3p, and Hap5p assemble in a single step to form the DNA-binding heterotrimer, which can bind target promoters containing the cognate CCAAT site. Once bound to DNA, the Hap2p/Hap3p/Hap5p complex then recruits Hap4p to facilitate activated transcription under the appropriate environmental conditions. HAP2, HAP3, and HAP5 have been shown to be constitutively expressed in yeast (12). Thus, Hap2p/Hap3p/Hap5p may be constitutively bound at target promoters poised for the interaction with Hap4p and subsequent gene activation; however, constitutive DNA binding by Hap2p/Hap3p/Hap5p has not been demonstrated. If Hap2p/Hap3p/Hap5p were constitutively bound to DNA, then the induction of HAP4 mRNA and protein (Fig. 6) in response to the lack of glucose would permit a rapid reprogramming of gene expression when dictated by changes in nutrient availability. Consistent with the model, previous studies have shown that constitutive overexpression of HAP4 on glucose-containing medium results in the transcriptional induction of many Hap2p/Hap3p/Hap4p/Hap5p-regulated genes (31), implying that Hap2p/Hap3p/Hap5p is competent to bind DNA in the presence of glucose and that Hap4p is limiting for activated transcription. While these studies would imply that posttranslational modification of Hap4p is not necessary for activation on glucose-containing medium, we are cautious in making this assumption, since overexpression may override the normal posttranslational regulatory controls.

ACKNOWLEDGMENTS

We thank Fred Winston for generously providing yeast strains and Leonard Guarente for providing plasmids, yeast strains, and the anti-TBP antisem. D.S.M. expresses his appreciation to Leonard Guarente for his advice and encouragement with this research. This work was supported by startup funds from the University of Arkansas and NIH grant R01AI51470 to D.S.M.

REFERENCES

1. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1994. Current protocols in molecular biology. Green Publishing Associates and Wiley-Interscience, New York, N.Y.

2. Baxevanis, A. D., G. Arens, E. N. Moudrianakis, and D. Landsman. 1995. A variety of DNA-binding and multimeric proteins contain the histone fold motif. Nucleic Acids Res. 23:2695–2691.

3. Bellorini, M., D. K. Lee, J. C. Dantonel, K. Zemzoumi, R. G. Roeder, L. Tora, and R. Mantovani. 1997. CCAAT binding NF-Y-TBP interactions: NF-YB and NF-YC require short domains adjacent to their histone fold motifs for association with TBP basic residues. Nucleic Acids Res. 25:2174–2181.

4. Bourgarel, D., C. C. Nguyen, and M. Bolotin-Fukuhara. 1999. HAP4, the glucose-repressed regulated subunit of the HAP transcriptional complex involved in the fermentation-respiration shift, has a functional homologue in the respiratory yeast Kluyveromyces lactis. Mol. Microbiol. 31:1205–1215.

5. Brachmann, C. R., A. Davies, G. J. Cost, E. Caputo, J. L. Fr Hieter, and J. D. Boeke. 1998. Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast 14:115–132.

6. Bucher, P. 1990. Weight matrix descriptions of four eukaryotic RNA polymerase II promoter elements derived from 502 unrelated promoter sequences. J. Mol. Biol. 212:563–578.

7. Clifton, P. F., L. W. Hillier, L. Fulton, T. Graves, T. Miner, W. R. Gish, R. H. Waterston, and M. J. Stalston. 2001. Survey of Escherichia genomes to identify functional elements by comparative DNA sequence analysis. Genome Res. 11:1175–1186.

8. Courty, A., and R. Tjian. 1988. Analysis of Sp1 in vivo reveals multiple transcriptional domains, including a novel glutamine rich activation motif. Cell 55:887–899.

9. Cours, F., S. N. Maity, S. Sinha, and B. de Crombrugghe. 1996. The transcriptional activity of the CCAAT-binding factor CBF is mediated by two distinct activation domains, one in the CBF-B subunit and the other in the CBF-C subunit. J. Biol. Chem. 271:14485–14491.

10. Dang, V. D., C. Bohn, M. Bolotin-Fukuhara, and B. Daigam-Fornier. 1996. The CCAAT box-binding factor stimulates ammonium assimilation in Saccharomyces cerevisiae, defining a new cross-pathway regulation between nitrogen and carbon metabolism. J. Bacteriol. 178:1842–1849.

11. Dang, V. D., M. Valens, M. Bolotin-Fukuhara, and B. Daigam-Fornier. 1996. Cloning of the ASN1 and ASN2 genes encoding asparagine synthetases in Saccharomyces cerevisiae: differential regulation by the CCAAT-box-binding factor. Mol. Microbiol. 22:681–692.

12. Delisi, J. J., V. R. Iyer, and P. O. Brown. 1997. Exploring the metabolic and genetic control of gene expression on a genomic scale. Science 278:680–686.

13. Edwards, D., J. A. Murray, and A. G. Smith. 1998. Multiple genes encoding the conserved CCAAT-box transcription factor complex are expressed in Arabidopsis. Plant Physiol. 117:1005–1022.

14. Flores, C. L., C. Rodriguez, T. Petit, and C. Gancedo. 2000. Carbohydrate and energy-yielding metabolism in non-conventional yeasts. FEMS Microbiol. Rev. 24:507–529.

15. Forsburg, S. L., and L. Guarente. 1988. Yeast HAP2 and HAP3: transcriptional regulatory locus of HAP4: a third component of the CCAAT-bound HAP2/HAP3 heteromer. Genes Dev. 3:1166–1178.

16. Gancedo, J. M. 1998. Yeast carbon catabolite repression. Microbiol. Mol. Biol. Rev. 62:334–361.

17. Gietz, R. D., R. H. Schiestl, A. R. Willems, and R. A. Woods. 1995. Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast 11:355–360.

18. Gietz, R. D., and A. Sugino. 1988. New yeast- Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene 74:527–534.

19. Guan, K. L., and J. E. Dixon. 1991. Eukaryotic proteins expressed in Escherichia coli: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. Anal. Biochem. 192:262–267.

20. Guarente, L., and M. Ptashne. 1981. Fusion of Escherichia coli lacZ to the cytochrome c gene of Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA 78:2199–2203.

21. Gusmaroli, G., C. Tonelli, and R. Mantovani. 2001. Regulation of the CCAAT-binding NF-Y subunits in Arabidopsis thaliana. Gene 264:173–185.

22. Guthrie, C., and R. G. Fink. 1991. Guide to yeast genetics and molecular biology. Academic Press, San Diego, CA.

23. Hahn, S., and L. Guarente. 1988. Yeast HAP2 and HAP3: transcriptional activators in a heteromic complex. Science 240:317–321.

24. Hahn, S., J. Pinkham, R. Wei, R. Miller, and L. Guarente. 1988. The HAP3 regulatory locus of Saccharomyces cerevisiae encodes divergent overlapping transcripts. Mol. Cell. Biol. 8:655–663.

25. Harlow, E., and D. Lane. 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

26. Hoffman, C. S., and F. Winston. 1987. A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of Escherichia coli. Gene 57:267–272.
27. Horvath, A., and H. Riezman. 1994. Rapid protein extraction from Saccharomyces cerevisiae. Yeast 10:1305–1310.

28. Iizuka, N., L. Najita, A. Franuszco, and P. Sarnow. 1994. Cap-dependent and cap-independent translation by internal initiation of mRNAs in cell extracts prepared from Saccharomyces cerevisiae. Mol. Cell. Biol. 14:7522–7330.

29. Kim, I. S., S. Sinha, B. de Crombrugghe, and S. N. Maity. 1998. The molecular biology of the CCAAT-binding factor. Mol. Cell. Biol. 18:3377–3385.

30. Klein, C. J., L. Olsson, and J. Nielsen. 1998. Glucose control in Saccharomyces cerevisiae: the role of Mig1 in metabolic functions. Microbiology 144:13–24.

31. Lascaris, R., H. J. Bussemaker, A. Boorsma, M. Piper, H. van der Spek, L. Grivell, and J. Blom. 2003. Hap4p overexpression in glucose-grown Saccharomyces cerevisiae induces cells to enter a novel metabolic state. Genome Biol. 4:R3.

32. Li, X. Y., M. G. Mattei, Z. Zaleska-Rutczynska, R. Hoefft van Huijsduijnen, F. Figueroa, J. Nadeau, C. Benoist, and D. Mathis. 1991. One subunit of the transcription factor NF-Y maps close to the major histocompatibility complex in murine and human chromosomes. Genomics 11:630–634.

33. Liang, S. G., and S. N. Maity. 1994. Pathway of complex formation between DNA and three subunits of CBF/NF-Y. Photocross-linking analysis of DNA-protein interaction and characterization of equilibrium steps of subunit interaction and DNA binding. J. Biol. Chem. 273:31590–31598.

34. Maity, S. N., S. Sinha, E. C. Rutehouser, and B. de Crombrugghe. 1992. Three different polypeptides are necessary for DNA binding of the mammalian heteromeric CCAAT-binding factor. J. Biol. Chem. 267:16574–16580.

35. Mantovani, R. 1999. The molecular biology of the CCAAT-binding factor NF-Y. Gene 239:15–27.

36. Mantovani, R. 1999. Cloning of yeast HAP5: a novel subunit of a heterotrimeric complex required for CCAAT binding. Mol. Cell. Biol. 17:7088–7018.

37. McNabb, D. S., K. A. Tseng, and L. Guarente. 1999. The Saccharomyces cerevisiae Hap5p homolog from fission yeast reveals two conserved domains that are essential for assembly of heterotrimeric CCAAT-binding factor. Mol. Cell. Biol. 19:6562–6570.

38. McNabb, D. S., Y. Xing, and L. Guarente. 1995. Cloning of yeast HAP5: a novel subunit of a heterotrimeric complex required for CCAAT binding. Genes Dev. 9:47–58.

39. Olesen, J. T., and L. Guarente. 1990. The HAP2 subunit of yeast CCAAT transcriptional activator contains adjacent domains for subunit association and DNA recognition: model for the HAP2/3/4 complex. Genes Dev. 4:1714–1729.

40. Pinkham, J. L., J. T. Olesen, and L. P. Guarente. 1987. Sequence and nuclear localization of the Saccharomyces cerevisiae HAP2 protein, a transcriptional activator. Mol. Cell. Biol. 7:578–585.

41. Riego, L., A. Avendano, A. DeLuna, E. Rodriguez, and A. Gonzalez. 2002. GDH1 expression is regulated by GLN3, GCM4, and HAP4 under respiratory growth. Biochem. Biophys. Res. Commun. 290:79–85.

42. Romier, C., F. Cocchiarella, R. Mantovani, and D. Moras. 2003. The NF-Y/NF-C structure gives insight into DNA binding and transcription regulation by CCAAT factor NF-Y. J. Biol. Chem. 278:1336–1345.

43. Sambrook, J., E. G. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

44. Schneider, B. L., W. Seufert, B. Steiner, Q. H. Yang, and A. B. Futcher. 1995. Use of polymerase chain reaction epitope tagging for protein tagging in Saccharomyces cerevisiae. Yeast 11:1265–1274.

45. Schuller, H. J. 2003. Transcriptional control of nonfermentative metabolism in the yeast Saccharomyces cerevisiae. Curr. Genet. 43:139–160.

46. Seino, A., Y. Yanagida, M. Aizawa, and E. Kohatake. 2003. Translational control by internal ribosome entry site in Saccharomyces cerevisiae. Biochim. Biophys. Acta 1681:166–174.

47. Sinha, S., I. S. Kim, K. Y. Sohn, B. de Crombrugghe, and S. N. Maity. 1996. Three classes of mutations in the A subunit of the CCAAT-binding factor CBF delineate functional domains involved in the three-step assembly of the CBF-DNA complex. Mol. Cell. Biol. 16:326–337.

48. Sinha, S., S. N. Maity, J. Lu, and B. de Crombrugghe. 1995. Recombinant rat CBF-C, the third subunit of CBF/NF-Y, allows formation of a protein-DNA complex with CBF-A and CBF-B and with yeast HAP2 and HAP3. Proc. Natl. Acad. Sci. USA 92:1624–1628.

49. Sinha, S., S. N. Maity, M. F. Seldin, and B. de Crombrugghe. 1996. Chromosomal assignment and tissue expression of CBF-C/NFY-C, the third subunit of the mammalian CCAAT-binding factor. Genomics 37:260–263.

50. Stebbins, J. L., and S. J. Triezenberg. 2004. Identification, mutational analysis, and coactivator requirements of two distinct transcriptional activation domains of the Saccharomyces cerevisiae Hap4p protein. Eukaryot. Cell 3:339–347.

51. Sybrina, B., K. Giardi, Y. F. Li, W. G. Bao, M. Bolotin-Fukuhara, and A. Delahodde. 2005. A new Hansenula polymorpha HAP4 homologue which contains only the N-terminal conserved domain of the protein is fully functional in Saccharomyces cerevisiae. Yeast 22:1221–1229.

52. Triezenberg, S. J., K. L. LaMarco, and S. L. McKnight. 1988. Evidence of DNA/protein interactions that mediate HSV-1 immediate early gene activation by VP16. Genes Dev. 2:730–742.

53. Walker, G. M. 1998. Yeast: physiology and biotechnology. John Wiley & Sons, Ltd., West Sussex, England, United Kingdom.

54. Winston, F., C. Dollard, and S. L. Ricupero-Hovasse. 2005. Construction of a set of convenient Saccharomyces cerevisiae strains that are isogenic to S288C. Yeast 21:593–599.

55. Xing, Y., J. D. Fikes, and L. Guarente. 1994. Subunit interaction in the CCAAT-binding heteromeric complex is mediated by a very short alpha-helix in HAP2. Proc. Natl. Acad. Sci. USA 91:3099–3103.

56. Zitomer, R. S., and C. V. Lowry. 1992. Regulation of gene expression by oxygen in Saccharomyces cerevisiae. Microbiol. Rev. 56:1–11.