The *Saccharomyces cerevisiae* Hap5p Homolog from Fission Yeast Reveals Two Conserved Domains That Are Essential for Assembly of Heterotetrameric CCAAT-Binding Factor

DAVID S. MCNABB, KELLY A.-S. TSENG, AND LEONARD GUARENTE*

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

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The CCAAT-binding factor is an evolutionarily conserved heteromeric transcription factor that binds to CCAAT box-containing upstream activation sites within the promoters of numerous eukaryotic genes. The CCAAT-binding factor from *Saccharomyces cerevisiae* is a heterotetramer that contains the subunits Hap2p, Hap3p, Hap4p, and Hap5p and that functions in the activation of genes involved in respiratory metabolism. Here we describe the isolation of the cDNA encoding the *Schizosaccharomyces pombe* homolog of Hap5p, designated php5*. We have shown that Php5p is a subunit of the CCAAT-binding factor in fission yeast and is required for transcription of the S. pombe cycl*1* gene. Analysis of the evolutionarily conserved regions of Hap5p, Php5p, and the mammalian homolog CBF-C revealed two essential domains within Hap5p that are required for DNA binding and transcriptional activation. One is an 87-amino-acid core domain that is conserved among Hap5p, Php5p, and CBF-C and that is required for the assembly of the Hap2p-Hap3p-Hap5p heterotrimer both in vitro and in vivo. A second domain that is essential for the recruitment of Hap4p into the CCAAT-binding complex was identified in Hap5p and Php5p.

The transcriptional activation of gene expression is mediated by the binding of distinct regulatory factors to specific cis-acting DNA sequence elements, referred to as enhancers or upstream activation sites, located within the promoters of eukaryotic genes. Many of the DNA-binding transcriptional activators interact with specific DNA sequence elements as either homodimers or heterodimers and can be classified into several groups according to the structural motifs that are necessary for dimerization (41). Other transcriptional activators are heteromeric complexes consisting of multiple polypeptides that interact in a specific manner to recognize cis-acting promoter sequences. Examples of such protein complexes include the herpes simplex virus activator VP16 that forms a complex with cellular DNA-binding proteins, including Oct-1, to activate the transcriptional machinery (13, 34, 44); the interferon-stimulated complex that binds specifically to the interferon-stimulated response element (9, 24, 28, 29); the GA-binding protein complex that is required for VP16-mediated activation of herpes simplex virus immediate-early genes (27, 55); and the core binding protein complex that promotes T-cell-specific expression of several genes (57). In all of these cases, one polypeptide from the complex can bind to DNA in a site-specific manner in the absence of the other proteins from the complex.

The CCAAT-binding factor is a heteromeric DNA-binding complex that binds to promoter elements containing the pentanucleotide CCAAT sequence. In the yeast *Saccharomyces cerevisiae*, the CCAAT-binding factor contains four subunits, designated Hap2p, Hap3p, Hap4p, and Hap5p (11, 18, 35, 38), that are required for the transcriptional activation of numerous nuclear genes whose products are involved in mitochondrial functions (12, 61). The *HAP2*, *HAP3*, and *HAP4* genes were initially identified because mutations in these genes abolish CCAAT box-dependent transcriptional activation in vivo (11, 19, 42, 43). The gene encoding the fourth subunit, Hap5p, was recently identified in a two-hybrid screen via its interaction with the Hap2p-encoding gene (35). Yeast strains containing *hap2*, *hap3*, *hap4*, or *hap5* null alleles fail to grow on media containing nonfermentable carbon sources such as lactate or glycerol (11, 19, 35, 43), a phenotype characteristic of the respiratory defect. Moreover, cell extracts prepared from *hap2*, *hap3*, or *hap5* mutants no longer exhibit CCAAT-binding activity in vitro (35, 38). Through the use of size variants, Hap2p, Hap3p, and Hap5p were shown to be present in the DNA-bound complex and to remain stably associated in the absence of DNA (18, 35, 38). Furthermore, recombinant Hap2p, Hap3p, and Hap5p were shown to bind specifically to a CCAAT box-containing probe in mobility shift assays, with the DNA-binding activity being dependent on the presence of all three subunits (35). Thus, the CCAAT-binding factor is unique among heteromeric transcription factors in that it absolutely requires the three heterologous subunits for DNA-binding activity (31, 35, 53).

The final subunit of the complex, Hap4p, contains an acidic activation domain (11) that strongly activates transcription when fused to a heterologous DNA-binding domain (39). In strains containing a *hap4* null allele, genes that contain a CCAAT box are not activated (11, 39); however, this defect can be overcome by fusing the Gal4p activation domain to Hap2p (39) or the B42 activation domain to Hap5p (35). These results indicate that Hap4p is not essential for the interaction of the Hap2p-Hap3p-Hap5p heterotrimer with the CCAAT box in vivo but is required for transcriptional activation.

The polypeptides encoded by *HAP2*, *HAP3*, and *HAP5* have been both structurally and functionally conserved over evolution. Genes encoding homologs of Hap2p, Hap3p, and Hap5p have been isolated from numerous organisms (5, 22, 30, 32, 40, 53, 56, 59). Moreover, Hap2p and Hap3p can functionally substitute for the corresponding subunits of the human CCAAT-binding factor to restore DNA binding at the CCAAT box (6). Correspondingly, CBF-C, the rat homolog of Hap5p,
can functionally replace this subunit in mobility shift assays with recombinant Hap2p and Hap3p (53). The high degree of structural and functional conservation of the CCAAT-binding factor has been useful in delimiting the minimum functional domains of the subunits. For example, the various homologs of Hap2p and Hap3p contain strongly conserved regions of 60 and 93 amino acid residues, respectively (30, 59), and these core domains within Hap2p and Hap3p are sufficient for complementation of hap2Δ and hap3Δ mutants, respectively (39, 59).

The evolutionary divergence of the fission yeast Schizosaccharomyces pombe from the more commonly studied budding yeast S. cerevisiae has made it an attractive organism for comparative evolutionary analyses. In fact, S. pombe has been judged to be as evolutionarily divergent from S. cerevisiae as it is from metazoan eukaryotic lineages (46). Thus, S. pombe is an ideal organism for examining the evolutionarily conserved domains of the various CCAAT-binding factor subunits. For example, the S. pombe homologs of Hap2p and Hap3p, termed Php2p and Php3p, respectively, show amino acid sequence homology that is restricted to regions of the polypeptides that were defined by deletion studies as the minimum functional domains (39, 40, 59). Therefore, to define the essential functional region of Hap5p, we cloned the cDNA for the gene encoding the S. pombe homolog of Hap5p (designated php5+, for pombe HAP5) by in vivo complementation of a hap5+ mutant of S. cerevisiae. We subsequently examined the function of the php5+ gene product in both S. pombe and S. cerevisiae. Through these analyses, we identified a conserved core domain that is present in Hap5p, Php5p, and CBF-C and that is required for assembly of the heterotrimeric Hap2p-Hap3p-Hap5p DNA-binding component of the CCAAT-binding factor both in vitro and in vivo. In addition, we identified a second domain that is conserved in Hap5p and Php5p but is lacking in CBF-C. We demonstrate that this domain is necessary for the recruitment of Hap4p into the CCAAT-binding complex of S. cerevisiae. The presence of the Hap4p recruitment domain in Php5p raises the intriguing possibility that Hap4p homologs may exist in other eukaryotic organisms containing homologs of Hap2p, Hap3p, and Hap5p.

### Materials and Methods

**Yeast strains and methods.** The yeast strains used in these studies are listed in Table 1. The S. cerevisiae strains are isogenic derivatives of BWG1-7a (16), and the S. pombe strains are derivatives of 972 (h−). S. cerevisiae DMY116 was generated by disrupting HAP5 in strain JO1-1a with the 5′-hisG fragment of plasmid pDM212 (35). Strain DMY118 was constructed by disrupting HAP4 in strain DMY110 with the 5′-hisG fragment of plasmid pKs::HAP4hisG (35). S. pombe DMP100 is a php5 mutant derivative of JFP38 that was generated by transforming JFP38 with the 3.9-kb NotI php1::ura4+ fragment of plasmid pDM268.

S. cerevisiae strains were grown on rich, synthetic complete, and 5-fluoro-orotic acid media prepared as previously described (50, 51) and supplemented with 2% glucose or 2% lactate. S. pombe strains were grown on YEC medium (0.5% yeast extract, 0.2% Casamino Acids, either 3% glucose or 3% glycerol–0.1% glucose) supplemented with 250 μg each of uracil and adenine per ml (23), on SC-Ura (50) containing 3% glucose, and on synthetic minimal medium containing either 3% glucose or 3% glycerol–0.1% glucose and the appropriate auxotrophic supplements at 250 μg/ml. For S. cerevisiae, DNA transformations were performed by the lithium acetate method (14), and a modification of this method was used for S. pombe DNA transformations (23).

**Cloning and sequencing of the php5+ cDNA.** A cDNA library prepared with S. pombe mRNA and vector pBD20 (5) was introduced into S. cerevisiae DMY110 through eight independent transformation reactions, and the Ura+ transformants were selected on SC-Ura. The transformants were rescued (21) from a single isolate from each pool and introduced into Escherichia coli XL1 blue (Stratagene) by electroporation. Sequencing was performed by the dideoxy chain termination method (49) with the Sequenase system (U.S. Biochemicals). The templates for sequencing were single-stranded phage DNA containing the polymerase chain reaction (PCR) fragments of plasmid pDM223 generated by disrupting HAP4 in strain DMY110 with the 5′-hisG fragment of plasmid pKs::HAP4hisG (35). S. pombe strains were transformed using the lithium acetate method (14), and a modification of this method was used for S. pombe DNA transformations (23).

### Table 1. Strains used in this study

| Strain               | Genotype         | Reference or source |
|----------------------|------------------|---------------------|
| Saccharomyces cerevisiae |
| BWG1-7a              | MATa ura3-52 leu2-3,112 his4-519 ade1-100 | 16 |
| JO1-1a               | BWG1-7a hap5::hisG | 39 |
| DMY110               | BWG1-7a hap5::hisG | 39 |
| DMY16                | BWG1-7a hap5::hisG | 39 |
| DMY118               | BWG1-7a hap5::hisG | 39 |
| Schizosaccharomyces pombe |
| JFP38                | h− ura4-D18 leu1-32 ade6-M210 | 40 |
| JFP47                | JFP38 php2Δ::ura4+ | 40 |
| DMP100               | JFP38 php5::ura4+ | 40 |

### Table 2. Primer sequences used for PCR

| Primer | Sequence of oligonucleotide |
|--------|-----------------------------|
| D22205 | 5′-GCCGGGGCGCCGCGGGCTAATCAGTGATCGTAACTGCGTGAATTCGCGATACCTTGGTTGCGATGC-3′ |
| D22206 | 5′-GCCGGGGATTCGCGGGTTAAGCTATGCGTAACTTCGCGATACCTTGGTTGCGATGC-3′ |
| D20352 | 5′-GCCGGGGATTCGCGGGTTAAGCTATGCGTAACTTCGCGATACCTTGGTTGCGATGC-3′ |
| dDM0015 | 5′-GCCGGGGATTCGCGGGTTAAGCTATGCGTAACTTCGCGATACCTTGGTTGCGATGC-3′ |
| dDM0016 | 5′-GCCGGGGATTCGCGGGTTAAGCTATGCGTAACTTCGCGATACCTTGGTTGCGATGC-3′ |
| dDM0017 | 5′-GCCGGGGATTCGCGGGTTAAGCTATGCGTAACTTCGCGATACCTTGGTTGCGATGC-3′ |
| dDM0018 | 5′-GCCGGGGATTCGCGGGTTAAGCTATGCGTAACTTCGCGATACCTTGGTTGCGATGC-3′ |
| dDM0027 | 5′-GCCGGGGATTCGCGGGTTAAGCTATGCGTAACTTCGCGATACCTTGGTTGCGATGC-3′ |
| dDM0028 | 5′-GCCGGGGATTCGCGGGTTAAGCTATGCGTAACTTCGCGATACCTTGGTTGCGATGC-3′ |
| dDM0029 | 5′-GCCGGGGATTCGCGGGTTAAGCTATGCGTAACTTCGCGATACCTTGGTTGCGATGC-3′ |
| dDM0030 | 5′-GCCGGGGATTCGCGGGTTAAGCTATGCGTAACTTCGCGATACCTTGGTTGCGATGC-3′ |
| dDM0032 | 5′-GCCGGGGATTCGCGGGTTAAGCTATGCGTAACTTCGCGATACCTTGGTTGCGATGC-3′ |
| dDM0033 | 5′-GCCGGGGATTCGCGGGTTAAGCTATGCGTAACTTCGCGATACCTTGGTTGCGATGC-3′ |
| dDM0034 | 5′-GCCGGGGATTCGCGGGTTAAGCTATGCGTAACTTCGCGATACCTTGGTTGCGATGC-3′ |
DMY110. The disrupted allele of php5+ was generated by subcloning the 2.5-kb Not1 fragment containing php5+ from pDM242 into the Not1 site of Bluescript KS+ (Stratagene) to create plasmid pDM243. To remove the Clal and HindIII sites from the polinker, pDM243 was digested with HindIII and Xhol, the cohesive ends were blunt filled with the Klenow fragment, and into the Not1 site added. The plasmid was subsequently digested with Clal, the ends were made flush with the Klenow fragment, and a HindIII linker was added. The plasmid was then digested with HindIII, and a 1.6-kb HindIII fragment containing the used *HAP1* gene from pUC8-ura4 (3; a generous gift from Charles Hoffman, Boston College, Boston, Mass.) was ligated into the site to create plasmid pDM268. Plasmid pDH24-2aL contains the complete coding region of HAP4 fused in frame at codon 258 of HAP2. The plasmid was constructed by PCR amplification of HAP4 from pH2H4-2 (2) cloned in the presence of [35S]methionine and during translation. For the in vitro protein-protein interaction studies, the Novagen single-tube protein translation system was used. The in vitro transcription-translation reactions, programmed with 2 μM of pDM378 or pDM379, were carried out according to the manufacturer’s protocol. For in vitro protein-protein interaction studies, Hap4p was labeled with [35S]methionine during translation. For mobility shift assay, Hap4p was not radiolabeled, however, parallel reaction was precluded in the presence of [35S]methionine to verify the translation products generated.

**DNA binding assays and gel electrophoresis.** The UAS2U1 probe containing the CCAAT box was previously described (35). The DNA probe was end labeled with [α-32P]ATP (6,000 Ci/mmol; Amersham Corp.) with the Klenow fragment. All DNA-binding reaction mixtures with yeast extracts contained 20 to 30 μg of protein, 12 mM HEPES-NaOH (pH 7.9), 60 mM KCl, 4 mM Tris-HCl (pH 7.9), 1 mM EDTA, 12% glycerol, 4.2 mM β-mercaptoethanol, 3.0 μg of poly(dI-dC), 3.0 μg of denatured salmon sperm DNA, and 0.5 to 1.0 ng of the UAS2U1 probe in a final volume of 20 μL. For reaction mixtures containing purified recombinant Hap2p, Hap3p, or Php5p, the conditions were identical except that each subunit was present at approximately 100 ng, and the nonspecific competitor was 200 ng of poly(dI-dC). For reaction mixtures containing in vitro translation extracts along with recombinant proteins, 2.0 μg of poly(dI-dC) was used as the nonspecific competitor. Reaction mixtures were incubated at 23°C for 30 min. The protein-DNA complexes were resolved by gel electrophoresis and autoradiography as performed previously described (35).

**Isolation of RNA and Northern blot analysis.** S. pombe strains were grown to a density of 3 × 10^7 to 7 × 10^7 cells/mL in YEC medium containing either 3% glucose or 3% galactose (48). The strains were then harvested, and the RNA was isolated from the cells by acid guanidinium thiocyanate-phenol-chloroform extraction and ethanol precipitation. The RNA samples were then size fractionated on 1% agarose gels and transferred to nylon membranes (Amersham). The membranes were hybridized with uniformly labeled DNA fragments as described (48). The membranes were washed under high-stringency conditions (48). The probes used for hybridization were the 0.85-kb EcoRI fragment of the S. pombe cycl1+ gene (5) from plasmid pH777 and the 2.1-kb HindIII fragment of the S. cerevisiae leu1+ gene from pYK311 (25; a generous gift from Fred Winston, Harvard Medical School, Boston, Mass.). The probes were purified by agarose gel electrophoresis and radiolabeled with [α-32P]dCTP (Amersham) by use of a random primer labeling kit (U.S. Biochemicals) according to the manufacturer’s protocol. The transcript levels were quantitated on a Molecular Dynamics PhosphorImager.

**Nucleotide sequence accession number.** Our DNA sequence of php5+ has been deposited in GenBank under accession number U68525.

**RESULTS**

**Isolation of the gene encoding the S. pombe homolog of Hap5p.** Previous studies demonstrated that hap5 mutants are unable to grow on rich medium containing lactate as the sole carbon source (Lat) (35). This Lat− phenotype provided a convenient selection by which to isolate the gene encoding the S. pombe homolog of Hap5p by in vivo complementation. Thus, an S. pombe cDNA library (5) was introduced into S. cerevisiae DMY110, and Lat− transformants were selected by growth on lactate medium. Four independent Lat− transformants were identified. To verify that the Lat− phenotype was conferred by a plasmid-encoded gene, the transformants were streaked on medium containing 5-fluoro-orotic acid to select for plasmid loss, and the Ura− segregants were tested for growth on lactate medium. All four transformants concomitantly lost the Ura− and Lat− phenotypes, demonstrating that the library plasmid was required for complementation. The plasmids were subsequently isolated from the four transformants, and restriction mapping studies indicated that all four contained a 2.5-kb EcoRI fragment of the S. pombe php5+ gene. Plasmid pDM242, used for further characterization, and the gene was designated php5+, for pombe HAP5. The complementation of DMY110 conferred by php5+ is shown in Fig. 1. Strain DMY110 was transformed with pDB20 (vector), pDM233 (HAP5), or pDM242 (php5+), and the transformants
were streaked on rich medium containing lactate or lactate as the carbon source. As a control, parent strain BWG1-7a containing pDB20 was also tested. As expected, all of the strains grew equally well on rich glucose medium (Fig. 1A), demonstrating the viability of the transformants. On rich lactate medium (Fig. 1B), DMY110 containing pDB20 had a Lat+ phenotype, as previously reported (35), whereas DMY110 containing the plasmids encoding either HAP5 or php5+ had a Lat− phenotype.

To further characterize the php5+ cDNA, we determined its complete nucleotide sequence. The php5+ open reading frame is predicted to encode a polypeptide of 415 amino acids with an estimated molecular weight of 46,644. Analysis of the predicted amino acid sequence of Php5p revealed that it contains a region (amino acid residues 71 through 102) that has 40% identity and 62% similarity to amino acid residues 115 through 146 of Hap5p (53), as shown in Figs. 2A and B. These results indicate that this conserved domain is likely to be critical for the function of these proteins. Interestingly, a second juxtaposed region of Php5p (amino acid residues 71 through 102) that has 40% identity and 62% similarity to amino acid residues 115 through 146 of Hap5p was identified (Fig. 2A and C); however, this region of homology is absent from CBF-C. The functional significance of these domains will be addressed in subsequent sections, but the comparison of the predicted amino acid sequences of Hap5p, CBF-C, and Php5p seemed to delineate two specific regions that may represent functionally important domains. No additional amino acid sequence homology was identified outside of these regions; however, the C terminus of Php5p was found to be rich in glutamine, serine, and proline residues. The relevance of this observation for S. pombe is unknown; however, the C terminus of CBF-C is also rich in glutamine residues (53), and recent in vitro studies indicated that this domain functions in transcriptional activation (8).

Functional analysis of php5+ in S. pombe. Previous studies demonstrated that php2 mutants are unable to grow on minimal medium containing 3% glycerol–0.1% glucose as the carbon source (40). To determine whether php5 mutants shared a similar phenotype, php5+ cDNA was used to generate a disrupted allele of php5+ by insertion of ura4+ within the coding sequence as described in Materials and Methods. php5 mutants were subsequently tested for their ability to grow on minimal medium containing a nonfermentable carbon source. Strain DMP100 (php5::ura4+) grew well on minimal medium containing glucose (Fig. 3A) but was unable to grow on medium containing 3% glycerol–0.1% glucose as the carbon source (Fig. 3B). Parent strain JFP38 grew well on both media (Fig. 3A and B), whereas JFP127 (php2Δ::ura4+) failed to grow on medium containing 3% glycerol–0.1% glucose (Fig. 3B) as previously described (40). Thus, php5 mutants displayed a phenotype identical to that of php2 mutants, suggesting that php5+ encodes a component of the S. pombe CCAAT-binding factor. It should also be emphasized that php2 and php5 mutant strains formed small colonies on medium containing 3% glycerol–0.1% glucose due to the presence of the glucose; however, these colonies did not continue to grow after the glucose was depleted, even after prolonged incubation (36).

To further address whether Php5p is a component of the S. pombe CCAAT-binding factor, DNA mobility shift assays were performed with cell extracts prepared from strain DMP100 (php5::ura4+) or JFP38 (php5+) grown in rich glucose medium. Whole-cell extracts prepared from JFP38 demonstrated CCAAT-binding activity (Fig. 4, lane 2), whereas those prepared from DMP100 did not show DNA-binding activity (Fig. 4, lane 3), suggesting that Php5p is essential for the assembly and DNA-binding activity of the CCAAT-binding complex. To determine whether CCAAT-binding activity could be reconstituted by the addition of recombinant Php5p to the DMP100 extract, full-length php5+ was expressed in E. coli as a GST fusion protein from plasmid pDM283 and purified as described in Materials and Methods. The addition of purified GST-Php5p to the DMP100 extract restored CCAAT-binding activity (Fig. 4, lane 4), whereas those prepared from DMP100 did not show DNA-binding activity (Fig. 4, lane 3), suggesting that Php5p is essential for the formation of the CCAAT-binding factor.GST-Php5p fusion alone was unable to bind to the CCAAT box-containing probe (36). Together with the data described above, these results indicate that Php5p represents a bona fide subunit of the CCAAT-binding factor from S. pombe.

php2 and php5 mutants are defective in cyc1+ transcription in S. pombe. The phenotype of php2 and php5 mutants indicates that the CCAAT-binding factor may be involved in the regulation of genes involved in respiratory metabolism. Moreover, it has been noted that the S. pombe cyc1+ gene, encoding cytochrome c, contains four putative CCAAT-binding sites within its promoter (10). Thus, it seemed plausible that mutations abolishing CCAAT-binding activity might alter the transcription of the cyc1+ gene. To address this possibility, Northern blot analysis was performed with total RNA isolated from JFP38 (php5+), JFP127 (php2Δ::ura4+), and DMP100 (php5::ura4+) grown in rich medium containing either 3% glucose or 3% glycerol–0.1% glucose as the carbon source. It should be noted that php2 and php5 mutants failed to grow in minimal medium containing glycerol as the carbon source; however, these strains were able to grow in rich medium with glycerol.

FIG. 1. Complementation of a hap5 mutant of S. cerevisiae with the php5+ gene. Yeast strains BWG1-7a (HAP5) and DMY110 (hap5::hisG) were transformed with plasmids pDB20 (vector), pDM233 (HAP5), and pDM242 (php5+) and streaked on rich medium containing glucose (A) and rich medium containing lactate (B). The strains were subsequently incubated at 30°C for 3 days.

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Northern blot hybridization of total RNA with a cyc1-specific probe revealed that the cyc1 mRNA was abundant in cells grown in glucose medium (Fig. 5, lane 1) and was induced an additional sevenfold when the cells were grown in medium containing 3% glycerol–0.1% glucose (Fig. 5, lane 4). These results indicate that cyc1 transcription is induced when cells are grown under conditions that require higher levels of respiratory activity.

Northern analysis of total RNA isolated from either php2 or php5 mutants demonstrated a substantial decrease (three- to fourfold) in cyc1 mRNA when the cells were grown in glucose medium (Fig. 5, lanes 2 and 3); however, the inducibility of cyc1 transcription was maintained when the cells were grown in glycerol medium (Fig. 5, lanes 5 and 6). As a control for RNA loading, the Northern blots were subsequently rehybridized with a probe specific for leu1 mRNA (Fig. 5).

These results suggest that the S. pombe CCAAT-binding factor is required for the maintenance of constitutive levels of cyc1 transcription when cells are grown in fermentable carbon sources. Thus, the functional roles of the S. cerevisiae and S. pombe CCAAT-binding factors are evolutionarily conserved in that they both regulate the activities of genes involved in respiratory metabolism. However, the mechanisms by which these transcription factors regulate respiratory gene transcription are likely to have diverged significantly.

The evolutionarily conserved core domain of Hap5p and Php5p is sufficient for CCAAT-binding factor assembly and DNA-binding activity in vitro. Previous studies demonstrated that CBF-C and Hap5p are both capable of interacting with Hap2p and Hap3p to form a heterotrimeric DNA-binding complex (35, 53), and in a previous section we demonstrated that Php5p must interact with Hap2p and Hap3p in vivo, since the php5 gene complements a hap5 strain (Fig. 1). Thus, the minimum core domain of Hap5p that is sufficient for assembly and DNA binding of the Hap2p-Hap3p-Hap5p heterotrimer must encompass a region that is highly homologous among CBF-C, Hap5p, and Php5p. The data shown in Fig. 2B suggests that an 87-amino-acid domain between amino acid residues 154 and 240 of Hap5p is the only region of conservation among all three proteins. Thus, we anticipated that this region must be essential for the assembly and DNA-binding activity of the heterotrimer. To test this hypothesis, amino acid residues 154...
to 242 of Hap5p and amino acid residues 103 to 191 of Php5p were expressed in E. coli as GST fusion proteins and purified as described in Materials and Methods. The proteins were used in mobility shift assays along with recombinant Hap2p and Hap3p core domains that had been expressed and purified as previously described (35). The addition of GST along with recombinant Hap2p and Hap3p failed to reconstitute CCAAT-binding activity (Fig. 6, lane 2), whereas the addition of GST-Hap5p (p92, amino acid residues 80 to 242), which has been shown to assemble with recombinant Hap2p and Hap3p to bind to a CCAAT box-containing probe (35), reconstituted DNA-binding activity (Fig. 6, lane 3). In addition, GST-Php5p (amino acids 2 to 415) also resulted in CCAAT-binding activity when combined with recombinant Hap2p and Hap3p (Fig. 6, lane 6). When the GST-Hap5p core (amino acids 154 to 242) or the GST-Php5p core (amino acids 103 to 191) was combined with recombinant Hap2p and Hap3p, CCAAT binding was also observed (Fig. 6, lanes 4 and 5). These data suggest that the minimal core domain of Hap5p that is required for the interaction with Hap2p and Hap3p lies between amino acid residues 154 and 242. Moreover, the homologous domain of Php5p (amino acid residues 103 to 191) provides the same functional role and, by analogy, one would predict that amino acid residues 37 to 123 of CBF-C would also be functional for the assembly and DNA-binding activity of the mammalian CCAAT-binding complex. In fact, recently published studies have demonstrated by deletion analysis that this domain of CBF-C is sufficient for the assembly of the CBF-A/B/C heterotrimer in vitro (26).

The conserved core domain of Hap5p and Php5p is sufficient for CCAAT-binding activity in vivo but is defective in the recruitment of Hap4p into the CCAAT-binding complex. The data presented in the previous section indicated that the do-

FIG. 3. S. pombe strains containing a php5::ura4+ null allele are unable to grow on minimal medium containing 3% glycerol-0.1% glucose as the sole carbon source. Isogenic yeast strains JFP38 (wild type), JFP47 (php2Δ::ura4+), and DMP100 (php5::ura4+) were streaked on minimal medium containing 3% glucose (A) or 3% glycerol-0.1% glucose (B) as the sole carbon source. The strains were subsequently incubated at 30°C for 5 days.

FIG. 4. The php5+ gene product is required for the assembly and DNA-binding activity of the CCAAT-binding factor. DNA mobility shift assays were performed with DNA-binding reaction mixtures containing a radiolabeled CCAAT box-containing probe incubated with crude extracts (ext.) prepared from strain JFP38 (php5+), or DMP100 (php5::ura4+). Purified recombinant GST or GST-Php5p was included in the DNA-binding reaction mixtures as indicated. The position of the CCAAT-binding complex is indicated on the left. As a control, the binding reaction mixture in lane 1 contained unbound probe only.

FIG. 5. S. pombe strains containing mutations in php2+ and php5+ are defective in cyc1+ transcription. Northern blot analysis was performed with total RNA isolated from S. pombe JFP38 (php2+), JFP47 (php2Δ::ura4+), JFP47 (php5+), and DMP100 (php2+ php5::ura4+) grown in rich medium (YEC) containing either 3% glucose or 3% glycerol-0.1% glucose. The membrane was hybridized with a radiolabeled probe specific for cyc1+ or leu1+ mRNA. The leu1+ mRNA was used to normalize RNA loading.
were constructed. The in vivo complementation of a Hap2p-Hap3p-Hap5p complex to assemble and bind to DNA mutant by these fusion proteins would reflect the ability of the domain and expressed from the constitutive Php5p core domains, respectively, fused to the B42 activation mids pDM279 and pDM280, which encode the Hap5p or Hap5p can partially bypass the Hap4p requirement (35), demonstrated that the fusion of the B42 activation domain to stimulate target gene expression (11). Since previous studies because Hap4p contains the activation domain required to functionally complement the Lat− phenotype of strain DMY116 (hapat::hisG) was used as the recipient of the LEU2-based plasmid encoding the Hap2-Hap4p chimera and the URA3-based plasmid encoding the Hap5p or Php5p core domain. The positive controls included plasmids pDM233 (HAP5), pDM242 (php5+), and pDM298 (HAP2); as negative controls, plasmid vectors Yep351 (LEU2) and pDB20 (URA3) were used. The plasmids were introduced into strain DMY116 in various combinations (Fig. 7A). The transformants were subsequently replica plated to rich glucose medium (Fig. 7B) or rich lactate medium (Fig. 7C), and complementation was scored after 3 days. As expected, all of the strains grew well on glucose medium, demonstrating the viability of the transformants. On medium containing lactate as the carbon source, the positive control strains, containing HAP2/HAP5 or HAP2/php5−, grew well, demonstrating that the hap2 and hap5 mutations could be complemented by the plasmid-borne genes. Moreover, HAP2−4/HAP5 and HAP2−4/php5− also complemented the mutations, showing that the fusion of HAP4 to HAP2 did not deleteriously affect cell growth. When plasmids expressing the HAP5 or php5+ core domain (termed HAP5C or php5C, respectively) were introduced into DMY116 harboring the plasmid expressing only HAP2, no complementation was observed; however, when these plasmids were introduced into DMY116 containing HAP2-4, complementation was obvious. These data suggest that the inability of HAP5C and php5C to complement a hap5 strain is due to the inability of the core domains to recruit Hap4p into the CCAAT-binding complex.

Identification of a conserved domain within Hap5p and Php5p that is required for the recruitment of Hap4p into the CCAAT-binding complex. From the studies described in the previous section, we reasoned that Hap5p and Php5p must contain an additional domain that is required for Hap4p to interact with the Hap2p-Hap3p-Hap5p heterotrimer. Alternatively, it was conceivable that Hap5p contains such a domain and that Php5p contains an activation domain that stimulates transcription, thereby obviating the Hap4p requirement. A more extensive analysis of the homology among Hap5p, Php5p, and CBF-C revealed a second region of conservation between Hap5p and Php5p that was absent from CBF-C (Fig. 2C). Initially, we did not focus on this domain, since it was not found in CBF-C; however, the experiments described in the previous section led us to suspect that this region might be important for Hap4p recruitment into the CCAAT-binding complex. To test this hypothesis, plasmids pDM294 and pDM295, which express amino acid residues 115 to 242 of Hap5p and amino acid residues 71 to 191 of Php5p, respectively (termed HAP5C4 and php5C4), were generated. These plasmids were introduced into strain DMY118 (hap4Δ::hisG hap5::hisG) with either plasmid vector Yep351 or plasmid pSLF406L, containing HAP4 (Fig. 8A), and the transformants

Php5p fusion proteins were found to partially complement the Lat− phenotype of DMY110, suggesting that the core domains of these proteins could function in vivo in the assembly and DNA-binding activity of Hap2p-Hap3p-Hap5p (36). Although it remained plausible that the fusion of the core domains to the B42 activation domain stabilized the proteins and allowed their import into the nucleus, the more intriguing possibility was that the Hap5p and Php5p core domains failed to recruit Hap4p into the CCAAT-binding complex. To examine the latter possibility, we constructed plasmid pH2H4-2pL, expressing a HAP2-HAP4 gene fusion (termed HAP2-4) from the HAP2 promoter. The expression of a Hap2-Hap4p chimera should obviate the need for Hap4p to be recruited independently into the CCAAT-binding complex, thereby allowing us to assay whether the Hap5p or Php5p core domain is capable of assembling the CCAAT-binding complex in vivo. The yeast strain DMY116 (hap2Δ hap5::hisG) with either plasmid vector Yep351 or plasmid pDM295, which express amino acid residues 115 to 242 of Php5p and full-length GST-Php5p were also included as positive controls. As a negative control, the binding reaction mixture in lane 1 contained unbound probe only. The positions of the CCAAT-binding complexes are shown on the left.

FIG. 6. The conserved core domains of Hap5p and Php5p are sufficient for assembly of the heterotrimeric CCAAT-binding complex in vitro. To examine whether the Hap5p or Php5p core domain alone is sufficient for the assembly of the complete heterotrimeric CCAAT-binding factor (Hap2p-Hap3p-Hap5p-Hap5p complex) in vivo, plasmids pDM281 and pDM282, which express only the Hap5p and only the Php5p core domains, respectively, from the ADH1 promoter, were constructed. Surprisingly, neither of these plasmids was able to functionally complement the Lat− phenotype of strain DMY110. The inability of these small polypeptides to complement DMY110 could have resulted from the lack of protein expression or stability, the failure of the proteins to localize to the nucleus, or the failure of Hap2p-Hap3p-Hap5p to interact with Hap4p, thereby resulting in the loss of complementation because Hap4p contains the activation domain required to stimulate target gene expression (11). Since previous studies demonstrated that the fusion of the B42 activation domain to Hap5p can partially bypass the Hap4p requirement (35), plasmids pDM279 and pDM280, which encode the Hap5p or Php5p core domains, respectively, fused to the B42 activation domain and expressed from the constitutive ADH1 promoter, were constructed. The in vivo complementation of a hap5 mutant by these fusion proteins would reflect the ability of the Hap2p-Hap3p-Hap5p complex to assemble and bind to DNA independently of Hap4p. Thus, these plasmids were introduced into strain DMY110, and both the B42-Hap5p and B42-
were replica plated to rich glucose (Fig. 8B) or lactate (Fig. 8C) medium. All of the transformants grew well on rich glucose medium; however, the control strains, containing HAP4 or Php5 alone, failed to grow on rich lactate medium. The Lat 

\( ^2 \) phenotype of DMY118 containing only Php5 ruled out the possibility that Php5 contains an activation domain that is functional in \( S. \) cerevisiae. A Lat 

\( ^1 \) phenotype was observed when DMY118 contained HAP4/HAP5 or HAP4/Php5, demonstrating complementation of the strain with the wild-type genes. The HAP5C and Php5C alleles were again unable to complement the Lat 

\( ^2 \) phenotype in the presence of HAP4; however, complementation of the Lat 

\( ^1 \) phenotype was observed with the HAP5C4 and Php5C4 alleles when HAP4 was coexpressed.

The data described above are consistent with the hypothesis that the regions between amino acid residues 115 and 146 of Hap5p and amino acid residues 71 and 102 of Php5p are required for the recruitment of Hap4p into the Hap2p-Hap3p-Hap5p complex. To test this hypothesis biochemically, Hap4p was synthesized in a rabbit reticulocyte lysate and assayed for its association with the Hap2p-Hap3p-Hap5p complex by DNA mobility shift assays (Fig. 9). The Hap2p-Hap3p-Hap5p complex in each reaction mixture contained either GST-Hap5p core (lacking the Hap4p interaction domain) or GST-Hap5p (p92, containing the Hap4p interaction domain). The data demonstrated that Hap4p is capable of interacting with the Hap2p-Hap3p-Hap5p complex containing GST-Hap5p (p92) (Fig. 9, lane 7) but not with the complex containing GST-Hap5p core (Fig. 9, lane 6). Since the in vitro translation of Hap4p resulted in a doublet with a mobility similar to that of the faint background bands observed with a control reticulocyte lysate in the DNA-binding reactions (Fig. 9, lanes 4 and 5), a truncated form of Hap4p, designated Hap4ΔCp, was also translated in parallel and assayed for its association with Hap2p-Hap3p-Hap5p. The data demonstrated that Hap4ΔCp...
also interacts with the Hap2p-Hap3p-Hap5p complex containing GST-Hap5p (p92) (Fig. 9, lane 9) but not with the complex containing GST-Hap5p core (Fig. 9, lane 8). Moreover, the change in the mobility of the Hap2p-Hap3p-Hap4ΔCp-Hap5p complex relative to that of the Hap2p-Hap3p-Hap4p-Hap5p complex conclusively showed that Hap4p is associated with Hap2p-Hap3p-Hap5p. In other experiments (data not shown), neither Hap4p nor Hap4ΔCp alone was found to bind to the CCAAT box-containing probe, whereas the background bands seen in the presence of Hap2p-Hap3p-Hap5p were also observed with a control reticulocyte lysate alone (36). Thus, the results demonstrated that amino acid residues 115 to 146 of Hap5p and, by analogy, amino acid residues 71 to 102 of Php5p are essential for the interaction of Hap4p with the Hap2p-Hap3p-Hap5p complex.

To determine whether this small domain alone is sufficient for the recruitment of Hap4p to a heterologous promoter, we generated LexA fusion plasmids containing amino acid residues 115 to 146 of Hap5p or residues 71 to 102 of Php5p fused to the C terminus of LexA (residues 1 to 202) in plasmid pEG202 (17). The plasmid was introduced into a hap5 mutant of S. cerevisiae (35), implying that Php5p can functionally interact with Hap2p, Hap3p, and Hap4p to form an active complex. The predicted amino acid sequence of php5+ revealed regions that were highly homologous to the amino acid sequences of Hap5p and CBF-C, suggesting that these conserved regions are important for the protein-protein and protein-DNA interactions that are required for DNA binding and transcriptional activation by the CCAAT-binding factor. Apart from the evolutionary conservation of Php5p, Hap5p, and CBF-C, other independent lines of evidence indicated that Php5p is a subunit of the S. pombe CCAAT-binding factor. First, php5 mutant strains were unable to grow on minimal medium containing glycerol as the carbon source, a phenotype shared by php2 mutants (40). Second, cell extracts prepared from php5 mutants no longer displayed CCAAT-binding activity; however, the DNA-binding activity could be restored by the addition of recombinant GST-Php5p to the extracts.

What is the function of the CCAAT-binding factor in fission yeast? The respiratory defect associated with php2 and php5 mutants suggested that the CCAAT-binding factor may function in a manner analogous to that in budding yeast, namely, to regulate genes involved in respiratory metabolism. To address this issue, we examined the transcription of the cyc1+ gene in S. pombe to determine whether php2 and php5 mutants display defects in the transcriptional activation of this gene, whose product is involved in respiratory metabolism. The data indicated that the CCAAT-binding factor is important for the transcription of the cyc1+ gene; however, the mechanism by which this transcription factor functions in fission yeast appears to be different from that in S. cerevisiae. For example, the activity of the CCAAT-binding factor in S. cerevisiae is induced when cells are grown in nonfermentable carbon sources via the transcriptional regulation of HAP4 (11). In contrast, the fission yeast complex appears to function in the maintenance of constitutive levels of cyc1+ transcription in fermentable carbon sources, and an additional regulatory factor(s) is required for the induction of cyc1+ transcription in nonfermentable carbon sources. This additional factor(s) must function independently of the CCAAT-binding factor, since the induction of cyc1+ transcription in nonfermentable carbon sources is maintained in the absence of CCAAT-binding activity (i.e., php2 and php5 mutant strains).

If the S. pombe CCAAT-binding factor does function as a constitutive regulator of transcription, this role would be analogous to the functional role of its mammalian counterpart. In mammalian cells, the CCAAT-binding factor functions as a proximal promoter factor that works synergistically with other highly regulated activators to control gene expression (33, 37, 38). Therefore, the recruitment of Hap4p into the CCAAT-binding complex may also be important for the regulation of other genes in S. pombe. Further studies will be required to determine whether the CCAAT-binding factor is involved in the transcription of these other genes.
Thus, it is possible that the CCAAT-binding factor from fission yeast is a general regulatory factor that functions in conjunction with other gene-specific activators to facilitate transcriptional activation. Although it remains to be determined whether the fission yeast CCAAT-binding factor is strictly involved in the regulation of respiratory genes or whether it serves as a general regulator of a diverse number of genes, the php2 and php5 mutants do not display a broad range of growth phenotypes that would be consistent with such a general function. Our future analyses of the S. pombe CCAAT-binding factor and its target genes should prove important for our understanding of both transcriptional activation in fission yeast and the evolution of the CCAAT-binding factor function in eukaryotic cells. Moreover, additional studies on the cis-acting elements within the cycl1 promoter and the factors that bind to these elements should prove informative with respect to our understanding of respiratory gene regulation in fission yeast and to our overall understanding of transcriptional regulation in S. pombe.

Hap5p and Php5p contain two distinct functional domains.

To initiate further genetic and biochemical studies on the protein-protein and protein-DNA interactions necessary for the function of the CCAAT-binding factor, we have delimited the minimum functional domain of Hap5p that is essential for the assembly and DNA-binding activity of the complex. The 87-amino-acid domain that is conserved among Hap5p, Php5p, and CBF-C is essential, both in vitro and in vivo, for the assembly and DNA-binding activity of the Hap2p-Hap3p-Hap5p heterotrimeric complex. Structurally, this region is particularly interesting since it encompasses amino acid sequences that are homologous to the primary sequence of the histone fold motif of histone H2A (4, 26). Taken together with the fact that the minimum functional domain of Hap5p contains sequences that are homologous to the primary sequence of the histone fold motif of histone H2B (4, 54), this fact strongly suggests that Hap3p and Hap5p form a heterodimer via this protein-protein interaction motif. It should be noted that the Hap3p-Hap5p heterodimer must also interact with Hap2p, which does not contain sequences homologous to histone fold sequences. In fact, previous studies delimited a 15-amino-acid α-helical domain of Hap2p (termed the subunit association domain) that mediates its interaction with Hap3p and Hap5p (60). Although the subunit(s) that directly contacts Hap2p is presently unclear, previous studies with the mammalian CCAAT-binding factor demonstrated that the formation of the CBF-A-CBF-C complex is a prerequisite for the interaction of CBF-B (53). This finding suggests a model whereby the putative histone-fold motifs within Hap3p and Hap5p mediate their heterodimerization to create a hybrid protein surface that is capable of binding to Hap2p through the subunit association domain. The Hap2p-Hap3p-Hap5p interaction must then form a protein surface competent for DNA binding. Although it is presently unclear which of the three subunits actually makes direct DNA contact, the isolation of altered specificity mutations in HAP2 has strongly suggested that the Hap2p subunit must contact DNA (59). Furthermore, a HAP2 mutation that changes arginine to leucine at amino acid 199 causes Hap2p to recognize CCAC with a higher affinity than the conical CCAAT, suggesting that arginine 199 is involved in discriminating the fifth base position of the CCAAT box. We have also identified a second domain within Hap5p and Php5p that is required for the recruitment of Hap4p into the CCAAT-binding complex of S. cerevisiae. This domain was clearly absent from CBF-C, suggesting either that higher eukaryotes lack the Hap4p subunit or that the subunit is recruited to the CCAAT-binding factor through a different mechanism.

Since the mammalian homologs of Hap2p (CBF-B) and Hap5p (CBF-C) contain glutamine-rich activation domains (8), like that of SP1 (7), it is not surprising that the mammalian complex lacked a homolog to Hap4p. It is important to emphasize that homologs of Hap4p have not been identified in any organism other than S. cerevisiae. Therefore, the evolutionary conservation of the Hap4p recruitment domain within Php5p raises the possibility that a Hap4p homolog may exist in S. pombe. Using a strategy analogous to that described for the cloning of php5 (5), we have been unsuccessful in isolating a HAP4 homolog from S. pombe by functional complementation of a hap4Δ mutant (36); however, the conservation of the Hap4p recruitment domain within Php5p has provided the strongest evidence to date that Hap4p homologs may exist in other organisms. As the sequences of Hap5p homologs from other eukaryotes become available, it will be of interest to examine whether this domain is conserved. It is conceivable that the Hap4p recruitment domain represents an evolutionary marker as to whether other organisms contain Hap4p homologs.

The identification of the Hap4p recruitment domain within Hap5p and Php5p has provided us with a clue as to how Hap4p may interact with the Hap2p-Hap3p-Hap5p heterotrimer. We have demonstrated that the Hap4p recruitment domain is necessary for the interaction of Hap4p with the Hap2p-Hap3p-Hap5p complex; however, this domain alone does not appear to be sufficient for Hap4p interaction with the complex. It is possible that Hap4p contacts other regions of the Hap2p-Hap3p-Hap5p heterotrimer along with the Hap4p recruitment domain. In fact, mutations in HAP3 that abolish the interaction of Hap4p with the Hap2p-Hap3p-Hap5p heterotrimer have been identified (59). It is possible that Hap4p directly contacts both Hap3p and Hap5p or, alternatively, that mutations in HAP3 that abolish Hap4p binding actually alter the conformation of Hap5p within the heterotrimer, thereby indirectly inhibiting the ability of Hap4p to interact with the complex. The continued structure-function analysis of the protein-protein and protein-DNA interactions required for the function of the CCAAT-binding factor will likely lead to answers that will be extremely informative in terms of our overall knowledge of DNA-binding proteins and their role in cellular physiology.

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