

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

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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*<sup>+</sup>. 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* *cycl1*<sup>+</sup> 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,

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TABLE 1. Strains used in this study

Strain	Genotype	Reference or source
<i>Saccharomyces cerevisiae</i>		
BWG1-7a	<i>MATa ura3-52 leu2-3,112 his4-519 ade1-100</i>	16
JO1-1a	BWG1-7a <i>hap2Δ</i>	39
DMY110	BWG1-7a <i>hap5::hisG</i>	35
DMY116	BWG1-7a <i>hap2Δ hap5::hisG</i>	This study
DMY118	BWG1-7a <i>hap5::hisG hap4Δ::hisG</i>	This study
<i>Schizosaccharomyces pombe</i>		
JFP38	<i>h<sup>-</sup> ura4-D18 leu1-32 ade6-M210</i>	40
JFP47	JFP38 <i>php2Δ::ura4<sup>+</sup></i>	40
DMP100	JFP38 <i>php5::ura4<sup>+</sup></i>	This study

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<sup>+</sup>*, for pombe *HAP5*) by in vivo complementation of a *hap5* mu-

tant of *S. cerevisiae*. We subsequently examined the function of the *php5<sup>+</sup>* 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<sup>-</sup>*). *S. cerevisiae* DMY116 was generated by disrupting *HAP5* in strain JO1-1a with the *hap5::hisG* fragment of plasmid pDM212 (35). Strain DMY118 was constructed by disrupting *HAP4* in strain DMY110 with the *hap4Δ::hisG* fragment of plasmid pKS::HAP4ΔhisG (35). *S. pombe* DMP100 is a *php5* mutant derivative of JFP38 that was generated by transforming JFP38 with the 3.9-kb *NoI* *php5::ura4<sup>+</sup>* 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<sup>+</sup>* cDNA.** A cDNA library prepared with *S. pombe* mRNA and vector pDB20 (5) was introduced into *S. cerevisiae* DMY110 through eight independent transformation reactions, and the *Ura<sup>+</sup>* transformants were selected on SC-Ura. The transformants were scraped into eight separate pools, replated on rich lactate medium, and incubated at 30°C for 5 days. Positive (*Lat<sup>+</sup>*) colonies were identified in four of the pools. The plasmids 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 phagemids derived from Bluescript KS(+/-) or SK(+/-). Homology searches were performed with the BLAST network (1) at the National Center for Biotechnology.

**Plasmids and plasmid constructions.** The primers used for PCRs are listed in Table 2. Plasmids pDM233 (*HAP5*), pG2C (*GST-HAP2* core domain), pG3C (*GST-HAP3* core domain), and pDM215 (*GST-HAP5*, p92, amino acid residues 80 to 242) were previously described (35). Plasmid pDM242 contains the 2.5-kb cDNA encoding *php5<sup>+</sup>* that was cloned by complementation in *S. cerevisiae*

TABLE 2. Oligonucleotides used for the PCR

Primer	Sequence of oligonucleotide
D22205	5'-GCCCGGGCGCGCCGCTAGCTATCTAGATCTGTTAACGTCGTTATTCGTG-3'
D22206	5'-CGCGGGATCCC GGGTTAACGTCATGACCGCAAAGACTTTTCTACTACAGG-3'
D20352	5'-CCCGGGATCCGCGCCGCTAAAATACTTGACCTTTAAAAAATC-3'
oDM0015	5'-GGCGAATTCGACTTCAAATCACATTCC-3'
oDM0016	5'-CCGGAATTC AAGCTTCCATTA ACTCTCTCATTGTGG-3'
oDM0017	5'-GGCGAATTCGCTGTAAAAACTCTGCATCTTCCC-3'
oDM0018	5'-CCGGAATTC AAGCTTAAATTGTTATCTTTAGATATAATATC-3'
oDM0027	5'-GCCAAGCTTACGATGGACTTCAAATCACATTCC-3'
oDM0028	5'-GCCAAGCTTACGATGGCTGTAAAAACTCTGCAT-3'
oDM0029	5'-GGCGAATTCATCATAACCAAGACCAACACACATGC-3'
oDM0030	5'-GGCGAATTCATAAAAGGCATATCCCAACGGC-3'
oDM0032	5'-GCCAAGCTTCCACGATGTTTAGGAACGTTGGTCAGGG-3'
oDM0033	5'-GCCAAGCTTCCACGATGTTTGATAATGTTACTCAAGGT-3'
oDM0076	5'-GCCGGATCCTGACCGCAAAGACTTTTCTAC-3'
oDM0077	5'-GCCCGGGCCGCTAAAATACTTGACCTTTAAAAAATC-3'

DMY110. The disrupted allele of *php5*<sup>+</sup> was generated by subcloning the 2.5-kb *NotI* fragment containing *php5*<sup>+</sup> from pDM242 into the *NotI* site of Bluescript KS<sup>+</sup> (Stratagene) to create plasmid pDM243. To remove the *Clal* and *HindIII* sites from the polylinker, pDM243 was digested with *HindIII* and *XhoI*, the cohesive ends were made blunt with the Klenow fragment, and a *NotI* linker was 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 1.8-kb *HindIII* fragment containing the *ura4*<sup>+</sup> 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 pSLF406L contains the complete coding region of *HAP4* fused in frame at codon 258 of *HAP2*. The plasmid was constructed by PCR amplification of *HAP4* with primers D22206 and D20352 and digestion with *SmaI-NotI*. A 1.5-kb *EcoRI-PvuII* fragment containing the promoter and coding region of *HAP2* to codon 258 was isolated from pJP103 (42) and ligated, along with the *HAP4* fragment, into pRS316 (52) that had been digested with *EcoRI-NotI*. The resulting plasmid was digested with *SacI-NotI*, and the *HAP2-HAP4* fusion was subcloned into pRS425 (52). Plasmid pSLF406 was constructed by ligating the 1-kb *XbaI-PstI* fragment of pSLF413 into the *XbaI-PstI* sites of pSLF405 (11). Plasmid pSLF406L was prepared by digesting pSLF406 with *SacI-PstI*, isolating the 2.9-kb *HAP4* fragment, and ligating it into the *SacI-PstI* sites of Yep351 (20). Plasmids pDM279 and pDM280 contain the *HAP5* and *php5*<sup>+</sup> core domains, respectively, fused to the B42 activation domain under the control of the *ADHI* promoter. These plasmids were constructed as follows: pRS424 was digested with *SacI-Clal*, the ends were made blunt with the Klenow fragment, and a *BglII* linker was ligated. The plasmid was subsequently digested with *BglII*, and the 2.9-kb *BamHI* fragment containing the *ADHI* promoter-B42-*HAP5* (p92)-*ADHI* terminator from pDM207 (35) was ligated into the *BglII* site to create plasmid pDM263. The *HAP5* and *php5*<sup>+</sup> core domains were generated by PCR with primers oDM0015 and oDM0016 for *HAP5* and primers oDM0017 and oDM0018 for *php5*<sup>+</sup>. The PCR-generated products and pDM263 were digested with *EcoRI*, gel purified, and ligated, and the correct insert orientation was verified by restriction enzyme analysis. Plasmids pDM275 and pDM276 contain the same PCR-generated *php5*<sup>+</sup> and *HAP5* core domains, respectively, fused to glutathione *S*-transferase (GST) for expression in *E. coli*. These plasmids were constructed by digestion of pDM215 (35) with *EcoRI*, and the *EcoRI*-digested PCR products of the *HAP5* and *php5*<sup>+</sup> core domains were ligated into the vector. The recombinant proteins expressed from these plasmids did not interact strongly with recombinant Hap2p and Hap3p core domains in DNA mobility shift assays (36). We reasoned that the close proximity of the GST moiety to the Hap5p and Php5p core domains may have prevented the proper folding of the proteins or sterically inhibited the interaction. To circumvent this problem, plasmids pDM284 and pDM285 were generated. To construct these plasmids, the *HAP5* and *php5*<sup>+</sup> core domains were excised from pDM276 and pDM275, respectively, by digestion with *BamHI-PstI*, and the fragments were ligated into pGEX-KT (15) digested with *BamHI-PstI*. The recombinant proteins expressed from these plasmids contained a glycine linker separating the GST moiety from the Hap5p and Php5p core domains, thereby allowing stronger interactions among Hap2p, Hap3p, and GST-Hap5p or GST-Php5p. Plasmid pDM283 contains the complete open reading frame of *php5*<sup>+</sup> (minus the initiator ATG) fused to GST in the *E. coli* expression plasmid pGEX-3X (Pharmacia). The plasmid was constructed by digestion of pDM243 with *EcoRI* and ligation of the 1.7-kb fragment of *php5*<sup>+</sup> into *EcoRI*-digested pGEX-3X, and the correct orientation of the *php5*<sup>+</sup> gene was verified by restriction analysis. For the in vivo expression of the *HAP5* and *php5*<sup>+</sup> core domains (designated *HAP5C* and *php5C*), plasmids pDM281 and pDM282, respectively, were constructed. The *HAP5C* and *php5C* alleles were generated by PCR with primers oDM0027 and oDM0016 for *HAP5C* and the primers oDM0028 and oDM0018 for *php5C*. The oDM0027 and oDM0028 primers insert an artificial initiator ATG immediately upstream of the *HAP5C* and *php5C* coding regions. These PCR-generated DNA fragments were digested with *HindIII* and ligated into the *HindIII*-digested vector pDB20 (5), thereby placing *HAP5C* and *php5C* under the control of the *ADHI* promoter. For in vivo expression of *HAP5C4* and *php5C4*, plasmids pDM294 and pDM295, respectively, were constructed in a manner identical to that of pDM281 and pDM282, except that the primers used for PCR amplification were oDM0032 and oDM0016 for *HAP5C4* and oDM0033 and oDM0018 for *php5C4*. Plasmid pDM287 was constructed by PCR amplification of the *cycl1*<sup>+</sup> gene from *S. pombe* genomic DNA with primers oDM0029 and oDM0030, subsequent digestion of the PCR product with *EcoRI*, and ligation into the *EcoRI* site of Bluescript KS<sup>+</sup>. The identity of the *cycl1*<sup>+</sup> gene was verified by restriction analysis. Plasmid pDM298 contains the 2.1-kb *EcoRI* fragment of *HAP2* from pJP103 (42) cloned into the *EcoRI* site of Yep351 (20). For the in vitro transcription-translation of *HAP4*, plasmids pDM378 and pDM379 were constructed. Plasmid pDM378 was constructed by PCR amplification of the coding region of *HAP4* (amino acids 2 to 554) with primers oDM0076 and oDM0077, subsequent digestion of the PCR product with *BamHI-NotI*, and ligation with *BamHI-NotI*-digested pCITE2c (Novagen). Plasmid pDM379, containing a truncated allele of *HAP4* (amino acids 2 to 329), was generated in an identical manner, except that primers oDM0076 and D22205 were used for PCR amplification.

**Preparation of yeast extracts, recombinant proteins, and in vitro transcription-translation.** *S. pombe* strains were grown to a density of approximately 10<sup>7</sup> cells/ml in YEC medium containing 3% glucose. The cells were harvested, and

protein extracts were prepared by disruption of the cells with glass beads and ammonium sulfate precipitation as previously described (35). Expression of the recombinant CCAAT-binding factor subunits as GST fusion proteins in *E. coli* DH5 $\alpha$  and subsequent purification of the proteins from bacterial lysates with glutathione-Sepharose beads (Pharmacia) were carried out as previously described (35). Cleavage of the fusion proteins with thrombin was performed as previously described (15). To express Hap4p for DNA mobility shift assays and in vitro protein-protein interaction studies, the Novagen single-tube protein translation system was used. The in vitro transcription-translation reactions, programmed with 2  $\mu$ g of pDM378 or pDM379, were carried out according to the manufacturer's protocol. For in vitro protein-protein interaction studies, Hap4p was labeled with [<sup>35</sup>S]methionine during translation. For mobility shift assays, Hap4p was not radiolabeled; however, parallel reactions were carried out in the presence of [<sup>35</sup>S]methionine to verify the translation products generated.

**DNA-binding assays and gel electrophoresis.** The UAS2UP1 probe containing the CCAAT box was previously described (35). The DNA probe was end labeled with [ $\alpha$ -<sup>32</sup>P]dATP (6,000 Ci/mmol; Amersham Corp.) with the Klenow fragment. All DNA-binding reaction mixtures with yeast extracts contained 20 to 30  $\mu$ g 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  $\beta$ -mercaptoethanol, 3.0  $\mu$ g of poly(dI-dC), 3.0  $\mu$ g of denatured salmon sperm DNA, and 0.5 to 1.0 ng of the UAS2UP1 probe in a final volume of 20  $\mu$ l. For reaction mixtures containing purified recombinant Hap2p, Hap3p, Hap5p, 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  $\mu$ g of poly(dI-dC) was used as the nonspecific competitor. Reaction mixtures were incubated at 23°C for 30 to 45 min. The protein-DNA complexes were resolved by gel electrophoresis and autoradiography performed as previously described (35).

**Isolation of RNA and Northern blot analysis.** *S. pombe* strains were grown to a density of 5  $\times$  10<sup>6</sup> to 7  $\times$  10<sup>6</sup> cells/ml in YEC medium containing either 3% glucose or 3% glycerol-0.1% glucose as the carbon source. The cells were harvested by centrifugation, and total RNA was prepared by the glass bead-acid phenol method as previously described (2). Approximately 30  $\mu$ g of each RNA sample was loaded, separated by formaldehyde-1% agarose gel electrophoresis (48), 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 (48). The probes used for hybridization were the 0.85-kb *EcoRI* fragment of the *S. pombe cycl1*<sup>+</sup> gene (47) from plasmid pDM287 and the 2.1-kb *Clal* fragment containing the *leu1*<sup>+</sup> 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 [ $\alpha$ -<sup>32</sup>P]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*<sup>+</sup> has been deposited in GenBank under accession number U88525.

## 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<sup>-</sup>) (35). This Lat<sup>-</sup> 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<sup>+</sup> transformants were selected by growth on lactate medium. Four independent Lat<sup>+</sup> transformants were identified. To verify that the Lat<sup>+</sup> 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<sup>-</sup> segregants were tested for growth on lactate medium. All four transformants concomitantly lost the Ura<sup>+</sup> and Lat<sup>+</sup> 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 clones contained an identical 2.5-kb cDNA insert. One of these plasmids, pDM242, was used for further characterization, and the gene was designated *php5*<sup>+</sup>, for *pombe HAP5*. The complementation of DMY110 conferred by *php5*<sup>+</sup> is shown in Fig. 1. Strain DMY110 was transformed with pDB20 (vector), pDM233 (*HAP5*), or pDM242 (*php5*<sup>+</sup>), and the transformants

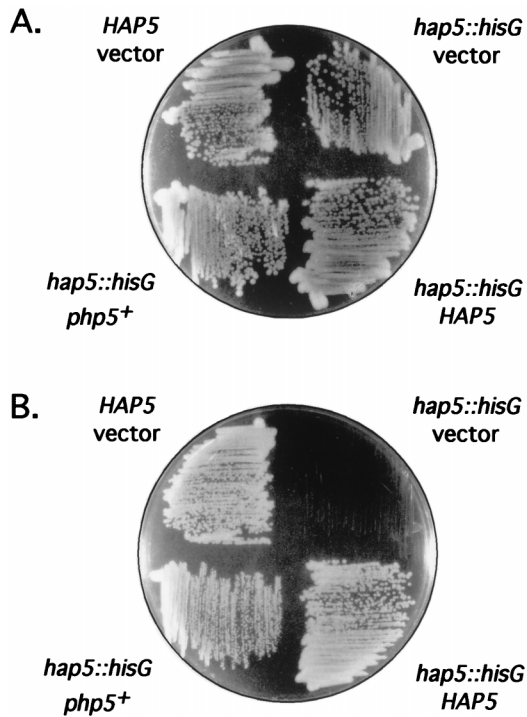


FIG. 1. Complementation of a *hap5* mutant of *S. cerevisiae* with the *php5*<sup>+</sup> gene. Yeast strains BWG1-7a (*HAP5*) and DMY110 (*hap5::hisG*) were transformed with plasmids pDB20 (vector), pDM233 (*HAP5*), and pDM242 (*php5*<sup>+</sup>) and streaked on rich medium containing glucose (A) and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

were streaked on rich medium containing glucose 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<sup>-</sup> phenotype, as previously reported (35), whereas DMY110 containing the plasmids encoding either *HAP5* or *php5*<sup>+</sup> had a Lat<sup>+</sup> phenotype.

To further characterize the *php5*<sup>+</sup> cDNA, we determined its complete nucleotide sequence. The *php5*<sup>+</sup> 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 103 through 191) that has significant sequence homology to Hap5p (amino acid residues 154 through 240; Fig. 2A and B), with 63% identity and 80% similarity over the region. In addition, amino acid residues 37 through 123 of CBF-C, the rat homolog of Hap5p (53), has 62% identity and 80% similarity to Php5p over the same region (Fig. 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

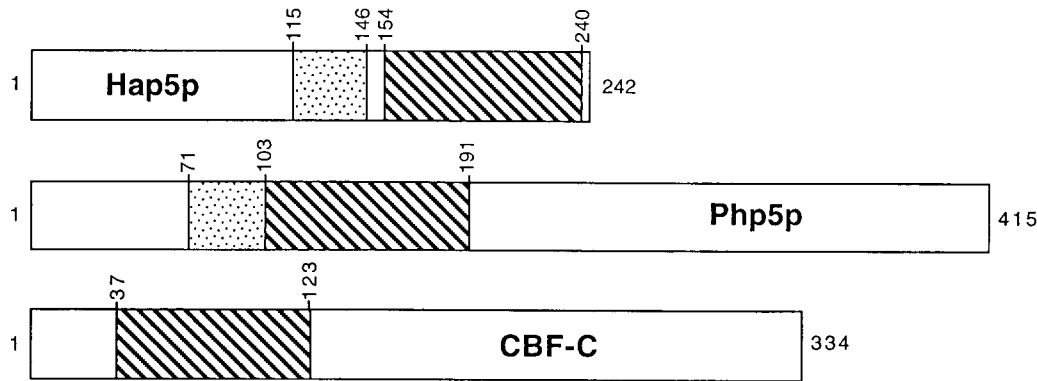
these regions; however, the C terminus of Php5p was found to be rich in glutamine, serine, and proline residues. The relevance of this observation for *S. pombe* is unknown; however, the C terminus of CBF-C is also rich in glutamine residues (53), and recent in vitro studies indicated that this domain functions in transcriptional activation (8).

**Functional analysis of *php5*<sup>+</sup> 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*<sup>+</sup> cDNA was used to generate a disrupted allele of *php5*<sup>+</sup> by insertion of *ura4*<sup>+</sup> 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*<sup>+</sup>) 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 JFP47 (*php2Δ::ura4*<sup>+</sup>) 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*<sup>+</sup> 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*<sup>+</sup>) or JFP38 (*php5*<sup>+</sup>) 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*<sup>+</sup> 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 5), whereas the addition of purified GST did not reconstitute DNA-binding activity (Fig. 4, lane 4). Moreover, the 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*<sup>+</sup> 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*<sup>+</sup> 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*<sup>+</sup> gene. To address this possibility, Northern blot analysis was performed with total RNA isolated from JFP38 (*php5*<sup>+</sup>), JFP47 (*php2Δ::ura4*<sup>+</sup>), and DMP100 (*php5::ura4*<sup>+</sup>) 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

A.



B.

*S. cerevisiae* 154-DFKSHSLPFAIRIRKVMKTDEDV\***KMISAEAPIIFAKACEIFITELTMRAW**-202  
*S. pombe* 103-AVKTLHLPLARIKKVMKTDDVDV**NKMISAEAPFLFAKGSEIFIAELTMRAW**-152  
 Rat 037-DFRVQELPLARIKKIMKLEDEV\***KMISAEAPVLFAKAAQIFITELTLRAW**-086  
 INVARIANT LP ARI K MK D DV KMISAEAP FAK IFI ELT RAW

*S. cerevisiae* 203-CVAERNKRRTLQKADIAEALQKSDMFD**FLIDV**VP RRPL-240  
*S. pombe* 152-LHAKKNQRRTLQ**RS**DIANA**VSKSEMYDFLID**IISKDNN-191  
 Rat 087-IHTEDNK**RR**TLQ**RND**IA**MAITKFDQDFLID**IVPRDEL-123  
 INVARIANT N RR TLQ DIA A K DFLID

C.

*S. cerevisiae* 115-FRN**V**QGLV**G**HYKEIMIR**YWQ**ELINEIESTNE-146  
*S. pombe* 071-FDN**V**TQGLV**G**SDAQALAE**YWQ**KTIDTLEHDDQ-102  
 INVARIANT F NV QGLVG YWQ I E

FIG. 2. Schematic representation of the evolutionarily conserved domains of Hap5p, Php5p, and CBF-C. (A) Diagram depicting the relative locations of the conserved domains in Hap5p, Php5p, and CBF-C. The numbers on each diagram show the relative positions of the amino acid residues within each polypeptide. The amino acid sequence alignments of the domains shown as hatched regions and the stippled regions are shown in panels B and C, respectively, with the invariant amino acid residues indicated in boldface type. The GenBank accession numbers for the nucleotide sequences of *HAP5*, *php5*<sup>+</sup>, and the CBF-C gene are U19932, U88525, and U17607, respectively.

(YEC) at a lower rate than wild-type strains (36). Northern blot hybridization of total RNA with a *cyc1*<sup>+</sup>-specific probe revealed that the *cyc1*<sup>+</sup> 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*<sup>+</sup> 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*<sup>+</sup> mRNA when the cells were grown in glucose medium (Fig. 5, lanes 2 and 3); however, the inducibility of *cyc1*<sup>+</sup> 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*<sup>+</sup> mRNA (Fig. 5).

These results suggest that the *S. pombe* CCAAT-binding factor is required for the maintenance of constitutive levels of *cyc1*<sup>+</sup> transcription when cells are grown in fermentable carbon sources. Moreover, the lack of CCAAT-binding activity in *php2* (40) and *php5* (Fig. 4) strains suggests that other regulatory factors must be required for the additional stimulation of *cyc1*<sup>+</sup> transcription when cells are grown in nonfermentable

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*<sup>+</sup> 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

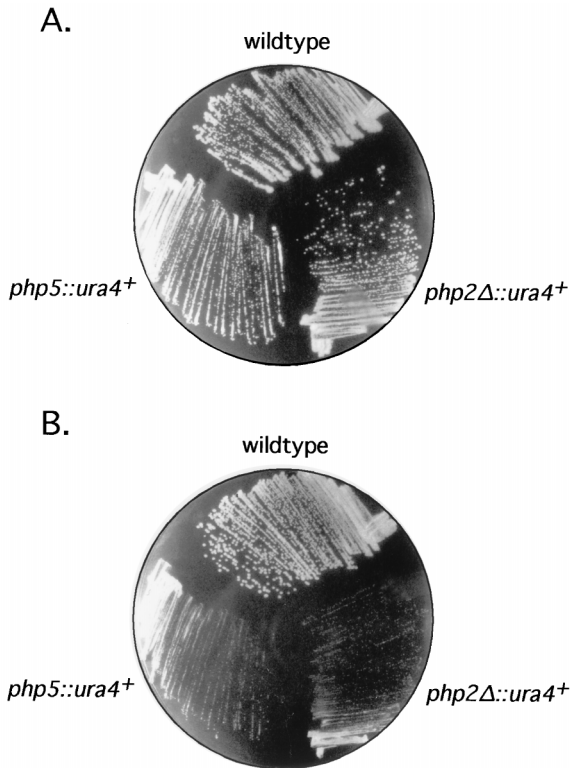


FIG. 3. *S. pombe* strains containing a *php5::ura4+* null allele are unable to grow on minimal medium containing 3% glycerol-0.1% glucose as the sole carbon source. Isogenic yeast strains JFP38 (wild type), JFP47 (*php2Δ::ura4+*), and DMP100 (*php5::ura4+*) were streaked on minimal medium containing 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.

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).

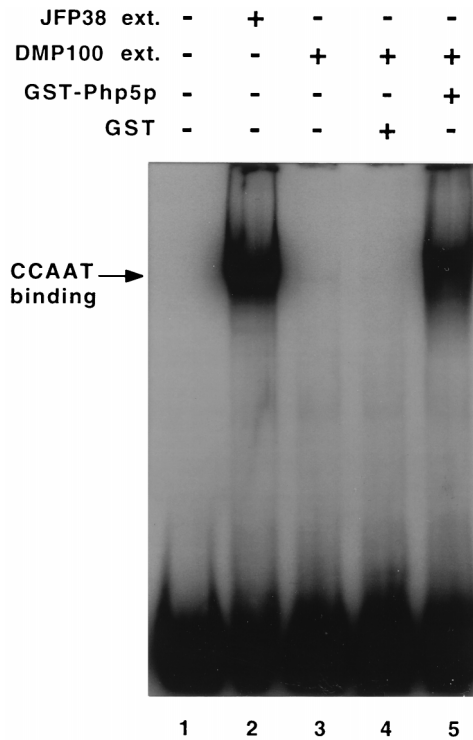


FIG. 4. The *php5+* gene product is required for the assembly and DNA-binding activity of the CCAAT-binding factor. DNA mobility shift assays were performed with DNA-binding reaction mixtures containing a radiolabeled CCAAT box-containing probe incubated with crude extracts (ext.) prepared from strain 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.

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-

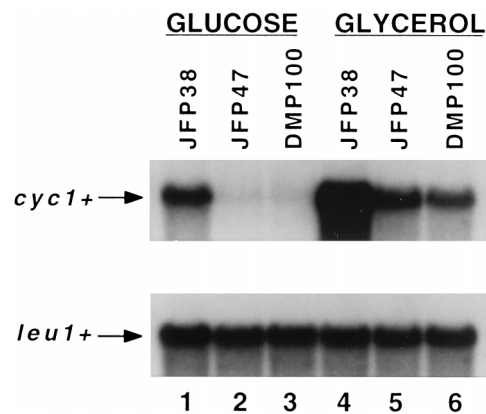


FIG. 5. *S. pombe* strains containing mutations in *php2+* and *php5+* are defective in *cyc1+* transcription. Northern blot analysis was performed with total RNA isolated from *S. pombe* JFP38 (*php2+ php5+*), JFP47 (*php2Δ::ura4+ php5+*), and DMP100 (*php2+ php5::ura4+*) grown in rich medium (YEC) 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.

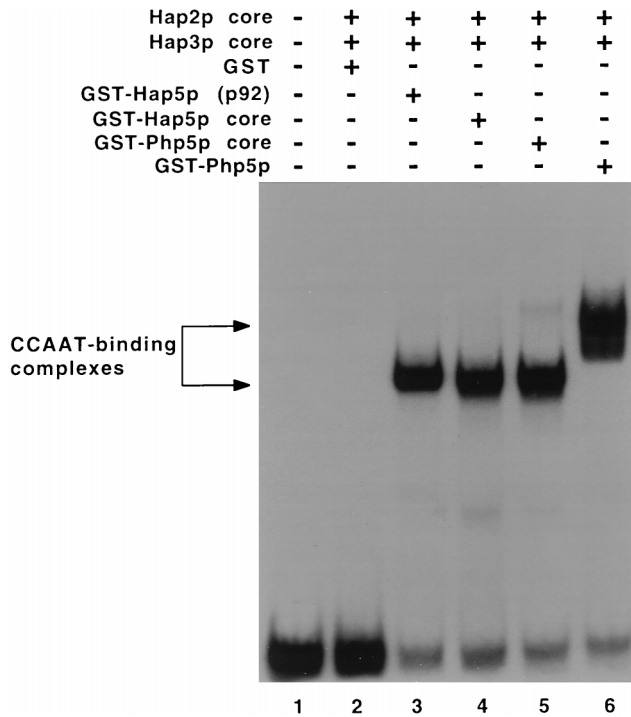


FIG. 6. The conserved core domains of Hap5p and Php5p are sufficient for CCAAT-binding factor assembly and DNA-binding activity in vitro. DNA mobility shift assays were performed with DNA-binding reaction mixtures containing a radiolabeled CCAAT box-containing probe incubated with purified recombinant CCAAT-binding factor subunits. The minimum functional core domains of Hap2p and Hap3p were synthesized in *E. coli* as GST fusion proteins, and the GST moiety was subsequently removed by thrombin cleavage. The reaction mixtures containing the minimum functional core domains of Hap5p and Php5p as GST fusion proteins are indicated (core). In addition, GST-Hap5p (p92) 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.

main that is conserved among Hap5p, Php5p, and CBF-C is 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 heterotetrameric CCAAT-binding factor (Hap2p-Hap3p-Hap4p-Hap5p complex) in vivo, plasmids pDM281 and pDM282, which express only the Hap5p and only the Php5p core domains, respectively, from the *ADHI* promoter, were constructed. Surprisingly, neither of these plasmids was able to functionally complement the Lat<sup>-</sup> 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 *ADHI* 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-

Php5p fusion proteins were found to partially complement the Lat<sup>-</sup> 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 pH2H4-2 $\mu$ L, 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 (*hap2 $\Delta$  hap5::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<sup>+</sup>*), 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<sup>+</sup>*, 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<sup>+</sup>* 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<sup>+</sup>* 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 (*hap4 $\Delta$ ::hisG hap5::hisG*) with either plasmid vector Yep351 or plasmid pSLF406L, containing *HAP4* (Fig. 8A), and the transformants

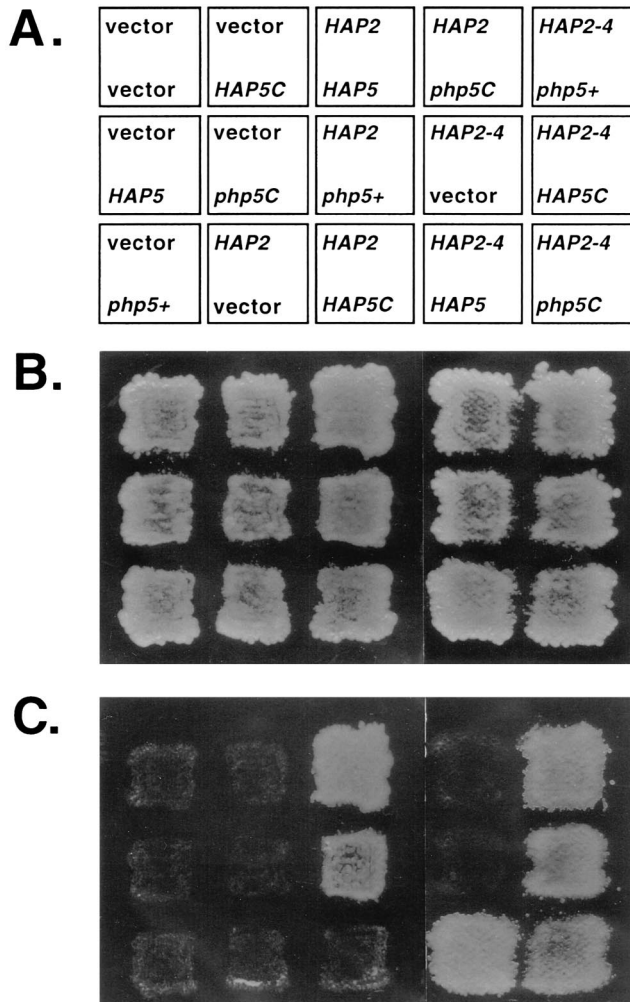


FIG. 7. The conserved core domains of Hap5p and Php5p are sufficient for CCAAT-binding activity in vivo but are defective in the recruitment of Hap4p. (A) *S. cerevisiae* DMY116 (*hap2Δ hap5::hisG*) was transformed with various combinations of *LEU2* plasmids Yep351 (vector), pDM298 (*HAP2*) and pH2H4-2 $\mu$ L (*HAP2-HAP4*) and *URA3* plasmids pDB20 (vector), pDM233 (*HAP5*), pDM242 (*php5+*), pDM281 (*HAP5C*), and pDM282 (*php5C*). The upper and lower labels in each square of panel A indicate the *LEU2*- and *URA3*-based plasmids, respectively, that were introduced into DMY116. The transformants were plated on SC-Ura-Leu and then replica plated to rich medium containing 2% glucose (B) or 2% lactate (C) as the sole carbon source. The strains were subsequently incubated for 3 days at 30°C.

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*, *HAP5*, or *php5+* alone, failed to grow on rich lactate medium. The Lat<sup>-</sup> phenotype of DMY118 containing only *php5+* ruled out the possibility that Php5p contains an activation domain that is functional in *S. cerevisiae*. A Lat<sup>+</sup> 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<sup>-</sup> phenotype in the presence of *HAP4*; however, complementation of the Lat<sup>-</sup> 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

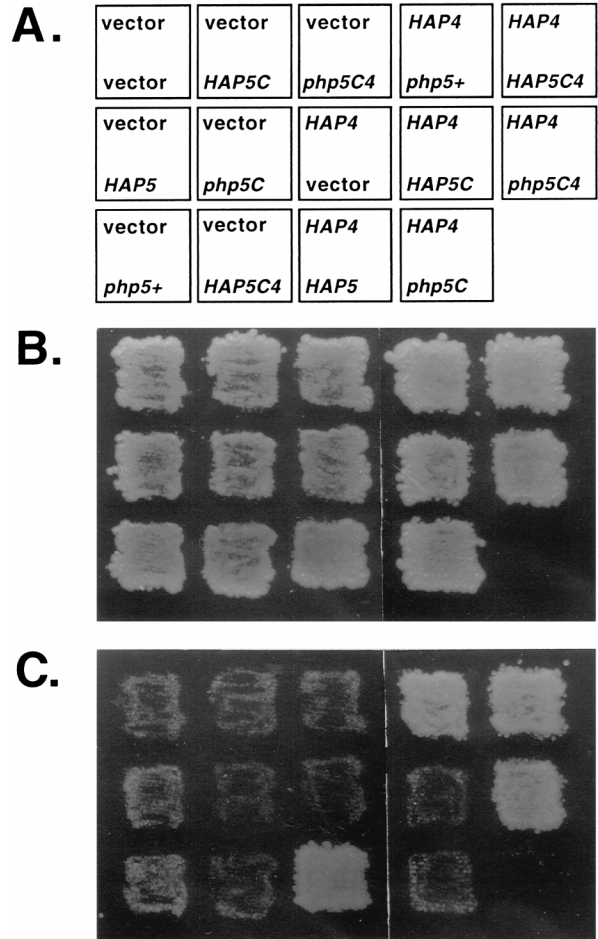


FIG. 8. Identification of a conserved domain within Hap5p and Php5p that is required in vivo for the recruitment of Hap4p into the CCAAT-binding complex. (A) *S. cerevisiae* DMY118 (*hap4Δ::hisG hap5::hisG*) was transformed with various combinations of *LEU2* plasmids Yep351 (vector) and pSLF406L (*HAP4*) and *URA3* plasmids pDB20 (vector), pDM233 (*HAP5*), pDM242 (*php5+*), pDM281 (*HAP5C*), pDM282 (*php5C*), pDM294 (*HAP5C4*), and pDM295 (*php5C4*). The upper and lower labels in each square of panel A indicate the *LEU2*- and *URA3*-based plasmids, respectively, that were introduced into DMY118. The transformants were plated on SC-Ura-Leu and then replica plated to rich medium containing 2% glucose (B) or 2% lactate (C) as the sole carbon source. The strains were subsequently incubated for 3 days at 30°C.

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 Hap4 $\Delta$ Cp, was also translated in parallel and assayed for its association with Hap2p-Hap3p-Hap5p. The data demonstrated that Hap4 $\Delta$ Cp



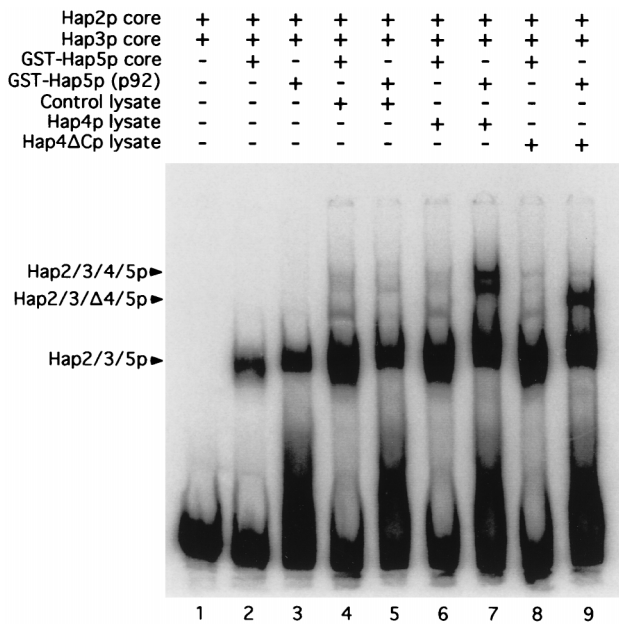


FIG. 9. Interaction of Hap4p with the Hap2p-Hap3p-Hap5p complex. DNA mobility shift assays were performed with DNA-binding reaction mixtures containing a radiolabeled CCAAT box-containing probe incubated with purified recombinant Hap2p core, Hap3p core, and either GST-Hap5p core or GST-Hap5p (p92). Rabbit reticulocyte lysates containing Hap4p, Hap4ΔCp, or an unprogrammed reticulocyte lysate were added to the binding reaction mixtures as indicated. The free probe is at the bottom of the gel, and the positions of the various complexes are indicated on the left.

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-Hap4p-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 *hap4 hap5* yeast strain containing an integrated *lexA-lacZ* reporter, along with a 2- $\mu$ m plasmid that constitutively overexpresses *HAP4* from the *ADH1* promoter. As a positive control, we fused the full-length *HAP4* gene to LexA in an identical manner. We failed to detect the recruitment of Hap4p to the LexA site of the reporter, suggesting that this small domain of Hap5p or Php5p alone is not sufficient to direct Hap4p to a heterologous site (36). Furthermore, we have also been unable to detect the interaction of Hap4p with Hap5p using *in vitro* GST pulldown assays. In these studies, <sup>35</sup>S-radiolabeled Hap4p was incubated with GST-Hap5p core or GST-Hap5p (p92), and their interaction with Hap4p was analyzed by sodium dodecyl sulfate-

polyacrylamide gel electrophoresis (36). Taken together with the results described above, the results indicate that this domain is necessary but not sufficient for the recruitment of Hap4p into the CCAAT-binding complex.

Thus, the analysis of Hap5p and Php5p has allowed us to delimit two distinct functional domains of Hap5p that are essential for the assembly of the heterotetrameric CCAAT-binding complex in *S. cerevisiae*. Additional analyses will be required to determine whether these domains are sufficient for the function of Php5p in *S. pombe* or whether additional regions of the protein are necessary.

## DISCUSSION

In this report, we describe the identification of *php5*<sup>+</sup> cDNA, encoding the third subunit of the CCAAT-binding factor from fission yeast. The *php5*<sup>+</sup> gene was cloned by functional complementation of the respiratory defect of 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*<sup>+</sup> 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*<sup>+</sup> 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*<sup>+</sup> 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*<sup>+</sup> transcription in fermentable carbon sources, and an additional regulatory factor(s) is required for the induction of *cyc1*<sup>+</sup> transcription in nonfermentable carbon sources. This additional factor(s) must function independently of the CCAAT-binding factor, since the induction of *cyc1*<sup>+</sup> 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,

45, 58). 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 *cyc1*<sup>+</sup> 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 Hap3p 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  $\alpha$ -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 CCAAC 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*<sup>+</sup>, we have been unsuccessful in isolating a *HAP4* homolog from *S. pombe* by functional complementation of a *hap4* $\Delta$  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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