The *Schizosaccharomyces pombe* Homolog of *Saccharomyces cerevisiae* HAP2 Reveals Selective and Stringent Conservation of the Small Essential Core Protein Domain

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The fission yeast *Schizosaccharomyces pombe* is immensely diverged from budding yeast (*Saccharomyces cerevisiae*) on an evolutionary time scale. We have used a fission yeast library to clone a homolog of *S. cerevisiae* HAP2, which along with HAP3 and HAP4 forms a transcriptional activation complex that binds to the CCAAT box. The *S. pombe* homolog *php2* (*S. pombe* HAP2) was obtained by functional complementation in an *S. cerevisiae* *hap2* mutant and retains the ability to associate with HAP3 and HAP4. We have previously demonstrated that the HAP2 subunit of the CCAAT-binding transcriptional activation complex from *S. cerevisiae* contains a 65-amino-acid "essential core" structure that is divisible into subunit association and DNA recognition domains. Here we show that *Php2* contains a 60-amino-acid block that is 82% identical to this core. The remainder of the 334-amino-acid protein is completely without homology to HAP2. The function of *php2* in *S. pombe* was investigated by disrupting the gene. Strikingly, like HAP2 in *S. cerevisiae*, the *S. pombe* gene is specifically involved in mitochondrial function. This contrasts to the situation in mammals, in which the homologous CCAAT-binding complex is a global transcriptional activator.

Investigation of the fission yeast *Schizosaccharomyces pombe* has led to the surprising discovery of extensive evolutionary divergence from the more commonly studied budding yeast *Saccharomyces cerevisiae*. Indeed, based on the degree of conservation of important RNAs (such as 5S RNA) and protein sequences (such as the histones and tubulins), the fission yeast *S. pombe* has been judged to be as evolutionarily divergent from the budding yeast as it is from metazoan eucaryotic lineages (53).

Transcriptional activation proteins have been shown to be composed of small discrete functional domains for site-specific DNA binding and for transcriptional activation (27, 36, 37). Site-specific DNA binding commonly occurs through various DNA binding motifs such as the helix-turn-helix (reviewed in reference 7), zinc finger (reviewed in references 5 and 30), leucine zipper (32, 33), and helix-loop-helix (42) structures. Although less is known about the structure of transcriptional activation regions, they are often enriched in specific amino acids such as aspartate/glutamate (36, 37), glutamine (10), or proline (40). Regions of transcriptional activation proteins not directly involved in either DNA binding or transcriptional activation are frequently dispensable for functional complementation in vivo (4, 48).

The *S. cerevisiae* HAP2 protein exists as a heterotrimeric complex with the HAP3 and HAP4 proteins (17, 44). The HAP2/3/4 complex binds to the sequence CCAAT located upstream of *CYC1* and other genes involved in mitochondrial electron transport in *S. cerevisiae*. As a consequence, *hap2*, *hap3*, and *hap4* mutants fail to adequately express such genes, resulting in an inability to grow on nonfermentable carbon sources, for which oxidative phosphorylation is essential for energy production. Amazingly, the HAP2/3/4 complex appears to have been conserved over the millions of years of evolutionary divergence between yeasts and hu-

mans. Chromatographic fractionation of human cells has identified a similar multicomponent CCAAT-binding complex (8). Cooperative binding between the human and yeast subunits has demonstrated that the human HAP2 homolog is contained in a fraction designated CP1B, while the human homolog of HAP3 occurs in fraction CP1A (9). These studies revealed that both protein-protein interactions between the subunits and protein-DNA interactions allowing site-specific binding have been conserved between yeasts and humans by the subunits of this complex transcriptional activator. We have exploited this apparently widespread conservation of the CCAAT-binding complex to clone a fission yeast homolog of HAP2 by direct complementation of an *S. cerevisiae* *hap2* null mutant for growth on a nonfermentable carbon source.

We have demonstrated (45) that the budding yeast HAP2 protein contains a small "essential core" protein structure encompassing both HAP3/4 subunit association and CCAAT DNA recognition domains (44). The remaining 75% of the HAP2 protein is functionally dispensable. Here we demonstrate that only the functionally important essential core region is conserved in a homolog from the evolutionarily divergent fission yeast *S. pombe*. Furthermore, the degree of sequence conservation in this region suggests that both the subunit association and DNA recognition domains have relatively constrained protein structures despite their lack of homology to known dimerization and/or DNA-binding motifs. The function of this gene in *S. pombe* was studied by gene disruption. Our studies indicate that the regulatory role of the CCAAT transcription complex, while altered in mammals, has been conserved in divergent microbes.

MATERIALS AND METHODS

Yeast strains. *S. pombe* SP401 (*h90 swi5 leu1-32 his3*) was used to prepare extracts for DNA binding assays. Complementation and function of *php2* in *S. cerevisiae* was assessed

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by using JO1-1a, a *hap2* null derivative of BWG 1-7a (*MATa leu2-3,112 his4-519 ade1-100 ura3-52* [21]) containing a chromosomal deletion of the *HAP2* gene obtained by the two-step gene transplacement technique (51). The *hap3::HIS4* null strain SHY40 has been described before (24). Strain JO2-1, a *hap2 hap3* double mutant, was derived from JO1-1a by the one-step gene disruption technique described previously (24). The *hap4* null strain used, SLF401, has been described before (17).

To construct a strain with a disruption of *php2*, the entire coding sequence was removed from pJO161 by digestion with *NheI* and *Sall*, which cleave 133 bp upstream and 56 bp downstream of the *php2* coding sequence, respectively. The deleted fragment was replaced with the *S. pombe ura4* gene (19). A DNA fragment bearing *ura4* flanked by the *php2* sequences was used to transform JFP46 (*h⁺/h⁻ ura4-D18/ura4-D18, leu1-32/leu1-32 ade6-M210/ade6-M216*) to *Ura⁺* by electroporation (3). Diploids with one wild-type and one disrupted copy of *php2* were identified by Southern analysis (not shown), and five tetrads from a representative diploid were analyzed after sporulation. In each tetrad, four viable spores were observed, and the *Ura* and *Ade* phenotypes segregated 2:2 (the phenotypes of the *ade6-M210* and *ade6-M216* mutations are red and pink colonies, respectively, on rich medium plates).

Extract preparation and DNA-binding assays. *S. pombe* extracts were prepared essentially as described previously for *S. cerevisiae* extracts (44) except that cells were grown on YEL-glucose or YEL-glycerol medium (23). UAS2 and UAS2 UP1 probes were prepared as described previously (44). The sequence and preparation of the UAS2 UP1 oligonucleotide have been described before (9). Gel shift DNA-binding assays were done as described previously (44) except that 30 μ g of total *S. pombe* protein (Bio-Rad assay) and 1 μ g of salmon sperm DNA and 1 μ g of poly(dI:dC) competitors were used in each binding reaction mix. Reaction mixes contained 20 to 25 μ g of extract protein.

Cloning and subcloning of *php2*. JO1-1a was transformed with *S. pombe* *Sau3A* and *HindIII* partial genomic libraries (41) by spheroplasting and plated on minimal glucose (no leucine) selective medium (57). Transformants were scraped, resuspended in 1 $\times$ SD (45), and plated on a rich lactate medium for selection of *HAP2⁺*. Lactate-positive clones were obtained at a frequency of approximately 1 in 10,000 from each library. *S. pombe* clones complementing the *S. cerevisiae ura3-52* mutation (presumably *S. pombe ura4* clones) were obtained at a frequency of 1 in 10,000 from the *Sau3A* partial library and at a frequency of 1 in 1,000,000 from the *HindIII* partial library. No clones complementing the lactate growth defect of a *hap3* null strain were obtained from either library. Clones were recovered by the yeast teeny prep technique (57) and purified by transformation into *Escherichia coli* HB101* (*hsdR hsdM supE44 ara14 galK2 lacY1 proA2 rspL220 xyl-5 mtl-1 recA13 mcrB*). Isolates which retransformed JO1-1a to *Lac⁺* all had the structures shown for pA11a (from the *Sau3A* partial library) or pB11a (from the *HindIII* partial library) by restriction mapping. DNA isolation and manipulation techniques were, in general, done as described by Sambrook et al. (54). Subcloning analysis was performed with vector YE352 (*URA3 Amp^r* [2 μ m] [25]).

Sequencing. The dideoxy chain termination technique (55) and Sequenase reagents (U.S. Biochemicals) were used to sequence the *php2* gene. The published sequence includes analysis of both DNA strands and the junctions of all M13 cloning sites. The *php2* DNA sequence and predicted amino

acid sequence shown in Fig. 3 were generated with the PUBLISH program of the National Biomedical Research Foundation—Protein Identification Resource (NBRF-PIR).

Northern analysis. *S. pombe* RNA was loaded at 5 μ g per lane. The Northern (RNA blot) gel, transfer, and probe hybridizations were done essentially as described in Forsburg and Guarente (17). Probes 1 and 2 (see Results section) were prepared by random hexamer labeling (U.S. Biochemicals) of the appropriate gel-purified *php2* DNA restriction fragments.

β -Galactosidase assays. JO1-1a was transformed with the indicated plasmids by the lithium acetate transformation protocol (57). Vectors pAAH5 (2) and pJP300 (49) have been described. Transformants were grown selectively in minimal medium (SD plus glucose but without uracil or leucine) and assayed for β -galactosidase activity as described previously (20).

Computer analysis. Protein sequence homology searches were conducted with the Massachusetts Institute of Technology Whitaker College VAX, using the NBRF FASTP and University of Wisconsin Genetics Computer Group (UWGCG) protein sequence data bases. DOTMATRIX is a graphic matrix display program which utilizes the RELATE (NBRF-PIR) homology algorithm to find regions of sequence similarity. Matches were scored (window size = 25, minimum score = 25) by using the mutation data matrix (MD), which identifies amino acid identities as well as evolutionarily frequent substitutions (12). PEPLLOT is a protein secondary structure prediction algorithm (UWGCG).

RESULTS

CCAAT-binding activity in *S. pombe*. In the yeast *S. cerevisiae*, the HAP2/3/4 CCAAT binding activity can be detected in whole-cell extracts by using a radiolabeled UAS2 UP1 DNA probe in a gel shift DNA-binding assay (17, 44). This probe contains the sequence CCAAT in the binding site. The CCAAT-binding activity detected is vastly affected by the carbon source on which the cells are grown prior to extract preparation. At least 20-fold more binding activity is detected with lactate (a nonfermentable carbon source)-grown cells than with glucose-grown cells. Furthermore, DNA binding is greatly reduced when the wild-type UAS2 sequence (containing CCAAC instead of CCAAT) is used as a probe. In order to determine whether a similar CCAAT binding activity occurs in *S. pombe*, extracts were prepared from fission yeast grown in medium containing either the fermentable carbon source glucose or the nonfermentable carbon source glycerol. As shown in Fig. 1, gel shift DNA-binding assays with radiolabeled *CYC1* UAS2 probes revealed the presence of a UAS2 binding activity which was greatly affected by the UAS2 UP1 mutation (compare lanes 1 and 2) but relatively unaffected by the carbon source (compare lanes 2 and 5) in these *S. pombe* extracts. A small oligomeric probe containing the UAS2 UP1 CCAAT box and only 27 bp of flanking sequence (−226 to −195 of *CYC1*) was similarly retarded by these extracts (lanes 3 and 6). These results are consistent with there being a CCAAT binding activity in *S. pombe* which is similar to *S. cerevisiae* HAP2/3/4 and HeLa CP1 (9). Unlike the budding yeast CCAAT binding activity, however, the fission yeast activity is apparently not responsive to carbon source.

Cloning *php2*. In order to determine whether a functional homolog of the *S. cerevisiae* HAP2 gene occurs in *S. pombe*, we transformed a *hap2* null strain with a library containing genomic DNA from *S. pombe* cloned into the shuttle vector

FIG. 1. CCAAT DNA-binding activity detected in *S. pombe*. Gel retardation DNA binding assays with labeled DNA probes and crude whole-cell *S. pombe* extracts as indicated. Probes were 90-bp *Xho-Sma* fragments of UAS2 (CCAAC) (lanes 1 and 4) and UAS2 UP1 (CCAAT) (lanes 2 and 5) or a small UAS2 UP1 CCAAT-containing oligonucleotide (lanes 3 and 6). Extracts were prepared (44) from log-phase *S. pombe* cells grown on either the fermentable carbon source glucose (lanes 1 to 3) or the nonfermentable carbon source glycerol (lanes 4 to 6).

pWH5. pWH5 contains the 2 μ m autonomously replicating sequence (ARS) and *LEU2* gene from *S. cerevisiae* and is capable of replicating in either fission or budding yeast (61). Two versions of this library were used, one containing *Sau3A* partial genomic fragments cloned into the *Bcl*I site of pWH5 and the other containing *Hind*III partial genomic fragments cloned into the *Hind*III site of pWH5 (41). *LEU2*⁺ transformants were plated onto a medium containing lactate as the sole carbon source. Since the HAP2/3/4 complex is required for the transcriptional activation of many genes involved in electron transport and oxidative phosphorylation, *S. pombe* HAP2 homologs were identified directly by their ability to restore growth to the *S. cerevisiae* *hap2* null strain on this nonfermentable carbon source. Twelve independent clones were analyzed from each of the two *S. pombe* libraries. Restriction mapping revealed a single class of insert from each of the two libraries (pA11a and pB11a, Fig. 2). Both isolates retransformed the *hap2* strain to Lac⁺. Subcloning into another yeast high-copy-number vector narrowed the HAP2-complementing region to a 1.2-kb *Nhe*I-*Sal*I fragment (pJO168, Fig. 2). The gene was named *php2* for *S. pombe* HAP2 homolog, using the lowercase letter nomenclature system accepted for *S. pombe* (31).

Sequence of *php2*. The complete nucleotide sequence of the 1.77-kb *Ssp*I-*Ssp*I *php2* fragment is shown in Fig. 3. A single 334-amino-acid open reading frame (ORF) extending from nucleotide 481 to nucleotide 1482 was found within the sequence spanning the smallest complementing subclone (pJO168). The predicted amino acid sequence of this ORF revealed a protein with an expected molecular mass of 35 kDa (Fig. 3). The calculated codon bias index for this ORF was only 0.023, indicating a lack of preferred codon utilization and suggesting that this gene is expressed at a relatively low level in *S. pombe* (52). Interesting features of the amino acid sequence include an unusually basic (33% Lys or Arg)

FIG. 2. Restriction map and subcloning of *php2* (*S. pombe* HAP2 homolog). All sites for *Hind*III (H), *Eco*RI (R), *Nhe*I (Nh), *Sph*I (Sp), *Xba*I (Xb), *Pst*I (P), *Sal*I (S), and *Bam*HI (B) are shown on the 6.5-kb *Hind*III-*Hind*III fragment obtained from isolate pB11a, which was cloned from the *Hind*III partial genomic *S. pombe* library. The two *Ssp*I sites indicated delimit the region sequenced and are not unique. pA11a was isolated from the *Sau*3A partial genomic *S. pombe* library. Subcloning of a 2.7-kb *Eco*RI-*Eco*RI fragment derived from pA11a is shown below the restriction map. Complementation was evaluated by assessing growth on lactate after transformation into an *S. cerevisiae* *hap2* null strain (JO1-1a). A single ORF occurring in the smallest complementing subclone (pJO168) is boxed. Northern probes 1 and 2 are indicated above the restriction map.

Ssp I 10 30 50 70 90
 AATATTGTACGAGGTAAAGTCTCCCTCTCAACTACATCGCATACAACATTGAAAACGTGTTAAAAAGATATTGGTATAACGTGTTCCAGTGCCATA
 110 130 150 170 190
 CGCTACAATGCAACCTCTGTGTATTATATTTTCGACTGTAGCTTTTTTTTAACCCAATTTCATAAGAAAAAGTTATGCGATTTCAGTTAGTGACATTATC
 210 230 250 270 290
 ATATGTGTACATAAGATTATATATTCGTTTGTGACAAAAATGCACTGCAAGCCGTCCTCACTATTGCAATCCTGGAAAGATCGACAATAATCGTTATACA
 310 330 350 **Nhe I** 370 390
 CATTTCACGATATGTAAGAAATAGCTGCTTACCATTGGTAGTCTGCTAGCGCCTGCGGAGTTACAAACACCTAAACCAACCAACCGACCAACGAGACGAA
 410 430 450 470 490
 TATACCGTAACGAGCAGAAATTTGATTCCCTAGACTCTCCCAATAGTTAGAGCAATTGAAATAAGAATAGTCATTTTCCAAATGAATCCATATGAGCGGT
 M N P Y E P V
 510 530 550 570 590
 CGAGGGGCTTTACGTGAATGCAAGCAATACCATCGAATTTTGAACGTAAGAGAGCTCGCGCAAGTTTGAAGAGAGATTGCGTGGTGTGACAGACTAG
 E G L Y V N A K Q Y H R I L K R R E A R A K L E E R L R G V Q T T
 610 630 **Sph I** 650 670 690
 AAAAAACCATTTTACATGAATCAAGGCATAAGCATGCTATGCGACGACCTCGTGGTCTGGTGGCCGATTCTTGAATGCTGACAAAGATCAAAATTA
 K R F Y L H E S R H K H A M R R P R G P G G R F L T A D K V S K L R
 710 **Pst I** 730 750 770 790
 GAGCGCAGGAAGCTGCAAGGAGCTGCTAATGGAGGAAGTACAGGAGACGATGTTAATGCCACAAATGCCAATGATGCCACCGTGCCTGCAACCGTTTC
 A Q E A A E A A A N G G S T G D D V N A T N A H D A T V P A T V S
 810 830 850 870 890
 TTCTGAAGTGACTCACACGTCTGAAGGATATGGGATAGCAATGATTCAAGACCTAGCTCTATCTCCAACTCGTCTGAGTCTCCAGCTCTATCAATTC
 S E V T H T S E G Y A D S N D S R P S S I S N S S E S P A P I N S
 910 930 950 970 990
 GCCACAGCATCTATGCTCTCTGCTAACATTAATCTTGGAAACAACATTAATCTCTCCAAATGTCAGAGGCGAGCTAGACATGAGTGGTAAATATGGCATGT
 A T A S H S P A N N T S G N N I T S P N V R G E L D M S G N I A H S
 1010 1030 1050 1070 1090
 CGGGTGGGCCCACTAATACCGCTTCCACCTCGGGGCCAGTGCCTCATGATATGACAGTGTCCCAAGAGCCGACTCAACACATCGAATCTTATGTCAG
 G G P T N T A S T S G P V P H D H T V L P Q T D S N T S N L H S S
 1110 1130 1150 1170 1190
 TGGTTCTCAACTAGGCAGTTTGTCTACAGCTTCTACCAATGGCAACAACCTCTACTACTACTACTCTGTCAGCTGCTCATCCAGGGTCAATTCATAAA
 G S Q L G S F A T A S T N G N N S T T T T T S S A A H P G S F H K
 1210 1230 1250 1270 1290
 GGTACTAATGATTATTCATCTACACTAGCTGGAAATGAGCATAGTGTCTTCTCTGCTCTGATGTGATCAGATGATAGCCTGTCTCCAGGTGCAGCTT
 G T N D Y S S T L A G N E H S A F P G L D V Y H D D S V S A G A A F
 1310 1330 1350 1370 1390
 TTATACCACACAATCAATGGATTTCGATTGATCACCTTGTATGTCATGATCTTACAGCCACAGGTCTCCCTGTACTACAGCGAGGATATCGATCCTCT
 I P H N P H D S I D H L D V N D P T A T G L P V L P A S D I D P L
 1410 1430 1450 1470 1490
 TAATTTAACAGGCAATACCCAAAGATTCTATGATAATGGTCAGCAACATATCCCTCACACGGTCTTTCAGGAACCATGAAGTAGTCAACTAATTAAGGC
 N L T G N T Q D S H I I G Q Q T Y P S H G S S G T H K *
 1510 1530 **Sal I** 1550 1570 1590
 TGAGAGAGATACAAAACAATCACAAGACTGTGTATAGGTTGTGACCTAACCGACAAAGTTCAAATTTAGGACTGCAATGTTGTTGGTATTTTGTGAA
 1610 1630 1650 1670 1690
 GAAACAAGTCGAAATCACTCAGTCATGGCCTTTTTCATATCATATTATCTCATTATTTTTTGTACACCGATTATTTATCCATCAATTTGTTTGTACT
 1710 1730 1750 1770 **Ssp I**
 TTTTCGTCTGCGCAATTTTCTTTTATTAACTCATATCCCTCTGCTGCTTTTCTTATTCCAATAATATT

FIG. 3. DNA sequence of the *php2* gene and predicted amino acid sequence of Php2. Overlapping DNA sequence from both strands of *php2* was obtained from M13 clones carrying appropriate restriction fragments by the Sequenase/dideoxy chain termination method. Amino acid homology to *S. cerevisiae* HAP2 is boxed. Restriction sites shown in Fig. 2 are indicated.

FIG. 4. Northern blot analysis of *php2*. Hybridization of total *S. pombe* RNA to probe 1 and to probe 2 (see Fig. 2) is shown. λ DNA size markers are indicated, as is the 2.2-kb *php2* transcript detected with each probe.

amino acid content in the 60 amino acids extending between residues 15 and 74. This same feature of the *S. pombe php2* amino terminus occurs within the carboxyl terminus of *S. cerevisiae HAP2*. The extent and significance of this similarity will be explored below. The remainder of the Php2 protein is unusually enriched in the hydroxyl-containing amino acids serine and threonine (30% in the 234-amino-acid region extending from residue 101 to residue 334). In particular, there is a repeat of five threonines in a row flanked by serine residues between residues 224 and 231. Subsequent analysis of this transcript has revealed that it contains a long 3' untranslated sequence (16a).

Transcription of the *php2* ORF in *S. pombe*. In order to confirm that the deduced 334-amino-acid ORF is indeed expressed in *S. pombe*, Northern blot analysis of *php2* mRNA was performed with two nonoverlapping DNA probes. Probe 1 is a 0.71-kb *SspI-PstI* which spans the first 77 amino acids of the *php2* ORF, while probe 2 is a 0.48-kb *PstI-SspI* fragment which spans the last 64 amino acids of the ORF (Fig. 2). Northern blot analysis with either probe 1 or probe 2 revealed a single *php2* transcript of ~2.2 kb (Fig. 4), confirming that the entire deduced *php2* ORF is transcribed in *S. pombe*.

Activity of *php2* in *S. cerevisiae*. In budding yeast the HAP2 protein associates with the HAP3 and HAP4 proteins to form a heteromeric CCAAT binding transcriptional activation complex which is responsive to carbon source and heme levels. In order to determine whether the *php2 S. pombe* HAP2 homolog has retained these properties, we examined more closely the function of *php2* in *S. cerevisiae*. The *php2* gene did not bypass the requirement for *HAP3* and *HAP4*, as indicated by the lack of complementation for growth on lactate of the *php2* gene in either a *hap3* null strain, a *hap2 hap3* double null strain, or a *hap4* null strain (results not shown). This implies that the ability of the *php2* gene product to recognize and associate with the other subunits of the budding yeast CCAAT binding complex has been conserved and is necessary for functional complementation. The ability of the Php2/HAP3/HAP4 transcriptional activator to respond to carbon source was tested by assaying levels of activation from UAS2-*lacZ* and UAS2 UP1-*lacZ* fusions (22) in a *hap2* null strain. As shown in Table 1, the Php2/HAP3/HAP4 transcriptional activator induced the UAS2 UP1-*lacZ* fusion approximately 30-fold more strongly in lactate than in glucose. Furthermore, the Php2/HAP3/HAP4 complex retains its ability to discriminate between the UAS2 CCAAC sequence and the UAS2 UP1 CCAAT sequence (compare β -galactosidase units in lactate medium from UAS2 and

TABLE 1. *php2* function in *S. cerevisiae*^a

Plasmid	β -Galactosidase activity			
	UAS2 (CCAAC)		UAS2 UP1 (CCAAT)	
	Glucose	Lactate	Glucose	Lactate
pAAH5 (control)	1	NG ^b	2	NG
pA11a (<i>php2</i>)	1	63	11	305
pB11a (<i>php2</i>)	1	50	10	306
pJP300 (<i>HAP2</i>)	4	186	120	478

^a β -Galactosidase activity (Miller units) was detected from UAS2-*lacZ* (pLGΔ265 [22]) and UAS2 UP1-*lacZ* (pLGΔ265UP1 [22]) fusions in the *S. cerevisiae hap2* null strain JO1-1a cotransformed with the indicated plasmid and grown on either glucose or lactate. pAAH5 is a negative (*HAP2* minus) control, while pJP300 is a positive (*S. cerevisiae HAP2*) control. pA11a and pB11a are the original *S. pombe php2* isolates.

^b NG, No growth on lactate in the absence of *S. pombe* or *S. cerevisiae HAP2* activities.

UAS2 UP1). Levels of activation from Php2/HAP3/HAP4 in glucose were undetectable with the UAS2-*lacZ* fusion and significantly lower than HAP2/HAP3/HAP4 with the UAS2 UP1-*lacZ* fusion. These results may be due to less than optimal expression of the *php2* gene in *S. cerevisiae* under nonselective growth conditions (i.e., in glucose). Nevertheless, the function of Php2/HAP3/HAP4 largely parallels that of the HAP2/HAP3/HAP4 transcriptional activation complex.

Homology of Php2 to HAP2. A computer search for proteins homologous to Php2 with the FASTP data base searching program (35) and NBRF Protein (version 20.0) and New (version 38.0) sequence data bases revealed significant homology only between the Php2 protein and the *S. cerevisiae HAP2* transcriptional activator (optimized score = 268, ktup = 2). All other proteins in the data base scored lower than expected for proteins of significant biological relatedness (35).

A DOTMATRIX homology alignment (NBRF-PIR) between the Php2 and HAP2 proteins revealed an exceptional degree of homology between residues 11 to 67 of Php2 and residues 162 to 217 of HAP2, as indicated by the diagonal line in the upper left-hand quadrant of Fig. 5. This region of strong homology corresponds to the unusually basic (Lys and Arg rich) protein sequence noted above for the amino terminus of Php2 and the carboxyl terminus of HAP2. Two other small patches of homology correspond to evolutionarily conservative permutations of an Ala/Thr/Ser-rich sequence. Figure 6 shows the area of extensive homology in enlarged detail. A single-amino-acid gap must be introduced into the HAP2 sequence between residues 186 and 187 in order to optimize alignment between the two proteins. Residues 11 to 67 of Php2 have 82% identity with residues 162 to 217 of HAP2. If evolutionarily conservative changes are included (12), 91% homology is seen over this region, and the homology alignment extends another 45 residues downstream in each protein, with 78% evolutionarily conservative changes occurring over this region (not shown). The highly conserved region of homology in HAP2 corresponds closely to the functionally defined essential core domain of this protein (45), which contains CCAAT recognition and subunit association domains.

Secondary structure predictions made with a Chou-Fasman-derived algorithm (see Materials and Methods) suggest similar protein configurations for the conserved essential core regions of HAP2 and the Php2 homolog (results not shown). HAP2 residues 161 to 173 are predicted to form two

FIG. 5. Computer-generated DOTMATRIX homology plot between Php2 and HAP2. Numbers indicate amino acid residues starting from the amino termini of Php2 (*S. pombe* HAP2, x axis) and HAP2 (*S. cerevisiae* HAP2, y axis). Dots correspond to the center of a 25-amino-acid window scoring a critical level of protein-protein homology (see Materials and Methods).

β -strand structures of five and six residues separated by a break. This region is followed by an apparently dispensable (45) α -helical region extending from residue 177 to residue 187. These protein structures are contained within the delineated subunit association domain of HAP2 (Fig. 6). Notably, this region contains no homology to previously described dimerization motifs associated with DNA binding proteins such as the leucine zipper (33), helix-loop-helix (42), or three-stranded coiled-coil (58) oligomerization motifs. The remainder of the protein sequence shows little propensity for forming either α -helix or β -pleated sheet structures, although residues 201 to 206 may form a short α -helix. Notably, there is no homology to helix-turn-helix (7, 46), homeodomain (56), or zinc finger (30) DNA-binding motifs in this region. Nor is there overt homology to putative DNA-contacting regions associated with leucine zipper (27, 32), helix-loop-helix (11, 34), or coiled-coil (60) proteins.

Role of *php2* in *S. pombe*. In order to determine the role of the *php2* gene in *S. pombe*, a gene disruption was constructed in a diploid strain of *S. pombe*, JFP46, that bore the *ura4/ura4* genotype. The *ura4*⁺ marker was inserted into a plasmid bearing a *php2* insert between the *Nhe*I and *Sall* sites, which lie 133 nucleotides upstream and 56 nucleotides downstream of the coding sequence, respectively. The resulting construct was linearized at the outside boundaries of the *S. pombe* insert and used to transform JFP46 to Ura⁺. Transformants were checked by Southern blotting to verify that the *php2* gene had been disrupted in one of the two chromosomal homologs (data not shown). A diploid bearing the disruption was sporulated and gave rise to four viable spores, which grew into colonies on glucose medium. In each tetrad, the two segregants which were Ura⁺ displayed little to no growth defect. Thus, *php2* is not essential for growth in *S. pombe*. Next, we checked whether the segregants would grow on glycerol medium, which is a measure of mitochondrial function. In five of five tetrads, the two Ura⁺ segregants were glycerol-negative and the Ura⁻ segregants were glycerol-positive (Fig. 7). These results indicate that disruption of *php2* in *S. pombe* results in a block to mitochondrial function and the petite phenotype, just as does disruption of *HAP2* in *S. cerevisiae*.

The disrupted strain provided a means to verify that the CCAAT-binding complex from *S. pombe* observed in Fig. 1 corresponded to the HAP complex of *S. cerevisiae*. Figure 8 shows a gel shift experiment that was performed with an extract from the *php2*-deleted strain. The CCAAT-binding complex was absent, while other complexes formed with this probe were identical to the extract from the wild-type strain. Thus, we conclude that the CCAAT-binding complex from *S. pombe* corresponds to the HAP complex in *S. cerevisiae*.

DISCUSSION

We have cloned a functional homolog of the *S. cerevisiae* *HAP2* gene from an *S. pombe* genomic library by direct complementation. *php2* is apparently expressed adequately in *S. cerevisiae* from its native promoter despite differences in the mechanism of mRNA initiation which make heterologous transcription inefficient in most cases (52). The Php2 protein, expressed in *S. cerevisiae*, retains the ability to assemble with HAP3 and HAP4, the other subunits of the *S.*

FIG. 6. Amino acid sequence alignment of *S. pombe* Php2 residues 11 to 68 (top) and *S. cerevisiae* HAP2 residues 162 to 218 (bottom). Identical amino acids (aa) are indicated by an asterisk, and evolutionarily conservative substitutions (12) are marked by an open circle. The sequence shown spans the HAP2 essential core (45), including subunit association (black box with broken end at amino terminus) and CCAAT recognition (white box) regions. The carboxyl end of the subunit association domain occurs somewhere within the shaded region. The positions of subunit association and CCAAT recognition domains implied from Php2-HAP2 homology (see Discussion) are shown below.

FIG. 7. Disruption of *php2* in *S. pombe*. A diploid strain of *S. pombe* in which one of the two copies of *php2* was disrupted with *ura4* was constructed as described in Materials and Methods and Results. The diploid was sporulated, and five tetrads were dissected. The four segregants of a typical tetrad are shown. Two segregants (A and C) are *Ura*⁺ (i.e., bear the disruption) and fail to grow on minimal medium with glycerol as the carbon source. These segregants grow at a slightly reduced rate on plates with glucose as the carbon source. The other two segregants (B and D) are *Ura*⁻ and glycerol positive. Glycerol plates were prepared as described before (26).

cerevisiae CCAAT transcriptional activator. The resulting Php2/HAP3/HAP4 complex, like the native HAP2/HAP3/HAP4 complex, retains the ability to respond to growth conditions by increasing transcriptional activation in a non-fermentable carbon source. Thus, either the HAP2 subunit is not significantly involved in this process or this function has been retained through evolution in Php2. Evidence from previous experiments (45) suggests that the role of the HAP2 subunit in the HAP2/HAP3/HAP4 complex is primarily structural rather than regulatory. The HAP2 protein provides surfaces for association with the other subunits of the complex and for site-specific DNA binding. The Php2/HAP3/HAP4 complex also retains the ability to discriminate between the UAS2 sequence CCAAC and the canonical CCAAT sequence occurring in UAS2 UP1. If the HAP2 subunit makes a specific contact(s) with the UP1 A · T base pair, then the protein surface(s) responsible must have been conserved through evolution in the Php2 homolog.

The sequence of Php2 reveals selective conservation of a

FIG. 8. CCAAT-binding activity in *php2*-disrupted strain. Extracts were prepared from the wild-type strain of *S. pombe* grown in glucose or glycerol or from the *php2*-disrupted strain grown in glucose, and the UAS2 UP1 probe and gel retardation analysis were as described in the legend to Fig. 1. Lanes 1 and 3 show the wild type grown in glucose and glycerol, respectively, and lane 2 shows the *php2*-disrupted strain.

functionally important structure of the *S. cerevisiae* HAP2 carboxyl terminus within the amino terminus of *S. pombe* Php2. Amino acids 11 to 67 of Php2 show 82% identity with HAP2 residues 162 to 217. We have demonstrated that this region of HAP2 constitutes an essential core domain of the protein which provides all the necessary protein structures for the *in vivo* function of HAP2 (45). The conservation of amino acid sequence in this region is notably not accompanied by a conservation of DNA sequence. Only 40% of the identical amino acids in this region utilize identical codons. This is close to the value expected if codon usage for these identical amino acids were entirely random in both *php2* and HAP2 (32%). The only other region of possible homology between Php2 and HAP2 is in the 45 amino acids carboxyl to the essential core homology, in which 78% of the aligned residues represent evolutionarily conservative substitutions but virtually none of these are identical. The significance of this clustering of conservative changes is not immediately apparent due to the dispensability of this region of HAP2 in *S. cerevisiae*, but it may represent an augmenting but nonessential extension of the DNA recognition component of the essential core structure (discussed below). We have recently constructed cDNA libraries from *S. pombe* and HeLa cells to systematize cloning by functional complementation. With these libraries, we have isolated the TFIID gene from *S. pombe* (16) and the human counterpart of HAP2 (3a).

The irregular distribution of homology between Php2 and HAP2 demonstrates well the inverse relationship between the rate of evolution of an amino acid sequence and the functional constraints upon the corresponding protein structure *in vivo*. Thus, both exceptionally slow and exceedingly rapid rates of sequence divergence, corresponding to functionally essential and functionally dispensable protein domains, respectively, are evident within this single transcriptional regulatory protein. The Php2/HAP2 essential core appears to be evolving at an even slower rate than the relatively constrained cytochrome *c* molecule, suggesting that this region consists of a rigidly constrained protein structure. In contrast, the remainder of the Php2/HAP2 sequences have diverged to such an extent that no discernible similarity is evident.

The essential core of *S. cerevisiae* HAP2 has been functionally divided into a subunit association domain (residues 154 to 197), which is sufficient for assembly of HAP2 with the other subunits of the CCAAT heterotrimeric complex, and a CCAAT recognition domain (residues 198 to 218), which is necessary for site-specific DNA binding by the HAP2/3/4 complex (45). The division between subunit association and DNA recognition domains occurs somewhere between residues 178 and 197 of HAP2 (gray box, Fig. 6). Interestingly, this region encompasses a relatively unconserved sequence in the center of the Php2/HAP2 homology (HAP2 residues 187 to 191). This gap in the homology includes a single-amino-acid insertion in Php2. We infer that this region of the protein defines a "spacer" separating the subunit association and DNA recognition domains that is relatively unconstrained in sequence and structure. Indeed, an insertion-deletion mutant of HAP2 in which residues 180 to 184 were replaced with two different residues is functional (45).

The biological role of the CCAAT-binding activation complex appears to have been altered over evolution. While the HAP2/3/4 complex in *S. cerevisiae* is dedicated to the transcription of genes involved in mitochondrial function, the role of the mammalian HAP2/3 homolog CP1 has been

diversified to include regulation of the heat shock HSP70 gene, major histocompatibility class II genes, α -globin and β -globin genes, and the histone H1 gene (9, 13). Recent experiments also suggest, surprisingly, that mammalian CP1 may be responsible for serum induction of the Rous sarcoma virus long terminal repeat via a tyrosine kinase-mediated pathway (14). As many as 80 cellular genes are inducible by serum (1), including the HSP70 gene, which is known to contain a CP1-binding CCAAT box. CP1 thus may have evolved to become a general "housekeeping" transcriptional activator with a possible role in the control of cell proliferation and differentiation. Amazingly, the human cytochrome *c* gene shows remarkable similarities in structure to yeast *CYC1*, including an upstream CCAAT box which is critical to promoter function (15), suggesting that the function of HAP2/3/4 in yeast cells may have been conserved in mammalian CP1. The CCAAT regulatory element is also exploited in viral systems, including the adenovirus major late promoter and herpes simplex virus thymidine kinase gene (39).

It was thus of interest to determine the role of the *S. pombe* homolog *php2* in that organism. A disruption of the gene was constructed in a diploid strain of *S. pombe*. Sporulation of the diploid gave rise to two segregants per tetrad that bore the disruption and were viable. However, these segregants were unable to grow on medium supplemented with glycerol as a carbon source, suggesting that, like *HAP2* in *S. cerevisiae*, *php2* in *S. pombe* is specifically dedicated to the transcription of genes involved in mitochondrial function. We speculate more generally that, in divergent microorganisms, the cellular role of conserved transcriptional activators may also have been conserved. It remains to be determined whether carbon source plays any role whatever in regulating the activity of the complex in *S. pombe*. Clearly, the level of CCAAT-binding activity is not regulated in *S. pombe*, in contrast to the situation in *S. cerevisiae*. The cellular role of the CCAAT-binding complex may have been altered in metazoans to accommodate the increased complexity or multicellular organisms. The modular structure of transcriptional activators often segregates sequences involved in regulatory responses from those that mediate DNA binding and transcriptional activation. This partitioning of functional domains would facilitate the alteration of the regulatory region over evolution to yield a new response without changes in the other critical parts of the protein.

Counterparts of other *S. cerevisiae* activators, such as GCN4 and MCM1, also exist in mammalian cells (Jun and SRF, respectively [6, 28, 29, 43, 47, 59]). While much of the primary structure and the DNA site recognition have been conserved for these activators, their regulatory role has been altered, as for HAP2. It would be of obvious interest to isolate counterparts of these genes from *S. pombe* to determine whether their role in gene control has been conserved. More broadly, the ability to clone functional homologs in *S. cerevisiae* and determine their role in their normal host by gene disruption will be a powerful approach to studying how gene control has been tailored to fit the needs of different eucaryotic organisms.

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