Schizosaccharomyces pombe pfh1+ Encodes an Essential 5′ to 3′ DNA Helicase That Is a Member of the PIF1 Subfamily of DNA Helicases

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The Saccharomyces cerevisiae Pif1p DNA helicase is the prototype member of a helicase subfamily conserved from yeast to humans. S. cerevisiae has two PIF1-like genes, PIF1 itself and RRM3, that have roles in maintenance of telomeric, ribosomal, and mitochondrial DNA. Here we describe the isolation and characterization of pfh1+, a Schizosaccharomyces pombe gene that encodes a Pif1-like protein. Pfh1p was the only S. pombe protein with high identity to Saccharomyces Pif1p. Unlike the two S. cerevisiae Pif1 subfamily proteins, the S. pombe Pfh1p was essential. Like Saccharomyces Pif1p, a truncated form of the S. pombe protein had 5′ to 3′ DNA helicase activity. Point mutations in an invariant lysine residue in the ATP binding pocket of Pfh1p had the same phenotype as deleting pfh1+, demonstrating that the ATPase/helicase activity of Pfh1p was essential. Although mutant spores depleted for Pfh1p proceeded through S phase, they arrested with a terminal cellular phenotype consistent with a postinitiation defect in DNA replication. Telomeric DNA was modestly shortened in the absence of Pfh1p. However, genetic analysis demonstrated that maintenance of telomeric DNA was not the sole essential function of S. pombe Pif1p.

INTRODUCTION

The Saccharomyces cerevisiae PIF1 is a nonessential gene that encodes a 5′ to 3′ DNA helicase (Lahaye et al., 1991) that was first identified because it is required for the stable maintenance of mitochondrial DNA (Foury and Kolodnyński, 1983). In addition, there is a nuclear form of Pif1p that affects telomeres (Schulz and Zakian, 1994; Zhou et al., 2000). Telomere length is inversely proportional to the amount of Pif1p: cells lacking Pif1p have long telomeres, whereas cells overexpressing Pif1p have short telomeres. The catalytic activity of Pif1p is required for its effects on telomeres as point mutations that eliminate the helicase activity of Pif1p have the same phenotypes as null alleles (Zhou et al., 2000). Pif1p also inhibits telomerase-mediated addition of telomeric DNA to spontaneous and induced double strand breaks (Schulz and Zakian, 1994; Zhou et al., 2000; Mangahas et al., 2001; Myung et al., 2001). Because Pif1p is associated with telomeric DNA in vivo, its effects on telomeres are likely direct (Zhou et al., 2000).

The S. cerevisiae PIF1 is the founding member of a helicase subfamily with homologues found in other fungi, Caenorhabditis elegans, Drosophila melanogaster, and Homo sapiens (Zhou et al., 2000; Bessler et al., 2001). S. cerevisiae has a second PIF1-like gene, RRM3, that encodes a protein that is 38% identical to Pif1p over a 485-amino acid region. Like PIF1, RRM3 is not essential; moreover, cells lacking both genes are also viable (Ivessa et al., 2000). RRM3 was first identified because its mutation increases recombination in the ribosomal DNA (rDNA; Keil and McWilliams, 1993). However, the effects of Rrm3p on rDNA replication are probably a secondary consequence of defects in rDNA replication (Ivessa et al., 2000). In the absence of Rrm3p, replication stalls at multiple sites within the rDNA. Separation of converging forks is especially impaired in rrm3 cells. The effects of Rrm3p on rDNA replication require its catalytic activity and are probably direct, because Rm3p is rDNA associated in vivo (Ivessa et al., 2000). Rrm3p also affects fork progression within subtelomeric and telomeric DNA. As in rDNA, sister chromatid separation within telomeric regions is delayed in an rrm3 strain (A.S. Ivessa, J.-Q. Zhou, V.P. Schulz, E.K. Monson, and V.A. Zakian, unpublished results).

Most helicases contain seven short motifs spread throughout a 300–500 amino acid region (Ellis, 1997). Because these motifs are short and degenerate, their presence alone is not sufficient to confer significant sequence similarity on proteins containing them. For example, S. cerevisiae has 134 open...
reading frames (ORFs) with helicase-like features (Shiratori et al., 1999), yet Rrm3p is the only S. cerevisiae protein with significant similarity to Pif1p by the criterion of a TBLASTN search (Zhou et al., 2000; Bessler et al., 2001). Helicase subfamilies, such as the Pif1 subfamily, are defined as a group of proteins that have highly significant sequence similarity to each other but not to other helicases. The functional significance of the sequence similarity that defines helicase subfamilies is not known. The sequence similarity might reflect common functions, recognition of a common nucleic acid substrate, or interaction with a common cofactor.

As part of our goal to understand the functions of the PFI1 subfamily of DNA helicases, we isolated and characterized a PFI1-like gene from the fission yeast Schizosaccharomyces pombe, called pfh1+ (PFI1 homologue). (Previously, pfh1 was called rph1; Zhou et al., 2000; Bessler et al., 2001.) S. pombe and S. cerevisiae are distantly related, about as similar to each other as is to humans (Russell and Nurse, 1986), yet many genes involved in telomere replication and chromosomal maintenance are conserved between them. Although the 3 S. pombe chromosomes are each much larger than any of the 16 S. cerevisiae chromosomes, other features of genome organization are similar. For example, in both organisms telomeres are ~300 base pairs (bp) in length and have an irregular sequence, C13A/TG1~3A, in S. cerevisiae (Shampay et al., 1984) and C2~3TGTA2/T2ACAG2~3 in S. pombe (Sugawara, 1989; Hiraoka et al., 1998). Likewise, ribosomal DNA is organized and replicated similarly in both organisms (Brewer and Fangman, 1988; Linskens and Huberman, 1988; Sanchez et al., 1998). In contrast, the S. cerevisiae mitochondrial DNA is much larger (~75 kb; reviewed in Poon and Schatz, 1991) than in S. pombe (~19 kb; Lang and Wolf, 1984), and unlike wild-type S. pombe, S. cerevisiae can live without mitochondrial DNA.

Our analysis of Pfh1p showed that it had roughly equivalent similarity to both Pif1p and Rrm3p, ~60% similar over a ~450 amino acid region that contains the seven helicase motifs. We purified a truncated form of Pfh1p and demonstrated that, like Pif1p (Lahaye et al., 1991), it had 5' to 3' DNA helicase activity. Unlike both of its Saccharomyces homologues, pfh1+ was essential, and its catalytic activity was required for its essential function. Although cells lacking Pfh1p had telomeres that were ~50 bp shorter than wild-type cells, maintenance of telomeric DNA was not the sole essential function of Pfh1p.

**MATERIALS AND METHODS**

**General Methods and Nomenclature**

All S. pombe strains were isogenic to wild-type strain 972 h−. Strains constructed by integrative transformation (Rothstein, 1983) were confirmed by Southern blotting. Adhering to the conventions appropriate for each yeast species, the wild-type and mutant versions of the S. cerevisiae PFI1 gene were designated, respectively, PFI1 and pfh1, whereas the wild-type and mutant versions of the S. pombe PFI1-like gene were designated, respectively, pfh1+ and pfh1−. Deletion alleles are noted by a delta symbol in S. cerevisiae and by a "D" in S. pombe.

**Identification and Analysis of pfh1+**

The S. pombe pfh1+ was identified using nested degenerate PCR. S. pombe genomic DNA was first amplified using the FIE/DML primer TCGAATTCTC/TATA/C/GATGATC/T/T and the QAYV1 primer CAACAGTCTA/G/CATATC/T/GATCCTC/T/T, where I stands for inosine. Reaction products of 500-1300 bp were isolated and reapplied using the GAGVM primer TCAATTCC- GCCICCA/A/GGTTAATG and the GQVT primer CAAGGCCTTA/A/GGTACCT/TGTAGC/T/TGT. The major PCR product of 450 bp was isolated, cloned, and sequenced. To obtain the rest of the pfh1+ gene, the PCR restriction fragment was used as a probe to screen a S. pombe genomic library (generously provided by Wayne Wahls of Vanderbilt University). Nested deletions of pfh1+ for sequencing were generated as described (Davies and Hutchinson, 1991). Sequencing was done with a combination of manual Sequenase (USB/Amersham) and automated dye terminator reactions. Both strands of the pfh1+ gene were fully sequenced. The pfh1+ sequence was analyzed using GCG software (Group, 1994). The sequence was deposited in the database (Accession number AF074444).

The splicing of the 41-bp intron was confirmed by sequencing a pfh1+ cDNA. The pfh1+ cDNA was obtained by amplifying a S. pombe cDNA library (Becker et al., 1991) using the primer 5' ACATT-TAAATAACAGCAAG and 5' GATCCTAAGAACGAC. The PCR product was checked, and the region of the cDNA that contains the intron was sequenced. The 5' end of the pfh1+ gene was also identified by amplification of the S. pombe cDNA library. The library was first amplified with an ADC promoter primer 5' CTTTT-TCTTGCAAGATTACGACATACC and pfh1+ primer SP403 5' TTAGTACATGCAAACCTCTG. This PCR product was reamplified using the ADC primer and primer 5' TCAAAAAACGGGGCATGGAGG. The PCR product was cloned and sequenced.

**Construction and Analysis of pfh1-D1: ura4+ Mutant Strains**

The plasmid pVS110 contains a 2.9-kb Sna3A genomic DNA fragment generated by partial digestion with Sna3A that begins 35 bp 5' of the start codon and extends to the Cln1 site at the end of the sequence. In the deletion plasmid, the two EcoRV internal pfh1− fragments, a 1492-bp region, were replaced with a 1.8-kb SphI fragment containing ura4+. The integration was performed using diploid strain 585 with the genotype his3-D1his3-D1 leu1-32 / leu1-32 his4-D18/ura4-D18 ade6-m210 / ade6-M216 +/− selecting for Ura− transformants. Different diploid strains were used for mating of the two EcoRV internal pfh1− fragments. The diploid strain was confirmed by mating strains of genotype his3-D1 leu1-32 ura4-D18 ade6-m210 +/− and his3-D1 leu1-32 ura4-D18 ade6-M216 +/−, which were kindly provided by Kathy Gould (unpublished strains). For sporulation experiments, overnight cultures of diploid strains were grown in YEA medium. For sporulation, 100 μl of each culture was spread onto YEPE medium for 2 days at 30°C. The plates were scraped, and cells were resuspended in water to a final cell density of ~2–5 × 107 cells/ml. Glucoselase (Dupont, Wilmington, DE) was added to 0.5%, and cells were incubated overnight at 30°C, and then spores were harvested. For germination, 106 spores were inoculated into 50 ml EMM medium that contained sodium glutamate (EMMG) instead of ammonium chloride as the nitrogen source. Spores derived from the pfh1-D1::ura4+/pfh1− heterozygote 584 were inoculated into EMMG without uracil. In this medium, only spores that had the pfh1-D1::ura4+ deletion were able to germinate and grow. Wild-type spores derived from the pfh1+ pfh1− homozygous control were inoculated into EMMG with uracil. Samples of the germinating spores were taken at various time points, harvested, fixed in 70% ethanol, digested with RNase A, and either stained with Yo-Yo 2 (Molecular Probes, Eugene, OR) for conical microscopy or with propidium iodide for fluorescence-activated cell sorting (FACS) analysis (Alfa et al., 1993).

For Southern blot analysis, DNA was prepared by a glass bead procedure (Runge and Zakian, 1989), digested with HindI or ApaI, and run on a 1% agarose gel. The blots were hybridized sequentially with a telomeric probe (from plasmid pSPT16; Sugawara, 1989), a probe for the entire S. pombe mitochondrial genome (from plasmid...
The viability of a ptp1-1::ptp1-D1::ura4+ double mutant was tested by using ptp1+ strain PTP52 (h- ura3-161 ade6-266 ptp1-1 rho) obtained from T. Fox (Haffer and Fox, 1992) with a strain of genotype h- his3-D1 leu-32::ura4-D18 ade6-M210 pht1-D1::ura4+/pVS117. Single amino acid changes (K337A and K337R) in Pht1p were made using the Quick-Change Site-Directed Mutagenesis Kit (Strategene, La Jolla, CA) and VP6003 plasmid, a his3+ plasmid containing pH1. To make K337A, oligonucleotides K337A5 and K337A3 were used (K337A5: 5′-gct gga aca ggC GCC tct gtt ctc ct-3′; K337A3: 5′-ag gag aac aga GGC Gcc tgt tcc agc-3′). (Mutated residues are capitalized.) This pair of oligonucleotides generated a SfsI site near K337 in pht1 without changing any additional amino acids, which facilitated identification of the K337A mutation. To make K337R, oligonucleotides K337R5 and K337R3 were used (K337R5: 5′-ct gga aca ggt CGa tcG gtt ctc ctc cg-3′; K337R3: 5′-cg gac gag acc Gac tgc ttc acg-3′). This pair of oligonucleotides generates a PvuI site near K337 without changing any additional amino acids.

To determine if pht1+ was essential in cells that lack telomere DNA, we used strains CF199 (h- leu1-32 his3-D3 ura4-D18 ade6-M210; the wild-type control) and CF448 (isogenic to CF199 except for trt1::his3+ and having circularized chromosomes), both kindly supplied by T. Nakamura (Nakamura et al., 1998). Strains were transformed with a 2.9-kb EcoRI/PstI/Bgl fragment from pVS110 which contained the pht1::ura4+ deletion/disruption cassette with or without the LEU2 plasmid pREP41X-pht1+ (the S. cerevisiae LEU2 gene complements a s. pombe leu1–32 strain.)

**Purification and Analysis of Pfh1p-h**

To express active recombinant Pfh1p, a 1608-bp fragment that encoded amino acids 255-789 of Pfh1p was isolated from the pht1+ cDNA, cloned into the Sall site of pEG(KT) (Mitchell et al., 1993) to generate pEG(KT)-Pfh1-h, and transformed into a protease-deficient S. cerevisiae strain BCY123 (Bennett et al., 1998). Expression of the GST-Pfh1p-h fusion protein was carried out using minor modifications of methods described in Bennett et al. (1998). Purification was carried out at 4°C. Cells were harvested, washed, and resuspended in 8 volumes of ice-cold lysing buffer (50 mM Tris-HCl, pH 7.8, 500 mM NaCl, 4 mM MgCl2, 40 μM/m gDNase I, 10 mM dithiothreitol (DTT), 0.1% Triton X-100, 0.004% 1-octanole) and a mixture of protease inhibitors (10 μg/ml aprotinin, 5 μg/ml leupeptin, 10 μg/ml pepstatin A, 100 μg/ml bacitracin, 250 μg/ml soybean trypsin inhibitor, 0.4 mM phenylmethylsulfonyl fluoride, and 10 mM benzamidine hydrochloride). Cells were then subjected to two passages through a cell disruptor (EmulsiFlex-C5; Avestin, Ottawa, Ontario, Canada). After centrifugation (15,000 × g, 15 min), the supernatant was brought to 50% saturation with ammonium sulfate and left on ice for 30 min. The precipitate was collected by centrifugation (27,000 × g, 30 min), suspended in 20 ml PBS supplemented with 5 mM DTT, 0.5% Triton X-100, 0.001% 1-octanole, and protease inhibitor mix. The soluble fraction was recovered by centrifugation (27,000 × g for 30 min) and loaded onto a 4 ml gluthathione sepharose 4B column (1.6 × 2) equilibrated with PBS, at a flow rate of 20 ml/h. The column was washed with 20-bed volumes of the same buffer and 20-bed volumes(s) of the same buffer containing 1 M NaCl. Protein was eluted with 10 ml of elution buffer (50 mM Tris-HCl, pH 8.8, 30 mM reduced glutathione, 50 mM NaCl, 10 mM DTT, 0.1% Triton X-100, 0.001% 1-octanole). The eluate was digested with 200 U of thrombin overnight and loaded onto a 1-ml heparin column equilibrated with 50 mM Tris-HCl buffer, pH 7.8 (200 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.002% Triton X-100) at a flow rate of 30 ml/h. The column was washed with the equilibration buffer and eluted with linear NaCl gradient from 200 mM to 1 M in the same buffer and 1-ml fractions were collected. Recombinant Pht1p-h eluted between 700 and 800 mM NaCl, as determined by Coomassie blue-stained SDS-PAGE and immunoblot analysis. The peak fractions were pooled and concentrated by Centricron (Millipore, Bedford, MA) centrifugation. The Pht1p antibody used to follow expression and purification of Pfh1p-h was made against amino acids 447–708 of Pfh1p expressed in Escherichia coli as a GST-fusion protein. The fusion protein was purified according to the Pharmaceutical Biotechnology protocols (Piscataway, NJ) and was used both to raise polyclonal serum in rabbits and to make an affinity column for the purification of the antisera as described in Koff et al. (1992).

ATPase reactions were carried out in 20 μl ATPase buffer (25 mM HEPES, pH 7.6, 5 mM MgCl2, 2 mM ATP, 1 mM DTT, 100 μg/ml BSA, 200 μg/ml M13 single-strand DNA, with 200 ng of recombinant Pht1p-h or 100 ng of recombinant Pif1p), for 30 min at 37°C. Each reaction contained 0.5 μCi of [γ-32P]ATP. Reactions were stopped by the addition of 1 μl of 0.5 M EDTA, and 0.5 μl of each reaction was spotted on polyethyleneimine (PEI) cellulose plate (Baker, Sanford, ME). The plate was developed in 0.8 M LiCl and dried with hot air. The ATP hydrolysis was visualized on a Molecular Dynamics Phosphoimager (Sunnyvale, CA). For helicase assays, a 25-mer (5′-GTTGATAAAACGACCCAGCTGAAT-3′) and 36-mer (5′-CGTATATGGCTGACGCGTCTGAT-3′) oligonucleotides were annealed to single-stranded M13mp7 or M13mp18 DNA. For the helicase assay, 10 pmol of the 36-mer was 32P-labeled at its 5′ end using 14 polymerase kinase and [γ-32P]ATP, and 2.5 pmol was annealed with equal molar single-stranded M13m18 virion DNA in a 75-μl reaction mixture. For the helicase polarity assay, 2.5 pmol each of the 32P-labeled 36-mer and of the 32P-labeled 25-mer were annealed with equal molar single-stranded M13mp7 DNA linearized by digestion with EcoRI. The labeled substrates were purified with the Chroma Spin-1000 column (Clontech, Palo Alto, CA). Helicase assays (20 μl each) contained 20 mM HEPES, pH 7.6, 5 mM MgAc2, 4 mM ATP, 100 μg/ml M13 single-strand DNA, with 200 ng of recombinant Pht1p-h or 100 ng of recombinant Pif1p, and carried out for 15–30°C. The amount of Pfh1p-h used is comparable to that used to detect helicase activity of the S. cerevisiae Pif1p (Lahaye et al., 1991; 1995; Zhou et al., 2000) as well as other S. cerevisiae DNA helicases (Sung et al., 1988; Sedman et al., 2000) and the Mcm complex from S. pombe (Lee and Hurwitz, 2001) and other organisms (You et al., 1999; Chong et al., 2000). Products were analyzed by electrophoresis in a 10% polyacrylamide (89 mM Tris borate, pH 8.3, 2 mM EDTA) and visualized on a Molecular Dynamics Phosphoimager.

**RESULTS**

**Isolation of a PIFI-like Gene from S. pombe**

We identified a PIFI-like gene from the fission yeast S. pombe using nested degenerate PCR. To obtain a clone that contained the entire S. pombe PIFI-like gene, hereafter called pfi1+, for PIFI homologue, the PCR restriction fragment was used as a probe to screen a S. pombe genomic library (Fikes
et al., 1990). Both strands of the pfh1+ gene were sequenced, and the sequence was deposited in the database (GenBank Accession number AF074944). The DNA sequence and predicted protein sequence are shown in supplementary material.

The conceptual translation of the pfh1+ gene generated an 805 amino acid protein with one small 41-base intron. The splicing of this intron was confirmed by sequencing a cDNA obtained from a random library of S. pombe cDNA clones. There were two out of frame AUG codons in the 5′ end of the mRNA before the first AUG of the pfh1+ ORF. The presence of the two upstream AUG codons suggests that pfh1+ is either translationally regulated or poorly translated, because eukaryotic translation usually initiates at the first AUG in an mRNA (Kozak, 1992). There are three MluI sites (MCB, MluI cell cycle box) 5′ of the start of the pfh1+ ORF. MluI sites are found in the promoter regions of many S. cerevisiae and S. pombe DNA synthesis genes and often confer cell cycle regulated transcription (reviewed in McIntosh, 1993).

When the predicted protein sequence of S. pombe Pfh1p was compared with the translated DNA database using the TBLASTN program (Altschul et al., 1990), it displayed highly significant similarity to each of the previously described (Bessler et al., 2001) Pif-like proteins (Table 1). Because our last analysis of PIF1-like genes (Bessler et al., 2001), several additional Pif1-like proteins were reported. Neurospora crassa and the basidiomycete Phanerochaete chrysosporium both encode two Pif1-like proteins. Like multicellular eukaryotes (but unlike S. cerevisiae, N. crassa, and P. chrysosporium), S. pombe encoded only a single Pif1-like protein. Although the helicase domain of Pfh1p was ≥36% identical to each of the other Pif1-like proteins (Zhou et al., 2000), its amino terminal ~330 and its carboxyl terminal 52 amino acids had no sig-

Table 1. Proteins homologous to pfh1+

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The predicted protein sequence of pfh1+ was compared to the translated DNA database using the default program and parameters of the NCBI server. The expectation score indicates the probability that similarity occurred by chance. All proteins with an expectation score equal to or smaller than 8 x 10^{-50} are shown.

The S. pombe Pfh1p Is a 5′ to 3′ DNA Helicase

To determine if Pfh1p is also a DNA helicase, we purified and characterized recombinant Pfh1p. Initially we expressed full-length Pfh1p in E. coli, but this protein had poor solubility and was often degraded. Therefore, a truncated version of Pfh1p consisting of amino acids 255–789 of the 805 amino acid protein, a region that contained all seven helicase motifs, was fused at its amino terminus to GST and expressed in S. cerevisiae from a galactose-inducible promoter. Proteins were resolved by 8% SDS-PAGE and detected by Coomassie blue staining (A) or, in a separate gel, by immunoblotting with affinity-purified anti-Pfh1p antibodies (B). Lane M, contained prestained protein size markers (NEB). The covalent coupling of the dye to the proteins affects their behavior in SDS-PAGE gels such that the 83-kDa marker has a slower mobility than expected from its mass. The lanes contain total cell extract from an uninduced culture (lane 2), total cell extract from a galactose-induced culture (lane 3), 50% ammonium sulfate precipitate from the induced culture (lane 4), extract from induced cells after fractionation on glutathione sepharose (GST column, lane 5), and extract from induced cells after thrombin digestion to remove the GST moiety and fractionation on Heparin sepharose (lane 6).
The purified recombinant Pfh1p-h had Mg\(^{2+}\)-dependent ATPase activity (Figure 2A) that was stimulated by single-strand DNA (Figure 2A, lane 5). Helicase activity of recombinant Pfh1p-h was demonstrated by its ability to displace a \(^{32}\)P-labeled 36-mer oligonucleotide annealed to single-stranded circular M13 DNA, a reaction that was both Mg\(^{2+}\)- and ATP-dependent (Figure 2, B and C). Because the ATPase and helicase activities were not detectable until the GST-Pfh1p-h was thrombin cleaved, these enzymatic activities were due to Pfh1p-h itself, rather than to a minor contaminant.

The polarity of the recombinant Pfh1p-h helicase was established using a substrate in which kinase-labeled 25- and 36-mer oligonucleotides were annealed at, respectively, the 5' and 3' ends of linearized single-stranded M13 DNA (Figure 2C). A 5' to 3' DNA helicase will displace the 36-mer, whereas a 3' to 5' DNA helicase will remove the 25-mer. Like purified recombinant Pif1p (Figure 2C, lane 6), recombinant Pfh1p-h displaced the 36-mer but not the 25-mer from the M13 DNA (Figure 2C, lane 5). Thus, Pfh1p-h is a 5' to 3' DNA helicase.

**The S. pombe pfh1\(^{+}\) Gene Is Essential**

Integrative transformation was used to delete 1492 bp from one of the two copies of pfh1\(^{+}\) in a diploid strain. The deletion was marked by insertion of the ura4\(^{-}\) gene in an otherwise ura4\(^{-}\) strain. The pfh1\(^{+}\)/pfh1-D1::ura4\(^{-}\) diploid was sporulated, and 100 random spores were all found to be ura4\(^{-}\). When tetrads from a pfh1\(^{+}\)/pfh1-D1::ura4\(^{-}\) diploid were dissected, only two of the four spores formed large colonies, and the large colonies were all ura4\(^{-}\), confirming that pfh1\(^{+}\) was an essential gene (Figure 3A). The pfh1-D1::ura4\(^{-}\) spores formed microcolonies with 1–10 cells in which the individual cells were often elongated (as in Figure 3B).

We constructed versions of pfh1\(^{+}\) that were expressed from the thiamine-repressible nmt promoter (Basi et al., 1993; Maundrell, 1993). These plasmids were introduced into the pfh1\(^{+}\)/pfh1-D1::ura4\(^{-}\) diploid strain, and the diploid sporulated. Even when the weakest nmt1 promoter was used, the nmt1-driven pfh1\(^{+}\) gene was able to complement the pfh1-D1 strain in medium containing thiamine. This nmt-driven pfh1\(^{+}\) gene's ability to rescue the lethality of the pfh1-D1::ura4\(^{-}\) strain showed that the phenotypes attributed to loss of Pfh1p were not due to reduced expression of a neighboring gene and also demonstrated that very little Pfh1p was needed for viability.

**S. pombe Cells Lacking Pfh1p Proceed through S Phase but Have Shorter Telomeres and an Elongated Cellular Phenotype**

To obtain larger numbers of pfh1-D1::ura4\(^{-}\) cells, the pfh1\(^{+}\)/pfh1-D1::ura4\(^{-}\) diploid was sporulated, and then spores were grown in liquid medium lacking uracil. As a control, a pfh1\(^{+}\)/pfh1-D1::ura4\(^{-}\)/ura4\(^{-}\) diploid strain was treated in the same way. As demonstrated by others, ura4\(^{-}\) spores do not germinate in medium lacking uracil (see, for example, Waseem et al., 1992). As expected, no germinated.
spores were seen in the wild-type culture. In contrast, the pfh1-D1::ura4+/H11001 culture germinated, and at least some cells divided one or more times, producing a culture in which the cell number was two- to threefold higher than in the starting culture. Because only the pfh1-D1::ura4+/H11001 cells could divide in the absence of uracil, these methods yielded a population of cells depleted for Pfh1p.

The DNA of the pfh1-D1::ura4+/H11001 cells was stained, and cells were observed by confocal microscopy (Figure 3B). For comparison, we sporulated a pfh1+/pfh1+ strain, but otherwise isogenic diploid strain in media containing uracil (Figure 3C). By 20 h postsporulation, pfh1-D1::ura4+ cells appeared elongated compared with wild-type cells, indicative of cells that are able to grow but not divide (Waseem et al., 1992; see, for example, Kelly et al., 1993). This difference in cellular morphology was apparent at ~14 h postsporulation. At various
times during germination, samples of wild-type and the pfh1-D1::ura4+ mutant cells were stained with propidium iodide and analyzed by FACS. Like wild-type cells, DNA replication was detectable 10 h postgermination in the pfh1-D1::ura4+ cells (Figure 4). By 20–22 h, the vast majority of cells in both the wild-type and mutant cultures had a 2C DNA content. Thus, upon depletion of Pfh1p, arrested cells accumulated with replicated genomes resembling the steady-state distribution of wild-type cultures.

We also examined telomere length in DNA from pfh1-D1::ura4+ mutant cells (Figure 5A). S. pombe has six telomeres, each bearing ~300 bp of telomeric repeats (Sugawara, 1989). Five of the six telomeres also have at least 19 kb of subtelomeric DNA. The other telomere, which generates a 0.9-kb fragment after Hinfl digestion (Figure 5A, right-hand lanes) is immediately abutted to the rDNA locus (Sugawara, 1989). Telomeric DNA from pfh1-D1::ura4+ cells was 40–60 bp shorter than telomeric DNA from pfh1+ cells (Figure 5A). The telomere adjacent to the rDNA that generates the 0.9-kb Hinfl fragment appeared to be less affected than the other telomeres. Although the effect on telomere length was small, it was reproducible, being seen in DNA prepared from two independent sporulations, restricted with three different enzymes (Hinfl and Apal, Figure 5A, and EcoRI), and run on multiple gels. Similar amounts of telomere shortening were seen 16, 20, and 24 h after germination (Figure 5A). The shortening of DNA was specific to telomeres, because rehybridization of the same gels with a subtelomeric or other internal sequences (Figure 5, B and C) showed that the mobility of these sequences was not affected. There was no detectable change in rDNA (Figure 5B) or mitochondrial DNA (Figure 5D) in pfh1-D1::ura4+ cells.

Maintenance of Telomeric DNA Is Not the Sole Essential Function of Pfh1p

Although most S. pombe cells die when they lack telomerase, a small subset of cells survives. In most of these survivors, the three S. pombe chromosomes lose the simple repeats at the very ends of the chromosomes as well as much of the subtelomeric TAS elements and fuse end to end to generate circular chromosomes (Naito et al., 1998; Nakamura et al., 1998). If replication of telomeric DNA were the sole essential function of Pfh1p, it should be possible to delete pfh1+ in a strain with circular chromosomes.
Because cells with circular chromosomes are not viable in meiosis (Naito et al., 1998; Nakamura et al., 1998), we could not sporulate a pfh1+/pfh1-D::ura4+ diploid having circular chromosomes to obtain the desired strain. Rather, we used two alternative approaches. In the first experiment, we used a trt1+ survivor strain CF448 (Nakamura et al., 1998) that had been shown previously to have circularized chromosomes. We transformed this haploid strain with the pfh1D1::ura4+ fragment that was used previously to disrupt pfh1+ in diploid cells, selecting for Ura+ cells. As a control for transformation efficiency, we transformed the same strain with both the pfh1D1::ura4+ and a LEU2 pfh1+ plasmid, selecting for Ura+ Leu+ cells. Although we obtained only a few Ura+ transformants with the pfh1 disruption fragment alone, and Southern analysis revealed that none of these rare Ura+ transformants had integrated at the pfh1+ locus. In the second approach, we disrupted the trt1+ gene as described (Nakamura et al., 1998) in a pfh1-D1::ura4+ haploid yeast that carried a LEU2 plasmid with the pfh1+ gene. We isolated survivors and then asked if these survivors could lose the pfh1+ LEU2 plasmid. Out of 700 survivors, none generated subclones that were able to grow on plates lacking leucine. In contrast, 17% of the survivors generated in a trt1- pfh1+ strain lost a LEU2 pfh1+ plasmid. Thus, pfh1+ is essential even in strains that lack telomeric DNA.

The Inviability of pfh1-D1::ura4+ Cells Was Not Due to Lack of Mitochondrial DNA

Mitochondrial DNA is lost at a high rate in S. cerevisiae cells that lack Pfh1p (Foury and Kolodny, 1983; Schulz and Zakian, 1994). Because wild-type S. pombe cannot survive without mitochondrial DNA (Munz et al., 1989), if Pfh1p were needed to maintain mitochondrial DNA, it would explain the inviability of pfh1-D1::ura4+ strains. Although wild-type S. pombe cells require mitochondrial DNA, cells lacking mitochondrial DNA are viable in a ptp1-1+ strain (Haffter and Fox, 1992). Thus, if the only essential function of Pfh1p is to maintain mitochondrial DNA, a ptp1-1- pfh1-D1::ura4+ strain should be viable. To test this possibility, a ptp1-1- haploid strain was mated to a pfh1-D1::ura4+ strain that carried the pfh1+ gene on a plasmid. Twenty-five of the 33 tetrads that were dissected from this cross had only two viable spores, and the two nonviable spores often gave rise to cells with an elongated phenotype similar to that of the pfh1-D1::ura4+ cells shown in Figure 3B. The four tetrads with more than two viable spores were due to the pfh1+ plasmid being retained in a pfh1-D1::ura4+ spore. Because 14 of 30 viable spore progeny had the ptp1-1+ mutation, ptp1-1- was not genetically linked to pfh1-D1::ura4+. Because the ptp1-1- mutation did not rescue a pfh1-D1::ura4+ strain, maintenance of mitochondrial DNA cannot be the sole essential function of pfh1+. The presence of mitochondrial DNA in pfh1-D1::ura4+ cells supports this interpretation (Figure 5D).

Although S. cerevisiae rrm3Δ strains have no evident mitochondrial defect, deleting RRM3 in a pfi1Δ strain partially suppresses the loss of mitochondrial DNA caused by deleting PFI1. These results suggest that Rrm3p has some role in mitochondrial DNA metabolism, at least in the absence of Pfi1p (V.P. Schulz and V.A. Zakian, unpublished results). Although Pfh1p did not appear to be required to maintain mitochondrial DNA, it is possible that Pfh1p, like the S. cerevisiae Rrm3p, has some role in mitochondria. To assess if Pfh1p might function in mitochondria, we used computer programs to predict its likely subcellular localization. The subcellular localization program PSORT II (http://psort.nibb.ac.jp) predicted that the S. pombe Pfh1p as well as the S. cerevisiae Pfi1p and Rrm3p are targeted to both the nucleus and to mitochondria. In addition, we scanned Pfh1p for mitochondria targeting signals using MitoProt II (http://www.mips.biochem.mpg.de/proj/medgen/mitop/). Both PSORT II and MitoProt II base the likelihood of mitochondrial DNA targeting on the amino acid composition of the N-terminal region of the protein. MitoProt II predicts the probability of localization to the mitochondria to be 0.9877, 0.9326, and 0.9264 for Pfi1p, Rrm3p, and Pfh1p, respectively. Importantly, other helicases not believed to be found in the mitochondria such as Sgs1p and Rqh1p, the S. cerevisiae and S. pombe homologues of the Bloom’s and Werner’s syndrome

Table 2. The ATPase/helicase activity of Pfh1p is essential

<table>
<thead>
<tr>
<th>S. pombe strain/LEU2 plasmid</th>
<th># His+ colonies</th>
<th># His+ Leu+ colonies</th>
<th>Percentage of colonies that lost LEU2 plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>612/pfh1+</td>
<td>188</td>
<td>140</td>
<td>74.4</td>
</tr>
<tr>
<td>612/pfh1-K337R</td>
<td>320</td>
<td>0</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>612/pfh1-K337A</td>
<td>309</td>
<td>0</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>613/pfh1+</td>
<td>204</td>
<td>200</td>
<td>98.0</td>
</tr>
<tr>
<td>613/pfh1-K337R</td>
<td>388</td>
<td>0</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>613/pfh1-K337A</td>
<td>147</td>
<td>0</td>
<td>&lt;0.7</td>
</tr>
</tbody>
</table>

The pfh1-D::ura4+ S. pombe strains 613, which carried a LEU2 plasmid with pfh1+ under the control of its own promoter, or 613, which carried a LEU2 plasmid with pfh1+ under the control of the nmt1+ promoter, were transformed with a his3+ plasmid carrying either wild type pfh1+ or mutant alleles pfh1-K337A, or pfh1-K337R. The first column indicates the S. pombe strain and the identity of the LEU2 plasmid it contained. Leu+ His+ colonies were streaked twice to medium containing leucine and lacking histidine. The resulting His+ colonies (row 2) were replica plated to determine the fraction of His+ Leu+ cells (row 3). The percentage of colonies that lost the LEU2 plasmid is also indicated (row 4).
helicases, were not predicted to possess mitochondria targeting sequences by these programs.

The ATPase/Helicase Activity Is Required for Its Essential In Vivo Function

Some genes that encode DNA helicases, for example the S. cerevisiae RAD3 and DNA2 genes, are essential for viability yet the helicase activity of their products is not required for their essential function (Sung et al., 1988; Formosa and Nittis, 1999; Budd et al., 2000). To determine if the helicase activity of Pf1p is essential, we used site-directed mutagenesis to modify an invariant lysine in the ATP-binding domain of motif I to either alanine (K337A) or arginine (K337R). Mutations in this residue eliminate the activity of all helicases in which they have been tested (Gorbunova and Koonin, 1993), including Pf1p (Zhou et al., 2000). A his3+ plasmid carrying either the wild-type pfhl+ gene or one of the two point mutation alleles, pfhl-K337A or pfhl-K337R, were introduced into two different pfhl-D1::ura4+ strains, VPS612 and VPS613. The two S. pombe strains were identical except that VPS612 had a LEU2 plasmid containing the wild-type pfhl+ gene under the control of its own promoter and VPS613 had a LEU2 plasmid containing the pfhl+ gene under the control of the nmt1 promoter (the S. cerevisiae LEU2 gene complements an S. pombe leu1− strain). Leu+ His+ transformants were isolated and then streaked twice on medium lacking histidine but containing leucine. Growth on medium containing leucine allowed cells to lose the LEU2 plasmid. However, Leu− cells could only be generated if the his3+ plasmid could supply the essential function of Pf1p. S. pombe strains VPS612 or VPS613 carrying the wild-type pfhl+ his3+ plasmid readily lost the LEU2 plasmid: in these strains, 74% or 98% of the cells were Leu− after cells were streaked twice on medium containing leucine. In contrast, Leu− cells were not recovered when the his3+ plasmid had either the pfhl-K337R or pfhl-K337A alleles (Table 2). Thus, the K337A and the K337R alleles were unable to supply the essential function(s) of Pf1p.

DISCUSSION

The S. pombe pfhl+ gene encodes an 805 amino acid protein with very high similarity to all other members of the Pf1p subfamily of DNA helicases (Table 1). Although Pf1p had the seven motifs that characterize helicases, it had no significant similarity by the criterion of a TBLASTN search to other S. pombe helicases. S. cerevisiae has two PIF1-like genes, PIF1 itself and RRM3. Neither of the S. cerevisiae genes is essential, and pfhl rrm3 cells are also viable (Iovina et al., 2000). Because pfhl rrm3 S. cerevisiae cells are viable, the essentiality (Figure 3A) of the S. pombe pfhl+ gene was unexpected.

We purified a truncated form of Pf1p (Figure 1) and demonstrated that it had both ATPase (Figure 2A) and 5′ to 3′ DNA helicase activity (Figure 2D). Because alleles with point mutations in the ATP binding motif of Pf1p were indistinguishable from null alleles (Table 2), the ATPase/helicase function of Pf1p was essential. Cells lacking Pf1p underwent essentially a complete or nearly complete round of DNA replication (Figure 4) before arresting as elongated cells (Figure 3B). Although elongated cell morphology is not a highly specific phenotype, many S. pombe mutants with postinitiation defects in DNA replication have an appearance similar to that of pfhl-D1::ura4+ cells (Muris et al., 1996; Gould et al., 1998).

Although the S. cerevisiae Pif1 and Rrm3 proteins affect mitochondrial DNA and rDNA (see Introduction), we saw no difference in the structure of mitochondrial DNA or rDNA in mutant versus wild-type cells by Southern hybridization (Figure 5). This level of Southern analysis would detect the types of differences seen in the mitochondrial DNA of most mitochondrial-deficient S. cerevisiae strains but would not detect more subtle changes in abundance or structure of either ribosomal or mitochondrial DNA (Figure 5, B and D). Cytological observations also showed that many Pf1p-depleted cells contained mitochondrial DNA (Figure 3B), and the lethality of the pfhl-D1::ura4+ mutation was not bypassed by the ptf1-1 mutation, which allows growth of S. pombe cells lacking mitochondrial DNA (Haffter and Fox, 1992). Taken together, these data argue that maintenance of mitochondrial DNA is unlikely to be the essential function of Pf1p. However, given that Pf1p was predicted to localize to both mitochondria and nuclei, it might have a nonessential role in mitochondrial DNA metabolism.

Cells lacking Pf1p exhibited a modest decrease in telomere length (Figure 5A). Because this decrease was manifest by 16 h, persisted for at least 8 h, and was not seen with nontelomeric DNA fragments, it was unlikely to be due to DNA degradation in dying cells. The extent of telomere shortening in pfhl-D1::ura4+ cells was similar to that seen in several mutants defective in both DNA replication and DNA damage checkpoints (Dahlen et al., 1998) or for mutants in either of the two S. pombe ATM-like genes (Naito et al., 1998). Despite the role of Pf1p in maintaining wild-type length telomeres, eliminating telomeric DNA did not eliminate the requirement for Pf1p. Thus, although Pf1p may affect telomeres, maintenance of telomeric DNA cannot be its sole essential function.

Our data suggest that Pf1p is essential because it plays a vital role in chromosomal DNA replication. The strongest support for this hypothesis is the essentiality (Table 2) of the 5′ to 3′ DNA helicase activity it encodes (Figure 2). A terminal phenotype similar to that of mutants with defects in DNA replication (Figure 3B) is consistent with a role for Pf1p in chromosome replication. An appealing possibility...
is that Pfh1p, like the S. cerevisiae Rrm3p (Ivessa et al., 2000), is required to separate converged replication forks in specific regions of the genome.

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