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* Pfh1p.

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 Kolodnyski, 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 recombination 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 Rrm3p 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

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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 PIF1 subfamily of DNA helicases, we isolated and characterized a PIF1-like gene from the fission yeast *Schizosaccharomyces pombe*, called *pfh1*⁺ (PIF1 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 each is to humans (Russell and Nurse, 1986), yet many genes involved in telomere replication and chromosome 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, C₁₋₃A/TG₁₋₃ in *S. cerevisiae* (Shampay *et al.*, 1984) and C₂₋₈TGTA₂/T₂ACAG₂₋₈ 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 Pon 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* PIF1 gene were designated, respectively, PIF1 and *pif1*⁻, whereas the wild-type and mutant versions of the *S. pombe* PIF1-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/DMNL

primer TCGAATTCT(C/T)AT(A/C/T)GIATG(C/T)TIA and the QAYVAL primer CAAAGCTTA(A/G)IGCIAC(A/G)TAIGC(CT)TG, where I stands for inosine. Reaction products of 500-1300 bp were isolated and reamplified using the GAQVM primer TCGAATTCG-GIGCICA(A/G)GTIATG and the QGQTL primer CAAAGCTTA(A/G)IGT(C/T)TGICC(C/T)TG. 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 AF074944).

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-TAATAAACACAGAAG and 5' GATCCTAAAAGAACCAGCAC. The resulting PCR product was cloned, and the region of the insert 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-TCTGCAGAAATATTTCAAGCTATACC and *pfh1*⁺ primer SP1403 5' TTAGTACATGGTAAACTCGT. This PCR product was reamplified using the ADC primer and primer 5' TCAAAAACGGGCAGTGAGGG. The PCR product was cloned and sequenced.

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

The plasmid pVS110 contains a 2.9-kb *Sau3A* genomic DNA fragment generated by partial digestion with *Sau3A* that begins 35 bp 5' of the start codon and extends to the *Clal* 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-D1/his3-D1 leu1-32-/leu132- ura4-D18/ura4-D18 ade6-m210-/ade6-M216- h⁺/h⁻* by selecting for *Ura*⁺ transformants. This diploid strain was made by mating strains of genotype *his3-D1 leu1-32- ura4-D18 ade6-m210- h⁺* and *his3-D1 leu1-32- ura4-D18 ade6-M216- h⁻*, 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 μ l of each culture was spread onto YEPD 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 $\times$ 10⁷ cells/ml. Glusulase (Dupont, Wilmington, DE) was added to 0.5%, and cells were incubated overnight at 30°C, and then spores were harvested. For germination, 10⁸ 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 confocal 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 *HinfI* 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

pDG3; Del Giudice, 1981), a 288-bp probe that detects the *rhp6⁺* gene that was made by PCR amplification of genomic DNA (Reynolds *et al.*, 1990), and to a 2.4-kb rDNA probe made by PCR amplification. Probes were labeled with [α - 32 P]dCTP using the RTS RadPrime DNA labeling system (Life Technologies, Rockville, MD).

Complementation analysis of the *pfh1-D1::ura4⁺* deletion strain was performed by plasmid swapping. A 6.1-kb *S. pombe* genomic DNA fragment was inserted into Bluescript KSII vector. The 6.1-kb insert began 1.7 kb upstream of the first AUG of *pfh1⁺* and ended 1.9 kb after the stop codon. The 6.1-kb fragment was released from the Bluescript KSII vector by digestion with *NotI* and *PstI*, treated with T4 DNA polymerase to generate blunt ends, and inserted into the *SmaI* site of pBG2 (Burke and Gould, 1994), a plasmid containing the *S. pombe his3⁺* gene and *ars1*. This plasmid, called pVS117, was transformed into a heterozygous *pfh1-D1::ura4⁺/pfh1⁺* diploid strain, and the diploid was sporulated to yield a *pfh1-D1::ura4⁺* haploid strain. The haploid strain was transformed with either of two plasmids, pREP41X or pREP81X, that carry the *pfh1⁺* gene under the control of the thiamine-regulated *nmt* promoters (Basi *et al.*, 1993).

The viability of a *ptp1-1⁻ pfh1-D1::ura4⁺* double mutant was tested by mating *S. pombe* strain PTP25 (*h⁺ ura1-161⁻ ade6-216⁻ ptp1-1⁻ rho⁺*) obtained from T. Fox (Haffter and Fox, 1992) with a strain of genotype *h⁻ his3-D1 leu1-32⁻ ura4-D18 ade6-M210⁻ pfh1-D1::ura4⁺/pVS117*. Single amino acid changes (K337A and K337R) in Pfh1p were made using the Quick-Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and VPS603 plasmid, a *his3⁺* plasmid containing *pfh1⁺*. 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 *SfoI* site near K337 in *pfh1* 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 gag gag aac CGa tCG acc tgt tcc ag-3'). This pair of oligonucleotides generates a *PvuI* site near K337 without changing any additional amino acids.

To determine if *pfh1⁺* was essential in cells that lack telomeric DNA, we used strains CF199 (*h⁻ leu1-32 his3-D1 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/BglI* fragment from pVS110 which contained the *pfh1::ura4⁺* deletion/disruption cassette with or without the *LEU2* plasmid pREP41X-*pfh1⁺* (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 *pfh1⁺* cDNA, cloned into the *SalI* 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 lysis buffer (50 mM Tris-HCl, pH 7.8, 500 mM NaCl, 4 mM MgCl₂, 40 µg/ml DNase I, 10 mM dithiothreitol (DTT), 0.1% Triton X-100, 0.004% 1-octanol) 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 lysed by two passes 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-octanol, and protease inhibitor mix. The soluble fraction was recovered by centrifugation (27,000 × g for 30 min) and loaded onto a 4 ml glutathione 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 volume(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-octanol). 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 Pfh1p-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 Centricon (Millipore, Bedford, MA) centrifugation. The Pfh1p 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 Pharmacia 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 antiserum 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 Pfh1p-h or 100 ng of recombinant Pif1p), for 30 min at 37°C. Each reaction contained 0.5 µCi of [γ - 32 P]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 polyethylimine (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 Phosphorimager (Sunnyvale, CA). For helicase assays, a 25-mer (5'-GTTGTAAAACGACGGCCAGTGAATT-3'), and 36-mer (5'-CGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATT-3') oligonucleotides were annealed to single-stranded M13mp7 or M13mp18 DNA. For the helicase assay, 10 pmol of the 36-mer was 32 P-labeled at its 5' end using T4 polynucleotide kinase and [γ - 32 P]ATP, and 2.5 pmol was annealed with equal molar single-stranded M13mp18 virion DNA in a 75-µl reaction mixture. For the helicase polarity assay, 2.5 pmol each of the 32 P-labeled 36-mer and of the 32 P-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 BSA, 5% glycerol, 1 mM DTT, 2 µl DNA substrate, and 200 ng of recombinant Pfh1p-h protein (or 100 ng recombinant Pif1p) and carried out for 10 min at 37°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, 1993; 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 Phosphorimager.

RESULTS

Isolation of a PIF1-like Gene from *S. pombe*

We identified a *PIF1*-like gene from the fission yeast *S. pombe* using nested degenerate PCR. To obtain a clone that contained the entire *S. pombe PIF1*-like gene, hereafter called *pfh1⁺*, for *PIE1* 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 *Mlu*I sites (MCB, *Mlu*I cell cycle box) 5' of the start of the *pfh1*⁺ ORF. *Mlu*I 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 Pif1p-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-

Figure 1. Purification of recombinant Pfh1p-h. Amino acids 255–789 of the 805 amino acid Pfh1p were fused at the 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).

nificant homology to these portions of other Pif1 subfamily members.

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 to generate Pfh1p-h (h stands for helicase). DNA encoding the GST-Pfh1p-h fusion protein was introduced into *S. cerevisiae* under the control of a galactose-inducible promoter. Protein extracts were prepared from both galactose- and glucose-grown cells and examined by Western analysis using affinity-purified anti-Pfh1p antibodies (Figure 1). The galactose-grown cells (induced) contained an ~84-kDa protein, the appropriate size for the GST-Pfh1p-h fusion protein, that was not detected in control extracts (Figure 1B, compare uninduced in lane 2, to induced by galactose addition in lane 3). Because the GST-Pfh1p fusion showed no helicase activity, the 58.8-kDa truncated Pfh1p-h freed of the GST moiety by thrombin cleavage was purified to near homogeneity as described in MATERIALS AND METHODS (Figure 1A, lane 6). The faint Coomassie blue staining bands in the prepara-

Table 1. Proteins homologous to *pfh1*⁺

Accession Number ^a	Gene	Expectation Score
Contig 1.441	<i>N. crassa</i>	1×10^{-149}
X05342	<i>S. cerevisiae</i> PIF1	1×10^{-117}
U00062	<i>S. cerevisiae</i> RRM3	1×10^{-105}
Scaffold.152	<i>P. chrysosporium</i>	1×10^{-101}
AB015041	<i>C. elegans</i>	5×10^{-76}
AE003579	<i>D. melanogaster</i>	2×10^{-70}
AF108138	<i>H. sapiens</i>	1×10^{-61}
AF083915	<i>Chilo iridescent virus</i>	9×10^{-56}
X74788	<i>C. maltosa</i>	4×10^{-54}
Scaffold.162	<i>P. chrysosporium</i>	2×10^{-54}
Contig 1.464	<i>N. crassa</i>	8×10^{-50}

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 8×10^{-50} are shown.

^a There are no accession numbers for the Pif1-like proteins from *N. crassa* or the basidiomycete *P. chrysosporium*. However, these sequences can be obtained from a search of, respectively, <http://www.genome.wi.mit.edu/annotation/fungi/neurospora> and <http://www.jgi.doe.gov/programs/whiterot.htm>.

Figure 2. Recombinant Pfh1p-h has single-strand DNA-stimulated ATPase and 5' to 3' DNA helicase activities. Truncated Pfh1p (called Pfh1p-h; h for helicase) was purified as shown in Figure 1 and used in enzyme assays. (A) ATPase activity assay. Reactions were carried out in ATPase buffer as described in MATERIALS AND METHODS, the products were developed in a polyethylimine (PEI) cellulose plate and visualized on a Molecular Dynamics Phosphorimager. Lane 5 contains [γ-³²P]ATP, M13 single-strand DNA, Mg²⁺, and recombinant Pfh1p-h. The other lanes were the same except lane 1 contained recombinant Pif1p, prepared as described in Zhou *et al.* (2000), in place of Pfh1p-h; lane 2, contained BSA in place of Pfh1p-h; lane 3 had no Mg²⁺, and lane 4 had no single-strand DNA. (B) Helicase activity assay and (C) Polarity assay. Kinase-labeled 25-mer oligonucleotide, kinase-labeled 36-mer oligonucleotide, and single-stranded M13 DNA were used to make the substrate for the helicase assays shown at the right. The products were resolved in 10% PAGE and visualized on a Molecular Dynamics Phosphorimager. Lane 1 shows heat denatured substrate. Lane 5 contains [γ-³²P]ATP, Mg²⁺, and recombinant Pfh1p-h. The other lanes are the same, except lane 2 contains BSA instead of Pfh1p-h; lane 3 lacks ATP; lane 4 lacks Mg²⁺, and lane 6 contains recombinant Pif1p in place of Pfh1p-h.

tion of purified Pfh1p-h (Figure 1A) were detected by the anti-Pfh1p antibodies (Figure 1B), indicating that they were likely degradation products of GST-Pfh1p-h.

The purified recombinant Pfh1p-h had Mg²⁺-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 ³²P-labeled 36-mer oligonucleotide annealed to single-stranded circular M13 DNA, a reaction that was both Mg²⁺- 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*⁺ *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

Figure 3. Phenotypes of cells lacking Pfh1p. (A). The *pfh1*⁺/*pfh1-D1::ura4*⁺ diploid strain was sporulated, and the four haploid spore progeny from each of seven tetrads were separated on rich medium, arranged top to bottom, and allowed to grow for 3 days at 30°C. Viability segregated 2⁺:2⁻ and viable spores were always Ura⁻. Spores that failed to form visible colonies often divided one or, more rarely, several times to form microcolonies containing up to 10 cells. (B) A *pfh1*⁺/*pfh1-D1::ura4*⁺ diploid strain was sporulated. Spores were germinated and grown in medium lacking uracil, in which *pfh1*⁺ cells cannot grow owing to their being Ura⁻. DNA was stained using the fluorescent dye Yo-Yo 2 (Molecular Probes, Eugene, OR), and cells were examined by fluorescence microscopy at various times after sporulation. The sample shown was taken 28 h after sporulation. At 28 h, the final cell number was two to three times higher than that of the starting culture, indicating that many *pfh1-D1::ura4*⁺ cells had divided one or two times. The speckled fluorescent dots are mitochondria (Haffter and Fox, 1992). (C) Analysis is as in B, except that a wild-type diploid was sporulated, and cells were germinated and grown in medium containing uracil.

Figure 4. Cells lacking Pfh1p arrest with a 2C DNA content. Spores derived from a *pfh1-D1::ura4*/*pfh1*⁺ heterozygote were inoculated into germination medium lacking uracil so that only *pfh1-D1::ura4*⁺ mutant spores could germinate. Control spores derived from a *pfh1*⁺/*pfh1*⁺ homozygote (wild-type) were inoculated into germination medium containing uracil such that all spores could germinate. Samples of cells were harvested at various time points after inoculation, stained with propidium iodide, and analyzed by FACS for DNA content.

spores were seen in the wild-type culture. In contrast, the *pfh1*⁺/*pfh1-D1::ura4*⁺ 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*⁺ 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*⁺ cells was stained, and cells were observed by confocal microscopy (Figure 3B). For comparison, we sporulated a *pfh1*⁺/*pfh1*⁺, 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

Figure 5. Deletion of *pfh1*⁺ is associated with shortening of *S. pombe* telomeres. DNA was isolated from *pfh1*⁺ and *pfh1-D1::ura4*⁺ cells after 16, 20, or 24 h of germination. Panel A contains DNA digested with *ApaI* (left lanes) or *HinfI* (right lanes) and probed with a *TaqI* fragment from pSPT16 that contains *S. pombe* telomeric sequences (but not subtelomeric sequences). For *ApaI*, the wild-type and mutant samples are grouped, whereas mutant and wild-type samples are alternated for the *HinfI*-digested samples. In *ApaI*-digested DNA, there is an *ApaI* site very near the tract of telomeric repeats in five of the six telomeres, such that the broad band of ~300 bp contains most of the telomeres. *HinfI* digested DNA places five of the six telomeres on fragments of differing sizes. (B–D) The same *HinfI*-digested samples from A, after analysis with the following probes: (B) a 2.4-kb PCR fragment from *S. pombe* rDNA. This probe hybridizes to multiple *HinfI* fragments, seven of which are smaller than 360 bp. Shown are the three largest hybridizing fragments of 1077 bp and a doublet of 825 and 856 bp. (C) A 288-bp PCR fragment from the *S. pombe rhp6*⁺ gene, which hybridizes to a unique, nontelomeric 2.1-kb *HinfI* fragment. (D) *S. pombe* mitochondrial DNA from plasmid pDG3 (Del Giudice, 1981), which hybridizes to multiple *HinfI* fragments. The positions of molecular weight markers are indicated.

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 *HinfI* 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 *HinfI* 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 (*HinfI* and *ApaI*, 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.

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

<i>S. pombe</i> strain/ <i>LEU2</i> plasmid	# His ⁺ colonies	# His ⁺ Leu ⁻ colonies	Percentage of colonies that lost <i>LEU2</i> plasmid
612/ <i>pfh1</i> ⁺	188	140	74.4
612/ <i>pfh1</i> -K337R	320	0	<0.3
612/ <i>pfh1</i> -K337A	309	0	<0.3
613/ <i>pfh1</i> ⁺	204	200	98.0
613/ <i>pfh1</i> -K337R	388	0	<0.2
613/ <i>pfh1</i> -K337A	147	0	<0.7

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 two times 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).

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 *pfh1*D1::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 *pfh1*D1::ura4⁺ fragment and a *LEU2* *pfh1*⁺ plasmid, selecting for Ura⁺ Leu⁺ cells. Although we got hundreds of transformants when we cotransformed with the *pfh1*⁺ plasmid, in two independent experiments, 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 Pif1p (Foury and Kolodinsky, 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* *rrm3*Δ strains have no evident mitochondrial defect, deleting *RRM3* in a *pif1*Δ strain partially suppresses the loss of mitochondrial DNA caused by deleting *PIF1*. These results suggest that Rrm3p has some role in mitochondrial DNA metabolism, at least in the absence of Pif1p (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* Pif1p 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 mitochondria 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 Pif1p, 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


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1   CTTAGTATTGATTGTTTCTACATACAGTATATAATTTATTAATTGATAGCATTATTATTACAGACTTCCGAGGATATGGTATCGTAGACCGGTT 100
101 TTATCAAAATATTATAAAATTAATAACTTACTGTAAACACGGGTTAATATTGCTTCGATTGTAACATATTATAACAAAAGTCAATTTATTGTCATTGCAT 200
201 TGCATAGGCCCGAAAGCAAATTTATGCAAGTTTAACTTAAGGAACGACTAATGTAAAAGGACTTTATGATAATTACAGTATTGAATGATAAAAAATTG 300
301 TGCAAAAGTTGTGAAGTAAAAACACGTGCTGGTGGTGACAGACGTGACCGGCTTGACATACCGGCTTTCATTGTCTCGTCCACTACCACCGTAAAT 400

401 TTGATTTTATTAGTATAGCGCTTTTCATTCTGTACCTCCTTGTCAACAGCGGTTTCCTCGACATGACAAATTTCTTCAACCGGTATCCCAATTAATAC 500
501 AATCAACAAAGGGATACTTCCAACCTCTTATGCTTGGACCGCTTATCATAGGTGTAGGTTCGAGGTGCGCATAAGTCAGTAGCAGATTGTTGTTAAAT 600
601 CAAACTGAAATTTGTTTCTTGTGCTTTCTTCGATCAAACAATTTGGGTGATCTATTTCGTTTACGGTTTCTTTTTTAGACAACCTGCGTACAGATC 700

701 GCCTTCCAGTTTCTTGAAGTAATTCGATGAATGTTTTCTGTCGCAATCTCTTTATAAAATTTAGTCACTCTTTTAGGAAACGTATCCCGTCATGTTTCAA 800
801 GAGCTCAGCAGAAAGCAGCTTGTCTCATACACAAAACGAGTCATCGCATCAACCTCCTTGAACAAATTGGGCGGTTTTCTGTCGCTTCTTTAACTT 900
901 CAATTCCTCTCGCTCCTCTACTAATGATGATCAGCAAAACATTTCTAGCCAGTCTGATAACCTTCCGTCATCTCCAATAACCTTACCAGCCAAAAGAGGT 1000
1001 CGTTCTGTCTAGCTTAAACAACTTGATAACACCGTGGGATTGATGTTTCGAAACCTTCACTGCCCCGTTTTTGAAAATTTCTGGCTTGGGTAGTAAAT 1100
1101 ACTCCACTGAAATAGCGGCTCTATATTGATGAAAATGACTTTGATGATGATCTACTTTAGAAAACGATATTGACCAAAAACCAATTCCTTGGTC 1200
1201 TTCATCCCCTATCGAGCATACTAAGTTAACTAAATCAATGCTCTCGAGCGAAAGCGGTCAAAAAATCATCTCTCAAAAATTTACGAAGACCATACTTCT 1300
1301 GAAAAAGGCGCAAGTAGCGTAATATCTAGTAATATTGCGAGGCGAGGAATAAAACGCTCACGAACCTTCTTGGGCGAGTGGATCCTTACAGATATGGAG 1400
1401 ATCGTATCTTAAAGAACCGCACTTCAGCTGATATCAGTCAACACTGTATCTAATGATTCTTCAAATAAGCTTAGCAATGGACGAAGCAGTTCTTT 1500
1501 GGATAGTCTGGCTAAAAACGCATGAGTAAATCTAAGTCTACCCACAGATATCTAAAAATTTTCTGTTCTTTAACTCTGCTTCCAAATCGCCAATA 1600
1601 GGATCTTCTTTGTTTAAAGACAAGTGATTCTCGTAAAAATCTGTCCCTAGCATATTCCTTTCCGATGAGCAAAAAGAAATTTAGACATGGTTGTTGAGC 1700
1701 AACCAACAGTATATTTTTTACGGGCTCTGCTGGAACAGGTAATCTGTTCTCCCGAAAAATTAATGAAGTCCTAAAATCTAAATATAGAAAGCAATC 1800
1801 TGACAGAGTTGCTGTTACCGCTTCTACAGGCTTGGCAGCCTGTAATATCGGCGGTGTAACACTTCACTCCTTTGCTGGGCTAGGCTTCTGCTGTAATCT 1900
1901 GTCGACCTCCTGGTATCTAAAAATAAGAAAAATAAGAGTGTTAATCGTTGGTTGGCACCAGAGTTTGGATCATTGATGAGGTAACCAATTTAAGT 2000
2001 GAGTTTATATTACTAATAACTATAGTTTCTATGGTGCATGTAATGGAACAAGTTAGAAGAGGTTGCGCGTGTATTTCGCAAGAGATAGTAAGCCA 2100
2101 TTTGGTGAATTCACTGGTTTAACTGGGATTTTTTTCAGTTACCTCCTGTTCCGAAAAATGGGAAAGAAATCGAAATCTGTTTTCGAATCTCAGACAT 2200
2201 GGAAAAGCGCACTTGATTTTACAATAGGATTGACTCACGTTTTTTCGGCAAAAGGATGAGGAATTTGTCAAATGCTTAAATGAATTGCGCTCGGAAAT 2300
2301 ATCTGATGAATCGGTTAGAAAATTCAAAGTCTTAAATAGAACGATTGAATATGAAGATGGGCTTCTTCCGACAGAGTTATTTCCACACGTTATGAGGTT 2400
2401 GAGCGTTGCAACGATATGCGTATGCAACAAATTAATCAAAATCCTGTCACGTTTACCCTATTTGACTCGGGTACTGTTGAGATAAAGAAATTCGTGACC 2500
2501 GATTACTGCAAGGGTGCATGCTCCTGCCACTTTAGTGTAAAGGTAATGCTCAGGTAATGCTTATAAAGAATATAGATGATCAGTTAGTAAATGGATC 2600
2601 TCTTGGAAAAGTTATAGGTTTCATAGATGATGAGACCTATCAAATGAAAAAAGGATGCTGAAATGAGGGAAGAAATGCTTTTGAATGTATAGTTTA 2700
2701 GACATTTCAACCTTTGATCTTCCAGACGTTAAGCAAAAGAAATAAAATTTGATTGCAATGCGTAAAGCTTCATCCACGCAATAAATGGCCATTAGTTC 2800
2801 GTTTTAACTTCCAATGGAGGTGAACGTACAATTTGTTGACAAACGGGAACTTGAATATTGAGCTTCCCAATGGTGAGGTTCAAGCTTCTAGAAGCCA 2900
2901 AATTCCTCTTATTTTGGCTTATGCGATATCTATTACAAGGCACAAGGACAGACTTTGGATCGAGTCAAGGTTGATTGGGTCGTGTTTTGAGAAAGGG 3000
3001 CAAGCGTATGTTGCTTATCTCGTCAACTACTCAAGAAGTCTCCAGGTTCTTAATTTTTCGCTCGCAAGGTGATGGCACACCCCAAGTCGTGCAAT 3100
3101 TCATAAAACAACCTGGCTTCTGTTAATGGTTTGGCGATACGTAATGAAAACAAGGCTCCAGTTCAAAATGAGGGGAGTCAAAAAATAAGTAAAAATCAATTT 3200
3201 TACGAGTTTACCATGTACTAAAGATTTATTCATAACGGCTGAATATATATGTTGAAGTGACAGTATTTAGACTAATTAGGTAATTTTATTCATAG 3300
3301 AAATAATTTTAAATATATATTCATATATTTAATTTCTTCTTTTATTAATTTTGAAGCTTACATTTATTTATTTTTCCTATGGTGCTAATTCATTT 3400
3401 CTATAACAGCGGTTATCTCTGAGGATGTTTAAATTTGTACAGGAAGTAATTTGTACAAGGCCACCTTCTAGTAATCTTTAACTATCGCATAAACGTGTA 3500
3501 TAAACGAACAGGGTGCACCTGCTTCAATCAGTCTCTTAACTTTAAATGACCAACACCTTGACCATGCAATAAACTGAACATTTAATCCACTTTCAAT 3600
3601 CAGAACCTTGAATTCG 3616

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Supplemental Figure 1

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 Pfh1p 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 (Gorbalenya and Koonin, 1993), including Pif1p (Zhou *et al.*, 2000). A *his3*⁺ plasmid carrying either the wild-type *pfh1*⁺ gene or one of the two point mutation alleles, *pfh1*-K337A or *pfh1*-K337R, were introduced into two different *pfh1*-D1::ura4⁺ strains, VPS612 and VPS613. The two *S. pombe* strains were identical except that VPS612 had a *LEU2* plasmid containing the wild-type *pfh1*⁺ gene under the control of its own promoter and VPS613 had a *LEU2* plasmid containing the *pfh1*⁺ 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 two times 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 Pfh1p. *S. pombe* strains VPS612 or VPS613 carrying the wild-type *pfh1*⁺ *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 *pfh1*-K337R or *pfh1*-K337A alleles (Table 2). Thus, the K337A and the K337R alleles were unable to supply the essential function(s) of Pfh1p.

DISCUSSION

The *S. pombe* *pfh1*⁺ gene encodes an 805 amino acid protein with very high similarity to all other members of the Pif1p

subfamily of DNA helicases (Table 1). Although Pfh1p 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 *pif1 rrm3* cells are also viable (Ivessa *et al.*, 2000). Because *pif1 rrm3* *S. cerevisiae* cells are viable, the essentiality (Figure 3A) of the *S. pombe* *pfh1*⁺ gene was unexpected.

We purified a truncated form of Pfh1p (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 Pfh1p were indistinguishable from null alleles (Table 2), the ATPase/helicase function of Pfh1p was essential. Cells lacking Pfh1p 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 *pfh1*-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 Pfh1p-depleted cells contained mitochondrial DNA (Figure 3B), and the lethality of the *pfh1*-D1::ura4⁺ mutation was not bypassed by the *ptp1-1* 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 Pfh1p. However, given that Pfh1p was predicted to localize to both mitochondria and nuclei, it might have a nonessential role in mitochondrial DNA metabolism.

Cells lacking Pfh1p 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 *pfh1*-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 Pfh1p in maintaining wild-type length telomeres, eliminating telomeric DNA did not eliminate the requirement for Pfh1p. Thus, although Pfh1p may affect telomeres, maintenance of telomeric DNA cannot be its sole essential function.

Our data suggest that Pfh1p 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 Pfh1p in chromosome replication. An appealing possibility

Supplemental Figure 1 (facing page). Supplemental Material: Sequence of the *S. pombe* *pfh1*⁺ gene. The transcription initiation site is in bold, underscored and indicated by an arrow. Upstream out of frame AUG's are indicated by an M and are underlined. The first AUG of the *pfh1*⁺ ORF is underlined and in bold. Nucleotides in bold that are underlined by dotted lines are matches to MCB (*Mlu* cell cycle box) sequence. The amino acid residues in each of the seven helicase motifs are underscored by dotted lines and denoted by the appropriate roman numeral (the seven motifs are named I, Ia, II-VI). The lysine residue in the ATP binding pocket of motif I that was mutated is boxed. Intron junctions are noted by In, and splicing signals are underlined and in bold. Motif II is split by an intron. The brackets indicate the region of Pfh1p that was expressed as a GST fusion and used for the biochemical assays in Fig. 1 and 3. The region deleted in the *pfh1*-D1::ura4⁺ deletion mutation is denoted by Δ's. The sequence ends with the *Cla*I site (denoted with italics) used to clone *pfh1*⁺ into plasmid pVS110 (see MATERIALS AND METHODS).

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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