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Research Article

Biochemical and genetic characterization of Hmi1p, a yeast DNA helicase involved in the maintenance of mitochondrial DNA

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Abstract

The *HMI1* gene encodes a DNA helicase that localizes to the mitochondria and is required for maintenance of the mitochondrial DNA (mtDNA) genome of *Saccharomyces cerevisiae*. Identified based on its homology with *E. coli uvrD*, the *HMI1* gene product, Hmi1p, has been presumed to be involved in the replication of the 80 kb linear *S. cerevisiae* mtDNA genome. Here we report the purification of Hmi1p to apparent homogeneity and provide a characterization of the helicase reaction and the ATPase reaction with regard to NTP preference, divalent cation preference and the stimulatory effects of different nucleic acids on Hmi1p-catalysed ATPase activity. Genetic complementation assays indicate that mitochondrial localization of Hmi1p is essential for its role in mtDNA metabolism. The helicase activity, however, is not essential. Point mutants that lack ATPase/helicase activity partially complement a strain lacking Hmi1p. We suggest several possible roles for Hmi1p in mtDNA metabolism. Copyright © 2005 John Wiley & Sons, Ltd.

Keywords: **DNA helicase; ATPase; mitochondria; mtDNA**

Introduction

The 80 kb *Saccharomyces cerevisiae* mitochondrial DNA (mtDNA) genome exists within the organelle in a predominantly linear concatemeric form and encodes several proteins required for oxidative phosphorylation and ATP synthesis (Costanzo and Fox, 1990; Maleszka *et al*., 1991). Loss of the mitochondrial genome or of crucial mtDNA genes results in respiration deficiency, therefore mechanisms must exist to maintain the integrity of the mitochondrial genome (Chen and Clark-Walker, 2000). One method by which the mitochondrial genome can be lost is through failure to replicate mtDNA. Alternatively, a failure in transmission of the mtDNA to daughter cells can result in loss of the mitochondrial genome. Although well understood in terms of its genetic contribution to the cell, little is known regarding the mechanism by which this linear genome is replicated and maintained in yeast.

Current mechanisms proposed for *S. cerevisiae* mtDNA replication involve both rolling circle and recombination models (Lockshon *et al*., 1995; Bendich, 1996; Ling and Shibata, 2002). Studies by Maleszka *et al*. (1991) in a similar yeast, *Torulopsis glabrata,* suggest that mtDNA undergoes replication via a rolling circle mechanism (although not exclusively). Electron microscopy reveals putative rolling-circle intermediates in which circular molecules have a single-stranded tail or lariat structure. However, these studies do not address the mechanism by which replication is initiated. Studies of *MHR1*, whose gene product is involved in partitioning mtDNA into bud cells, have shown that the predominant form of mtDNA in bud cells

is a circular monomer, suggesting that a circular form of the mtDNA genome is transmitted from the mother cell to the daughter (Ling and Shibata 2002). This may suggest that the concatemeric mtDNA found within the mother cell is the rolling circle replication intermediate that will subsequently be cleaved to yield monomeric circular molecules (representing the heritable units) that are transmitted to the bud cell. How this may occur remains unknown. There is also evidence to suggest that recombination events play an important role in mtDNA replication. For example, the Holliday junction resolving and stabilizing proteins, Cce1p and Abf2p, respectively, are important for maintenance of mtDNA (Kleff *et al*., 1992; MacAlpine *et al*., 1998; Zelenaya-Troitskaya *et al*., 1998). Furthermore rho$^-$ mitochondria do not require specific origins of replication to propagate their genome (Kleff *et al*., 1992; MacAlpine *et al*., 1998; Lecrenier and Foury, 2000). This may suggest that a rolling-circle replication mechanism can be initiated by a recombination event or that recombination-based replication involves intermediates that resemble rolling circle structures. Mhr1p has been shown to participate in the generation of D-loop structures by pairing duplex DNA with homologous single-stranded DNA (ssDNA), and this activity may play a role in initiating rolling circle replication (Ling and Shibata, 2002).

Several of the proteins that are believed to be directly involved in the replication of the mtDNA genome have been purified and described biochemically. *MIP1* was shown to encode the mitochondrial DNA polymerase and *RIM1* encodes the mitochondrial ssDNA-binding protein (Foury, 1989; Van Dyck *et al*., 1992). Both gene products are required for the maintenance of mtDNA, as evidenced by deletion studies (Genga *et al*., 1986; Van Dyck *et al*., 1992). A DNA helicase directly involved in replication of *S. cerevisiae* mtDNA has yet to be established, despite the presence of two helicases that appear to have a role in mtDNA metabolism. The *PIF1* gene has been shown to encode a helicase with roles in yeast mtDNA repair and recombination (Foury and Kolodynski, 1983). However, *pif1*Δ cells still contain functional mitochondria and are able to respire at normal temperatures (Lahaye *et al*., 1991). For these reasons, Pif1p is not believed to function as the primary helicase involved in mtDNA replication, although it apparently has an ancillary role in mtDNA metabolism. Indeed, recent data indicate that Pif1p is involved in the repair of DNA damage induced by reactive oxygen species in a role that apparently does not involve recombination (O'Rourke *et al*., 2002; Doudican *et al*., 2005).

The other known mtDNA helicase, *HMI1*, was identified as a protein with significant sequence homology with the *E. coli uvrD* gene encoding DNA helicase II (Sedman *et al*., 2000). The *S. cerevisiae* superfamily 1 helicase encoded by *HMI1* has been shown to localize to the mitochondria and is required for the maintenance of the yeast mitochondrial genome (Sedman *et al*., 2000). When this nuclear gene is disrupted, the resulting cell progeny display a petite colony phenotype. This phenotype is generally associated with mitochondrial malfunction and the inability to generate ATP via oxidative phosphorylation (reviewed in Chen and Clark-Walker, 2000). This mitochondrial defect can further be demonstrated by the failure of these colonies to grow on media lacking a fermentable carbon source, since yeast are facultative anaerobes and do not require functional mitochondria for growth (reviewed in Shadel 1999). Hmi1p has been shown to unwind duplex DNA and has no role in the transcription of mtDNA genes (Sedman *et al*., 2000). Furthermore, cells lacking *HMI1* 'lose' their mitochondrial DNA to become rho^0 (Lietzel, 2000; Sedman *et al*., 2000). Despite these characteristics, Hmi1p is not believed to be the yeast mtDNA replicative helicase, because rho$^-$ genomes can be maintained in *hmi1*Δ strains (Sedman *et al*., 2000). Recently, it has been demonstrated that *hmi1*Δ rho$^-$ strains suffer significant shortening of the concatemeric mtDNA compared to *HMI1* rho$^-$ strains (Kuusk *et al*., 2005). In addition, genetic studies show that a mutant form of Hmi1p, in which a conserved residue found within the Walker A box and known to be necessary for ATPase activity in other helicases has been altered, complements an *hmi1*Δ mutant (Sedman *et al*., 2005). From these data it was concluded that Hmi1p stimulates the formation of concatemeric mtDNA, although the mechanism by which this occurs remains unknown.

To further elucidate the function of Hmi1p in the cell and its role in mtDNA maintenance, we have purified Hmi1p and characterized its helicase and ATPase activities. In addition, single point mutants designed to eliminate ATP hydrolysis have been engineered and the mutant proteins were purified to

test their activity *in vitro* and *in vivo* in comparison to the wild-type. Complementation assays using a point mutant lacking ATPase/helicase activity and a mutant lacking the mitochondrial localization signal indicate that Hmi1p is essential for maintenance of mtDNA, but the helicase activity of the protein appears to be at least partially dispensable.

Materials and methods

Bacterial strains, plasmids, and nucleic acids

E. coli RDK1896(DE3) is an exoI$^-$, exoIII$^-$, endoI$^-$ and recJ$^-$ strain kindly provided by Richard Kolodner (University of California at San Diego, Ludwig Institute). *E. coli* DH5α was from Invitrogen and was used in all cloning steps. The expression plasmid, pTYB4, was from New England Biolabs and modified as described below. Poly(dT) was from US Biochemical Inc. M13mp18 ssDNA was prepared as previously described (Lechner and Richardson, 1983). rRNA was from Boehringer-Mannheim and nucleotides were from Amersham Pharmacia Biotech.

To construct pEG(KG)*HMI1, HMI1* was amplified by polymerase chain reaction (PCR) from CBOO1 genomic DNA, prepared as described (Ausubel 1998) using primers AKL01 and AKL02, which provided *Bam*H1 sites at each end of the gene (Table 1). The amplified *HMI1* gene was then inserted into pEG(KG) at the *Bam*H1 site and orientation was determined by restriction site analysis. pEG(KG) was the kind gift of R. Deschenes (Mitchell and West, 1994). pYE*HMI1* was obtained by excising *HMI1* from pEG(KG)*HMI1* using *Bam*HI and inserting the *HMI1* fragment into pYE12. pYE12 was constructed by digestion of pEG(KG) with *Sac*I to remove the *GST* gene and the multiple cloning site. The resulting plasmid, pYE12, retains the *Bam*H1 and *Hin*dIII restriction sites and allows galactose-inducible expression of a gene without a GST tag.

pYE12*hmi1K32M* was constructed using Quikchange Site Directed Mutagenesis (Stratagene) and primers AKL03 and AKL04 (Table 1). Amplification of the *HMI1* gene, using pMal*HMI1* as the target with AKL03 and AKL04, DpnI treatment and transformation, were performed according to the manufacturer's recommended protocol. Primers AKL03 and AKL04 change codon 32 from AAA (K) to ATG (M) and introduce a *Sma*I site 5′ to codon 32. The introduction of a *Sma*I site allowed rapid screening of transformants by restriction digest to identify plasmids containing *HMI1* with the desired mutation. The *hmi1K32M* gene was excised from pMal*hmi1K32M* using *Bam*HI and inserted into pYE12 at the unique *Bam*HI

Table 1. Oligonucleotides

	Oligonucleotide sequence (5′ → 3′)
HK1	AGATCTTTAACAACATTATG
HK2	CATGGTTATAATGTGCAGCG
HK5	TACTATCAACTGCTTTGTTT
HK6	GGTGTTGGAACGTACTTGCA
AKL01	TGTGGATCCATGGACAAGCTAACTCCATC
AKL02	GTGGGATCCTATATACGTCTGAAAACGC
AKL03	GCGGGCCCGGGCTCAGGAATGACGCTAACGCT
AKL04	AGCGTTAGCGTCATTCCTGAGCCCGGGCCCGC
AKL05	AAAGGATCCTCAATAAAATCCAAAATTTTTACG
pET11d sequencing primer	GGAATTGTGAGCGGATAACAATTCCCC
8xHIS for intein	AATCTGCAGTCAGTGATGGTGATGGTGATGGTGATGT
CtermHmi1dmls	TGTGTCCAGCTGTGCTCTATAAAATCCAAA
Hmi1Nco18His	TTTTTTCCATGGCTCATCACCATCACCATCACCATCAC
20-mer	ATTCAAAAGGGTGAGAAAGG
40-mer	CAGGAGGCCGATTAAAGGGATTTTAGACAGGAACGGTACG
91-mer	AGTAGCACCATTACCATTAGCAAGGCCGGAAACGTCACCAATGAAA-CCATCGATAGCAGCACCGTAATCAGTAGCGACAGAATCAAGTTTG
K32S FOR	CGGGCCCGGGCTCAGGAAGCACGCTAACGCTAC
K32S REV	GTAGCGTTAGAGTGCTTCCTGAGCCCGGGCCCG
K32A FOR	CGGGCCCGGGCTCAGGAGCAACGCTAACGCTAC
K32A REV	GTAGCGTTAGCGTTGCTCCTGAGCCCGGGCCCG

site. Orientation was determined by restriction site analysis.

The pYE12*hmi1*Δ*mls* plasmid, which contains *HMI1* lacking the mitochondrial localization signal (mls), was obtained by amplification of *HMI1* from CBOO1 genomic DNA using primers AKL01 and AKL05 (Table 1). AKL05 replaces R691 with a stop codon to truncate Hmi1p 16 residues from the C-terminus, which removes the mitochondria localization signal (mls). AKL05 also introduced a *Bam*H1 site on the 3′ end of the gene. The amplified DNA was digested with *Bam*H1 and inserted into pYE12 to generate pYE12*hmi1*Δ*MLS*.

Yeast strains

Saccharomyces cerevisiae KCY3-2D (Table 2) was derived from W303 by single-step gene replacement (Sikorski and Hieter, 1989). Primers HK6 and HK5 (Table 1) were used to amplify the region from −300 to 181 of YOL095c. Primers HK2 and HK1 (Table 1) were used to amplify the region from 2081 to +65 3′ to the YOL095c sequence. These fragments were inserted into a yeast integrating plasmid (YIp) carrying *TRP1*. The inserted fragments target the YIp to the *HMI1* sequence without disrupting the first 66 amino acid residues of Hmi1p. 104 bp from the 3′ end of *HMI1* also remain in the genome. After integration of the linearized YIp, KCY1 was sporulated and tetrads were dissected to obtain KCY3-2D. KCY3-2D was crossed with W303a to obtain the heterozygous diploid strain (ALY01) used in complementation experiments. Heterozygosity at the *HMI1* locus was confirmed by Southern blots probed with a (^{32}P)DNA fragment containing *HMI1* and 249 base pairs upstream of the *HMI1* gene. pYE12, pYE12*HMI1*, pYE12*hmi1*Δ*mls* and pYE12*hmi1K32M* were transformed into ALY01 and selected on SD ura$^-$ to generate ALY02, ALY03, ALY05 and ALY04 respectively. Plasmids were transformed into yeast using the lithium acetate procedure, as described by Ausubel (1998). Yeast strains were grown on standard yeast media. SD was 6.7 g yeast nitrogen base without amino acids, with ammonium sulphate and 20 g/l dextrose plus the appropriate drop-out mixture of amino acids. Mitochondrial function was assessed on YPEG (10 g yeast extract, 20 g peptone, 20 ml ethanol, 20 ml glycerol, 20 g/l agar). Modified SGE (6.7 g yeast nitrogen base without amino acids, with ammonium sulphate, 3% glycerol, 2% ethanol, 0.1% dextrose plus the appropriate amino acids) or SR (6.7 g yeast nitrogen base without amino acids, with ammonium sulphate, 20 g/l raffinose plus the appropriate amino acids) media were used for induction of protein expression in yeast.

Antibody preparation

Anti-MBPHmi1p antiserum was prepared by Covance Inc., using a maltose-binding protein–Hmi1p fusion protein (MBP-Hmi1p). The fusion protein was expressed in *E. coli* and purified by ammonium sulphate precipitation, amylose affinity chromatography and Superose 12 chromatography. The partially purified protein was resolved from contaminants on a SDS-polyacrylamide gel and used directly as antigen. We observed cross-reactions with a variety of yeast proteins using this antiserum.

Table 2. Yeast strains

Yeast strains	Genotype	Source
CBOO1	MAT**a**, *leu2-3, trp1-1, ura3-52, prb1, pep4::URA3*	A. Sugino
W303	MAT**a**/MATα, *leu2-3,112/leu2-3,112 ura3-3/ura3-3 ade2-101/ade2-101 can1-100/can1-100 his3*Δ*-11,15/his3*Δ*-11,15 trp1-1/trp1 -1*	T. Petes
W303a	W303 MAT**a**	T. Petes
KCY1	W303 MAT**a**/α, *HMI1/hmi1::TRP1*	H. Klein, this study
KCY3-2D	W303 MATα, *hmi1::TRP1*; obtained by dissection from KCY1	H. Klein, this study
ALY01	KCY3-2D X W303a; *HMI1/hmi1::TRP1*	This study
ALY02	ALY01/pYE	This study
ALY03	ALY01/pYE*HMI1*	This study
ALY04	ALY01/pYE*hmi1K32M*	This study
ALY05	ALY01/pYE*hmi1*Δ*MLS*	This study

Genotype and source of yeast strains used in this study are indicated.

Expression vector construction

The pTYB4 expression plasmid was modified at nucleotide 7330 by the addition of a sequence encoding eight histidines for use as an extra purification tag. This was accomplished by amplifying the 1.7 kb region of the intein–chitin binding domain on pTYB4, using the pET11d sequencing primer and a primer complementary to the end of the chitin-binding domain (CBD) containing the engineered 8 histidine sequence and a *Pst*I restriction site to clone into the vector's *PstI* site at base 7330 (Table 1). The amplified product and the unmodified pTYB4 vector were digested with *Hin*dIII and *Pst*I. The fragment was inserted into the vector and verified by sequencing. *HMI1* was amplified from yeast genomic DNA via PCR using primers that contained an *Nco*I site and *Xho*I site to clone into pTYB4 (Table 1). The primer used to amplify the C-terminal end of the gene was constructed to remove the last 48 nucleotides, which correspond to the termination codon and the predicted C-terminal mls (Lee *et al*., 1999). The amplification product was digested with *Nco*I and *Xho*I, purified on an agarose gel, and ligated between the *Nco*I and *Xho*I sites on pTYB4-8His. The point mutants K32S, K32A and K32M were generated through PCR-facilitated Quikchange Site Directed Mutagenesis (Stratagene; see primers in Table 1). The mutated alleles of *hmi1* were inserted in the corresponding region on the pTYB4-8His *Hmi1*Δ mls, through a simple fragment replacement utilizing two restriction sites within the gene. All genes were verified by direct DNA sequencing to ensure the absence of unintended mutations.

Protein purification

All purification steps were conducted at 4 °C. The pTYB4-8His *HMI* Δmls plasmid was used to express recombinant Hmi1Δmls protein in RDK1896(DE3). The cultures were grown to OD_{600} ~1.0 in LB media containing 100 μg/ml ampicillin and 10 μg/ml tetracyline. Cells were induced for 24 h at 16 °C with 0.3 mM isopropyl-β, D-thiogalactopyranoside (IPTG). The lower temperature was required to maintain the solubility of the expressed protein. Cells were harvested by centrifugation and suspended in a lysis buffer containing 50 mM Hepes–NaOH, pH 7.0, 1 mM EDTA, 200 mM NaCl and 10% sucrose. The suspended cells were lysed by the addition of 150 μg/ml lysozyme for 1 h, followed by the addition of Triton X-100 to a final concentration of 0.1%. The NaCl concentration was raised to 500 mM and the cell suspension was sonicated to reduce the viscosity (three bursts at 14 s intervals). The lysate was clarified by centrifugation at 47 800 × *g* for 60 min. Polymin P was added to the soluble cell lysate to a final concentration of 0.3% (w/v) by the slow addition of 10% polymin P, pH 6.8, to precipitate nucleic acids. The polymin P precipitate was collected by centrifugation at 26 890 × *g* for 20 min. Solid ammonium sulphate was added to the supernatant to 33% saturation. The $(NH_4)_2S0_4$ precipitate was collected by centrifugation and suspended in a buffer containing 50 mM Hepes–NaOH, pH 7.0, 10 mM imidazole and 10% glycerol (buffer A). NaCl was added to a final concentration of 500 mM. The solution was batch bound to 2 ml TALON metal affinity resin (BD Biosciences) for 1 h and the column was washed to baseline with buffer A containing 500 mM NaCl (buffer B). The column was eluted with buffer B containing 350 mM imidazole and fractions containing the Hmi1-CBP–intein fusion protein were identified by SDS-polyacrylamide gel electrophoresis and Western blotting, using antibodies directed against Hmi1p. The fractions were pooled and batch bound to 2 mls chitin bead resin (New England Biolabs) for 1 h, poured into a column and washed to baseline with the chitin wash buffer (buffer C): 50 mM Hepes–NaOH, pH 7.0, 500 mM NaCl, 0.1 mM EDTA and 10% glycerol. The column was then quickly washed with 2 column volumes of buffer C containing 50 mM dithiothreitol (DTT) and incubated for ~40 h at 4 °C to induce intein cleavage. The column was eluted using buffer C and fractions containing Hmi1p were identified by SDS-polyacrylamide gel electrophoresis and Western blotting. Appropriate fractions were combined and dialysed against a storage solution containing 25 mM Hepes–NaOH, pH 7.0, 200 mM NaCl, 0.1 mM EDTA, 1 mM DTT and 50% glycerol. The protein was judged to be greater than 95% pure by SDS-polyacrylamide gel electrophoresis (see Figure 1) and was stored at −20 °C.

Partial duplex substrates

Partial duplex DNA substrates containing 22 bp, 42 bp or 93 bp of duplex DNA were prepared

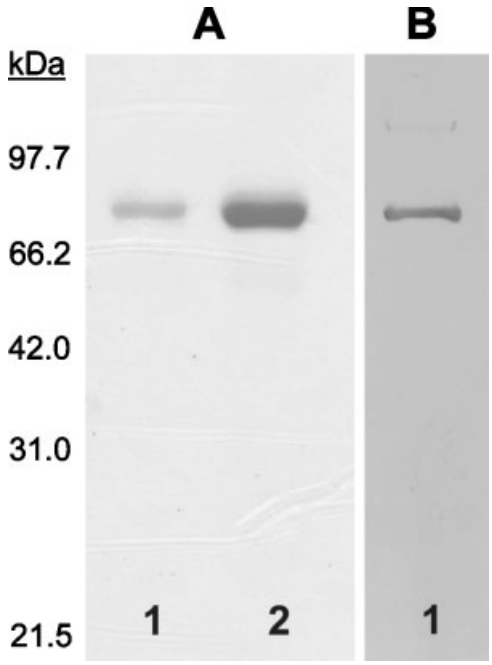

Figure 1. Analysis of purified Hmi1p. (A) Purified Hmi1p was resolved on a 9.6% SDS-polyacrylamide gel and stained with Coomassie blue. Lane 1, 1 μg purified Hmi1p; lane 2, 3 μg purified Hmi1p. (B) 1 μg purified Hmi1p was resolved on an identical gel and then transferred to nitrocellulose and probed with antibody directed against Hmi1p. The position of the molecular weight standards (Biorad), resolved in an adjacent lane on the gel, are indicated on the left

by mixing M13mp18 ssDNA with the appropriate oligonucleotide at equimolar concentrations of M13 ssDNA and oligonucleotide. The mixture was boiled for 5 min followed by slow cooling to promote annealing of the oligonucleotides to the M13 ssDNA. Products were 3′ end-labelled using the Klenow fragment of DNA polymerase I and (α-^{32}P)dCTP. Products of the extension reaction were phenol/chloroform extracted and the DNA was purified on an A5M (Biorad) column equilibrated with 10 mM Tris–HCl, pH 8.0, 100 mM NaCl and 1 mM EDTA. The fractions were collected dropwise to separate the partial duplex DNA substrate from unincorporated nucleotides. Fractions containing the partial duplex substrate were pooled and used directly in helicase activity assays. The final concentration of the pooled fractions was estimated at 20 μM DNA-Pi. Partial duplex substrates containing 25 bp, 30 bp and 35 bp of duplex DNA were prepared by mixing the appropriate oligonucleotide, labelled at the 5′-end using (γ-^{32}P)ATP and polynucleotide kinase, with an equimolar concentration of M13 ssDNA. Annealing and purification of the partial duplex substrate were as described above.

Helicase directionality substrate

The substrate used to determine the polarity of the Hmi1p unwinding reaction (see Figure 3A) was constructed by annealing the 91 base oligonucleotide to the M13mp18 ssDNA. This oligonucleotide anneals to the region between bases 2490 and 2581 on M13mp18 ssDNA. The 91 bp partial duplex DNA was digested to completion with *Cla*I, and DNA polymerase I (Klenow fragment) was used to extend all available 3′-OH ends in the presence of (α-^{32}P)dCTP and dGTP. The final product was purified as described above for the partial duplex substrates and resulted in a DNA molecule with a long internal ssDNA region and short (43 bp on the 3′-end and 52 bp on the 5′ end) duplex regions on each end.

Helicase Assays

Helicase reaction mixtures (20 μl) contained 25 mM Tris–HCl, pH 7.5, 6 mM $MgCl_2$, 20 mM NaCl, 1 mM DTT, 50 μg/ml bovine serum albumin, 2 mM ATP, the indicated amount of enzyme, and partial duplex substrate (2 μM DNA-Pi or ~0.25 nM circular molecules). The reactions were incubated at 30 °C for 10 min and quenched with 10 μl stop buffer, containing 38% glycerol, 50 mM EDTA, 0.3% sodium dodecyl sulphate and dyes. The reaction products were resolved on 8% non-denaturing polyacrylamide gels and visualized using a PhosphorImager (Molecular Dynamics).

ATPase assays

Assays designed to measure the ATP hydrolysis reaction catalysed by Hmi1p were set up in the same manner as the helicase assays, substituting partial duplex DNA with 30 μM M13mp18 ssDNA and ATP with the indicated concentration of (γ-^{32}P)ATP. The reactions were incubated at 30 °C for 10 min unless otherwise stated. Reactions were quenched with 280 μl 20 mM phosphoric acid solution containing 1 mM EDTA and 5% Norit® activated carbon. Mixtures were incubated on ice for 15 min before centrifugation at 16 110 × g for 10 min. A 200 μl aliquot of each supernatant was

removed and added to 3 ml scintillation fluid for quantification in a scintillation counter.

Genetic analysis

ALY01 was sporulated using standard procedures (Ausubel 1998) and tetrads were dissected onto YPD and YPEG to determine the mitochondrial phenotype. The sporulation media was modified from standard sporulation media by the addition of 0.0005% adenine (Ausubel, 1998).

For complementation studies, the plasmids pYE12, pYE12*HMI1*, pYE12*hmi1*Δ*mls* and pYE12*hmi1K32M* were transformed into ALY01, a diploid strain heterozygous at the *HMI1* locus, to generate ALY02, ALY03, ALY05 and ALY04. ALY02, ALY03, ALY04 and ALY05 were sporulated and dissected onto YPD, after which the plates were incubated at 30 °C for 48 h, shifted to 4 °C for 48 h and scored for red/white colour. The dissection plates were then replica-plated to YPD, SD ura$^-$, SD trp$^-$ and YPEG media. After overnight incubation at 30 °C, the tetrads were scored for growth on the respective media. Growth on media lacking tryptophan revealed spores that contained the Δ*hmi1::TRP1* allele. Growth in the absence of uracil indicated spore colonies that contained pYE12-derived plasmids. Δ*hmi1::TRP1* spores containing pYE12-derived plasmids were scored for retention of functional mitochondria, as indicated by their ability to grow on YPEG media.

Results

Purification of recombinant Hmi1p

The *HMI1* gene was amplified from yeast genomic DNA using PCR primers that excluded the last 48 bp of the gene, which encodes the mitochondrial localization signal (MLS). Since the MLS is cleaved *in vivo* to generate the mature protein product upon entrance into the mitochondrial matrix (Lee *et al*., 1999), it was not included in the protein purified and analysed here. The purification of Hmi1p was a three-step process that utilized two affinity columns. A fusion of Hmi1p with an intein–chitin-binding domain (CBD) containing a C-terminal eight-histidine affinity tag was precipitated from the cell lysate using ammonium sulphate and was partially purified using a TALON metal affinity column (BD Biosciences), as described in Materials and methods. The peak fractions containing the fusion protein were pooled and bound to chitin resin. A 40 h incubation with 50 mM DTT induced cleavage of the intein–CBP affinity tag and resulted in the release of the Hmi1Δmls product. We will refer to this protein as Hmi1p. The purified protein had a relative molecular mass of 80 kDa, consistent with the predicted molecular weight of the *HMI1* gene product, and was judged to be >95% pure, based on analysis of an SDS-polyacrylamide gel stained with Coomassie blue. The protein was confirmed to be the product of the *HMI1* gene, using a polyclonal antibody generated against Hmi1p (Figure 1). The protein preparation used in the studies described below was substantially more pure than previous preparations of Hmi1p (Sedman *et al*., 2000; Kuusk *et al*., 2005; Sedman *et al*., 2005).

Hmi1p helicase activity

Prior to this study, the helicase activity associated with Hmi1p had not been thoroughly characterized, due to the lack of significant amounts of protein. We also note that a more rigorous characterization of Hmi1p was recently published using recombinant protein (Kuusk *et al*., 2005). With significant amounts of purified recombinant protein available, we sought to provide an initial characterization of both the helicase reaction and the ATPase reaction catalysed by Hmi1p. Initially, helicase activity was measured using a series of partial duplex DNA substrates containing duplex regions with lengths of 22 bp, 42 bp and 93 bp (Figure 2A). Purified Hmi1p effectively unwound the 22 bp partial duplex substrate. Complete unwinding of this DNA substrate was observed in a 10 min incubation with 20 nM Hmi1p. At the same protein concentration, Hmi1p was able to unwind only ~35% of the 42 bp and <10% of the 93 bp partial duplex substrates. Time-course reactions (Figure 2B) using 20 nM Hmi1p and the 22 bp partial duplex substrate suggested that unwinding of this duplex was complete in <15 min. Similar experiments with the 42 bp and 93 bp partial duplex substrates indicated that increasing the length of the incubation did not dramatically improve the fraction of the substrate unwound. It is interesting to note that unwinding of the 93 bp partial duplex was only slightly less than unwinding of the 42 bp partial duplex, suggesting that once a critical length of

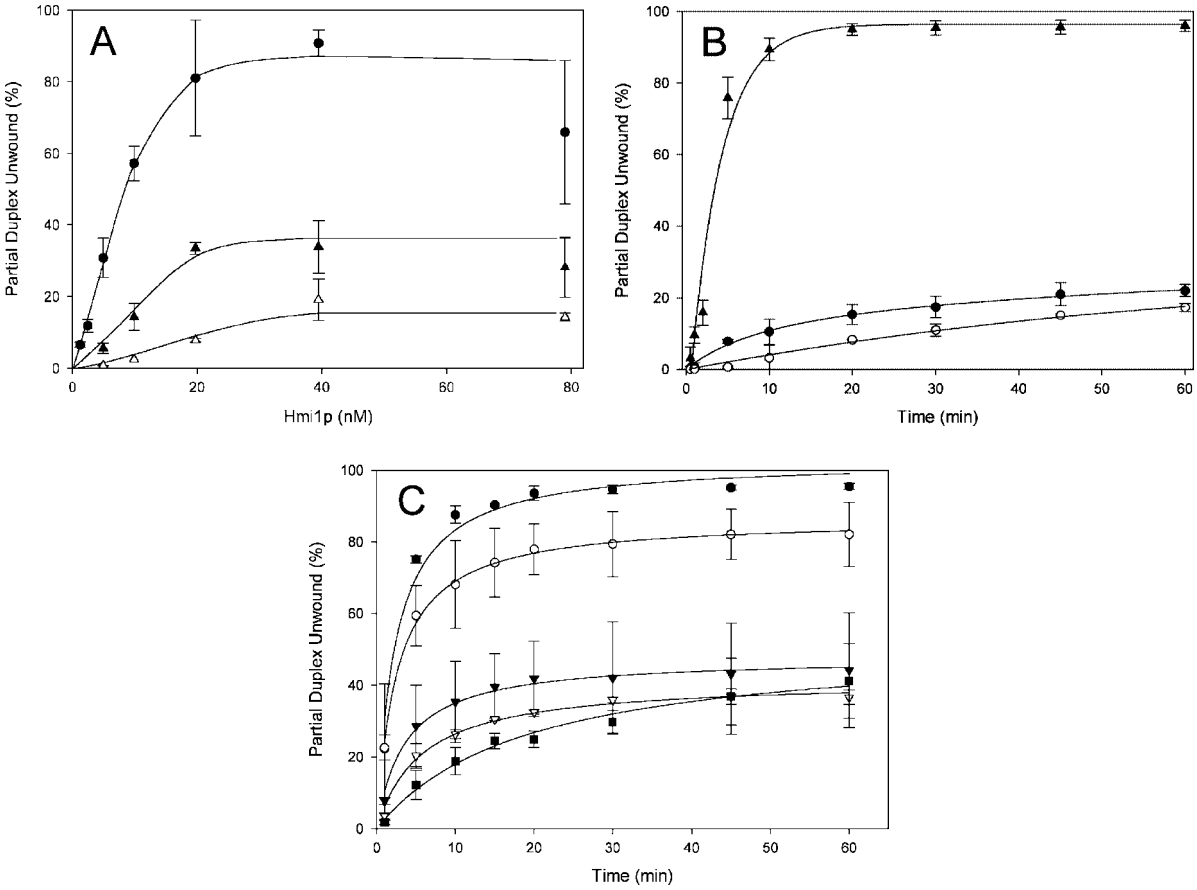

Figure 2. Hmi1p-catalysed unwinding of partial duplex substrates. (A) Helicase activity assay showing a titration of Hmi1p from 1 nM to 80 nM on 22 bp (closed circles), 42 bp (closed triangles) and 93 bp (open triangles) partial duplex DNA substrates. Reactions were incubated for 10 min under standard helicase reaction conditions (see Materials and methods) and products were resolved on native polyacrylamide gels. (B) Helicase assay showing the unwinding activity of 20 nM Hmi1p over the course of 60 min. Closed triangles, 22 bp partial duplex; closed circles, 42 bp partial duplex; open circles, 93 bp partial duplex. (C) Helicase assay testing the unwinding activity of 40 nM Hmi1p on partial duplexes 22–42 bp in length over a period of 60 min. Closed circles, 22 bp partial duplex; open circles, 25 bp partial duplex; closed inverted triangle, 30 bp partial duplex; open inverted triangle, 35 bp partial duplex; closed square, 42 bp partial duplex. The curves represent the best fit of the data to a rectangular hyperbola. The error bars represent the deviation about the mean in at least three separate experiments

duplex DNA is encountered, unwinding is reduced dramatically. This is in contrast to the proportional decrease in unwinding as duplex length increases that has been reported for the UvrD helicase from *E. coli* (Matson and George, 1987). The significance of this observation is not understood at present. Apparently, the protein encounters some barrier to unwinding of longer partial duplex substrates that cannot be overcome by increasing either the length of the incubation or the protein concentration.

To more accurately determine the length dependence of the steady-state unwinding reaction, helicase assays were conducted using partial duplex DNA substrates containing 25 bp, 30 bp and 35 bp of duplex DNA (Figure 2C). In terms of sequence, these substrates represent 5 bp incremental decreases in the length of the 40 bp oligonucleotide used in construction of the 42 bp partial duplex substrate. We observed significant unwinding of the 25 bp partial duplex DNA, but unwinding of both the 30 and 35 bp partial duplex DNAs was reduced to levels comparable to that of the 42 bp substrate. Thus, a kinetic barrier to unwinding duplex regions greater than about 25 bp seems to exist. The reason for this is unknown

but could reflect protein dissociation from the substrate, allowing reannealing of the two strands to occur. Alternatively, the protein might be blocked in some way and unable to progress further than 25–30 bp as it translocates through duplex DNA. Efforts to unwind longer partial duplex DNAs in the presence of the mitochondrial single-stranded DNA binding protein RIM1, or the related *E. coli* SSB, have not been successful (data not shown).

Hmi1p shares significant similarity (23% identical, 39% similar) at the amino acid level with *E. coli* UvrD. UvrD, also known as DNA helicase II, catalyses the $3' \rightarrow 5'$ unwinding of duplex DNA (Matson, 1986). For this reason, Hmi1p has been assumed to catalyse an unwinding reaction with a $3' \rightarrow 5'$ polarity, but this has not been directly demonstrated. To directly determine the polarity of the Hmi1p-catalysed helicase reaction, the partial duplex substrate shown in Figure 3A was constructed. This linear DNA contains a long internal region of ssDNA (>7100 nucleotides), on which the protein can load, and duplex regions of different lengths at each end. A helicase that binds the internal ssDNA and translocates $3' \rightarrow 5'$ will catalyse unwinding of the 52 bp duplex at the 5′-end of the linear molecule. A helicase that binds and translocates $5' \rightarrow 3'$ will catalyse displacement of the 43 nucleotide DNA fragment.

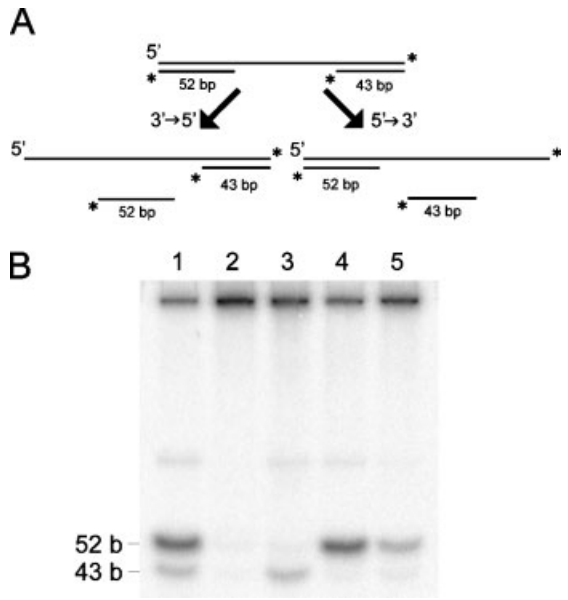

Figure 3. Hmi1p unwinds DNA with a $3' \rightarrow 5'$ polarity. (A) Schematic depicting the DNA substrate and the possible products generated as a result of the helicase polarity experiment. The substrate was prepared as described in Materials and methods. The asterisks represent radioactive label at each 3′-end. (B) Helicase reaction mixtures were as described in Materials and methods and incubations were for 10 min at 30 °C. Lanes 1 and 2, no enzyme; lane 1, boiled to denature the substrate; Lanes 3, 4 and 5 contained TraI (10 nM), UvrD (10 nM) and Hmi1p (40 nM), respectively. The products were resolved on a 6% non-denaturing polyacrylamide gel

The polarity of the unwinding reaction catalysed by Hmi1p was tested using this substrate. As controls, two helicases, helicase I, an enzyme with $5' \rightarrow 3'$ polarity, and helicase II, an enzyme with $3' \rightarrow 5'$ polarity, were also tested using this DNA substrate. As expected, helicase I catalysed the unwinding of the 43 nucleotide DNA fragment, indicating a $5' \rightarrow 3'$ polarity. Helicase II catalysed the unwinding of the 52 bp duplex region, consistent with its known $3' \rightarrow 5'$ polarity. Hmi1p catalysed the unwinding of the 52 bp duplex region, consistent with unwinding of duplex DNA in a $3' \rightarrow 5'$ direction (Figure 3B, lane 5). We conclude that Hmi1p translocates in a $3' \rightarrow 5'$ direction with respect to the DNA strand on which it is bound, as it catalyses the unwinding of duplex DNA.

We also tested a series of reaction conditions and different co-factors to determine an optimal set of conditions for measuring the helicase activity of Hmi1p *in vitro*. A helicase, by definition, catalyses the unwinding of double-stranded nucleic acid in a reaction that requires NTP hydrolysis. The unwinding activity of Hmi1p was measured in the presence of each of the eight canonical (d)NTPs individually, using the 22 bp partial duplex substrate (data not shown). As expected, no unwinding was detected in the absence of an NTP. Both ATP and dATP supported the unwinding reaction and, under these conditions, resulted in the unwinding of approximately 65% of the DNA substrate. Even though there was no significant difference between dATP and ATP, ATP consistently supported the helicase reaction slightly better than dATP. UTP and both forms of CTP were poor co-factors in the helicase reaction, supporting the unwinding of approximately 10% of the DNA substrate. No unwinding was observed with dTTP or either form of GTP. The dependence of the Hmi1p-catalysed unwinding reaction on ATP concentration was investigated using the 22 bp partial duplex substrate, and revealed an apparent K_M for ATP of 90 μM, with optimal unwinding at a final ATP concentration of 2 mM (data not shown).

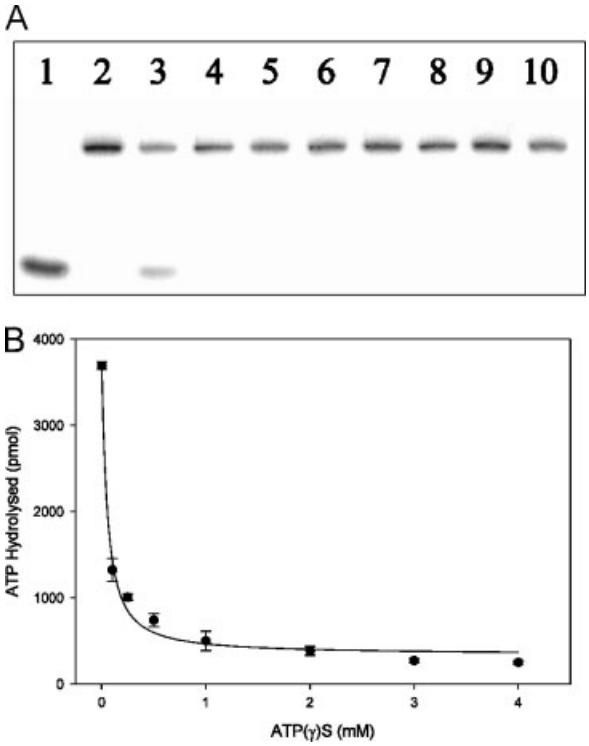

Figure 4. ATP(γ)S, AMP-PNP, and AMP-PCP are inhibitors of Hmi1p helicase activity. (A) Helicase activity assays were conducted as described in Materials and methods, using the 22 bp partial duplex substrate. All reactions were incubated for 10 min at 30 °C and contained 40 nM Hmi1p. Lane 1, no enzyme and boiled for 3 min; lane 2, no enzyme; lane 3, 2 mM ATP; lane 4, no ATP; lane 5, 2 mM ATP + 2 mM ATP(γ)S; lane 6, 2 mM ATP(γ)S; lane 7, 2 mM ATP + 2 mM AMP-PNP; lane 8, 2 mM AMP-PCP; lane 9, 2 mM ATP + 2 mM AMP-PCP; lane 10, 2 mM AMP-PCP. (B) A titration of ATP(γ)S from 0 mM to 4 mM in an Hmi1p-catalysed ATPase assay containing 2 mM (^{32}P)ATP and 160 nM Hmi1p. A hyperbolic decay curve was fitted to the data. (C) ATPase activity assays were conducted as described in Materials and methods, using 98 nM Hmi1p and the indicated concentrations of ATP. Incubation was at 30 °C for 10 min. Error bars represent the standard deviation about the mean of three independent experiments. A rectangular hyperbola, as described by the Michaelis–Menten equation, was fitted to the data

For this reason, all subsequent experiments were conducted using 2 mM ATP as the energy co-factor.

To demonstrate directly the dependence of the Hmi1p-catalysed unwinding reaction on the hydrolysis of ATP, several ATP analogues were evaluated for their ability to support or inhibit the helicase reaction (Figure 4A). Each of these ATP analogues is either poorly hydrolysed [ATP(γ)S] or is non-hydrolysable (AMP-PNP and AMP-PCP). The helicase reaction was dependent on the presence of ATP, as expected (Figure 4A, lane 3), and none of the ATP analogues supported the unwinding reaction (Figure 4A, lanes 6, 8 and 10), suggesting that ATP hydrolysis was essential. In addition, each of the analogues inhibited the helicase reaction when the analogue was added to a reaction that contained ATP.

We chose to explore further the impact of ATP(γ)S on the ATPase reaction catalysed by Hmi1p. To ensure that the protein bound this ATP analogue, the mechanism of inhibition was determined. An inhibitor that binds the protein at the active site is expected to demonstrate competitive inhibition, which was the case for ATP(γ)S (data not shown). To determine an apparent K_i for inhibition by ATP(γ)S, this analogue was titrated into an Hmi1p-catalysed ATPase reaction (Figure 4B). The data were well described by a hyperbolic decay curve with an apparent K_i of 170 μM in the presence of 2 mM ATP. Thus, ATP(γ)S is an effective competitive inhibitor of both the unwinding and ATPase reactions catalysed by Hmi1p and ATP hydrolysis is required for unwinding.

Helicase reactions also typically require the presence of a divalent cation co-factor. A titration of $MgCl_2$ from 0 mM to 27 mM, in a helicase reaction using the 22 bp partial duplex substrate, revealed that upon addition of $MgCl_2$ there was a pronounced increase in unwinding as the $MgCl_2$ concentration was increased with maximal helicase activity between 6 and 12 mM $MgCl_2$ (data not shown). However, since there was no significant difference between 6 mM and 12 mM $MgCl_2$, 6 mM $MgCl_2$ was used for all subsequent reactions. No activity was observed in the absence of $MgCl_2$, suggesting that this is an essential co-factor.

The ATPase activity of Hmi1p

The unwinding reaction catalysed by a helicase requires energy which is usually supplied by the hydrolysis of an NTP. In addition, nucleic acid co-factors often stimulate the NTPase activity of helicases. To evaluate the effect of various nucleic acid co-factors on the ATPase activity of Hmi1p, ATP hydrolysis was measured in the presence of several nucleic acid co-factors with differing secondary structure and at varying concentrations (Figure 5). The nucleic acid co-factors tested were circular M13mp18 ssDNA, supercoiled plasmid DNA,

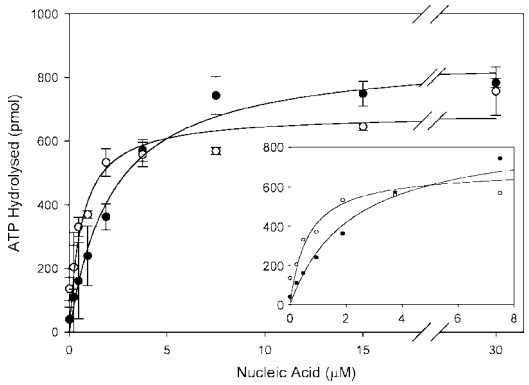

Figure 5. ssDNA stimulates Hmi1p ATPase activity. (A) ATPase activity assays were conducted as described in Materials and methods, using the indicated concentrations of either M13mp18 ssDNA (closed circles) or poly(dT) (open circles). The reactions were incubated for 10 min at 30 °C and contained 50 nM Hmi1p. (Inset) The region from 0.2 μM to 7.5 μM M13 ssDNA and poly(dT) is shown. Error bars represent the standard deviation about the mean. Rectangular hyperbolas have been fitted to the data

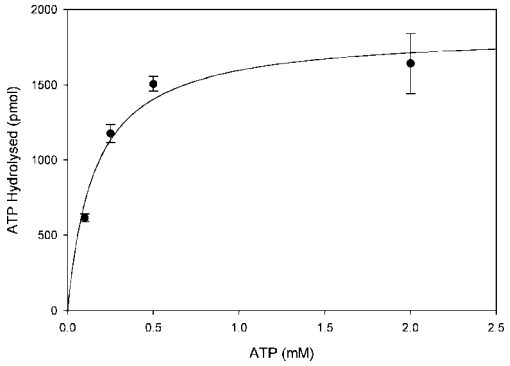

Figure 6. Dependence of the ATPase reaction on ATP concentration. ATPase activity assays were conducted as described under Materials and methods using 98 nM Hmi1p and the indicated concentrations of ATP. Incubation was at 30 °C for 10 min. Error bars represent the standard deviation about the mean of three independent experiments. A rectangular hyperbola, as described by the Michaelis–Menten equation, was fitted to the data

rRNA, linear dsDNA and poly(dT). Each co-factor was added to an Hmi1p-catalysed ATPase reaction at concentrations in the range 0–30 μM nucleotide phosphate. Both M13mp18 ssDNA and poly(dT) stimulated the Hmi1p-catalysed ATPase reaction to similar extent. The DNA concentration required for half-maximal stimulation of ATP hydrolysis was 2.2 μM DNA for M13mp18 ssDNA and 0.6 μM DNA for poly(dT), suggesting that both a circular DNA and a linear DNA were good ATPase activators. Hmi1p-catalysed ATPase activity was not supported by rRNA, linear dsDNA or supercoiled dsDNA, suggesting that the protein does not productively interact with RNA or with duplex DNA (data not shown).

Several divalent cations were also tested in ATPase reactions using Hmi1p and M13 ssDNA as the nucleic acid co-factor (data not shown). A total of five divalent cations were tested as Hmi1p co-factors: $MgCl_2$, $MnCl_2$, $CaCl_2$, $ZnCl_2$ and $CuCl_2$. Each divalent cation was tested in an ATPase reaction at a final concentration of 8 mM. ATP hydrolysis in the presence of $MgCl_2$ was two-fold higher than in the presence of either $MnCl_2$ or $CaCl_2$. $ZnCl_2$ and $CuCl_2$ were poor divalent cation co-factors in the Hmi1p-catalysed ATPase reaction.

ATPase reactions containing M13mp18 ssDNA were used to determine the steady-state kinetic parameters for DNA-stimulated ATP hydrolysis catalysed by Hmi1p (Figure 6). The dependence of the ATP hydrolysis reaction on ATP concentration was well described by a rectangular hyperbola. Assuming Michaelis–Menten kinetics for this reaction, the K_m value was 150 μM ATP and a k_{cat} of 72.7 min^{-1} was observed.

Genetic characterization of *hmi1* mutants

The haploid W303aΔ*hmi1::TRP1* strain, KCY3-2D, formed petite colonies on rich media and failed to grow on YPEG media (data not shown). Together, these phenotypes suggest a lack of mitochondrial function, similar to the results reported by others (Lee *et al*., 1999; Sedman *et al*., 2000). KCY3-2D and W303α cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) to determine whether disruption of *HMI1* caused an observable effect on mtDNA (data not shown). When KCY3-2D cells were stained with DAPI, the strain appeared to completely lack mtDNA (i.e. the strain was rho^0). The mtDNA was clearly present in W303α cells, as evidenced by the punctate DAPI staining pattern around the perimeter of the cells.

Since the loss of mtDNA is an irreversible event, it was not possible to simply introduce a plasmid carrying *HMI1* into *hmi1*Δ haploid cells to evaluate the ability of various mutants to complement the *HMI1* deletion. Complementation experiments must be performed under circumstances in which mtDNA is available for *Hmi1p* or mutant forms of *Hmi1p* to maintain. Therefore, KCY3-2D and

W303α were mated to obtain ALY01, a diploid strain heterozygous (*HMI1/hmi1*Δ) at the *HMI1* locus. ALY01 formed grande colonies on rich media and exhibited a wild-type growth phenotype when streaked on YPEG media. The W303 background is *ade2* and respiring *ade2* colonies are red. The red colouration depends on active respiration and in the absence of respiration white colonies are formed. This colour phenotype allowed for a rapid assay of mitochondrial function on rich media (Greenleaf *et al*., 1986; Malaney *et al*., 1997). Since the heterozygous diploid grew on YPEG media and developed the red colouration indicative of active respiration, one copy of the wild-type allele of *HMI1* is sufficient to ensure the presence of functional mitochondria. Thus, the petite phenotype associated with *hmi1*Δ strains is recessive to *HMI1*.

When the heterozygote was sporulated and the tetrads dissected, the *hmi1*Δ spore colonies exhibited the expected mitochondrial defect, as evidenced by the 2 : 2 segregation of red and white colonies on YPD. To confirm the genotype of each spore colony, the tetrads were replica-plated to media lacking tryptophan, to score for the *hmi1::TRP1* deletion allele. The tetrads were also replica-plated to media lacking a fermentable carbon source, to determine which spore colonies were capable of respiration. Of 48 ALY01 tetrads dissected, the ability to grow on medium lacking tryptophan and the ability to grow on medium lacking a fermentable carbon source were never observed in the same haploid colony. These results indicate that disruption of the *HMI1* gene is responsible for the petite phenotype.

The heterozygote ALY01 was also sporulated and dissected directly onto YPEG media. In this case, two spores in each tetrad were expected to be respiration-competent and to grow normally on YPEG media. This is exactly what was observed. The remaining two spores formed microcolonies. Even after extensive incubation at 30 °C, the microcolonies did not grow into normal-sized colonies. The number of cells in the colonies was determined as described in Materials and methods. The microcolonies contained an average of 4700 cells, while the grande colonies contained an average of 1.3×10^6 cells.

As indicated above, the grande spores that grew on the YPEG plates were respiration-proficient. When cells from these spore colonies were stained with DAPI, mtDNA was clearly visible (Figure 7A). The cells from the microcolonies, on the other hand, appeared heterogeneous with respect to the presence of mtDNA (Figure 7B); some cells in each microcolony contained mtDNA, while others did not. Since all the cells in the microcolony were respiration-deficient, the mtDNA present in a subpopulation of the microcolony cells is likely to be rho$^-$. Therefore, the cells in the microcolonies were either rho^0 or rho$^-$ with respect to the mitochondrial genome. In either case the cells lacked a fully functional mitochondrial genome. However, it is important to note that some rho$^-$ mtDNA genomes must be able to replicate in the absence of Hmi1p. Similar data has been reported by others (Sedman *et al*., 2000, 2005).

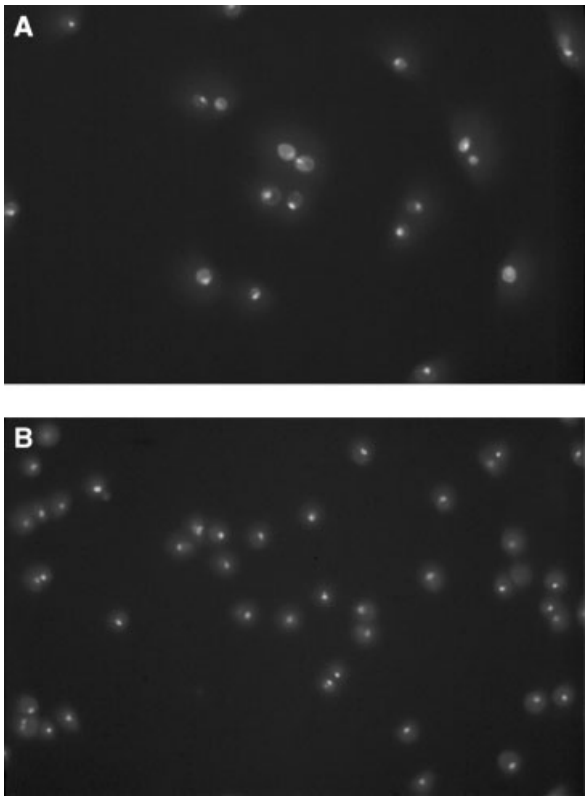

Figure 7. Examination of the spore products from the tetrad dissection of *HMI1/hmi1::TRP1* yeast cells on YPEG media by DAPI staining. (A) The heterozygous strain was sporulated and directly dissected onto YPEG media. The resulting spore colonies were dispersed in water and plated onto YPD. The cells were stained with DAPI using standard techniques and visualized using fluorescence microscopy. The mtDNA is visible as punctate staining at the cell periphery. (B) When the microcolonies were stained with DAPI, rho$^-$ and rho^0 cells were visible

The importance of mitochondrial localization and the role of the helicase activity associated with Hmi1p were tested in genetic complementation assays, using plasmids expressing *HMI1* and *hmi1* mutants in the strain heterozygous at the *HMI1* locus. Maintenance of functional mtDNA was evaluated by examining the spore products of a heterozygous strain containing plasmid copies of wild-type *HMI1* or the appropriate mutant. When ALY03, the heterozygous strain carrying pYE12*HMI1* (wild-type *HMI1*), was sporulated and dissected on YPD media, the four spores were two red and one or two sectored or red (data not shown). The sectored pattern observed in one or two of the spore colonies presumably resulted from the loss of plasmid-borne *HMI1*, since there was no selection for maintenance of the plasmid. Loss of pYE*HMI1* results in loss of the ability to respire and the concomitant loss of the ability to produce the red colouration. Therefore, plasmid loss is seen as a variegated pattern in the spore colony. When the dissected tetrads were replica-plated to YPEG media to directly assess mitochondrial function, all four spore colonies were able to grow (Table 3). The tetrads segregated 2 : 2 for growth on SD Trp^-, indicating that two spores were Δ*hmi1::TRP1* and two spores were *HMI1*. Spore colonies that grew on SD Ura^- retained the pYE*HMI1* plasmid. Thus, the presence of pYE*HMI1* allowed Δ*hmi1::TRP1* spore colonies to retain mitochondrial function. When ALY02 containing pYE12 (vector plasmid) was sporulated and dissected on YPD, the red and white phenotype segregated 2 : 2, as expected (data not shown). Trp^+, Ura^+ spores from ALY02 failed to grow on YPEG, indicating that the empty vector does not allow Δ*hmi1::TRP1* cells to retain functional mitochondria (Table 3).

To test the importance of localization of Hmi1p to the mitochondria, the *HMI1* gene was altered by site-directed mutagenesis to remove the C-terminal MLS. This altered gene, *hmi1ΔMLS*, was introduced into ALY01 (heterozygous at the *HMI1* locus) and its ability to complement the loss of *HMI1* was tested. When ALY05, containing pYE12*hmi1ΔMLS*, was sporulated and dissected on YPD, the spores segregated 2 : 2 in the red : white assay, suggesting that two spores lacked functional mitochondria. When these spore colonies were replica-plated to YPEG, the Δ*hmi1::TRP1*/pYE*hmi1ΔMLS* spores failed to grow (Table 3). Thus, pYE12*hmi1ΔMLS* does not complement the mitochondrial defect seen in the Δ*hmi1::TRP1* strain. The *HMI1*/pYE12*hmi1 ΔMLS* spores formed red colonies and contained functional mitochondria. To determine the location of the truncated Hmi1p, mitochondrial and cytosolic fractions were isolated from *hmi1::TRP1*/pYE*hmi1Δmls* cells. Hmi1Δmls was present in the cytosolic fraction, as determined by Western blot, but no Hmi1Δmls could be detected in the mitochondrial fraction. In simultaneous experiments with *hmi1::TRP1*/pYE*hmi1K32M* and *hmi1::TRP1/*pYE*HMI1*, Hmi1p was found in the mitochondrial fraction from both strains (data not shown). We conclude that complementation of the mitochondrial defect requires the C-terminal MLS. This, in turn, suggests that Hmi1p must localize to the mitochondria to function.

To evaluate the role of the helicase reaction catalysed by Hmi1p in maintenance of the mtDNA genome, three point mutants were constructed and evaluated. Point mutants were generated at the highly conserved lysine residue (K32) in the Walker A box of *HMI1*. Previous studies have shown that other helicases containing a similar mutation are able to bind the ATP but

Table 3. Plasmid-dependent retention of functional mitochondria in Δ*hmi1::TRP1* spores

Strain	$YPEG^+$	$YPEG^-$	Total	Complementation
ALY02(pYE12)	0	150	150	No
ALY03(*HMI1*)	113	19	132	Yes
ALY04(K32M)	22	62	84	Partial
ALY05(ΔMLS)	2	128	130	No

Strains heterozygous at the *HMI1* locus and carrying the indicated plasmid (pYE12, pYE12*HMI1*, pYE12*hmi1K32M*, pYE12*hmi1ΔMLS*) were generated as described in Materials and methods. The strains were sporulated and dissected on YPD media. Spore colonies were replica-plated to SD trp^-, SD ura^- and YPEG media. Spores that grew on SD trp^-, indicating that they were Δ*hmi1::TRP1*, and on SD ura^-, indicating that they contained the plasmid, were scored for their ability to grow on YPEG media. The total number of Trp^+, Ura^+ spores scored for functional mitochondria for each strain is indicated. The number of respiration-proficient and respiration-deficient spores from the strains were compared using the Fisher exact test method. Two-tailed p values >0.05 were not considered significant. The two-tailed p value for the number of $YPEG^+$ spores from ALY02 (the negative control) and ALY03 (*HMI1*) is <0.0001. The two-tailed p value for the number of $YPEG^+$ spores from ALY02 (the negative control) and ALY04 (containing pYE*hmi1K32M*) is <0.0001. The two-tailed p value for the number of $YPEG^+$ spores from ALY04 (*hmi1K32M*) and ALY03 (*hmi1*) is <0.0001. The p value for the number of $YPEG^+$ spores from ALY02 (the negative control) and ALY05 (*hmi1ΔMLS*) was 0.2147

fail to catalyse ATP hydrolysis, which abrogates the helicase reaction (Zavitz and Marians, 1992; George *et al*., 1994; Budd *et al*., 1995; Hishida *et al*., 1999). The conserved lysine at position 32 was changed to an alanine, serine and methionine as described in Materials and methods. Hmi1p-K32M was insoluble when overexpressed in *E. coli*. Two of the mutant proteins, Hmi1p-K32A and Hmi1p-K32S, were soluble when expressed in *E. coli* and were purified in the same manner as the wild-type protein. Neither purified protein exhibited significant helicase activity when tested using a partial duplex DNA substrate (data not shown). Thus, as expected, the conserved lysine in the Walker A box is essential for helicase activity. It should also be noted that this result makes it extremely unlikely that a contaminant in our preparation of Hmi1p was responsible for the helicase activity associated with the purified protein.

The *hmi1K32M* allele was introduced into the *HMI1/hmi1*Δ heterozygote on a multicopy plasmid (pYE12*hmi1K32M*) and its ability to complement the loss of *HMI1* was tested. When ALY04 was sporulated and dissected on YPD, the spore colonies were either red or sectored. When the tetrads were replica-plated to YPEG, 22 of 84 Δ*hmi1::TRP1*/pYE12*hmi1K32M* spores were able to grow (Table 3). The remaining 62 Δ*hmi1::TRP1*/pYE12*hmi1K32M* spores were unable to grow on YPEG. It appears that pYE12 *hmi1K32M* partially complements the mitochondrial defect present in the Δ*hmi1::TRP1* strain. This suggests that complementation of the mitochondrial defect does not require the helicase activity associated with Hmi1p. However, the number of Δ*hmi1::TRP1*/pYE12*hmi1K32M* spores that were capable of growth on YPEG media was significantly different from that seen when pYE12*HMI* was used in the complementation test. This indicates that the active helicase confers some advantage to the cell in terms of mtDNA maintenance. However, an active helicase is apparently not essential for maintenance of mtDNA.

Discussion

Here we report the purification of a yeast DNA helicase, Hmi1p, and provide an initial biochemical characterization of its DNA helicase and ATPase activities. In addition, the results of genetic complementation studies using two *hmi1* mutants are reported. Taken together, the data demonstrate that Hmi1p is a DNA-stimulated ATPase with $3' \rightarrow 5'$ DNA helicase activity that is involved in the maintenance of mtDNA. However, the molecular role of this protein in mtDNA metabolism is still not resolved.

A truncated form of Hmi1p, lacking the MLS which is cleaved *in vivo*, was expressed in *E. coli* and purified. The helicase activity associated with Hmi1p requires ATP hydrolysis, as demonstrated by inhibition of the unwinding reaction by ATP(γ)S, a competitive inhibitor of the DNA-stimulated ATPase reaction catalysed by Hmi1p. The purified protein demonstrates optimal unwinding activity at 2 mM rATP with half-maximal reaction velocity at an ATP concentration of 90 μM. We have also directly demonstrated a $3' \rightarrow 5'$ unwinding polarity, using a DNA substrate that is able to detect both $5' \rightarrow 3'$ and $3' \rightarrow 5'$ unwinding. The unwinding reaction catalysed by Hmi1p can be characterized as 'limited', based on the ability of the protein to unwind 22 and 25 bp partial duplex DNAs, while unwinding of partial duplex substrates in excess of 30 bp is dramatically reduced. Increasing the protein concentration or the length of incubation did not significantly improve this result, suggesting that there is a block of some kind to unwinding of longer duplex regions. Efforts to stimulate the unwinding reaction by including either *E. coli* SSB or the mitochondrial single-stranded DNA binding protein RIM1 did not improve the result. Thus, Hmi1p, in the absence of other proteins, catalyses the unwinding of a relatively short duplex region. It is possible that the protein interacts with other proteins in the mitochondrion and this may stimulate unwinding. Proteins involved in DNA metabolism often form complexes and work in conjunction with other proteins. Further work will be required to identify proteins that interact with, and perhaps modulate the activity of, Hmi1p.

The ATPase activity of Hmi1p was strongly stimulated by the addition of a nucleic acid cofactor. Both M13 ssDNA and poly(dT) proved to be effective in this regard, while RNA and several duplex DNA molecules were not effective. This suggests that Hmi1p does not interact with RNA, consistent with the fact it is not required for transcription of the mtDNA genome (Sedman *et al*., 2000). Both linear and circular ssDNA

stimulate the ATPase reaction to the same extent. However, the DNA concentration required for one-half maximal stimulation (K_{eff}) is somewhat different for these two effectors of the ATPase reaction. A four-fold lower concentration of the linear poly(dT) was required for one-half maximal stimulation than circular M13 ssDNA. Previous studies with other helicases have suggested that proteins that translocate processively along ssDNA exhibit a significantly lower K_{eff} for circular DNA than for linear DNA (Matson and George, 1987; Lahue and Matson, 1988). This is based on the fact that a processive translocase would not dissociate from a circular molecule as frequently as from a linear molecule. Applying the same interpretation would suggest that Hmi1p is not a processive translocase, consistent with the poor unwinding of long duplex regions. Thus, it is possible that this protein exhibits low processivity as a translocase and a helicase in the absence of additional proteins. This interpretation is offered with caution, since careful kinetic experiments to directly address the issue of processivity have not been performed.

Genetic studies have shown that disruption of the *HMI1* gene resulted in a haploid strain that lacked mitochondrial function. The failure of *hmi1*Δ colonies to grow on non-fermentable carbon sources and the lack of visible mtDNA when stained with DAPI augment the conclusion that disruption of *HMI1* affects mtDNA maintenance. A diploid strain heterozygous at the *HMI1* locus exhibited no apparent phenotype with regard to mitochondrial function, indicating that the *hmi1*Δ allele was recessive to the wild-type allele. Sporulation and tetrad dissection of the *HMI1/hmi1*Δ heterozygote on rich media resulted in two grande and two petite colonies. The cells in petite colonies (*hmi1::TRP1*) exhibited one of two patterns with regard to DAPI staining of mtDNA, either rho^0 (i.e. lacked mtDNA) or a mixture of rho^0 and rho^- cells. The grande colonies contained mtDNA. Thus, in the absence of Hmi1p yeast cells were not able to maintain rho^+ mitochondrial genomes; however, some rho^- mitochondria were present. A similar result has been obtained by others (Sedman *et al*., 2000, 2005) consistent with the notion that rho^+ and rho^- mitochondrial genomes may be maintained differently as many *trans*-acting factors and *cis*-acting sequences required for rho^+ maintenance are not required for maintenance of rho^- mitochondrial genomes (Fangman *et al*., 1990; Piskur, 1994; Lorimer *et al*., 1995; Graves *et al*., 1998; MacAlpine *et al*., 1998, 2000; Van Dyck and Clayton, 1998; Sedman *et al*., 2000).

Although *hmi1*Δ mutant cells cannot support the maintenance of rho^+ mitochondrial genomes, these cells can sustain rho^- mitochondrial genomes at least for some period of time. When the *hmi1/HMI1* heterozygote was sporulated and dissected on YPEG, *hmi1*Δ spores formed microcolonies. Even after extensive growth at 30 °C, these remained microcolonies containing an average of 4700 cells, while grande colonies grown for the same time contained 1.32×10^6 cells. Approximately 12 generations were required to generate 4700 cells, while approximately 20 generations were required to generate 1.32×10^6 cells. The absence of Hmi1p affects mtDNA maintenance before 12 generations occur, as cells from the microcolony are rho^0 or rho^-. The effect of Hmi1p loss on mtDNA is rapid, considering that the number of mitochondrial genomes in yeast cells varies depending on a variety of conditions, with an upper limit of approximately 50 mitochondrial genomes/cell (Dujon, 1981; Ulery and Jaehning, 1994). The fast development of the mitochondrial phenotype suggests that Hmi1p plays an important, integral role in mtDNA metabolism.

The petite phenotype associated with *hmi1*Δ strains was prevented by expressing *HMI1* from a plasmid. In addition, spore colonies containing the pYE*HMI1* plasmid were able to grow when replica-plated to YPEG media, indicating normal mitochondrial function. This result confirms that the petite phenotype of Δ*hmi1::TRP1* cells is caused by the lack of Hmi1p. DAPI staining of Δ*hmi1::TRP1*/pYE*HMI1* cells revealed mtDNA (data not shown), providing further confirmation that Hmi1p is required for maintenance of rho^+ mitochondria.

Hmi1p localizes to mitochondria and recent work indicates that the MLS of Hmi1p is located at the C-terminal end of the protein (Lee *et al*., 1999; Sedman *et al*., 2000). Therefore, a C-terminal truncation of Hmi1p was constructed and tested for Hmi1p function in Δ*hmi1::TRP1* cells. Exogenous *hmi1*Δ*MLS* supplied on a plasmid did not allow retention of mtDNA, although exogenous Hmi1p with an intact MLS did allow retention of mtDNA. The failure of *hmi1*Δ*MLS* to sustain functional mitochondria indicates that Hmi1p requires the C-terminus for full *in vivo* function; removal of the

C-terminal 16 residues abrogates localization of Hmi1p to the mitochondria. This, in turn, leads to the failure of Δ*hmi1::TRP1* cells containing the *hmi1ΔMLS* allele to sustain rho$^+$ mitochondria. The results presented here, and those previously published (Sedman *et al*., 2000), are entirely consistent and indicate that localization of Hmi1p to the mitochondria requires the C-terminus of Hmi1p and that mitochondrial localization is essential for function.

The involvement of Hmi1p in rho$^+$ mtDNA maintenance, combined with the *in vitro* helicase activity of the purified protein, suggests that Hmi1p might affect rho$^+$ mtDNA maintenance through its helicase activity. To test the importance of the helicase activity in mtDNA maintenance, a point mutant was constructed that was designed to eliminate the unwinding activity of the helicase. Unexpectedly, the plasmid-encoded Hmi1p-K32M allowed maintenance of functional mitochondria in a significant fraction of the deletion spores. However, when compared with the wild-type gene, *hmi1K32M* did not allow retention of the wild-type phenotype as well as *HMI1* (see Table 3), indicating a partial restoration of function by Hmi1p-K32M. Apparently the unwinding activity of Hmi1p is not essential for rho$^+$ mitochondrial maintenance. However, the ability of Hmi1p to function in rho$^+$ mtDNA maintenance is clearly improved when the protein is fully functional.

Maintenance of the mitochondrial genome depends on several processes including transcription, recombination and replication, and segregation to the daughter cell (Pon, 1991). Hmi1p does not appear to affect mitochondrial transcription, as similar levels of mitochondrial RNA transcripts occur in both wild-type and deletion strains (Sedman *et al*., 2000). Thus, Hmi1p could impact mtDNA maintenance through a role in recombination or replication. Several possibilities are considered below. Replication of rho$^+$ mtDNA is thought to be initiated by mitochondrial RNA polymerase-dependent priming, similar to that of mammalian mitochondria (Shadel and Clayton, 1997; Lee and Clayton, 1998). However, it has also been suggested that replication of mtDNA may be initiated using recombination intermediates (Lockshon *et al*., 1995; Bendich, 1996; Zelenaya-Troitskaya *et al*., 1998). Perhaps Hmi1p plays a role in mtDNA recombination. In wild-type cells Hmi1p might regulate mitochondrial recombination by disrupting non-homologous regions. In the absence of Hmi1p, recombination of the mitochondrial genome would increase to such a level that the mitochondrial genome would be destabilized by the presence of an increased number of unresolved recombination junctions and, in some cases, the mitochondrial genome would be lost resulting in rho^0 cells. In other cases recombination would rapidly generate rho$^-$ genomes consisting of short repeated sequences that present easy targets for recombination. Although attractive, this model seems less likely, since it does not readily account for partial restoration of function by the *hmi1K32M* allele. In this model one would predict that a mutant form of Hmi1p, incapable of unwinding, would not be able to retain functional mitochondria or would affect the stability of mtDNA in the heterozygous strain.

Several helicases that function specifically during initiation of replication have been described, including PriA from *E. coli*. PriA binds DNA at primosome assembly sequences (PAS) or D-loops and recruits other components of the primosome to a complex that restarts collapsed replication forks (Ng and Marians, 1996; Jones and Nakai, 1999; Liu and Marians, 1999; Liu *et al*., 1999; Jones and Nakai 2000; Sandler 2000; Sandler and Marians 2000). It is thought that the helicase activity of PriA allows the proteins to provide the ssDNA required for a more stable replisome or primosome complex (Boehmer and Lehman, 1997; Jones and Nakai, 2000). However, helicase-deficient mutants, which bind DNA and recruit other components of the primosome normally, allow almost wild-type levels of function (Zavitz and Marians, 1992). Thus, the helicase activity of PriA is not required for its function *in vivo*.

Both the wild-type *HMI1* and helicase-deficient *hmi1K32M* results are consistent with a model in which Hmi1p participates in replication in a manner similar to the function of PriA in replication restart. Wild-type Hmi1p would bind mtDNA and recruit essential replication factors but would not necessarily participate in the elongation phase of replication. In the absence of Hmi1p, some recombination-initiated replication would allow establishment of and replication of rho$^-$ genomes. The ability of *hmi1*Δ cells to support rho$^-$ genomes suggests that Hmi1p is unlikely to be directly involved in the elongation phase of mtDNA replication. While initiation of replication or replication restart might occur more efficiently if Hmi1p were able to provide unwound ssDNA, the

essential role of Hmi1p in mtDNA maintenance may be in recruiting other proteins necessary for replication. Although deficient in helicase activity, the Hmi1p-K32M mutant would still be capable of binding DNA and assembling replication proteins on either a collapsed replication fork or the origin of replication. A role for Hmi1p in replication restart or in the initiation of mtDNA replication is consistent with the observed results.

The currently available data suggest that Hmi1p is not the replicative helicase in yeast mitochondria. It has been shown that *HMI1* is required for mtDNA maintenance but that the helicase/ATPase function is dispensable. Perhaps the primary role of Hmi1p is to help maintain the integrity of the replication complex. For example, PriA is involved in replication restart at stalled forks in *E. coli* and is able to function in primosome assembly, even with disabled ATPase function. Cells lacking Hmi1p may undergo fork stalling during replication and ultimately generate double-stranded breaks, resulting in the fragmented genome seen recently by Sedman *et al*. (2005). Furthermore, they observed that *HMI1* was not required for the maintenance of rho$^-$ genomes, which might undergo less fork stalling due to the relatively small genome size (often 1 kb), resulting in a much higher viability rate and giving the impression that Hmi1p is not the replicative helicase.

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