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BIOCHEMICAL PROPERTIES OF HMI1P, A DNA HELICASE FROM *SACCHAROMYCES CEREVISIAE* MITOCHONDRIA

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which will be referred to in the text by their Roman numerals:

I Sedman, T., **Kuusk, S**., Kivi, S., Sedman, J. (2000). A DNA helicase required for maintenance of the functional mitochondrial genome in Saccharomyces cerevisiae. *Mol Cell Biol*. 2000 20(5):1816–1824.

II **Kuusk, S.**, Sedman, T., Sedman, J. (2002). Recombinant yeast mtDNA helicases. Purification and functional assays. In Copeland, W.C: (ed.) *Methods in Molecular Biology: Mitochondrial DNA. Methods and Protocols*. Humana Press Inc., Totowa, New Jersey, 197:303–316.

III **Kuusk, S.**, Sedman, T., Jõers, P., Sedman, J. (2005). Hmi1p from Saccharomyces cerevisiae mitochondria is a structure-specific DNA helicase. *J Biol Chem*. 2005 280(26):24322–24329.

IV **Kuusk, S**., Sedman, T. Sedman, J. Yeast mitochondrial DNA helicase Hmi1p stimulates synthesis of one strand of mtDNA through specific binding to ori sequences. Manuscript.

ABBREVIATIONS

2D	two-dimensional
bp	basepair
D loop	displacement loop
dsDNA	double-stranded DNA
EMSA	electrophoretic mobility shift assay
GST	glutathione-S-transferase
HS	hypersuppressive
kb	kilo basepair
mtDNA	mitochondrial DNA
nt	nucleotide
RDR	recombination dependent replication
SF	superfamily
SSB	single-stranded DNA binding protein
ssDNA	single-stranded DNA
ts	temperature-sensitive
wt	wild type

1. INTRODUCTION

Helicases are enzymes that unwind duplex molecules of nucleic acids, being thus essential in DNA and RNA metabolism. To meet the requirements given by different processes of nucleic acid metabolism, helicases have acquired diverse substrate recognition and unwinding properties. Therefore, by describing the biochemical properties of a helicase, possible roles of the protein within the nucleic acid metoblism can be proposed.

In budding yeast *Saccharomyces cerevisiae* only five helicases have been described to date to have function in mitochondrial nucleic acid metabolism. There are RNA helicases with determined functions in mRNA splicing (Mss116p), intron degradation (Suv3p) and ribosome biogenesis (Mrh4p). Of DNA helicases two are shown to localize in mitochondria — Pif1p and Hmi1p. Pif1p is the repair and recombination helicase, the exact function of Hmi1p is still to be studied. Unsolved is also the question of the DNA helicase participating in the main process of DNA metabolism, replication.

Trying to find answers to these two questions, I have concentrated during my studies on the DNA helicase in *S. cerevisiae* mitochondria with the function to be specified — Hmi1p. Although no conclusive solutions found, we have come much closer to the truth through this work. To make my interpretations of our results more understandable, background information about several aspects of helicases and processes they are involved in is given in Section 2. General characterization and classification of helicases together with crystal structure analysis and an overview of hypotheses for the mechanism of function makes the first part of Section 2. In the second part, there is a short overview of *S. cerevisiae* mitochondrial DNA maintenance, the process Hmi1p is demonstrated to be involved in. This is followed by a review of all the DNA helicases described to date to function in mitochondria through the whole group of eukaryotes. In the third part of Section 2 the closest homologues of Hmi1p are described. The results of my experimental work with Hmi1p are discussed in Section 3 of this thesis.

2. REVIEW OF LITERATURE

2.1. Helicases

2.1.1. General characterization

Helicases are enzymes that hydrolyze nucleoside triphosphates and use the derived free energy to translocate along RNA or DNA and unwind the duplex nucleic acid. All organisms contain several helicases with different biochemical properties. Helicases generally function *in vivo* as integral components of macromolecular machines that are engaged in processes of nucleic acid metabolism (reviewed in Delagoutte and von Hippel, 2002; Delagoutte and von Hippel, 2003; von Hippel and Delagoutte, 2003; Rocak and Linder, 2004; Tuteja and Tuteja, 2004a; Tuteja and Tuteja, 2004b).

Helicases can be classified by the nature of their nucleic acid substrate, which can be DNA, RNA or DNA/RNA hybrid duplex. DNA helicases are involved in replication, recombination, repair and transposition. RNA helicases are involved in translation, splicing and ribosome biogenesis. RNA/DNA helicases are mostly involved in transcription, but also in telomere biogenesis. Helicases interact with single-stranded nucleic acids and move along them in certain orientation in regard to the sugar-phosphate backbone. Therefore helicases can be characterized by the orientation of this movement, being either 3'-5' or 5'-3' directional. However, for those helicases that start unwinding from the blunt end of duplex nucleic acid it is rather difficult to verify, on which strand the enzyme moves. Helicases can be distributive or processive according to the length of the duplex nucleic acid they can unwind before dissociating from the substrate. Generally, RNA helicases are rather distributive while some DNA helicases, especially those that function on replication forks, can be very processive being able to unwind several thousand base pairs (bp) of DNA before dissociating. On the basis of their quaternary structure helicases are either monomeric, dimeric or hexameric. Some helicases contain subdomains with primase activity and there are some proteins that acquire the helicase activity in the presence of additional factors (reviewed in Matson and Bean, 1995).

2.1.2. Structural classification

Based on amino acid sequence comparison helicases are divided into 3 superfamilies and 2 smaller independent families (Table 1) (Gorbalenya and Koonin, 1993). The conserved sequence motifs in primary structure characteristic to helicases are called helicase motifs. These motifs are distributed along sequence regions ranging in length from 200 to 700 amino acids. In different superfamilies the number and sequence of conserved motifs differs, but within one superfamily all proteins share the same motifs. The sequence between the conserved motifs is rather diverse as similarities between different proteins can be seen only between close homologues. Superfamily 1 (SF1) is the largest family containing monomeric and dimeric RNA and DNA helicases. Helicases belonging to this superfamily have 7 conserved helicase motifs (Gorbalenya et al., 1988; Hodgman, 1988). To this superfamily, the subfamily of UvrD-like helicases belongs also Hmi1p, the helicase this thesis is concentrated on. Superfamily 2 (SF2) is closely related to SF1, both superfamilies having similar conserved motifs I and II. The other five motifs differ between SF1 and SF2 proteins (Gorbalenya et al., 1989; Linder et al., 1989). In addition, SF2 helicases contain an eighth motif — the Q motif that lies upstream of motif I (Tanner et al., 2003). SF2 consists mainly of RNA helicases, and as a subfamily also Snf2-like chromatin remodeling proteins (Laurent et al., 1992; Bork and Koonin, 1993). Superfamily 3 (SF3) contains helicases from RNA and DNA viruses containing only 3 helicase motifs in their primary structure (Gorbalenya et al., 1990). Family 4 is a smaller one consisting of hexameric helicases that have additional primase domains or are associated with primases. The most famous helicase of this type is the bacterial replicative helicase DnaB (Ilyina et al., 1992). Family 5 contains transcription termination factor Rho-like hexameric DNA/RNA helicases, which show significant sequence similarity to proton-translocating ATPases (Gorbalenya and Koonin, 1993). Recently it has been shown that some helicases, for example the RuvB-like branch migrating enzymes and MCM proteins, belong to the AAA+ family of chaperone-like ATPases rather than any of the described helicase families (Neuwald et al., 1999).

Table 1. Helicase superfamilies and families: conserved sequence motifs and some better-studied subfamilies[1]

		Superfamily 1	Superfamily 2	Superfamily 3	Family 4	Family 5
conserved sequence motifs[2]	motif Q	–	Y Y V W W T I Fx$_{16}$GFccPSPLQ	–	–	–
	motif I (Walker A)	G A T ++xAxPGoGKS	T ++xxxoGxGKS	T Pxx+++xGPxGSGKS	T +xsxxsxGKS	S ssx+GKT
	motif Ia	xx+xxxoo	x+++xPoo	–	s+++sxExs	–
	motif II (Walker B)	+++DExo	+++DExH	EE ++x+DD	++++Dxu	VV II VLL+uD
	motif III	++++GDxoQ	TGS +x+SATxxx	T T +++SxxxxxSN	ux++ooxxxs+++xso	–
	motif IV	xx+xooxR	Y ++Fxxoxo	–	S A Gsxuxoxsoxxu	–
	motif V	T K I xxS+xxxQG+o+ooV	T +xSxxxxxG+o+xo+	–	–	–
	motif VI	A T VG+SRxoo	QxxGRxxR	–	–	–
some subfamilies		UvrD-like, Pif1, UL5-like, Tra1, Sen1-like, Hel4, (+) RNA viral helicases	eIF-4A-like, UvrB, PriA-like, Rad3-like, Ercc3-like, UL9-like, RecQ, Snf2-like	T antigen-like, Rep-like, α primase-like, 2C-like	DnaB-like, bacterial and bacteriophage primase-helicases	Rho, Fli1, V-ATPases, F-ATPases

[1] The table is drawn according to (Gorbalenya et al., 1990; Ilyina et al., 1992; Gorbalenya and Koonin, 1993; Opperman and Richardson, 1994; Leipe et al, 2000; Tanner et al., 2003).

[2] c = polar residue (D, E, H, K, R); + = hydrophobic (I, L, V, M, F, Y, W, C, A); o = hydrophilic (D, E, H, K, R, N, Q, S, T); u = I, L, V, M; s = small (A, C, D, G, N, P, S, T, V); x = any

2.2. Structure and mechanism of function of SF1 helicases

2.2.1. Structure

By now the crystal structures of PcrA from *Bacillus stearothermophilus* and Rep from *Escherichia coli* have been solved for the helicases from SF1 (Subramanya et al., 1996; Korolev et al., 1997). In addition, the crystal structure of the RecBCD complex from *E. coli* has been solved (Singleton et al., 2004), two components of which — RecB and RecD — are SF1 helicases (Boehmer and Emmerson, 1992; Hsieh and Julin, 1992; Gorbalenya and Koonin, 1993). However, as most of the studies on helicase structure and function have been done on PcrA and Rep helicases, I discuss only these proteins here.

Both Rep and PcrA share the similar domain structure containing four domains — 1A, 1B, 2A and 2B (Fig. 1A). Domains 1A and 2A have the same fold, which is also very similar to the central region structure of the *E. coli* recombinase RecA (Story et al., 1992). Domains 1B and 2B are formed of the amino acid sequence extensions from 1A and 2A correspondingly. Their structure is mainly α helical and more heterogeneous. It has been shown that they both comprise a helix-hairpin-helix motif that is commonly involved in nonspecific double-stranded DNA (dsDNA) binding (Shao and Grishin, 2000). The ATP binding site is situated in a cleft between domains 1A and 2A that is lined with conserved helicase motifs (Fig. 1B).

The Rep helicase was crystallized in complex with single-stranded DNA (ssDNA) and ADP (Korolev et al., 1997). The complex consisted of 2 helicase molecules on the same DNA, one in open conformation and the other in the closed one, where the 2B domain was rotated 130^{o}. For PcrA the crystal structures have been solved for the apoenzyme (Subramanya et al., 1996), in complex with the nonhydrolyzable ATP analogue ADPNP (Soultanas et al., 1999b) and two different complexes with DNA (Velankar et al., 1999). Of the complexes of PcrA with DNA, the one with sulfate ion and DNA can be considered an enzyme product complex while the other one with DNA and ADPNP can be considered an enzyme substrate complex. The conformation of the enzyme differs significantly in different complexes and from the apoenzyme as well. The main difference lies in the orientation of 1B and 2B domains. They are in the position similar to the open conformation of the Rep helicase in the apoenzyme but in the position similar to the closed conforamtion in the product complex. In the substrate complex the positions of the domains 1B and 2B are again different, the alteration resulting from the closure of the cleft between domains 1A and 2A. SsDNA is bound in both substrate and product complexes along a groove on the top of 1A and 2A domains, while dsDNA ahead of the ssDNA-dsDNA junction is bound to 1B and 2B domains. The path taken by the complementing DNA strand is not so clearly determined, but it is presumed to

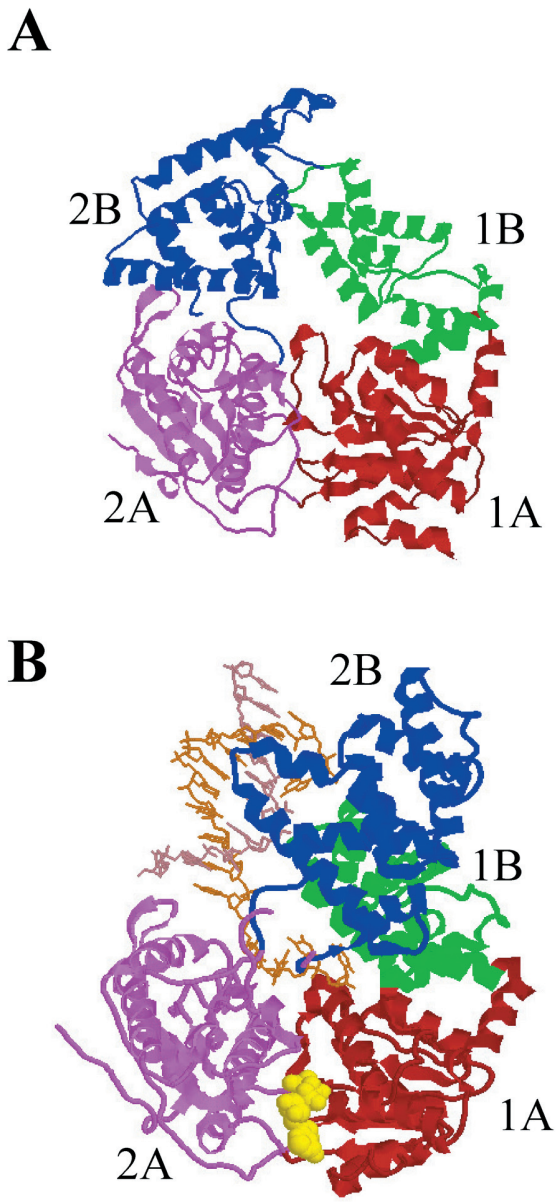

Figure 1. Ribbon diagrams of the structures of the helicase PcrA from *Bacillus stearothermophilus* A – apoenzyme and B – complex with DNA and ADPNP. Domain 1A – red, 1B – green, 2A – magneta and 2B – blue. ADPNP is depicted in yellow; one strand of DNA is displayed in gold and the other one in pink. The diagrams were drawn using Protein Explorer (http://www.umass.edu/microbio/chime/pe/protexpl/frntdoor.htm) on the basis of the PDB files 1pjr.pdb (Subramanya et al., 1996) and 3pjr.pdb (Velankar et al., 1999) from the protein data bank at http://www.rcsb.org/pdb/.

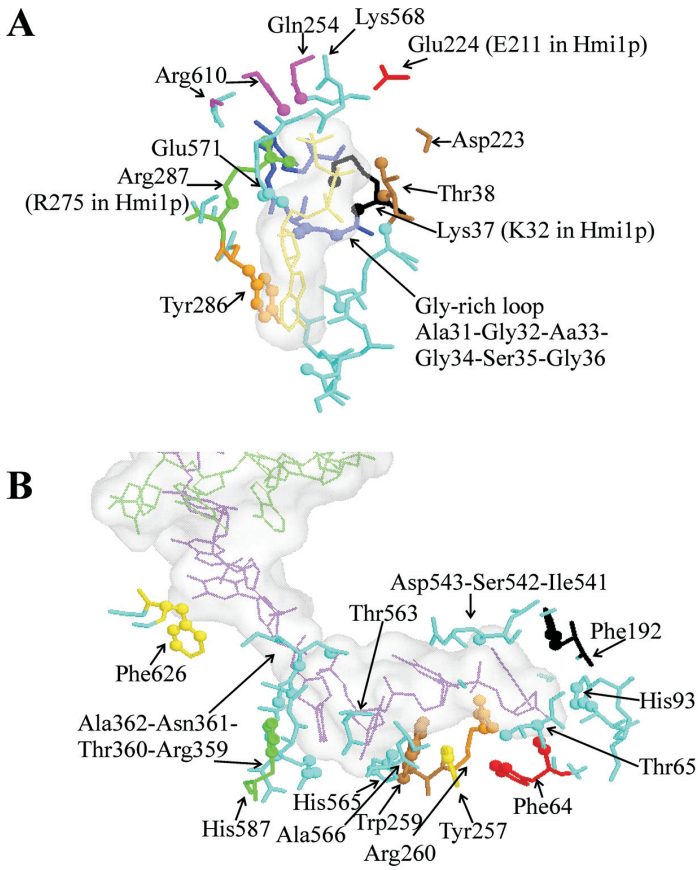

Figure 2. Details of the ATP binding site (A) and ssDNA binding site (B) of the helicase PcrA from *Bacillus stearotermophilus* in complex with ADPNP and DNA. A – ATP – yellow with van der Waal's radius – grey. Residues that make contact with ADPNP or Mg^{2+} ion are coloured, other residues in the viscinity are light blue. The positions of respective amino acid residues in Hmi1p are indicated for those which were mutated to generate enzymatically inactive proteins. B – One strand of DNA – magneta, the second – green; van der Waal's radii of both – grey. The amino acid residues that are in contact with bases of DNA are coloured; other amino acids in the viscinity are light blue. The diagrams were drawn using Protein Explorer (http://www.umass.edu/microbio/chime/pe/protexpl/frntdoor.htm) on the basis of the PDB file 3pjr.pdb (Velankar et al., 1999) from the protein data bank at http://www.rcsb.org/pdb/. C – The sequences of the conserved helicase motifs of the same protein together with the positions of the first and last amino acid residue of a given motif.

continue from the dsDNA to the surface of the 2A domain, although it may well be that the helicase does not contact the second strand at all.

The most important amino acids concerning the binding of ATP and DNA as well as the catalytic events belong to the conserved helicase motifs being thus conserved through evolution (Gorbalenya and Koonin, 1993) (Fig. 2). The role of these amino acids has been studied by measuring the activities of mutant PcrA proteins in parallel with crystal structure analyses of the same proteins (Subramanya et al., 1996; Bird et al., 1998; Dillingham et al., 1999; Soultanas et al., 1999b; Velankar et al., 1999; Soultanas et al., 2000; Dillingham et al., 2001). Motifs I and II, also called Walker A and B motifs, form the NTP binding and hydrolysis complex. These motifs are present not only in helicases but also in a variety of other proteins that use NTP hydrolysis to carry out their respective functions (Walker et al., 1982; Smith and Rayment, 1996; Leipe et al., 2003; Leipe et al., 2004). The triphosphate part of ATP is bound by the glycine rich loop of Walker A motif (blue on Fig. 2A, sequence on Fig. 2C). The bottom of the ATP binding pocket is formed of motif IV that provides a linkage between domains 1A and 2A. The only amino acid to interact with the nitrogenous base part of ATP is tyrosine 286 from motif IV (orange on Fig. 2A). This may be different in other helicases, as it has been shown, that while PcrA is able to hydrolyse all dNTP-s and NTP-s (Bird et al., 1998), most of the helicases are more specific for certain nucleotides. This indicates that the base part of the bound NTP is more specifically recognized in such proteins. The Mg^{2+} ion is coordinated by threonine 38 from Walker A motif and aspartate 223 from Walker B motif (both brown on Fig. 2A). The catalytic amino acid residue is proposed to be glutamate 224 from Walker B motif (red on Fig. 2A), which activates the water molecule that attacks the γ phosphate group during the hydrolysis of the bound nucleotide. In the substrate complex additional contacts are formed between γ phosphate and glutamine 254 from motif III (magneta on Fig. 2A), arginine 287 from motif IV (green on Fig. 2A) and arginine 610 from motif VI (magneta on Fig. 2A). These interactions stabilize the complex with the cleft between domains 1A and 2A being closed. No amino acid residues from different sides of the cleft contact each other in the substrate complex. Upon hydrolysing the γ phosphate these contacts are broken and the cleft opens up again. Therefore these amino acid residues are proposed to work as sensors that transmit ATP hydrolysis-induced conformational changes from the active site to the DNA binding site.

The protein-DNA interactions are formed mainly through hydrophobic interactions between aromatic side chains stacking against the nitrogenous bases of ssDNA (Dillingham et al., 2001) (Fig. 2B). The ssDNA binding site in domain 2A is formed of histidine 587 (green on Fig. 2B) and phenylalanine 626 (yellow on Fig. 2B). In domain 1A the ssDNA-binding site is formed of tyrosine 257 (yellow), tryptophane 259 (brown) and arginine 260 (orange) from motif III, phenylalanine 64 from motif Ia (red) and phenylalanine 192 (black). In the substrate complex four nitrogenous bases of ssDNA extend across the

centre of the protein. In the product complex, however, the same region is occupied by five bases. The nitrogenous base of the additional nucleotide (nt) in the product complex occupies a pocket on domain 1A that is not accessible in the substrate complex as the side chain of the conserved phenylalanine 64 has rotated to fill the pocket. The position and/or contacts with ssDNA of phenylalanines 626 and 64 and tyrosine 257 are altered by the ATP hydrolysis cycle, meaning that presumably these amino acid residues coordinate translocation and unwinding with ATP hydrolysis. DsDNA is bound only by domains 1B and 2B and the amino acid residues making contacts with dsDNA are not conserved. By changing these amino acids it is possible to uncouple the helicase activity from ssDNA translocation activity (Soultanas et al., 2000). Thus the less conserved domains 1B and 2B might contribute specificity for different nucleic acid substrates while the more conserved domains 1A and 2A, that also contain all the conserved helicase motifs, comprise the helicase motor, which hydrolyses NTP and translocates along ssDNA.

2.2.2. Mechanism of function

Before any crystal structures of helicases were solved, there were two main hypotheses for the mechanism of unwinding double-stranded nucleic acids by helicases — the rolling and the inch-worm mechanism. The rolling mechanism was proposed by T. Lohman on the basis of studies of the Rep helicase (Wong and Lohman, 1992). This mechanism suggests that the helicase operates by an alternation in the affinity of the enzyme for dsDNA and ssDNA, controlled by binding and hydrolysis of ATP (Fig. 3A). It requires at least a dimeric enzyme with the monomers working cooperatively in the unwinding reaction. Both monomers should be unable to bind ssDNA and dsDNA simultaneously. The inch-worm mechanism was first proposed by Yarranton and Gefter (Yarranton and Gefter, 1979). This model proposes that the helicase functions by moving along DNA and splitting the duplex as it translocates. The inch-worm mechanism can be used by monomeric as well as oligomeric enzymes. A third model, the so called passive model has been under discussion (Lohman, 1993). According to this model, the helicase does not unwind DNA actively but rather traps the fluctuations at the fork, when the last basepair (bp) is in the unwound state. The rate of opening up of a single bp at the ssDNA-dsDNA junction is estimated to be up to 1000 times per second (Chen et al., 1992). Thus, this model does not explain the rate of DNA unwinding of the processive helicases, like the RecBCD complex from *E. coli,* that can unwind DNA at the rate greater than 1 kb/s (Roman and Kowalczykowski, 1989; Bianco et al., 2001). Also different biochemical and structural studies have shown, that at least some SF1 helicases actively destabilize the nucleic acid duplex (Amaratunga and Lohman, 1993; Velankar et al., 1999; Soultanas et al., 2000).

The studies of crystal structures allowed a more detailed understanding of the helicase mechanism. Based on these studies PcrA was proposed to use the inch-worm rather than the rolling mechanism (Bird et al., 1998; Velankar et al., 1999; Soultanas et al., 2000). The general model proposes that in response to ATP binding and hydrolysis the cleft between 1A and 2A domains opens and closes respectively. This brings along the movement of nitrogenous bases of the bound ssDNA and increases and decreases the affinity of different domains of the protein toward ssDNA (Fig. 3B). First of all, protein binding to ssDNA causes the domain swivelling to make the complex that is described as the product complex. Upon ATP binding the cleft between 1A and 2A closes. As a result, the affinity of 1A domain for ssDNA is weakened, since only 4 nitrogenous bases of ssDNA can bind. Therefore 1A domain can move one base forward along ssDNA. When ATP is hydrolyzed, the cleft opens up again. At that position the affinity of 2A domain for ssDNA is weakened, while 1A holds it tightly. Thus 2A can move along ssDNA now in the 5' direction and rebind to repeat the translocation cycle. When the protein reaches dsDNA, 2B domain binds duplex DNA adjacent to the fork. The conformational changes upon binding ATP set up a surface to bind duplex DNA by moving domains 1B and 2B into appropriate conformations. In this conformation the DNA duplex is distorted at the junction region with a slight unwinding resulting from this. When ATP is hydrolyzed the affinity of 2B for dsDNA becomes weaker, allowing thus dsDNA to slide over the protein.

The mechanism of action of UvrD and Rep helicases has been described in detail by kinetic studies. Pre-steady-state single turnover kinetics experiments and single-molecule fluorescence studies show that the monomers of both enzymes can translocate along ssDNA but for unwinding dsDNA they need to dimerize (Ali et al., 1999; Cheng et al., 2001; Ha et al., 2002; Maluf et al., 2003; Fischer et al., 2004). A model was offered, where the first monomer in the dimer is the one that contacts dsDNA and destabilizes it, while the second monomer behind works as the translocator pushing the first monomer on as well (Ha et al., 2002). Fluorescence resonance energy transfer experiments with fluorescence labelled Rep confirmed that the protein is in the closed conformation upon binding ssDNA and without ATP in complex. The reaction conditions were optimized for the Rep monomer binding and as no ATP was included, it was concluded to be the inhibited conformation that cannot unwind dsDNA (Rasnik et al., 2004). Mutant RepΔ2B that lacks 2B domain has the helicase activity also as a monomer, suggesting that 2B inhibits the helicase activity of the monomeric protein. Thus it was proposed, that 2B domain has the regulatory role in helicase action (Cheng et al., 2002; Brendza et al., 2005).

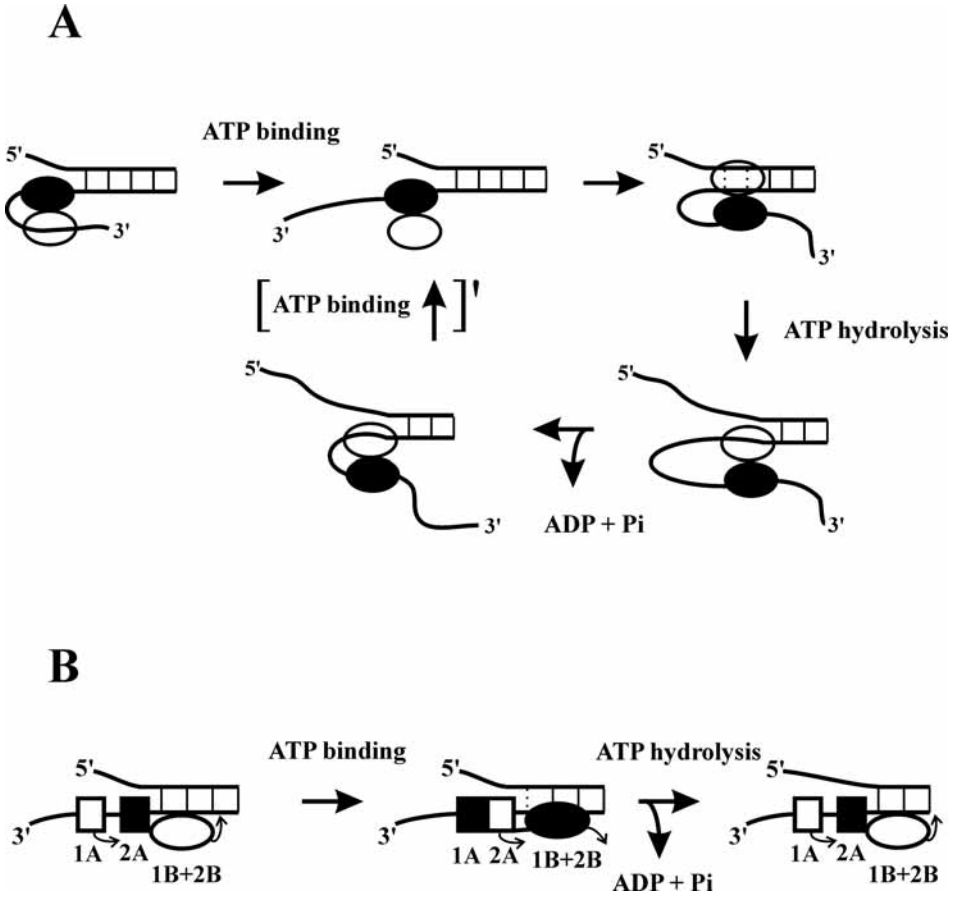

Figure 3. Schematic models of proposed helicase mechanisms. A — The rolling mechanism. The different monomers of the helicase are depicted as full and empty ellipses to show the proposed movement of the monomers in the dimer. B — The inch-worm mechanism modified according to the crystal structure studies of PcrA. Black boxes and ellipses indicate the domains that are in tight interaction with nucleic acid, white boxes and ellipses represent the domains that interact only weakly with the nucleic acid and are thus able to move along it. Dotted lines on both figures display hydrogen bonds that are broken during the indicated steps.

2.2.3. Step size

There have been long discussions as to what the step size of a helicase would be, if the step size indicates, how many base pairs are unwound upon the hydrolysis of one ATP molecule. It has been shown that different helicases protect different number of nucleotides. For the PcrA monomer it has been shown to be 6 nt of ssDNA and 4 bp of dsDNA using the DNAse I cleavage protection assay (Soultanas et al., 2000). The Rep dimer is shown to bind to 16 nt of ssDNA in crystal (Korolev et al., 1997). However, the observed unwinding step sizes are always smaller than the region the protein is able to protect. This may be due to conformational features, but also because a helicase may slip back after one unwinding step. Kinetics measurements by Lohman and coworkers with UvrD showed the step size to be 5 bp (Ali and Lohman, 1997). Later, using a modification of a stopped-flow fluorescence assay, they showed that monomeric UvrD translocates 3,7 nt between two successive rate-limiting steps (Fischer et al., 2004). The crystal structure analyses suggest that during the hydrolysis of 1 molecule of ATP the PcrA helicase moves 1 nt, so the step size is 1 (Velankar et al., 1999). This result has also been supported by pre-steady-state kinetics analyses of PcrA (Dillingham et al., 2000). It remains to be studied, whether the step size can vary so much between different helicases or some of these results do not reflect the actual step size.

2.3. Mitochondrial DNA and its replication in *S. cerevisiae*

2.3.1. Organization of mitochondrial DNA

Mitochondrial DNA (mtDNA) of *S. cerevisiae* varies in length between 75 and 80 kb depending on the strain. It encodes for seven major subunits of energy-transducing complexes, one ribosomal protein, several minor proteins involved in intron splicing and gene conversion, large and small rRNAs, 24 tRNA-s and an RNA involved in tRNA processing. In the intergenic region there are 7–8 replication origins, 4 of which are active (Foury et al., 1998). MtDNA accounts for approximately 5–15% of total cellular DNA in yeast, which corresponds to 20–50 copies of mtDNA per haploid cell (Williamson et al., 1971; Nagley and Linnane, 1972; Dujon, 1981). The majority of mtDNA is in the form of a network of linear and branched molecules, although a small subset can be in circular form as well (Maleszka et al., 1991; Maleszka and Clark-Walker, 1992; Bendich, 1996).

S. cerevisiae is a facultative aerobe, meaning that it can produce energy by respiration as well as fermentation. For fermentation a functional mitochondrial genome is dispensable while for respiration it is essential. This makes yeast an ideal organism for studying factors involved in mitochondrial genome main-

tenance. In case the mtDNA replication is disabled and cannot ensure the maintenance of a full-length mitochondrial genome, mtDNA is either lost totally or maintained only partly, which is not functional and referred to as rho$^-$ (Ephrussi et al., 1949). Such rho$^-$ mtDNA consists of short segments of the original genome, which are repeated to give an amount of DNA comparable to the wild type (wt) level (Fukuhara, 1969). Depending on the nature of the segment, rho$^-$ mitochondrial genomes are either neutral or hypersuppressive (HS). Neutral mitochondrial genomes, when mated with wt strains, are lost in the progeny. HS mitochondrial genomes, however, when mated with wt strains, have the potential to outcompete the wt mitochondrial genome, giving the progeny mainly with rho$^-$ mtDNA (Ephrussi et al., 1955). HS mitochondrial genomes contain active replication origins in their repeats and thus are thought to have a replication adventage over other mtDNA-s (Blanc and Dujon, 1980; de Zamaroczy et al., 1981).

2.3.2. Mechanisms of replication

MtDNA is replicated independently of the nuclear DNA throughout the cell cycle (Williamson and Moustacchi, 1971; Sena et al., 1975). In *S. cerevisiae* mitochondria different modes of DNA replication are in use. To date strand-asymmetric bidirectional replication, recombination dependent replication (RDR) and as a subset of RDR, rolling circle replication have been described to take place (Maleszka et al., 1991; MacAlpine et al., 1998; Lecrenier and Foury, 2000; Williamson, 2002). The rolling circle mode of replication has been proposed to be essential for the segregation of mtDNA into daughter cells (Ling and Shibata, 2002; Ling and Shibata, 2004). In the mitochondria of nondividing cells the other modes of replication — RDR and the bidirectional replication are proposed to be in use. For maintaining a functional mitochondrial genome all replication mechanisms are required, but in case of rho$^-$ mtDNA probably only one of the mechanisms in use is able to maintain the genome. Thus, studying different rho$^-$ mitochondrial genomes as well as the influence of different factors on the replication of rho$^-$ mtDNA should make it possible to distinguish between different modes of replication and the roles of factors involved in them.

The defined replication origin regions called ori/rep sequences consist of three conserved G/C boxes A, B and C, separated from each other by A/T rich heterogeneous sequences (Fig. 4). The G/C rich boxes A and B together with the intervening A/T rich sequence form a hairpin structure (de Zamaroczy et al., 1979; de Zamaroczy et al., 1984). In front of the C box there is a transcription initiation site. In inactive origins this site is interrupted (de Zamaroczy et al., 1981; Baldacci and Bernardi, 1982). From active origins bidirectional mode of replication is initiated (Baldacci et al., 1984). It may well be that the hairpin structures are used also as initiation sites for rolling circle replication as the initiation sites for rolling circle replication in bacterial plasmids are known to

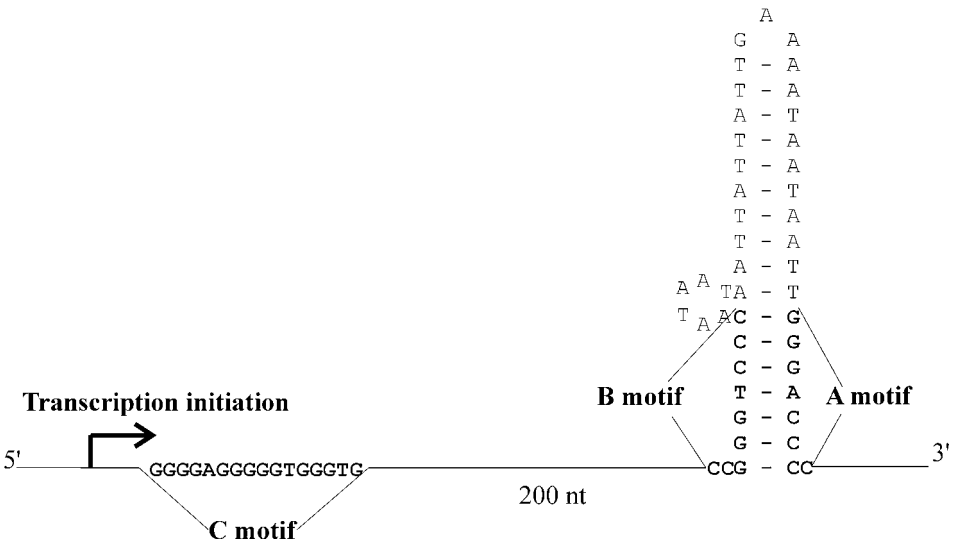

Figure 4. Schematic diagram of yeast mitochondrial ori2 region. Conserved G/C rich boxes A, B and C are indicated in bold letters. The exact sequence of the potential stem-loop structure formed of boxes A and B together with the intervening sequence is given. The promoter in front of box C is with an arrow indicating the direction of RNA synthesis.

contain hairpin structures as well. In the strand-asymmetric bidirectional mode of replication the leading strand synthesis is initiated by an RNA primer that is transcribed by the mitochondrial RNA polymerase Rpo41p from the promoter in front of the C box (Greenleaf et al., 1986; Kelly et al., 1986; Xu and Clayton, 1995; MacAlpine et al., 2001). Mtf1p, the specificity factor of the RNA polymerase is required for promoter recognition (Schinkel et al., 1987; Jang and Jaehning, 1991). The RNA-DNA transition has been described to be in the middle of the C box, from where the DNA is synthesized by the mitochondrial DNA polymerase Mip1p (Baldacci et al., 1984; Genga et al., 1986; Foury, 1989; Graves et al., 1998). Other proteins described to be involved in replication are the ssDNA binding protein (SSB) — Rim1p and mitochondrial isoform of Cdc9p, the ligase (Van Dyck et al., 1992; Willer et al., 1999). The initiation of the second strand in the strand asymmetric bidirectional mode of replication is still under discussion since a mitochondrial primase is unknown. Some early works have presented the purification of primase activity from yeast mitochondria, but so far no specific gene has been defined to encode a protein bearing such activity (Desai et al., 1989). Similarly, the mitochondrial topoisomerase activity has been isolated from yeast, but it is not clear, which gene is it encoded by (Ezekiel et al., 1994; Wang et al., 1995; Tua et al., 1997). During RDR the invading 3' strands are probably used for priming replication.

Recombination hotspots are described to be G/C rich clusters that are quite widespread in the otherwise extremely A/T rich mitochondrial genome of *S. cerevisiae* (Dieckmann and Gandy, 1987). The proteins involved in recombination are the recombinase Mhr1p, the Holliday junction cutting endonuclease Cce1p and the helicase Pif1p (Foury and Kolodynski, 1983; Foury and Lahaye, 1987; Kleff et al., 1992; Ezekiel and Zassenhaus, 1993; Ling et al., 1995; Ling et al., 2000). The general DNA synthesis machinery is the same for all modes of replication as deletion of Mip1p and Rim1p results in rho^{o} cells that have no mtDNA (Genga et al., 1986; Van Dyck et al., 1992). The rolling circle mode of replication is not well described to date. The initiation is either recombination-based or from the hairpin structures of the ori/rep sequences as mentioned above. The participating proteins involved beside the DNA polymerase and SSB are still under discussion. One of the factors required either for the template preparation or initiation is probably the recombinase Mhr1p (Ling and Shibata, 2002; Ling and Shibata, 2004). Depletion of most of the proteins participating in the replication of mtDNA result in rho^{-} cells rather than rho^{o}, showing that the majority of these proteins are necessary for effective replication of the full-length genome while some DNA synthesis can occur also without them. It can therefore be concluded that such factors participate in only one of the modes of replication while in the other modes they are dispensable.

2.4. Mitochondrial DNA helicases

In different organisms rather different mitochondrial DNA helicases have been identified. Altogether three DNA helicases have been described to date to function in mitochondria — Twinkle, Pif1p and Hmi1p. They are all encoded by nuclear genes and transported into mitochondria. Out of these, Twinkle has been described mainly in multicellular eukaryotes from the nematode *Caenorhabditis elegans* to human, but also in the malaria-causing parasite *Plasmodium falciparum*. Pif1p is the most conserved of the three being present from yeast to human. The mitochondrial localization, however, has been determined only for the yeast homologue. Hmi1p has been described in the evolutionarily close yeasts from the family of *Saccharomycetaceae* (Richard et al., 2005). In addition, a study of the mitochondrial proteome of *S. cerevisiae* points to Rrm3p being one more DNA helicase in mitochondria (Prokisch et al., 2004). Rrm3p is a close homologue of Pif1p and its described functions in nucleus are in the same processes as those of Pif1p (Ivessa et al., 2000; Ivessa et al., 2002). However, as no detailed studies have been published on the mitochondrial function of Rrm3p I do not discuss this protein here further.

2.4.1. Twinkle

Twinkle was first identified as one out of three loci associated with a human hereditary disease, the autosomal dominant progressive external ophthalmoplegia. Twinkle is a hexameric helicase that has sequence similarity to the bacteriophage T7 gene 4 protein and other hexameric helicases/primases of the helicase family 4 (Spelbrink et al., 2001). It is a 5'-3' directional DNA helicase that requires either a forked structure with longer 5' ssDNA and shorter 3' ssDNA or a 5' flap structure for initiating the unwinding reaction. The helicase activity of Twinkle is strongly enhanced by mitochondrial SSB (Korhonen et al., 2003). Twinkle is proposed to be the replicative helicase in mammalian mitochondria since it forms a minimal mtDNA replisome *in vitro* together with the mitochondrial DNA polymerase and the mitochondrial SSB (Korhonen et al., 2004). In addition, Twinkle has a function in controlling the copy number of mtDNA (Tyynismaa et al., 2004).

2.4.2. Pif1p

According to structural classification, Pif1p belongs to SF1, where the homologues from different organisms form a separate Pif1 subfamily (Bessler et al., 2001). The PIF1 gene has two translation initiation sites (Schulz and Zakian, 1994). From the first one a longer isoform is generated, which has a characteristic mitochondrial targeting signal at the N terminus (Foury and Lahaye, 1987). From the second ATG codon a shorter isoform is made that is targeted to nucleus and has a role in telomere biogenesis as well as in rDNA replication (Schulz and Zakian, 1994; Ivessa et al., 2000). Pif1p is a 5'-3' directional distributive DNA helicase that is functional in the monomeric state and is preferentially recruited to fork-like DNA structures (Lahaye et al., 1991; Lahaye et al., 1993). It was recently demonstrated, that Pif1p unwinds preferentially RNA/DNA duplexes with 5' DNA overhang, this being important for the function of the nuclear isoform of the helicase in removing telomerase from the DNA ends (Boule et al., 2005).

Deletion of Pif1p affects the stability of mtDNA upon exposing to UV light and on growing at 37^{o}C but not at 28^{o}C. The suppressivity of rho^{-} clones is also significantly increased in Pif1p deficient strains, indicating either a rise in the efficiency of the replication of rho^{-} mitochondrial genomes or a decline in the stability of the functional mitochondrial genome (Foury and Kolodynski, 1983; Foury and Lahaye, 1987; Lahaye et al., 1991). Later similar phenotypes were seen with the strain lacking only the first ATG codon of the PIF1 ORF, which is deficient only in the mitochondrial isoform of Pif1p (Schulz and Zakian, 1994). Based on these phenotypes Pif1p has been suggested to be required in mtDNA recombination and repair. In recombination the role of Pif1p has been studied mainly with different rho^{-} strains. Pif1p has been shown to enhance sequence-

or structure-specific recombination between rho$^+$ and rho$^-$ mtDNA. The Pif1p specific sequence determinants are not fully described though (Foury and Kolodynski, 1983; Foury and Van Dyck, 1985). The role of Pif1p in repair processes has been proposed, on the basis of the inhibitory role of Pif1p on the telomere synthesis in the nucleus, to inhibit similarly the replication progression in order to allow time for repair to occur (Schulz and Zakian, 1994; Zhou et al., 2000; Doudican et al., 2005). Pif1p may also promote tolerance of mtDNA damage through its recombinational activities or other unknown functions. Genetic interaction studies have discovered one more role for Pif1p in mtDNA metabolism, in regulating the mtDNA copy number (O'Rourke et al., 2002; O'Rourke et al., 2005; Taylor et al., 2005). It has been shown that at least in mammalian cells the mtDNA copy number is influenced by alterations in the cellular dNTP pools (Nishino et al., 1999; Mandel et al., 2001; Saada et al., 2001). dNTP pools are under the control of the conserved Mec1/Rad53 pathway, which is activated upon DNA damage (Elledge, 1996; Zhao et al., 1998; Zhao et al., 2001; Zhao and Rothstein, 2002). Pif1p is proposed to regulate the copy number of mtDNA in the Mec1/Rad53 pathway dependent manner being thus the link between alterations in the dNTP pools and mtDNA copy number in yeast (Taylor et al., 2005).

2.4.3. Hmi1p

The other DNA helicase described in the mitochondria of *S. cerevisiae* is Hmi1p. Hmi1p is encoded by the nuclear nonessential ORF YOL095c, in chromosome XV. By sequence homology Hmi1p belongs to SF1, the UvrD subfamily. The C terminus of Hmi1p contains a mitochondrial targeting signal peptide that is cleaved off after the protein is transported into mitochondria (Lee et al., 1999). The copy number of Hmi1p in wt cells is very small (T. Sedman, unpublished data). Due to this also yeast mitochondrial proteome studies have not reported Hmi1p (Ohlmeier et al., 2004; Prokisch et al., 2004). The overexpression experiments, however, demonstrate clearly the mitochondrial localization (Sedman et al., 2000). Also it has been demonstrated, that the mitochondrial targeting sequence deficient Hmi1pΔCterm is not able to complement the lack of wt Hmi1p (Monroe et al., 2005).

The biochemical properties of Hmi1p are discussed in the Section 3 (Results and Discussion). Deletion of the HMI1 gene has a more severe effect on mtDNA metabolism than the deletion of PIF1, since Hmi1p deficient mutants do not retain functional mtDNA. After disrupting the HMI1 ORF, most of the cells lose mtDNA while about 20% of the cells retain rho$^-$ mtDNA. Both neutral and HS rho$^-$ mitochondrial genomes can be maintained in Hmi1p deficient cells (Sedman et al., 2000; Monroe et al., 2005). We have shown that Hmi1p has a significant influence on the rho$^-$ mtDNA metabolism as well. The HS rho$^-$ concatemeric mtDNA molecules are much longer in the presence of

Hmi1p compared to the mtDNA molecules from mitochondria without Hmi1p. This shortening of the HS rho$^-$ mtDNA molecules is linked to transcription since the absence of Hmi1p has much less effect in the mitochondrial RNA polymerase Rpo41p deficient mutants. Moreover, we have shown that this specific effect is independent of the enzymatic activity of Hmi1p. Such longer mtDNA molecules are detected also in the mitochondria of the cells expressing only enzymatically inactive mutants of the helicase. The main difference between the cells containing wt Hmi1p and mutant Hmi1p was seen in the amount of ssDNA. Namely an ssDNA arc was missing on the 2-dimensional (2D) agarose gel electrophoresis pictures with mtDNA from the mutant strains (Sedman et al., 2005). This specific arc has earlier been described to contain only ssDNA originating from the transcript strand in respect to the promoter in front of the C box of the ori/rep sequence (MacAlpine et al., 2001). The helicase activity of Hmi1p has also a function in wt mtDNA metabolism, although the effect of mutations in single amino acid residues is not as strong as the effect of disrupting the HMI1 gene. MtDNA in the cells containing only the enzymatically inactive mutant protein is less stable (Sedman et al., 2005; Monroe et al., 2005). In addition, the role of Hmi1p in the metabolism of functional wt mtDNA has been studied using a temperature-sensitive (ts) mutant of Hmi1p. We demonstrated that the cells containing the ts mutant of Hmi1p lost mtDNA through fragmentation during growth under restrictive temperatures (Sedman et al., 2005). This is similar to the effect of Hmi1p on HS rho$^-$ mtDNA metabolism. These results lead to the conclusion, that Hmi1p is necessary for efficient and processive DNA synthesis in yeast mitochondria. It is further most probable, that Hmi1p has two different functions in the maintenance of mtDNA, one of which requires the enzymatic activity of the protein while the other does not.

Although Hmi1p has been described to have different effects on mtDNA metabolism, the exact role of the helicase has remained ambiguous. Most likely Hmi1p is involved in one or two modes of replication used in the mitochondria of *S. cerevisiae*. However, it cannot be the sole replicative helicase as in Hmi1p deficient strains rho$^-$ mtDNA is still maintained. We have ruled out the participation of Hmi1p in transcription from the promoter in front of ori/rep sequences (Sedman et al., 2000). To further elucidate the role of Hmi1p in mtDNA maintenance we purified the recombinant Hmi1p as well as its enzymatically inactive mutant and described ATPase, helicase and DNA binding activities of these proteins. These results and their biological significance are discussed below in Section 3.

2.5. Homologues of Hmi1p, biochemical properties and biological functions

By primary structure analysis the closest homologues of Hmi1p are the bacterial PcrA, UvrD and Rep helicases as well as Srs2p from yeast. Therefore Hmi1p can be placed in the subfamily of UvrD-like helicases in SF1 (Gorbalenya and Koonin, 1993). As the sequence homology is often accompanied with similarities in functions, I discuss here the biochemical properties as well as biological functions of the closest well studied homologues of Hmi1p — UvrD and Rep from Gram-negative bacteria, PcrA from Gram-positive bacteria and Srs2p from *S. cerevisiae*.

2.5.1. Prokaryotic members of the UvrD subfamily

Helicases of the UvrD subfamily are all either monomeric or dimeric DNA helicases, but there are some differences in their biochemical properties due to adaptations to their different biological functions. Most of the helicases from this subfamily are 3'-5' directional. As an exception, PcrA possesses both 3'-5' and 5'-3' directional activities. The 3'-5' directional unwinding is much more efficient on simple substrates, while on substrates with hairpins and flap structures the 5'-3' directional unwinding appeared to be more efficient (Chang et al., 2002; Anand and Khan, 2004). These results were obtained with PcrA from *Staphylococcus aureus*, but supposedly homologues from different Gram-positive bacteria share at least partly the same properties, as has been already shown for *Bacillus stearotermophilus, Bacillus cereus* and *Bacillus anthracis* homologues (Bird et al., 1998; Naqvi et al., 2003; Anand et al., 2004). As another exception UvrD has been shown to unwind DNA/RNA heteroduplexes *in vitro*, the biological significance of this function remainig open though (Matson, 1989). The requirements for efficient and productive substrate binding are rather different for different proteins. UvrD can start unwinding from a nick in one strand or from a double-stranded break. A nicked duplex is also a natural substrate of UvrD in the repair processes (Langle-Rouault et al., 1987; Van Houten et al., 1988; Runyon and Lohman, 1989; Runyon et al., 1990). PcrA is shown to bind preferentially substrates with hairpin structures and a flap in one end, these being the preferential structutres for starting the unwinding reaction as well (Anand and Khan, 2004). With the Rep helicase only a few of such experiments have been performed with results indicating that 3' flap structures are more efficiently unwound than forked structures (Heller and Marians, 2005). The processivities of Rep and PcrA are rather low, the unwinding efficiencies dropping significantly at DNA duplexes over 50–70 bp in length. However, the replication initiator proteins encoded by the bacteriophage φX174 or plasmid pC221 DNA respectively have been shown to enhance the

processivity of these helicases strongly (Yarranton and Gefter, 1979; Arai and Kornberg, 1981; Soultanas et al., 1999a). The only processive helicase of this subfamily is UvrD, which can unwind up to 3 kb at high protein/DNA ratios (Runyon and Lohman, 1989). Still, the unwinding activity of UvrD can be increased by adding SSB to the reaction to trap the unwound strands of the substrate (Matson and George, 1987). In addition, the stimulatory effect on the unwinding activities of these helicases has been demonstrated for two *E. coli* ribosomal proteins. L14 stimulates activities of Rep and UvrD, while L3 stimulates that of PcrA (Smith et al., 1989; Yancey and Matson, 1991; Soultanas et al., 1998). However, the biological significance of this phenomenon has remained unclear.

Rep and UvrD from Gram-negative bacteria are not essential, but double mutants lacking both proteins are inviable (Washburn and Kushner, 1991). For years it was believed that the two helicases together carry out some function, essential for the cell. Recently this interpretation was challenged by X. Veaute et. al, who proposed, that in Rep helicase deficient mutants intermediates are formed, which are toxic for the cell if not resolved by the UvrD helicase (Veaute et al., 2005). PcrA, the only member of this subfamily of helicases from Gram-positive bacteria, is essential (Iordanescu, 1993a). The *E. coli* double mutant without UvrD and Rep helicases is viable in the presence of *B. subtilis* PcrA, this suggesting that PcrA can provide the function of either UvrD or Rep or both in *E. coli* (Petit et al., 1998).

UvrD is the most abundant DNA helicase in *E. coli*, the copy number being approximately 5000 molecules per cell (Klinkert et al., 1980). It is DNA damage inducible with the copy number increasing more than three-fold after induction of the SOS system (Siegel, 1983). The deletion mutant of UvrD shows several different phenotypes including sensitivity to UV and ionizing radiation, inability to carry out methyl-directed mismatch repair, higher homologous recombination frequencies and improper transposition (Ogawa et al., 1968; Smirnov and Skavronskaya, 1971; Horii and Clark, 1973; Siegel, 1973; Zieg et al., 1978; Arthur and Lloyd, 1980; Lorenzo et al., 1990). Based on different genetic interaction as well as biochemical studies it has been verified that UvrD participates in methyl-directed mismatch repair and nucleotide excision repair systems and also has a role in recombination processes (reviewed in Modrich, 1989; Mendonca et al., 1995; Sancar, 1996). In the repair systems UvrD acts at the late stage unpairing the damaged or mutated strand from its template before the synthesis of a new DNA strand starts (Orren et al., 1992). In recombination UvrD was shown to dismantle the RecA nucleoprotein filament working thus as an antirecombinase (Petranovic et al., 2001; Flores et al., 2005; Veaute et al., 2005).

Rep is not highly abundant in *E. coli*, the copy number being estimated around 50 molecules per cell (Scott and Kornberg, 1978). The role of the Rep helicase in DNA metabolism is under discussion still. According to the most prevailing hypothesis, Rep raises the efficiency of replication fork movement

since in deletion mutants of Rep the replication fork movement is approximately twice slower than in the wt cells (Lane and Denhardt, 1975). However, in Rep deficient mutants the overall rate of DNA synthesis is unaffected because of compensatory overinitiation (Lane and Denhardt, 1974). In addition, in the Rep deficient strains the chromosome is susceptible to breakage as a consequence of replication pausing (Michel et al., 1997). One possibility is that Rep is involved in removing proteins from the DNA ahead of the replication fork. It has been shown, that Rep can displace bound proteins from DNA (Yancey-Wrona and Matson, 1992). Another hypothesis was proposed based on single molecule experiments. Individual Rep monomers were observed to move on ssDNA in the 3' to 5' direction ATP hydrolysis dependently, until they reached a blockade, from where the protein abruptly snapped back close to its initial position, followed by further cycles of translocation and snapback. Such repetitive shuttling was observed on ssDNA bound by an oligonucleotide on the 5' end, streptavidin, a stalled replication fork and an Okazaki fragment analogue. By the same mechanism the presence of Rep delayed formation of a filament of the recombination protein RecA on ssDNA. Thus Rep was proposed to keep DNA ahead of the replication fork clear of proteins and toxic recombination intermediates via such shuttling (Myong et al., 2005). In the absence of Rep such intermediates may form with much higher frequency and be the cause of inviability in the cells lacking UvrD. In addition, Rep has been shown to be involved in one more pathway of chromosomal DNA metabolism, namely an alternative PriC dependent pathway of direct, non-recombinogenic replication restart. In case of gaps in the leading strand the lagging strand replication may proceed alone for a while. Rep participates in solving such kind of stalled replication forks by unwinding the nascent lagging strand to enable PriC to load DnaB, the replicative helicase, this being the key step in replisome assembly (Heller and Marians, 2005).

The role of PcrA in chromosomal DNA metabolism has not been studied as thoroughly as those of Rep and UvrD. Complementation experiments with UvrD deficient *E. coli* mutants have demonstrated that PcrA can suppress the deficiency of UvrD in UV repair but not in mismatch repair (Petit et al., 1998). The discovery, that pcrA deletants are viable in the presence of mutations in the recF, recO or recR genes makes it likely that PcrA is, similarly to UvrD, involved in resolution of toxic recombination structures or stalled replication forks that may impede the replication fork progression (Petit and Ehrlich, 2002). RecFOR proteins remove SSB from ssDNA and load RecA, the recombinase (Fernandez et al., 2000). In addition, the chromosomal DNA synthesis was slightly affected in strains lacking PcrA (Petit et al., 1998). However, the question, which is the essential proecess of DNA metabolism PcrA is involved in, remains at the moment unanswered.

A function additional to the one in the chromosomal DNA metabolism, which is shared by all bacterial helicases from the UvrD subfamily, lies in replication of rolling circle plasmids and bacteriophages. Through this function

Rep and PcrA were defined initially, notably Rep as a protein required for the replication of phage φX174 and PcrA as an essential factor for replicating rolling circle plasmids (Denhardt et al., 1967; Iordanescu and Bargonetti, 1989; Iordanescu and Basheer, 1991; Iordanescu, 1993a). For UvrD the participation in rolling circle replication was only recently reported (Bruand and Ehrlich, 2000). The most intriguing fact in this context is that UvrD participates in the replication of rolling circle plasmids in Gram-negative bacteria, while Rep in that of bacteriophages in the same organisms. Moreover, it has been demonstrated, that Rep cannot complement UvrD in replication of rolling circle plasmids and UvrD cannot complement Rep in bacteriophage replication (Denhardt et al., 1967; Bruand and Ehrlich, 2000). In Gram-positive bacteria PcrA has been demonstrated to be involved in plasmid replication, while no information about participation of the helicase in phage replication is available.

Regarding the mechanism of replication, there is much in common between phages and plasmids, although there are also some significant differences (reviewed in Novick, 1998; Khan, 2005). One difference between the plasmids and phages is in the leading-strand replication origins. The plasmid leading-strand replication origins are usually G/C rich and have the ability to form cruciform structures (Gruss and Ehrlich, 1989; Novick, 1989). In contrast, those of phages are usually A/T rich sequences surrounded by G/C rich stretches (Baas and Jansz, 1972; Fiddes et al., 1978). The initiation of the rolling circle replication of plasmids and phages is rather similar. The plasmid or phage encoded specific initiator proteins that belong to the strand-transferase superfamily bind to the leading-strand replication origin and introduce a sequence-specific nick at a nearby site (Eisenberg et al., 1977; Koepsel et al., 1985; Koepsel et al., 1986; Koonin and Ilyina, 1993). Simultaneously with the nicking event or even previous to it, the host helicase is recruited to the replication origin. This may happen either by protein-protein interactions between the replication initiator protein and the helicase or protein-DNA interactions between the helicase and the replication origin. It is also possible that both types of interactions are involved (Eisenberg et al., 1977; Arai et al., 1981a; Iordanescu, 1993b; Chang et al., 2002; Anand et al., 2005). Such specific interactions are most probably the reason, why Rep and UvrD cannot complement each other in plasmid and phage replications. Next, the replisome is assembled and the synthesis of leading strand occurs, facilitated by the helicase mediated unwinding. The helicase is likely in physical interaction with the replication initiator protein, which stimulates the unwinding activity of the helicase (Yarranton and Gefter, 1979; Soultanas et al., 1999a). The lagging strand replication occurs subsequent to and independently of the leading strand replication. The lagging strand is initiated by an RNA primer that is synthesized independently of the initiator protein (Arai et al., 1981a; Arai et al., 1981b; Birch and Khan, 1992). The main difference between the plasmid and phage replication lies in the initiation and termination regulation as the plasmid replication is rather controlled by the host cell cycle while the phage is set up to

produce as many progeny as possible during the lytic cycle (reviewed in Novick, 1998). It has been proposed, that the structure of plasmid replication origins has an important function in the regulation of reinitiation (Jin et al., 1997). The role of helicases, though, in making this difference has not been described.

2.5.2. Eukaryotic members of the UvrD subfamily

In budding yeast *S. cerevisiae* there are two proteins described from the UvrD subfamily of helicases — Srs2p and Hmi1p. From the evolutionary point of view they are probably formed by a gene duplication event as the presence of Hmi1p is characteristic only for the family of *Saccharomycetaceae* (Richard et al., 2005). In other yeast families as well as in *Arabidopsis thaliana*, the model organism of plants, only Srs2p is described. In the model organisms of multicellular animals — *C. elegans, D. melanogaster, M. musculus, H. sapiens* — none of the proteins belonging to this subfamily have been described to date.

Srs2p is a 3'-5' directional DNA helicase that is shown to start unwinding from 3' ssDNA, although under optimized conditions also blunt-ended substrates can be unwound. Srs2p alone is rather distributive, unwinding efficiently dsDNA up to 40 bp in length. In the presence of RPA, the yeast nuclear SSB, the processivity of Srs2p is much higher, reaching up to 500 bp (Rong and Klein, 1993; Van Komen et al., 2003). Recently it has been shown that Srs2p is able to specifically unwind hairpins formed of triplet repeats. The authors propose that this property defines the function of Srs2p in the DNA metabolism, in stopping the expansions of specific DNA triplet repeats. This property is significant in terms of evolution as well, since the bacterial homologues PcrA and possibly also UvrD recognize hairpins at the plasmid replication initiation sites. It has been demonstrated though, that UvrD is much less efficient than Srs2p in unwinding the structures formed of trinucleotide repeats (Bhattacharyya and Lahue, 2004; Bhattacharyya and Lahue, 2005).

The biological function of Srs2p is pleiotropic and to some extent still under discussion. Srs2p deficient cells are viable, but they are sensitive to UV irradiation, although only in the G1 phase (Aboussekhra et al., 1989). They also show hyper-recombination phenotype that occurs spontaneously or after radiation exposure. This indicates the involvement of Srs2p in repair and recombination (Aguilera and Klein, 1988; Rong et al., 1991). Srs2p was recently shown to negatively control the recombination by dismantling Rad51p filaments formed on ssDNA, which are required for initiation of the recombination process (Krejci et al., 2003; Veaute et al., 2003). Rad51p is the yeast homologue of RecA. This function of Srs2p is similar to the proposed functions of PcrA as well as UvrD in resolving toxic recombination intermediates in different bacteria. The role of Srs2p in repair and recombination pathways has

been studied using a variety of genetic interaction experiments with double- and triple-knockouts of different repair, recombination and replication proteins. Together they show that Srs2p acts as a molecular switch between the error-free post-replication repair and homologous recombination (reviewed in Barbour and Xiao, 2003). Srs2p channels the repair of damage-induced DNA lesions into the Polη or Polζ dependent translesion synthesis or recombination dependent damage avoidance pathway and prevents the incorrect homologous recombination pathway (Schiestl et al., 1990; Ulrich, 2001). Srs2p has been shown to act at two different checkpoints during the cell cycle — the G2/M and the intra-S-phase DNA damage checkpoints. At the G2/M checkpoint Srs2p is required to recover from the checkpoint mediated arrest (Vaze et al., 2002). This is likely done by resolving recombination intermediates or removing some other complexes from DNA that activate the checkpoint-mediated cell cycle arrest. In the intra-S-phase DNA damage checkpoint Srs2p has been shown to be a component in the functional checkpoint pathway. Here Srs2p is phosphorylated in a DNA damage checkpoint dependent manner and the phosphorylated Srs2p is required for activating other members of the checkpoint pathway (Liberi et al., 2000). The activated Srs2p participates in regulating replication over DNA lesions as described above. Also Srs2p prevents unwanted recombination during replication in the S phase even in the absence of exogenous DNA damage (Pfander et al., 2005). Taking together, it seems that one of the main functions of Srs2p is unwinding of Rad51p filaments or recombination intermediates from ssDNA, preventing thus recombination. This ability of the protein is applied to many different steps of replication and repair by activating and recruiting it to the site needed by different interacting proteins.

One more known function of Srs2p is to regulate the length of conversion tracts during a specific type of replication/recombination, being a selective inhibitor of the expansion of trinucleotide repeats. Trinucleotide repeats fold into hairpins that are the intermediates in the mutation process. Srs2p is proposed to unwind these hairpins before they become fixed as expansions (Bhattacharyya and Lahue, 2004; Bhattacharyya and Lahue, 2005). The unwound hairpins either reanneal with the template strand or are cleaved by nucleases and thereby the excess DNA is removed. However, the exact pathway, how this helicase is recruited to trinucleotide repeat sites, remains open.

Hmi1p, the other protein of the UvrD subfamily in *S. cerevisiae*, is a mitochondrial DNA helicase that is required for the maintenance of functional mtDNA as described above. The biochemical characterization of this particular helicase is the focus of the experimental part of my thesis.

3. RESULTS AND DISCUSSION

3.1. Objectives

The role of the helicase Hmi1p in the metabolism of mtDNA in *S. cerevisiae* has been studied in our lab for several years already. We have characterized the protein using different *in vitro* methods to give this way insight into the processes Hmi1p might be involved in. The major precondition for such an approach is that most of the components of the specific system should be purified to make the process reproducible *in vitro*. Nevertheless, the properties of the helicase itself may also point to one or other process in mtDNA metabolism it might be involved in, when compared to the properties of other better described helicases.

3.2. Purification of the recombinant Hmi1p (I, II, III)

To purify Hmi1p in milligram amounts, Hmi1p was overexpressed in *E. coli* as a fusion protein with glutathione-S-transferase (GST). After fractionating the cell lysate on glutathione-agarose column the GST-tag was removed by thrombin cleavage and Hmi1p was further purified through subsequent steps of ion exchange cromatography on Q and S Sepharose columns (Fig. 3A in I). The purified protein was kept at –75^{o}C, where the protein has been stable for at least 3 years. The yield was approximately 0,4–0,5 mg from 1 liter of bacterial culture. The protein concentration was measured with Bradford method and thus the result reflects the total protein concentration in the preparation (Bradford, 1976). However, the protein was estimated to be more than 90% pure as judged by the SDS-polyacrylamide gel electrophoresis (Fig. 5). Moreover, the glycerol gradient analysis of the protein showed no aggregation and all the ATPase activity copurified with the protein peak (Fig. 1C in III). Thus the approximate biochemical properties of Hmi1p can be calculated from the results of different assays using the measured protein concentrations.

Recently also Dr. Matson's group purified recombinant Hmi1p using C-terminal cleavable intein-chitin-binding domain with eight-histidine affinity tag. They estimated the purity of the protein to be more than 95%, but the yield was not indicated (Monroe et al., 2005).

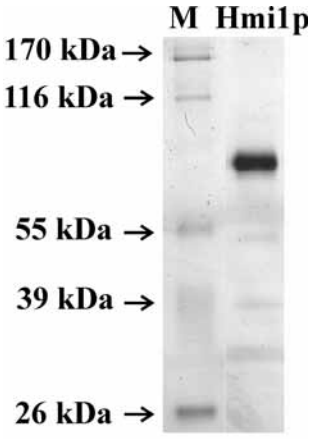

Figure 5. Purified recombinant Hmi1p. 0,2 μg of the protein preparation was separated in SDS-polyacrylamide gel and silver stained. M – molecular mass protein standards.

3.3. Preparation of enzymatically inactive mutants of Hmi1p (III)

For the preparation of negative controls for all experiments described below, we introduced three point mutations separately into the HMI1 gene to make them encode for three different enzymatically inactive proteins. Such negative controls are essential components of different enzymatic assays to confirm that detected enzymatic activities are not due to some contaminants in the protein preparation. The mutations were introduced to the highly conserved amino acids. Lysine 32 in helicase motif I was changed to methionine (K32M), glutamate 211 in helicase motif II was changed to glutamine (E211Q) and arginine 275 in motif IV was changed to methionine (R275M) (Fig. 6). Similar mutations have been used earlier with other helicases to render them enzymatically inactive (Pause and Sonenberg, 1992; George et al., 1994; Brosh and Matson, 1995; Gross and Shuman, 1995; Hall and Matson, 1997; Soultanas et al., 1999b). The crystal structure analysis of PcrA, the closest homologue of Hmi1p in bacteria, suggests that E211 is the catalytic residue (Fig. 2A, red) (Subramanya et al., 1996). Studies of different helicases with mutations in the conserved lysine in motif I indicate that the role of this amino acid residue may vary between different enzymes being either in binding or hydrolysing ATP (Sung et al., 1988; Rozen et al., 1989; George et al., 1994; Jindal et al., 1994). The crystal structure analysis of PcrA indicates that this conserved lysine is in contact with the β phosphate of ATP (Fig. 2A, black). The R275 analogue from PcrA makes contact with the γ phosphate of ATP in the crystal structure of the substrate complex (Fig. 2A green), but as the ATP binding properties of the enzyme with such mutation were similar to those of the wt enzyme, it was proposed that R275 participates most likely in polarising the γ phosphate group,

increasing thus the susceptibility of the phosphorous atom to the nucleophilic attack (Soultanas et al., 1999b).

Out of these three different mutants only one — Hmi1p(E211Q) — could be purified by the same method as the wt Hmi1p (Fig. 1A in III). The GST fusion proteins of the two other mutants were both in the insoluble fraction after cell lysis. This can be explained by differences in the biochemical properties of the mutated proteins. E211, being the catalytic residue, is required for attacking the water molecule in the active site, but makes no direct contact with the bound nucleotide. The residues K32 and R275, on the other hand, make contacts with β and γ phosphates of the ATP molecule. These contacts may be necessary for the GST-Hmi1 fusion protein to be able to take a stable conformation in *E. coli*. Another reason for the insolubility of the mutants may lie in the choice of amino acids used for the replacement of the original residues. It may well be, that some other non-functional amino acid residue at these positions would not have rendered the protein insoluble. However, we did not try different replacements, leaving thus this question open. We also did not try any other purification scheme with the insoluble mutants, since in order to prevent a rise of other contaminating proteins the purification protocol needed to be unchanged.

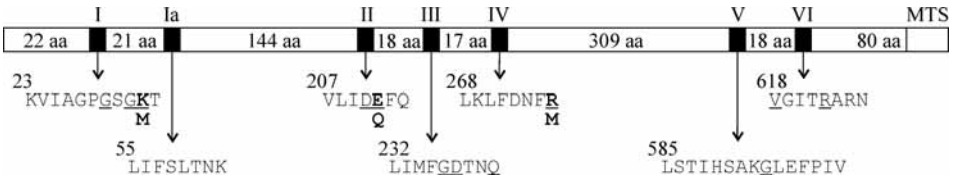

Figure 6. Schematic representation of HMI1 ORF. The black boxes indicate the conserved helicase motifs, the distance between the motifs is in amino acid residues — aa. MTS indicates the mitochondrial targeting sequence. Below are the sequences of the conserved motifs with the positions of first amino acid residues indicated and residues conserved in more than 75% of helicase superfamily 1 proteins underlined (Gorbalenya and Koonin, 1993). Bold are the residues that were mutated during our studies, the replacing residues are below the sequence.

3.4. ATPase activity of Hmi1p (II, III)

To measure the kinetic parameters of Hmi1p for the hydrolysis of ATP, a spectrophotometric ATPase assay was performed. In this assay the ATP hydrolysis is linked to the oxidation of NADH, which can be monitored continuously (Runyon et al., 1993). The reactions were performed at four different ATP concentrations in the presence or absence of an oligonucleotide ssDNA cofactor at saturating concentrations ($10\times K_M$). The concentration of ssDNA cofactor

required for half-maximal stimulation of ATP hydrolysis was shown by others to be 0,6–2,2 μM in nucleotides depending on the cofactor. We did not test different ssDNA cofactors, but others have shown that linear and circular ssDNA are both good ATPase activators, while linear and circular dsDNA and RNA did not support ATPase activity of Hmi1p (Monroe et al., 2005). The recombinant wt Hmi1p preparation exhibited ssDNA dependent ATPase activity with kinetic parameters for ATP hydrolysis being: K_M = 0,185±0,037 mM and k_{cat} = 13±1 s^{-1}. The ATPase activity without ssDNA was below the limits of detection at all ATP concentrations used. The recombinant mutant Hmi1p(E211Q) preparation did not show detectable ATPase activity neither in the presence nor absence of ssDNA. Thus the measured ATPase activity is that of Hmi1p and not of any of the contaminating proteins. The same parameters for Hmi1p were estimated by others to be K_M = 0,15 mM and k_{cat} = 1,2 s^{-1} (Monroe et al., 2005). The reason for such a big difference in k_{cat} remains unclear. The kinetic parameters for ATP hydrolysis have been determined also for most of the well-studied members of the UvrD subfamily of helicases (Table 2). As can be seen, K_M is quite similar for all the helicases, while k_{cat} is more than ten fold higher for Rep and UvrD than for Srs2p, PcrA and Hmi1p. The similarity in K_M indicates that the optimal ATP concentration is similar to all these proteins. This is not surprising, as the ATP concentration in different organisms is rather similar. The difference in catalytic activities may reflect the fact that PcrA, Srs2p and Hmi1p need some cofactors, in the presence of which these helicases acquire the activity similar to the activity of Rep and UvrD. The other possibility is that these results represent the true situation in cell and for some reason these three helicases do not need to be more active to carry out their functions.

Table 2. Kinetic parameters for ATP hydrolysis in UvrD subfamily

Protein (organism)	K_M (mM)	k_{cat} (s^{-1})	Reference
PcrA (*B. stearotermophilus*)	0,225	25	(Soultanas et al., 1999b)
Rep (*E. coli*)	0,065	425	(Lohman et al., 1989)
UvrD (*E.coli*)	0,11	416	(Matson and George, 1987)
Srs2p (*S. cerevisiae*)	not determined	50	(Van Komen et al., 2003)
Hmi1p (*S. cerevisiae*)	0,185	13	Our data, III
Hmi1p (*S. cerevisiae*)	0,150	1,2	(Monroe et al., 2005)

3.5. Helicase activities of Hmi1p (I, II, III)

In helicase assays dsDNA unwinding activity of Hmi1p was followed on a variety of DNA substrates under varying experimental conditions. In these assays substrates prepared of synthetic oligonucleotides were used. One of the oligonucleotides was radioactively labelled to make it possible to detect the results of unwinding reactions using gel electrophoresis followed by auto-radiography.

Preliminary helicase assays showed that Hmi1p could unwind a DNA duplex of 28 nt in length in the presence of ATP and Mg^{2+} ion (Fig. 3B in I). We have not tested RNA substrates, but it has been demonstrated, that RNA does not support the ATPase activity of Hmi1p, suggesting thus that Hmi1p does not interact productively with RNA (Monroe et al., 2005). The helicase activity was detected only in recombinant wt Hmi1p preparations while no unwinding activity could be detected in preparation of the recombinant mutant Hmi1p(E211Q) (Fig. 1B in III). Thus, like with ATPase activity, detected helicase activities were caused by the Hmi1 helicase and not by any contaminating proteins.

3.5.1. Nucleotide cofactor usage

As helicases use energy derived from nucleotide hydrolysis to unwind duplex DNA, helicase assays can be used to assess nucleotides that can be hydrolysed by a helicase. To determine the cofactor usage of Hmi1p, recombinant Hmi1p preparation (for simplicity referred to as Hmi1p below) and a substrate containing 23 bp dsDNA with a 22 nt 3' ssDNA overhang were incubated in the presence of different NTP-s and dNTP-s (Fig. 2B in III). The concentration of NTP-s in the reaction was 1 mM, which is more than five times above K_M measured for ATP. The highest enzymatic activity of Hmi1p was detected in the presence of ATP and dATP, these nucleotides being thus the preferred cofactors. Incubation in the presence of dCTP and CTP resulted in less than 20% unwinding activity compared to ATP. None of the other nucleoside triphosphates could support the helicase activity of Hmi1p above the limits of detection. In comparison, PcrA is able to hydrolyze all different NTP-s and dNTP-s, while UvrD is able to hydrolyze more or less effectively only ATP, dATP, CTP, GTP and dGTP (Matson and George, 1987; Bird et al., 1998). The difference in cofactor usage can be explained by differences in the nucleotide-binding pocket of different enzymes (Fig. 2A). If the protein has no or only limited contact with the base and sugar parts of the bound nucleotide all NTP-s and dNTP-s can be hydrolysed. On the other hand, if several such contacts exist, only one specific nucleotide can be bound.

3.5.2. Processivity

The processivity of Hmi1p was estimated with a heterogeneous substrate, where the length of the double-stranded part of the DNA ranged between 20 and 200 nt. The unwinding of the substrate by Hmi1p was assessed in the course of 30 min (Fig. 3 in III). The release of 30, 50, 80 and 120 nt oligonucleotides in length was quantified by phosphorimaging analysis. During 30 min of incubation solely 30-mers were completely unwound, while only about 60% of the 50-mers were displaced in the same time. The unwinding of 80-mers and 120-mers was far less effective, being 10–20% after 30 min of reaction. Similar results were also obtained by others (Monroe et al., 2005). It has been demonstrated with different members of the UvrD subfamily, the eukaryotic — Srs2p — as well as the bacterial proteins — Rep and PcrA — that they are distributive helicases by themselves. However, cofactors like ssDNA binding proteins and/or bacteriophage or plasmid encoded replication initiator proteins have been found to strongly enhance the processivity of these helicases (Yarranton and Gefter, 1979; Soultanas et al., 1999a; Van Komen et al., 2003). Thus it remains to be studied, whether some mitochondrial proteins can influence the processivity of Hmi1p as well. My preliminary experiments indicate though, that at least Rim1p, the yeast mitochondrial SSB does not raise the processivity of Hmi1p significantly. It might nonetheless well be, that Hmi1p participates in a process during mtDNA metabolism not requiring any processive action at all.

3.5.3. Directionality

The directionality of the helicase activity of Hmi1p was determined in two ways. First, it was tested using two different substrates in two separate experiments. As can be seen on Fig. 3 in Ref. II, Hmi1p could unwind efficiently only substrates with 3’ ssDNA overhangs. Unwinding substrates with 5’ ssDNA overhangs was of very low efficiency and could be detected at a high protein/DNA ratio. Second, the directionality was assessed in a competition assay with a substrate consisting of linearized pUC119 ssDNA with oligonucleotides hybridized to both ends of the molecule. Hmi1p could unwind only oligonucleotides from the 5’ end of the phagemid ssDNA (Fig. 2A in III). No oligonucleotide displaced from the 3’ end of the linearized pUC119 ssDNA could be detected. These results confirm that Hmi1p is a 3’-5’ directional DNA helicase. Similar results have been reported also by others (Monroe et al., 2005). In addition, the second assay indicates that Hmi1p can start its unwinding reaction internally from the ssDNA stretch, no 3’ ssDNA end being required. Regarding directionality, Hmi1p acts similar to its homologues from the UvrD subfamily with the exception of PcrA, which has also shown 5’-3’ directional unwinding activity to some extent (Chang et al., 2002; Anand and Khan, 2004).

3.5.4. Requirements for the higher order structure of the substrate

The substrate structure requirements for the helicase activity of Hmi1p were characterized using oligonucleotide substrates with different structures. Of all the different substrates used, Hmi1p could unwind substrates that contained long enough 3' ssDNA ends or stretches of ssDNA (Fig. 4 in III). Substrates that contained only 5' ssDNA ends, a nick or a 5 nt gap in one strand were not unwound. Neither could Hmi1p unwind Holliday junction structures (my unpublished results). The substrates with different structures had different requirements for the length of the 3' ssDNA to be efficiently unwound by Hmi1p. The obtained results can be summarized as follows: i) on simple duplex DNAs with 3' ssDNA overhangs a ssDNA overhang of at least 19 nt in length was needed for the productive targeting of Hmi1p (Fig. 5 in III); ii) on forked substrates the maximum unwinding activity was observed already with two 9 nt overhangs (Fig. 6 in III); iii) on 3' flap structures an even shorter 3' ssDNA tail of 2–5 nt was sufficient for the helicase activity of Hmi1p (Fig. 7 in III). The activity of Hmi1p was also tested on displacement loop (D loop) substrates, which were found to be unwound by Hmi1p very efficiently (Fig. 4 in III). All of these structures, however, contain longer stretches of ssDNA, from where the unwinding could start. Thus it cannot be decided from this experiment, whether D loops are the native substrates of Hmi1p or not.

When comparing these properties of Hmi1p with properties of other helicases, many similarities can be found. Regarding the results with simple dsDNA with 3' ssDNA overhangs, Hmi1p behaves similarly to PriA, a bacterial DNA helicase, that requires at least a 16 nt 3' overhang for stable binding (Nurse et al., 1999). Although PriA is not a homologue of Hmi1p by sequence, it has some functional redundancy with the Rep helicase, supposedly in loading the replicative helicase in two alternative replication restart pathways (Kogoma et al., 1996; Seigneur et al., 1998; Liu and Marians, 1999; Sandler, 2000; Heller and Marians, 2005). Thus it is possible that Hmi1p has some similar function in yeast mitochondria.

The less stringent requirements for a 3' overhang on the forked substrates indicate that Hmi1p is a structure specific helicase. The results on forked substrates with one short (2 nt) and one long (at least 9 nt) overhang differed depending on which strand was longer and which was shorter. Such substrates were unwound with about half of the maximum efficiency, but unexpectedly, the substrate with longer 5' overhang was slightly more efficiently unwound than the substrate with longer 3' overhang (Fig.-s 6A and 6B in III). This might possibly be due to the sequence difference that may influence the fork structure recognized by Hmi1p in this case.

Finally, the results with flap substrates, that a 5 nt 3' ssDNA tail can support effective unwinding, confirm the hypothesis that Hmi1p recognizes a higher order structure of DNA. The 2–5 nt 3' tails, that were found to be enough for Hmi1p to start unwinding dsDNA, cannot promote stable binding by

themselves. Thus it can be concluded, that Hmi1p is probably targeted to 3' flap structures. In this property Hmi1p is somewhat similar to the Rep helicase, since it was recently demonstrated that Rep unwinds 3' flap structures more efficiently than forked structures (Heller and Marians, 2005). A further homologue, PcrA has also been demonstrated to be a structure specific helicase, but does not unwind forked substrates at all. It works preferentially on substrates that have a hairpin structure with a short ssDNA overhang at one end (Anand and Khan, 2004).

3.6. Specific binding of Hmi1p to yeast mitochondrial DNA replication origins (IV)

An insight into the native location of Hmi1p activity in the yeast mitochondria was tried to give with electrophoretic mobility shift assay (EMSA), which is used for studying DNA binding properties of proteins. In this assay complexes consisting of proteins and radioactively labelled DNA are formed and separated in non-denaturing polyacrylamide gels. The mobility of the complexes differs from that of the free components.

The EMSA-s were performed using different oligonucleotides with sequences from yeast mitochondria. One nonmitochondrial sequence served as control. Hmi1p could bind substrates with different hairpin structures, but most efficiently those, containing the hairpin formed of A and B boxes of one strand of the mitochondrial ori/rep sequence (Fig. 2A and Table 1 in IV). Specific binding was seen with the template strand in regard to the promoter in front of the C box. Transcript strand binding was even less effective than binding of one non-related hairpin structure. To confirm, that Hmi1p is the protein that forms the complex with hairpin structures and not any of the contaminating proteins in the Hmi1p preparation, GST-Hmi1 fusion protein was used in addition. As expected, the complex with the fusion protein migrated slower in the gel than the one with Hmi1p. Thus Hmi1p is the protein that forms the specific complex. This binding activity of Hmi1p was dependent on ATP binding, but not hydrolysis, as non-hydrolyzable ATP analogues ADPγS and ADPNP could support complex formation (Fig. 2B in IV). The mutant Hmi1p(E211Q), which can bind ATP but not hydrolyze it, was able to form the complex as well.

To further characterize the specificity of Hmi1p binding, the Hmi1p-ori template strand complex was subjected to hydroxyl radical treatment (Fig. 2C in IV). As a result, a clear difference in the hydroxyl radical cleavage pattern was seen in comparison with the cleavage pattern of the template strand oligonucleotide control. The main difference could be observed in the stem part of the hairpin as indicated on Supplementary Fig. 1 in Ref. IV, while for the single-stranded parts the cleavage pattern was unchanged. A similar experiment was performed with the Hmi1p-ori transcript strand complex. There the

difference in the cleavage pattern when compared to the oligonucleotide control was much less clear. That was an expected result, since the complex formation with this oligonucleotide was much weaker. The cleavage pattern was changed again in the stem part of the hairpin of the oligonucleotide, confirming thus, that Hmi1p binds specifically to hairpin structures. The most surprising difference, when comparing the hydroxyl radical footprint experiments, was seen between the cleavage patterns of oligonucleotides of the complementary strands of the ori/rep sequence without the bound Hmi1p. While the transcript strand oligonucleotide was cut rather uniformly along the whole oligonucleotide with some weaker and stronger cutting sites at the hairpin structure, the template strand oligonucleotide had a distinct cleavage pattern with some hypersensitive and some absolutely hidden nucleotides, especially in the hairpin part. Thus we suggest that the hairpin of the template strand oligonucleotide takes a specific conformation, which the hairpin of the transcript strand oligonucleotide takes at only a very low efficiency. This particular conformation is probably recognized specifically by Hmi1p. The mitochondrial heat shock protein Hsp60p has been demonstrated to bind the template strand of the ori/rep sequence as well (Kaufman et al., 2000). However, Hsp60p binding is of much lower specificity than Hmi1p binding, since Hsp60p can be outcompeted of the specific complex with non-specific plasmid dsDNA, while Hmi1p cannot (my unpublished results). Taken together it can be concluded, that this particular conformation taken by the hairpin of the template strand ori/rep sequence, has higher affinity for different DNA binding proteins. But as the specific binding of Hmi1p was detected also in the presence of competitor DNA, it is most possible, that in yeast mitochondria such Hmip-ori template strand hairpin interaction may occur.

The specificity for hairpins has also been described for the other enzymes of the UvrD subfamily, notably Srs2p and PcrA. For PcrA it has been shown, that hairpin structures at one end of the dsDNA enhance the DNA binding and unwinding efficiencies in case there are both a 3' flap and a 5' ssDNA overhang present (Anand and Khan, 2004). This binding, however, was sequence independent, since different sequences with the same structures were bound and unwound at equal efficiencies. Such structures form as intermediates during plasmid rolling circle replication and thus these properties of the PcrA helicase most probably find use in this process (Gruss and Ehrlich, 1989; Novick, 1989). Srs2p has been shown to unwind hairpin structures formed of stretches of trinucleotide repeats. This ability is quite specific for Srs2p as for example UvrD unwound these structures much less efficiently (Bhattacharyya and Lahue, 2004; Bhattacharyya and Lahue, 2005). So it can be proposed, that this particular subfamily of helicases has a common trait in showing specificity for hairpin structures, which has found use in different and unrelated processes of DNA metabolism.

3.7. Possible roles of Hmi1p in mtDNA metabolism

Considering the above described characteristics of the DNA binding and unwinding activities of Hmi1p together with the results of *in vivo* experiments reported in several of our studies (see Chapter 2.4.3. for review), it is most probable, that Hmi1p has two different roles in yeast mtDNA metabolism. One of the functions requires the helicase activity of the protein, while the other may depend only on the DNA binding properties.

The helicase activity of Hmi1p may be involved in recombination processes, since during recombination 3' flap structures may form to which Hmi1p is preferentially targeted. Active recombination has been demonstrated to take place in yeast mitochondria, resulting in a network of branched mtDNA molecules. Furthermore, this process has been proposed to be used for initiating replication. The helicase participating in mtDNA recombination is Pif1p (Foury and Kolodynski, 1983; Foury and Van Dyck, 1985; Lahaye et al., 1991). Thus, hypothesizing, that Hmi1p participates in recombination as well, it should be possible to stop recombination in yeast mitochondria upon disrupting both genes. This is true in case no additional helicase is able to replace the missing Hmi1p and Pif1p in the process. We demonstrated that in the absence of these two helicases neutral rho$^-$ mitochondrial genomes could not be maintained, while the replication of HS rho$^-$ mitochondrial genomes was unaffected (I). It can be proposed, that neutral rho$^-$ mitochondrial genomes are replicated by RDR and in that type of replication Hmi1p or Pif1p can work as the main replicative helicase, while in the absence of both no replication occurs. The HS rho$^-$ mtDNA, on the other hand, can be replicated by some other mechanism independent of Pif1p and Hmi1p.

The properties of the helicase activity of Hmi1p are similar to the properties of PriA and Rep in the requirement for at least 19 nt 3' ssDNA end to start unwinding and the considerably less stringent requirements for the 3' flap substrates to be unwound (Nurse et al., 1999; Heller and Marians, 2005). Thus it can be hypothesized that Hmi1p has an analogous function to these proteins in yeast mitochondria. PriA and Rep share the same function in alternative pathways of stalled replication fork restart. They are both involved in unwinding the lagging strand and reloading the replication complex at places, where the leading strand synthesis is stopped due to lesions in the template strand (Heller and Marians, 2005). Hmi1p might have the same function at places of DNA damage in mitochondria. According to this hypothesis, double-stranded breaks would accumulate in the absence of Hmi1p, which would result in fragmentation of mtDNA. Such an effect was indeed detected in mitochondria of cells lacking Hmi1p (Sedman et al., 2005).

The third possibility is that the helicase activity of Hmi1p is required for the rolling circle mode of replication in mitochondria. This can be proposed based on the function of the bacterial homologues from the UvrD subfamily in the rolling circle replication of plasmids and bacteriophages. In addition 2D agarose

gel electrophoresis analysis of mtDNA from the cells containing only enzymatically inactive mutants of Hmi1p showed that in this specific strain an arc of transcript strand ssDNA, present in wt mitochondria, was missing (Sedman et al., 2005). This can be an intermediate of rolling circle replication, as this replication mechanism includes the initial overproduction of one strand.

Hmi1p may be necessary at some step of replication elongation as has been proposed for its bacterial homologue, Rep (Lane and Denhardt, 1975; Yancey-Wrona and Matson, 1992; Michel et al., 1997). It is possible that Hmi1p may facilitate replisome progression through regions containing hairpin or other secondary structures. This hypothesis is in accordance with the ability of Hmi1p to bind specifically to hairpin structures. It is also supported by the results of the experiments with a ts mutant of Hmi1p, where mtDNA became fragmented after shifting the culture to restrictive temperatures. Such fragmentation may possibly be the result of ineffective elongation in the absence of Hmi1p. Moreover, 2D agarose gel electrophoresis analysis of different rho$^-$ strains showed that in the presence of Hmi1p the DNA molecules in mitochondria are longer than in the cells lacking Hmi1p. This role is independent of the helicase activity, since such longer molecules were also detected in mitochondria of cells containing only the ATPase deficient mutants of the protein (Sedman et al., 2005). Similarly, the binding of hairpins by Hmi1p was ATP hydrolysis independent but required the binding of ATP.

The binding of the hairpin in the ori/rep region by Hmi1p may be required at some step of the initiation of the rolling circle replication. The leading strand is initiated from hairpins in this type of replication of bacterial plasmids (Gruss and Ehrlich, 1989; Novick, 1989). Moreover, PcrA, the closest homologue of Hmi1p, has been shown to be required for the initiation of plasmid rolling circle replication. Therefore it is possible, that also in yeast mitochondria hairpins serve as initiation sites for the leading strand in rolling circle mode of replication, and Hmi1p has a function in that process. One piece of data supporting this hypothesis comes from 2D agarose gel electrophoresis experiments. HS rho$^-$ mtDNA-s from strains expressing Hmi1p or not were treated with restriction enzymes cutting only once per repeat and separated in 2D gels. On these gels distinctive Hmi1p-specific spikes of partially ssDNA were detected. The ssDNA part of these molecules originated only from the transcript strand in regard to the promoter in front of the C box. We also demonstrated that these specific molecules were present in strains expressing only enzymatically inactive Hmi1p(E211Q), indicating that this kind of effect does not require the helicase activity of Hmi1p (IV). Thus the presence of the hairpin binding activity of Hmi1p enhances the synthesis of the transcript strand, which is later on used as a template to synthesize the new template strand. Based on these results it can be hypothesized, that in the absence of Hmi1p no overproduction of one strand occurs, suggesting that no rolling circle replication takes place.

Whether some of these hypotheses represent the true roles of Hmi1p in the yeast mitochondria remains to be revealed by future investigations.

4. CONCLUSIONS

We have purified recombinant Hmi1p from *E. coli* and characterized the protein with different *in vitro* methods. NTP hydrolysing, DNA binding and unwinding properties of Hmi1p have the following characteristics:

- K_M for ATP hydrolysis is 0,180 mM and k_{cat} is 13 s^{-1}
- Hmi1p can hydrolyze efficinetly ATP and dATP. CTP is also hydrolysed, but at much lower rates.
- Hmi1p is a rather distributive DNA helicase efficiently unwinding DNA duplexes of less than 50 bp in length.
- Hmi1p is a 3'–5' directional DNA helicase, that requires at least 19 nt long 3' ssDNA tails to start unwinding reaction.
- Hmi1p starts unwinding from forked structures with less stringent requirements for the length of ssDNA strands, ends of 9 nt in length ensuring the efficient unwinding.
- On flap-structured substrates already a 5 nt 3' ssDNA overhang supports efficient unwinding.
- Hmi1p binds specifically to hairpin structures of the template strands of yeast mitochondrial ori/rep sequences. Hmi1p is also able to bind specifically to different other hairpin structures but with much lower efficiency.
- The specific Hmi1p-hairpin complex formation is dependent on the presence of ATP but independent of ATP hydrolysis.

These results suggest that Hmi1p has two different roles in mtDNA metabolism — one requiring its helicase activity and the other depending on the specific binding to the ori/rep sequences. The helicase activity dependent role of Hmi1p in the mtDNA metabolism of *Saccharomyces cerevisiae* is proposed to be either in recombination, replication restart at stalled replication forks or in the rolling circle mode of replication. The ATP hydrolysis independent role of Hmi1p in mitochondrial DNA metabolism is proposed to be in either replication fork progerssion through hairpin structures or the initiation of rolling circle replication.

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SUMMARY IN ESTONIAN

Pärmi *Saccharomyces cerevisiae* mitokondriaalse helikaasi Hmi1p biokeemilised omadused

Helikaasid on ensüümid, mis kasutades nukleosiid trifosfaatide hüdrolüüsist saadud energiat keeravad lahti kaksikahelalist nukleiinhapet. Helikaasid osalevad peaaegu kõigis nukleiinhapete metabolismi protsessides — replikatsioonis, rekombinatsioonis, transkriptsioonis, translatsioonis, splaissingus, ribosoomide biogeneesis jne.

Pärmi *Saccharomyces cerevisiae* mitokondriaalses nukleiinhapete metabolismis osalevatest helikaasidest on praeguseks kirjeldatud 3 RNA ja 2 DNA helikaasi. RNA helikaasid osalevad mRNA splaissingus (Mss116p), intronite degradatsioonis (Suv3p) ja ribosoomide biogeneesis (Mrh4p). DNA helikaasidest Pif1p funktsioon on seotud rekombinatsiooni ja reparatsiooniga. Lisaks osaleb Pif1p mitokondriaalse DNA (mtDNA) koopia arvu kontrollis. Teine DNA helikaas pärmi mitokondris on Hmi1p. Hmi1p roll mtDNA metabolismis ei ole veel päris selge. Me oleme näidanud, et Hmi1p on vajalik funktsionaalse mtDNA alalhoidmiseks, kuid ei ole vajalik mittefunktsionaalse lühikestest kordustest koosneva rho$^-$ mtDNA alalhoidmiseks. Temperatuuri-tundlike mutantide abil on selgunud, et Hmi1p inaktiveerumisel mtDNA fragmenteerub. Sarnane mõju on Hmi1p-l mittefunktsionaalstele rho$^-$ mtDNA molekulidele — Hmi1p juuresolekul on mtDNA molekulid tunduvalt pikemad kui rakkudes, kus Hmi1p puudub.

Käesolevas doktoritöös uurisime rekombinantse Hmi1p biokeemilisi omadusi, et sel kombel kindlaks teha, millised võiksid olla selle helikaasi rollid pärmi mitokondris.

Tulemusena saime teada, et Hmi1p on 3'–5' suunaline distributiivne DNA helikaas, mis vajab substraadiga seondumiseks kas 3' polaarsusega üksikahelalise DNA otsa või pikemat lõiku üksikahelalist DNAd ilma vaba otsata. Hmi1p hüdrolüüsib efektiivselt ainult ATP-d ja dATP-d, kuid madala efektiivsusega ka CTP-d. Hmi1p on struktuurispetsiifiline helikaas, sest olenevalt substraadi struktuurist vajab Hmi1p erineva pikkusega 3' polaarsusega üksikahelalise DNA otsa, et alustada substraadi lahtikeeramist. Kõige pikemat, 19 nukleotiidi pikkust 3' polaarsusega üksikahelalise DNA otsa on vaja lihtsa kaksikahelalise substraadiga, millel on 3' üksikahelaline DNA ots. Kahvelsubstraadi puhul on piisavaks 9 nukleotiidi pikkuse 3' ja 5' üksikahelalise DNA otsa olemasolu. 3' *flap* substraadi puhul, kus 3' üksikahelaline ots algab kaksikahelalise DNA keskelt, piisab ainult 5 nukleotiidi pikkusest 3' polaarsusega üksikahelalise DNA otsast.

Et uurida Hmi1p rolli pärmi mtDNA metabolismis, vaatlesime Hmi1p sidumist DNA-le elektroforeetilise liikuvuse muutumise katses. Tulemusena tegime kindlaks, et Hmi1p seob spetsiifiliselt erinevaid juuksenõela struktuure, kusjuures kõige efektiivsemalt seondub Hmi1p pärmi mtDNA replikatsiooni

initsiatsiooni järjestuste ehk ori/rep järjestuste ühest ahelast moodustunud juuksenõeladele. Spetsiifiliselt siduvaks ahelaks on ori/rep järjestuse ees paikneva promootori suhtes matriitsahel. Lisaks näitasime, et spetsiifiliseks juuksenõel-struktuuride sidumiseks on vajalik ATP sidumine, kuid see on sõltumatu ATP hüdrolüüsist.

Võrreldes *in vitro* meetoditega saadud tulemusi erinevate *in vivo* meetoditega kirjeldatud Hmi1p sõltuvate efektidega pakume välja hüpoteesi, et Hmi1p-l on kaks erinevat funktsiooni pärmi mtDNA metabolismis — üks helikaassest aktiivsusest sõltuv ja teine, mis sõltub ainult spetsiifilisest DNA sidumisest. Helikaassest aktiivsusest sõltuvaks rolliks võiks olla kas osalemine rekombinatsioonis, replikatsiooni initsiatsioonis DNA katkete kohas või veereva ratta replikatsioonis. DNA spetsiifilisest sidumisest sõltuvaks rolliks võiks olla kas replikatsioonikompleksi aitamine liikumisel üle juuksenõela struktuuride või osalemine veereva ratta replikatsiooni initsiatsioonil.

Milline neist hüpoteesidest kajastab Hmi1p tegelikku rolli pärmi mitokondriaalses metabolismis jääb välja selgitamiseks edasiste katsete käigus.

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PUBLICATIONS

Yeast mitochondrial DNA helicase Hmi1p stimulates synthesis of one strand of mtDNA through specific binding to ori sequences

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ABSTRACT

Hmi1p is a *Saccharomyces cerevisiae* mitochondrial DNA helicase that is essential for the maintenance of functional mitochondrial DNA (mtDNA). Hmi1p has also an effect on the replication of nonfunctional ρ^- mtDNA. In both wild type and ρ^- strains Hmi1p is required for processive synthesis of mtDNA. Here we show, using restriction enzyme digested ρ^- mtDNAs separated in 2-dimensional agarose gels, that Hmi1p stimulates specifically synthesis of one strand of mtDNA. *In vitro* experiments reveal that Hmi1p binds strand- and structure-specifically to ori elements in mtDNA. Based on these results we suggest that ρ^- mtDNA can be replicated by a specific Hmi1p-dependent mechanism, which includes initial overproduction of one strand.

Keywords: helicase, mitochondrial DNA, replication, *Saccharomyces cerevisiae*

INTRODUCTION

The 80 kb mitochondrial (mt) genome of the yeast *Saccharomyces cerevisae* is maintained as a mixture of molecules of different topology. The majority of mitochondrial DNA (mtDNA) forms a network of linear and branched molecules, while a small subset may exist in circular form (Maleszka et al., 1991). Different modes of replication have been proposed to be in use in yeast mitochondria, including rolling circle replication (RC), strand-asymmetric bidirectional replication and recombination dependent replication. Replication initiation has been proposed to be either transcription or recombination based (Lecrenier and Foury, 2000; Maleszka et al., 1991; Williamson, 2002). Currently, however, no consensus exists, how the different modes of replication are used for synthesis and segregation of mtDNA.

Replication of mt genome depends on a number of protein factors encoded by the nuclear genome. The role of some proteins, such as Mip1p, the catalytic

subunit of DNA polymerase γ, has been well established (Foury, 1989). However, there are other accessory factors required for the stable maintenance of mt genome, the role of which is less clear. This list includes mitochondrial DNA helicases.

Helicases are enzymes that use the energy of NTP hydrolysis to unwind double-stranded DNA (dsDNA). In *S. cerevisiae* at least two DNA helicases — Pif1p and Hmi1p — are imported into mitochondria and have functions in mtDNA metabolism (Foury and Lahaye, 1987; Sedman et al., 2000). Pif1p influences mtDNA recombination and repair but is not essential for the maintenance of mt genome at regular growth conditions (Lahaye et al., 1991). In contrast, wild type (wt) mt genome is unstable in strains with the HMI1 ORF disrupted. However, ρ^- mt genomes of tandem short repeats are efficiently maintained in such strains, indicating that Hmi1p cannot be the sole replicative helicase (Sedman et al., 2000; Monroe et al., 2005). We have shown that Hmi1p also has an effect on the replication of hypersuppressive (HS) ρ^- mtDNA. In the absence of Hmi1p HS ρ^- mt genomes are mostly organized as concatemers with 3–5 repeats per one molecule, while in the presence of the helicase mtDNA molecules contain generally more than 10 repeats. This is similar to the effect of Hmi1p on the wt mt genome replication, since the cells containing a temperature-sensitive mutant of Hmi1p lose mtDNA through fragmentation during growth under restrictive temperatures (Sedman et al., 2005).

Hmi1p is a 3'–5' directional distributive and structure specific DNA helicase (Kuusk et al., 2005). However, our recent studies revealed, that the helicase activity of Hmi1p was not a prerequisite for the wt mt genome maintenance. Furthermore, enzymatically inactive mutants of Hmi1p can stimulate the synthesis of HS ρ^- long concatemeric mtDNA molecules (Sedman et al., 2005). The helicase activity is still not entirely dispensable, since mtDNA is less stable in strains expressing only enzymatically inactive Hmi1p. Also the topology of HS ρ^- mtDNA is affected in such mutant strains, a specific arc of ssDNA being absent on blots of 2-dimensional (2D) agarose gels (Sedman et al., 2005). Earlier it has been demonstrated, that this specific arc consists of ssDNA molecules of only transcript strand in regard to the promoter in front of the ori sequence (MacAlpine et al., 2001). These results raise a possibility that Hmi1p has two different roles in mtDNA metabolism. One of these is helicase activity dependent while the other may be a structural one, mediated by specific protein-DNA or protein-protein interactions.

To explore the role of Hmi1p in mtDNA metabolism we analyzed HS ρ^- mt genome replication intermediates with 2D agarose gel electrophoresis. We demonstrate that Hmi1p stimulates specifically the synthesis of the transcript strand of mtDNA. This stimulation does not require the enzymatic activity of the protein. Our experiments demonstrate that Hmi1p forms a strand and structure specific complex with the hairpin formed of a subset of mitochondrial ori sequences *in vitro*.

RESULTS AND DISCUSSION

Hmi1p stimulates synthesis of transcript strand of HS ρ⁻ mtDNA

For more detailed understanding of the role of Hmi1p in mtDNA metabolism, we analyzed mitochondrial replication intermediates of the HS ρ⁻ strains HS61(+HMI1) and HS61(*hmi1*Δ), where a 0.83 kb ori2 containing mtDNA fragment is maintained (Sedman et al., 2000; Sedman et al., 2005). 0.3 kb ori is a characteristic sequence region of wt as well as HS ρ⁻ yeast mt genomes (de Zamaroczy et al., 1979). Ori contains 3 conserved GC rich elements, boxes A, B and C, with intervening heterogeneous AT rich sequences, and a promoter in front of box C (Fig. 1B). Boxes A and B together with bordering sequences can potentially form a fold structure with a small loop and a sidebulge (de Zamaroczy et al., 1984). The samples of purified mtDNAs were separately digested with two restriction enzymes — DraI and EcoRV — that cut only once per HS61 mt genome repeat (Fig. 1B). DraI cuts the repeat upstream of the promoter sequence (defined here as position 1) and EcoRV next to box B of ori (nt 282). The digestion products were separated with the neutral-neutral 2D agarose gel electrophoresis (Fig. 1A). Most of the digested mtDNA from both strains moved in 2D gels as a 1N spot that corresponds to the HS61 mt genome repeat size (Fig. 1D-G). Spots corresponding to 2N and 3N size fragments could result from the digestion of DNA molecules with some of the restriction enzymes' recognition sites single-stranded or containing DNA-RNA hybrid. As the repeat size of the HS61 genome is only 0.83 kb, the traditional replication intermediates known as the Y arc and the bubble arc cannot be resolved from the dsDNA arc (Brewer and Fangman, 1987). However, recombination intermediates were detected as a strong restriction enzyme cleavage resistant arc emerging from the 2N spot. Altogether three Hmi1p dependent differences could be detected. First, the ssDNA arc that runs below the dsDNA arc was visible only on gels with digested mtDNA from the HS61(+HMI1) strain. Second, different molecules running above the dsDNA arc were more abundant in the HS61(*hmi1Δ*) strain. Similar differences were also detected previously on blots with uncut mtDNAs from the same strains (Sedman et al., 2005). The most interesting difference between the digested mtDNA-s from different strains was the presence of distinctive spikes in the region between the dsDNA and the ssDNA arcs that were detected only on the blots with the HS61(+HMI1) mtDNA (Fig.-s 1D-G). The spikes started from 1N, 2N and 3N spots on the dsDNA arc and pointed towards the ssDNA arc. When HS61(+HMI1) mtDNA was treated with DraI and S1 nuclease prior to analysis on gels, the spikes disappeared (Fig. 1H). As the spikes connected the arcs of dsDNA and ssDNA molecules and were sensitive to S1 treatment, we propose that they represent partially ssDNA molecules. On blots with DraI digested mtDNA the spikes appeared to end on the arc of ssDNA molecules (Fig. 1D). Thus the mtDNA of the HS61(+HMI1) strain should contain a substantial fraction of molecules where the consecutive DraI sites are double-stranded but the region between

them largely single-stranded (Fig. 1C). When EcoRV was used for digesting mtDNA, the spikes were shorter, starting again from 1N, 2N and 3N spots on the dsDNA arc, but terminating between the dsDNA and the ssDNA arcs (Fig. 1F). This suggests that, in contrast to DraI, EcoRV cleaves only repeats with a substantial fraction of the DNA between the consecutive EcoRV sites being double-stranded (Fig. 1C). The gels with DraI digested mtDNA were also analyzed with strand-specific probes (Fig. 1I and J). The lower ends of the spikes, apparently mostly single-stranded molecules, hybridized more efficiently with the transcript strand probe, while only a very weak signal could be detected when using the template strand probe. Thus, the single-stranded regions in the spikes correspond to the transcript strand of mtDNA. This result indicates that in the presence of Hmi1p the synthesis of transcript strand is significantly more processive than that of the template strand. In addition it can be concluded that the transcript strand may be the leading strand during the synthesis of HS ρ^- mtDNA. Based on the differences seen on blots with DraI and EcoRV treated mtDNAs, we can hypothesize that the initiation of the synthesis of the other strand is from somewhere between the DraI and EcorV sites. This region includes conserved box C and the promoter (Fig. 1C). In the absence of Hmi1p no overproduction of one strand could be seen, indicating some different mode of replication to be in use. This may be recombination dependent replication since in the Hmi1p deficient strains mitochondrial recombination intermediates are more abundant than in strains expressing Hmi1p.

Using long exposition times in analysis of blots of intact HS ρ^- mtDNA separated in 2D agarose gels MacAlpine and coworkers were able to detect a presence of small amounts of partially single-stranded replication intermediates. These molecules are visible as spikes that extend from single-stranded circular molecules to the dsDNA arc (MacAlpine et al., 2001). Relatively strong signal corresponding to spikes observed here suggests therefore that the spikes originate mostly from high molecular weight mtDNA. To assess the nature of this high molecular weight mtDNA we analyzed the restriction enzyme digested mtDNA from the HS61(*hmi1*E211Q) strain, where only the enzymatically inactive mutant of Hmi1p — Hmi1pE211Q — is expressed. We have shown previously that the ATPase and helicase activities of Hmi1p are not required for the synthesis of long concatemeric HS ρ^- mtDNA molecules, whereas the population of ssDNA together with circular ssDNA molecules were absent (Sedman et al., 2005). Therefore, by analyzing mtDNA from the HS61(*hmi1*E211Q) strain, it is possible to ascertain, whether the Hmi1p specific spikes on the blots of digested mtDNAs originate from long concatemeric HS ρ^- mtDNA molecules or not. As in case of HS61(+HMI1) but not in HS61(*hmi1*Δ) a 2D gel analysis of restricted mtDNA from the HS61(*hmi1*E211Q) strain revealed the presence of the partially ssDNA spikes (Fig. 1K). This indicates that very likely the partially single-stranded structures are generated during the synthesis of long concatemeric molecules. In addition the result shows that this

Hmi1p specific effect is not dependent on the helicase activity of Hmi1p but rather mediated by protein-DNA or protein-protein interactions.

We have demonstrated earlier that the helicase activity dependent effect of Hmi1p on mtDNA topology is strand-specific as well. Hmi1p is required for the synthesis of ssDNA molecules that have been shown by others to be of only transcript strand (MacAlpine et al., 2001; Sedman et al., 2005). Thus the helicase activity dependent and independent activities of Hmi1p are interconnected, being both in stimulating the synthesis of the transcript strand of mtDNA.

Hmi1p forms a strand-specific complex with ori *in vitro*

In order to define the biochemical mechanism underlying the observed effect of Hmi1p in stimulating the synthesis of partly single-stranded replication intermediates, we performed DNA binding assays with the purified recombinant Hmi1p. Since ori is the most characteristic region of HS ρ^- mt genomes, we tested the binding of Hmi1p to synthetic oligonucleotides containing conserved elements of ori region. Hmi1p could form a specific complex with the template strand oligonucleotide AB containing the AB fold with bordering single-stranded regions (Fig. 2A, lane 2). The complex formation was strand-specific as the complementary transcript strand oligonucleotide BA bound Hmi1p with much lower efficiency (Fig. 2A, lane 4; Table 1). With nonstructured oligonucleotide GTC, used as a negative control, no significant complex formation was detected (Fig. 2A, lane 6). To exclude the possibility that the observed shift in electrophoretic mobility represents a complex with some contaminating bacterial protein in our Hmi1p preparation, we tested recombinant GST-Hmi1 fusion protein in the same assay. In accordance with the larger mass of the GST fusion protein GST-Hmi1p-AB complex had slower mobility than Hmi1p-AB complex (Fig. 2A, lane 1). Next we tested oligonucleotides with defined mutations in the AB fold structure for complex formation. The introduced mutations a) destroyed the sidebulge, making thus the double-stranded hairpin structure continuous (ABm1); b) displaced the sidebulge structure to the other side of the hairpin (ABm2); c) changed the sequence of the terminal loop (ABm3) (Supplementary Fig. 1). All three mutant oligonucleotides could form complexes with Hmi1p with the binding affinity about 15% weaker than for AB-Hmi1p complex (Fig. 2B, lanes 8, 10 and 12; Table 1). Among the mutants ABm1, that lacked the sidebulge, showed the lowest binding efficiency. Finally, we tested oligonucleotides with mitochondrial sequences outside the ori region — VAR1 and IS. These oligonucleotides could form different smaller hairpin structures (Supplementary Fig. 1). The complex formation was detected with both oligonucleotides, but with much lower efficiencies than with AB (Fig. 2A lanes 14 and 16; Table 1). Thus it can be concluded that Hmi1p binds specifically to different hairpin structures, but most efficiently to the AB fold of the template strand of ori sequence.

Since the stimulation of synthesis of partly single-stranded replication intermediates by Hmi1p was not dependent on the helicase activity, we addressed

this issue also for the specific binding of Hmi1p to the template strand hairpin of the ori sequence by performing DNA binding assays in the presence of nonhydrolyzable ATP analogues as well as with the enzymatically inactive recombinant mutant protein. The Hmi1p-AB complex formation required the presence of nucleotide cofactor, but was ATP hydrolysis independent, since nonhydrolyzable ATP analogues ADPNP and ATPγS could support the complex formation (Fig. 2B, lanes 1–4). The complex formation, albeit at a slightly reduced level, was also detected with mutant Hmi1p(E211Q), that binds ATP but does not hydrolyze it (Fig. 2B, lanes 5–8). Thus we propose that the other function of Hmi1p that does not require the enzymatic activity is through the complex formation with the template strand of the AB fold of ori sequence. Since this is similar to the stimulation of the synthesis of the partially single-stranded molecules, it is most probable, that through this complex formation the stimulation of transcript strand synthesis takes place.

To characterize the specificity of Hmi1p complex formation with the template strand of ori region, the Hmi1p-AB complex was subjected to hydroxyl radical treatment (Fig. 2C). For complex formation the same oligonucleotides were used as in DNA binding assays. A clear difference in the cleavage pattern of the Hmi1p-AB complex was seen compared to the cleavage pattern of the AB oligonucleotide control (Fig. 2C, lanes 1–3). The main difference was in the stem part of the hairpin (Supplementary Fig. 2) while in the single-stranded parts the cleavage pattern was unchanged. Similar experiment was performed with the Hmi1p-BA complex, the complex of Hmi1p with the transcript strand of ori (Fig 2C, lanes 5–7). Here the difference in the cleavage pattern was much less clear, since the complex formation with this oligonucleotide was much weaker. The cleavage pattern was changed in the stem part of the hairpin again, confirming thus that Hmi1p binds specifically to hairpin structures. The most surprising difference, when comparing the hydroxyl radical treatment experiments, was seen between the cleavage patterns of AB and BA without the bound Hmi1p (Fig 2C lanes 3 and 7). While BA was cut rather uniformly along the whole oligonucleotide with some weaker and stronger cleavage sites in the part with hairpin structure, AB had a distinct cleavage pattern with some hypersensitive and some absolutely hidden nucleotides, especially in the hairpin part. Thus we suggest that the hairpin of the AB oligonucleotide takes a specific conformation, which the hairpin of the BA oligonucleotide takes at only a very low efficiency. Probably this particular conformation is also recognized specifically by Hmi1p. The same strand of ori has been shown to be specifically bound also by mitochondrial chaperone Hsp60p (Kaufman et al., 2000).

Speculation

Of the three different modes of replication described to date to be in use in yeast mitochondria — rolling circle replication, bidirectional replication from ori sequences and recombination dependent replication — only the mechanism of rolling circle replication includes the initial overproduction of one strand. Thus we hypothesize, that Hmi1p is required for rolling circle replication to take place. This is similar to the roles of the closest homologues of Hmi1p from bacteria — UvrD, Rep and PcrA — that all participate in replication of RC plasmids or bacteriophages (Bruand and Ehrlich, 2000; Denhardt et al., 1967; Iordanescu, 1993).

METHODS

2D agarose gel electrophoresis: Strains are described in Supplementary Table 1. Growth conditions and mtDNA preparation were as described previously (Sedman et al., 2005). The DNA samples were digested with 20 U of DraI or EcoRV 3 hr at 37°C. S1 treatment was performed with 1 U of the enzyme 1 min at 37°C. First dimension electrophoresis was in 0.8% TBE agarose gels run at 0,8 V/cm 17 hr at room temperature. Second dimension electrophoresis was in 1.4% TBE agarose gels with 300 ng/ml ethidium bromide run at 5 V/cm 3 hr at 4°C. DNA was blotted to nylon membranes and mtDNA was detected with PCR probe A, prepared with primers A1 and A2 and α^{32}P dCTP in the reaction. Probes B and C were used for strand-specific probing. For preparing probes B and C PCR was made using primers B and C. The amplified fragment was purified through agarose gel electrophoresis and used as a template in PCR reactions containing only one primer, either B or C, and α^{32}P dCTP, to give probes B and C. Primer descriptions and sequences are in Supplementary Table 2.

DNA binding assay: Oligonucleotides used for binding are in Supplemetary Table 3. Hmi1p was purified as described previously (Kuusk et al 2005). GST-Hmi1p was purified similarly with the thrombin cleavage step omitted. 20 µl DNA binding reactions contained 30 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT, 0.1 mg/ml BSA, 1 nM ^{32}P 5'-end-labeled oligonucleotide, 15 µM (in nucleotides) competitor pRS304 dsDNA, 1 mM ATP, ADPNP or ATP-γS as indicated and 10 nM Hmi1p. Reactions were incubated at 25°C for 25 min, 10% glycerol was added, and complexes were separated in 6% polyacrylamide TBE gels run at 4°C.

Hydroxyl radical footprint assay: To DNA binding reactions, done as described above, 0.09% H_2O_2, 6 mM Na-ascorbate and 2 µl of freshly made mix of 1.2 mM $Fe(NH_4)_2(SO_4)_2$ and 2.4 mM EDTA were added. Cleavage was performed for 3 min at 25°C and reactions were stopped by adding 10 mM

thiourea, 200 mM sodium acetate, 10 ng dextrane, 15 mM EDTA and 3 volumes of ethanol. DNA was precipitated and separated in 6 M urea, 10% polyacrylamide TBE gels. As markers ^{32}P labeled oligonucleotides AB and BA were subjected to G and A specific Gilbert-Maxam reactions.

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Table 1. Quantification of the DNA binding assays

Oligonucleotide	Description	% of substrate in complex
AB	ori template strand	42.2
BA	ori transcript strand	13.3
ABm1	ori template strand, no bulge	30.4
ABm2	ori template strand, 5' bulge	37.5
ABm3	ori template strand, mutated hairpin	35.1
GTC	no structure	2.4
VAR1	mitochondrial gene	18.9
IS	mitochondrial intergenic sequence	12.9

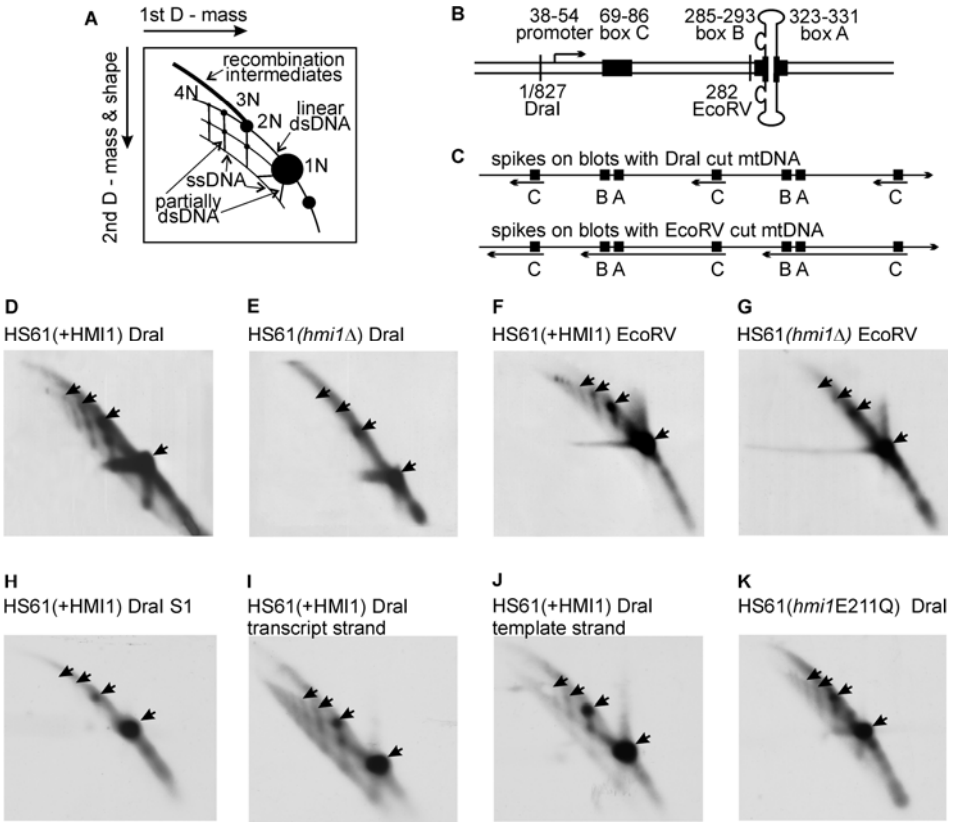


Figure 1. 2-dimensional gel electrophoresis of restriction enzyme treated hyper-suppressive ρ^- mtDNA. **(A)** Diagram of restriction enzyme treated mtDNA resolved in neutral-neutral 2D agarose gels. **(B)** HS61 mtDNA repeat with DraI and EcoRV sites indicated. **(C)** mtDNA molecules that form spikes on blots with mtDNA treated with different restriction enzymes. **(D-K)** mtDNA of strains as indicated treated with restriction enzymes as indicated resolved in 2D agarose gels. 1N, 2N, 3N and 4N spots are indicated with arrows **(I, J)** the same blot as on **(D)** probed with strand-specific probes. **(I)** — transcript strand, **(J)** — template strand.

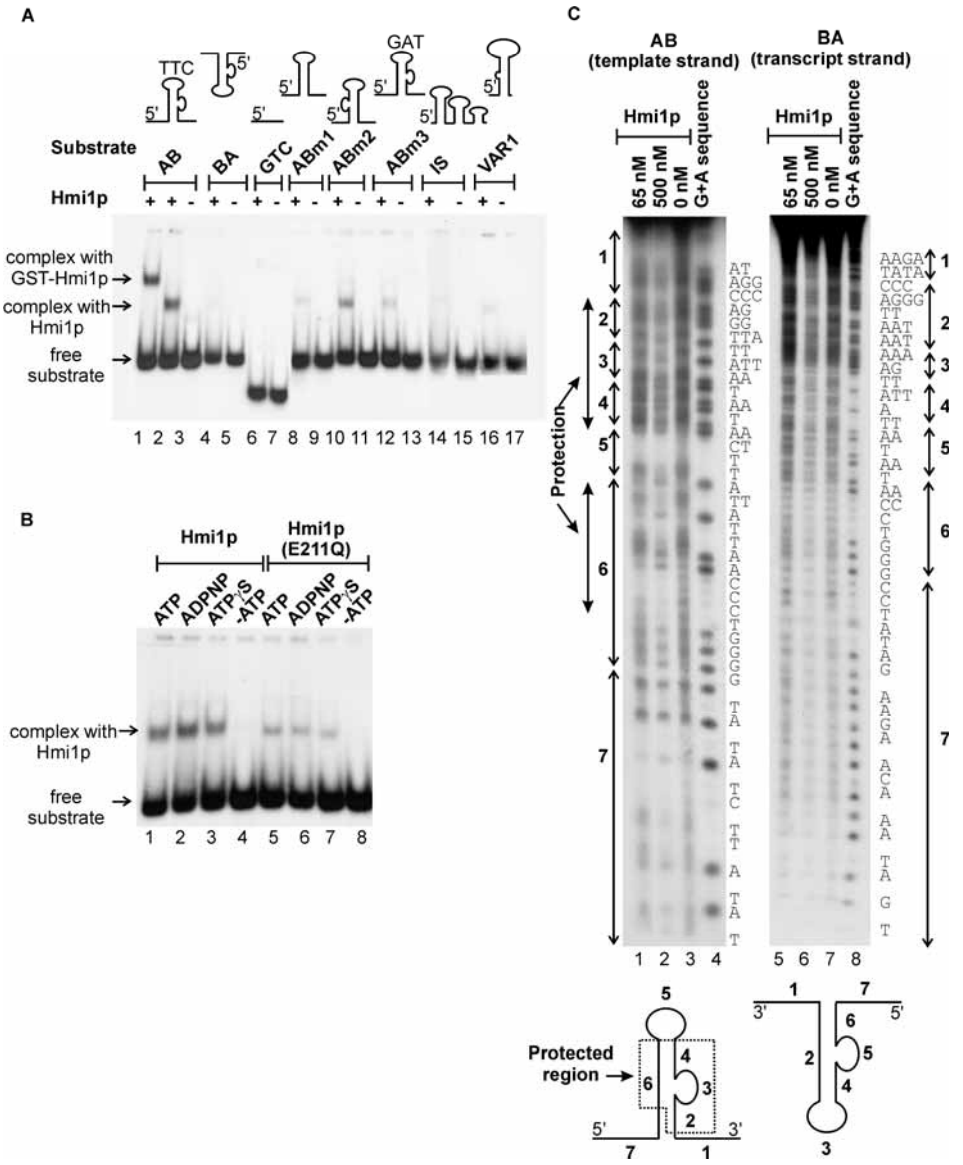


Figure 2. Hmi1p binds to the hairpin structure of the template strand of yeast mitochondrial ori2 sequence *in vitro*. **(A, B)** DNA binding assays. **(A)** Oligonucleotides with secondary structures as schematically indicated above the figure were used. Structure of the transcript strand oligonucleotide is inverted. For exact sequences and secondary structures see Supplementary Information. On lane 1 GST-Hmi1 fusion protein was used. **(B)** Hmi1p or Hmi1p(E211Q) and the oligonucleotide AB were incubated in the presence of ATP or its analogues as indicated. **(C)** Hydroxyl radical footprint assay. G+A sequence — 5' end-labeled oligonucleotides subjected to G and A specific cleavage. Protected area is indicated on scheme below the figure. Exact sequence of the protected area is on Supplementary Figure 2.

SUPPLEMENTARY INFORMATION

Supplementary Table 1. Strains

Strain	Description	Reference
HS61(+HMI1)	W303–1A (*hmi1*::TRP1 ρ^- +pRS315-HMI1)	(Sedman et al., 2005)
HS61(*hmi1Δ*)	W303–1A (*hmi1*::TRP1 ρ^- +pRS315)	(Sedman et al., 2005)
HS61(*hmi1*E211Q)	W303–1A (*hmi1*::TRP1 ρ^- +pRS315-*hmi1*E211Q)	(Sedman et al., 2005)

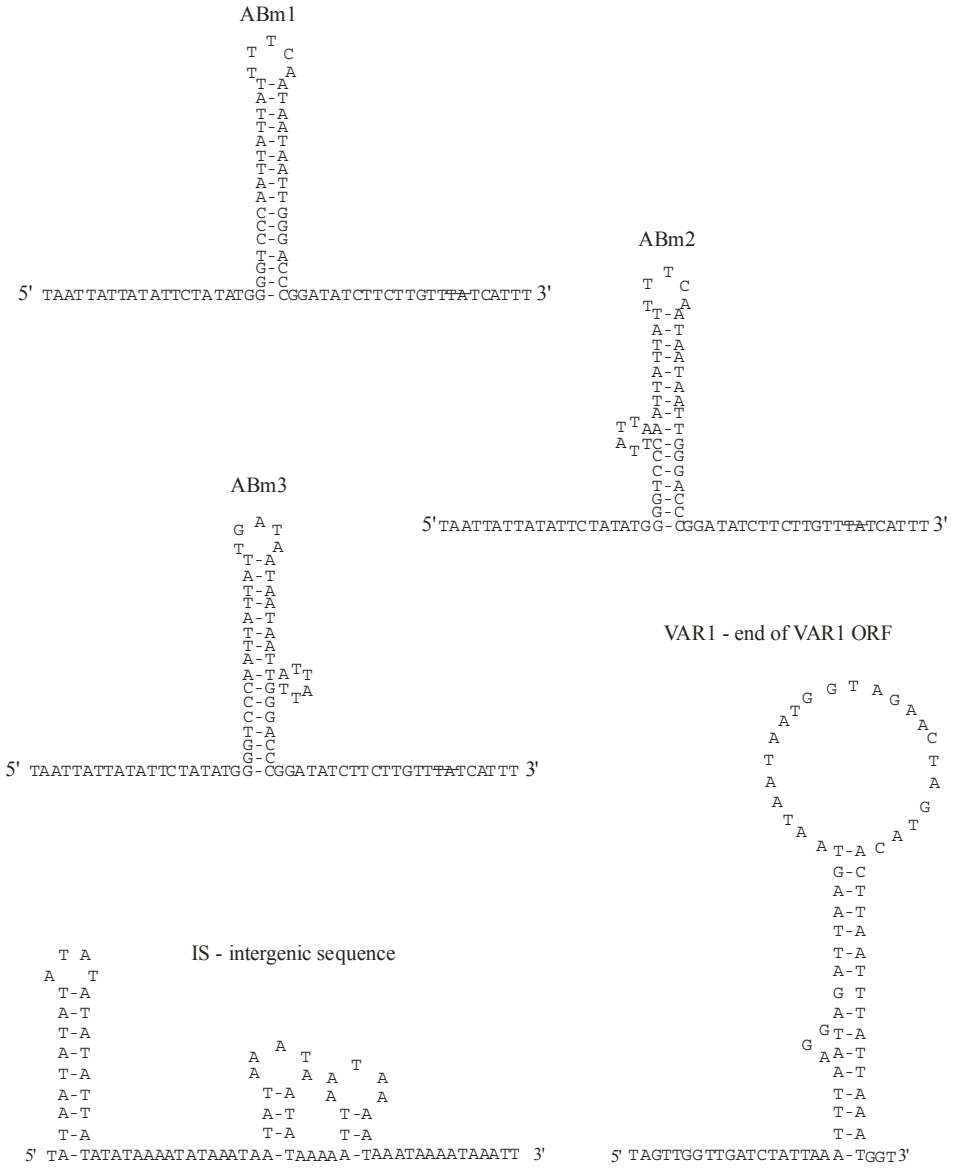
Supplementary Table 2. Oligonucleotides used for making mtDNA specific probes

Oligonucleotide	Description	Sequence (5'→3')
A1	HS61 mtDNA nt 66–83, transcript strand	TAGGGGGAGGGGGTGGGT
A2	HS61 mtDNA nt 310–332, template strand	GGGGGTCCCAATTATTATTTTC
B	HS61 mtDNA nt 728–752, template strand	TTATATAAATTTATAATAATTATAA
C	HS61 mtDNA nt 448–472, transcript strand	TAAATTTAATAATTATATATATTTT

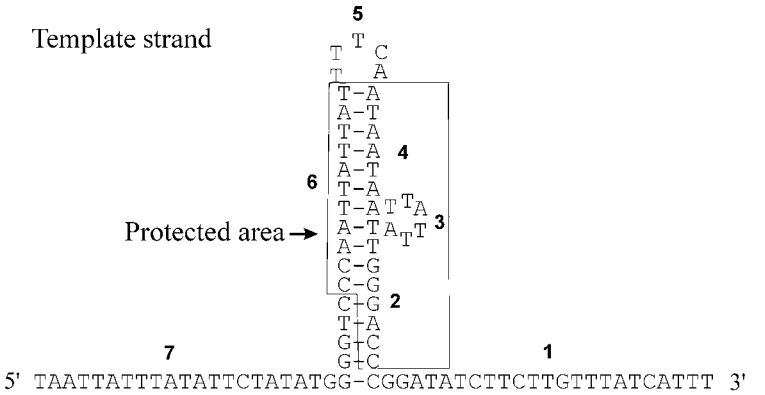
Supplementary Table 3. Oligonucleotides used in DNA binding assays

Oligonucleotide	Description	Sequence (5'→3')
AB	mt ori2, template strand	TAATTATTTATATTCTATATGGGGTCCCAATTATTATTTTCAAT AATAATTATTATTGGGACCCGGATATCTTCTTGTTTATCATTT*
BA	mt ori2, transcript strand	AAATGATAAACAAGAAGATATCCGGGTCCCAATAATAATTATTA TTGAAAATAATAATTGGGACCCCATATAGAATATAAATAATTA*
ABm1	mt ori2, template strand, no bulge	TAATTATTTATATTCTATATGGGGTCCCAATTATTATTTTCAAT AATAATTGGGACCCGGATATCTTCTTGTTTATCATTT*
ABm2	mt ori2, template strand, 5' bulge	TAATTATTTATATTCTATATGGGGTCCCTTATTAAATTATTATT TTCAATAATAATTGGGACCCGGATATCTTCTTGTTTATCATTT*
ABm3	mt ori2, template strand, mutated loop	TAATTATTTATATTCTATATGGGGTCCCAATTATTATTGATAAT AATAATTATTATTGGGACCCGGATATCTTCTTGTTTATCATTT*
GTC	$(GTC)_{24}$	GTCGTCGTCGTCGTCGTCGTCGTCGTCGTCGTCGTCGTCGTCGT CGTCGTCGTCGTCGTCGTCGTCGTCGTC
IS	mt intergenic sequence, nt 10001–10080	TATAATATATATATATATATTATATATAAAATATAAATATATAA ATAATATAAAAATTAATAAAATAAATAAAATAAATT
VAR1	mt VAR1 ORF, nt 971–1050	TAGTTGGTTGATCTATTAAATTTAAAGGTAGATTAAGTAATAAT AATGGTAGAACTAGTACACTTAATTTATTAAATGGT

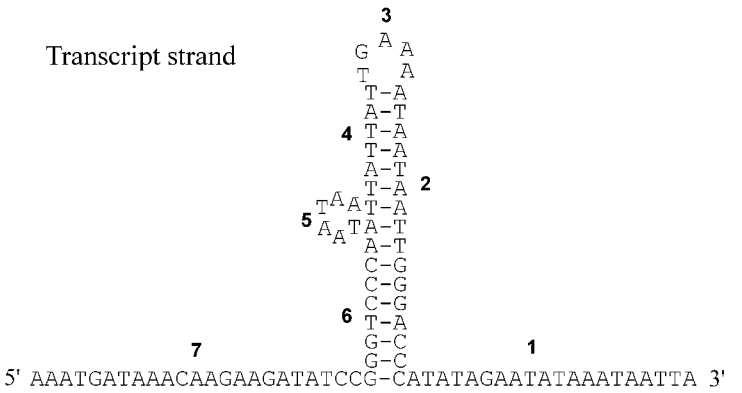
* underlined are the A and B boxes of the mitochondrial ori sequence.



Supplementary Figure 1. Secondary structures of ABm1, ABm2, ABm3, IS and VAR1 oligonucleotides.



1 - CGGATATCTTCTTGTTTATCATTT
2 - TTGGGACCC
3 - TTATTA
4 - ATAATAA
5 - TTTCA
6 - GGTCCCAATTATTAT
7 - TAATTATTTATATTCTATATGG



1 - CATATAGAATATAAATAATTA
2 - ATAATAATTGGGACC
3 - TGAAA
4 - TTATTAT
5 - TAATAA
6 - GGTCCCAA
7 - AAATGATAAACAAGAAGATATCCA

Supplementary Figure 2. Secondary structures of AB and BA oligonucleotides. Below the structure the exact sequences corresponding to the numbers on schemes of Fig. 2C are given. The protected area as detected in hydroxyl radical treatment assays is indicated with box.

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Scientific work

Since 1996 I have been working in the group of Prof. Juhan Sedman. My main research topic has been the mitochondrial DNA metabolism in baker's yeast *Saccharomyces cerevisiae.* I have characterized the biochemical properties of Hmi1p, a yeast mitochondrial DNA helicase. Also I have studied the functions of the same protein in yeast mitochondria.

List of Publications

1. T. Sedman, **S. Kuusk**, S. Kivi, J. Sedman. A DNA helicase required for maintenance of the functional mitochondrial genome in *Saccharomyces cereviviae*. Mol. Cell. Biol., 2000 20:5 1816–1824.
2. **Kuusk S**, Sedman T, Sedman J. Recombinant yeast mtDNA helicases. Purification and functional assays. In *Methods in Molecular Biology: Mitochondrial DNA.* Ed. B. Copeland, (Humana press Inc) 2002; 197: 303–316.

3. **Kuusk S**, Sedman T, Jõers P, Sedman J. Hmi1p from Saccharomyces cerevisiae mitochondria is a structure-specific DNA helicase. J Biol Chem. 2005 Jul 1; 280(26): 24322–24329.
4. Sedman T, Jõers P, **Kuusk S**, Sedman J. Helicase Hmi1 stimulates the synthesis of concatemeric mitochondrial DNA molecules in yeast Saccharomyces cerevisiae. Curr Genet. 2005 Apr; 47(4): 213–222.

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Teadustegevus

Alates 1996 aastast olen töötanud Prof. Juhan Sedmani grupis. Minu põhiliseks uurimisteemaks on olnud pagaripärmi *Saccharomyces cerevisiae* mitokondriaalse DNA metabolism. Olen kirjeldanud pärmi mitokondriaalse helikaasi Hmi1p biokeemilisi omadusi ning uurinud selle valgu funktsiooni pärmi mitokondris.

Publikatsioonide nimekiri

1. T. Sedman, **S. Kuusk**, S. Kivi, J. Sedman. A DNA helicase required for maintenance of the functional mitochondrial genome in *Saccharomyces cereviviae*. Mol. Cell. Biol., 2000; 20:5 1816–1824.
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4. Sedman T, Jõers P, **Kuusk S**, Sedman J. Helicase Hmi1 stimulates the synthesis of concatemeric mitochondrial DNA molecules in yeast Saccharomyces cerevisiae. Curr Genet. 2005 Apr; 47(4): 213–222.