DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
111

DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
111

CHARACTERIZATION OF THE YEAST *SACCHAROMYCES CEREVISIAE* MITOCHONDRIAL DNA HELICASE HMI1

TIINA SEDMAN

TARTU UNIVERSITY
PRESS

Department of General and Microbial Biochemistry, Institute of Molecular and Cell Biology, University of Tartu, Estonia

Dissertation was accepted for the commencement of the degree of Doctor of Philosophy (in Molecular Biology) on September 22th, 2005 by the council of the Institute of Molecular and Cell Biology, University of Tartu

Opponent: Associate Professor Josef Nosek, PhD (Department of Biochemistry, Comenius University, Slovakia)

Commencement: October 21, 2005

The publication of this dissertation is granted by the University of Tartu

ISSN 1024–6479

ISBN 9949–11–149–8 (trükis)
ISBN 9949–11–150–1 (PDF)

Tartu Ülikooli Kirjastus
www.tyk.ee
Tellimus nr 422

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

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

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):1816–24.

II. Kuusk S., **Sedman T.**, Sedman J. (**2002**) Recombinant Yeast mtDNA Helicases. Purification and functional assays. *Methods Mol Biol*. 2002; 197:303–16. Mitochondrial DNA. *Methods and Protocols,* edited by *William C. Copeland,* 2002.

III. **Sedman T.,** Joers P., Kuusk S., Sedman J. (**2005**) Helicase Hmi1 stimulates the synthesis of concatemeric mitochondrial DNA molecules in yeast *Saccharomyces cerevisiae*. *Curr Genet.* 2005 47(4):213–22.

My contribution to the articles referred in the current thesis is as follows:

Ref. I – designed and performed the experiments, analysed the experimental data and participated in writing of the paper.

Ref. II – participated in protein purification and in writing of the paper.

Ref. III – designed and performed the experiments, analysed the experimental data and participated in writing of the paper.

ABBREVIATIONS

CSBs	–	conserved sequence blocks
DAPI	–	4′,6′-diamidino-2-phenylinole
D-loop	–	displacement loop
dsDNA	–	double stranded DNA
FITC	–	fluorescein isothiocynate
HA	–	hemagglutinin
HS rho$^-$	–	hypersuppressive rho$^-$
HSP	–	heavy strand promoter
H-strand	–	heavy strand
L-strand	–	light strand
mtDNA	–	mitochondrial DNA
O_H	–	heavy strand origin
O_L	–	light strand origin
OXPHOS	–	oxidative phosphorylation system
RC	–	rolling circle
RDR	–	recombination dependent replication
RI	–	replication intermediates
ssDNA	–	single-stranded DNA
2D	–	two-dimensional
ts	–	temperature sensitive

INTRODUCTION

Maintenance of the functional mitochondrial genome is essential for most eukaryotic organisms. Mutations in mitochondrial DNA (mtDNA) can impair the oxidative phosphorylation system and thus lead to human diseases and aging of the organism (reviewed in Wallace, 1992). Preservation of the mitochondrial genetic information throughout the lifetime requires flawless replication, segregation, repair and expression of the organelle genome. Most of our current understanding of these basic processes comes from studies of the yeast *Saccharomyces cerevisiae*. This particular yeast has been successfully used as a model for studies of the nuclear control of integrity and transmission of mtDNA. Since many of the nuclear factors that control maintenance of the mitochondrial genome have been conserved through evolution, the experimentally simple yeast system provides a valuable tool to track and characterize the key players.

The DNA unwinding enzymes, DNA helicases that are present in all organisms are involved in replication, repair, recombination and transcription. All these processes are required for faithful propagation of the mitochondrial genome. Currently, the list of isolated and partially characterized mitochondrial DNA helicases is rather short. Some functional aspects of the *S. cerevisiae* PIF1 helicase and the human Twinkle helicase have been described. In the present thesis I have attempted to characterize the novel *S. cerevisiae* mitochondrial DNA helicase Hmi1. In the literature overview section, I present some background information on mitochondrial DNA metabolism necessary for the understanding of the experimental work. Hopefully, the results summarized in my thesis will have an impact on the list of characterized mitochondrial DNA helicases and add novel information on nuclear factors required for the stability of mtDNA.

1. REVIEW OF LITERATURE

Mitochondria from all organisms have a genome that is distinct from that of the nucleus. According to the endosymbiotic hypothesis, mitochondrial genomes are remnants of the process of gene migration toward the nucleus (reviewed in Martin and Muller, 1998 and Gray et al., 1999). As a consequence, the mitochondrial genome encodes only a small number of genes required for oxidative phosphorylation. Also, rRNAs and tRNAs used by the protein synthesis machinery of the mitochondrion are encoded by the mitochondrial genome. All these proteins and RNAs are presumed to be essential for the respiratory function of the organelle, and therefore the integrity of mitochondrial genetic information must be maintained to ensure the cell-energy production. The functional state of mtDNA depends on a great number of factors involved in gene expression, DNA replication, repair and segregation of the mitochondrial genome. These processes are controlled by the cell nucleus, because the majority of mitochondrial proteins (95–98%) are encoded by nuclear genes and are imported into the mitochondria (reviewed in Neupert, 1997). Relatively little information is available on nuclear genes, that may directly or indirectly control maintenance and integrity of the mtDNA in higher eukaryotic organisms, however, we know more about these genes in the budding yeast *S. cerevisiae.* This yeast is a facultative aerobe that can grow by fermentation in the absence of mitochondrial gene expression and even in the absence of the intact mtDNA. Consequently, *S. cerevisiae* provides an excellent model system for classical and molecular genetics to identify the genes involved in mitochondrial genome maintenance. In this respect, important differences exist between yeast and higher eukaryotes: yeast is a unicellular facultative aerobe, it does not stably maintain a heteroplasmic state and the structure of its mtDNA differs remarkably from that of higher eukaryotes. Regardless of these facts, many of the nuclear factors that control the integrity of mtDNA and its transmission have been conserved throughout evolution. Thus, studying nuclear factors that control mitochondrial genome stability in yeast may help to find relevant nuclear genes in higher eukaryotes.

1.1. Organization of mtDNA

The mitochondrial genome carries genetic information for a small number of essential polypeptides of the oxidative phosphorylation system and of mitochondrion-specific rRNAs and tRNAs. Despite the differences in size, gene organization and expression mode, the basic gene content and function of mtDNA is extremely well conserved throughout evolution (reviewed in Attardi and Schatz, 1988).

The complete nucleotide sequence of mtDNA from many organisms is known (reviewed in Attardi, 1985). In vertebrate cells, mtDNA is present in multiple copies (usually 10^2–10^4 copies/cell). The mitochondrial genomes range in size from ~ 16 to 18 kb and can be isolated as closed circular molecules. The vertebrate mtDNA encodes 13 polypeptide components of the respiratory chain enzyme complexes located in the inner membrane of the mitochondrion. All these proteins are presumed to be essential for oxidative phosphorylation and hence for the production of cellular ATP. The mtDNA molecules also encode two ribosomal RNAs and a set of 22 transfer RNAs. Overall, the vertebrate mitochondrial genome is extremely compact, with each of the protein and rRNA genes directly flanked by at least one tRNA gene (Ojala et al., 1980; Bibb et al., 1981; Montoya et al., 1982).

The human mtDNA has been sequenced entirely and it is the most extensively investigated mitochondrial genome. It is a double-stranded, closed-circular molecule of 16,569 bp (Anderson et al., 1981). For several decades the human mtDNA was taken as a prototype of all other vertebrate and also of the yeast *S. cerevisiae* mtDNA.

The mtDNA of *S. cerevisiae* is almost five times larger than human mtDNA; the complete length of the mtDNA from the laboratory strain FY1679 was determined to be 85,8 kb (Foury et al., 1998). MtDNA accounts for 5–15% of the total cellular DNA in most *S. cerevisiae* strains (Nagley and Linnane, 1972). This corresponds to approximately 25–50 molecules of mtDNA per haploid cell. *S. cerevisiae* mtDNA encodes seven proteins of the oxidative phosphorylation machinery, two rRNAs, 24–25 tRNAs, one protein of the mitochondrial ribosomal small subunit, and one 9S RNA participating in the maturation of tRNAs. It contains up to 20 additional ORFs, most of them are located within introns (Fonty et al., 1978; Foury et al., 1998). An unusual feature of the yeast mtDNA is the high AT content; the average GC content is only 18% of the total nucleotide composition. It is also interesting that the AT and GC base pairs are highly clustered. About 50% of all mtDNA sequences are made of long AT-rich sequences, which range in size from 150 bp to 1500 bp. In contrast, the GC-rich sequences (~ 50–80 bp long), also called “GC clusters”, constitute only 2–3% of the total mtDNA. A large number (>150) of these GC clusters are dispersed throughout the mitochondrial genome, mostly in the intergenic regions (Grossman et al., 1971; Bernardi, 1976; Prunell and Bernardi, 1977; Fonty et al., 1978). The remaining part of mtDNA sequence is composed of genes with a relatively moderate GC content. It has been shown that the GC rich repeats are the preferential sites of recombination in mtDNA (Dieckmann and Gandy, 1987; Zinn et al., 1988).

Studies of mammalian mtDNAs generated a belief that mitochondrial genomes in all organisms are organized similarly. Based on genetic and restriction mapping data, it was generally accepted that the mitochondrial genome of *S. cerevisiae* and of other organisms as well is circular. This was supported by the hypothesis that mitochondria had evolved from bacteria. Most bacterial

genomes are known to be circular. For some time, it was suggested that large (~80 kb) circular mtDNA molecules were broken during extraction, and therefore appeared linear during electrophoresis analysis. Nonetheless, after nearly three decades this appeared to be a false track, and is now referred to as the "broken-circle theory" (reviewed in Williamson, 2002). The use of pulse-field gel electrophoresis, electron microscopy, mapping of fragments produced by digestion of mtDNA with restriction enzymes and moving microscopic pictures of stained mtDNA made it possible to test the theory of broken circles. Using these approaches, *S. cerevisiae* mtDNA was found to be a heterogeneous mixture of polydisperse linear molecules of various lengths (from 60 kb to several hundreds of kilobases), branched molecules and a minor fraction of circular molecules (Skelly and Maleszka, 1989; Maleszka et al., 1991; Maleszka and Clark-Walker, 1992; Fukuhara et al., 1993; Backert et al., 1995; Bendich, 1996; Jacobs et al., 1996). It is now clear that in general, linear mtDNA molecules are found in a variety of taxonomic groups, including fungi, plants and apicomplexan parasites and that small circular mitochondrial genomes occur in most cases in higher animals and kinetoplastids (reviewed in Nosek et al., 1998 and Nosek and Tomaska, 2003).

The two DNA strands of the mammalian mitochondrial genome can be distinguished according to their base content and can be separated after denaturation in cesium chloride density-gradient centrifugation. The strands are named based on their relative buoyant density as the heavy-strand (H-strand) and the light-strand (L-strand). The mtDNA molecule includes a triple-stranded regulatory region called the displacement loop (D-loop), in which a nascent H-strand DNA segment of 500–700 nt remains annealed to the parental L-strand. The D-loop is a major non-coding segment that supposedly contains the main regulatory elements for mtDNA transcription and replication. In mammals, the D-loop contains the unidirectional H-strand replication origin (O_H) and the promoters for light-strand (LSP) and heavy-strand (HSP) transcription (Chang and Clayton, 1984). Additionally, the D-loop structure from different animals contains three conserved GC-rich sequence blocks (CSB-s 1, 2, 3), termination-associated sequences and tRNA-like secondary structures. The CSBs are located near the transcription initiation site from where the RNA primer for mtDNA replication is synthesized. The second non-coding sequence in mammalian mtDNA is the origin of the L-strand (O_L), which is a region about 30 nt long that is located approximately 11 kb downstream of the heavy-strand origin (reviewed in Schmitt and Clayton, 1993).

The mitochondrial genome of the yeast *S. cerevisiae* contains seven or eight GC- rich conserved sequence elements called *ori* or *rep*. The *ori/rep* sequences are about 300 bp long and each of these regions contains three 100% conserved GC-rich blocks, A, B and C that are separated by AT-stretches. The *ori/rep* sequences also contain a nonanucleotide promoter adjacent to the *ori/rep* sequences block C (fig.1). However, only three or four of the *ori/rep* sequences

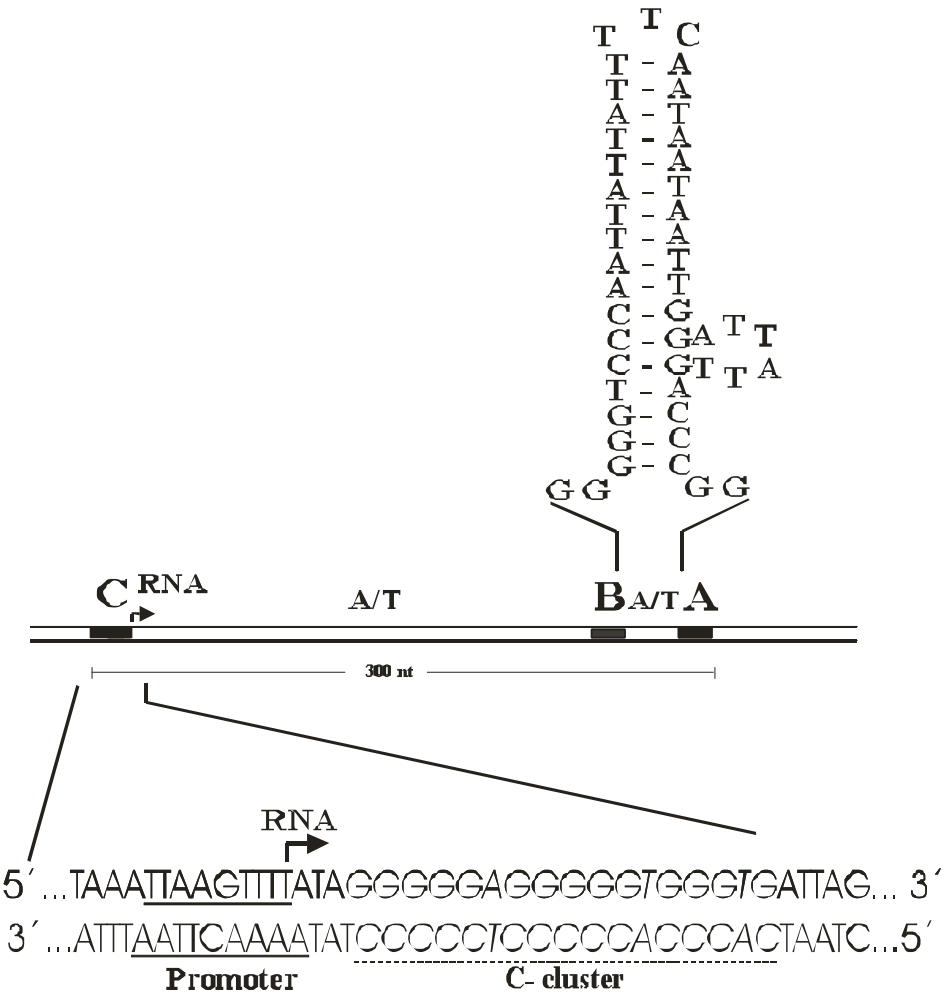

Figure 1. Schematic diagram of the *ori*2 and the DNA sequence near its C cluster. Conserved *ori* sequences are composed of three GC clusters C, B and A, separated by A/T stretches. The C cluster is in italics and underlined. The promoter element located upstream of the C cluster is underlined and the bent arrow marks the transcription start site and also the 5´-ends of the primers for promoter strand. The position of RNA synthesis is determined on the similarities with the model of the yeast replication initiation at the *ori*5 (Graves et al., 1998). The potential stem loop structure is formed by A and B cluster and an A/T rich sequence between them (based on the work by de Zamaroczy et al., 1984).

contain an uninterrupted canonical mitochondrial promoter motif that is thought to be active. The transcription start site is located upstream of GC cluster C in the active origins *ori* 1, 2, 3 and 5 (Baldacci and Bernardi, 1982; de Zamaroczy et al., 1984). A 34-bp inverted repeat formed by A and B blocks and an AT-rich region between them has a potential to form a stem-loop structure. By structural analogy with mammalian mtDNA, the yeast *ori/rep* sequences have been regarded as active origins of mtDNA replication, however, unlike O_H and O_L in mammalians, these *ori*s are thought to be bi-directional (Baldacci et al., 1984).

1.2. Rho$^-$ mutants

S. cerevisiae is one of the few eukaryotic organisms that can survive complete loss or extensive alterations of its mtDNA when a fermentable carbon source is provided. The mutants carrying a defective mitochondrial genome or no mtDNA at all are called cytoplasmic petite (small) yeast (Ephrussi et al., 1949). Petite yeast colonies grow slower than wild-type ones and are smaller in size on a respiratory medium, as the mutants are incapable of oxidative phosphorylation. They produce ATP by glycolysis and fermentation of glucose to ethanol, which provides them with much less ATP per mole of glucose than oxidative phosphorylation. Cytoplasmic petite mutations are irreversible and pleiotropic since the mutants are not able to perform mitochondrial protein synthesis. The mitochondrial mutants are called either rho^0 (without mtDNA) or rho$^-$ (truncated versions of mtDNA), while cells with wild type (wt) mitochondrial genome are called rho$^+$ (Ephrussi et al., 1949; Dujon, 1981). The frequency of spontaneous mutations from rho$^+$ to rho$^-$/rho^0 averages 1–2% per cell generation in many laboratory strains, but can be increased by drugs like ethidium bromide (Slonimski et al., 1968) acriflavine (Marcovich, 1951) or UV irradiation (Deutsch et al., 1974), starvation (Barclay and Little, 1978), high and low temperatures (Butow et al., 1973), and in brief, by any condition under which the yeast grows poorly.

Irrespective of the size of the deletion, the mass of mtDNA in rho$^-$ petites is approximately the same as the mass of mtDNA in wild-type cells. Thus, the number of the retained rho$^-$ mtDNA molecules per cell must be correspondingly amplified. If only a very short sequence is present, the amplification factor is considerablely high (e.g. 100 times or more) (Nagley and Linnane, 1972). There are two major types of arrangements of concatemeric repeats of rho$^-$ mtDNA; either the sequence is directly repeated with head-to-tail junctions (tandem rho$^-$) or the arrangement is of the inverted type (palindromic rho$^-$). Other versions of arrangements, including mixed repeats, can also be found in some rho$^-$ mutants (Morimoto et al., 1975; Lazowska and Slonimski, 1976; Morimoto et al., 1977). The bulk of mtDNA purified from rho$^-$ strains is composed of polydisperse linear concatemers and of branched molecules. Additionally, some rho$^-$ strains

contain a certain amount of small circular mtDNA molecules. The number of circles depends on the mutant strain, and some rho$^-$ mutants do not exhibit a considerable amount of circles (Lazowska and Slonimski, 1976). The same authors have concluded that small circles and linear fragments contain the same basic sequence and that the circles are either of the repeat unit size or oligomers of this unit. In some rho$^-$ mutants, two or more multimeric series of circles coexist in the cell. In each series the monomers are more frequent than dimers, which are in turn, more frequent than trimers, etc. Thus, the frequency of a given oligomer is inversely proportional to the number of repeat units it contains (Lazowska and Slonimski, 1976).

1.3. Suppressiveness of the rho$^-$ mutants

When two genetically marked rho$^+$ *S. cerevisiae* strains are mated, the resulting diploid segregates the different parental and recombinant mtDNAs within a few cell divisions. The resulting zygotic colonies are homoplasmic, containing individual cells with either one or the other of the input rho$^+$ genomes (Dujon et al., 1974; Dujon, 1981). Matings between a rho$^+$ and different rho$^-$ strains can result in uniparental inheritance, delivering rather biased results compared to the outcomes of rho$^+$ x rho$^+$ crosses (Perlman, 1976; Birky et al., 1978). Petite genomes can be classified according to their behavior in genetic crosses with wt yeast cells and are characterized according to their suppressiveness that is being an inherent characteristic of individual rho$^-$ mitochondrial genomes. Some rho$^-$ strains give rise to zygotic diploid clones which all contain rho$^+$ cells. Such strains are called neutral petites. For most rho$^-$ strains, some fraction of the diploid progeny of rho$^+$ x rho$^-$ crosses inherits the rho$^-$ mtDNA and those are called suppressive strains (Ephruss and Grandchamp, 1965). A mutant that produces 25% rho$^-$ diploid cells is said to be 25% suppressive. The extent of suppressiveness generally depends on the fragment of the wt mtDNA retained in the petite mutant (Dujon, 1981). In some cases, the diploid progeny of rho$^+$ x rho$^-$ crosses composes almost exclusively of petite mutants. Rho$^-$ mutants that exhibit >95% zygotic suppressiveness are called hypersuppressive (HS rho$^-$). All HS mitochondrial genomes consist of tandem repeats of a short DNA fragment containing one of the active *ori/rep* sequences (*ori*s 1, 2, 3 and 5). Low or moderate suppressiveness likely results from random segregation of mtDNA molecules over cell division, while hypersuppressivity suggests that rho$^-$ mtDNA molecules are preferentially transmitted to the diploid progeny, despite the inherent growth advantage for rho$^+$ cells (Blanc and Dujon, 1980; Dujon, 1980; de Zamaroczy et al., 1981). It is still not clear whether the preferential inheritance of HS rho$^-$ genomes is caused by a replication and /or segregation advantage, or some other mechanism. Models have been proposed suggesting that the *ori/rep* sequences are putative origins of replication and that

the high density of *ori* sequences in a HS rho$^-$ strain provides a replication advantage over rho$^+$ or neutral rho$^-$ genomes (de Zamaroczy et al., 1979; de Zamaroczy et al., 1981; Baldacci et al., 1984). It has also been demonstrated that the uniparental inheritance in HS rho$^-$ x rho$^+$ crosses does not involve extremely preferential segregation of HS rho$^-$ mtDNA into the first zygotic buds compared with mtDNA of the neutral rho$^-$ strain. However, eight generations after mating, most of the diploid progeny cells of rho$^+$ x HS rho$^-$ crosses contain rho$^-$ mtDNA. This indicates that HS rho$^-$ mtDNA does not have an immediate segregation advantage in early zygote maturation (MacAlpine et al., 2001).

The degree of suppressiveness depends on the intact promoter element, since an alteration within the conserved nonanucleotide promoter motif in the active *ori5* blocks the preferential transmission of rho$^-$ genomes to the progeny in crosses with rho$^+$ cells (MacAlpine et al., 2001). The authors proposed that promoter dependent RNA primed replication influences the biased inheritance of HS rho$^-$ mtDNA. Early experiments demonstrated that mutants with deletions that include the promoter and the GC block C exhibit dramatically reduced inheritance bias (de Zamaroczy et al., 1981). However, it has been shown that preferential transmission of HS rho$^-$ genomes is maintained in the absence of transcription since the deletion of the gene encoding the catalytic subunit of mtRNA polymerase RPO41 did not change the biased transmission of HS rho$^-$ genome in matings between HS rho$^-$ and neutral petites (Lorimer et al., 1995).

As for now, only two nuclear genes have been described which influence the biased transmission of the rho$^-$ genomes. A point mutation in the gene encoding the helicase PIF1 increases supperssivity of all types of rho$^-$ mutants (Foury and Kolodynski, 1983) and mutations in the recombination junction-resolving enzyme Cce1 reverse the preferential inheritance of HS rho$^-$ genomes (Lockshon et al., 1995).

1.4. Mitochondrial DNA replication

MtDNA is replicated independently from nuclear DNA replication. Its replication takes place continuously throughout the cell cycle while nuclear DNA is replicated during the S phase (Bogenhagen and Clayton, 1977). The electron microscopic images of replication intermediates generated a generally accepted view that mammalian mtDNA replication proceeds via a strand-asynchronous, asymmetric displacement mechanism. The replication starts from a unidirectional O_H origin in the D-loop region and thereby generates a long single-stranded DNA (ssDNA) region. This could subsequently expose the O_L origin, localized two-thirds of the genome away, where the initiation of the opposite strand synthesis takes place (fig.2) (reviewed in Clayton, 1982 and Clayton, 1991).

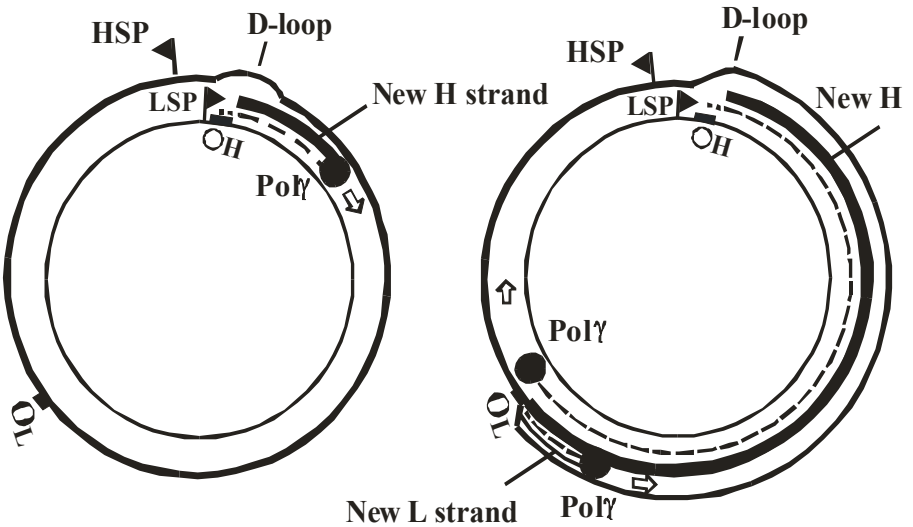

Figure 2. Schematic representation of human mtDNA replication (based on the work by Clayton, 1982). The two human mtDNA strands are designed as H and L strands. Replication origins O_H and O_L are unidirectional. The O_H is located immediately downstream of the LSP; O_L is physically separated from O_H on the DNA molecule. The LSP, HSP and O_H are located in the D-loop region. An RNA primer (tightly dotted line) is synthesized from LSP using L strand as template. The RNA primer is used by DNA polymerase γ to extend a new H strand (the open arrow shows the direction of replication). The L strand synthesis is initiated after the replication fork passes O_L and exposes the parental H strand in this region as a single strand.

The strand-asymmetric replication model prevailed for decades, but was questioned recently. It was suggested that mammalian mtDNA replication might possibly proceed via coupled leading and lagging strand synthesis (Holt et al., 2000; Yang et al., 2002; Bowmaker et al., 2003). The relevancy of strand-asymmetric and strand-coupled mechanisms has to be elucidated in future studies.

The replication mechanism in yeast mitochondria is still partially hypothetical. Mammalian mtDNA replication origins and yeast *ori/rep* sequences have structural and sequence homology, generating the idea that the yeast mtDNA replicates in a similar way. According to the strand-asymmetric model, mammalian mtDNA replication is dependent on mitochondrial transcription, since RNA transcripts initiated at the light strand promoter by mitochondrial RNA polymerase serve as primers for the synthesis of the H-strand DNA (reviewed in Clayton, 1991). RNA primers attached to the D-loop region of mouse mtDNA have been described (Chang and Clayton, 1985, 1986). By analogy, the transcription dependent initiation of yeast mtDNA replication at an *ori/rep* sequence has been proposed and some biochemical evidence supports

that model. S1 nuclease based technique was used for HS petites to identify RNA primers and RNA/DNA transition sites. The RNA 5' ends were mapped to the promoter and RNA/DNA transition was localized to the region of the GC cluster C (Baldacci et al., 1984). Also, *in vitro* capping of the RNA primers with guanylyltransferase to map the 5' ends for both strands demonstrated that the promoter strand is indeed primed from the promoter and that the majority of primers extend to the middle of cluster C. For the non-promoter strand, priming was also mapped near cluster C, but outside of the conserved *ori/rep* region (Graves et al., 1998) (fig. 3). Similar results were presented by MacAlpine

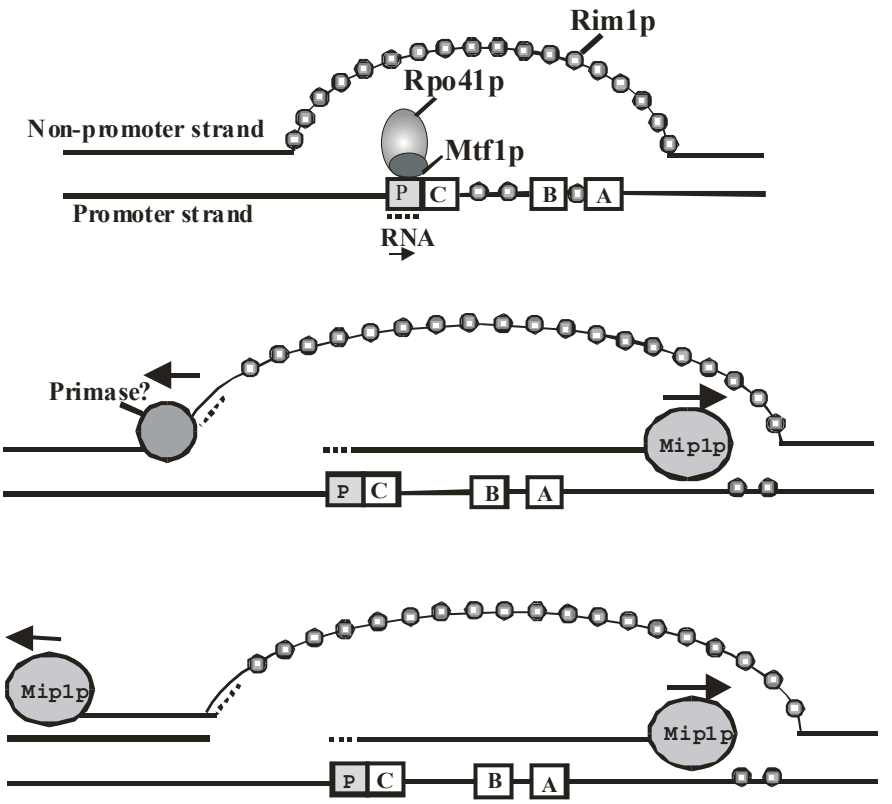

Figure 3. Yeast *S. cerevisiae* mtDNA replication initiation model. **A**. Replication origins contain GC clusters A, B, C and promoter (P). **B**. RNA polymerase RPO41 makes a short RNA primer (dotted line). The non-promoter strand primer would be synthesized by a primase, upstream of the promoter element. **B** and **C**. These RNA primers are used by Mip1 polymerase to elongate new DNA strands (continuous lines). The identities of nuclear factors involved in this process are Rpo41p – mitochondrial RNA polymerase, Mtf1p – mitochondrial transcription factor, Mip1p – mtDNA polymerase γ, Rim1p – ssDNA binding protein. The arrow shows the direction of replication. The RNA primers positions are based on the work by Graves et al. (1998).

et al., who demonstrated the presence of RNA-DNA hybrids that mapped to the promoter of an active *ori*5 (MacAlpine et al., 2001). No such RNA-DNA hybrids were detected in a rho$^-$ strain with a mutated *ori*5 promoter, even though the mitochondrial transcripts were present at comparable levels in both strains. This result indicated that the *ori*5 promoter is not the only site for the transcription initiation in this fragment of mtDNA; however, only transcripts initiated from the wt *ori*5 promoter were capable of priming the mtDNA replication.

The analysis of mtDNA isolated from neutral rho$^-$ and hypersuppressive rho$^-$ strains on two-dimensional (2D) gel electrophoresis resolved linear, oligomeric circular and supercoiled mtDNA molecules (MacAlpine et al., 2001). The major difference between the two mtDNAs was the presence of strand specific ssDNA molecules in the HS rho$^-$ strain while these molecules were absent in the neutral rho$^-$ strain. All ssDNA molecules originated from the strand of mtDNA that would be displaced by transcription from the HS mtDNA *ori*5 promoter. Based on these results the authors proposed that the transcription initiated at the *ori*5 promoter would generate transcripts through the GC box C. Subsequent elongation of the nascent strand displaces the complementary strand, resulting in the release of the ssDNA molecules (MacAlpine et al., 2001). These results are in accordance with the mammalian mtDNA asymmetric strand displacement model (Clayton, 1982).

Synthesis of the non-promoter strand (L-strand) during mammalian mtDNA replication is initiated at the O_L region that is thought to form a stable stem-loop structure when exposed as a single strand (fig. 2). This initiation requires primase activity, which can generate short RNA primer molecules (Wong and Clayton, 1985). Similarly, in *S. cerevisiae,* the non-promoter strand synthesis initiation has been suggested to be carried out by a primase (Baldacci et al., 1984). However, both in mammals and in yeast the corresponding protein or the gene encoding for primase has not been isolated so far.

While the existence of transcription-dependent mtDNA replication has been established in HS rho$^-$ and rho$^+$ *S. cerevisiae,* it is intriguing that cells lacking mitochondrial RNA polymerase Rpo41p are still able to maintain the rho$^-$ mtDNAs (Fangman et al., 1990; van Dyck and Clayton, 1998). Both hyper-suppressive and neutral rho$^-$ genomes are maintained in the *rpo41*Δ strain. It is likely that the yeast contains enzymatic machinery that provides an alternative mtDNA replication initiation system for rho$^-$ genomes, were Rpo41p is not involved. In addition, a petite genome consisting of only AT base pairs, with repeats as small as 35 bp is stably maintained (Fangman et al., 1989). Thus, neither Rpo41p or *ori/rep* sequences are required for rho$^-$ mtDNA replication and therefore the priming of mtDNA synthesis in yeast mitochondria might utilize a different mechanism.

It has been suggested that alternatively to the transcription-driven replication model, the replication of yeast mtDNA could involve a recombination-dependent mechanism (Zelenaya-Troitskaya et al., 1998). The mtDNA of yeast

undergoes continuous intra-mitochondrial recombination (Williamson and Fennell, 1974; Sena et al., 1976) and therefore the priming of mtDNA replication by recombination is quite plausible. The corresponding recombination-dependent replication (RDR) model proposes that DNA replication is initiated by recombination via priming from the 3' ends of single-strands that have invaded duplex DNA. RDR has been shown to be used for the replication of some *E. coli* plasmids and bacteriophage DNAs (Asai et al., 1994; Formosa and Alberts 1986). The yeast *S. cerevisiae* mtDNA preparations contain polydisperse linear molecules along with branched structures that were shown to accumulate in *cce1* mutants. These mutants lack activity of the enzyme that cleaves four-way DNA junctions, which are the equivalents of Holliday recombination intermediates (Lockshon et al., 1995). It has also been speculated that double-stranded breaks that are detected near GC clusters may represent recombination hot spots and therefore be involved in the initiation of a recombination-dependent replication (Dieckmann and Gandy, 1987; Zinn et al., 1988).

Additional support to the hypothesis that recombination may play a role in yeast mtDNA replication comes from studies of the apicomplexan parasite *Plasmodium falciparum.* The mtDNA of the malaria parasite has been shown to undergo massive recombination, similar to the process seen in yeast. It has been shown that the mtDNA replication of this parasite is largely achieved by a mechanism that is dependent on the recombination of linear tandem arrays that are possibly generated by rolling circle (RC) replication (Preiser et al., 1996). The RC mode of mtDNA replication has been also reported for several fungi, including *Torulopsis glabrata*, *Schizosahharomyces pombe* (Maleszka et al., 1991; Han and Stachow, 1994), and for mitochondrial plasmid mp1 from a higher plant *Chenopodium album* (Backert et al., 1995; 1996a; 1996b). These studies demonstrated that the replication intermediates (RIs) in mitochondria contain an unexpectedly large amount of long linear molecules, in size of several genome units. Furthermore, a large fraction of ssDNA molecules and dsDNA molecules containing ssDNA regions were found and some of the RIs exist as circular molecules with single- or double-stranded tails. It has also been observed that the mtDNA of rho$^-$ *S. cerevisiae* strains contain polydisperse linear mtDNA molecules along with circular molecules with long linear branches (Locker et al., 1974). The presence of such RIs in mitochondria of different organisms supports the possibility that the rolling-circle process generates mtDNA in *S. cerevisiae* as well in other fungi.

1.5. Nuclear genes of *S. cerevisiae* involved in mtDNA maintenance

Mutations that can lead to either complete loss of mtDNA or to production of defective rho$^-$ genomes have been identified in many yeast nuclear genes. They mostly encode proteins that are directly involved in mitochondrial genome replication, recombination, transcription and repair. Such examples include the mtDNA polymerase Mip1p, the DNA ligase Cdc9p and the ssDNA binding protein Rim1p. Additionally, the RNA polymerase Rpo41p is thought to be directly involved in mtDNA replication as it generates primers for the initiation of replication. Proteins involved in recombination include the DNA helicase Pif1p, the DNA binding protein Abf2p, the cruciform cutting endonuclease Cce1p, and the recombinase Mhr1p. Msh1p and Mgm101p are involved in mtDNA repair. The following section of this literature overview mainly focuses on above-mentioned proteins. However, a large number of other nuclear proteins are required for the integrity and maintenance of the mitochondrial genome in *S. cerevisiae.* For example, this list includes proteins acting in mitochondrial protein synthesis, nucleotide biosynthesis, mitochondrial inheritance and distribution. Proteins required for mitochondrial protein expression and for the import of proteins and metabolites into mitochondria also belong to this list (reviewed in Contamine and Picard, 2000).

1.6. Enzymes directly involved in mtDNA replication

MIP1

The MIP1 gene encodes the catalytic subunit of the yeast mtDNA polymerase (Foury, 1989). Mip1p is a large, 140 kDa protein that possesses 5'→ 3' DNA polymerase activity and 3'→ 5' exonuclease proofreading activity (Foury, 1989; Foury and Vanderstraeten, 1992). Deletion of this gene in a haploid *S. cerevisiae* strain leads to the loss of mtDNA (Foury, 1989). The heterozygous diploids containing a single copy of the MIP1 gene also generate rho$^-$ and rho^0 cells at elevated temperatures, indicating a gene dosage effect (Hu et al., 1995; Lecrenier and Foury, 1995).

CDC9

CDC9, the *S. cerevisiae* gene for DNA ligase, encodes two distinct polypeptide products that are translated from separate in-frame initiation codons. It has been shown that the longer form is targeted into mitochondria where it is required for mtDNA stability. The shorter polypeptide product is directed to the nucleus and is essential for nuclear DNA replication and cell viability (Willer et al., 1999). Inactivation of the mitochondrial Cdc9p leads to the depletion of mtDNA in

both dividing and stationary yeast cultures implicating the involvement of this enzyme in mtDNA replication and in stabilization of nonreplicating mtDNA (Wu et al., 1999; Donahue et al., 2001).

RIM1

Similarly to MIP1 and CDC9 gene deletions, *rim1*Δ cells become rho^0. Rim1p is the mitochondrial single-stranded DNA binding protein (SSB) in *S. cerevisiae* that was originally isolated as a multicopy suppressor of Pif1 helicase mutations. The primary structure of the RIM1 gene product is homologous to the SSB in *E. coli* and to the mitochondrial SSB in *Xenopus laevis* (van Dyck et al., 1992).

RPO41/MTF1

The catalytic subunit of the mitochondrial RNA polymerase Rpo41p has been thought to act directly in mtDNA replication through initiation of DNA strand synthesis or indirectly through mitochondrial protein synthesis. Inactivation of Rpo41p leads to the production of petite cells and both, hypersuppressive and neutral petite genomes are stably maintained in *rpo41* strains (Fangman et al., 1990). Thus, transcription is essential for the maintenance of rho$^+$ genomes but is dispensable for the maintenance of rho$^-$ genomes.

The initiation of transcription in yeast mitochondria by the catalytic subunit of the mitochondrial RNA polymerase requires an additional factor for promoter recognition. This factor is called Mtf1p (Schinkel et al., 1987). The disruption of MTF1 leads to the petite phenotype but it is not known whether rho^0 or rho$^-$ petites are produced (Lisowsky and Michaelis, 1988).

The aforementioned enzymes are strictly required for the maintenance of wt mtDNA. Several other replication enzymes and the corresponding genes still have to be characterized in yeast *S. cerevisiae* mitochondria. Such unidentified enzymes include a replicative DNA helicase, a primase and topoisomerases. Identification of these factors could provide more insight into the yeast mtDNA replication process as well as into the repair and recombination of the yeast mitochondrial genome.

1.7. Proteins involved in the repair and/or recombination of mtDNA

MSH1

The search for genes involved in mtDNA repair led to the identification of the MSH1 gene. The yeast MSH1 gene encodes a mitochondrial protein that is a homologue of the MutS protein of the bacterial MutHLS DNA mismatch repair system. Msh1p possesses ATPase activity and recognizes DNA structures

containing mismatches *in vitro* (Chi and Kolodner, 1994). Disruption of the MSH1 locus leads to rapid accumulation of rho$^-$ mutations, which are mostly hypersuppressive (Reenan and Kolodner, 1992). The heterozygous msh1/MSH1 strain exhibits a high rate of point mutations and mtDNA instability (Chi and Kolodner, 1994).

PIF1

Pif1p was the first DNA helicase described and characterized in yeast mitochondria. The descriptive name for PIF1 stands for "petite integration frequency", since *pif1* mutations prevent high-frequency recombination between rho$^+$ and tandem rho$^-$ genomes. Such tandem rho$^-$ mutants were called PIF-dependent. In contrast, the high frequency recombination was not affected in crosses with palindromic rho$^-$ mutants and thus, these mutants were named PIF-independent (Foury and Kolodynski, 1983). Sequence analysis of a number of related tandem rho$^-$ genomes located in the *ery* region of the 21S rRNA gene led to the identification of the recombinogenic signal. The signal was localized to the junctions between two tandem units in all PIF-dependent clones and was composed of a 41bp A+T sequence that itself exhibited a perfect palindrome over 26 bp. Similar but not identical recombination signal was found in the *oli1* region and therefore the authors proposed that the signal is possibly not sequence-specific but rather related to the topology of rho$^-$ DNA. The signal sequence may form the cruciform type of secondary structure and it has been proposed that PIF1 helicase may be involved in the initiation of recombination by melting these structures (Foury and van Dyck 1985). In addition to the effect on recombination frequencies, *pif1* mutations dramatically increase the suppressiveness of homozygous rho$^+$ *pif11* x rho$^-$ *pif1* crosses. High transmission of rho$^-$ mtDNA to diploid progeny was observed when diploids were homozygous for *pif1* mutations and this occurred with all types of rho$^-$ (head-to tail or inverted types) genomes. The high transmission frequency was not dependent on the size or the sequence of the repeat (Foury and Kolodynski, 1983). *pif1* mutations are highly pleiotropic, since in addition to their specific effect on recombination frequency and suppressivity, Pif1p is required for the repair of mtDNA after UV light and ethidium bromide treatments. Pif1p is also required for the maintenance of mtDNA at elevated temperatures, since disruption of the PIF1 ORF causes loss of functional mtDNA at 37^o, but not at 25ºC (Foury and Kolodynski, 1983; Foury and Lahaye, 1987). Pif1p was overexpressed and purified from mitochondrial lysates and it was shown that the protein is a single-stranded DNA-dependent ATPase and DNA helicase that unwinds duplex DNA in a 5'$\rightarrow$ 3'direction. Long duplex DNA-structures were poorly unwound by Pif1p, indicating that the protein is a distributive enzyme. Thus, the PIF1 helicase is required for a specific type of recombination and repair in mitochondria, but it is not essential for the replication of yeast mtDNA since the protein is not required for the maintenance of mtDNA at normal

growth temperatures (Lahaye et al., 1991; 1993). Interestingly, it was demonstrated later that Pif1p also acts in the nucleus, inhibiting telomere elongation and *de novo* telomere formation (Schulz and Zakian, 1994). PIF1 encodes two forms of helicase through the alternative use of two AUG codons: the longer form localizes in the mitochondria and the shorter form is directed to the nucleus (Lahaye et al., 1991).

ABF2

Abf2p, a member of the mitochondrial HMG protein family was isolated through its ability to bind yeast autonomously replicating sequences (Diffley and Stillman, 1991). Abf2p is required for the maintenance of the mitochondrial genome since cells with a null allele of ABF2 rapidly loose their rho^+ mtDNA. However, when *abf2*Δ cells are grown on a nonfermentable carbon source such as glycerol, the rho^+ mtDNA is maintained indefinitely (Diffley and Stillman, 1991). It has also been demonstrated that Abf2p is not required for the maintenance of mtDNA in HS rho^- cells (Zelenaya-Troitskaya et al., 1998). Abf2p is the most abundant DNA binding protein in yeast mitochondria; there is enough of that protein to bind mtDNA in intervals of 30 bp (Diffley and Stillman, 1992). Abf2p has been described as a histone-like DNA packaging protein that bends DNA and introduces negative supercoiles (Fisher et al., 1992). The level of recombination intermediates is reduced in *abf2*Δ cells and the moderate overproduction of Abf2p increases the copy number of mtDNA, indicating that Abf2p may have a role in recombination and mtDNA copy number control (MacAlpine et al., 1998; Zelenaya-Troitskaya et al., 1998). Several studies have demonstrated that Abf2p is directly associated with mtDNA within mitochondrial nucleoid structures. Each individual nucleoid is estimated to contain 3–4 copies of mtDNA and it has been proposed to be the inheritance unit of mtDNA (Lockshon et al., 1995). *In organello* formaldehyde crosslinking studies demonstrated that in the nucleoid enriched fraction up to 22 proteins are crosslinked to mtDNA; among them are Abf2p, Rim1p, Mgm101p (Kaufman et al., 2000) and Rpo41p (Chen et al., 2005).

MGM101 and MMM1

Previous studies had already shown that Mgm101p is indeed a component of highly enriched nucleoids and that this protein is required for mtDNA maintenance as disruption of the MGM101 gene leads to loss of mtDNA (Meeusen et al., 1999). It has been demonstrated that MGM101 is required for the maintenance of rho^+ and neutral rho^- genomes but not for maintenance of HS rho^- genomes (Zuo et al., 2002). The *mgm101* cells are highly sensitive to damages of mtDNA induced by UV irradiation, gamma rays, and H_2O_2 and these results led to the conclusion that Mgm101p might function in the repair of oxidatively damaged mtDNA (Meeusen et al., 1999). Mgm101p has been shown to coimmunoprecipate with the outer membrane protein Mmm1 from

cross-linked mitochondrial fractions (Meeusen and Nunnari, 2003). These authors demonstrated the existence of self-replicating two membrane-spanning structures (TMS) and proposed that Mgm101p and Mmm1p function together within this proteinaceous structure that spans the outer and inner mitochondrial membranes. Fluorescence deconvolution microscopy studies of cells coexpressing Mgm101p and Mmm1p demonstrated that subsets of nucleoids were labeled with these proteins and that the localization signal of these proteins partially overlapped. In contrast, the DNA packaging protein Abf2 labeled the entire population of nucleoids. Since the mtDNA polymerase Mip1p was also localized to the subset of nucleoids that were labeled with Mgm101 and Mmm1 proteins, the authors proposed that TMS is associated specifically with nucleoid structures where mtDNA is replicating (Meeusen and Nunnari, 2003).

MHR1

The relationship between recombination and repair of mtDNA was evident in *mhr1* mutants. Some of the mutant *mhr1* cells exhibited enhanced UV-induced production of rho$^-$ progeny and showed temperature sensitivity; the others displayed deficiency in gene conversion-type recombination (Ling et al., 1995). *In vitro* studies demonstrated that Mhr1p has the activity to pair ssDNA and homologous double-stranded DNA (dsDNA) to form heteroduplex joints, indicating that Mhr1p plays a role in homologous mtDNA recombination (Ling and Shibata, 2002). The same authors proposed that the Mhr1p-dependent pathway mainly forms linear mtDNA concatemers and that these concatemers could be the intermediates that are processed into circular monomers upon mtDNA partitioning into buds. This assumption was based on the observation that linear concatemers were predominant in mother cells and nondividing cells, whereas circular monomers were the major form of mtDNA in growing buds (Ling and Shibata, 2002). The finding that overexpression of Mhr1p accelerates the vegetative segregation of mitochondrial alleles in growing heteroplasmic zygotes while the mutated Mhr1p causes its delay, indicates that MHR1 is acting in the production of homoplasmic cells from heteroplasmic ones (Ling and Shibata, 2004).

MGT1/CCE1

The nuclear gene MGT1 was identified through a mutation reversing the outcome of rho$^+$ × HS rho$^-$ matings. Deletion of the MGT1 gene switches the transmission bias; rho$^+$ mtDNA rather than HS rho$^-$ mtDNA is inherited in most zygotes (Zweifel and Fangman, 1991). The MGT1 gene was independently identified as CCE1 gene using a screen for yeast mutants defective in the cleavage of recombination junctions (Kleff et al., 1992). Deletion of CCE1 causes only a slight increase in petite production; mostly rho^0 cells are produced. The CCE1 gene encodes a mitochondrial enzyme that cleaves four-way DNA

junctions, which are equivalents of the Holliday recombination intermediates (Kleff et al., 1992). Cce1p localizes to the mitochondria where the protein is associated with the inner membrane (Ezekiel and Zassenhaus, 1993). Deletion of the CCE1 gene had a dramatic effect on the distribution of mtDNA. The DAPI-staining of *cce1* rho$^-$ cells revealed only a few bright nucleoids instead of the more numerous and diffused nucleoids found in CCE1 rho$^-$ cells. The decrease in the number of nucleoid structures would obstruct the segregation of the mtDNA into the daughter cells and thus increase the fraction of daughter cells that become rho^0 (Lockshon et al., 1995). The HS rho$^-$ mtDNA isolated from *cce1*Δ and CCE1 cells was digested with restriction endonucleases and the topology of the DNA fragments was analyzed by 2D gel electrophoresis. The analysis demonstrated an increased level of branched DNA structures in the *cce1*Δ strain compared to the CCE1 strain. Both X forms and Y structures were present in the *cce1*Δ rho$^-$ strain indicating that tandemly repeated rho$^-$ mtDNA molecules were linked together by recombination junctions into larger networks. The analysis of CCE1 deletions in rho$^+$ strains revealed similar increased level of branched DNA structures (Lockshon et al., 1995). Thus, the large networks of mtDNA created by unresolved recombination structures in the *cce1*Δ strains contribute to a decrease in the number of nucleoids and also to a brighter staining of these structures.

1.8. Concluding remarks

Despite decades of intensive efforts, molecular processes ensuring the stability of mtDNA are not completely understood. Replication is one of the crucial processes in the maintenance of the mitochondrial genome. The strand-asymmetric replication model for the mammalian mtDNA was proposed as early as in the beginning of the 1980s. However, recently that mode of replication was challenged and the coupled leading-and lagging-strand mtDNA replication was proposed. Thus, the relevance of either one of these models remains to be determined in future studies. The replication process of mtDNA in *S. cerevisiae* is even more questionable. Several models for the initiation of replication and for the propagation of the mtDNA have been proposed, including a hypothesis of RDR, transcription dependent replication and RC models. Not only mechanisms for replication, but also recombination, repair and segregation of mtDNA have been underinvestigated. Identification and isolation of novel nuclear factors that participate in metabolic processes mentioned above, may help in understanding how these processes control the mtDNA stability.

2. RESULTS AND DISCUSSION

2.1 Aims of the study

Maintenance and integrity of the *S. cerevisiae* mitochondrial genome requires a large number of nuclear factors involved in a broad variety of metabolic processes. Deletion of genes and the search for mutations causing development of rho^0 or rho^- phenotypes have revealed a number of players essential for rho^+ mtDNA maintenance. An important group of these proteins encoded by nuclear genes are involved in replication, transcription, recombination and repair of mtDNA. Some gene products involved in these processes have been characterized; however, more information is needed to supplement the gaps in the present understanding of the faultless propagation of the yeast mtDNA. DNA helicases play an essential role in the aforementioned metabolic processes. The first thoroughly studied mitochondrial DNA helicase from *S. cerevisiae* was the product of the nuclear PIF1 gene (Foury and Lahaye, 1987; Lahaye et al., 1991). In the present thesis I, together with my colleagues, characterize a novel yeast mitochondrial DNA helicase Hmi1p encoded by the yeast *S. cerevisiae* nuclear genome open reading frame YOL095c. The results of my work are described in following sections.

2.2. Hmi1p is an ATP dependent 3'→ 5' DNA helicase (I, II)

DNA helicases are enzymes that use the energy obtained from hydrolysis of nucleoside triphosphates (NTPs) to unwind the double-stranded DNA. In the course of these theses, I have revealed some functions of a novel DNA helicase encoded by the ORF YOL095c of the *S. cerevisiae* nuclear genome. The gene has been named HMI1 for "Helicase in Mitochondria" (Yeast Genomic Database) and it encodes a 80-kDa protein with seven conserved helicase motifs (I, fig. 1A). According to the classification by Gorbalenya and Koonin (1993), Hmi1p belongs to the helicase superfamily I. Our *in silico* analyses ascertained that the Hmi1 protein possesses similarity with several prokaryotic helicases including Rep and UvrD from *E. coli* and PcrA from *S. aureus*. The analyses of eukaryotes revealed HMI1 homologues only in evolutionarily close yeasts from the class of *Saccharomycetales*. Based on its structural homology we thought that Hmi1p could possess similar activities in yeast cells as the bacterial helicases Rep, UvrD and PcrA in bacterial cells do. The Rep protein is required for the replication of a number of ssDNA bacteriophages, including ϕX174 and M13 (Takahashi et al., 1979). PcrA helicase is involved in the replication of the plasmid pT181 (Iordanescu and Bargonetti, 1989; Iordanescu, 1993) and also in UV repair (Petit et al., 1998). This particular helicase may also play a role in

replication of chromosomal DNA, as the protein is essential for cell viability (Iordanescu, 1993). UvrD, also called DNA helicase II, is required in both methyl-directed mismatch repair (Grilley et al., 1993; Modrich, 1994) and UvrABC-mediated nucleotide excision repair (Caron et al., 1985). Neither Rep nor UvrD are essential in *E. coli* (Colasanti and Denhardt, 1987), however double mutants of *rep* and *uvrD* are lethal for bacteria (Fassler et al., 1985). *rep* mutations cause reduced rate of replication fork movement and therefore the Rep helicase likely plays a role in chromosomal DNA replication in *E. coli* (Lane and Denhardt, 1975). The Rep and UvrD proteins are both ssDNA-stimulated ATPases that catalyze unwinding of the duplex DNA with 3'→ 5'polarity (Kornberg et al., 1978; Yarranton and Gefter, 1979; Matson, 1986) while the PcrA helicase possess both 3'→ 5'and 5'→ 3' activities (Dillingham et al., 2001; Chang et al., 2002).

In order to clarify the role of Hmi1p in *S. cerevisiae*, we first decided to check if the protein encoded by the YOL095c ORF is a DNA helicase and for this purpose we overexpressed and purified the recombinant protein in *E. coli* by using the pGEX4T1-based expression system (I fig. 3A). When partially dsDNA was used as a substrate to analyze the activity of the protein, a 28-mer oligonucleotide was displaced from the DNA duplex (I fig. 3B). The purified recombinant Hmi1 protein had ATPase activity and this reaction was stimulated by ssDNA (II fig. 2). The II fig. 3 A demonstrates that the purified Hmi1p had a preference for duplex DNA with flanking 3' ssDNA. Additionally, the recombinant Pif1 helicase which possesses ssDNA stimulated ATPase and 5'→ 3' DNA helicase activities was included as a control in II fig. 2 and 3.

Thus, the purified recombinant Hmi1p has ssDNA stimulated ATPase and 3'→ 5' DNA helicase activities. As mentioned earlier, the bacterial homologues of Hmi1p, Rep and UvrD, possess similar ATPase and helicase activities, and in this respect these helicases resemble each other.

2.3. Hmi1p is essential for mitochondrial respiratory activity (I)

In oder to understand the Hmi1 helicase functions in yeast *S. cerevisiae*, we decided analyze the phenotypic effects of the HMI1 gene disruption. The internal fragment of HMI1 ORF containing the conserved helicase motifs II–IV was replaced with the *S. cerevisiae* TRP1 gene (I fig. 1A). It is likely that such replacement would destroy the helicase activity of the Hmi1 protein. We disrupted the HMI1 gene in both haploid W303-1A and diploid W303-1 yeast strains. The haploid W303-1A cells carrying only the disrupted copy of the HMI1 gene were able to grow on glucose containing synthetic complete media (SCM) but failed to grow on nonfermentable carbon sources (glycerol or glycerol/ethanol) indicating that the mutant strain had a mitochondrial

respiratory defect. The diploid HMI1/*hmi1*:: TRP1 strain with one copy of the disrupted HMI1 gene grew equally well on glucose and glycerol media, demonstrating that the observed defect was not dominant. After the sporulation of HMI1/*hmi1*:: TRP1 heterozygotes and tetrad analysis we got four viable spores on glucose media, of which two were respiratory competent and two respiratory deficient (I fig. 1 B left panel and C). Some proteins, such as Abf2p, are not required for the maintenance of the rho^+ genome when propagated on glycerol medium (Diffley and Stillman, 1991). To rule out the possibility that mtDNA was lost during propagation on glucose medium, we dissected tetrads directly on glycerol containing media. On these plates only two spores were viable (I fig. 1B right panel) indicating that Hmi1p is required for the maintenance of the rho^+ mtDNA when propagated on nonfermentable media. In addition, at a lower growth temperature (24°C), again only two spores out of four were able to grow. According to Lahaye et al. (1991), deletion of the second mitochondrial DNA helicase Pif1 affects the stability of mtDNA at 37°C but not at lower temperature (28°C), and therefore the Pif1 helicase is nonessential at regular growth conditions (Lahaye et al., 1991). The requirement of Hmi1 helicase in yeast respiratory competence appears to be more crucial because HMI1 is indispensable at normal growth temperature. Unlike *abf2* cells, which loose mtDNA when propagated on glucose medium, but retain the *rho*$^+$ genome on glycerol medium (Diffley and Stillman, 1991), the loss of the respiratory competency of *hmi1*Δ cells cannot be avoided on selective medium.

2.4. Hmi1p is not required for rho^- mtDNA maintenance (I)

The observed phenotypic effects of the HMI1 gene disruption prompted us to ask whether the integrity of mtDNA is affected in the *hmi1*Δ strain. The total DNA extracted from W303-1A and *hmi1*Δ strains was digested with restriction endonucleases, resolved on an agarose gel and probed with specific mitochondrial and nuclear probes (I fig. 2). The absence of COX2 signals on *hmi1*Δ DNA lanes indicated that the mutant strain lacked functional rho^+ mtDNA. Next, we stained the mutant *hmi1*Δ and W303-1A cells with DNA specific dye 4′,6′-diamidino-2-phenylinole (DAPI) and compared the specific mitochondrial nucleoid-staining pattern in these strains (I fig. 2B). Analyzes of the wt W303-1A cells revealed characteristic DAPI staining of small fluorescent extranuclear spots corresponding to mtDNA. On the other hand, similar staining pattern was lost in the majority of haploid *hmi1*Δ cells. However, some cells retained their mtDNA. We analyzed the cells from several colonies obtained by selection for TRP1 integration and found that in some colonies up to 30% of cells maintained the mtDNA. We also stained haploid *hmi1*Δ cells obtained through sporulation of the heterozygous HMI1/*hmi1*:: TRP1 diploid strain. In this case, the fraction of cells containing nucleoid structures was noticeably

lower, only 1–5%. However, all these cells failed to grow on glycerol-containing media indicating that these cells contain rho$^-$ mtDNA. A similar phenomenon has been observed in *rpo41*Δ strains. Disruption of the RPO41 gene in vegetative haploid cells led mainly to the accumulation of rho$^-$ genomes (Fangman et al., 1990), while cells obtained from haploid spores, which inherited the *rpo41* mutation through meiosis, were mainly rho^0 (Greenleaf et al., 1986).

Thus, the disruption of HMI1 led to a petite phenotype and both rho^0 and rho$^-$ cells were generated. We subcloned some of these rho$^-$ cells and four of those rho$^-$ strains were analyzed more thoroughly. Sequencing of the isolated rho$^-$ genomes showed that all four strains contained small head-to-tail tandem repeats. Crossing the wt W303 cells with rho$^-$ *hmi1*Δ strains revealed the following results. Two isolates exhibited >95% zygotic suppressiveness (HS61 and HS41), indicating that the mitochondrial genome of these mutants was hypersuppressive, and the other two isolates behaved as neutral (SK035 and SK048), since their diploid progeny had almost exclusively rho$^+$ mtDNA (the strains are described in I table 1). Taken together, these results demonstrate that the Hmi1 helicase is essential for the stability of wt rho$^+$ mtDNA but is not required for maintenance of the rho$^-$ mtDNA, and both hypersuppressive and neutral rho$^-$ genomes can be maintained in *hmi1*Δ strains.

The fact that HMI1 does not seem to be required for the maintenance of rho$^-$ mtDNA lead to the question whether the second mitochondrial DNA helicase PIF1 is essential in the *hmi1*Δ background for the stability of these rho$^-$ genomes. In order to assess this, we disrupted the PIF1 ORF in the four characterized rho$^-$ isolates and analyzed the presence of the mtDNA using *in situ* DAPI staining. Both hypersuppressive rho$^-$ strains retained their mtDNA in *hmi1*Δ *pif1*Δ cells. Unexpectedly, we did not obtain viable colonies of SK048 *pif1*Δ strain and currently we do not have the explanation for that phenomenon. The other neutral strain SK035 *pif1*Δ had lost mtDNA in all analyzed cells. The mitochondrial genome of the SK035 strain contains a fragment of the 21S rRNA gene. The putative PIF1-dependent recombinogenic signal is proposed to be present in this region (Foury and van Dyck 1985), and therefore that signal may play a role in the maintenance of this particular rho$^-$ genome. We have also analyzed the PIF1 disruption effects in SK035 cells transformed with pRS315-REP22, where the HMI1 gene is provided on a centromeric plasmid. Disruption of the PIF1 gene in SK035 pRS315-REP22 cells revealed that the rho$^-$ mtDNA was stably maintained (unpublished data). Thus, the Hmi1 and Pif1 helicases are dispensable for the stability of the analyzed HS rho$^-$ mtDNA, but either Hmi1p or Pif1p is needed for stable maintenance of the neutral SK035 mitochondrial genome.

2.5. Hmi1 helicase is localized in mitochondria (I)

The phenotypic effects caused by the disruption of the HMI1 gene prompted us to ask whether the Hmi1 helicase is transported into mitochondria. The Hmi1 protein was tagged with a hemagglutinin (HA) epitope at the N-terminus of the protein (HA-Hmi1) and expressed using the yeast expression vector pYCAH (Alexandre et al., 1993). The tagged Hmi1p is functional as we were able to get four viable spores on glycerol plates when the pYCAH-HMI1 plasmid was transformed into the heterozygous HMI1/hmi1:: TRP1 diploid strain prior to sporulation. Therefore, the hybrid protein expressed by pYCAH-HMI1 plasmid appeared to be suitable for localization studies. The W303-1A strain was transformed with the pYCAH-HMI1 plasmid. From these cells, mitochondria were isolated by differential centrifugation and further fractionated into outer membrane, intermembrane space, inner membrane, and matrix fractions (Daum et al., 1982). These fractions were analyzed by western blotting using the anti-HA antibody 12CA5 (HA), or antibodies against Tom40p (outer membrane protein), cytochrome c peroxidase (intermembrane space protein), or Mge1p (peripheral inner membrane protein) (I fig. 5A). The majority of HA-Hmi1 protein in cells was recovered in the mitochondrial fraction (I fig. 5A lane 1). The subfractions revealed that the Hmi1 protein copurifies with the inner membrane (I fig. 5A lanes 3 and 5) but not with intermembrane space or outer membrane (I fig. 5A lanes 2 and 4). To analyze specificity of the association of the Hmi1 protein with the inner membrane, mitochondria were extracted with 0,1 M sodium carbonate (pH 11,5) (I fig. 5B lanes 2 and 3) or as the control with TE (pH 8,0) (I Fig. 5B, lanes 4 and 5). The extraction with 0,1M sodium carbonate should make peripheral inner membrane proteins, but not integral membrane proteins soluble and neither of these proteins should dissolve during extraction with TE. Soluble and membrane-bound fractions were separated by centrifugation and visualized by western blotting using anti-HA antibody 12CA5. Antibodies against Yta10p (integral inner membrane protein) and Mge1p (peripheral inner membrane protein) served as controls. Hmi1p and Mge1p were recovered in the 0,1 M sodium carbonate supernatant (I fig. 5B lane 3) while the integral inner membrane protein Yta10p was recovered in the pellet fraction (I fig. 5B lane 2). Extraction with TE (pH 8.0) did not dissolve Hmi1p or other proteins investigated in this experiment (I fig. 5B lanes 4 and 5). These results demonstrated that Hmi1p is a mitochondrial matrix protein that is loosely associated with the inner membrane, but is not an integral membrane protein.

The biochemical fractionation analysis was complemented with *in situ* analysis using indirect immunostaining with the anti-HA antibody. W303-1A cells were transformed with the pYCAH-HMI1 plasmid and the localization of the HA-tagged Hmi1p was analyzed using staining of the cells with DAPI and simultaneous immunofluorescence detection of the HA-tag. Similar DAPI

staining was observed in W303-1A cells and in samples of cells transformed with pYCAH-HMI1 (I fig. 6 panels 1 and 3). The specific FITC staining of the HA tag colocalized with DAPI-stained mtDNA in pYCAH-HMI1 transformed cells but not in W303-1A control cells (I fig. 6 panels 2 and 4), showing that HA-Hmi1 protein expressed in these cells is transported into mitochondria. *In situ* staining experiments and biochemical fractionation were performed also with cells that lack endogenous Hmi1 protein. The results obtained were identical to those of the aforementioned experiments, confirming that Hmi1p localizes into mitochondria *in vivo*.

Thus, based on the fractionation and *in situ* immunostaining experiments we could show that Hmi1p is localized in mitochondria, and that the helicase is the part of the mitochondrial matrix being loosely associated with the inner membrane.

2.6. The unique C-terminal import signal targets the Hmi1 helicase into mitochondria *in vivo* (I)

So far we demonstrated that the Hmi1 protein is targeted into mitochondrial matrix. The next question was to elucidate the nature of the mitochondrial targeting signal of the Hmi1 helicase. Most proteins that are transported into mitochondrial matrix bear an N-terminal targeting signal and these proteins traverse mitochondrial membranes in N-to C-terminal direction (N→C). The N-terminal mitochondrial localization signal is usually situated within a stretch of 20–60 amino acids and forms an amphipathic α-helix. One side of the helix has many positively charged and hydroxylated amino acid residues and is devoid of negatively charged residues. The other side of the helix forms a hydrophobic surface (von Heijne, 1986). We found several acidic residues at the N-terminal region of the Hmi1 protein (I fig. 7A) and therefore we concluded that probably the N-terminus of this helicase does not contain the mitochondrial-targeting signal. However, there are no negatively charged residues in C-terminal segment of the Hmi1 protein and the 16 residues starting from Arg-691 are predicted to form an amphipathic α-helix. One side of the C-terminal helix is rich in basic and hydroxylated residues and the other side is hydrophobic (I fig. 7A and B). The C-terminal targeting signal is rather uncommon for mitochondrial proteins and, in fact, until 1999 no reports had been published describing the backward import of proteins into the mitochondrial matrix *in vivo*. However, it had been demonstrated *in vitro* that it is possible to direct artificial chimeras into mitochondria in the reverse direction (C→N) (Folsch et al., 1998). To clarify if the C-terminal segment of the Hmi1 protein indeed resembles the mitochondrial import signal, we made deletions in the HMI1 gene removing 32 (construct pYCAH-ΔC33Gly) or 14 (construct pYCAH-ΔC15Ala) amino acid residues from the C-terminus. The endpoints of

these deletion mutations are pointed out with arrows below the C-terminal segment in I fig. 7A. W303-1A cells were transformed with these constructs and the intracellular localization of the deleted proteins was analyzed by western blotting of whole-cell extracts and mitochondrial fractions. As I fig. 7 demonstrates, only the full-length Hmi1 protein is detected in the mitochondrial fraction (I fig. 7C lane 4) while ΔC33Gly and ΔC15Ala are not (I fig. 7C lanes 5 to 6). Our unpublished results have revealed that the deletion of only 5 residues from the C-terminal part of Hmi1 helicase (ΔC6Ser) does not completely abolish the import into mitochondria *in vivo*. These experiments indicate that the critical structural determinants of the Hmi1p import signal reside somewhere between residues Ala-693 and Val-703. The biochemical fractionation was complemented with staining of the cells with DAPI and with simultaneous immunofluorescence detection of HA tagged proteins (I fig. 6 panels 5 to 8). The DAPI staining of cells transformed with tagged mutants Hmi1 (ΔC 33Gly) and Hmi1 (ΔC15Ala), showed a characteristic spotted pattern (I fig. 6 panels 5 and 7). However, the specific FITC staining of HMI1 deletion mutants did not show a distinct colocalization pattern confirming that their transport into mitochondria was strongly impaired (I fig. 6 panels 6 and 8).

To further confirm the signaling role of the C-terminal part of the Hmi1 protein, the amino acid residues 616 to 706 of the Hmi1p were fused to mouse dihydrofolate reductase (DHFR) carrier protein and intracellular localization of this fusion protein was analyzed. As revealed by western blot analyses, the DHFR and the fusion protein DHFR Hmi1(616-706) were expressed at similar levels (I fig. 7D lanes 1 and 3), and were detected in crude mitochondrial fraction (not shown). However, when isolated crude mitochondria were treated with proteinase K, the DHFR Hmi1(616-706) fusion protein was protected from the cleavage but DHFR was not (I fig. 7D lanes 2 and 4). Thus, only the fusion protein was transported into the mitochondria *in vivo*. Additional experiments to determine the length of the C-terminal segment that targets the protein into mitochondria showed that at least 46 C-terminal amino acid residues of the Hmi1 helicase are needed for translocation (unpublished results). The results presented here are in accordance with *in vitro* studies, which demonstrated that the C-terminal segment of HMI1 targets the DHFR protein into mitochondrial matrix (Lee et al., 1999).

The C-terminal part of the Hmi1 protein contains several positively charged amino acid residues. Two arginines are located at positions 704 and 705 (I fig. 7A). These Arg residues were mutated to negatively charged aspartic acid residues and the intracellular location of the mutated Hmi1Asp12 protein was subsequently analyzed. As demonstrated in I Fig. 7D, only the wt Hmi1 protein was protected against proteinase K treatment, but not the mutant Hmi1(Asp12), indicating that import of the mutant protein into mitochondria was strongly impaired (I fig. 7D lanes 6 and 8).

The presented data demonstrate that Hmi1p has a unique C-terminal transport signal. To summarize briefly, the following facts support the

conclusion. First, deletion of the last 15 C-terminal amino acid residues from Hmi1p or introduction of two negatively charged residues into the C-terminus of the protein clearly blocks its import. Second, the C-terminal segment of Hmi1p targets hybrid DHFR protein into the mitochondria *in vivo*.

2.7. The Hmi1 helicase is not required for the transcription in mitochondria (I)

It is well known that mitochondrial transcription and gene expression are required for the stability of mtDNA (Myers et al., 1985) and that helicases are involved in aforementioned metabolic processes (reviewed in Matson et al., 1994). Previous experiments demonstrated that Hmi1 is a mitochondrial DNA helicase, however, the exact role of Hmi1p in mitochondria remained unclear. The structural homologues of Hmi1p, Rep and UvrD from *E. coli*, and PcrA from *S. aureus* are known to act as DNA helicases (Kornberg et al., 1978; Dillingham et al., 2001). Thus, it is unlikely that the Hmi1 helicase is needed for mitochondrial RNA processing or translation. However, the helicase activity could be required for mitochondrial transcription and therefore we wanted to elucidate whether the Hmi1 helicase plays a role in this process. Furthermore, similar mitochondrial DNA instability and production of neutral and HS rho$^-$ mtDNA clones in *hmi1*Δ and *rpo41*Δ strains imply the possibility that Hmi1p may function in some step of mitochondrial transcription or gene expression. In order to test this possibility, we analyzed mitochondrial RNA transcription in hypersuppressive *hmi1*Δ strains (HS61 and HS41). The mtDNA repeats of these rho$^-$ genomes contain the sequence of *ori*2, which has a promoter close to the conserved GC box C (fig. 1). Total RNA was prepared from these strains and the mitochondrial RNA was analyzed by dot blot hybridization using ^{32}P-labeled *ori*2 fragment as the probe (I fig. 4). Strong hybridization signals were observed in both HS *hmi1*Δ strains indicating active transcription from the *ori*2 promoter in these strains. We also transformed these rho$^-$ strains with pRS315-REP22 to test whether reintroduction of Hmi1p has an effect on mitochondrial transcription. We did not detect differences between RNA samples originating from either *hmi1*Δ or *hmi1*Δ pRS315-REP22 transformed cells. Thus, transcription is active in analyzed HS *hmi1*Δ strains and therefore the Hmi1 helicase is not essential for mitochondrial transcription. However, we do not rule out the possibility that disruption of the HMI1 gene has some different effect on transcription that was not detected in this assay system.

2.8. Hmi1p stimulates the synthesis of long concatemeric rho⁻ molecules (III)

The results described in previous chapters demonstrated that the Hmi1 protein is a DNA helicase that is transported into yeast mitochondria and is required for wt mtDNA stability but is dispensable for rho⁻ genome maintenance. Next, we asked whether the Hmi1 helicase has an effect on mitochondrial DNA topology in rho⁻ strains. MtDNA from rho⁻ petites has been analyzed by two-dimensional (2D) gel electrophoresis where the first dimension separates DNA molecules by mass, and the second dimension separates molecules by mass and shape (Fangman et al., 1989). According to MacAlpine et al., (2001) the yeast cells maintain the HS rho⁻ mtDNA as a heterogeneous mixture of DNA molecules that differ in size and topology. These authors demonstrated that 2D electrophoresis enables distinction of relaxed and supercoiled circular molecules from double- and single-stranded linear molecules and from molecules having a more complex topology (MacAlpine et al., 2001). To clarify the role of Hmi1p in mtDNA metabolism, we used the 2D gel system to separate various molecular species of mtDNA isolated from the strains HS61*hmi1*Δ and HS61 pRS315-REP22 (HS61HMI1). The mtDNA isoforms isolated from these strains differed mainly in two aspects (III fig. 2). First, a large fraction of concatemeric linear dsDNA molecules in HS61HMI1 appeared longer than 20 kb while the same type of molecules in HS61*hmi1*Δ were generally shorter than 10 kb. Second, the arc of ssDNA molecules was clearly visible in HS61HMI1. Contrary to this, the ssDNA molecules were absent in HS61*hmi1*Δ (III fig. 2 B and C). We also noticed slight differences in the population of branched molecules in both strains. There were more branched DNA molecules in HS61HMI1 and also these molecules were more uniformly distributed compared to HS61*hmi1*Δ. Further, denaturing alkaline conditions were used to analyze the heavily branched DNA molecules that did not migrate out of the gel wells during neutral electrophoresis. Denatured mtDNA molecules that originated from the HS61HMI1 strain were noticeably longer than those isolated from the HS61*hmi1*Δ strain (III fig. 2D). Thus, Hmi1 helicase stimulates the synthesis of long concatemeric rho⁻ mtDNA molecules.

Interestingly, DAPI staining of these strains demonstrated that the mtDNA is packed into few and large nucleoid structures in HS61HMI1 strain, while a number of smaller nucleoids were detected in HS61*hmi1*Δ strain. In one focal plane, 7(±2) nucleoids were visible in HS61HMI1 cells whereas 25(±5) nucleoids could be identified in HS61 *hmi1*Δ cells (III fig. 1B). Direct measurements of the amount of total mtDNA in both strains revealed similar values (III fig 1C). These results suggest that increase in the number of nucleoid structures correlates with the increase of smaller mtDNA concatemers. It has been demonstrated that deletion of the Holliday recombination junction resolving enzyme Cce1p causes aggregation of mtDNA and consequently

packing of these molecules into fewer brightly stained nucleoids (Lockshon et al., 1995). The production of long concatemeric rho$^-$ mtDNA molecules in the HS61HMI1 strain also causes reduction in the number of nucleoids and increase of their size. This indicates that the parting of mtDNA into nucleoids depends on the topology of the mtDNA.

The shorter mtDNA concatemers and the absence of ssDNA molecules in HS61*hmi1*Δ cells raised the question whether this phenomenon is unique or if other defects in the mtDNA maintenance system generate similar effects or suppress the defects caused by disruption of the HMI1 gene. It has been demonstrated that deletion of the mitochondrial RNA polymerase RPO41 is tolerated by rho$^-$ genomes (Fangman et al., 1990). It was therefore possible to test if blocking of transcription in mitochondria has an effect on the topology of rho$^-$ mtDNA in the HS61*hmi1*Δ strain. For this purpose the ORF encoding for RPO41 was disrupted in the strains HS61HMI1 and HS61*hmi1*Δ. Subsequent analysis of mtDNA isolated from these strains by 2D gel electrophoresis showed that ssDNA molecules were absent in both HS61*hmi1*Δ *rpo41*Δ and HS61HMI1*rpo41*Δ cells (III fig. 5). This result is in accordance with the data presented by MacAlpine and coauthors who demonstrated that the formation of ssDNA replication intermediates depends on the transcription from the active *ori* (*ori*5) promoter (MacAlpine et al., 2001). The dsDNA linear concatemers in the HS61*hmi1*Δ *rpo41*Δ strain were significantly longer than in the HS61*hmi1*Δ strain, but shorter than in the HS61HMI1 strain (III fig. 5). These results demonstrate that shortening of mtDNA molecules in the HS61*hmi1*Δ strain was influenced by active transcription in the mitochondria.

2.9. ATPase activity of Hmi1p is not essential for wt mtDNA maintenance and is dispensable for the synthesis of long concatemeric rho$^-$ molecules (III)

We demonstrated in references I and II that Hmi1p is a DNA helicase. To further characterize the properties of the Hmi1 helicase, we asked if the ATPase activity is essential for its biological function. A major characteristic feature of the helicase proteins of superfamily I is the presence of 7 conserved helicase motifs I, Ia, II, III, IV, V, VI (Gorbalenya and Koonin, 1993) (I fig. 1A). These conserved residues are essential for NTP hydrolysis and DNA unwinding. Two site-directed mutations were made to investigate their impact on Hmi1p functions. The lysine residue 32 in the helicase motif I was altered to methionine and the glutamate residue 211 in the helicase motif II to glutamine (III fig. 3A). These residues are highly conserved in different helicases; K32 coordinates the binding of ATP β and γ phosphate groups, and E211 is the putative ATP hydrolyzing residue (Walker et al., 1982). Identical mutations in other DNA helicases have been made to generate catalytically inactive

helicases. For example, the altered *E. coli* helicases UvrD K32M and PriA K230R have been shown to be deficient in ATPase activity and unwinding reactions (Zavitz and Marians, 1992; George et al., 1994). We have demonstrated that the recombinant Hmi1 E211Q protein lacks detectable ATPase and helicase activities (Kuusk et al., 2005).

Surprisingly, the plasmid shuffling experiments (III fig. 3D) indicated that the ATPase activity of the Hmi1 helicase was not essential for respiratory activity and wt mtDNA could be maintained in strains that contained only the mutated Hmi1p. However, in this assay system the wt mtDNA was clearly lost more frequently in strains that contained the mutated Hmi1p.

To test how enzymatic activity of the Hmi1 helicase affects the topology of rho$^-$ mtDNA, we analyzed mtDNAs purified from HS61(*hmi1*K32M) and HS61(*hmi1*E21Q) strains by 2D electrophoresis (III fig. 3E). The strains with the mutant Hmi1p contained long concatemeric mtDNA molecules similar to the strain with wt Hmi1 helicase. The only notable difference between the strains harboring the mutant Hmi1p and the strain HS61HMI1 was a dramatic decrease in the ssDNA molecule population in mutated strains (III, compare fig. 3E and fig. 2B). From these experiments we concluded that ATPase and helicase activities of Hmi1p are not required for the synthesis of long concatemeric rho$^-$ DNA molecules, however, these enzymatic activities of the Hmi1 helicase are required for the production of ssDNA molecules in the HS rho$^-$ strain. Additionally, we have analyzed the mtDNAs isolated from the neutral rho$^-$ SK035HMI1 and the ATPase defective SK035(*hmi1*K32M) and (*hmi1*E211Q) strains (unpublished data). All these strains contained long concatemeric mtDNA molecules similarly to the HS rho$^-$ strains, however, the ssDNA arc was not detectable in any of these neutral isolates. These ssDNA molecules were also absent in mtDNA samples isolated from HS61*hmi1*Δ *rpo41*Δ and HS61HMI1*rpo41*Δ strains (III fig. 5). Thus, transcription from the *ori*2 promoter and enzymatically active Hmi1 helicase is needed for the accumulation of ssDNA in rho$^-$ strains.

The purified Hmi1 protein possesses ATPase and 3'→ 5' helicase activities (I fig. 3B; II fig. 2 and 3); however, these enzymatic activities of Hmi1p are not essential for the synthesis of long concatemeric mtDNA molecules. It is also notable that the mutants Hmi1p(K32M) and Hmi1p(E211Q) partially supported the maintenance of wt mtDNA. In contrast, disruption of the HMI1 gene caused disappearance of the wt mtDNA. A similar phenomenon, where enzymatic activity of a helicase protein is nonessential, has been observed for *E. coli* PriA helicase. Deletion of the PriA is associated with very low viability (Nurse et al., 1991), but PriA mutants lacking helicase activity are viable and resistant to DNA damage. Even more intriguing is the fact that the ATPase deficient PriA protein is still capable of catalyzing the primosome assembly, which is its main function (Zavitz and Marians, 1992). The Hmi1 and PriA helicases do not share extensive sequence homology**,** however it is possible that these helicases have some functional homology. Both helicases bind dsDNA with a 3' overhang and

possess a 3'→ 5' helicase activity (Nurse et al., 1991). Also, PriA has some redundancy with Rep helicase (Seigneur et al., 1998), which is the closest structural homologue of the Hmi1p.

2.10. The Hmi1 helicase is required for the synthesis of full-length wt mtDNA (III)

Shortening of the concatemeric mtDNA molecules in the knockout strain HS61*hmi1*Δ prompted us to ask if the phenomenon is unique for the rho$^-$ strain or a similar effect can be observed in the case of rho$^+$ mtDNA. In order to clarify the question, we analyzed the mtDNA isolated from the yeast strain carrying a temperature sensitive (ts) mutant allele *hmi1-5*. The cells carrying the mutant allele gradually loose respiratory competence when incubated at 37^o C. The mtDNA molecules, prepared from the *hmi1-5* and wt W303-1A strains grown in glucose or in glycerol medium at 37^o C and at 28^o C, were separated on an one-dimensional gel (III fig. 4B). A large fraction of linear fragmented molecules was present in samples that originated from the *hmi1-5* strain compared to W303-1A, the wt strain, and fragmentation was even more significant in cells grown at 37^o C. At elevated temperature almost all DNA molecules in the strain *hmi1-5* were shorter than 20 kb. These data suggest that the Hmi1 helicase is required for the effective synthesis of full-length mtDNA in wt rho$^+$ cells. However, degradation or preferential transmission of fragmentized molecules to the daughter cells may also explain the defects observed in the *hmi1-5* strain.

Thus, the Hmi1 helicase is required for the wt mtDNA synthesis and for the effective production of long concatemeric rho$^-$ DNA molecules. However, the helicase activity of Hmi1p is essential neither for the wt mtDNA maintenance nor for the production of these long concatemeric rho$^-$ mtDNA molecules. On the other hand, the Hmi1 helicase is dispensable for the replication of rho$^-$ mitochondrial genomes since rho$^-$ mtDNA is maintained in the *hmi1*Δ strain. Two mitochondrial helicases have been described in the yeast *S. cerevisiae*, Pif1p and Hmi1p. We demonstrated that HS rho$^-$ DNA is stable in a *hmi1*Δ *pif1*Δ strain indicating that neither of those helicases is essential for HS rho$^-$ genome replication. The role of the Pif1 helicase has been suggested to be in mtDNA recombination and repair (Foury and Kolodynski, 1983; Foury, 1985). The evidence presented here demonstrates that the Hmi1 protein is strictly required for the maintenance of wt mtDNA. However its enzymatic activity is dispensable and therefore we think that the Hmi1p is probably not a replication fork helicase in yeast mitochondria.

The mammalian genome encodes a mitochondrial helicase called Twinkle, which has no homologue in yeast. Twinkle shares sequence homology with the bacteriophage T7 gp4 helicase/primase (Spelbrink et al., 2001). *In vitro*,

Twinkle displays 5'→ 3' helicase activity and forms a minimal mtDNA replisome together with the mtDNA polymerase γ and mtSSB (Korhonen et al., 2003; Korhonen et al., 2004). The data supports the idea that Twinkle is a replicative helicase. While Twinkle is likely the mammalian mitochondrial replicative helicase, the yeast helicase required for mtDNA replication still remains unidentified. Replication of the *S. cerevisiae* mtDNA is probably initiated from multiple sites of the 85 kb genome and the mtDNA molecules frequently undergo recombination (Williamson and Fennell, 1974; Sena et al., 1976). In contrast, the existence of a recombination process in mammalian mtDNA has been debated and only few repots have been published describing this process in mammalian mitochondria (Thyagarajan et al., 1996). Replication of the mammalian mtDNA can start unidirectionally from two origins (Clayton, 1982), and the mammalian mitochondrial genome is almost four times smaller (~16 kb) than the *S. cerevisiae* mtDNA. Thus, it is likely that the strategy for mtDNA replication differs between yeast and mammals. In yeast mitochondria, priming of the mtDNA replication by recombination has been proposed (Zelenaya-Troitskaya et al., 1998). Indeed, the analyses of yeast rho$^-$ mtDNA on 2D gels in the present study revealed replication intermediates (III fig. 2), which might have been generated through a recombination process coupled by rolling circle replication. Our *in vitro* studies demonstrated that the Hmi1 helicase is targeted to specific structures with 3' single-stranded overhangs, ssDNA – dsDNA junctions, and forked structure substrates with both 3' and 5' overhangs. In the case of flap structured substrates that mimicked the chain displacement structures, a short 3' ssDNA was required for efficient unwinding (Kuusk et al., 2005). These results refer to the possibility that Hmi1p possesses a role in mitochondrial recombination. It is also possible that Hmi1p may be required for replication elongation similarly to that was proposed for its bacterial homologues (Lane and Denhardt, 1975) or may facilitate replisome progression through regions containing secondary structures. This hypothesis is supported by results, which demonstrated that in the ts mutant *hmi1-5* strain wt mtDNA becomes fragmented (III fig. 4). Future experiments are needed to generate a detailed mechanistic picture of mtDNA replication in yeast and to clarify the function of Hmi1p in *S. cerevisiae* mitochondrial DNA maintenance.

CONCLUSIONS

During the course of investigation of the mitochondrial DNA helicase Hmi1 we have characterized several aspects of its functionality and its role in mitochondrial DNA metabolism.

The most important conclusions can be drawn as follows:

1. The Hmi1 helicase possesses ssDNA stimulated ATPase and 3'→ 5' DNA helicase activities.

2. Hmi1p is required for the maintenance of the rho^+ mitochondrial genome at normal (30^oC) growth temperature but is not required for rho^- mtDNA maintenance. Disruption of the HMI1 gene produces rho^0, neutral rho^- and hypersuppressive rho^- petite cells.

3. The unique C-terminal import signal targets the Hmi1 helicase into mitochondria *in vivo*. The protein localizes into the mitochondrial matrix where the protein is loosely associated with the inner membrane but it is not an integral membrane protein.

4. Hmi1p is not required for the transcription in mitochondria.

5. The Hmi1 helicase stimulates synthesis of long concatemeric rho^- DNA and ssDNA molecules.

6. ATPase activity of Hmi1p is not essential for the maintenance of wt mtDNA and is dispensable for the synthesis of long concatemeric rho^- molecules.

7. The Hmi1 helicase is required for the effective synthesis of full-length mtDNA in the wt rho^+ cells.

REFERENCES

Alexandre, C., Grueneberg, D.A., Gilman, M.Z. (1993) Studying heterologous transcription factors in yeast. *Methods: A Companion to Methods in Enzymology*, **5**, 147–155.

Anderson, S., Bankier, A.T., Barrell, B.G., de Bruijn, M.H., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlich, D.P., Roe, B.A., Sanger, F., Schreier, P.H., Smith, A.J., Staden, R. and Young, I.G. (1981) Sequence and organization of the human mitochondrial genome. *Nature*, **290**, 457–465.

Asai, T., Bates, D.B. and Kogoma, T. (1994) DNA replication triggered by double-stranded breaks in E. coli: dependence on homologous recombination functions. *Cell*, **78**, 1051–1061.

Attardi, G. (1985) Animal mitochondrial DNA: an extreme example of genetic economy. *Int Rev Cytol*, **93**, 93–145.

Attardi, G. and Schatz, G. (1988) Biogenesis of mitochondria. *Annu Rev Cell Biol*, **4**, 289–333.

Backert, S., Dorfel, P. and Borner, T. (1995) Investigation of plant organellar DNAs by pulsed-field gel electrophoresis. *Curr Genet*, **28**, 390–399.

Backert, S., Dorfel, P., Lurz, R. and Borner, T. (1996a) Rolling-circle replication of mitochondrial DNA in the higher plant Chenopodium album (L.). *Mol Cell Biol*, **16**, 6285–6294.

Backert, S., Lurz, R. and Borner, T. (1996b) Electron microscopic investigation of mitochondrial DNA from Chenopodium album (L.). *Curr Genet*, **29**, 427–436.

Baldacci, G. and Bernardi, G. (1982) Replication origins are associated with transcription initiation sequences in the mitochondrial genome of yeast. *EMBO J*, **1**, 987–994.

Baldacci, G., Cherif-Zahar, B. and Bernardi, G. (1984) The initiation of DNA replication in the mitochondrial genome of yeast. *EMBO J*, **3**, 2115–2120.

Barclay, B.J. and Little, J.G. (1978) Genetic damage during thymidylate starvation in Saccharomyces cerevisiae. *Mol Gen Genet*, **160**, 33–40.

Bendich, A.J. (1996) Structural analysis of mitochondrial DNA molecules from fungi and plants using moving pictures and pulsed-field gel electrophoresis. *J Mol Biol*, **255**, 564–588.

Bernardi, G. (1976) Organization and evolution of the mitochondrial genome of yeast. *J Mol Evol*, **9**, 25–35.

Bibb, M.J., Van Etten, R.A., Wright, C.T., Walberg, M. W., Clayton, D.A. (1981) Sequence and gene organization of mouse mitochondrial DNA. *Cell*, **26**, 167–180.

Birky, C.W., Jr., Demko, C.A., Perlman, P.S. and Strausberg, R. (1978) Uniparental inheritance of mitochondrial genes in yeast: dependence on input bias of mitochondrial DNA and preliminary investigations of the mechanism. *Genetics*, **89**, 615–651.

Blanc, H. and Dujon, B. (1980) Replicator regions of the yeast mitochondrial DNA responsible for suppressiveness. *Proc Natl Acad Sci U S A*, **77**, 3942–3946.

Bogenhagen, D. and Clayton, D.A. (1977) Mouse L cell mitochondrial DNA molecules are selected randomly for replication throughout the cell cycle. *Cell*, **11**, 719–727.

Bowmaker, M., Yang, M.Y., Yasukawa, T., Reyes, A., Jacobs, H.T., Huberman, J.A. and Holt, I.J. (2003) Mammalian mitochondrial DNA replicates bidirectionally from an initiation zone. *J Biol Chem*, **278**, 50961–50969.

Butow, R.A., Ferguson, M.J. and Cederbaum, A. (1973) Low-temperature induction of respiratory deficiency in yeast mutants. *Biochemistry*, **12**, 158–164.

Caron, P.R., Kushner, S.R. and Grossman, L. (1985) Involvement of helicase II (uvrD gene product) and DNA polymerase I in excision mediated by the uvrABC protein complex. *Proc Natl Acad Sci U S A*, **82**, 4925–4929.

Chang, D.D. and Clayton, D.A. (1984) Precise identification of individual promoters for transcription of each strand of human mitochondrial DNA. *Cell*, **36**, 635–643.

Chang, D.D. and Clayton, D.A. (1985) Priming of human mitochondrial DNA replication occurs at the light-strand promoter. *Proc Natl Acad Sci U S A*, **82**, 351–355.

Chang, D.D. and Clayton, D.A. (1986) Identification of primary transcriptional start sites of mouse mitochondrial DNA: accurate in vitro initiation of both heavy- and light-strand transcripts. *Mol Cell Biol*, **6**, 1446–1453.

Chang, T.L., Naqvi, A., Anand, S.P., Kramer, M.G., Munshi, R. and Khan, S.A. (2002) Biochemical characterization of the Staphylococcus aureus PcrA helicase and its role in plasmid rolling circle replication. *J Biol Chem*, **277**, 45880–45886.

Chen, X.J., Wang, X., Kaufman, B.A. and Butow, R.A. (2005) Aconitase couples metabolic regulation to mitochondrial DNA maintenance. *Science*, **307**, 714–717.

Chi, N.W. and Kolodner, R.D. (1994) Purification and characterization of MSH1, a yeast mitochondrial protein that binds to DNA mismatches. *J Biol Chem*, **269**, 29984–29992.

Clayton, D.A. (1982) Replication of animal mitochondrial DNA. *Cell*, **28**, 693–705.

Clayton, D.A. (1991) Replication and transcription of vertebrate mitochondrial DNA. *Annu Rev Cell Biol*, **7**, 453–478.

Colasanti, J. and Denhardt, D.T. (1987) The Escherichia coli rep mutation. X. Consequences of increased and decreased Rep protein levels. *Mol Gen Genet*, **209**, 382–390.

Contamine, V. and Picard, M. (2000) Maintenance and integrity of the mitochondrial genome: a plethora of nuclear genes in the budding yeast. *Microbiol Mol Biol Rev*, **64**, 281–315.

Daum, G., Bohni, P.C. and Schatz, G. (1982) Import of proteins into mitochondria. Cytochrome b2 and cytochrome c peroxidase are located in the intermembrane space of yeast mitochondria. *J Biol Chem*, **257**, 13028–13033.

de Zamaroczy, M., Baldacci, G. and Bernardi, G. (1979) Putative origins of replication in the mitochondrial genome of yeast. *FEBS Lett*, **108**, 429–432.

de Zamaroczy, M., Faugeron-Fonty, G., Baldacci, G., Goursot, R. and Bernardi, G. (1984) The ori sequences of the mitochondrial genome of a wild-type yeast strain: number, location, orientation and structure. *Gene*, **32**, 439–457.

de Zamaroczy, M., Marotta, R., Faugeron-Fonty, G., Goursot, R., Mangin, M., Baldacci, G. and Bernardi, G. (1981) The origins of replication of the yeast mitochondrial genome and the phenomenon of suppressivity. *Nature*, **292**, 75–78.

Deutsch, J., Dujon, B., Netter, P., Petrochilo, E., Slonimski, P.P., Bolotin-Fukuhara, M. and Coen, D. (1974) Mitochondrial genetics. VI. The petite mutation in Saccharomyces cerevisiae: interrelations between the loss of the p+ factor and the loss of the drug resistance mitochondrial genetic markers. *Genetics*, **76**, 195–219.

Dieckmann, C.L. and Gandy, B. (1987) Preferential recombination between GC clusters in yeast mitochondrial DNA. *EMBO J*, **6**, 4197–4203.
Diffley, J.F. and Stillman, B. (1991) A close relative of the nuclear, chromosomal high-mobility group protein HMG1 in yeast mitochondria. *Proc Natl Acad Sci U S A*, **88**, 7864–7868.
Diffley, J.F. and Stillman, B. (1992) DNA binding properties of an HMG1-related protein from yeast mitochondria. *J Biol Chem*, **267**, 3368–3374.
Dillingham, M.S., Soultanas, P., Wiley, P., Webb, M.R. and Wigley, D.B. (2001) Defining the roles of individual residues in the single-stranded DNA binding site of PcrA helicase. *Proc Natl Acad Sci U S A*, **98**, 8381–8387.
Donahue, S.L., Corner, B.E., Bordone, L. and Campbell, C. (2001) Mitochondrial DNA ligase function in Saccharomyces cerevisiae. *Nucleic Acids Res*, **29**, 1582–1589.
Dujon, B. (1980) Sequence of the intron and flanking exons of the mitochondrial 21S rRNA gene of yeast strains having different alleles at the omega and rib-1 loci. *Cell*, **20**, 185–197.
Dujon, B. (1981) *Mitochondrial Genetics and Function*. Cold Spring Harbor Laboratory Press.
Dujon, B., Slonimski, P.P. and Weill, L. (1974) Mitochondrial genetics IX: A model for recombination and segregation of mitochondrial genomes in saccharomyces cerevisiae. *Genetics*, **78**, 415–437.
Ephruss, B. and Grandchamp, S. (1965) [Studies on the Suppressivity of Respiratory-Deficient Yeast Mutants. I. Existence at the Cell Level of Various "Degrees of Suppressivity".]. *Heredity*, **20**, 1–7.
Ephrussi, B., Hottinguer, H., Chimenes, A.M. (1949) Action de l' acriflavine sur les levures. I. La mutation 'petites'-colonies. *Ann. Inst. Pasteur.*, **76**, 351–367.
Ezekiel, U.R. and Zassenhaus, H.P. (1993) Localization of a cruciform cutting endonuclease to yeast mitochondria. *Mol Gen Genet*, **240**, 414–418.
Fangman, W.L., Henly, J.W. and Brewer, B.J. (1990) RPO41-independent maintenance of [rho-] mitochondrial DNA in Saccharomyces cerevisiae. *Mol Cell Biol*, **10**, 10–15.
Fangman, W.L., Henly, J.W., Churchill, G. and Brewer, B.J. (1989) Stable maintenance of a 35-base-pair yeast mitochondrial genome. *Mol Cell Biol*, **9**, 1917–1921.
Fassler, J.S., Tessman, I. and Tessman, E.S. (1985) Lethality of the double mutations rho rep and rho ssb in Escherichia coli. *J Bacteriol*, **161**, 609–614.
Fisher, R.P., Lisowsky, T., Parisi, M.A. and Clayton, D.A. (1992) DNA wrapping and bending by a mitochondrial high mobility group-like transcriptional activator protein. *J Biol Chem*, **267**, 3358–3367.
Folsch, H., Gaume, B., Brunner, M., Neupert, W. and Stuart, R.A. (1998) C- to N-terminal translocation of preproteins into mitochondria. *EMBO J*, **17**, 6508–6515.
Fonty, G., Goursot, R., Wilkie, D. and Bernardi, G. (1978) The mitochondrial genome of wild-type yeast cells. VII. Recombination in crosses. *J Mol Biol*, **119**, 213–235.
Formosa, T. and Alberts, B. M. (1986). DNA synthesis dependent on genetic recombination:
characterization of a reaction catalyzed by purified bacteriophage T4 proteins. *Cell* **47**, 793–806.
Foury, F. (1989) Cloning and sequencing of the nuclear gene MIP1 encoding the catalytic subunit of the yeast mitochondrial DNA polymerase. *J Biol Chem*, **264**, 20552–20560.

Foury, F., and E. van Dyck. (1985) A PIF1-dependent recombinogenic signal in the mitochondrial DNA. *EMBO J*, **4**, 3525–3530.
Foury, F. and Kolodynski, J. (1983) pif mutation blocks recombination between mitochondrial rho+ and rho- genomes having tandemly arrayed repeat units in Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A*, **80**, 5345–5349.
Foury, F. and Lahaye, A. (1987) Cloning and sequencing of the PIF gene involved in repair and recombination of yeast mitochondrial DNA. *EMBO J*, **6**, 1441–1449.
Foury, F., Roganti, T., Lecrenier, N. and Purnelle, B. (1998) The complete sequence of the mitochondrial genome of Saccharomyces cerevisiae. *FEBS Lett*, **440**, 325–331.
Foury, F. and Vanderstraeten, S. (1992) Yeast mitochondrial DNA mutators with deficient proofreading exonucleolytic activity. *EMBO J*, **11**, 2717–2726.
Fukuhara, H., Sor, F., Drissi, R., Dinouel, N., Miyakawa, I., Rousset, S. and Viola, A.M. (1993) Linear mitochondrial DNAs of yeasts: frequency of occurrence and general features. *Mol Cell Biol*, **13**, 2309–2314.
George, J.W., Brosh, R.M., Jr. and Matson, S.W. (1994) A dominant negative allele of the Escherichia coli uvrD gene encoding DNA helicase II. A biochemical and genetic characterization. *J Mol Biol*, **235**, 424–435.
Gorbalenya, A.E., and E. V. Koonin. (1993) Helicases: amino acid sequence comparisons and structure-function relationships. *Curr. Opin. Struct. Biol.*, **3**, 419–429.
Graves, T., Dante, M., Eisenhour, L. and Christianson, T.W. (1998) Precise mapping and characterization of the RNA primers of DNA replication for a yeast hypersuppressive petite by in vitro capping with guanylyltransferase. *Nucleic Acids Res*, **26**, 1309–1316.
Gray, M.W., Burger, G. and Lang, B.F. (1999) Mitochondrial evolution. *Science*, **283**, 1476–1481.
Greenleaf, A.L., Kelly, J.L. and Lehman, I.R. (1986) Yeast RPO41 gene product is required for transcription and maintenance of the mitochondrial genome. *Proc Natl Acad Sci U S A*, **83**, 3391–3394.
Grilley, M., Griffith, J. and Modrich, P. (1993) Bidirectional excision in methyl-directed mismatch repair. *J Biol Chem*, **268**, 11830–11837.
Grossman, L.I., Cryer, D.R., Goldring, E.S. and Marmur, J. (1971) The petite mutation in yeast. 3. Nearest-neighbor analysis of mitochondrial DNA from normal and mutant cells. *J Mol Biol*, **62**, 565–575.
Han, Z. and Stachow, C. (1994) Analysis of Schizosaccharomyces pombe mitochondrial DNA replication by two dimensional gel electrophoresis. *Chromosoma*, **103**, 162–170.
Holt, I.J., Lorimer, H.E. and Jacobs, H.T. (2000) Coupled leading- and lagging-strand synthesis of mammalian mitochondrial DNA. *Cell*, **100**, 515–524.
Hu, J.P., Vanderstraeten, S. and Foury, F. (1995) Isolation and characterization of ten mutator alleles of the mitochondrial DNA polymerase-encoding MIP1 gene from Saccharomyces cerevisiae. *Gene*, **160**, 105–110.
Iordanescu, S. (1993) Characterization of the Staphylococcus aureus chromosomal gene pcrA, identified by mutations affecting plasmid pT181 replication. *Mol Gen Genet*, **241**, 185–192.
Iordanescu, S. and Bargonetti, J. (1989) Staphylococcus aureus chromosomal mutations that decrease efficiency of Rep utilization in replication of pT181 and related plasmids. *J Bacteriol*, **171**, 4501–4503.

Jacobs, M.A., Payne, S.R. and Bendich, A.J. (1996) Moving pictures and pulsed-field gel electrophoresis show only linear mitochondrial DNA molecules from yeasts with linear-mapping and circular-mapping mitochondrial genomes. *Curr Genet*, **30**, 3–11.

Kaufman, B.A., Newman, S.M., Hallberg, R.L., Slaughter, C.A., Perlman, P.S. and Butow, R.A. (2000) In organello formaldehyde crosslinking of proteins to mtDNA: identification of bifunctional proteins. *Proc Natl Acad Sci U S A*, **97**, 7772–7777.

Kleff, S., Kemper, B. and Sternglanz, R. (1992) Identification and characterization of yeast mutants and the gene for a cruciform cutting endonuclease. *Embo J*, **11**, 699–704.

Korhonen, J.A., Gaspari, M. and Falkenberg, M. (2003) TWINKLE Has 5' –> 3' DNA helicase activity and is specifically stimulated by mitochondrial single-stranded DNA-binding protein. *J Biol Chem*, **278**, 48627–48632.

Korhonen, J.A., Pham, X.H., Pellegrini, M. and Falkenberg, M. (2004) Reconstitution of a minimal mtDNA replisome in vitro. *EMBO J*, **23**, 2423–2429.

Kornberg, A., Scott, J.F. and Bertsch, L.L. (1978) ATP utilization by rep protein in the catalytic separation of DNA strands at a replicating fork. *J Biol Chem*, **253**, 3298–3304.

Kuusk, S., Sedman, T., Joers, P. and Sedman, J. (2005) Hmi1p from Saccharomyces cerevisiae mitochondria is a structure specific DNA helicase. *J Biol Chem*.

Lahaye, A., Leterme, S. and Foury, F. (1993) PIF1 DNA helicase from Saccharomyces cerevisiae. Biochemical characterization of the enzyme. *J Biol Chem*, **268**, 26155–26161.

Lahaye, A., Stahl, H., Thines-Sempoux, D. and Foury, F. (1991) PIF1: a DNA helicase in yeast mitochondria. *EMBO J*, **10**, 997–1007.

Lane, H.E. and Denhardt, D.T. (1975) The rep mutation. IV. Slower movement of replication forks in Escherichia coli rep strains. *J Mol Biol*, **97**, 99–112.

Lazowska, J. and Slonimski, P.P. (1976) Electron microscopy of analysis of circular repetitive mitochondrial DNA molecules from genetically characterized rho-mutants of Saccharomyces cerevisiae. *Mol Gen Genet*, **146**, 61–78.

Lecrenier, N. and Foury, F. (1995) Overexpression of the RNR1 gene rescues Saccharomyces cerevisiae mutants in the mitochondrial DNA polymerase-encoding MIP1 gene. *Mol Gen Genet*, **249**, 1–7.

Lecrenier, N. and Foury, F. (2000) New features of mitochondrial DNA replication system in yeast and man. *Gene*, **246**, 37–48.

Lee, C.M., Sedman, J., Neupert, W. and Stuart, R.A. (1999) The DNA helicase, Hmi1p, is transported into mitochondria by a C-terminal cleavable targeting signal. *J Biol Chem*, **274**, 20937–20942.

Ling, F., Makishima, F., Morishima, N. and Shibata, T. (1995) A nuclear mutation defective in mitochondrial recombination in yeast. *EMBO J*, **14**, 4090–4101.

Ling, F. and Shibata, T. (2002) Recombination-dependent mtDNA partitioning: in vivo role of Mhr1p to promote pairing of homologous DNA. *EMBO J*, **21**, 4730–4740.

Ling, F. and Shibata, T. (2004) Mhr1p-dependent concatemeric mitochondrial DNA formation for generating yeast mitochondrial homoplasmic cells. *Mol Biol Cell*, **15**, 310–322.

Lisowsky, T. and Michaelis, G. (1988) A nuclear gene essential for mitochondrial replication suppresses a defect of mitochondrial transcription in Saccharomyces cerevisiae. *Mol Gen Genet*, **214**, 218–223.

Locker, J., Rabinowitz, M. and Getz, G.S. (1974) Electron microscopic and renaturation kinetic analysis of mitochondrial DNA of cytoplasmic petite mutants of Saccharomyces cerevisiae. *J Mol Biol*, **88**, 489–507.

Lockshon, D., Zweifel, S.G., Freeman-Cook, L.L., Lorimer, H.E., Brewer, B.J. and Fangman, W.L. (1995) A role for recombination junctions in the segregation of mitochondrial DNA in yeast. *Cell*, **81**, 947–955.

Lorimer, H.E., Brewer, B.J. and Fangman, W.L. (1995) A test of the transcription model for biased inheritance of yeast mitochondrial DNA. *Mol Cell Biol*, **15**, 4803–4809.

MacAlpine, D.M., Kolesar, J., Okamoto, K., Butow, R.A. and Perlman, P.S. (2001) Replication and preferential inheritance of hypersuppressive petite mitochondrial DNA. *EMBO J*, **20**, 1807–1817.

MacAlpine, D.M., Perlman, P.S. and Butow, R.A. (1998) The high mobility group protein Abf2p influences the level of yeast mitochondrial DNA recombination intermediates in vivo. *Proc Natl Acad Sci U S A*, **95**, 6739–6743.

Maleszka, R. and Clark-Walker, G.D. (1992) In vivo conformation of mitochondrial DNA in fungi and zoosporic moulds. *Curr Genet*, **22**, 341–344.

Maleszka, R., Skelly, P.J. and Clark-Walker, G.D. (1991) Rolling circle replication of DNA in yeast mitochondria. *EMBO J*, **10**, 3923–3929.

Marcovich, H. (1951) [Effect of acriflavine on yeasts. VIII. Determination of the active component and study of euflavine.]. *Ann Inst Pasteur (Paris)*, **81**, 452–468.

Martin, W. and Muller, M. (1998) The hydrogen hypothesis for the first eukaryote. *Nature*, **392**, 37–41.

Matson, S.W. (1986) Escherichia coli helicase II (urvD gene product) translocates unidirectionally in a 3' to 5' direction. *J Biol Chem*, **261**, 10169–10175.

Matson, S.W., Bean, D.W. and George, J.W. (1994) DNA helicases: enzymes with essential roles in all aspects of DNA metabolism. *Bioessays*, **16**, 13–22.

Meeusen, S. and Nunnari, J. (2003) Evidence for a two membrane-spanning autonomous mitochondrial DNA replisome. *J Cell Biol*, **163**, 503–510.

Meeusen, S., Tieu, Q., Wong, E., Weiss, E., Schieltz, D., Yates, J.R. and Nunnari, J. (1999) Mgm101p is a novel component of the mitochondrial nucleoid that binds DNA and is required for the repair of oxidatively damaged mitochondrial DNA. *J Cell Biol*, **145**, 291–304.

Modrich, P. (1994) Mismatch repair, genetic stability, and cancer. *Science*, **266**, 1959–1960.

Montoya, J., Christianson, T., Levens, D., Rabinowitz, M., Attardi, D.(1982) Identification of initiation sites for heavy-strand and light-strand transcription in human mitochondrial DNA. *Proc Nat. Acad Sci USA*, **79**, 7195–7199.

Morimoto, R., Lewin, A., Hsu, H.J., Rabinowitz, M. and Fukuhara, H. (1975) Restriction endonuclease analysis of mitochondrial DNA from grande and genetically characterized cytoplasmic petite clones of Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A*, **72**, 3868–3872.

Morimoto, R., Lewin, A. and Rabinowitz, M. (1977) Restriction cleavage map of mitochonrial DNA from the yeast Saccharomyces cerevisiae. *Nucleic Acids Res*, **4**, 2331–2351.

Myers, A.M., Pape, L.K. and Tzagoloff, A. (1985) Mitochondrial protein synthesis is required for maintenance of intact mitochondrial genomes in Saccharomyces cerevisiae. *EMBO J*, **4**, 2087–2092.

Nagley, P. and Linnane, A.W. (1972) Cellular regulation of mitochondrial DNA synthesis in Saccharomyces cerevisiae. *Cell Differ*, **1**, 143–148.
Neupert, W. (1997) Protein import into mitochondria. *Annu Rev Biochem*, **66**, 863–917.
Nosek, J. and Tomaska, L. (2003) Mitochondrial genome diversity: evolution of the molecular architecture and replication strategy. *Curr Genet*, **44**, 73–84.
Nosek, J., Tomaska, L., Fukuhara, H., Suyama, Y. and Kovac, L. (1998) Linear mitochondrial genomes: 30 years down the line. *Trends Genet*, **14**, 184–188.
Nurse, P., Liu, J. and Marians, K.J. (1999) Two modes of PriA binding to DNA. *J Biol Chem*, **274**, 25026–25032.
Nurse, P., Zavitz, K.H. and Marians, K.J. (1991) Inactivation of the Escherichia coli priA DNA replication protein induces the SOS response. *J Bacteriol*, **173**, 6686–6693.
Ojala, D., Merkel, C., Gelfand, R. and Attardi, G. (1980) The tRNA genes punctuate the reading of genetic information in human mitochondrial DNA. *Cell*, **22**, 393–403.
Perlman, P.S. (1976) Genetic analysis of petite mutants of Saccharomyces cerevisiae: transmissional types. *Genetics*, **82**, 645–663.
Petit, M.A., Dervyn, E., Rose, M., Entian, K.D., McGovern, S., Ehrlich, S.D. and Bruand, C. (1998) PcrA is an essential DNA helicase of Bacillus subtilis fulfilling functions both in repair and rolling-circle replication. *Mol Microbiol*, **29**, 261–273.
Preiser, P.R., Wilson, R.J., Moore, P.W., McCready, S., Hajibagheri, M.A., Blight, K.J., Strath, M. and Williamson, D.H. (1996) Recombination associated with replication of malarial mitochondrial DNA. *EMBO J*, **15**, 684–693.
Prunell, A. and Bernardi, G. (1977) The mitochondrial genome of wild-type yeast cells. VI. Genome organization. *J Mol Biol*, **110**, 53–74.
Reenan, R.A. and Kolodner, R.D. (1992) Characterization of insertion mutations in the Saccharomyces cerevisiae MSH1 and MSH2 genes: evidence for separate mitochondrial and nuclear functions. *Genetics*, **132**, 975–985.
Schinkel, A.H., Koerkamp, M.J., Touw, E.P. and Tabak, H.F. (1987) Specificity factor of yeast mitochondrial RNA polymerase. Purification and interaction with core RNA polymerase. *J Biol Chem*, **262**, 12785–12791.
Schmitt, M.E. and Clayton, D.A. (1993) Conserved features of yeast and mammalian mitochondrial DNA replication. *Curr Opin Genet Dev*, **3**, 769–774.
Schulz, V.P. and Zakian, V.A. (1994) The saccharomyces PIF1 DNA helicase inhibits telomere elongation and de novo telomere formation. *Cell*, **76**, 145–155.
Seigneur, M., Bidnenko, V., Ehrlich, S.D. and Michel, B. (1998) RuvAB acts at arrested replication forks. *Cell*, **95**, 419–430.
Sena, E., Welch, J. and Fogel, S. (1976) Nuclear and mitochondrial DNA replication during zygote formation and maturation in yeast. *Science*, **194**, 433–435.
Skelly, P.J. and Maleszka, R. (1989) Isolation of mitochondrial DNA using pulsed field gel electrophoresis. *Nucleic Acids Res*, **17**, 7537.
Slonimski, P.P., Perrodin, G. and Croft, J.H. (1968) Ethidium bromide induced mutation of yeast mitochondria: complete transformation of cells into respiratory deficient non-chromosomal "petites". *Biochem Biophys Res Commun*, **30**, 232–239.
Spelbrink, J.N., Li, F.Y., Tiranti, V., Nikali, K., Yuan, Q.P., Tariq, M., Wanrooij, S., Garrido, N., Comi, G., Morandi, L., Santoro, L., Toscano, A., Fabrizi, G.M., Somer, H., Croxen, R., Beeson, D., Poulton, J., Suomalainen, A., Jacobs, H.T., Zeviani, M. and Larsson, C. (2001) Human mitochondrial DNA deletions associated with

mutations in the gene encoding Twinkle, a phage T7 gene 4-like protein localized in mitochondria. *Nat Genet*, **28**, 223–231.

Zavitz, K.H. and Marians, K.J. (1992) ATPase-deficient mutants of the Escherichia coli DNA replication protein PriA are capable of catalyzing the assembly of active primosomes. *J Biol Chem*, **267**, 6933–6940.

Zelenaya-Troitskaya, O., Newman, S.M., Okamoto, K., Perlman, P.S. and Butow, R.A. (1998) Functions of the high mobility group protein, Abf2p, in mitochondrial DNA segregation, recombination and copy number in Saccharomyces cerevisiae. *Genetics*, **148**, 1763–1776.

Zinn, A.R., Pohlman, J.K., Perlman, P.S. and Butow, R.A. (1988) In vivo double-strand breaks occur at recombinogenic G + C-rich sequences in the yeast mitochondrial genome. *Proc Natl Acad Sci U S A*, **85**, 2686–2690.

Zuo, X.M., Clark-Walker, G.D. and Chen, X.J. (2002) The mitochondrial nucleoid protein, Mgm101p, of Saccharomyces cerevisiae is involved in the maintenance of rho(+) and ori/rep-devoid petite genomes but is not required for hypersuppressive rho(-) mtDNA. *Genetics*, **160**, 1389–1400.

Zweifel, S.G. and Fangman, W.L. (1991) A nuclear mutation reversing a biased transmission of yeast mitochondrial DNA. *Genetics*, **128**, 241–249.

Takahashi, S., Hours, C., Chu, A. and Denhardt, D.T. (1979) The rep mutation. VI. Purification and properties of the Escherichia coli rep protein, DNA helicase III. *Can J Biochem*, **57**, 855–866.

Thyagarajan, B., Padua, R.A. and Campbell, C. (1996) Mammalian mitochondria possess homologous DNA recombination activity. *J Biol Chem*, **271**, 27536–27543.

van Dyck, E. and Clayton, D.A. (1998) Transcription-dependent DNA transactions in the mitochondrial genome of a yeast hypersuppressive petite mutant. *Mol Cell Biol*, **18**, 2976–2985.

van Dyck, E., Foury, F., Stillman, B. and Brill, S.J. (1992) A single-stranded DNA binding protein required for mitochondrial DNA replication in S. cerevisiae is homologous to E. coli SSB. *EMBO J*, **11**, 3421–3430.

Walker, J.E., Saraste, M., Runswick, M.J. and Gay, N.J. (1982) Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J*, **1**, 945–951.

Wallace, D.C. (1992) Diseases of the mitochondrial DNA. *Annu Rev Biochem*, **61**, 1175–1212.

Willer, M., Rainey, M., Pullen, T. and Stirling, C.J. (1999) The yeast CDC9 gene encodes both a nuclear and a mitochondrial form of DNA ligase I. *Curr Biol*, **9**, 1085–1094.

Williamson, D.H. (2002) The curious history of yeast mitochondrial DNA. *Nature rev*, **3**, 475–481.

Williamson, D.H. and Fennell, D.J. (1974) Apparent dispersive replication of yeast mitochondrial DNA as revealed by density labelling experiments. *Mol Gen Genet*, **131**, 193–207.

von Heijne, G. (1986) Mitochondrial targeting sequences may form amphipathic helices. *EMBO J*, **5**, 1335–1342.

Wong, T.W. and Clayton, D.A. (1985) In vitro replication of human mitochondrial DNA: accurate initiation at the origin of light-strand synthesis. *Cell*, **42**, 951–958.

Wu, X., Braithwaite, E. and Wang, Z. (1999) DNA ligation during excision repair in yeast cell-free extracts is specifically catalyzed by the CDC9 gene product. *Biochemistry*, **38**, 2628–2635.

Yang, M.Y., Bowmaker, M., Reyes, A., Vergani, L., Angeli, P., Gringeri, E., Jacobs, H.T. and Holt, I.J. (2002) Biased incorporation of ribonucleotides on the mitochondrial L-strand accounts for apparent strand-asymmetric DNA replication. *Cell*, **111**, 495–505.

Yarranton, G.T. and Gefter, M.L. (1979) Enzyme-catalyzed DNA unwinding: studies on Escherichia coli rep protein. *Proc Natl Acad Sci U S A*, **76**, 1658–1662.

SUMMARY IN ESTONIAN

Pärmi *Saccharomyces cerevisiae* mitokondriaalne DNA helikaas Hmi1

Mitokonder on eukarüootse raku organell, milles toimub palju erinevaid metaboolseid protsesse, oluliseim neist on ATP tootmine oksüdatiivse fosforüleerimise süsteemi (OXPHOS) abil (Attardi and Schatz, 1988). Mitokondril on oma iseseisev geneetiline süsteem, mis kodeerib vähese hulga hädavajalikke polüpeptiide, mis on vaja OXPHOS süsteemi toimimiseks (Foury et al., 1998). Valdav enamus mitokondri valkudest kodeeritakse tuumas, sünteesitakse tsütoplasmas ja seejärel transporditakse mitokondritesse (Neupert, 1997). Teadmine, et mutatsioonid mtDNAs põhjustavad geneetilisi haigusi, on suurendanud huvi protsesside vastu, mis tagavad mitokondriaalse genoomi stabiilsuse (Wallace, 1992). Nende bioloogiliste protsesside molekulaarsete mehhanismide mõistmiseks on püütud identifitseerida ja iseloomustada erinevaid valke, sealhulgas ka helikaase, mis osalevad mtDNA säilitamise protsessides. DNA helikaasid on ensüümid, mis harutavad lahti DNA kaksikahelaid, kasutades selleks nukleosiidtrifosfaatide hüdrolüüsil vabanevat energiat. Helikaasid funktsioneerivad erinevates rakulistes protsessides, mis vajavad üksikahelalist DNAd, näiteks DNA replikatsioonis, reparatsioonis, rekombinatsioonis ja RNA transkriptsioonis. Mitokondriaalsetest DNA helikaasidest on isoleeritud ja iseloomustatud tänaseks vaid kolm. Hiljuti kirjeldatud TWINKLE on arvatavasti imetajate mitokondriaalne replikatiivne 5'→ 3' DNA helikaas (Korhonen et al., 2003; Korhonen et al., 2004). Mutatsioonid selles valgus põhjustavad inimestel rasket mitokondriaalset haigust adPEO (autosomal dominant progressive external ophthalmoplegia), millega kaasnevad ulatuslikud deletsioonid mtDNAst (Spelbrink et al., 2001). Mudelorganismi pärmi *S. cerevisiae* mitokondriaalsetest helikaasidest on nüüdseks isoleeritud ja iseloomustatud 2 helikaasi. Esimesena kirjeldatud Pif1p osaleb mtDNA rekombinatsioonis ja reparatsioonis (Foury and Kolodynski, 1983). Tuuma lokaliseerununa inhibeerib Pif1p telomeeride pikenemist ja telomeeride *de novo* formeerumist (Schulz and Zakian, 1994). Teiseks identifitseeritud *S. cerevisiae* mitokondriaalseks helikaasiks on käesolevas doktoritöös iseloomustatud valk Hmi1p. Hmi1p kodeeritakse *S. cerevisiae* XV kromosoomi avatud lugemisraamilt YOL95c. Hmi1p on homoloogne mitmete prokarüootsete helikaasidega, sealhulgas *E. coli* valkudega Rep ja UvrD. Seitsme konserveerunud helikaasi motiivi järgi HMI1 geenis paigutub see valk Gorbalenya ja Koonini klassifikatsioonis (Gorbalenya and Koonin 1993) helikaaside superperekonda I. Käesoleva doktoritöö peamised tulemused on järgmised.

1. YOL95c ORFi deleteerimine põhjustas funktsionaalse rho^+ mtDNA kadumise normaalsel kasvutemperatuuril (30^oC) ja seda kasvatamisel nii

fermentatiivsel glükoosi sisaldaval kui ka mittefermentatiivsel glütserooli sisaldaval söötmel. *hmi1*Δ tüve mtDNA analüüs näitas, et enamusest rakkudest kadus mtDNA (rho^0 rakud) ja vähestes rakkudes säilis defektne rho^- mtDNA. Seega on Hmi1 valk vajalik metsiktüüpi rho^+ mtDNA säilitamiseks rakus, kuid ei ole vajalik defektsete rho^- mitokondriaalsete genoomide alalhoidmiseks.

2. Hmi1 valk puhastati *E. coli* rakkudest kasutades pGEX4T1 üleekspressioonisüsteemi. Rekombinantne Hmi1 valk omas ATPaasset aktiivsust ja oli võimeline lahti harutama kaksikahelalisi DNA molekule 3'→ 5' suunaliselt. DNA ahelate eraldamine Hmi1 helikaasi poolt sõltus ATP hüdrolüüsist.
3. Valgu lokalisatsiooni katsed näitasid, et Hmi1 helikaas transporditakse pärmi mitokondri maatriksisse, kus ta jääb nõrgalt seondunuks mitokondri sisemembraaniga, olemata sealjuures integraalne membraanivalk.
4. Hmi1 helikaas on esimene senikirjeldatud valkudest, mille mitokondri maatriksisse impordisignaal paikneb valgu C-terminaalses osas. Valdavalt paikneb valku mitokondri maatriksisse suunav signaal tema N-terminuses. Deletsioonid ja mutatsioonid Hmi1 helikaasi transpordisignaalis omasid drastilist mõju valgu lokalisatsioonile mitokondrisse. Hmi1p C-terminaalne signaaljärjestus oli võimeline transportima DHFR (dihydrofolate reductase) kandjavalgu mitokondrisse *in vivo*.
5. Hmi1 helikaas ei ole vajalik mitokondriaalseks transkriptsiooniks.
6. MtDNA analüüs kahedimensionaalsel geelelektroforeesil (2D) näitas, et Hmi1 helikaas stimuleerib pikkade lineaarsete, konkatemeersete rho^- mtDNA ja ssDNA molekulide sünteesi.
7. Üllatuslikult selgus, et helikaasi konserveerunud motiividesse sisse viidud mutatsioonid K32M ja E211Q, mis blokeerisid Hmi1p ATPaasse ja helikaasse aktiivsuse, ei mõjutanud Hmi1 valgu võimet stimuleerida pikkade konkatemeersete DNA molekulide sünteesi. Samuti selgus, et Hmi1p ATPaasne aktiivsus on oluline, kuid mitte hädavajalik metsiktüüpi rho^+ mtDNA alalhoiuks.
8. Katsed temperatuuritundliku mutandiga *hmi1-5* näitasid, et Hmi1 helikaas on lisaks pikkade rho^- mtDNA molekulide sünteesimisele veel hädavajalik efektiivseks täispikkusega metsiktüüpi rho^+ mtDNA sünteesiks.

Kirjeldatud andmetest selgub, et Hmi1p on 3'→ 5' DNA helikaas, mis on hädavajalik mtDNA säilitamiseks pärmi *S. cerevisiae* rakus. Samas on selle valgu ATPaasne aktiivsus küll oluline, kuid siiski mitte hädavajalik metsiktüüpi mitokondriaalse genoomi säilitamiseks, mis omakorda viitab sellele, et Hmi1 ei ole replikatsioonikahvlis paiknev helikaas. Siiski on Hmi1 helikaasil võime stimuleerida pikkade lineaarsete rho^- molekulide sünteesi ning tagada täispika rho^+ mtDNA süntees, kusjuures Hmi1p ATPaaset aktiivsust ei ole selleks vaja. Seega võib oletada, et mõne Hmi1 helikaasi funktsiooni täitmiseks on vaja helikaaset ja ATPaaset aktiivsust, ning mõneks teiseks funktsiooniks aga mingit muud Hmi1 valgu omadust. Teatud määral sarnaneb Hmi1p *E. coli* PriA

helikaasile. PriA valgu deleteerimine põhjustab *E. coli* rakkude väga madala eluvõime (Nurse et al., 1991), samas on mutandid, millel puudub helikaasne aktiivsus eluvõimelised ja taluvad DNA kahjustusi. Lisaks on ATPaasi defektne PriA helikaas võimeline katalüüsima primosoomiks kutsutava replikatsiooni taasalustamiseks moodustatava valkude kompleksi koostamist, mis on ka selle valgu peamiseks funktsiooniks *E. coli* rakkudes (Zavitz and Marians, 1992). Hmi1p ja PriA ei ole järjestuse põhjal homoloogid, samas võivad nende valkude funktsioonid olla sarnased. Mõlemad helikaasid seonduvad dsDNAle, millel on 3' üleulatuv ssDNA ots ning mõlemal on 3'→ 5' helikaasne aktiivsus (Nurse et al., 1999; Kuusk et al., 2005). Samuti on teada, et PriA ja Rep helikaas omavad funktsionaalset sarnasust (Seigneur et al., 1998) ning Rep helikaas on Hmi1p lähim homoloog. Edaspidised katsed võiksid selgitada täpsemalt Hmi1 helikaasi funktsiooni mitokondriaalse DNA metabolismis.

ACKNOWLEDGEMENTS

My greatest gratitude belongs to Juhan for supporting and believing in me all these years. Undoubtedly, without you I wouldn't manage it.

I wish to thank all my colleagues in the Institute of Molecular and Cell Biology, and especially in our laboratory for help, advice and discussions, for good and friendly atmosphere.

My special thanks to all co-authors, Silja Kuusk, Priit Jõers, Sirje Kivi.

Thanks to always-helpful Maie Loorits for keeping our laboratory running.

I thank all my students for keeping me busy.

Special thanks to my brother, dr. Urmas Arumäe providing me with reprints.

A lot of thanks to dr. Arne Stenlund for giving me the opportunity to work at the Cold Spring Harbor Laboratory for several years (1992–1996). During these years I learned numerous basic techniques in molecular and cellular biology and most importantly I was guided to the scientific way of thinking.

I would like to thank Joachim Gerhold, other members of our lab and Laura Sedman for careful reading of the manuscript and for fixing of misspellings.

Most importantly, I owe much to my family for their love, friendship and support.

This work was supported by grants from the Estonian Science Foundation.

PUBLICATIONS

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):1816–24.

Kuusk S., **Sedman T.**, Sedman J. (**2002**) Recombinant Yeast mtDNA Helicases. Purification and functional assays. *Methods Mol Biol*. 2002; 197:303–16. Mitochondrial DNA. *Methods and Protocols,* edited by *William C. Copeland,* 2002.

Sedman T., Joers P., Kuusk S., Sedman J (**2005**) Helicase Hmi1 stimulates the synthesis of concatemeric mitochondrial DNA molecules in yeast *Saccharomyces cerevisiae*. *Curr Genet.* 2005 47(4):213–22.

CURRICULUM VITAE

Tiina Sedman

Date and place of birth:	December 9, 1959 in Häädemeeste, Estonia
Citizenship:	Estonian
Family status:	married, three children
Work address:	Institute of Molecular and Cell Biology of Tartu University, Vanemuise 46, Tartu 51014, Estonia
Phone:	+372 7 375 037
E-mail:	tsedman@ut.ee
Home address:	Pallase pst. 126-4, 51013, Tartu, Estonia

Education and professional employment

1978	finished Häädemeeste Seconday School
1983	graduated as microbiologist from Tartu University, Faculty of Biology and Geograpy
1989–1992	laboratory technician in Tartu University, Department of Molecular Biology
1992–1996	research technician at Cold Spring Harbor Laboratory, USA
Since 1996	Ph. D. student in Tartu University, Institute of Molecular and Cell Biology
2000–2001	research scientist in Estonian Biocentre
2000	MSc degree in molecular biology, Tartu University, the title of the thesis “Identification of the Novel Mitochondrial DNA helicase Hmi1”.
Since 2002	research scientist in Tartu University, Institute of Molecular and Cell Biology, Department of General and Microbial Biochemistry

Scientific work

Since 1996 I have been working in the lab of prof. Juhan Sedman. I am studing nuclear factors that control maintenance and integrity the of yeast *S. cerevisiae* mitochondrial DNA. My main research subject has been the mitochondrial DNA helicase Hmi1 and the topology of mitochondrial DNA.

List of publications

Sedman T., Sedman J. and Stenlund, A. (1997) Binding of the E1 and E2 proteins to the origin of the bovine papillomavirus. *J. Virol. 71*, 2887–96.

Kurg R., Parik J., Juronen E., **Sedman T**., Abroi A., Liiv I., Langel Ü., Ustav M. (1999) Effect of Bovine Papillomavirus E2 Protein-Specific Monoclonal Antibodies on Papillomavirus DNA Replication. *J.Virol. 73*, 4670–77.

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*. 20, 1816–24.

Kuusk S., **Sedman T.**, Sedman J.(2002) Recombinant Yeast mtDNA Helicases. Purification and functional assays . *Methods Mol Biol*. 197, 303–16. Mitochondrial DNA. *Methods and Protocols,* edited by *William C. Copeland,* 2002.

Sedman T., Joers P., Kuusk S., Sedman J (2005) Helicase Hmi1 stimulates the synthesis of concatemeric mitochondrial DNA molecules in yeast *Saccharomyces cerevisiae*. *Curr Genet.* 47, 213–22.

Kuusk, S., **Sedman, T.**, Joers, P. and Sedman, J. (2005). Hmi1p from *Saccharomyces cerevisiae* mitochondria is a structure specific DNA helicase. *J Biol Chem.* 280, 24322–9.

ELULOOKIRJELDUS

Tiina Sedman

Sünniaeg ja koht:	9, detsember 1959
Kodakondsus:	Eesti
Perekonnaseis:	Abielus, kolm last
Aadess tööl:	Molekulaar ja Rakubioloogia Instituut, Tartu Ülikool Vanemuise 46, 51014, Tartu, Eesti
Telefon:	+372 7 375 037
E-mail:	tsedman@ut.ee
Kodune aadress:	Pallase pst. 126–4, 51013, Tartu, Eesti

Haridus ja erialane teenistuskäik

1978	Häädemeeste Keskool
1983	Tartu Ülikooli bioloogia- geograafia teaduskond, lõpetatud mikrobioloogina
1989–1992	Tartu Ülikool, vanemlaborant molekuaarbioloogia osakonnas
1992–1996	Cold Spring Harbor laboratoorium, USA, tehnik dr. Arne Stenlundi laboratooriumis
1996–	Tartu Ülikool, Molekulaar ja Rakubioloogia Instituut, doktorantuur
2000–2001	Eesti Biokeskus, teadur
2000	Tartu Ülikooli teadusmagistrikraad molekulaarbioloogias, magistritöö pealkiri "Identification of the Novel Mitochondrial DNA helicase Hmi1".
2002–	Tartu Ülikool, Molekulaar ja Rakubioloogia Instituut, teadur üldise ja mikroobibiokeemia õppetoolis

Teadustegevus

Alates 1996 aastast olen töötanud prof. Juhan Sedmani töögrupis keskendudes pagaripärmi *S. cerevisiae* mitokondriaalse DNA stabiilsust tagavate protsesside, ning nendes protsessides osalevate valkude uurimisele. Peamisteks uurimisobjektideks on olnud mitokondriaalne DNA helikaas Hmi1. Lisaks sellele olen viimasel ajal keskendunud mitokondriaalse DNA topoloogiaga seotud küsimustele.