Comparison of replication structures in mutant and wild-type mitochondrial DNA of the yeast *Saccharomyces cerevisiae*

By

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Submitted in Partial Fulfillment of the Requirements

For the Degree of

Master of Science

In the

Biological Sciences

Program

Youngstown State University

December, 2006

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Abstract

Mitochondrial genomes vary greatly in structure and size from organism to organism. The mechanisms for replicating mitochondrial DNA (mtDNA) are not yet completely understood. *Saccharomyces cerevisiae*, baker's yeast, is a model organism utilized to study mtDNA replication. An issue arises using it for research since two forms are commonly used: the wild-type form, called rho$^+$, and a form that has undergone massive mtDNA deletions, called rho$^-$. Since rho$^-$ cells only contain a fragment of the wild-type mitochondrial genome amplified to an equivalent mass, it is of concern that these strains may replicate in an unusual manner, akin to an SOS response. Using neutral/neutral two-dimensional (2-D) gel electrophoresis, this study presents evidence to support a mtDNA replication mechanism that is common to both rho$^+$ and rho$^-$ strains. Both intact and restriction enzyme-digested mitochondrial genomes were examined.

The most widely- accept model of mtDNA replication in *S. cerevisiae* is the strand-asynchronous asymmetric model. This mechanism would generate primarily single-stranded replication intermediates. 2-D gel analysis of rho$^+$ and rho$^-$ strains revealed both single-stranded and double-stranded replication intermediates along with potential RNA-dependent species. This suggests that more models of replication are occurring in addition to the strand-asynchronous one. These potential models include a rolling circle mechanism and also one that is dependent on recombination. Recombination-dependent replication has been previously reported in mammals, but this is the first evidence presented for it in yeast.

Acknowledgments

I would like to thank the following people who aided me over the course of this project:

Dr. Lorimer: Thank you for exposing me to the world of research as an undergraduate and seeing me through graduate school as well. Your patience and expertise have been invaluable.

Dr. Asch: Thank you for serving on my committee and your help with molecular genetics and technical processes.

Dr. Cooper: Thank you for serving on my committee, helping me rediscover the world of fungus and all of your advice along the way.

My parents, Jim and Debbie and my sister, Brittany: You guys are truly blessings, without your love and support none of this would have been possible. Thank you for staying late nights in the lab and learning more than you ever wanted to know about genetics.

My fiancée, Paul Burke: You support has carried me thorough and kept me focused. Thank you for being there for the triumphs and rough patches. I love you.

Thank You!!

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Introduction

Mitochondria are membrane-bound organelles, roughly 0.2 - 1 μm in diameter and 1 – 5 μm in length, located in the cytoplasm of eukaryotic cells. Like chloroplasts in plants, mitochondria contain a double membrane structure that separates the internal matrix from the intermembrane space. The inner membrane consists of many cristae, or infoldings, that greatly increase the membrane's surface area and project into the matrix. The enzymes responsible for the oxidation reactions of the electron transport chain are housed in this membrane, while the mitochondrial genome is located in the matrix (reviewed in Schmid, 1992). Mitochondria play a central role in cellular respiration by generating energy in the form of adenosine triphosphate (ATP), and are thus referred to as the "powerhouses" of the cell. They also play a role in apoptosis (programmed cell death), ion homeostasis, and intermediary metabolism, which are enzyme-mediated processes that construct cellular components using energy derived from nutrients (reviewed in Burger et al., 2003).

Both mitochondria and chloroplasts are suggested to have evolved from an endosymbiotic relationship between a bacterium and a primitive eukaryotic cell. This endosymbiont theory proposes that more than one billion years ago, eukaryotic cells did not contain mitochondria, but instead existed in an anaerobic state. A free-living aerobic bacterium that possessed its own complete genome was endocytosed by one of these primitive cells, which then exploited the bacterium's oxidative phosphorylation system to produce ATP for its own use. Thus, this is why mitochondria contain their own genomes. Over time, most of the genes encoded by the bacterium, now known as the mitochondrion, were transferred to the nucleus of the eukaryotic cell, leaving behind only those encoding a few proteins involved in the oxidative phosphorylation pathway and tRNAs and rRNAs

utilized in protein synthesis (reviewed in Saccone et al., 2000). This means that even though mitochondrial DNA (mtDNA) is transmitted from an existing parent (maternal) cell to a new daughter cell intact, there is still nuclear involvement (reviewed in Burger et al., 2003).

Mitochondrial genomes can differ greatly from organism to organism in physical structure and size of coding information. While it is accepted that most mitochondrial genomes exist in a circular form, linear genomes have been reported in *Plasmodium* (an obligate parasite causing malaria), fungi, and some cnidarian animals. The smallest mitochondrial genome belongs to *Plasmodium* with 6 kb (kilobases) (reviewed in Burger et al., 2003). The genus to which cucumbers belong, *Cucumis* contains some of the largest with between 1500 and 2300 kb (reviewed in Havey et al., 2002). The average eukaryote contains 15 – 60 kb of mtDNA that encodes 40 – 50 genes; however, there is no correlation between the amount of genes encoded and the genome size (reviewed in Burger et al., 2003). Animals require large amounts of ATP. Perhaps due to that they appear to have undergone evolutionary pressure to reduce the size of the genome while retaining full respiration capabilities. Typically, animal mtDNA exists in small circular structures of 14 – 42 kb. Therefore, in order to encode as much genetic information as possible in such a small space, these genomes lack introns, and instead contain genes located only a few nucleotides apart, in a continuous fashion, or even with overlapping reading frames. Also, there is little recombination between genes, so they are highly conserved (reviewed in Leblanc et al., 1997). The human mitochondrial genome of ~16.5 kb is typical of mammals and does not contain introns (reviewed in Lecrenier and Foury, 2000).

Plant mtDNA, on the other hand, exists in much larger and more complex genomes due to the presence of introns and other non-coding DNA. Frequent gene rearrangements

also occur due to recombination (reviewed in Leblanc et al., 1997). Differences also exist among members of the same species. For example, five types of maize mtDNA have been identified. Each of these genomes can ultimately be mapped as structures called master chromosomes, which are circular molecules containing 47 genes encoded by 700 kb, 570 kb, or 540 kb of DNA (reviewed in Fauron et al., 1995).

Other organisms contain mitochondrial genomes organized in a manner somewhere in between that of plants and animals. For example, most fungal mtDNA consists of 17 – 180 kb arranged in circular molecules that contain introns. Multiple organizations of mitochondrial genomes can also exist in the same cell. For example, kinetoplastid trypanosomes organize their mtDNA into mini-circles made up of 0.6 – 2.5 kb and maxi-circles made up of 20 – 35 kb (reviewed in Leblanc et al., 1997). Protists such as *Acanthamoeba castellanii* and *Dictyostelium discoideum* exhibit a phenomenon called gene fusion in which the genes *cox1* and *cox2* (encoding the subunits of cytochrome oxidase) form a single open reading frame, instead of each having their own (reviewed in Burger et al., 2003).

Another example of how there can be differences in mtDNA within a single type of organism is that of yeast. Two types of yeast are commonly used as model organisms: fission and budding. Both are classified in the Ascomycota fungal division. Throughout the course of evolution, however, their lineages diverged. Filamentous yeasts are classified as euascomycetes, budding yeasts as hemiascomycetes, and fission yeasts as archiascomycetes based on rRNA sequence data (Nishida and Sugiyama, 1994). More recent data based on mtDNA comparison indicates that fission yeast should be classified at the base of the same branch as hemiascomycetes because they diverged after hemiascomycetes and

euascomycetes separated, not before as previously thought (Bullerwell et al., 2003). Despite their shared evolutionary history, these two eukaryotes have different reproduction processes and mitochondrial genome sizes. Fission yeasts, such as *Schizosaccharomyces pombe*, reproduce via ascospore production (Prillinger et al., 1990). Budding yeasts like *Saccharomyces cerevisiae*, however, reproduce by the mating of two parent cells to form a zygote that later produces mitotic buds to give rise to new daughter cells (reviewed in Berger and Yaffe, 2000). The complete sequence of mtDNA of each yeast has been determined. *S. pombe* contains a smaller mitochondrial genome of 19, 431 bp (Wood et al., 2002) whereas that of *S. cerevisiae* contains ~85, 700 bp (Foury et al., 1998).

Purpose for studying mtDNA

Like chromosomal DNA, mutations, deletions, and rearrangements can occur in mtDNA, leading to loss of function and possibly disease. In humans, it is estimated that once mitochondrial function drops below 15% of its normal capacity, disease results. This is known as the threshold effect. Since mitochondria also require genetic information from nuclear DNA in order to function properly, disease can result from disruptions here as well (reviewed in Kakuda, 2000). In general, mitochondrial disorders in children are more likely to be nuclear in origin, whereas in adults, the defect usually lies within the mtDNA (reviewed in Chinnery and Turnbull, 2000).

Approximately 1 in 15,000 adults are affected by pathogenic changes in mtDNA (reviewed in Chinnery and Turnbull, 2000). Most of these mtDNA defects detrimentally affect the oxidative phosphorylation system (OXPHOS). Human diseases caused by abnormalities in the OXPHOS due to mtDNA mutations include chronic progressive external

ophthalmoplegia (CPEO), mitochondrial encephalopathy lactic acidosis and stroke-like episodes (MELAS), myoclonic epilepsy and ragged-red fibers (MERRF), and Leber hereditary optic neuropathy (LHON) (Orth and Schapira, 2001). Clinical manifestations of MELAS include mental impairment, myopathy, epilepsy, short stature, stroke-like episodes, hearing disturbance, and neuropathy (Huang et al., 2002). Many of these disorders lead to muscular problems such as spasms of varying degrees, seizures, loss of coordination and cardiac myopathy, which can be fatal (reviewed in Chinnery and Turnbull, 2000). Data also suggests that there is a higher frequency of liver disease in patients with mitochondrial defects, whether they are inherited or acquired (Hassanein, 2004). MtDNA links to Parkinson's disease and Alzheimer's disease have also been reported (Orth and Schapira, 2001).

MtDNA dysfunction can also be acquired. One example of this may occur during the use of nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs). These drugs are currently used as part of highly active antiretroviral therapy (HARRT) to combat human HIV infection. HIV is a retrovirus that must transcribe its single-stranded RNA genome into double-stranded DNA upon entry into a target cell using the enzyme reverse transcriptase (reviewed in White, 2001). NRTIs are nucleoside and nucleotide analogs that can be used as target substrates for reverse transcriptase. Chemical modification of these analogs eliminates the 3'OH normally found on nucleotides that is necessary to form a phosphodiester bond with the 5' PO_4 of the next one during transcription. Thus, not only do NRTIs compete for incorporation with naturally occurring nucleotides in the cell, they also terminate elongation prematurely. A problem arises because they can also be used as substrates for the DNA polymerases responsible for normal cellular functions (reviewed in Kakuda, 2000).

DNA polymerase γ is the polymerase responsible for mtDNA replication. Since it can act in both RNA- and DNA-dependent manners, DNA polymerase γ is particularly susceptible to interference by NRTIs. This can in turn lead to inhibition of mtDNA replication and subsequent disease. Mitochondrial DNA polymerase γ inhibition of NRTIs in current use has been ranked: zalcitabine ≥ didanosine ≥ stavudine > lamivudine > zidovudine > abacavir. In fact, it has been reported that zalcitabine interferes with DNA polymerase γ more efficiently than it does reverse transcriptase, its intended target (reviewed in Kakuda, 2000). Interference with mtDNA replication contributes to the clinical toxicity of these NRTIs. Depletion of mtDNA due to their use in HIV treatment has been linked to myopathy (especially dangerous in the heart), fatal lactic acidosis, neuropathy, and renal toxicity (reviewed in White, 2001). Thus, it is important to understand the process of mtDNA replication not only to combat mitochondrial diseases themselves, but also to design safe and effective antiviral drugs.

Yeast

Saccharomyces cerevisiae, commonly known as baker's yeast, is one of the favored model organisms for mtDNA replication studies. It is a eukaryote with a completely sequenced genome, a short generation time, and the ability to exist in a haploid or a diploid state. Also, cellular activities such as DNA replication, RNA transcription and translation, and metabolism are conserved from yeast to humans (reviewed in Barrientos, 2003). *S. cerevisiae* is also relatively cheap and easy to grow since it is a facultative anaerobe capable of growing via respiration or, if mtDNA is diminished or nonexistent, fermentation (reviewed in Berger and Yaffe, 2000).

The mitochondrial genome of *S. cerevisiae* used to be considered to exist in circular molecules that are broken during DNA extraction, either physically or enzymatically. Thus, they appear as linear molecules when electrophoresed. Testing this theory of broken circles reveals linear molecules of about 60 to several hundred kb in size, with only a low percent of circular molecules. It remains unclear whether the circular molecules are functional and the linear molecules are merely involved as replication intermediates or if the circular molecules serve no functional role (reviewed in Lecrenier and Foury, 2000).

Strains of *S. cerevisiae* with mutant mtDNA can provide information about mtDNA separation and replication. Subjecting the wild-type (rho^+) mitochondrial genomes to massive deletions yields respiratory-deficient cells that, when grown on a fermentable medium, form petite colonies. These cells are referred to as rho^- mutants (reviewed in Lecrenier and Foury, 2000). Rho^- mutants may contain only AT base pairs and can retain a fragment as small as 35 bp of the original mitochondrial genome (Fangman and Dujon, 1984). The retained fragment is amplified as tandem repeats until a mass equivalent to that of the rho^+ genome is achieved. If all of the cell's mtDNA is deleted, it is referred to as rho^o (reviewed in Lecrenier and Foury, 2000).

Mating the different types of strains together produces progeny cells of predictable types. When two rho^+ strains are mated, they produce diploids that segregate over very few generations into pure parental or recombinant rho^+ genomes. These cells start out in a heteroplasmic state and then transition to homoplasmy (reviewed in Berger and Yaffe, 2000). When a rho^+ and a rho^- strain are mated, the cells segregate into pure homoplasmic states as the rho^+ strains did before in some cases. In other cases, however, the rho^- genome is preferentially transmitted so the resulting zygotic clones contain only rho^- genomes and the

rho$^+$ genomes are eliminated. The fraction of clones in which the rho$^-$ genome is preferentially inherited is referred to as suppressiveness, of which there can be varying degrees. The range of suppresssiveness from neutral, which is when less than 5% of zygote colonies are rho$^-$, to hypersuppressive, when greater than 95% of the zygote colonies are rho$^-$ (De Zamaroczy et al., 1981).

DNA replication

In general, chromosomal DNA replicates according to a semiconservative model in which each strand of the double helix is used as a template to synthesize a new one. Regardless of chromosomal shape, this mechanism usually operates in a bi-directional fashion from an origin of replication via replication forks. Replication forks are made up of a leading strand that is replicated continuously and a lagging strand that is replicated discontinuously.

In circular molecules, like plasmids, the general mode of replication is called θ. It is schematically represented in Figure 1C (Bowmaker et al., 2003). In this method, the priming sites of leading and lagging strand synthesis are close together within the origin of replication and elongation can occur in either a unidirectional or bi-directional fashion (Helinski et al., 1985). Whether or not this simplified version of chromosomal replication is also the true replication mechanism in mtDNA has been the topic of much study and debate.

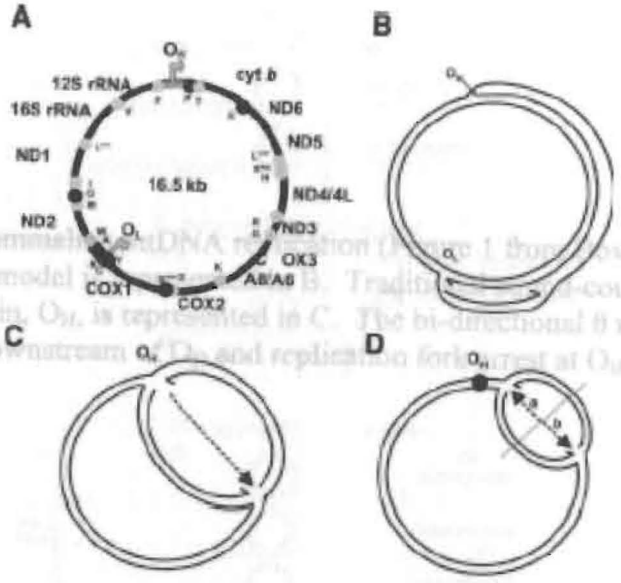

A
B
C
D
12S rRNA
16S rRNA
ND1
ND2
cyt b
ND6
ND5
ND4/4L
ND3
COX3
AB/A6
COX2
COX1
16.5 kb

Figure 1. Models of mammalian mtDNA replication (Figure 1 from Bowmaker et al., 2003). The strand asymmetric model is represented in B. Traditional strand-coupled unidirectional replication from an origin, O_H, is represented in C. The bi-directional θ model with an origin of replication located downstream of O_H and replication fork arrest at O_H is represented in D.

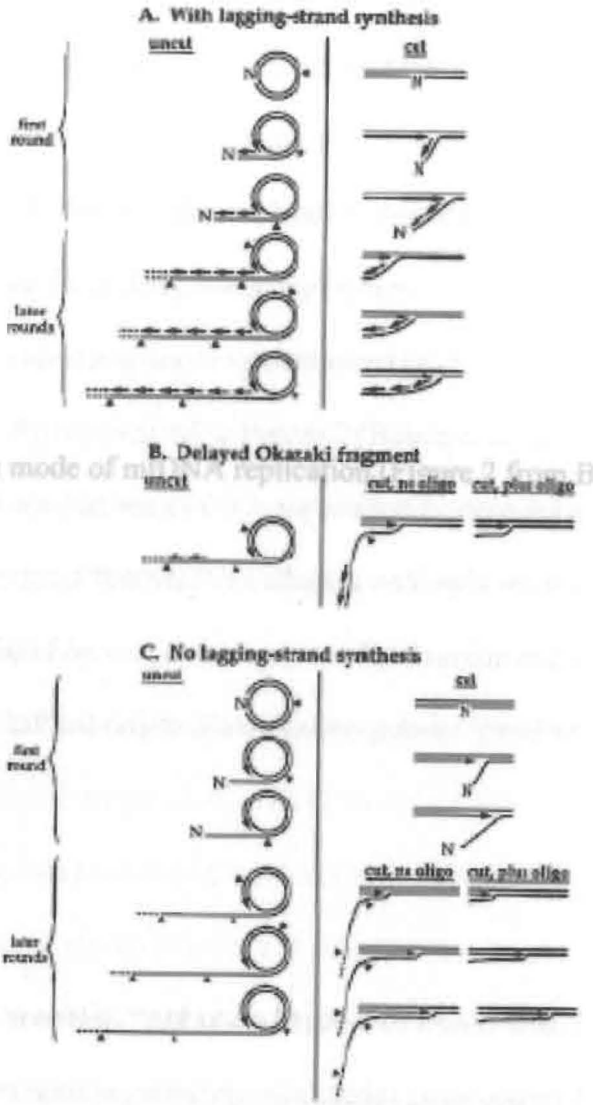


Figure 2. Rolling circle mode of minute virus of mice DNA replication (Figure 2 from Belanger et al., 1996).

Figure 2. Rolling circle mode of mtDNA replication (Figure 2 from Belanger et al., 1996).

An alternative to the θ mode of replication called σ, or rolling circle (RC), has been proposed. In this type of replication, priming events for the two strands are independent and occur at different origins (reviewed in del Solar et al., 1993). First, a strand- and site-specific nick is introduced in the double-stranded replication origin (dso) to generate a free 3′-OH end that serves as a primer for leading strand replication. The result is two full-sized circular products: one double-stranded and one single-stranded (Koepsel et al., 1985). This mode of replication is schematically represented in Figure 2 (Belanger et al., 1996).

Another unusual mechanism of DNA replication is recombination-dependent. When a replication fork encounters a "barrier," it collapses and replication stops. The process of replication can be reinitiated by enzymes that normally function during recombination. This occurs when there is no defined origin of replication present (reviewed in Kowalczykoski, 2000).

mtDNA Replication

The most widely accepted, "orthodox," model of mammalian mtDNA replication involves a strand-asynchronous asymmetric mechanism proposed by David A. Clayton in the early 1980's. The two strands of the double-stranded circular mammalian genome, approximately 16 kb in size, are designated heavy (H) and light (L) due to the differences in their A+G and C+T content during CsCl buoyant density centrifugation (reviewed in Clayton, 2003). Each strand has its own major promoter involved in transcription: light-strand promoter (LSP) and heavy-strand promoter (HSP) (reviewed in Lecrenier and Foury, 2000). These two promoters direct the expression of most, if not all, of the mitochondrial genes and are located in the displacement-loop (D-loop) region of the genome along with O_H

(Chang and Clayton, 1984). The strand-asynchronous model proposes that initiation of H strand replication occurs at a single site (O_H) and continues unidirectionally until it encounters the light strand origin of replication (O_L), located roughly two-thirds of the way around the genome. Replication of the light strand then proceeds in the opposite direction, also in a continuous manner (Clayton, 1982). Therefore, mammalian mtDNA replication depends on H strand synthesis as its first step (Chang and Clayton, 1985). The strand asynchronous model is schematically represented in Figure 1B (Bowmaker et al., 2003).

MtDNA replication in yeast is not yet completely understood. The most widely accepted mode of replication involves two types of sequences: *rep* and *ori*. The *rep* sequences consist of three blocks referred to as A, B, and C, are GC rich, and contain an intact promoter. The *ori* sequences contain only A, B, and C blocks with an interrupted, presumably non-functional promoter (reviewed in Schmitt and Clayton, 1993). The promoter has been hypothesized to act by brining in RNA polymerase which in turn acts as a primase for transcription (Chang and Clayton, 1985). Later, however, this hypothesis was concluded to be incorrect and that RNA polymerase is not necessary for priming or biased inheritance (Lorimer et al., 1995). Although they lack most of the wild-type mitochondrial genome's sequence, hypersuppressive *S. cerevisiae* strains contain *ori* sequences, which are considered active origins of replication (reviewed in Lecrenier and Foury, 2000). Homology between mammalian origins of replication and *S. cerevisiae rep* sequences has led to a universal model of mtDNA in which these sequences are conserved from yeast to man (reviewed in Schmitt and Clayton, 1993). One functional difference exists between the conserved sequences: O_H and O_L are unidirectional, whereas it is believed that *ori* sequences are bi-directional (reviewed in Lecrenier and Foury, 2000).

Recently, the universal model of mtDNA replication has been challenged and evidence for alternative modes has been presented. Holt et al. discovered evidence for a synchronous mode of replication of both leading and lagging strands that initiates at O_H and continues around the circular molecule in a single direction. They employed neutral/neutral two-dimensional agarose gel electrophoresis to reach this conclusion. Arcs representing partially single-stranded replication intermediates (RIs) like those expected from the universal strand-asynchronous asymmetric model were observed. Also present were arcs of double stranded RIs like those predicted by conventional unidirectional replication involving leading and lagging strands. This means that mtDNA replication may occur according to several different models in addition to the "orthodox" model depending on the cell type or stage of development. It also may not be conserved from yeast to mammals (Holt et al., 2000). Subsequent studies indicate a possibility that the intermediates that would support the orthodox model may be present due to incorporated ribonucleotides and RNase H activity in the samples (Bowmaker et al., 2003 and Yang et al., 2002).

There is also further evidence that mammalian mtDNA may not operate as much like the strand asynchronous model as previously thought. In detailed mapping studies on rat, mouse, and human mtDNA using neutral/neutral 2-D gel electrophoresis, support was lent to the hypothesis that initiation of mtDNA replication occurs downstream of O_H and proceeds in a strand-coupled (θ) fashion (Bowmaker et al., 2003). Since one of the replication forks arrests at O_H, it serves as a terminus for replication instead of an initiator. This is schematically represented in Figure 1D (Bowmaker et al., 2003).

Neutral/Neutral 2-D Gel Electrophoresis

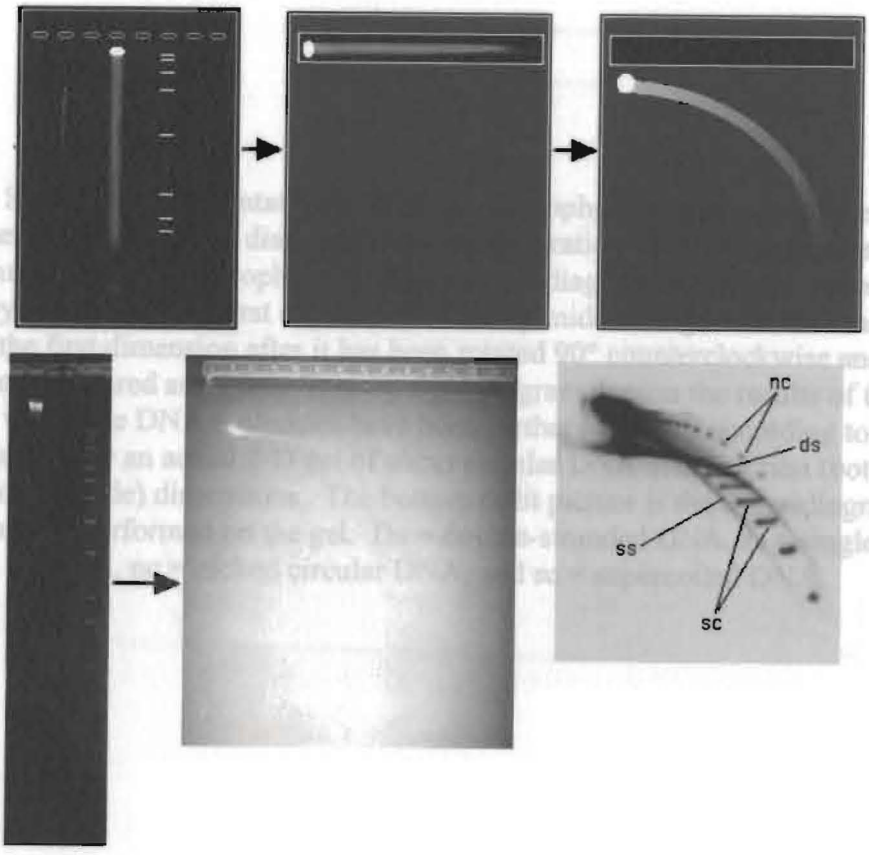
One method employed to study mtDNA replication is that of neutral/neutral two dimensional (2-D) gel electrophoresis. It is based on the fact that linear and nonlinear DNA molecules migrate at different rates through gels. Small DNA molecules move faster through the gel matrix than larger ones. If the molecules are of the same mass, the nonlinear duplex DNA migrates slower than the linear form. In the first dimension, molecules are separated according to mass, or size, them in the second dimension they are separated according to shape. This is demonstrated in Figure 3. When DNA of any type is cut by restriction endonucleases, it generates linear fragments of specific sizes. Fragments may behave differently during replication. If a fragment contains an origin of replication, then two replication forks move in opposite directions through the fragment. If a fragment does not contain an origin of replication, then only one replication fork moves through it in a unidirectional fashion (Brewer and Fangman, 1987).

In the first dimension, DNA samples are electrophoresed through a low percentage agarose gel at a low voltage (therefore the molecules move slowly through the gel) and in the absence of ethidium bromide (EtBr). EtBr is not used because it is a dye that intercalates into the DNA molecules, making them more rigid, and therefore possibly causing changes in the sizes of the molecules and interfering with results. The first dimension separates molecules according to their sizes. The lane of the gel containing the DNA is then excised, rotated 90° counterclockwise and the second-dimension gel poured surrounding it. In the second dimension, the DNA molecules are separated based on shape using a much higher voltage and a higher percentage of agarose. EtBr is included in this dimension to ensure that the

shapes of the DNA molecules remain defined in order to amplify any differences (Lorimer, 2002). It also allows the DNA to be visualized using ultraviolet light.

Each of the proposed mechanisms of mtDNA replication produces intermediates of distinct shapes that can be observed using neutral/neutral 2-D gel electrophoresis. Example results are best illustrated in Figure 4, from Brewer and Fangman (1987), using a theoretical restriction fragment 1kb in length. In the first example, simple Y, the mode of DNA replication involves a single replication fork that starts at one end of the molecule and moves to the other (Figure 4A). It will produce an arc that accounts for all of the possible RIs. Molecules just beginning replication fall at the far right end of the arc, in the 1 kb linear position. Molecules finishing replication are linear and about 2 kb in length so they are found at the far left side of the arc. RIs in intermittent stages of replication fall within the arc in between depending on which end of the molecule the replication fork is closer to. The top of the arc represents the slowest moving molecules and thus those least like the linear DNA; in this case, that would represent the half-replicated RI (Figure 4A). The half-replicated fragment will measure 1.5 kb in length and consist of three branches of equal length: two of the replication fork and one of the DNA still to be replicated (Brewer and Fangman, 1987).

A second replication form, the bubble, occurs when replication is initiated from the center of the fragment and moves in opposite directions (Figure 4B). As each side of the bubble moves towards opposite ends of the DNA molecule, RIs produced begin to approach 2 kb in size and are less and less linear in structure. Therefore, they migrate in the same fashion in the first dimension as their simple Y counterparts, but in the second dimension their migration is much different. This means that the part of the arc produced by molecules just starting replication resembles that of the simple Y-arc towards the 1 kb position. As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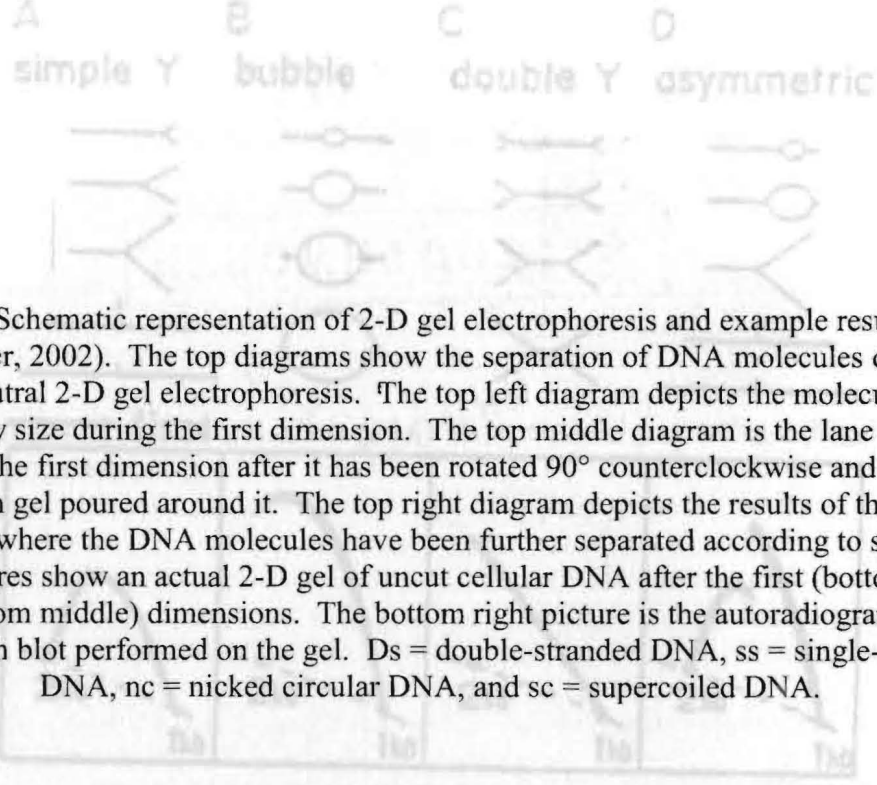


Figure 3. Schematic representation of 2-D gel electrophoresis and example results (from Lorimer, 2002). The top diagrams show the separation of DNA molecules during neutral/neutral 2-D gel electrophoresis. The top left diagram depicts the molecules being separated by size during the first dimension. The top middle diagram is the lane containing DNA from the first dimension after it has been rotated 90° counterclockwise and the second dimension gel poured around it. The top right diagram depicts the results of the second dimension, where the DNA molecules have been further separated according to shape. The bottom pictures show an actual 2-D gel of uncut cellular DNA after the first (bottom left) and second (bottom middle) dimensions. The bottom right picture is the autoradiogram results of a Southern blot performed on the gel. Ds = double-stranded DNA, ss = single-stranded DNA, nc = nicked circular DNA, and sc = supercoiled DNA.

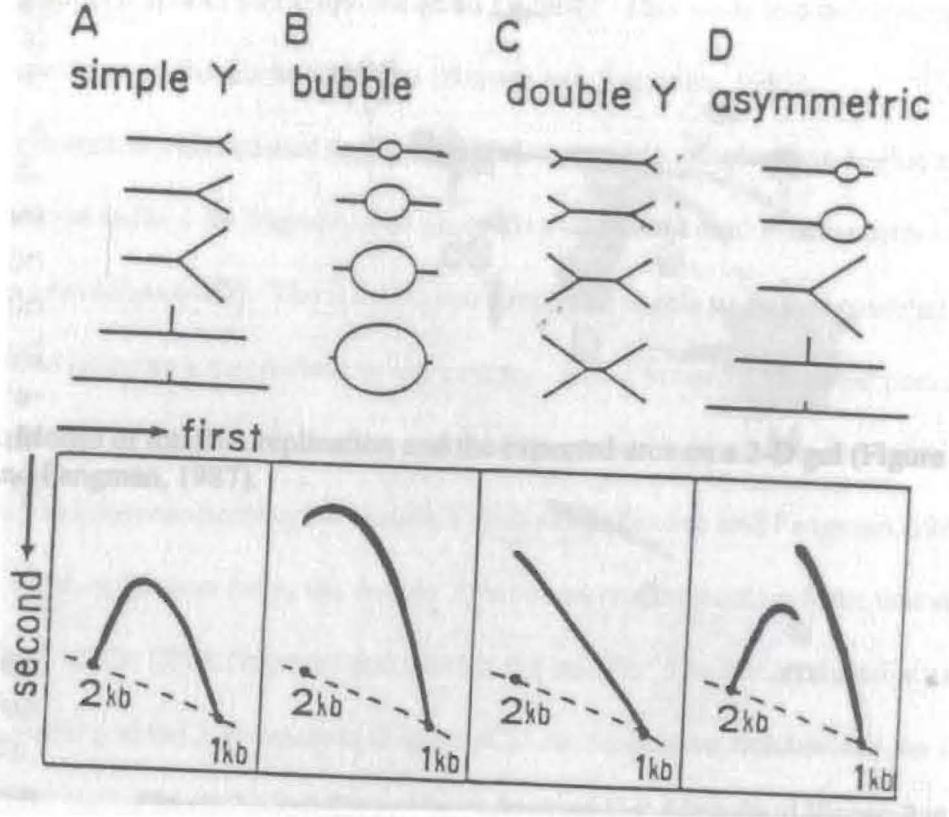

A
simple Y
B
bubble
C
double Y
D
asymmetric
first
second
2kb
1kb
2kb
1kb
2kb
1kb
2kb
1kb

Figure 4. Modes of mtDNA replication and the expected arcs on a 2-D gel (Figure 1 from Brewer and Fangman, 1987).

replication continues, however, the bubble grows and deviates farther from the linear form, generating a much greater steric hindrance on mobility. This leads to a much steeper arc that does not converge on the linear 2 kb spot (Brewer and Fangman, 1987).

Asymmetric bidirectional replication is also possible. Replication begins at a non-central position in the 1 kb fragment and proceeds as a bubble until it encounters one of the restriction sites (Figure 4D). The RIs become simple Ys at this point and continue replication to generate a discontinuous gel pattern. Both 1 kb and 2 kb linear positions are present, but there is a gap between the portion of the pattern representing the bubble arc (right side) and the representing the simple Y (left side) (Brewer and Fangman, 1987).

A third replication form, the double Y, involves two replication forks that start at opposite ends of the DNA fragment and meet in the middle. The arc produced is more like a steep line starting at the 1 kb position (Figure 4C). As replication finishes and the forks meet in the middle, the molecule resembles an "X," which moves slower in the second dimension, so this arc also does not converge at the 2 kb point (Brewer and Fangman, 1987). The rolling circle mode of replication also generates distinct patterns on a 2-D gel: a double- or single-stranded eyebrow curve which is demonstrated in Figure 5 (Belanger et al., 1996).

Objections to the use of 2-D gel electrophoresis to study mtDNA replication intermediates have also been presented. Some question whether this technique can offer truly valid results contrary to the accepted strand asynchronous model. These objections include the possible presence of artifacts caused by ribonucleotide incorporation if mtDNA molecules are actually undergoing transcription instead of replication. Also, the majority of

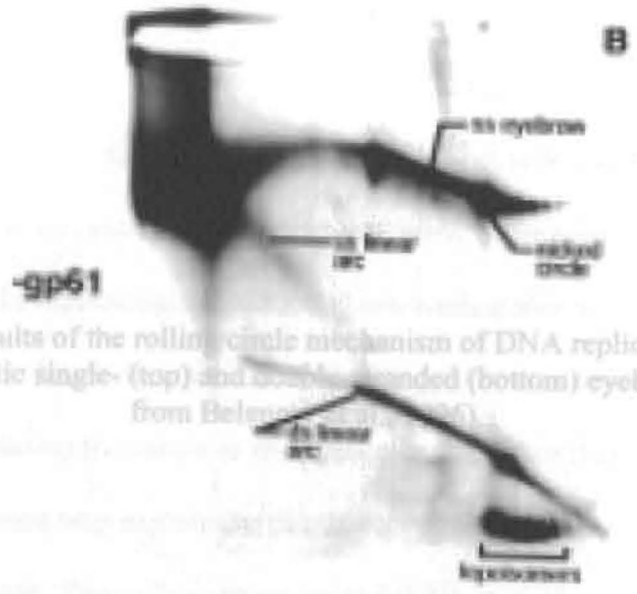


Figure 5. 2-D gel results of the rolling circle mechanism of DNA replication. These results feature the characteristic single- (top) and double-stranded (bottom) eyebrow arcs (Figure 3B from Bel

Figure 5. 2-D gel results of the rolling circle mechanism of DNA replication. These results feature the characteristic single- (top) and double-stranded (bottom) eyebrow arcs (Figure 3B from Belanger et al., 1996).

RIs generated may be fragile and become destroyed during the course of electrophoresis, leaving behind structures that represent only a minority of these intermediates (reviewed in Bogenhagen and Clayton).

Experimental Design

The issue that arises with mtDNA replication research utilizing *S. cerevisiae* is that since rho$^-$ strains exist as obligate anaerobes and lack most of the mitochondrial genome of rho$^+$ strains, they may be replicating in an unusual mechanism akin to an SOS response. I examined this possibility by cutting rho$^-$ mtDNA with specific restriction enzymes and looking at the corresponding fragments in rho$^+$ genomes. If in fact they prove to operate by different modes, this could help explain the phenomenon of biased inheritance among hypersuppressive mutants. The cells were grown to log phase to ensure that there are many replication forms present to observe instead of cells that are merely maintaining their mtDNA as would be the case with stationary phase cells. The rho$^-$ strains that I used were HS82d and HS3324, the rho$^+$ (parental) strains were BS127 and BS132, and the rho^o strains were N28 and N23-3. A map of the mitochondrial genome with mutants indicated is found in Figure 6. HS82d, N23-3, and BS127 have the same nuclear background that differs from that HS3324, N28, and BS132 (the latter three have the same nuclear background). These strains were chosen in order to have an example of both small and large sized-mutants. Two of each type of mutant, hypersuppressive and neutral, were chosen from different locations of the genome. N23-3 and N28 lack promoters while HS3324 and HS82d contain intact ones.

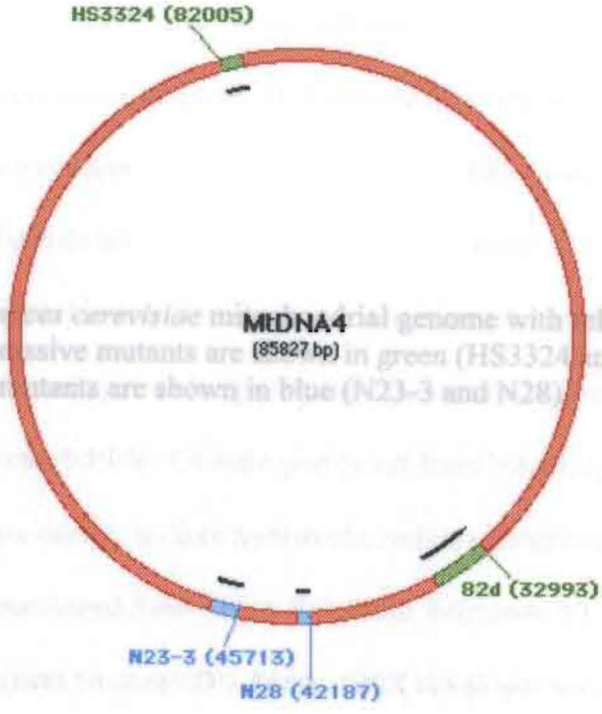

HS3324 (82005)
MtDNA4
(85827 bp)
82d (32993)
N23-3 (45713)
N28 (42187)

Figure 6. *Saccharomyces cerevisiae* mitochondrial genome with relevant mutant sites indicated. Hypersuppressive mutants are shown in green (HS3324 and HS82d). Neutral mutants are shown in blue (N23-3 and N28).

Materials

Agarose, acid-washed glass beads, sodium acetate, Polaroid flim Type 667, Tris base, Triton X-100, RNase A, siliconized glass wool, sephadex G-50 beads and yeast extract were purchased from Sigma Chemical Company, St. Louis, MO. Boric acid, cholorform, dextrose, ethylenediaminetetraacetic acid-disodium salt (EDTA), isoamyl alcohol, phenol, sodium chloride and sodium dodecyl sulfate were purchased from Ameresco®, Solon, OH. 200 proof ethanol and hydrochloric acid were purchased from pharmco™ Products Inc., Brookfield, CT. Restriction enzymes along with their respective buffers, dNTPs, RNase I, RNase H and random primer NEBlot kit were purchased from New England Biolabs, Inc. Ethidium bromide, sodium citrate, sodium hydroxide, sodium phosphate monobasic, sodium azide and peptone were purchased from Fisher Scientific, Fairlawn, NJ. α^{32}P dATP was purchased from New England Nuclear (DU Pont). GBX developer and fixer and Kodak Biomax MR scientific imaging film were purchased from Eastman Kodak Company, Rochester, NY. Whatman paper was purchased from Whatman International Ltd., Maidstone, England. Positively charged nylon membrane was purchased from Roche Diagnostics Corporation, Indianapolis, IN.

Strains and Media

All *S. cerevisiae* strains utilized in this project were derived from A364a and previously described by Lorimer et al. (1995) and Lockshon et al. (1995). These strains were: BS127 (rho$^+$), BS132 (rho$^+$), HS82d (rho$^-$), HS3324 (rho$^-$), N28 (rho$^\circ$), N23-3 (rho$^\circ$). Cells were stored in 80% glycerol at -80°C for long terms and in 5 ml liquid cultures refrigerated at 4°C for shorter terms.

Methods

Determining fragment sizes

All of the fragment sizes of wild-type and mutant yeast strains were determined using the Gene Construction Kit™ version 1.28.

Total DNA Isolation (Large Scale Smash and Grab)

Total cellular DNA of the yeast was isolated using a procedure adapted from Hoffman and Winston (1987). 0.1 ml of each strain was first grown in 5 ml YEPD (1% yeast extract and 2% bactopeptone autoclaved in Yamato Autoclave SM 32 at 121°C for 15 min. then 2% dextrose added) overnight in a spinning incubator (STABILTHERM® Dry Tape Bacteriological Incubator, Gravity Convection) at 30°C to ensure a fresh culture. 1 ml of this culture was then grown in 50 ml YEPD (in a 250 ml Erlenmeyer flask) in a shaking water bath (Gyrotary Water Bath Shaker, model G76) at 30°C. Cellular density readings were taken at 650 nm using a spectrophotometer until a desired density of ~1.0 (~1.8 x 10^7 cells), which corresponds to log phase growth, was obtained. The cuvettes used were made of polystyrene with a path length of 1 cm. The log phase culture was then diluted into 1 L YEPD and again grown until the optimal density was reached to ensure the cells were in log phase of growth. Once the cells reached log phase, 10 ml NaN_3 and 30 ml 0.5M EDTA were added to rapidly kill the cells and stop DNA replication.

Cells were then pelleted by spinning the culture in 250 ml Sorvall bottles for 5 min at 4,000 g (6,500 rpm) at 4°C in a Sorvall RCB Plus SLA-1500 Rotor. After the supernatant was discarded, the pellets were then resuspended and combined in 20 ml 1X TE (10 mM Tris, 1 mM EDTA pH 8) into a 50 ml conical tube. The tubes were spun in a Beckman

Coulter Allegra™ UR refrigerated centrifuge at 15°C at 2,000 rpm for 5 min and the supernatant discarded. 3 ml acid-washed glass beads, 2 ml cold lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-Cl pH 8.0, and 10 mM EDTA), and 2 ml PCIA (50% phenol, 48% chloroform, 2% isoamyl alcohol) were added to the pellet and vortexed for 30 sec followed by incubation on ice for 30 sec. The vortexing and ice incubation step was repeated ten more times once the pellet was no longer visible in the bottom of the tube (~50 times total). The mixture was then centrifuged again at 2,000 rpm for 5 min to separate the mixture into three layers: a layer of beads and PCIA on the bottom, an opaque white interface in the middle, and a clear aqueous layer on the top. The aqueous layer was then transferred to a new 15 ml conical tube. 2 ml 10X TE was added to the original tube, vortexed, and centrifuged for 5 min at 2,000 rpm. The resulting aqueous layer was combined with the previous aqueous layer.

To this combined sample, 4 ml PCIA was added followed by 30 sec of vortexing, and centrifuged again at 2,000 rpm for 5 min. The resulting aqueous (top) layer was transferred to a new conical tube. This step was repeated until the opaque white material at the interface between the two layers was gone. To the resulting aqueous layer (in a new 15 ml conical tube) 1/10 volume (~400 µl) 3M sodium acetate (NaOAc) and 2.5 volumes (~10ml) 100% ethanol (EtOH) were added, followed by mixing and freezing at -20°C for at least 30 min to allow the DNA to precipitate. After freezing, the DNA was pelleted out by spinning at 20,000 g (14,000 rpm) in a Sorvall RCB Plus SA-600 rotor at 4°C. After discarding the supernatant, the pellet was gently washed with cold 75% EtOH to remove salts. The pellets sat on the bench at room temperature overnight in order to dry. Once dry, they were

resuspended in 1X TE and aliquoted into 1.5 ml Eppendorf tubes. One tube of each strain was also treated with RNase A (1 µl). All samples were stored at -20°C.

Restriction Enzyme Digests

A restriction enzyme digest was performed on each mutant strain and its corresponding wild-type parental strain. Restriction enzymes were selected on the basis that they did not interfere with the *rep* sequences of any of the mutant strains and generated fragments of predictable sizes. Figure 7 shows a map of the *S. cerevisiae* mitochondrial genome containing the relevant sites of rho⁻ mutants and selected restriction enzyme cut sites. HS82d, BS127, and N23-3 were cut with the restriction enzymes *BstY1* and *EcoRI*. This digest was carried out in *EcoRI* buffer. 15 µl sterile water, 2 µl DNA, 2 µl *EcoRI* buffer, and 1 µl *EcoRI* were combined in a 1.5 ml Eppendorf tube and incubated at 37°C for two hours. 1 µl *BstY1* was then added to the mix and incubated at 60°C for two more hours. HS3324, BS132, and N28 were cut with the restriction enzymes *SacII* and *SphI*. 15µl sterile water, 2 µl DNA, 1 µl NEB buffer 2, 1 µl NEB buffer 4, 1 µl *SacII* and 1 µl *SphI* were mixed in a 1.5 ml Eppendorf tube and incubated at 37°C for two hours. The resulting DNA fragments were run on a 1-D minigel.

For 2-D gels, a higher concentration of mtDNA was cut by increasing the amount of DNA in roughly the same volume. After digestion, an equal volume of PCIA was added, the samples vortexed and then centrifuged for 5 min. The aqueous (top) layers were transferred to new Eppendorf tubes. One-tenth volume 3M NaOAc was added to each sample along with 2.5 volumes 100% EtOH to precipitate the DNA. Samples were incubated for at least 2 hours at -20°C to allow DNA precipitation, then centrifuged for 10 mins. The resulting

supernatants were discarded and the remaining pellets were washed with cold 75% EtOH. After drying, the pellets were resuspended in 15 µl 1X TE each and run on a 2-D gel.

Gel Electrophoresis

Minigel

A 1-D minigel was run in order to check the concentration and size of the DNA fragments prior to running the 2-D gels. The gel was a 0.8% agarose minigel made by putting 0.4 g agarose in 50 ml 1X TBE (Tris base, boric acid, and EDTA) in a 250 ml Erlenmeyer flask. This solution was then heated in a microwave until the agarose was completely dissolved. Once the solution had cooled for ~15 min, 5 µl ethidium bromide (EtBr) was added to it and then poured into the gel tray to solidify. Once the gel was solid, it was placed into a Fotodyne minigel apparatus that was then filled with 1X TBE and 10 µl EtBr. After adding 5 µl blue juice (tracking dye) to each DNA sample, 12 µl was then loaded per well. 5 µl of marker (λ phage DNA cut with *HindIII* and *EcoRI*) was loaded into the first well. The gel was run at 60V (applied by a Fotodyne source) for 2 hours. The gel was then placed onto a UV lightbox (Foto/Phoresis I by Fotodyne) and a picture of it taken using a Fotodyne Inc. Foto Phoresis I System camera with an orange filter at aperture 5.6, shutter speed 2.

2-D Gel Electrophoresis

A neutral/neutral 2-D gel was run as previously described by Lorimer (2002). The percent agarose and parameters of the first and second dimensions were determined based on the concentration of the resulting DNA fragments from the 1-D minigel. The first dimension gel was made of 0.5% agarose in 1X TBE (1.5 g agarose in 300 ml 1X TBE). The gel was allowed to harden for 30 min at room temperature and then for an additional 15 min in a refrigerator to ensure its hardening. 5 µl blue juice was added to each DNA sample during this time. The gel was then placed into an Ellard Instrumentation Ltd Gel box with an electrode-to-electrode path length of 35 cm containing 1X TBE buffer (no EtBr). The gel was run for 30 hrs at 25 V (0.7 V/cm), voltage applied by an E-C Appartus Corporation power source. Subsequently, the gel was stained in 1X TBE buffer plus 30 µl EtBr for 20 min. and visualized on a large UV light box (Fotodyne Inc., model 3-3500). A razor blade was used to excise the lanes of the gel containing DNA, which were then slid onto a new tray.

The second dimension gel was prepared based on the first dimension gel. A higher percentage agarose gel, 1.8%, containing 5% EtBr (7.2 g agarose in 400 ml 1X TBE + 20 µl EtBr) was then prepared. The pieces of the first gel were sealed using warm gel solution from the second gel preparation so they did not slide when the second gel was poured. After allowing them to cool, the rest of the gel was poured and allowed to harden. The second dimension gel was run in 1X TBE buffer also containing 5% EtBr for 6 hours at 200 V (5 V/cm) in the cold room (4°C). A pump was used during electrophoresis to keep the buffer circulating throughout the gel box. A picture was taken of the gel again using the Photo Phoresis System 1 camera. The gel was then trimmed leaving a space ~1 cm between its

edge and the first dimension gel pieces, then flipped over and put into a glass tray to perform a Southern blot.

Southern Blot

The DNA from the 2-D gel was transferred to a nitrocellulose membrane using a modification of the Southern blot procedure (Southern, 1975). The gel was placed facedown in a glass tray and rocked for 15 min in 200 ml BI (1/4 N HCl) to remove purine bases using a New Brunswick Scientific Classic Series C1 Platform Shaker. The first solution was then poured off and this process repeated a second time. Once the BI solution was poured off for the second time, 200 ml BII (1/2 M NaOH, 1M NaCl) was added and rocked for 10 min to denature the DNA and break the strands where they had been depurinated. The solution was poured off and the step repeated once more. Finally, 400 ml BIII (1/2 M Tris pH 7.5, 3M NaCl) was then added, rocked for 30 min., and drained off.

A piece of Whatman paper and a piece of positively charged membrane were cut to the same size as the gel facedown. The membrane was dipped into deionized water, then BIII solution and placed onto the gel. The Whatman paper was also wetted with BIII solution and placed on top of the membrane. A stack of paper towels was then placed on top of the Whatman paper until it reached at least one inch higher than the sides of the dish. A weight was placed on top of the paper towels to draw up the solutions and allow the DNA to stick to the membrane. After sitting overnight, the paper towels and Whatman paper were removed from the stack and then discarded. The membrane was then removed and

crosslinked using a UV Crosslinker (Fisher biotech UV Crosslinker, FB-UVXL-1000) set on optimal crosslink mode.

Probes

The probes were made according to manufacturer's recommendations using the NEBlot kit. 50 ng of specific DNA in 11 µl (no more than 5 µl TE, with the rest being water) was denatured at 95°C for 5 min, briefly placed on ice and spun. 2.5 µl 10X labeling buffer (NEBlot kit), 8 µl dNTP mix (with 1:100 dATP), and 2 µl [^{32}P] dATP were combined in a screw-cap tube and incubated at room temperature overnight. Three spin columns per probe were prepared using a 1cc syringe barrel. A few millimeters of siliconized glass wool was packed into the bottom of the syringe barrel that was then filled with sephadex G-50 beads (10 g Sephadex G-50, 160 ml sterile water, 0.32 ml 1M Tris-HCl pH 8.0, and 0.4 ml 0.2 M EDTA). After tapping to start the flow, the liquid was permitted to drip out and the barrel was filled to the top with beads by repeated pipetting.

The syringe was then placed into a 15 ml conical tube and spun in an IEC Clinical Centrifuge for 5 min at a setting of 3 to produce ~9 ml of packed beads. The liquid was removed from the conical tube and discarded. Each probe was then pipetted onto the sephadex in the spin column and 300 µl STE (sodium chloride, Tris, EDTA) was loaded on top of the probe. The mixture was spun through the spin column for 5 min at a setting of 3. DNA molecules that have been newly synthesized will contain α^{32}P dATP and also will be larger, thus passing through the column to end up in the liquid at the bottom of the tube. ^{32}P dATP that does not get incorporated will get caught in the G-50 Sephadex and remain in the column. Counts in the syringe were checked and compared to counts in the probe using a

Geiger counter. Percent incorporation was calculated to be the number of counts in the liquid in the 15 ml tube after spinning divided by the counts of the liquid plus that remaining in the column at the same distance from the Geiger counter. Approximately 50% incorporation was considered ideal, with under 20% and over 60% being unsuccessful. Columns were disposed of in radioactive waste.

Probing the blot

The membranes from the blots were each prehybridized by rolling them up and placing them into hybridization tubes, using a spacer sheet if more than one blot was to be probed at the same time. Cambridge prehyb solution, consisting of 0.25 M sodium phosphate monobasic (pH 7.2) and 7% SDS brought to a pH of 7. 2 with NaOH, was used to wet each blot which were then incubated at 60°C rolling in the hybridization oven (Techne Hybridizer HB-1D) for 45 min. 12 ml of the prehyb solution was then added to each 15 ml tube along with 300 µl of 5 mg/ml SSS DNA and placed into boiling waterfor 5 min. Once the original prehyb solution was poured off of the blots, the probes were added and incubated at 60°C rolling overnight. The probes were then poured back into 15 ml conical tubes and refrigerated. The blots were washed with 100 ml 2X SSC (sodium chloride and sodium citrate) with 1% SDS (sodium dodecyl sulfate) rotating at 60°C for 30 min. Once this solution was poured off, the blots were taken out of the hybridization tube and placed into a tray where they were washed two times with 0.2 X SSC 1% SDS for 15 min. All excess liquid was removed and the blots wrapped in plastic (SaranWrap).

Exposing the probe

The wrapped blots were then placed into cassettes with Kodak BioMax MR film with or without screens. The cassettes were placed into the -80°C freezer for a few days.

Developing the film

The film of each blot was developed in a dark room using three trays: one containing cool water, one containing fixer and one containing developer. First the film was slid into the tray containing GBX developer (103 ml diluted with water to 473 ml) and shaken gently until arcs were seen (~1 – 3 min). The film was then placed into the water for ~3-5 min and then moved into the GBX fixer (103 ml diluted with water to 473 ml) until it became transparent. The film was then placed back into the water for an additional 3-5 min, taken out and allowed to air dry.

Results

Isolating DNA

MtDNA is not replicated in concert with the cell cycle, therefore there is no point in synchronizing the cells (reviewed in Berger and Yaffe, 2000). Nonetheless, rapidly growing cells are replicating more DNA quicker than slow-growing or stationary cells. It is then fairly obvious that log phase cells, which are growing the most rapidly, will have more replicating DNA on average. Cells were therefore grown to late log phase in order to optimize the amount of replication forms present. Total DNA was isolated from the cells using the large scale smash and grab method as described in materials and methods. Sodium azide was added to halt replication and preserve replication forms with as little structural damage as possible by rapidly killing the cells. Sodium acetate was also added to aid in subsequent precipitation.

Restriction enzymes were used to focus on specific fragments of the mitochondrial genome for electrophoresis. Four enzymes were chosen that cut both within the wild-type and mutant mitochondrial genomes at sites located outside of regions containing presumed replication origins. BS127, 82d, and N23-3 were digested with *BstYI* and *EcoRI* while BS132, HS3324, and N28 were digested with *SacII*and *SphI*. Double digests were carried out in buffers that allow optimal activity of both enzymes according to New England Biolabs Inc. *BstYI* and *EcoRI* digests were performed in *EcoRI* buffer due to the star activity of *EcoRI*. This means that in its unique buffer, this enzyme remains restricted to its specific cut site but in other buffers, it may cut randomly throughout the genome. *BstYI* required a 60°C incubation temperature, while *EcoRI* required 37°C. *SacII* and *SphI* digests were performed

in New England Buffer 4 at 37°C. A map of the *S. cerevisiae* mitochondrial genome with relevant mutant origins and restriction enzyme cut sizes is found in Figure 7.

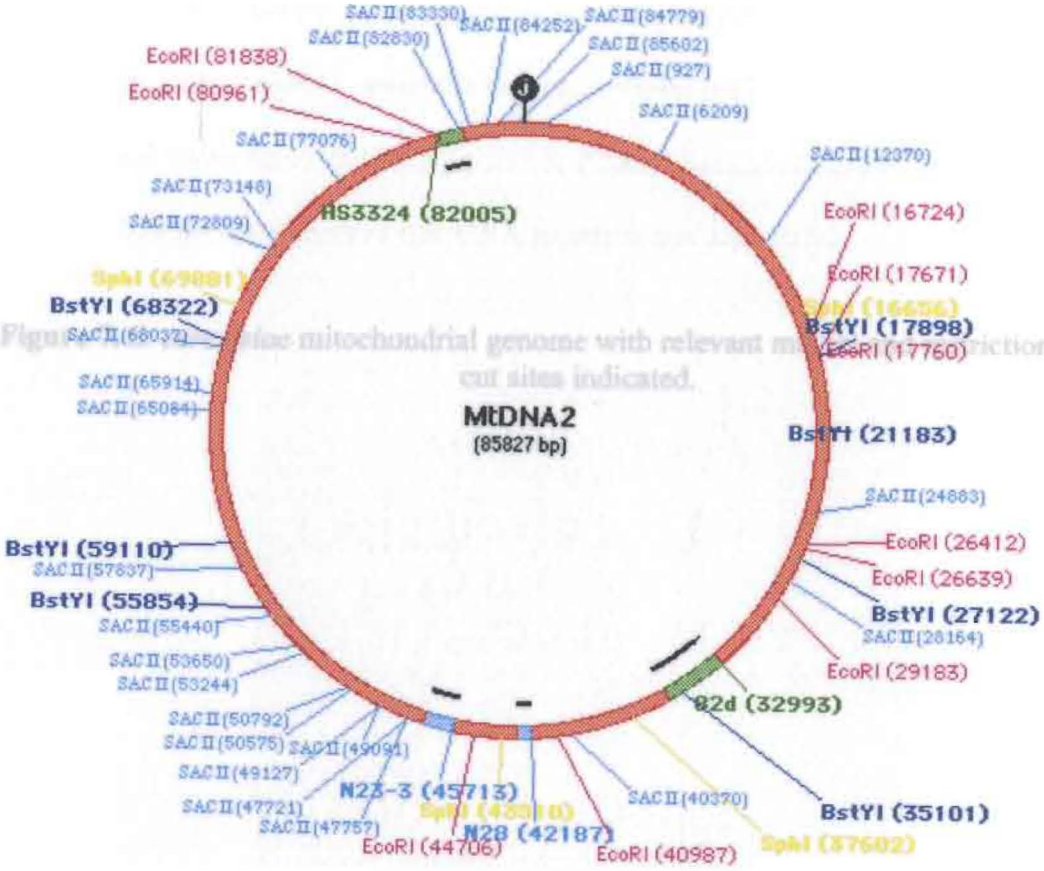
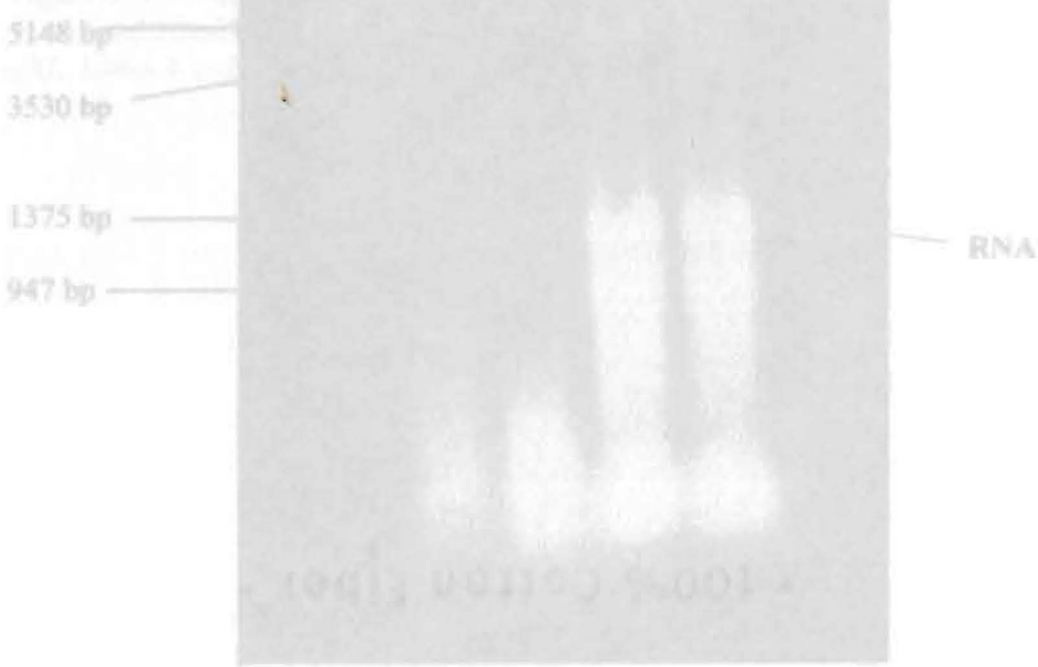

MtDNA2
(85827 bp)
HS3324 (82005)
N28-3 (45713)
N28 (42187)
82d (32993)
BstYI (68322)
BstYI (59110)
BstYI (55854)
BstYI (17898)
BstYI (21183)
BstYI (27122)
BstYI (35101)
EcoRI (81838)
EcoRI (80961)
EcoRI (16724)
EcoRI (17671)
EcoRI (17760)
EcoRI (26412)
EcoRI (26639)
EcoRI (29183)
EcoRI (44706)
EcoRI (40987)
SacII (83330)
SacII (84252)
SacII (84779)
SacII (52830)
SacII (85602)
SacII (927)
SacII (6209)
SacII (12370)
SacII (77076)
SacII (73146)
SacII (72809)
SacII (68037)
SacII (65914)
SacII (65084)
SacII (57837)
SacII (55440)
SacII (53650)
SacII (53244)
SacII (50792)
SacII (50575)
SacII (49091)
SacII (49127)
SacII (47721)
SacII (47757)
SacII (24863)
SacII (28164)
SacII (40370)

Figure 7. *S. cerevisiae* mitochondrial genome with relevant mutant and restriction enzyme cut sites indicated.

A 0.8% agarose mini-gel of both mutant and wild-type restriction enzyme digests is shown in Figure 8. Bands representing 2 micron DNA plasmids commonly found in yeast appear at about 5000 bp in both wild-type and mutant strains. Bright bands located at about 1400 bp are seen in lanes 3 and 4 which are BS132 and HS3324 respectively. These bands are representative of rRNAs present in the total cellular nucleic acid samples. Lanes 2 and 3, however, lack these bands indicating possible RNase contamination of the BS127 and 82d samples. This gel demonstrates that DNA isolation was successful.



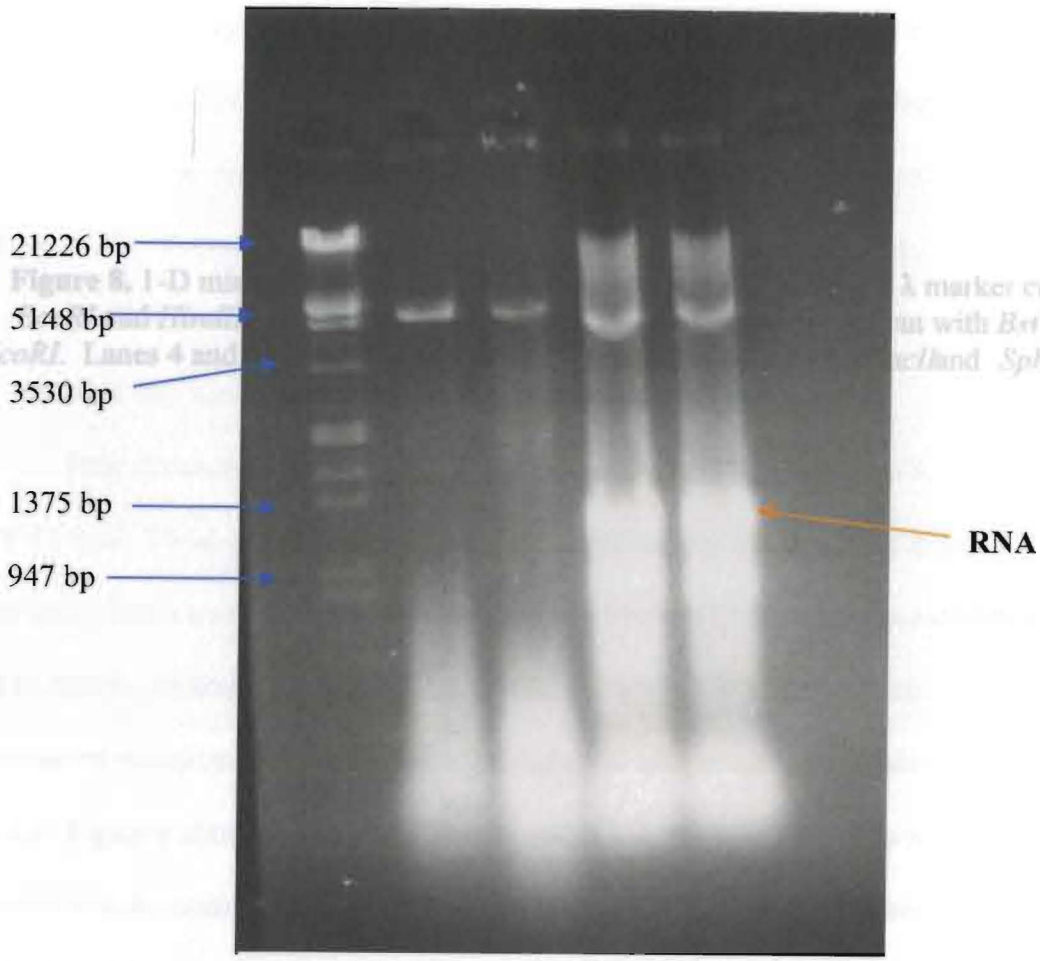

21226 bp
5148 bp
3530 bp
1375 bp
947 bp
RNA

Figure 8. 1-D minigel of cut wild-type and mutant strains. Lane 1 is λ marker cut with *EcoRI* and *HindII*. Lanes 2 and 3 are BS127 and 82d, respectively, cut with *BstYI* and *EcoRI*. Lanes 4 and 5 are BS132 and HS3324, respectively, cut with *SacII*and *SphI*. There is possible RNase contamination in lanes 2 and 3.

Neutral/Neutral 2-D Gel Electrophoresis and Southern Blots

Neutral/neutral 2-D gel electrophoresis was performed on both wild-type and mutant *S. cerevisiae* strains in cut and uncut forms as described in materials and methods. In order to obtain high enough concentrations of DNA to allow replication intermediates (RIs) to be accurately viewed on a 2-D gel, the volume of mtDNA being cut had to be increased several-fold. This in turn required an additional step of PCIA extraction, purification and resuspension. Previous research has demonstrated that phenol-chloroform extraction did not have any effect on resulting 2-D gel arcs (Yang et al., 2002). This step, therefore, should not have caused any loss of or damage to replication forms.

First dimension gels consisted of 0.5% agarose and were electrophoresed for 30 hours at 0.7 V/cm. These conditions allowed the molecules to be sufficiently separated based on size only. Since a much higher voltage (5 V/cm) was applied to a higher percentage agarose gel (1.8%) in the second dimension, breakage of fragile RIs was a concern. EtBr, which was included to visualize DNA bands on a UV lightbox, also acted to maintain the shape of these forms. Figure 9 shows a 2-D gel of BS132 and HS3324 as visualized on a UV light box. Similar arcs are seen in both the BS132 and HS3324 gels. The visible arcs of dsDNA of random sizes from approximately 100 bp to the limit of mobility at over 20 kb. These arcs are not very steep, so these conditions are more favorable for visualizing rare forms, replication intermediates that contain branches and rise above the arc of linear forms after Southern blotting.

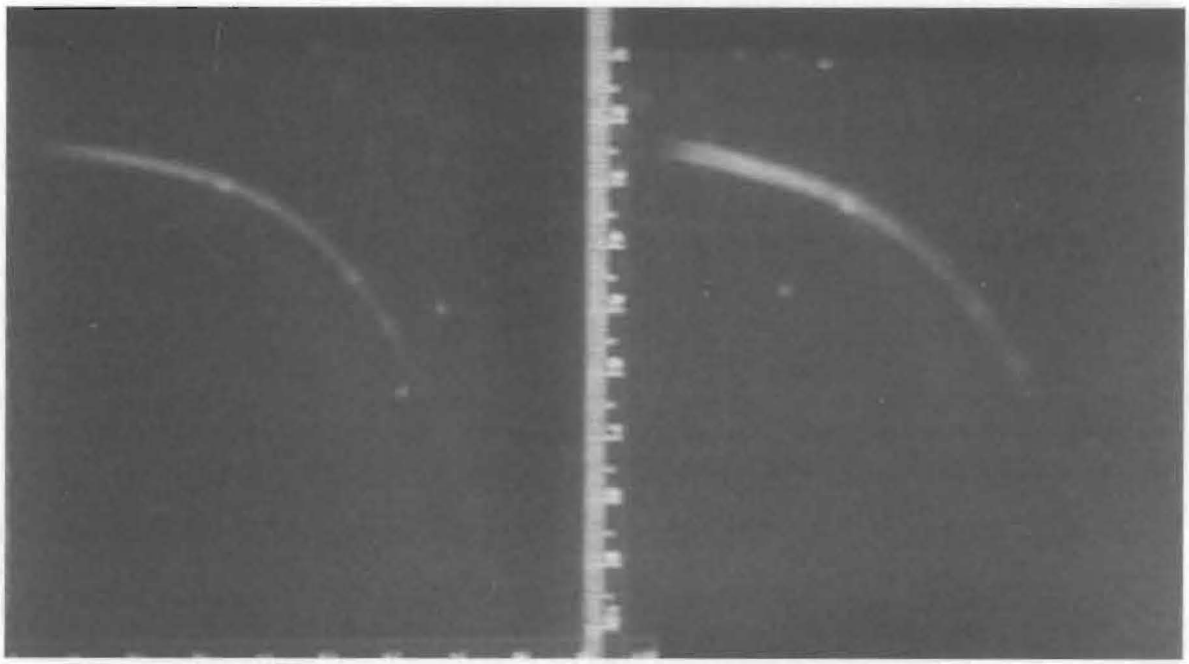
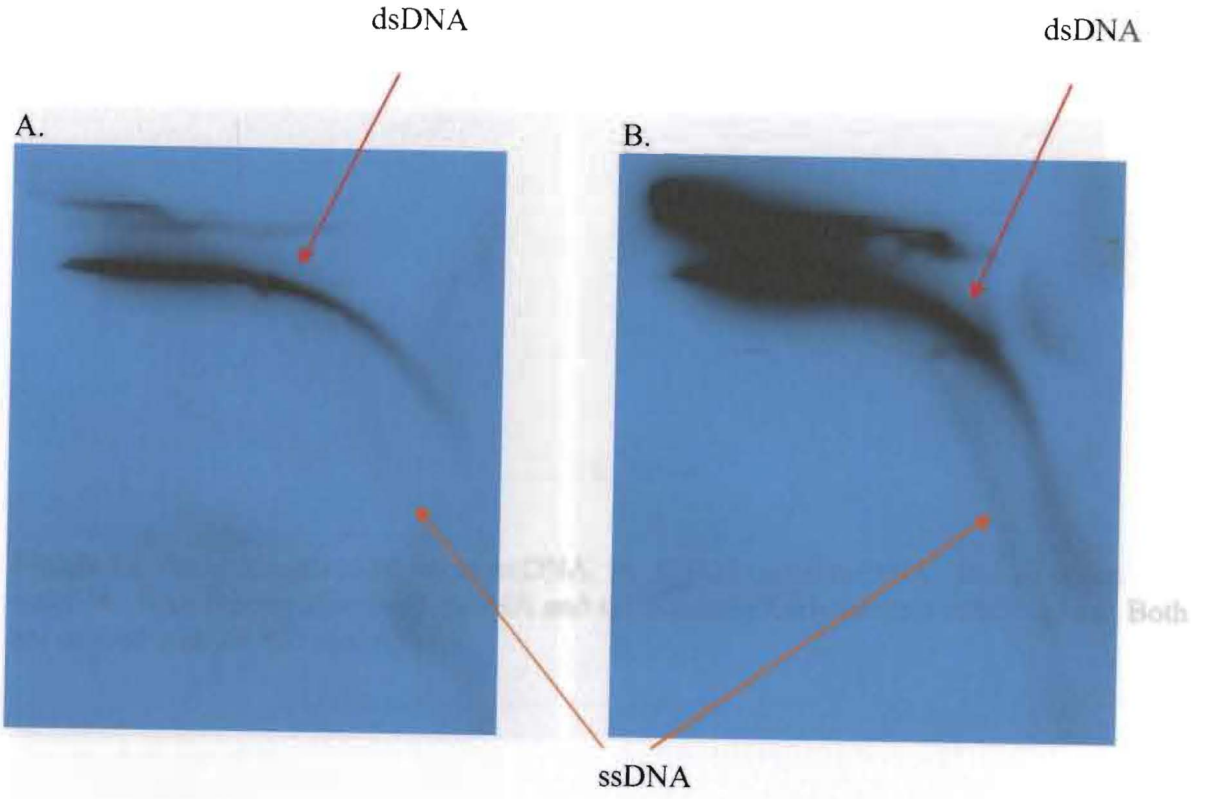


Figure 9. 2-D gel of BS132 (left) and HS3324 (right) both cut with *SacII* and *SphI*.

Southern blots were performed on each 2-D gel. Probes specific for relevant mutants were used to obtain autoradiograms. BS132 blots were probed with either HS324 or N28 radiolabelled fragments. BS127 blots were probed with either probes specific for N23-3 or 82d. 8-3-specifc probes were used to detect 82d fragments because 82d is a subdeletion of 8-3.

Wild-type vs. mutant mtDNA

Autoradiograms of wild-type and mutant mtDNA in both cut and uncut forms support a common replication mechanism. Figure 10 shows uncut rho^+ and rho^- mitochondrial genomes. Arcs of dsDNA are clearly present in both A and B. There are possibly more complex forms located above the double-stranded arc in 82d (Figure 10B) due to recombination of the repetitive genome, but due to the relatively high exposure, they are not visible. Also present in both are arcs of ssDNA. Figure 11 shows these same strains, but in the cut form. DsDNA arcs are once again clearly present, but the ssDNA arcs are not. This could be because ssDNA is either not present, present only in very low levels, or has been degraded during the course of incubation with the restriction enzyme due to its fragile nature. Since the same samples were used for both cut and uncut gels, degradation is the most likely case.


dsDNA
dsDNA
A.
B.
ssDNA

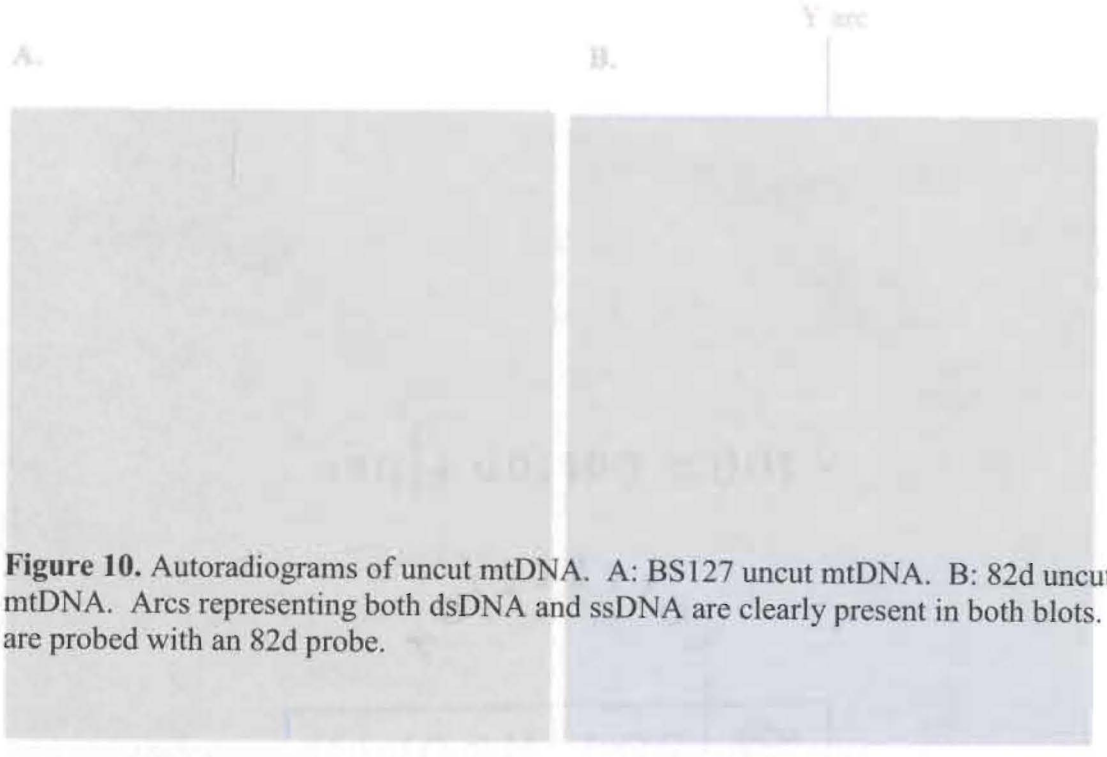


Figure 10. Autoradiograms of uncut mtDNA. A: BS127 uncut mtDNA. B: 82d uncut mtDNA. Arcs representing both dsDNA and ssDNA are clearly present in both blots. Both are probed with an 82d probe.

A.

B.

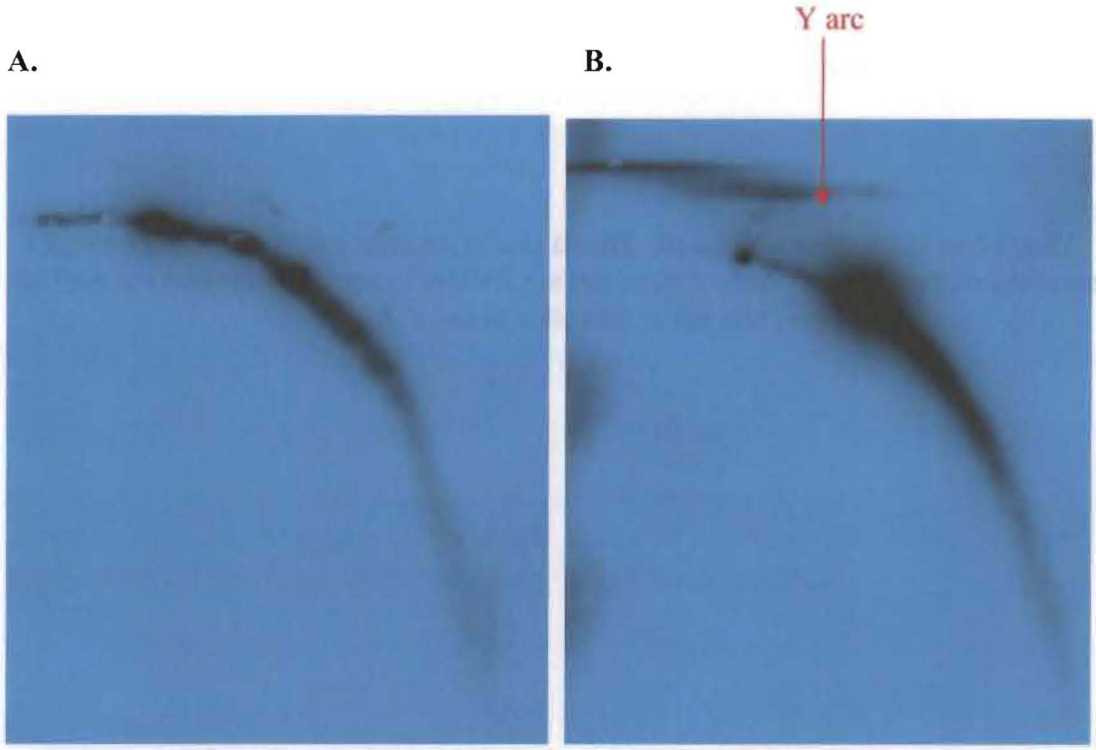


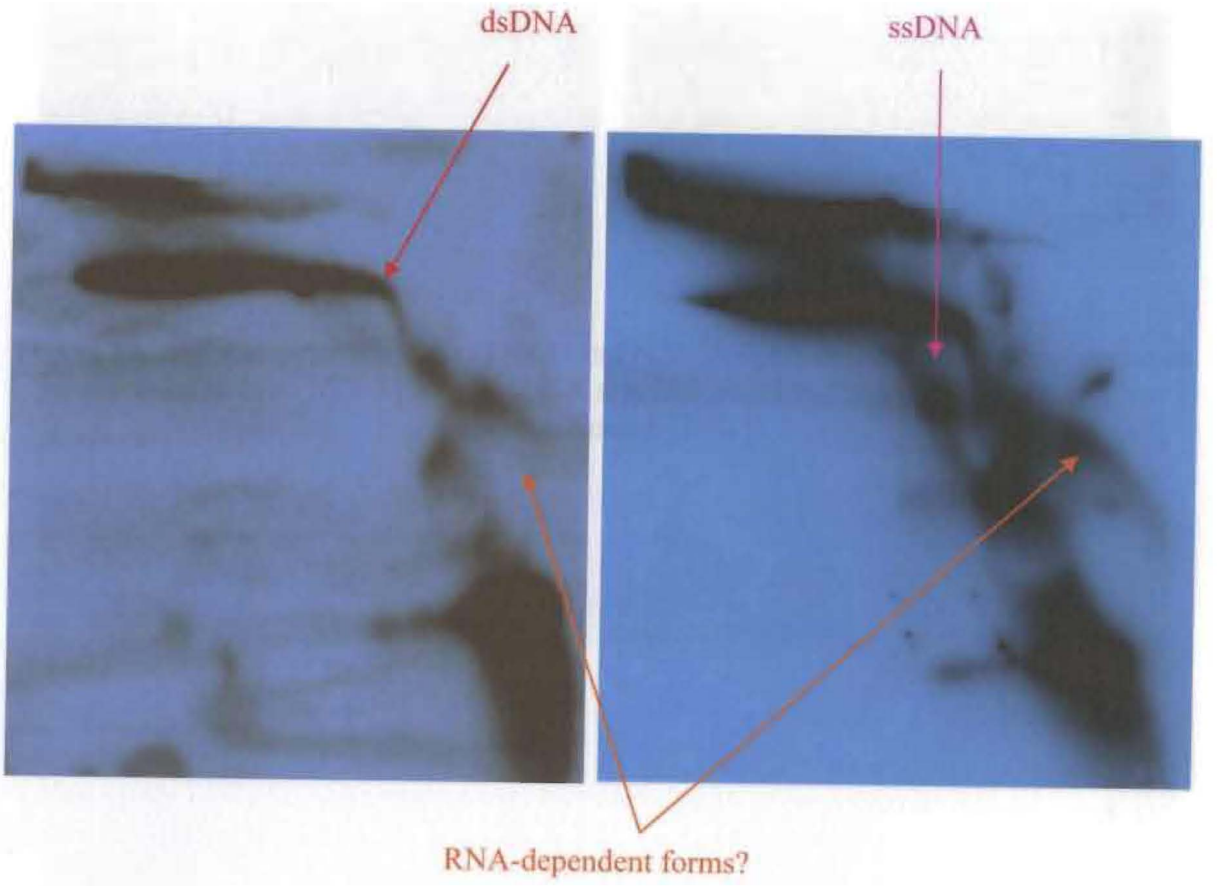
Figure 11. A: BS127 cut with *BstYI* and *EcoRI*. B: 82d cut with *BstYI* and *EcoRI*. Arcs of dsDNA are prominent. Arcs of ssDNA are not readily observed. 1n and 2n spots are visible. A Y arc is indicated in the 82d prep.

Cut vs. uncut mitochondrial DNA

In order to determine if mtDNA replication occurs in the same manner in both cut and uncut forms, the resulting Southern blots were compared. These results support a basic mechanism of replication common to both forms. As seen in Figures 10 and 11, dsDNA arcs are the predominant RIs in both. As previously mentioned, ssDNA arcs are not as prominent in the cut forms because they may be present in levels too low for detection, are not present at all, or have been degraded. A Y-arc is clearly present in the 82d cut, but not in the BS127 cut. One reason this arc is missing is due to the difficulty in achieving a high enough concentration of the wild-type mtDNA to see forms that would cause the arc. Restriction enzyme digestion of BS127 may not have been complete due to its high concentration, which would result in spots of various fragment sizes on the arc, as seen.

Uncut genomes

The results of the BS127 and N23-3 uncut 2-D gels yielded some unusual forms (Figure 12). These forms are located at a position on the blot indicating that they are smaller and more complicated. They are also located away from the arcs of dsDNA and ssDNA, meaning that they are possible RNA-dependent. If this was the case, then treating these genomes with RNase A prior to 2-D gel electrophoresis would eliminate the forms. The results of RNase A treatment of BS127 and N23-3 are shown in figure 13. The unusual forms were eliminated, leaving behind only arcs of dsDNA and ssDNA.


dsDNA
ssDNA
RNA-dependent forms?

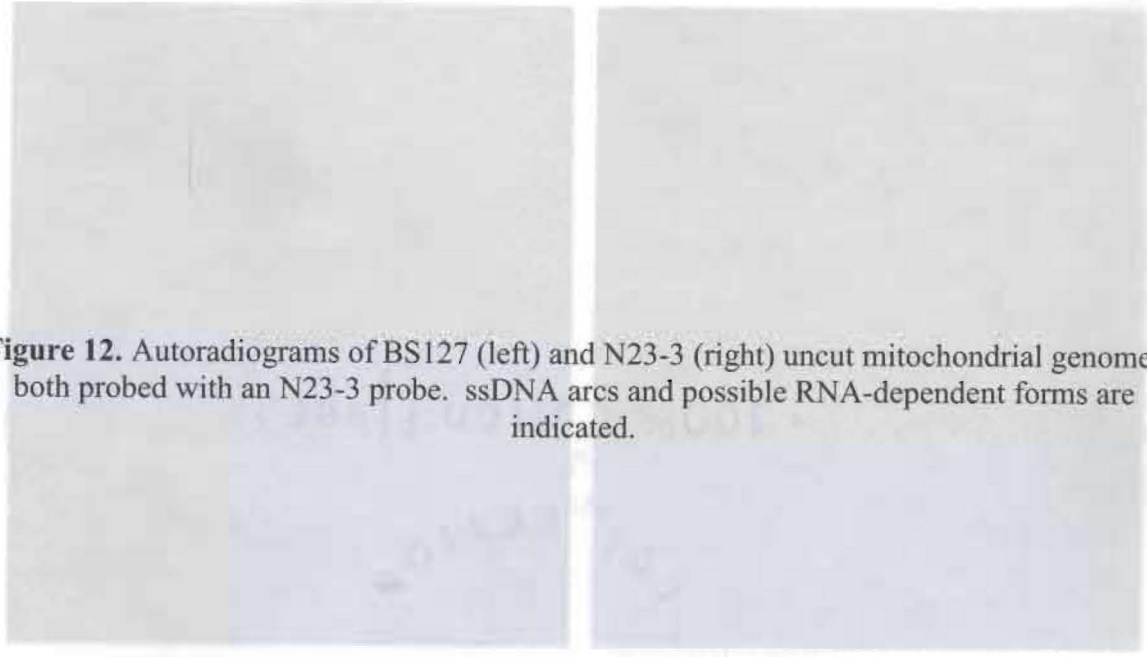
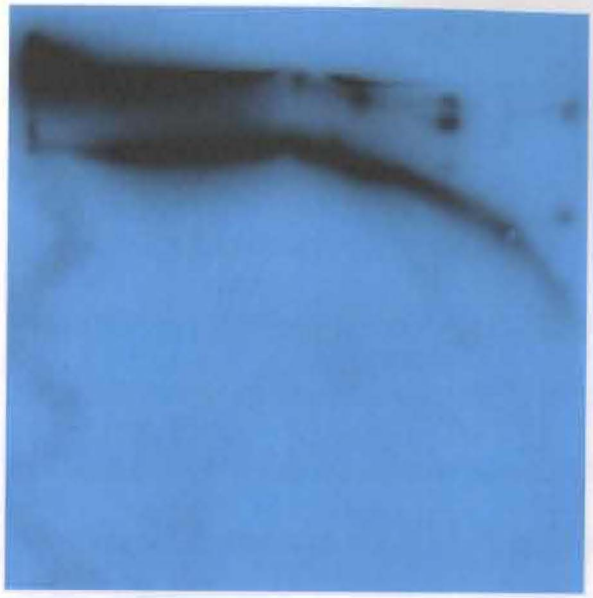


Figure 12. Autoradiograms of BS127 (left) and N23-3 (right) uncut mitochondrial genomes both probed with an N23-3 probe. ssDNA arcs and possible RNA-dependent forms are indicated.

Figure 13. BS127 (left) and N23-3 (right) uncut mitochondrial genomes both treated with RNase A prior to 2-D gel electrophoresis. Possible RNA-dependent forms in untreated samples have been eliminated. Arcs of dsDNA and ssDNA are still present.

To further characterize these unusual forms, N23-3 mtDNA was treated with RNase H and RNase I. The commercially obtained RNase I is a more pure RNase then RNase A thought both degrade ssRNA. According to New England Biolabs, Inc., RNase I is strand-specific and will cut at any RNA nucleotide, while RNase A will only cut at unpaired cytosine or uracil bonds. RNase H specifically degrades RNA:DNA complexes. One N23-3 uncut sample was treated with RNase I in New England Buffer 3 (Figure 14A) and a second sample was treated with RNase H in RNase H buffer (Figure 14C). As an RNase H control, another N23-3 sample was treated in the same way as the RNase H sample, but the enzyme was not added (Figure 14B). These samples were incubated at 37°C for 30 min, PCIA purified and precipitated before being resuspended in 1X TE buffer. A final control was an untreated N23-3 sample, also PCIA purified and resuspended in 1X TE buffer (Figure 14D). With each RNase treatment, no forms matching the previous unusual ones are seen. Also, arcs presumed to be ssDNA are still present which demonstrates that this arcs is indeed made up of ssDNA. It is still possible that the forms are RNA:DNA complexes since RNase H degrades them. Unfortunately, the untreated sample also lacks these forms. This could be due to differences in conditions during blotting or RNase contamination of the sample or buffers. Since they were not duplicated in the new N23-3 sample, it is hard to precisely identify them.

A. RNase H



B. RNase H control

C. RNase I

D. Untreated control

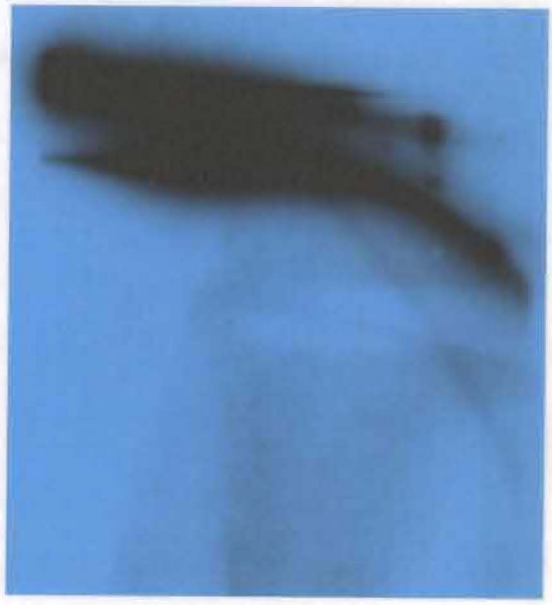


Figure 14. N23-3 uncut mtDNA treated with RNase H (A) and RNase I (C). Controls are RNase H conditions without the RNase H (B) and untreated N23-3 sample (D).

Besides the unusual forms seen in the BS127 and N23-3, only one good arc of replication intermediates (Y arc in Figure 11B) was seen, and even it was faint. This is due in large part to issues with obtaining high enough concentrations of mtDNA to see forms and allow electrophoresis to proceed without overloading the gel. Since rho$^-$ mitochondrial genomes contain repeated fragments, yet have the same mass of DNA as the rho$^+$ genomes, these cells contain about 80x as much of the target sequence. This causes problems when comparing wild-type and mutant cells, because relative concentrations must be obtained. Wild-type mtDNA concentrations had to be dramatically increased in order to have enough DNA to see rare forms on the gels. This process can lead to incomplete restriction digests and overloading of the gel during electrophoresis. Both of these situations do not yield clear results. Blotting issues can also account for unclear arcs. Bubbles in the membrane during blotting and exposure to the radioactive probe also may have led to unclear arcs. What remains clear is that ssDNA and dsDNA arcs were consistent in all wild-type and mutant samples in both cut and uncut forms.

Discussion

Several technical issues arose throughout the course of this project. The first obvious issue was comparing wild-type and mutant strains of *S. cerevisiae.* A mutant strain contains about 80x as many copies of the same DNA fragment sequence as does the wild-type, so copy-number during probing became an issue. This is why autoradiogram exposures of mutant strains are so much darker than those of wild-type strains—the same probe will have many more target sequences to detect. Therefore, mtDNA concentrations had to be equalized. The bulk of the work during my research was spent addressing this problem. Wild-type DNA amounts had to be drastically increased in order to see rare forms, also taking care not to compromise their analysis by overloading the experiment. 2-D gel conditions then had to be manipulated to obtain arcs that allowed replication intermediates to be observed. Issues also became evident during blotting. One of the solutions, BII, may actually be removing RNA, although the DNA should be fixed to the membrane by this point. A much larger issue was the possibility of RNase contamination, as mentioned in Figure 8. RNA may be vital for the stability of replication intermediates, as has been demonstrated in rats (Yang et al., 2002) and *Schizosaccharomyces pombe* (Lorimer, unpublished results). Forms may have been lost during this procedure. Also bubbling of the membrane interfered with the clarity of results. There were also issues with high amounts of background showing up on the autoradiograms.

According to the strand-asynchronous model, mtDNA replication commences at the D-loop region with continuous replication of the H strand of the genome. Once the replication origin for the L strand is uncovered roughly two-thirds of the way around the genome, it is replicated in the opposite direction (Clayton, 1982). Thus, this asymmetric,

asynchronous process would generate mostly single-stranded RIs. My 2-D gel electrophoresis results show arcs of ssDNA intermediates in both wild-type and mutant uncut *S. cerevisiae* mtDNA (Figures 10, 12 and 13). This possibly means that some replication occurs according to Clayton's proposed model.

A second mode of replication involving dsDNA intermediates may also be taking place in *S. cerevisiae* mtDNA. DsDNA Y-arcs can be seen in addition to the ssDNA arcs in both rho^+ and rho^- uncut genomes. Treating the mtDNA with RNase A does not eliminate these arcs, supporting that they do indeed consist of DNA. Since the strand-asynchronous model will not generate many dsDNA RIs, another mechanism must account for the observed results. These mechanisms may include rolling circle or be recombination-dependent. More than one mechanism of mtDNA replication has not only been observed in yeast but also in mammals. Holt et al. (2000) presented evidence of RIs sensitive to S1 nuclease, a nuclease that degrades ssDNA, and also S1-resistant RIs in mouse liver cells. Those sensitive to S1 were hypothesized to the result of the strand-displacement model, while the S1-resistant intermediates were the result of a strand-coupled mechanism. My results are consistent with their findings.

Rolling circle replication is a strong possibility for the mechanism of mtDNA replication that is occurring since it can generate both single- and double-stranded results—both of which were seen. During this process, long strands of newly-replicated DNA are produced, some of which may be single-stranded (leading strand synthesis) and others double-stranded (lagging strand synthesis). RNA-dependent forms may arise during priming of lagging strand synthesis.

Another candidate for the mode of mtDNA replication observed is recombination-dependent. Since the mitochondrial genome of *S. cerevisiae* is highly repetitive, especially in the case of rho⁻ cells, one strand of DNA undergoing synthesis can easily invade another and recombine in order to finish replication. This would generate long single-stranded replication intermediates as well as long double-stranded ones. These events could involve RNA priming, which would account for the RNA-dependent forms seen. Priming could also be absent, with simultaneous transcription instead causing the RNA-dependent forms.

The ssDNA arcs also lend further support to a recombination-dependent mechanism of mtDNA replication based on previously observed action of 2 genes: MGT1 and MHR1. The product of the MGT1 gene (also called CCE1) resolves recombination structures in yeast mtDNA (Lockshon et al., 1995). Mutant strains lacking both MGT1 and MHR1, a gene involved in strand invasion, cannot maintain any mtDNA. This suggests that mtDNA inheritance, and therefore replication, is recombination-dependent (Ling and Shibata, 2002). In both wild-type and mutant strains, ssDNA arcs were always present, possibly due to this process of recombining.

In order to further investigate how more specific regions of *S. cerevisiae* mtDNA replicates, genomes were digested into smaller pieces with a combination of *BstYI* and *EcoRI* or *SacII* and *SphI*. The arcs of dsDNA clearly present in all digests (Figure 11) indicated that these portions of the genomes also utilize primarily double-stranded intermediates. Faint ssDNA arcs are present as well. This deficiency of ssDNA can be attributed to it only being present in low levels or degradation during the extraction and enzymatic cleavage processes. Since the same DNA preps were run uncut and then cut, degradation is the most logical

explanation. These results support a mechanism of mtDNA replication other than a strand-asynchronous one.

Even though more than one mechanism may be responsible for *S. cerevisiae* mtDNA replication, it appears that they function in identical manners in both rho$^+$ and rho$^-$ genomes. When 2-D gel results of rho$^-$ mutants were compared to their rho$^+$ counterparts, similar arcs of both dsDNA and ssDNA were observed (Figure 10). Since rho$^-$ cells are not replicating their mtDNA in a unique manner, studies utilizing these genomes can be compared to ones using rho$^+$ cells without concern. The massive deletions do not appear to have a detrimental effect on the process by which mtDNA replicates.

Unexpected forms were observed in the uncut N23-3 and BS127 mtDNA samples (Figure 12). These forms were located low and to the right of the dsDNA arc, indicating that they are small, complicated forms. It was hypothesized that these forms were RNA-dependent. Treatment of BS127 and N23-3 cells with RNase A eliminated these forms (Figure 13). To further explore this phenomenon, N23-3 was also treated with RNase I, which degrades ssRNA, and RNase H, which degrades RNA:DNA complexes. These RNases also eliminated these forms (Figure 14). Therefore, these forms may represent a mode of replication involving RNA intermediates that can complex with DNA. One example of this would occur with extensive ribonucleotide incorporation, like that observed in rat mtDNA (Yang et al., 2002). These forms have not been previously reported in *S. cerevisiae.* Another possibility is a mode of replication involving multiple priming events using RNA intermediates. Unfortunately, as seen in the RNase H control (Figure 14D) these forms were not duplicated as I expected. Since this sample was not treated with any RNase, these forms

still should have been apparent. Whether they were merely artifacts of the DNA preparation or true RIs was not evident. Contamination with other RNases is also a possibility.

Future studies can be performed to further investigate the possibility of RNA-dependent species in *S. cerevisiae* mtDNA as potentially presented here. These could include treating other samples with specific RNases as well as S1 nuclease. Treatment can also be done before and after restriction enzyme digestion to see if these forms are also present in mtDNA fragment replication and if adding the enzymes at different points makes a difference. Furthermore, 2-D gels can also be run under strict RNase-free conditions then blotted with and without RNA removal to see if RNA-dependent forms are being lost during this process.

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