Replication Analysis of Mitochondrial DNA in the Yeast *Saccharomyces cerevisae*

By

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Abstract

The mechanisms of mitochondrial DNA (mtDNA) replication are not completely understood. The major current model of mtDNA replication involves an asynchronous, asymmetric process called D-loop replication. The D-loop model of replication requires mtTFA and RNA polymerase for initiation of replication. The nuclear gene, (RPO41) encodes catalytic subunit of the RNA polymerase. Massive deletions in mtDNA (rho$^-$) can produce small mtDNA genomes that contain presumed replication origins, called *ori/rep* sequences or not. Those rho$^-$ genomes that have *ori/rep* sequences exhibit a replication/segregation advantage and are called hypersuppressive (HS rho$^-$). Those without ori/rep sequences are called neutral, rho$^-$. All rho$^-$ mutants with deleted RPO41 decrease the transcription to non-detectable levels, but mtDNA is stably maintained. This indicates that D-loop mode is not only replication mechanism found in *Saccharomyces cerevisae* rho$^-$ cells. Two dimensional gel electrophoresis of HS and neutral rho$^-$ mtDNA reveals evidence that another mode of replication is possibly found in these cells. The presence of long strands of single stranded mtDNA in rho$^-$ and wildtype cells grown to log phases may indicate rolling-circle mechanism of replication in *Saccharomyces cerevisae* cells.

Mammalian cells have been found to have two modes of mtDNA replication. An asymmetric-asynchronous D-loop mechanism of mtDNA replication is found in cells that maintain their copy number of mtDNA. Another mode of mtDNA replication was identified in cells that need to reamplify the copy number of mtDNA. Analogous to these findings this study results suggests different modes of replication in cells that are rapidly increasing their mtDNA copy number (cells in log phase), possibly through a rolling-circle mechanism of replication. Another mode of replication is found in stationary cells that simply maintain their mtDNA copy number, possibly by a D-loop mode of replication.

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Introduction

Mitochondria are the only organelles besides the nucleus, and chloroplasts in plant cells, that have their own genetic material, DNA. Mitochondria are the cell organelles that are necessary for cell respiration. Mitochondrial function in the cell respiration requires expression of mitochondrial genes. Mitochondrial DNA (mtDNA) is organized in a DNA-protein complex within mitochondria that are called nucleoids. Mitochondria are typically tubular or sausage shaped organelles about the size of bacteria.

Mitochondria are found in all eukaryotic cells. They have two membranes: an outer smooth membrane and an inner folded membrane, with christae. Thus, mitochondria have an inner space called the matrix, as well as the space between inner and outer membrane, the intermembrane space. On the surface of the inner membrane are submerged proteins that carry out oxidative metabolism, that is the oxygen-requiring process by which energy in macromolecules is stored in ATP. Most mitochondrial proteins are synthesized outside the organelle on free cytoplasmic ribosomes that are not bound to the endoplasmic reticulum. These proteins are released in the cytosol and taken up by mitochondria. Proteins cross phospholipid bilayers but they can cross membrane only in an unfolded state. To do this they must bind to chaperonens, proteins that unfold other proteins, to cross the mitochondrial membrane (reviewed in Ryan et. al., 1997). Proteins produced in the cytoplasm are directed into specific organelles by signal sequences of amino acids.

The growth and division of mitochondria is not coupled to nuclear division. Mitochondria grow by incorporation of proteins and lipids, the process that occurs continuously during the all phases of the cell cycle. As mitochondria increase in size, one or more daughters pinch off in a manner similar to the way in which bacterial cells grow and divide.

Origin of Mitochondria; Symbiosis and Endosynthesis

It is believed that mitochondria are descendents of endosymbiotic prokaryotes (Fridovich, 1974). This theory proposes that today's eukaryotic cells evolved by symbiosis in which one species of prokaryotes (an eubacteria) was surrounded and lived inside of another species of prokaryote (an archeabacteria). Those engulfed prokaryotes provided their hosts with certain advantages associated with their special metabolic activities. So it is believed that mitochondria have originated as bacteria that were capable of carrying out oxidative metabolism. Chloroplast originated in a similar manner from photosynthetic bacteria.

The endosymbiotic theory is supported with several lines of evidence. First both mitochondria and chloroplasts have two membranes: presumably the inner membrane evolved from plasma membrane of engulfed bacteria and the outer membrane evolved from the plasma membrane of an endocytotic vessicle or the endoplasmic reticulum of the host cell. Mitochondria are about same size as bacteria and most mitochondria and chloroplasts have circular genomes like bacteria and not like nuclear chromosomal DNA, which is divided into linear chromosomes. Mitochondria also divide by simple fission as do bacteria. Mitochondrial ribosomes resemble prokaryotic ribosomes and differ from

cytoplasmatic ribosomes in their RNA and protein compositions, their size, and their sensitivity to certain antibiotics. For example, chloramphenicol blocks protein synthesis by bacterial and most mitochondrial ribosomes but not cytoplasmatic ribosomes. Conversely cyclohexamide inhibits protein synthesis by eukaryotic cytoplasmic ribosomes but does not affect protein synthesis by mitochondrial ribosomes or bacterial ribosomes.

Mitochondrial DNA is not inherited through Mendelian genetics in which the same amount of genetic material is inherited from both parent cells. In a normal human somatic cell there are between 1,000-5,000 copies of the mtDNA genome (reviewed in Howell, 1999). In a mature oocyte cell there are about 100,000 copies and in a mature sperm cell about 1,000 copies of the mtDNA. In mammals, mtDNA is inherited strictly maternally. Paternal mtDNA enters the oocyte, but it does not make a physiologically or evolutionary significance to a genetic contribution. It is suggested that the function of paternal mtDNA is only in support of sperm motility.

Yeasts are eukaryotes and they are facultative anaerobes that means they can produce ATP either through the process of oxidative phosphorylation or fermentation. They can reproduce either through asexual or sexual reproduction. The yeast *Saccharomyces cerevisae* cells with wild-type mtDNA, (rho^+) inherit their mtDNA biparentally, where an equal amount of mtDNA in the zygote is from each parent. However, in certain types of mutant yeast cells mitochondrial DNA is inherited uniparentally. Some of these mutants when crossed to wildtype cells produce almost only mutant offspring. These mutants are called hypersuppressive, rho^- (HS) because in these crosses the wild-type mtDNA is "suppressed" producing only of mutant cells.

However when certain other mutants are crossed to wildtype they produce primarily wildtype yeast cells. These are called neutral, rho$^-$ mutants because they do not "suppress" the inheritance of rho$^+$ genome (Dujon, 1981). A wide range of variation exists between these two extremes. These variations in mtDNA inheritance are caused by changes in the mtDNA sequence. Every mutant, rho$^-$ mtDNA has its own characteristic level of suppressivenss.

Why do we study mtDNA?

Even though the first mitochondrial diseases were discovered in 1962, and mtDNA was not discovered until 1963 (reviewed Wallace, 1999) in the early 1950's was suggested that there was some genetic material found in the cytoplasm, called a "cytoplasmic factor" (Ephrussi et. al., 1955). The first studies published on the relationship between human disease and mutations in mtDNA were in 1988 (Wallace, 1999). Mitochondria are the main source of energy, ATP, in a eukaryotic cell. All cells require ATP. That means if there is a defect in the mtDNA then a cell may not produce enough energy and that may lead to the pathophysiology of that cell. Diseases caused by mutations in mtDNA produce symptoms due to mitochondrial respiratory chain dysfunction (reviewed in Howell, 1999). MtDNA defect can be caused by any type of mutation such as insertions, deletions, or copy number variations. These mutations cause pathologies mainly in cell and tissues with high energy such as muscle or nerve cells (reviewed in Wallace, 1999). Since mitochondria are involved in oxidative phosphorylation, development, apoptosis (programmed cell death), and aging, defects in mtDNA can cause diseases in any of these processes (Fernandez-Moreno, 2000).

One of the most common human diseases caused by deletion is Kearns-Syre syndrome (KSS) and chronic progressive external ophthalmoplegia (CPEO). These mtDNA diseases are caused by deletion called a "common deletion". The common deletion was discovered that occurs between two 13-bp direct repeats situated 4,977bp apart (Zullo, 2002). The origin of the common deletion is still unknown but it was found out that this deletion is associated with reduced energy capacity. In 40% of patients with mitochondrial myopathies large deletions in their mtDNA were found (Harding and Hammans, 1992). Many of these deletions occur during oogenesis.

Other mitochondrial diseases are caused by point mutations. Examples include following mtDNA diseases: MEALS (mitochondrial encelopathy with lactic acidosis and stroke-like episodes), MERRF (myoclonic epilepsy with ragged red fibers), and NARP/MILS (neuropathy, ataxia, and retinitis pigmentosum/maternally inherited Leigh syndrome) and others. MELAS and MERRF are two the most commonly found mitochondrial diseases. MELAS is caused by an A-G transition at nucleotide 3243 in $tRNA^{Leu(UUR)}$ and MERRF is an A-G transition at nucleotide 8334 in the $tRNA^{Lys}$ gene (reviewed in Harding and Hammans, 1992). The main characteristics of these two diseases are that both of them affect multiple systems and are heterogenous. The patients with these disorders can range from mildly impaired to increased chance of death, dependent on the percent of the mtDNA in the cells that is mutant. Most mutations are produced by errors in DNA replication leading to changes in DNA sequences.

Studies done on mtDNA suggest that the main reason for mtDNA defects is that mtDNA mutates at much higher rate than the nuclear DNA. This is due to mtDNA polymerase being more error-prone and/or also does not posses all repair systems as

nuclear DNA polymerase (Howell, 1999). MtDNA is also exposed too much higher endogenous oxidative damage than the nuclear DNA (Beckman and Ames, 1999). One piece of evidence that mtDNA suffers from much higher endogenous oxidative damage is that mtDNA has 53 adducts (chemical addition to a nucleotide) (Shibutani, 2001) per 10^5 nucleotides and nuclear DNA has only 3.2 adducts per 10^5. MtDNA is expected to have a higher level of steady-state oxidative DNA damage than nuclear DNA because of several reasons; proximity of mtDNA to the electron transport chain, lack of protective histones, lack of some DNA repair activities of the mtDNA polymerase, and high rate of mtDNA oxidation presumably high energy use could exacerbate the problem.

MtDNA molecules can be heteroplasmic, which means that some of the mtDNA molecules are mutant and some are wild-type (Shadel, 1999). Over a number of human generations the percentage of the mutant molecules can increase and the percentage of wild-type molecules can decrease. This occurs until this ratio comes to the certain point that is called the threshold. The threshold occurs when the percentage of mutant mtDNA molecules is so high that the cell can no longer respire aerobically, can only carry out anaerobic respiration.

The other reason for studying mtDNA is also because a typical human somatic cell contains between 1,000-5,000 copies of mtDNA molecules and a mature oocyte can contain up to 100,000 mtDNA molecules (Howell, 1999). So the average cell contains ~2,000 copies of mtDNA molecules. There are only two copies of nuclear DNA molecules in each cell. So mtDNA can be used as a phylogenetic tool because there is much higher possibility that an old specimen will have preserved mtDNA and not

preserved nuclear DNA. Following mtDNA through generations maternal inheritance can be followed also.

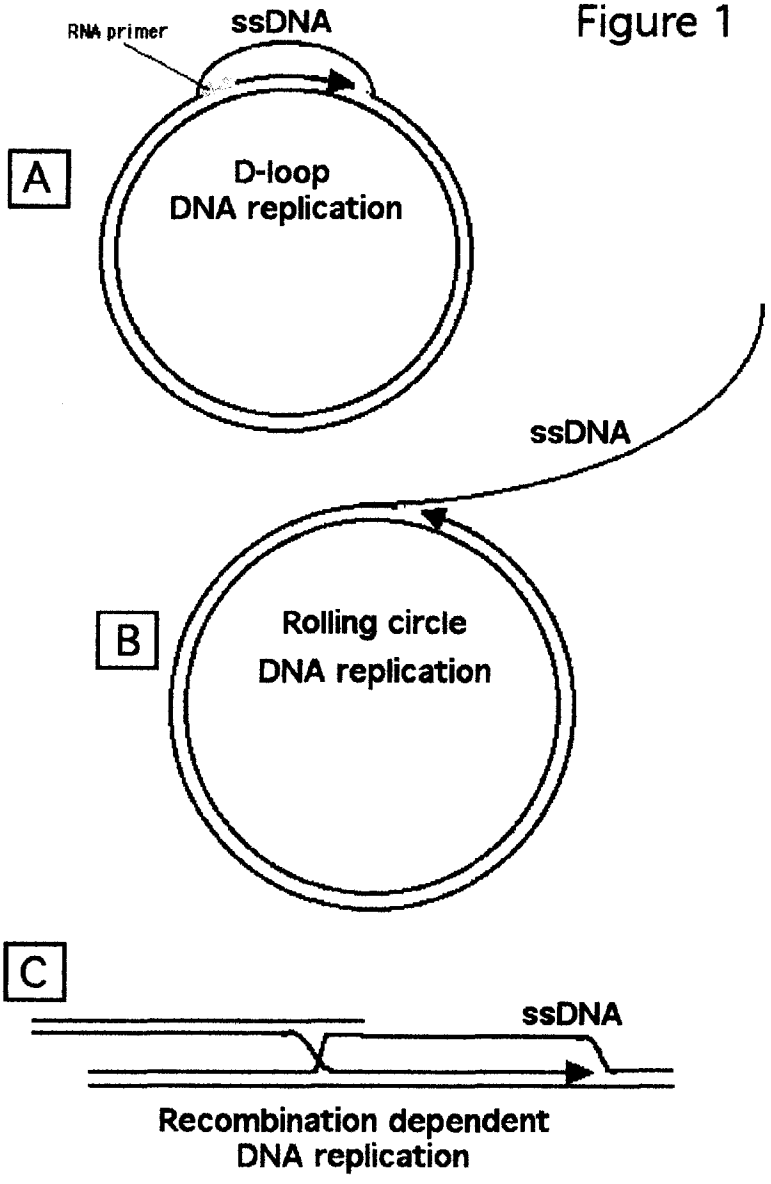
Yeast as a Model

In this study mitochondrial DNA replication intermediates were examined in the budding yeast (*Saccharomyces cerevisiae*). There are a number of advantages of yeast as a genetic model, including: these are eukaryotic cells, they are single-celled, and easy to grow. Yeast cells can grow as haploids and diploids making genetic analysis easier. Haploid meiotic products of a single meiosis can be isolated and grown into colonies of haploid cells for genetic analysis. Yeast cells have short generation time of only 90 minutes so a high number of cells can be obtained in a short time.

The differences between *Saccharomyces cerevisae* and human mtDNA are the following: yeast mtDNA genome is 85.8kbp long and human is only 16.5kb (reviewed in Lecrenier and Foury, 2000). Yeast mtDNA is much larger than human mtDNA because of presence of high number of introns and noncoding sequences in yeast mitochondrial genome. In human mtDNA there is no introns in mtDNA. Human mtDNA is entirely circular. In yeast mtDNA both linear and circular molecules of mtDNA are found. However it is not certain whether the circles are functional units and linear units are just replication intermediates or circular forms are not important for mitochondrial function.

In yeast cells four different modes of mtDNA replication have been suggested (Figure 1). First, the D-loop model, which is asynchronous and asymmetric producing an intermediate with a newly replicated double stranded and single stranded loop (Clayton et. al., 1984.). The second mechanism that is suggested to occur in yeast is a rolling

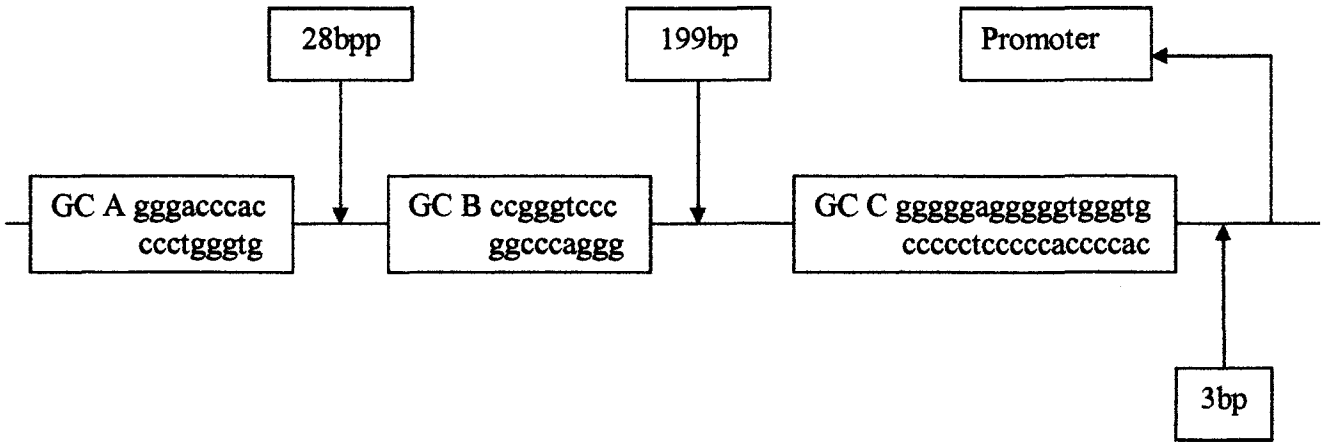
Figure 1


RNA primer
ssDNA
A
D-loop
DNA replication
ssDNA
B
Rolling circle
DNA replication
C
ssDNA
Recombination dependent
DNA replication

circle replication with an introduction of a nick in the on of the strands of double stranded DNA and producing a long pieces of ssDNA (Malasezka, 1991). The third possible way of mtDNA replication is standard bi-directional mode of replication (Grawes et. al., 1998). Fourth is recombination dependent replication, with priming caused by strand invasion (MacAlpine et. al., 2001).

In mammalian cells two different modes of replication have been found, asymmetric-asynchronous mode of replication, D-loop (Schmitt and Clayton, 1993) and coupled leading and lagging strand synthesis (Holt et.al., 2000). In the D-loop mode of replication the origin of replication in human cells of mtDNA is unidirectional. Replication initiates at the heavy-strand replication origin, O_H located at the light strand promoter (LSP) in the noncoding region. This non-coding region makes up the displacement loop or D-loop. The D-loop contains three GC rich sequence bocks, CSBI, CSBII, and CSBIII conserved among most mammalian species. The light-strand replication origin O_L is outside of D-loop, very distant from O_H and only 30bp from a cluster of tRNA genes (reviewed in Lecrenier and Foury, 1999). D-loop replication is initially unidirectional and it first starts at O_H using LSP that is located on the opposite strand. Only when O_H replicates 2/3 over the circle of mtDNA O_L starts to replicate (Schmitt and Clayton, 1993). There is also another mode of replication found in mammalian mtDNA, the standard coupled leading and lagging strand synthesis (Holt et. al., 2000). From the results of this study it was suggested that in mammalian cells that reamplify their mtDNA from a depleted state, rapidly increasing the copy number, use coupled leading and lagging strand synthesis. In mammalian cells that just maintain the copy number appear to use asymmetric asynchronous mode of replication.

The presumed replication origins of yeast *Saccharomyces cerevisae* were discovered through analysis of cells containing mutant mitochondrial genomes (reviewed in Lecrenier and Foury, 1999). Wild-type cells have seven to eight *ori/rep* sequences that are suggested to be origins of replication (reviewed in MacAlpine, 2001). Out of these eight *ori/rep* sequences only four: *ori1*, *ori2*, and *ori3*, and *ori5* are considered active origins of replication. The other four *ori/rep* sequences are inactive origins of replication because they have a short insertion that disrupts the *rep* sequence promoter. Rho^- mutants have undergone massive deletions, some rho^- mutants may have only 35bp of the original wild-type 80,000bp mitochondrial genome. There are different kinds-of rho^- mutants. Hyperssuppresive mtDNA mutants are one class of rho^- mutants. Hypersuppressive mutants are defined by their pronounced replication/segregation advantage over wild-type (rho^+) mtDNA. This appears to be due to the fact that they retain an *ori/rep* sequnce and therefore have more *ori/rep* sequences per kb of mtDNA than the wild-type. An active *ori/rep* sequence contains a nonanucleotide promoter for the RPO41-encoded mitochondrial RNA polymerase and three GC-rich sequences called boxes A, B, and C (Figure 2). In yeast cells replication appears to occur bidirectionaly starting at the promoter which is located close to GC cluster C of the *ori/rep* sequence (Lecrenier and Foury, 2000). The position of the promoter of an *ori/rep* sequence is similar to the heavy strand origin of replication, O_H in humans and the GC-rich regions, A box and B box, which forms an inverted repeat along with A/T rich region between them, are similar to light origin of replication, O_L in humans. It was thought that the second, non-promoter was primed by primase in *ori/rep* region (Grawes et.al., 1998). Another similarity between mammalian and yeast mitochondria is that there is also a


28bpp
199bp
Promoter
GC A gggacccac
ccctgggtg
GC B ccgggtccc
ggcccaggg
GC C gggggaggggtgggtg
ccccctccccacccccac
3bp

version of an enzyme that may cleave the nascent transcript to make the primer for mtDNA replication. In human cells this enzyme is called MRP (Schmmitt and Clayton, mtDNA when grown in media containing a fermentable carbon source.

In both mammalian and yeast cells it has been thought that the primer for the initiation of mtDNA synthesis is made from a nascent RNA transcript. This would require that trancription be coupled to replication of mtDNA. The nuclear gene *RPO41*, mitochondrial RNA polymerase is the only gene that encodes the catalytic subunit of mitochondrial RNA polymerase. So if transcription is coupled to replication than in cells with deleted *RPO41* it would be expected that mtDNA would fail to replicate. In all rho- cells examined to date, HS, neutral, and all in between, when the *RPO41* gene was removed abolishing transcription but mtDNA is still replicated (Fangman et. al., 1990, Lorimer et. al., 1995). But in these cells mtDNA is maintained. This indicates that transcription is not absolutely required for initiation of replication. One possibility is that a different type of replication exists in rho^- cells. Or, there might be a different mode of replication in the cells with and without *RPO41*.

To look for mitochondrial DNA replication intermediates can be done through two different processes:

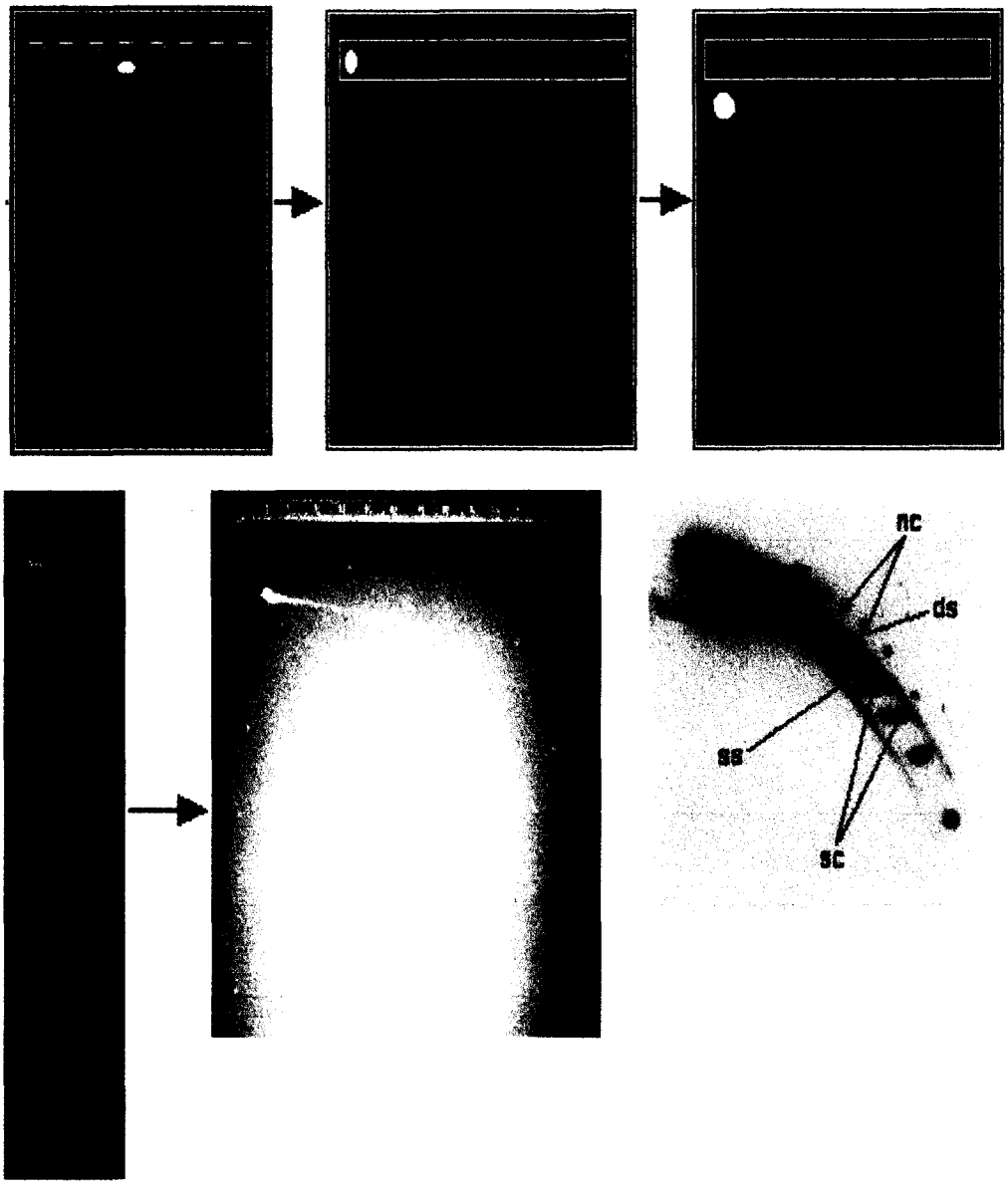
1. Direct analysis of replication intermediates
2. Biochemical characterization and genetic identification of all components involved mtDNA replication

Yeast mutant mtDNA among many different mutants can be HS rho^- or rho^- neutral. Hypersuppressive rho^- have up to 80% of deleted rho^+ genome but they have tandem repeats of *ori/rep* sequences. So these cells have many more *ori/rep* than the

wild-type. Also the characteristic of these sequences is that when the wild-type is crossed to HS rho$^-$ progeny is HS rho$^-$ (Grawes et. al., 1998). But when wild-type, rho$^+$ is crossed to rho$^-$ neutral than progeny is rho$^+$. This indicates that there is something in HS rho$^-$ that makes them preferably inherited. Neutral rho$^-$ do not contain any rep sequences. The studies that have been done by now suggest that *ori/rep* sequences are the origins of replication.

The goal of this study was to look at replication intermediates of mtDNA of yeast *Saccharomyces cerevisae* to determine mode of replication. Different methods have been used to look for replication intermediates. One of the first methods used for this type of analysis was electron microscopy. In this study pulse field gel electrophoresis (PFGE), was used (Malasezka et. al., 1991). Using this method it was found that bacteriophage T4, replicates through a rolling-circle mechanism. In late '80s a new method for looking of replication intermediates of DNAwas developed by Brewer and Fangman (Brewer et. al., 1988). This method was called two dimensional gel electrophoresis, 2D gel electrophoresis (Figure 3).

This method was originally adopted and modified from Bell and Byers, 1983 (Bell and Byers, 1983) where conditions were developed in which branched DNA fragments move in one dimension according their size and in second dimension they are slowed down according the number, length, and topology of their branches (reviewed in Brewer et. al, 1988). Brewer and Fangman used this method originally to look for replication origins on ARS plasmids in *Saccharomayces cerevisae* (Brewer and Fangman, 1987).


nc
ds
ss
sc

The first dimension of the 2D gel is run under low concentration of agarose and low voltage at room temperature for a long time usually between 16 and 24 hours. This first dimension separates DNA molecules on the basis of their size. The second dimension is run at substantially higher concentration of agarose and high voltage, five times larger than the first dimension in addition with the presence of edithium bromide, an intercalacting agent that makes DNA more rigid. Because of the high percentage of agarose and high voltage, the gel is subjected to substantially more resistance. To prevent excessive heating the gel is run at 4°C for shorter period of time, between three to five hours. The second dimension separates DNA on the basis of shape. Thus, the electrophoretic mobility of a DNA molecule in the 2D gel depends on both, its shape and size (Friedman and Brewer, 1995). This is the most often seen when circular DNA molecules are analyzed on an agarose gel because neither supercoiled nor the nicked circles move same as the linear molecules of the same size. Also branched molecules move differently in agarose gels depending on the length of the branches. Its migration is also influenced by agarose concentration and the currency of the electric field.

Two dimensional gel electrophoresis has been used to look for replication intermediates in many of different systems. As an example, this method has been used to look at rolling circle replication of T4 bacteriophage in the presence and absence of bacteriophage T4 primase (Belanger et. al., 1996). This phage encodes all of its replication proteins in its relatively small genome and therefore it is a very useful model for studying the mechanism of DNA replication. Bacteriophage T4 primase makes the duplex of DNA tails and in the absence of T4 primase single-stranded DNA tails are

obtained. So in the presence of T4 primase duplex tails are observed and in the absence of T4 primase single-stranded tails are observed.

Two dimensional gel electrophoresis has been also used to investigate populations of plasmid DNAs with different topological conformations in pBR322 among others (Martin-Paras et. al., 1998). In that study was looked for different topological conformations that pBR322 can adopt *in vivo* in bacterial cells and also in eukaryotic cells of *Xenopus eggs* extracts. To notice any difference in signals at undigested samples of DNA and also samples treated with DNase I and topoisomerase I and II were analyzed.

Two dimensional gel electrophoresis has been also used to study replication intermediates of mammalian mitochondrial DNA (Holt et. al. 2000). Mammalian cells appear to operate two modes of replication of mitochondrial DNA. This was suggested because one class of intermediates was resistant to single strand nuclease digestion and showed mobility of coupled leading and lagging replication intermediates forming a standard Y arc. These cells were recovering from transient mtDNA depletion and that indicates that this mode of replication occurs in cells that amplify the copy number of mtDNA. A bidirectional mode of mitochondrial DNA replication was found in both mouse liver and placenta cell, indicating that coupled leading and lagging mode of replication may occur in all mammalian cells. Another type of replication intermediates was sensitive to single-strand nuclease treatment. Also these cells were just maintaining the copy number of mtDNA. These cells did not form a Y arc because they were modified by single-strand-specific nuclease treatment. So this suggested the different mode of replication present in mammalian cells, perhaps the orthodox, strand-asynchronous mode of mtDNA replication.

All these studies showed that two dimensional gel electrophoresis is a powerful technique for studying of replication intermediates of DNA replication. In this study two dimensional gel electrophoresis was used to look for replication intermediates of mithochondrial DNA in yeast *Saccharomyces cerevisae.*

For yeast cells it was proposed, and widely accepted, that mtDNA replicates only through one mechanism in which a newly transcribed RNA is cleaved and used as a primer to start the replication, D-loop model. This suggested that in yeast replication is coupled to transcription and dependent on transcription (Shmitt and Clayton, 1993). However it was seen that when the nuclearly encoded gene RNA polymerase-gene, RPO41 is deleted transcription was reduced to non-detectable levels but the replication still occurred (Fangman et. al., 1990). This suggested that perhaps yeast has an alternative mode of replication, one present in the presence of *RPO41* and another present in the absence of *RPO41.* In the presence of *RPO41* yeast could replicate through the orthodox asynchronous asymmetric mode of replication (Schmitt and Clayton, 1993). It is not certain what the alternative mode of replication occurs in cells without *RPO41.* Some studies suggest that this mode of replication could be recombination dependent since recombination in mitochondria is a very active process (MacAlpine et. al., 2001). Others studies suggest that the alternative mode of replication could be a rolling circle mechanism since there is production of long strands of single stranded DNA and linear mtDNA exist in head to tail repeats (Lorimer et. al., 1995). Also rolling circle replication was seen in DNA replication of yeast mitochondrial DNA using pulse field gel electrophoresis and electron microscopy (Malasezka et. al., 1991).

Two different modes of replication occur in mammalian cells that amplify their mtDNA and in the cells, one for rapid amplification of their mtDNA and another that just maintain the copy number of mtDNA (Holt et. al. 2000). We suggest that in yeast since was seen that if two different modes of replication occur one mode of replication may occur in cells that are in log phase when rapid total number of cells increase the most rapidly therefore the amount of mtDNA increases. The other mode replication could be expected in stationary phase when the number of cells does not increase and therefore the amount of mtDNA increase slowly if at all.

Experimental Design

I) The nuclear gene *RPO41* is the only gene that encodes for mtRNA polymerase. If the initiation of replication in yeast *Saccharomyces cerevisiae* requires the cleavage of a nascent transcript than the mutants without gene *RPO41* (rho$^-$, *Δrpo41*) would not maintain their mtDNA. However, it was already shown that in these mutants transcription rate decreases to non-detectable levels, but the mtDNA is stably maintained (Fangman et. al., 1990). This could be explained by the presence of the high number of *rep* sequences in HS rho$^-$ Δrpo41 mutants. *Rep* sequences are presumably the origins of replication in *S. cerevisiae*. This indicates that in *S. cerevisiae* HS rho$^-$ Δrpo41 mutants might be present another mechanism of DNA replication except the mechanism that requires a primer which is made from newly transcribed RNA. In this study we used HS3324 and N28, each with intact *RPO41* or with the *RPO41* gene deleted *(Δrpo41)*. Samples of these cells were grown to log phases and mtDNA replication intermediates examined by 2D gel electrophoresis. If a different mode of replication is used *RPO41* cells compared to *Δrpo41* cells different replication intermediates could be found on 2D gels in log and stationary phases.

II) In mammalian cells two different modes of mtDNA replication occur (Holt et. al., 2000). One mode of replication, (D-loop) is found in cells that just maintain their mtDNA and bi-directional replication in cells that have to reamplify mtDNA. If two different mechanisms of replication exist in yeast mtDNA depending on the presence or absence of *RPO41* than it might be that one mechanism occurs in cells grown in log phase and another mechanism in cells grown in stationary phase. Therefore different replication intermediates could be seen in cells same strain growing in the log phase

versus stationary phases. Julia Sable started work on these experiments in our lab in 1998. Here we expand those preliminary findings. We used three different types of cells to test this hypothesis: rho^-, HS 3324 and two rho^+ wildtypes BS 127 and BS 132.

Materials:

Ethanol was purchased from Aaper Alcohol and Chemical Company, Shelbyville, KY; isopropanol was purchased from Baxter Healthcare Corporation, McGraw Park, IL; restriction endonucleases (*EcoRI, and HindIII*) were purchased from New England Biolabs, RNase was purchased from Boehringer Mannheim, Indianopolis, IN; bacto-tryptone and bacto-agar were purchased from Difco Laboratories, Detroit, MI; agarose was purchased from EM Science, Cherry Hill, NJ; edithium bromide and sodium citrate were purchased from Fisher Scientific, Fairlawn, NJ; Polaroid film, yeast extract, sodium chloride, ampicilin, calcium chloride, dextrose, Trizma base, ethylenediaminetetraacetic acid-disodium salt (EDTA), sodium hydroxide, sodium dodecyl sulfate (SDS), phenol, chloroform, sodium acetate, magnesium chloride, boric acid, DAPI stain, and glycerol were purchased from Sigma Chemical Company, St. Louis, MO; dNTPs and random primer NEblot Kit were purchased from New England Biolabs, Inc., α-^{32}P dATP was purchased from New England Nuclear (Du Pont), S1 nuclease was purchased from Boehringer Mannheim GmbH, Germany, GBX developer and fixer were purchased from Estman Kodak Company, Rochester, New York.

Methods

Strains and Media

All *S. cerevisae* strains used in this study were derived from A364a and have been previously described (Lorimer et. al., 1995). The YEPD plates were made of 5g yeast extract, 10g bactopeptone, 450ml water, and 10g of bactoagar was added before autoclaving in Yamato Autoclave SM 32 at 121°C for 21 minutes and than 50ml of 80% glucose was added. Plates were inoculated with HS3324, ΔHS3324, N28, ΔN28, BS127/HS3324, BS127, and BS132 cells and grown in the incubator at 30°C for 2 days. The colonies where transferred to 5ml YEPD liquid media and grown in the incubator for 24 hours. 1ml of each cell type was transferred into 250ml YEPD media and grown in a shaking water bath (Gyrotary Water Bath Shaker, model G76) at 30°C until they achieved the desired optical density. The cells were grown to log and stationary phases. The optical density of cells was measured using Beckman DU-50 Spectrometer using visible lamp a wavelength of 660nm. Cuvettes used to measure the optical density are 1cm path length made of polystyrene. The log phase cells were grown until they achieved optical density of 0.9 (1×10^7) and the stationary phase cells were grown for 1-2 days after they achieved optical density above 1.0 (3×10^8). The grown cells were transferred into 250ml centrifuge bottles with 20ml of frozen EDTA in the bottom and spun at 15,000rpm for 10 minutes in Sorvall RC5B Plus SLA-1500 Rotor at speed 10,000 rpm for 15 minutes and the temperature of 4°C.

DAPI Stain

DAPI (4',6-diamino-2-phenylindole dihydrochloride) stain of all strains was performed to look for the presence of mitochondrial DNA. O.5ml of methanol and 0.1ml of PBS (phosphate buffer saline) was added 0.1ml of cell grown in liquid YEPD media or a colony from a plate. Than the mixture was spun for 5 seconds at 13,000rpm in a Sorvall Biofuge Microfuge Pico. The supernatant was discarded and the pellet was resuspended in 0.5ml PBS. The mixture was spun again at 13,000rpm and for 5 minutes. The supernatant was discarded and the pellet was resuspended in the 10μl of DAPI solution (2μg/ml). 90μl of PBS was added to the mixture and the mixture was placed on a clean slide and covered with a coverslip on the top. The cells were examined using a fluoroscent microscopre, Olympus Provis, AX70.

DNA Extraction

DNA extraction was done using a modification of a small scale procedure (adapted from Hoffman and Winston, 1987) adding 2.0ml of lysis buffer, 2.0ml of glass beads (washed previously in HCl), and 2.0ml of PCIA (phenol chloroform isoamyl alcohol) in the ratio of 50% saturated phenol, 48% chloroform, and 2% isoamyl alcohol. The mixture was vortexed approximately 30 times for 30 seconds at a time keeping them on ice between vortexing. Cells were periodically examined under a binocular Bausch and Lomb microscope for evidence of cell wall breakage. When about 90% cells had broken cell walls they were transferred into the 15ml conical tubes and the mixture was centrifuged for 5 minutes at 2,000rpm in the Beckman GPR centrifuge using GH3.7 rotor at the temperature of 15°C. The aqueous top layer was transferred to a new tube and 2ml

of TE (10mM Tris pH 8.8, 1mM EDTA) was added and the sample vortexed again then centrifuged. The aqueous layer was added to that from the first extraction. The combined aqueous layers were extracted with an equal volume of PCIA and the samples centrifuged again. PCIA extractions were repeated until there was no opaque layer at the interphase between the aqueous and organic (bottom) layer. The final aqueous layer was transferred to a new tube and 1/10 volume of NaOAc (sodium acetate) and 2.5 volumes of EtOH (ethyl alcohol) was added and samples mixed and the nucleic acids allowed to precipitate in the freezer at –20°C. Isolated DNA was taken from the freezer and transferred into a round bottom tube with a purple cap and centrifuged in Sorvall RC5B Plus, SA-600 Rotor at 24,000 rpm for 15 minutes and 4°C. The supernatant was discarded and the pellet was washed twice with 75% cold ethanol to remove salts. The pellets were dried out in the Labconco Centrivap Concentrator for 15 minutes. To the dry pellets 400µl of 1XTE was added.

Gel Electrophoresis

Analytical Minigel

1-D gels were run to see the concentration, integrity, and size of the isolated DNA. 0.8% agarose gels were made using 0.4g agarose and 50ml 1XTBE (for 5X 51.5g, 0.42M Tris, 27.5g, 0.44M Boric acid, and 4.65g, 0.016M EDTA) and microwaved until the agarose was completely melted (Maniatis et. al., 1982). The gel was poured and run using a Fotodyne minigel apparatus. Two µl (50ng) of the DNA preparation, 5µl sterile water, and 3µl of dye totaling 10µl was loaded. In the first well 10µl of marker λ phage DNA cut with *HindIII and EcoRI* was loaded. The gel was run at 60V for 2 hours in the

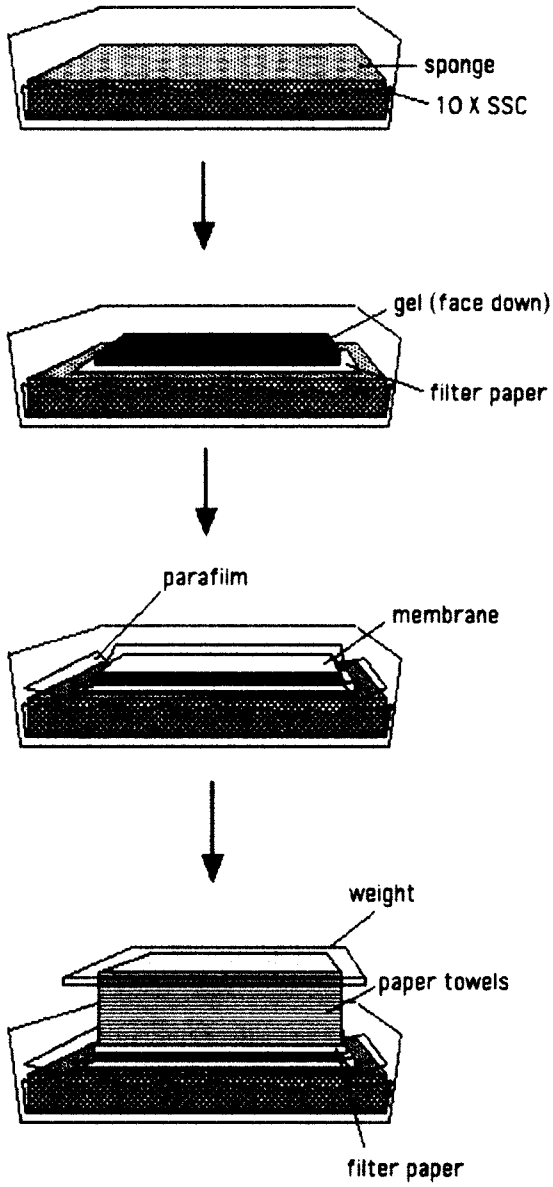
presence of TBE buffer and 15µl EtBr (edithium bromide). A picture was taken to use for further analysis, to estimate the amount to be used in the 2-D gels, and to check the concentration and integrity of the DNA preparation using Fotodyme INC. Foto Phoresis I System camera using orange filter, at aperture 5.6 and shutter speed 2 and Foto/Prep I UV light by Fotodyne.

Two Dimensional Gel Electrophoresis

To analyze replication structures I used two dimensional Fangman/Brewer gel electophoresis (Brewer and Fangman, 1988). The first dimension of the 2D gel was 0.6% agarose gel that was made using 1.5g agarose and 250ml 1XTBE. Wells were loaded with 12.5µl (2µg) of DNA and 2.5µl of dye. The gel was run at 35V (1 volt per 1 cm the length of the gel) at room temperature for 20 hours in the presence of 1XTBE. The gel was than stained in the 500ml of 1XTBE and 35µl of EtBr for 30 minutes and the picture of the gel was taken using Foto/PrepI by fotodyne UV light. The second dimension of 2D gel, 1.4% agarose gel with 3.5g agarose, 250ml 1XTBE, and 15µl edithium bromide was made. The bands from the first dimension were cut out and rotated 90° counterclockwise, placed in the gel tray and thee second dimension gel was poured around the lanes from the 1st dimension. The gel was run at 5V per 1cm of the length of the gel for 3 hours at 4°C in pre-chilled TBE and with the presence of 60µl EtBr per liter of TBE. The pictures of the second dimension gel were than taken and Southern Blot was than performed.

Southern Blot

DNA was transferred to nitrocellulose membrane using a modification of the Southern blot (Figure 4) (Southern, 1975) previously reported (Lorimer, 2002). The gel was placed down in a tray and shaken in the BI which (¼ N HCl, concentrated HCl is 37% with molecular weight of 36.5g/mol concentrated HCl is ~10M and since it is a reasonably strong acid, ~10N diluted to 1/40 for ¼ normal for 400ml, 10ml HCl + 390ml water) for 15 minutes using Junior Orbit Shaker Lab-line. This solution, BI removes some purine bases. The first solution was poured out and the process was repeated. 200ml of the second solution was added, BII (1/2M NaOH, 1M NaCl: Made from stock solutions 10M NaOH and 5M NaCl for 400ml use 300ml of water, 20ml of 10M NaOH, and 80ml 5M NaCl) and rocked 15 minutes. The BII solution was poured out and the process was repeated. Solution II denatures the DNA and breaks strands where they were already depurinated. Then BII was poured out and solution BIII (1M Tris pH 7.5, add NaCl, for 400ml 200ml 1M Tris pH 7.5, 70.13 g NaCl, and add H_2O to 400ml) was added. The sample was rocked for 30 minutes. During this time a sponge (cut to fit a tray) was saturated with 10X SSC (1.5M NaCl, 0.15M sodium citrate and pH of 7.0 calibrated with 10M NaOH). A piece of Whatman 4mm paper was cut slightly larger than the gel was placed on the top of the sponge (Figure 4). The gel was placed face down on the paper. A piece of positively charged membrane was cut to the size of the gel and marked the date with a pencil on the bottom corner. The membrane was wetted with deionized water and then in the BIII or 10X SSC. The wet membrane was faced down on the gel, so the bottom corner of the membrane was on the bottom corner of the gel. A wetted sheet of Whatman 4mm paper was placed on the top of the membrane.


sponge
10 X SSC
gel (face down)
filter paper
parafilm
membrane
weight
paper towels
filter paper

The gel was surrounded with Parafilm (flat on sponge). A stack of paper towels was placed on the top of Whatman paper until at least an inch higher than the dish. A small weight (bottle) was placed on the top of the tray to apply some pressure to the stack. The gel was let sit overnight. The membrane was crosslinked using UV Crosslinker, Fisher biothech UV Crosslinker, FB-UVXL-1000 using optimal crosslinking mode.

Probes

The probes were made using NEBlot kit according to manufacturer's recommendations with the additional modification of adding a small amount of unlabeled dATP. 50ng of specific DNA in 11µl (no more than 5µl TE, the rest water) was denatured at 90°C for 5 minutes. Two and a half µl of 10X buffer, 6µl dNTPs, 2µl 1/10 dATP µl, 2.5µl α-^{32}P dATP, and 1µl of Klenow were added. The mixture was incubated at room temperature overnight. The spin column was prepared using the barrel of a 1cc syringe, without needle or plunger, packed with a minimal amount of silane tread glass wool in the bottom, the columns filled with hydrated sephadex G-50 beads (10g Sephadex G-50, 160ml sterile water, 0.32ml 1M Tris-HCl pH 8.0, and 0.4ml 0.2M EDTA). The liquid was allowed to drip out. The spin column was placed in 15ml conical tube and for 5 minutes in the IEC Clinical Centrifuge for 5 minutes at speed of 5. This produced 0.9 ml of packed beads. The liquid was removed from the conical tube. The label mix was poured into the spin column, 150µl of STE (sodium chloride, Tris, EDTA) was loaded on the top of the probe. The mixture was spun through spin column for 5 minutes at setting 5. Newly synthesized DNA molecules containing α^{32}-P dATP pass through the column, ending up in liquid in the bottom of the tube. Unincorporated

32P dATP remains caught in the G-50 sephadex in the column. After the spinning the probe the incorporation of activity was measured using a Gaiger counter. The activity was measured in both, the syringe and the probe. Ideally, the percent of the incorporation would be approximately 50%.

Probing the Blot

The blot was rolled up and placed in a hybridization tube. If more than one blot was used spacer sheets were used to separate membranes. Thirty ml 6X SCP (20X SCP 0.9M Na_2HPO_4 , 0.02M EDTA, and 2.0M NaCl with pH of 6.8 calibrated with concentrated HCl) with 1% sarkosyl (25% sarkosyl, 250g sarkosyl, 750ml water, and filter sterilized) were added to the tube with the blots were prehybridized at 60°C rolling for 30 minutes in the Techne Hybridizer HB-1D. During this time 12ml 6X SCP was boiled with 1% sarcosyl and 150µl (10mg/ml) ssDNA. Than all probe was added and boiled for 5 minutes. For HS3324 RPO41, HS3324 Δrpo41, BS127, BS127/HS3324, and BS132 purified mitochondrial DNA from HS3324 cells was used as a probe and for N28 RPO41 and N28 Δrpo41 purified mitochondrial DNA was used as a probe. The prehybridizing mix was discarded from the hybridization tube and the probe mix was added. The whole mixture with the probe was hybridized overnight rotating at 60°C. The next day the probe was poured off into a 15ml conical tube and stored at 4°C. The blots were washed with 100ml 2X SCP with 1% SDS (sodium dodecyl sulfate), rotating at 60°C for 30 minutes. The washing solution was discarded. The blots were taken out from the tube, placed into a tray, and washed twice with 0.2 X SCP 1% SDS for 15

minutes. The washing solution was discarded and the blots were wrapped into plastic wraps.

Exposing the Probe

BioMax film and wrapped blot were placed into a cassette in the dark room. The cassette was than placed in the freezer at –80°C for couple of days.

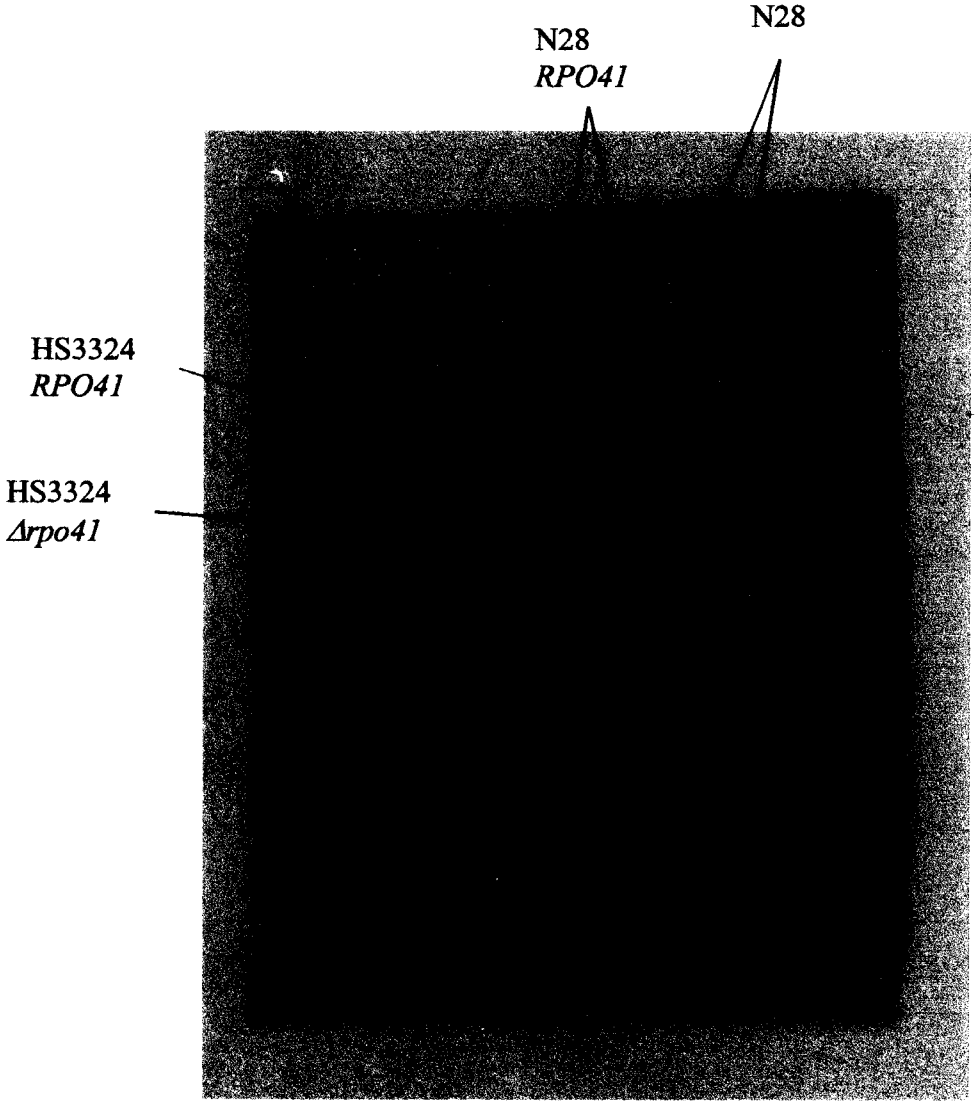
Developing the Film

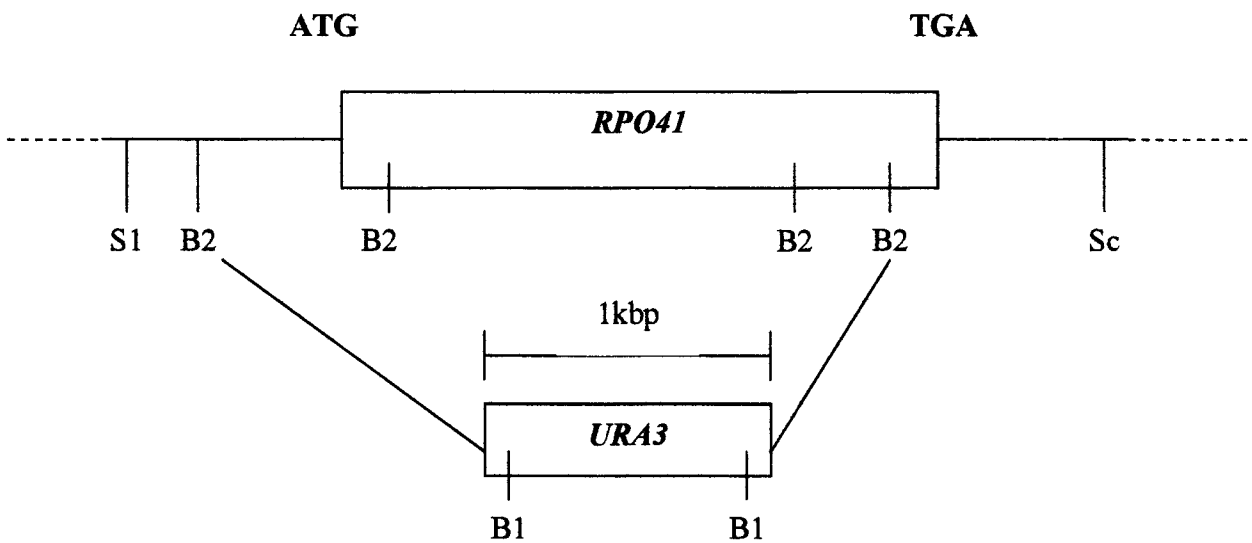
Three different trays were used to develop the film. The first one was filled with Kodak GBX developer and replenisher, the second one water, and the third one with Kodak GBX fixer and replenisher. The film was first placed into a developer between 1 and 5 minutes or until forms become visible under the safe-light. The film was then transferred in the water tray for 5 minutes and than a fixer tray for 5 minutes. After being in the fixer for 5 minutes the film was rinsed with water and airdried.

Results

The D-loop model is based on cleavage of a nascent transcript to provide an RNA primer for DNA replication. Previous work clearly showed that the catalytic subunit (*RPO41*) of the only mitochondrial RNA polymerase is not necessary for maintenance of rho$^-$ mtDNA (Lorimer et. al., 1995). Nonetheless it is possible that multiple mechanisms of mtDNA replication may occur. If so, than *RPO41* cells may replicate their mtDNA differently than Δrpo41 cells do. To examine this *RPO41* and Δrpo41 cells with different rho$^-$ mtDNAs were grown to log phase to maximize the number of replication intermediates. Total nucleic acids were isolated from the cells. These samples were run on single dimension gels to verify the status of their *RPO41* genes and compare the amounts of total mtDNA. Figure 5 shows nucleic acids from the *RPO41* and *Δrpo41* strains used. The Δrpo41 construct uses the *URA3* gene in place of the *RPO41* gene as a selectable marker. The *URA3* gene contains a PstI site, while the *RPO41* gene does not. Therefore the samples were cut with PstI, and analyzed by gelelectrophoresis followed by Southern blot analysis using the 3' flanking region of the *RPO41* gene as a probe. Each Δrpo41 strain showed the 3kbp band produced by the PstI site in the *URA3* gene, while the RPO41 equivalents revealed 10kbp band because the *RPO41* gene has no PstI site.

To check if the samples had indeed deleted the *RPO41* gene, a Southern blot and autoradiography was performed on cellular DNA cut with PstI (Figure 5a). It was clearly seen that HS3324 *Δrpo41* and N28 *Δrpo41* have deleted *RPO41* gene since they have smaller molecular weight band hybridizing to the *RPO41* 3' flank probe (Figure 5b). In order to see if different mechanisms of replication do indeed occur in cells with and without *RPO41* two dimensional gel electrophoresis was performed on HS3324 with


N28
RPO41
N28
HS3324
RPO41
HS3324
Δrpo41


ATG
TGA
RPO41
S1
B2
B2
B2
B2
Sc
1kbp
URA3
B1
B1

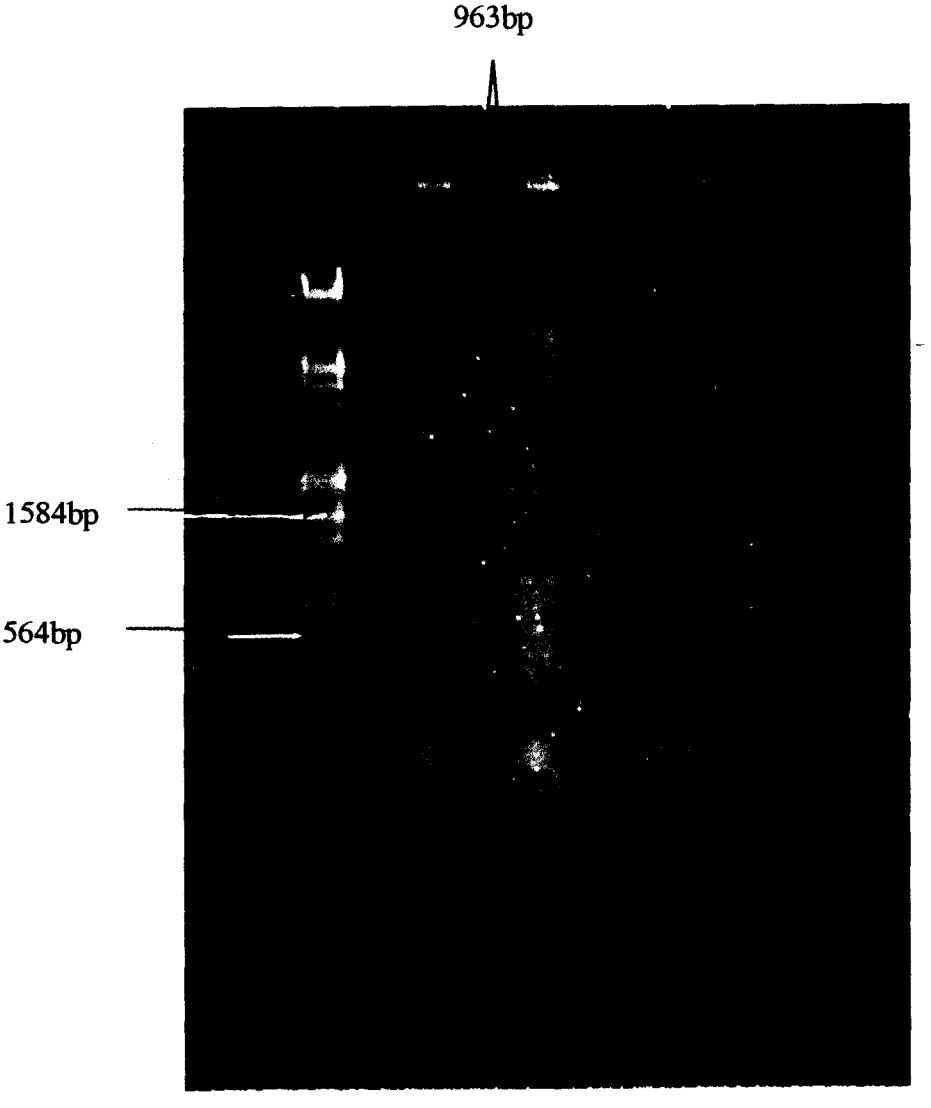
and without *RPO41* and N28 with and without *RPO41*.

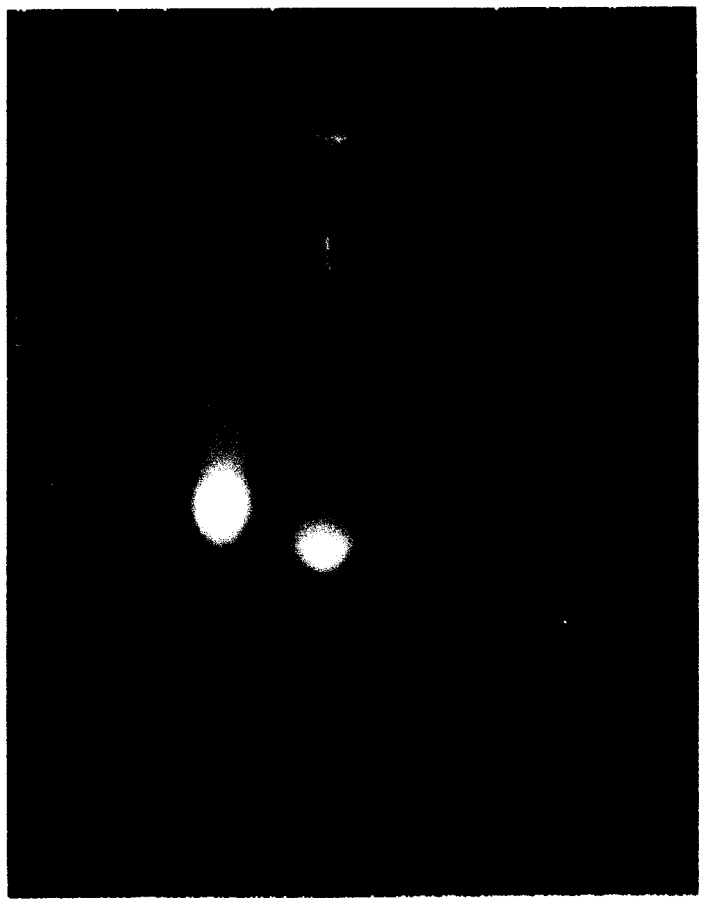
After isolating the total cellular DNA from HS3324 *RPO41* and HS3324 *Δrpo41* samples were cut with *EcoRV* which linearizes the HS3324 genome, and run on the one dimensional gel electrophoresis to check the size and amount of the isolated DNA. After taking the picture of the one dimensional gel it was observed that the HS3324 samples have the appropriate mtDNA size of 960bp (Figure 6) and that the mtDNA looks roughly equivalent in size and relative amount.

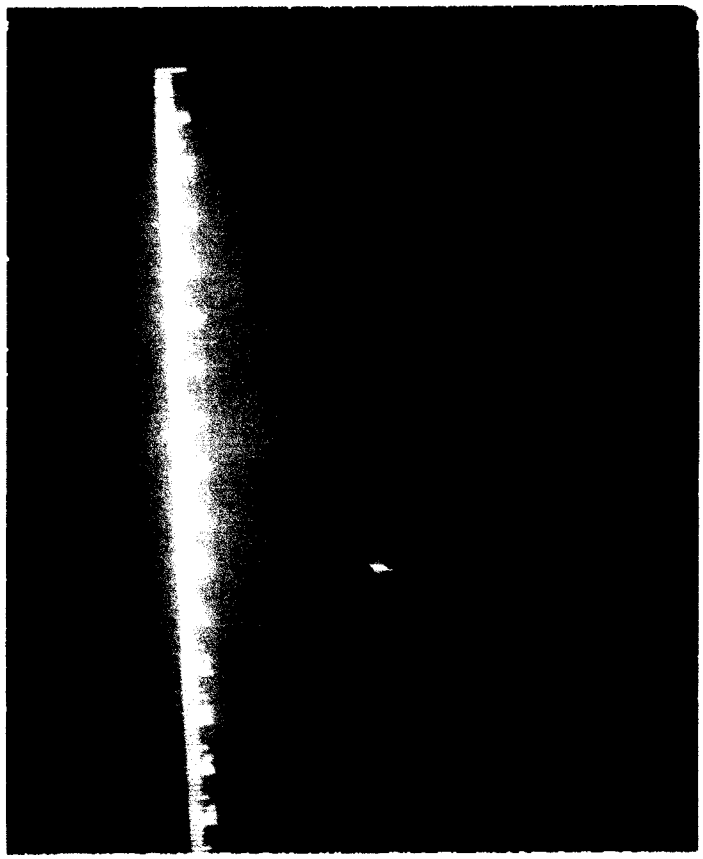
The HS3324 samples were treated with RNase since they contained a lot of RNA (Figure 6). After treating HS3324 samples with RNase to amount of RNA decreased in the samples to at most about ¼ of the original level of RNA (Figure 7).

In order to separate DNA on the basis of size, HS 3324 sample were run in the first dimension of the two dimensional gel electrophoresis. After running the first dimension gel the picture was taken and the bands of the HS3342 samples with and without gene *RPO41* were observed (Figure 8). In order to separate DNA on the basis of shape, the second dimension gel was run using the bands from the first dimension gel rotated 90° counterclockwise. After taking a picture of second dimension gel dots, DNA, of both samples were observed (Figure 9).

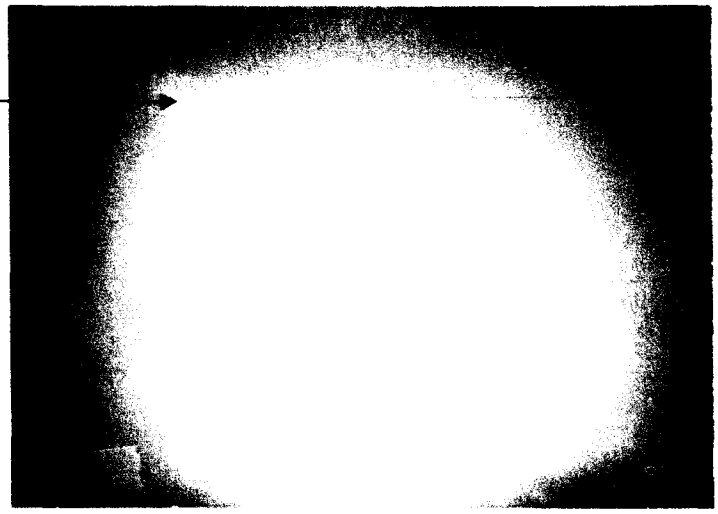
The Southern blot of the two dimensional gel with HS3324 *RPO41* and HS3324 Δrpo41 was performed and the membrane was probed using HS3324 mtDNA as the probe. Both samples HS3324 *RPO41* and HS3342 *Δrpo41* showed the presence of double stranded, single stranded, nicked, and supercoiled DNA (Figure 10).


963bp
1584bp
564bp

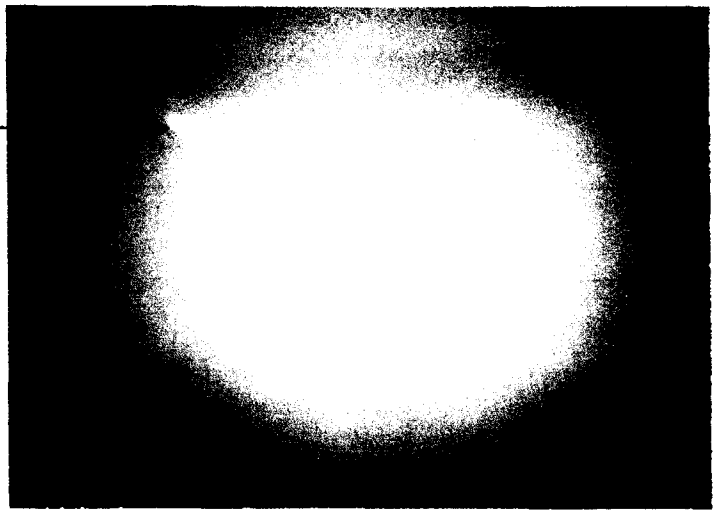


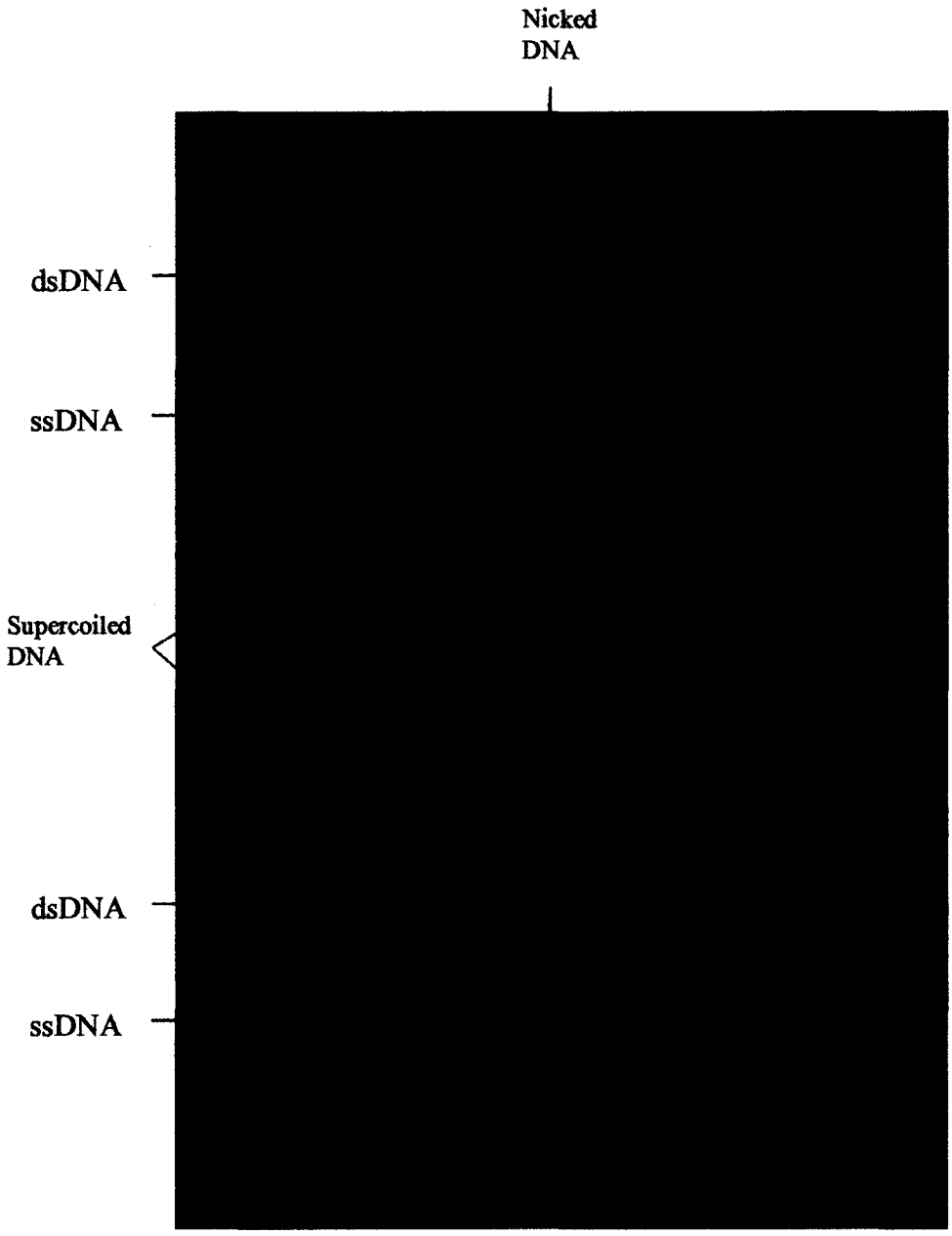


HS3324
RPO41



HS3324
Δrpo41

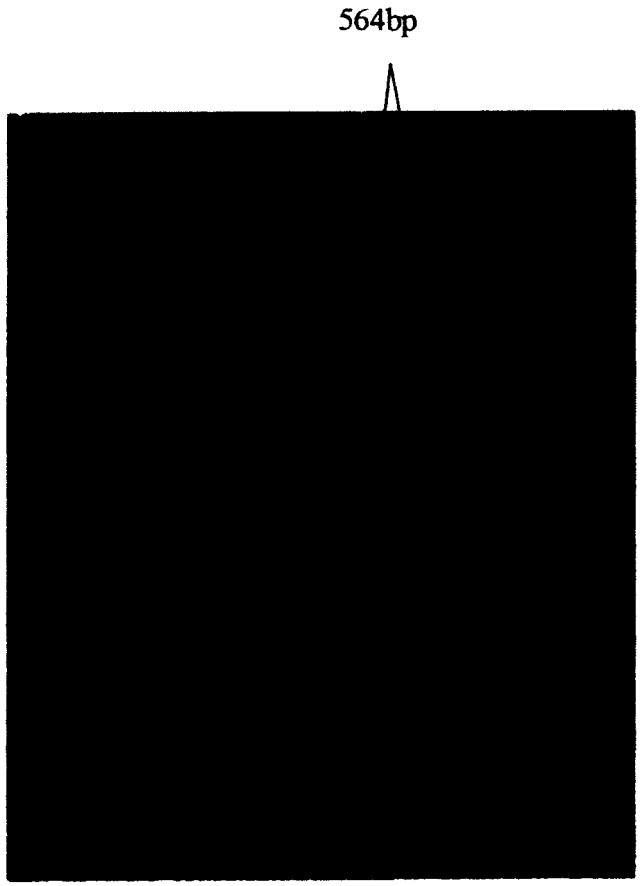


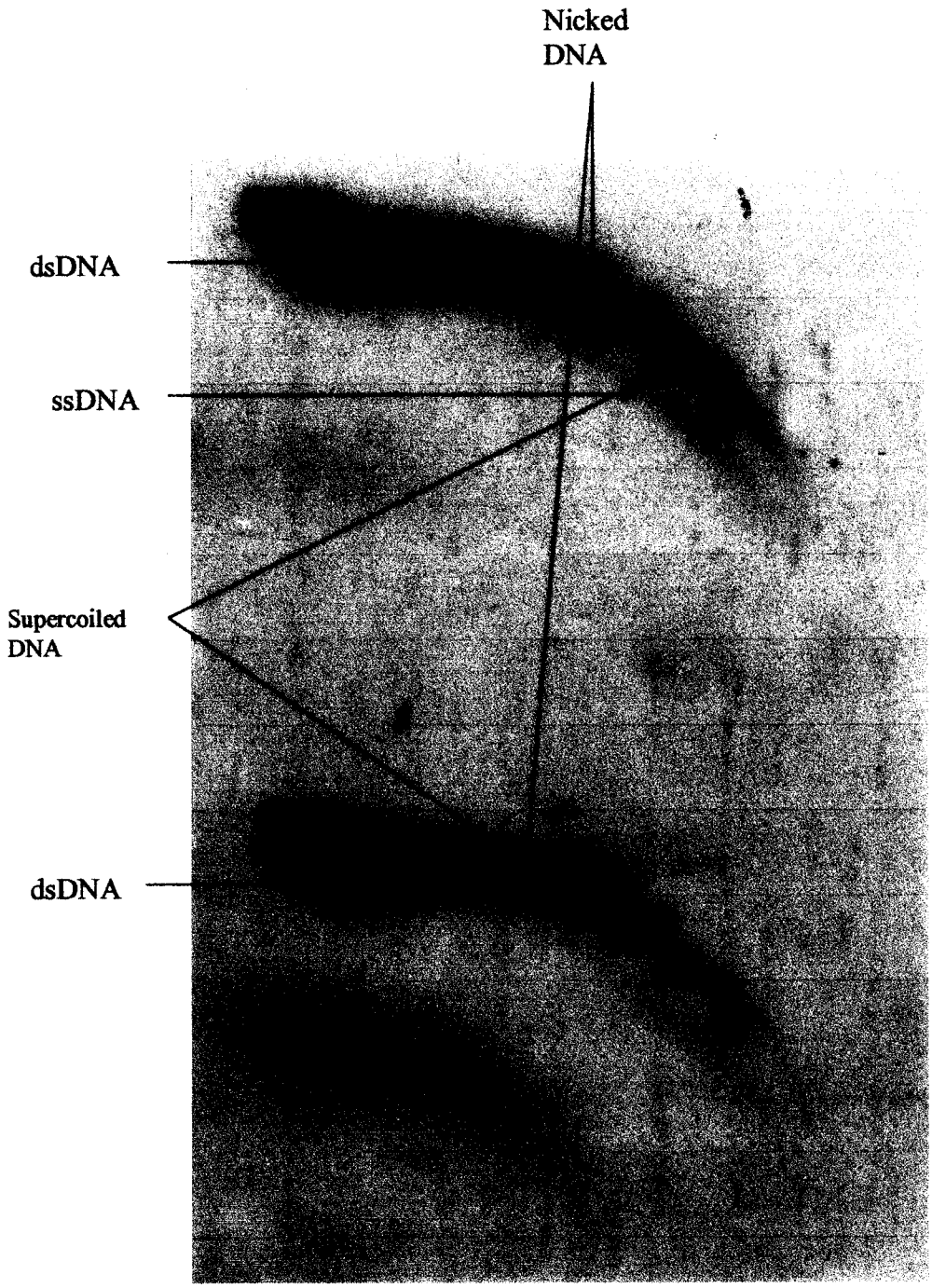

Nicked
DNA
dsDNA
ssDNA
Supercoiled
DNA
dsDNA
ssDNA

These results suggest that both samples with and without deleted gene *RPO41* replicate though the same mechanism since both of them showed the presence of the same structures. Since these results suggest that hypersuppressive cells with and without the *RPO41* gene have same mechanism of replication, the rho$^-$ neutral cells were analyzed to see if they reveal different mechanism of replication, since they have no *ori/rep* sequences.

Total cellular DNA was isolated from N28, rho$^-$ neutral cells with and without the *RPO41* gene. The isolated DNA was cut with HindIII that linearizes the N28 genome and the samples were run on the one dimension gel to determine the sizes of the isolated DNA. After taking the picture of the one dimension gel it was seen that the isolated DNA contained a band 564bp long, which is appropriate for N28 comparing them to the marker sizes (Figure 11). The two dimension gel was run on N28 *RPO41* and N28 Δrpo41 and the Southern blot was than performed. The blot was probed using a N28 mtDNA probe. The autoradiogram showed the presence of equivalent amounts of the same structures in both samples of N28, rho$^-$ neutral DNA (Figure 12). The observed structures of both samples were double stranded, single stranded, nicked, and supercoiled DNA.

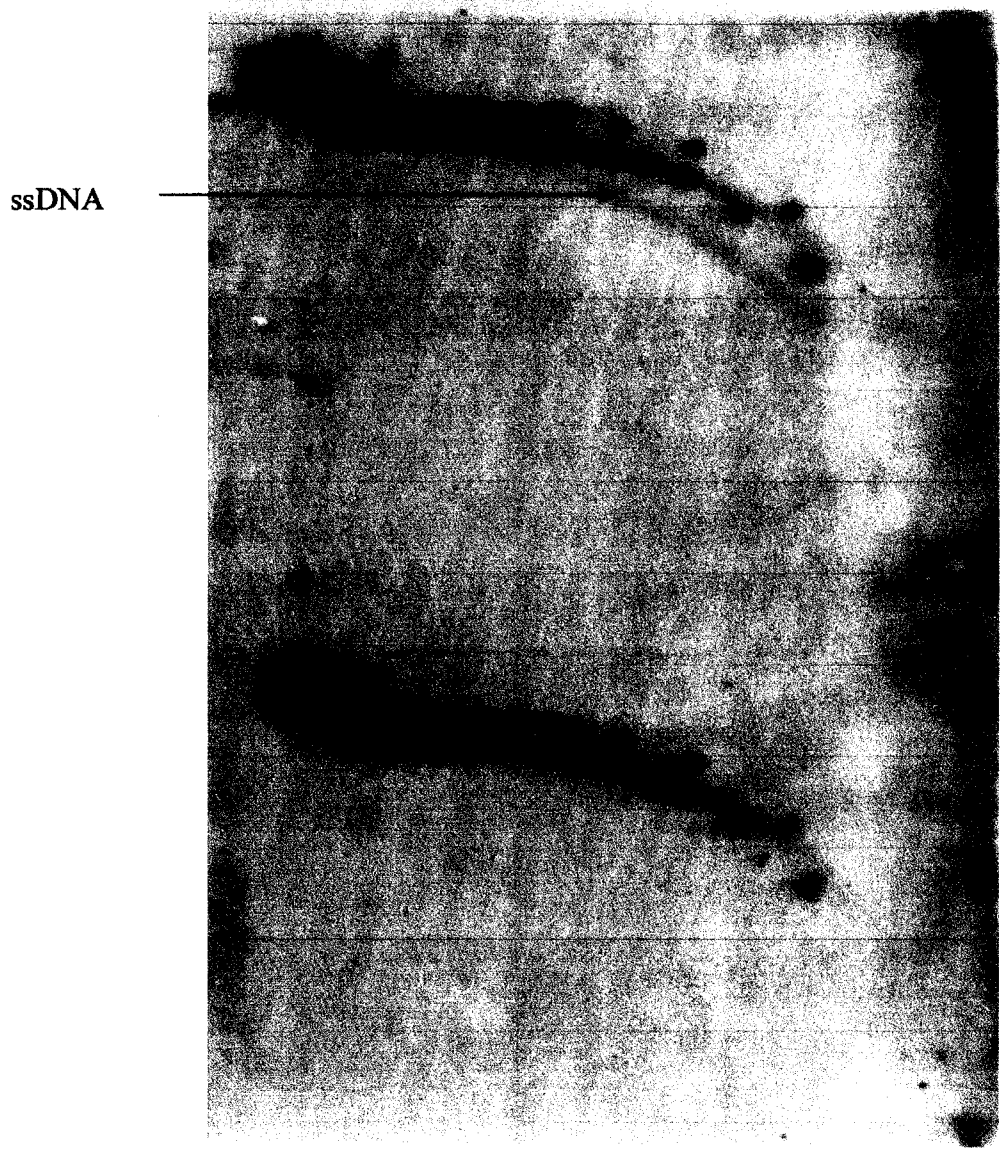
The presence of long single stranded DNA molecules in the samples supports the possibility of a rolling circle mechanism of replication in rho$^-$ hypersuppressive and neutral cells. To check if the assumed single-stranded DNA were indeed single stranded DNA the samples HS3324 *RPO41* was treated with SI nuclease which cuts the single stranded DNA. After running HS3324 with and without SI on two dimensional gel, performing the Southern blot and autoradiography, the autoradiograph showed the actual


564bp


Nicked
DNA
dsDNA
ssDNA
Supercoiled
DNA
dsDNA

presence of single stranded DNA (Figure 13). The sample treated with SI nuclease showed no presence of single stranded DNA. Also as supercoiled forms are clearly apparent in both, SI was used also at an appropriate level nicking of supercoiled forms did not occur to an observable degree.

Since the same replication intermediates are observed in both hypersuppressive and neutral rho$^-$ genomes with and without mitochondrial polymerase the same mechanism of replication exists in cells with and without *RPO41* gene. This also confirms and furthers previous results that *RPO41* in not necessary for maintenance of mtDNA (Lorimer et. al., 1995).

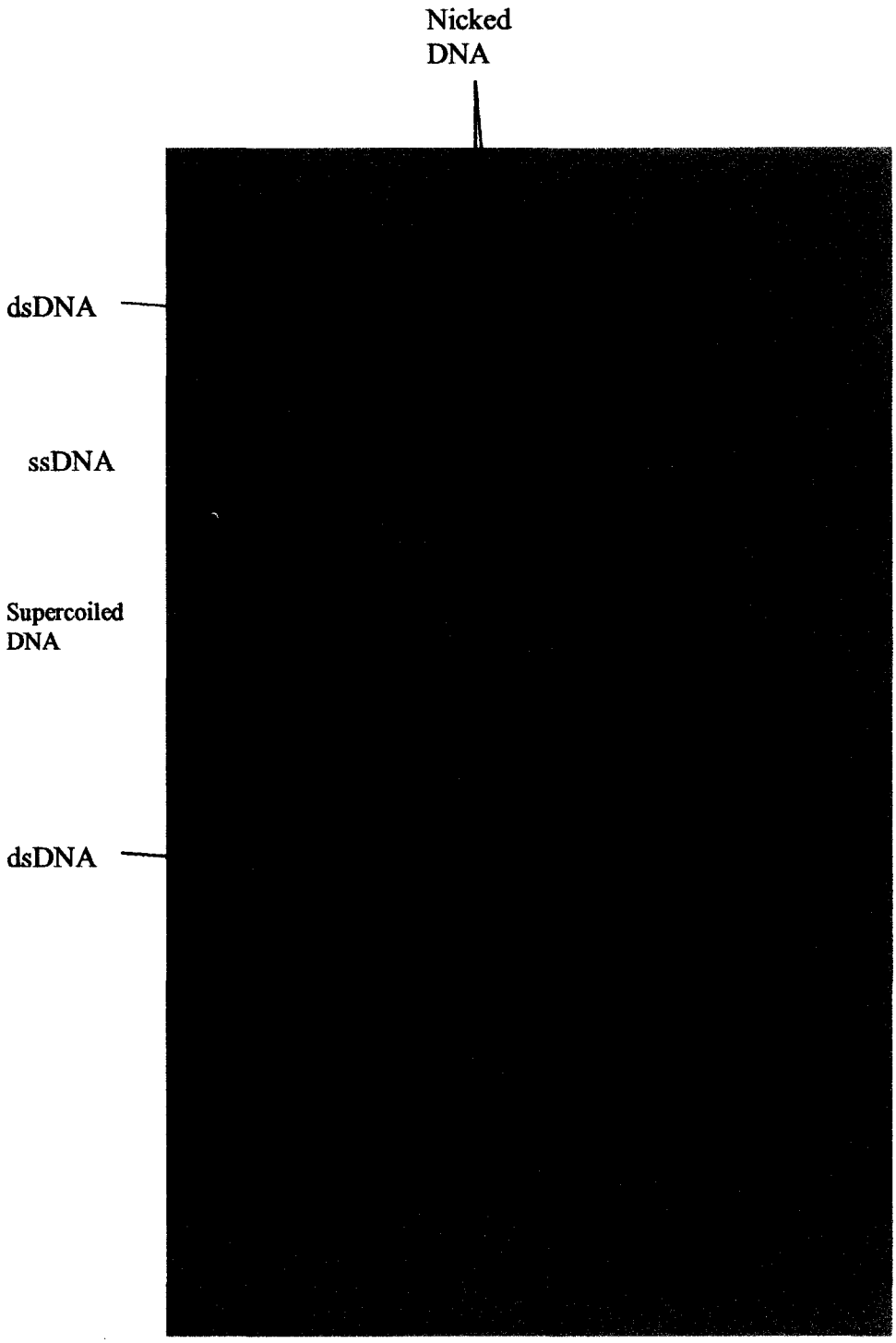

ssDNA

II) Determination of different growth dependent modes of replication

It was already seen that in mammalian cells at least two different modes of replication of mitochondrial DNA occur (Holt et. al., 2000). One mode of replication, an asymmetric-asynchronous D-loop mode of replication is found in the cells that just maintain their DNA copy number. Another mode of replication of mammalian mitochondrial DNA, coupled leading and lagging strand synthesis is found in the cells that need to reamplify their copy number. Analogous to this finding one mode of replication could be found in yeast *Saccharomyces cerevisae* log phase when cells replicate the most and produce the most DNA. Another mode of replication could be found in the cells grown in the stationary phase when cells need to do not increase their copy number of DNA but rather just maintain the copy number. Therefore rho$^-$ HS3324/BS127 where BS127 is a nuclear background and HS3324 is the mtDNA mutant put into that nuclear background and rho$^+$ cells BS127 and BS132 were grown to log and stationary phases.

The hypersuppressive cells, HS3324 were grown in log and stationary phases. The total cellular DNA from both samples was isolated and run on a 2D gel. A Southern blot was then performed and the membrane was probed using HS3342 mtDNA. HS3342 grown in log phase show the presence of the double stranded, single stranded, nicked, and supercoiled DNA (Figure 14). However on the sample of HS3324 grown to the stationary phase double stranded, nicked, and supercoiled DNA was observed, but not single stranded DNA.

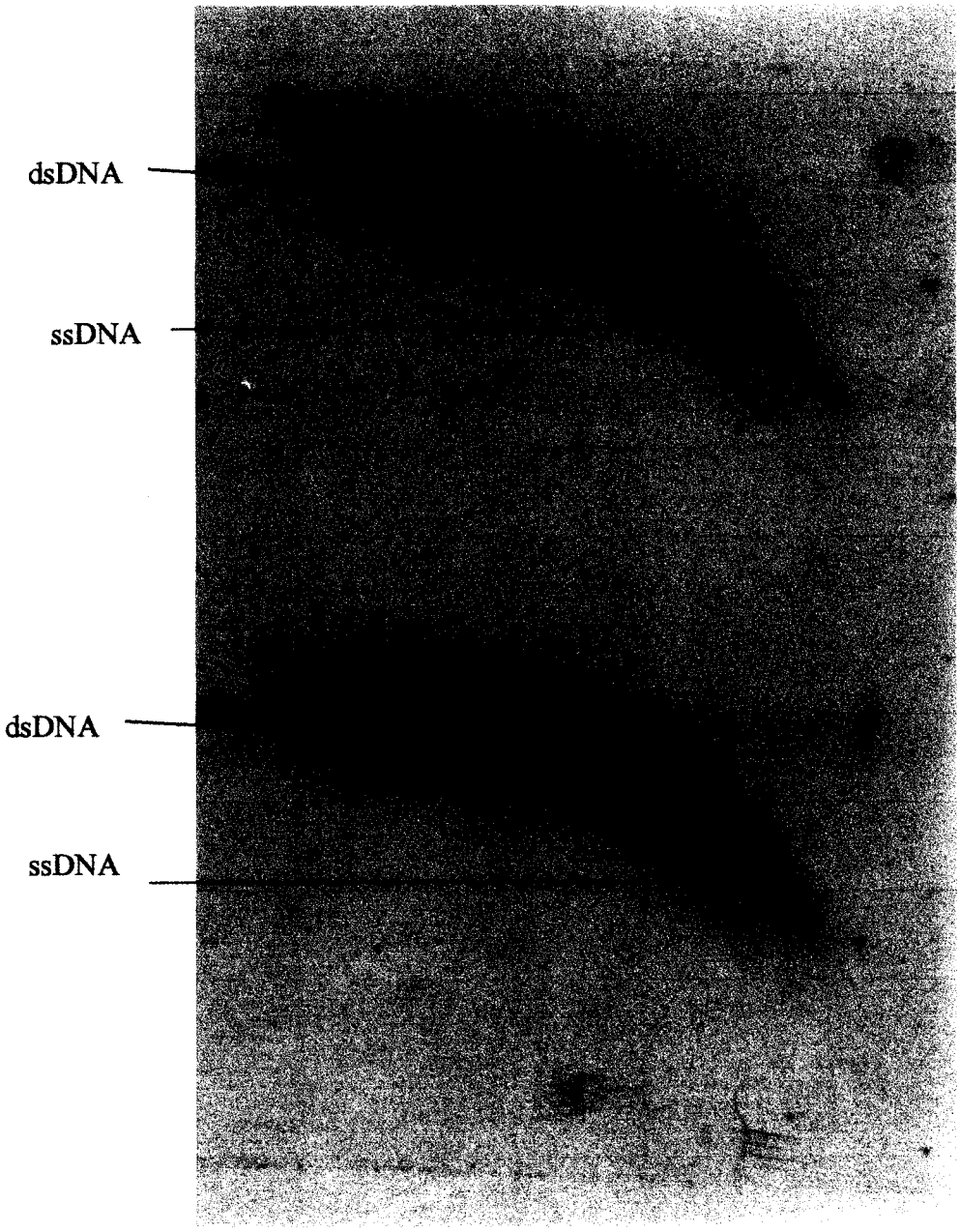
The wildtype strain, BS127, was grown to log and stationary phases. Total cellular DNA was extracted from both samples and run on 2D gel. The Southern blot

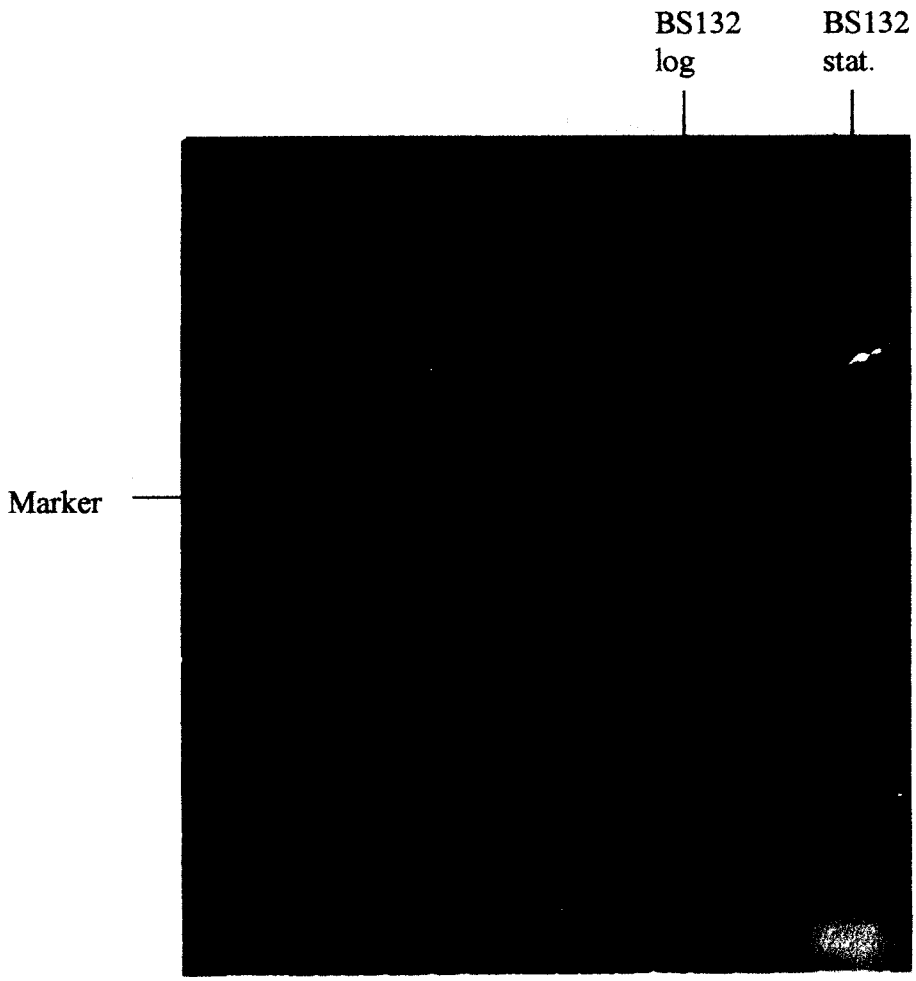

Nicked
DNA
dsDNA
ssDNA
Supercoiled
DNA
dsDNA

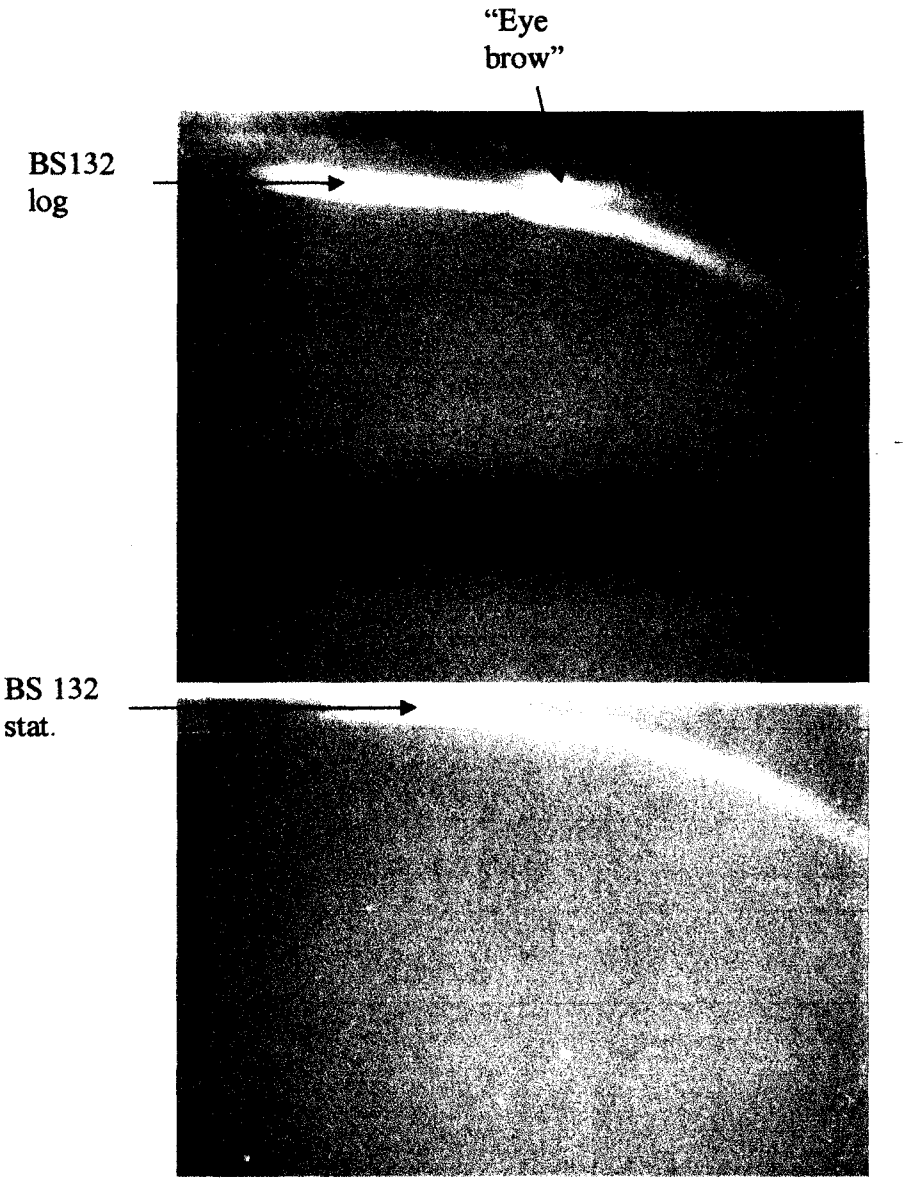
was then performed. The autoradiography was performed using a mtDNA fragment as a probe. The autoradiogram of both samples shows the presence of the same structures, double stranded and single stranded DNA (Figure 15). The nicked and supercoiled DNA could not be observed because these are widltype cells whose genome is about 70,000bp so those structures cannot be observed.

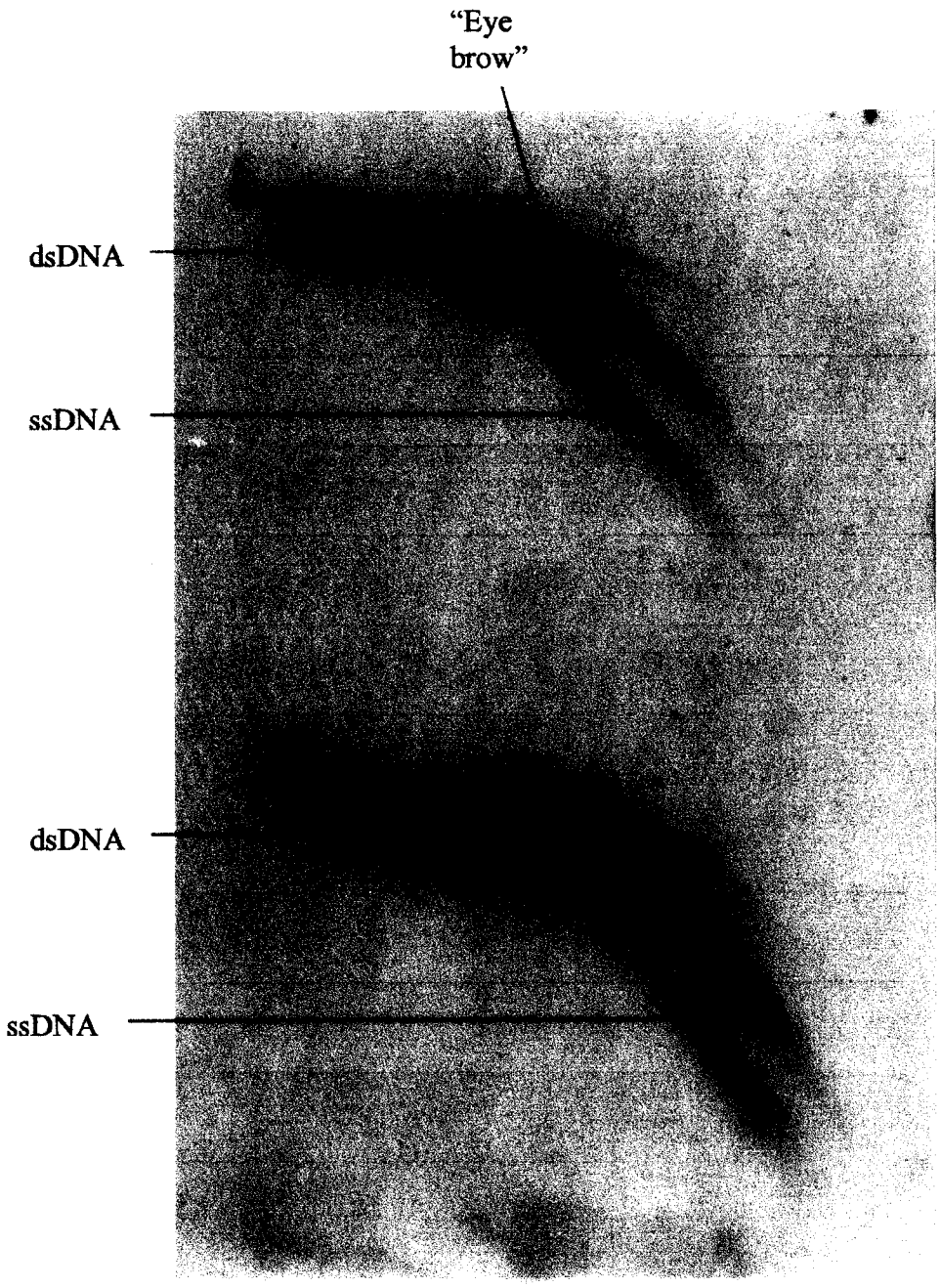
The wildtype cells BS132 were also grown in the log and stationary phases. The total cellular DNA from both samples was isolated and run on 2D gel. The picture of the first dimension shows the presence of log and stationary DNA of BS132 (Figure 16). The picture of the second dimension gel was taken and a potential eyebrow structure was observed in the BS132 log cells (Figure 17). The eyebrow structure was not observed in the BS132 stationary phase, which indicates the characteristic presence of the eyebrow structure in the wildtype cells grown in log phase. To determine if this is really an eyebrow structure the BS132 cells were grown again and the whole preparation of DNA was repeated. When the autoradiogram was developed the eyebrow structure was observed again (Figure 18). The eyebrow structure is caused by circular molecules of DNA with linear tails of varying length, which indicates that rolling circle mechanism is indeed a possible mechanism of replication in rho^+ cells grown in the log phase when cells replicate the most and produce the most DNA.

The autoradiogram of the BS132 log and stationary phase cells (Figure 18) was made using HS3324 mtDNA as a probe. The autoradiogram of both samples showed the presence of double and single stranded DNA but only BS132 cells grown in log phase showed the presence of the eyebrow structure. This autoradiogram also confirmed the


dsDNA
ssDNA
dsDNA
ssDNA


BS132
log
BS132
stat.
Marker

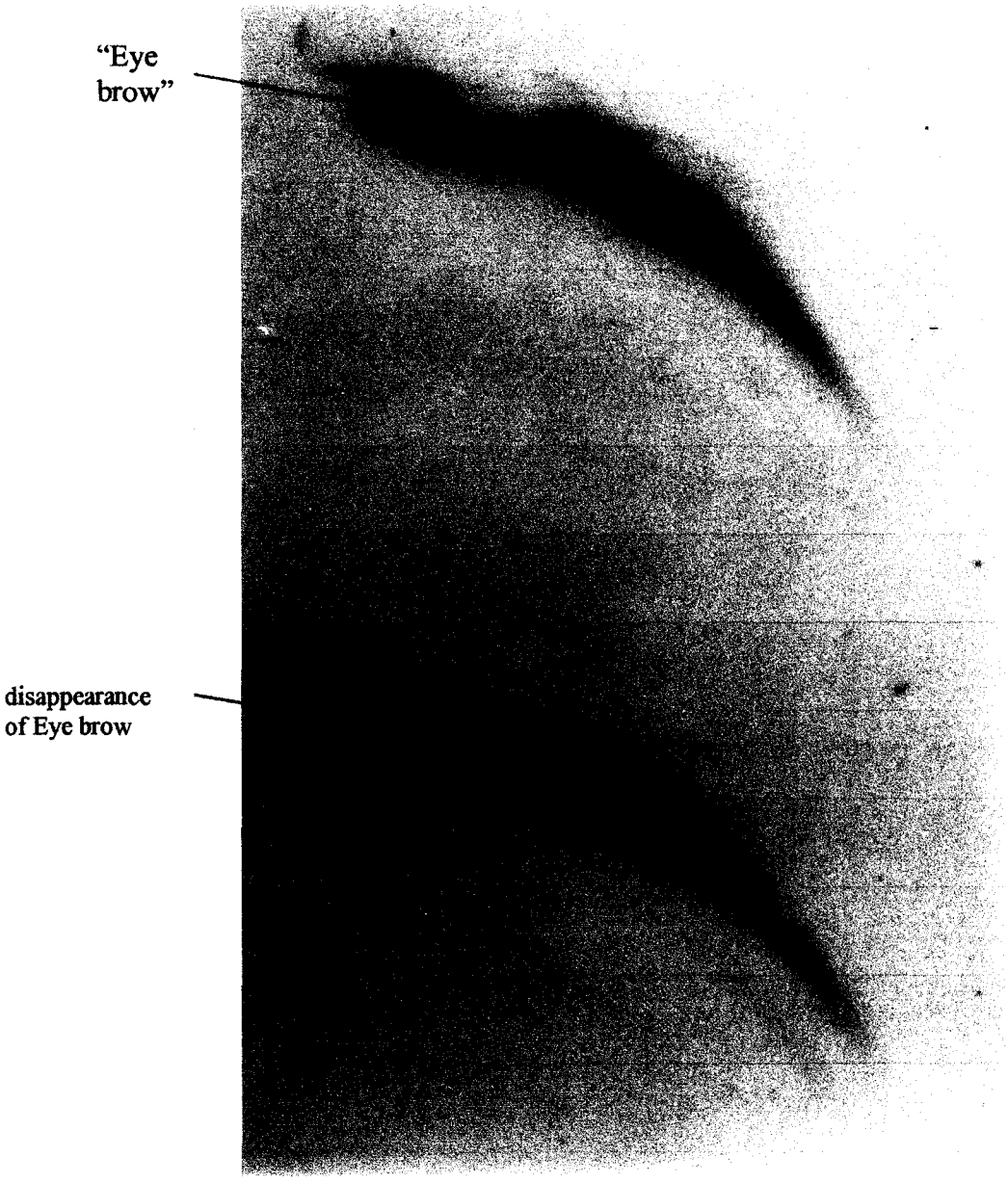

"Eye
brow"
BS132
log
BS 132
stat.


"Eye
brow"
dsDNA
ssDNA
dsDNA
ssDNA

characteristic presence of an eyebrow structure in the wildtype cells grown in the log but not stationary phases.

To confirm the presence of single stranded DNA in the wildtype cells BS127 and BS132 the BS132 cells grown in the log phase were treated with S1 nuclease, which cuts single stranded DNA. The samples of BS132 log phase DNA not treated with S1 and treated with S1 were run on 2D gel and then the Southern blot was performed. The autoradioram was made using specific DNA and α-^{32}P dATP. The autoradiogram (Figure 19) showed the presence of double stranded and single stranded DNA in both samples of BS132 not treated and treated with S1 nuclease. However the eyebrow structure disappeared in the sample that was treated with S1 nuclease. This indicated that the eyebrow structure apparently contains at least some single stranded DNA.

The presence of the single stranded DNA in the BS127, BS132, and HS3324/BS127 samples grown in the log phases and the finding of an eyebrow structure in the BS132 sample grown in the log phase that has some single stranded DNA indicates that a rolling circle mechanism may indeed occur in wildtype yeast cells that are rapidly increasing the amount of DNA.


"Eye
brow"
disappearance
of Eye brow

Discussion

I) Involvement of the gene RPO41 in the initiation of replication of yeast Saccharomyces cerevisae

The origins of replication in Yeast *Saccharomyces cerevisae* are presumed to be *ori/rep* sequences (reviewed in MacAlpine et. al., 2000). The wildtype cell contains seven to eight *ori/rep* sequences but only four are considered active origins of replication. The *ori/rep* sequences contain three GC rich boxes and a promoter very close to the GC box C (Figure 1). The active *ori/rep* sequences differ from inactive *ori/rep* sequences by the insertion of a short sequence in inactive origins of replication that disrupts a promoter. The nuclear gene *RPO41* in *Saccharomyces cerevisae* encodes the catalytic subunit of the mitochondrial mtRNA polymerase (Fangman et. al, 1990). Surprisingly, mutants with disrupted *RPO41* gene were respiratory deficient but they maintained their mtDNA (Lorimer et. al. 1995).

In this study dependence of replication structures of the mitochondrial genome on mitochondrial RNA polymerase was examined. To check for the involvement and the role of RPO41 in the initiation of replication of mitochondrial DNA in *Saccharomyces cerevisae* hypersuppressive cells, HS3324 and rho$^-$ neutral cells N28 were used.

Since the same potential replication structures were identified in cells with and without RPO41 it might be suggested that *RPO41* may not have major role in the initiation of replication of yeast mitochondrial DNA.

There have been four different modes of mtDNA replication hypothesized for yeast mitochondrial DNA (Figure 2). They are: 1. The D-loop model which is asynchronous and asymmetric. 2. Bi-directional DNA replication which is symmetrical

and synchronous. 3. Rolling-circle replication produces head to tails repeats of the genome and can produce long single-strand molecules, and 4. Recombination dependent replication in that DNA have to be first recombined and than replicated.

The D-loop model in mammals involves bidirectionnal, asymmetric-asynchronous DNA replication. In this model there is an origin of replication on each strand, and they are separated from each other by 2/3 of the genome (Clayton, 1984). This mode of mtDNA replication requires mtTFA and RNA polymerase (*RPO41*) for the initiation of transcription. The nascent RNA transcript is cleaved by a site-specific ribonucleoprotein endoribonuclease (MRP) in mammalian cells (Chang et. al. 1985). It is proposed that the same process occurs in yeast cells where MRP cleavage of a nascent transcript occurs on the *ori/rep* promoter to make a primer for DNA (Stohl and Clayton, 1992). If this were the only replication mechanism there would not be mtDNA replication if RPO41 was deleted from the genome. Early studies report mtDNA loss in rho$^-$, Δrpo41 cells (Dick and Clayton, 1998). In fact it was already seen that mtDNA replication still occurs even when RPO41 is deleted (Fangman et. al., 1990).

The mitochondrial RNA polymerase may still be involved in mtDNA replication if there is more than one mechanism. If that is the case, there should be different intermediates in cells with and without *RPO41*. In fact we observed the same replication intermediates in cells with and without *RPO41* (Figure 10). Cells with and without RPO41 both contained dsDNA, ssDNA, supercoiled, and nicked DNA in equilibrium proportions. Since the same structures were observed it is suggested that the same mechanism of replication occurs in the cells with and without *RPO41*, and it is possible that this mechanism is not D-loop.

We suggest another mode of mtDNA replication, rolling-circle. This mode of replication was identified in the phage T4 DNA replication system (Balenger et. al, 1996). This mechanism occurs by introduction of a nick in one of the strands of dsDNA and producing long strands of ssDNA (Figure 20). We have identified long strands of ssDNA in both cells samples with and without *RPO41* by treating samples with S1 nuclease (Figure 13). This supports the hypothesis that rolling-circle mechanism occurs in yeast cells.

There is also the possibility that recombination-dependent replication occurs in yeast cells (MacAlpine et. al., 2001, Lockshon et al., 1995). Since the yeast mtDNA has a high recombination index it might be that replication initiates from 3'OH ends produced by strand-invasion mtDNA.

II) Different Modes of Mitochondrial DNA Replication in Yeast *Saccharomayces cerevisae*:

A second mode mitochondrial DNA replication was discovered recently (Holt et. al., 2000). One mode of replication, the asymmetric-asynchronous, D-loop mechanism was discovered in cells that just maintains their copy number of mitochondrial DNA. Another mode of replication was found in cells that have to rapidly increase the copy number of mitochondrial DNA. This appeared to be coupled leading and lagging strand synthesis.

Since little is known about *in vivo* mitochondrial DNA replication in mammalian and yeast cells we wanted to see if there are different modes of replication of mitochondrial DNA in wild type yeast cells grown in different phases. It was possible

that in the cells in log phase a different mode of replication would be found than in the cells grown in stationary phases.

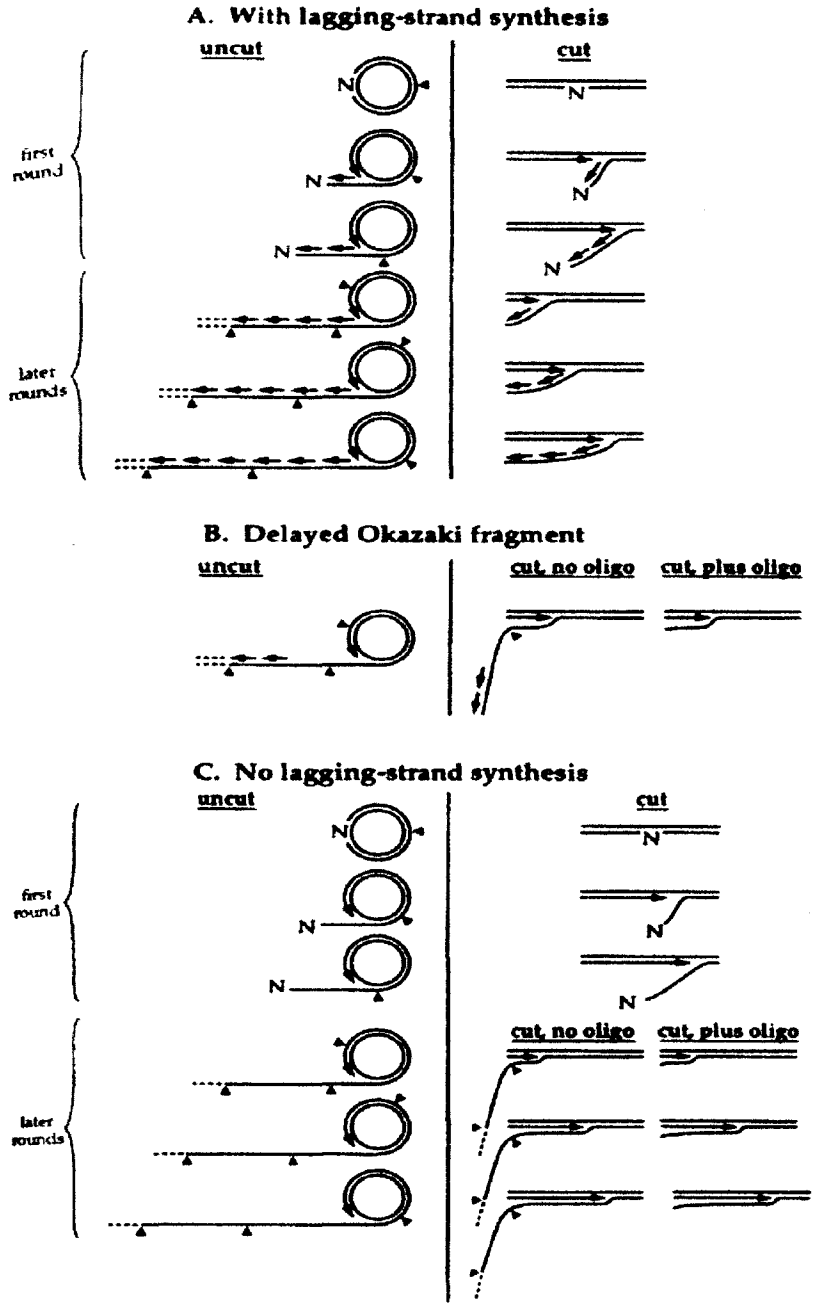
We grew wildtype and rho$^-$ cells to both log and stationary phases and looked for differences in replication intermediates. Two different wildtype cells strains were used for this: BS127 and BS132 that have different mating types and different nuclear markers.

In BS127 there were both double stranded and single stranded DNA in both log and stationary phases but there was more ssDNA in log than in the stationary phase. There were equal amounts of double and single stranded DNA in log and stationary phases. However in BS132 cells double and single stranded DNA forms were found in cells in log and stationary phases. In cells grown to log phase another structure running in a position indicating both high molecular weight and complex structure was discovered. The structure appears to be similar to a structure found in T4 phage replication, characteristic of rolling-circle called "an eye brow". The eyebrow structure was originally found in 2D gels of the phage T4 (Belanger et. al., 1996). More double stranded DNA in BS132 cells grown in stationary phases was found than in the BS132 grown in the log phase.

These results indicate that there are possibly two modes of replication occurring in yeast cells grown to log as opposed to the stationary phase. Since the higher amount of ssDNA and the presence of the "eye brow" structure was identified in the wildtype cells in the log phase than in the stationary phase it could be proposed that rolling-circle replication occurs rapidly growing cells. Another mechanism could occur in cells in stationary phase, which just have to maintain the copy number of mtDNA. The smaller

fragment of ssDNA in stationary cells may support the D-loop model. Recombination dependent replication may also produce short ssDNA molecules. Rolling-circle DNA replication is known to produce long strands of ssDNA (Figure 20). Since we have seen long strands of ssDNA and a potential eyebrow structure in cells grown in log phase and these structures were not identified in the stationary phase, rolling-circle is possibly the method of replication of rho^+ mtDNAin log phase. The cells grown in the stationary phase did not produce long strands of ssDNA so there is probably another mechanism of replication occurring in these cells. Since the D-loop model (Clayton, 1984) does not require long strands of ssDNA it is believed that this is the model of replication occurring in the wildtype yeast cells in stationary phase when they are simply maintaining their DNA.

These results are analogous to those identified in the mammalian cells that need to increase the copy number of mitochondrial DNA that replicate through the coupled leading and lagging strand synthesis (Holt et. al, 2000). Yeast cells that are grown in log phase are dramatically increasing their mtDNA copy number and may replicate mtDNA through one mechanism, the rolling circle mechanism, that increases the copy number of mitochondrial DNA a lot in a short time. Another, second mode of replication is found in mammalian cells that just need to maintain the copy number of mitiochondrial DNA, perhaps using the standard D-loop asymmetric-asynchronous mode of replication. Using Fangman/Brewer 2-D gels we looked for the involvement of the RPO41 gene in the replication of yeast *Saccharomyces cereviase* and for different modes of mitochondrial DNA replication depending on growth rate. Based on these results and previously done studies, it appears that in yeast cells mtDNA replication may occur


A. With lagging-strand synthesis
uncut
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later rounds
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B. Delayed Okazaki fragment
uncut
cut, no oligo
cut, plus oligo
C. No lagging-strand synthesis
uncut
cut
first round
later rounds
cut, no oligo
cut, plus oligo

through rolling-circle mechanism. However it is possible that the wildtype yeast cell grown in the stationary replicate probably through the D-loop asymmetric-asynchronous mode of replication since the long strand of ssDNA were not identified (Clayton, 1984).

For the future studies

To obtain an even better insight about different modes of replication in yeast cells, cells with and without *RPO41* could be grown in different phases, log and stationary. For example HS3324 with deleted *RPPO41* could be grown in the log and stationary phases and compare the replication intermediates in both of these cells. Also rho$^-$ neutral cells N28 *RPO41* could be grown in the log and stationary phase or rho$^-$ neutral cells N28 with deleted *RPO41* could be grown in log and stationary phase to compare the replication intermediates. Also the possibility of strand specificity of ssDNA should be checked. To check for strand specificity a single stranded probe that is complementary to the desired strand should be used. In order to see the exact structures during the replication of different cells grown in different phases transmission electron microscopy (TEM) could be performed as well.

References:

Beckman B. K. and Ames N. B. 1999. Endogenus oxidative damage of mtDNA. *Mutation Research* 424, 51-58

Belanger G. K., Mirzayan C., Kreuzer E. H., Albers M. B., and Kreuzer N. K. 1996. Two-dimensional gel analysis of rolling circle replication in the presence and absence of bacteriophage T4 primase. *Nucleic Acids Research.* Vol.24, No.11, 2166-2175

Bell, L and Byers, B. 1983. Separation of branched from linear DNA by two-dimensional gel electrophoresis. Analytical Biochemistry. Vol. 130, no. 2: 527-35

Brewer J. B., Sena P. E., and Fangman L. W. 1988. Analysis of Replication Intermediates by Two-dimensional Agarose Gel Electrophoresis. *Cancer Cells 6, Eukaryotic DNA Replication, Cold Spring Harbor Laboratory* 0-87969-308-8

Brewer J. B. and Fangman L. W. 1987. The Localization of Replication origins on ARS plasmids in *Saccharomyces cerevisae. Cell.* Vol.51 463-471

Chang D. D. and Clayton A. D. 1985. Priming of human mitochondrial DNA replication occurs at the light-strand promoter. *Proceedings of National Academy of Sciences.* Vol.82 351-355

Clayton, D. A. 1984. Transcription of the mammalian mitochondrial genome. *Annu. Rev. Biochem.* 53:573-94

Dyck V. E. and Clayton A. D. 1998. Transcription-Dependent DNA Transaction in the Mitochondrial Genome of a Yeast Hypersuppressive Petite Mutant. *Molecular and Cellular Biology.* Vol.18, No.5 2976-2985

Dujon, B. 1981. Mitochondrial genes and function, p. 505-635. In J. Broach, E. Jones, and J. Strathern (ed). The molecular biology of the yeast Saccharomyces. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Ephrussi, B., De Marerie-Hottinguer, H., and Roman, H. 1955. Suppressiveness; A New Facror in the Genetic Determinism of the Synthesis of Respiratory Enzymes in Yeast. *Proceedings of National Academy of Sciences.* 1065-71

Fangman L. W., Henly W. J., and Brewer J. B. 1990 RPO41-Independent maintenance of (rho$^-$) Mitochondrial DNA in *Saccharomyces cerevisae. Molecular and Cellular Biology.* Vol.10, No.1 10-15

Fernandez-Moreno A. M., Bornstein B., Petit N., and Garesse R. 2000. The Pathophysiology of Mitochondrial Biogenesis: Towards Four Decades of Mitochondrial DNA Research. *Molecular Genetics and Metabolism* 71, 481-495

Friedman L. K. and Brewer J. B. 1995. The Analysis of Replication Intermediates by Two-Dimensional Agarose Gel Electrophoresis. *Methods in Enzymology.* Vol.262, 613-627

Fridovich, I. 1974. Evidence for the symbiotic origin of mitochondria. *Life Sience.* Vol. 14, no.5: 819-826

Grawes T., Dante M., Eisenhour L., and Christianson W.T. 1998. Precise mapping and characterization of the RNA primers of DNA replication for a yeast hypersuppressive petite bi in vitro capping with guanylyltransferase. *Nucleic Acids Research.* Vol. 26, No. 5, 1309-1316

Harding E. A. and Hammans R. S. 1992. Deletions of the Mitochondrial Genome. J. Inher. Mateb. Dis. 15, 487-498

Hoffman, C. S. and Winston, F. (1987) A Ten-minute DNA prepatarion from yeast efficiently releases autonomous plasmids for transformation of Escherichia coli. *Gene* Vol.57, no.2-3 267-272

Holt J. I., Lorimer E. H., and Jacobs T. H. 2000. Coupled Leading- and Lagging-Strand Synthesis of Mammalian Mitochondrial DNA. *Cell* Vol.100 515-524

Howell N. 1999. Human Mitochondrial Diseases: Answering Questions and Questioning Answers. *Review of Cytology.* Vol.186, 49-114

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.* 81:947-55

Lorimer E. H., Brewer J. B., and Fangman L.W. 1995 A Test of the Transcription Model for Biased Inheritance of Yeast Mitochondrial DNA. *Molecular and Cellular Biology.* Vol.15 No.9 4803-4809

Lorimer H: 2D Gel Electrophoresis of mtDNA. In *Methods in Molecular Biology, Mitochondrial DNA Methods and Protocols.* Edited by Copeland C.W. Humana Press. 2002. 187-196

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

MacAlpine M. D., Kolesar J., Okamoto K., Butow A. R., and Perlman S. P. 2001 Replication and preferential inheritance of hypersuppressive petite mitochondrial DNA. *The EMBO journal* Vol.20 No.7 1807-1817

MacAlpine M. D., Perlman S. P., and Butow A. R. 2000 The numbers of individual mitochondrial DNA molecules and mitochondrial DNA nucleoids in yeast are co-

regulated by the general amino acid control pathway. *The EMBO Journal* Vol.19 No.4 767-775

Maleszka, R., Skelly P.J., and Clark-Walker G.D. 1991. Rolling circle replication of DNA in yeast mitochondria. *The EMBO Journal* Vol.10 no.12 pp.3923-3929

Maniatis, T., Fritsch E. F., and Sambrok J. *Molecular Cloning.* New York: Cold Spring Harbor Publications, 1982

Martin-Parras L., Lucas I., Martinez-Robles L. M., Hernandez P., Krimer B.D. Hyrien O., and Schvarzman B. J. 1998. Topological complexity of different populations of pBR322 as visualized by two-dimensional agarose gel electrophoresis. *Nucleic Acids Research.* Vol.26, No.14, 3424-3432

Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. Journal of Molecular Biology 98, 503-517

Schmitt E.M. and Clyton A. D. 1993. Conserved features of yeast and mammalian mitochonrial DNA replication. *Current opinion in Genetics and Development.* 3:769-774

Shadel S. G. 1999. Insights from Model Systems: Yeast as a model for Human mtDNA Replication. *Am. J. Human Genetics.* 65, 1230-1237

Shibutani, S., Reardon, T.J., Suzuki, N., and Sancar A. 2001. Excision of Tamoxifen_DNA Adducts by the human Nuceotide Excision Repair System. *Cancer Research.* 60:2607-2610

Stohl, L.L., and Clayton, D.A. 1992. Saccharomyces cerevisae contains an RNase MRP that cleaves at a conserved mitochondrial RNA sequence implicated in replication priming. *Mol Cell Biol.* 12:2561-9

Ryan, M.T., Naylor, D.J., Hoj, P.B., Clark, M.S., and Hoogenraad, N.J. 1997. The Role of molecular chaperones in mitochondrial protein import and folding. *International Review of Cytology.* Vol.174:127-93

Wallace C.D. 1999. Mitochondrial Diseases in Man and Mouse. *Science.* Vol.283 1482-1488

Zullo, J.S. *In Situ* PCR of the Common Human mtDNA Deletion. In *Methods in Molecular Biology, Mitochondrial DNA Methods and Protocols.* Edited by Copeland C.W. Humana Press. 2002. 187-196