Study of inheritance of mitochondrial DNA in similar sized rho$^-$, hypersuppressive and neutral, in the absence of nuclear gene, MGT1 (mitochondrial genome transmission)

By

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Abstract

Mitochondrial DNA is essential for respiration in eukaryotes and essential for life in many organisms including humans. The mechanisms underlying the maintenance and inheritance of both normal and deletion mutants (ρ^-) of mitochondrial DNA (mtDNA) in *Saccharomyces cerevisiae* have not been completely understood. The mtDNA sequence retained in the deletion mutants of *S. cerevisiae* and the nuclear gene, *MGT1*, which encodes a recombination junction-resolving endonuclease, are thought to be two important factors in the inheritance and maintenance of an individual mutant mtDNA. There is preliminary evidence that *MGT1* may be an absolute requirement for maintenance of some mutant mtDNAs but not for others possibly indicating sequence dependent recombination in yeast mtDNA.

Hypersuppressive (HS) ρ^- and neutral (N) ρ^- are two types of deletion mutants of yeast mtDNA. Both retain only a short fragment of mtDNA, which is amplified to a mass that is equivalent to that of the wild-type mtDNA (ρ^+). HS ρ^- have a replication/segregation advantage over N ρ^- and wild-type ρ^+ mtDNAs that is due to the presence of rep/ori sequences that N ρ^- lack. In these experiments, when HS ρ^- is mated with N ρ^- in the absence of *MGT1* gene, the resulting cells are all HS ρ^-. This indicates that *MGT1* gene may not have rep sequence dependent action on preferential inheritance.

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Introduction

Mitochondria

Mitochondria are found in most eukaryotic cells. Mitochondria are about the size of *E.coli* bacterium. These organelles cover twenty-five percent of the volume of the cytoplasm. They fall in the group of larger organelles that includes nucleus, vacuoles, and chloroplast. Mitochondria are the only organelles to have DNA besides the nucleus and chloroplasts. The mitochondrion has two different membranes, an outer membrane and an inner membrane. These two membranes are separated by an intermembrane space and enclose a central compartment known as the matrix. The inner membrane of mitochondria contains many infoldings, known as cristae, which protrude into the matrix. These infoldings enhance the ability of mitochondria to generate ATP by providing more surface area. The inner membrane also contains many protein-rich intramembrane particles. The outer membrane is smooth and contains transmembrane channel proteins. The inner membrane and the matrix are the most important sites where oxidation of pyruvate and fatty acids to CO_2 and H_2O takes place. Synthesis of ATP from ADP and Pi also occurs in inner membrane and matrix (reviewed in Lodish et. al., 2001).

Mitochondria are responsible for the production of the majority of energy in all eukaryotic cells. They are the site where most of the energy in the form of ATP is made in the cells by the process of oxidative phosphorylation (reviewed in Saraste, 1999). The oxidation energy reactions of mitochondria produce 34 of a total of 36 ATPs in a typical eukaryotic cell. Therefore, these tubular organelles are also known as the "powerhouse" of eukaryotic cells. Mitochondria are also considered the major source of reactive oxygen superoxides since it is the most important site of oxidative reaction metabolism in the

cell. These reactive oxygen superoxides, which are the toxic byproducts of respiration, are thought to play an important role in mitochondrial mutations and the process of aging. Mitochondria also play a vital role in the regulation of programmed cell death or apoptosis. Mitochondria initiate apoptosis by the opening of an inner membrane channel, mitochondrial permeability transition pore (mtPTP). The opening of the mitochondrial permeability transition pore leads to several events in cell, which includes the loss of mitochondrial transmembrane potential, swelling of the inner membrane of mitochondria and release of cytochrome c, the flavoprotein AIF (apoptosis-inducing factor), and caspases that are important factors for the apoptotic cell death (reviewed in Smith et. al., 2000). Mitochondria can also trigger apoptosis by altering the electron transport chain, oxidative phosphorylation, and adenosine triphosphate (ATP) production (reviewed in Green and Reed, 1998). Understanding of any or all of these mechanisms may aid in explaining how defects in mitochondria can lead to human degenerative diseases, cancer, and aging.

Origin and evolution of mitochondria

Mitochondria are considered to be products of endosymbiosis. Endosymbiosis is a condition where one organism lives inside another. It is a mutually beneficial relationship with one serving as a host cell and the dependent organism residing within the host. Therefore, symbiogenesis is considered an important evolutionary process, as it creates new organelles, tissues, physiology and other features arising from a symbiotic association. According to the endosymbiosis theory, it is believed that mitochondria are the descendents of a free-living α-proteobacteria that established a symbiotic relationship

with an anaerobic, hydrogen-dependent, autotrophic archaebacterium ((Margulis and Chapman, 1998; reviewed in Nosek and Tomáška, 2003). The bacterium that was engulfed by the eukaryote lost or transferred most of its genes to the genome of the eukaryote (reviewed in Emelyanov, 2001). This may be the reason why many genes required for mitochondrial function, such as, oxidative phosphorylation reside in the nuclear genome (reviewed in Gray et al., 1999) and selective import of mitochondrial proteins that are coded in the nuclear genome are selectively imported back into mitochondria (reviewed in Smith et. al., 2000). There is other molecular evidence that support the endosymbiosis theory. The mitochondrion has its own DNA and it replicates its genome independently of the cell cycle control of nuclear genome replication. Mitochondrial DNA is also not organized into nucleosomes by histones and the same characteristic is also seen in bacteria. Mitochondrial translation is inhibited by chloramphenicol and erythromycin, which are also the inhibitors of bacterial translation. Chloramphenicol and erythromycin, however do not affect the cytoplasmic protein synthesis of eukaryotes (Hartwell et al., 2000). The double membrane structure of mitochondria, its circular genome, and the existence of tRNAs and rRNAs point its origin through endosymbiosis (reviewed in Smith et. al., 2000). All of this evidence together suggests that mitochondria may have evolved from an endosymbiont bacterium.

Mitochondrial DNA

Mitochondria are the only organelles to have their own genetic material besides the nucleus and chloroplasts in plant cells. The nuclear genes encode most of the mitochondrial and chloroplast proteins. These proteins are made on cytosolic ribosomes

and are imported into the organelle post-translationally (reviewed in Gray et. al., 1999; Fernández-Silva et. al., 2003).

Mitochondrial DNA was discovered in 1963 by Nass and Nass (reviewed in Fernández -Moreno et. al., 2000). The mitochondrial DNA (mtDNA) is located in the mitochondrial matrix and is sometimes found attached to the inner mitochondrial membrane (Lodish et. al., 2001). In different organisms, the size of mtDNA, the number and nature of proteins they encode, and also the mitochondrial genetic code itself are quite different. However the mtDNA of all mammals have same basic structure, genetic content and organization. Therefore, human mitochondrial DNA that has been completely sequenced can be taken as a model for all other mammals. Human mitochondrial DNA is among the smallest mtDNA known. It is a circular double-stranded molecule of 16.5 kb. The two strands of mtDNA are known as heavy (H) and light (L) strands due to their buoyant density in cesium chloride gradient. Since these two strands have different G + A content, it is possible to separate them in cesium chloride on the basis of their density (reviewed in Fernández-Silva et. al., 2003).

The human mitochondrial genome is very compact and does not have introns. It has very few intergenic spaces with overlapping genes (reviewed in Fernández -Moreno et al., 2000). It codes for 13 polypeptides which are the subunits of the mitochondrial respiratory chain, two rRNA genes and 22 tRNA genes that are required for their translation. It frequently has a triple stranded structure know as a displacement loop (D-loop). The region where D-loop occurs is a non-coding region about 1 kb long and has the main mitochondrial regulatory functions that are involved in the control of replication and transcription. The D-loop contains what has been presumed to be the origin of

replication for the heavy strand (O_H) and transcription promoters of both heavy and light strand. The presumed origin of replication for the light strand (O_L) is located farther away from the O_H at about two-third of the genome (reviewed in Shoubridge, 2001). Substantial evidence has led to a model that describes human mtDNA replicating unidirectionally and asynchronously from the origins of replication for O_H and O_L. Replication of mtDNA starts at O_H and is catalyzed by DNA poly γ and accessory proteins. The priming of H-strand is found to be served by RNA transcripts generated at the light-strand promoter (reviewed in Shadel, 1999). This shows that there is a connection between replication of mtDNA and the transcription of genes.

The mitochondrial genome of *Saccharomyces cerevisiae* is about 80kb, which is almost five times larger than the human mitochondrial genome. In an average cell of *S. cerevisiae*, mtDNA accounts for 15% of the total cellular DNA, which is about 50 copies of mtDNA molecule in haploid cells and 100 in diploid (reviewed in Gingold, 1988). Unlike human mtDNA, which is circular, mtDNA of *S. cerevisiae* has recently been found to be linear (reviewed in Nosek, 2003). The mitochondrial DNA of *S. cerevisiae* codes for seven proteins that are the subunits of oxidative phosphorylation, one protein of the mitochondrial ribosomal small subunit, two rRNAs, one 9S RNA and 24-25 tRNAs (reviewed in Pon et. al., 1991). Despite its large genome size, only one-fifth of its total mtDNA codes for genes (reviewed in Piškur, 1994).

MtDNA is inherited uniparentally in higher eukaryotic cells meaning that it is inherited only from one parent and that of the other parent is permanently lost. In most mammals, it is inherited only from the mother (reviewed in Howell, 1999; Smith et. al., 2000). This shows that mtDNA is inherited in non-Mendalian fashion (reviewed in

Pišhur, 1994), in which nuclear DNA is inherited from both parents. The ratio of mtDNA molecules in ovum to sperm is 100,000 to 1,000. The presence of higher number of mtDNA in ovum could be one of the reasons for the maternal inheritance of mtDNA (reviewed in Shoubridge, 2001). It has been shown that sperm mtDNA is found in the mid piece of the tail and is eliminated during the process of fertilization while the nuclear DNA that is found in the head of the sperm is donated by the sperm to the egg (reviewed in Berdanier et. al., 2001). While some studies have reported the entrance of oocytes by sperm mtDNA (reviewed in Howell, 1999; Smith et. al., 2000), an ubiquitin-dependent mechanism destroys or eliminates the few sperm mtDNA that is able to penetrate or enter the oocytes (reviewed in Fernández-Silva et. al., 2003). The sperm or paternal mtDNA thus is insignificant in making any physiological and evolutionary genetic contribution (reviewed in Howell, 1999).

Mitochondrial Diseases

In 1962, the first patient was identified who had a disease resulting from mitochondrial defects. Defects in mitochondria compromise the energy supply of the cell and these defects could be caused by pathogenic mutations in genes that are encoded in the mitochondrial or nuclear genome (reviewed in Fernández –Moreno et al., 2000). Now we know of many diseases in humans that are associated with defects in mitochondria or mitochondrial DNA. These diseases can be caused by point mutations or rearrangement mutations (deletions and insertions) in mtDNA. These diseases are seen in organs like brain and muscles where there is a high demand for energy (reviewed in Lecrenier and Foury, 2000). A few examples of mitochondrial diseases are Leigh's syndrome,

myotonic epilepsy and ragged-red fiber disease (MERRF), mitochondria encephalomyopathy, lactic acidosis and stroke-like syndrome (MELAS) and mitochondria encephalomyopathy. Diseases such as diabetes mellitus, Alzheimer's disease and Parkinson's are also associated with mitochondrial mutations (reviewed in Berdanier et. al., 2001).

There are several reasons for the manifestation of a high rate of mutation in mtDNA. MtDNA lacks histone proteins, which means its structure receives less protection by proteins. This can make it more prone to free radical damage and DNA damaging agents. Also, the close proximity of the mtDNA to the inner mitochondrial membrane also makes it able to mutate faster than the nuclear DNA. This results from fact that inner membrane is the site of the production of the reactive oxygen species during oxidative phosphorylation. Although much of the mutations are caused by reactive oxygen species generated during oxidative phosphorylation, environmental factors can also contribute to this process. It also appears that DNA repair mechanisms of mtDNA are not as efficient as that of nuclear DNA (reviewed in Berdanier et. al., 2001; Fernández-Silva et. al., 2003).

In a human somatic cell, there are about 100-500 copies of mtDNA. This number is even higher in mature oocytes, somewhere around 100,000 (reviewed in Howell, 1999). If all of the copies of mtDNA have either normal sequence or mutated sequence, the cell is referred to as homoplasmic (reviewed in Berdanier et. al., 2001). However if all of the copies of DNA have some mutated sequence and some normal sequence, then the cell is said to have a condition called heteroplasmy (reviewed in Wallace, 1999). In a heteroplasmic cell with a low mutation level the effect of the mutation is not seen,

because the normal mtDNA can complement the effect produced by the mutation. But when a heteroplasmic cell has mutant mtDNAs whose percentage is higher than a threshold value (a particular value), the normal mtDNA cannot complement the mutation that inhibits oxidative metabolism. This is where the onset of disease can be noticed (reviewed in Berdanier et. al., 2001; Fernández –Moreno et. al., 2000).

Yeast, *Saccharomyces cerevisiae* as a genetic model

Saccharomyces cerevisiae is a budding yeast, which is commonly referred to as baker's yeasts. It is a eukaryote that has been used to make bread and alcoholic beverages for many years. It is one of the most widely studied organisms in the areas of genetics and biochemistry. There are several advantages of yeast as a genetic model for studying mitochondrial mutations. The main advantage of yeasts is that they are facultative anerobes, meaning that they can produce ATP either through aerobic respiration using its mitochondria or by fermenting sugars, bypassing respiration. So, in the absence of respiration, yeasts can survive and grow well on a fermentable carbon source, such as glucose, even without their mtDNA (reviewed in Lecrenier and Foury, 2000). This means that mutant cells that are incapable of respiration can be easily grown in the laboratory and are readily available for study as long as they are provided with a fermentable carbon source. This also makes it easier to select mutant cells from wild-types. Petite mutants can be identified if they are grown on a differential medium containing 0.1% glucose, 2%glycerol. The petite colonies grow on glucose but cannot grow on nonfermentable substrates such as glycerol, which makes it easier to differentiate mutant colonies from wild-type colonies that grow on glycerol (Fukuhara and Rabinowitz, 1979)

Saccharomyces cerevisiae has a very short generation time of about ninety minutes. This makes it easy to grow many cells in a short period of time. Yeast cells can also be grown as haploid or diploid, which makes genetic analysis much easier. *Saccharomyces cerevisiae* has been used for studying transmission or segregation of mitochondrial DNA, which is difficult to do in higher eukaryotes. It is also the first eukaryote to have its nuclear and mitochondrial genomes sequenced completely in 1996. Thus, yeast is a good model system for studying mitochondrial genetics, especially mtDNA mutations and effects of such mutations.

Petite mutants

When yeast cells are first grown in liquid media and then plated onto solid media, both containing glucose as an energy source, different types of colonies are formed. The majority of the colonies is large (grande), capable of respiration and have wild-type (ρ^+) mtDNAs. On the other hand, small colonies arise spontaneously at a rate of around 1 percent and are named as petites (ρ^-) mutants. These petite mutants do not have active mitochondria and thus are incapable of respiration. They can survive only by fermenting carbon containing substrates (reviewed in Gingold, 1988). These mutants have mtDNAs that have lost most of their (~ 80 kb) wild-type (ρ^+) genome. The fragment remaining in the petite mtDNA genome is very small and amplified to a mass that is equivalent to that of ρ^+ mtDNA. Thus, a small fragment of mtDNA found only once in the wild-type (ρ^+) genome, is included many times in the mtDNAs of petite (ρ^-) mutants. Sometimes petite cells have no mtDNA at all and are referred as Rho^0 (ρ^0) (reviewed in Piškur, 1994; Ginggold 1988). Mutants in yeast mtDNA can also be achieved by mutagenesis and

many mutants are available for study. These mutants also retain a few hundred to a few thousand base pairs of the original eighty thousand base pair genome and are very important in studies on inheritance of yeast mitochondrial genome.

When petite cells (ρ^-) are mated to wild-type (ρ^+) cells, several inheritance patterns are observed. In some $\rho^- \times \rho^+$ matings, the zygotic clones consist of a majority of pure ρ^+ cells. On the other hand, in some $\rho^- \times \rho^+$ matings, the zygotic clones consist almost entirely of ρ^- cells and the ρ^+ mtDNA is not transmitted to progeny, although it was originally present in the zygote. These cells are referred to as suppressive petite cells (Ephrussi et. al, 1955). The percentage of the zygotic clones that contain exclusively ρ^- cells is defined as degree of suppressiveness. The suppressiveness of ρ^- is an intrinsic heritable property of each ρ^- mitochondrial genome and thus differs from one mutant to another (Blanc and Dujon, 1980). The petite cells that produce >95% ρ^- cells when mated to cells that have ρ^+ mtDNA are referred to as hypersuppressive (HS) cells. On the other hand, petite cells that produce <5% ρ^- progeny when mated to a ρ^+ strain are termed as non-suppressive or neutral petites. A ρ^- mutant can fall into either of these two classes or fall somewhere in between these two extremes (reviewed in Piskur, 1994; Gingold, 1988).

The hypersuppressive ρ^- cells have a short mtDNA fragment, which is less than 5 kb and is amplified in head-to-tail repeats until it is equal to the mass of the wild-type. This small piece of DNA contains one of three or four highly conserved sequences called rep or ori sequences that are found at different places in the genome of ρ^+ mtDNA (Blanc and Dujon, 1980; de Zamaroczy et al., 1981). They are called rep or ori because they are believed to be primary replication origins for the yeast mitochondrial ρ^+ genome (Blanc

and Dujon, 1980). The ori/rep sequences consist of a 300 bp segments that are homologous in all HS ρ^- mutants (reviewed in Piskur, 1994). The mitochondrial genome of *S. cerevisiae* has seven to eight copies of these ori/rep sequences. Among these ori /rep sequences, there are only four (ori1, ori2, ori3 and ori5) that are thought to be "active oris" with a functional promoter. These "active" oris are the only ones found in the mitochondrial genome of hypersuppressive ρ^- strain (de Zamaroczy et al., 1981). The other remaining oris are termed as "inactive" as their promoter is disrupted with a short DNA insertion (Baldacci and Bernardi, 1982). Each ori/rep contains three 100% conserved GC-rich blocks designated as A, B, and C, separated by an AT-rich region (de Zamaroczy et al., 1981) and a functional nonanucleotide promoter for mitochondrial RNA polymerase that is encoded by a nuclear gene *RPO41* (Greenleaf et al., 1986). A promoter is found upstream of GC cluster C that allows transcription towards the Boxes A and B (Baldacci and Bernardi, 1982). Boxes A and B are separated by a short stretch of AT, but when combined can form a 34bp inverted repeat which is 100% conserved in all rep sequences. The AB palindrome can form a stem-loop structure which shares a strong similarity with that of the human O_L mtDNA origin (Bernardi et al., 1982; de Zmaroczy et al., 1981). The rep/ori activity is dependent on the integrity of GC clusters A and B. The rep/ori sequence becomes inactive when GC cluster C and its surrounding area is deleted (de Zamaroczy et al., 1981; de Zamaroczy et al., 1984).

It is thought that the presence of multiple copies of rep/ori sequences in amplified HS (ρ^-) genome provides preferential inheritance of hypersuppressive ρ^- mtDNA in

Figure 1. rep sequences in wild-type (ρ^+) and hypersuppressive, HS3324 (ρ^-) cells. Wild-type (ρ^+) mtDNA contains three active rep sequences. Hypersuppressive, HS3324 (ρ^-) has a small mtDNA fragment less than 5kb consisting of one of the three rep sequences found in (ρ^+), which is amplified in head-to-tail repeats to equal the mass of wild-type (ρ^+) mtDNA.

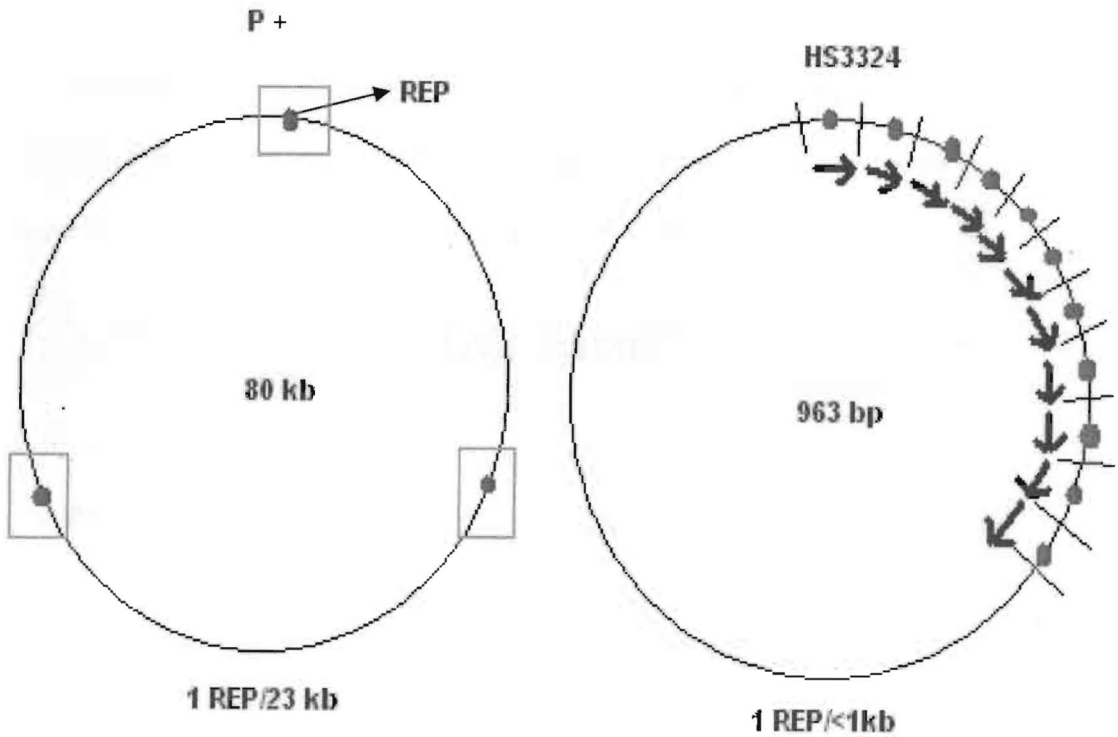
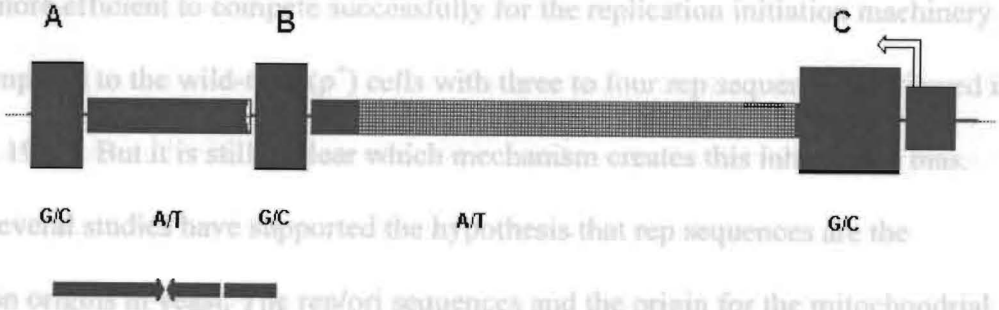

P +
REP
80 kb
1 REP/23 kb
HS3324
963 bp
1 REP/<1kb

Figure 2. rep sequence DNA. Black areas represent the transcriptionally active rep sequences that are 100% conserved; gray areas are less highly conserved. The open arrow shows the promoter and the direction of transcription. The filled arrows indicate the inverted repeat formed by GC elements A and B and the AT sequence in between them.


A
B
C
G/C
A/T
G/C
A/T
G/C

crosses with ρ^+ mtDNA (Blanc and Dujon, 1980; de Zamaroczy et al., 1981). On the other hand, neutrals (ρ^-) mtDNAs do not have such rep sequences and they do not exhibit preferential inheritance in ρ^- x ρ^+. Some studies have shown that these ori/rep sequences function as origins of mtDNA replication in yeast. So it has been presumed that the high density of rep sequences in HS ρ^- genomes confers a replication advantage in the preferential transmission of HS genomes in crosses with ρ^+ genomes. It is believed that RNA transcripts are created at the rep sequence which aids in the process of DNA synthesis. The higher frequency of rep sequences in HS mtDNA has been thought to make it more efficient to compete successfully for the replication initiation machinery when compared to the wild-type (ρ^+) cells with three to four rep sequences (reviewed in Gingold, 1988). But it is still unclear which mechanism creates this inheritance bias.

Several studies have supported the hypothesis that rep sequences are the replication origins in yeast. The rep/ori sequences and the origin for the mitochondrial leading, heavy strand DNA replication in the D-loop region of vertebrate mtDNA have some similar structural features (Schmitt and Clayton, 1993). First, the rep/ori sequence contains a consensus promoter which is similar to that of the H-strand origin of replication of mammalian mitochondrial DNA (de Zamaroczy et al., 1981). Second, a short palindromic sequence found in the rep sequence is similar in structure to that of the L-strand origin of replication of mammalian mtDNA (Bernardi, 1982). Third, studies have reported that polynucleotide contains an RNA-DNA junction within sequence complementary to a rep sequence (Baldaacci et al., 1984; Graves et al. 1994). Fourth, a stable RNA- DNA heteroduplex has been made using *in vitro* transcription from yeast

ori/rep promoter and it has been shown that yeast mtDNA polymerase was able to use this duplex to prime in vitro DNA synthesis (Xu and Clayton, 1995).

Due to the structural homology between rep sequences and replication origin of mammalian mtDNA, a universal model of mtDNA replication initiation mechanism was proposed. This model states that yeast rep sequence has two origins of replication. Replication is initiated at the first origin, which is similar to the origin of the heavy-strand of human mitochondrial DNA. The primers required for replication are created by the cleavage of transcripts that are initiated at the promoter, which is upstream of GC cluster C. The second strand starts its replication at a palindrome, which is formed by the GC-rich sequences (blocks A and B) and A-T rich sequences in between them (reviewed in Schmitt and Clayton, 1993). This second origin is similar to the light-strand origin of human mtDNA. A DNA primase is believed to prime the second strand (Baldacci et al. 1984). All these studies have provided some evidence that yeast rep sequence indeed functions as an origin of replication.

It has been shown that the maintenance of wild-type (ρ^+) mtDNA genome is dependent upon translation (Myers et al., 1985). But the action of the ori/rep promoter in the replication of the wild-type (ρ^+) genome is still poorly understood. The ρ^- deletion mutants can maintain their genome in the absence of a rep/ori sequence. This was demonstrated in several studies done by Fangman et al. They identified several ρ^- genomes that were stable in the absence of an ori/rep sequence in their genomes (Fangman et al., 1989). And later they also showed that rho$^-$ genomes, both hypersuppressive and non-suppressive, (neutral) carrying a disrupted gene of *rpo41*, were maintained in the haploid strains (Fangman et al., 1990). This shows that replication of

rho$^-$ (ρ^-) mtDNA does not seem to require *RPO41*. It may also suggest that when the rep sequence is absent in rho$^-$ genome, yeast can use some other sequence for mtDNA replication or an alternative mechanism that is not dependent on a specific sequence. It is thought that a recombination-dependent replication mechanism could be one of the alternatives, since there is a possibility of recombination in rho$^-$ genomes due to their repetitive nature (reviewed in Shadel, 1999).

Similarly, Lorimer and co-workers have demonstrated that mtRNA polymerase is not required for the biased transmission of hypersuppressive ρ^- genomes and that cell without *RPO41* were able to maintain this inheritance. The replication of the hypersuppressive mitochondrial genome was maintained in the strains with deleted *RPO41*. In other words, the study showed that *RPO41* primed replication is not essential for hypersuppressiveness. This suggests that the high frequency of rep sequences found in the ρ^- genomes does not confer a replication advantage in the preferential transmission of HS ρ^- genomes, but rather provides a segregation advantage over other mtDNA (Lorimer et al., 1995). This hypothesis is similar to the action of deleted/over expressed *MGT1/CCE1* (mitochondrial genome transmission / cruciform cutting endonuclease) gene, which suggests that *MGT1/CCE1* that resolves the recombining mtDNAs could play an important role in the transmission and segregation of mtDNA (reviewed in Contamine and Picard, 2000).

MGT1/CCE1 and its role in the bias transmission of mtDNA

Homologous recombination is a process in which information is exchanged between two chromosomes that are in homologous alignment. It plays an important role in different functions such as genetic diversity, DNA damage repair and proper chromosomal segregation at meiosis (Whitby and Dixon, 1997; reviewed in West 1985). In *Saccharomyces cerevisiae*, recombination of mtDNA takes place at a high rate (reviewed in Gingold, 1988). The process of recombination starts by synapsis of chromosomes and proceeds to cross-exchange between two DNA single strands. This creates a 4-armed, heteroduplex intermediate known as a Holliday junction (Meselson and Radding, 1975; Orr-Weaver and Szostak, 1985). It is crucial to resolve these branched DNA molecules, as they are necessary for the viability of cells.

The resolution of Holliday junctions is achieved by specific endonucleases that recognize and cleave these four-way DNA junctions. In 1992, Kleff et al. discovered a *CCE1*, which is a structural gene for cruciform-cutting endonuclease 1 in yeast. It was identified through a screen for yeast mutants that were deficient in the cleavage of Holliday junctions, using cruciform structures that resemble Holliday junctions as the substrate (Kleff et al., 1992). Later *CCE1* was found to localize to the mitochondria of yeast *S. cerevisiae* and the cruciform cutting endonuclease activity was not seen in the mitochondria of the mutant strains that lacked this gene (Ezekiel and Zassenhaus, 1993).

The *CCE1* gene codes for a 41 kDa protein and is located on the left of the centromere of chromosome XI. It was found that *CCE1* was allelic to a nuclear gene *MGT1* (mitochondrial genome transmission), since the location of *CCE1* mutation coincided with the map position of the *MGT1* mutation on chromosome X1 near met14.

It was demonstrated that cce1 mutant was resistant to hypersuppressiveness of mitochondria and mgt1 mutant lacked cruciform cutting endonuclease activity, which further provided evidence that *CCE1* and *MGT1* were allelic (Kleff et al., 1992). The gene product of *CCE1* and *MGT1* has been shown to cleave Holliday junction analogs and is presumed to be associated with the replication or segregation advantage mechanism in yeast mtDNAs (reviewed in Piškur, 1994).

The *MGT1* gene was originally identified by Zweifel and Fangman and was found to be necessary for the biased transmission of the hypersuppressive rho$^-$ genome. When the *mgt1* mutation was carried by only one haploid parent, whether it has ρ^+ or ρ^-, the percentage of ρ^- diploid clones was substantially reduced. But when the mutation was present in both ρ^+ and ρ^- haploid parents, there was a complete switch in the biased transmission of petite mtDNA molecule and only wild-type mtDNA was inherited in most zygotic progeny. This suggested that *MGT1* plays an important role for the preferential transmission of ρ^- genome when in competition with ρ^+ genome (Zweifel and Fangman, 1991).

Further study by Lockshon et al. demonstrated that cells that lacked *MGT1* (*Δmgt1*) had an increased ratio of mtDNA molecules linked presumably by unresolved recombination junctions. This caused the mtDNA molecules in *Δmgt1* cells to aggregate into a smaller number of cytological structures known as chondriolites when compared to the diffuse, large number of chondriolites normally seen in *MGT1* ρ^- cells. The deletion of the *MGT1* gene had less effect on ρ^+ cells and only 10% of *Δmgt1* ρ^+ cells had large chondriolites. The existence of more pronounced branched networks of mtDNA in the *Δmgt1* ρ^- cells was supported by gel electrophoresis. The DNA from *MGT1* ρ^- cells

traveled through the gel while the DNA from *Δmgt1* ρ^- cells was stuck in the well. The two-dimensional gel electrophoresis further revealed the presence of more linked mtDNA structures in *Δmgt1* ρ^- cells than in *MGT1* ρ^- cells. The study also found that the over expression of *MGT1* gene product decreased the frequency of mtDNA to be linked by recombination junction (Lockshon et al, 1995).

All the aforementioned results showed that the *MGT1* gene has the ability to resolve recombining mtDNA genomes in *Saccharomyces cerevisiae* and to prevent it from forming highly branched mtDNA networks, linked by Holliday junctions. Thus, this function of *MGT1* gene to resolve recombination junctions is believed to have a crucial role in the segregation of mitochondrial DNA in yeast. The clustering of mitochondrial genomes caused by unresolved holiday junctions in *Δmgt1* cells has been thought to reduce the number of mtDNA units segregating independently. The decrease in the number of heritable units of mtDNA in the absence of *MGT1* gene activity was expected to be higher in rho$^-$ genomes than in rho$^+$ genomes because the high density of tandemly repeated small fragment of rho$^-$ genomes is seen to have a higher chance of recombining homologous sequences (Lockshon et al, 1995).

MHR1 in *S. cerevisiae* has been isolated as a gene necessary for mitochondrial DNA recombination (Ling et al., 1995) and DNA repair in wild-type (ρ^+) cells (Ling et al., 2000). *In vitro* the *MHR1* gene product has been demonstrated to function in pairing up single-stranded DNA with homologous double-stranded DNA resulting in heteroduplex joints. Interestingly, cells with only *mhr1-1* mutations were able to maintain rho$^-$ mtDNA but were found to lose mtDNA if they lacked both *MGT1* and *MHR1* (Ling and Shibata, 2002).

Although recombination resolution was proposed to be the sole source of the change in biased inheritance caused by the loss of the *MGT1* gene in yeast cells, as suggested by Lockshon et al., some rho$^-$ cells lose all of their mtDNA in the absence of *MGT1*, while others maintain their genomes well. The ability to maintain the mitochondrial genome in the absence of *MGT1* was found to vary in different deletion mutants of *S. cerevisiae* mtDNA. Here are a few examples of deletion mutants with different abilities to be maintained in the absence of *MGT1* gene. In the absence of *MGT1*, the two neutral (rho$^-$) genomes, M4 with a 3.2-kb repeat and N28 with a 598-bp repeat, do not maintain their mtDNA, while 23-3, a 1350-bp deletion mutant with also neutral (rho$^-$) genome, maintains its mtDNA well. Similarly, HS8-3, a 4.6-kb deletion mutant, maintains mtDNA very poorly, whereas HS82d, which is a subdeletion derived from HS8-3, maintains mtDNA relatively well in the absence of *MGT1*. Also, a hypersuppressive (rho$^-$) genome, HS3324, with 963-bp repeat is maintained well in the absence of *MGT1* (Lorimer-unpublished data). The action of the *MGT1* does not seem to correlate with the size of the deletion mutants, as mutants with different genome sizes are either maintained or fail to be maintained in the absence of *MGT1*. It is worth making a note here that both hypersuppressives and neutrals have some mtDNA genomes that are maintained in the absence of *MGT1* while others are not maintained well and hypersuppressive (rho$^-$) genomes contain rep sequences, while neutral does not. Thus, there has to be something within the sequence retained in the mutant mitochondrial genome that affects the *MGT1* function, or the action of *MGT1* may be specific to the mutant mitochondrial genome. This could be an interesting study as rho$^-$ mtDNA can arise from almost anywhere in the rho$^+$ genome and often does not contain rep sequences.

Cruciform-cutting endonuclease (CCE) binds to X-junctions of DNA in a strongly structure-specific manner. It also exhibits sequence selectivity for the cleavage of four-way DNA junctions that arise from the recombination of mitochondrial genomes (Evans and Kolodner 1988; White and Lilley, 1996). Rep sequences contain palindromic regions which might form cruciform structures. Cruciform structures are equivalent to Holliday junctions in structure, which are involved in recombination (Symington and Kolodner1985; Jensch et al., 1989). There is indirect evidence that the 100% conserved A/B palindromic region, which could form a stem and loop structure (Bernardi et al., 1982; de Zmaroczy et al., 1981) may be involved in hypersuppressivenesss (Zweifel and Fangman, 1991). A study has shown that ρ^- mtDNAs that are generated spontaneously when they lack all or part of GC cluster A have been shown to be temperature sensitive in their ability to be preferentially transmitted (Goursot et al., 1988). This shows that the cluster A is important in the preferential transmission of mtDNA. Therefore, *MGT1* may have an important role in the preferential transmission of HS ρ^- mtDNAs by promoting recombination-dependent replication starting from the A/B palindromic region in the rep sequence.

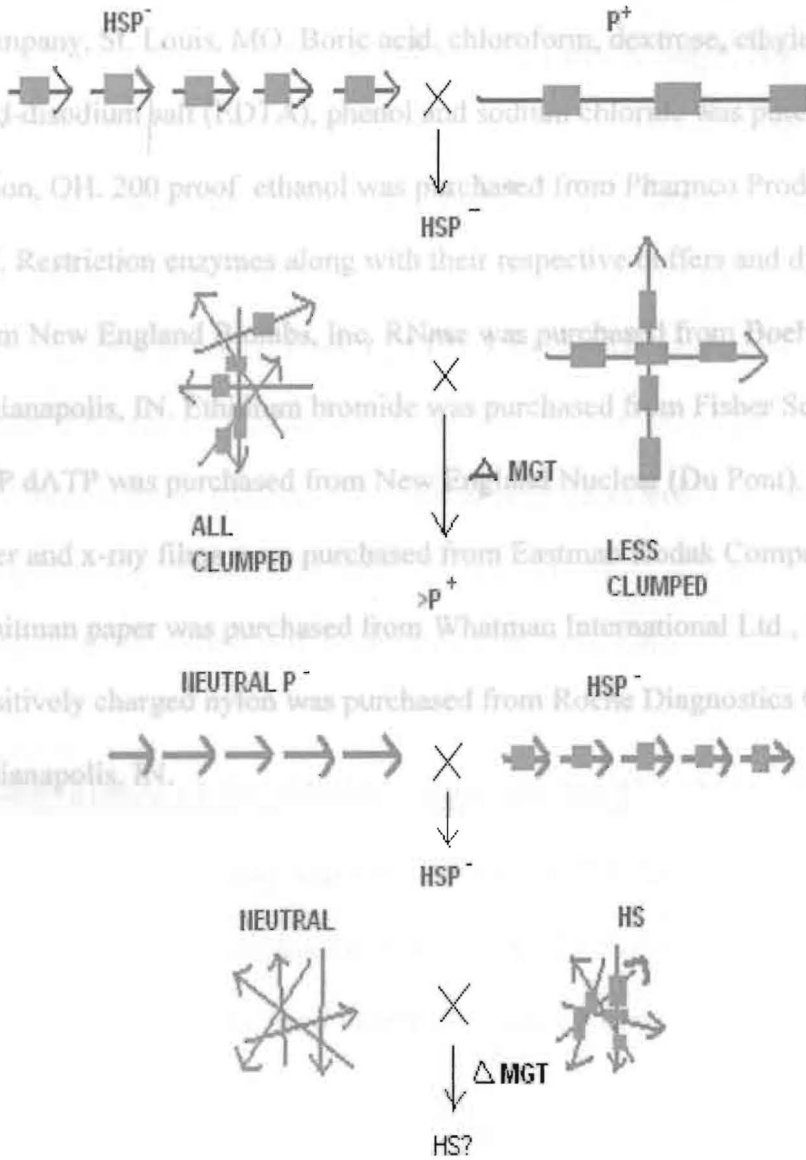
Experimental Design

The inheritance and maintenance of individual mutant mtDNA are dependent on several factors. Two such important factors are the nuclear genes and the DNA sequence that is retained in the mutants. The nuclear gene, *MGT1*, which encodes a recombination junction-resolving endonuclease, has been found to be involved in the inheritance of mtDNA and is required for the preferential transmission of mtDNA deletion mutants (Lockshon et al., 1995). There is preliminary evidence that some mutant mtDNAs may absolutely require this gene for their maintenance while others don't, suggesting that there might be sequence dependent replication in yeast mtDNA (Lorimer-unpublished data).

My project tests the hypothesis which states that the knockout of the *MGT1* gene influences the biased inheritance of the hypersuppressive deletion mutants. This hypothesis states that the head-to-tail repeats of short DNA sequences of hypersuppressive mtDNAs recombine more than the rho^+ genomes, with far less repetitive DNA sequences and, in the absence of *MGT1* gene, the HS mtDNA forms a large, tangled network which reduces the number of mitochondria segregating into daughter cells during cell division. This hypothesis has been tested in matings involving hypersuppressive and wild-type cells. The results have shown that in the absence of *MGT1* gene product, the rho^+ genome is preferentially transmitted to the diploid progeny when rho^+ is crossed to HS rho^- (Lockshon et al., 1995). But hypersuppressive mtDNAs with high density of rep sequences have never been crossed to similar sized neutral rho^- that lack rep sequence. So, if the decrease in the inheritance of HS rho^- mtDNAs in the absence of *MGT1* is due to excessive aggregation compared, to less aggregation as seen

in the larger mitochondrial genome of rho^{+}, then the mtDNAs of a neutral cell should be affected equally by the absence of *MGT1,* as it also has head-to-tail repeats of DNA fragments. In this study, HS mtDNAs will be crossed to a neutral mitochondrial genome having *Δmgt1*. If the matings of *Δmgt1*HS rho^{-} with *Δmgt1*neutral rho^{-} produce substantially lower HS rho^{-} mtDNAs, then we can propose that *MGT1* gene also has rep sequence-specific effect on preferential inheritance. There has already been some evidence for such a sequence-dependent effect. If only one parent has the *MGT1* gene in a mating test, preferential inheritance is greatly reduced no matter which parent has *MGT1*. So, it is likely that rep sequences have some preferential sites for the resolvase and the structure of mtDNA prior to mating may not have an important role in the preferential transmission of the HS genome.

Figure 3. Wild-type (ρ^+) x HS3324 (ρ^-) and N (ρ^-) x HS3324 (ρ) matings in the presence and absence of MGT1. The arrows indicate the mtDNA genome . The solid boxes on the arrows show the rep sequence.


HSP⁻
P⁺
HSP⁻
ALL
CLUMPED
Δ MGT
LESS
CLUMPED
>P⁺
NEUTRAL P⁻
HSP⁻
HSP⁻
NEUTRAL
HS
Δ MGT
HS?

Materials

Agarose, acid-washed glass beads, yeast extract, amino acids, peptone, Tris HCl, lithium acetate and Poloroid film Type 667 were purchased from Sigma Chemical Company, St. Louis, MO. Boric acid, chloroform, dextrose, ethylenediamine-tetraacetic acid-disodium salt (EDTA), phenol and sodium chloride was purchased from Ameresco, Solon, OH. 200 proof ethanol was purchased from Pharmco Products Inc.; Brookfield, CT. Restriction enzymes along with their respective buffers and dNTPs were purchased from New England Biolabs, Inc. RNase was purchased from Boehringer Mannheim, Indianapolis, IN. Ethidium bromide was purchased from Fisher Scientific, Fairlawn, NJ. α^{32}P dATP was purchased from New England Nuclear (Du Pont). GBX developer, GBX fixer and x-ray films were purchased from Eastman Kodak Company, Rochester, NY. A Whitman paper was purchased from Whatman International Ltd., Maidstone, England. Positively charged nylon was purchased from Roche Diagnostics Corporation, Indianapolis, IN.

Methods

Strains and media

All *Saccharomyces cerevisiae* strains used in this study were derived from A364a. The following stains BS127 rho$^+$, BS132 rho$^+$, BS127*Δmgt1*, BS132*Δmgt1*, BS127/HS3324 rho$^-$, BS127/HS3324*Δmgt1*, BS127/23-3 rho$^-$ and BS127/23-3*Δmgt1* rho$^-$ were used in this study. The procedures used to make the following strains BS127*Δmgt1*, BS127/HS3324 rho$^-$, BS127/HS3324*Δmgt1*, BS127/23-3 rho$^-$ and BS127/23-3*Δmgt1* were described in Lockshon et al., 1995. Frozen cells were streaked on YEPD (1% yeast extract, 2% peptone supplemented with 2% dextrose) plates and were incubated at 30°C for 2 days to form colonies. The colonies were transferred into 5ml YEPD liquid media and grown at 30°C for 18-24 hours. Both YEPD plates with colonies and colonies grown in liquid media were stored in the refrigerator until used.

Construction of SS1/HS3324 and SS1/HS82d (Mat α)

SS1/ HS3324 rho$^-$ was made by mating BS132α rho$^+$ with BS127/HS3324a rho$^-$ and SS1/HS82 rho$^-$ was made by mating BS132α rho$^+$ with BS127 for a short period of time as described in the hypersuppression test method (Zweifel and Fangman, 1991). BS132α rho$^+$ and BS127/HS3324 rho$^-$ were grown overnight in YEPD (1% yeast extract, 2% peptone supplemented with 2% dextrose). Each culture of haploid cells was combined with 4ml of sporulation media (1% KAC, .05% dextrose, .10% yeast extract supplemented with .03% ade, arg, his, iso, leu, lys, met, phe, thr, trp, ura, val) and vortexed briefly. Cells were collected onto a 25mm diameter Millipore filter disk and filters were incubated at 30°C for 3 hours on YEPD plates. Filter paper was transferred

from the plate into a tube containing 5ml of sporulation media and incubated at 30°C for 48 hours. Next, the tube with filter paper was vortexed gently and spun at 2k rpm for 5 minutes using a 15ml conical tube. The supernatant was discarded and 100µl of sorbitol buffer was added to the pellet in the tube. One microliter zymolase was added to the tube and allowed to sit for 20 minutes. Cells were diluted by adding 1ml sorbitol and spreaded on trp free plate to make individual colonies. The plates were incubated at 30°C for 48 hours for spore formation. The plates were replica plated onto BS127 specific media (tyr free) and onto glycerol plate. Colonies that did not grow on both tyr free and glycerol plates but grew on the original plate were selected and grown in YEPD liquid media. The cells were DAPI stained and looked at under fluorescence microscopy for the presence of mitochondrial chondriolites. Next, total cellular DNA was isolated by a smash and grab technique and cut with EcoRV. Gel electrophoresis was performed followed by the Southern blot to confirm the correct mitochondrial genome of SS1/HS3324 and SS1/HS82d.

Knockout of *MGT1* gene (Yeast transformation)

The removal of the *MGT1* gene was started by making competent yeast cells. The strains whose *MGT1* gene was later removed were grown overnight in 5ml YEPD. The overnight cells were then used to inoculate 100 ml of YEPD and were allowed to grow until they reached the mid-log phase. Five milliliters of sterile water was added to the cells and cells were pelleted at 5K for 3 minutes. The supernatant was discarded; the pellet was dissolved in 1 ml sterile water and spun at 5K for 5 minutes. The supernatant was removed and the pellet was resuspended in 1.5ml 1xTE-LiAc and transferred into an

eppendorf tube. The cells were pelleted again, the supernatant discarded and the pellet dissolved in 300µl 1xTE-LiAc. Next the cells were rotated at 23°C for 1 hour. While the cells were rotating, 10µl of SSS DNA (salmon sheared sperm DNA) was boiled for 15 minutes at 95°C. The denatured DNA was spun for 5 seconds and cooled on ice for 3 minutes. Four microliter of pΔcce1 was added to the SSS DNA and 50µl of competent cells (cells in 1x-LiAc) were added. Then 300µl of sterile 40% polyethylene glycol solution was added, incubated at 30°C for 30 minutes, heat shocked for 15 minutes at 42°C and spun for 5 seconds. 1 ml YEPD was added to the tube, incubated for another 45 minutes at 30°C and spun for 30 seconds. The supernatant was removed and the pellet was dissolved in 500µl 1XTE. 300µl, 100µl and 50µl of cells were plated on –leu plates and incubated at 30°C. Colonies were grown in liquid –leu and cells were DAPI stained for the presence of the mitochondrial chondrialites. Total cellular DNA was isolated as described below and gel electrophoresis was performed followed by a Southern Blot (Southern, 1975) to ensure the correct removal of the *MGT1* gene.

Hypersuppression Test

The biased inheritance of the rho$^-$ genome was measured by using a modified procedure that has been previously described (Zweifel and Fangman, 1991). Haploid cells were grown in liquid medium until they reached mid-log phase. Rho$^+$ strains were grown in medium containing 3% glycerol (YEPG) while rho$^-$ strains were grown in 2% glucose (YEPD). Each haploid parental strain (3 X 10^6) was combined in 4 ml YEPD and vortexed briefly. Cells were filtered onto 250r 24mm diameter glass fiber filters. The filters were incubated cell side up for 3 hours at 30°C on YEPD plates. Each filter was

put into tube containing 5 ml YEPD, vortexed, sonicated, plated on medium (His free, Trp free, 2% glucose) selective for diploids. The zygotic colonies were replica plated onto YEPD and YEPG plates if the crosses contained a rho^+ parent. Colonies that did not have significant growth on YEPG plate after 3 days were counted as respiration deficient.

DNA Extraction: Small Scale Smash and Grab

Total cellular DNA was isolated using a procedure known as "smash and grab" (adapted from Hoffman and Winston, 1987). The cells were grown overnight and pelleted for 1 minute using Sorvall Biofuge, *pico*. Next, 0.3 ml acid-washed glass beads were added to the pellet. The pellet was suspended in 0.2 ml lysis buffer (2%Triton X-100, 1%SDS, 100mM NaCl, 10mMTris-HCl [pH8.0] and 10mM EDTA) and 0.2 ml PCIA (phenol chloroform isoamyl alcohol) in the ratio of 50% equilibrated phenol, 48% chloroform and 2% isoamyl alcohol. The tube was vortexed using Fisher Vortex Genie 2 until the pellet was dissolved and vortexed 30-second more to break open most of the cells. The sample was centrifuged at 13,000 rpm for 5 minutes causing three layers to be formed in the tube. The top layer had an aqueous portion containing total cellular DNA, a second layer had an opaque white band consisting of cellular debris and the last layer had a pellet of insoluble glass beads. The aqueous layer was transferred to a new tube. The organic layer in the original tube was back extracted by adding 0.2ml 10x TE (100 mM Tris HCl [pH 8.0], 10 mM EDTA), vortexed for 30 seconds and centrifuged at 13,000 rpm for 5 minutes. The aqueous layer was extracted and added to the first aqueous layer. Next, 0.4 ml PCIA was added to the combined aqueous layer, vortexed 30 seconds and centrifuged at 13,000 rpm for 5 minutes. The aqueous layer was transferred to a new

tube. This step was repeated until the white interface was no longer seen. Then, 40 µl of 3M sodium acetate and 1 ml 100% ethanol were added to the aqueous layer and the sample was stored in the freezer for at least 30 minutes. The sample was centrifuged at 13,000 rpm for 10 minutes to pellet the DNA. The DNA pellet was washed with 75% cold ethanol after discarding the supernatant. The pellet was dried using a speed vacuum, (Labconco Centrivap Concentrator) for about 10 minutes using heat. The DNA was dissolved in 20 µl 1x TE and 1 µl Rnase to cut any remaining RNA. The sample was stored in the freezer until needed.

Restriction digest

The DNA isolated from smash and grab was cut with restriction enzyme to visualize the size of the fragments in an agarose gel. Different restriction enzymes were used for different samples. 5 µl of DNA sample was added to a tube that had 2 µl of an appropriate New England Biolabs buffer which is unique to each enzyme and 1 µl of restriction enzyme. The volume of the mixture was brought to 20 µl by adding sterile water. The entire mixture was incubated at 37° C for two hours. The samples that were cut with restriction enzymes were stored in the freezer until used.

Gel Electrophoresis

Gel electrophoresis was performed to visualize the concentration and the integrity of DNA isolated from smash and grab. This procedure was also used to see the size of the DNA fragments cut with restriction enzyme. A 0.8% agarose gel was made using 0.4g agarose, 50ml 1X TBE (Tris, boric acid and EDTA) and 5 µl of ethidium bromide for a

small gel and 1.6g agarose and 200 ml 1X TBE and 12 µl of ethidium bromide for a large gel. Ethidium bromide was used to visualize the bands on a UV box. The gel was poured and run using Fotodyne gel electrophoresis box. The first well in the gel was loaded with 5µl of marker (λ phase DNA cut with HindIII and EcoRI) and other wells were loaded with cut or uncut DNA samples that had DNA tracking dye in them. Small gels were run at 60 volts for 2 hours and the large gels were run at 120 volts for 4 to 5 hours in the presence of 1X TBE buffer and ethidium bromide (10 µl for small gel and 45 µl for large scale). After this time, a gel was visualized on a UV box. A Foto/phoresis I UV box by Fotodyne was used to visualize the small gel whereas Foto/Prep I UV box by the same company was used see the large gel. Finally, a picture was taken with Foto/phoresis I system camera by Fotodyne Inc. using an orange filter.

Southern Blot

DNA from gels was transferred to a positively charged, nylon membrane using a procedure known as a Southern Blot (modified from Southern, 1975).The gel was placed face down in a tray containing about 200ml of ¼ N HCL solution (B1) and rocked for fifteen minutes using platform shaker by New Brunswick Scientific Company, Incorporated. The solution BI removes purine bases. Solution BI was poured off and the step was repeated one more time. Next, about 200 ml of solution BII (1/2 M NaOH, 1 M NaCl) was added to the tray and rocked for 15 minutes (no longer). Solution BI was poured off and the process was repeated one more time. Solution BII denatures the DNA and breaks strands where they have been depurinated. The solution BII was discarded and solution BIII (1/2 M Tris pH 7.5, 3 M NaCl) was poured into the tray. The gel was

rocked for 30 minutes in this solution. The solution BIII was poured off after 30 minutes and the gel was placed face up in the tray. A piece of positively charged nylon membrane was cut to the size of the gel and marked with date and initials on bottom corner with pencil. The membrane was wetted with deionized water and then with solution BIII or 10xSCC. The wet membrane was placed on top of the gel facing down, so that the bottom corner of the membrane was on the bottom corner of the gel. A Whatman 3mm chromatography paper was cut slightly bigger than the gel, wetted with solution BIII and placed on top of the nylon membrane. A stack of paper towels was placed on top of the filter paper, about an inch higher than the dish and a small weight was placed on top of the tray to apply some pressure to the stack. The gel was allowed to sit an overnight and the membrane was cross-linked with UV Crosslinker FB-UVXL-1000 by Fisher Scientific. The blot was hybridized with an appropriate mtDNA sequence. Finally the radioactive probe was used to label the blot. The blot was radiolabeled by random priming with α-^{32}P dATP using the NEBlot kit of New England Biolabs according to manufacturer's recommendations.

Stripping procedure

Blots were stripped of probes by adding boiling 0.1% SDS, shaking until cooled to room temperature and repeating until radioactivity no longer easily detected by a Geiger counter.

Exposing the probe

The plastic-wrapped blot and the film were placed on a cassette in the dark room. The cassette was put in the freezer at -80° C for a few days.

Developing the film

Three different trays, white, red and black were used to develop the film. The Kodak GBX developer and replenisher were added to the white tray, cool water was allowed to run onto the red tray and the black tray was filled with the Kodak GBX fixer and the replenisher. The film was put in the white tray with the developer and rocked gently until the DNA bands were visible under the safe light. The film shouldn't be left in the developer for more than 5 minutes. Next, the film was transferred to the red tray with cool running water and left it immersed in the water for 5 minutes. Then the film was placed in the black tray with replenisher and rocked for 5 minutes, during which film had started turning dark. Finally the film was rinsed with cool water for three minutes and let it air dried.

DAPI (4', 6-diamidino-2-phenylindole) Stain

The DAPI stain is a method that allows seeing the total DNA content of a cell using fluorescence microscopy. The DAPI stain intercalates with the DNA helical structure found both in the nucleus and the mitochondria and fluoresces blue under UV light. Thus, DAPI staining of all yeast strains used in the experiments was done to visualize the presence of mtDNA in the cell. Cells were prepared for DAPI stain using methanol fixation (modified by Dan Drum from Williamson and Fennell, 1979). The

staining procedure was started by placing 500 microliters of an overnight culture in an eppendorf tube and spinning the tube in a centrifuge at 13,000 rpm for 1 minute. The supernatant was discarded and the pellet was suspended in 500 microliter of 100% methanol and centrifuged at 13,000 rpm for 1 minute. The supernatant was discarded and the pellet was suspended in 500 microliter of 1xPBS (phosphate buffered saline) and centrifuged at 13,000 rpm for 1 minute. After discarding the supernatant, this step was repeated two more times. Each sample was suspended in 10 microliters of freshly made DAPI stain mix (10% DAPI and 90% PBS) for ten minutes. Next, each sample was diluted with 500 microliters of 1xPBS and spun at 13,000 rpm for 1 minute. The supernatant was discarded and the pellet was resuspended in 25 microliter of 1xPBS. 10 microliter of each sample was placed onto a slide, and covered with a coverslip that transmits UV light. The slide was viewed under the oil immersion lens of the fluorescent microscope, Olympus Provis, AX70 under UV light.

Results

Confirming the construction of a new strains (*MAT* α):SS1/HS3324 and SS1/ HS82d

A new *S. cerevisiae* mating type (*MAT* α) was created, as both the hypersuppressive and the neutral cells available in Dr. Lorimer's Laboratory belong to a same mating type (*MAT* a). BS132 rho$^+$ (α strain) was mated with BS127/HS3324 (a strain) as described in the hypersuppression test method (Zweifel and Fangman, 1991). The mated cells were plated on trp free plates to form individual colonies. Similarly, BS132 rho$^+$ (α strain) and BS127/HS82d (a strain) were also mated and plated on trp free plates. The resulting plates with colonies were replica plated onto tyr free and glycerol plates. Only those colonies that grew on original plates (trp free) but not on tyr free and glycerol plates were selected and grown in liquid YEPD. Mating tests were then performed to see which of the isolated resulting clonal strains were alpha strains and could mate with a strains. Total cellular DNA was isolated, cut with EcoRV and analyzed by gel electrophoresis (Figure2) and Southern blot (Figure 3) to see the presence of correct mtDNA genome size. MtDNA from BS127/HS3324 and BS127/HS82d strains was cut with EcoRV and was loaded onto the gel as a control for the size and specificity of the mitochondrial probe. The mtDNA genome size of BS127/HS3324 and BS127/HS82d are 963bp and 2.8 kb respectively. In Figure 4, the second lane was loaded with total cellular DNA from SS1/HS82d and showed a fragment whose size was approximately equal to 2.8 kb. This fragment also appeared to have a same size as that of the fragment from control mtDNA (BS127/HS82d) which was loaded on the fourth lane. Similarly, the total cellular mtDNA from five samples of SS1/HS3324 were loaded in

Figure 4. A Gel electrophoresis of total nucleic acid isolated from SS1/HS82d and SS1/HS3324 stained with ethidium bromide. Total cellular DNA from SS1/HS82d and SS1/HS3324 was cut with EcoRV. The size marker, EcoRI/HindIII was loaded in the first lane. There were two samples of SS1/HS82d and five samples of SS1/HS3324 loaded in lanes 2 to 3 and 7 to11, respectively, in the gel. BS127/HS82d and BS127/HS3324 were run in the gel as controls in lanes 4 and 12, respectively.

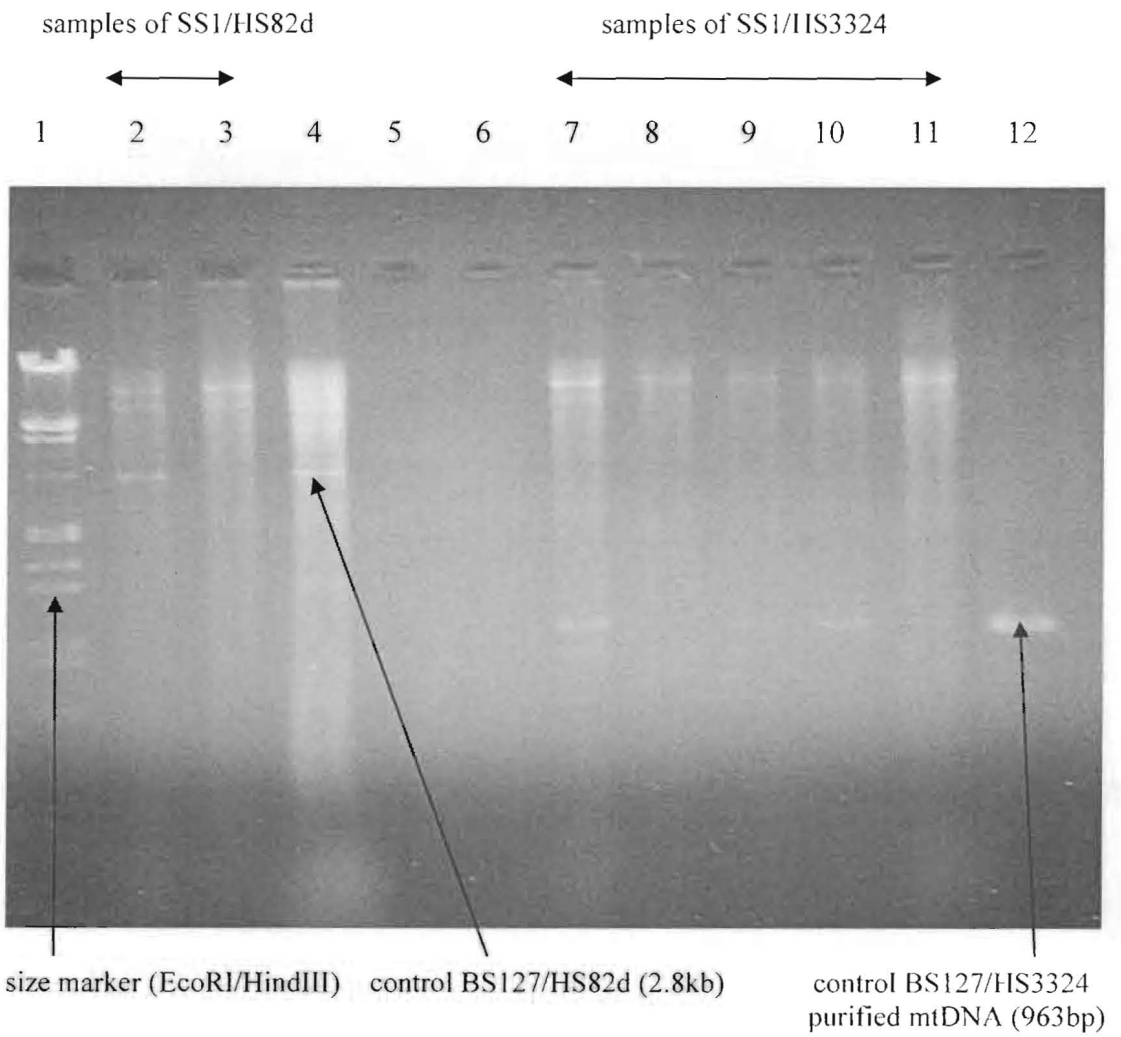
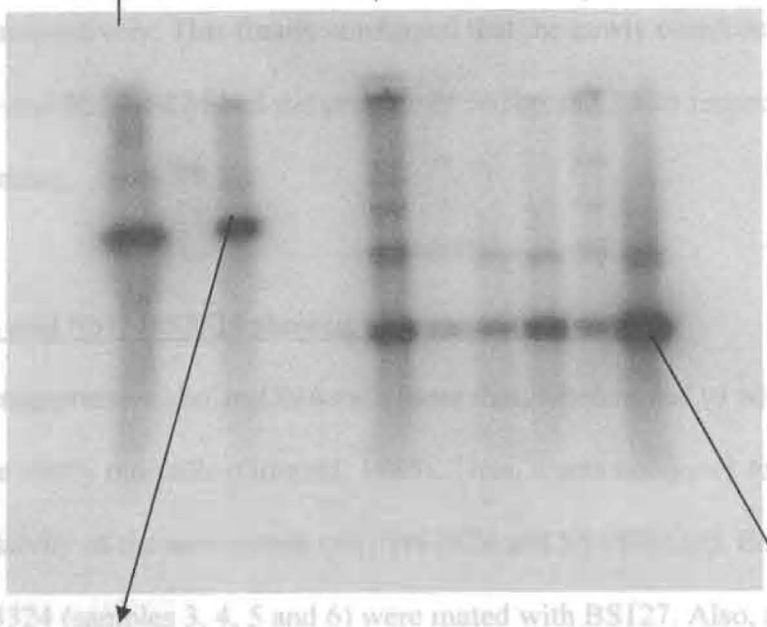

samples of SS1/HS82d
samples of SS1/HS3324
1 2 3 4 5 6 7 8 9 10 11 12
size marker (EcoRI/HindIII)
control BS127/HS82d (2.8kb)
control BS127/HS3324
purified mtDNA (963bp)

Figure 5. Confirmation of new strains SS1/HS3324 and SS1/HS82d. An autoradiogram of a Southern Blot of total nucleic acids isolated form SS1/HS3324 and SS1/HS82d cut with EcoRV and probed with mtDNA of BS127/HS82d and BS127/HS3324, respectively. Samples marked with * show the correct size of the new strains.


SS1/HS82d
SS1/HS3324
control (BS127/HS82d)
3*
4*
5*
6*
7*
control (BS127/HS3324)

lanes 7, 8, 9, 10 and 11. An mtDNA fragment of about 963 bp was observed in each lane. These fragments have the same size as that of the mtDNA fragment from BS127/HS3324 which was run in the gel as a control. Finally, a Southern blot was performed to confirm the correct size of the new strains. The Southern blots were hybridized with an appropriate random primed labeled probe using HS3324 and HS82d mtDNA. It was clearly seen in an autoradiogram (Figure 5) that the probes containing mtDNA from BS127/HS3324 and BS127/HS3324 hybridized to mtDNA from SS1/ HS3324 and SS1/HS82d, respectively. This finally confirmed that the newly constructed strains of SS1/HS3324 and SS1/HS82d had the genome of 963bp and 2.8kb respectively and were the correct strains.

SS1/HS3324 and SS1/ HS3324 showed hypersuppressiveness

Hypersuppressive rho$^-$ mtDNAs are those that, when mated to wild-type rho$^+$ cells, produce >95% rho$^-$ cells (Gingold, 1988). Thus, it was necessary to determine the hypersuppressivity of the new strains (SS1/HS3324 and SS1/HS82d). Both SS1/HS82d and SS1/HS3324 (samples 3, 4, 5 and 6) were mated with BS127. Also, a neutral ρ^-, BS127/23-3 was mated with BS127 ρ^+ to check if BS127/23-3 was still a neutral ρ^-. The mated cells were plated on -his -trp plates to select for zygotes and their diploid clonal colonies. Then the plates were replica plated on YEPD (1% yeast extract, 2% peptone supplemented with 2% dextrose) and YEPG (1% yeast extract, 2% peptone supplemented with 3% glycerol) respectively. The wild-type ρ^+ cells that are able to respire can grow both on YEPD and YEPG, but the mutants ρ^- that are unable to respire can grow only on YEPD by the process called fermentation. Therefore, colonies on each plate were counted

to determine the degree of suppressiveness of SS1/HS3324, SS1/HS82d and BS127/23-3. The suppressiveness was calculated as a percentage of the total zygotic clones that contained almost exclusively ρ^- mtDNA after the mating between the rho$^-$ and wild-type cells (Blanc and Dujon, 1980). In the mating between SS1/HS3324 (#s 3, 4, 5 and 6) and BS127, all of the diploid colonies contained only rho$^-$ (HS3324) mtDNA (Table 1). Similarly, when SS1/HS82d was crossed to BS127, all of the zygotic colonies contained only rho$^-$ (HS82d) mtDNA (Table 1). The inheritance of the rho$^-$ (HS3324) mtDNA was 100% in all these mating. This demonstrated that the new strains (SS1/HS3324 and SS1/HS82d) were indeed

Table1. Biased inheritance in HS ρ^- x wild-type ρ^+ and neutral ρ^- x wild-type ρ^+ mating.

Mating	% Zygotic colonies containing		Suppressiveness (%)
	ρ^+ mtDNA	ρ^- mtDNA	
SS1/HS82d x BS127	0	28	100
SS1/HS3324(#3) x BS127	0	72	100
SS1/HS3324(#4) x BS127	0	69	100
SS1/HS3324(# 5) x BS127	0	65	100
SS1/HS3324(# 6) x BS127	0	32	100
BS127/23-3 x BS132	80	0	0

hypersuppressive and were preferentially inherited when crossed with wild- type (BS127) cells. In BS127/23-3 x BS132 mating, all of the diploid colonies contained only wild-type (BS127) mtDNA. This result confirmed that BS127/23-3 was still a neutral cell because

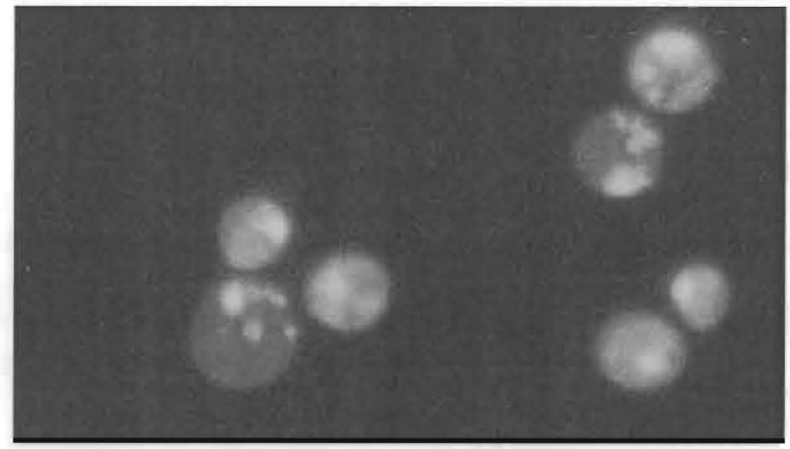
the neutral cells are those rho$^-$ cells that produce <5 % ρ^- progeny when mated to a ρ^+ strain (reviewed in Piskur, 1988).

Deletion of *MGT1* gene in SS1/HS3324, SS1/HS82d and BS127/23-3

The *MGT1* gene has been found to be necessary for the biased transmission of the hypersuppressive rho$^-$ genome. When the *MGT1* mutation was present in only one haploid parent, whether it was rho$^+$ or rho$^-$, the percentage of diploid clones was substantially decreased. When the mutation was carried by both haploid parents, there was a complete switch in the biased transmission of rho$^-$ mtDNA (Lockshon et al., 1995).

A *Δcce*::*LEU2* null mutation was created by using the construct described in Kleff et al., 1992. The *MGT1* open reading frame was deleted and replaced with *LEU2* as a selectable marker in *Δmgt1*, the null alleles of *MGT1* (Lockshon et al., 1995). The samples of SS1/HS3324, SS1/HS82d and BS127/23-3, whose *MGT1* gene was deleted and replaced with *LEU2*, were first plated on –leu plates. If the *MGT1* gene had been successfully replaced with *LEU2*, the cells should grow on –leu plates. The growth on –leu plate indicates the restoration of leucine synthesis pathway in *Δmgt1* cells, as the original cells did not have *LEU2* gene. Thus, the colonies that grew on –leu plates were selected and grown in –leu liquid medium for DAPI stain. The samples of SS1/HS3324*Δmgt1*, SS1/HS82d*Δmgt1* and BS127/23-3*Δmgt1* were DAPI stained and looked under the Fluorescence microscopy for the presence of mtDNA chondriolites. Previous studies have shown that the deletion of *MGT1* caused mtDNAs to aggregate into a few numbers of large chondriolites in BS127/HS3324 and BS127/23-3, as compared to the large number of small chondriolites in *MGT1* rho$^-$ cells (Lockshon et al., 1995). In Figure 6, a few bright chondriolites could be seen in SS1//HS3324*Δmgt1* rho$^-$ cells

Figure 6. Distribution of chondriolites in SS1/HS3324 *Δmgt1* cells. Cells were grown overnight in glucose medium, stained with DAPI and visualized using fluorescence microscope.



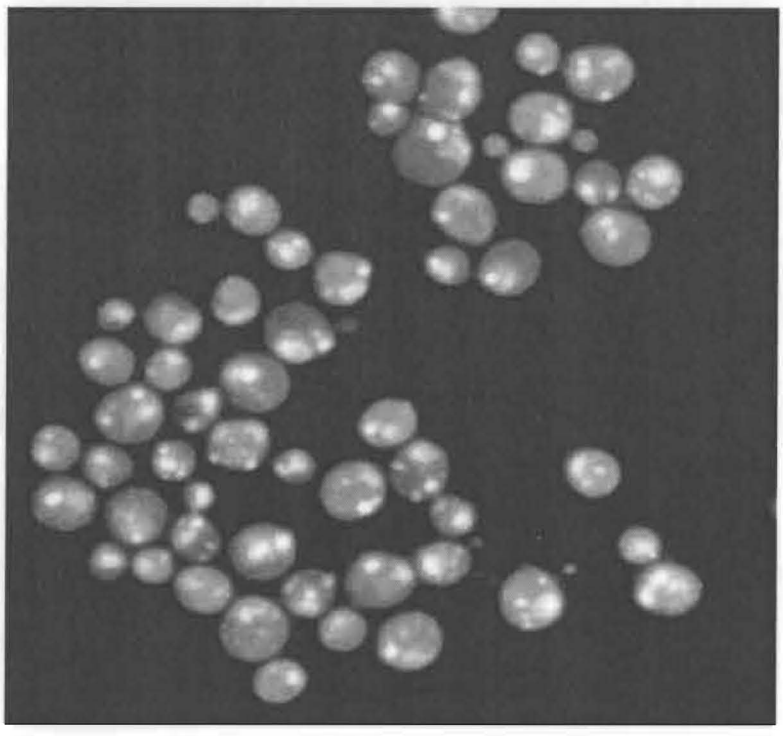
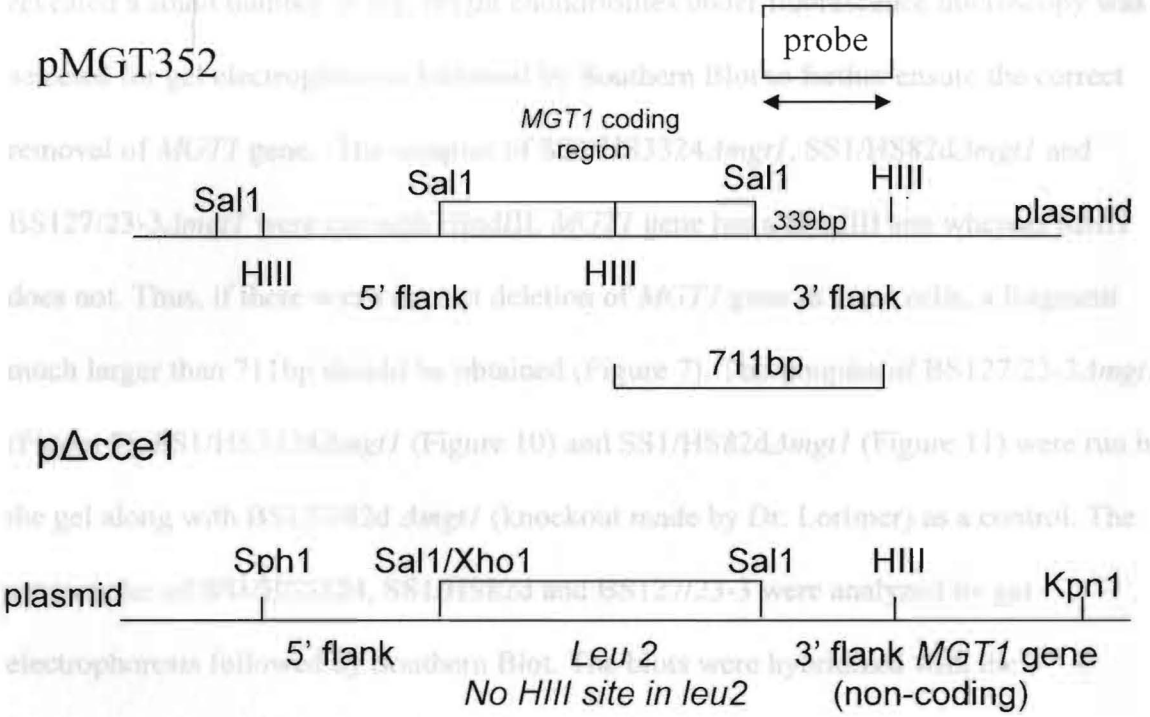


Figure 7. A restriction map of pMGT352 and pΔcce1.

A plasmid, pMGT352 has a *MGT1* gene. The coding region of this gene is replaced with *Leu2* in pΔcce1. The 3' flanking, non-coding region of *MGT1* was used as a probe to determine the correct removal of *MGT1* gene.



Note: HIII stands for HindIII

indicating the correct deletion of *MGT1* gene. Therefore, in this study, each sample which revealed a small number of big, bright chondriolites under fluorescence microscopy was selected for gel electrophoresis followed by Southern Blot to further ensure the correct removal of *MGT1* gene. The samples of SS1/HS3324*Δmgt1*, SS1/HS82d*Δmgt1* and BS127/23-3*Δmgt1* were cut with HindIII. *MGT1* gene has a HindIII site whereas *LEU2* does not. Thus, if there was a correct deletion of *MGT1* gene in these cells, a fragment much larger than 711bp should be obtained (Figure 7). The samples of BS127/23-3*Δmgt1* (Figure 8), SS1/HS3324*Δmgt1* (Figure 10) and SS1/HS82d*Δmgt1* (Figure 11) were run in the gel along with BS127/82d *Δmgt1* (knockout made by Dr. Lorimer) as a control. The cut samples of SS1/HS3324, SS1/HS82d and BS127/23-3 were analyzed by gel electrophoresis followed by Southern Blot. The blots were hybridized with the 3' flanking region of *MGT1*, which served as a probe. The autoradiogram (Figure 9) showed that BS127/23-3 *Δmgt1* (sample # 4, 6, 8 and 9) loaded in lanes 3, 5, 6 and 7 had a correct deletion of *MGT1* gene since they had a mtDNA fragment that is of the same size as shown by BS127/82d *Δmgt1*, which was loaded in lane 15 as a control. Similarly, SS1/HS3324 *Δmgt1* (sample #s 3, 6 and 7) in lanes 12, 13 and 14 revealed that they had a successful removal of *MGT1* gene when its mtDNA band was found to have the same size when compared to the control BS127/82d *Δmgt1* (Figure 10). Also, SS1/HS82d *Δmgt1* had several samples (#s14, 16, 17, 20, 21, 22, 23, 24, 26, 28 and 29) that showed the correct deletion of *MGT1* gene (Figure 11).

Figure 8. A gel electrophoresis of BS127/23-3 *Δmgt1* cells. Total nucleic acids were isolated from BS127/23-3 *Δmgt1* cells and cut with HindIII. Seven samples of BS127/23-3*Δmgt1* (4, 5, 6, 8, 9, 10 and 11) was loaded in the gel and ran for two and half hours. BS127/HS82d*Δmgt1* was also loaded in the gel as a control.

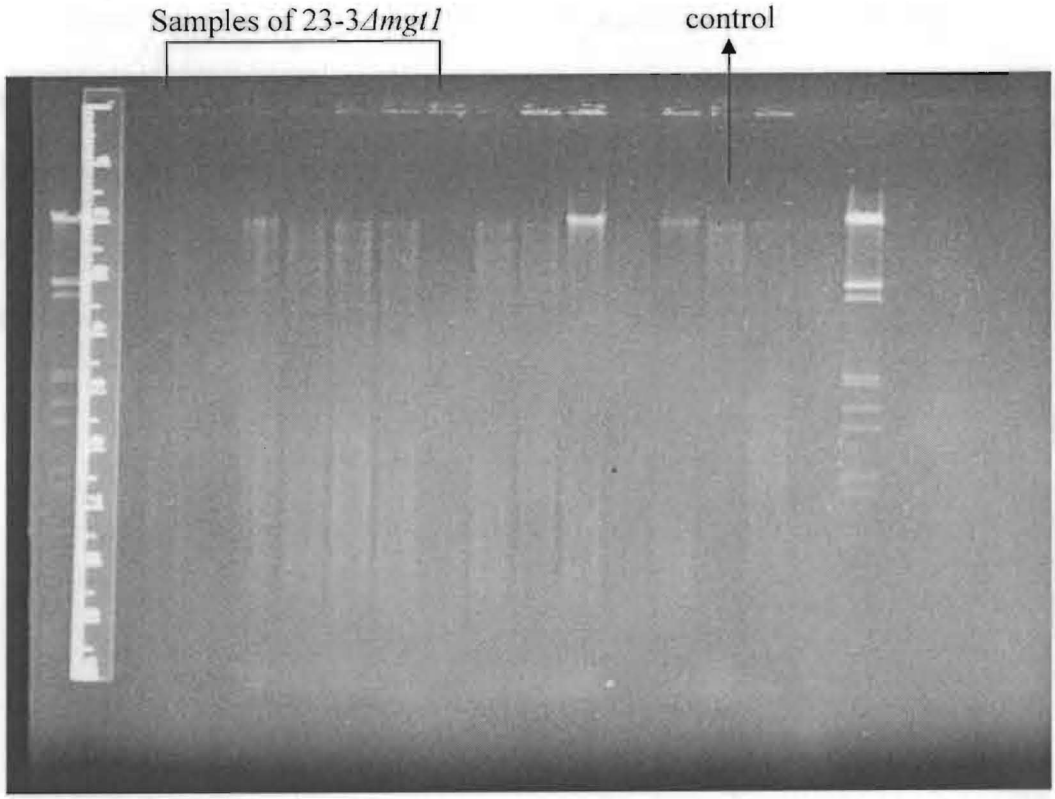

Samples of 23-3Δmgt1
control
4 5 6 8 9 10 11
(samples)

Figure 9. An autoradiogram of BS127/23-3*Δmgt1* cells. Total nucleic acids were isolated from BS127/23-3*Δmgt1* cells, cut with HindIIII, separated by gel electrophoresis, transferred by Southern blotting and probed with 3' flanking region of *MGT1* gene fragment. Seven samples of BS127/23-3*Δmgt1* (4, 5, 6, 8, 9, 10 and 11) were loaded in lanes 3 to 9 respectively. Samples designated with * have the correct deletion of *MGT1* gene.

*4 5 *6 *8*9 10 11 (samples)

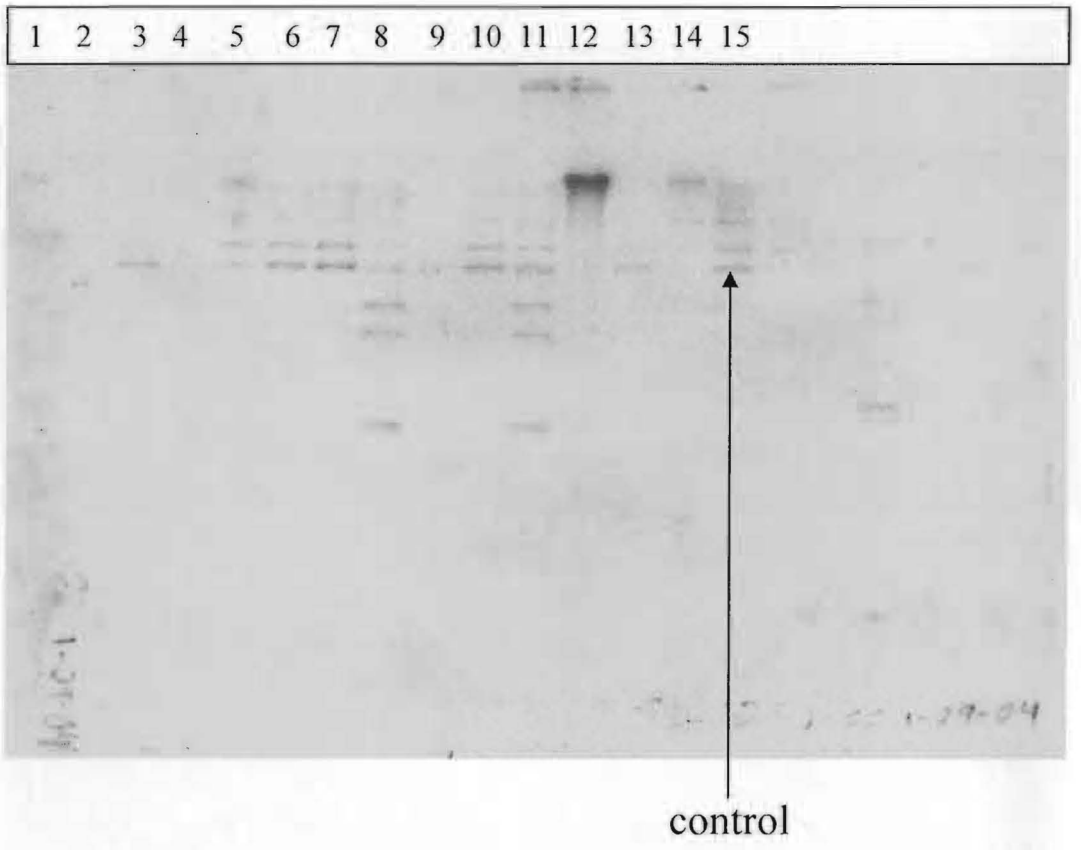


Figure 10. A gel electrophoresis (top) and an autoradiogram (bottom) of SS1/HS3324*Δmgt1* cells. Total nucleic acids were isolated from BS127/HS3324*Δmgt1* cells, cut with HindIIII, separated by gel electrophoresis, transferred by Southern blotting and probed with 3'flanking region of *MGT1* gene fragment. Seven samples of SS1/HS3324*Δmgt1* (1, 2, 3, 6, 7, 8 and 10) were loaded in lanes 10 to 16 respectively. BS127/HS82d*Δmgt1* was loaded in lane 24 as a control. Samples designated with * have the correct deletion of *MGT1* gene.

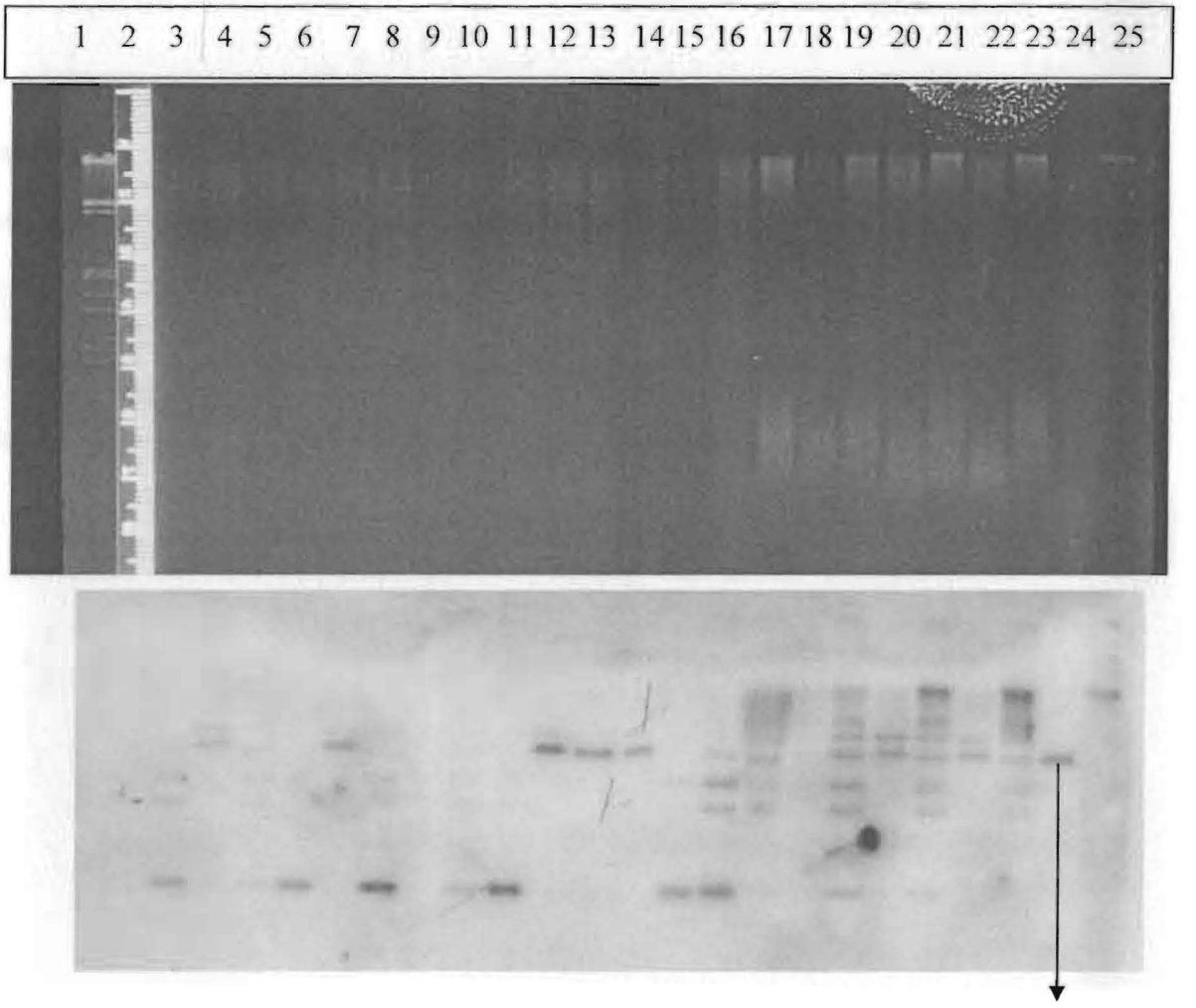
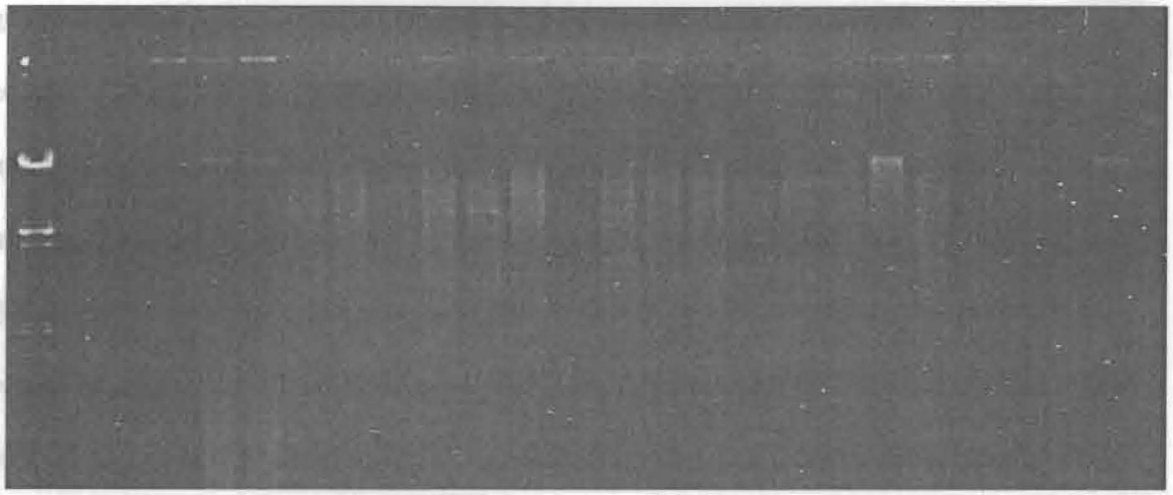
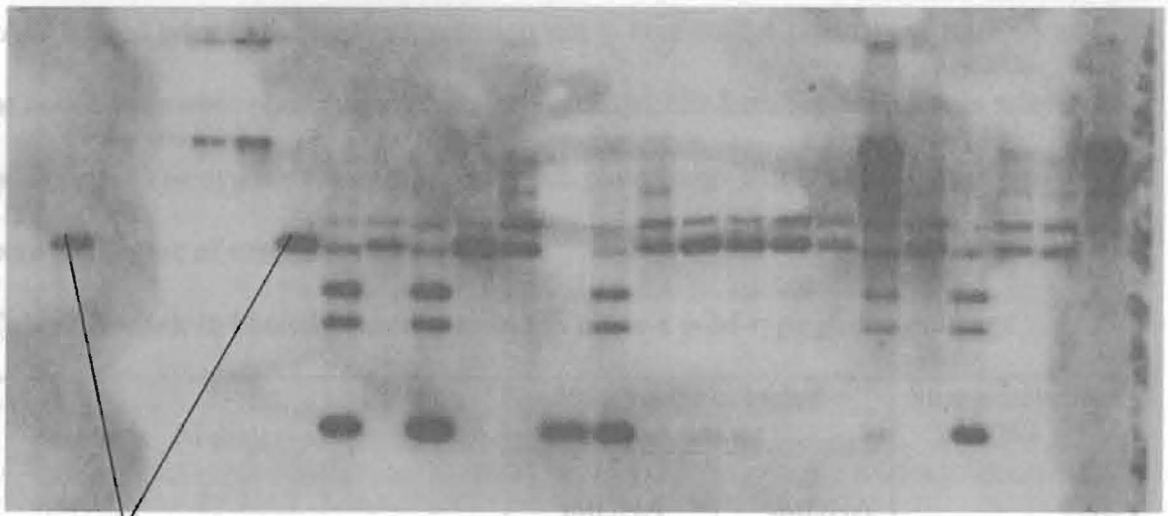

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
1 2 *3 *6 *7 8 10
control

Figure 11. A gel electrophoresis (top) and an autoradiogram (bottom) of SS1/HS82d*Δmgt1* cells. Total nucleic acids were isolated from BS127/HS82d*Δmgt1* cells, cut with HindIIII, separated by gel electrophoresis, transferred by Southern blotting and probed with 3'flanking region of *MGT1* gene fragment. Eighteen samples of SS1/HS82d*Δmgt1* were loaded in lanes 8 to 25, respectively. BS127/HS82d*Δmgt1* was loaded in lanes 2 and 7 as a control. Samples designated with * have the correct deletion of *MGT1* gene.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25





control 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

* * * * * * * * *

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Confirming a switch in the biased inheritance of rho$^-$ mtDNA when SS1/HS82d*Δmgt1* and SS1/HS3324*Δmgt1* were mated with BS127*Δmgt1*

When an *MGT1* mutation was carried by only one parent, whether it was ρ^+ or ρ^-, there was a substantial reduction in the percentage of diploid clones having an HS rho$^-$ genome. On the other hand, when both haploid parents carry the mutation, there was a complete switch in the biased inheritance of ρ^-, and approximately 90% of wild-type mtDNA was inherited in the zygotic clones. Thus, it was necessary to determine if the mating of the newly constructed SS1/HS82d*Δmgt1* and SS1/HS3324*Δmgt1* with BS127*Δmgt1* would yield primarily wild-type mtDNA and the biased inheritance of ρ^- would be reversed. SS1/HS82d*Δmgt1* (#s 17 and 23) and SS1/HS3324*Δmgt1* (#s 3 and 6) were mated to BS127*Δmgt1* using the hypersuppression test method. Each sample was DAPI stained prior to the hypersuppression test to confirm the presence of mtDNA and the mated cells were plated on medium (2% glucose, His free, Trp free) that is selective for diploids. The zygotic colonies were replica plated onto YEPD and YEPG plates to score the degree of suppressiveness.

Table2. Switch in biased inheritance in HS ρ^-Δm x wild-type ρ^+ Δm mating.

Mating	% Zygotic colonies containing:		Suppressiveness (%)
	ρ^+ mtDNA	ρ^- mtDNA	
SS1/HS3324*Δm (#3) x BS127Δm	136	28	17
SS1/HS3324*Δm (#6) x BS127Δm	72	7	9
SS1/HS82dΔm (#17) x BS127Δm	6	0	0
SS1/HS82dΔm (#23) x BS127Δm	25	2	7

Note: Δm is used for *Δmgt1*.
* SS1/HS3324 (#6) from Table 2.

When SS1/HS3324*Δmgt1* (#s 3 and 6) were mated with BS127*Δmgt1*, most of the diploid clones contained rho$^+$ mtDNA (BS127) and the suppressiveness was reduced to 17% and 9% respectively (Table 2) when compared to the 100% suppressivity in the presence of intact *MGT1* (Table 1). Similarly, in the mating of SS1/HS82d*Δmgt1* (#s 17 and 23) x BS127*Δmgt1*, most of the diploid progeny had only wild-type mtDNA (BS127) and the suppressiveness was reduced to 0% and 7% respectively (Table 2) from 100%, which was in the presence of *MGT1* (Table 1). This showed a complete switch in the biased inheritance of rho$^-$ genome when the *MGT1* mutation was carried by both haploid parents and only wild-type mtDNA was inherited.

SS1/HS3324*Δmgt1* x BS127/ 23-3*Δmgt1* produced hypersuppressive cells

The biased inheritance of the hypersuppressive rho$^-$ genome has been shown to be influenced by the deletion of the nuclear gene, *MGT1* (Zweifel and Fangman 1991). The *MGT1* gene product is involved in resolving the Holliday junctions which arise from the recombination of mtDNAs (Kleff et al., 1992). In the absence of *MGT1* gene, the hypersuppressive mtDNAs have been shown to form large, tangled structures, which decrease the number of mtDNA segregating into the daughter cells. The formation of the highly branched mtDNA networks are attributed to the presence of head-to-tail repeats of DNA sequences in the HS genomes that are linked by unresolved Holliday junctions. The deletion of the *MGT1* also had the same effects on a neutral rho$^-$ genome (23-3), which also has head-to-tail repeats of DNA fragments (Lockshon et al., 1995). So it is logical and interesting to look into the outcomes of the crossings involving the

hypersuppressive rho$^-$ mtDNAs with high density of rep sequences to similar sized neutral rho$^-$s that do not have rep sequences in the absence of *MGT1* gene.

The hypersuppressive rho$^-$ SS1/HS3324*Δmgt1* was mated with neutral rho$^-$ BS127/23-3*Δmgt1* by using the modifications of hypersuppression (HS) test method that has been previously described in Zweifel and Fangman, 1991. The mated cells were plated on his free and trp free plates and incubated for three days. The colonies were grown in YEPD liquid medium. There were eighteen samples of SS1/HS3324*Δmgt1* x BS127/ 23-3*Δmgt1* and one sample of each BS127/23-3 and HS3324 were loaded onto the gel. BS127/23-3 and HS3324 were used as controls. The total cellular DNA of each sample and controls were extracted by using "smash and grab" technique. All the samples and controls were cut with DraI and incubated for two and half hours before running in the gel. The samples of SS1/HS3324*Δmgt1* x BS127/ 23-3*Δmgt1* were loaded in lanes 2 to 19, respectively (Figure 12). The controls, BS127/ 23-3 and HS3324 were run in the gel in lanes 20and 21, respectively. The presence of bands can be seen in Figure 12. Thus, to verify whether these bands belong to HS3324 or 23-3 strains, a Southern blot was performed followed by autoradiography. The Southern blot membrane was probed with BS127/HS3324 (Figure 13A). Two prominent mtDNA bands of approximately 1900bp and 950bp could be seen in lane 21 where HS3324 was loaded as a control. The similar bands could be noticed in all the sample lanes of SS1/HS3324*Δmgt1* x 23-3*Δmgt1* except in lane 7. This suggested that cells that were loaded in these lanes showing two mtDNA bands were all hypersuppressive. But these cells, the diploid progeny of SS1/HS3324*Δmgt1* x 23-3*Δmgt1* cross, could also be 23-3 since it takes couple of generations for cells to be homoplasmic. The Southern blot

Figure 12. Gel electrophoresis of total cellular nucleic acid isolated from SS1/HS3324Δm x BS127/23-3Δm. Total cellular DNA from eighteen samples was cut with DraI and loaded onto the gel from lane 2 to 19. Controls, 23-3 and HS3324 were loaded in lanes 20 and 21. Size marker was loaded in the first lane.

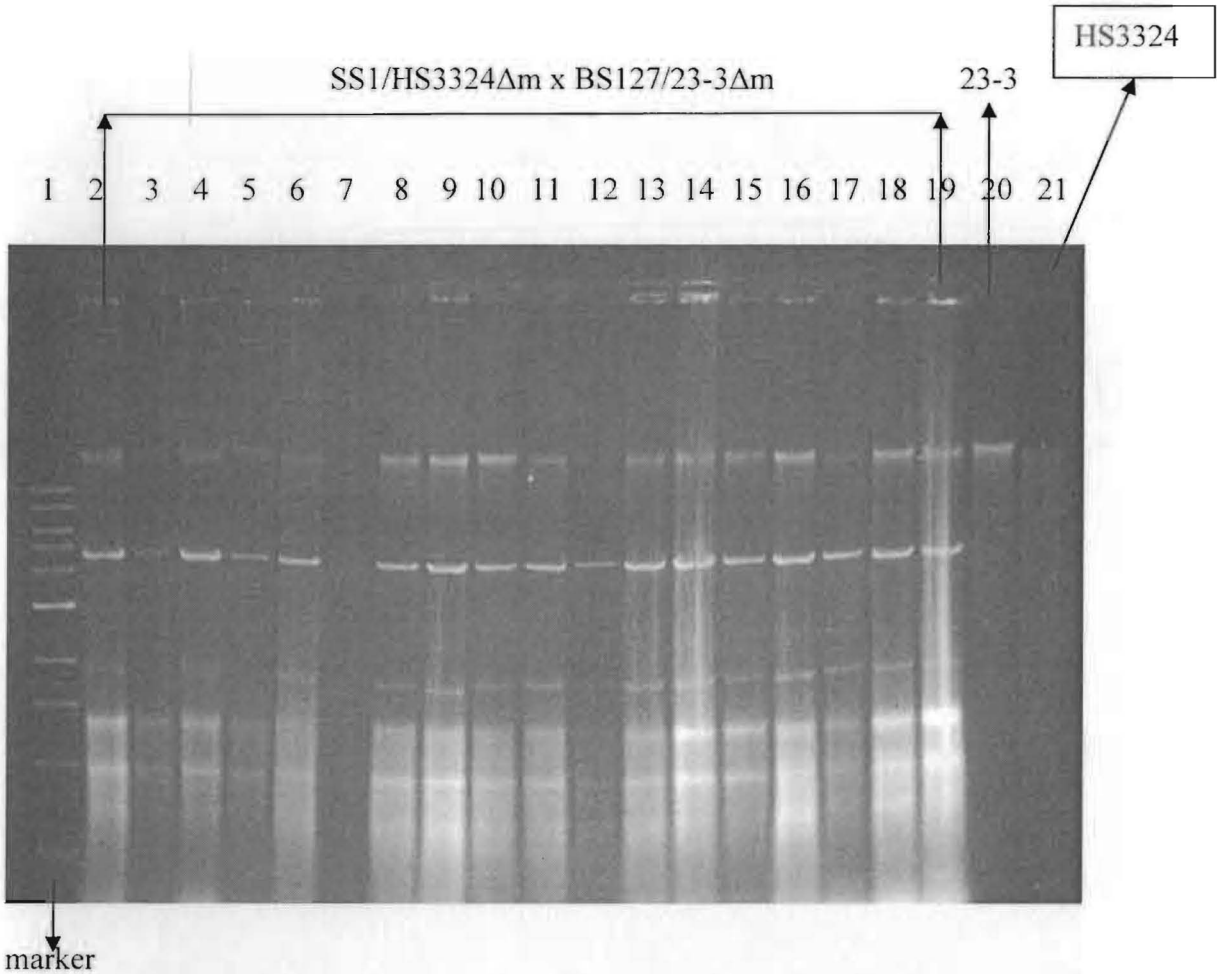
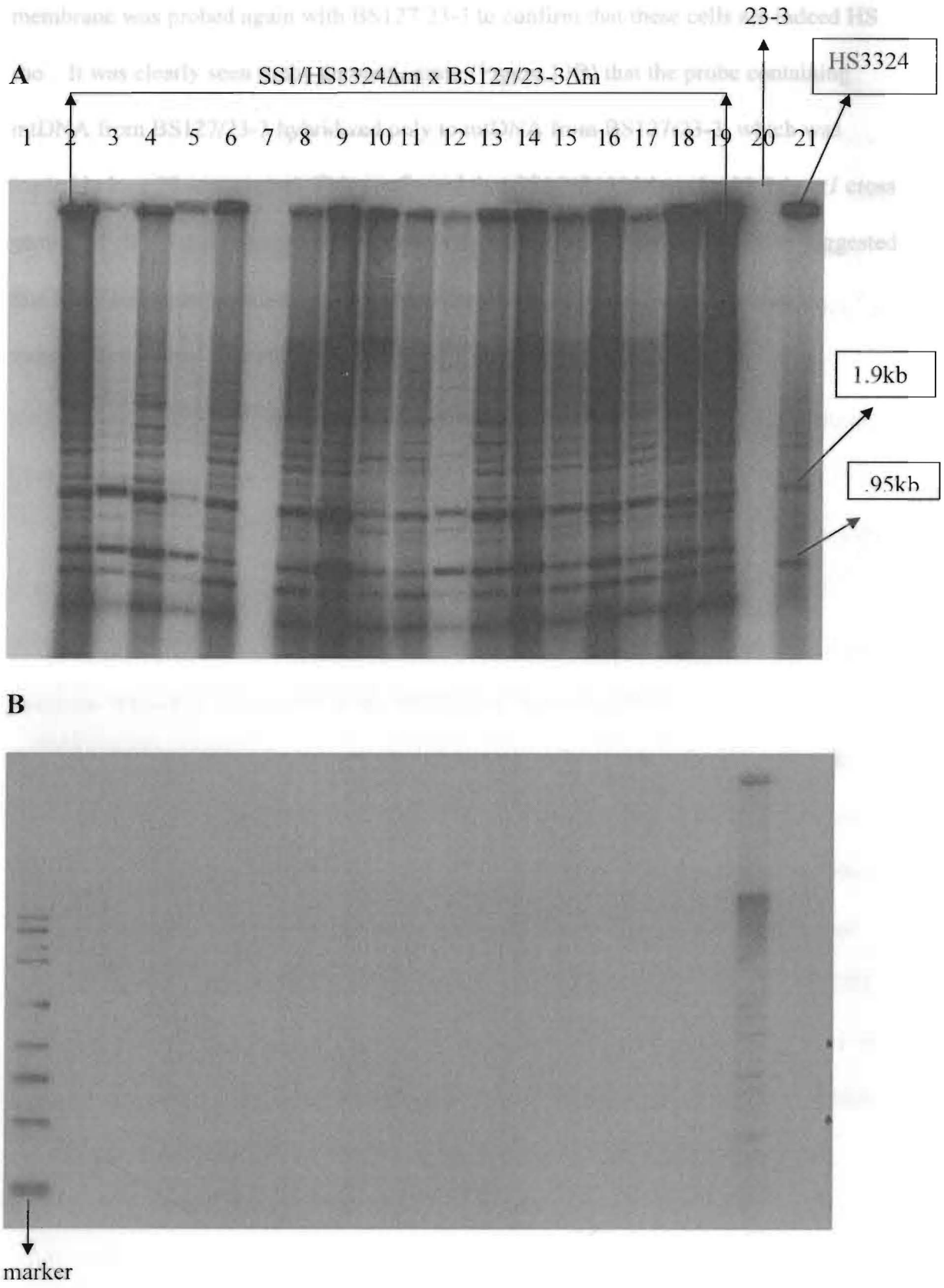

HS3324
SS1/HS3324Δm x BS127/23-3Δm
23-3
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
marker

Figure 13. Autoradiogram of cells that were obtained after SS1/HS3324Δm x BS127/23-3Δm matings. The18 samples of the progeny of SS1/HS3324Δm x BS127/23-3Δm matings were loaded in lanes 2 to19, respectively. Also, N23-3 (lane20) and HS3324 (lane 21) were loaded in the gel as controls. Total nucleic acids were isolated from each sample, cut with DraI and transferred by Southern blotting and probed with HS3324 **(A)** and then stripped and probed with 23-3 mtDNA **(B)**.


A
SS1/HS3324Δm x BS127/23-3Δm
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
23-3
HS3324
1.9kb
.95kb
B
marker

membrane was probed again with BS127/23-3 to confirm that these cells are indeed HS rho$^-$. It was clearly seen in the autoradiogram (Figure 13B) that the probe containing mtDNA from BS127/23-3 hybridized only to mtDNA from BS127/23-3, which was loaded in lane 20 as a control. This confirmed that SS1/HS3324*Δmgt1* x 23-3*Δmgt1* cross generated all HS rho$^-$. The generation of mostly HS rho$^-$ in this experiment also suggested that MGT1 may not have any rep sequence dependent action in the preferential transmission of rho$^-$ genome.

Discussion

The *MGT1* gene is presumed to be associated with replication or a segregation advantage mechanism, which has been attributed to the mechanism for mtDNA genome transmission of *Saccharomyces cerevisiae* (reviewed in Piskur1994) .This gene had been found to be necessary for the biased transmission of the hypersuppressive rho$^-$ genome. When the *MGT1* mutation was present in only one haploid parent, whether it was rho$^+$ or rho$^-$, the percentage of the diploid clones were substantially decreased. When the mutation was carried by both haploid parents, there was a complete switch in the biased transmission of rho$^-$ mtDNA (Lockshon et al., 1995).

The *MGT1* gene product has been shown to resolve Holliday junctions. Lockshon and coworkers demonstrated an increased ratio of linked mtDNA in cells that were without the *MGT1* gene and this was presumed to be caused by the unresolved Holliday junctions. Therefore, the ability of the *MGT1* gene to resolve Holliday junctions and to avoid forming highly branched networks of mtDNA molecules is believed to have an important role in the segregation of mtDNA. It has been thought that the clustering of mitochondrial genomes caused by the unresolved Holliday junctions would reduce the number of heritable units of mtDNA segregating independently in *Δmgt1* cells. It was expected that the reduced number of heritable units of mtDNA in the absence of *MGT1* would be higher in rho$^-$ genomes than in rho$^+$ genomes and this has been attributed to the presence of a high density of tandemly repeated small fragment of rho$^-$ genomes, which is seen to have higher chance in recombining homologous sequences (Lockshon et al., 1995).

Although Lockshon et al. suggested that a change in the biased inheritance in yeast cells is caused by the lack of *MGT1* gene due to unresolved Holliday junctions, there are some rho$^-$ cells that lose all of their mtDNA in the absence of *MGT1*, while others maintain their genomes very well. Different deletion mutants of *S. cerevisiae* mtDNA have been found to vary in their ability to maintain the mitochondrial genome in the absence of *MGT1*. Both hypersuppressives and neutrals have some mtDNA genomes that are maintained in the absence of *MGT1* while others are not maintained so well. This study mated similar sized neutral and hypersuppressive rho$^-$ cells in the absence of *MGT1* and analyzed the result of the mating to determine if there is a possibility of a sequence dependent effect on preferential inheritance in yeast mtDNA. Both hypersuppressive and neutral mtDNAs are small and repetitive, but hypersuppressive contains a rep sequence that neutrals lack. We assume that in the absence of *MGT1*, neutral mtDNAs should be affected equally, like hypersuppressive mtDNAs, as neutral mtDNAs also have head-to-tail repeats of DNA fragments. Thus, the neutral mtDNAs should recombine and form tangled networks of HS mtDNA in the loss of the recombination junction resolvase and fail to migrate into daughter cells in cell division.

In this study, BS132/HS3324*Δmgt1* was mated with BS127/N23-3*Δmgt1*. The mating produced all hypersuppressive rho$^-$ cells. If this experiment had produced a low percent of HS rho$^-$ mtDNAs, then we could have proposed that the *MGT1* gene has a rep sequence-specific effect on preferential inheritance. But our result showed that this is not the case. Why were the diploid progeny all HS and not neutral? Finding a reasonable answer to this question is very difficult. It seems that presence of rep sequence in the genome of BS132/HS3324*Δmgt1* has benefited it in hypersuppressiveness as compared to

BS127/N23-3*Δmgt1*, but the role of rep sequence in this mechanism is puzzling and unclear. MacAlpine et al. had provided strong evidence that priming by promoter for DNA replication is a necessary factor for hypersuppressiveness. They had demonstrated that priming of RNA from a nonanucleotide promoter is a mode of replication in HS rho$^-$ genome. But they could not see any such mode of replication in the neutral rho$^-$ genome (MacAlpine et al., 2001). It is obvious that further study and analysis is required to determine the mechanism behind preferential transmission of the HS rho$^-$ genome in SS1/HS3324*Δmgt1* x BS127/N23-3*Δmgt1* crosses. This was the first study done in Lorimer's Laboratory involving the mating of SS1/HS3324*Δmgt1* with BS127/N23-3*Δmgt1*. Thus, future studies should include repeats of this experiment to determine if the matings of SS1/HS3324*Δmgt1* with BS127/N23-3*Δmgt1* would result in all or mostly hypersuppressive cells. Also, another hypersuppressive cell, SS1/HS82d*Δmgt1* could be crossed to BS127/N23-3*Δmgt1* to check if they would also produce mostly hypersuppressive cells. If all these above mentioned matings produce the same result, mostly hypersuppressive cells, only then can we propose that *MGT1* gene may not have rep sequence-specific effect on preferential inheritance.

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