A Novel Mitochondrial DNA Replication Intermediate in the Yeast *Saccharomyces cerevisiae*

By

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ABSTRACT

Mitochondria are small organelles that function as the biological powerhouses of the cell by acting as the principal site of ATP production in aerobic cells. They contain their own genome (mtDNA) that replicates independently from chromosomal DNA in the nucleus. Several human degenerative diseases including neuropathies, myopathies, diabetes and some cancers have been recently discovered that involve large-scale rearrangements of mtDNA. MtDNA mutations are also the cause of many inherited human diseases such as MELAS, LOHN and MERRF. It has also been proposed that the aging process is influenced by accumulated damage (referred to as common deletions) in mtDNA.

Very little is known concerning the relative transmission of normal or mutant mtDNAs, or for that matter, control of mitochondrial DNA replication itself. In the yeast *Saccharomyces cervisiae,* Mutant yeast mtDNAs that contain deletions termed (ρ-) can create a phenotype called hupersuppressive (HSρ-) that possess a huge replication advantage over wild-type mtDNA and are preferentially inherited. Our studies using 2-D gel DNA electrophoresis to analyze mtDNA replication intermediate structures from both wild-type and mutant strains during log phase growth, when mtDNA production is high, show novel replication intermediates of single stranded and supercoiled mtDNA forms. The current theory of mtDNA replication in yeast is based on a mammalian D loop model (Clayton, 1985). This method is asynchronous asymmetrical replication that does not include single stranded mtDNA or supercoiled DNA forms. From our data we suggest that there are alternative mtDNA replication mechanisms, i.e. Rolling Circle or recombination dependant replication, in yeast, which account for these novel forms.

II. Introduction

Mitochondria

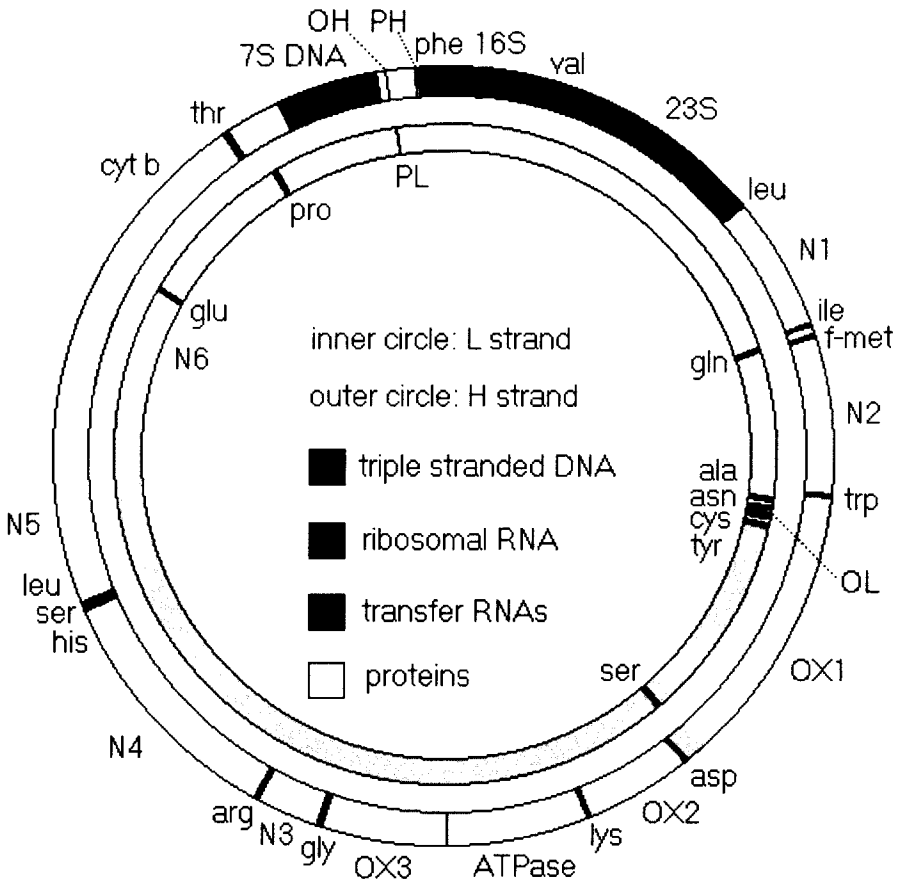
Mitochondria are complex, membrane-bound organelles found in virtually all eukaryotic cells, where they play an essential role in aerobic energy metabolism. They are thought to have evolved from an ancestral endosymbiotic relationship between Eubacteria and Archaea with remnants of each ancestral genome seen today including components for protein synthesis. Yeast contains its own mitochondrial DNA (mtDNA) that replicates autonomously from nuclear DNA and has very high levels of recombination. The mtDNAs of most eukaryotes code for the rRNAs and all of the tRNAs necessary for translation of the thirteen proteins encoded by mtDNA **[Figure 1a]**. While these proteins are necessary for respiration, numerous additional cellular factors necessary for both maintenance of mtDNA and for the metabolic function of the organelle are encoded by the nuclear genome These nuclearly encoded proteins are assembled in the cytoplasm, and transported into the mitochondrion. The theory is that mitochondrial genes have steadily migrated over millions of years from mtDNA to nuclear DNA, leaving only gene products that must be made in the mitochondria in the mitochondrial genome (Margulis and Bermudes, 1985). It appears that transporting RNA (tRNAs and rRNAs) is very difficult to accomplish outside of the mitochondria so they are still encoded by the mtDNA, as are several membrane bound subunits of cyclic oxidaseB (COB) and ATPase.

Yeast mtDNA has a circular genetic map (**Figure 1b**) but exists as both unit (or multi-unit) circles, as well as heterogeneous linear molecules. It is approximately 80kb and has very active recombination and very complicated segregation. (i.e.) individual

cells have one pure genome type but its origin can be from either parent or a recombinant through a poorly understood segregation mechanism (Piskur, 1994; Piskur, 2001). In mammalian cells, the mtDNA genome consists of a 16.6kb closed circular dsDNA molecule. It has no detectable recombination and its inheritance is strictly maternal (Harding et al., 1992).

Mutations in mtDNA cause an assortment of degenerative neurological diseases in humans, most of which are caused by large-scale rearrangements of mtDNA or point mutations in mtDNA. Many degenerative diseases and age related diseases have been associated with these changes in mtDNA such as cancer, Parkinson's disease, Alzheimer's, and late-onset diabetes mellitus (Wallace et al., 1992). Mitochondrial DNA damage has also been linked to degenerative problems and is associated with the aging process itself. MtDNA mutations increase with age and inherited mutations tend to become worse with successive generations (Wallace, 1999). This may be caused by mtDNA mutations increasing the oxidative stress on cells. A reduction in respiration activity has been reported due to oxygen free radical damage to mtDNA, which in turn results in free radical damage in other areas of the cell and nuclear DNA (Davidson and Schiestl, 2001). There is also a correlation between the incidence of age-related cancers and the preferential replication of mutant mtDNA genomes over full-size mitochondrial genomes (Wallace et al., 1999). Several other inherited human diseases are caused by mtDNA mutations such as mitochondrial encephalitic lactic acidosis syndrome (MELAS), myoclonic epilepsy and ragged red fibers (MERF), and Leber's optic neuropathy (LON). These diseases all tend to increase in severity with age (Wallace et al., 1998).

A


OH
PH
7S DNA
phe
16S
val
23S
leu
N1
ile
f-met
N2
trp
OL
OX1
ser
asp
OX2
lys
ATPase
OX3
gly
N3
arg
N4
his
ser
leu
N5
glu
N6
thr
cyt b
pro
PL
gln
ala
asn
cys
tyr
inner circle: L strand
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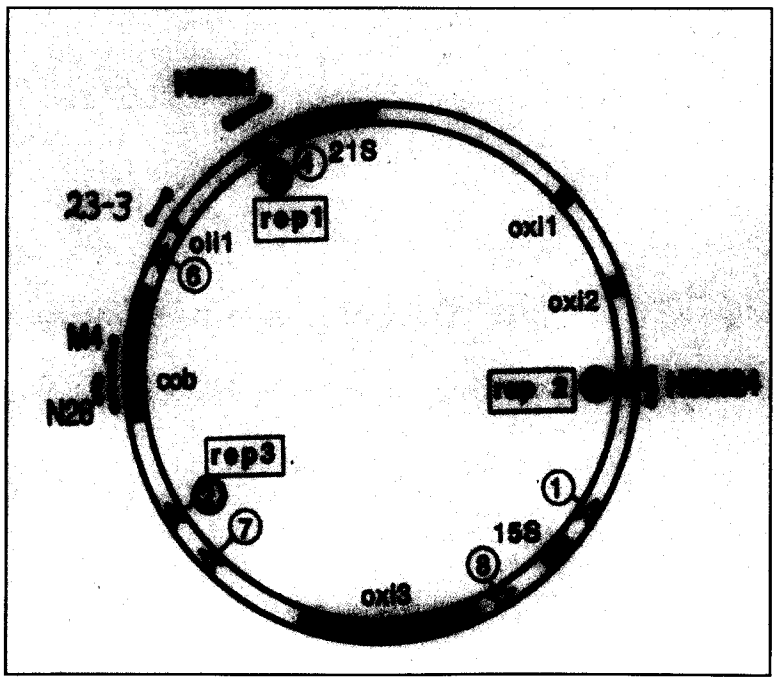
triple stranded DNA
ribosomal RNA
transfer RNAs
proteins

B


21S
rep1
oxi1
oli1
oxi2
cob
rep 2
rep3
15S
oxi3

Yeast as A Model System

The yeast *Saccharomyces cerevisiae* is an excellent system for genetic analysis of mtDNA because it is a facultative anaerobe. Therefore, as yeast prefers to grow anaerobically, cells with defective mitochondrial genomes, or even lacking mtDNA, will continue to grow well on a fermentable carbon source such as glucose. Mutants in yeast mtDNA arise both spontaneously and by mutagenisis and are incapable of respiration typically due to large deletions in the wild type mtDNA (Bugeja et al., 1989).

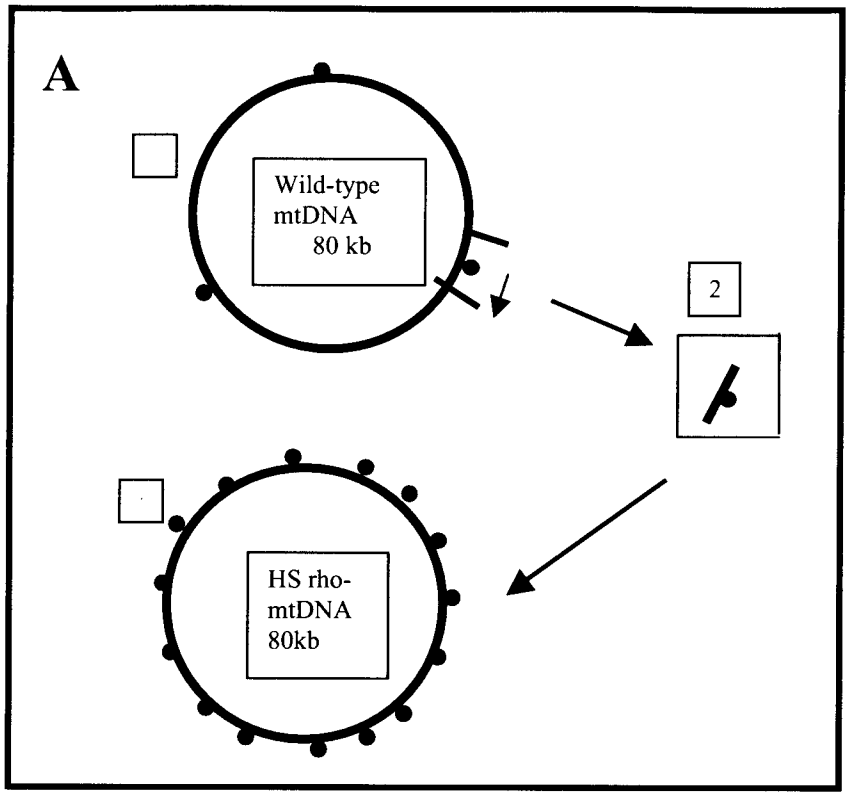
By using the power of yeast genetics, it is possible to observe abnormal mtDNA in a living system. It has been a model genetic organism for decades and many powerful systems of mutants are available for use. Homologous recombination occurs readily in yeast and allows for easy elimination/replacement of nuclear genes. In addition, the yeast chromosomal genome was the first eukaryote sequenced in its entirety in 1996. And thus, the yeast *Saccharomyces cerevisiae* provides us with a model system for studying mtDNA functions. With a better understanding of the mechanisms of mtDNA replication in normal and abnormal conditions in yeast, it may be possible to discover the mechanisms and causes of mitochondrial diseases in humans, and possibly some of the degenerative changes associated with aging in humans.

When yeast cells are plated on solid media several types of colonies will form. The majority of the colonies will become large (grande), can respire and contain wild type ($\rho+$) mtDNAs. Respiratory-deficient deletion mutants of mtDNA arise spontaneously at a rate of 1-2% and are referred to as petities or (ρ^-) mutants (Bugeja et al., 1989). These petite strains contain mtDNA that have sustained massive deletions and

only a small fragment of the ~80-kb mtDNA genome is retained which is referred to as the Rep sequence or ori. The remaining DNA (Rep sequence) is amplified so that the mass of mtDNA in the petite genome is equivalent to that in a wild-type cell **[Figure2a]**. These petites or ρ- genomes exhibit unusual inheritance patterns when they are mated to ρ+ cells. Some ρ- mutants give rise to diploid progeny composed almost exclusively of ρ- when crossed with cells containing ρ^+ mtDNA. Others produce zygotes that grow into colonies that are almost entirely ρ+. There is a wide range of ρ- mtDNA inheritance bias between these two extremes. The percent of the zygote-derived colonies that are ρ- is referred to as suppressivenees. The suppressivenees of a ρ- is an inherent characteristic of each of each ρ- genome. Mutants that produce <10% ρ- diploid progeny (<10% supressivity) are referred to as Neutral ρ-. Other ρ- mutants produce >90% ρ- diploid progeny (90% supressivity). These mutants, termed hypersuppressive (HS) ρ- mutants are thought to present a replicative and/or segregation advantage over ρ^+ DNA. Superficially, this seemed to be due to an amplification of replication origins. However, ρ- mtDNA can come from virtually all over the wild type genome, which would imply that multitudes of replication origins (oris) exist. Additionally, Neutral ρ- replicate well, but do not preferentially pass on their genomes when mated to ρ+ cells. While the basis for this biased inheritance is still unclear, molecular characterization of HS genomes has provided important insights.

The Standard Mammalian mtDNA Replication Model

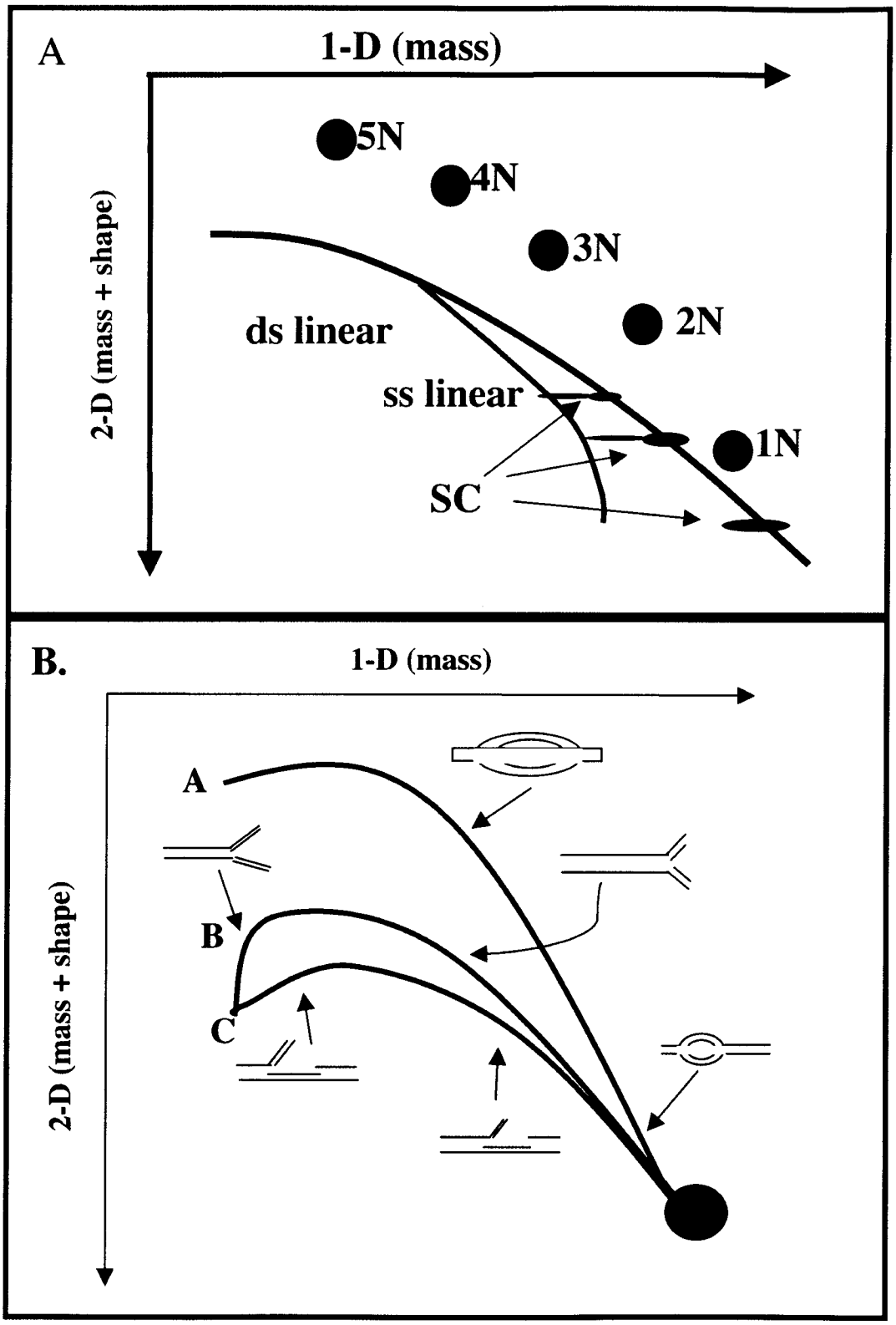
MtDNA replication mechanisms in *S. cerevisiae* are less well understood than those of mammals probably due to yeast developing alternative ways of replication. Many assumptions of *S. cerevisiae* mtDNA replication are directly based on the


A
Wild-type
mtDNA
80 kb
2
HS rho-
mtDNA
80kb

Mammalian mtDNA replication, which may or may not be relevant to *S. cerevisiae* mtDNA replication. It is also important to include that the majority of work in mammals has been performed in a highly purified, biochemical *in vitro* system that may or may not Accurately reflect the *in vivo* replication of either mammal or yeast.

Early progress in understanding the nature of mtDNA replication in mammals was based on several important technical achievements. The first was examination of mtDNA molecules by electron microscopy (EM), most of which contained a persistent displacement loop of roughly uniform size comprising less than 1/10 the diameter of the circular molecule (Kasamatsu et al., 1971). The second was the use of alkaline CsCl density gradients to separate the two complementary strands of mtDNA based on their differing (G/C) content (Kasamatsu et al., 1971) and the use of EtBr/CsCl gradients to separate different replicative intermediates (Berk and Clayton, 1974), the nature of each was confirmed by EM. The third was recognition of distinct thymidine kinases (TKs) in the nucleus and mitochondrion (Berk and Clayton, 1973). Cells lacking the nuclear TK enzyme will incorporate radiolabeled thymidine almost exclusively into mtDNA, forming the basis for a series of critical *in vivo* pulse-labeling experiments.

The fourth technique that has proven invaluable to the field of DNA replication is 2-D neutral/neutral gel-electrophoresis. This technique allows for the separation of restriction fragments on the basis of size (MW) in one dimension and on the basis of shape in the other dimension **[Figure 3a]**. The fragment of interest is then identified by Southern hybridization and random-primed labeling with a complementary strand. The primary forms of replicative intermediate identifiable this way are bubbles that form at origins of replication in this fragment; replication forks, which pass through the fragment;


A
1-D (mass)
2-D (mass + shape)
5N
4N
3N
2N
1N
ds linear
ss linear
SC
B.
1-D (mass)
2-D (mass + shape)
A
B
C

for forks moving in opposite directions which converge in the fragment (Brewer and Fangman, 1987) **[Figure 3b]**.

The three common apparent replicative intermediates separated by EtBr/CsCl centrifugation were shown by EM to be closed circular molecules with a loop of ssDNA forming a D-loop of roughly uniform size (lower band). Others had expanded D-loops varying in size but larger than the uniform D-loops (intermediate band). The third common form found was gapped circular molecules (upper band). After *in vitro* pulse labeling of mtDNA with [^{3}H]-thymidine, D-loop intermediates were shown to have incorporated label predominantly into the H-strand while gapped circular molecules contained label almost exclusively in the L-strand (Berk and Clayton, 1974). These data support a model in which replication is initiated by (heavy) H-strand synthesis, which proceeds a considerable distance around the circular molecule before initiation of (light) L-strand synthesis in the opposite direction, accounting for the predominance of H-strand labeling in D-loop intermediates. Upon completion of H-strand synthesis but well before completion of L-strand synthesis, the daughter molecules separate, accounting for the predominance of L-strand labeling in the resulting gapped circular molecules. After separation, synthesis of the daughter L-strand is completed and both molecules are ligated into covalently closed circles ((Berk and Clayton, 1976)). Detailed electron microscopic evidence for simultaneous leading- and lagging-strand synthesis existed at the time (Wolstenholme et al., 1974) but was dismissed for lack of biochemical support (Clayton, 1982).

The uniformity, stability, and persistence of the small D-loops and the ability to separate these species from parental strands by denaturation permitted mapping of the

origin of heavy strand replication (O_H). It was believed that these short H-strands (isolated as a 7S species on sucrose gradients) represented the stable remnants of abortive replication initiation at O_H so that, unlike the closed circular parental strands, 7S DNA would have free 5' and 3' ends, which could be preferentially end-labeled. Isolation of the denatured 7S strands on sequencing gels showed that they consisted of several discrete species ranging in size from 500-580nts in the mouse and 555-61nts in humans (Gillum and Clayton, 1978). After digestion with *HaeIII*, the 5' ends of the mouse strands were shown to be of uniform length, with the observed heterogeneity laying at the 3' end. Maxam and Gilbert sequencing of the 5' end of the molecules indicated a rough homogeneity and also identified a narrow window for the initiation of H-strand replication **[Figure 4a]**.

A related strategy allowed for the identification of the light strand origin of replication (O_L) in mouse cells, this time using long-labeled mtDNA isolated from the mid-region of the density gradient, where nascent L-strand synthesis has begun in some molecules. Recovered mtDNA was restriction enzyme digested followed by S1 nuclease, to remove any region of single-strandedness remaining after restriction. These digests were compared with restriction-digested non-replicative mtDNA and a novel species was mapped to a region of approximately 50nts, located between the genes for $tRNA^{Asn}$ and $tRNA^{Cys}$, which was sequenced as part of a 340bp restriction fragment spanning the putative O_L by the method of Maxam and Gilbert (Martens and Clayton, 1979). Inspection of this sequence identified an extended region of dyad symmetry with the potential to form a prominent stem-loop structure at precisely the point identified as O_L. This observation coincided with EM data showing the L-strand transcription does not

begin until H-strand synthesis passes O_L. Displacement of the parental H-strand by daughter H-strand synthesis provides the ideal opportunity for formation of single-stranded secondary structure by the template H-strand in the region of O_L, implicating this region in the initiation of L-strand synthesis. In this way, synthesis of the daughter H-strand through the region of O_L serves as the initiating event for L-strand replication, coupling replication of both strands to a single event: productive initiation of H-strand replication.

Now that the origins had been identified, the focus shifted to identifying the mechanisms responsible for strand initiation at O_H and O_L. The stark differences between initiation at O_H (initiates by presumed unwinding of duplex parental strands) and at O_L (initiates from a displaced, single-stranded template with probable high-order secondary structure) suggested different mechanisms at these two origins. Alkali-labile sites had been identified throughout the mtDNA molecule, but especially in the vicinity of O_H and O_L, suggesting a role for ribonucleotide primer at both origins (Brennicke and Clayton, 1981) and (Martens and Clayton, 1979). Publication of the human (Anderson et al., 1981) and mouse (Bibb et al., 1981) mtDNA sequences and identification of conserved coding regions showed that the only significant stretch of non-coding mtDNA was a 1kb segment encompassing the D-loop region between the genes for $tRNA^{Phe}$ and $tRNA^{Pro}$, making this the logical site not just for initiation of replication, but also of transcription, perhaps for both strands.

Development of an *in vitro* transcription system based on purified human KB cell mitochondrial extracts (Walberg and Clayton, 1983) permitted the identification of both the H- and L-strand promoters and transcription start sites by deletion mapping and

primer extension (Chang and Clayton, 1984). The major mtDNA molecular form they found was a stable three-stranded DNA structure that contains a displacement loop (D-loop).

This D-loop region occurs within the larger non-coding region referred to as the "D-loop regulatory region" which contains both heavy and light strand promoters separated by less than 150bps and the O_H which makes this the control site for mtDNA replication and transcription. The light strand promoter (LSP) was located upstream of O_H revealed the possibility that truncated L-strand transcripts act as primers for H-strand DNA synthesis, either by regulated interruption of transcription or by processing of longer transcripts by an endo- or exo-ribonuclease. Transcripts initiated at the L-strand promoter (LSP) are *templated* by the L-strand; the sequence of the so-called "L-strand transcript" is the ribonucleotide equivalent of the H-strand DNA sequence. Further *in vivo* studies using primer extension before and after DNase or RNase digestion showed strong evidence of covalent linkages between RNAs initiating at the LSP and nascent H-strand DNA (Chang et al., 1985). The transition between RNA and DNA was localized to a window of 90bp containing three conserved sequence blocks (CSBs), suggesting that these CSBs might be cis-acting regulatory elements controlling the RNA-DNA transition **[Figure 4b]**.

Biochemical fractionation of purified mouse mitochondria identified an extract capable of processing longer, O_H-containing L-strand transcripts into shorter RNAs suitable for H-strand DNA priming at the observed RNA-DNA transition site, O_H (Chang and Clayton, 1987a). Description of a RNA component in this ribonuclease complex (Chang and Clayton, 1987b), cloning of the nuclear-encoded RNA moiety (Chang and

Clayton, 1987a), and detailed purification and biochemical characterization of the RNase MRP (mitochondrial RNA processing) complex showed that it cleaves L-strand transcripts at precisely the points at which RNA-DNA transitions had been observed previously (Lee and Clayton, 1997) **[Figure 4c]**.

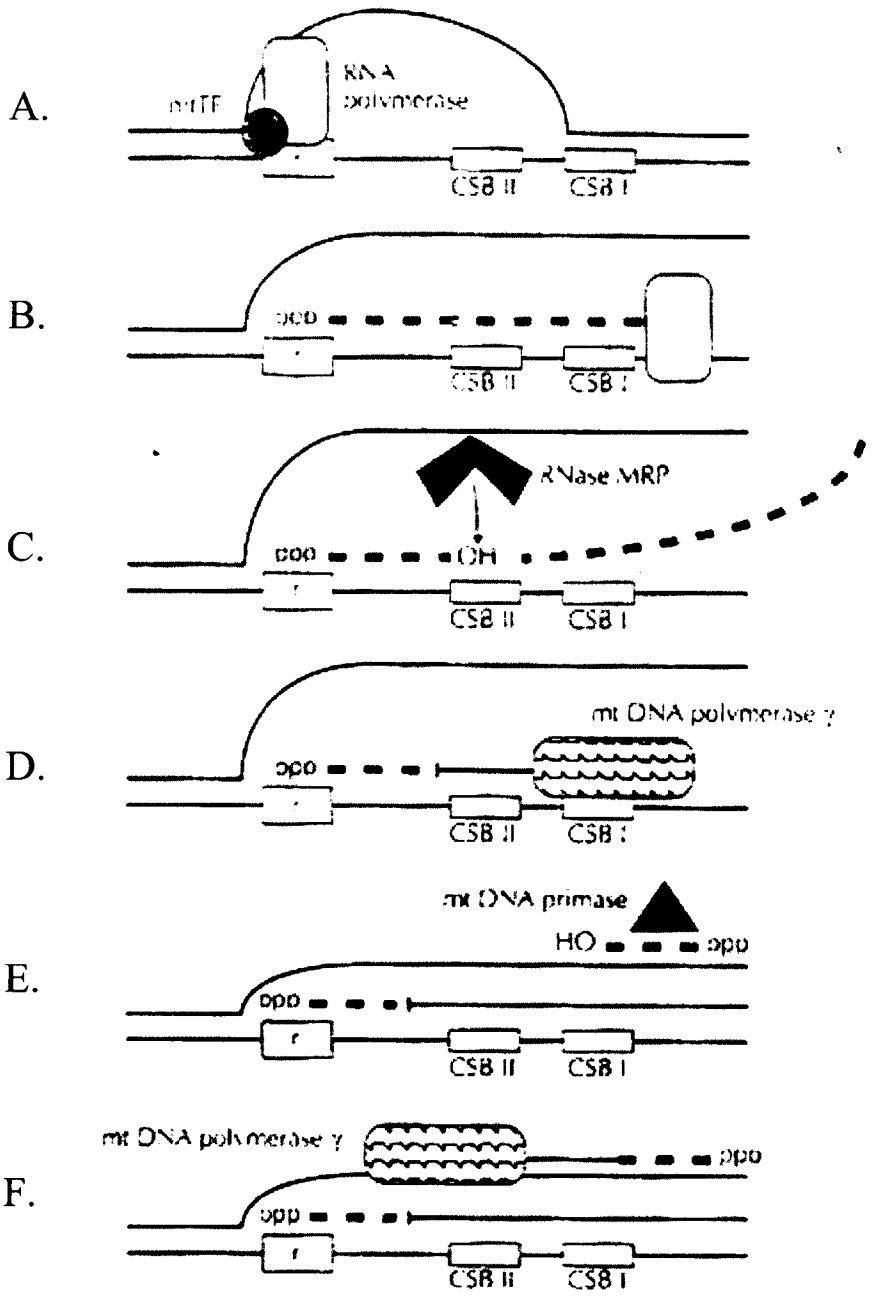
The RNase MRP holoenzyme has a number of interesting and unusual characteristics, although the details of how it chooses a particular transcript for processing into primer remain elusive. In particular, its preferred substrate is a specialized and unusually stable triple-helix consisting of the two parental mtDNA strands and the L-strand transcript undergoing processing (Lee and Clayton, 1998). Furthermore, the same enzyme seems to be involved in the processing of 5.8S rRNA in the nucleus (Schmitt and Clayton, 1993). The RNA moiety is ~270 nucleotides long and contains a decamer sequence identical to a conserved sequence adjacent to the cleavage site for RNase MRP (Chang and Clayton, 1989), presumably involved in targeting the enzyme complex to its precise substrate. Finally, identification of the complete subunit composition of the homologous RNase MRP holoenzyme in *S. cerevisiae* shows that it contains nine subunits, eight of which it shares in common with the nuclear enzyme RNase P (Chamberlain et al., 1998), an enzyme responsible for post-transcriptional processing of nucleus-encoded tRNAs. BLAST searches of the protein and six-way translated nucleotide databases at GenBank reveal putative human homologues for most of these subunits, although the related genes have yet to be cloned in any mammal.

Priming at O_L has more in common with nuclear DNA synthesis than with the type of priming found at O_H. The most important similarity is the L-strand DNA synthesis proceeds from a short RNA primer laid down by a putative primase specialized

for this function **[Figure 4e]**. The activity of this primase was examined *in vitro* using fractionated extracts from human epidermal carcinoma KB cells and was shown to initiate DNA synthesis at the recognized origin of L-strand mtDNA replication by polymerization of RNA primers about 10nts long (Wong and Clayton, 1985a). The reaction required an ssDNA template and was absolutely dependent on the presence of rNTPs in addition to dNTPs (Wong and Clayton, 1985a). Further characterization *in vitro* showed that replication initiation on a cloned ssDNA containing the region surrounding O_L would only start in the presence of rATP, suggesting that priming initiates in the loop of this region's major stem-loop (specifically a region of six thiamine's in the template comprising the 3' end of the loop) and ends near the base of the stem (Wong and Clayton, 1985b).

DNA replication in mitochondria is catalyzed by DNA polymerase γ, the only DNA polymerase yet shown to enter the mitochondrion of human cells (Hubscher et al., 1979) **[Figure 4d and f]**. Pol γ consists of two subunits, a 140kD catalytic subunit (Ropp and Copeland, 1996) and a 54kD accessory subunit (Carrodeguas and Bogenhagen, 2000) whose function is still being investigated. The catalytic subunit shows high processivity and high fidelity, the latter attributable in part to an intrinsic 3' to 5' exonuclease activity (Wang et al., 1991). The accessory subunit has structural homology to aminoacyl-tRNA synthetases; this homologous region has been hypothesized to act as a primer recognition factor, but specific data to support this hypothesis are lacking (Fan et al., 1999).

Little is known about the regulation of mtDNA synthesis, though initiation of H-strand synthesis at O_H begins the replication cycle in all models postulated to date. Because of their roles in creating primers for H-strand DNA synthesis, mitochondrial

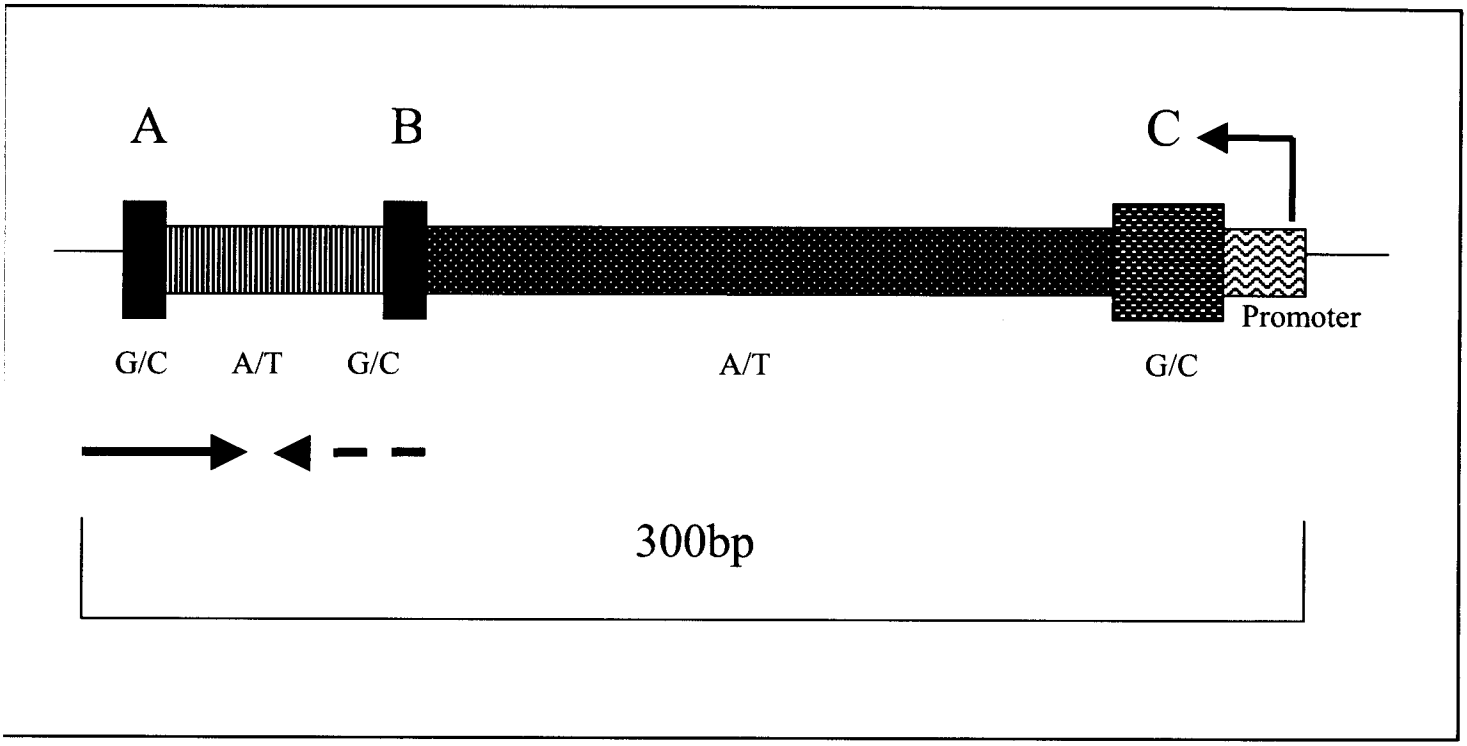


Schmitt and Clayton *Current Opinion in Genetics and Development 1993, 3:769-774*

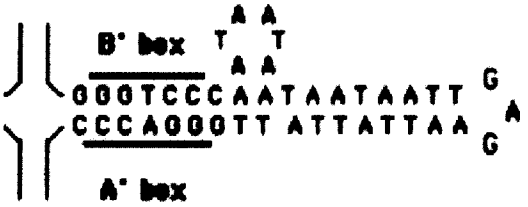
RNA polymerase, mtTFA (the transcription factor responsible for initiation of transcription on both strands) and RNase MRP are possible targets for regulation. In humans, mtRNA polymerase requires an additional 24kDa transcription factor, h-mtTFA to maintain a high level of specific initiation. H-mtTFA contains domains that are homologous to the nuclear high mobility group (HMG) proteins and have the ability to wrap, bend and unwind DNA (Fisher et al., 1992). It's thought that transcription activation in human mitochondria begins with direct binding of h-mtTFA to the upstream LSP and HSP sequences. It has been shown *in vivo* that h-mtTFA has been shown to be the critical factor for mtDNA replication (Larsson et al., 1998). H-mtTFA supposedly forms a complex with DNA, promoting a bend in the structure of the promoter-activating sequences and thus stimulating RNA polymerase transcription initiation activity. Mutagenisis studies have revealed a great deal about the cis-acting sequences involved in primer processing by RNase MRP (Bennett and Clayton, 1990), but the factors or circumstances responsible for choosing a particular L-strand transcript for processing into an H-strand primer are still unknown. As noted earlier, a large number of nascent H-strands terminate prematurely near a specific site ~500nts downstream of O_H, leading to formation of the stable triplex D-loops observed in a large proportion of non-replicating mtDNA molecules. The termination region contains several conserved DNA sequences, and these sequences were used as bait in a series of UV-crosslinking experiments to identify a ~48kD factor in bovine mitochondria that binds preferentially to this sequence (Madsen et al., 1993). To date, this factor has not been cloned or characterized further in a way that supports a regulatory role.

MtDNA Replication in the Yeast *Saccharomyces cerevisiae*

Many, but not all, aspects of the yeast mtDNA replication mechanism discovered to date exhibit homology to those found in mammalian mtDNA replication. All HS ρ- genomes contain a homologous region containing one of the three or four (depending on the parental strand) active Rep/ori sequences that are present in the [*rho*$^+$] genome. In *S. cerevisiae*, there are 7or 8 (depending on the strain) rep/ori sequences, 1, 2, 3 and 5 have been identified in HS rho- and are termed are "active oris"(de Zamaroczy et al., 1981) all of which contain similar organization and sequence (de Zamaroczy et al., 1984); (Faugeron-Fonty et al., 1984). The other ori's are called "inactive" and contain a short insertion within the promoter, These Rep sequences are believed to be the normal origins for yeast mtDNA replication (Blanc and Dujon, 1980) and are comprised of a 300-bp segment sharing about 80% homology. Embedded within each ori are three 100% conserved GC-rich blocks (GC clusters A, B, and C) (de Zamaroczy et al., 1981), as well as a functional nonanucleotide promoter for the RPO41- encoded mitochondrial RNA polymerase (Greenleaf et al., 1986) **[Figure 5]**. The RPO41 promoter, along with the C box, allows transcription towards the inverted repeat. The first two boxes (A and B) are separated by an A/T region, which is not as highly conserved (~80%). When boxes A and B are combined, they create a 100% conserved 34bp inverted repeat. This palindromic A/B box could form a hairpin structure with strong structural similarity to that of the human O_L **[Figure 6]**. The integrity of GC clusters in A and B is required for *rep/ori* activity; similarly, deletion of GC cluster (C) and its surrounding region inactivates the *rep/ori* sequence (Faugeron-Fonty et al., 1984).


A
B
C
Promoter
G/C
A/T
G/C
A/T
G/C
300bp

Human O_L



Yeast A/B palindrome

The replication origins were defined by using the hypersuppressive petites found in *S.cerevisiae* (Blanc and Dujon, 1980; Markussen et al., 1995) (de Zamaroczy et al., 1981). Like the human LSP, yeast contains a promoter at one end of the Rep region that may be processed and used as a primer for transcript initiation. Also like human mtDNA, a stable RNA: DNA duplex was found while performing in-vitro assays on cloned yeast *ori* using purified yeast mtDNA polymerase (Xu and Clayton, 1995). Baldacci et al., had previously discovered that the RNA primers were initiated from the Rep promoter (in vivo) in hypersuppressive mitochondrial genomes, with RNA-DNA transitions occurring at the boundary of the GC cluster C and REP sequence (Baldacci et al., 1984). Similar results have been shown using in-vitro-capping of replication intermediates but with some discrepancies as the location of the RNA-DNA transition site which were found in their studies with in cluster C (Graves et al., 1998). The mtRNA polymerase RP041 is proposed as the enzyme in yeast that synthesizes the RNA primers for "leading strand" DNA strands. This transcription dependant mechanism for ρ+ genomes widely accepted, but Fangman et al., showed that in ρ- genomes, the RPO41 gene is not required (Fangman et al., 1990). It is therefore very likely that yeast has kept the enzymatic machinery to provide an alternative mtDNA replication mechanism for rho- genomes that is not transcription dependent. Also critical to this idea of a second mechanism is the finding of that in RPO41-deleted strains, the replication of HS mitochondrial genomes was maintained as well as their biased inheritance. Since the yeast Rep sequence contains an inverted repeat that could form a stem-loop very similar in structure to that of the human O_L, **[Figure 6]** the initiation of the ρ- strand replication may be achieved by the action of a primase or by alternative recombination mechanisms (see discussion).

Yeast also contains a 19kDa protein, Abf2p that contains HMG boxes and 21% homology with human mtTFA and also bends and introduces negative supercoils in DNA. It is present at much higher levels than h-mtTFA and by analogy it is referred to as sc-mtTFA and it 's thought to function more in mtDNA stability, copy number and transmission (MacAlpine et al., 1998).

Yeast RPO41p requires an additional 40kDa transcription factor, sc-mtTFB (aka MTF1) for binding to the promoter and specificity of the catalytic subunit (Schinkel et al., 1987); (Xu and Clayton, 1992). To date, no known human homologue of sc-mtTFB has been found.

Yeast also contains a site-specific RNase MRP (Schmitt and Clayton, 1992). *In vitro* it has been shown to cleave RNAs initiated from origins of replication and could possibly be extended further by DNA polymerase activity to process RNA primers. The existence/ location of this RNase MRP is a large point of debate in this field and has been further refuted by Graves et al. (1998). The yeast RNase MRP is clearly involved in nuclear ribosomal processing but there is evidence both for and against MRP cleavage producing a functional primer in mitochondria.

Second strand synthesis in yeast is not preceded by a transcription promoter, suggesting that the primers that initiate replication on this strand are not synthesized from the RNA polymerase but from another source. In humans, a mitochondrial primase creates the RNA primer necessary for the mtDNA polymerase at human O_L and is thought to co-purify with Pol γ activity (Wong and Clayton, 1985b). In yeast a primase has also been proposed for primer synthesis on the non-r strand (Baldacci et al., 1984) but as to date no primase has been cloned. It is important to add here is that the non-r strand

initiation site from *ori* 5 was relocated just upstream from the *ori* sequence which questions the priming model for this DNA strand (Graves et al., 1998).

Other factors required for mtDNA replication in *S.cerevisiae* include single-stranded-binding (SSB) protein which is encoded by the nuclear gene RIM1(Van Dyck et al., 1992). It functions as a homotetramer and is essential for mtDNA maintaince but not for cell viability. Mitochondrial helicases, topoisomerases and DNA ligases, all three of which are typical DNA replication factors, are not yet completely characterized in yeast. A helicases, Pif1p, is localized both in the nucleus and the mitochondria and is important for DNA repair and recombination (Foury and Lahaye, 1987); (Lahaye et al., 1991). It is thought that the TOP1 topoisomerase may be responsible for *S.cerevisiae*'s topoisomerases activity. DNA ligase cdc9p is required for nuclear replication to ligate Okazaki fragments from the lagging strand and has been shown to have sub-localization in the mitochondria (Willer et al., 1999).

Using yeast as a model system and comparing human and yeast mtDNA replication may provide insight into normal and abnormal human mitochondrial function. In particular, examining rep sequence and the role that Rep sequences have in the preferential transmission of HS (ρ-) mtDNA over wild type (ρ+) may help provide answers to questions about transmission of mutant human mtDNA. The conclusion that Rep sequences are the origins of mitochondrial replication is based on the presumption that the HS phenotype is a result of the high frequency of tandemly repeated Rep sequences out-competing the relatively few present in the ρ+ genome for DNA replication machinery. For example, in a 80kb segment of a 1kb repeat length HS ρ-, there would be 80 Rep sequences versus ρ+, where there are only three Rep sequences

per 80kbp. Interestingly, it has recently been shown using a FISH technique that some ρ+ is present after multiple generations in these types of crosses proving that ρ+ can replicate in the presence of HS ρ- although apparently not enough to maintain a respiration competent phenotype (MacAlpine et al., 2001). Additional evidence to support the idea that Rep sequences are acting as replication origins: 1.) The Rep sequences contain a short palindromic sequence (Yeast A/B palindrome) that is very similar in its hairpin structure to the Human O_L (Baldacci and Bernardi, 1982). 2. The Rep sequences must contain an active promoter to support HS (Baldacci and Bernardi, 1982). In human mtDNA, a RNA-DNA hybrid formation has been found that is believed to be a precursor to a primer that acts in initiation of mtDNA replication (Xu and Clayton, 1996). There have been reports of a polynucleotide displaying an RNA-DNA junction within sequences complementary to a rep sequence (Baldacci et al., 1984), and (Graves et al., 1998). Alternatively, it has also been suggested that preferential segregation could be causing HS or, more strongly emphasized, that there are maybe multiple DNA replication mechanisms in yeast mtDNA (Lorimer et al., 1995).

Clearly Rep sequences are not the only sites in the rho+ genome that function as origins of replication. A large number of ρ- deletion mutants (Neutral ρ-) are able to sustain themselves in the absence of a Rep sequence (Fangman et al., 1989). Further studies have shown that rho- genomes, which lack the mtRNA polymerase (RP041), continue to replicate their DNA (Fangman et al., 1990). This finding lead to the possibility of the existence of at least two mechanisms for replication initiation; one using RNA Polymerase and promoters (RP041) in the Rep sequences and another that utilizes some non-Rep sequence. There is also the possibility that transcription may not be

involved in replication initiation at all. If there is a second replication system, it could use a rolling circle and/or recombination-dependent DNA replication model. The recombination-dependent model has been supported by Myers et al who showed that there is a requirement for protein synthesis for stable ρ+ maintenance (Myers et al., 1985).

Hypothesis Section

The previous research of Dr. Lorimer was to determine how HS mtDNA was being preferentially transmitted in *Saccharomyces cerevisiae* and what trans-acting protein factors might interact with the Rep sequence to cause it. The Rep sequence contained two potential origin elements, the promoter for mtRNA polymerase encoded by the nuclear gene RP041 and the inverted repeat region that could be cleaved by a endonuclease encoded by another nuclear gene MGT1. MGT1 is required for the preferential transmission of mtDNA deletion mutants (Lockshon et al., 1995), but neither RPO41 or MGT1 is required for mtDNA maintenance (Fangman et al., 1990). It was thought that both genes might be essential for alternate mechanisms of mtDNA replication, each acting from separate sites within the Rep sequence. However, it appears that report that mtDNA is maintained in cells missing both genes. ρ⁻ maintain well in the absence of both, if they can tolerate being Δmgt1 (Lorimer- unpublished data).

The original hypothesis was that biased inheritance of HS ρ- DNA was caused by preferential replication of the larger number of Rep sequences that act as origins. If the RNA polymerase RP041 was required to form primers at the Rep sequence to initiate mtDNA replication as Clayton has suggested, it might also be required for

preferential transmission of HS ρ- DNA. The experiment was done to cross HS ρ- to neutral ρ– to see if HS does indeed contain a replication advantage. The results showed all ρ- strains tested was stable in the absence of RP041, all RP041 null strains showed no detectable mtRNA and the biased inheritance was unchanged. Thus, biased inheritance of yeast mtDNA is independent of the RP041 mtRNA polymerase and is not required for mtDNA synthesis (Lockshon et al., 1995; Lorimer et al., 1995; MacAlpine et al., 2001). This suggests that there has to be an alternate mechanism for mtDNA replication that is not RNA polymerase dependent in HS rho- DNA.

Preliminary data obtained by Dr. Lorimer on HS ρ-strains indicated evidence of ssDNA replication intermediates as well as supercoiled DNA with possible log phase dependence in 2-D gels. This is were I began my research by repeating the 2-D gels in ρ- strains to determine what, if any, growth phase dependence they may exhibit as well as the authentication of several different native replication forms such as ssDNA and supercoiled DNA. I also wanted to test for the presence of ssDNA replication intermediates in log phase wild type (ρ+) strains to prove that these replication forms were not exclusive to mutated mtDNA. If the presence of ssDNA is verified in both mutant and wild type log phase cells it should provide evidence that alternative mechanisms of mtDNA replication do exist and that the current model (which does not account for presence of such molecules) should be modified.

Materials and Methods

Constructs:

The following *S.cerevisiae* strains BS127 wt ρ+ and BS127/HS3324ρ- and (Lorimer et al., 1995) were used in the following experiments. Frozen stocks were streaked onto fresh YEPD [1% yeast extract and 2% peptone supplemented with 2% glucose] plates using a sterile inoculating loop and were allowed to grow at 37°C overnight to form colonies. BS127 and BS132 are both *ade1*, *ade2* mutants. These mutant yeasts are red in color when respiration competent, plated and allowed to grow an appropriate medium. Plates were stored at 4°C for further use. A single colony was placed into 5ml of YEPD liquid media [1% yeast extract and 2% peptone supplemented with 2% glucose] and grown overnight at 30°C in a rolling incubator.

Using the fresh overnight cells a dilution was chosen to obtain both log phase (a period of high mtDNA replication) and stationary phase (a period of little or no mtDNA replication) preps. Determination of growth phase was calculated by taking an OD reading between 660λ and 680λ that was approximately 1x 10^7cells/ml for log phase and 3 x 10^8cells /ml for stationary phase (approximately 24 hours after growth had ceased). Large volumes of media (500mls) were used to obtain enough cells for harvesting log phase DNA and the timing of dilutions was varied according to when the yeast needed to be harvested. Cells were spun down in a Sorval GSA rotor in 250ml centrifuge tubes containing frozen EDTA at 15,000rpm (7,000G) for 10 minutes. The pellets were washed once with DI water and, transferred to a microfuge tube and spun again at 15,000rpm for 10 min. The water was removed and the pellets were stored at -20°C until further use.

Total cellular DNA was isolated by a modified disruption procedure referred to as a 'Smash and Grab' (modified and scaled up from (Hoffman and Winston, 1987). The cell pellet was suspended in Lysis Buffer (2%Triton X-100, 1%SDS, 100mM NaCl, 10mM Tris-HCL [pH 8.0] and 1mM EDTA). The detergents help to soluablize the membrane, the NaCl adds ions to solubilize DNA, the Tris-HCL ensures proper pH and EDTA is a chelator to keep the DNA intact. Equal volumes of acid washed glass beads (Sigma G-9268) and a 50:50 mixture of equilibrated phenol/chloroform with isoamyl alcohol (1:25) were added to the tube to further disrupt and dissolve the proteins and cell membranes present. The tubes were vortexed for 30-second intervals and placed on ice in between for 10-20 times. A small sample was taken from the tube and diluted with water to check for ghosts (percentage of dead/lysed cells). Ghosts appear as pale cells with irregular outlines as opposed to intact cells that are refractive, round and have clearly defined cell walls. The vortexing was preformed until >80% lysis. The tubes were centrifuged at 20,000 rpm (10,000G) for 10 minutes in a swinging bucket rotor (IEC Centra-7R). The resulting layers contained an aqueous portion (total cellular DNA), a white band (cellular debris) and a pellet that contained the insoluble portion (glass beads). The aqueous layer was extracted by pipette and transferred to a new microfuge tube. The organic layer was back extracted by adding 1X TE (10mM Tris-HCL, pH 8.0 and 1mM EDTA), vortexed for 30s and centrifuged for 10 min at 20,000rpm. The aqueous layer from this spin was added to the original aqueous and DNA was precipitated using 100% Ethanol (added at 2.5 times the final volume) and 3M NaOAc (added at 1/10th the final volume). The tube was placed at -20°C for an hour and was spun at 20,000rpm for 5 minutes. The EtOH was removed and the pellet was washed using cold 70% EtOH and

the pellet is left to dry overnight. Pellets were resuspended in 20µl 1XTE and stored at 4°C.

If required, the DNA pellet was also resuspended in 20µl of TE and treated with 1µl of RNase H (Sigma) for one hour at 25°C. The DNA was again treated with phenol chloroform and precipitated with ethanol as described above.

2-Dimensional Gel Electrophoresis Procedure

Using the Fangman /Brewer method of 2-D gel electrophoresis (Brewer and Fangman, 1987) a large first dimension 0.4% agarose gel (0.8g Fisher Brand EEC grade; dissolved in 250µl 1X TBE [2.5mM NaOH, 89.2mM Tris Base, 88.9mM Boric Acid and 1.98mM EDTA]) (Maniatis et al., 1982) was made in a 20x24.8cm tray with a 40 µl comb. This percentage of agarose allows for good separation at low voltage and longer amounts of time. The solidified gel was inserted into a BRL large format gel box that contains 1X TBE as the running buffer.

For the 1st dimension, gel loading dye was added (3µl)[10 mM Tris (pH to 7.5) ,50 mM EDTA (pH to 8.0 to dissolve), to10µl of total cellular DNA and loaded into a well. Lambda DNA marker (5µl) [1µg λ cut with Eco RV and HindIII] was loaded in the first well of all first dimension gels. The gel was run at 0.8V/cm for 17 hours and then stained with Ethidium Bromide to visualize the bands on an UV box. The 1-D bands were cut vertically as close to the band as possible and are placed on a new gel tray in a horizontal orientation, rotated 90° counterclockwise, across the top of the tray with the well-end facing the left-hand side of the box. The second dimension gel was then prepared as a 1-% agarose gel (1.0g agarose, 18µl ethidium bromide solution and 250ml of 1XTBE). The ethidium allows for separation based on shape of the DNA forms by

intercalating between the bases and stiffening the DNA. The 1-D gel slice was anchored to the tray with molten 1-% agarose placed around the edges. The remaining agarose solution is poured around the gel slab and is allowed to solidify. The gel is placed into the same large gel box with a 1X TBE running buffer that contains 157µl of ethidium bromide and is attached to a peristaltic Mini-Pump (VWR) to re-circulate the buffer while the gel is running. The second dimension is run at 4°C at [5.914V/cm] and visualized by UV.

The second dimension gel was transferred to nitrocellulose membrane (S&S Nytran Plus) using capillary action (Southern, 1975). The Blot was then hybridized with an appropriate cloned mtDNA sequence. The plasmid pCT10 contains HS3324 mtDNA cloned into the EcoRV site in pBR322, pN28 contains N28 mtDNA cloned into the HindIII site in pUC18. The blot was then radiolabeled by random priming with [α-^{32}P] dATP using the NEBlot system of New England Biolabs according to manufacturers specifications. Hybridizations were carried out at 65°C in hybridization buffer (6X SCP, 1% Sarkosyl, 100µg/ml non-specific carrier DNA) using a 20X SCP stock (0.9 M sodium phosphate pH 6.8, 2 M NaCl, 0.02 M EDTA), 1mM EDTA and 0.2mg/ml sheared/denatured salmon sperm DNA (Sigma). Stringent washes were performed at 65°C in 1M $NaPO_4$, pH 7.0 containing 2% SDS.

Denaturation of ssDNA molecules

For control boiling experiments 5µg of BS127 ρ+ log phase was incubated at 95°C for 10min and placed directly on ice. 2-D gel electrophoresis was done using the same conditions as stated previously and blot was hybridized with a N28 probe.

S1Nuclease treatment of ssDNA

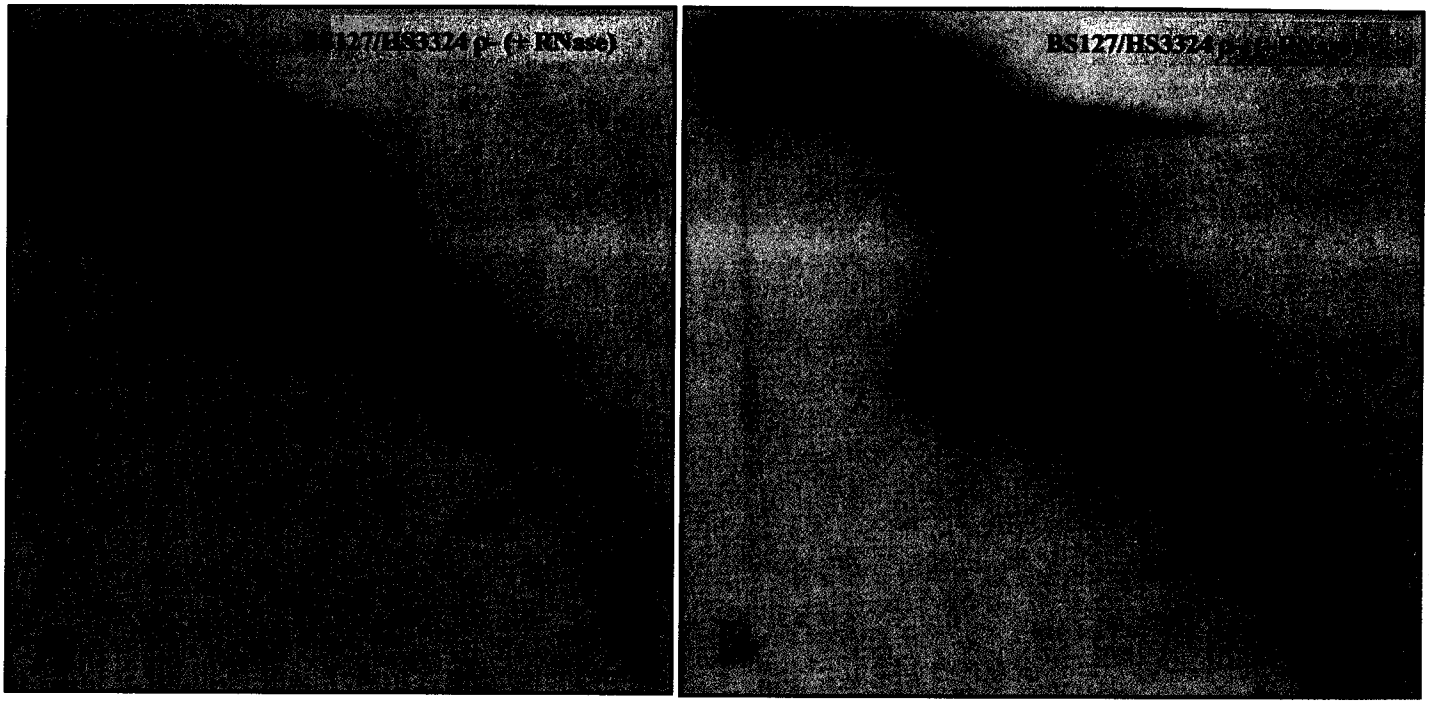
To determine the optimal concentration of S1 nuclease, 10μg of BS127 ρ+ mtDNA was suspended in 100μl of S1 Buffer (50mM NaOAc, 200mMNaCl, 1mM $ZnSO_4$ and 0.5% glycerol pH 5.7) and treated with either 1000U or 500U of S1 Nuclease (Sigma) at 37°C. Aliquots were obtained at t=0, 4, 15 and 60 minutes and stopped with stopping buffer (50mM EDTA and 1M Tris HCl) and placed directly on ice. Linear mtDNA was phenol/chloroform extracted, EtOH purified and run on a 1-D gel at 0.8V/cm for 17h. The 1-D was transferred to nitrocellulose and probed with a N28 probe.

Results

Single-stranded molecules in mtDNA of the HS petite HS3324ρ- show log phase dependence

The mechanism causing the preferential inheritance of HS ρ⁻ mtDNA is still unknown. At this point the most likely cause seems to be a replication advantage. Therefore, it is logical to look if HS ρ- has unique forms of replication that could cause the hypersuppressive phenotype by a replication advantage. Preliminary data suggested the presence of single stranded DNA (ssDNA) in 2-D gels of the yeast strain HS3324ρ-. The first set of experiments was done to confirm these results using the same HS3324ρ- strain in a 2-D gel under growth conditions of either log phase (a period of most rapid cellular, and presumably mtDNA, replication) or stationary phase (a period of little or no cellular/mtDNA replication). **Figure 3a** illustrates the patterns normally obtained by a 2-D gel after southern blotting (Reynolds et al., 1989). Total DNA was prepared using a "smash and grab" technique breaking cell walls with acid washed glass beads in the presence of lysis buffer and phenol/chloroform. After the resulting nucleic acids were extracted, they were and loaded onto a gel without prior restriction enzyme disruption. The purpose of this type of process was to isolate the DNA quickly and with minimal manipulation, so as to obtain all the forms of nucleic acids present in as close to their native state as possible. For log phase, total DNA preps were either treated with RNase for one hour prior to running on a gel or run directly **[Figure7A and B]**. Blots were hybridized with a random primed labeled probe for HS3324 ρ-.

For both log and stationary growth phases, we detected a strong double stranded (ds) linear DNA form, both monomeric and oligimeric circular forms of the repeat unit,

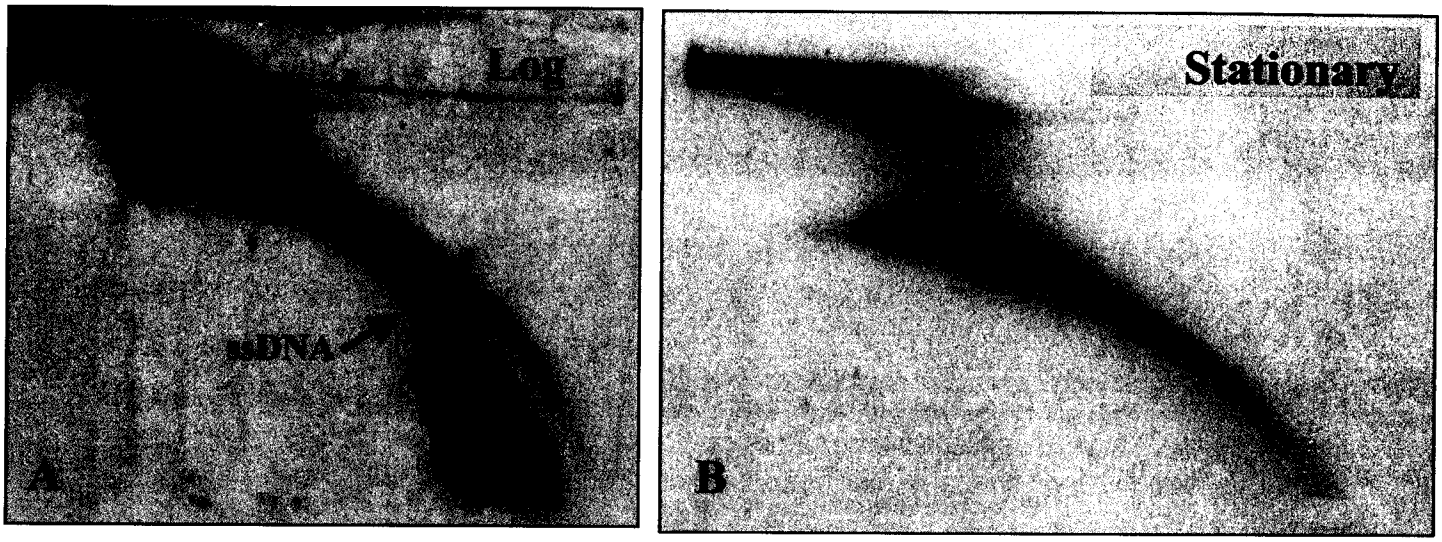

BS127/HS3324 p- (+ RNase)
BS127/HS3324

both as presumably nicked relaxed circles and a variety of supercoiled forms exhibiting different linking numbers. (Note: linking numbers involve one size of a circle with different amount of supercoiling). The integrity of the DNA preparation could be judged in part by the presence and ratio of supercoiled circles compared to relaxed circles. Preparations that experience significant DNA degradation lost both higher molecular weight forms and supercoiled circles. The resulting autoradiograms differ from one another in two ways: first, the stationary phase **[Figure 8]** had a higher number of supercoiled forms and second, the log phase **[Figure 7A and B]** showed a distinct arc below the linear dsDNA corresponding to an area where ssDNA should run (Backert et al., 1997a). This data suggests that log phase BS127/HS3324 ρ- creates ssDNA while the stationary phase apparently does not which leads one to believe that this is a valid form of active replication used by ρ- strains.

Single-stranded molecules in mtDNA of the BS127 ρ+ (wt) show log phase dependence

If we want to argue that the presence of ssDNA molecules is indeed a valid replication intermediate and not an artifact of a ρ- mutant strain, we needed to test for the presence of ssDNA in a wild type strain. Using the BS127 ρ+ (wt) strain, 2-D gels were run with total DNA preps grown under the conditions of either log or stationary growth phases and blots were hybridized with and random primed with a N28 ρ+ probe **[Figure 9A and B]**. Again there was the presence of a strong ds-linear DNA molecule in both growth phases but no supercoiled, oligomers or ds circular molecules were present. Due to the large size (80kb) of the wild-type genome these forms would not be seen.

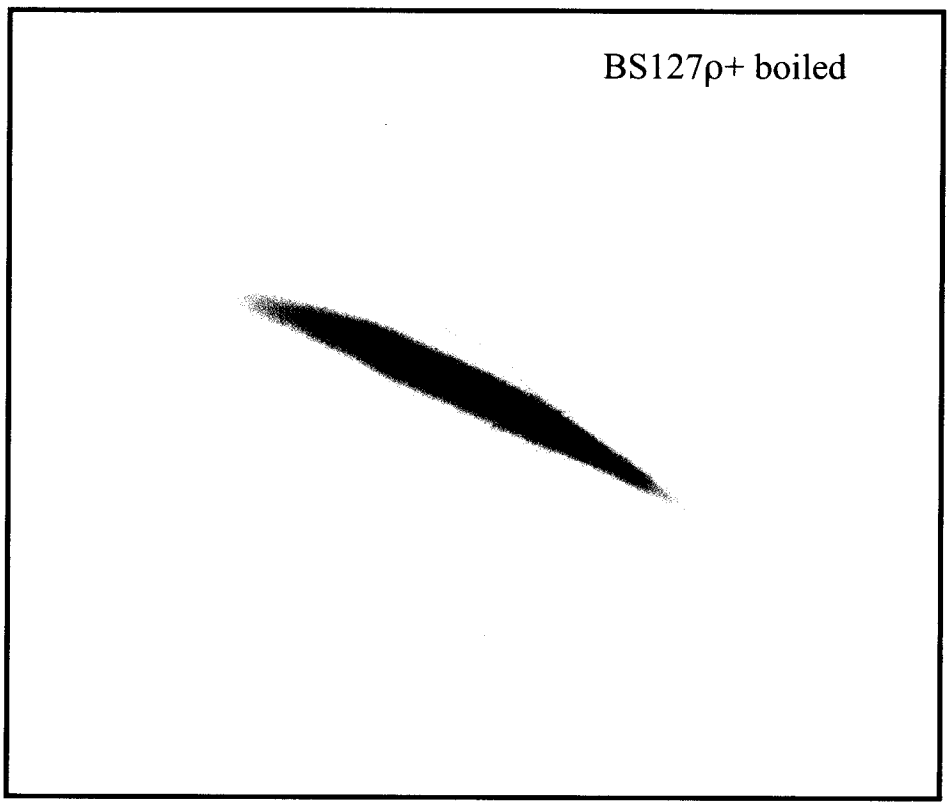
Figure 8 – BS127/HS3324 ρ- stationary phase shows no ssDNA molecules and have a higher number of closed circular and supercoiled topoisomers than log phase.


Log
ssDNA
A
Stationary
B

expected to be seen under these 2-D gel conditions. The log phase showed the strong presence of an ssDNA molecule falling just below the linear dsDNA arc but no similar arc was observed in stationary phase. Thus, supporting the idea that that ssDNA is a valid replicative form for both ρ+ and ρ- genomes.

Testing the presence of ssDNA in BS127 ρ+

In order to confirm and characterize the ssDNA replicative intermediates found in log phase growth, we compared the patterns of native and denatured DNA samples from log phase preps and log phase preps treated with single strand specific nuclease S1. For denaturation studies, ρ+ log phase preps were harvested and boiled at 95°C for 5 minutes to abolish the presence of dsDNA forms. Blots were hybridized with the same random primed probe (N28) **[Figure 10]**. The boiled log phase prep shows only ssDNA. For S1 nuclease treatment, a log phase prep of ρ+ was obtained and treated with increasing amounts of S1 to observe the elimination of ssDNA in a 1-D gel. An optimal amount of S1 nuclease was obtained for treatment of log phase preps in a 2-D gel for future studies. The 2-D gel was not performed in this set of experiments but has since been performed by another student in the lab confirming the absence of ssDNA in S1 treated log phase preps. In addition, MacAlpine et al., have also shown S1 nuclease depletion of ssDNA forms in another ρ- strain, HS40 (MacAlpine et al., 2001).


BS127ρ+ boiled

Discussion

Our results clearly identify a novel mode of replication of both HS ρ- and ρ+ mtDNA. This type of replication shows strong growth phase dependence with ssDNA intermediates being prevalent in log phase preparations for both mutant ρ^- and wild type ρ^+ strains and was later shown to be depleted in the presence of S1 endonuclease. Heat denaturation and later S1 experiments, performed by Bojana Stevic, confirmed that this growth rate dependent form is indeed single stranded mtDNA. The ssDNAs in ρ^- strains includes monomer and oligimeric circles as well as longer linear molecules. We also observe nicked or partially circular dsDNA, which could be intermediates in the synthesis of the complementary strand. Another group, Mac Alpine et al, have discovered similar results using another hypersuppressive ρ- strain HS40. They observe similar patterns in 2-D gels with a strong ssDNA arc in their case only detectable only with a sense strand probe. They also tested a Neutral ρ- (VAR 1) and saw no ssDNA intermediates but did detect the presence of supercoiled topoisomers which we observe in our stationary phase preps (MacAlpine et al., 2001). We have seen ssDNA in neutral ρ^- mtDNAs however. They further characterized the ssDNA intermediates with S1 nuclease and Exo 1 (3'exo) treatment and saw depletion of ssDNA forms. The Exo1 treatment is a 3'-5' single strand specific nuclease and only ssDNAs with an exposed 3'end will be digested. The Exo1 treatment showed circular ss monomers and oligomers of the rep sequence. The authors interpret these results as either possible sigma molecules undergoing rolling-circle replication (but they were unable to detect 5'ends) or, more likely, as molecules undergoing asymmetric strand displacement replication. All ssDNA replication intermediates found in the MacAlpine study correspond to the strand of mtDNA that

would be displaced by transcription. This combined with the discovery of RNA-DNA hybrids in an ori5 HS petite (Graves et al., 1998) suggest that ssDNA intermediates may come from RNA-primed replication.

Alternative Yeast mtDNA Replication Mechanisms

In yeast, D-loops like those seen in mammals, are rarely detected and theta-type mechanisms with a leading and lagging strand have not been studied as of yet. Thus the yeast mtDNA replication mechanism is still fairly hypothetical. The only well studied portion of the mechanism being the initiation steps, which are similar to mammalian. A major difference between yeast in mammalian mtDNA replication is that the ori in yeast are potentially bi-directional whereas in man the origins are unidirectional and spatially separated. Graves et al., (1998) have observed discrete 5' DNA ends near the oris of HS yeast but there is no indication of initiation efficiency of the non-(r) coding strand compared to the coding strand (r).

Other types of yeasts *Schizosaccharomyces pombe* and *Torulopsis glabrata* both exhibit a rolling-circle mode of replication (Han and Stachow, 1994; Maleszka et al., 1991). This type of replication is suggested to be responsible for the large amounts of large linear molecules in preparations of mtDNA and has been suggested that *S. cerevisiae* might have a similar mechanism responsible for the ssDNA forms in 2-D gels. In addition to yeast, the higher plant *Chenopodium album* (L.) exhibits rolling-circle mtDNA replication as observed by EM studies as lariat shaped loops ((Backert et al., 1997b). MtDNA in plants consists of giant catenanes of minicircles and maxicircles, with individual molecules also replicating unidirectionally via theta-like intermediates

(Carpenter and Englund, 1995). Plants, like yeast, are suggested to have additional mechanisms of replication such as recombination-dependent replication.

Yeast mtDNA may have multiple mechanisms of replication, one of which may also be a transcription dependent replication mechanisms used in ρ+ genomes. Some *E Coli* plasmids have a recombination-dependent replication system, referred to as SOS or iSDR replication (Asai et al., 1994). This replication is primed from the 3'end of the invading DNA strand and further supports the idea of a transcription dependent mechanism. Other evidence to support this mechanism come from ρ- genomes that are highly recombinogenic and do not require origins of replication for maintenance. In addition in ρ+ genomes, Abf2 is required for stable maintaince. This protein is responsible for stabilizing Holliday junctions and further provides linkage between mitochondria stability and recombination.

Models of mtDNA Replication

The current model of animal mtDNA replication is referred to as the D-loop model, a bi-directional, asynchronous- asymmetrical mechanism relying on one origin at each strand displaced from each other by 2/3 of the genome (Clayton, 1984). This mechanism is very different from the traditional bi-directional replication mechanism found in nuclear DNA **[See Figure 4]**. In general, the experimental evidence used to support this model has resulted from work done in highly purified mitochondrial cell systems and also assumes that analogous systems operate in both yeast and human mtDNA. These sorts of studies may not reflect the true nature of yeast mtDNA *in vivo*.

The D-loop model requires at least two polypeptides required for transcription initiation; mtTFA and RNA polymerase (RPO41). Several transition sites (primer RNA

to DNA synthesis) have been found in mammalian mtDNA downstream of the promoter and are referred to as conserved sequence blocks (CSBs) (Chang and Clayton, 1985). Transcription begins upstream of two conserved sequence blocks (CSB's) at the O_H promoter which is very A/T rich. Mitochondrial RNA transcribed from the promoter acts as a primer for first strand synthesis. The proposed yeast mitochondrial replication model follows the same idea that the RNA transcribed from the Rep sequence acts to prime the first (promoter) strand for DNA replication (Baldacci and Bernardi, 1982; Graves et al., 1998). Preliminary data suggested the absence of single stranded DNA (ssDNA) in 2-D gels of HS3324 ρ- lacking RPO41 (Lorimer unpublished data). This would support a mechanism of DNA replication appears to involve RNA priming from the canonical nonanucleotide mitochondrial promoter on one side of the Rep sequence, perhaps leading to asymmetric strand displacement. However, additional studies have found ss mtDNA in cells lacking the mitochondrial RNA polymerase (Stevic and Lorimer unpublished data).

In addition to our own findings, evidence to support the initiation step refers to the fact that inactive (not HS) ori or Rep sequences have a short DNA insertion that disables promoter function (de Zamaroczy et al., 1981).

The idea of RNA-primed replication beginning at the ori sites was controversial because of evidence that the RP041 was neither required for the replication of ρ- mtDNAs not for the preferential transmission of mtDNA to a HS ρ- strain (Fangman et al., 1990; Lorimer et al., 1995). The standard cross of ρ+ to ρ- was not testable in this paper because ρ+ is unstable in the presence of RPO41. To overcome this obstacle, MacAlpine et al used reverse genetics to mutate the ori5 promoter in HS ρ- mtDNA and

found that it is necessary for hypersupressiveness in crosses with a ρ+ strain. In order to compare the Lorimer study to their own they crossed mutated and intact ρ- deletion mutants to a neutral ρ- and our strain HS3324 which contains a wild-type ori5. They discovered that the low (16%) suppressiveness observed when the mutated deletion sORI5p-476 is crossed to a neutral provides the genome with a sufficient advantage to out-compete a neutral ρ- but not enough to compete with a ρ- which contains a wild-type ori5. It is ill unclear what causes preferential transmission in ρ- x ρ- crosses, a bias in replication or segregation.

MacAlpine et al also showed that mutating the promoter eliminated transcripts from this particular ori but the steady state level of transcripts was unaltered. This supports the idea that ρ- mutants lacking a promoter accumulate transcripts due to alternative initiation sites (Zassenhaus et al., 1984). These findings of alternative transcripts are dependent on Rpo41p and thus a hierarchy of promoters must exist in yeast mtDNA that is yet uncharacterized. Using primer extension and capping experiments on mutated *ori*5 promoters, MacAlpine et al showed elimination of 5' ends in transcripts. This suggests that there are potentially many other sites for initiation of transcription in the absence of a promoter. This also does not explain how come the reduction of suppressiveness in the inactivated promoter strains. This leaves the problem of if the transcript is there then why can't that mechanism still work, and how come there is still hypersuppressiveness w/o the RPO41?

A site-specific ribonucleoprotein endoribonuclease (MRP) has been located in mammalian mitochondria that cleaves primer RNA termini *in vitro* near a number of these *in vivo* transition sites (Bennett and Clayton, 1990; Chang and Clayton, 1987b; Lee

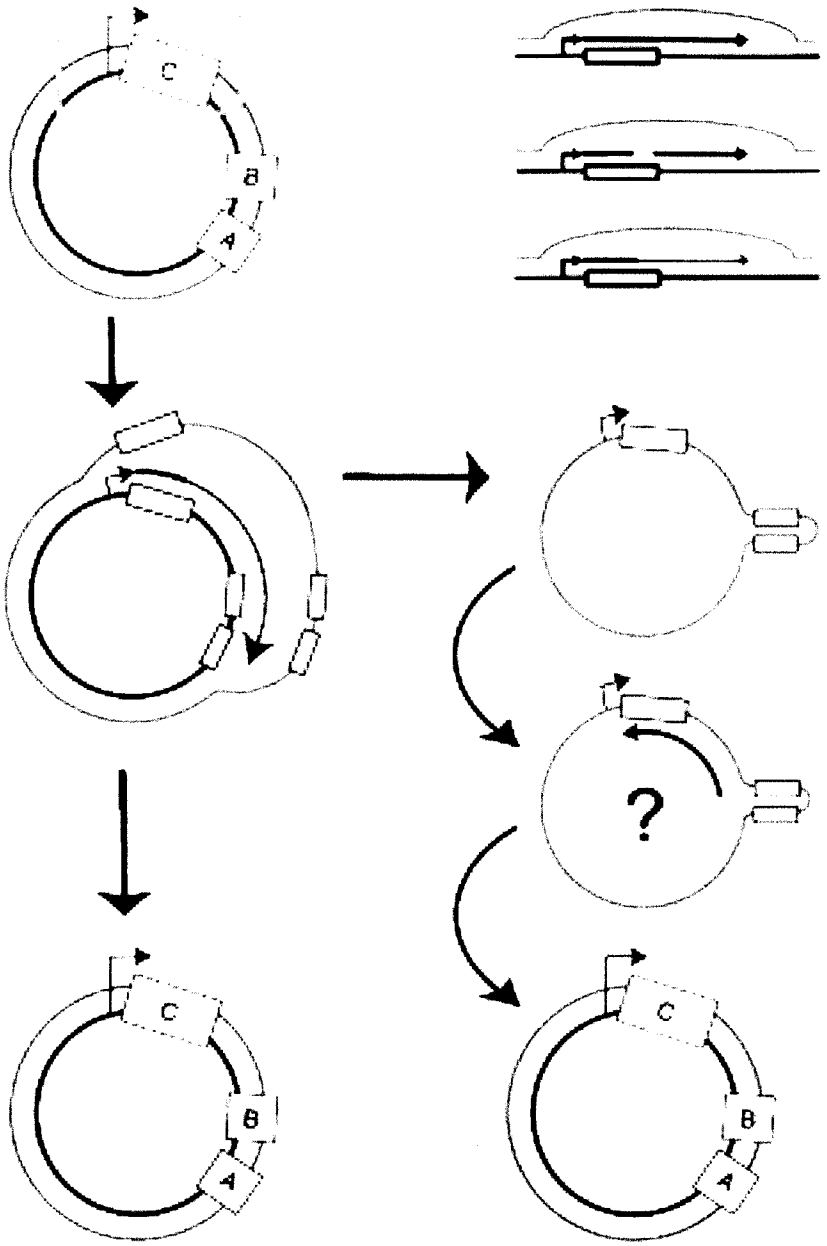
and Clayton, 1997). It is also thought to have an RNase-H like activity. MRP is also thought to recognize an RNA-DNA hybrid formation as the substrate for attachment to the transcript, thus showing the importance of the degree of retention of RNA on the mtDNA template strand. MRP supposedly cleaves the RNA transcript at the LSP (light strand promoter) to create a 3'OH end that is used by mtDNA polymerase γ to initiate leading strand synthesis unidirectionally using processed transcripts as primers until 2/3 around the genome forming a single-stranded displacement loop (Chang et al., 1985). The yeast model is thought to work similarly with MRP cleavage occurring on the transcript from the Rep promoter to make a primer for promoter strand replication (Stohl and Clayton, 1992).

An inverted repeat in the O_L probably could form a hairpin stem-loop because genome is very A/T rich and complementary which makes them potentially full of such structures. The hairpin supposedly t functions as the primer for second strand synthesis (Wong and Clayton, 1986)((Hixson et al., 1986). There is a great deal of sequence similarity to the yeast A/B palindrome and the O_L showing the relatedness of the two systems and the relevance of studding mtDNA replication in yeast (**Figure 6**).

After polymerization past the O_L in mammalian mtDNA, a DNA primase recognizes the hairpin formation then binds and cleaves to produce a primer for DNA polymerase to use for second strand synthesis (Wong and Clayton, 1985b). This mechanism does not produce a traditional lagging strand or Okazaki fragments. This site also does not contain a promoter. It is important to clarify that this is a very basic model of mtDNA replication done in a highly purified system that does not account for the function of helicases, ssDNA binding proteins, ligases nucleotide transporters, kinases

and synthtases. The second strand synthesis mechanism is asynchronous-discontinuous replication with each strand rolling back to it's own origin and terminating by supposedly running onto its own end. In the proposed yeast model, primase attachment is believed to be in a Rep sequence after first strand synthesis exposes the second strand origin (that contains the A/B palindrome) to initiate second strand synthesis. Yeast presumably follows the same mechanism and is believed to resolve it's recombination structures by a possible cruciform cutting endonuclease MGT1 (Lockshon et al., 1995). This supports the recombination dependent DNA replication model.

MacAlpine et al propose an updated model for ori5-dependent mtDNA replication **[Figure 11]** adapted from (MacAlpine et al., 2001) that results in asymmetric strand displacement that could explain the ssDNA that both our group and theirs have observed. Transcription initiates from the nonanucleotide promoter and transcripts (shown in red) are processed in the C box to create a 3'OH end that primes DNA replication (shown in blue). The elongation of the nascent primed DNA strand displaces the sense (complementary) strand of mtDNA (shown in green), which eventually releases a ssDNA circle. Our arcs of linear ssDNA should come from events initiated on larger oligomers with large displaced circles that are broken during our preps. Initiation of lagging strand synthesis of the ssDNA circle should form at or near the hairpin formed by the A and B boxes (analogous to the animal model of light strand synthesis).



MacAlpine et al 2001 . *Embo J.* 20:1807-17

RECENT OBSERVATIONS AND FUTURE DIRECTIONS

With some refinements of detail, this is where the state of knowledge about mammalian mtDNA replication stood until 2001 when an analysis of human and mouse mtDNA by pulse-field (2-D) gel electrophoresis provided convincing evidence for mtDNA replication by standard coupled leading- and lagging strand synthesis of a type quite similar to that found in the nucleus (Holt et al., 2000). In most of the cell types examined, orthodox (asynchronous) DNA synthesis was still the predominant form, and selective degradation of the partially single-stranded products of orthodox mtDNA synthesis was necessary in order to visualize the intermediates of standard synthesis described in this report. These observations raise the possibility that two fundamentally different types of DNA replication are going on in mitochondria from the same tissue, if not from the same cell or organelle. Further evidence from this report suggests that strand-synchronous replication is the *dominant* mode in cells that are replenishing their supply of mtDNA after experimental depletion. This finding raises an array of further questions pertaining to the necessity for both forms of synthesis, the regulation of synthesis in different cells of organelles, and the nature of the *trans*-acting factors, if any, involved in making the choice.

2-D neutral/neutral gel-electrophoresis allows for the separation of restriction fragments on the basis of size (MW) in one dimension and on the basis of shape in the other dimension (Brewer and Fangman 1987). The fragment of interest is then identified by Southern hybridization. The primary forms of replicative intermediate identifiable this way are bubbles that form at origins of replication in this fragment; replication forks

which pass through the fragment; or forks moving in opposite directions which converge in the fragment. The replication bubble initiating at O_H moved out of one end of the fragment but not both, indicating that strand-synchronous replication, like orthodox strand-asynchronous replication, is unidirectional. Also in common with the orthodox model, strand-synchronous replication begins at a single origin in the vicinity of O_H and moves in the direction of H-strand synthesis, i.e., the H-strand is the leading strand and the L-strand is the lagging strand. However, no evidence of a specific bi-directional origin of replication in yeast has been found using 2-D gel electrophoresis. All studies published to date have revealed only linear molecules, Y-arcs, and recombination structures.

Future experiments should include to further confirm the ssDNA replication intermediates by checking the strandedness of our ssDNA with a sense specific ssDNA probe and to investigate the potential rolling circle structures observed in the BS127 ρ+ experiments completed after this thesis. Also interesting is the role of the nuclear gene MGT1 in the resolution of ρ^- mtDNA. Current experiments in the lab include sequence dependent maintenance of ρ^- mtDNA in the absence of MGT1. MGT1 may be critical for replication if this is linked to possible a site-specific recombination mechanism proving again that there is more than one potential mechanism for yeast mtDNA replication.

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