A Role for Recombination Junctions in the Segregation of Mitochondrial DNA in Yeast

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Summary

In S. cerevisiae, mitochondrial DNA (mtDNA) molecules, in spite of their high copy number, segregate as if there were a small number of heritable units. The rapid segregation of mitochondrial genomes can be analyzed using mtDNA deletion variants. These small, amplified genomes segregate preferentially from mixed zygotes relative to wild-type mtDNA. This segregation advantage is abolished by mutations in a gene, *MGT1*, that encodes a recombination junction–resolving enzyme. We show here that resolvase deficiency causes a larger proportion of molecules to be linked together by recombination junctions, resulting in the aggregation of mtDNA into a small number of cytological structures. This change in mtDNA structure can account for the increased mitotic loss of mtDNA and the altered pattern of mtDNA segregation from zygotes. We propose that the level of unresolved recombination junctions influences the number of heritable units of mtDNA.

Introduction

The mitochondrial genomes found in eukaryotic cells are multiple-copy genetic systems. A given cell may be either homoplasmic, with the hundreds to thousands of mitochondrial DNA (mtDNA) molecules being identical, or heteroplasmic if more than one genetic type exists within it. Heteroplasmy may arise by the fusion of two cells with genetically different mitochondrial genomes, or by mutational alteration of one or more of the mtDNA molecules within a cell. Regardless of how heteroplasmy is generated, homoplasmy is quickly restored through the rapid segregation of mitochondrial genomes during subsequent mitotic divisions (reviewed by Birky et al., 1982; Birky, 1994). Since normal cellular respiration requires that cells produced by mitotic division receive a sufficient number of functional mtDNA molecules, the accumulation of defective copies of mtDNA during development can have

serious consequences. For example, a number of inherited human diseases are characterized by the transmission of a mixture of mutant and wild-type mtDNA molecules by the egg and a subsequent clonal increase in mutant mtDNA relative to normal mtDNA molecules after fertilization (Simonetti et al., 1992; reviewed by Brown and Wallace, 1994). Compromised cellular respiration leads to pathologies, especially in tissues with high respiration demand such as muscle and nerve. The mechanism that allows the accumulation of mtDNA molecules with point mutations or deletions is not well understood, and one of the fundamental questions associated with mitochondrial inheritance is how a small number of mutant mtDNA molecules can become predominant in a somatic cell line that contains numerous copies of the mitochondrial genome.

The segregation of mtDNAs is not easily studied in higher eukaryotes, since it is difficult to generate cells with known quantities of differently marked mitochondrial genomes and then follow their inheritance during cell divisions. To avoid these limitations, the yeast Saccharomyces cerevisiae has been extensively used as a model system for studying the transmission of mtDNA. A zygote with mixed mtDNA types is readily produced by mating two haploids with genetically marked mitochondrial genomes. Upon mating, the homoplasmic haploid cells fuse to form heteroplasmic zygotes containing approximately 50 copies of each mitochondrial genome. As indicated by a high frequency of recombination, there is rapid mixing of mtDNA types. However, these zygotes segregate pure parental and recombinant genomes within only a few cell divisions (reviewed by Dujon, 1981). This rapid generation of cells with pure mtDNA types by random segregation suggests that the number of heritable units of mtDNA must be considerably less than the number of mitochondrial genomes in the zygote. Early cytological analysis of the distribution of mtDNA in yeast demonstrated that the number of mtDNA staining bodies, called chondriolites, is smaller than the actual number of mtDNA molecules determined by biochemical analyses (Williamson, 1976; Williamson et al., 1977). This apparent aggregation of mtDNAs, possibly promoted by the high frequency of mtDNA recombination, was proposed to contribute to reducing the number of heritable units.

Additional information regarding mitochondrial genome segregation in S. cerevisiae has been provided by respiration-deficient deletion variants of the mtDNA (reviewed by Dujon, 1981; Gingold, 1988; Piskur, 1994). These mutants, termed *rho⁻*, arise by deletions of the wild-type (*rho⁺*) mtDNA genome that eliminate most of the ~80 kb of *rho⁺* mtDNA sequence. The mtDNA sequences retained in these *rho⁻* genomes are amplified to a mass of mtDNA equivalent to that found with the *rho⁺* mitochondrial genome. A short mtDNA sequence found only once in the 80 kb *rho⁺* genome is present numerous times as tandem repeats within large molecules in a *rho⁻* cell. When *rho⁻* cells are mated to *rho⁺* cells, the two genomes rapidly segregate to produce pure homoplasmic types. Some of

the zygotic clones contain only cells with the rho⁻ mtDNA. The fraction of such clones in which the rho⁺ genome is not transmitted is defined as the degree of suppressiveness. Suppressiveness values depend on the particular rho⁻ variant and can range from less than 5% (nonsuppressives) to greater than 95% (hypersuppressives). Hypersuppressive rho⁻ mtDNAs consist of a short mtDNA fragment (<5 kb) that includes one of several highly related ~300 bp sequences, called rep sequences, found at a number of locations in the rho⁺ mitochondrial genome. The preferential inheritance of hypersuppressive rho⁻ mtDNA in competition with rho⁺ mtDNA is thought to be a consequence of an advantage conferred by the increased density of these rep sequences in the amplified hypersuppressive rho⁻ mtDNAs (Blanc and Dujon, 1980; De Zamaroczy et al., 1981). There is some evidence that rep sequences serve as replication origins, but it is not yet clear whether the inheritance bias is due to preferential replication, segregation, or some other mechanism.

We previously identified a nuclear gene, MGT1 (mitochondrial genome transmission), through a mutation that reverses the outcome of rho⁺ × hypersuppressive rho⁻ matings (Zweifel and Fangman, 1991). When both haploid parents carry the recessive allele mgt1-1, hypersuppressive rho⁻ mtDNA is rarely found in the diploid clones. Thus, in the absence of the MGT1 gene, there is a switch in the transmission bias; rho⁺ mtDNA rather than the hypersuppressive rho⁻ mtDNA is inherited in most zygotic progeny. The MGT1 gene was independently identified as CCE1 (cruciform cutting endonuclease) through a screen for yeast mutants defective in the cleavage of recombination junctions, using cruciform structures as the substrate (Kleff et al., 1992). Its gene product had previously been shown to cleave two DNA strands at the base of a four-way DNA junction, but to be incapable of cleaving at a three-way DNA junction (Evans and Kolodner, 1988). The MGT1/CCE1 gene product has been shown to be localized to the mitochondrion (Ezekiel and Zassenhaus, 1993).

The ability of the MGT1 gene product to resolve recombination junctions supports the idea that recombination among mtDNAs may play a role in the segregation of mitochondrial genomes. Here, we present evidence that deletion of the MGT1 gene increases the fraction of mtDNA molecules that are linked together by recombination junctions and aggregates mtDNA into fewer chondriolites. We propose that the resulting reduction in the number of heritable units of mtDNA in the absence of MGT1 activity accounts for an elevated loss of mtDNA from mitotic cells and the altered postzygotic segregation of mitochondrial genomes in rho⁺ × rho⁻ matings.

Results

Deletion of MGT1 Alters the Cytoplasmic Distribution and Mitotic Stability of mtDNA

The MGT1 gene was originally identified by the mgt1-1 mutation that reduces suppressiveness in matings between rho⁺ cells and hypersuppressive rho⁻ cells (Zweifel and Fangman, 1991). In the work reported here, we used alleles, called Δmgt1, in which the MGT1 open reading

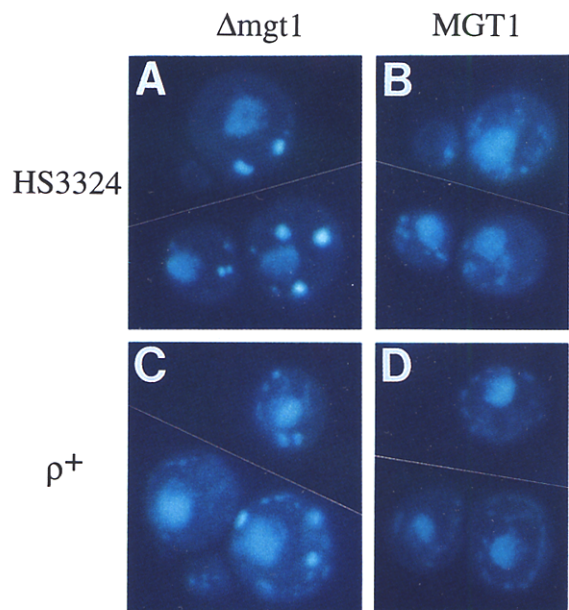Figure 1. Distribution of Chondriolites in MGT1 and Δmgt1 Cells

Cells growing exponentially in glucose medium were stained with DAPI and then examined with the fluorescence microscope. (A) Δmgt1 rho⁻ HS3324 cells, (B) MGT1 rho⁻ HS3324 cells, (C) Δmgt1 rho⁺ cells, (D) MGT1 rho⁺ cells.

frame was deleted and replaced by either the LEU2 or URA3 gene to observe the null phenotype (see Experimental Procedures). In matings between a rho⁺ MGT1 haploid and a hypersuppressive rho⁻ MGT1 haploid, greater than 95% of the diploid clones formed by the zygotes contain only rho⁻ mtDNA and are thus respiration deficient. When both haploid parents in a mating contain the Δmgt1 allele, the percent of respiration-defective diploid clones decreases to less than 1%, demonstrating that in the absence of the MGT1 gene product there is greatly reduced transmission of the hypersuppressive rho⁻ mtDNA to the zygotic progeny.

In conjunction with mating tests for suppressiveness, we examined the distribution of mtDNA in the haploid cells by 4′,6-diamidino-2-phenylindole (DAPI) staining and fluorescence microscopy. In addition to the nucleus, this DNA stain reveals chondriolites, distinctly staining foci indicative of mtDNA, in the cytoplasm of yeast cells (Williamson and Fennell, 1979). Deletion of the MGT1 gene has a dramatic effect on the cytoplasmic staining of rho⁻ cells: the mtDNA is aggregated into a small number of large chondriolites. MGT1 rho⁻ cells, such as those with the rho⁻ HS3324 mtDNA, typically have many chondriolites (Figure 1B), suggesting multiple discrete units of mtDNA. In the majority of Δmgt1 rho⁻ cells (Figure 1A), only a few very bright chondriolites are seen, instead of the more diffuse punctate pattern found in MGT1 rho⁻ cells. The high intensity fluorescence of chondriolites in the Δmgt1 cells suggests that the amount of mtDNA per rho⁻ cell is unaffected by mutation of MGT1. Indeed, as found previously for the mgt1-1 allele, quantitative hybridization analysis shows that isogenic MGT1 and Δmgt1 strains contain, within

10%, the same amount of HS3324 mtDNA per cell (see Experimental Procedures). The pronounced aggregation into a smaller number of cytological structures was also observed with two other rho⁻ mtDNAs: the hypersuppressive HS8-3 and the nonsuppressive 23-3. The Δmgt1 allele has a more subtle effect on the cytoplasmic staining of rho⁺ mtDNA. The diffuse punctate mtDNA staining found in MGT1 rho⁺ cells (Figure 1D) is still present in Δmgt1 rho⁺ cells. However, a few (less than 10%) of the Δmgt1 rho⁺ cells also contain large chondriolites (Figure 1C). Although the limited resolution of the DAPI staining prevents us from making a precise quantitative measurement of the number of chondriolites in rho⁺ and rho⁻ cells, our cytological observations suggest that deletion of MGT1 affects the mtDNA distribution in rho⁻ cells to a greater extent than the mtDNA distribution in rho⁺ cells.

If the relative number of chondriolites seen by DAPI staining reflects the units of mtDNA that are available for segregation into progeny cells, then we expect that transmission of the mitochondrial genome in Δmgt1 cells will be hampered during mitotic cell divisions. Owing to the random partitioning of a smaller number of segregating units, a Δmgt1 strain would produce daughter cells without mtDNA at an elevated frequency compared with an MGT1 strain. Consistent with this idea is the observation that deletion of MGT1 slightly elevates the frequency of respiration-deficient cells in rho⁺ cultures (Kleff et al., 1992). To determine the effect of deleting MGT1 on the maintenance of rho⁻ mtDNA, individual clones of rho⁻ strains were examined by DAPI staining and fluorescence microscopy. Three rho⁻ strains were included in this study: two hypersuppressive rho⁻ strains (HS3324 and HS8-3) and a nonsuppressive rho⁻ strain (23-3). Colonies from cells with either the MGT1 allele or the Δmgt1 allele were used to inoculate liquid medium containing glucose, grown for approximately ten generations, harvested, stained with DAPI, and examined by fluorescence microscopy. Only those cultures that contained some cells with chondriolites, and thus must have arisen from a cell that contained mtDNA, were included in the final calculations. Ten such cultures were examined for each strain, with a minimum of 150 cells scored for each. For the MGT1 rho⁺ strain, the fraction of cells without detectable chondriolites was low (2.3% ± 0.7%), and this fraction increased about 3-fold (7.6% ± 1.9%) when MGT1 was deleted. In the MGT1 background, the fraction of cells without detectable chondriolites was low for all three of the rho⁻ strains examined (between 2%–4%). The fraction of cells without chondriolites in the Δmgt1 background was increased about 3-fold for HS3324 (10% ± 4.0%), over 20-fold for HS8-3 (81% ± 16%), and about 10-fold for 23-3 (43% ± 8%). Although there is considerable variation among the different rho⁻ strains in the fraction of cells lacking chondriolites, in part due to different nuclear backgrounds, all of these cultures showed clearly elevated levels of mtDNA loss in response to the MGT1 deletion. Since the Δmgt1 mutation causes cytoplasmic aggregation and increased mitotic loss of both hypersuppressive and nonsuppressive rho⁻ mtDNAs, a rep sequence is not required for these effects.

The effect of the MGT1 on maintenance of mtDNA was confirmed by measuring directly the rate of mtDNA loss per division for rho⁺ cells and for HS8-3 rho⁻ cells. This analysis was done using four strains whose nuclear genomes are identical except for their MGT1 alleles. Mother and daughter cells were separated on an agarose plate by microdissection and allowed to form colonies. These colonies were then analyzed by DAPI staining. A loss event was detected as a division in which one cell produced a colony of cells containing chondriolites and the other produced a colony of cells without chondriolites. In MGT1 rho⁺ cells, <1% of divisions resulted in mtDNA losses (Table 1). No increase in loss rate was detected in isogenic Δmgt1 rho⁺ cells; however, about 3% of these cell divisions resulted in a clone in which the mtDNA was poorly transmitted such that the colony had only 3%–20% of cells with detectable chondriolites. Such divisions were not observed (<1%) in the MGT1 rho⁺ strain. Deletion of MGT1 had a much more dramatic effect on the HS8-3 rho⁻ mtDNA. In MGT1 HS8-3 rho⁻ cells, mtDNA was lost in 3% of cell divisions, and the rate increased 10-fold (to 32%) in the Δmgt1 HS8-3 rho⁻ cells (Table 1). These observations on the loss of mtDNA during mitotic cell cycles support the idea that a large increase in the aggregation of rho⁻ mitochondrial genomes increases the probability that a daughter cell will receive no mtDNA during the random partitioning of cytoplasmic components associated with mitotic cell division. The microdissection results with the four isonuclear strains indicate the maintenance of rho⁻ mtDNA is more profoundly affected than that of rho⁺ mtDNA.

Deletion of MGT1 Influences the Electrophoretic Mobility of mtDNA

Recombination of mtDNA in S. cerevisiae occurs at a very high rate in both vegetative cells and zygotes (Williamson and Fennell, 1974; Sena et al., 1986; reviewed by Gingold, 1988). The level of recombination junctions will depend upon the rates of their formation and resolution. We propose that the MGT1 resolvase enzyme contributes to the resolution of these structures in the mitochondrion and may thus be involved directly in determining the number of segregating units identified cytologically as chondriolites. The model presented in Figure 2 illustrates that in the MGT1 cells there is a low level of recombination junctions present in the mtDNA. However, in the absence of MGT1

Table 1. Microdissection Analysis of mtDNA Losses per Cell Division

Genotype	Number of Divisions Analyzed[a]	Number of Losses[b]	Losses per Division
MGT1 rho⁺	185	0	<0.01
Δmgt1 rho⁺	146	0	<0.01
MGT1 HS8-3 rho⁻	71	2	0.03
Δmgt1 HS8-3 rho⁻	87	28	0.32

[a] Only those divisions are included in which at least one cell produced a colony of cells containing chondriolites.
[b] Divisions in which one cell produced a colony of cells containing chrondriolites and the other cell produced a colony with <0.2% of cells having detectable chondriolites.
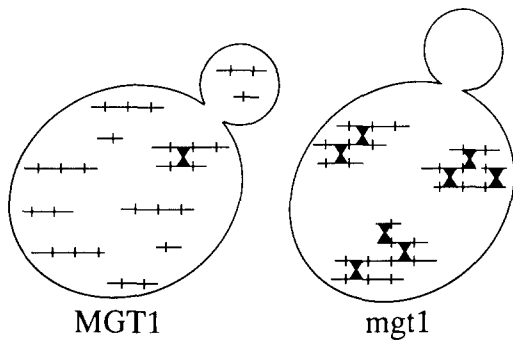
Figure 2. A Model for the Generation of mtDNA Networks by Recombination Junctions in mgt1 Mutant Cells

Two budding yeast cells (MGT1 and mgt1) are depicted. The vertical bars indicate repeat units of rho⁻ mtDNA, and hourglass symbols indicate recombination junctions. In wild-type MGT1 cells, there is a low steady-state level of recombination junctions. In the absence of MGT1 activity (mgt1), there is a pronounced increase in the number of unresolved recombination junctions and a corresponding decrease in transmission of mtDNA to the bud.

activity, the level of unresolved recombination junctions increases markedly. As a consequence, more mtDNA molecules are physically linked together as part of a branched network, and thus, the number of independently segregating units of mtDNA in the cell decreases. Because much of the rho⁻ mtDNA is present as large, tandemly repeated arrays (Fangman et al., 1989), a single unresolved recombination junction could tie together a very large number of repeats. Furthermore, the rho⁻ mtDNA is expected to be more affected by decreased levels of the MGT1 resolvase than the rho⁺ mtDNA because the amplification of short mtDNA sequences retained in rho⁻ cells will increase the chance that homologous sequences will pair and initiate recombination.

The possibility that the MGT1 gene product influences the frequency of recombination junctions in mtDNA molecules was examined directly using agarose gel electrophoresis. Purified mtDNA was isolated from MGT1 HS8-3 rho⁻ and Δmgt1 HS8-3 rho⁻ cells and separated on agarose gels. Figure 3 is an autoradiogram of uncut and EcoRV-digested HS8-3 rho⁻ mtDNA probed with the 2.0 kb EcoRV fragment from the HS8-3 repeat. Analysis of uncut HS8-3 rho⁻ mtDNA from MGT1 cells reveals a band of monomeric 4.6 kb circles and a prominent, broad smear of DNA molecules (Figure 3, lane 1). As observed previously for rho⁻ mtDNAs (Fangman et al., 1989), the broad smear consists of a continuum of linear molecules, which are tandem arrays of the rho⁻ mtDNA sequence, with sizes extending to >20 kb. When uncut HS8-3 rho⁻ mtDNA from Δmgt1 cells is examined, in contrast with the mtDNA from MGT1 cells, a large fraction is retained in the well (Figure 3, lane 2). Quantitation of the radioactivity shows 30% of the total sample in lane 2 to be retained in the well. Since even very large linear molecules enter the gel during electrophoresis (Fangman, 1978) and since protease and RNase treatments did not reduce the amount of material in the well (data not shown), we conclude that the mtDNA remains in the well as a result of some structural change. Further-
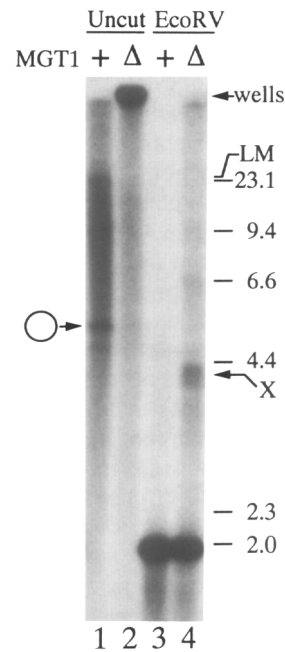

Figure 3. Effect of the Δmgt1 Mutation on the Mobility of HS8-3 rho⁻ mtDNA in Agarose Gels

Lanes 1 and 2 contain uncut mtDNA isolated from MGT1 (+) and Δmgt1 (Δ) cells. Lanes 3 and 4 contain EcoRV-digested mtDNA. Southern blots were probed with the 2.0 kb fragment from the 4.6 kb HS8-3 repeat. The position of the 4.6 kb nicked circular monomer DNA is indicated by a circle with an arrow pointing to lane 1. The faint band in lane 1 just below the monomer nicked circle is the 4.6 kb linear molecule that presumably arose by breakage of monomer circles. The positions of size markers are indicated in kilobases on the right. LM (limiting mobility) is the migration position of all linear molecules larger than about 25 kb (Fangman, 1978). X indicates a species of DNA with an apparent size of 4.0 kb. It corresponds to the X-shaped structures in Figure 5.

more, the DNA in the well does not behave as catenated circles, since treating the samples with topoisomerase II fails to reduce the amount of material retained there (see Experimental Procedures). Digestion with EcoRV, which cleaves the HS8-3 rho⁻ mtDNA into two fragments (2.0 kb and 2.6 kb), releases a large amount of the material from the well (Figure 3, lanes 3 and 4). In addition to the unit length 2.0 kb fragment, there is the appearance of a new band of HS8-3 rho⁻ mtDNA whose mobility shows its size to be equivalent to that of a 4.0 kb fragment. This material is not eliminated by further incubation with EcoRV. Its apparent mass is consistent with a structure in which two DNA molecules, each of about 2.0 kb, are joined by a recombination junction.

The retention of large amounts of mtDNA in the wells of the agarose gel was also observed for HS3324 rho⁻ mtDNA and 23-3 rho⁻ mtDNA isolated from Δmgt1 cells. In these two cases, gel analysis showed that even for MGT1 cells some uncut mtDNA was retained in the well, although the fraction retained was higher still for mtDNA from Δmgt1 cells (data not shown). A higher level of branched molecules in HS3324 and 23-3 mtDNA from MGT1 cells compared with HS8-3 mtDNA from MGT1 cells is not surprising, considering the relative sizes of their
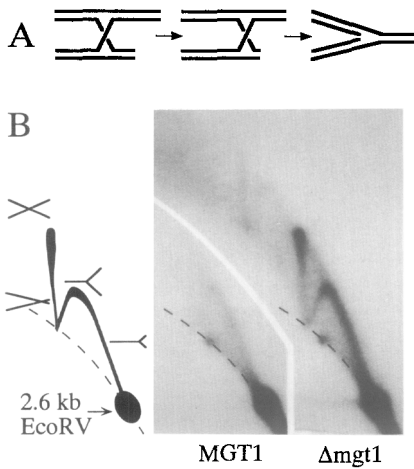
Figure 4. An Increase in Branched mtDNA Structures in *rho⁻ Δmgt1* Cells

(A) Generation of Y-shaped structures by branch migration of recombination junctions. When one of the branches in an X-shaped molecule is shorter than its homolog, migration of the crossover to the break results in a stable Y structure.

(B) Increase in branched molecules of HS8-3 *rho⁻* mtDNA in *Δmgt1* cells. The left panel is a cartoon of the expected 2D gel patterns for X and Y DNA structures. The first dimension of electrophoresis is left to right; the second dimension is top to bottom. An arc of Y molecules and a spike of X molecules are shown as filled lines. The dashed line describes the arc of linear molecules. The spot of linear 2.6 kb molecules is indicated. The center and right panels show 2D gel analysis of the 2.6 kb EcoRV fragment from HS8-3 mtDNA probed with the 2.6 kb EcoRV fragment. Center panel, HS8-3 mtDNA from *MGT1* cells; right panel, HS8-3 mtDNA from *Δmgt1* cells.

respective repeats. Since *rho⁻* mtDNAs are all present at about the same mass per cell, the 963 bp sequences of HS3324 mtDNA are present at about a 5-fold greater abundance, and the 1350 bp sequences of 23-3 mtDNAs are present at about 3-fold greater abundance compared with the 4.6 kb HS8-3 repeat. The elevated level of linked HS3324 and 23-3 mtDNA molecules can be accounted for by the increased opportunity for homologous recombination.

Linked mtDNA Structures Identified by Two-Dimensional Electrophoresis

Our model predicts that the anomalous band of mtDNA produced by restriction enzyme digestion (Figure 3, lane 4) contains X-shaped molecules: two linear DNA molecules joined by a recombination junction. To identify linked structures, two-dimensional (2D) gel electrophoresis was performed on restriction-digested mtDNA samples. The first dimension of electrophoresis minimizes the effect of shape on mobility and thus separates the DNA molecules mainly by differences in mass. The second dimension exploits the difference in shape between DNA molecules by using higher voltage, higher agarose concentrations, and ethidium bromide. The dashed arc illustrated in the left panel of Figure 4B shows the migration of linear molecules, which have the simplest structure and therefore the fastest mobility in the second dimension. Branched molecules, which are complex in structure, migrate slower

than linear molecules in the second dimension, and unique X-shaped and Y-shaped DNA molecules can be easily distinguished by their mobility pattern on a 2D gel (Brewer and Fangman, 1987; Brewer et al., 1988; Friedman and Brewer, 1995). The population of X-shaped molecules, with the recombination junction at different positions along the restriction fragment, migrate with about the same mobility in the first dimension, since they are of the same mass. In the second dimension, these isomers are resolved into a nearly vertical spike. The position of a molecule on the spike depends on the distance of the crossover from the ends. DNA molecules with the recombination junction in their middle migrate most slowly, and those with the junction at one of their ends migrate most rapidly, since they have only very short branches. Y-shaped molecules, with two equal-sized branches, range in size from 1 unit length to 2 unit lengths in mass and are resolved as an arc in the second dimension (Figure 4B, left panel). Low levels of both X and Y structures were originally observed in restriction-digested HS8-3 *rho⁻* mtDNA by electron microscopy (Sena et al., 1986). These two types of nonlinear molecules were subsequently resolved by 2D gel electrophoresis (Brewer et al., 1988). Since an appreciable amount of random ends are present in a population of *rho⁻* mtDNAs (Fangman et al., 1989), branch migration along X-shaped molecules will produce Y-shaped molecules (Figure 4A). Therefore, both types of branched structures are expected to be elevated in mtDNAs isolated from *Δmgt1* cells. We wished to determine whether the absence of the *MGT1* gene product causes X and Y structures to accumulate, as might be expected if resolution of recombination junctions is impaired.

HS8-3 *rho⁻* mtDNA was isolated from *MGT1* and *Δmgt1* cells, digested with EcoRV, and separated by 2D gel electrophoresis. After transfer to a nylon membrane, the mtDNA was probed with the 2.6 kb EcoRV fragment. Branched structures are greatly elevated in *Δmgt1* cells, relative to the spot of unbranched 2.6 kb molecules (Figure 4B, center and right panels). The most prominent nonlinear structure is the nearly vertical spike of 5.2 kb molecules that we interpret to represent the population of X-shaped molecules resulting from two mtDNA fragments held together by a recombination junction. In addition to the increase in X forms in the *Δmgt1* cells, there is also a substantial increase in Y-shaped molecules. An increase in branched structures, both X forms and Y structures, was consistently found on probing a number of different restriction fragments along the 4.6 kb HS8-3 repeat. This observation suggests that recombination junctions are distributed throughout the 4.6 kb repeat of the *rho⁻* genome. 2D gel analyses of HS3324 *rho⁻* mtDNA and 23-3 *rho⁻* mtDNA also showed a large increase in branched structures upon deletion of the *MGT1* gene (data not shown).

If the overall density of recombination junctions is increased in *Δmgt1* cells, then the probability of a given *rho⁻* repeat being linked by junctions to more than one other repeat should also increase. Restriction enzyme digestion of such multiply branched molecules should produce higher molecular weight structures more complex than simple Ys and Xs. Such complex structures are indeed
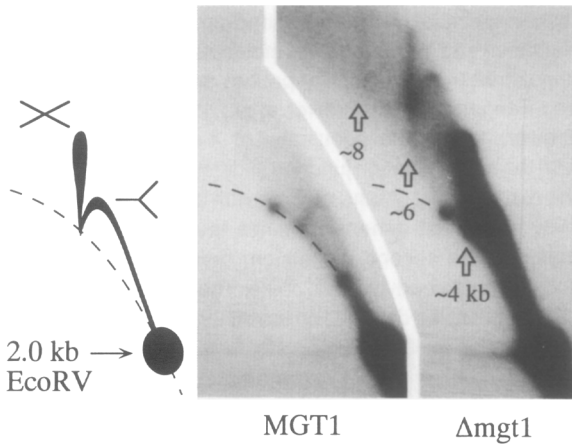
Figure 5. Complex Branched Structures in Δmgt1 Cells

(Left) A cartoon of the expected 2D gel patterns for simple Xs and Ys (see Figure 4).

(Center and right) 2D gel analysis of sequences in the 2.0 kb EcoRV fragment from HS8-3 mtDNA probed with the 2.0 kb EcoRV fragment from the HS8-3 repeat. HS8-3 mtDNA from MGT1 cells (center panel). HS8-3 mtDNA from Δmgt1 cells (right panel). The open arrows indicate vertical spikes; those labeled ~6 and ~8 are thought to be composed of three and four 2 kb fragments, respectively, joined by recombination junctions.

Figure 6. Increase in X- and Y-Shaped Molecules in rho⁺ mtDNA upon Disruption of MGT1

2D gel analysis of EcoRV-digested rho^+ mtDNA probed with the 2.0 kb EcoRV fragment from the HS8-3 repeat. rho^+ mtDNA from MGT1 cells (left panel). rho^+ mtDNA from Δmgt1 cells (right panel). Arrows indicate the spike of Xs and the arc of simple Ys.

revealed by longer exposures of the 2D gel blots. For example, probing for sequences in the 2.0 kb EcoRV fragment of HS8-3 rho^- mtDNA reveals discrete spikes and arcs of material migrating more slowly than simple Ys and Xs in the first and second dimensions (Figure 5, right panel). The patterns are similar in shape to those for the arc of 2.0–4.0 kb simple Ys and the spike of 4 kb Xs. The sizes of the two additional vertical patterns (arrows in Figure 5, right), estimated using linear markers present in the first dimension gel, are about 3.6 kb, 5.5 kb, and 7.6 kb. These sizes are close to the 4 kb, 6 kb, and 8 kb values expected for complexes composed of two, three, and four 2 kb restriction fragments linked by recombination junctions. Very low levels of these complex structures can also be observed after long exposure of 2D gels containing digested HS8-3 rho^- mtDNA from MGT1 cells. These results suggest that the multiple, tandemly repeated rho^- molecules of mtDNA are linked together by recombination junctions into a large network of DNA.

The DAPI staining results with the Δmgt1 rho^+ cells (see Figure 1C) revealed some cells with chondriolites that were larger and brighter than those in MGT1 rho^+ cells. To determine whether the Δmgt1 allele also affects the level of branched structures in the rho^+ mitochondrial genome, we examined the 2D gel pattern produced from mtDNA isolated from MGT1 rho^+ and Δmgt1 rho^+ cells. 2D gel analysis of EcoRV-digested rho^+ mtDNA, probed with the 2.0 kb fragment from HS8-3 rho^- mtDNA, shows that there is indeed an increased level of X and Y structures in rho^+ mtDNA isolated from Δmgt1 cells compared with MGT1 cells (Figure 6). The rho^+ genome, however, accumulates less branched material than does the HS8-3 rho^- genome (Figures 4 and 5) upon deletion of MGT1. The higher level of recombination junctions in the HS8-3
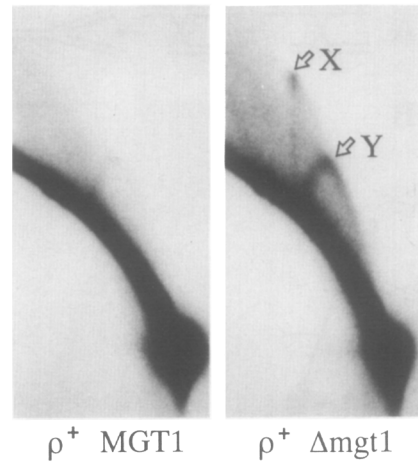
rho^- genome (4.6 kb unit length) can be explained by a higher rate of formation of recombination junctions in the small amplified genome.

Overexpression of MGT1 Reduces the Frequency of Branched Structures and Affects the Inheritance of a Neutral rho^- Genome

If eliminating the MGT1 gene product increases the level of branched molecules, then overproducing the MGT1 gene product would be expected to decrease the steady-state level of X- and Y-shaped molecules. We tested this hypothesis by transforming cells with a multiple copy 2 μm plasmid vector containing a functional MGT1 gene that results in a large increase in resolvase activity (Kleff et al., 1992). This plasmid (here called pMGT352) was transformed into the MGT1 HS8-3 rho^- strain. After growth under selection for maintenance of the plasmid, the mtDNA was purified, digested with EcoRV, and separated on a 2D gel similar to the experiments presented above. As a control, mtDNA was isolated from HS8-3 rho^- cells that were transformed with the plasmid vector alone (YEp352). Figure 7 compares the 2D gel patterns for the 2.0 kb EcoRV fragment of HS8-3 mtDNA isolated from the YEp352 transformants and for HS8-3 mtDNA isolated from the pMGT352 transformants. Equal amounts of the two samples were loaded, as seen by the equivalent intensities of the spot of linear 2.0 kb EcoRV fragment. A comparison of the two panels demonstrates that there are fewer X-shaped molecules in mtDNA from cells overexpressing MGT1, indicating that the level of MGT1 gene product influences the steady-state level of mtDNA recombination junctions in the cell. The presence of a substantial amount of simple Y molecules, even upon overexpression of MGT1, may mean that in wild-type MGT1 cells these structures are produced mainly by replication.
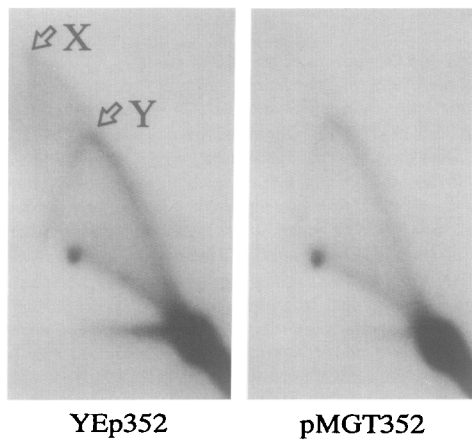
YEp352   pMGT352

Figure 7. Decrease in X-Shaped Molecules due to Overproduction of the *MGT1* Gene Product

2D gel analysis of sequences in the 2.0 kb EcoRV fragment from HS8-3 *rho⁻* mtDNA. The left panel shows HS8-3 *rho⁻* mtDNA isolated from *MGT1* cells containing the multiple copy vector alone (YEp352). Arrows indicate the spike of Xs and the arc of simple Ys. The right panel shows HS8-3 *rho⁻* mtDNA isolated from *MGT1* cells containing the multiple copy vector with the *MGT1* gene inserted (pMGT352).

In matings between *rho⁺* and *rho⁻* cells, the two mtDNAs entering the zygote compete for transmission during the postzygotic divisions. The reduction in branched *rho⁻* mtDNA molecules seen when *MGT1* is overexpressed suggests that the transmission of *rho⁻* mtDNA in a *rho⁺* × *rho⁻* mating might be augmented by increasing the availability of the *MGT1* gene product. Since hypersuppressive *rho⁻* genomes are already transmitted at a high frequency, we chose to study the effects of *MGT1* overproduction on the inheritance of a nonsuppressive *rho⁻* strain. Normally, the nonsuppressive *rho⁻* genomes are rarely inherited in matings with *rho⁺* cells; fewer than 5% of the zygotic clones are made up entirely of *rho⁻* cells. Overproduction of the *MGT1* resolvase activity in a nonsuppressive *rho⁻* strain should further reduce the level of recombination junctions in the *rho⁻* mtDNA. This reduction would create more segregating units of *rho⁻* mtDNA that could compete with the *rho⁺* genome for inheritance during postzygotic divisions. To test this prediction, the nonsuppressive 23-3 *rho⁻* strain was transformed with either the 2 μm vector (YEp352) or with the vector plus the *MGT1* gene insert (pMGT352). Each of these transformed strains was mated to the *rho⁺* strain, then grown in liquid medium selective for the diploids for approximately 20 generations to allow segregation of pure mtDNA types. Diploid cells were then plated on selective plates, and at least 200 colonies were scored for respiration competence or deficiency by replica plating to media containing glycerol. Growth on the nonfermentable carbon source was used to indicate whether the diploid colony contained *rho⁺* mtDNA. As expected for the poor inheritance of a nonsuppressive *rho⁻* genome, the frequency of respiration-deficient diploid colonies was low (2.6%) after outgrowth of zygotes from the *rho⁺* × YEp352 *rho⁻* mating. However, in the mating between the *rho⁺* strain and the pMGT352 *rho⁻* strain, the fraction of respiration-deficient diploid colonies was considerably higher

(23%). Inheritance of the 23-3 *rho⁻* mtDNA in these respiration-deficient diploid clones was verified by DNA hybridization analysis from ten independent colonies. We conclude that the presence of a multicopy plasmid containing the *MGT1* gene can enhance the zygotic inheritance of a nonsuppressive *rho⁻* genome. This result is consistent with our model that the activity of the *MGT1* gene product governs the level of recombination junctions, thereby influencing the number of heritable units of the *rho⁻* genome.

Discussion

Deletion of the *MGT1* gene in yeast cells affects a variety of mtDNA phenotypes, including mtDNA structure, cytological distribution, loss from the cell, and inheritance in mating. All of these observations can be explained by the cruciform cutting activity associated with the *MGT1* gene product. We propose that the loss of *MGT1* enzymatic activity results in an increase in unresolved recombination junctions. These branched structures effectively create large mtDNA networks, as indicated by the DNA molecules that fail to leave the well during electrophoresis. These networks of mtDNA molecules contribute to the increased brightness of cytoplasmic chondriolites and to the decrease in their numbers. If the networks are the heritable units of mtDNA that are transmitted during mitotic cell division, then a decrease in the number of units would increase the probability that daughter cells receiving no mtDNA will be produced. In *rho⁻* mtDNAs, in which amplification results in a high concentration of the short retained sequences, there will be a higher rate of homologous recombination. Thus, in the absence of the *MGT1* gene activity, the tandem multimers of *rho⁻* genomes experience a greater decline in segregating units than do the *rho⁺* genome, and this decrease in numbers results in a higher rate of loss.

The *MGT1* gene was identified genetically through the effect of the mutant allele, *mgt1-1*, on the transmission bias of *rho⁻* mitochondrial genomes that contain a *rep* sequence when they compete in a zygote with the wild-type *rho⁺* genome. The null allele, *Δmgt1*, gives a similar loss of inheritance bias, indicating that the preferential inheritance of hypersuppressive *rho⁻* genomes requires the wild-type *MGT1* cruciform cutting endonuclease. From this simple result, it is tempting to speculate that the *MGT1* enzyme interacts directly with the *rep* sequences to bring about the biased inheritance. However, our analysis of *rho⁻* mitochondrial DNAs in *Δmgt1* strains shows that the structure of hypersuppressive and nonsuppressive genomes are similarly affected. It is therefore unlikely that the *MGT1* enzyme plays a direct role in the creation of the inheritance bias, but contributes indirectly by its effects on the structure of the mitochondrial DNAs that are segregating in the zygote. In the *Δmgt1* cells, the number of *rho⁻* mtDNA segregating units is greatly reduced, while the number of *rho⁺* mtDNA segregating units is less affected. This highly unfavorable ratio of segregating units cannot be compensated by the transmission advantage normally provided by the high density of *rep* sequences in hypersuppressive *rho⁻* mtDNAs. Thus, in the absence of

the *MGT1* gene product, the *rho*+ genome is preferentially transmitted to the diploid progeny during postzygotic divisions. Consequently, there is a decreased probability of the hypersuppressive *rho*⁻ mtDNA being inherited in postzygotic divisions when in competition with the *rho*+ genome.

Even in the *MGT1* cell, the level of recombination junctions is presumably high enough to contribute to the aggregation of mtDNA molecules. Such an aggregation may help to explain the paradox of rapid segregation of the large number of mitochondrial genomes during postzygotic cell divisions. It is possible that the generation of pure mtDNA types in *rho*+ × *rho*+ matings is due, at least in part, to the mtDNA molecules being linked together by recombination junctions creating a smaller number of heritable units. The finding that overproduction of the *MGT1* gene product increases the inheritance of a neutral *rho*⁻ mtDNA in a mating with a *rho*+ mtDNA supports this idea. An important prediction is that increasing and decreasing the level of the *MGT1* resolvase in *rho*+ × *rho*+ matings will extend and decrease, respectively, the number of divisions required to generate cells with pure mtDNA types. We are currently investigating this possibility.

The role that the resolution of recombination junctions plays in the segregation of high copy number genomes is not unique to yeast mtDNA. The segregation of extrachromosomal elements is well documented for a number of bacterial plasmids (reviewed by Nordstrom and Austin, 1989). In these examples, plasmid transmission is augmented by maintaining the number of randomly partitioning units at a maximum level. The resolution of a site-specific recombination junction within a dimeric circular plasmid generates two monomer plasmids, thus ensuring a maximum copy number of independently segregating plasmid molecules. In the yeast mitochondrial system, the rate of resolution of recombination junctions also appears to determine the number of potential segregating units. In addition, we propose that the rapid transition from heteroplasmy to homoplasmy in an apparent multicopy genetic system may occur because a low level of unresolved recombination junctions results in fewer independently segregating mtDNA molecules. The genetic drift associated with a limited number of randomly segregating molecules may also explain how a small number of defective genomes can establish themselves in a mitotically dividing cell line over a short period of time.

The connection between the recombination and segregation of mtDNA established in this work may exist in other organisms in which mtDNA recombination occurs. Although few organisms allow the genetic analysis of segregation of mtDNA with the ease of yeast zygotes, molecular analyses of the kind reported here could provide evidence in other systems for DNA networks that are held together by recombination junctions. Recombination is currently believed not to occur in the mtDNA of somatic cells of mammals (Wallace, 1992). However, the data on this point are not conclusive, and 2D gel analyses would provide a direct and sensitive assay for the detection of recombination junctions in dividing cells.

Experimental Procedures

Plasmids and Disruption of the *MGT1* Gene

Plasmid pMGT352 (originally termed pSub8-2) consists of the 2.1 kb SphI–SacI fragment containing the *MGT1* gene inserted in plasmid YEp352 (Kleff et al., 1992). Chromosomal null alleles of *MGT1*, called *Δmgt1*, were made by recombinational replacement of the wild-type gene with recombinant plasmids in which the open reading frame was deleted and replaced with a selectable marker. A previously described construct was used to make *Δmgt1::LEU2* null alleles (Kleff et al., 1992). The chromosomal *mgt1::URA3* null alleles were made with plasmid pURA3Δmgt that was created by the following procedure. First, the NarI–AatII fragment was removed from pMGT352 (Kleff et al., 1992). Second, the recircularized plasmid was digested with SalI to remove the *MGT1* coding sequence and was treated with T4 DNA polymerase to generate blunt ends. Third, the 1.4 kb FspI–HpaI fragment containing the *URA3* gene was inserted into the blunt end site. To generate chromosomal *mgt1::URA3* null alleles, pURA3Δmgt was cut with SphI and SacI and used to transform *ura3* yeast strains. The gene replacements were confirmed by showing that a 1200 bp HaeIII fragment replaced the wild-type 2440 bp HaeIII fragment upon probing with the SphI–SalI *MGT1* fragment isolated from pMGT352.

Yeast Strains and Mitochondrial Genomes

All of the isogenic *Δmgt1* strains used in this study were generated by recombinational replacement (see above). The *MGT1 rho*+ strain used was 127 (*MATα ade1 ade2 ura3 leu2 trp1 CYH*ʳ CANʳ SAPʳ, an A364A derivative supplied by R. Sclafani), and the *Δmgt1 rho*+ strain was 127 *mgt1::LEU2*. The *MGT1 rho*⁻ HS8-3 strain was SG72-7b (*MATα leu2 trp1 ura3 ade1*), which was made by crossing 127 × α-4 *rho*⁻ 8-3 (*MATα trp4 leu1*, Sena et al., 1986) and sporulating the zygotes. The *Δmgt1 rho*⁻ HS8-3 strain was SG72-7b *mgt1::LEU2*. A *rho*+ strain that is isonuclear with the *MGT1 rho*⁻ HS8-3 strain was made from SG72-7b by cytoduction (Berlin et al., 1991). A *Δmgt1 rho*+ derivative of this cytoductant was then made by introducing the *mgt1::LEU2* allele. The *MGT1 rho*⁻ HS3324 strain was constructed by cytoduction of the *rho*⁻ genome from strain KL14-4a/HS3324 (Blanc, 1984) into the 127 nuclear background. The *Δmgt1 rho*⁻ HS3324 strain was 127 *mgt1:: LEU2*/HS3324. The *MGT1 rho*⁻ 23-3 strain was DL1/23-3 (*MATα ade1 ura3 lys2 trp1*), and the *Δmgt1 rho*⁻ 23-3 strain was DL1 *mgt1::URA3*/ 23-3. The strain DL1 was created by selecting a 5-fluoroorotic acid–resistant mutant from a nonmutagenized culture of strain 23-3 (*MATα ade1 lys2 trp1*; Ooi and Nagley, 1986) containing the mitochondrial genome here called 23-3.

The HS3324 *rho*⁻ genome consists of a 963 bp repeat that contains *rep2* (Fangman et al., 1989). The HS8-3 *rho*⁻ genome consists of a 4.6 kb repeat that contains *rep1* (Sena et al., 1986). The neutral 23-3 *rho*⁻ genome consists of a 1350 bp repeat that includes the *oli1* gene and flanking regions (Ooi and Nagley, 1986).

Cytology, Suppressiveness Testing, and Gel Electrophoresis

Cells were prepared for DAPI staining using methanol fixation (Williamson and Fennell, 1979). Suppressiveness tests (Zweifel and Fangman, 1991), isolation of mtDNA (Huberman et al., 1987; Fangman et al., 1989), and 2D agarose gel electrophoretic analysis (Brewer and Fangman, 1987; Friedman and Brewer, 1995) have been described previously. Southern blots of agarose gels were hybridized with ^{32}P-labeled probes made by random-primed synthesis of gel-purified cloned mtDNA fragments. Plasmids containing the 2.0 kb and 2.6 kb EcoRV fragments of the HS8-3 mitochondrial genome were created by inserting these fragments into the EcoRV site of pBluescript SK(+) (Stratagene). Plasmid pEW4 contains the 2.0 kb fragment, and pEW6 contains the 2.6 kb fragment. The amount of DNA retained in the wells of 1D gels was determined by loading samples in molten low melting agarose to eliminate loss from the wells following electrophoresis. Comparison of the amount of mtDNA per cell in isogenic *MGT1* and *Δmgt1* strains was made by quantitation of Southern blots of DNA prepared from exponentially growing cultures using a phosphorimager (Molecular Dynamics, Incorporated). The amount of mtDNA was determined relative to the amount of nuclear DNA in a gel lane by probing

for a mtDNA sequence and for the nuclear 2.3 kb EcoRV fragment containing ARS1. Four independent samples were analyzed for each strain.

Topoisomerase II Experiment

Trypanosome kinetoplast DNA (kDNA), which consists of a network of catenated circles (Marini et al., 1980), was used as a positive control. A mixture of 70 ng of purified, uncut HS8-3 mtDNA from Δmgt1 cells (see Figure 3, lane 2) and 50 ng of kDNA (a gift from T. deVos) was reacted with 2 U of topoisomerase II (a type II topoisomerase from fly embryos, purchased from United States Biochemical Corporation). The reaction conditions were 10 mM Tris–HCl, 50 mM NaCl, 50 mM KCl, 5 mM MgCl$_2$, 0.1 mM EDTA, 15 µg of BSA/ml, 1 mM ATP (pH 7.9) in a total volume of 10 µl for 90 min at 37°C. Probing after electrophoresis (as in Figure 3) showed that no detectable HS8-3 mtDNA was released from the well, whereas 77% of the kDNA was released. The faster migrating kDNA was shown to be circular DNA by the effect that altering the agarose concentration had on electrophoretic mobility.

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