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Analysis of Repeat-Mediated Deletions in the Mitochondrial Genome of *Saccharomyces cerevisiae*

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ABSTRACT

Mitochondrial DNA deletions and point mutations accumulate in an age-dependent manner in mammals. The mitochondrial genome in aging humans often displays a 4977-bp deletion flanked by short direct repeats. Additionally, direct repeats flank two-thirds of the reported mitochondrial DNA deletions. The mechanism by which these deletions arise is unknown, but direct-repeat-mediated deletions involving polymerase slippage, homologous recombination, and nonhomologous end joining have been proposed. We have developed a genetic reporter to measure the rate at which direct-repeat-mediated deletions arise in the mitochondrial genome of *Saccharomyces cerevisiae*. Here we analyze the effect of repeat size and heterology between repeats on the rate of deletions. We find that the dependence on homology for repeat-mediated deletions is linear down to 33 bp. Heterology between repeats does not affect the deletion rate substantially. Analysis of recombination products suggests that the deletions are produced by at least two different pathways, one that generates only deletions and one that appears to generate both deletions and reciprocal products of recombination. We discuss how this reporter may be used to identify the proteins in yeast that have an impact on the generation of direct-repeat-mediated deletions.

THE mitochondrion of eukaryotic cells is well known as the powerhouse of the cell. The mitochondrial genome is essential for respiration and codes for components of the electron transport chain. Also encoded within the mitochondrial genome are ribosomal and transfer RNAs necessary for the synthesis of these proteins. All of the remaining proteins that act in the mitochondria are encoded in the nuclear genome, translated in the cytoplasm, and imported into the mitochondria. The budding yeast *Saccharomyces cerevisiae* is a facultative anaerobe that can grow in the absence of respiration when provided with a fermentable carbon source and can therefore tolerate large deletions of its mitochondrial genome. In addition, yeast mitochondria can be transformed with DNA that can integrate into the mitochondrial genome. This has made *S. cerevisiae* an excellent model system for the study of genes responsible for maintenance of an active respiration state.

Yeast strains can undergo large deletions of their mitochondrial DNA. Such strains are termed ρ^-, and analysis of the deletion junctions suggests that at least some of these strains arise by direct-repeat-mediated deletion events (Dujon 1981). In humans, mitochondrial DNA deletions and point mutations accumulate in an age-dependent manner (Cortopassi and Arnheim 1990; Chomyn and Attardi 2003). Specifically, a 4977-bp deletion flanked by direct repeats at its junctions has been commonly observed in aging individuals. This deletion has been found to accumulate in spontaneous Kearns-Sayre syndrome and chronic external ophthalmoplegia patients (Schoffner *et al.* 1989). In addition, a 50-bp mitochondrial D-loop deletion has been detected in gastric adenocarcinomas. Multiple deletions in a single muscle mitochondrial DNA region have been detected in patients with mitochondrial myopathies. All of these deletions were found to be flanked by directly repeated sequence (Zeviani *et al.* 1989; Burgart *et al.* 1995). In fact, 66% of the mitochondrial DNA deletions reported so far have direct repeats at their junctions, implying that direct-repeat-mediated deletions give rise to these excisions (Bianchi *et al.* 2001). Zeviani *et al.* (1989), in their study of human mitochondrial deletions, demonstrated that a trait associated with these lesions displayed Mendelian inheritance and proposed that a mutant *trans*-activating factor was responsible for these deletions. Mitochondrial DNA deletions in mitochondrial ophthalmoplegia patients are believed to result from a dominant mutation in an autosomal gene located in a region that overlaps the coding region for the mitochondrial transcription factor MtTFA (Suomalainen *et al.* 1995). Studies in human tissues displaying repeat-mediated deletion of mitochondrial DNA have suggested that polymerase slippage during replication accounts for the deletion (Schoffner *et al.* 1989). However, no specific proteins involved in this process have been identified, nor

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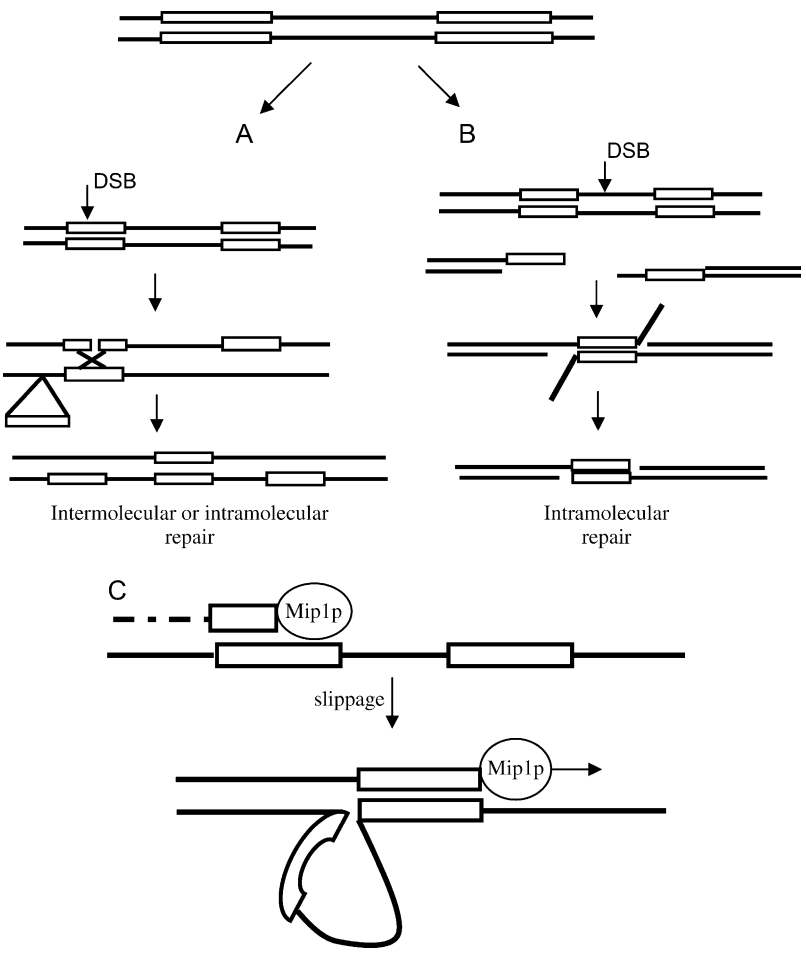

FIGURE 1.—Possible pathways for repeat-mediated deletions. The initiating event for repeat-mediated deletion events is a DSB. (A) A DSB in the repeat sequence initiates a homologous recombination-based repair pathway. If the repeat is repaired using the homologous sequence from the other repeat, it would result in a deletion in the repaired strand. Such repair events can occur within the same mitochondrial genome (intramolecular) or between two mitochondrial genomes (intermolecular). (B) A DSB in the intervening sequence between the directly repeated sequences may be repaired using SSA. This repair pathway will always be intramolecular. (C) Polymerase slippage could also account for repeat-mediated deletion events. During replication, if the nascent strand dissociates during the replication of the first repeat sequence and reanneals at the second repeat sequence, continued synthesis will result in a deletion of one direct repeat and the intervening sequence in the newly replicated strand.

have any pathways been specifically shown to function in the generation of these deletions in the mitochondria.

Deletions and rearrangements of prokaryotic and eukaryotic genomes are often associated with repeated sequences. Studies in *Escherichia coli* and yeast nuclear DNA have explored factors that cause and affect direct-repeat-associated deletions in the nuclear genome. These studies have identified more than one mechanism that can act on directly repeated sequences to generate a deletion of the intervening nonhomologous DNA. In the yeast nucleus, two pathways, single-strand annealing (SSA) and unequal sister chromatid exchange during homologous recombination (SCE), have been identified, both of which are initiated by double-strand breaks (DSBs; PRADO *et al.* 2003). When a DSB occurs in the repeat sequence, a pathway of homologous recombination-based repair is initiated. In SCE, a DSB is generated in the repeat sequence resulting in ends that can invade a homologous sequence on the sister chromatid. If the invasion occurs in misaligned sequences, it can lead to a deletion event (Figure 1A). When a DSB occurs in the intervening sequence between the repeats, SSA can be initiated. In this pathway, the break is processed to generate 3′ single-strand ends, which reveals the homologous repeat sequences. These repeat sequences can now anneal, leaving nonhomologous flaps. The flaps are then cleaved and the ends ligated, resulting in the deletion of one of the repeats as well as the intervening sequence (Figure 1B). Other than these two pathways, repeat-mediated deletion events can also arise as a result of polymerase slippage. This model predicts that, during replication, the nascent and template strands can transiently dissociate. If these strands reassociate such that the first repeat on the nascent strand anneals to the second repeat on the template strand, as shown in Figure 1C, continued synthesis will result in a deletion of one of the repeats and the sequences between the repeats on the newly synthesized strand.

In spite of decades of evidence for mitochondrial DNA recombination in yeast, no proteins essential for this process have been identified, although a handful have been shown to have activities consistent with a role in recombination. *MHR1* was first identified in a screen for mutants with deficiencies in mitochondrial recombination during mating (LING *et al.* 1995). The Mhr1 protein was subsequently shown to promote pairing of single-strand DNA with homologous double-strand DNA *in vitro*. This protein was not essential for recombination to occur, and the *mhr1-1* phenotype indicated the presence of another pairing protein (LING and SHIBATA 2002). The product of the *NUC1* gene is a protein responsible for the major 5′ to 3′ exonuclease

TABLE 1

Strains used in this study

Strain	Relevant genotype	Reference
DFS188	*MAT***a** *ura3-52 leu2-3,112 lys2 his3 arg8::hisG*	Sia *et al.* (2000)
DFS160	*MAT*α *ade2-101 leu2*Δ *ura3-52 arg8*Δ*::URA3 kar1-1* [ρ^0]	Steele *et al.* (1996)
TF236	*ino1::HIS3 arg8::hisG pet9 (op1) ura3-52 lys2 cox3::arg8^m-1*	Bonnefoy and Fox (2000)
EAS748	DFS188 Rep96::*ARG8^m*::*cox2*	This study
NPY066	DFS160 pEAS74 ρ$^+$	This study
NPY049	DFS188 Rep66::*ARG8^m*::*cox2*	This study
NPY040	DFS188 Rep48::*ARG8^m*::*cox2*	This study
NPY087	DFS188 Rep39::*ARG8^m*::*cox2*	This study
NPY088	DFS188 Rep33::*ARG8^m*::*cox2*	This study
NPY075	DFS188 Rep21::*ARG8^m*::*cox2*	This study
NPY048	DFS188 Rep96mut1::*ARG8^m*::*cox2*	This study
NPY058	DFS188 Rep96mut2::*ARG8^m*::*cox2*	This study
NPY046	DFS188 Rep96mut3::*ARG8^m*::*cox2*	This study
NPY007	DFS188 *mip1-D347A*	Phadnis and Sia (2004)
NPY068	DFS188 *mip1-D347A* Rep96*::ARG8^m::cox2*	This study
NPY025	DFS188 Rep96::*ARG8^m::cox2 mhr1-*Δ*::URA3*	This study
NPY026	DFS188 Rep96::*ARG8^m::cox2 cce1-*Δ*::URA3*	This study
NPY027	DFS188 Rep96::*ARG8^m::cox2 nuc1-*Δ*::URA3*	This study
NPY089	DFS188 Rep96::*ARG8^m::cox2 din7-*Δ*::URA3*	This study

activity in yeast (Zassenhaus *et al.* 1988) and has been proposed to be required for initiation of strand invasion (Zassenhaus and Denniger 1994). The Cce1 protein is a mitochondrial resolvase, an enzyme that cleaves recombination intermediate structures (Kleff *et al.* 1992; Ezekiel and Zassenhaus 1993). Deletion of the *CCE1* gene results in accumulation of branched structures that may be unresolved Holliday junctions and alters the dynamics of mitochondrial DNA segregation (Lockshon *et al.* 1995). The *DIN7* gene codes for a mitochondrial protein homologous to the *S. cerevisiae* Exo1p, Rad2p, and the flap endonuclease Rad27p (Mieczkowski *et al.* 1997; Fikus *et al.* 2000). Overexpression of Din7p results in a twofold increase in recombination between mutations at the *cytb* gene that are ~1 kb apart (Koprowski *et al.* 2003). Although these proteins have biochemical activities consistent with a role in recombination, their loss has not been demonstrated to result in a substantial defect in this process *in vivo*.

Our understanding of mitochondrial DNA metabolism has lagged well behind our understanding of similar processes in the nucleus. Recent progress in the development of molecular tools has made a more detailed study possible. We have generated a genetic reporter for measuring the rate of direct-repeat-associated deletions in the mitochondrial genome of *S. cerevisiae*. We report here experiments that demonstrate a very high rate of direct-repeat-mediated deletion in wild-type yeast mitochondria. We show that the size of the repeat and heterology between repeats affects the rate of recombination. We have analyzed the pathways by which these events occur and propose that as in the nucleus, multiple pathways are likely to be involved in the generation of these deletions.

MATERIALS AND METHODS

Strains and plasmids: The yeast strains used in this study are listed in Table 1. *S. cerevisiae* strains used in this study are isogenic with DFS188 (Sia *et al.* 2000). The 96-bp repeat recombination reporter plasmid was constructed by amplification of the first 99 bp of *COX2* coding sequence followed by the *ARG8^m* gene (primers available on request) into *Bam*HI-*Afl*II sites of pBluescriptSK+ to generate pEAS72. The entire *COX2* gene lacking the start codon was then cloned into the *Afl*II-*Eco*RI sites of pEAS72 to generate pEAS74. The 66-, 48-, 39-, 33-, and 21-bp repeat reporter plasmids were generated by replacing the *Bam*HI-*Afl*II fragment of pEAS74 with PCR fragment consisting of the first 66, 48, 39, 33, or 21 bp of *COX2* coding sequence fused to *ARG8^m*, to generate pEAS79, pEAS80, pEAS81, pEAS82, and pEAS83, respectively. Heterologous repeats were generated by site-directed mutagenesis of pEAS74, using the Quikchange system (Stratagene, La Jolla, CA) to generate plasmids pEAS76 (point mutations in the second repeat), pEAS85 (mutations in first repeat), and pEAS77 (mutations in both repeats). The *mhr1*-Δ, *cce1*-Δ, *nuc1*-Δ, and *din7*-Δ strains were constructed by one-step gene replacement of the wild-type gene with the *URA3* marker in the recombination reporter strain using standard methods (Sherman 1991).

Mitochondrial transformations and screening: Strain DFS160 (ρ^0 *kar1-1*) was biolistically transformed with the reporter plasmids and pRS415 bearing the *LEU2* marker. Mitochondrial transformation and mitochondrial genome integration were carried out by standard methods (Bonnefoy and Fox 2002). Nuclear transformants were selected on synthetic medium lacking leucine. Transformants were replica plated to a lawn of TF236 tester strain and incubated 8 hr to overnight to allow mating to occur and then replica plated to synthetic medium lacking arginine to identify mitochondrial transformants. Once identified, mitochondrial transformants were mated to DFS188 to integrate the reporters into the wild-type *COX2* locus. Haploid strains with the wild-type DFS188 nuclear background and carrying the recombination reporter were selected. The presence of the recombination reporter construct in the mitochondrial genome was verified by PCR and Southern blot analysis.

Measurement of mitochondrial mutation rates: Recombination reporter strains were incubated overnight at 30° in synthetic medium lacking arginine. Single colonies from the overnight culture were isolated on YPD medium after growth at 30° for 3 days. Twenty independent colonies were resuspended in 100 μl of sterile water except for the 21-bp reporter strain, for which 20 independent colonies were inoculated in 5 ml YPD and incubated overnight at 30°. Appropriate dilutions of each individual suspension were plated on YPD and YPG medium, respectively. The frequency of respiring colonies relative to viable colonies was determined after growth at 30° for 3 days. The rate of recombination per cell division was calculated using the method of the median (Lea and Coulson 1949). The experiment was performed twice for each strain.

DNA analysis: Cells were grown in synthetic medium lacking arginine and diluted into YPD medium. DNA was extracted at intervals after dilution as described (Hoffman and Winston 1987), and appropriate restriction endonuclease digested DNA and undigested DNA samples were analyzed on Southern blots. Blotted samples were probed with the 96 bp of *COX2* directly repeated sequence generated by PCR or with a 1.2-kb fragment of *ARG8*^{m}, gel isolated after digestion of pEAS72 with *Hin*dIII and *Afl*II. For the PCR assay, approximately equal amounts of genomic DNA (determined spectrophotometrically) from each of the time-point samples were used as template to amplify reciprocal products of recombination.

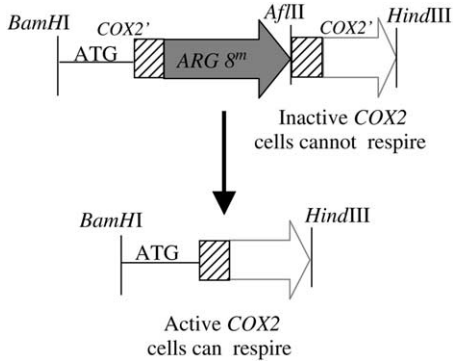

Figure 2.—The structure of the *ARG8*^{m} recombination reporter construct. The hatched boxes represent 96 bases of *COX2* sequence immediately 3′ of the ATG start. The shaded arrow represents the *ARG8*^{m} coding sequence. The entire *COX2* coding sequence lacking the start codon follows the *ARG8*^{m} gene resulting in 96 bases of directly repeated sequence flanking the *ARG8*^{m} gene. This results in an inactive *COX2* gene, after integration of the construct at the wild-type *COX2* locus in the mitochondrial genome. The cells are phenotypically Arg$^+$ and respiration deficient. These Arg$^+$ cells can undergo a repeat-mediated deletion event resulting in an active *COX2* gene and the deletion of one of the repeats along with the *ARG8*^{m} gene. The recombinants generated are respiration proficient.

RESULTS AND DISCUSSION

Homologous recombination in yeast mitochondria has been well accepted for some time; however, the presence of such activity in vertebrate mitochondria has been controversial. Several recent studies provide evidence for mitochondrial DNA recombination in mammalian cells. *In vitro* assays using mitochondrial extracts indicate that mammalian mitochondria possess homologous recombination and SSA activities (Thyagarajan *et al.* 1996). In addition, mitochondrial DNA molecules that appear to be generated by such recombination processes have been studied in cultured cells (Holt *et al.* 1997). Most recently, Kraytsberg *et al.* (2004) looked for the presence of recombinant mitochondrial genomes in the muscle tissue of an individual who had inherited paternal mitochondrial DNA. These researchers identified a number of different recombinant genomes, which consisted of both maternal and paternal mitochondrial DNA. Common breakpoints were also found, indicating hotspots for recombination (Kraytsberg *et al.* 2004). In addition, several studies have implicated direct-repeat-mediated recombination in age-related mtDNA instability in humans (Chomyn and Attardi 2003). However, studies have been hindered by the drawbacks of the mammalian cell as a model system for studying mtDNA recombination. Due to the almost universal maternal inheritance of mitochondrial DNA in mammalian cells, it is difficult to trace recombination events. Also, active mitochondria within mammalian cells cannot be transformed with exogenous DNA, making the use of mammalian cells for studying mitochondrial DNA recombination difficult. Using the genetic tools available in *S. cerevisiae*, we have constructed a reporter to study direct-repeat-mediated deletion events in the yeast mitochondrion.

Construction of the reporter: The nuclear *ARG8* gene of yeast encodes an enzyme required for arginine biosynthesis. This step in arginine synthesis occurs in the mitochondrial matrix. The Arg8 protein is normally translated in the cytoplasm and imported into the mitochondrion. Steele *et al.* (1996) generated a reporter in which the *ARG8* gene was mutated to display the genetic code of a mitochondrially encoded gene (Steele *et al.* 1996). When expressed as a translational fusion with a mitochondrial gene, this variant, *ARG8*^{m}, can complement a nuclear *ARG8* deletion. Our reporter consists of a translational fusion of *ARG8*^{m} with the first 99 bp of the *COX2* coding sequence. The entire *COX2* gene, lacking the ATG start codon, immediately follows this gene fusion. This results in the functional *ARG8*^{m} gene flanked by 96-bp direct repeats. Integration of this construct at the *COX2* locus of the mitochondrial genome causes nuclear *arg8*-Δ cells to be phenotypically Arg$^+$ and respiration deficient due to interruption of the *COX2* gene (Figure 2). Recombination or slippage events that occur between the repeated sequences will restore the *COX2* gene and the ability of the strains to respire, allowing the cells to grow on medium containing glycerol as the sole carbon source. These events also result in the deletion of *ARG8*^{m} (Figure 2). We calculated the rate of deletion events per cell division in this reporter using fluctuation analysis (Lea and Coulson 1949). In wild-type yeast, respiration-proficient recombinants arose at a high rate of 3.7×10^{-4} recombination events/cell division (Figure 3, construct Rep96). Southern blot analysis of mtDNA

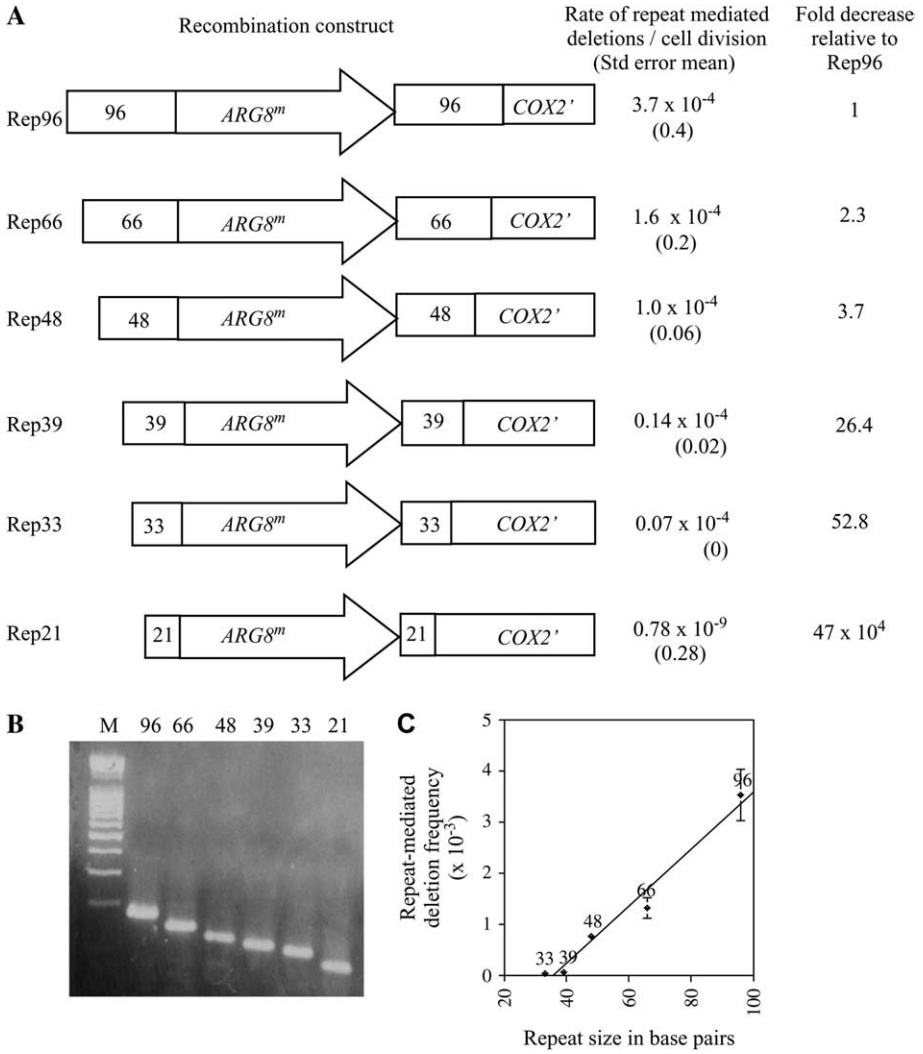

FIGURE 3.—Effect of repeat length on deletion rate. (A) Recombination reporter constructs are shown to the left, and the rates of repeat-mediated deletion events per cell division are shown to the right. Standard error values are indicated in parentheses. The fold-decrease in deletion rates relative to the Rep96 construct is also indicated. Construct Rep96 is the recombination reporter with 96 bases of directly repeated sequence on either side of the *ARG8*^{m} gene. Constructs Rep66, Rep48, Rep39, Rep33, and Rep21 are recombination reporters with 66, 48, 39, 33, and 21 bases of directly repeated sequence flanking the *ARG8*^{m} gene, respectively. (B) The length of the repeats in each construct was verified by PCR amplifying the repeat using a primer upstream of the *COX2* start codon and reverse primer within the *ARG8*^{m} gene. The amplified product was visualized on a 2% agarose gel. (C) The deletion frequencies as a function of the length of homologous sequence are shown. Due to the low standard error values for the Rep33-, Rep39-, and Rep48-bp repeat constructs, their error bars are not visible.

isolated from 17 independent recombinants showed that all carried *COX2* alleles indistinguishable from wild type. In these recombinants, there was no evidence for heteroplasmy or mixed mitochondrial genotypes (not shown).

Effect of repeat size: Studies involving direct-repeat recombination in the nucleus demonstrate that recombination rate decreases with repeat tract length. Nuclear studies on direct-repeat recombination have shown a linear relationship between the frequency of recombination and the length of the homology between the repeated sequences up to a minimal size (SHEN and HUANG 1986; SUGAWARA *et al.* 2000). This minimal size is called the minimal efficient processed segment (MEPS). In the yeast nucleus, the MEPS for the SSA pathway has been found to be 33 bp (SUGAWARA *et al.* 2000). To study the effect of repeat size on repeat-mediated deletions, we generated mitochondrial reporters identical to the 96-bp repeat plasmid, but with repeats of 66, 48, 39, 33, and 21 bp (Figure 3, A and B). The various construct lengths were verified by PCR analysis using primers that amplified the repeat sequence (Figure 3B). We found that the rate of recombination shows a linear decrease with respect to the size of the repeat down to 33 bp; at 21 bp, this linear relationship is lost (Figure 3, A and C). Our data predict that the yeast mitochondrial MEPS is between 33 and 21 bp (Figure 3A).

Effect of heterology between repeats: Studies of direct and inverted repeats in mitotic recombination in the nuclear DNA of yeast have also shown that heterology in the repeats reduces the rate of recombination between them. It has been shown that the mismatch repair proteins Msh2p and Msh6p bind to the mismatches and prevent recombination between divergent sequences (CHAMBERS *et al.* 1996; EVANS and ALANI 2002). In several studies, mismatches resulting in as little as 1% heterology were sufficient to significantly reduce mitotic recombination (YUAN and KEIL 1990; DATTA *et al.* 1997). The yeast nuclear studies using direct repeats with ~3% heterology showed a sixfold reduction in SSA activity (SUGAWARA *et al.* 2004). We have therefore generated recombination reporters with mismatches in the repeats of the Rep96 strain. As previously described, the repeats in our system consist of *COX2* coding sequence.

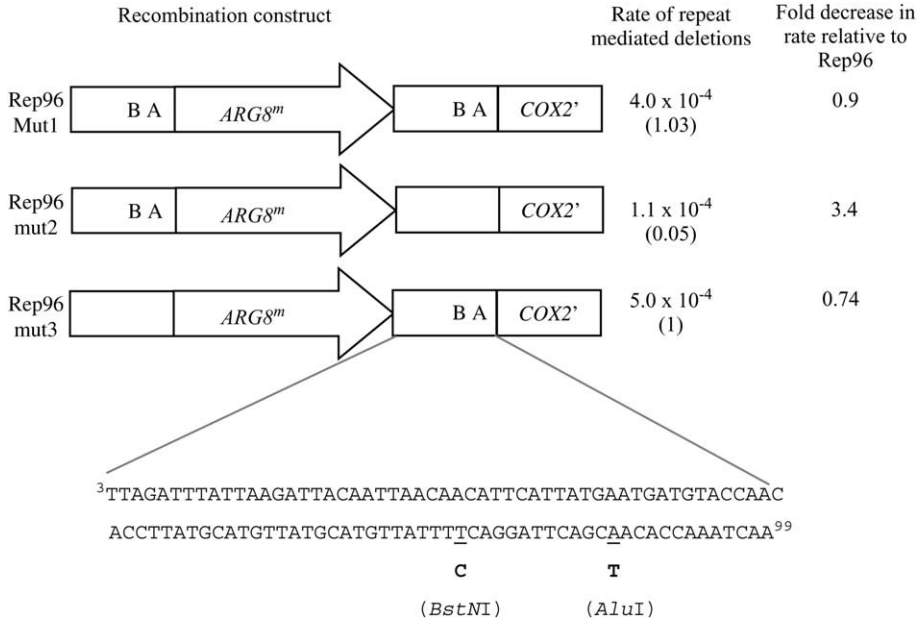

FIGURE 4.—Effect of heterology on repeat length. Heterology reporter constructs are to the left, and the rates of repeat-mediated deletion events per cell division are to the right. Standard error values are indicated in parentheses. The fold-decrease in deletion rates relative to Rep96 is also indicated. The Rep96 mut1 strain carries two point mutations in both repeats, resulting in no heterology between the repeats. The strains with the Rep96 mut2 and Rep96 mut3 constructs carry two point mutations in the first repeat or the second repeat, respectively, resulting in ~2% heterology in both reporters. The sequence of the repeat is shown. The underlined letters show the positions of the polymorphisms. The introduced nucleotide changes at those positions are indicated by the letters in boldface type below. Nucleotide positions are indicated in superscript.

Therefore, the mismatches introduced must not alter the function of the expressed Cox2p. We examined the codon preference in mitochondrial genes and identified amino acids for which at least two different codons were used at the same frequency. We introduced our mismatches in the third position of these codons to create silent mutations that generate codons used at the same frequency as the originals. These mutations also resulted in easily detectable restriction site polymorphisms. Polymorphism B introduces a *Bst*NI restriction site at nucleotide position 75 in the *COX2* gene, and polymorphism A introduces an *Alu*I site at nucleotide position 82 in the *COX2* coding sequence (Figure 4). Using site-directed mutagenesis, we introduced these silent polymorphisms into either the first or the second repeat to generate reporters with ~2% heterology. To ensure that these polymorphisms did not interfere with *COX2* expression following recombination, we introduced the same polymorphisms into both repeats (Figure 4, construct Rep96 mut1). Using fluctuation analysis, we determined that the recombination rate in the reporter with polymorphisms in both repeats was not significantly different from that of the original reporter (Figure 4). This result suggests that the mutations do not affect recombination rate or expression of *COX2* in the recombined form. Mutations in the first repeat reduce the rate of recombination about threefold relative to strains with identical repeats (Figure 4, construct Rep96 mut2), whereas the same mutations when present in the second repeat (Figure 4, Rep96 mut3) had no effect on the deletion rate. The threefold reduction in the rate of recombination observed for Rep96 mut2 was significant as determined by an unpaired *t*-test comparing the Rep 96 mut2 rate to the Rep96 deletion rate ($P = 0.039$). Using the same unpaired *t*-tests, we confirmed that the rates for Rep96 mut1 and Rep96 mut3 were not significantly different from the Rep96 deletion rate ($P = 0.66$ and 0.31, respectively).

To determine the sequence retained in the recombinants and the position of the recombination junction, we amplified DNA sequences spanning the single repeat in the respiring recombinants. We analyzed the fragment sizes after digestion of the PCR product with either *Bst*NI or *Alu*I, the novel restriction sites generated by the introduced mutations (Figure 5). Analysis of 95 independent recombinants derived from the strain with polymorphisms in the second repeat showed that 94% of the respiring recombinants retained the *Alu*I mutation; a subset of those, 92%, also retained the *Bst*NI mutation. The remaining 6% contained the wild-type sequence at both positions. Conversely, in the strain with the polymorphisms in the first repeat, 74% of the recombinants retained the wild-type sequence, 26% retained the *Bst*NI mutation, and 13% retained both the *Alu*I and the *Bst*NI mutations (Table 2). Therefore, we see retention of either the wild-type sequence or the mutated sequence depending on the proximity to the *COX2* coding region. These results suggest that the repeat sequences nearest to *COX2* are preferentially retained. This result also rules out the possibility that we are preferentially selecting mutant recombinants that may confer a growth advantage. Additionally, these data demonstrate that sequences spanning the two mutations participate in the recombination, since 13% carry one polymorphism and not the other.

We find that the presence of an ~2% heterology reduces the rate of recombination threefold or has no effect at all, depending on where the polymorphisms lie. One explanation for the different effects of heterology, depending on the location of the polymorphisms, is that different mispairs may be generated in the recombination intermediates. For example, if the SSA pathway is initiated to generate the deletion, the top strand of the

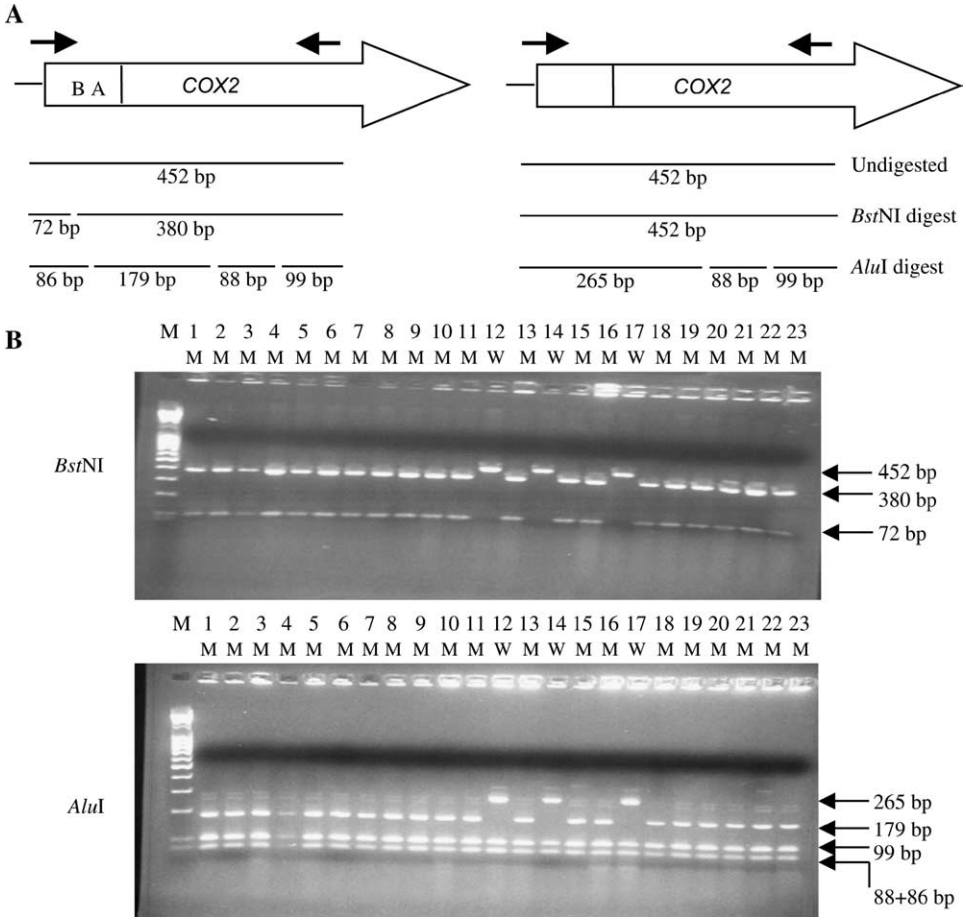

FIGURE 5.—Analysis of sequences retained by recombinants. (A) The recombinants arising from strains Rep96 mut3 harboring either the mutant or the wild-type sequence are shown. Sequences retained at the *COX2* locus in the recombinants were analyzed by PCR amplification of 452 bp of the *COX2* gene using primers denoted by arrows. Expected sizes of bands after digestion of PCR product with respective restriction enzymes for both types of recombinants are also shown. (B) The amplified product was digested with *Bst*NI or *Alu*I and digestion products were analyzed on a 2% agarose 0.5X TBE gel. A representative gel for 23 samples from that analysis is shown; M, presence of the mutation; W, absence of the mutation.

first repeat will pair with the complementary bottom strand of the second repeat. In the presence of heterology, the mismatches produced in the paired intermediates will differ between the Rep96 mut2 and Rep96 mut3 constructs. Specifically, C-A and T-T mismatches are predicted to be generated in Rep96 mut2, while T-G and A-A mispairs would be produced by this mechanism in Rep96 mut3. It is possible that the individual mismatches or the combination of the two mismatches are recognized more efficiently in the Rep96 mut2 strain by mitochondrial mechanisms for heteroduplex rejection. This would result in rejection of recombination between the heterologous sequences and reduction in the rate of deletion events.

We also find that the repeat sequence closest to *COX2* is preferentially retained. Although it is difficult at this stage to rationalize the preference for the directionality of the retained sequence, the preference may be influenced by the direction of the movement of the replication fork. Previously, polymerase slippage during replication has been implicated in repeat-mediated deletion of mitochondrial DNA (SCHOFFNER *et al.* 1989).

Analysis of deletion products: To identify the predominant pathway by which these deletion events arise, we analyzed the products of deletion events at different times following release of cells from selection for the functional Arg8 protein. Deletions generated by the homologous recombination-based pathway would give rise to two types of reciprocal products: a circle generated by intramolecular recombination or an *ARG8*^{m} duplication generated by intermolecular recombination, in addition to the restored *COX2* product as shown

TABLE 2

Analysis of sequences retained by recombinants

	% recombinants			
Sequence retained	*Alu*I polymorphism	*Bst*NI polymorphism	*Alu*I and *Bst*NI polymorphisms	Wild type at both positions
Mutations in second repeat ($N = 95$)	94	92	92	6
Mutations in first repeat ($N = 100$)	13	26	13	74

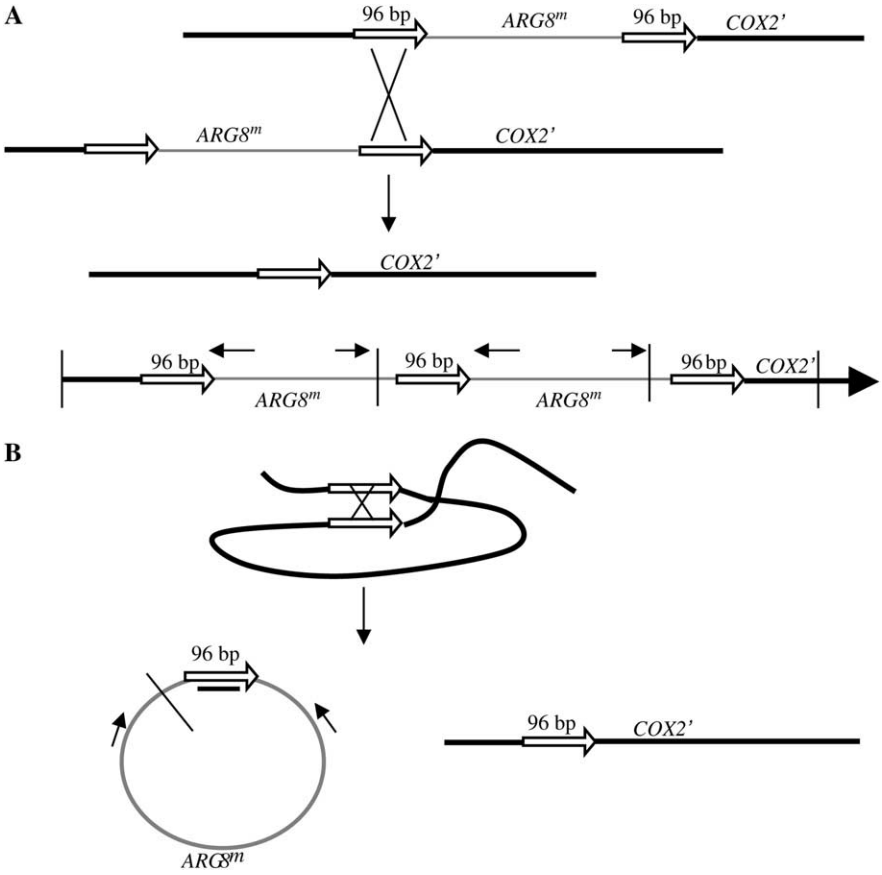

FIGURE 6.—Products of the homologous recombination-based pathway. If a DSB occurs in the repeat sequence, the homologous recombination-based repair pathway can be activated. (A) If recombination occurs between two mitochondrial genomes (intermolecular), it will generate the restored *COX2* deletion product and a reciprocal product harboring a duplication of the repeat and the intervening sequence as shown. (B) If recombination occurs within the same mitochondrial genome (intramolecular), it will generate the restored wild-type *COX2* along with a circle containing one repeat sequence and the *ARG8* ^{m} gene.

in Figure 6. The SSA pathway or the polymerase slippage pathway would restore the *COX2* gene without the generation of additional products. If homologous recombination was the only pathway by which these deletions arose, we expected to detect the reciprocal products of such events in equimolar quantities to the deletion product. Using Southern blot analysis, we looked for the molecular products of direct-repeat-mediated deletion events. To analyze products generated by the repeat-mediated deletion events, cells were grown in synthetic medium lacking arginine. At time T_0, cells were diluted into rich medium, releasing selection for the reporter and allowing the cells to undergo repeat-mediated deletions. DNA was extracted at time intervals and analyzed by Southern blot. The possible products of deletion events and their pattern on the Southern blot are shown in Figure 7A. The full-length intact reporter construct generates 2.75- and 0.62-kb fragments. Deletion products restoring the wild-type *COX2* coding sequence are observed as a 1.97-kb fragment. Reciprocal products of intermolecular or intramolecular recombination are predicted to generate additional 1.4-kb fragments. Southern blots probed with either the repeat sequence (Figure 7B) or the *ARG8*^{m} gene (Figure 7C) revealed no reciprocal products of homologous recombination although the deletion product was clearly visible, as early as 45 min after the shift to nonselective medium. We then used a more sensitive PCR-based assay to determine whether reciprocal recombination products are present in the preparation of total cellular DNA previously analyzed by Southern blotting (Figure 8). Primers were designed such that an amplification product of 1.1 kb would be observed only if reciprocal products of homologous recombination events were present in the sample (Figure 8A). These primers were diverged with respect to the sequences in the original Rep96 construct and the *COX2* gene and will not generate a product unless homologous recombination has occurred. As shown, we observed the amplification of a DNA band of expected size for the reciprocal products of homologous recombination. Cloning and sequencing of the PCR fragment confirmed the amplification product to be a corresponding product of a homologous recombination event containing the *ARG8*^{m} gene (not shown). Whether these reciprocal products are generated by intermolecular or intramolecular recombination cannot be determined by this assay. Since we did not detect reciprocal products of homologous recombination by Southern blot hybridization, although the deletion product was observed as early as 45 min after the shift, and since homologous recombination events were detectable by PCR amplification of the reciprocal products, we concluded that homologous recombination-based deletion events do occur, albeit at a lower frequency. As observed in the nucleus, we propose that more than one pathway might be used to generate such deletions. The first may

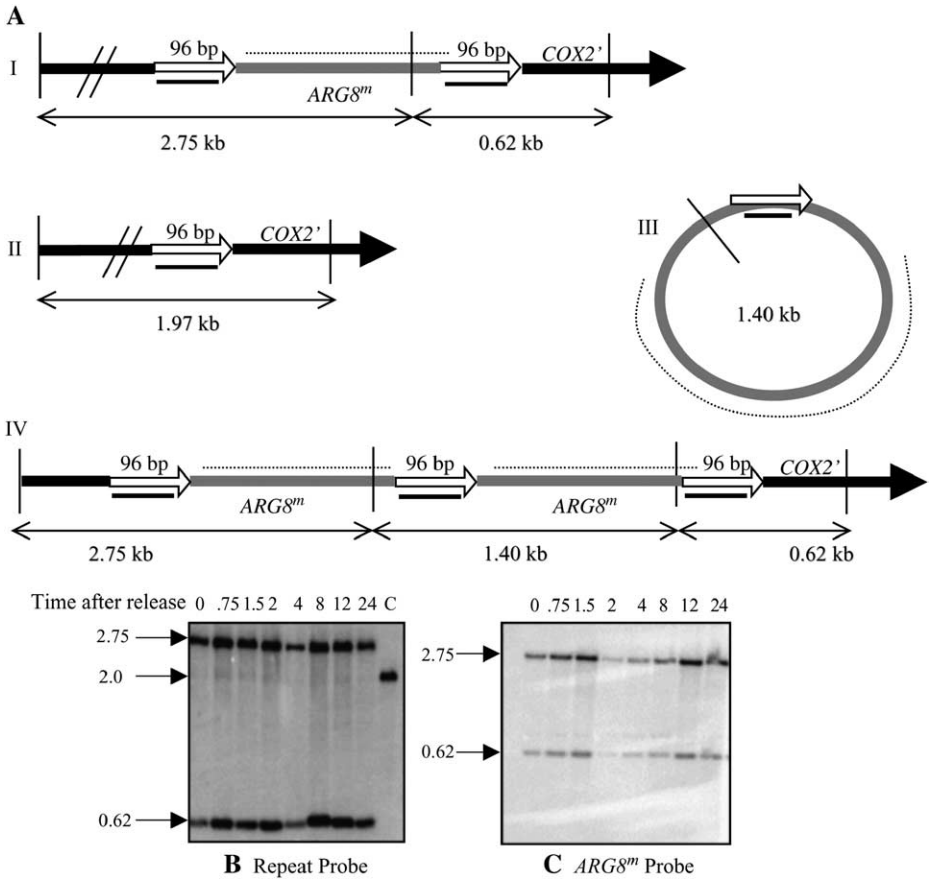

FIGURE 7.—Analysis of products of direct-repeat-mediated deletion. (A) Possible products of direct-repeat-mediated deletion. The vertical lines indicate restriction sites for *Eco*RV, the restriction enzyme used to digest the genomic DNA samples. Thick horizontal lines represent the *COX2* 96-bp repeat probe. The dotted horizontal line represents the 1.2-kb *ARG8*^{m} probe. Horizontal double-sided arrows on the bottom indicate sizes of restriction fragments visualized by either of the probes. (I) The full-length recombination reporter construct reveals bands of 2.75 kb and 0.62 kb. Reciprocal products of homologous recombination reveal a 1.97-kb band for the active *COX2* (II) gene along with a 1.4-kb circle (III), the reciprocal product in case of intramolecular recombination events. (IV) Reciprocal products of intermolecular recombination events are visualized as bands of 2.75, 1.4, and 0.62 kb. Arg$^+$ cells were diluted into YPD medium and DNA samples extracted at indicated time intervals. Southern blot analysis of repeat-mediated deletion events as a time course after release from arginine selection using the *COX2* 96-bp repeat sequence as probe (B) reveals a 0.62-kb band and a 2.75-kb band of the original reporter. Within 45 min of growth in YPD, the 1.97-kb restored *COX2* deletion product can be detected, designated by the arrow at 2.0 kb. The wild-type *COX2* gene is used as a control for the recombinant band (B, lane C). Probing with *ARG8*^{m} sequence (C) displayed 2.75- and 0.62-kb bands, revealing no reciprocal products of homologous recombination.

involve SSA and/or polymerase slippage pathways that would be predicted to generate the deletion products without the concomitant generation of other DNA species. The second may utilize a recombination-dependent pathway that generates reciprocal products in addition to the deletion. Our data suggest that at least two pathways are acting to generate these deletions.

Effect of disruption of candidate genes: To identify factors important for repeat-mediated deletion, we disrupted or mutated putative yeast mitochondrial recombination genes. We then tested the effect of these gene disruptions on the rate of deletions in the Rep96 reporter strain. An exonuclease mutant of the mitochondrial DNA polymerase (Mip1p) was examined for its effect on the rate of deletion events. The *mip1-D347A* mutation results in a 500-fold increase in point mutation rate at two loci in the mitochondrial genome (FOURY and VANDERSTRAETEN 1992) and therefore displays a high mutator activity. We found that the deletion rate in *mip1-D347A* mutants was 4.4-fold lower than that in Rep96 (0.84×10^{-4}). It is possible that most polymerase slippage events occur during the process of proofreading when the mitochondrial polymerase stalls at a misincorporated base. Mip1p-D347A has dramatically reduced exonuclease activity as determined by *in vitro* assays (FOURY and VANDERSTRAETEN 1992). It could be that this mutation reduces the stalling of the polymerase at a misincorporated base, thereby reducing slippage events. Alternatively, the D347A substitution might increase polymerase processivity, which may also be predicted to reduce slippage events. However, *in vitro* evidence suggests that this mutant protein has reduced processivity relative to the wild-type protein (FOURY and VANDERSTRAETEN 1992). Finally, it is possible that in addition to disruption of proofreading exonuclease activity, this mutant is impaired in interactions with other proteins that play a role in the generation of repeat-mediated deletions. This result implicates a DNA polymerase-dependent pathway in the generation of repeat-mediated deletions.

We also tested other candidate genes that are involved in homologous recombination in the mitochondrion through *in vitro* or phenotypic assays; *MHR1*, a putative single-strand invasion protein; *CCE1*, a mitochondrial cruciform resolvase; *NUC1*, a mitochondrial exonuclease; and *DIN7*, a mitochondrial *FEN1* (Flap endonuclease) homolog. The *cce1*-Δ (4.0×10^{-4} events/cell division, 1.08-fold relative to Rep96), *nuc1*-Δ (4.5×10^{-4}, 1.2-fold relative to Rep96), *din7*-Δ (3.1×10^{-4}, 0.8-fold relative to Rep96), and *mhr1*-Δ (2.7×10^{-4}, 0.7-fold relative to Rep96) strains did not show any significant effect on the

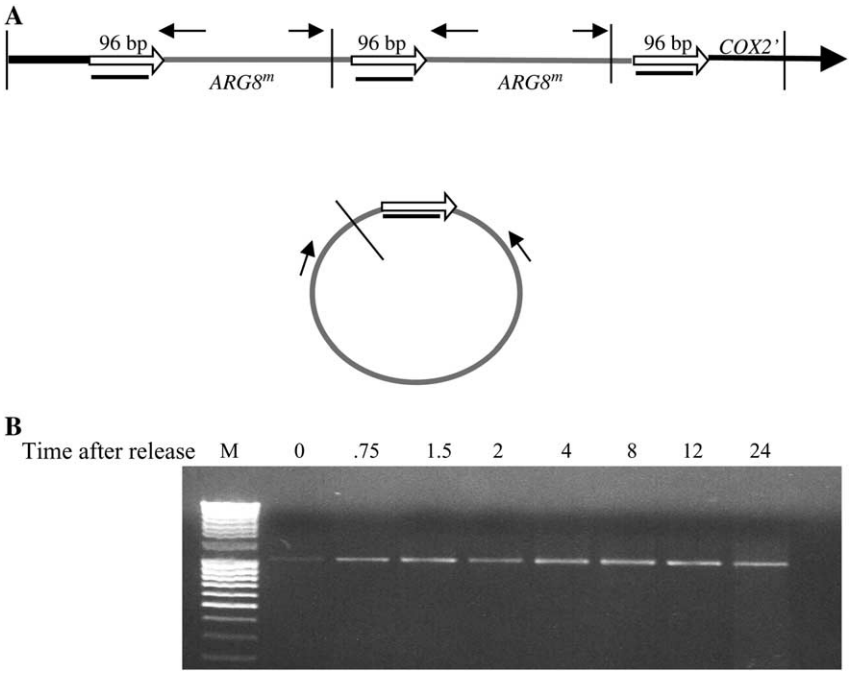

FIGURE 8.—PCR-based analysis of reciprocal products of homologous recombination. (A) Reciprocal products of homologous recombination are shown. Arrows show position of primers used for the PCR amplification. (B) Approximately equal amounts of genomic DNA from samples for each of the time intervals used for the Southern blot analysis were used as a template to amplify reciprocal products of intermolecular or intramolecular recombination. Such products result in the amplification of a 1.1-kb band.

rate of repeat-mediated deletions. It is important to note that none of the candidate genes we tested have been shown to be essential for recombination events, and no mutants have ever been tested for mitochondrial recombination phenotypes in mitotic haploid yeast. We therefore believe that redundant functions exist or that novel proteins are involved in generating repeat-mediated deletions. This reporter gives us the tool to identify such proteins.

In conclusion, our results demonstrate the use of a novel genetic reporter for studying the mechanism of direct-repeat-mediated deletions of the mitochondrial genome. This reporter will be used in a genome-wide screen to identify factors involved in such deletion events. This will allow us to define the mechanism by which these deletions arise and characterize specific protein participants of these pathways in the yeast mitochondrion. If this screen is successful, mammalian homologs of these proteins may exist. These homologs can be used in the study of mitochondrial DNA deletions and pathways involved in giving rise to such deletions in humans.

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