Analysis of microsatellite mutations in the mitochondrial DNA of *Saccharomyces cerevisiae*

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In the nuclear genome of *Saccharomyces cerevisiae*, simple, repetitive DNA sequences (microsatellites) mutate at rates much higher than nonrepetitive sequences. Most of these mutations are deletions or additions of repeat units. The yeast mitochondrial genome also contains many microsatellites. To examine the stability of these sequences, we constructed a reporter gene (*arg8*^{m}) containing out-of-frame insertions of either poly(AT) or poly(GT) tracts within the coding sequence. Yeast strains with this reporter gene inserted within the mitochondrial genome were constructed. Using these strains, we showed that poly(GT) tracts were considerably less stable than poly(AT) tracts and that alterations usually involved deletions rather than additions of repeat units. In contrast, in the nuclear genome, poly(GT) and poly(AT) tracts had similar stabilities, and alterations usually involved additions rather than deletions. Poly(GT) tracts were more stable in the mitochondria of diploid cells than in haploids. In addition, an *msh1* mutation destabilized poly(GT) tracts in the mitochondrial genome.

Microsatellites are regions of DNA (usually 20–60 bp in length) with repetitions of small numbers of base pairs (1, 2). Microsatellite alterations occur at a rate much higher than the mutation rate in nonrepetitive DNA (3). These alterations are likely to reflect DNA polymerase slippage (1). During DNA replication, a transient dissociation of the DNA strands, followed by incorrect reassociation, results in one or more unpaired repeat units on either the template or the nascent strand (4). If these unpaired loops are not repaired, another round of replication will result in a tract that is shorter (if the unpaired repeats are on the template strand) or longer (if the unpaired repeats are on the nascent strand) than the original tract.

The instability of microsatellites observed in wild-type yeast strains is elevated by several different types of mutations, most of which affect DNA synthesis or DNA repair (1, 5). Mutations affecting DNA polymerase or polymerase cofactors presumably destabilize microsatellites by increasing the rate of DNA polymerase slippage. The simplest interpretation of the destabilizing effect of mutations of the DNA mismatch repair system is that most of the DNA loops created by DNA polymerase slippage are repaired in wild-type strains.

The mtDNA of the yeast *Saccharomyces cerevisiae* is approximately 75–85 kb in size and extremely AT-rich (82%) (6). Consequently, most of the microsatellites found in the mtDNA are AT-rich [for example, poly(AT) repeats]. There are about 50 copies of mtDNA per haploid yeast cell and about 100 copies per diploid cell (7). The mtDNA polymerase (DNA polymerase γ) is encoded by the nuclear *MIP1* gene (8, 9). DNA polymerase γ, which has an associated proofreading exonuclease activity, replicates DNA *in vitro* with high fidelity compared with a number of other DNA polymerases (10).

In the nuclear genome, most misincorporated bases that escape correction by the proofreading exonuclease are corrected by DNA mismatch repair (5). In yeast, one of the homologs of the bacterial MutS protein (responsible for recognition of the DNA mismatch) is Msh1p, and yeast strains with an *msh1* mutation lose mitochondrial function within about 20 generations (11). Diploid strains heterozygous for the *msh1* mutation have a 7-fold elevated frequency of point mutations in the mtDNA (12). These results indicate that Msh1p has roles in the repair and maintenance of mtDNA.

Microsatellite stability is a sensitive indicator of genomic stability. Genomic instability is associated with several different human diseases. For example, global microsatellite instability, resulting from a defect in DNA mismatch repair, is associated with hereditary nonpolyposis colorectal cancer (13). Instability of the mtDNA is associated with several types of neuromuscular diseases (14), and large deletions of mtDNA are associated with the aging process, age-related disorders such as diabetes, and long-term treatment with certain drugs (15). Below, we describe a system allowing the measurement of microsatellite instability in the mtDNA of yeast.

Materials and Methods

Construction of Plasmids and Yeast Strains. *ARG8* is a nuclear gene encoding an enzyme that functions in the mitochondria. Steele *et al.* (16) constructed a derivative of *ARG8*, *ARG8*^{m}, that could be expressed when transformed into the mitochondria, replacing the mitochondrial *COX3* gene (GenBank accession no. U31093). Both the promoter and the first 9 amino acids of *cox3*::*ARG8*^{m} are derived from *COX3*. The first 22 aa of the fusion protein are removed by posttranslational processing (16).

The *cox3*::*ARG8*^{m} gene has an *Acc*I site 20 bp downstream of the translational start site that we used to insert various microsatellites. The plasmid pDS24, containing the *cox3*::*ARG8*^{m} gene (16), was treated with *Acc*I and ligated to various double-stranded oligonucleotides. The resulting plasmids and the oligonucleotides used in their construction were pEAS14, 5′-AG$(TA)_{15}$C and 5′-CTG$(TA)_{15}$; pEAS15, 5′-AG$(TA)_{16}$C and 5′-CTG$(TA)_{16}$; pEAS16, 5′-A$(GT)_{16}$C and 5′-CTG$(AC)_{15}$A; pEAS17, 5′-A$(GT)_{17}$C and 5′-CTG$(AC)_{16}$A; pEAS22, 5′-A$(GT)_{16}$AC and 5′-CTGT$(AC)_{15}$A; pEAS23-1 and pEAS23-2, 5′-AG$(TA)_{16}$GTC and 5′-CTGAC$(TA)_{16}$; and pEAS42, 5′-AG$(TA)_{17}$GTC and 5′-CTGAC$(TA)_{17}$. The ligated products were transformed into *Escherichia coli* strain DH5α.

These plasmids were introduced, in a cotransformation experiment with the *LEU2*-containing vector pRS315, into the *rho*^{0} yeast strain DFS160 (α *ade2-101 leu2 ura3-52 arg8*::*URA3 kar1-1* [*rho*^{0}]) by microprojectile bombardment (16). Leu$^+$ transformants that also contained the pEAS plasmids in their mitochondria were identified by their ability to give rise to respiring recombinants when mated to strain GW22, which has a deletion mutation in the 5′ untranslated leader of *COX3* (16). For each

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Abbreviation: 5-FOA, 5-fluoroorotate.

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of the plasmids, a positive transformant was mated to the *rho*$^+$ strain DFS188 (**a** *ura3-52 leu2-3,112, lys2 his3 arg8*::*hisG*). Cytoductants were identified that had the nucleus of DFS160 and the *cox3*::*ARG8*^{m} gene with the repetitive tracts integrated into the mitochondrial DNA. Cytoductants with in-frame insertions in the *cox3*::*ARG8*^{m} gene were identified by their Arg$^+$ phenotype. Cytoductants with out-of-frame insertions (*cox3*::*arg8*^{m} genes) were identified by their inability to grow on medium containing glycerol as a carbon source and their ability to produce respiring recombinants when mated to *rho*$^-$ strains carrying wild-type *COX* genes. These two types of cytoductants then were crossed with a *rho*^{0} derivative of DFS188 to obtain cytoductants with the *rho*$^+$ *cox3*::*ARG8*^{m} (or *cox3*::*arg8*^{m}) mitochondria and the nuclear background of DFS188. The resulting strains (plasmids used to generate the insertions indicated in brackets) were isogenic except for the microsatellite insertion: CAB142 [pEAS14, in-frame poly(AT)], CAB183-1 [pEAS23-1, poly(AT), +1 reading frame], MD70 and CAB188-1 [pEAS23-2, poly(AT), +1 reading frame], CAB152 [pEAS15, poly(AT), +2 reading frame], CAB172 [pEAS17, in-frame poly(GT)], CAB162 [pEAS16, poly(GT), +1 reading frame], CAB193-1 [pEAS22, poly(GT), +2 reading frame], MD72 [pEAS42, in-frame poly(AT)]. Strains CAB183-1, MD70, and CAB188-1 were independently constructed strains with the same mitochondrial reporter genes. Although both CAB142 and MD72 had in-frame insertions in the reporter genes, the lengths of the poly(AT) tracts and the DNA sequences flanking the poly(AT) repeats were different in the two strains.

A diploid strain (EAS469) isogenic with CAB193-1 was constructed by mating CAB193-1 to EAS464, an α-mating-type *rho*^{0} derivative of DFS188. This derivative was prepared by using a galactose-inducible *HO* gene to switch the mating type of DFS188 (as described by ref. 17) and then growing this strain in medium containing ethidium bromide (18). A diploid strain (EAS468), isogenic with DFS188, was constructed by mating DFS188 to EAS464. Similarly, diploids isogenic with CAB152 and CAB183-1 were constructed by mating EAS464 to CAB152 (diploid EAS565) and CAB183-1 (diploid EAS569), respectively.

The *MSH1* gene was deleted to generate EAS513. Oligonucleotides with 5′ sequences homologous to *MSH1* and 3′ sequences homologous to the *kanMX* gene (5′-CAATAATATAGATGGTACATAACATATGCGCAAGAAAACGTAAAGGCCACCGTACGCTGCAGGTCGAC and 5′-CAAATTATATACAATTAATAGTTGTATTCAAAAGTTATCCCAATATTTCGCATCGATGAATTCGAGCTCG) were used to amplify the *kanMX* gene of pFA6a-kanMX4 (19). We transformed strain EAS464 with the amplified fragment and selected for transformants on rich medium supplemented with 200 μg/ml Geneticin. To generate diploid strains heterozygous for the *msh1* mutation (EAS514, EAS515, EAS567, and EAS571), we mated EAS513 to DFS188, CAB193-1, CAB152, and CAB183-1, respectively.

The assay for microsatellite instability in nuclear DNA utilized a plasmid (pSR348) containing the *GAL1,10* promoter fused to a *URA3* hybrid gene and the selectable *LEU2* marker (20). Previously, we inserted an in-frame 33-bp poly(GT) into a *Bgl*II site located within the fusion gene to generate the plasmid pMBW1 (20). We constructed a plasmid (pMD52), identical to pMBW1 except for a substitution of a 33-bp poly(AT) tract for the poly(GT) tract, by annealing two oligonucleotides [5′-GATCGTCGACA$(TA)_{16}$CACTCGAG and 5′-GATCCTCGAGTG$(TA)_{16}$TGTCGAC] and ligating the double-stranded product into *Bgl*II-digested pSR348. Plasmids pMBW1 and pMD52 were treated with *Eco*RI and transformed into the haploid strain AMY125 (α *ade5 his7-2 leu2-3,112 trp1-289 ura3-52*) to generate the transformants MBW1-33 (20) and MD53-33, respectively, and into the haploid strain DFS188 to generate PG107 and PG108, respectively. These strains contained the fusion reporter genes flanked by duplicated *LEU2* sequences as a consequence of integration of the plasmids into the *LEU2* locus.

Measurement of Mutation Rates of Mitochondrial Genes. The yeast strains CAB152, CAB162, CAB183-1, MD70, CAB188-1, CAB193-1, EAS469, and EAS515 were phenotypically Arg$^-$ because they contained a nuclear *arg8*::*hisG* mutation and a frameshift mutation in the mitochondrial *cox3*::*ARG8*^{m} reporter gene. To determine the rate of alterations that restore the *cox3*::*ARG8*^{m} reading frame, we measured the frequency of Arg$^+$ colonies in 12–20 independent cultures. We used the method of the median (21) to calculate rates from the frequency data.

Because derivatives of our starting strains that lose the reporter gene (by becoming *rho*$^-$) would be incapable of generating Arg$^+$ colonies, we checked for retention of the reporter gene in at least one colony from each culture used in the rate measurements. One method was to mate the colonies to the *rho*$^-$ strain MCC259 (**a** *ade2-101 ura3-52 kar1-1* [ρ^- *COX3*]). Although this strain is deleted for most of the mitochondrial genome, it retains the *COX3* locus. Consequently, if the strains being tested retain the intact *cox3*::*arg8*^{m} reporter gene and other mitochondrial sequences, the mated cells should be able to give rise to respiratory-proficient recombinants. The alternative method was to perform PCR by using primers specific for the reporter gene. None of the approximately 80 colonies tested lost the *cox3*::*arg8*^{m} reporter gene. Mutations resulting in resistance to erythromycin (*ery*^{R}) map within the mitochondrial gene encoding 21S ribosomal RNA (reviewed by ref. 22). We measure the rate of *ery*^{R} mutations by standard methods (9).

Measurement of Mutation Rates of Nuclear Microsatellites. The yeast strains MBW1-33 and MD53-33 are phenotypically Ura$^+$ because the repetitive tracts in the *URA3* fusion gene are in-frame with the *URA3* coding sequence. These strains fail to grow on medium containing 5-fluoroorotate (5-FOA). We measured the rate of appearance of 5-FOA-resistant (5-FOA^R) derivatives by using methods described previously (20). Because the reporter gene is flanked by homologous sequences (used for integration into the *LEU2* locus), some of the 5-FOA^R colonies result from loss of the entire reporter construct by homologous recombination. To distinguish these events from isolates with microsatellite alterations, we determined whether purified 5-FOA^R derivatives were capable of reversion to the Ura$^+$ phenotype. Strains in which the reporter is lost were unable to revert to Ura$^+$.

Analysis of the Length of Mitochondrial and Nuclear Microsatellites. The lengths of the mitochondrial microsatellites in the Arg$^+$ derivatives were determined by performing PCR amplification in the presence of labeled nucleotide (dATP) by using primers (5′-ATTATTTAATTTTATTATATAGTTTTGAGG and 5′-GATGTGAATCTTCTTGATGATGTTG) that flank the repeated tracts. The products were analyzed on 6% denaturing polyacrylamide gels as described previously (20). Ambiguities and new classes of alterations were verified by DNA sequence analysis. Mitochondrial DNA was prepared for sequence analysis as described in ref. 18. The lengths of the nuclear microsatellites in the 5-FOA^R colonies were determined as described previously (20) by using the primers (5′-CCAACGTGGTCATTTAATGAGC and 5′-GCTTGAACTCGTCTAATTTG).

Results

Experimental Rationale. Tracts of poly(GT) are common in the nuclear genome of the yeast *S. cerevisiae* (23). The yeast mtDNA has many poly(AT) tracts whereas GT tracts are rare and short (6). To examine the stability of these microsatellite DNA sequences in the yeast mitochondrial genome, we constructed a

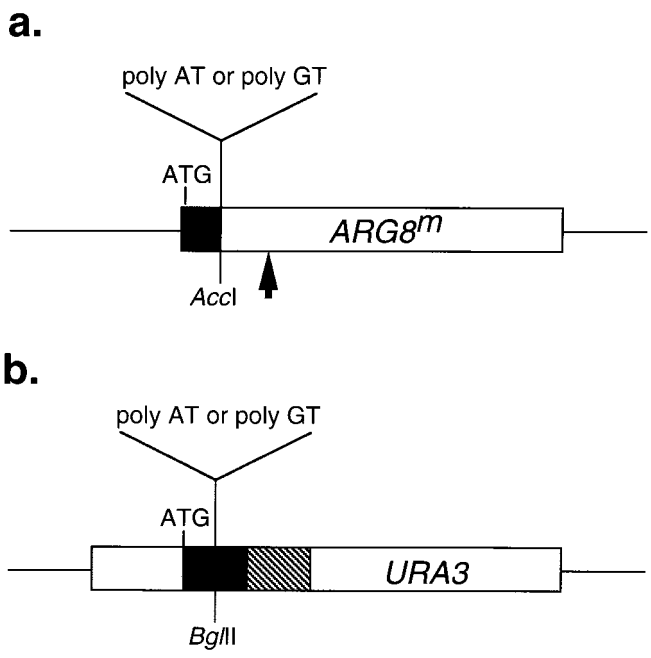

Fig. 1. Reporter genes for monitoring mitochondrial (*a*) or nuclear (*b*) microsatellite stability in yeast. (*a*) Reporter gene for monitoring mitochondrial microsatellite stability (*cox3*::*arg8*^{m}). *ARG8*^{m} is a derivative of the yeast *ARG8* gene in which the codon usage has been altered to be consistent with mitochondrial expression (16). This gene is expressed by using the *COX3* transcriptional and translational signals in mtDNA (thin line). *COX3* protein coding sequences are represented by the solid box. The fusion protein is processed posttranslationally at the site indicated by the arrow. Microsatellite sequences [in-frame or out-of-frame insertions of poly(AT) or poly(GT)] were inserted at the designated *Acc*I site. (*b*) Reporter gene for monitoring nuclear microsatellite stability (20). The components of this fusion gene encoding a protein with wild-type Ura3p activity are *GAL1,10* promoter (shaded box), N-terminal amino acids derived from *LYS2* (solid box), *HIS4*-derived amino acids (striped box), and *URA3*-derived sequences (open box). Microsatellite sequences [in-frame insertions of poly(AT) or poly(GT)] were inserted at the designated *Bgl*II site. These plasmids were integrated into the *LEU2* locus in a strain with a *ura3* mutation.

mitochondrial reporter gene (*arg8*^{m}) in which alterations in the length of the microsatellite affected the functional expression of the Arg8 protein. The nuclear *ARG8* gene encodes acetylornithine aminotransferase, an enzyme that functions in the mitochondria (24, 25). A derivative of *ARG8*, *ARG8*^{m}, was constructed to function as a mitochondrial gene (16). This gene is expressed as a translational fusion with the first eight codons of the mitochondrial-encoded *COX3* gene. Inserting this *cox3*::*ARG8*^{m} fusion gene at the *COX3* locus in the mtDNA of a strain with a mutation in the nuclear *ARG8* gene results in a respiratory-deficient strain that is phenotypically Arg^{+}.

We constructed derivatives of *cox3*::*ARG8*^{m} in which microsatellite sequences [poly(GT) or poly(AT)] were inserted into the coding sequence 20 bp from the initiating codon (Fig. 1*a*). Insertions at this position are unlikely to affect a catalytic domain of the enzyme, because the Arg8p precursor is processed at a position downstream of the insertion to yield the mature enzyme (16). Consistent with this expectation, when we transformed the mitochondria of an *arg8*::*hisG* yeast strain with *cox3*::*ARG8*^{m} derivatives containing in-frame microsatellite insertions of poly(AT) or poly(GT), the resulting transformants were Arg^{+}.

We also made derivatives of *cox3*::*ARG8*^{m} containing insertions of poly(AT) or poly(GT), creating frameshifts that will be translated in the +1 or +2 frame. All four genes had microsatellite insertions of 32 bp, with the reading frame adjusted by altering the sequences flanking the repeats. When these genes were transformed into the mitochondria of an *arg8*::*hisG* yeast strain, the strains were Arg^{-}, as expected. Consequently, we could select alterations in the microsatellite that restored the correct reading frame by plating the transformed strains on medium lacking arginine.

A similar approach previously was used in *E. coli* (26) and, for microsatellites in nuclear genes, in yeast (27) and mammalian cells (28). In these studies, the most common microsatellite alterations were the minimal changes in repeat units required to restore the correct reading frame. By using insertions of the same size that generate frameshifts to both the +1 and +2 reading frames, we can determine the relative frequency of single-repeat additions and deletions.

Rates and Types of Tract Alterations in Mitochondrial Repeats. We measured the rate of reversion to the Arg^{+} phenotype by determining the frequency of Arg^{+} derivatives in multiple independent cultures and using the method of the median (21) to calculate rates. These rates are shown in Table 1. To determine what fraction of the Arg^{+} derivatives had an altered microsatellite, we analyzed the lengths of the microsatellites by PCR and/or DNA sequencing. The rate of Arg^{+} derivatives was multiplied by this fraction to determine the rate of microsatellite alterations (Table 1).

Surprisingly, we observed wide variation in the rates of tract alterations. The poly(AT) tract that generates the +1 frameshift (in strains CAB183-1, MD70, and CAB188-1) gave the lowest rate of tract alterations to the correct reading frame, 2×10^{-10} per cell division. It should be noted that this low rate was observed in three strains that were constructed independently by using two independently constructed (but, presumably identical) plasmids, pEAS23-1 (CAB183-1) and pEAS23-2 (MD70 and CAB188-1). The same length poly(AT) tract that results in a +2 frameshift (strain CAB152) altered at a rate 800-fold higher. We examined the types of alterations in DNA derived from 25 independent Arg^{+} colonies that had the poly(AT) tract resulting in the +1 frameshift and 23 independent Arg^{+} colonies that had the poly(AT) tract resulting in the +2 frameshift. As expected, most (17/23) of the Arg^{+} derivatives of the strain with the tract generating the +2 frameshift had 2-bp deletions, although five larger deletions were also observed (Table 2). From the data of Tables 1 and 2, we calculate that the rate of 2-bp deletions for the poly(AT) tract is 1.2×10^{-7} per cell division. None of the sequenced tracts from the Arg^{+} derivatives of the strain with the poly(AT) tract that generates a +1 frameshift had the expected 2-bp additions. By PCR analysis, most of the strains had 1-bp deletions (Table 2). In 15 Arg^{+} derivatives examined by sequence analysis, 12 had a 1-bp deletion from a run of three As located about 10 bp downstream of the tract; one derivative had a 22-bp deletion within the tract. From these data, we calculate the rate of 2-bp insertions of the poly(AT) tract to be less than 2×10^{-10} per cell division.

One explanation for the lack of 2-bp insertions in strains CAB183-1, MD70, and CAB188-1 is that a 2-bp insertion does not result in a functional Arg8 protein. To rule out this possibility, we constructed a strain (MD72) in which the reporter gene contained a tract that was identical to that expected as a consequence of a 2-bp insertion in CAB183-1, MD70, or CAB188-1. Nine of nine independent isolates were Arg^{+}.

The poly(GT) tracts were much less stable than the poly(AT) tracts (Table 1). The poly(GT) tract in the +2 reading frame (strain CAB193-1) gave the highest rate of alterations, 4.0×10^{-6} per cell division. This rate was about 25-fold higher than that observed for the poly(AT) tract in the same frame. Most alterations were single-repeat deletions (Table 2). The calculated rate of single-repeat deletions was 3.9×10^{-6} per cell division. The rate of alterations was considerably lower for the

Table 1. The stability of microsatellites in the mtDNA of *S. cerevisiae*

Microsatellite	Rates of mutation to Arg^+ (per cell division)*	Fraction of Arg^+ revertants with tract changes	Rates of changes in tract (per cell division)†	Tract stability relative to $(AT)_{16}$ (+1 frame)
$(AT)_{16}$ (+1 frame)	3.7 (0.4–7.0) $\times 10^{-9}$ 2.4 (0–5.2) $\times 10^{-9}$ 7.6 (7.6–17) $\times 10^{-9}$ 5.9 (4.8–7.1) $\times 10^{-9}$ **4.9 $\times 10^{-9}$**	1/25	2.0×10^{-10}	1
$(AT)_{16}$ (+2 frame)	1.4 (0.7–2.0) $\times 10^{-7}$ 2.0 (1.6–4.2) $\times 10^{-7}$ **1.7 $\times 10^{-7}$**	22/23	1.6×10^{-7}	800
$(GT)_{16}$ (+1 frame)	3.9 (1.8–7.1) $\times 10^{-7}$ 3.6 (2.0–5.1) $\times 10^{-7}$ **3.8 $\times 10^{-7}$**	21/22	3.6×10^{-7}	1800
$(GT)_{16}$ (+2 frame)	5.2 (3.6–6.5) $\times 10^{-6}$ 2.8 (2.1–3.9) $\times 10^{-6}$ **4.0 $\times 10^{-6}$**	33/33	4.0×10^{-6}	20,000

*Rates for all microsatellites, except for the first two $(AT)_{16}$ (+1 frame) values, were calculated by the method of the median (21). The rates for the first two $(AT)_{16}$ (+1 frame) microsatellites were determined by fluctuation analysis (21). For each microsatellite, the data for two separate experiments are shown (each involving about 20 independent cultures), with numbers in parentheses indicating 95% confidence limits. The rates in boldface represent averages of two or more experiments.

†The rates of tract alterations were calculated by multiplying the average rate of Arg^+ reversion by the fraction of revertants with altered microsatellites.

poly(GT) tract in the +1 reading frame (strain CAB162) than in the +2 reading frame (Table 1), and most of the observed changes did not represent single-repeat additions (Table 2). The calculated rate of single-repeat additions was 10^{-7} per cell division, about 40-fold lower than the rate of single-repeat deletions.

In summary, for both poly(AT) and poly(GT) yeast mitochondrial microsatellites, single-repeat deletions occur much more frequently than single-repeat additions. In addition, poly(GT) tracts are much less stable than poly(AT) tracts. In previous studies of poly(GT) tracts on plasmids within the yeast nucleus (27, 29), we found that single-repeat additions were more common than single-repeat deletions. As described below, we confirmed this result for poly(GT) tracts within the yeast chromosomal DNA and also examined the stability of poly(AT) tracts.

Tract Alterations of Nuclear Repeats. To monitor the stability of nuclear microsatellites, we used a strain described previously (20) in which in-frame poly(AT) or poly(GT) tracts 33 bp in length were inserted into the coding sequence of a reporter gene with wild-type *URA3* activity (Fig. 1*b*); this gene was integrated into chromosome III. Additions or deletions of the microsatellite sequences leading to an out-of-frame insertion were selected on medium containing 5-FOA. We measured the rates of formation of 5-FOA^R derivatives; the types of alterations were determined by PCR analysis (Table 3). The rates and types of alterations for poly(AT) (strain MD53-33) and poly(GT) (strain MBW1-33) were not significantly different. For both microsatellites, single-repeat additions were significantly more frequent than single-repeat deletions. We calculated the rate of single-repeat deletions in nuclear poly(AT) and poly(GT) tracts as 5×10^{-7} per division and 1.7×10^{-6} per division, respectively, and the rates of single-repeat additions as 1.2×10^{-5} per division and 1×10^{-5} per division, respectively.

We also monitored tract stability by using strains derived from DFS188, the same nuclear background used in the mitochondrial experiments. The rates of alteration were consistent with the previous results: 1.3×10^{-5} per division for the poly(AT) tracts (strain PG108) and 1.0×10^{-5} per division for the poly(GT) tracts (strain PG107). Both strains also displayed a strong bias for additions of 2 bp; seven of seven alterations in the poly(AT) tract and eight of nine alterations in the poly(GT) tract were additions of 2 bp. Because additions were more frequent than deletions in these wild-type yeast strains, poly(AT) and poly(GT) nuclear microsatellites 33 bp in length would be expected to expand in size, whereas, based on our observations, tracts of the same size present in mtDNA would be expected to contract.

Table 2. Types of microsatellite alterations in mtDNA

	No. of dinucleotide tracts with additions or deletions of base pairs								
Microsatellite	−22	−20	−14	−8	−4	−2	0	+2	Total
$(AT)_{16}$ (+1 frame)	1						24*		25
$(AT)_{16}$ (+2 frame)		1	1	3		17	1†		23
$(GT)_{16}$ (+1 frame)					15		1†	6	22
$(GT)_{16}$ (+2 frame)			1			32			33

Tracts in independent Arg^+ revertants were examined by PCR or DNA sequence analysis as described in *Materials and Methods*.

*In 14 of these 24 Arg^+ strains, the reporter genes were sequenced, and 12 had a deletion of one A in a run of three As located about 10 bp downstream of the dinucleotide tract. In 10 additional strains, PCR analysis indicated a 1-bp deletion, but the genes were not sequenced.

†No changes were detected within the dinucleotide tract or in the short run of As located downstream of the tract.

Tract Alterations in Diploid Strains of Yeast. As described above, the most unstable mitochondrial microsatellite was poly(GT) in the +2 reading frame, altering at a rate of 4×10^{-6} per division in the haploid CAB193-1. To determine whether the rate of alterations was affected by ploidy, we constructed an isogenic diploid strain with the poly(GT) tract in this reading frame (EAS469). The rate of alterations was 2.3×10^{-8} per division, about 170-fold less than observed in the haploid strain. The types of alterations were similar to those in the haploid: 13 single-repeat deletions, 2 two-repeat additions, and 1 seven-repeat deletion. We also examined the rate of tract alterations in diploid strains [EAS565 (+2 reading frame) and EAS569 (+1 reading

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frame)] with the poly(AT) reporter genes. The rate of alterations for EAS565 was $1.5 \times 10^{-9} \pm 1.2 \times 10^{-9}$ per cell division, about 100-fold lower than that observed in the comparable haploid strain CAB152 (Table 1); of five tracts sequenced, four were 2-bp deletions and 1 was an 8-bp deletion. In EAS569, the rate of tract alterations was very low (2.7×10^{-10} per cell division), about 20-fold less than observed in the isogenic haploid strains; the only Arg^+ derivative obtained in this experiment had a 1-bp deletion.

We also measured the rate of mutation to Ery^R in haploid (DFS188) and diploid (EAS468) strains isogenic to those used for measuring microsatellite mutations except that they contained wild-type mtDNA. The mutation rate was about 5-fold lower in the diploid (6.7×10^{-8} per division) than in the haploid (3×10^{-7} per division) strain. In a previous analysis of the stability of a nuclear poly(GT) microsatellite, Wierdl *et al.* (3) found no significant difference between haploid and diploid cells.

Effect of the *msh1* Mutation on Microsatellite Stability. The nuclear-encoded *MSH1* gene of yeast shares homology with the bacterial *mutS* gene, and yeast strains heterozygous for an *msh1* mutation have a 7-fold elevation in the frequency of mutation to Ery^R, a measure of the frequency of point mutations in the mtDNA (12). Because mutations in other *mutS* homologs destabilize nuclear microsatellites (1), we constructed strains to test the effect of an *msh1* deletion on the mitochondrial microsatellites. Because strains lacking functional Msh1p lose mitochondrial function within approximately 20 generations (11), these experiments were carried out by using diploid yeast strains heterozygous for a deletion of *MSH1*. These strains contained either wild-type mtDNA (EAS514), to allow measurement of the rate of mutation to Ery^R, or strains with reporter genes containing microsatellite insertions [EAS515, poly(GT), +2 frame; EAS567, poly(AT), +2 frame; EAS571, poly(AT), +1 frame] to measure the rate of microsatellite alterations.

As observed previously (12), the heterozygous diploid had a slightly elevated rate of mutation to Ery^R, 1.4×10^{-7} per division in EAS514 vs. 6.7×10^{-8} per division in the isogenic control EAS468. In addition, the rate of alteration of the poly(GT) tract in EAS515 was 8.0×10^{-7}, a 35-fold elevation over the rate observed for the wild-type diploid (EAS469). The spectrum of alterations in the repetitive tract was not significantly different from that of the wild-type strain. We observed 16 single-repeat deletions, 1 two-repeat addition, 1 seven-repeat deletion, and 1 ten-repeat deletion. In contrast to the results with the poly(GT) microsatellite, no significant destabilization of the poly(AT) tract was observed in either heterozygous diploid. The rates of alterations per cell division were $2.1 \times 10^{-9} \pm 1.4 \times 10^{-9}$ for EAS567 [heterozygous for *msh1* mutation, poly(AT), +2 frame] compared with 1.5×10^{-9} for EAS565 (isogenic wild-type diploid), and 6.1×10^{-10} for EAS571 [heterozygous for *msh1* mutation, poly(AT), +1 frame] compared with 2.7×10^{-10} for EAS569 (isogenic wild-type diploid). Because the rates observed in strains with the poly(AT) reporter are very low, they are less accurate than those observed for the poly(GT) reporter. For example, only 1 of 20 cultures for EAS569 and only 2 of 20 cultures for EAS571 had any Arg^+ derivatives; both of the EAS571 Arg^+ derivatives had 1-bp deletions.

Discussion

We have developed a method of measuring microsatellite instability in yeast mtDNA. Our analysis allows several conclusions. First, poly(AT) tracts are considerably more stable than poly(GT) tracts. Second, single-repeat deletions for both the poly(AT) and poly(GT) tracts are much more frequent than single-repeat additions. Third, in contrast to the results obtained for the microsatellites in the mtDNA, poly(AT) and poly(GT) tracts in the nuclear DNA have similar levels of instability; in addition, for these tracts, single-repeat additions are more common than single-repeat deletions. Fourth, mitochondrial microsatellites are more stable in diploid than in haploid cells. Fifth, diploid strains heterozygous for an *MSH1* deletion display elevated rates of mutation to Ery^R as well as destabilization of the poly(GT), but not the poly(AT), microsatellites. These conclusions will be discussed further below.

We used the rate of Arg^+ derivatives per cell division to calculate the rate of microsatellite stability. As in other studies of mitochondrial mutations, we do not have sufficient information to determine the rate of mutation per mtDNA molecule. Previous studies (22) indicate that mutations, presumably arising in a single mtDNA molecule of the approximately 50 mtDNA molecules in the haploid genome, rapidly achieve fixation. This fixation process probably involves both unequal segregation of mtDNA molecules between mother and daughter cells and repeated cycles of gene conversion between mutant and wild-type mtDNA (22, 30). In our studies, all Arg^+ isolates examined by PCR or DNA sequence analysis had only one species of mtDNA, indicating fixation of the microsatellite alteration within the cell. We do not know what fraction of the mtDNA molecules in the cell needs to contain an in-frame *cox3*::*ARG8*^{m} gene for the cell to be Arg^+. It is likely, however, that the fixation of the in-frame gene is not a direct requirement of the selection process, because *cox3*::*ARG8*^{m} expression yields substantial amounts of Arg8p (16).

Our analysis of the types of changes of microsatellites in mtDNA shows two interesting effects. Deletions are much more common than additions for both poly(AT) and poly(GT) tracts, and poly(AT) tracts (which are common in yeast mtDNA) are much more stable than the rare poly(GT) tracts. As discussed in the Introduction, it is likely that microsatellite alterations reflect DNA polymerase slippage events (1). By this model, there is a

Table 3. Rates and types of changes for nuclear poly(AT) and poly(GT) microsatellites

Microsatellite	Rate of mutation to 5-FOA^R (per cell division)	Rate of changes in tract (per cell division)*	Types of alterations				
			−2	0	+2	Other†	Total
$(AT)_{16.5}$	5.1 (4.5–6.4) $\times 10^{-5}$ 2.7 (2.6–3.1) $\times 10^{-5}$ **3.9 $\times 10^{-5}$**	1.3×10^{-5}	1	1	23	1	26
$(GT)_{16.5}$	4.0 (2.4–5.5) $\times 10^{-5}$ 2.4 (1.9–3.3) $\times 10^{-5}$ **3.2 $\times 10^{-5}$**	1.2×10^{-5}	4	0	24	1	29

*These rates were determined by multiplying the average rate of mutation to 5-FOA^R by the proportion of 5-FOA^R derivatives that had altered tracts. Most of the 5-FOA^R derivatives that did not have a tract alteration had lost the reporter gene as a consequence of mitotic recombination.

†In this class are two 5-FOA^R strains in which the tract had gained more than five repeats.

transient dissociation of primer and template strands, followed by a misaligned reassociation, resulting in unpaired repeats in either the primer or template strands. If the mismatch resulting from these unpaired repeats is not corrected, either by the proofreading exonuclease or postreplicative DNA mismatch repair, they result in additions (if the unpaired repeat is on the primer strand) or deletions (if the unpaired repeat is on the template strand). Consequently, the differences in stability of the poly(AT) and poly(GT) tracts, as well as differences in the relative frequencies of additions and deletions, are likely to reflect biases in the types of mutations caused by the mtDNA polymerase γ (or associated cofactors), substrate affinities of the proofreading exonuclease associated with DNA polymerase γ, and/or substrate preferences of postreplicative DNA mismatch repair in the mitochondria. For example, DNA polymerase slippage might occur equally frequently for both poly(AT) and poly(GT) tracts, but displaced AT repeats could be corrected more efficiently by postreplicative DNA mismatch repair.

The poly(AT) sequence, a common mitochondrial microsatellite (6), in the +1 reading frame is remarkably stable, undergoing Arg^+ reversions at a rate of less than 10^{-9} per division. Three arguments indicate that this stability is not an artifact. First, most (23 of 25) of the observed alterations in *cox3*::*arg8*^{m} containing this poly(AT) tract are those expected to restore the correct reading frame. Second, the reporter gene with the sequence expected for an insertion of 2 bp to the poly(AT) tract is Arg^+. Third, given the relative stabilities of the poly(AT) and poly(GT) tracts in the +2 reading frame, and the relative stabilities of the poly(GT) tracts in the +1 and +2 reading frames, it is expected that the poly(AT) tract that generates the +1 reading frame will exhibit the highest level of stability.

When located in the nucleus, the poly(AT) and poly(GT) tracts alter at approximately the same rate; additions are significantly more common than deletions, the opposite bias to that observed in the mitochondria. This result argues that the types of changes observed with different microsatellites do not reflect intrinsic structural features of the DNA, but interactions between specific DNA sequences and organelle-specific properties of DNA replication and/or DNA repair. The bias in favor of additions over deletions for the poly(GT) nuclear microsatellite is a property of the DNA mismatch repair machinery because, in the absence of DNA mismatch repair, deletions and additions occur with equal frequency (29, 31).

The poly(GT) and poly(AT) microsatellites were about 100-fold more stable in diploid cells than in haploid cells. The rate of mutation to erythromycin resistance was about 7-fold lower in diploid than in haploid cells. Although the reasons for the haploid–diploid difference in mutation rate is unclear, it could reflect a difference in the dynamics of mtDNA segregation or other processes involved in fixation of mtDNA mutations rather than a difference in DNA replication and/or DNA mismatch repair. Because diploid cells contain about twice as many mtDNA molecules as haploid cells (7), if a certain fraction of the mtDNA molecules must have an in-frame insertion for the cell to be phenotypically Arg^+, then a higher mutation rate would be observed in haploids. Alternatively, there may be cell-type-specific regulation of mtDNA replication or repair.

A heterozygous *msh1* mutation destabilized the poly(GT) (+2 frame) tract 35-fold, but did not destabilize the poly(AT) (+2 frame) tract. Because Msh1p is homologous to the bacterial mismatch repair protein MutS and binds to mismatches in DNA *in vitro* (12), a reasonable interpretation of this effect is that Msh1p is involved in the repair of 2-bp GT or CA loops resulting from DNA polymerase slippage on poly(GT) tracts. The lack of effect of *msh1* on the stability of the poly(AT) tract might indicate that DNA polymerase γ has a lower rate of slippage on poly(AT) tracts than on poly(GT) tracts. Further evidence relevant to the mechanism by which repetitive tracts in the mtDNA change in length could be obtained by isolating mutants that have elevated levels of microsatellite instability.

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1. Sia, E. A., Jinks-Robertson, S. & Petes, T. D. (1997) *Mutat. Res.* **383,** 61–70.
2. Tautz, D. & Schlotterer, C. (1994) *Genet. Dev.* **140,** 965–972.
3. Wierdl, M., Dominska, M. & Petes, T. D. (1997) *Genetics* **146,** 491–498.
4. Streisinger, G., Okada, Y., Emrich, J., Newton, J., Tsugita, A., Terzaghi, E. & Inouye, M. (1966) *Cold Spring Harbor Symp. Quant. Biol.* **31,** 77–84.
5. Kolodner, R. (1996) *Genes Dev.* **10,** 1433–1442.
6. Foury, F., Roganti, T., Lecrenier, N. & Purnelle, B. (1998) *FEBS Lett.* **440,** 325–331.
7. Grimes, G. W., Mahler, H. R. & Perlman, P. S. (1974) *J. Cell Biol.* **61,** 565–574.
8. Foury, F. (1989) *J. Biol. Chem.* **264,** 20552–20560.
9. Foury, F. & Vanderstraeten, S. (1992) *EMBO J.* **11,** 2717–2726.
10. Roberts, J. D. & Kunkel, T. A. (1996) in *DNA Replication in Eukaryotic Cells*, ed. DePamphilis, M. L. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 217–247.
11. Reenan, R. A. G. & Kolodner, R. D. (1992) *Genetics* **132,** 975–985.
12. Chi, N.-W. & Kolodner, R. D. (1994) *J. Biol. Chem.* **269,** 29984–29992.
13. Marra, G. & Boland, C. R. (1995) *J. Natl. Cancer Inst.* **87,** 1114–1125.
14. Brown, M. D. & Wallace, D. C. (1994) *J. Bioenerg.* **26,** 273–289.
15. Tritschler, H.-J. & Medori, R. (1993) *Neurology* **43,** 280–288.
16. Steele, D. F., Butler, C. A. & Fox, T. D. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 5253–5257.
17. Herskowitz, I. & Jensen, R. E. (1991) in *Guide to Yeast Genetics and Molecular Biology*, eds. Guthrie, C. & Fink, G. R. (Academic, San Diego), pp. 132–146.
18. Fox, T. D., Folley, L. S., Mulero, J. J., McMullin, T. W., Thorsness, P. E., Hedin, L. O. & Costanzo, M. C. (1991) in *Guide to Yeast Genetics and Molecular Biology*, eds. Guthrie, C. & Fink, G. R. (Academic, San Diego), Vol. 194, pp. 149–165.
19. Wach, A., Brachat, A., Pohlmann, R. & Philippsen, P. (1994) *Yeast* **10,** 1793–1808.
20. Wierdl, M., Greene, C. N., Datta, A., Jinks-Robertson, S. & Petes, T. D. (1996) *Genetics* **143,** 713–721.
21. Lea, D. E. & Coulson, C. A. (1949) *J. Genet.* **49,** 264–285.
22. Dujon, B. (1981) in *Molecular Biology of the Yeast Saccharomyces: Life Cycles and Inheritance*, eds. Strathern, J. N., Jones, E. W. & Broach, J. R. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 505–635.
23. Walmsley, R. M., Szostak, J. W. & Petes, T. D. (1983) *Nature (London)* **302,** 84–86.
24. Heimberg, H., Boyen, A., Crabeel, M. & Glansdorff, N. (1990) *Gene* **90,** 69–78.
25. Jauniaux, J.-C., Urrestarazu, L. A. & Wiame, J.-M. (1978) *J. Bacteriol.* **133,** 1096–1107.
26. Levinson, G. & Gutman, G. A. (1987) *Mol. Biol. Evol.* **4,** 203–221.
27. Henderson, S. T. & Petes, T. D. (1992) *Mol. Cell. Biol.* **12,** 2749–2757.
28. Farber, R. A., Petes, T. D., Dominska, M., Hudgens, S. S. & Liskay, R. M. (1994) *Hum. Mol. Genet.* **3,** 253–256.
29. Strand, M., Prolla, T. A., Liskay, R. M. & Petes, T. D. (1993) *Nature (London)* **365,** 274–276.
30. Birky, C. W. J. & Skavaril, R. V. (1976) *Genet. Res.* **27,** 249–265.
31. Sia, E. A., Kokoska, R. J., Dominska, M., Greenwell, P. & Petes, T. D. (1997) *Mol. Cell. Biol.* **17,** 2851–2858.