Mutations in the Yeast MRF1 Gene Encoding Mitochondrial Release Factor Inhibit Translation on Mitochondrial Ribosomes*

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Although the control of mitochondrial translation in the yeast Saccharomyces cerevisiae has been studied extensively, the mechanism of termination remains obscure. Ten mutations isolated in a genetic screen for read-through of premature stop codons in mitochondrial genes were localized in the chromosomal gene encoding the mitochondrial release factor mRF1. The mrf1–13 and mrf1–780 mutant genes, in contrast to other alleles, caused a non-respiratory phenotype that correlated with decreased expression of mitochondrial genes as well as a reporter ARG69™ gene inserted into mitochondrial DNA. The steady-state levels of several mitochondrially encoded proteins, but not their mRNAs, were dramatically decreased in mrf1–13 and mrf1–780 cells. Structural models of mRF1 were constructed, allowing localization of residues substituted in the mrf1 mutants and offering an insight into the possible mechanism by which these mutations change the mitochondrial translation termination fidelity. Inhibition of mitochondrial translation in mrf1–13 and mrf1–780 correlated with the three-dimensional localization of the mutated residues close to the PST motif presumably involved in the recognition of stop codons in mitochondrial mRNA.

The mitochondrial genetic system of the yeast Saccharomyces cerevisiae has been a subject of intensive studies. Seven genes in the yeast mitochondrial DNA (mtDNA) encode integral membrane proteins that are assembled together with subunits encoded in the nuclear DNA into multicentric respiratory complexes. Maintenance and expression of mtDNA require approximately 200 proteins encoded in the nuclear DNA and imported to mitochondria from the cytosol (1, 2). Among them are mitochondrial ribosomal proteins, general translation factors, and translational activators. Current analysis of the proteome of yeast mitochondria allowed for identification of 65 mitoribosomal proteins (2); however, only ~60% of these have recognizable homology with bacterial ribosomal proteins (3). Multiple mitoribosome-specific proteins in yeast and mammals demonstrate the considerable divergence of mitochondrial ribosomes from their prokaryotic ancestors (4, 5). The mechanisms that control mitochondrial translation are difficult to study because no in vitro system for the expression of translation products encoded in mtDNA has yet been established. In contrast to the biochemical difficulties, the yeast S. cerevisiae, being a facultative anaerobe, offers distinct advantages for mitochondrial genetic studies. Several hundred point mutations or small deletions called mit™ have been genetically mapped and assigned to genes in mtDNA. We have long been involved in a search for suppressors of mit™ mutants that act at the level of mitochondrial translation. Analogous suppressor approach applied to chromosomal mutants allowed identification of proteins responsible for translation fidelity in the yeast cytosolic system. Ribosomal proteins from the decoding center of the ribosome and translation termination factors represented two classes of proteins that control decoding of the stop signal (6, 7).

An ochre mit™ mutation cox2-V25, localized in the coding region of the mitochondrial COX2 gene, was used for suppressor screen. One dominant suppressor, NAM9–I (8), and 10 recessive suppressors (9) were identified. The cloning of the NAM9–I gene identified a structural component of the mitochondrial ribosome homologous to the S4 ribosomal protein from bacteria and eukaryotes that controls translation fidelity (8, 10). Here we show that all of the remaining recessive nuclear suppressors are mutations in a gene specifying the mitochondrial translation termination factor mRF1 (11).

mRF1 is the only release factor that recognizes stop codons in mitochondrial mRNAs (11, 12). In eukaryotic cytosol, also a single factor, eRF1, recognizes all three stop codons. In contrast, prokaryotes have two codon-specific factors, RF1 for UAA/UAG and RF2 for UAA/UGA. Eukaryotic eRF1s and prokaryotic RFs show no detectable similarity at the level of amino acid sequences, but their shapes mimic tRNA as seen from their crystal structures (13, 14). Functional mimicry of release factors and tRNA is supported by biochemical and genetic studies that point to tripeptide “anticodons” in bacterial RF1 and RF2 (15, 16).

The yeast mrf1 mutants described here are, to our knowledge, the first identified informational suppressors of mit™ mutations that act by changing mitochondrial translation termination fidelity. We constructed mRF1 structural models that allowed localization of the amino acids substituted in the mutants and offered a clue to the possible mechanism of suppression. Interestingly, molecular analysis showed that some mrf1 mutations block translation of mitochondrially encoded proteins.

EXPERIMENTAL PROCEDURES

Media and Growth Conditions—Rich media contained YPD (1% yeast extract, 2% peptone, and 2% glucose) or 2% galactose (YPGal) or media and growth conditions

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‡ The abbreviations used are: mtDNA, mitochondrial DNA; Cox2 and 3, cytochrome c oxidase subunits 2 and 3; RF, release factor; mRF, mitochondrial RF.
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2% glycerol (YPGly). Minimal medium SC (0.67% yeast nitrogen base without amino acids) was supplemented with 2% glucose, amino acids, uracil, and adenine as required (17). All of the reagents were from Difeo. For determination of the respiratory (Gly) phenotype, yeast strains were grown on YPD plates, replica-plated on YPGly, and incubated for 3 days at 30 °C. To estimate accumulation of [rho]– mutations, initially [rho]– strains were plated for single colonies on YPD and replica-cropped with a [rho]– testor followed by scoring of diploids on YPGly. For in vivo labeling and isolation of mitochondria, yeast were grown at 30 °C in liquid YPGly containing 0.3% glucose and harvested right after the point of glucose exhaustion as determined with Gluconostix (Bayer).

Yeast strains, Plasmids, and DNA Manipulations—S. cerevisiae strains used in this study are listed in Table 1. Cytoduction, generation of R13 and standard genetic manipulations were performed following published procedures (17). As reported before, R13 and R780 had the Gly phenotype with wild type mtDNA but leaky growth on YPGly with the mit– mutant mtDNA (9). Genetic analysis was done to confirm the phenotype of R13, referred to here as the mit–13 mutant. 10 full tetrads from the cross-R13 [rho]– × W303-1B were analyzed. Clear 2:2 segregation of the mit–13 phenotype (Gly–) was observed. All of the ascospores were made [rho]– and then crossed back with the target mit– mutation cox2-V25 (strains CD11 and CD112). Only two clones per tetrad, those identified previously to carry mit–13, yielded Gly– because of suppression of cox2-V25.

A yeast genomic library in a pRS315 vector (18) was kindly supplied by M. Wysoczak (Institute of Biochemistry and Biophysics, Polish Academy of Sciences). Yeast cells were transformed by the lithium acetate method (19). MRF1 was subcloned as the Sac-Sall fragment in pRS306, pRS314, and pRS315, resulting in pRS306-MRF1, pRS314-MRF1, and pRS315-MRF1, respectively. For chromosomal integration in the MRF1 locus, pRS306-MRF1 was cut with EcoRI before transformation.

The isogenic wild type MRF1 strain was generated by two step gene replacement (20). pRS306-MRF1 was integrated in the MB143-3D/13 genome, resulting in the Ura– phenotype. Homologous recombination, forced by growth of the integrant on SC medium supplemented with 5-fluoroorotic acid (2 mg/ml), resulted in excision of the URA3 plasmid and loss of either the MRF1 or mit–13 allele. The Gly– clone corresponding to as MB143-3D. An analogous approach was subsequently applied to generate the isogenic mit–13–80 mutant (MB143-3D/780) using the PCR-amplified mit–13–80 allele of R780 subcloned in pRS306.

All mit1 alleles were amplified by PCR using Mastercycler gradient (Eppendorf), specific primers 5'–TCGGCCGTCGACATTGTCGCATCGA –G–3' and 5'–AAATACAAACCTTATATGGTTGATGC–3', TaqDNA pol ymerase (Promega), and genomic DNA prepared from strains R12, R13, R16, R705, R726, R742, R752, R763, R780, R838, R136, B145, MB143-3D/13, MB168-5A, and MB165-2D (see Table 1). For each mutant, five PCR reactions were pooled and the PCR fragment was cloned in pGEM-T Easy vector (Promega) and sequenced. The presence of each mutation was confirmed in three independent clones.

MB168-2D and MB168-5A were spores from the cross-R780 × W303-2C. MB168-2D was subsequently crossed with JCS855 to generate MB172-1B (mit–13–80, kar–1). M114-1D (mit–13–80 kar–1 arg8–URA3) was selected from the spore progeny of the cross-MB172-1B × DFS160. Cytoduction was applied to transfer mitochondria containing the ARGS reporter gene from donor cells (DFS189) into the nuclear mit–13–80 background of recipient cell MJ14-1D [rho]– harboring the Ycp314-MRF1 plasmid. Cytoductants were red as a result of the ade2–1 nuclear marker of MJ14-1D. The presence of the ARGS mitochondrial reporter gene was confirmed by a cross with the tester strain AK5-13B (recombination of mtDNA carrying the point cox2-V25 mit– mutation with mit–13–80 mit–13–80 cox2-ARGS– deletion gives wild type mtDNA, leading to the recovery of the Gly– phenotype).

In Vivo Labeling of Mitochondrial Translation Products—Cells grown as described above were suspended in buffer P (40 mM KPi, pH 7.0, 0.45% glucose, 0.0077% Complete supplement mixture-methionine (BIO101)) and incubated on a shaker for 30 min at 30 °C. Cycloheximide (20 μg/ml) was used to reduce the final concentration of 20 μM. After centrifugation, the cells were washed in CM buffer (40 mM KPi, pH 7.0, 100 μg/ml cycloheximide), supplemented with 20 μM methionine, and collected. To a second aliquot of [35S]methionine incorporation, cells were filtered under vacuum on GF/A glass microfiber filters (Whatman), washed with 10% trichloroacetic acid, 95% ethanol, and acetone, and dried. Radioactivity was measured in a scintillation counter. To determine mitochondrial translation products, total protein was extracted from collected cells as described by Taffe and Schatz (21). Mitochondrial translation products were analyzed by 12% SDS-PAGE followed by autoradiography.

Analysis of Mitochondrial Protein and RNA—Yeast were grown as described above. Mitochondria were purified according to Glick and Pon (22). The Nycodenz purification step was omitted. Protein concentration was estimated with a Bio-Rad protein assay using bovine serum albumin as a standard.

Mitochondrial proteins were separated on SDS-PAGE, transferred to Hybond C-Extra (Amersham Biosciences), and hybridized with antibody according to instructions. The monoclonal antibodies against Cox2 and Cox3 were from Molecular Probes. All of the other antibodies were from the collection of G. Schatz (Biozentrum, Basel, Switzerland). Secondary antibody coupled to horseradish peroxidase (DAKO) was visualized by chemiluminescence using the ECL detection kit (Amersham Biosciences) followed by autoradiography. Films were quantified using ImageQuant, version 1.1 with local average background correction (Molecular Dynamics, Sunnyvale, CA).

RNA was isolated from mitochondria using the TRIzol reagent (Invitrogen). 10 μg of each RNA sample was resolved by electrophoresis in 1% agarose gel containing 0.925% formaldehyde in NBF buffer (0.5 M boric acid, 10 mM sodium citrate, 50 mM NaOH). The RNAs were transferred from gel to a Hybond N* membrane (Amersham Biosciences) and hybridized with probes according to instructions. 5'-TTAAGCCCAACCGGATCCCTCCGAGTAAGCTGA-3' and 5'-GTCTTGTTATCTATCATGGAAAATTTAGGATACCTTGACGATG-3' oligonucleotides labeled with [α-32P]dATP and T4 polynucleotide kinase were used as probes for 16 S RNA and COX2 mRNA, respectively. PCR fragment synthesized with 5'-TATGGCTTCACGATTCTCCAATG-3' and 5'-CATGTAAGAAGACTGACTGA-3' primers and labeled with [α-32P]dATP using random primer system served as a probe for COX3 mRNA. After hybridization, blots were washed 15 min in 6× SSC at room temperature and exposed to film or to a PhosphorImager plate. RNA was quantified using the laser densitometer GelScan XL (Phar macia). COX2 and COX3 mRNA levels, measured for two independent blots, were corrected for 16 S rRNA levels.

Cyclochrome Spectra—Cells were grown on YPGal plates. Cyclochrome absorption spectra were recorded for whole cells in liquid nitrogen as described previously (23).

Three-dimensional Models of mRF1—A three-dimensional model of S. cerevisiae mRF1 protein in the conformation unbound to the ribosome was generated with the MODELER program (24) using the Escherichia coli RF2 (1QGE) crystal structure (14) as template. The N- terminal mitochondrial targeting sequence was not considered in the model. The critical step in the modeling, generation of the sequence-to-structure alignment, was based on the results of secondary structure prediction and tertiary-fold recognition carried out with the Meta Server (24) using the consensus alignment approach and three-dimen sional assessment (25). In addition, a Co trace model of RF1 bound to the ribosome was derived from the low resolution structure of E. coli RF2 in a complex with the ribosome (26).

RESULTS

1. Identification of mit– Suppressor Mutations as Mitochondrial Gene Specifying the Mitochondrial Translation Termination Factor—Several recessive suppressors of the mit– nonsense mutation cox2-V25 were isolated previously and characterized genetically (9) but not identified up to now. Specificity studies involving a collection of several hundred point mutations or small deletions in mtDNA revealed that the suppressors act on <20 mutations localized in different mitochondrial genes. The action spectra and growth phenotypes of the suppressors were similar but not identical, indicating that they are not identical mutations. 2 of 10 suppressors were temperature-sensitive, and these two mutants (designated previously as R13 and R780) did not grow on a medium containing non-fermentable carbon when combined with wild type mtDNA (9). To clone the gene able to complement the Gly– phenotype of the R13 mutant, the wild type yeast single copy genomic library was introduced into MB143-3D/13 cells (see Table I and “Experimental Procedures”). Screening of 12,000 Leu+ transformants for complementation of the respiratory defect resulted in two clones that showed a plasmid-dependent phenotype. Another analysis revealed two overlapping plasmids that have the
MRF1 gene in common. MRF1 is known to encode the mRF1 release factor involved in decoding of stop signals during termination of protein synthesis on mitochondrial ribosomes (11).

To confirm that R13 and possibly other recessive suppressors are alleles of MRF1, we first mapped these mutants by genetic recombination. The wild type strain W301-1B with the MRF1 locus was crossed with R13 and several other suppressors, including the CK311/B145 strain carrying the nam3–1 mutation (27). Diphtheria were sporulated, and >10 tetrads from each cross were analyzed for segregation of Ura+ and suppressor phenotypes. In none of the crosses did these traits co-segregate, providing strong evidence that the examined suppressor mutations are alleles of MRF1.

All mrf1 alleles were amplified and sequenced on both strands. This confirmed that all of the previously isolated recessive suppressors of mit- cox2-V25 contained single point mutations in the mrf1-coding region, resulting in the following amino acid substitutions: mrf1–12 (V163D); mrf1–780 (G164C); mrf1–136 (S216Y); mrf1–763 (V226F); mrf1–838 (R228G); mrf1–13 (R231G); mrf1–705 (A247P); mrf1–16 (R313I); mrf1–752 (Q349R); and mrf1–145 (S352I). Note that mrf1–145 and mrf1–136 represent nam3–1 and nam3–2, respectively. The remaining mrf1 allele numbers correspond to the R suppressors.

2. Localization of Suppressor Mutations in mRF1 Domains—The strong sequence conservation between the release factors from prokaryotes and mitochondria and the known crystal structure of E. coli RF2 (14) allowed us to build a reliable three-dimensional model of the S. cerevisiae mitochondrial mRF1 protein (Fig. 1A). Moreover, on the basis of a low resolution structure of the E. coli RF2 termination complex (22), a Ca trace model for mRF1 in a complex with the ribosome was also derived (data not shown).

mRF1 is composed of four domains (Fig. 1), largely corresponding to those predicted by Pol (11). The N-terminal helical domain 1, shown in brown, is apart from the compact structure formed by the remaining domains. The highly conserved domain 2 (blue) is supposed to be responsible for stop codon recognition. Domain 4 (magenta) is considered as a subdomain of domain 2. Domain 3 (green) contains the absolutely conserved GGQ motif responsible for hydrolysis of the peptidyl-tRNA bond (9). The highly conserved Arg-313 in domain 3 is located close to the GGQ loop and possibly interacts directly with the tRNA molecule. Mutating this charged residue to the hydrophobic isoleucine in mrf1–16 may destabilize the interactions of domain 3 with tRNA, decreasing catalytic activity of mRF1 toward the peptidyl-tRNA bond.

The PST motif in domain 2 of mRF1 corresponds to the tripeptide anticodons identified in the RFs of E. coli (15), resembling rather the PAT motif of RF1 than the SPF motif of RF2. This is consistent with the fact that the RF2-specific UGA codon is not a stop signal in yeast mitochondria (28). We believe that the PST directly binds to the stop codons in mitochondrial mRNA. In addition, in the model of mRF1 in complex with the ribosome, the PST motif contacts helix 44 of 16 S rRNA. Helix 44 is involved in the interaction between the ribosomal subunits, possibly important for the signaling from the stop codon to the peptidyl transferase center (29). As shown in Fig. 1, domain 2 contains the majority of the amino acid substitutions caused by our mrf1 mutations. These mutations can be divided into three groups. The first comprises mrf1–780 (G164C) and mrf1–13 (R231G), which cause lack of growth on media requiring respiration and inhibit mitochondrial translation (see results below). Interestingly, the substituted residues, Gly-164 and Arg-231, are located most closely to the PST motif (Fig. 1). Gly-164 is one of the two conserved glycines that form a tight loop from the middle β-strand in domain 2 to the central α-helix of mRF1. This structural feature seems to be important for the tight packing of mRF1 onto the ribosome as well as for PST loop maintenance and orientation. Replacement of Gly-164 with a bulky amino acid side chain probably causes several sterical clashes and destabilizes the correct orientation of the PST loop. The positively charged side chain of the highly conserved Arg-231 seems to stabilize the conformation of the PST loop and, in addition, could be directly involved in contact with 16 S rRNA. The R231G substitution would destroy these interactions.

The S216Y substitution, in a position occupied by small amino acids in homologous RFs, replaces the second group of mutations in mRF1 domain 2. Ser-216 is buried in the mRF1 structure in a tight pocket formed by surrounding residues. Conversion of the serine side chain into the bulkier tyrosine could destroy internal packing, leading to a local conformational change of the backbone, possibly affecting the linker between domains 1 and 2 and, consequently, the relative orientation of these domains.
FIG. 1. Localization of suppressor mutations in mRF1. A, ribbon diagram of a three-dimensional model of *S. cerevisiae* mRF1. Domain 1 is shown in brown, domain 2 is shown in blue, domain 3 is shown in green, and domain 4 is shown in magenta. The locations of functional PST and GGQ motifs are labeled. The positions of amino acid substitutions that change translation fidelity in the *mrf1* mutants are marked as red spheres. Critical residues, corresponding to the *mrf1*–13 and *mrf1*–780 mutations, are underlined. B, multiple sequence alignment of several mitochondrial mRF1 and *E. coli* RF1 and RF2 proteins for domains 2 and 3. Residues conserved in >60% of all of the sequences are highlighted in black (identical) and gray (similar). The positions of residues substituted in the analyzed *mrf1* mutants are marked in red with mutated residues shown above the sequence. The locations of secondary structure elements, α-helices (H) and β-strands (E) in *E. coli* RF2, are marked below the sequences. Color shading of secondary structure elements corresponds to that in the structural diagram.
The substitutions representing mutations from the third group, V163D, V226F, R228G, and A247P, cluster on the surface of the β-sheet of domain 2. Interestingly, in the structure of mRF1 complexed with the ribosome, these usually (when unbound to ribosome) solvent-exposed residues interact with the long loop-connecting domains 3 and 4 (data not shown). This linker between the domains contains two other amino acid substitutions detected in our study, Q349R and S352I, that interact directly with the mutations located on the surface of the β-sheet of domain 2 but only when mRF1 adopts the conformation observed in the complex with the ribosome. As a consequence, the V163D, V226F, R228G, A247P, Q349R, and S352I substitutions may influence the movement of domains 1 and 3 with respect to the functional unit formed by domains 2 and 4 and alter mRF1 interaction with the ribosome. In addition, change in physicochemical properties of the domain 2 surface caused by these mutations may also affect its direct binding to the ribosome (ribosomal protein S12 and 16 S rRNA).

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3. mrf1 Mutant Cells Maintain Wild Type mtDNA and Mitochondrial Ribosomes—The non-respiratory phenotype of mrf1–13 and mrf1–780 point to an essential role of Gly-164 and Arg-231 for mRF1 activity. These two mutants do not grow on medium containing glycerol or other non-fermentable carbon sources if wild type mtDNA is present (9). In contrast, other mrf1 alleles do not cause respiratory defects.

Because mRF1 is required for the maintenance of wild type mitochondrial genome, the loss of mtDNA could explain the respiratory deficiency of mrf1–13 and mrf1–780 cells. To determine mtDNA maintenance, mrf1–13 and mrf1–780 were crossed to a wild type [rho0] strain. The resulting diploids grew on non-fermentable carbon sources, indicating that the mutants are recessive and that the cells retained mtDNA (Fig. 2A).

The fraction of accumulated [rho–] mutations, determined as described under “Experimental Procedures,” is 22% in mrf1–13 and 51% in mrf1–780, whereas the control strain and the remaining mrf1 alleles show 1–4% [rho–]. It is obvious that stronger defects in translation termination lead to increased accumulation of [rho–], although ~50% of cells with mtDNA retained is enough to ensure expression of mitochondrial genes in a cell population.

Unstable mitochondrial ribosomes in cells with a non-functional release factor could be an alternative reason of the respiratory deficiency of mrf1–13 and mrf1–780 mutants. The steady-state levels of selected mitochondrial ribosomal proteins were examined to rule out the effect of ribosome degradation. It is known that Mrp13, Mrp20, and Mrp49 are relatively unstable in the absence of ribosome assembly (30, 31) and free Mrp7 is relatively stable (32). As presented in Fig. 2B, the levels of all of the mitochondrial ribosomal proteins tested, Mrp7, Mrp13, Mrp20, and Mrp49 are not affected in mrf1–13 and mrf1–780 cells (Fig. 2B). This shows that despite the presence of a non-functional release factor, the mitochondrial ribosome is assembled and not degraded in the mrf1–13 and mrf1–780 cells.

4. Inhibition of Mitochondrial Translation Mediated by Mutated Release Factor mRF1—We compared the rate of synthesis of mitochondrial gene products in wild type (MB143-3D), mrf1–13 (MB143-3D/13), and mrf1–780 (MB143-3D/780) cells by pulse labeling with [35S]methionine in the presence of cycloheximide to inhibit cytoplasmic protein synthesis. Proteins were then analyzed by SDS-PAGE and autoradiography (Fig. 3A). The rate of label incorporation by the mrf1–13 and mrf1–780 cells was significantly lower than that in the control strain (Fig. 3B). Moreover, the synthesis of Cox subunits was decreased. Labeling of Cox2 was not visible, and labeling of Cox1 was reduced in the mutants relative to wild type. It was difficult to estimate Cox3 labeling because this band overlapped Atp6, but subsequent study showed that the level of Cox3 was decreased also (see below). Thus, mrf1–13 and mrf1–780 cells have a decreased level and, additionally, an altered pattern of mitochondrial protein synthesis.

The cytochrome spectrum of mutant mitochondria was determined for frozen cells (Fig. 3C). The absorption bands corresponding to cytochrome b and to cytochromes aa3 (which reflects the amount of cytochrome c oxidase) were not visible in the mrf1–13 and mrf1–780 mutants. In contrast, the band of cytochrome c+c3 was unaffected, presumably because no mitochondrial encoded component was involved.

Western blot analysis of mutant cell extracts revealed considerably reduced levels of the mitochondrially encoded proteins Cox2, Cox3, and Atp6 (Fig. 3D). Reduction of Cox2 and Cox3 was by ~90%, and only traces of these proteins were
detectable in mrf1–13 and mrf1–780 cells at steady state. The defect of Cox2 and Cox3 synthesis was not the result of an mRNA synthesis or stability defect, because the steady-state levels of COX2 and COX3 mRNAs were similar in the mrf1–13, mrf1–780, and wild type strains (Fig. 3E).

To further monitor translation of the mitochondrial COX
locus, we took advantage of the synthetic mitochondrialy located ARG8m gene kindly provided by T. Fox and successfully used in his laboratory as a reporter for mitochondrial gene expression (33). Arg8 protein is normally encoded in the nucleus, synthesized in the cytoplasm, and imported into the mitochondrial matrix where it participates in arginine biosynthesis. The synthetic ARG8m when inserted to mtDNA encodes the same enzyme within the mitochondrial matrix and is able to complement chromosomal arg8-Δ deletion. To check whether mutant release factor inhibits translation of the reporter gene, we performed a cytoduction experiment. We introduced mitochondria containing ARG8m in the COX3 coding region to the recipient mrf1-780 arg8-Δ cells devoid of mtDNA and containing a plasmid with MRF1 (Fig. 4 and see “Experimental Procedures”). The resulting cytoductants were replica-plated on a medium lacking arginine. In the presence of the MRF1 gene supplied by the plasmid, the cytoductant was Arg+ , indicating efficient translation of the reporter cox3:ARG8m gene. The effect of mrf1-780 was observed when the complementing pRS314-MRF1 plasmid was lost on a non-selective medium. mrf1-780 inhibited translation of cox3:ARG8m, which resulted in weak growth on a medium lacking arginine. The results of this experiment (Fig. 4) verify that the mrf1-780 mutation inhibits translation of mitochondrial mRNAs.

**DISCUSSION**

Translation seems to be crucial for regulation of mitochondrial gene expression (34), but the role of the termination step in this regulation has not been studied so far. We identified ten alleles of the S. cerevisiae chromosomal gene encoding mitochondrial release factor mRF1 that change the fidelity of stop codon recognition. Only two alleles show a non-respiratory phenotype in the presence of wild type mtDNA, and this phenotype correlates with inhibition of mitochondrial translation.

It is known that mitochondrial translation in yeast is controlled at the initiation and elongation steps, although the mechanisms of this control are poorly understood. The scanning model does not function in start codon selection in yeast mitochondrial mRNA, and initiation might be analogous to that of internal ribosome entry in eukaryotic cytoplasmic translation (35). The major distinguishing feature is that mitochondrial initiation requires the action of membrane-bound translational activator proteins that recognize targets in the 5’-untranslated leaders and seem to mediate individual mRNA-mitoribosome interactions (34, 36). Yeast mitoribosomes are also sensitive to secondary mRNA structures within coding sequences. Translation of COX2 is inhibited by ribosome pausing at negative regulatory sequences identified in the coding region that are antagonized by a positive sequence specifying the pre-Cox2 leader peptide (37, 38).

The data presented here show that mitochondrial translation can also be inhibited at the termination step. Such inhibition was observed in the presence of specific mutations, mrf1-13 and mrf1-780, in the nuclear gene encoding the mitochondrial release factor, mRF1. The overall rate of translation on mitochondrial ribosomes was reduced in mrf1-13 and mrf1-780 cells, but the degree of inhibition varied among individual mitochondrially encoded polypeptides, being the most pronounced for COX genes. Decreased mitochondrial translation of COX1 and COX2 was previously reported for two other mrf1 mutants (11). Similar gene-specific effects on mitochondrial gene expression were reported previously for mutations in other general components of mitochondrial translation system (10, 39–41). Additionally, we showed that the steady-state levels of Cox2 and Cox3 were decreased in mrf1-13 and mrf1-780 over 20-fold, whereas the levels of the respective mRNAs were not affected. A connection of this gene-specific effect with co-translational cytochrome oxidase assembly could be excluded because translation of the ARG8m reporter gene inserted into the COX3 locus was also decreased. Additionally, translation inhibition, some post translational effects were also observed. Although COB translation occurred efficiently in mrf1-13 cells, cytochrome b was not observed in the spectrum. Moreover, whereas mrf1-13 and mrf1-780 showed Gly- phenotype with wild type mtDNA, the same mutations acted as suppressors of premature stop codons in mtDNA and caused leaky growth on YPGly with mit- mutant mtDNA (9).

Although the detailed genetic analysis reported here fully confirms this phenotype, we do not understand the molecular basis of observed effect. Similarly, the pet309 allele isolated as a suppressor of a cox3 initiation codon mutation caused the Gly- phenotype with the wild type mtDNA but leaky growth with mutant mtDNA.

The molecular mechanism of translation inhibition in the

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2 L. S. Folley, D. F. Steele, and T. D. Fox, personal communication.
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presence of mutated release factor is unknown. The level of mRF1 protein is not decreased in the mrf1–13 and mrf1–780 mutants (data not shown); therefore, simple titration of the factor could be excluded. Instead, it is likely that mutations change the interaction of mRF1 with the mitoribosome as it was shown previously for two other mrf1 mutants (12). One could expect a slowing down of the codon recognition step in the mrf1–13 and mrf1–780 mutants because of amino acid substitutions close to the PST motif that is suggested to act as a peptidyl-tRNA hydrolysis, and, consequently, inhibition of translation.

Newly synthesized nascent polypeptide chains emerge from the ribosome through the exit tunnel. As revealed by recent cryoelectromagnetic studies, the structure of the exit tunnel in mitoribosomes is distinctly different from the tunnels in cytoplasmic ribosomes. The tunnel has a wide opening allowing access of proteins that are not defined so far (42). It is probable that the nascent mitochondrial polypeptide chains interact with the release factor and this interaction stabilizes an intermediate termination complex. One could assume that particular amino acid substitutions in mutated mRF1 increase the stability of these interactions leading to ribosomal stalling. Moreover, the degree of inhibition could be differentially modulated by the C-terminal sequences and that is a possible explanation why mrf1–13 and mrf1–780 differently affect translation of the various mitochondrialy synthesized polypeptides. Interestingly, a mechanism of translation termination inhibition, mediated by direct interaction of the nascent polypeptide chain with the eRF1 release factor, has been described for cytomegalovirus (43).

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