Functional Characterization of Yeast Mitochondrial Release Factor 1*

Received for publication, December 29, 1999, and in revised form, March 15, 2000
Published, JBC Papers in Press, March 22, 2000, DOI 10.1074/jbc.M910448199

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The yeast Saccharomyces cerevisiae mitochondrial release factor was expressed from the cloned MRF1 gene, purified from inclusion bodies, and refolded to give functional activity. The gene encoded a factor with release activity that recognized cognate stop codons in a termination assay with mitochondrial ribosomes and in an assay with Escherichia coli ribosomes. The noncognate stop codon, UGA, encoding tryptophan in mitochondria, was recognized weakly in the heterologous assay. The mitochondrial release factor 1 protein bound to bacterial ribosomes and formed a cross-link with the stop codon within a mRNA bound in a termination complex. The affinity was strongly dependent on the identity of stop signal. Two alleles of MRF1 that contained point mutations in a release factor 1 specific region of the primary structure and that in vivo compensated for mutations in the decoding site rRNA of mitochondrial ribosomes were cloned, and the expressed proteins were purified and refolded. The variant proteins showed impaired binding to the ribosome compared with mitochondrial release factor 1. This structural region in release factors is likely to be involved in codon-dependent specific ribosomal interactions.

Release factors (RFs) are proteins involved in the decoding of stop signals during translational termination. There are two prokaryotic class I RFs, RF1 and RF2, which recognize UAA/UAG and UUA/UAGA, respectively, within termination signals (1), and one class II RF, RF3, which is involved in recycling the other two factors (2). RF1 and RF2 are structurally related (3), whereas RF3 is distinct (4). In the eukaryotic cytosol, two RFs have been identified: eukaryotic RF1, which recognizes all three stop signals (5), and the G protein eukaryotic RF3 (6), which stimulates eukaryotic RF1 activity. They are structurally and functionally distinct compared with their prokaryotic counterparts (7). An organellar RF was first isolated from rat liver mitochondria (8), and genes coding for putative organellar RFs have been cloned from the yeasts Saccharomyces cerevisiae and Kluyveromyces lactis (9, 10) and from humans (11). The S. cerevisiae gene encodes a product of 413 amino acids with a calculated molecular mass of 46.74 kDa, a larger protein than either E. coli RF1 or RF2. This could be explained by a small number of insertions and the presence of extra amino acids at the N terminus (9). The N terminus derived from the gene sequence has a composition suggestive of a cleavable mitochondrial targeting sequence.

The most functionally conserved sites between prokaryotic and mitochondrial ribosomes are the decoding site and peptidyltransferase center, and the most highly conserved primary sequence in the RFs might reflect an interaction with either of these two centers (12). The alignment of amino acid sequences of yeast mRF1 and class I prokaryotic RFs (9) shows the highest percentage of identity in the central and the C-terminal regions, particularly within a stretch of 43 amino acids between position 280 and 322 of mRF1 (79 and 70% with RF1 and RF2, respectively). The alignment reveals 15 positions at which the mRF1 is identical to prokaryotic RF1s but differs from RF2, whereas there is only one residue at which mRF1 is identical to RF2 but differs from RF1 (9). A group of four such positions is in cluster B (12), suggesting that this region might play a role in some RF1-specific trait. The higher resemblance between mRF1 and RF1 would support the idea that yeast mRF1 is also an RF1-type protein, particularly because the RF2-specific UGA codon is not a stop signal in yeast mitochondria (13). In yeast mitochondrial genes, the most common termination codon is UAA, although amber mutations and frameshift mutations terminated by UAG have been described (14, 15). Only one factor of the RF1 type would be sufficient in yeast mitochondria as well as in mammalian mitochondria, and indeed, the S. cerevisiae genome contains no obvious reading frame that could encode an RF2-like protein.

Two mutant alleles of MRF1 have been detected and subsequently cloned and sequenced in the yeast S. cerevisiae with defective growth phenotype (16). Both alleles had single amino acid changes that mapped in the same region of the putative RF with high sequence similarity between mitochondrial and bacterial RFs (16). The leaky phenotype of these yeast mutants suggested that both variant factors were able to catalyze translational termination, albeit with a reduced efficiency. Introduction of the wild-type MRF1 gene into mRF1-mutant strains resulted in full complementation of the mutant phenotype. Multiple copies of the gene for mRF1-R231K also restored growth on glycerol to almost wild-type level. In contrast, there was no complementation of the mRF1-P233L allele (16).

These studies suggested that the yeast MRF1 gene encoded a mitochondrial RF, although supporting biochemical studies have been lacking. In this study, we report biochemical evidence that the MRF1 gene encodes an RF1-like factor with...
activity on both mitochondrial and bacterial ribosomes responding to UAA and UAG termination codons. The two variant proteins are defective in ribosome binding, consistent with the observation that mutations in the decoding site region of the small subunit rRNA of mitochondria can restore the phenotype in yeast carrying these mutations (16).

**MATERIALS AND METHODS**

**Bacterial Strains and Plasmid DNA Used**

The *E. coli* BL21 strains were grown at 37 °C in LB (Luria Broth) medium transformed with plasmid pET-3a and its derivatives. The yeast strain *S. cerevisiae* D273 was grown at 28 °C in rich galactose medium (2% galactose, 2% Bacto-Peptone Diﬀo), and 1% yeast extract.

**Polymerase Chain Reaction of Inserts for mRF1**

The open reading frames of both full-length mRF1, without the targeting sequence and the mRF1-mutant genes (mRF1-R231K and mRF1-P233L) were amplified by polymerase chain reaction. For full-length mRF1, the two primers used were as follows: N terminus (YMRF1), 5'-CTG GCA cat ATG TOG CTT TCA AAG TTC-3'; C terminus (YMRF3), 5'-CTG GCT TTG GAA Atc AAG CTC-3'. For the short version of mRF1 and the two mutant alleles, the two primer sequences used were as follows: N terminus, (YMRF2), 5'-AGC AAC cat Atg Aaa CCA ACA TAC ACA GAA-3'. The N and C termini have *Ndel*/*BamHI* overhangs, respectively (underlined). Polymerase chain reaction conditions were 94 °C for 5 min, 30 cycles at 46 °C for 1 min, 72 °C for 3 min, and then 94 °C for 0.5 min.

**Expression of Constructs in E. coli**

Fleshly transformed individual colonies were grown overnight in the medium supplemented with the desired antibiotic. For protein expression, 250 ml or 1 liter of LB containing the appropriate antibiotic was inoculated with an overnight culture (A600 of ~0.05), and when the A600 reached about 0.6, 1 m mole isopropyl-1-thio-β-galactopyranoside was added to the culture to induce expression of the genes under the control of T7 promoter.

**Protein Purification**

**Isolation and Solubilization of Inclusion Bodies**—The cells from 1-liter cultures of *E. coli* BL21 containing pET-3a/MRF1, pET-3a/mRF1-R231K, or pET-3a/mRF1-P233L plasmid were harvested 3 h after induction by centrifuging at 5,000 × g for 20 min. These cells were frozen at −80 °C and resuspended in 7 ml/mg sonication buffer (0.05 M sodium phosphate buffer, pH 8, and 0.3 M NaCl). Lysozyme (1 mg/ml) was then sonicated three times for 10 s each and centrifuged for 20 min at 12,000 × g at 4 °C. The pellet was washed twice with storage buffer (0.2 mM Tris-HCl, pH 7.8, 5 mM KC1, and 1 mM dithiothreitol), once with storage buffer containing 1.5% (v/v) deoxycholate, and then with storage buffer again to remove the residual deoxycholate. This pellet was finally resuspended and homogenized in storage buffer containing 8 μM urea in a Dounce homogenizer.

**Refolding of Denatured Protein**—The solubilized protein was refolded by diluting the protein concentration in storage buffer without urea; the denatured protein was diluted very slowly (3 m/h) to a concentration of 0.2 mM in storage buffer containing 0.05% (v/v) Tween 20 at RT. Aquacide II and/or ultrafiltration were used to concentrate the diluted protein.

**Isolation of Mitochondria**

*S. cerevisiae* D273 was grown to late logarithmic phase. Cells were harvested by centrifuging at 1000 × g for 10 min, and the mitochondria were purified as explained in Ref. 17 with the following modifications. The cells were washed with water; suspended to 5 g of wet weight/ml in 0.1 mM Tris-H2SO4, pH 9.4, and 10 mM dithiothreitol; and incubated for 10 min at 30 °C. After washing with water and 1.2 mM sorbitol, cells were suspended in 1.2 mM sorbitol, 20 mM K HPO4/KH2PO4, pH 7.4 (0.15 g of wet weight cells/ml), Zymolyase 50000 (SEIKAGAKU) (5 mg/g of cells, wet weight) was added, and the suspension was incubated at 30 °C with gentle shaking until spheroplasts formed (about 2 h). The spheroplasts were washed with 1.2 mM sorbitol twice and then suspended in homogenization buffer (0.6 mM mannitol, 10 mM Tris-HCl, pH 7.4, 1 mM methyl-2-methylnitrosourea fluoride and 1 mM bovine serum albumin) to a concentration of 0.15 g of spheroplasts (wet weight)/ml. After chilling on ice, they were homogenized, and the suspension was clarified at 4000 × g for 10 min at 4 °C to remove the cellular debris. After a 15-min centrifugation at 20,000 × g, the mitochondria were pelleted. The isolated mitochondrial ribosomes were resuspended in homogenization buffer (0.6 mM mannitol, 0.5 mM EDTA, pH 6.7) and centrifuged at 5000 × g for 10 min at 4 °C to remove residual cellular debris. Finally, the mitochondria were pelleted at 20,000 × g for 15 min at 4 °C.

**Isolation of Mitochondrial Ribosomes**

The mitochondrial membranes were digested with 0.1% Triton X-100 (18), and the suspension was clarified at 30,000 × g for 30 min. The supernatant containing the mitochondrial matrix components was ultracentrifuged at 200,000 × g for 18 h. In an alternative method, crude mitochondrial ribosomes were isolated from mitoplasts. The mitochondrial pellet was resuspended in 5 volumes of breaking buffer (110 mM Tris-HCl, pH 7.4, 50 mM NH4Cl, and 10 mM Mg(OAc)2) and left in suspension on ice while being stirred very gently on a magnetic stirrer. Shocked mitochondria were sedimented at 48,000 × g for 20 min. The pellet was stored at −80 °C or used fresh for the following steps: the mitoplast was suspended at 4 mg/ml of breaking buffer and left as a suspension on ice to allow further swelling of the mitochondrial matrix for 5 min. A 15% volume of breaking buffer containing 1.8 μg sucrose and 8 μM ATP was added to the suspension and lysis was accomplished by strokes in a loose Dounce homogenizer. After 5 min on ice, the suspension was ultrasonicated. Total mitochondrial membranes were sedimented at 35,000 rpm in a Beckman type 40 rotor for 60 min. The supernatant represents the total mitochondrial matrix. Either the matrix was layered on a sucrose gradient or the ribosomes were pelleted by ultracentrifuging at 60,000 rpm in a Beckman Ti75 rotor for 90 min. The resuspended ribosomal pellet was used directly for functional assays or further purified on a 15–50% (w/v) sucrose gradient in a buffer containing 10 mM Tris-HCl, pH 7.4, 0.5 mM NH4Cl, and 10 mM Mg(OAc)2. The high salt was included to remove inhibitory substances and achieve an active preparation of ribosomes.

To determine the yield and purity of the mitochondria, the presence of lactate dehydrogenase enzyme has been used as a marker for cytoplasmic contamination. The activity of citrate synthetase was used as a marker for the mitochondrial matrix, and the rate of this activity was measured as described in Daum et al. (19). Protein concentration was assayed using the Lowry protein assay (20).

**Analysis of Ribosomes by Sucrose Gradient**

The gradient of 15–50% (w/v) sucrose was prepared, and the ribosomes were layered on top of the gradient. All samples were centrifuged at 20,000 rpm in SW-41 Beckman rotor for 20 h at 4 °C. The fractions were collected from the bottom of the tube. The A260 of each fraction was measured.

**In Vitro Termination Assay**

This assay was performed as described by Caskey et al. (21). Briefly, 70 S ribosomes (5 pmol) were incubated with [3H]Met-tRNA (2.5 pmol) and AUG (250 pmol) in binding buffer (20 mM Tris-HCl, pH 7.4, 10 mM Mg(OAc)2, 150 mM NH4Cl) at 30 °C for 20 min. This complex was incubated at 20 °C for 30 min with 20 pmol of release factor and 2000 pmol of stop codon. Reaction mixtures were stopped by the addition of 0.1 M HCl. Released [3H]Met was extracted into 1 ml of ethyl acetate by vortexing for 15 s. After separating the phases by centrifuging for 1 min at 1000 × g, radioactivity in 750 μl of the 1 ml organic phase was measured in a scintillation counter. The background was subtracted, using data from an assay excluding release factor and codon. The amount of released [3H]Met with bacterial ribosomes minus RF1 was approximately 0.05 pmol, and for mitochondrial ribosomes, it was approximately 0.14 pmol.

**Release Factor Ribosome Binding Assay Using an Airfuge**

In this assay, a complex of 50 pmol of ribosomes, 2 nmol of codon (stop or sense), and 20 pmol of RF was prepared in binding buffer (50 mM Tris-HCl, pH 7.2, 20 mM Mg(OAc)2, 100 mM NH4Cl) with 4% ethanol and incubated at 4 °C for 20 min. The 200-μl total volume complex was processed with an Airfuge at 30 psi (180,000 × g) for 20 min to pellet the ribosome-bound protein. Four 50-μl aliquots were collected from the top (unbound protein) to the bottom (including loose pellet containing the protein bound to 70 S ribosomes) of the Airfuge tube and analyzed by SDS-PAGE and Western blotting, with Ni-NTA-HRP conjugate detecting the His-tagged proteins. The density of each band was measured by laser densitometry.
Release Factor Ribosome Binding Assay Using a Spun Column

A complex similar to the above was prepared in a total volume of 50 μl. The complex was loaded onto a S300 spun column (Amersham Pharmacia Biotech) and centrifuged at 1500 × g for 2 min. 50 μl of binding buffer was loaded onto the column and centrifuged. This step was repeated five times, and each time, 50-μl fractions were collected. All fractions were analyzed by SDS-PAGE, followed by Western blotting against Ni-NTA-HRP conjugate, and the density of each band was measured by laser densitometer.

Site-directed Cross-linking and Analysis

The cross-linking reaction was prepared, and the level of cross-linked RF was measured as described (22).

RESULTS

Expression of Putative Yeast mRF1 in E. coli—Two DNA constructs expressed two forms of a putative S. cerevisiae mRF1 in E. coli. Plasmid pYMRF1 contained the entire reading frame of the MRF1 gene and produces pre-mRF1, a protein that contains the putative mitochondrial targeting signal at its N terminus. The characteristics of the N-terminal amino acid sequence (23) and sequence alignments between mRF1 and S. cerevisiae constructs expressed two forms of a putative yeast mRF1 in E. coli. If the MRF1 gene encodes a functional release factor, then it should function to release the nascent polypeptide from mitochondrial ribosomes. An in vitro assay to measure the hydrolysis of a model peptide, first described for bacterial ribosomes and factors by Caskey et al. (21), was investigated to test the mRF1 activity in a homologous in vitro assay with yeast mitochondrial components. Fig. 3A shows the sedimentation of ribosomes from both yeast mitochondria and E. coli in separate isokinetic sucrose gradients. A small yield of yeast mitochondrial ribosomes (70 S) was recovered from the matrix fraction at a similar position in the gradient to E. coli 70 S ribosomes (fraction 5 in each case).

Active preparations of mitochondrial ribosomes formed small amounts of a termination complex with fMet-tRNA.AUG. The activity of mRF1 was determined in the presence or absence of UAA, with the very limited amounts of active ribosomes available for these studies (Fig. 3B) and with the more abundant and robust E. coli ribosomes (Fig. 3B). Release of fMet from the termination complex mediated by mRF1 was observed in each case and was dependent on the stop codon, although limited codon-independent activity was measured.
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with bacterial ribosomes. This represented the first direct demonstration that the putative mitochondrial protein encoded by the mRF1 gene was indeed a release factor. The results also confirmed that the mRF1 formed a functionally active tertiary structure after protein refolding in vitro, using the procedure outlined in Fig. 2A. mRF1 was five times more active than the pre-mRF1 prepared in the same way (data not shown). This suggests that the extra sequence on this protein may indeed be the “dispensable” targeting sequence that facilitates transfer of the factor to the organelle before subsequent cleavage. Control extracts from the same bacterial strain without the mRF1 expression vector showed no detectable endogenous release factor activity, suggesting that functional activity did not result from minor contamination of E. coli RF1 in the mRF1 protein preparation.

Codon Specificity of mRF1 and Affinity for E. coli Ribosomes—The low yield and difficulty in obtaining active mitochondrial ribosomes necessitated that most studies had to be carried out with heterologous bacterial ribosomes. Despite this, the binding of the mRF1 to E. coli ribosomes in response to the stop codons UAA, UAG, UGA, or UGG was specific, with only the expected mitochondrial stop codons UAA and UAG stimulating the formation of stable termination complexes (Fig. 4A). A titration of E. coli RF1 and mRF1 with UAA determined that there was a ~100-fold difference between the specific release activities of RF1 and mRF1 on bacterial ribosomes (Fig. 4B), although the affinity of refolded mRF1 for bacterial ribosomes (as shown in Fig. 4A) was of the same order as that seen with RF1 under the same conditions (data not shown). Without codons, the ribosomes bound mRF1 with ~10% occupancy without any codon, however with the cognate codons UAA and UAG, they bound mRF1 with up to 50% occupancy at a 3-fold excess of factor. These values are comparable to those obtained with highly purified and active E. coli RF1.

Release activity was seen for E. coli RF1 (Fig. 4C) and mRF1 (Fig. 4D) with E. coli ribosomes in the presence of UAA and the relatively rare stop codon UAG, but in this assay, there was also additional stimulation of mRF1 with the tryptophan codon UGA. The other sense codons UGG and CUA modestly reduced the activity found with no codon (Fig. 4D). These effects were specific for mRF1 (Fig. 4C).

Characterization of Two mRF1 Variants—Two yeast strains that carry mutations in the MRF1 gene encoding the mRF1 characterized in the current study were described by Pel et al. (16). These mutations provoke gene-specific defects in mitochondrial translation. The studies hinted that the mutations might be located in a RF domain involved in ribosome binding. The position of these two mutations is indicated in Fig. 5A by the Δ symbol. mRF1-R231K is at a position that is highly conserved among bacterial factors, whereas mRF1-P233L is at a position conserved in the RF1 family. The mRF1 genes from the two mutants were cloned into the bacterial expression vector (PET-3a); the proteins expressed in E. coli and purified from inclusion bodies as described above for the wild-type factor. The expressed proteins migrated as if they had a molecular mass of 45 kDa, near to the expected size (~42 kDa). Similar to
the wild-type protein, both mRF1-R231K and mRF1-P233L had a His6 tag placed at the C termini of the protein that was used to detect the protein specifically.

Activities of mRF1-R231K and mRF1-P233L Variant Proteins—The functional activities of both variant proteins were compared with the wild-type protein in the homologous and, in more detail, the heterologous in vitro assays. The mRF1-R231K protein showed higher activity than mRF1-P233L, but both activities were lower than that for mRF1 (Fig. 5B). This paralleled the previous genetic studies that suggested that mRF1-R231K had some residual activity. Further characterization used E. coli ribosomes. As with mitochondrial ribosomes, mRF1-R231K showed partial activity. However, mRF1-P233L in this heterologous assay also had partial activity at higher concentrations of protein (Fig. 5C). This paralleled the previous genetic studies that suggested that mRF1-R231K had some residual activity. Further characterization used E. coli ribosomes. As with mitochondrial ribosomes, mRF1-R231K showed partial activity. However, mRF1-P233L in this heterologous assay also had partial activity at higher concentrations of protein (Fig. 5C). The mRF1-R231K and mRF1-P233L bound to E. coli ribosomes, but with lower affinity than that of wild-type mRF1 (Fig. 5D). The mRF1-P233L binding affinity to this ribosome was lower than that of mRF1-R231K, accounting for the relative release activities of the proteins.

mRF1 Cross-links to the Stop Codon—Small designed mRNAs containing a photoactivable thio-U residue in the first position of UAA, or UAG, or UGA for stop codons, or UGG for a sense codon control, were used to consider whether mRF1 was close to the stop codon. Termination complexes were formed with mRF1 using E. coli ribosomes, with the stop codon of mRNA positioned in the A-site by a P-site bound deacylated tRNAGlu. Control complexes with E. coli RF2 were formed in parallel. By subjecting the mRNA in the cross-linked termination complex to ribonuclease (RNase) T1 digestion, which specifically cuts to the 3’ side of G nucleotides, the mRNA analogue was cleaved into fragments of defined length.

Fig. 6 shows that mRF1 cross-linked to the thio-U in all mRNAs but not as effectively as RF2 to the mRNA. The most intense band for mRF1 cross-link was, surprisingly, the band with mRNA containing the UGA codon, and the least intense band was the one with UGG. Despite the UGA being a noncognate codon for mRF1, the factor must still form a complex with the mRNA containing this codon and be in an orientation in which the cross-linking of the thio-U to the factor occurs with relatively high efficiency. This is consistent with UGA being able to stimulate the fMet release by mRF1 in the model release assay with bacterial ribosomes.

Efficiency of Cross-linking mRF1-R231K and mRF1-P233L to the Stop Codon—To consider the closeness and orientation of mRF1-R231K and mRF1-P233L to the stop codon, cross-link patterns were investigated for mRF1-R231K and mRF1-P233L after digestion of the complexes with RNase T1. mRF1-R231K cross-links were detected with the UGA-containing mRNA and to a lesser extent with the UAG containing mRNA. A mRF1-P233L cross-link was detected only with the UGA-containing mRNA. The lower affinity of the variants for the ribosome may explain the lower yield of cross-links, although the three proteins may also be orientated in the active center differently with respect to the mRNAs.

DISCUSSION

Mitochondria use a genetic code different than that used for cytoplasmic protein synthesis in eukaryotic cells. Proteins involved in decoding mRNAs signals should reflect that mito-
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The mitochondrial factor functioned on bacterial ribosomes. How-
however, if the overall charge is examined according to the seven
structural domains (A–G) assigned on theoretical grounds (7), the
major charge differences are found in domains A and B
charged with increasing amounts of mRF1-R231K and mRF1-P233L compared with increasing amounts of mRF1, with E. coli
termination complexes. D, the ribosomal binding activity of mRF1 was compared with that of the two variant proteins. A complex of ribosome release factor
and UAA was analyzed by Western blotting against Ni-NTA-HRP conjugate. The relative binding affinity of the three proteins was determined by laser densitometry. Data are expressed as a relative ratio (mRF1 variant:mRF1).

Two forms of the putative yeast mRF1 protein were ex-
pressed, one reflecting the coding information of the gene and the other with the putative N-terminal mitochondrial targeting
signal removed. Unlike that found for overexpressed bacterial
factors, insoluble proteins resulted. Differences in the charge
ratio and specific residues on the protein surface may explain
mRF1 aggregation. Table I shows the overall charge (in bold-
face) of functional mRF1, RF1, and RF2. mRF1 has an overall
charge closer to neutrality, particularly in the N-terminal two-
thirds of the protein, and a net positive charge of 5 in the
C-terminal region. In contrast, the two bacterial factors have a
significant net negative charge (–18 and –27 for RF1 and RF2,
respectively), the majority of which is found in the N-terminal
region (Table I).

The mitochondrial protein was resolubilized and refolded so
that it had functional activity on both mitochondrial and bac-
terial ribosomes. As predicted, this protein functioned as a
release factor and exhibited RF1-like activity, recognizing
UAA, the stop codon used in yeast genes, as well as UAG, the
unused codon. Given the different charge ratios for bacterial
and mitochondrial factors, it was perhaps a surprise that the
mitochondrial factor functioned on bacterial ribosomes. How-
ever, if the overall charge is examined according to the seven
structural domains (A–G) assigned on theoretical grounds (7), the
major charge differences are found in domains A and B

Fig. 5. Alignment of E. coli and mitochondrial RFs indicating the mutant site in mRF1 and activities of these mutants compared with the wild-type. A, alignment of E. coli class I RFs and mitochondrial RF. Δ indicates the mutant site. The triplet amino acid sequence implicated in the anticodon region of the RF (36) is indicated by ***, and the initiation factor 1 motif (28) is indicated by the solid line. B, release activity of mRF1-R231K and mRF1-P233L variants compared with wild-type mRF1 in an in vitro assay on mitochondrial ribosomes. C, release activity with increasing amounts of mRF1-R231K and mRF1-P233L compared with increasing amounts of mRF1, with E. coli termination complexes. D, the ribosomal binding activity of mRF1 was compared with that of the two variant proteins. A complex of ribosome release factor
and UAA was analyzed by Western blotting against Ni-NTA-HRP conjugate. The relative binding affinity of the three proteins was determined by laser densitometry. Data are expressed as a relative ratio (mRF1 variant:mRF1).

Fig. 6. Autoradiographs of cross-linked complexes after SDS-
PAGE separation and transfer to a nitrocellulose membrane. E.
coli RF2, mRF1, and variant mRF1 cross-linked complexes after RNase
T1 digestion. The radiolabeled mRNAs contained the stop codons
shown. The arrows show the cross-links from the thio-U of the stop
signal to the RFs and ribosomal protein S1. A maximum of 15% of added
RF formed cross-linked complexes. E. coli ribosomes were used in all
termination complexes. A control lacking ribosomes showed no cross-
linked complexes. The cross-links from the mRNA to ribosomal protein
S1 reflect the orientation of the components of the complex with respect
to different stop codons (40).

Table I. These regions of the proteins have not been implicated
as being involved in the critical functions of codon recognition
and peptidyl-tRNA hydrolysis. Most likely, domains C and D contain the RF anticodon region (27, 28), and domain E contains the peptidyl-tRNA hydrolysis region (29). These structural domains have more similar charge ratios between the bacterial and mitochondrial factors (Table I), but more important, there are significant amino acid similarities within domains C, D, and E of the different factors that suggest why the mitochondrial factor is able to function on bacterial ribosomes.

The current models for factor binding to the ribosome invoke
an initial binding event that is a low discrimination step with
respect to codon, followed by movement to a high discrimina-
tion functional state dependent on a cognate stop codon (30).2

The second codon-dependent discrimination step may be
perturbed with the mitochondrial factor on bacterial ribosomes,
but in the examples in which we achieved mRF1 activity with
mitochondrial ribosomes, function was completely dependent
on cognate codon.

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For RF to function as a tRNA on ribosomes, the factor has to bind to a region of the active center at the subunit interface that includes the decoding site of the small ribosomal subunit. The recent x-ray crystallographic solutions of ribosome structure have shown that the most striking feature of the subunit interface is a long ~100-A helix that runs vertically along the 30 S subunit body (31, 32). The penultimate stem (32) encompassing the decoding site has a 30° bend around residues 1492 and 1493 (33, 34) (Fig. 7A). The decoding region represented by helix 44 of the E. coli ribosome (35) and the equivalent region of the 15 S yeast mitochondrial rRNA are displayed in Fig. 7, with the positions of the tRNA protected and bend residues (1492 and 1493 for 16 S rRNA, and 1581 and 1582 for 15 S rRNA) marked with arrows in each case. Although the primary sequences of these regions are very different for the two ribosomes, they can form similar secondary and, presumably, tertiary structures. In this study, we examined two variants of the mRF1 with amino acid changes at two residues (231 and 233) in domain D of mRF1 (see Fig. 5A). These changes altered in vivo mitochondrial protein synthesis (9). Of particular interest was the isolation of second-site mutations within helix 44 of the 15 S rRNA (Fig. 7B, circles) that restored function.4 This implied that there was interaction between this part of domain D in mRF1 and the decoding site rRNA that was important for the release factor function. In this study, we have shown that these variant factors do indeed bind to the native ribosome with lower affinity than the wild-type type, and this is reflected in their overall lower activity in termination reactions. Site-directed cross-links from the first position of the stop codon to the factor in a termination complex reflected this lower affinity. The two changes in mRF1 represented by these variants are within the narrow region that is believed to be the anticodon mimicry region of the bacterial RFs. It has been shown that the first and third amino acids of the tripeptide sequence (RF1, 158–190 (PAT), and RF2, 205–207 (SPF)) (Fig. 5A) discriminate the second and third bases of stop codon, respectively (36). mRF1 (233–235; PST) has a sequence similar to RF1, which could explain the discrimination of UAA and UAG from UGA, as well as reduced activity of mRF1-P233L, in which the first residue of the tripeptide is altered. In addition, they are within the region of the RFs that shows high degree of homology with initiator factor 1 (residues 52–69) (28), RF1

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