Temperature-sensitive mutation in yeast mitochondrial ribosome recycling factor (RRF)

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ABSTRACT

The yeast protein Rrf1p encoded by the *FIL1* nuclear gene bears significant sequence similarity to *Escherichia coli* ribosome recycling factor (RRF). Here, we call *FIL1* Ribosome Recycling Factor of yeast, *RRF1*. Its gene product, Rrf1p, was localized in mitochondria. Deletion of *RRF1* leads to a respiratory incompetent phenotype and to instability of the mitochondrial genome (conversion to *rho⁻*/*rho⁰* cytoplasmic petites). Yeast with intact mitochondria and with deleted genomic *RRF1* that harbors a plasmid carrying *RRF1* was prepared from spores of heterozygous diploid yeast. Such yeast with a mutated allele of *RRF1*, *rrf1-L209P*, grew on a non-fermentable carbon source at 30 but not at 36°C, where mitochondrial but not total protein synthesis was 90% inhibited. We propose that Rrf1p is essential for mitochondrial protein synthesis and acts as a RRF in mitochondria.

INTRODUCTION

Mitochondrial gene expression involves a complex translation system. In *Saccharomyces cerevisiae* more than 120 genes have been identified that code for components required for mitochondrial protein synthesis (1–5). The components of the mitochondrial translation machinery are distinct from those of the cytoplasmic system, except for three aminoacyl tRNA synthetases (6–8). The mitochondrial translation system is known to resemble the prokaryotic translation system (9). However, a number of unusual features of the mitochondrial translation system are not found in the prokaryotic translation system. For example, the mitochondrial genetic code differs in various aspects from the universal one (10), and the structural features of the mitochondrial ribosome are unusual (1,2,11). A stringent block of mitochondrial protein synthesis destabilizes the mitochondrial genome (12). The mitochondrial translation factors have been cloned either through their homology with bacterial translation factors [EF-Tu (13), human ribosome recycling factor (RRF) and RF1 (14)] or through the complementation of respiratory incompetent mutants [IF2, EF-G (15) and RF1 (16)]. However, no EF-Ts have been detected in yeast mitochondria (17). Since no functional *in vitro* mitochondrial translation system with natural mRNA exists (18), the mechanisms of mitochondrial protein synthesis are poorly understood.

The RRF (formally called ribosome releasing factor) is an essential component of the translation machinery in prokaryotes, where it was first characterized in 1970 in our laboratory (19,20). It catalyzes the disassembly of post-termination complexes consisting of ribosome, mRNA and tRNA, allowing the recycling of the components for another round of translation. In the absence of RRF, the ribosome stays on the mRNA and starts downstream unscheduled translation (21,22). RRF is also involved in the fidelity of translation (23). Recent work indicates that RRF binds to A/P sites of the ribosome (24,25) and is translocated on the ribosome to release tRNA (26) bound at the P/E site (27). RRF has been identified in all prokaryotic organisms examined so far except for Archaeons (for reviews see 28–31). The spinach RRF homolog has been shown to be localized in the chloroplast (32), where a stoichiometric amount of RRF homolog is associated with the 50S ribosomal subunit (33).

Kanai *et al.* isolated a yeast mutant that failed to derepress expression of the *S.cerevisiae* isocitrate lyase gene (*ICL1*) in acetate medium and then identified the gene, *FIL1*, that complemented this mutation. This gene was named *FIL1* (factor for isocitrate lyase derepression) (34). In this paper, we call the gene *FIL1 RRF1* because, as pointed out by Kanai *et al.*, it is clearly a yeast homolog of the *Escherichia coli* gene coding for RRF (*frr*). According to yeast conventions, the protein encoded by *RRF1* is called Rrf1p.

In this communication, we show that Rrf1p is a mitochondrial protein that is essential for mitochondrial protein

Table 1. Strains and plasmids

Strain	Genotype	Source
H1402	MATα ino1 ura3-52 leu2-3,112 HIS4-lacZ	(65)
Strain RRF	Wild-type haploid stain with intact *RRF1* [for example, WY344 (*RRF1*)]	
Strain Δ*rrf1*	Haploid cells lacking *RRF1* [for example, WY347 (Δ*rrf1*/ *rho*⁻)]	
WY344 (*RRF1*)	H1402 in which the *TRP1* gene was disrupted using a method described in (66).	This study
	MATα ino1 ura3-52 leu2-3,112 trp1::*URA3* HIS4-lacZ	
WY347 (Δ*rrf1*/*rho*⁻)	WY344 in which the *RRF1* gene was disrupted using a method described in (38).	This study
	MATα ino1 ura3-52 leu2-3,112 trp1::*URA3* HIS4-lacZ *RRF1*::TRP1	
DS413	MATa/α ura3-52/ura3-52 leu2-3,112/leu2-3,112 ade2-101/+ his3Δ200/his3Δ200 +/trp1Δ1	D. Roof[a]
ET1 (*RRF1*/Δ*rrf1*)	DS413 in which one allele of the *RRF1* gene was disrupted using a method described in (41).	This study
	MATa/α ura3-52/ura3-52 leu2-3,112/leu2-3,112 ade2-101/+ his3Δ200/his3Δ200 +/trp1 Δ*rrf1*/*RRF1*::kan^r	
ET2 (Δ*rrf1*/ *rho*⁺)	Haploid meiotic progeny of ET1. MATa ura3-52 leu2-3,112 ade2-101 his3Δ200trp1 Δ*rrf1*::kan^r	This study

Plasmid	Markers	Source
pRS415-ADHp	ARS_CEN6 LEU2 *ADH1* promoter (low copy number, centromeric)	D. Roof[a]
p*RRF1*W1	pRS415-ADHp carrying *RRF1* (wild type)	This study
p*rrf1*-K67R	pRS415-ADHp carrying *rrf1-K67R*	This study
p*rrf1*-L206E	pRS415-ADHp carrying *rrf1-L206E*	This study
p*rrf1*- L209P	pRS415-ADHp carrying *rrf1-L209P*	This study
p*rrf1*- I228P	pRS415-ADHp carrying *rrf1-I228P*	This study
pRS426	2 μm origin *URA3* (high copy number) plasmid	(39)
p*RRF1*W2	pRS426 *ADH1* promoter carrying *RRF1* (wild type)	This study
pET28a	*Escherichia coli* expression plasmid	Novagen
pET28a-t*RRF1*	pET28a carrying a PCR amplified fragment coding for the 194 C-terminal fragment Rrf1p	This study

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synthesis and for the maintenance of the respiratory function of mitochondria. A haploid yeast dependent on plasmid-borne *RRF1* for growth on non-fermentable carbon source was created. Using this strain, we changed a leucine residue at position 209 of Rrf1p to proline and obtained a temperature-sensitive yeast strain. Mitochondrial protein synthesis of this strain was severely reduced at 37°C while total cytoplasmic protein synthesis was not affected under the same conditions.

MATERIALS AND METHODS

Strains, plasmids and genetic manipulations

The yeast strains used are listed in Table 1. They are derivatives of WY344 and DS413 and the haploid of DS413. The *rho*⁰ strains were prepared by ethidium bromide treatment (35). Genetic techniques used are described by Sprague (36). The *E.coli* were DH5α, BL21(DE3)pLysS (Novagen) and NMS22 mutS (Pharmacia Biotech).

Media

YEP medium (1% yeast extract, 2% Bacto-Peptone) or synthetic minimal medium containing the required nutrients (0.7% yeast nitrogen base with ammonium sulfate) was supplemented with glucose (2%), glycerol (3%) or ethanol (2%) as noted (37).

Construction of Δ*rrf1 rho*⁻ (WY347) and Δ*rrf1 rho*⁺ (ET2) strains

The *RRF1* ORF was replaced with *TRP1* (tryptophan synthase gene) or *kanMX* (kanamycin resistance gene), resulting in Δ*rrf1 rho*⁻, as described in Baudin *et al*. (38). The *TRP1* gene was amplified by PCR from the plasmid PRS314 (39). Stable transformants with the amplified fragment were prepared as in

Ausubel *et al*. (40). Replacement of *RRF1* by *TRP1* was confirmed by PCR. Δ*rrf1 rho*⁺ was prepared as in Wach *et al*. (41) by inserting *kanMX* into *RRF1* and sporulating. The *kanMX* gene was amplified by PCR with pFA6a-*kanMX*6 (41–43). Diploid yeast strain DS413 was transformed with this fragment as in Ausubel *et al*. (40) and a geneticin (Gibco BRL)-resistant clone, strain ET1, Δ*rrf1*::kan*MX*/*RRF1* diploids, were selected.

Expression of recombinant truncated Rrf1p (His₆-partial Rrf1p) in *E.coli* and production of antibody against Rrf1p

Histidine-tagged truncated Rrf1p (194 C-terminal residues of Rrf1p) was expressed in BL21(DE3)pLysS *E.coli* harboring pET28a-t*RRF1* [pET28a (Novagen) carrying t*RRF1* (truncated *RRF1*)]. His₆-partial Rrf1p was used to obtain a rabbit polyclonal antibody (Cocalico Biologicals Inc.) against Rrf1p.

Low copy and multi-copy expression of Rrf1p

Yeast total DNA (strain WY344) for PCR was isolated as in Ausubel *et al*. (40). The amplified fragment was ligated into the BamHI site of the *LEU2*/*CEN* shuttle vector pRS415-ADHp (a gift from D. Roof), creating p*RRF1*W1. For multi-copy expression, p*RRF1*W1 was sub-cloned into the XhoI and SacI sites of the 2 μm *URA3* shuttle vector pRS426 (a gift from D. Roof), generating p*RRF1*W2.

Complementation of Δ*rrf1* cells

The plasmid p*RRF1*W2 was introduced into Δ*rrf1*::*kanMX*/ *RRF1* diploids (strain ET1), which were then sporulated (40) and subjected to random spore analysis. We selected *ade2* cells which turn red on low adenine-containing medium if respiratory competent (44,45). The Δ*rrf1* haploid, *RRF1*, replaced with *kanMX* in spores containing p*RRF1*W2, was

selected for resistance to geneticin and the ability to grow without uracil (p*RRF1*W2 carries the selectable marker *URA3*).

Site-directed mutagenesis of Rrf1p

Mutations were introduced using the unique site elimination (USE) procedure (Mutagenesis Kit; Pharmacia Biotech) developed by Deng and Nickoloff (46). ADHp-*RRF1* (p*RRF1*W1) was mutagenized using both a target mutagenic primer introducing the desired mutation in *RRF1* and a USE selection primer introducing a mutation in the unique XhoI site of the plasmid polylinker. The elimination of the XhoI site subsequently served as the basis for selection of mutated plasmids. The target mutagenic primers used to introduce the different mutations were as follows: CTTTAGAAATT-CAAA**G**ACAGAAAATG for the mutation Rrf1p-K67R, GCTGAGAGGGAT**G**A**G**GAAAAACTG for the mutation Rrf1p-L206P, GGGATTTGGAAAAAC**C**GCATAAGGAT-TACG for the mutation Rrf1p-L209P and GTTGAAAA-AAGC**CC**TGTAAAATGA for the mutation Rrf1p-I228P. Underlined characters indicate the nucleotide positions changed by the site-directed mutagenesis. The phenotypes of the mutant alleles were tested by the 'plasmid shuffle' procedure (47).

Immunofluorescence and DAPI staining

Yeast cells were fixed and prepared for immunofluorescence microscopy as described (48). Rrf1p was detected using a rabbit antibody against Rrf1p and FITC-conjugated goat anti-rabbit secondary antibody (Sigma). DNA was stained with 4,6-diamino-2-phenylindole (DAPI) (Sigma) as described (48).

Preparation of cellular extracts, isolation of mitochondria and detection of Rrf1p

Crude yeast extracts were prepared according to Kolodziej and Young (49). Mitochondria were isolated as in Daum *et al.* (3). The protein Rrf1p was detected by western blot, using immunoaffinity purified rabbit antibody against His_6-partial Rrf1p (dilution 1/250) and goat anti-rabbit alkaline phosphatase-conjugated antibody (dilution 1/7500; Sigma). Porin protein was detected using a rabbit antibody against *S.cerevisiae* porin (a gift of D. Pain) and FITC-conjugated goat anti-rabbit antibodies (Sigma).

Labeling of mitochondrial translation products *in vivo*

Mitochondrial translation products were labeled *in vivo* as described (50). Cells were incubated at 30°C and cyclo-heximide was added to a final concentration of 150 μg/ml. After further incubation for 2 min, the incubation temperature was shifted to 37°C and labeling with [^{35}S]methionine (40 μCi, 1175 Ci/mmol) (NEN) was performed for 80 min and stopped by adding unlabeled methionine (10 mM). Total cell proteins were extracted by alkaline lysis (51). Proteins were separated by SDS–PAGE (12% acrylamide) and visualized by auto-radiography and Coomassie staining.

[^{35}S]methionine incorporation into whole cells

Cells (grown at 29°C in glucose medium) were diluted to $OD_{540} = 0.05$ and [^{35}S]methionine 20 μM (39.5 μCi in 5 ml) was added and incubated at 29°C. When the OD_{540} reached 0.45, the culture was split for incubation at 37 and 29°C. The incorporation of [^{35}S]methionine into the hot TCA-insoluble fraction was counted.

Homology modeling

The structural model was built using the software provided by Tripos and Associates after amino acid sequence alignment with *Thermotoga maritima* RRF. There is ~20% sequence identity and ~52% sequence similarity. Individual side chain replacements were made to the 1DD5 structure to reflect the yeast RRF sequence, as previously described (52). A loop engraftment was used to modify the template in the region of the *T.maritima* RRF between residues 102 and 107 (FPSPTT). The corresponding yeast sequence is LPPPTT. In searching the structural database, a small loop was found in 15-lipoxygenase (database code 1LOX) with the identical sequence LPPPTT and a suitable geometry for insertion into the structural template. After loop grafting, explicit hydrogens were added and the final structure was subjected to four cycles of dynamics (200 steps)/minimization (50 steps) followed by minimization to energy convergence. Minimization was done by the conjugate gradient method using the Kollman all-atom force field and Gasteiger–Huckle charges. The final structure was checked for appropriate bond lengths, chirality and dihedral angles using the ProTable module of the Tripos software.

RESULTS

Irreversible damage of the mitochondrial genome by *RRF1* deletion

The Δ*rrf1* haploid strain, in which *RRF1* was disrupted by replacing the complete *RRF1* ORF with the *TRP1* gene, grew on rich glucose medium but slower than the wild-type (data not shown). The Δ*rrf1* strain, however, did not grow on rich glycerol medium. These two observations suggest that the cells deleted for *RRF1* are respiratory incompetents. Although data were not shown, Kanai *et al.* stated that a similar strain did not grow on acetate (34). The expression of Rrf1p in wild-type (*RRF1* cells) and mutant *S.cerevisiae* (Δ*rrf1* cells) was examined in crude cell extracts by western blot using anti-Rrf1p antibodies as shown in Figure 1A. A protein with an apparent molecular mass of 23 kDa was detected in the wild-type strain *RRF1* and in a strain, Δ*rrf1*, transformed with a centromeric low copy number plasmid carrying the DNA corresponding to *RRF1* (p*RRF1*W1). This band was absent in Δ*rrf1* transformed with the control plasmid (pRS415-ADHp). The apparent molecular mass of Rrf1p (23 kDa) was smaller than the size deduced from the *RRF1* nucleotide sequence (26.4 kDa). This difference in size suggests that the Rrf1p N-terminal signal sequence acts as a removable mitochondrial targeting sequence (34). The yeast *RRF* gene carried by p*RRF1*W1 (low copy number plasmid), despite its expression, was unable to restore the capacity to grow on ethanol in the strain Δ*rrf1* (Fig. 1B, lower left corner).

We then mated Δ*rrf1* haploids with haploids that contained wild-type *RRF1* but no mitochondrial DNA (*rho^0* strain). The diploids generated were unable to grow on non-fermentable carbon sources (data not shown). Furthermore, the mitochondrial protein porin and the mitochondrial DNA of Δ*rrf1*
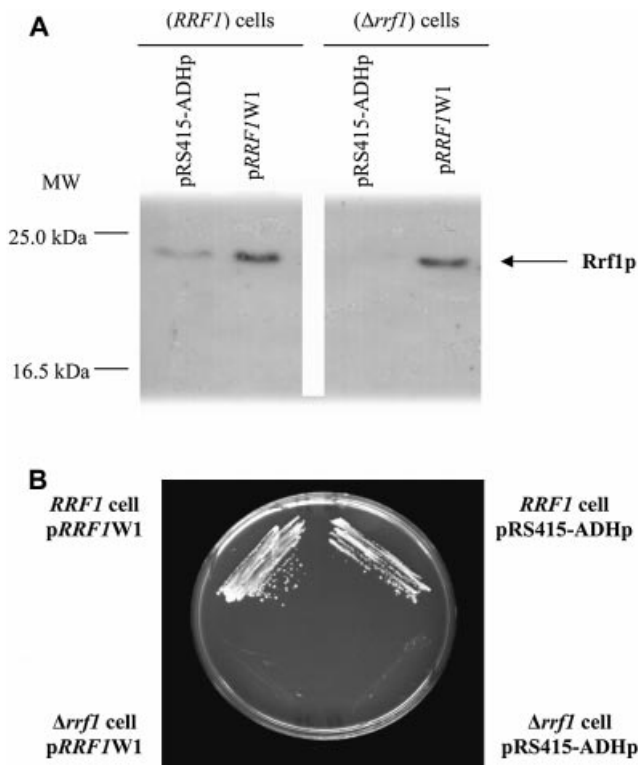
Figure 1. Haploid yeast with a *RRF1* deletion does not grow on glycerol, a phenotype that cannot be overcome by cytoplasmic Rrf1p. (**A**) Immunodetection of Rrf1p in Δ*rrf1* haploid cells (WY347) transformed with a centromeric copy of the *RRF1* gene. Total protein extracts from four different strains were analyzed, shown from left to right: wild-type *RRF1* haploid cells (WY344, designated *RRF1* cells) harboring a centromeric control plasmid carrying no *RRF1* (pRS415-ADHp); WY344 harboring a centromeric plasmid carrying the *RRF1* gene (designated pRRF1W1); Δ*rrf1* haploid cells (WY347, designated Δ*rrf1* cells) harboring pRS415-ADHp or harboring pRRF1W1. Protein extracts (50 µg) were analyzed in 12% gels by western blotting using an antibody against His$_6$-partial Rrf1p. (**B**) Lack of growth of Δ*rrf1* haploid cells harboring a plasmid carrying *RRF1* on a non-fermentable carbon source. Four different strains were streaked on an agar plate containing ethanol selective medium. Wild-type *RRF1* haploid cells (WY344, designated *RRF1*) harboring a centromeric control plasmid carrying no *RRF1* (pRS415-ADHp) (top right). WY344, harboring a centromeric plasmid carrying the *RRF1* gene (pRRF1W1) (top left). Δ*rrf1* haploid cells (WY347, designated Δ*rrf1*) harboring pRS415-ADHp (bottom right). WY347 harboring pRRF1W1 (bottom left). The photograph was taken after 3 days growth at 30°C.

haploids were stained with DAPI and fluorescent labeled anti-porin. The results showed that mitochondria-like particles were present in Δ*rrf1* haploids, but the mitochondrial DNA appeared more diffuse and less abundant than in the control strain (data not shown). These results suggest that, in Δ*rrf1* cells, the mitochondrial DNA present may be irreversibly damaged and additional *RRF1* is unable to restore the mitochondrial function necessary for growth on a non-fermentable carbon source.

RRF1 rescues the respiratory deficiency of Δ*rrf1* mutants that contain intact mitochondrial DNA

Since the simple addition of a plasmid carrying *RRF1* (pRRF1W1) to the strain Δ*rrf1* did not complement the null disruption as indicated above, we concluded that the lack of

RRF1 led to a loss of respiration-competent mitochondria. Rrf1p expression also could not restore respiration by the mitochondria. To circumvent this problem, we generated a haploid Δ*rrf1* strain maintained by *RRF1* on a plasmid from a heterozygous diploid strain using the sporulation technique. All Δ*rrf1* haploids harboring pRRF1W2 obtained were able to utilize a non-fermentable carbon source (data not shown). Therefore, as predicted, pRRF1W2 was able to complement the strain Δ*rrf1* (haploid) if prepared in this fashion. The high copy number plasmid (pRRF1W2) was then exchanged with a low copy number centromere-based plasmid (pRRF1W1). Figure 2A shows that these haploid cells with (upper middle section) and without (upper right section) extra-chromosomal *RRF1* grew on the plate containing glucose. As shown in Figure 2B (upper middle sections of plates 1–3), the Δ*rrf1* haploids carrying pRRF1W1 (Δ*rrf1 rho*⁺ pRRF1W1) were able to grow on a non-fermentable carbon source. The control strain without wild-type *RRF1* (Δ*rrf1 rho*⁺, pRS415-ADHP) did not grow on the glycerol media (upper right sections of plates 1–3). This demonstrates that complementation is possible only when the plasmid with wild-type *RRF1* is introduced before mitochondrial function is compromised.

Intracellular localization of Rrf1p

To determine the subcellular location of Rrf1p, mitochondria and post-mitochondrial supernatant fractions were isolated from wild-type diploid cells (DS413). The presence of Rrf1p in each fraction was examined by immunoblotting with affinity purified Rrf1p polyclonal antibodies. Western blot analysis revealed Rrf1p in mitochondria, but not in the post-mitochondrial supernatant (Fig. 3I). In a separate experiment, anti-Put2p serum (a gift from D. Pain) detected Put2p, a protein known to localize to mitochondria, only in the mitochondrial fraction obtained by the same method (data not shown). This established the validity of our biochemical fractionation method for the isolation of mitochondria used in Figure 3I. The size of Rrf1p detected in the mitochondria was the same as that of Rrf1p detected in the crude extract, 23 kDa. This suggests that cleavage of the mitochondrial signal peptide takes place in the mitochondria and that the major percentage of Rrf1p is in the mitochondria.

To further confirm mitochondrial localization of Rrf1p, Rrf1p was detected *in situ* by immunofluorescence microscopy of wild-type cells and cells overexpressing Rrf1p. Cells were treated with polyclonal anti-Rrf1p antibodies and with DAPI. As shown in Figure 3II, in the cells overexpressing Rrf1p, Rrf1p co-localized with mitochondrial DNA stained with DAPI. It should be emphasized that Rrf1p was detected exclusively in mitochondria. There was no fluorescent signal associated with either the nuclei or cytoplasm. The signals associated with anti-Rrf1p in wild-type cells expressing native levels of Rrf1p were very faint, but the results indicate a similar mitochondrial distribution as in cells containing overexpressed Rrf1p (data not shown).

Isolation of a temperature-sensitive *RRF1* allele

To establish the role of Rrf1p in mitochondrial protein synthesis, it was desirable to obtain a temperature-sensitive allele of *RRF1*. We reasoned that, because of the sequence similarity between Rrf1p and *E.coli* RRF, site-specific mutagenesis of *RRF1* at residues corresponding to
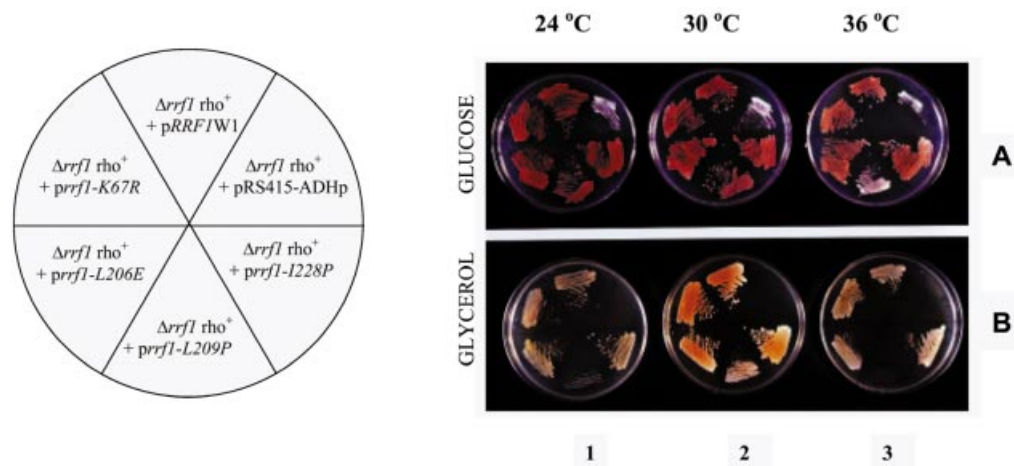
Figure 2. Complementation of Δ*rrf1 rho*⁺ haploid cells with a plasmid carrying *RRF1*. Evidence that *rrf1-L209P* gives temperature-sensitive growth. Δ*rrf1 rho*⁺ haploid cells harboring various plasmids carrying mutated *RRF1* were created as described in Materials and Methods. These various strains are indicated in the circle with the pie cut (left). (**A**) Cells harboring each of the plasmids as shown in the left panel were streaked onto synthetic glucose (3%) medium lacking leucine and grown at 24, 30 and 36°C. (**B**) The same strains were grown overnight in synthetic glucose medium minus leucine, streaked onto YPG plates (containing 3% glycerol) and incubated at 24, 30 and 36°C. Δ*rrf1 rho*⁺ haploid cells (ET2) are *ade2-101*. Such adenine-deficient cells are known to accumulate red pigments in the presence of low adenine when they are respiratory competent. Note that Δ*rrf1 rho*⁺ + p*rrf-L209P* could not grow on glycerol at 36°C (lower middle streak, plate 3) while the strain harboring pRS415-ADHp (no *RRF1*) did not grow in glycerol medium at all temperatures tested (plates 1–3, upper right streak). This indicates that Δ*rrf1 rho*⁺ + pRS415-ADHp was converted to *rho*⁻ because of the lack of Rrf1p.
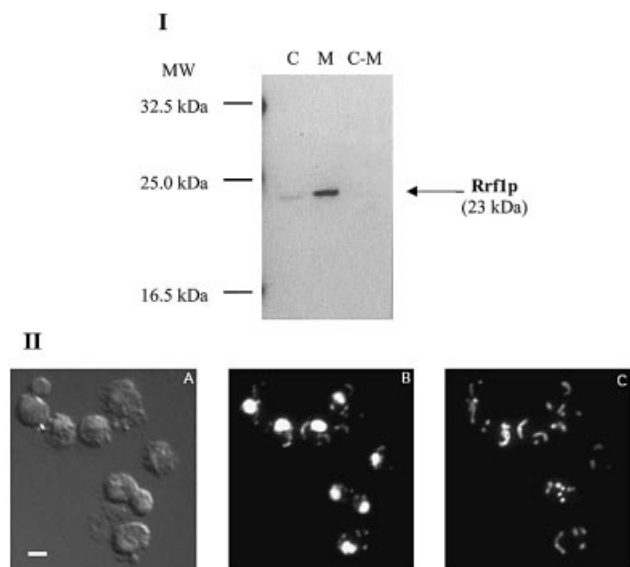

Figure 3. (**I**) Rrf1p is localized in the fraction containing mitochondria. Western blot analysis of crude yeast extracts (designated column C), isolated mitochondria (column M) and post-mitochondrial supernatant fraction (C-M) obtained from wild-type strain DS413. Proteins (40 μg) of each preparation were analyzed by western blotting using antibody against His₆-partial Rrf1p. (**II**) Cytological localization of Rrf1p to mitochondria. A representative microscopic field of Δ*rrf1 rho*⁺ haploid cells (strain ET2) harboring a high copy number plasmid carrying *RRF1* (p*RRF1*W2) is shown. (**A**) Differential interference contrast view. (**B**) DAPI staining showing the locations of nuclear and mitochondrial DNA. (**C**) Indirect immunofluorescence using antibodies to His₆-partial Rrf1p and FITC-conjugated secondary antibody. Bar represents 1 μm.

temperature-sensitive alleles of *E.coli frr* may also produce temperature-sensitive yeast strains. Twelve temperature-sensitive mutations of *E.coli frr* gene have been characterized (21). The position and the amino acid change for each of these mutations are known. Out of the 12 mutations, four were selected using two criteria: (i) the amino acid residue at the mutated position was conserved among most RRF homolog sequences and (ii) the corresponding mutation in *RRF1* requires the modification of less than 3 nt. The four selected mutations were *frr4*, *frr7*, *frr15* and *frr17* in *E.coli* (21) and correspond to L163P, V160E, K21R and L182P, respectively.

We constructed the alleles *rrf1-K67R*, *rrf1-L206E*, *rrf1-L209P* and *rrf1-I228P*, each of which was confirmed by sequencing to be otherwise identical to *RRF1*. Low copy number centromere plasmids each bearing either *rrf1-K67R* (p*rrf1*-K67R), *rrf1*-L206E (p*rrf1*-L206E), *rrf1-L209P* (p*rrf1*-L209P) or *rrf1-I228P* (p*rrf1*-I228P) were prepared. Each of these plasmids was introduced into haploid Δ*rrf1*::*kanMX* (*ade2*⁻) (*rho*⁺) cells (strain ET2) harboring p*RRF1*W2 by the 'plasmid shuffle' scheme. Haploid Δ*rrf1* cells transformed with wild-type *RRF1* (p*RRF1*W1), as described in the preceding section, provided the respiratory positive control for analysis of the *RRF1* mutants. Haploid Δ*rrf1* cells transformed with the empty plasmid (pRS415-ADHp) was a respiratory negative control.

At 24, 30 and 36°C, cells harboring either p*rrf1*-K67R, p*rrf1*-L206E, p*rrf1*-L209P or p*rrf1*-I228P grew as well as the cells containing p*RRF1*W1 on media containing a fermentable carbon source (glucose) and low levels of adenine. As shown in Figure 2A, they turn pink, as expected for respiratory competent *ade2* strains growing on low adenine-containing medium (45). However, cells deleted for *RRF1* (upper right corner of Fig. 2A, *RRF1 rho*⁺ + pRS415-ADHp) and cells harboring p*rrf1*-L209P grown at 36°C (lower middle section of plate 3 of Fig. 2A) were white. This suggested that expression of *rrf1-L209P* led to a respiratory deficiency at 36°C but not at 30°C. Supporting this conclusion, cells containing p*rrf1*-L209P did not grow on glycerol plates at 36°C (Fig. 2B, plate 3, lower middle section) but grew at lower
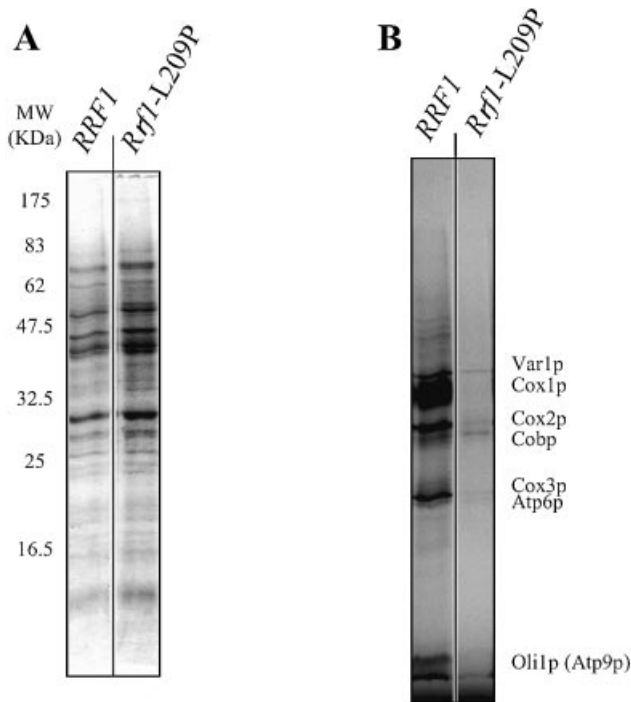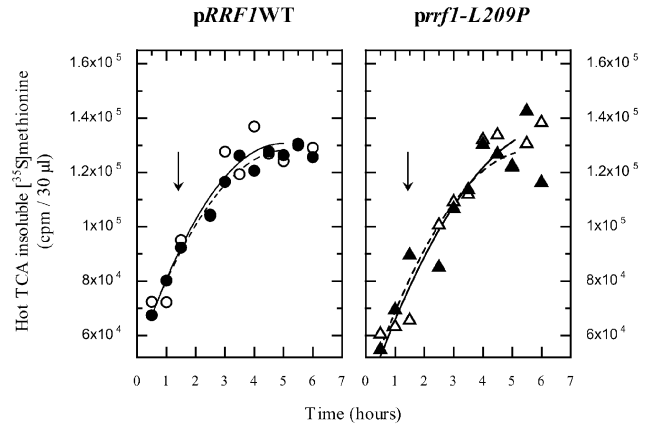
Figure 4. Cells with mutant Rrf1p (L209P) synthesize much less mitochondrial protein than those with wild-type Rrf1p. Haploid Δ*rrf*/*rho*+ cells expressing wild-type Rrf1p (ET2 + p*RRF1*W1) and thermosensitive Rrf1p (ET2 + p*rrf1*-L209P) were grown overnight at 30°C in minimal glucose medium lacking methionine until mid-exponential phase. Cells were then labeled with [^{35}S]methionine for 80 min in the presence of cycloheximide at 37°C. Whole cell extracts (~0.5 OD$_{600}$ units) were separated on a 12% polyacrylamide gel. The gel was Coomassie stained, dried and exposed to X-ray film. (**A**) and (**B**) are Coomassie staining and autoradiography, respectively. The mitochondrial proteins revealed by autoradiography were identified as Cox1p, Cox2p, Cox3p, Cobp (cytochrome b), Atp6p and Oli1p (Atp9p) (subunits 6 and 9 of the F$_0$F$_1$-ATPase, respectively) and Var1p (variant ribosomal protein). The positions of the molecular weight markers are indicated on the left (in kDa).

Figure 5. Incorporation of [^{35}S]methionine into total proteins by haploid cells expressing thermosensitive Rrf1p at a permissive (29°C) and a non-permissive temperature (37°C) are similar. Translation activities of haploid Δ*rrf1 rho*+ cells expressing wild-type Rrf1p (ET2 + p*RRF1*W1, left panel) and thermosensitive Rrf1p (ET2 + p*rrf1*-L209P, right panel) were examined. Overnight cultures in glucose were diluted to 0.05 OD$_{540}$ and [^{35}S]methionine was added. Cells were incubated at 29°C until the OD$_{540}$ reached 0.45. Then (indicated by arrow), one sample was exposed to 37°C (closed circles or triangles and dot line) while the other was kept at 29°C (open circles or triangles and solid line). The incorporation of radioactive methionine into the hot TCA-insoluble fraction after the temperature shift is shown.

temperatures (plates 1 and 2). The control strain, *RRF1 rho*+ + pRS415-ADHp, did not grow on glycerol at any of the temperatures tested, confirming the requirement of *RRF1* for growth on glycerol (Fig. 2B, upper right section of plates 1–3).

In contrast, plasmids carrying mutated copies of *RRF1* [p*rrf1*-K67R (upper left section of the plates, Fig. 2B), p*rrf1*-L206E (lower left section) and p*rrf1*-I228P (lower right section of the plates)] complemented the null disruption of *RRF1* at 24, 30 and 36°C. These all grew on glycerol-containing media (Fig. 2B) and turned pink on glucose plates at each temperature, indicating their respiratory competence.

A strain carrying p*rrf1*-L209P stops mitochondrial but not cytoplasmic major protein synthesis at the non-permissive temperature

The effect of *rrf1-L209P* mutation on mitochondrial translation activity was examined by *in vivo* labeling of mitochondrial protein with [^{35}S]methionine after inhibiting cytoplasmic protein synthesis with cycloheximide (53). After [^{35}S]methionine incorporation at 37°C, the whole cell extract was subjected to gel electrophoresis. Because cycloheximide inhibits cytosolic translation, only proteins synthesized in

mitochondria are labeled (Fig. 4B). At 37°C, the total proteins shown by Coomassie blue staining (Fig. 4A) of the extracts of cells carrying wild-type *RRF1* are very similar to those of cells carrying *rrf1-L209P*. On the other hand, a striking difference was observed between cells with wild-type and mutant Rrf1p; the mutant showed a very low level of [^{35}S]methionine incorporation into seven mitochondrial proteins, as identified in Figure 4B. Autoradiography shows that mitochondrial translation activity at 37°C is at least 90% less in cells expressing *rrf1-L209P* compared to wild-type cells (Fig. 4B). At the semi-permissive temperature (30°C) similar but less inhibition of synthesis of three of these proteins was observed (data not shown).

In contrast, cytoplasmic translation activity is not appreciably affected in the *rrf1-L209P* mutant, as shown by the measure of total [^{35}S]methionine incorporation at both permissive and non-permissive temperatures. At different time points, aliquots of cells were taken and the corresponding incorporated radioactivity was measured (Fig. 5). This is consistent with Figure 2A, where cells with *rrf1-L209P* still grew on glucose at 37°C, although at a somewhat reduced rate.

DISCUSSION

Kanai *et al*. (34) identified a gene named *FIL1* (factor for isocitrate lyase depression). This gene, tentatively renamed here *RRF1*, has sequence similarity to *E.coli RRF* and the putative N-terminal mitochondrial targeting sequence. As shown in the present paper and stated by Kanai *et al*., the null *RRF1* mutants are viable but cannot grow on a non-fermentable carbon source. Kanai *et al*. further showed that cytochrome c oxidase was severely reduced in the null *RRF1* mutants.

Isocitrate lyase is a key enzyme in the glyoxylate cycle that plays a vital role in producing succinate for mitochondria.

When yeast are placed on a non-fermentable carbon source, a message from functional mitochondria is transmitted to the nucleus to initiate the synthesis of the enzyme(s) necessary for succinate production for mitochondria. Since functional mitochondria are required to communicate non-availability of fermentable sugars to the nucleus (54), it is understandable that *FIL1* turned out to code for yeast RRF because, as shown in this paper, *RRF1* is essential for the function of mitochondria.

Interestingly, of the many genes necessary for mitochondrial maintenance, only mitochondrial RRF was identified as causing the phenotype, derepression of isocitrate lyase. Theoretically, any gene specifically required for the maintenance of mitochondria should exhibit the same phenotype and can be '*FIL1*'. Since Kanai *et al*. did not indicate whether or not they identified another mitochondrial maintenance gene as '*FIL1*', it would be of interest to examine whether other genes involved in mitochondrial protein synthesis could become '*FIL1*'. This may reveal a possible second role of RRF other than as a protein synthesis factor in mitochondria.

In this paper, we propose that Rrf1p, the yeast homolog of bacterial RRF, functions in mitochondrial protein synthesis in a manner similar to bacterial RRF. Mitochondrial localization of Rrf1p is supported by four observations. (i) Immuno-chemical studies revealed Rrf1p in isolated mitochondria but not in post-mitochondrial supernatants. (ii) Rrf1p could only be detected *in situ* in mitochondria by immunofluorescence. (iii) The molecular mass of Rrf1p detected in yeast crude extract and in yeast isolated mitochondria is 23 kDa, a size similar to that predicted from the cDNA sequence, assuming that Rrf1p undergoes maturation in mitochondria. (iv) The N-terminal sequence of the protein deduced from the cDNA sequence is identified as a mitochondrial targeting signal (55).

The following observations also support the above proposal regarding the function of Rrf1p. (i) Deletion of *RRF1* yielded a phenotype (*rho⁻*) very similar to those exhibited by deleting other mitochondrial proteins needed for mitochondrial protein synthesis [e.g. threonine and tryptophan tRNA synthetases, mitochondrial EF-G and mitochondrial ribosomal protein (12)]. Our results indicate that *RRF1* encodes a component essential for yeast mitochondrial DNA maintenance as observed by Myers *et al*. (12). (ii) The Δ*rrf1* strain expressing Rrf1p-L209P exhibited a respiratory deficient phenotype at the non-permissive temperature (37°C) because this strain did not grow at 37°C. However, it grew at the lower culture temperature (Fig. 2B). (iii) Mitochondrial protein synthesis was markedly reduced at the non-permissive temperature in the Rrf1p-L209P strain but not in the wild-type strain. No significant inhibition of cytoplasmic protein synthesis was observed with the mutant strain (Figs 4 and 5).

The structural biology of Rrf1p also supports the above proposal. First, Rrf1p has sequence homology with *E.coli* RRF. Both proteins are of similar size, sharing 23% identical residues (34). The crystal structure of RRF was first solved by us (*T.maritima*) (56) and confirmed by others with RRF from other organisms (57–59). We have also obtained the solution structure of *Aquifex aeolicus* RRF (60) using a novel NMR technique. Based on the crystal structure of *T.maritima* RRF (accession no. 1DD5), a 3-dimensional structure of Rrf1p was built using an homology modeling approach. The modeled Rrf1p structure as shown in Figure 6, just like other RRFs, is

Figure 6. Predicted surface of Rrf1p. A Connolly surface was calculated as described in Materials and Methods for the Rrf1p protein using a theoretical sphere of 2.4 Å. The green color indicates the portion corresponding to the D loop of tRNA.

remarkably similar to that of tRNA (56). It is important to recall that mammalian mitochondrial tRNA has no or a small D loop (61). The modeled yeast RRF structure does contain the portion corresponding to the D loop, shown in green.

Second, the mutation *rrf1-L209P* resulted in temperature-sensitive yeast on a non-fermentable carbon source. This is due to temperature-sensitive protein synthesis in mitochondria (Fig. 4). The corresponding mutation in *E.coli* RRF, L163P, also resulted in a temperature-sensitive *E.coli* (21). This finding supports the notion that Rrf1p has a structure very similar to that of *E.coli* RRF, since changing Leu209 to Pro influences the structure of both proteins in a similar way. In addition, although the function and cytological localization of the human RRF homolog remain obscure, it has been cloned using its sequence similarity to *E.coli* RRF and has been suggested to be a mitochondrial enzyme (14).

Our proposal can be widened to state that all eukaryotic RRF homologs localize in organelles containing ribosomes and function as a ribosome recycling factor. In our previous paper, we showed that mature RRFHCP (the RRF homolog in chloroplasts) was found in the chloroplasts of spinach (32). The chloroplast RRF homolog is very similar to Rrf1p in the following aspects. (i) The molecular mass is 26.5 kDa. (ii) A larger precursor with an N-terminal signal sequence guides the protein to chloroplasts. (iii) RRFHCP was exclusively localized to chloroplasts and does not exist in the cytoplasm. (iv) The fact that RRFHCP appears to compete with *E.coli* RRF in *E.coli* strongly suggests both functional and structural homology between these proteins.

As stated above, Rrf1p appears to function in mitochondria as RRF does in *E.coli*. However, we should hasten to point out that there may be important functional and structural

differences between Rrf1p and bacterial RRF. For example, the sequence identity between bacterial RRF and the eukaryotic homolog is rather low. In addition, as shown in Figure 2B, *rrf1*-K67R, *rrf1*-L206E and *rrf1-I228P* did not yield a temperature-sensitive phenotype despite the fact that these mutations resulted in a temperature-sensitive bacterial RRF. Clearly, subtle but distinct differences in the structure of Rrf1p and bacterial RRF exist. In addition, the protein synthesis machinery of mitochondria is not exactly the same as that in bacteria (2,5). This may be related to the evolution and diversification of mitochondrial genomes (62). Different ribosomal proteins are present in mitochondrial ribosomes compared to bacterial ribosomes (2,11,63,64). These considerations lead us to believe that enough structural and functional differences may exist between eukaryotic mitochondrial and prokaryotic RRF to make it possible to devise antibacterial agents targeted against bacterial RRF. Finally, our present results confirm the notion that the disassembly of post-termination complexes in the cytoplasm of eukaryotes must take place under the control of factors other than RRF homologs.

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