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Translational readthrough of a termination codon in the yeast mitochondrial mRNA *VAR1* as a result of mutation in the release factor mRF1✪

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Yeast mitochondrial DNA codes for eight major polypeptides. Translation of he mitochondrially encoded polypeptides in strains with mutated mitochondrial release factor, mRF1, was found to result in the synthesis of a novel protein, V2. Different *mrf1* alleles were associated with different efficiency of V2p synthesis. Translation of V2p was enhanced by paromomycin. Comparative analysis of peptides resulting from protease digestion indicated that V2p is a derivative of Var1p. According to our hypothesis, V2p represents a readthrough product of the natural stop codon in *VAR1* mRNA.

An effective way of investigating the expression of genes in mtDNA is based on studying the control exerted by the nucleus over mitochondrial functions. This is due to replication, transcription and translation apparatus in mitochondria being composed mostly of polypeptides encoded by nuclear DNA. The yeast *Saccharomyces cerevisiae,* being a facultative anaerobe, offers distinct advantages for mitochondrial genetic studies. Several hundred respiratory deficient point mutations or small deletions, called *mit*$^-$, have been genetically mapped and assigned to genes in mtDNA. Respiration of *mit*$^-$ strain

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Abbreviations: mRF1, mitochondrial release factor; mtDNA, mitochondrial DNA; *mit*$^-$, point mutants in mitochondrial DNA; SDS/PAGE; sodium dodecylsulphate polyacrylamide gel elecrophoresis

could be restored by nuclear mutations, thus providing phenotypes amenable to fast and simple screening. A systematic search for nuclear suppressors of *mit*$^-$ resulted in the identification of several chromosomal genes that have a well-defined impact on mitochondrial functions (Herbert *et al.*, 1988; Asher *et al.*, 1989; Altamura *et al.*, 1992; Boguta *et al.*, 1992; Ekwall *et al.*, 1992).

We have studied suppressors of *mit*$^-$ mutants that act at the level of mitochondrial translation. Two nuclear mutants, *nam3-1* and *nam3-2*, carrying a suppressor of the *cob*-M2001 *mit*$^-$ *ochre* mutation localized in the maturase coding region and preventing splicing of intron bi2 in the mitochondrial *COB* gene, were isolated (Kruszewska & Slonimski, 1984a). Genetic studies indicated an allele-specific, gene non-specific omnipotent mode of suppression that could result from decreased translation fidelity of premature stop codons (Kruszewska & Slonimski, 1984b; Zagorski *et al.*, 1987b). This hypothesis was in part verified by direct biochemical data (Boguta *et al.*, 1988; Mieszczak & Zagorski, 1987b). Recent studies identified *nam3-1* and *nam3-2* as alleles of the nuclear gene *MRF1* encoding the mitochondrial release factor corresponding, respectively, to *mrf1-145* ^{352}S→I and *mrf1-136* ^{216}S→Y (Towpik *et al.*, 2004). Construction of a structural model of the mRF1 molecule allowed for localization of the substituted serines, ^{352}S and ^{216}S, in the vicinity of the codon recognition motif. This localization confirms the genetic data indicating the effect of *mrf1-136* and *mrf1-145* on the fidelity of termination.

Here we analyse precisely the proteins synthesized in *mrf1-136* and *mrf1-145* mitochondria, searching for polypeptides possibly representing the products of reading through the natural stop codons. We observed the synthesis of a novel protein, V2, which is an extended form of Var1p. Thus, mutations in the mRF1 encoding gene allow mitoribosomes to misread a premature as well as a natural stop codon.

MATERIALS AND METHODS

Strains, media and growth conditions. The yeast strains used in these studies, derived from P.P. Slonimski's collection, are listed in Table 1.

Media used for yeast growth were YPD (2% glucose, 2% peptone, 1% yeast extract) or YPGly (2% glycerol, 2% peptone, 1% yeast extract). The growth rate in liquid cultures at 30°C was monitored by A_{600} measurements. For isolation of mitochondria and *in vivo* labeling, yeast cells were harvested in the mid exponential phase of growth.

In vivo labeling of mitochondrial translation products. Cells grown as described above were suspended in low sulphate me-

Table 1. *Saccharomyces cerevisiae* strains used in this study

Strain	Nuclear genotype	Mitochondrial genotype
CK01	*MATa kar1-1 leu1 can*^{r}	wt, *rho*$^+$
CK311	*MATa kar1-1 leu1 can*^{r}	*cob*-M2101, *mit*$^-$
CK247	*MATa kar1-1 leu1 can*^{r}	*cob*-M3041, *mit*$^-$
CK311/B136*	*MATa kar1-1 leu1 mrf1-136 can*^{r}	*cob*-M2101, *mit*$^-$
CK311/B145**	*MATa kar1-1 leu1 mrf1-145 can*^{r}	*cob*-M2101, *mit*$^-$

*Previous name of the same mutant was *nam3-2*; ** previous name of the same mutant was *nam3-1*

dium and incubated for 2 h at 30°C on a shaker before the addition of cycloheximide to a final concentration of 600 μg ml^{-1}. After 4 min 0.5 mCi/ml $Na_2[^{35}S]O_4$ was added and samples were collected after 1 h. The labeling reaction was terminated by chilling the cells on ice and simultaneous addition of unlabeled sulphate. After centrifugation the cells were resuspended in a buffer containing 250 mM mannitol, 20 mM Tris/HCl, pH 7.5, 1 mM EDTA and 1 mM phenylmethylsulphonyl fluoride (PMSF). Mitochondria were isolated as described by Claisse *et al.* (1980). Samples of mitochondrial proteins (50 000–200 000 c.p.m. each) were analysed by SDS/PAGE on gel slabs containing an exponential gradient (10–15%) of polyacrylamide. The gels were fixed, dried, and exposed to Kodak X-ray films, as described by Claisse *et al.* (1977).

Translation in isolated mitochondria. Isolation of mitochondria and labeling of their translation products with $Na_2[^{35}S]O_4$ were based on the method of McKee and Poyton (1984) with the modifications proposed by Zagorski *et al.* (1987a) and Kozlowski & Zagorski (1988). Paromomycin was added to the translation mixtures at 400 μg ml^{-1}. Analysis by SDS/PAGE was as described above.

Peptide maps. Peptide maps were based on the method of Cleveland *et al.* (1977) with the modifications described by Claisse *et al.* (1980). Selected labeled bands were cut out from dried SDS/PAGE slabs. Proteins were treated with *S. aureus* V8 protease (Miles Laboratories) using, respectively, 0.1 μg/sample or 50 μg/sample for limited or extensive digestion and the peptides were analysed by 15% SDS/PAGE. For double digests, co-migrating labeled fragments generated in the *S. aureus* primary digests were cut out from dried gel and submitted to *B. subtilis* nagarse (Serva) proteolysis directly on a second 15% acrylamide slab gel, according to Claisse *et al.* (1980). The gels were infiltrated with the scintillator PPO (2,5-diphenyloxazole, Sigma Chemical Co.) as described by Bonner and Laskey (1974), dried, and exposed to Kodak X-ray films for one day to several weeks or months.

RESULTS

Changes in the fidelity of mitochondrial translation result in the biosynthesis of a novel polypeptide, V2

Mitochondrial *mit*$^-$ nonsense mutations *cob*-M2101 and *cob*-M3041, localized in the *COB* gene region coding for maturase bi2, arrest the splicing of *COB* and *COX1* genes pre-mRNAs (Kruszewska & Slonimski, 1984b). This is directly reflected in the synthesis of mitochondrial polypeptides since mutant cells do not synthesize Cobp and Cox1p (Jacq *et al.*, 1982). *mrf1136* and *mrf1-145*, previously isolated as nuclear mutations that suppress the respiratory deficiency of *cob*-M2101, *cob*-M3041 and several other *mit*$^-$ nonsense mutations localized in different mitochondrial genes, were subsequently identified as alleles of a gene encoding the mitochondrial release factor (Kruszewska & Slonimski, 1984a; Towpik *et al.*, 2004).

To determine the effect of the *mrf1* suppressor mutations on the pattern of proteins synthesized in mitochondria of the *cob*-M2101 mutant, the cells were labeled with $Na_2[^{35}S]O_4$ in the presence of cycloheximide to inhibit cytoplasmic protein synthesis. Proteins were then analyzed by SDS/PAGE and autoradiography (Fig. 1). The wild-type CK01 *S. cerevisiae* cells, grown on media containing glucose or glycerol, synthesize a basic set of mitochondrial polypeptides: Var1p, a mitochondrial ribosomal protein, Cox1p, Cox2p and Cox3p, subunits of cytochrome oxidase, Cobp, the apocytochrome *b* subunit of coenzyme Q – cytochrome *c* reductase, and Atp6p, Atp8p and Atp9p, subunits of the F_0F_1-ATPase.

In the *mit*$^-$ cells carrying the *cob*-M2101 mutation both Cob and Cox1 polypeptides are absent but their synthesis is fully restored by

mrf1-136 or *mrf1-145* suppressor mutations, especially in cells grown on YPGly (Fig. 1B). A novel polypeptide V2, migrating a little slower than Var1p, is present among the mitochondrial translation products in *mrf1-136* cells. In contrast to the accumulation of Cox1p, the synthesis of V2p in *mrf1-136* mutant cells does not depend on the carbon source (compare Fig. 1A and 1B). This observation points against V2p being a precursor of Cox1p or a mistranslation product of the *COX1* gene.

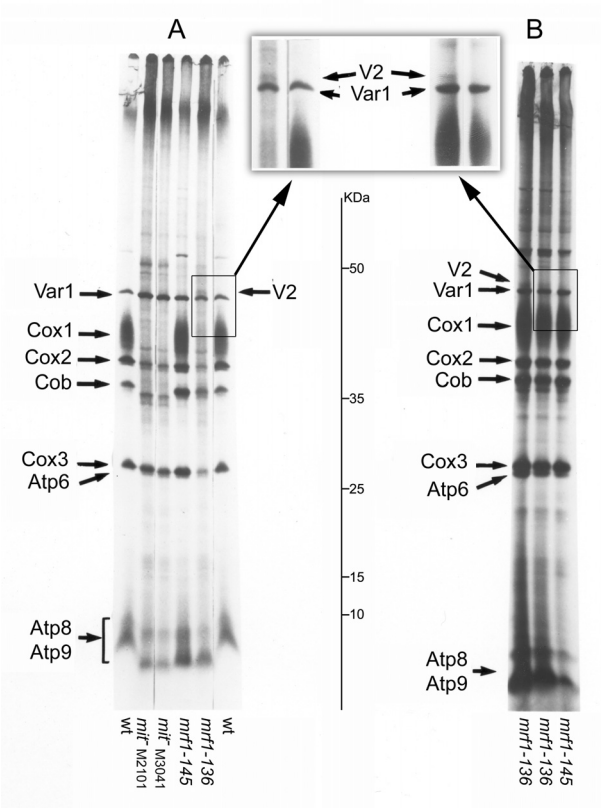

Figure 1. Synthesis of a novel polypeptide, V2, migrating a little slower than Var1p is observed in the *mrf1-136* mutant.

The wt, *mit*⁻ and *mrf1* strains used were isogenic. Lanes from left represent: CK01, CK311- *cob*-M2101, CK247-*cob*-M3041, CK311/B136 and CK311/B145 (see Table 1 for the strain details). *In vivo* labeling of mitochondrially synthesized proteins was performed in the presence of $Na_2[^{35}S]O_4$ and cycloheximide. Cells were disrupted by glass beads and the extracts were analyzed on 10–15% gradient SDS/PAGE followed by autoradiography. Cox1, Cox2, Cox3 are subunits 1, 2, 3 of cytochrome *c* oxidase, respectively; Cob, apocytochrome b; Atp6, Atp8 and Atp9 are subunits of the F_0F_1-ATPase; Var1, mitoribosomal protein. **A**. Cells grown in glucose medium (YPD). **B**. Cells grown in glycerol medium (YPGly). Lanes 1 and 2 in B represent the results of independent experiments. Respective fragments of gels A and B are shown enlarged.

Thus, decreasing the fidelity of stop codon recognition by mutated release factor allows for restoration of the wild type pattern of mitochondrial translation in an *ochre mit*⁻ but also results in the synthesis of an additional polypeptide.

To confirm that the appearance of V2p is really the result of an alteration in translation, we assessed whether its synthesis is affected by paromomycin, an antibiotic that changes translation fidelity of cytosolic as well as of mitochondrial ribosomes (Wilhelm *et al.*, 1978; Dujardin *et al.*, 1984; Zagorski *et al.*, 1987a). Translation in the presence of $Na_2[^{35}S]O_4$ was monitored in mitochondria incubated either with or without paromomycin (see Materials and Methods) after isolation from the wild type, *mit*⁻ as well as from the *mrf1-136* and *mrf1-145* cells. The products of mitochondrial translation *in organello* were analysed by SDS/PAGE and autoradiography (Fig. 2). In contrast to the wild type mitochondria, mitochondria isolated from *mrf1-145* cells synthesized V2p after addition of paromomycin. In *mrf1-136*, V2p was synthesized spontaneously but its synthesis was apparently intensified by paromomycin. This points towards the synergism of *mrf1* mutations and the antibiotic in promoting V2p synthesis, making it plausible that V2p represents a product of mistranslation.

V2p is a derivative of Var1 polypeptide

Since *mrf1* mutants decrease the fidelity of stop codon recognition (Towpik *et al.*, 2004) we assumed that the V2 polypeptide may have resulted from reading through of the natural termination codon in *VAR1* mRNA. This hypothesis was substantiated by direct compari-

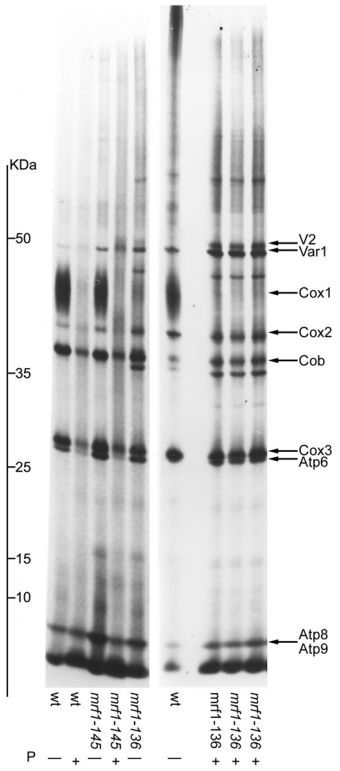

Figure 2. Paromomycin promotes readthrough of *VAR1* mRNA.

Mitochondria were isolated from the isogenic strains CK01 (wt), CK311/B136 (*mrf1-136*) and CK311/B145 (*mrf1-145*) grown in YPD. Translation in isolated mitochondria was carried out in the presence of [^{35}S]methionine and, as indicated, either without (P –) or with (P +) paromomycin (400 μg/ml). Mitochondrial proteins were analyzed on 10–15% gradient SDS/PAGE followed by autoradiography.

son of peptide maps of original Var1p and V2p.

Radiolabeled polypeptides were synthesized in isolated mitochondria and separated by SDS/PAGE. Var1, V2 and Cox1 proteins were isolated from the gels and submitted to digestion with *S. aureus* V8 protease. Spontaneously synthesized or paromomycin-induced V2p, both after partial and extensive digestion created patterns almost identical with those obtained for Var1p (Fig. 3A). The peptide patterns obtained with V2p and Var1p, synthesized in the wild-type and suppressor strains, could almost be superimposed. In contrast, the patterns for V2p and Cox1p were entirely different. This further refuted

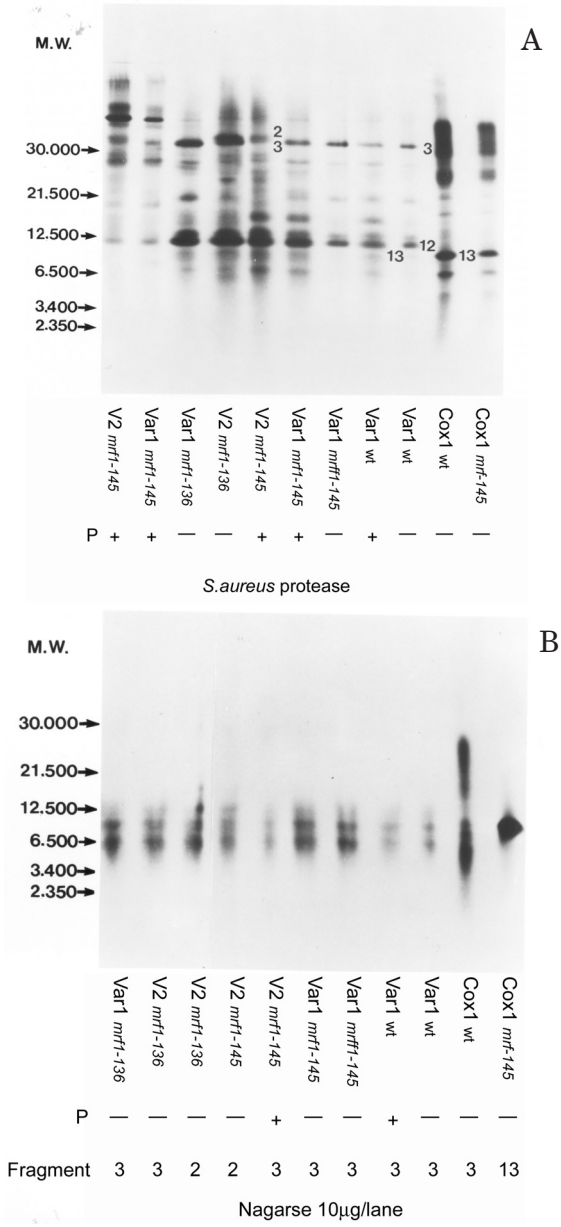

Figure 3. V2p shares the peptide pattern with Var1 protein.

Radiolabeled Var1, V2 and Cox1 polypeptides were synthesized in mitochondria from CK01 (wt), CK311/B136 (*mrf1-136*) and CK311/B145 (*mrf1-145*) cells, incubated, as indicated by P, with paromomycin (400 μg/ml), separated by electrophoresis and isolated from the gels. **A**. Var1p, V2p and Cox1p were subjected to limited (0.1 μg/sample, first two lanes from the left) or extensive (50 μg/sample, remaining lanes) digestion with *S. aureus* V8 protease. The products of proteolysis were analyzed on 15% SDS/PAGE followed by autoradiography. The numbers correspond to co-migrating fragments. **B**. Co-migrating peptide fragments were further analyzed by Nagarse proteolysis (10 μg/sample) directly on a second 15% SDS/PAGE, followed by autoradiography.

the possibility that V2p arose as a by-product of disturbed *COX1* gene expression in *mrf1* strains.

The identity of peptides co-migrating in the primary digests of V2p and Var1p was further confirmed by the second digestion. In this respect, peptides No. 2 and 3 from *S. aureus* V8 protease digests were isolated and treated with Nagarse. In all strains, Var1p-derived peptides were the same as those detected in the V2p-derived products (Fig. 3B). Control digest of a Cox1p-derived peptide migrating in the vicinity of Var1 peptide No. 3 confirmed that the peptide detected in V2p and Var1p is specific for the *VAR1* gene product. In conclusion, the peptide map analysis indicates that the V2 product is a derivative of mitochondrial Var1 polypeptide.

DISCUSSION

Yeast mitochondrial ribosomes synthesize eight major polypeptides, seven of which constitute subunits of membrane respiratory complexes (Foury *et al.*, 1998). The eighth product of the mitochondrial genome, a soluble protein Var1, is a mitoribosomal protein (Terpsta *et al.*, 1979). Here we examined the products of mitochondrial translation in the release factor mutants, *mrf1-136* and *mrf1-145*, that affect the fidelity of stop codon recognition (Towpik *et al.*, 2004). This led to observation of the synthesis of a novel protein, identified by finger printing as a Var1p derivative. We suggest that this novel protein is a readthrough product resulting from mistranslation of the regular stop codon in *VAR1* mRNA.

The *VAR1* gene is unusual in its base composition: it contains nearly 90% AT and a 46 bp GC-rich palindromic cluster (Butow *et al.*, 1985). Furthermore, *VAR1* is an expandable gene that participates in a recombinational event resembling exon shuffling, although no intron is present. Strain dependent variations of *VAR1* ORF differing in size by up to 7% were observed but this variation does not detectably interfere with its function (Butow *et al.*, 1985). In contrast to other yeast mitochondrial genes, no mutations were isolated in *VAR1* except for a deletion of a dodecamer sequence at the 3' end of the mRNA (Butow *et al.*, 1989). Little is known about *VAR1* expression. No translational activator specific for *VAR1* is known, although the 5' regions of *VAR1* mRNA apparently mislocalized the synthesis of Cox2 and Cox3 proteins from chimeric mRNA (Sanchirico *et al.*, 1998). Despite Var1p being the only mitochondrially encoded soluble protein, its synthesis seems to be localized in close proximity to the inner membrane, but at a site distinct from the site of synthesis of Cox2p and Cox3p (Fiori *et al.*, 2003). Membrane associated synthesis of Var1p could help initiate the ribosome assembly process, which would involve all remaining mitoribosomal proteins imported from the cytoplasm (Fiori *et al.*, 2003). A role for Var1p in mitoribosome assembly has been suggested previously (Davis & Ellis, 1995).

In this work we show the possibility of an alternative termination of Var1p biosynthesis. We have analysed mitochondrially synthesized proteins from *mrf1-136* and *mrf1-145* strains harboring, respectively, the $^{352}S \rightarrow I$ and $^{216}S \rightarrow Y$ amino acid substitutions in the mitochondrial release factor mRF1 (Towpik *et al.*, 2004). A novel polypeptide V2, migrating a little slower than Var1p, is present among the mitochondrial translation products in *mrf1-136* cells. An identical longer form of Var1p appears in the mutant *mrf1-145*, when protein synthesis is probed in the presence of paromomycin. This points towards a synergism of *mrf1* mutations and the antibiotic in promoting V2p synthesis, making it plausible that V2p represents a product of *VAR1* mistranslation. This hypothesis was substantiated by direct comparison of peptide maps of original Var1p and V2p.

The molecular mass of V2p calculated from SDS-gels exceeded that of Var1p by 1.2–2.3 kDa. We favour the idea that V2p is gener-

ated as a product of reading through the natural *ochre* terminator in *VAR1* mRNA. Translational suppression of the natural *VAR1 ochre* terminator with concomitant change in phase could allow reading into short ORFs and stopping at the next *ochre* terminators detected after 19 (phase +1) or 30 (phase –1) codons. The molecular mass of V2p indicates that its synthesis results from the +1 frameshift. Frameshifting is known to occur at a high rate at specific points during the expression of at least some mitochondrial genes (Fox & Weiss-Brummer, 1980). The change in phase at a natural stop codon, observed here, could be promoted by *mrf1* mutations in the release factor gene and by paromomycin.

To understand why V2p is the only readthrough product among mitochondrially synthesized proteins, we examined the sequences preceding and following the natural stop codon in the seven remaining mitochondrial polypeptides. All the stop codons were UAA and the termination regions, except for *COX3* mRNA, were AT-rich, but no consensus sequence was found. No specific motif was detected allowing for speculation that the stop codon in *VAR1* mRNA is especially susceptible to frameshifting. However, according to theoretical predictions, a +1 frameshift of all the other seven stop codons would result in minute extensions of the synthesized proteins. Such short extensions, even if they existed, would be not discerned by the SDS/PAGE.

We showed that the efficiency of V2p synthesis, reflecting the readthrough level, differs for the two *mrf1* alleles examined. The higher ambiguity of the *mrf1-136* ^{216}S→Y than the *mrf1-145* ^{352}S→I mutant is consistent with the spatial localization of the corresponding substituted amino acids. Ser216 is buried in the mRF1 structure in a tight pocket formed by the surrounding residues. Conversion of the serine side chain into the bulkier tyrosine could destroy the internal packing, leading to a local conformational change of the backbone possibly affecting the linker between domains 1 and 2 of mRF1, and, in consequence, the relative orientation of these domains (Towpik *et al.*, 2004). In contrast S352I substitution may only alter the mRF1 interaction with the ribosome.

We cannot exclude an alternative termination of Var1p biosynthesis occurring at low frequency in the wild type cell. The readthrough of the natural stop codon was documented for the yeast chromosomal gene *PDE2*. The synthesis of C-terminally extended Pde2 protein was important for the regulation of the cellular level of cAMP (Namy *et al.*, 2001). It is not known whether the extended form of Var1p has any physiological role.

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