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Balance between Transcription and RNA Degradation Is Vital for *Saccharomyces cerevisiae* Mitochondria: Reduced Transcription Rescues the Phenotype of Deficient RNA Degradation

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The *Saccharomyces cerevisiae SUV3* gene encodes the helicase component of the mitochondrial degradosome (mtEXO), the principal 3′-to-5′ exoribonuclease of yeast mitochondria responsible for RNA turnover and surveillance. Inactivation of *SUV3* (*suv3*Δ) causes multiple defects related to overaccumulation of aberrant transcripts and precursors, leading to a disruption of mitochondrial gene expression and loss of respiratory function. We isolated spontaneous suppressors that partially restore mitochondrial function in *suv3*Δ strains devoid of mitochondrial introns and found that they correspond to partial loss-of-function mutations in genes encoding the two subunits of the mitochondrial RNA polymerase (Rpo41p and Mtf1p) that severely reduce the transcription rate in mitochondria. These results show that reducing the transcription rate rescues defects in RNA turnover and demonstrates directly the vital importance of maintaining the balance between RNA synthesis and degradation.

INTRODUCTION

The degradation of RNA is an essential element in the expression of genetic information. It is required to control RNA abundance and thus gene expression and to eliminate aberrant or defective molecules that inevitably form during RNA synthesis and maturation (RNA surveillance) (Vasudevan and Peltz, 2003). The posttranscriptional mechanisms affecting mitochondrial gene expression, including RNA turnover, are of particular importance because transcriptional control is relatively simple and rudimentary. The single RNA polymerase (RNAP) of *Saccharomyces cerevisiae* mitochondria is composed of only two nuclear-encoded protein subunits—the core enzyme encoded by the *RPO41* gene and a transcription initiation factor encoded by the *MTF1* gene (Masters *et al*., 1987; Jang and Jaehning, 1991). The RNAP holoenzyme recognizes a simple nonanucleotide promoter sequence (Osinga *et al*., 1982; Mangus *et al*., 1994) and initiates synthesis of at least 13 primary multicistronic transcripts that undergo extensive processing to form mature RNAs (Christianson and Rabinowitz, 1983; Tzagoloff and Myers, 1986; Foury *et al*., 1998; Gagliardi *et al*., 2004; Schafer, 2005). Such organization leaves little room for regulation at the transcription initiation level and makes posttranscriptional processes, including RNA degradation, key control points for mitochondrial gene expression.

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The enzymes controlling RNA turnover in cells and organelles show great evolutionary divergence and are only partially conserved between mitochondria of different organisms (Gagliardi *et al*., 2004). The enzymatic activity responsible for turnover is, however, on the basic level, similar in all the systems discovered so far—it is that of a 3′-to-5′ processive exoribonuclease, either hydrolytic or phosphorolytic. In most cases, the exoribonuclease activity is contained in a larger multiprotein complex that, in addition to exoribonucleases, also contains RNA helicases, and in certain cases, endonucleases. A model example of such a complex is the eubacterial degradosome (Carpousis, 2002).

The first mitochondrial RNA degradation enzymatic complex was described in yeast *S. cerevisiae* and named mtEXO, or the mitochondrial degradosome (Margossian and Butow, 1996; Dziembowski *et al*., 2003). The mitochondrial degradosome is the main exoribonuclease in yeast mitochondria, which, unlike bacteria and animal or plant mitochondria, lack the phosphorolytic polynucleotide phosphorylase activity. Like the bacterial degradosome it contains an RNase and an RNA helicase; the subunit composition is, however, markedly different (Gagliardi *et al*., 2004). The yeast mitochondrial degradosome is composed of only two protein subunits—an RNR (RNase II-like) superfamily exoribonuclease encoded by the *DSS1/MSU1* (YMR287C) gene (Dmochowska *et al*., 1995) and an NTP-dependent RNA helicase related to the DExH superfamily, encoded by the *SUV3* (YPL029W) gene (Stepien *et al*., 1992). The two proteins are tightly associated and the activity of both is essential for the functioning of the complex. The mitochondrial degradosome is capable of unwinding dsRNA regions and subsequently degrading single-stranded RNA in a 3′-to-5′ direction (Dziembowski *et al*., 2003). The mtEXO complex

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

Name	Genotype		Origin
	Nuclear	Mitochondrial	
BWG1	*MAT* **a**, *his1, ade1, leu2, ura3*	*rho*$^+$	Stepien *et al.* (1992)
suv3Δ Δi	*MAT* **a**, *his1, ade1, leu2, ura3, suv3::URA3*	*rho*$^+$, intronless	Stepien *et al.* (1995)
W303/A/520	*MAT* **a**, *ade2, trp1, ura3, leu2, his3*	*rho*$^+$, intronless	J. Lazowska
WSU0 Δi	*MAT* **a**, *ade2, trp1, ura3, leu2, his3, suv3::URA3*	*rho*$^+$, intronless	This work
D273-10B/51	*MAT* α, *ade5*	*rho*^{0}	Groudinsky *et al.* (1981)
Y12799	*MAT* α, *his3, leu2, lys2, ura3, SUV3::kanMX4*	*rho*$^-$/*rho*^{0}	EUROSCARF
SUD1	*MAT* **a**, *ade2, trp1, ura3, leu2, his3, suv3::KanMX4*	*rho*$^+$, intronless	This work
SDB9	*MAT* **a**, *his1, ade1, leu2, ura3, suv3::URA3, su1-1*	*rho*$^+$, intronless	Stepien *et al.* (1995)
SUX1	*MAT* **a**, *ade2, trp1, ura3, leu2, his3, suv3::URA3, su1-2*	*rho*$^+$, intronless	This work
SUX3	*MAT* **a**, *ade2, trp1, ura3, leu2, his3, suv3::URA3, su2*	*rho*$^+$, intronless	This work
αKxNO41 Δi	*MAT* α, *Kar1-1, trp5, his4, ade6*	*rho*$^+$, intronless	Stepien *et al.* (1995)
SP55-11	*MAT* α, *lys2, ura3, suv3::URA3*	*rho*$^+$, intronless	P. P. Stepien
W303-1B/520	*MAT* α, *ade2, trp1, ura3, leu2, his3*	*rho*$^+$, intronless	This work
DSW3-12/A	*MAT* **a**, *ade2, trp1, ura3, leu2, his3, mtf1*^{su2}	*rho*$^+$, intronless	This work
DSW1-16/C	*MAT* **a**, *ade2, trp1, ura3, leu2, his3, rpo41*^{su1}	*rho*$^+$, intronless	This work

interacts with the mitochondrial ribosome (Dziembowski *et al*., 2003). Genetic interactions link the mitochondrial degradosome with the protein encoded by the *PET127* gene, which is involved in 5′ processing and turnover of mitochondrial RNAs (Wiesenberger and Fox, 1997; Wegierski *et al*., 1998), and with the 5′ untranslated region of the *CYTB* mRNA (Chen *et al*., 1999).

Inactivation of either the *SUV3* or *DSS1* gene gives a similar phenotype, corresponding to complete depletion of mitochondrial degradosome function. *SUV3*-deficient strains are all strictly respiratory deficient and rapidly lose wild-type mitochondrial DNA (mtDNA) converting to *rho*$^-$/*rho*^{0} forms. Introduction of intronless mtDNA (Seraphin *et al*., 1987) into the *suv3*Δ background (strain *suv3*Δ Δi) does not rescue the respiratory-deficient phenotype; however, it markedly improves the stability of such mtDNA to ~60% *rho*$^+$ in an overnight YP-glucose culture (Dmochowska *et al*., 1995; Golik *et al*., 1995; Stepien *et al*., 1995).

Among the plethora of molecular defects found in *suv3*Δ strains are overaccumulation of excised group I intronic sequences coupled with the destabilization of mature transcripts (Stepien *et al*., 1992, 1995; Golik *et al*., 1995; Margossian *et al*., 1996), accumulation of RNAs with abnormal 5′ and 3′ termini and of high-molecular-weight RNA precursors, variations in steady-state levels of mature transcripts, and disruption of mitochondrial translation (Dziembowski *et al*., 2003). Together, these phenotypes suggest that the mtEXO complex is the primary activity responsible for RNA degradation and surveillance in yeast mitochondria.

A spontaneous suppressor allowing the *suv3*Δ Δi strain to grow on respiratory carbon sources was first described in the original *suv3*Δ deletion mutant (Dmochowska *et al*., 1995; Golik *et al*., 1995; Stepien *et al*., 1995). The suppressor mutation, termed *suB9*, partially restored respiratory growth to the *suv3*Δ strain, as long as the mitochondrial genome did not contain the *LSU-rRNA* gene intron omega (Stepien *et al*., 1995) or more than three introns in *CYTB* and *COX1* genes (Golik *et al*., 1995). The *suB9* suppressor was found to be nuclear and monogenic, no further characterization was made at that time.

In this work, we show that mutations partially rescuing the phenotype associated with the loss of mitochondrial degradosome caused by disruption of the *SUV3* gene, including the *suB9* mutation, are point mutations in genes encoding subunits of the mitochondrial RNA polymerase that severely reduce the transcription rate. This is the first direct demonstration that maintaining the balance between RNA synthesis and degradation is crucial for the correct functioning of the mitochondrial genetic system.

MATERIALS AND METHODS

Strains, Media, and Classical Yeast Techniques

The *S. cerevisiae* strains used in this study are listed in Table 1. Standard yeast media and basic genetic methods were as described previously (Dujardin *et al*., 1980; Burke *et al*., 2000). The normal growth temperature was 30°C, and temperature sensitivity was tested at 36°C. The Singer MSM series 200 System micromanipulator (Singer Instruments, Watchet, Somerset, United Kingdom) was used for tetrad dissection and for the isolation of zygotes in isogenic crosses. Yeast were transformed using either the rapid or high-efficiency LiAc/SS-DNA/PEG protocol (Gietz and Woods, 2002).

Construction and Initial Characterization of suv3Δ *Strains*

The first *suv3*Δ mutants were constructed in the background of the BWG1 strain of *S. cerevisiae* (see Table 1 for all strain genotypes) as complete or partial deletions with the *URA3* cassette (Stepien *et al*., 1992). Because we planned to use such a strain as a starting point in the search for possible suppressors, we decided to recreate this system in the background of another, much better characterized strain W303, which has been extensively used in the study of nucleomitochondrial interactions. From a strain carrying the shorter (d2) deletion, we amplified the *URA3* cassette with ~200 base pairs and ~100 base pairs *SUV3* flanks at the 5′ and 3′ end, respectively, using primers 9090 and 9091. The PCR product was used to transform the W303/A/520 strain, which has the W303 nuclear background combined with the intronless mtDNA. The resulting strain, termed WSU0 Δi, behaved identically to the original *suv3*Δ strains, becoming respiratory deficient with a moderate decrease in the stability of the mitochondrial genome. To verify whether the partial nature of the deletion could have any influence on the phenotype, we also prepared an isogenic complete deletion strain by replacing the entire *SUV3* open reading frame (ORF) with the *KanMX4* selection module. We used DNA from the strain Y12799 originating from the *Saccharomyces* Genome Deletion Project (Winzeler *et al*., 1999; Giaever *et al*., 2002), which harbors the *SUV3* deletion, as a template for PCR using the SUV3_A and SUV3_D primers. This gave a product containing the *KanMX4* module with yeast genomic flanks upstream from the ATG and downstream from the termination codon of *SUV3*. Transformation of the W303/A/520 strain with this product gave a strain, termed, SUD1, carrying a deletion of the entire *SUV3* ORF in the W303 background with the intronless mtDNA. In all our phenotypic analyses and in subsequent suppressor experiments, this strain behaved exactly like the partial deletion generated previously. We can therefore conclude that all the *suv3*Δ strains we constructed and used were functionally equivalent and corresponded to a complete loss of *SUV3* gene function.

PCR Sequencing and Related Techniques

Yeast total DNA for PCR and plasmid isolation was prepared using the rapid phenol/glass beads protocol (Hoffman and Winston, 1987). Primers 9090

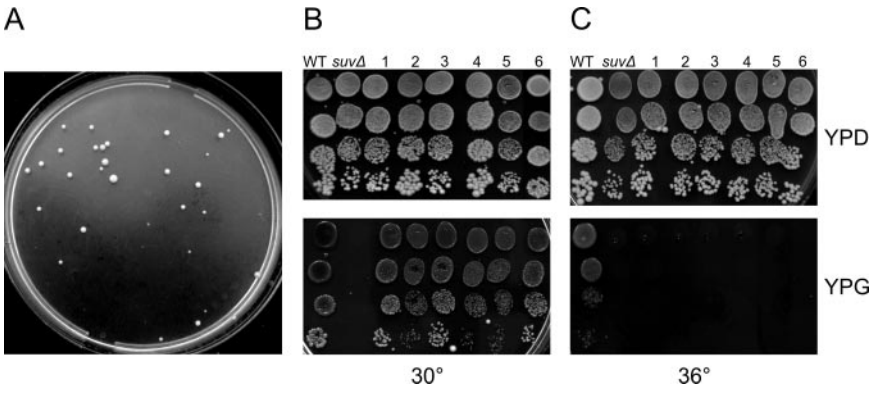

Figure 1. Analysis of the suppressors of the *suv3*Δ phenotype. (A) Appearance of the [Gly$^+$] pseudorevertant colonies in the intronless *suv3*Δ strain after 7 d of incubation on YPG at 30°C. (B) Selected pseudorevertant strains (numbered 1–6) grow at 30°C both on YPD (glucose) and on YPG (glycerol), albeit slower than the wild-type (WT) isogenic control W303/A/520 strain. The *suv3*Δ parental strain is strictly glycerol negative. (C) The pseudorevertant strains (1–6) fail to grow on YPG at 36°C, in contrast to the WT control. Four 10-fold serial dilutions, beginning with the 10^{-1} dilution of the saturated liquid YPD preculture are arranged vertically on each panel. Plates were grown for 3 (YPD) or 4 d (YPG).

(5′-AACTGCGGTTACATGGCCTA) and 9091 (5′-CTCGAAGATGAGAGGTGACC) were used to amplify the *suv3::URA3* d2 disruption cassette (Stepien *et al*., 1992). The *suv3::KanMX* deletion construct was amplified using primers SUV3_A (5′-TCAGAACACAATGTCCTTATTGAAA) and SUV3_D (5′-TATATTTTACTGCCCTTTGCTCAAC).

Primers MTF_A (5′-GATTATTGCGACTAATTTGAATGGT) and MTF_D (5′-CCTTTTCTTAAAGTTTTAGTTCCGC) were used to amplify the *MTF1* gene, and primers MTF_C (5′-CAGTAGTAAGGGAGGCATTTACAGA), MTF_B (5′-AGTTCTTGTTTCCAATACAGGACAG), MTF350F (5′-TATTTGTTCCTGAAGTTCAAT), and MTF690R (5′-GTAGGCCATATTTCCGCAGCA) were used to sequence the *MTF1* ORF.

Primers 976L (5′-CCACCAGCTTGTGAATAGGTT), 1764R (5′-CGAGCTTGTCGTTGAATGGA), 1724L (5′-ACTTCCATTCAACGACAAGC), 2427R (5′-CTCTCTTGCGGCTTCCGTTG), 2321L (5′-CACTGCCAACATTAGAGGAA), 3039R (5′-AGCTGGAGCTTCACCGTGAA), 2971L (5′-GTCGCTAAGGTATCTGTGCA), 3733R (5′-ACCACTTGAGACCAGAAGGT), 3714L (5′-ACCTTCTGGTCTCAAGTGGT), 4488R (5′-TAGTCCTAGTGGTGTTCGTCC), 4453L (5′-ATGTCATCCGTCATATGGAC), and 5210R (5′-TTGTAGTTCACGGCTCACGA) were used in pairs to amplify and sequence the *RPO41* gene.

Vectors and Genomic Libraries

The wild-type *S. cerevisiae* genomic library in the pRS200 (ARS-CEN,*TRP1*) plasmid vector (Sikorski and Hieter, 1989) constructed by P. Hieter was kindly donated by Dr. M. Johnston (Washington University, St. Louis, MO). YCplac111 (ARS-CEN, *LEU2*) and YCplac33 (ARS-CEN, *URA3*) (Gietz and Sugino, 1988) were used as vectors. Plasmids pJJ1148 and pJJ1149 (Cliften *et al*., 2000) containing the *RPO41* gene cloned in the YCplac33 (ARS-CEN, *URA3*) and YCplac111 vectors, respectively, were kindly donated by Dr. Judith Jaehning (University of Colorado, Denver, CO).

RNA Preparation and Northern Hybridization

RNA was prepared from yeast mitochondria purified from log phase liquid cultures by differential centrifugation as described previously (Dziembowski *et al*., 2003). RNA was run on a 0.8% agarose-formaldehyde gel and blotted onto nylon membrane as described previously (Tomecki *et al*., 2004). Amount of mitochondrial protein (quantified by the Bradford assay) in the starting preparation was used to normalize the amount of RNA in each lane. Oligonucleotide probes 14S (5′-TATAAGCCCACCGCAGGTTCCCCTACGGTAACTGTA) and *CYTB* (5′-TATCTATGTATTAATTTAATTATATATTATTTTATTAACTCTACCGAT) (Dziembowski *et al*., 2003) were used to detect the 14S rRNA and *CYTB* mRNA, respectively.

In Organello Transcription Assay

Isolation of yeast mitochondria and run-on transcription in organello was performed essentially as described previously (Krause and Dieckmann, 2004). Transcription reactions were performed in a buffer containing 50 mM HEPES-KOH, pH 8.0, 10 mM $MgCl_2$, 25 mM KOAc, 10 mM dithiothreitol, 125 μM CTP, ATP, and GTP each, 50 μCi of [α-^{32}P]UTP, 40 U of RiboLock RNase inhibitor (MBI Fermentas, Hanover, MD) and 5 μg of mitochondrial protein (as quantified by the Bradford assay) in a total volume of 50 μl. Two 2-μl aliquots were taken at each time point and spotted onto DEAE-cellulose filter discs (DE81; Whatman, Maidstone, United Kingdom). One of each pair of filters was washed four times in excess 0.4 M Na_2HPO_4 to remove unincorporated label, and the radioactivity was determined by liquid scintillation. The ratio of radioactive signal on the washed versus unwashed filter determined the incorporation for a given time point.

RESULTS

Spontaneous, Nuclear, Monogenic Suppressor Mutations Partially Restore Respiratory Competence in suv3Δ Strains Carrying the Intronless Mitochondrial Genome

In this work, we have undertaken a systematic study of spontaneous suppressors of the *suv3*Δ respiratory-deficient phenotype in the context of the intronless mitochondrial genome. Saturated liquid YPD cultures of the WSU0 Δi strain, described above, were plated on YPG plates at ~2.5 × 10^7 cells/plate and grown at 30°C for 7 d. Mitochondrial genome integrity was confirmed for each initial culture by crossing to the *rho*^{0} tester strain D273-10B/51. Colonies of respiratory-positive pseudorevertants were observed on each plate, with a median frequency of ~25 colonies/plate, which corresponds to an approximate mutation rate of 1 × 10^{-6} (Figure 1A). Both large and small pseudorevertant colonies were observed on each plate.

Subsequent testing showed that all the pseudorevertant strains grew on respiratory media (glycerol) at normal temperature (30°C), albeit visibly slower than the wild-type W303/A/520 strain (Figure 1B). Interestingly, the respiratory-positive phenotype was totally lost from the pseudorevertant strains grown at a higher temperature (36°), which means that the suppression is a temperature-sensitive phenomenon (Figure 1C). Growth on YPD media was not affected at either temperature. Four of the pseudorevertant strains, chosen randomly from among both large and small colony classes, and named SUX1, SUX2, SUX3, and SUX4, were selected for further detailed analysis.

To verify whether the suppressor mutation was carried in the nuclear genome or in the mitochondrial DNA, the four selected pseudorevertant clones were converted to rho^0 forms using ethidium bromide and fresh intronless mtDNA was introduced by cytoduction using the strain αKxNO41 (Seraphin *et al*., 1987) as a donor. In all cases, the respiratory-positive phenotype was maintained in the cytoductants, suggesting that the suppressor mutations occurred in the nuclear genome.

Each pseudorevertant strain was then crossed to the SP55-11 strain (*MAT*α, *suv3*Δ). The resulting diploids were homozygous for the *suv3*Δ deletion and heterozygous for the suppressor mutation. Each of the diploids failed to grow on respiratory media, indicating that the suppressor mutations were recessive.

To determine the number of different complementation groups among the suppressor mutations, the *MAT*α *suv3*Δ *su* spores obtained by outcrossing the pseudorevertant strains to the wild-type isogenic strain (see next section) were crossed to each of the original pseudorevertant strains.

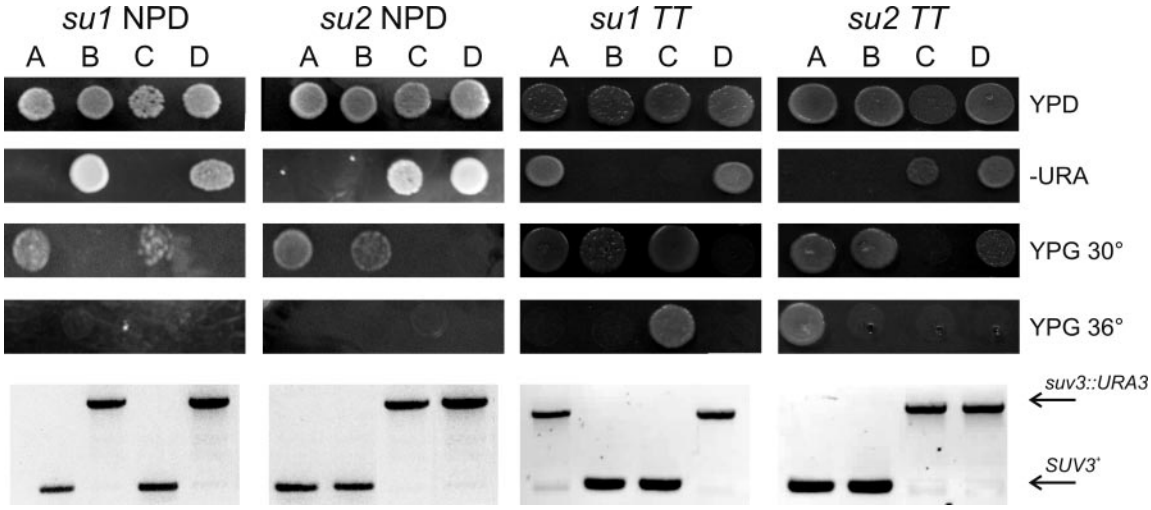

Figure 2. Analysis of typical TT and NPD tetrads resulting from the cross of pseudorevertant strains SUX1 (*su1* mutation) and SUX3 (*su2* mutation) with an isogenic wild-type strain W303-1B/520. Tetrads DSW1-16 and DSW1-2 were selected as *su1* NPD and *su1* TT, respectively. Tetrads DSW3-12 and DSW3-1 were selected as *su2* NPD and *su2* TT, respectively. Spores were precultured overnight in liquid YPD, 10^{-1} dilutions of each preculture were spotted on YPD (complete glucose), SC-URA (synthetic complete – uracil), and YPG (complete glycerol) and grown for 3 (YPD, SC-URA) or 4 d (YPG) at 30 and 36°C. Presence of the *suv3::URA3* disruption was also verified by PCR (primers 9090 and 9091), with the longer and shorter products corresponding to disruption and wild-type *SUV3* allele, respectively. In the NPD tetrads, spores DSW1-16/A and DSW1-16/C contain the *su1* mutation and the wild-type *SUV3*, whereas spores DSW3-12A and DSW13-12/B contain the *su2* mutation and the wild-type *SUV3*.

Because the suppressor mutations are recessive, the resulting diploids would be respiratory competent only if both suppressor mutations occurred at the same locus. The results of this analysis indicated that there were at least two different complementation groups of the suppressor mutations, one (larger colonies, strains SUX1 and SUX2) corresponding to the original *subB9* mutation, and the other (smaller colonies, SUX3 and SUX4) corresponding to another locus. They were given temporary names of *su1* and *su2*, respectively. The strains carrying the *su1* (SUX1) and *su2* (SUX3) mutations were selected for further analysis. To summarize, the genetic analysis of the spontaneous suppressors of the *suv3*Δ disruption showed that the suppressors were nuclear, recessive, and monogenic and they occurred at two distinct loci in the genome. The recessive character of the suppressor mutations practically eliminated the conventional strategy of cloning the suppressor alleles based on their capability of restoring respiration to a *suv3*Δ strain.

The Suppressors of the suv3Δ Disruption Are Temperature-sensitive Partial Loss-of-Function Mutations in the Mitochondrial RNA Polymerase Genes RPO41 and MTF1

We crossed the pseudorevertant strains to the isogenic *MATα* wild-type W303-1B/520 strain. Diploids were isolated by micromanipulation and, following sporulation, tetrads were analyzed. This was essentially a two-point cross of *suv3::URA3, su*× *SUV3*$^+$, *SU*$^+$. The *suv3*Δ spores are readily identified by their [Ura$^+$] phenotype, and the fact that the presence of the suppressor allele gives the *suv3*Δ spores ability to grow on glycerol at normal temperature facilitated the unambiguous assignment of each tetrad to one of the three classes. The ratio of PD:NPD:TT tetrads in each cross was close to 1:1:4, meaning that the suppressors were monogenic and not linked to the *SUV3* locus or to the centromere.

In the NPD tetrads from the crosses described above, two spores carry the *suv3*Δ allele with the wild-type allele of the suppressor (*SU1*$^+$ or *SU2*$^+$) and have the [Ura$^+$ Gly$^-$] phenotype, whereas the other two are *SUV3*$^+$ and carry the suppressor mutation. Two such NPD tetrads, DSW1-16 and DSW3-12, with the *su1* and *su2* mutations, respectively, were selected for further phenotypic analysis along with two TT tetrads DSW1-2 and DSW3-1. The spores were tested for uracil auxotrophy (as a marker of the *suv3::URA3* disruption) and for growth on glucose (YPD) and glycerol (YPG) media both at normal and elevated (36°C) temperature. Presence of the *suv3::URA3* disruption was additionally verified by PCR using primers 9090 and 9091. The results are shown in Figure 2. The *suv3*Δ, *SU*$^+$ spores are [Ura$^+$] and [Gly$^-$] at both temperatures, as expected. The [Ura$^-$] *SUV3*$^+$, *su* spores are [Gly$^+$] at the normal temperature, although their growth seems to be slower in comparison with a wild-type control. However, at the elevated temperature these spores become strictly [Gly$^-$]. This means that both suppressor mutations give a temperature-sensitive respiratory-deficient phenotype in the context of the wild-type *SUV3*$^+$ allele. In fact, the respiratory phenotype of these mutant strains is similar to that of the original pseudorevertant strain, regardless of the presence of the functional *SUV3* allele. The loss of respiratory function at the elevated temperature is irreversible, mutant cultures grown overnight in liquid YPD at 36°C do not recover a [Gly$^+$] phenotype upon subsequent transfer to 30°C, presumably because they lose mitochondrial DNA.

This temperature-sensitive respiratory-deficient phenotype was used to design a screen for cloning of the suppressor genes. Strain DSW3–12/A was transformed with a wild-type *S. cerevisiae* genomic library in the pRS200 (ARS-CEN,*TRP1*) plasmid vector (see *Materials and Methods*). The [Trp$^+$] transformants were screened for the ability to grow on YPG (glycerol) at 36°C. Ten such colonies were picked for further characterization and were all found to contain the same plasmid clone with an 8.4-kb insert corresponding to a fragment of chromosome XIII and covering two ORFs—*RRP5* (YMR229C) and *MTF1* (YMR228W). A 1.5-kb *Sac*I-*Eco*RI fragment containing

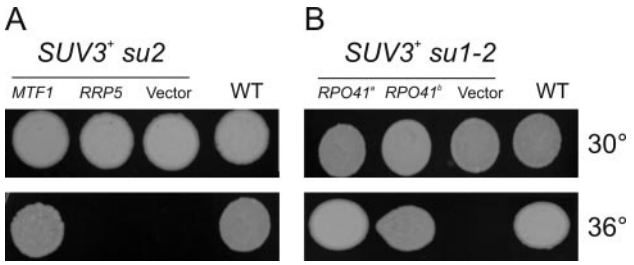

Figure 3. Rescue of the wild-type (WT) respiratory growth on YPG at 36°C in *SUV3*$^+$ strains carrying *su1* and *su2* mutations by low copy number plasmids carrying wild-type alleles of the respective genes. (A) Respiratory growth at 36°C in the *SUV3*$^+$ *su2* strain DSW3−12A is restored by pMTF1 carrying the *MTF1* gene, but not by pRRP5 or the empty vector (YCpLac111), to a level observed in the isogenic wild-type control W303/A/520 (WT). (B) Both plasmids carrying the wild-type *RPO41* gene on [a]YCpLac33 (pJJ1148) and [b]YCpLac111 (pJJ1149), but not the empty vector (YCplac111) restore WT growth at 36°C in the *SUV3*$^+$ *su1* strain DSW1-16/C.

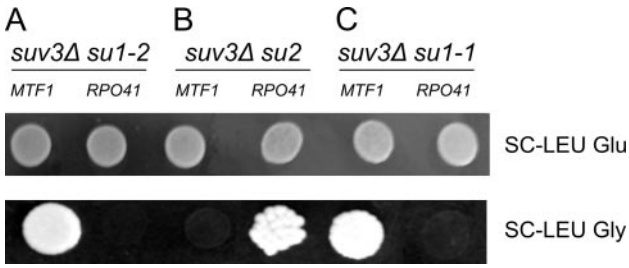

Figure 4. Plasmid-borne wild-type alleles of *MTF1* or *RPO41* genes reverse the recessive suppression in *suv3*Δ pseudorevertant strains with *su1* and *su2* suppressor mutations. The suppressor phenotype was tested on synthetic complete (SC –leucine) media with a respiratory carbon source (glycerol). (A) Wild-type *RPO41* (pJJ1149), but not *MTF1* (pMTF1), reverses the suppressor mutation phenotype in the SUX1 strain carrying the *su1-2* mutation. (B) Wild-type *MTF1* (pMTF1), but not *RPO41* (pJJ1149), reverses the suppressor mutation phenotype in the SUX3 strain carrying the *su2* mutation. (C) Wild-type *RPO41* (pJJ1149), but not *MTF1* (pMTF1), reverses the suppressor mutation phenotype in the SDB9 strain carrying the *su1-1* (*suB9*) mutation. Dilutions (10^{-1}) of a saturated preculture were incubated on plates for 5 d.

the *MTF1* ORF and a 6.9-kb *Bam*HI-*Eco*RI fragment containing the *RRP5* ORF were subcloned into the YCplac111 (ARS-CEN, *LEU2*) plasmid vector to yield pMTF1 and pRRP5, respectively. Transformation of the DSW3-12/A strain with the pRRP5 plasmid did not restore respiratory growth at 36°C, whereas transformation with the pMTF1 plasmid restored wild-type respiratory growth at either temperature (Figure 3A). This indicates that the *su2* mutation in DSW3-12/A occurred in the *MTF1* gene, which encodes the transcription factor of the mitochondrial RNA polymerase (Schinkel *et al*., 1987; Jang and Jaehning, 1991). Temperature-sensitive respiratory-deficient mutants with defects in *MTF1* had been previously described, along with similar mutations in the core mitochondrial RNA polymerase gene *RPO41* (Shadel and Clayton, 1995; Cliften *et al*., 1997, 2000; Karlok *et al*., 2002; Matsunaga and Jaehning, 2004). We decided therefore to verify directly whether the other suppressor mutation, *su1*, corresponded to the *RPO41* gene. The DSW1-16/C strain, carrying the *su1* mutation, was transformed with plasmids pJJ1148 or pJJ1149 (Cliften *et al*., 2000) containing the wild-type *RPO41* gene cloned in the YCplac33 (ARS-CEN, *URA3*) and YCplac111 vectors, respectively. All the obtained transformants were [Gly$^+$] at both 30 and 36°C (Figure 3B), indicating that the *su1* mutation occurred in the *RPO41* gene. Passage of the DSW1-16/C/pJJ1148 transformant on fluoroorotic acid media led to the loss of respiratory growth at 36°C concomitant with the loss of uracil prototrophy, which further strengthened the evidence for the association of the *su1* suppressor with a mutation in the *RPO41* gene.

To further verify the assignment of *su1* and *su2* suppressors to mutations in *RPO41* and *MTF1*, we transformed the original pseudorevertant strains (*suv3*Δ, *su*) with plasmids pJJ1149 and pMTF1, carrying wild-type alleles of the respective genes. Because the suppressor alleles were shown to be recessive, introduction of the wild-type allele of the cognate gene should reverse the suppression and revert the transformant to respiratory deficiency at normal temperature. The transformants were tested on synthetic complete media (SC-leucine) with glycerol. The results (Figure 4) indicate that transforming the SUX1 strain with pJJ1149 (*RPO41*) and transforming the SUX3 strain with pMTF1 results in reversion of the suppressor phenotype, indicating that the plasmid-borne genes are indeed wild-type alleles of the suppressors. The original suppressor strain SDB9 (Stepien *et al*., 1995) transformed with pJJ149 also reverts to the [Gly$^-$] phenotype, which demonstrates that the *suB9* mutation therein occurred in the *RPO41* gene.

Because transformation of the pseudorevertant strain with plasmids carrying wild-type alleles of *RPO41* or *MTF1* is a quick way of determining the nature of suppression, we applied this strategy to a batch of eight additional suppressors isolated in the initial screen and assigned them to mutations in either *RPO41* (5 colonies) or *MTF1* (3 colonies). Similarly, spontaneous suppressors obtained in the SUD1 strain (carrying the *KanMX4* deletion of the entire *SUV3* ORF) were assigned to either of these two genes using this strategy.

This suggests that spontaneous [Gly$^+$] suppressors arising in various *suv3*Δ strains carrying the intronless mitochondrial genome can be attributed to temperature-sensitive partial loss-of-function mutations in the *RPO41* and *MTF1* genes, encoding the two subunits of the mitochondrial RNA polymerase. The suppression occurs both with partial *URA3* disruption and with total *KanMX4* deletion alleles of *suv3*Δ

The Suppressor Mutations Are Novel Single Amino Acid Substitutions in RPO41 and MTF1

We selected the suppressor mutation in the *MTF1* gene contained in the SUX3 strain, and two suppressors attributed to mutations in *RPO41* (the SUX1 strain and the original SDB9 suppressor) for sequencing analysis. Fragments of genomic DNA corresponding to *MTF1* and *RPO41* genes were amplified and sequenced using primers described in *Materials and Methods*. Wild-type isogenic strains W303/A/520 and BWG1 were used as respective controls.

The SUX1 pseudorevertant strain was found to carry a single nucleotide substitution A1628G in the *RPO41* ORF (all numbers start from the A in the initiation codon of the ORF), corresponding to the E543G substitution in the translated amino acid sequence. The SDB9 strain, compared with the parental strain BWG1, contained the G2932T substitution in the *RPO41* gene, resulting in the V978F mutation in the amino acid sequence. Neither of these mutations has been previously described, they are both located in regions of the Rpo41p sequence displaying homology to the T3 and T7 phage RNA polymerases (Masters *et al*., 1987).

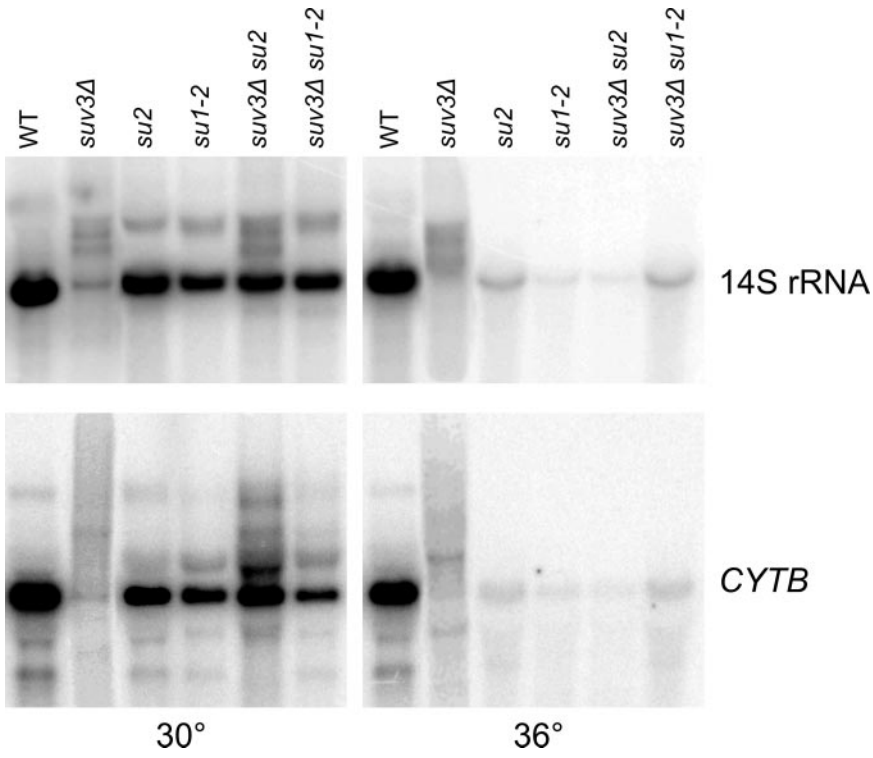

Figure 5. Northern blot analysis of steady-state levels of mitochondrial transcripts of the 14S rRNA and *CYTB* genes in wild-type W303/A/520 (WT), *suv3*Δ (WSU0 Δi), *su2* mutant in the *MTF1* gene (DSW3-12/A), *su1-2* mutant in the *RPO41* gene (DSW1-16/C), and pseudorevertant *suv3*Δ *su2* (SUX3) and *suv3*Δ *su1-2* (SUX1) strains. RNA was isolated from purified mitochondria, blotted as described in *Materials and Methods*, and hybridized with the 14S or *CYTB* oligonucleotide probes. Protein content of each original mitochondrial preparation was used to normalize RNA loading in each lane.

The *MTF1* gene in the SUX3 pseudorevertant, compared with the parental W303/A/520 strain contains a single mutation, C908T, resulting in a P303L substitution in the C-terminal region of the Mtf1p protein. The majority of the temperature-sensitive mutations in the *MTF1* gene known so far were located within or close to the regions displaying homology to the σ family of bacterial transcription factors (Shadel and Clayton, 1995; Cliften *et al*., 1997). Deletion of the C-terminal domain, beyond residue 291, resulted in a temperature-sensitive petite phenotype (Shadel and Clayton, 1995), corresponding to the effect of the P303L substitution observed in the DSW3-12/A spore.

Mutations in the RPO41 and MTF1 Genes Partially Suppressing the suv3Δ [Gly⁻] Phenotype Decrease the Transcription Rate of the Mitochondrial RNA Polymerase

Mutations in the mitochondrial RNA polymerase genes resulting in a similar, temperature-sensitive petite phenotype described previously (Shadel and Clayton, 1995; Cliften *et al*., 1997, 2000; Karlok *et al*., 2002; Matsunaga and Jaehning, 2004) resulted in a general slowing of mitochondrial transcription and a decrease in the abundance of mature RNAs. As the phenotype of the *suv3*Δ mutation involves dysfunction of mitochondrial RNA degradation and turnover (Golik *et al*., 1995; Stepien *et al*., 1995; Dziembowski *et al*., 2003), it was tempting to speculate that the suppressor mutations we discovered acted through lowering the transcription rate and reducing the RNA content in the organelle.

To verify this hypothesis, first we measured the steady-state levels of selected mitochondrial RNA transcripts by Northern hybridization. RNA obtained from purified mitochondria from the wild-type W303/A/520 (WT), *suv3*Δ (WSU0 Δi), *su2* mutant in the *MTF1* gene (DSW3-12/A), *su1-2* mutant in the *RPO41* gene (DSW1–16/C), and pseudorevertant *suv3*Δ *su2* (SUX3) and *suv3*Δ *su1-2* (SUX1) strains, grown at normal (30°C) and elevated (36°C) temperatures was separated on an agarose-formaldehyde gel and hybridized to 14S (SSU) rRNA and *CYTB* probes. The results are shown in Figure 5. As expected, the *suv3*Δ strain displays severe RNA processing abnormalities, resulting in very low levels of mature transcripts and accumulation of precursor species, including undefined high-molecular-weight smears as well as precursor bands. Strains carrying the suppressor mutations in *MTF1* (*su2*) and *RPO41* (*su1-2*) in the context of the wild-type *SUV3* allele exhibit a marked decrease in the steady-state levels of 14S and *CYTB* mature transcripts (to ~33 and 20% of the wild-type level, respectively). In the pseudorevertant strains combining the *suv3*Δ deletion with the suppressor mutations, the steady-state levels of mature transcripts are restored to the levels similar to those observed in strains carrying the suppressor mutations alone, thus explaining how these mutations can partially restore respiratory competence in the *suv3*Δ background. Some accumulation of precursors is still observed, in particular in the *suv3*Δ *su2* strain, but the proportion of mature transcript to precursor is much more favorable than in the *suv3*Δ strain.

At the elevated temperature the two mitochondrial transcripts in all strains carrying the suppressor mutations were present at extremely low levels, while in the wild-type control their level was still normal. These results correlate with the previously observed phenotype of slower growth on respiratory substrates at normal temperature, and complete respiratory-deficiency at the elevated temperature, discussed above. Interestingly, the effect of the *su2* mutation in the *MTF1* gene on mitochondrial transcription is less pronounced than that of the *su1* mutation in the *RPO41* gene, which correlates with the increased accumulation of residual RNA precursors in the *suv3*Δ *su2* strain compared with the *suv3*Δ *su1* strain and slightly slower respiratory growth.

To verify whether the lower abundance of the mitochondrial transcripts observed in the suppressor mutant strains was indeed related to a decrease in transcription, we mea-

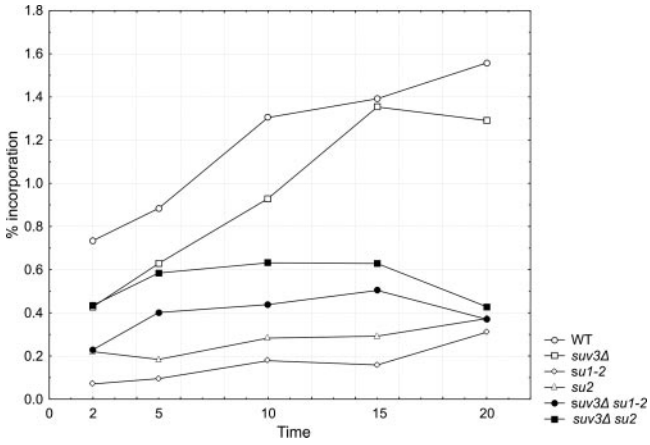

Figure 6. In organello transcription assay measuring labeled [α-^{32}P]UTP incorporation into purified mitochondria of the wild-type W303/A/520 (WT), *suv3*Δ (WSU0 Δi), *su2* mutant in the *MTF1* gene (DSW3-12/A), *su1-2* mutant in the *RPO41* gene (DSW1-16/C), and pseudorevertant *suv3*Δ *su2* (SUX3) and *suv3*Δ *su1-2* (SUX1) strains. Typical results of one from at least three independent experiments are shown for each strain as percent incorporation.

sured the mitochondrial transcription rates of the mutant strains using an in organello [α-^{32}P]UTP incorporation assay (Krause and Dieckmann, 2004). Mitochondria from wild-type W303/A/520 (WT), *suv3*Δ (WSU0 Δi), *su2* mutant in the *MTF1* gene (DSW3-12/A), *su1-2* mutant in the *RPO41* gene (DSW1-16/C), and pseudorevertant *suv3*Δ *su2* (SUX3) and *suv3*Δ *su1-2* (SUX1) strains, grown at a normal temperature to mid-log phase, were purified by differential centrifugation and incubated in transcription buffer containing [α-^{32}P]UTP and an RNase inhibitor (see *Materials and Methods*). The results, shown in Figure 6, demonstrate that although the wild-type strain shows typical rapid kinetics of the labeled precursor incorporation, the incorporation in the *su* mutant strains is much slower and never reaches wild-type levels, even upon prolonged incubation. Transcription in the *suv3*Δ strain is slightly lower than in the wild type, which may be related to the increased frequency of *rho*$^-$/*rho*^{0} petites. Pseudorevertant strains *suv3*Δ *su2* and *suv3*Δ *su1-2* show slight improvement over the strains containing the suppressor mutations alone, which may be explained by the reduced RNA degradation activity in *suv3*Δ mitochondria. At 10 min of incubation, the incorporation in *su1-2* (*rpo41*) and *su2* (*mtf1*) mutants is ~10 and 18% of the wild-type value, respectively.

We observed the same trend in several independent repetitions of the experiment, although absolute incorporation values were variable and the incorporation levels of the mutant strains were close to the sensitivity limit of the assay, which made a more rigorous statistical treatment of the quantitative results impossible. Positive incorporation in the mutant strains becomes more obvious when more than 5 μg mitochondrial preparation per reaction is used in the experiment; in such case, however, the wild-type preparation becomes overloaded and gives maximum signal already at the first time point.

These results demonstrate that the suppressor mutations in the *RPO41* and *MTF1* genes do indeed act by drastically slowing down the transcription rate in mitochondria, as demonstrated by the in organello incorporation assay. The effect on the steady-state level of mature RNAs seems slightly less pronounced, the two methods are, however, not directly comparable. Besides, more factors than just the transcription rate influence the steady-state level of transcripts in vivo which might explain the more moderate effect seen in the Northern hybridization assay. Although the effect of the suppressor mutations on transcription might seem drastic, respiratory competence of yeast mutants having <10% of the wild-type level of a mature mitochondrial transcript has been reported previously (Schmidt *et al*., 1998). In the *suv3*Δ background, decreased transcription rates result in partial restoration of mature RNAs and decrease in the accumulation of precursors, so that the mature transcripts become the dominant form again.

DISCUSSION

The RNA helicase encoded by the *SUV3* gene is a critical component of the yeast mitochondrial degradosome (Dziembowski *et al*., 2003) and is essential for the expression and maintenance of the mitochondrial genome. A prominent feature of *SUV3*-deficient yeast strains is the accumulation of aberrant RNA species. Some of them can be attributed to unprocessed intronic sequences (Stepien *et al*., 1995), but the abnormal accumulation of high-molecular-weight mitochondrial RNAs is also observed in the intronless strain (Dziembowski *et al*., 1998, 2003). Because some mature RNAs are still present in intronless mitochondria in degradosome-deficient strains, it has been suggested that impaired degradation of aberrant and unprocessed transcripts, rather than a block in the process of RNA maturation, is responsible for the molecular phenotype of *SUV3* or *DSS1*/*MSU1* deletion. In this model, the mitochondrial degradosome is responsible for the RNA surveillance function in the organelle.

The burden of aberrant and unprocessed RNA species could have a detrimental effect on the functioning of the mitochondrial genetic system. Accumulation of undegraded RNAs could disrupt the assembly of mitochondrial RNP complexes, and perturb the ribonucleotide pools. In particular, assembly and functioning of the mitochondrial ribosomes could be affected, because the accumulation of rRNA and *VAR1* precursors has been observed in degradosome-deficient strains (Dziembowski *et al*., 1998, 2003). Perturbations in mitochondrial translation are known to affect the stability of mitochondrial DNA (Myers *et al*., 1985), which would explain the elevated frequency of mitochondrial petites observed in *suv3*Δ strains (Stepien *et al*., 1992, 1995; Golik *et al*., 1995). Defects in the quality control of ribosomal RNAs leading to defects in ribosome maturation were found to cause lethality in *Escherichia coli* strains devoid of the two major exoribonucleases, RNase R and polynucleotide phosphorylase (Cheng and Deutscher, 2003). Although the polynucleotide phosphorylase has been found in human mitochondria (Piwowarski *et al*., 2003), it is conspicuously absent from the mitochondria of *S. cerevisiae*. The mtEXO complex is therefore the sole major exoribonuclease of yeast mitochondria and its inactivation could cause disturbances similar in extent to those observed in bacterial cells lacking the two major RNase enzymes.

In this study, we demonstrated that spontaneous suppressor mutations partially rescuing the functioning of the mitochondrial genetic system in degradosome-deficient *suv3*Δ strains occur in the *MTF1* (YMR228W) and *RPO41* (YFL036W) genes encoding the two subunits of the mitochondrial RNA polymerase (Kelly *et al*., 1986; Masters *et al*., 1987; Jang and Jaehning, 1991; Ulery and Jaehning, 1994). They are temperature-sensitive, recessive, partial loss-of-function (hypomorphic) mutations that reduce the transcrip-

tion rate at normal temperature and nearly abolish the mitochondrial RNA polymerase function at elevated temperature. At 30°C, mitochondrial transcription in mutant strains is <18% of wild-type level (as determined by nucleotide incorporation assay in organello), which also leads to reduced levels of mature transcripts (as detected by Northern blotting). It is also worth noting that the system we describe allows for positive selection of hypomorphic mutants in the RNA polymerase genes, which could be beneficial for studying the structure-function relationships in the RNAP protein subunits.

Partial rescue of the mitochondrial genetic function in the suppressor strains is linked to this decrease of mitochondrial transcription. Cells deficient in the mtEXO function cannot provide sufficient RNA degradation activity to cope with the production of unprocessed and aberrant RNAs associated with normal transcription rates. The pseudorevertant strains, with their reduced transcription rate, produce less RNA, including less unprocessed and/or aberrant transcripts, so that the mitochondrial genetic system can continue to function, albeit at a visibly lower level that translates to slower respiratory growth. In this model, decrease of the transcription rate caused by the suppressor mutations restores, at least partially, the balance between RNA synthesis and degradation disrupted by the genetic inactivation of the degradosome function. On the molecular level, this is manifested by shifting of the mature RNA/precursor ratios toward normal values, allowing sufficient expression of mitochondrially encoded genes. Although some overaccumulation of precursors is still observed in degradosome-deficient strains carrying the suppressor mutations, mature RNAs are the dominant form, like in wild-type mitochondria and unlike in the degradosome-deficient organelles.

It is possible that with the diminished transcriptional activity of suppressor mutants, the aberrant RNA species accumulate at a level that is so low that even without their removal, normally ensured by the degradosome, they do not interfere with mitochondrial function. Most likely, however, some residual exoribonuclease activity is responsible for the degradation of low levels of transcripts in suppressor strains. This activity could be provided by the RNase subunit of the mitochondrial degradosome encoded by the *DSS1/MSU1* gene, probably in association with another RNA helicase. An obvious candidate would be the Mss1p mitochondrial RNA helicase (Seraphin *et al*., 1989), which can partially rescue mitochondrial gene expression in *suv3*Δ strains when overexpressed from a multicopy plasmid (Minczuk *et al*., 2002).

Another explanation would postulate the existence of another exoribonuclease in yeast mitochondria, which alone cannot provide sufficient degradation activity to provide the RNA turnover and surveillance function with the normal transcript levels produced by the wild-type RNA polymerase, but it can nevertheless cope with the severely reduced RNA quantities in the suppressor mutants. In *E. coli*, there are eight 3′-to-5′ exoribonucleases (Deutscher and Li, 2001). Three of these, RNase II, RNase R, and polynucleotide phosphorylase, provide nonspecific processive activity required for the degradation of transcripts (Zuo and Deutscher, 2001; Cheng and Deutscher, 2005). The RNase component of the mitochondrial degradosome encoded by the *DSS1/MSU1* gene remains, however, the only yeast processive 3′-to-5′ exoribonuclease found in mitochondria. The protein encoded by the *NUC1* gene (Zassenhaus *et al*., 1988), once thought to be the main mitochondrial nuclease, was found not to be involved in RNA degradation (Dziembowski *et al*., 1998). The Ynt20p (Rex2p) 3′-to-5′ mitochondrial ribonuclease (Hanekamp and Thorsness, 1999) has a distributive activity, similar to the bacterial oligoribonuclease (Zuo and Deutscher, 2001) and is involved in the maturation of small RNAs (van Hoof *et al*., 2000). The activity of Ynt20p family nucleases seems to be limited to degradation of very short single-stranded oligonucleotides (Nguyen *et al*., 2000). None of the known proteins can be therefore considered as a candidate for the putative secondary 3′-5′ RNA degradation activity in yeast mitochondria that could supplement the mtEXO complex. Although this does not rule out the existence of such activity, no obvious candidate genes are apparent in the genome of *S. cerevisiae*. Alternatively, the 5′ end-directed degradation activity mediated by the product of the *PET127* gene (Krause and Dieckmann, 2004) could compensate for the lack of the 3′-to-5′ degradosome activity.

One of the particular characteristics of the mitochondrial genetic system is the close association between different levels of gene expression. RNA processing and translation have been shown to be physically coupled to transcription through a network of protein–protein and protein–RNA interactions (Rodeheffer *et al*., 2001; Bryan *et al*., 2002; Krause *et al*., 2004). Because the mitochondrial degradosome is associated with the ribosome (Dziembowski *et al*., 2003), it could also be a part of this network, offering another explanation for the genetic interaction between transcription and RNA degradation uncovered in this work.

The results of this study underscore the essential character of RNA degradation in the expression of genetic information. Mitochondria, with their simplified transcriptional control seem to be particularly dependent on posttranscriptional processes, including RNA degradation and surveillance. Our results clearly demonstrate that maintaining the balance between RNA synthesis and degradation is crucial to the functioning of the mitochondrial genetic system. Although the enzymatic machinery of mitochondrial gene expression shows considerable evolutionary divergence among phyla and is very different in higher eukaryotes in comparison with *S. cerevisiae*, a common theme of very simple transcription and complex posttranscriptional processing of RNAs is shared in mitochondria from yeasts to human. Understanding the mechanisms that maintain balance between the RNA synthesis and degradation is therefore a fundamental area of mitochondrial science.

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