Mitochondrial Translation: Elongation Factor Tu Is Essential in Fission Yeast and Depends on an Exchange Factor Conserved in Humans but Not in Budding Yeast

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Manuscript received October 20, 2004

Accepted for publication January 4, 2005

ABSTRACT

The translation elongation factor EF-Tu is a GTPase that delivers amino-acylated tRNAs to the ribosome during the elongation step of translation. EF-Tu/GDP is recycled by the guanine nucleotide exchange factor EF-Ts. Whereas EF-Ts is lacking in *S. cerevisiae*, both translation factors are found in *S. pombe* and *H. sapiens* mitochondria, consistent with the known similarity between fission yeast and human cell mitochondrial physiology. We constructed yeast mutants lacking these elongation factors. We show that mitochondrial translation is vital for *S. pombe*, as it is for human cells. In a genetic background allowing the loss of mitochondrial functions, a block in mitochondrial translation in *S. pombe* leads to a major depletion of mtDNA. The relationships between EF-Ts and EF-Tu from both yeasts and humans were investigated through functional complementation and coexpression experiments and by a search for suppressors of the absence of the *S. pombe* EF-Ts. We find that *S. cerevisiae* EF-Tu is functionally equivalent to the *S. pombe* EF-Tu/EF-Ts couple. Point mutations in the *S. pombe* EF-Tu can render it independent of its exchange factor, thereby mimicking the situation in *S. cerevisiae*.

MITOCHONDRIA are organelles specialized in the production of energy via the respiratory chain, which is a series of enzymatic complexes of dual origin. Several of these complexes contain, in addition to nuclear-encoded subunits, a few subunits encoded by the mitochondrial genome (mtDNA). The mtDNA also encodes all the RNAs (rRNAs and tRNAs), but at most only one protein component of the mitochondrial translation machinery. Consequently, most of the ribosomal proteins, factors involved in ribosome assembly, tRNA synthetases, and general or specific translation factors mediating the initiation, elongation, and termination steps of mitochondrial translation are encoded in the nucleus, synthesized in the cytoplasm, and imported into mitochondria.

The elongation step of mitochondrial translation, best documented in mammals, closely mimics the prokaryotic system (see SPREMULLI *et al.* 2004). Like its prokaryotic homolog, the mammalian EF-Tu GTPase hydrolyzes a molecule of GTP each time an amino-acylated tRNA is accommodated on the A site of the ribosome, and its recycling depends on the exchange factor EF-Ts. Another conserved GTPase, EF-G, catalyzes

the translocation step after peptide bond formation and is also involved in ribosome recycling, but is independent of an exchange factor. The budding yeast *Saccharomyces cerevisiae* has EF-Tu and EF-G orthologs (NAGATA *et al.* 1983; VAMBUTAS *et al.* 1991), but an equivalent of EF-Ts does not appear to be present, either as a biochemical activity (ROSENTHAL and BODLEY 1987) or as a homologous gene product. In *Schizosaccharomyces pombe*, the genome sequence predicts that all three factors are present.

The fission yeast *S. pombe* is strikingly different from the budding yeast *S. cerevisiae*, and in many respects mimics human cells more closely than *S. cerevisiae* does (ZHAO and LIEBERMAN 1995; FORSBURG 1999). This seems to be the case for various aspects of mitochondrial physiology. Mitochondrial distribution depends on microtubules in *S. pombe*, as in animal cells (WEIR and YAFFE 2004). Also, *S. pombe* is highly dependent on respiration and cannot survive the loss of mtDNA (*rho*⁰ mutants), unless specific nuclear mutations are present (HAFFTER and FOX 1992); in a similar way human cells cannot lose their mtDNA except in cell cultures containing specific supplements (KING and ATTARDI 1989). The *S. pombe* mtDNA closely resembles that of humans: it is very compact (19 instead of 75 kb for *S. cerevisiae*) and has a similar organization, with low intron content, limited transcriptional origins, and tRNA punctuation (SCHÄFER 2003). This suggests that transcription, RNA maturation, and translation could be similar in human and *S. pombe* mitochondria. This similarity is seen in

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general translation factors, as we were able to identify the complete human set in *S. pombe* by homology searches. Messenger-specific factors required for initiation of translation in *S. cerevisiae* seem to be absent from both the human and fission yeast nuclear genomes (COSTANZO *et al.* 2000). All these considerations have led us to use *S. pombe* as both a model and a tool for the study of mitochondrial translation.

Starting from a respiratory-deficient mutant, we found that *S. pombe* mitochondria, like human mitochondria, do have a nucleotide exchange factor EF-Ts that recycles EF-Tu. Using this couple of elongation factors, we have determined the consequences of defects in mitochondrial translation in *S. pombe*.

MATERIALS AND METHODS

Genetic techniques and strains: All strains are described in Table 1 and were grown at 28°. Media and genetic methods for *S. cerevisiae* and *S. pombe* were as reported (BONNEFOY *et al.* 1996, 2000). *S. pombe* asci were microdissected directly from the mixture of haploid, diploid, and sporulating cells. *S. pombe* transformation (OKAZAKI *et al.* 1990) was improved by (1) using single-strand salmon sperm DNA as a carrier, (2) regenerating cells in complete liquid medium overnight, and (3) plating the cells onto 5% glucose selective medium. Yeast genomic DNA was extracted as described (HOFFMAN and WINSTON 1987).

Genomic library screening: The ASD5 [Gly⁻] [Gal⁻] mutant was isolated in our lab by UV mutagenesis of the *S. pombe* strain Eg555 (a gift from R. Egel). A *his3* derivative of ASD5 (NB23-5B) was transformed with a pBG2-based genomic library (OHI *et al.* 1996) kindly provided by C. S. Hoffman. Among [His⁺] clones, a single cosegregating [Gal⁺] colony was obtained and its plasmid analyzed. The 0.9-kb complementing ORF was PCR amplified from Eg555 and ASD5 genomic DNA and the PCR products were sequenced.

Gene disruptions: For deletion of the *S. pombe tsf1* gene (*tsf1Sp*), the *Bgl*II *Hinc*II 734-bp internal fragment was replaced by either the 1.9-kb *Dra*I *ade6* gene (BONNEFOY *et al.* 2000; Figure 1B, line 5) or the 1.4-kb *KanR* gene that confers resistance to G418 (a gift from M. S. Longtine). The *S. pombe* predicted *tuf1* gene (ORF SPBC9B6.04c, called *tuf1Sp*), which contains one intron, was amplified as a 2.6-kb *Hind*III fragment and cloned into the *S. pombe* plasmid pON163 (WEILGUNY *et al.* 1991) or pKS⁻, where *KanR* was cloned into the *Eco*RI sites, creating a 1435-bp deletion. Flanking regions for recombination of the different constructs into strains NB205-6A and NB34-21A were 245 and 1329 bp for *tsf1Sp* and 812 and 283 bp for *tuf1Sp* at the 5' and 3' ends, respectively. A Δ *tuf1Sc* deletion was generated using a *KanR* PCR product with 50 bp of homology to *TUF1Sc* on each side. For all disruptions the integration was verified on both sides by PCR analysis.

Gene cloning and tagging: The *tuf1Sp* cDNA was amplified from an *S. pombe* cDNA library (LOLLIER *et al.* 1995) and cloned in the *S. cerevisiae* expression vector pFL61. The very end of the reading frame, fused to a two-glycine codon linker followed by the 6His-tag, was subcloned in the *Escherichia coli* pET3 vector and in the *S. pombe* expression vector pTG1754 (BONNEFOY *et al.* 1996) and fully complemented the Δ *tuf1Sp* mutant. A *LEU2* derivative of pTG1754 was constructed using a gap repair strategy (KELLY and HOFFMAN 2002) and used to express *tuf1Sp*. An intron-free V146I *tuf1Sp* plasmid was obtained in *S. cerevisiae* by gap repair of the pFL61 *tuf1Sp* plasmid digested

with *Ava*I and *Msc*I, with a PCR-amplified V146I *tuf1Sp* fragment. The *S. cerevisiae TUF1* gene (*TUF1Sc*), encoding EF-Tu_{sc}, was PCR amplified from genomic DNA and cloned in pFL61. The genes encoding both human elongation factors were amplified from a cDNA library (MINET *et al.* 1992) and subcloned into plasmids carrying the yeast factors to fuse the yeast targeting sequences to the human proteins. All constructs were verified by sequencing.

Suppressors of the *tsf1Sp* deletion: Since the *ura4* mutation decreases growth under nonfermentable conditions (our unpublished observations), the Δ *tsf1Sp::KanR* strain NB222 was transformed with a *ura4* empty vector before plating cells from 10 independent subclones on galactose medium to look for spontaneous reversion events. Such revertants appearing between 8 and 13 days were subcloned before testing the galactose and ethanol/glycerol growth at different temperatures to constitute phenotypic classes. At least one member from each class was crossed with a Δ *tsf1Sp::ade6* strain. All tested mutations segregated 2:2, some showing a linkage with the mating-type locus 293 kb away from *tuf1Sp* on chromosome 2. The *tuf1Sp* gene from these revertants and from others that had not been analyzed genetically was PCR amplified and fully sequenced. Revertants with a wild-type *tuf1Sp* gene represented at least two other complementation groups, but were not studied further.

S³⁵-labeling and cytochrome spectra: Mitochondrial proteins were labeled by a 3-hr incubation of whole cells with [³⁵S]methionine in the presence of 6 mg/ml cycloheximide, which specifically blocks cytoplasmic translation. Samples were run on a 16% acrylamide-0.5% bisacrylamide SDS gel, and the dried gel was exposed for 2 months up to 1 year at -70°. Low-temperature cytochrome spectra from *S. pombe* cell paste were recorded using a Cary 400 spectrophotometer after addition of sodium dithionite to fully reduce the cytochromes (CLAISSE *et al.* 1970). The absorption maxima were 603, 560, 554, and 548 nm for cytochromes *aa3*, *b*, *c1*, and *c*, respectively. The *S. pombe* cytochrome *c* peak always shows a 544-nm shoulder that disappears in a cytochrome *c* mutant (N. BONNEFOY, unpublished results).

Protein analysis and antibodies: Mitochondria were purified from *S. pombe* cells (BONNEFOY *et al.* 2000) grown in complete or minimal glucose medium. Total yeast proteins were extracted from exponential cultures (YAFFE 1991). Samples were run on 10 or 12% SDS-PAGE before Western blotting. Primary antibodies were anti-tetraHis: 1/1500, QIAGEN (Chatsworth, CA); anti-Arg8p: 1/4000, a gift from T. D. Fox (STEELE *et al.* 1996); anti-*S. pombe* Cox2p: 1/5000 (M. GAISNE and N. BONNEFOY, unpublished results). The 6-His-tagged EF-Tu_{sp} produced in *E. coli* was insoluble under all conditions tested and sedimented as the main pellet component, which was directly used to immunize rabbits. The serum obtained after three injections was diluted 5000-fold and specifically recognized one major 50-kD band present in purified mitochondrial or total proteins from wild-type yeast but not from Δ *tuf1Sp* cells.

RESULTS

***S. pombe* cells lacking the mitochondrial translation elongation factor EF-Ts_{sp} are respiratory deficient but viable:** Previously we showed that *S. pombe* mutants defective in respiratory chain biogenesis are unable to use either glycerol or galactose supplemented with 0.1% glucose as the sole carbon source (BONNEFOY *et al.* 1996, 2000). We isolated a collection of [Gly⁻] [Gal⁻] mutants after UV mutagenesis, one of which (ASD5) showed a

TABLE 1
S. pombe and *S. cerevisiae* strains used in this study

Name	Genotype		Origin
	Nuclear	Mitochondrial	
<i>S. pombe</i>			
Eg555	<i>h⁺ ade6-M216 leu1-32 ura4</i>	<i>rho⁺</i>	R. Egel
NB9-932	<i>h⁻ his3</i>	<i>rho⁺</i>	This study
NB205-1A	<i>h⁺ ade6-M216 leu1-32 his3Δ ura4-D18</i>	<i>rho⁺</i>	This study
NB205-6A	<i>h⁻ ade6-M216 leu1-32 his3Δ ura4-D18</i>	<i>rho⁺</i>	This study
NB208-15B	<i>h⁺ ade6-M210 leu1-32 his3Δ ura4-D18</i>	<i>rho⁺</i>	This study
SP1	<i>h⁺ ade6-M210 leu1-32 his3Δ ura4-D18</i> <i>h⁻ ade6-M216 leu1-32 his3Δ ura4-D18</i>	<i>rho⁺</i>	This study
PHP4	<i>h⁻ ade6-M210 ptp2-1</i>	<i>rho^o</i>	HAFFTER and Fox (1992)
PHP25	<i>h⁺ ade6-M216 ura1-161 ptp1-1</i>	<i>rho⁺</i>	HAFFTER and Fox (1992)
NB34-21A	<i>h⁻ ade6-M216 leu1-32 ura4 ptp1-1</i>	<i>rho⁺</i>	BONNEFOY <i>et al.</i> (2000)
ASD5	<i>h⁺ ade6-M216 leu1-32 ura4 tsf1Sp-1</i>	<i>rho⁺</i>	This study
NB23-5B	<i>h[?] leu1-32 his3 tsf1Sp-1</i>	<i>rho⁺</i>	This study
NB220	<i>h⁻ ade6-M216 leu1-32 his3Δ ura4-D18 Δtsf1Sp::ade6</i>	<i>rho⁺</i>	This study
NB222	<i>h⁻ ade6-M216 leu1-32 his3Δ ura4-D18 Δtsf1Sp::KanR</i>	<i>rho⁺</i>	This study
SP3	<i>h⁺ ade6-M210 leu1-32 his3Δ ura4-D18 Δtuf1Sp::KanR</i> <i>h⁻ ade6-M216 leu1-32 his3Δ ura4-D18 tuf1Sp</i>	<i>rho⁺</i>	This study
SP4	<i>h⁻ ade6-M216 leu1-32 ura4 ptp1-1 Δtuf1Sp::KanR</i>	<i>rho⁺d</i>	This study
SP7	<i>h⁺ ade6-M216 leu1-32 his3Δ ura4-D18 Δtsf1Sp::ade6</i>	<i>rho⁺</i>	This study
SP8	<i>h⁺ ade6-M216 leu1-32 his3Δ ura4-D18 Δtsf1Sp::ade6 + pSC3</i> <i>h⁻ ade6-M216 leu1-32 ura4 ptp1-1 Δtuf1Sp::KanR</i>	<i>rho⁺</i>	This study
<i>S. cerevisiae</i>			
CW04	<i>α ade2-1 ura3-1 his3-11,15 trp1 leu2-3,112 can1-100</i>	<i>rho⁺</i>	J. Banroques
W303-1B/A	<i>a ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100</i>	<i>rho⁺</i>	F. Lacroute
SC2	<i>α ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 Δtuf1Sc::KanR</i>	<i>rho⁻/°</i>	This study
SC5	<i>α ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 Δtuf1Sc::KanR</i> <i>a ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 TUF1Sc</i>	<i>rho⁺</i>	This study

The *S. pombe* strains NB9-932, NB205-6A, NB205-1A, and NB208-15B were generated through a series of crosses of our lab strains followed by tetrad dissection. The nature of the *his3* allele from NB9-932 is unknown, whereas the *his3Δ* allele in the three other strains was described (OHI *et al.* 1996) and is derived from strain CHP731 (a gift of C. S. Hoffman). SP1 is the diploid resulting from the cross of NB208-15B and NB205-6A. ASD5 is a UV-induced *tsf1Sp-1* point mutant derived from Eg555, and NB23-5B is a spore from the cross between ASD5 and NB9-932. NB220 and NB222 were generated by replacement of *tsf1Sp* by different markers genes in NB205-6A. SP3 and SP4 are derived from SP1 and NB34-21A, respectively, by disruption of *tuf1Sp*. SP4 contains low amounts of wild-type mtDNA (*rho⁺d*). SP7 is a spore from the cross of NB220 with NB205-1A, whereas SP8 is the transient diploid from the cross of SP4 to SP7 transformed with pSC3, which carries the *TUF1Sc* gene. The *S. cerevisiae* strains are all from the W303 background. SC2 comes from the disruption of *TUF1Sc* into CW04, and SC5 was generated by crossing SC2 with W303-1B/A.

spectral defect of the cytochromes *b* and *a + a3*, belonging to the complexes III and IV of the respiratory chain (Figure 1A). All tetrads from an ASD5-derived heterozygote showed a 2:2 cosegregation of the [Gal⁻] and spectral phenotype (Figure 1A).

The [His⁻ Gal⁻] spore, NB23-5B, was transformed with a library of *S. pombe* genomic DNA (OHI *et al.* 1996). A plasmid carrying a 2.3-kb insert containing two entire open reading frames (ORFs) from chromosome 2 restored the growth of NB23-5B on galactose medium. Subcloning or disruption of the plasmid insert showed that the complementing gene was SPBC800.07c (Figure 1B). We have called this gene *tsf1Sp*, since its predicted product is the only *S. pombe* protein showing substantial homology to both the *E. coli* and the human mitochon-

drial translation elongation factor EF-Ts (23% identity, 55% similarity, and 27% identity, and 61% similarity, respectively). Like the human protein, the predicted *S. pombe* protein contained an N-terminal extension typical of mitochondrial targeting sequences (Figure 2A). Strikingly, no homolog of EF-Ts could be identified in the *S. cerevisiae* genome by BLAST homology searches. Consistently, ROSENTHAL and BODLEY (1987) could not find any biochemical evidence for the presence of an exchange factor for the *S. cerevisiae* EF-Tu_{sc}, which was isolated almost 20 years ago (MYERS *et al.* 1985).

To demonstrate that we had cloned the wild-type gene mutated in the strain ASD5 and not a high-copy suppressor, we sequenced the *tsf1Sp* gene from ASD5 and the parent wild type. The mutant sequence contained an

FIGURE 1.—Analysis of the ASD5 mutant phenotype and identification of the corresponding wild-type gene and mutation. (A) Sister spores from the cross of ASD5 with a wild-type strain were grown on glucose before recording cytochrome spectra. Cytochrome *c*, *c1*, *b*, and *aa3* peaks are indicated. (B) The original complementing fragment (line 1), as well as deletion (lines 2–4) or disruption (line 5) derivatives were introduced into NB23-5B. Transformants were patched on glucose medium and replica plated onto galactose plates. The generic *S. pombe* ORF names are given at the top. Shaded boxes indicate ORFs, arrows show the gene orientations, and letters indicate restriction sites (S, *Sad*I; H, *Hinc*II; B, *Bgl*II). (C) The *tsf1Sp* gene nucleotide and deduced amino acid sequences in the wild-type (Eg555) and mutant (ASD5) strains are given in the vicinity of the mutation. Numbering refers to the start codon. (D) Mitochondrial translation products from a wild type (lane 1, Eg555) and two mutants (lane 2, *tsf1Sp-1* and ASD5; lane 3, Resp⁻, a respiratory mutant from our collection affected at a post-translational level) were radioactively labeled and separated by SDS-PAGE before phosphorimaging.

additional adenine in a stretch of nine A's (Figure 1C), resulting in a frameshift leading to a truncated 79-residue product instead of the wild-type 299-amino-acid protein. [Gal⁺] intragenic revertants of this mutation fell into two classes. Strong revertants had recovered the wild-type sequence by deletion of one A; weak revertants

FIGURE 2.—Complementation of the $\Delta tsf1Sp$ mutation by its human homolog. (A) The human (box with dark shading, Ts Hs) and *S. pombe* (box with light shading, Ts Sp) EF-Ts proteins present an N-terminal extension (marked with a dotted line) that is lacking in the *E. coli* factor (open box, Ts Ec). The solid boxes depict two regions of high homology that are known as the EF-Ts signature sequence. The *S. pombe* and *H. sapiens* sequences were fused at the very beginning of these signature sequences (arrows) to yield a chimera Ts Sp/Hs. (B) $\Delta tsf1Sp::KanR$ cells transformed with high-copy plasmids producing Ts Sp or Ts Sp/Hs compared to an empty vector were streaked onto complete ethanol glycerol medium. (C) The transformants from B were grown on either ethanol glycerol medium (Ts Sp, Ts Sp/Hs) or glucose (vector) before recording cytochrome spectra. Peaks as in Figure 1A.

had two additional adenine nucleotides inserted in the same stretch, restoring the reading frame by creating an additional lysine codon. We constructed disrupted versions of the *tsf1Sp* gene that were phenotypically identical to ASD5 and yielded diploid cells unable to use galactose when crossed to ASD5. This confirmed that the gene mutated in strain ASD5 is indeed SPBC800.07c, encoding EF-Ts_{Sp}, a homolog of the prokaryotic EF-Ts translation elongation factor, which would function in *S. pombe* mitochondria.

Mitochondrial translation products were labeled in

mutant and wild-type strains with [S^{35}]methionine in the presence of cycloheximide, which blocks cytoplasmic translation. Mutation of *tsf1Sp* dramatically decreased the labeling of all mitochondrial translation products, whereas another respiratory mutant generated substantial amounts of labeled proteins (Figure 1D). However, after a very long exposure, faint bands corresponding to the expected size for mitochondrial translation products were observed in the *tsf1Sp* mutant, suggesting that a low residual level of translation can still occur in mitochondria when *tsf1Sp* is mutated. These results support the idea that the cloned gene indeed encodes a translation factor.

The *tsf1Sp* gene encodes a functional homolog of human EF-Ts: To determine whether the sequence homology between EF-Ts_{sp} and its human counterpart reflected a functional conservation, we tested whether the human gene could complement the Δ *tsf1Sp* mutant. Since human mitochondrial targeting sequences are not always efficiently recognized by the yeast import machinery, we directly fused the first 42 residues of the fission yeast protein, specifying the targeting sequence, to residue 56 of the human protein (Figure 2A). This chimeric human gene, controlled by the endogenous *tsf1Sp* promoter, was introduced into the Δ *tsf1Sp* mutant and conferred strong growth on ethanol/glycerol, like the *bona fide* *S. pombe* *tsf1Sp* gene (Figure 2B). Similarly, both the *S. pombe* and human gene restored wild-type cytochrome spectra (Figure 2C).

Thus, under our expression conditions, the human EF-Ts exchange factor is able to replace its *S. pombe* counterpart to give wild-type growth and cytochrome spectra, suggesting that it can interact efficiently with the fission yeast EF-Tu_{sp} GTPase. In contrast, we found that a human EF-Tu protein fused to the *S. pombe* or *S. cerevisiae* EF-Tu targeting sequence and coproduced with its exchange factor could replace neither the *S. pombe* nor the *S. cerevisiae* homolog, suggesting that the human EF-Tu factor might not be able to interact with the required partners to deliver amino-acylated tRNAs to yeast mitochondrial ribosomes.

Absence of the EF-Tu_{sp} GTPase is lethal in *S. pombe*: Unlike the exchange factor EF-Ts, the GTPase EF-Tu is a central player in translation, which seems ubiquitous in mitochondria. A deleted version of the *tuf1Sp* gene was constructed on a plasmid using the G418 resistance marker and was tentatively integrated into both haploid and diploid *S. pombe* strains. Homologous [G418^R] recombinants could be isolated only in a diploid strain and yielded only [G418^S] spores. Thus, the loss of EF-Tu_{sp} is lethal in *S. pombe* whereas the lack of EF-Ts_{sp} is viable.

S. pombe is a petite-negative yeast, so the loss of mtDNA is lethal. CHEN and CLARK-WALKER (2000) had proposed that wild-type *S. pombe* cells cannot simultaneously lose the mitochondrially encoded subunits of the electron transport chain and the ATP synthase complex,

since one or the other of these complexes is required to generate the mitochondrial membrane potential essential for life. Thus, a stringent translational arrest in mitochondria is expected to have similar consequences to the complete loss of mitochondrial DNA. Because mutations in two as-yet-uncharacterized genes, *ptp1* and *ptp2*, allow *S. pombe* to tolerate the loss of its mtDNA (HAFFTER and FOX 1992; CHEN and CLARK-WALKER 2000), we reasoned that such mutants might also tolerate a complete block of mitochondrial translation. We used a haploid *ptp1-1* strain as recipient for the *tuf1Sp* disruption and could indeed isolate Δ *tuf1Sp::KanR* clones. However, these cells not only were [Gal⁻], but also grew very poorly on complete glucose plates and flocculated upon growth in liquid glucose medium (Figure 3A and data not shown). These phenotypes were reminiscent of *rho*^o derivatives of the *ptp* mutants. There was a dramatic decrease in all cytochromes, as observed for *rho*^o cells (Figure 3B). Thus, the consequences of a complete block of mitochondrial translation are similar to the effect of the loss of mtDNA, raising the question of the state of the mtDNA in the Δ *tuf1Sp* mutants.

Mitochondrial DNA is drastically depleted when EF-Tu_{sp} is absent: For unknown reasons, *S. cerevisiae* cells defective in general mitochondrial translation, such as strains lacking EF-Tu_{sc}, rapidly accumulate deleted molecules of mtDNA or even completely lose their mtDNA (MYERS *et al.* 1985; CHEN and CLARK-WALKER 2000; CONTAMINE and PICARD 2000).

To determine the fate of mtDNA in the absence of EF-Tu_{sp}, we first stained DNA molecules with DAPI in several independent *ptp1-1* Δ *tuf1Sp* transformants, but could detect only punctate structures characteristic of mtDNA in control strains (not shown). However, several mtDNA fragments could be amplified by PCR from whole genomic DNA of the Δ *tuf1Sp* clones, suggesting that at least some regions of mtDNA were still present. Southern blot analysis of genomic DNAs with the entire *S. pombe* mtDNA as a probe showed that in the Δ *tuf1Sp* clones, a dramatic mtDNA loss had occurred (lanes 3–6, Figure 3C), compared to the wild-type samples (lanes 2 and 7), which can eventually lead to a complete loss (lane 3), without producing detectable levels of rearranged mtDNA molecules. The depletion reflected the state of a homogenous cell population since subclones obtained after streaking the Δ *tuf1Sp* cells all contained low amounts of mtDNA. This 1–2% of residual mtDNA present in the Δ *tuf1Sp* clones showed a strictly wild-type profile.

To test whether this residual amount of mtDNA could still be functional upon recovery of the *tuf1Sp* gene, a series of *ptp1-1* clones deleted for *tuf1Sp* were crossed to a *rho*^o *ptp2-1* strain. Diploids were [Gal⁺], showing that the remaining low level of mtDNA present in the Δ *tuf1Sp* mutant was intact and could be reamplified to sustain respiration when EF-Tu_{sp} was present. We found that Δ *tsf1Sp* cells contained a wild-type amount of mtDNA

FIGURE 3.—Effects of EF-Tu_{Sp} absence on growth, cytochrome spectra, and mtDNA. (A) Serial dilutions of the mutant SP4 (bottom, *ptp1-1 Δtuf1Sp*) and its isogenic wild type NB34-21A (top, *ptp1-1*) were spotted onto media containing 5% glucose, 2% glucose supplemented with G418, or 2% galactose and 0.1% glucose. (B) Cells were grown on glucose before recording cytochrome absorption spectra. The *ptp2-1 rho*⁰ strain is PHP4 (HAFFTER and FOX 1992), other strains as in A, and peaks as in Figure 1A. (C) Caesium-chloride-purified wild-type mtDNA (lane 1; a gift of G. D. Clark-Walker) and total genomic DNA extracted from a heterozygous *Δtuf1Sp::KanR/tuf1Sp ura4-D1.8/ura4-D1.8* diploid strain (lane 2, SP3), several independent *ptp1-1 Δtuf1Sp::KanR ura4* strains (lanes 3–6, SP4), and the isogenic *ptp1-1 ura4* strain (lane 7, NB34-21A) were digested with *Hind*III and analyzed by Southern blotting under standard conditions. Probes were either (top) pDG3 [a pBR322-based plasmid that contains the whole *S. pombe* mtDNA (DEL GIUDICE *et al.* 1983)] or (bottom) the 1.8-kb *Hind*III *ura4* fragment (GRIMM *et al.* 1988). The blot hybridized to the complete mtDNA was exposed for either a few days (right) or several weeks (left). *Hind*III digestion of wild-type mtDNA gives 10 fragments of 4318, 4081, 3407, 2459, 2118, 1655, 1160, 134, 90, and 9 bp. Band sizes for the Raoul DNA marker (Appligene) are given in base pairs. Signals were quantified using a STORM phosphor imager (Molecular Dynamics, Sunnyvale, CA).

FIGURE 4.—Overexpression of the *tuf1Sp* gene suppresses the $\Delta tsf1Sp$ deletion. (A) Total protein extracts from a wild-type (Eg555), $\Delta tuf1Sp$ cells (SP4), or cells (NB34-21A) overexpressing *tuf1Sp* were analyzed by Western blotting using antibodies recognizing the EF-Tu_{Sp} and Cox2_{Sp} proteins. A cross-reacting band revealed in all lanes by the anti-EF-Tu_{Sp} antiserum (X) is used as loading control. (B) The $\Delta tsf1Sp::KanR$ strain (NB222) was transformed with a plasmid overexpressing a tagged version of *tuf1Sp*. Total (T, lane 1), postmitochondrial (S, lane 2), and mitochondrial (M, lane 3) protein fractions of this galactose-grown transformant were analyzed by Western blotting, using antibodies raised against the His-tag to detect EF-Tu_{Sp}-6His or against the *S. cerevisiae* mitochondrial matrix enzyme Arg8p (STEELE *et al.* 1996). The bottom of the two bands revealed by the anti-Arg8p antibody in lane 1 is absent in a $\Delta arg8$ strain and corresponds to the 47-kD *S. pombe* Arg8 protein (N. BONNEFOY, unpublished results). The top cross-reacting protein (Y) is absent in purified mitochondria, contrary to Arg8_{Sp}. (C) In addition to plasmids used in Figure 2A, the $\Delta tsf1Sp::KanR$ strain NB222 was transformed by a plasmid producing EF-Tu_{Sp} (Tu Sp). The transformants were streaked on minimal glucose (left) or complete ethanol glycerol (right) media.

(not shown), showing that even very weak residual translation is sufficient for mtDNA maintenance in *S. pombe*.

Overproduction of EF-Tu_{Sp} can compensate for the absence of its exchange factor: One explanation for the viability of $\Delta tsf1Sp$ and the lethality of $\Delta tuf1Sp$ mutants could be that the GTPase EF-Tu_{Sp} can still function at a low level without its exchange factor. To test this hypothesis, we overproduced EF-Tu_{Sp}. Overexpression of *tuf1Sp* in *S. pombe* indeed leads to increased accumulation of the protein (Figure 4A, compare lanes 3 and 1), which is fully imported into mitochondria (Figure 4B). The *S. pombe* Cox2 protein showed a similar pattern, suggesting that its synthesis was also increased as a consequence of EF-Tu_{Sp} overproduction. Overexpression of

FIGURE 5.—Heterologous complementation analysis. *S. pombe* *ptp1-1* Δ *tuf1Sp::KanR* Δ *tsf1Sp::ade6* cells containing plasmids producing the indicated *S. cerevisiae*, *S. pombe*, or *H. sapiens* EF-Tu or EF-Ts proteins were patched onto minimal glucose medium. Plates were replicated on galactose or glycerol medium (left). A heterozygous *S. cerevisiae* Δ *tuf1Sc::KanR* diploid was transformed with various plasmids producing the indicated proteins and sporulated. Mutant Δ *tuf1Sc* spores carrying the different plasmids producing the indicated EF-Tu or EF-Ts proteins from *S. cerevisiae* and/or *S. pombe* were patched onto minimal glucose medium and replica plated on nonfermentable glycerol medium (right). Tu, EF-Tu; Ts, EF-Ts; Sc, *S. cerevisiae*; Sp, *S. pombe*; Hs, *H. sapiens*.

tuf1Sp compensates for the deletion of *tsf1Sp* (Figure 4C). Elevated levels of EF-Tu_{Sp} probably sustain respiratory growth in the absence of EF-Ts_{Sp} by increasing the availability of GTP-bound EF-Tu_{Sp}. Thus EF-Tu_{Sp} can function, although at a reduced level, in the absence of its exchange factor. By extension, the remaining low EF-Tu_{Sp} activity due to the endogenous copy of the *tuf1Sp* gene probably ensures sufficient residual translation to maintain viability of the Δ *tsf1Sp* mutant but not growth on nonfermentable substrate.

EF-Tu_{Sc} functions independently of an exchange factor: The fact that *tsf1Sp* was partly dispensable when *tuf1Sp* was overexpressed may provide a key to understanding why *S. cerevisiae* is missing an EF-Ts exchange factor. The *TUF1Sc* gene could be naturally more highly expressed than its *S. pombe* counterpart. Alternatively, the *S. pombe* and *S. cerevisiae* GTPases could have different affinities for guanine nucleotides. It has been shown that EF-Tu_{Sc} has a higher affinity for GTP than for GDP, whereas the *E. coli* and *Homo sapiens* homologs, which depend on an exchange factor, have a higher affinity for GDP (MILLER and WEISSBACH 1970; ROSENTHAL and BODLEY 1987; CAI *et al.* 2000). The biochemical approach appeared difficult in *S. pombe* because EF-Tu_{Sp} aggregates, so we decided to use genetic strategies to study the relationships between both factors.

The heterologous complementation experiment in *S. pombe* was conducted by crossing a *ptp1-1* Δ *tuf1Sp::KanR* strain (SP4) to a Δ *tsf1Sp::ade6* mutant (SP7) transformed with a *ura4* plasmid expressing *TUF1Sc*. Fully respiring [*Ura*⁺ *G418*^R *Ade*⁺] segregants were obtained, showing that the *S. cerevisiae* gene could efficiently replace *tsf1Sp* and *tuf1Sp* at the same time (Figure 5). One of these segregants, carrying the *ptp1-1* mutation, was transformed

with a *LEU2* plasmid overexpressing *tuf1Sp* and the *ura4* *TUF1Sc* plasmid was cured using 5-fluoroorotic acid. The resulting strain clearly showed weaker growth on galactose medium. Coproduction of the *S. pombe* or human exchange factors improved growth, up to the level of the transformant producing EF-Tu_{Sc} alone (Figure 5). These data are consistent with the idea that the *S. cerevisiae* GTPase functions independently of an exchange factor.

To do the equivalent experiment in *S. cerevisiae*, we transformed a Δ *tuf1Sc* heterozygote with plasmids expressing the EF-Ts and/or EF-Tu factors from either yeast or humans and recovered spores carrying the different plasmids. This strategy was used because the Δ *tuf1Sc* allele would readily convert any haploid strain into a mixture of *rho*^o/*rho*[−] cells. The absence of EF-Tu_{Sc} in *S. cerevisiae* was complemented by production of EF-Tu_{Sp}. This complementation was improved when the *S. pombe* or the *H. sapiens* exchange factors were coproduced (Figure 5). Thus the *S. pombe* GTPase can only partially replace its *S. cerevisiae* counterpart and functions more efficiently with its cognate exchange factor.

Variants of EF-Tu_{Sp} can become independent of an exchange factor: To explain how closely related and functionally homologous GTPases like the *S. cerevisiae* and *S. pombe* EF-Tu factors could differ in their requirement for an exchange factor, we isolated suppressors able to compensate for the lack of *S. pombe* EF-Ts. Among 34 suppressors analyzed, 27 contained a 1-bp substitution in the *tuf1Sp* reading frame, creating a single-amino-acid replacement. A total of 16 different *tuf1Sp* suppressor mutations were recovered (Figure 6A). Fourteen of the mutations mapped into domain I of the EF-Tu_{Sp} factor, often in the vicinity of the nucleotide-binding site; two mutations mapped into domain III in a region of close contact with domain I (Figure 6B).

Since overexpression of *tuf1Sp* partially compensates for the Δ *tsf1Sp* mutation, we determined the level of EF-Tu_{Sp} in the suppressor strains. In the Δ *tsf1Sp* mutant, which is the parental strain of the suppressors, EF-Tu_{Sp} was nearly undetectable (Figure 6C, lane 2). This suggested that EF-Ts_{Sp} might act as a chaperone of EF-Tu_{Sp}, as in *E. coli* (KRAB *et al.* 2001). In the three suppressor strains, nearly wild-type (L354F and G168C) or even slightly higher than wild-type (V146I) levels of EF-Tu_{Sp} were restored (Figure 6C; compare lanes 3, 5, and 6 with lane 1), suggesting that the EF-Tu_{Sp} variants carry a mutation that can stabilize them. However, EF-Tu_{Sp} levels were dramatically higher in the *tuf1Sp* overexpression control (Figure 6C, lane 4) whereas the Cox2_{Sp}/EF-Tu_{Sp} ratio was at least 10-fold lower than that in the suppressors. This suggests that the EF-Tu_{Sp} variants are more active than the wild-type EF-Tu_{Sp} in a strain lacking the exchange factor.

The V146I variant gene was cloned in an *S. cerevisiae* expression plasmid and expressed in the *S. cerevisiae* Δ *tuf1* mutant (Figure 6D). The strong complementation

FIGURE 6.—Nature and localization of the EF-Tu_{Sp} mutations able to compensate for the absence of an exchange factor. (A) Serial 10-fold dilutions of Δ*tsf1Sp::KanR* (Δ, NB222), wild-type (NB205-6A), and revertant strains were spotted onto 2% galactose 0.1% glucose plates. In addition to the Δ*tsf1Sp::KanR* allele, each revertant carries a mutation in the *tuf1Sp* gene, given in the one-letter code with its position. (B) The EF-Tu_{Sp} variants from A are depicted with shaded circles on the crystal structure of the bovine EF-Tu (ANDERSEN *et al.* 2000) bound to GDP (indicated by a thick solid line) on the basis of sequence homologies with the *S. pombe* protein. The numbering in the shaded circles corresponds to the residue position in *S. pombe*. (C) Total protein extracts from a wild-type (NB205-6A, lane 1), Δ*tsf1Sp* (NB222) cells overexpressing (lane 4) or not (lane 2) the wild-type *tuf1Sp* gene, as well as cells from three suppressors from A (lanes 3, 5, 6) were analyzed by Western blotting as in Figure 4A. Signals were quantified using the ImageJ program. (D) The mutant gene encoding the V146I EF-Tu_{Sp} protein variant was subcloned in a *S. cerevisiae* expression vector and tested for complementation of the Δ*tuf1Sc* mutant as in Figure 5.

obtained clearly showed that the V146I EF-Tu_{Sp} variant had become independent of its exchange factor, even in a heterologous system.

DISCUSSION

The GTPase superfamily includes three types of GTPases: the small Rho-type G proteins, the heterotrimeric G proteins, and the translational GTPases. Apart from some of the translational GTPases, such as the initiation factor IF2, which delivers the initiator tRNA, and the elongation factor EF-G, all depend on nucleotide exchange factors. Unlike its *E. coli*, *S. pombe*, and higher eukaryote homologs, the *S. cerevisiae* EF-Tu_{Sc} also seems to lack an exchange factor (ROSENTHAL and BODLEY 1987). Both IF2 and EF-G contain additional domains that could play a role as an intrinsic exchange factor (AEVARSSON *et al.* 1994; LUCHIN *et al.* 1999). This

does not appear to be the case for EF-Tu_{Sc}, as sequence comparison of the EF-Tu factors from a number of organisms including *S. cerevisiae* and *S. pombe* could not highlight any obvious feature that could explain the autonomous recycling of EF-Tu_{Sc}.

We determined that the *S. pombe* EF-Tu_{Sp} is highly dependent upon its exchange factor, and thus this system offered a good opportunity to design a genetic strategy to understand the basis of EF-Ts factor loss in *S. cerevisiae*. We found that various single-amino-acid substitutions could confer independence toward its exchange factor on EF-Tu_{Sp}. Two nonexclusive hypotheses can be proposed for the mechanism of suppression: the mutations could modify the activity of EF-Tu_{Sp} or increase its stability. We have shown that overproduction of EF-Tu_{Sp} can compensate for the absence of its exchange factor, and suppressor effects of the overproduction of EF-Tu-type GTPases have also been observed

in *S. cerevisiae* either in the cytoplasmic system (CARR-SCHMID *et al.* 1999b) or in the mitochondrial tRNA mutants (RINALDI *et al.* 1997; FEUERMAN *et al.* 2003). However, the suppressor mutations do not augment the steady-state level of EF-Tu_{sp} enough to account for the significantly increased Cox2_{sp} synthesis. Thus we hypothesize that the main reason for the suppression is that the EF-Tu_{sp} variants have acquired or extended a new ability of self-exchange, *e.g.*, decreased affinity for GDP and/or increased affinity for GTP.

The nature and/or location of the suppressor mutations indeed points toward a crucial role of nucleotide binding in the suppression. Of 16 mutations, 14 are located in the GDP-binding domain I, mostly in close proximity of the G-nucleotide-binding sites. Position 94 (52 in *E. coli*) is located in one of the switch regions (SPREMULLI *et al.* 2004), alanine 215 (174 in *E. coli*) is directly part of the nucleotide-binding site (SONG *et al.* 1999), and aspartate 151 (155 in bovine) corresponds to a position connected to GDP through water molecules (ANDERSEN *et al.* 2000). Position 227 (G232 in bovine) has been proposed as a candidate to explain the weaker nucleotide binding of mammalian compared to prokaryotic EF-Tu (ANDERSEN *et al.* 2000). In addition, mutations in the nucleotide-binding site of the cytoplasmic eEF1A close to our mutant position 182 abolish the exchange factor requirement (CARR-SCHMID *et al.* 1999a), and position 168 corresponds to a conserved glycine whose mutation decreases the preference for GDP over GTP in *E. coli* (KNUDSEN *et al.* 1995). Finally, the two last suppressor mutations in EF-Tu_{sp} domain III are very close to domain I, according to both *E. coli* and bovine crystal structures (SONG *et al.* 1999; ANDERSEN *et al.* 2000), suggesting that they could alter domain I conformation to modulate nucleotide binding.

The subtle changes found in the EF-Tu_{sp} variants could make them functionally more similar to the EF-Tu_{sc} or to other self-recycling GTPases like EF-G or IF2, which display greater affinity toward GTP than do bacterial or human EF-Tu factors (ROSENTHAL and BODLEY 1987). This could explain why EF-Tu_{sc} does not seem to require a separate exchange activity, despite the absence of additional domains, and illustrates how the EF-Tu_{sc} gene could have evolved to allow the loss of the exchange factor EF-Ts without a drastic alteration in mitochondrial metabolism. Sequence searches revealed (GAILLARDIN *et al.* 2000) that EF-Ts loss actually seems to extend to the whole branch of hemi-ascomycete yeasts. Since G nucleotide exchange factors are known to perform an essential regulatory function in the GTPase cycle, hemi-ascomycetes might have developed another regulatory mechanism to compensate for the absence of EF-Ts. Another mitochondrial translation protein, the initiation factor IF3, also appears to be conserved in *E. coli*, *S. pombe*, and mammals (KOC and SPREMULLI 2002), but be missing in *S. cerevisiae* and probably in

the rest of the hemi-ascomycete branch. *E. coli* IF3 is a modulator of the initiation step of translation, which discriminates between different types of messenger RNAs, and its homolog might have a similar regulatory role in human and fission yeast mitochondria. However, in *S. cerevisiae* mitochondrial translation initiation is controlled by messenger-specific activators, conserved mainly in closely related yeasts (COSTANZO *et al.* 2000); these messenger-specific activators may compensate for the absence of IF3. Thus, the translation-factor losses in hemi-ascomycetes might reflect profound differences in the way mitochondrial translation is regulated compared to other eukaryotes.

In addition, our study has clearly shown that a complete block of mitochondrial translation is lethal in wild-type *S. pombe* and leads to a major depletion of mtDNA under conditions where viability can be maintained despite the translational block. Although *S. cerevisiae* strains with mitochondrial translational defects are viable, multiple deletions accumulate readily when mitochondrial translation is affected, and in general the extent of mtDNA deletion production depends on the stringency of the mutation (MYERS *et al.* 1985). The results we have obtained in *S. pombe* are reminiscent of results obtained with human cells. When human cells in culture were treated with thiamphenicol, a drug that inhibits mitochondrial translation, their mtDNA remained intact (SELWOOD *et al.* 2001). In cells from patients showing a mitochondrial translation defect of nuclear origin, no evidence of any large-scale rearrangement or mtDNA depletion was found (SASARMAN *et al.* 2002). Finally, mutant hamster cells affected in mitochondrial translation showed intact mtDNA but with a 50% decrease in the quantity of mtDNA (AU and SCHEFFLER 1997). However, in all three cases, mitochondrial translation was probably not completely blocked. The physiological state of these cell cultures was thus probably more similar to that of a $\Delta tsf1Sp$ mutant, which does contain wild-type amount of intact mtDNA, than to that of a $\Delta tuf1Sp$ mutant. This suggests that mitochondrial translation defects stringent enough to cause mtDNA depletion are probably unlikely to be found in humans, because they might correspond to drastic mutational events, not compatible with viability.

The fact that the mtDNA is intact in both sequence and quantity in the $\Delta tsf1Sp$ mutant shows that *S. pombe* mtDNA maintenance needs only a very limited level of mitochondrial translation. The $\Delta tsf1Sp$ mutant now provides the opportunity not only to analyze the relationships between mtDNA metabolism and mitochondrial translation, but also to study whether a dramatic mitochondrial translation decrease affects steps of mtDNA expression such as mRNA transcription and stability.

We are indebted to C. S. Hoffmann for the kind gifts of a *his3Δ* strain and the *S. pombe* gene bank constructed in the laboratory of K. L. Gould. We are grateful to T. D. Fox for the gift of strains and antibodies and for providing a plasmid constructed by L. Del Giudice

and coworkers. We thank A. Goffeau for useful advice concerning S³⁵-labeling in *S. pombe*, G. D. Clark-Walker and M. S. Longtine for the gift of DNAs, and R. Egel for a yeast strain. We also thank G. Dujardin and C. J. Herbert for excellent critical reading of the manuscript and helpful discussions, and C. J. Herbert for looking over the English. S.C. was a Ph.D. fellow of the Ministère de la Recherche et de la Technologie. This work was supported by a research grant from the Association Française contre les Myopathies to N.B.

LITERATURE CITED

- AEVARSSON, A., E. BRAZHNIKOV, M. GARBER, J. ZHELTONOSOVA, Y. CHIRGADZE *et al.*, 1994 Three-dimensional structure of the ribosomal translocase: elongation factor G from *Thermus thermophilus*. *EMBO J.* **13**: 3669–3677.
- ANDERSEN, G. R., S. THIRUP, L. L. SPREMULLI and J. NYBORG, 2000 High resolution crystal structure of bovine mitochondrial EF-Tu in complex with GDP. *J. Mol. Biol.* **297**: 421–436.
- AU, H. C., and I. E. SCHEFFLER, 1997 A respiration-deficient Chinese hamster cell line with a defect in mitochondrial protein synthesis: rapid turnover of some mitochondrial transcripts. *Somat. Cell Mol. Genet.* **23**: 27–35.
- BONNEFOY, N., M. KERMORGANT, P. BRIVET-CHEVILLOTTE and G. DUJARDIN, 1996 Cloning by functional complementation, and inactivation, of the *Schizosaccharomyces pombe* homologue of the *Saccharomyces cerevisiae* gene *ABC1*. *Mol. Gen. Genet.* **251**: 204–210.
- BONNEFOY, N., M. KERMORGANT, O. GROUDINSKY and G. DUJARDIN, 2000 The respiratory gene *OXA1* has two fission yeast orthologues which together encode a function essential for cellular viability. *Mol. Microbiol.* **35**: 1135–1145.
- CAI, Y. C., J. M. BULLARD, N. L. THOMPSON and L. L. SPREMULLI, 2000 Interaction of mammalian mitochondrial elongation factor EF-Tu with guanine nucleotides. *Protein Sci.* **9**: 1791–1800.
- CARR-SCHMID, A., N. DURKO, J. CAVALLIUS, W. C. MERRICK and T. G. KINZY, 1999a Mutations in a GTP-binding motif of eukaryotic elongation factor 1A reduce both translational fidelity and the requirement for nucleotide exchange. *J. Biol. Chem.* **274**: 30297–30302.
- CARR-SCHMID, A., L. VALENTE, V. I. LOIK, T. WILLIAMS, L. M. STARITA *et al.*, 1999b Mutations in elongation factor 1beta, a guanine nucleotide exchange factor, enhance translational fidelity. *Mol. Cell. Biol.* **19**: 5257–5266.
- CHEN, X. J., and G. D. CLARK-WALKER, 2000 The *petite* mutation in yeasts: 50 years on. *Int. Rev. Cytol.* **194**: 197–238.
- CLAISSE, M. L., G. A. PERE-AUBERT, L. P. CLAVILIER and P. P. SLONIMSKI, 1970 Method for the determination of cytochrome concentrations in whole yeast cells. *Eur. J. Biochem.* **16**: 430–438.
- CONTAMINE, V., and M. PICARD, 2000 Maintenance and integrity of the mitochondrial genome: a plethora of nuclear genes in the budding yeast. *Microbiol. Mol. Biol. Rev.* **64**: 281–315.
- COSTANZO, M. C., N. BONNEFOY, E. H. WILLIAMS, G. D. CLARK-WALKER and T. D. FOX, 2000 Highly diverged homologs of *Saccharomyces cerevisiae* mitochondrial mRNA-specific translational activators have orthologous functions in other budding yeasts. *Genetics* **154**: 999–1012.
- DEL GIUDICE, L., K. WOLF, F. MANNA and D. R. MASSARDO, 1983 Expression of cloned mitochondrial DNA from the petite negative yeast *Schizosaccharomyces pombe* in *E. coli* minicells. *Mol. Gen. Genet.* **191**: 91–98.
- FEUERMANN, M., S. FRANCISCI, T. RINALDI, C. DE LUCA, H. ROHOU *et al.*, 2003 The yeast counterparts of human 'MELAS' mutations cause mitochondrial dysfunction that can be rescued by overexpression of the mitochondrial translation factor EF-Tu. *EMBO Rep.* **4**: 53–58.
- FORSBURG, S. L., 1999 The best yeast? *Trends Genet.* **15**: 340–344.
- GAILLARDIN, C., G. DUCHATEAU-NGUYEN, F. TEKAIA, B. LLORENTE, S. CASAREGOLA *et al.*, 2000 Genomic exploration of the hemiascomycetous yeasts: 21. Comparative functional classification of genes. *FEBS Lett.* **487**: 134–149.
- GRIMM, C., J. KOHLI, J. MURRAY and K. MAUNDRELL, 1988 Genetic engineering of *Schizosaccharomyces pombe*: a system for gene disruption and replacement using the *ura4* gene as a selectable marker. *Mol. Gen. Genet.* **215**: 81–86.
- HAFFTER, P., and T. D. FOX, 1992 Nuclear mutations in the petite-negative yeast *Schizosaccharomyces pombe* allow growth of cells lacking mitochondrial DNA. *Genetics* **131**: 255–260.
- HOFFMAN, C. S., and F. WINSTON, 1987 A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. *Gene* **57**: 267–272.
- KELLY, D. A., and C. S. HOFFMAN, 2002 Gap repair transformation in fission yeast to exchange plasmid-selectable markers. *BioTechniques* **33**: 978, 980, 982.
- KING, M. P., and G. ATTARDI, 1989 Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. *Science* **246**: 500–503.
- KNUDSEN, C. R., I. V. KJAERGAARD, O. WIBORG and B. F. CLARK, 1995 Mutation of the conserved Gly94 and Gly126 in elongation factor Tu from *Escherichia coli*. Elucidation of their structural and functional roles. *Eur. J. Biochem.* **228**: 176–183.
- KOC, E. C., and L. L. SPREMULLI, 2002 Identification of mammalian mitochondrial translational initiation factor 3 and examination of its role in initiation complex formation with natural mRNAs. *J. Biol. Chem.* **277**: 35541–35549.
- KRAB, I. M., R. TE BIESEBEKE, A. BERNARDI and A. PARMEGGIANI, 2001 Elongation factor Ts can act as a steric chaperone by increasing the solubility of nucleotide binding-impaired elongation factor-Tu. *Biochemistry* **40**: 8531–8535.
- LOLLIER, M., L. JAQUET, T. NEDEVA, F. LACROUTE, S. POTIER *et al.*, 1995 As in *Saccharomyces cerevisiae*, aspartate transcarbamoylase is assembled on a multifunctional protein including a dihydroorotase-like cryptic domain in *Schizosaccharomyces pombe*. *Curr. Genet.* **28**: 138–149.
- LUCHIN, S., H. PUTZER, J. W. HERSHEY, Y. CENATIEMPO, M. GRUNBERG-MANAGO *et al.*, 1999 In vitro study of two dominant inhibitory GTPase mutants of *Escherichia coli* translation initiation factor IF2. Direct evidence that GTP hydrolysis is necessary for factor recycling. *J. Biol. Chem.* **274**: 6074–6079.
- MILLER, D. L., and H. WEISSBACH, 1970 Studies on the purification and properties of factor Tu from *E. coli*. *Arch. Biochem. Biophys.* **141**: 26–37.
- MINET, M., M.-E. DUFOUR and F. LACROUTE, 1992 Complementation of *Saccharomyces cerevisiae* auxotrophic mutants by *Arabidopsis thaliana* cDNAs. *Plant J.* **2**: 417–422.
- MYERS, A. M., L. K. PAPE and A. TZAGOLOFF, 1985 Mitochondrial protein synthesis is required for maintenance of intact mitochondrial genomes in *Saccharomyces cerevisiae*. *EMBO J.* **4**: 2087–2092.
- NAGATA, S., Y. TSUNETSUGU-YOKOTA, A. NAITO and Y. KAZIRO, 1983 Molecular cloning and sequence determination of the nuclear gene coding for mitochondrial elongation factor Tu of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **80**: 6192–6196.
- OHI, R., A. FEOKTISTOVA and K. L. GOULD, 1996 Construction of vectors and a genomic library for use with *his3*-deficient strains of *Schizosaccharomyces pombe*. *Gene* **174**: 315–318.
- OKAZAKI, K., N. OKAZAKI, K. KUME, S. JINNO, K. TANAKA *et al.*, 1990 High-frequency transformation method and library transducing vectors for cloning mammalian cDNAs by trans-complementation of *Schizosaccharomyces pombe*. *Nucleic Acids Res.* **18**: 6485–6489.
- RINALDI, T., R. LANDE, M. BOLOTIN-FUKUHARA and L. FRONTALI, 1997 Additional copies of the mitochondrial EF-Tu and aspartyl-tRNA synthetase genes can compensate for a mutation affecting the maturation of the mitochondrial tRNA^{Asp}. *Curr. Genet.* **31**: 494–496.
- ROSENTHAL, L. P., and J. W. BODLEY, 1987 Purification and characterization of *Saccharomyces cerevisiae* mitochondrial elongation factor Tu. *J. Biol. Chem.* **262**: 10955–10959.
- SASARMAN, F., G. KARPATI and E. A. SHOUBRIDGE, 2002 Nuclear genetic control of mitochondrial translation in skeletal muscle revealed in patients with mitochondrial myopathy. *Hum. Mol. Genet.* **11**: 1669–1681.
- SCHÄFER, B., 2003 Genetic conservation versus variability in mitochondria: the architecture of the mitochondrial genome in the petite-negative yeast *Schizosaccharomyces pombe*. *Curr. Genet.* **43**: 311–326.
- SELWOOD, S. P., A. MCGREGOR, R. N. LIGHTOWLERS and Z. M. CHRZANOWSKA-LIGHTOWLERS, 2001 Inhibition of mitochondrial protein synthesis promotes autonomous regulation of mtDNA expression and generation of a new mitochondrial RNA species. *FEBS Lett.* **494**: 186–191.
- SONG, H., M. R. PARSONS, S. ROWSELL, G. LEONARD and S. E. PHILLIPS,

- 1999 Crystal structure of intact elongation factor EF-Tu from *Escherichia coli* in GDP conformation at 2.05 Å resolution. *J. Mol. Biol.* **285**: 1245–1256.
- SPREMULLI, L. L., A. COURSEY, T. NAVRATIL and S. E. HUNTER, 2004 Initiation and elongation factors in mammalian mitochondrial protein biosynthesis. *Prog. Nucleic Acid Res. Mol. Biol.* **77**: 211–261.
- STEELE, D. F., C. A. BUTLER and T. D. FOX, 1996 Expression of a recoded nuclear gene inserted into yeast mitochondrial DNA is limited by mRNA-specific translational activation. *Proc. Natl. Acad. Sci. USA* **93**: 5253–5257.
- VAMBUTAS, A., S. H. ACKERMAN and A. TZAGOLOFF, 1991 Mitochondrial translational-initiation and elongation factors in *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **201**: 643–652.
- WEILGUNY, D., M. PRAETORIUS, A. CARR, R. EGEL and O. NIELSEN, 1991 New vectors in fission yeast: application for cloning the *his2* gene. *Gene* **99**: 47–54.
- WEIR, B. A., and M. P. YAFFE, 2004 Mmd1p, a novel, conserved protein essential for normal mitochondrial morphology and distribution in the fission yeast *Schizosaccharomyces pombe*. *Mol. Biol. Cell* **15**: 1656–1665.
- YAFFE, M. P., 1991 Analysis of mitochondrial function and assembly. *Methods Enzymol.* **194**: 627–643.
- ZHAO, Y., and H. B. LIEBERMAN, 1995 *Schizosaccharomyces pombe*: a model for molecular studies of eukaryotic genes. *DNA Cell Biol.* **14**: 359–371.

Communicating editor: M. JOHNSTON