Suppression of a Defect in Mitochondrial Protein Import Identifies Cytosolic Proteins Required for Viability of Yeast Cells Lacking Mitochondrial DNA

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ABSTRACT

The TIM22 complex, required for the insertion of imported polytopic proteins into the mitochondrial inner membrane, contains the nonessential Tim18p subunit. To learn more about the function of Tim18p, we screened for high-copy suppressors of the inability of *tim18*Δ mutants to live without mitochondrial DNA (mtDNA). We identified several genes encoding cytosolic proteins, including *CCT6*, *SSB1*, *ICY1*, *TIP41*, and *PBP1*, which, when overproduced, rescue the mtDNA dependence of *tim18*Δ cells. Furthermore, these same plasmids rescue the petite-negative phenotype of cells lacking other components of the mitochondrial protein import machinery. Strikingly, disruption of the genes identified by the different suppressors produces cells that are unable to grow without mtDNA. We speculate that loss of mtDNA leads to a lowered inner membrane potential, and subtle changes in import efficiency can no longer be tolerated. Our results suggest that increased amounts of Cct6p, Ssb1p, Icy1p, Tip41p, and Pbp1p help overcome the problems resulting from a defect in protein import.

WHILE mitochondria contain their own DNA (mtDNA) encoding a handful of proteins, the vast majority of mitochondrial proteins are synthesized on cytosolic ribosomes and imported post-translationally into the organelle (Jensen and Dunn 2002; Pfanner and Chacinska 2002). These proteins are sorted to four possible locations within the mitochondria: the outer membrane (OM), the intermembrane space (IMS), the inner membrane (IM), and the matrix. Several multisubunit transport machines in the OM and IM, called translocons, mediate protein import. Imported proteins first travel through a translocon in the OM, called the translocon of the outer membrane (TOM) complex. Many proteins are further transported through the IM by translocons of the inner membrane (TIM) complexes.

The TOM and TIM channels are best characterized in fungi such as *Neurospora crassa* and *Saccharomyces cerevisae*, although many of the identified components have homologs in higher eukaryotes (Hoogenraad *et al.* 2002). Precursors in the cytosol are recognized by one of several receptor proteins on the surface of mitochondria: Tom70p, Tom22p, and Tom20p. After binding to receptors, proteins cross the OM via the TOM translocon, which forms a pore in the mitochondrial OM. Mitochondrial precursors containing a cleavable presequence are then imported into the matrix by the action of the inner-membrane-localized TIM23 complex. The TIM23 complex consists of the Tim50, Tim23, and Tim17 proteins, which may form and/or regulate a protein-translocating pore (Truscott *et al.* 2001; Geissler *et al.* 2002; Yamamoto *et al.* 2002). In addition, Tim44p and mt-Hsp70 act as a molecular motor to ensure unidirectional movement of the precursor through the TIM23 channel (Neupert and Brunner 2002; Voos and Rottgers 2002). Once a precursor reaches the matrix, its presequence is removed by a processing peptidase (Gakh *et al.* 2002).

Many proteins that end up in the mitochondria use a different TIM complex for their import. In particular, a subset of polytopic membrane proteins, or proteins that pass through the membrane several times, is inserted into the IM by the TIM22 translocon. The movement of these proteins from the TOM translocon to the TIM22 translocon is dependent upon soluble complexes in the IMS, such as the TIM9/10 or TIM8/13 complex (Koehler *et al.* 1999). Within the TIM22 complex, the Tim22 protein, which is homologous to both Tim23p and Tim17p (Sirrenberg *et al.* 1996), is thought to form a pore through which polytopic proteins are inserted into the IM (Kovermann *et al.* 2002). Tim54p and Tim18p are membrane-spanning proteins that are also members of the TIM22 translocon, but their function in polytopic protein insertion is not yet known (Kerscher *et al.* 1997, 2000; Koehler *et al.* 2000).

An electrochemical gradient across the IM is essential for the activity of both TIM complexes (Schleyer *et al.* 1982; Zwizinski *et al.* 1983; Eilers *et al.* 1987; Martin *et al.* 1991). This potential is usually generated by the export of protons out of the matrix by the respiratory chain, components of which are encoded in the nucleus and by mitochondrial DNA. When the respiratory chain is damaged by mutation or when cells lose mtDNA, creating

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TABLE 1

Yeast strains and relevant genotypes

Strain	Genotype	Source
RJ867	*MAT***a** *leu2Δ0 met15Δ0 ura3Δ0 his3Δ200 trp1Δ63*	Brachmann *et al.* (1998)
RJ868	*MAT*α *leu2Δ0 met15Δ0 ura3Δ0 his3Δ200 trp1Δ63*	Brachmann *et al.* (1998)
RJ992	*MAT*α *his3Δ200 ura3-52 lys2Δ202 trp1Δ63 leu2Δ1 tim18::HIS3*	Kerscher *et al.* (2000)
RJ1263	*MAT*α *leu2Δ0 met15Δ0 ura3Δ0 his3Δ200 trp1Δ63 tim18::HIS3*	This study
RJ1577	*MAT***a** *leu2Δ0 met15Δ0 ura3Δ0 his3Δ200 atp2::kanMX^R*	Giaever *et al.* (2002)
RJ1578	*MAT*α *ade1 op1*	Peter Thorsness
RJ1581	*MAT*α *LEU2 ura3Δ0 his3Δ200 TRP1 op1*	This study
RJ1582	*MAT*α *leu2Δ0 met15Δ0 ura3Δ0 his3Δ200 trp1Δ63* ρ^0	This study
RJ1583	*MAT***a** *leu2Δ0 met15Δ0 ura3Δ0 his3Δ200 tom70::kanMX^R*	Giaever *et al.* (2002)
RJ1584	*MAT***a** *leu2Δ0 met15Δ0 ura3Δ0 his3Δ200 tip41::kanMX^R*	Giaever *et al.* (2002)
RJ1585	*MAT***a** *leu2Δ0 met15Δ0 ura3Δ0 his3Δ200 icy1::kanMX^R*	Giaever *et al.* (2002)
RJ1586	*MAT***a** *leu2Δ0 met15Δ0 ura3Δ0 his3Δ200 ssb1::kanMX^R*	Giaever *et al.* (2002)
RJ1587	*MAT***a** *leu2Δ0 met15Δ0 ura3Δ0 his3Δ200 pbp1::kanMX^R*	Giaever *et al.* (2002)
RJ1605	*MAT***a** *leu2Δ0 met15Δ0 ura3Δ0 his3Δ200 cyt1::kanMX^R*	Giaever *et al.* (2002)
RJ1606	*MAT***a** *leu2Δ0 met15Δ0 ura3Δ0 his3Δ200 coq4::kanMX^R*	Giaever *et al.* (2002)

Strains are ρ^+ unless otherwise noted.

"petite" cells, the IM potential is thought to be formed by the activities of an ATP/ADP transporter and of the matrix-localized F_1 ATPase (Dupont *et al.* 1985; Giraud and Velours 1997). The exchange of ATP^{-4} for ADP^{-3} in some way produces a sufficient potential for mitochondrial function. ATP/ADP transport is mediated by carrier proteins in the IM, with Aac2p being the most important (Chen and Clark-Walker 2000).

The Tim18 protein is a nonessential member of the TIM22 complex, but *tim18*Δ cells cannot live without mtDNA and are cold sensitive when grown on rich glucose medium (Kerscher *et al.* 2000). To further explore the function of the inner-membrane-localized Tim18 protein, we isolated high-copy suppressors of the *tim18*Δ mutant. Surprisingly, all of our suppressors encoded cytosolic proteins, including *CCT6*, *SSB1*, *TIP41*, *ICY1*, and *PBP1*. We present data indicating that a genetic pathway stretching from the cytosol to the mitochondrial matrix is required for viability of cells lacking mtDNA. We also speculate on how the proteins within this pathway might function when mitochondrial protein import is compromised.

MATERIALS AND METHODS

Strains and media: The genotypes of strains used in this study are indicated in Table 1. RJ992 was generated as previously described (Kerscher *et al.* 2000). *tim18*Δ strain RJ1263 was constructed by first mating RJ867 and RJ868, and then PCR-mediated gene disruption was used to delete one copy of *TIM18* in the diploid. After sporulation and tetrad dissection, the *tim18::HIS3* segregant RJ1263 was isolated. ρ^0 strain RJ1582 was obtained by growing RJ868 cells twice to saturation in YEPD containing ethidium bromide (EtBr). RJ1582 was identified as a single colony that failed to grow on YEP medium containing glycerol and ethanol as the sole carbon source and was subsequently shown to contain no mtDNA by microscopy after staining with 1 μg/ml 4′, 6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR) for 15 min. The *op1* strain RJ1581 was generated by crossing strain RJ1577 to strain RJ1578. Strains RJ1577, RJ1583, RJ1584, RJ1585, RJ1586, RJ1587, RJ1605, and RJ1606 were obtained from Research Genetics (Huntsville, AL).

Standard yeast genetic techniques and media were used (Adams *et al.* 1997), except that SD complete medium was made with individual amino acids and not casamino acids. Cycloheximide (Calbiochem, La Jolla, CA) and ethidium bromide (Sigma, St. Louis) were added to medium at 0.2 and 25 μg/ml, respectively. Sporulation was performed as follows: diploids were grown in GNA presporulation medium (5% dextrose, 3% Difco nutrient broth, 1% Difco yeast extract, 2% Difco bacto-agar; Saccharomyces Genome Database; http://genome-www.stanford.edu/Saccharomyces/) for three to five generations and then transferred to sporulation medium (0.01 g/ml potassium acetate, 2.5×10^{-5} g/ml zinc acetate).

Isolation and identification of *tim18*Δ suppressors: *tim18*Δ strain RJ992 was grown in YEPD to an OD_{600} of ~0.6 at 34° and then transformed with a 2μ-*URA3* library containing genomic yeast DNA inserts of ~6–8 kbp (a gift of Phillip Hieter, University of British Columbia). Ura^+ transformants were selected for growth upon SD-Ura medium containing ethidium bromide (SD-Ura + EtBr) at 34°. Fifty-six large- and medium-sized colonies were picked, and the plasmid DNA was isolated (Hoffman and Winston 1987) and electroporated into bacterial cells. PCR analysis using oligonucleotides complementary to the *TIM18* gene showed that 40 of the plasmids encoded Tim18p. One of the isolated plasmids carrying *TIM18*, called pM346, was used as a control in some of our studies. The remaining 16 plasmids were shown to suppress the *tim18*Δ mutation upon retransformation into yeast. Limited DNA sequencing of one or both ends of the genomic DNA (Biosynthesis and Sequencing Facility, Department of Biological Chemistry, The Johns Hopkins School of Medicine, Baltimore) allowed us to identify the chromosomal region contained within the DNA insert by searching Saccharomyces Genome Database (http://genome-www.stanford.edu/Saccharomyces/). Many of the plasmids contained the same or overlapping inserts, such that five different chromosomal regions were represented by the 16 plasmids.

Plasmid constructions: To localize the genes responsible for suppression, subclones from the five different genomic DNA inserts were introduced into the 2μ-*URA3* plasmid pRS426 (Sikorski and Hieter 1989) and transformed into *tim18*Δ

strain RJ992. Ura$^+$ transformants were streaked out onto SD-Ura plates containing 25 μg/ml EtBr to induce loss of mtDNA and allowed to grow at 34° for 3 days. Cells were then streaked onto SD-Ura medium lacking EtBr and the plates incubated at 34° for 4 days. If the plasmid suppressed the *tim18*Δ defect, then the cells would grow following the EtBr treatment; cells without functional suppressors would not grow.

The five plasmids that encode functional subclones were all constructed in similar ways. Genes were amplified from genomic yeast DNA using specific oligonucleotides and the polymerase chain reaction (PCR). The PCR product was digested with the appropriate restriction endonucleases and then inserted into the 2μ-*URA3* vector pRS426 (Sikorski and Hieter 1989). The oligonucleotides and enzymes used for each subclone are as follows. pM350, which carries *CCT6*, was constructed using oligos 528 (5′-cagcctcgagacgagaacggtaag catg-3′) and 529 (5′-cggcctcgaggcaactggcaatgactat-3′), followed by *Xho*I digestion. pM351, which carries *SSB1*, was made with oligos 530 (5′-cgcggatccgagtacacacgggacttg-3′) and 531 (5′-cgcggatccgcgttaccggcactgatt-3′) and *Bam*HI. For pM352, which carries *ICY1*, we used oligos 534 (5′-cgcggatccagctcgattt tcagcacc-3′) and 535 (5′-cgcggatccttccggtagctggtctta-3′) and *Bam*HI. pM353, which contains *PBP1*, was made using oligos 740 (5′-ccgctcgagatgagtcgcaccaagataag-3′) and 741 (5′-ccgctc gagtggcgattgaaatactgattac-3′) and *Xho*I. pM362, which carries *TOM70*, was constructed using oligos 705 (5′-ccgctcgaggg atagggatggacaatagc-3′) and 744 (5′-ataagaatgcggccgcgctccgca aattggcgaggg-3′) and both *Xho*I and *Not*I. pM354, which carries *TOM70* in a *URA3-CEN6* plasmid was made by inserting the *Xho*I/*Not*I *TOM70*-containing insert from pM362 into pRS316 (Sikorski and Hieter 1989).

RESULTS

Cytosolic proteins can suppress the mtDNA dependence of the *tim18*Δ mutant: As an attempt to determine the function of the Tim18 protein in mitochondrial protein import, we searched for genes that genetically interact with *TIM18*. Tim18p is not essential, but a strain deleted of *TIM18* exhibits at least two phenotypes. First, cells lacking Tim18p are cold sensitive on rich medium containing glucose as the carbon source. Second, *tim18*Δ cells are inviable after plating onto medium containing EtBr (Kerscher *et al.* 2000). This treatment causes the rapid loss of mtDNA (Goldring *et al.* 1970). Inability to grow after mtDNA loss is called a petite-negative phenotype (Chen and Clark-Walker 2000). We used the petite-negative phenotype of the *tim18*Δ mutant as the basis for our high-copy suppression screen.

We transformed a *tim18*Δ strain with a yeast genomic library on a 2μ-*URA3* vector. Plasmids containing the 2μ origin of replication are maintained at high copy, often resulting in the overexpression of genes carried on those plasmids (Rine 1991). We picked 56 colonies that grew on EtBr-containing medium and isolated the plasmid DNA from each strain. Plasmids from 40 colonies were found to contain the *TIM18* gene and were discarded. Sixteen plasmids that did not encode Tim18p were subjected to limited sequencing of the genomic DNA inserts and found to represent five different chromosomal regions. Subclones containing different open reading frames from the inserts were then tested for the ability to suppress the *tim18*Δ mutant as described in materials and methods. We identified five genes, *CCT6*, *SSB1*, *ICY1*, *TIP41*, and *PBP1* as high-copy suppressors of the *tim18*Δ lethality on EtBr-containing medium. As shown in Figure 1, when *tim18*Δ cells containing an empty vector were pregrown on medium with EtBr (Figure 1A) and then subcultured onto media lacking EtBr (Figure 1B), the *tim18*Δ strain failed to grow. In other words, the EtBr-induced mtDNA loss was incompatible with the lack of the Tim18 protein. However, if the *tim18*Δ mutant carried 2μ-*URA3* plasmids with *CCT6*, *SSB1*, *ICY1*, *TIP41*, or *PBP1*, the cells were able to grow after EtBr treatment nearly as well as *tim18*Δ cells with

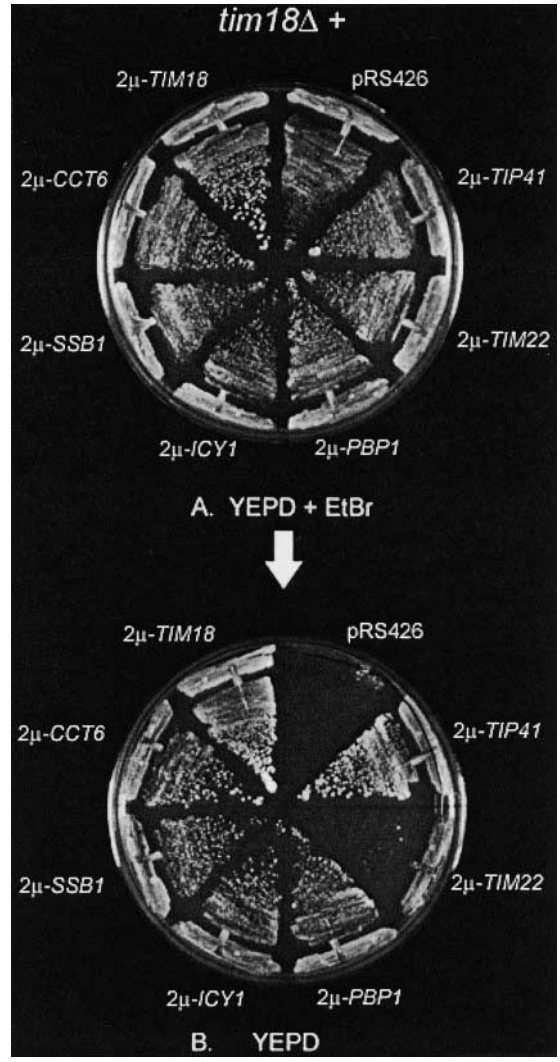


Figure 1.—Cytosolic proteins rescue the mtDNA dependence of *tim18*Δ cells. *tim18*Δ strain RJ992 was transformed with 2μ-*URA3* plasmids carrying *TIM18* (pM346), *CCT6* (pM350), *SSB1* (pM351), *ICY1* (pM352), *PBP1* (pM353), *TIM22* (pJH202; Kerscher *et al.* 1997), *TIP41* (pM345), or empty vector pRS426 (Sikorski and Hieter 1989). (A) Ura$^+$ transformants were streaked out onto SD-Ura plates containing 25 μg/ml EtBr to induce loss of mtDNA and allowed to grow at 34° for 3 days. (B) To identify strains killed by the EtBr treatment, cells from plate A were streaked to SD-Ura medium lacking EtBr, and the plates were incubated at 34° for 4 days.

a *TIM18*-containing plasmid. Although it was previously reported that increased levels of *TIM22* partially suppressed the *tim18*Δ mutant (Kerscher *et al.* 2000), we found that 2μ plasmids with *TIM22* did not allow *tim18*Δ cells to grow without mtDNA (Figure 1). Therefore, our results indicate that the mtDNA dependence of *tim18*Δ strains is not due strictly to lower steady-state levels of Tim22p.

Although Tim18p is a mitochondrial IM protein, we noted that none of the proteins encoded by our suppressors have been localized to mitochondria and all appear to be cytosolic proteins. Cct6p is a member of the TriC chaperonin complex and is thought to function in protein folding in the cytosol (Li *et al.* 1994; Hartl and Hayer-Hartl 2002). Ssb1p is a member of the Hsp70 chaperone family and has been shown to interact with nascent chains during their synthesis (Hundley *et al.* 2002). While little is known about Icy1p, this protein suppresses a mutation in the Tcp1 protein, which is a partner of Cct6p within the TriC complex (D. Ursic, unpublished observations). *TIP41* encodes a cytosolic protein proposed to function as a negative regulator of the yeast TOR-signaling pathway (Jacinto *et al.* 2001). Pbp1p interacts with a (poly)A-binding protein and, like Ssb1p, can be found associated with cytosolic ribosomes (Mangus *et al.* 1998). Deletions of *SSB1*, *ICY1*, *TIP41*, and *PBP1* are viable, while *CCT6* is an essential gene (Craig and Jacobsen 1985; Li *et al.* 1994; Mangus *et al.* 1998; Jacinto *et al.* 2001; Giaever *et al.* 2002).

In addition to suppressing the mtDNA dependence of *tim18*Δ cells, 2μ plasmids carrying *SSB1*, *PBP1*, and *ICY1* partially rescued the cold sensitivity of the *tim18*Δ mutant (C. Dunn, unpublished observations). *CCT6*- and *TIP41*-containing plasmids either did not suppress the cold-sensitive phenotype of *tim18*Δ or did so very weakly. Why genes such as *CCT6* or *TIP41* could suppress the mtDNA dependence of *tim18*Δ mutants, but not the cold sensitivity, is not clear.

Strains defective in other steps of the mitochondrial protein import pathway are also inviable without mtDNA: We found that the requirement for mtDNA is not restricted to *tim18*Δ cells and that other members of the import machinery are necessary for the viability of petite cells. For example, we found that cells lacking the mitochondrial outer membrane Tom70 receptor protein cannot live without mtDNA. Like *tim18*Δ cells, the *tom70*Δ mutant failed to grow after EtBr treatment (Figure 2A). We also found that the presence of Tim54p and a fully functional Tim10p, two other components of the TIM22 pathway, are required for petite cell viability (C. Dunn, unpublished observations). Since Tom70p, Tim10p, Tim54p, and Tim18p are all part of the TIM22 pathway, it is possible that dependence on mtDNA is limited to this import route. Supporting this idea, we find that cells with a deficient TIM23 pathway are viable after mtDNA loss, as a strain deleted of the outer membrane Tom20p import receptor and strains containing the mutant

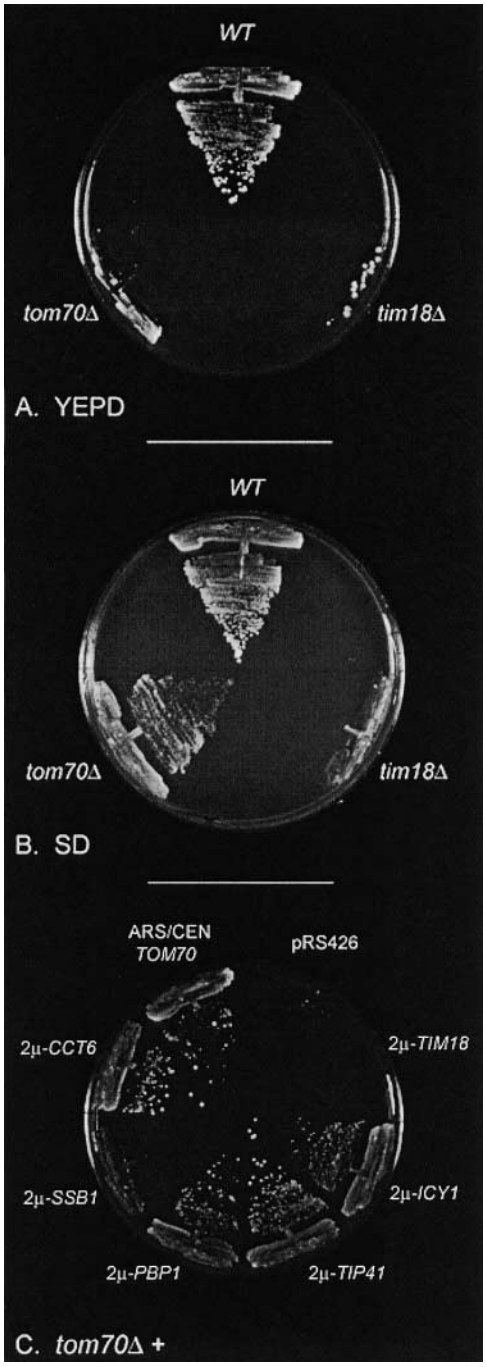


Figure 2.—*tom70*Δ cells require mtDNA when grown on rich medium. Wild-type (WT; RJ868), *tim18*Δ (RJ1263), and *tom70*Δ (RJ1583) were streaked to SD complete medium + EtBr and grown at 30° for 3 days (not shown). Strains were then streaked to YEPD (A) or SD complete (B) medium lacking EtBr and grown for 3 days at 30°. (C) *tom70*Δ strain RJ1583 was transformed with 2μ-*URA3* plasmids with *CCT6* (pM350), *SSB1* (pM351), *PBP1* (pM353), *TIP41* (pM345), *ICY1* (pM352), *TIM18* (pM346), *TOM70*-containing ARS/CEN plasmid pM354, or empty vector pRS426. Ura^+ transformants were pregrown on EtBr-containing medium and then streaked onto YEPD plates lacking EtBr.

Tim23-1 or Ssc1-2 proteins grow after EtBr treatment (C. Dunn and J. Emtage, unpublished observations). Nonetheless, further analyses are needed to show that mtDNA dependence is truly specific to one import pathway.

In contrast to the *tim18*Δ mutant, the mtDNA dependence of our *tom70*Δ strain was seen only on rich medium. *tom70*Δ cells were viable after EtBr treatment when grown on minimal media (Figure 2B). *tim18*Δ cells could not grow if forced to lose their mtDNA on either rich or minimal medium (compare A and B in Figure 2). A further indication that the mtDNA requirement for *tom70*Δ cells was not as strict as for the *tim18*Δ mutant was that a *tom70*Δ ρ^0 strain derived from the YPH499 background (Sikorski and Hieter 1989) could lose its mtDNA on both rich and minimal media (C. Dunn, unpublished observations), while the requirement of *tim18*Δ cells for mtDNA is not background specific.

For *tom70*Δ and other mutants (see below), a few survivor colonies appeared after EtBr treatment, even though the vast majority of cells died. *tom70*Δ survivors after EtBr treatment were found to have lost functional mtDNA, since they were no longer viable on nonfermentable medium. Preliminary results suggest that, in two cases, nuclear-encoded mutations allowed *tom70*Δ cells to live after mtDNA loss. However, since we tested only two survivors, it is also possible that some colonies simply adapted to mtDNA loss.

We found that our plasmid suppressors of the *tim18*Δ mutant also rescued the growth defect of *tom70*Δ cells after EtBr treatment. A *tom70*Δ strain carrying 2μ-*URA3* constructs with *CCT6*, *PBP1*, *ICY1*, or *TIP41* were able to grow after mtDNA loss (Figure 2C). For reasons that are not clear, *SSB1* only weakly suppressed the *tom70*Δ defect. We also noted that while *tim18*Δ and *tom70*Δ are both petite negative, and both mutants can be rescued by the same set of suppressor-containing plasmids, multiple copies of *TIM18* did not bypass the mtDNA requirement of *tom70*Δ (Figure 2C). Our observations support the view that a defect in any step in the polytopic protein import pathway, and not the loss of one protein *per se*, leads to mtDNA dependence.

Cells disrupted in *SSB1*, *PBP1*, *ICY1*, and *TIP41* are petite negative: Since *CCT6*, *SSB1*, *ICY1*, *TIP41*, and *PBP1* were found to suppress the lethality of both *tim18*Δ and *tom70*Δ after EtBr treatment, we asked if these genes are themselves required for cell viability in the absence of mtDNA. Our results show that Ssb1p, Icy1p, Tip41p, and Pbp1p are required for growth without mtDNA (Figure 3A). Since *CCT6* is an essential gene, we were unable to examine the relationship of Cct6p and mtDNA using this approach. Like the *tom70*Δ mutant, *ssb1*Δ, *icy1*Δ, *tip41*Δ, and *pbp1*Δ cells were viable after EtBr treatment when grown on minimal medium (Figure 3B). Hence the need for these proteins is not as strict as for the Tim18 protein.

The mtDNA dependence of a strain lacking one of the cytosolic suppressors can itself be rescued by multiple copies of the other suppressors. For example, the *ssb1*Δ mutant carrying an empty vector cannot grow after EtBr treatment on rich medium (Figure 3C). In contrast, 2μ

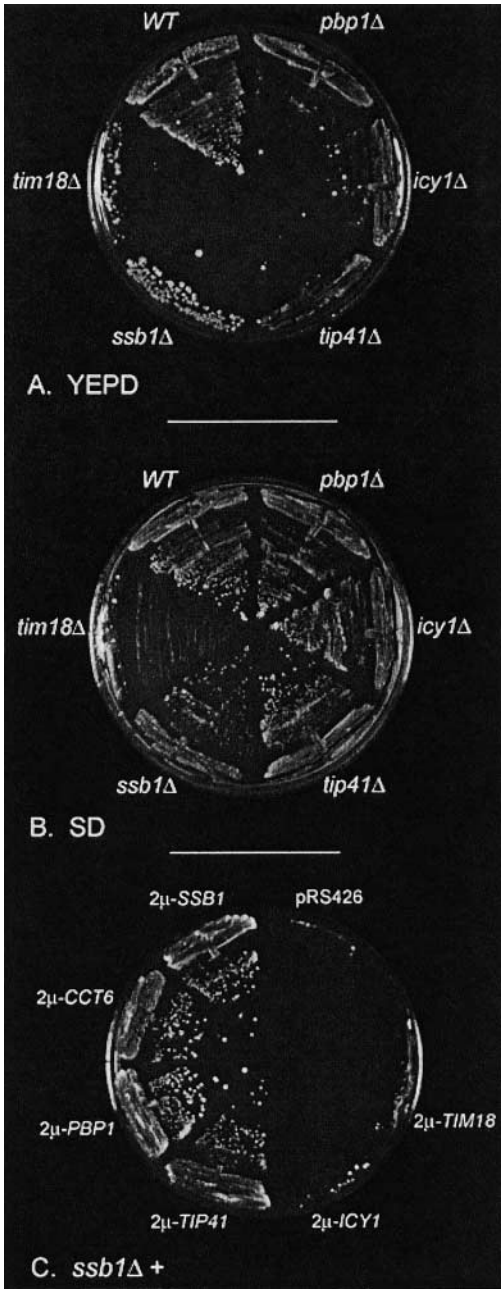


Figure 3.—*pbp1*Δ, *icy1*Δ, *tip41*Δ, and *ssb1*Δ mutants are petite negative. Wild-type (WT; RJ868), *tim18*Δ (RJ1263), *pbp1*Δ (RJ1587), *icy1*Δ (RJ1585), *tip41*Δ (RJ1584), and *ssb1*Δ (RJ1586) were pregrown on EtBr-containing minimal medium and then streaked onto either YEPD (A) or SD complete (B) medium lacking EtBr and grown for 3 days at 30°. (C) Rescue of the mtDNA dependence of *ssb1*Δ by *tim18*Δ suppressor plasmids. *ssb1*Δ strain RJ1586 was transformed with *SSB1* (pM351), *CCT6* (pM350), *PBP1* (pM353), *TIP41* (pM345), *ICY1* (pM352), *TIM18* (pM346), or empty vector pRS426. Ura^+ transformants were pregrown on YEPD medium with EtBr and then streaked to YEPD plates lacking EtBr.

TABLE 2

***tim18*Δ suppressors increase the growth rate of ρ^0 cells**

Plasmid	Fold increase in growth rate
2μ-*PBP1*	1.70 ± 0.37
2μ-*TIP41*	1.68 ± 0.35
2μ-*ICY1*	1.67 ± 0.32
2μ-*CCT6*	1.58 ± 0.31
2μ-*SSB1*	1.48 ± 0.26
2μ-*TIM18*	1.29 ± 0.28
Vector	1

ρ^0 strain RJ1582 was transformed with 2μ-*URA3* plasmids carrying *CCT6*, *SSB1*, *PBP1*, *ICY1*, *TIP41*, *TIM18*, or the empty vector pRS426 (Sikorski and Hieter 1989). The growth rate of log-phase cultures was determined by measuring the OD_{600} at different timepoints. Three independent experiments were performed for each strain, with the average increase in growth rate and one standard deviation from the mean shown.

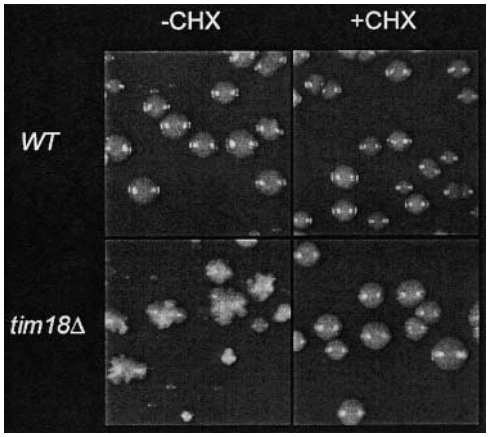


Figure 4.—Inhibiting protein synthesis suppresses the growth defect of *tim18*Δ cells lacking mtDNA. Wild type (WT; RJ868) and *tim18*Δ (RJ1263) cells were grown on YEPD medium lacking (−CHX) or containing (+CHX) 0.2 mg/ml cycloheximide at 30°.

plasmids with *CCT6*, *PBP1*, *TIP41*, and *SSB1* allowed *ssb1*Δ cells to grow without mtDNA. We noted that *ICY1* did not rescue *ssb1*Δ. Although the reasons are not apparent, it is possible that Icy1p requires Ssb1p for activity. Similar to our results with *tom70*Δ, we found that multiple copies of *TIM18* did not allow *ssb1*Δ cells to grow after EtBr treatment. We speculate that Tim18p, Tom70p, Ssb1p, Icy1p, Tip41p, Pbp1p, and Cct6p all participate in a common pathway and that each protein becomes even more crucial when cells lack mtDNA.

We also found that 2μ plasmids with *CCT6*, *PBP1*, *TIP41*, *ICY1*, and *SSB1* allowed cells lacking mtDNA, but otherwise wild type, to grow at faster rates. Strain RJ1582, which lacks mtDNA, was transformed with each plasmid, and the growth rate of cells was determined (Table 2). All isolated suppressors allowed RJ1582 to grow at a faster rate in comparison to cells carrying an empty vector. These observations provide additional evidence that these proteins play an important role in cells lacking mtDNA.

Slowing protein synthesis lessens the growth defect of *tim18*Δ cells: Our results showed that *tom70*Δ, *ssb1*Δ, *icy1*Δ, *tip41*Δ, and *pbp1*Δ mutants require mtDNA on rich medium, but not on minimal medium. Since the rate of translation is greater when cells are grown on rich media than on minimal media (Wehr and Parks 1969), we wondered if the problems caused by a deficient TIM22 pathway would be alleviated if the rate of protein synthesis were slowed by using sublethal amounts of the translation inhibitor cycloheximide. We were unable to test whether *tim18*Δ ρ^0 cells can grow in the presence of cycloheximide, because ρ^0 cells are quite resistant to the effects of cycloheximide as a result of activation of the pleiotropic drug resistance pathway (Hallstrom and Moye-Rowley 2000). However, we found that cycloheximide suppressed the growth defect of a ρ^+ *tim18*Δ mutant. As shown in Figure 4, instead of the smooth, round colony morphology seen with wild-type cells, the *tim18*Δ mutant forms colonies that are irregular and scallop shaped (Figure 4). Strikingly, if the *tim18*Δ mutant is grown on medium containing 0.2 μg/ml cycloheximide, single cells grow into perfectly smooth colonies (Figure 4). In similar studies, slowing protein synthesis with cycloheximide was found to suppress phenotypes associated with defects in the translocation of proteins into the endoplasmic reticulum (Ogg and Walter 1995; Wilkinson *et al.* 2001).

***tim18*Δ cells require a functional respiratory chain:** To pinpoint the reason that lack of mtDNA is incompatible with defects in mitochondrial protein import, we tested whether defects in nuclear-encoded electron transport proteins were tolerated by *tim18*Δ cells. We found that the *tim18*Δ mutation was lethal in combination with the lack of either cytochrome c_1 or a protein involved in ubiquinone biosynthesis. *tim18*Δ cells were crossed to a *cyt1*Δ strain or to a *coq4*Δ strain. We found that *tim18*Δ *cyt1*Δ and *tim18*Δ *coq4*Δ double mutants were initially viable when spores were allowed to germinate on glucose-containing medium. However, the double mutants failed to grow when they were subsequently streaked out for single colonies on YEPD medium (Figure 5). Why the lethality of *tim18*Δ *cyt1*Δ and *tim18*Δ *coq4*Δ mutants was delayed is not clear. *cyt1* and *coq4* deletion strains do not quickly lose mtDNA, as tested by DAPI staining of nucleoids in *coq4*Δ and *cyt1*Δ strains and by mating individual *coq4*Δ and *cyt1*Δ colonies to ρ^0 tester strains. Therefore, our observations indicate that the mtDNA dependence of *tim18*Δ cells is due to a general defect in electron transport.

Multiple copies of *CCT6*, *SSB1*, *ICY1*, *TIP41*, and *PBP1* rescue the mtDNA dependence of *atp2*Δ mutants, but not the need for ATP in the matrix: The results above raise the possibility that the lethality associated with loss of mtDNA may be due to a decrease in the electrochemical potential across the IM. When electron transport is compromised by loss of mtDNA, the IM potential is

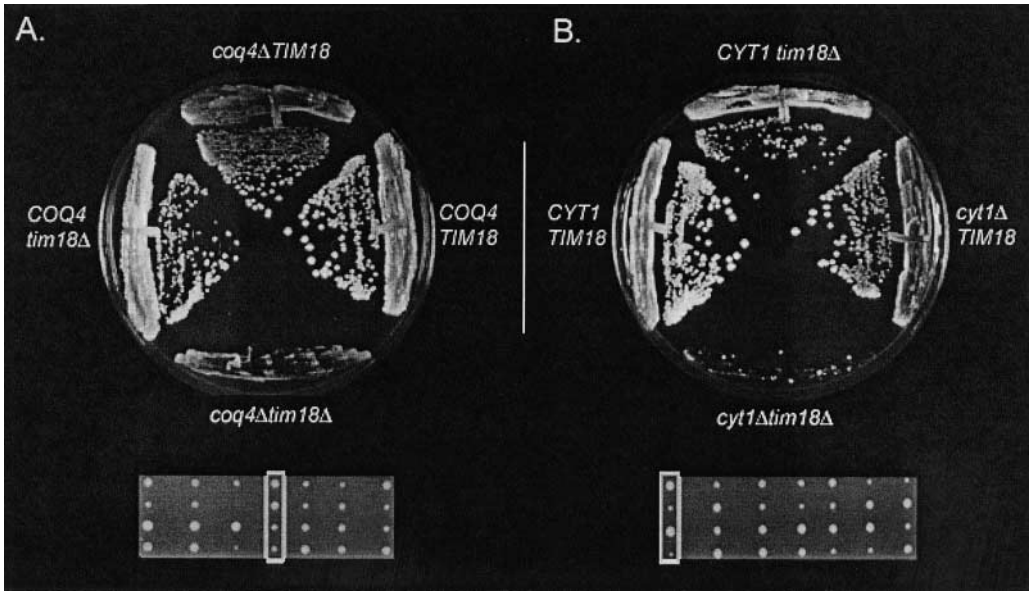


FIGURE 5.—*tim18*Δ is lethal in combination with nuclear-encoded defects in respiration. After tetrad dissection on YEPD at 34°, meiotic segregants were streaked onto YEPD medium and allowed to grow for 3 days at 30°. (A) Cells from the four spores of a tetratype tetrad produced by the *tim18*Δ (RJ1263) × *coq4*Δ (RJ1606) cross. The tetrad used for the analysis is indicated by the gray rectangle. (B) Cells from the four spores of a tetratype tetrad produced by the *tim18*Δ (RJ1263) × *cyt1*Δ (RJ1605). The tetrad used for the analysis is indicated by the shaded rectangle.

thought to be produced by the transport of ATP across the IM by the ATP/ADP carrier proteins, followed by ATP hydrolysis by the F_1 ATPase (CHEN and CLARK-WALKER 2000). Consistent with this view, *atp2*Δ mutants, lacking the β-subunit of the F_1 ATPase, and *op1* mutants, lacking a fully functional copy of the major ATP/ADP carrier Aac2p, are unable to grow without mtDNA (KOVACOVA *et al.* 1968; CHEN and CLARK-WALKER 1999).

We find that the high-copy suppressors of *tim18*Δ also rescued the petite-negative phenotype of *atp2*Δ mutants. While the *atp2*Δ mutant with an empty vector or with 2μ-*TIM18* does not grow without mtDNA, *atp2*Δ cells carrying *CCT6*, *SSB1*, *PBP1*, *TIP41*, or *ICY1*-containing plasmids grow at or near wild-type rates after treatment with EtBr (Figure 6A). Thus, the *tim18*Δ suppressors also rescue the petite-negative phenotype of cells lacking a protein known to be important for generating the IM potential, the *ATP2*-encoded ATPase subunit. These results therefore support our view that increased amounts of Ssb1p, Icy1p, Tip41p, Pbp1p, and Cct6p all alleviate the problems caused by a decreased mitochondrial potential in petite cells. We note that the mtDNA dependence of *atp2*Δ mutants is greatly reduced when cells are grown on minimal medium (Figure 6C), suggesting that other factors in addition to the F_1 ATPase can help generate the IM potential in cells lacking mtDNA.

The inner membrane protease Yme1p has been shown to be important for the growth of petite cells (THORSNESS *et al.* 1993). *YME1* genetically interacts with the F_1 ATPase and has been shown to increase the electrochemical potential in ρ^0 mitochondria (WEBER *et al.* 1995; KOMINSKY and THORSNESS 2000; KOMINSKY *et al.* 2002). We asked whether *YME1* might also interact genetically with *TIM18* or with any of its isolated suppressors. However, a strain lacking both Tim18p and Yme1p was viable (C. DUNN, unpublished results) and none of our isolated high-copy suppressors rescued the severe growth defect of a *yme1*Δ ρ^0 strain. Therefore, while both genes are required for growth of petite cells, no direct genetic link has yet been demonstrated between *YME1* and *TIM18*.

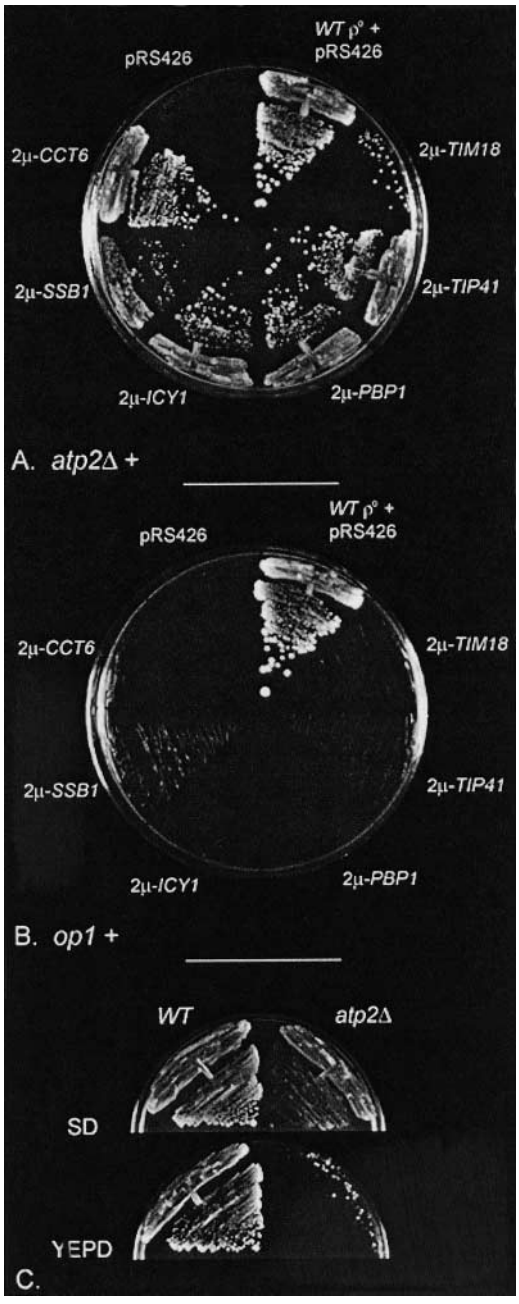
In addition, in contrast to *atp2*Δ, the mtDNA dependence of *op1* mutants is not rescued by our suppressor-containing plasmids. An *op1* strain carrying *CCT6*-, *SSB1*-, *PBP1*-, *TIP41*-, or *ICY1*-containing plasmids failed to grow at wild-type rates after treatment with EtBr (Figure 6B). Cells lacking mtDNA are acutely reliant upon the Aac2 protein, indicating an absolute need for mitochondrial ATP/ADP transport in petite cells.

DISCUSSION

To learn more about the role of the Tim18 protein, a member of the TIM22 inner membrane import complex, we isolated high-copy suppressors of the inability of *tim18*Δ mutants to grow in the absence of mtDNA. However, instead of finding new inner membrane components of the TIM22 complex, all of our suppressor-containing plasmids encode proteins thought to be located within the cytosol, such as the Cct6, Ssb1, Icy1, Tip41, and Pbp1 proteins.

We found that dependence upon mtDNA is not limited to the *tim18* mutant; mutations in genes encoding other mitochondrial protein import components can also generate a petite-negative phenotype. For example, cells lacking the TIM22 complex member Tim54p were unable to grow on medium that selects for mtDNA loss. In addition, a strain containing the *tim10-2* allele, encoding a defective intermembrane space member of the TIM22 complex, is petite negative. Furthermore, a *tom70*Δ disruption mutant lacking the outer membrane Tom70p receptor was inviable after treatment with ethidium bromide. Since Tim18p, Tim54p, Tim10p, and Tom70p all

function in the TIM22 pathway, which is required for the import of polytopic IM proteins, it is possible that this pathway is particularly sensitive to loss of mtDNA. Consistent with this possibility, we find that mutations in three members of the TIM23 pathway, which is required primarily for the import of presequence-containing proteins into the matrix, are not lethal in combination with mtDNA loss. A *tom20*Δ strain, lacking the Tom20p receptor, and the *tim23-1* mutant, defective in the inner membrane TIM23 complex protein Tim23p, are completely viable following growth on ethidium-bromide-containing medium. Moreover, a *ssc1-2* mutant, deficient in translocation through the TIM23 complex, can live in the absence of mtDNA. However, further studies are needed to determine if mtDNA dependence is truly specific to deficiencies within the TIM22 pathway.



Why are defects in mitochondrial protein import incompatible with mtDNA loss? Mitochondrial DNA encodes for proteins that function in electron transport. We argue that the lack of mitochondrial ATP synthesis is not the cause of the mtDNA dependence of import mutants, since *tim18*Δ, *tim54*Δ, *tim10-2*, and *tom70*Δ cells lacking mtDNA are dead even on glucose-containing medium. On glucose, sufficient levels of ATP are produced by glycolysis and mtDNA is not required (Chen and Clark-Walker 2000). Instead, we suggest that the petite-negative phenotype of the import mutants is caused by a lowered IM potential resulting from defective electron transport in cells lacking mtDNA. Supporting this idea, we found that *tim18*Δ is lethal in combination with mutations in two different nuclear-encoded components required for electron transport, cytochrome c_1 and Coq4p (Schneider and Guarente 1991; Belogrudov *et al.* 2001). In addition, suppressors of *tim18*Δ also suppress the petite-negative phenotype of a strain lacking *ATP2*, encoding a protein believed to play a role in generating mitochondrial potential in the absence of mtDNA (Giraud and Velours 1997; Chen and Clark-Walker 1999). Since the mitochondrial potential is essential for activity of the TIM22 complex, we suggest that when IM potential is lowered, changes in the efficiency of import caused by the loss of nonessential proteins, such as Tim18p, Tim54p, or Tom70p, or conditional defects in the function of an essential protein, such as Tim10p, become magnified. We propose that under these conditions imported proteins build up outside the mitochondria and are toxic to the yeast cell. Our model is supported by the properties of the high-copy suppressors of the petite-negative phenotype of the *tim18*Δ mutant.

Our suppressors seem to fall into at least two categories: cytosolic chaperones and proteins that may affect protein synthesis. *SSB1* and *CCT6* encode proteins known to function as cytosolic chaperones. Ssb1p is a member of the Hsp70 family (Boorstein *et al.* 1994) and Cct6p

Figure 6.—(A) The petite-negative phenotype of the *atp2*Δ mutant is rescued by *tim18*Δ suppressors. *atp2*Δ strain RJ1577 was transformed with 2μ-*URA3* plasmids containing *CCT6* (pM350), *SSB1* (pM351), *PBP1* (pM353), *TIP41* (pM345), *ICY1* (pM352), *TIM18* (pM346), and pRS426. ρ^0 strain RJ1582 transformed with pRS426 is also shown. Cells were pregrown on YEPD medium with EtBr and then streaked to YEPD plates lacking EtBr and grown for 4 days at 30°. (B) *op1* cells, lacking functional Aac2 protein, remain mtDNA dependent. *op1* strain RJ1581 was transformed with 2μ-*URA3* plasmids containing *CCT6* (pM350), *SSB1* (pM351), *PBP1* (pM353), *TIP41* (pM345), *ICY1* (pM352), and *TIM18* (pM346), pregrown on YEPD medium with EtBr and then streaked to YEPD medium lacking EtBr for 5 days at 30°. (C) *atp2*Δ cells lacking mtDNA grow slowly on minimal medium, but fail to grow on rich medium. Wild type strain RJ868 and *atp2*Δ strain RJ1577 were pregrown on either SD complete or YEPD medium containing EtBr for 3 days at 30° and then subcultured on the same medium lacking EtBr for 4 days at 30°.

is part of the heteroligomeric TriC chaperonin complex (Li *et al.* 1994). One explanation for how high-copy plasmids encoding *SSB1* and *CCT6* suppress the mtDNA dependence of some import mutants is that cytosolic chaperones can become limiting when mitochondrial protein import is compromised. Since mitochondrial precursor proteins bind to chaperones prior to their import into mitochondria (Beddoe and Lithgow 2002), precursors that build up outside the mitochondria may compete with other, essential cellular processes for chaperone activity and therefore lead to cell death. Under these conditions, increasing the amount of a limiting chaperone would restore viability.

Cytosolic Hsp70 proteins have been shown to facilitate the import of several different mitochondrial precursor proteins (Beddoe and Lithgow 2002), and it has been recently reported that Hsp70 proteins directly transfer some preproteins to the Tom70p receptor (Young *et al.* 2003). Thus, it is reasonable to assume that chaperones, such as Ssb1p, might bind mitochondrial precursors accumulating due to a defect in import. Supporting this idea, the bacterial Hsp70 protein dnaK has been found to tightly bind to membrane protein precursors when the activity of the Sec translocon is diminished (Qi *et al.* 2002). However, since Ssb1p is only one of several cytosolic Hsp70 proteins, it is not clear why other Hsp70 family members were not isolated in our genetic screen. Furthermore, we found that 2μ plasmids carrying *SSB2* did not suppress the mtDNA dependence of *tim18*Δ mutants (C. Dunn, unpublished observations). Since Ssb1p differs at only four amino acid residues from Ssb2p, further studies are needed to determine if Ssb1p truly plays some unique role in mitochondrial protein import.

In contrast to Hsp70 proteins, there is no compelling evidence that the TriC chaperonin complex plays a direct role in mitochondrial protein import. However, TriC has been shown to interact with peroxisomal (Pause *et al.* 1997) and endoplasmic reticulum (ER; Plath and Rapoport 2000) proteins prior to their import, so it would not be surprising if the chaperonin complex did facilitate the import of mitochondrial proteins. On the other hand, it is possible that the abnormal accumulation of mitochondrial proteins recruits chaperones not normally associated with protein import. Most of the substrates for the TIM22 pathway are polytopic membrane proteins, and these hydrophobic proteins would be particularly prone to aggregation in the cytosol. However, since TriC is composed of eight different subunits (Lin and Sherman 1997; Liou and Willison 1997), it is curious that only one TriC complex member was identified in our suppressor screen. Perhaps overexpressed Cct6p provides some chaperone activity in the absence of the other TriC subunits. Alternatively, Cct6p may have a more indirect role in suppressing problems with protein import, possibly by acting in a signaling pathway (Schmidt *et al.* 1996).

The *ICY1* gene was also identified as a high-copy suppressor in our studies. Since *ICY1* genetically interacts with the TriC complex, suppressing a mutation in the TriC subunit Cct1p (D. Ursic, unpublished observations), we include Icy1p in the same category with the chaperones Ssb1p and Cct6p. Although the function of Icy1p is not known, it is interesting to note that the expression of *ICY1* increases when mtDNA is lost (Epstein *et al.* 2001).

Proteins and conditions that appear to decrease the rate of protein synthesis make up our second category of suppressors of the petite-negative *tim18*Δ mutant. For example, high-copy *TIP41* was isolated in our screen. Tip41p is a negative regulator of the TOR-signaling pathway (Jacinto *et al.* 2001), and the TOR pathway functions in sensing nutrient levels and adjusting translation accordingly (Schmelzle and Hall 2000). Thus, it is possible that overexpression of Tip41p decreases translation. We find that cycloheximide suppresses a growth defect of the *tim18*Δ strain, also indicating that slowing protein synthesis can ameliorate a defect in protein import. Moreover, decreasing translation with cycloheximide or by mutation has been shown to rescue phenotypes associated with defective protein translocation into the ER (Ogg and Walter 1995; Wilkinson *et al.* 2001) and bacterial secretion (Lee and Beckwith 1986). The expression of many components of the cytosolic translational machinery are downregulated upon loss of mtDNA (Epstein *et al.* 2001), suggesting that modulating protein synthesis is a general cellular adaptation to mtDNA loss. If our model is correct, decreased translation would prevent the toxic accumulation of unimported mitochondrial proteins.

Although we have suggested that Ssb1p, Cct6p, and Icy1p suppress defects in protein import by binding to accumulated precursor proteins, we are also open to the possibility that overexpression of these proteins may instead regulate translation. For example, both Ssb1p (Hundley *et al.* 2002) and the TriC complex (McCallum *et al.* 2000) are thought to bind to nascent chains. Perhaps inappropriate amounts of Ssb1p, Cct6p, and/or Icy1p interact directly with the ribosome and slow protein synthesis.

PBP1 was also isolated as a suppressor in our genetic screen. Pbp1p interacts with a poly(A)-binding protein (Mangus *et al.* 1998) and is found, like Ssb1p, in the nucleus on ribosomes and in the cytosol (Mangus *et al.* 1998). As suggested for Ssb1p, increased levels of Pbp1p could rescue an import defect by either affecting protein synthesis or functioning as a chaperone. Strikingly, *PBP1* was first isolated in a screen that also identified Tim10p and Tim12p (Waldherr *et al.* 1993), proteins known to be part of the TIM22 import pathway. The actual role of Pbp1p, however, awaits further analysis.

Although we favor the idea that multiple copies of *CCT6*, *SSB1*, *ICY1*, *TIP41*, and *PBP1* prevent toxic accumulation of mitochondrial precursor proteins, there are additional possibilities that could explain their suppressor effects. For example, one or more of the suppressors

could in some way act to increase the mitochondrial potential when overexpressed, thus allowing mitochondrial import to occur more efficiently in cells lacking mtDNA. Evidence for this possibility is lacking since staining of ρ^0 cells containing the high-copy suppressors with the potentially dependent dye $DiOC_6$ did not show increased mitochondrial fluorescence (C. Dunn, unpublished observations). Alternatively, the suppressors may modulate a mitochondria-to-nucleus signaling pathway, such as the retrograde response pathway (Liu *et al.* 2001; Crespo *et al.* 2002). It is also possible that the suppressors all act to increase the import or stability of a single mitochondrial component. Future experiments are clearly needed to determine the mechanism by which *CCT6*, *SSB1*, *ICY1*, *TIP41*, and *PBP1* act in rescuing petite-negative cells.

It is interesting to note that not only do *SSB1*, *ICY1*, *TIP41*, and *PBP1* suppress the mtDNA dependence of some import mutants, but also disruption of any of these genes produces cells that are themselves petite negative. Our studies have therefore identified a genetic pathway, starting with the mitochondria IM proteins Tim18p and Tim54p, proceeding through the intermembrane space (Tim10p) and the outer membrane (Tom70p), and ending in the cytosol with the Cct6, Ssb1, Icy1, Tip41, and Pbp1 proteins. All of these components are required for yeast cell growth in the absence of mtDNA.

Our initial goal in the isolation of suppressors of *tim18*Δ was to further understand the role that Tim18p plays in import. Because *tim18*Δ mutants are dead in cells lacking a functional respiratory chain, we originally thought that Tim18p functions at a potentially dependent step in import. At first glance, our observations that mutants defective in nonmitochondrial proteins are also dependent upon mtDNA seem to argue against this possibility. However, we found that *tim18*Δ mutants are more sensitive to mtDNA loss than are most of our other mutants. *tim18*Δ cells are unable to grow on either rich or minimal medium containing ethidium bromide, whereas cells carrying *tom70*Δ, *ssb1*Δ, *icy1*Δ, *tip41*Δ, and *pbp1*Δ mutations are dead only on rich medium following mtDNA loss. Since strains grown on rich medium exhibit increased protein synthesis rates in comparison to those grown on minimal medium (Wehr and Parks 1969), these observations further support our conclusion that slowing translation helps cells cope with the loss of mtDNA. Nonetheless, the exquisite mtDNA dependence of *tim18*Δ mutants again raises the possibility that Tim18p may truly act in a potentially dependent manner. Studies to further examine this possibility are underway.

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