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# Overproduction of *PDR3* Suppresses Mitochondrial Import Defects Associated with a *TOM70* Null Mutation by Increasing the Expression of *TOM72* in *Saccharomyces cerevisiae*

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**Most mitochondrial proteins are synthesized with cleavable amino-terminal targeting signals that interact with the mitochondrial import machinery to facilitate their import from the cytosol. We previously reported that the presequence of the $F_1$-ATPase β subunit precursor (pre-$F_1$β) acts as an intramolecular chaperone that maintains the precursor in an import-competent conformation prior to import (P. Hajek, J. Y. Koh, L. Jones, and D. M. Bedwell, Mol. Cell. Biol. 17:7169–7177, 1997). We also found that a mutant form of pre-$F_1$β with a minimal targeting signal (Δ1,2 pre-$F_1$β) is inefficiently imported into mitochondria because it rapidly folds into an import-incompetent conformation. We have now analyzed the consequences of reducing the pre-$F_1$β targeting signal to a minimal unit in more detail. We found that Δ1,2 pre-$F_1$β is more dependent upon the Tom70p receptor for import than WT pre-$F_1$β is, resulting in a growth defect on a nonfermentable carbon source at 15°C. Experiments using an in vitro mitochondrial protein import system suggest that Tom70p functions to maintain a precursor containing the Δ1,2 pre-$F_1$β import signal in an import-competent conformation. We also identified *PDR3*, a transcriptional regulator of the pleiotropic drug resistance network, as a multicopy suppressor of the mitochondrial import defects associated with Δ1,2 pre-$F_1$β in a *tom70*Δ strain. The overproduction of *PDR3* mediated this effect by increasing the import of Δ1,2 pre-$F_1$β into mitochondria. This increased the mitochondrial ATP synthase activity to the extent that growth of the mutant strain was restored under the selective conditions. Analysis of the transcription patterns of components of the mitochondrial outer membrane import machinery demonstrated that *PDR3* overproduction increased the expression of *TOM72*, a little studied *TOM70* homologue. These results suggest that Tom72p possesses overlapping functions with Tom70p and that the pleiotropic drug resistance network plays a previously unappreciated role in mitochondrial biogenesis.**

The vast majority of mitochondrial proteins are directed to their final subcellular location with great specificity. Most proteins targeted to the mitochondrial matrix contain a cleavable amino-terminal presequence with basic and hydroxylated amino acids interspersed throughout their length (14, 15, 28, 29, 40, 56). The ability of these presequences to form an amphiphilic alpha-helical structure is thought to be a prime determinant of mitochondrial targeting (6, 47, 48, 58). Mitochondrial targeting signals facilitate efficient protein translocation across the outer mitochondrial membrane by mediating interactions with cytosolic cofactors and the translocase of the outer membrane (TOM) complex (1, 2, 4, 7, 11, 42). In *Saccharomyces cerevisiae*, the multisubunit TOM complex is thought to consist of a core comprised of Tom40p, Tom5p, Tom6p, and Tom7p. In addition, at least four outer membrane proteins, Tom20p-Tom22p and Tom70p-Tom37p, make up two receptor systems with partially overlapping specificity for precursor binding (2, 4, 18, 23, 26, 33, 38, 39).

Upon their synthesis on cytosolic polysomes, many precursors must be maintained in an extended conformation to be imported efficiently. It was previously shown that the presequence of the $F_1$-ATPase β subunit precursor (pre-$F_1$β) acts as an intramolecular chaperone by maintaining the preprotein in an import-competent state (21). The precursor may also be maintained in an import-competent conformation by molecular chaperones such as Hsp70 and Ydj1. A unique molecular chaperone, mitochondrial import stimulating factor (MSF), has been purified from reticulocyte lysate and shown to selectively bind mitochondrial precursors (3, 19). However, a yeast homologue has not been identified. Current models hypothesize that the MSF-precursor complex specifically docks with the Tom70p-Tom37p receptor complex in an ATP-dependent manner. The precursor is then sequentially delivered to Tom20p-Tom22p and the core TOM complex (7, 23, 27). After crossing the outer mitochondrial membrane, the targeting signal is inserted into the inner mitochondrial membrane in a Δψ-dependent manner (15, 37, 41). It then interacts with the translocase of the inner membrane complex, and its transport into the matrix is facilitated by an ATP-dependent motor formed by mitochondrial Hsp70p, Mge1p, and Tim44p (35, 41, 46, 53, 55).

Recent studies have sought to delineate the function and specificity of the receptor components in binding mitochondrial precursor proteins. Studies with purified cytosolic domains of Tom20p, Tom22p, and Tom70p indicate that these receptor subunits carry out a division of labor in binding mitochondrial proteins. In vitro experiments showed that the

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cytosolic domains of Tom20p and Tom22p preferentially bind precursors that contain amino-terminal targeting signals. In contrast, Tom70p interacts most strongly with precursors that have internal targeting signals. However, Tom70p has also been reported to stimulate the import of several precursors with amino-terminal presequences (24, 25). Strains containing knockouts of Tom20p, Tom37p, or Tom70p are viable but display mild growth defects on nonfermentable carbon sources. Furthermore, the overexpression of *TOM70* can restore the ability of a *tom20*Δ strain to grow on a nonfermentable carbon source. While the simultaneous disruption of *TOM70* and *TOM20* is lethal, growth can be restored by the overexpression of *TOM22* (27). These results suggest that the proteins encoded by these genes have overlapping and cooperative roles in mitochondrial binding and import.

The pleiotropic drug resistance (PDR) pathway controls the expression of a number of genes that mediate resistance to a broad range of structurally and functionally unrelated compounds. Recently, the PDR pathway was also reported to play a role in monitoring the functional status of mitochondria (22). Hallstrom and Moye-Rowley demonstrated that mitochondrial defects arising from the loss of a nuclear-encoded gene involved in electron transport activity or maintenance of the mitochondrial genome resulted in the increased expression of *PDR3*, a transcription factor that regulates the expression of many genes in the PDR pathway (22). In some cases, this stimulation of Pdr3p expression was mediated by the retrograde signaling pathway, which has been shown to respond to mitochondrial defects through the activation of several nuclear genes (31, 36). In addition, it was previously shown that a hyperactivating allele of *PDR3* results in an inability to respire on nonfermentable carbon sources (34). These results suggest a role for Pdr3p in regulating certain aspects of mitochondrial function.

In the present study, we utilized a well-characterized mutant form of pre-$F_1\beta$ with a minimal targeting signal to further examine the function of mitochondrial protein import receptors (5, 6, 21). Using an in vivo kinetic analysis, we found that the minimal targeting signal greatly increased the dependence of the pre-$F_1\beta$ precursor on Tom70p for mitochondrial import. Experiments using an in vitro mitochondrial protein import system suggested that this requirement for Tom70p is related to an inability to maintain this mutant precursor in an import-competent conformation. The resulting import defect was so severe that a *tom70*Δ strain expressing this mutant precursor exhibited a cold-sensitive phenotype on a nonfermentable carbon source. A multicopy suppressor screen revealed that the overexpression of *PDR3* stimulated the import of this defective precursor and restored growth under these conditions. Our results indicate that the import of this precursor is increased by the *PDR3*-mediated stimulation of *TOM72* expression, a little studied *TOM70* homologue.

## MATERIALS AND METHODS

**Strains and growth conditions.** The *S. cerevisiae* strains used in this study were SEY6215 (*MAT***a** *his3-Δ200 leu2-3,112 ura3-52 trp1-Δ901 atp2*Δ::*LEU2*), YDB210 (*MAT*α *his3-Δ200 leu2-3,112 ura3-52 trp1-Δ901 tom70*Δ::*HIS3 atp2*Δ::*LEU2*), YDB403 (*MAT***a** *his3-Δ200 leu2-3,112 ura3-52 trp1-Δ901 atp2*Δ::*LEU2 tom72*Δ::*hisG*), and YDB404 (*MAT*α *his3-Δ200 leu2-3,112 ura3-52 trp1-Δ901 tom70*Δ::*HIS3 atp2*Δ::*LEU2 tom72*Δ::*hisG*). The *TOM72* mutant strains were derived from SEY6215 and YDB210 using standard yeast genetic techniques (54). Yeast transformations were performed by the alkali cation technique (30, 50) and selected on SD medium (54) containing required supplements.

**Gene disruptions.** A gene disruption of *TOM70* was generated from pVH19 by deleting a 948-bp *Bgl*II fragment that included the AUG initiation codon of *TOM70*. The resulting 3.6-kb linear plasmid was ligated with a 1.8-kb *Bam*HI fragment containing *HIS3*. The resulting plasmid was digested with *Bam*HI and *Eco*RI to generate a 4.2-kb fragment that was transformed into SEY6215 by one-step gene disruption. Transformants were selected on SD plates for the histidine auxotrophic marker, and the correct integration event was verified by Southern blotting. The resulting strain was designated YDB210 for this study.

A gene disruption of *TOM72* was generated from pGAL-*TOM72* that was a generous gift from Trevor Lithgow. The 3.05-kb *Eco*RI-*Bam*HI fragment containing the *TOM72* structural gene and 788 bp of the 5′ untranslated region was subcloned into pSEY8. The gene disruption was constructed by deletion of a 799-bp *Xho*I-*Bst*XI fragment from the *TOM72* gene that included the AUG initiation site for the structural gene. The linear plasmid was treated with T4 DNA polymerase to generate blunt ends and religated. A 4.3-kb *Bam*HI insert containing *hisG-URA3-hisG* was then ligated into a *Bam*HI-digested plasmid containing the disruption of *TOM72*. The resulting plasmid was then digested with *Bam*HI and *Eco*RI, and the 6.5-kb fragment was isolated and purified by Genelute (Bio-Rad) and ethanol precipitation and then transformed into SEY6215 and YDB210 cells. Transformants were selected for the uracil auxotrophic marker and purified. Genomic DNA was prepared from each transformant, and the correct integration event was verified by PCR. The *URA3* gene was removed through the use of 5-fluoro-orotic acid. SEY6215 *tom72*Δ was designated YDB403 and YDB210 *tom72*Δ was designated YDB404 for these studies.

**Construction of plasmids.** The yeast shuttle plasmid pDB425 containing WT (WT) pre-$F_1\beta$ was constructed by cloning the 2.6-kb *Eco*RI-*Hin*dIII fragment containing the *ATP2* structural gene and its promoter region into Ycplac22 (16). pDB427 was constructed by replacing the 1.575-kb *Hin*dIII fragment of pDB425 with the 1.539-kb *Hin*dIII fragment of pDB51 encoding Δ1,2 pre-$F_1\beta$. The 2μm plasmid containing *TOM72*, pDB561, was constructed by cloning the 3.020-kb *Eco*RI-*Bam*HI fragment from the pGAL-*TOM72* plasmid into the *Eco*RI-*Bam*HI sites of pSEY8.

**Expression and purification of recombinant proteins.** Plasmids expressing WT pre-$F_1\beta$–dihydrofolate reductase (DHFR) (pDB421), Δ1,2 pre-$F_1\beta$–DHFR (pDB423), WT pre-$F_1\beta$–DHFR* (pDB507), and Δ1,2 pre-$F_1\beta$–DHFR* (pDB509) have been described (21). These precursors were expressed and purified from the *Escherichia coli* strain BL21(DE3). Purified precursors were stored in urea buffer (8 M urea, 20 mM Tris [pH 7.5], 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) until use (20).

**Mitochondrial protein import assays.** Mitochondria were prepared from the WT yeast strain D273-10B and the *tom70*Δ yeast strain HR1. Strains were grown on YMM (minimal salts supplemented with 0.1% glucose, 2% DL-lactate, and 0.3% yeast extract). Import assays were carried out as described previously (20, 21). Import reactions were stopped by a 10-fold dilution into ice-cold SEM buffer (0.25 mM sucrose, 1 mM EDTA, 10 mM morpholinepropanesulfonic acid [MOPS] [pH 7.2]) supplemented with 0.5 μg of valinomycin, 20 μg of oligomycin, and 8 μg of antimycin per ml. The samples were treated with 0.1 mg of proteinase K per ml for 30 min on ice followed by the addition of 1 mM phenylmethylsulfonyl fluoride. The samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and quantitation was performed using a PhosphorImager system (Molecular Dynamics). In control experiments, protease protection was not observed when 0.5 μg of valinomycin per ml and 20 μg of oligomycin per ml were present in the import reaction or when Triton X-100 was added during protease treatment. This confirmed that the protease-protected precursor proteins had undergone mitochondrial import.

**Screening of WT yeast genomic multicopy library.** The WT yeast genomic library was constructed by insertion of genomic *Sau*3a DNA fragments into the Yep24 2μm yeast shuttle vector and was a generous gift from Vytas Bankaitis. The library was transformed into YDB210 expressing pDB427 and transformants were screened on SMD plates selective for the auxotrophic markers. Each transformant was then streaked out on YP lactate plates for single colonies and incubated at 15°C for 10 days. More than 3,000 transformants were screened, 15 of which grew under the repressive conditions. Plasmids were then rescued, propagated in *E. coli*, and transformed back into YDB210 expressing pDB427. Transformants were grown under repressive conditions in order to confirm that the transforming plasmid conferred suppression of the growth defect on a nonfermentable carbon source, and 10 were confirmed to suppress the growth defect. The plasmids containing the complementing insert were subjected to restriction mapping. The *E. coli* transformants were screened for the presence of either the pDB427 or library plasmid by *Hin*dIII enzyme digests. Those transformants that contained the library plasmid were then subjected to *Bam*HI and

*Hin*dIII enzyme digestion. Of the 10 library plasmids screened by restriction mapping, 8 contained inserts with similar or identical restriction digests. The inserts were then sequenced by automated flourescent sequencing with primers that anneal to flanking regions of the plasmid insertion site. The sequences were then used to search the Saccharomyces Genome Database (Stanford University) by BLAST search to identify the genes contained within the insert. All eight inserts mapped to chromosome II between nucleotides 209,089 and 227,504. The library containing the smallest complementing insert was subjected to further restriction mapping in order to identify the gene conferring increased growth under the repressive conditions (see Fig. 2). Each subclone was then transformed into YDB210 expressing pDB427 to identify which gene suppressed the growth defect. In order to ultimately define the suppressive region of the insert, an 8.266-kb *Bam*HI-*Eco*RV fragment was subcloned into pSEY8. A 7.7-kb *Bgl*II fragment was then subcloned into pSEY8 resulting in the plasmid termed pDB562 and transformed into YDB210 expressing pDB427.

**Cell labeling and immunoprecipitation.** The *S. cerevisiae* strains SEY6215 and YDB210, transformed with pDB425 or pDB427 and pDB562, were grown in synthetic medium (49) containing 2% glucose to a cell density of 0.5 to 0.7 $A_{600}$ units/ml at 30°C. The cells were resuspended in fresh medium to 4 $A_{600}$ units/ml and incubated for 15 min at 30°C with shaking. The labeling reaction was initiated by the addition of 0.2 mCi of [$^{35}$S]EXPRESS protein labeling mix (DuPont NEN). The labeling reaction was terminated by the addition of either 0.1 mg of cycloheximide per ml and 0.02 mg of methionine per ml or 0.3% yeast extract per ml, 0.02 mg of methionine per ml, and 0.02 mg of cysteine per ml. Incubation with the label termination mix was continued throughout the chase period. To terminate the chase period, a 0.5-ml aliquot of cells was added directly to trichloroacetic acid (5% final concentration) and the mixture was incubated on ice for 30 min. Immunoprecipitations were carried out as described previously (5). The efficiency of import (mature $F_1\beta$ [%]) represents mature protein/(total precursor plus mature protein) × 100.

**Immunoassay blots and enzyme assays.** Mitochondria were prepared from the yeast strains as described previously (17). Published procedures were used to assay ATPase activity (44) and protein concentration (8). For immunoblot analysis of purified mitochondria, mitochondrial extracts were resuspended in SDS-PAGE sample buffer and boiled for 5 min. Ten micrograms of mitochondrial protein from each sample was analyzed by SDS-PAGE. Proteins were transferred to Immobilon-P membranes (Millipore) with a Trans-Blot apparatus (Bio-Rad). Following a 1-h incubation with 5% nonfat milk in phosphate-buffered saline–0.5% Tween 20, the blot was incubated with monoclonal antisera directed against pre-$F_1\beta$ and polyclonal antisera directed against porin for 2 h at room temperature with rocking. The blot was then treated for 1 h with rabbit antisera directed against mouse immunoglobulin G. The signal was then detected by incubation with $^{125}$I-protein A (Amersham) followed by autoradiography.

**Northern blot analysis.** Total cellular RNA was prepared by SDS-phenol extraction from strains grown on YMM at 30°C and was harvested at 1.2 to 1.5 $A_{600}$ units/ml (52). Equal amounts of RNA from each culture (20 μg) were subjected to agarose gel electrophoresis. Following transfer to nitrocellulose, *ATP2* mRNA was detected by using a 617-bp probe that spanned nucleotides 136 to 753 of the coding sequence. The DNA probe was amplified from the *ATP2* gene with primers DB697 (5′-GTTACCGCTGTCATTGGTGCCATT-3′) and DB69 (5′-CACTAAGGCGACCTTGGATTCACCTT-3′) by PCR and was labeled with [α-$^{32}$P]dATP by random hexamer priming. *TOM72* mRNA was detected by a probe corresponding to nucleotides 71 to 760 of the coding sequence that was amplified from genomic DNA with primers DB766 (5′-GGACTGCTG CTGTGGGTGCTTATT-3′) and DB767 (5′-TGCGAGCCTCTGCCTTCATCC TT-3′). *TOM37* mRNA was detected by a probe coresponding to nucleotides 366 to 797 of the coding sequence that was amplified from genomic DNA with primers DB764 (5′-TTCCAGACTAACGGACTACCAGC-3′) and DB765 (5′-TGAACGGATGCGGTTACCATCAG-3′). *TOM22* mRNA was detected by a probe corresponding to nucleotides 198 to 421 of the coding sequence that was amplified from genomic DNA with primers DB791 (5′-AGACATTGTCCCCC CAGGTAAGAG-3′) and DB792 (5′-TGCAGCATCTTTTTCACCTTGGGC-3′). *TOM40* mRNA was detected by a probe corresponding to nucleotides 53 to 647 of the coding sequence that was amplified from genomic DNA with primers DB789 (5′-CCCTTTCTCCTTTGACTGCGAAGC-3′) and DB790 (5′-CGCTA CCGTCAGTTCTGGAGTATA-3′). *TOM20* mRNA was detected by a probe corresponding to nucleotides 5 to 516 of the coding sequence that was amplified from genomic DNA with primers DB787 (5′-CCCAGTCGAACCCTATCTTA CTG-3′) and DB788 (5′-TCGTTAGCTTCAGCAACCGCATCA-3′). Following hybridization and washing, the radioactive bands were visualized by autoradiography.

## RESULTS

**A *tom70*Δ mutant inefficiently imports a derivative of pre-$F_1\beta$ with a minimal import signal.** We previously reported that the mitochondrial import signal of pre-$F_1\beta$ contains redundant targeting information within its amino-terminal 34 amino acids (5, 6). Δ1,2 pre-$F_1\beta$ is a mutant precursor that contains two small nonoverlapping deletions within its targeting signal, leaving a "minimal targeting signal" capable of facilitating import into mitochondria in vivo (Fig. 1A). Previous in vivo studies showed that this reduction to a minimal import signal has little effect on the steady-state levels of $F_1\beta$ protein (or ATPase activity) in mitochondria, and Δ1,2 pre-$F_1\beta$ is normally processed upon entry into the matrix (5).

We previously found that Δ1,2 pre-$F_1\beta$ is unable to bind to the mitochondrial surface or maintain an unfolded import-competent conformation as efficiently as WT pre-$F_1\beta$ (21). Other studies found that mitochondria isolated from *tom70*Δ strains import a subset of precursors, including WT pre-$F_1\beta$, less efficiently than WT mitochondria do (24). Moreover, upon dissipation of the electrochemical potential across the inner membrane, Tom70p enhanced the binding of pre-alcohol dehydrogenase III to the surface of mitochondria and maintained the bound precursor in an import-competent conformation (25). These observations led us to examine in greater detail the interaction between the redundant targeting information present in pre-$F_1\beta$ and how this information influenced its interaction with the Tom70p receptor.

We initially compared the import kinetics of WT pre-$F_1\beta$ and Δ1,2 pre-$F_1\beta$. Upon import into the mitochondrial matrix, the presequence is rapidly removed from both precursors (5), thus making it possible to use processing to follow the rate of import. Cells expressing either WT or Δ1,2 pre-$F_1\beta$ were pulse-labeled for 5 min at 30°C with [$^{35}$S]methionine-cysteine and chased for various lengths of time in the presence of cycloheximide to prevent further protein synthesis. The import of WT pre-$F_1\beta$ in WT cells was extremely efficient, with greater than 95% of precursor protein already processed to its mature form by the end of the pulse period (Fig. 1B). The initial rate of import of Δ1,2 pre-$F_1\beta$ was significantly lower than that of the WT precursor, suggesting that the loss of much of the targeting signal compromised the ability of the mutant precursor to efficiently recognize the import machinery (Fig. 1B). In addition, Δ1,2 pre-$F_1\beta$ import approached a plateau after the chase period had proceeded for 5 min. In a previous study, we found that the WT pre-$F_1\beta$ signal acts not only to engage the import machinery, but also functions as an intramolecular chaperone to maintain the precursor in an import-competent conformation (21). These results suggest that the mutant precursor may be able to maintain an import-competent state for only a limited period of time, with import decreasing as the precursor pool attains a folded state.

The minimal targeting signal present in Δ1,2 pre-$F_1\beta$ may also be compromised in its ability to mediate an interaction with cytosolic and/or membrane components of the mitochondrial import machinery. Since previous studies speculated that Tom70p acts to maintain precursors in an import-competent conformation, we next compared the import kinetics of Δ1,2 pre-$F_1\beta$ in WT and *tom70*Δ strains. Though the rapid import of WT pre-$F_1\beta$ is not dependent on the presence of Tom70p (Fig.

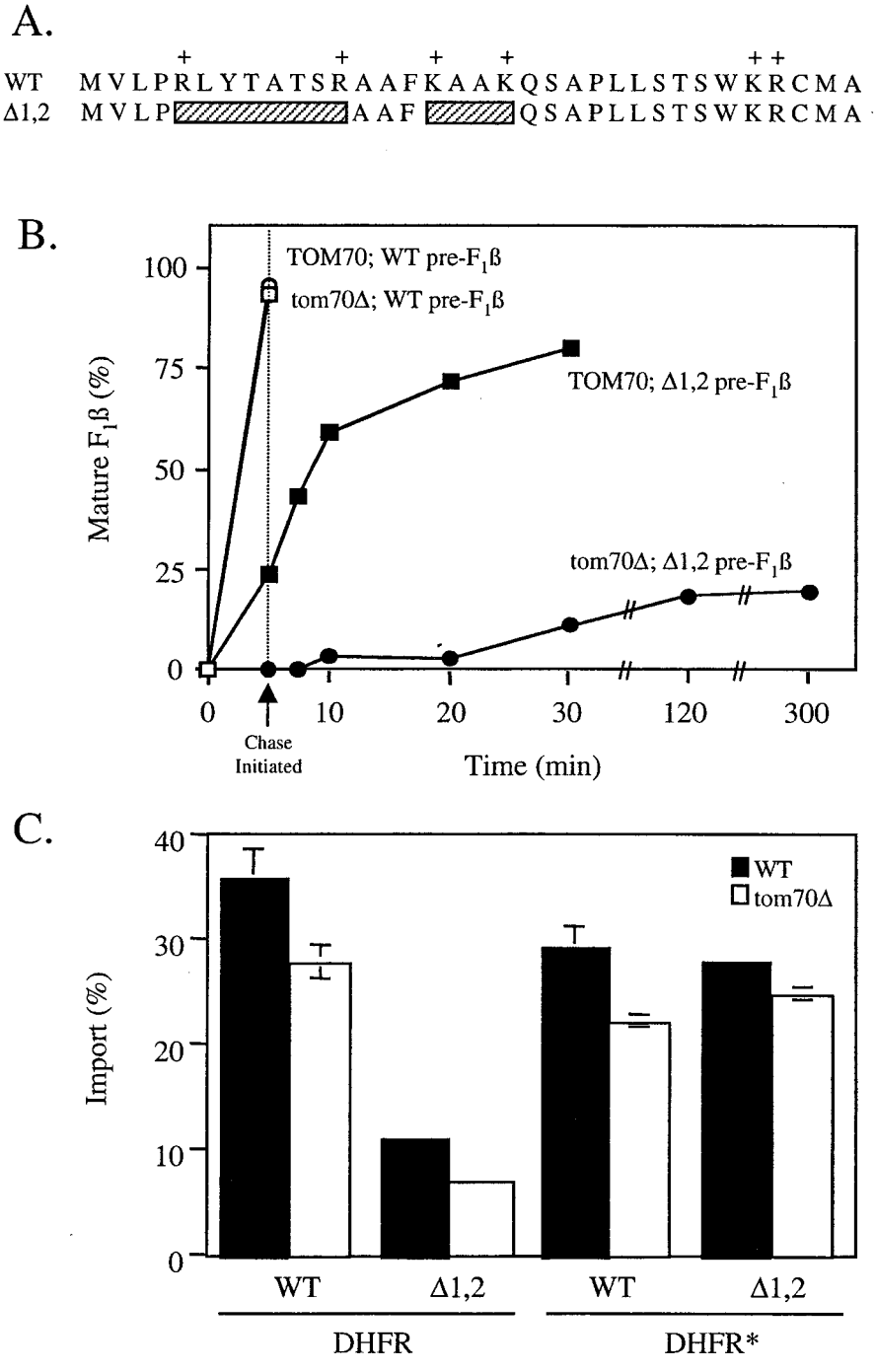


FIG. 1. Mitochondrial import mediated by WT and Δ1,2 pre-$F_1\beta$ targeting signals. (A) Amino acid sequences of the amino termini of WT pre-$F_1\beta$ and Δ1,2 pre-$F_1\beta$ as predicted from nucleotide sequences (5). Deletions are indicated by hatched boxes and basic residues are indicated by plus signs. (B) WT and *tom70*Δ cells expressing either WT or Δ1,2 pre-$F_1\beta$ were pulse-labeled for 5 min at 30°C, followed by chase times extending to 5 h. Cell lysates were subjected to immunoprecipitation with pre-$F_1\beta$-specific antiserum as described in Materials and Methods. (C) In vitro mitochondrial protein import of precursors containing WT or Δ1,2 pre-$F_1\beta$ targeting signals fused to the DHFR or DHFR* passenger proteins. Import reactions were carried out using WT or *tom70*Δ mitochondria as indicated.

1B), we found that the import of Δ1,2 pre-$F_1\beta$ was drastically reduced when Tom70p was absent. After 30 min of chase, only ≈10% of the precursor was imported. A total of only ≈20% was processed when the chase was continued for 5 h, suggesting that the majority of this precursor does not import into mitochondria. These results indicate that $F_1\beta$ becomes much more dependent on Tom70p for efficient import when the extended import signal is reduced to this minimal unit. Tom70p, in association with Tom37p, has previously been suggested to have a relatively minor role in mediating the import

of precursors with amino-terminal targeting signals. However, our results indicate that Tom70p can play a much more substantive role in facilitating import under certain conditions, possibly by maintaining the precursor in an import-competent conformation until import can occur.

To further test this possibility, we examined the ability of the WT and Δ1,2 presequences to import purified precursors into isolated WT or *tom70*Δ mitochondria. Since Δ1,2 pre-$F_1\beta$ is incapable of import into purified mitochondria (21), we examined the ability of the WT or Δ1,2 targeting signals to import fusion proteins containing either DHFR or a structurally destabilized form of DHFR (referred to subsequently as DHFR*). DHFR* contains three missense mutations (C7S, S42C, and D49C) that prevent it from folding into a stable three-dimensional structure. It has been shown that a construct containing the presequence of cytochrome oxidase subunit IV fused to this mutant form of DHFR exhibited an increased susceptibility to protease digestion and an increased rate of mitochondrial import, confirming that these mutations destabilized the DHFR moiety (57). In another study, we used purified pre-$F_1\beta$–DHFR and pre-$F_1\beta$–DHFR* fusion proteins to demonstrate that the redundant pre-$F_1\beta$ targeting signal functioned as an intramolecular chaperone that prevents precursor folding prior to import (21). We found that WT pre-$F_1\beta$–DHFR is imported into isolated WT mitochondria threefold more efficiently than the Δ1,2 pre-$F_1\beta$–DHFR construct (Fig. 1C). While the overall level of import was reduced slightly, we observed a similar threefold difference in the import of these precursors into *tom70*Δ mitochondria. These results indicate that the Δ1,2 presequence fused to DHFR exhibits an import defect similar to the in vivo defect observed with Δ1,2 pre-$F_1\beta$. To determine whether this defect is related to its inability to maintain the precursor in a loosely folded conformation, we next examined the import of precursors containing the DHFR* passenger protein. We found that the levels of WT and Δ1,2 pre-$F_1\beta$–DHFR* imported into WT and *tom70*Δ mitochondria were similar, indicating that the import defect associated with the Δ1,2 signal could be bypassed in either WT or *tom70*Δ mitochondria if precursor folding was prevented.

**Genetic screen for multicopy suppressors of the cold-sensitive growth defect.** $F_1\beta$ is an essential subunit of the $F_1$-ATPase complex, which carries out the generation of ATP by oxidative phosphorylation. As a result, *atp2*Δ strains are unable to grow on nonfermentable carbon sources such as lactate. We next asked whether the low level of pre-$F_1\beta$ import that occurred in a *tom70*Δ strain led to a growth defect on nonfermentable carbon sources. A *TOM70* strain expressing Δ1,2 pre-$F_1\beta$ exhibited only a slight growth defect when incubated on YP lactate plates at either 30 or 15°C (Fig. 2A). However, *tom70*Δ cells expressing Δ1,2 pre-$F_1\beta$ exhibited a much more severe cold-sensitive phenotype under these conditions (Fig. 2B). This is presumably due to the inability of the *tom70*Δ strain to assemble enough of the ATP synthase complex to sustain growth at 15°C.

Since a phenotype suitable for a genetic screen had not previously been obtained with a *tom70*Δ mutation, we decided to identify multicopy suppressors of the cold-sensitive growth defect on YP lactate plates. To do this, a *tom70*Δ strain expressing Δ1,2 pre-$F_1\beta$ was transformed with a WT yeast multicopy genomic library. Approximately 3,000 transformants were screened for the ability to grow on YP lactate plates at 15°C. The plasmids were rescued from 8 of 10 transformants by plasmid rescue and retransformed into the original mutant strain to confirm that the plasmid was responsible for suppression. The ends of the inserts were then sequenced and the insert was identified by BLAST searches against the *S. cerevisiae* genome. All eight transformants contained inserts that mapped to the same region of chromosome II, the smallest of which contained an 18.4-kb fragment. The insert contained the genes *HIR1*, *SLA1*, and *PDR3* and a TY element (Fig. 3A). Subcloning revealed that only restriction fragments containing the intact *PDR3* gene could complement the growth defect when transformed into the mutant strain (Fig. 3B). *PDR3* is a

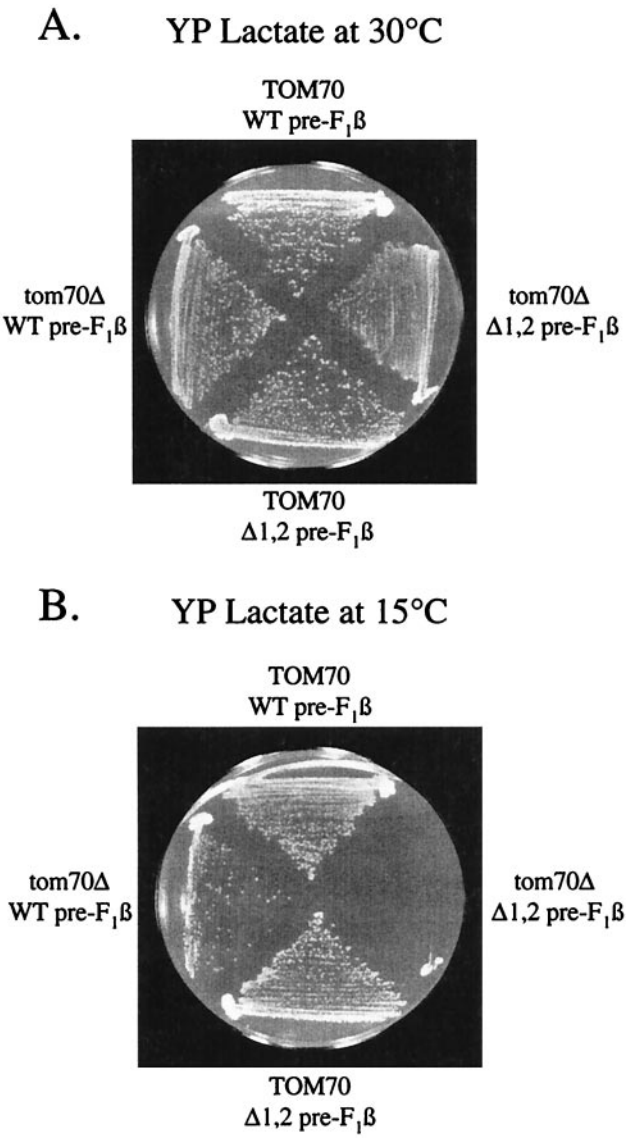


FIG. 2. Growth defects of yeast strains on a nonfermentable carbon source at low temperature. (A) WT cells and *tom70*Δ cells expressing either WT or Δ1,2 pre-$F_1\beta$ were grown on a YP lactate plate for 3 days at 30°C. (B) WT and *tom70*Δ cells expressing either WT or Δ1,2 pre-$F_1\beta$ were grown on a YP lactate plate for 8 days at 15°C.

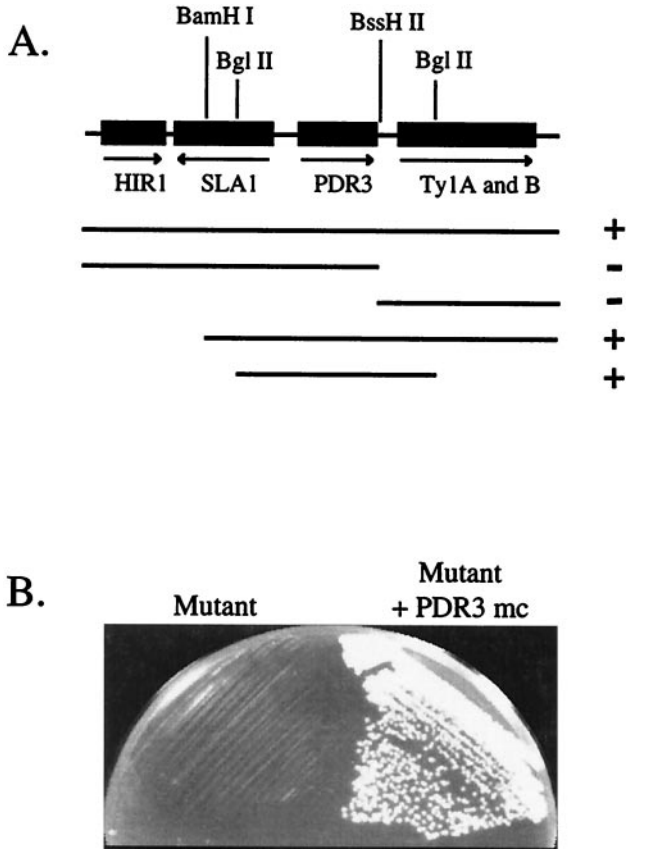


FIG. 3. Identification of a multicopy suppressor of the growth defect observed in a *tom70*Δ strain expressing Δ1,2 pre-$F_1\beta$. (A) Sequencing primers complementary to vector sequences on either side of the genomic insert were used to generate partial sequences, and the genes included in the complementing insert were identified by a BLAST search of the Saccharomyces Genome Database. Different fragments (indicated by solid black lines under the insert maps) were then tested for the ability to restore growth of the *tom70*Δ strain expressing Δ1,2 pre-$F_1\beta$, as determined on YP lactate plates at 15°C. Those clones that contained complementing inserts are indicated by a positive sign, while clones that did not complement the cold-sensitive phenotype are indicated by negative signs. (B) The *tom70*Δ strain expressing Δ1,2 pre-$F_1\beta$ was streaked on a YP lactate plate beside the same strain harboring a 2μm plasmid containing *PDR3*. The plate was incubated at 15°C for 8 days.

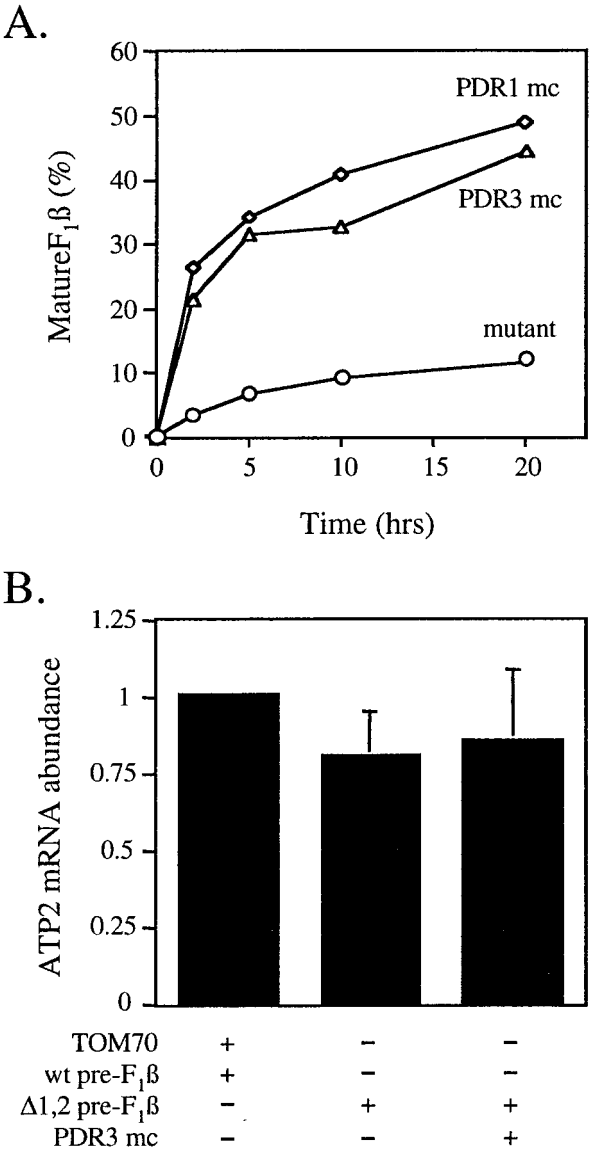


FIG. 4. Effect of *PDR3* and *PDR1* overexpression on Δ1,2 pre-$F_1\beta$ import. (A) *tom70*Δ cells expressing Δ1,2 pre-$F_1\beta$ and harboring 2μm plasmids containing either *PDR3* or *PDR1* were pulse-labeled for 5 min at 30°C and then chased for times extending to 20 h. Cell lysates were subjected to immunoprecipitation with a pre-$F_1\beta$-specific antiserum as described in Materials and Methods. (B) Northern blots were performed on mRNA isolated from WT and mutant cells and mutant cells overexpressing *PDR3*. The probe was directed against *ATP2* as described in Materials and Methods. Strains were grown at 30°C in YP lactate medium. The amount of mRNA was normalized to actin mRNA ($n = 3$).

transcription factor associated with the PDR pathway (12), which regulates the transcription levels of a wide variety of ATP binding cassette transporters that facilitate drug efflux. However, *PDR3* also regulates the expression of other genes that are not involved in drug resistance, such as the hexose transporter genes *HXT9* and *HXT11* (43). Since other expected multicopy suppressors, such as *TOM70* and *ATP2*, were not obtained in this genetic screen, it is possible that other suppressors of this growth defect remain to be identified.

**Multicopy *PDR3* or *PDR1* stimulates import of Δ1,2 pre-$F_1\beta$ in a *tom70*Δ strain.** We next examined how the expression of the *PDR3* gene from a multicopy plasmid affected the import kinetics of Δ1,2 pre-$F_1\beta$ in a *tom70*Δ strain. Since Pdr1p is a regulator of Pdr3p and both regulate many PDR genes in common, we examined the effects of expressing each of these genes from a multicopy plasmid. *tom70*Δ strains expressing Δ1,2 pre-$F_1\beta$ that carried either the *PDR3* or *PDR1* genes on a multicopy plasmid were pulse-labeled for 5 min with [$^{35}$S]methionine-cysteine and then chased for various lengths of time in the presence of cycloheximide to prevent further protein synthesis. We found that *PDR3* or *PDR1* overexpression resulted in a significant increase in the amount of Δ1,2 pre-$F_1\beta$ that was imported into mitochondria (Fig. 4A). While only 10 to 20% of Δ1,2 pre-$F_1\beta$ was imported into mitochondria in the *tom70*Δ

strain, 40 to 50% of the precursor protein was imported when multicopy plasmids expressing either *PDR3* or *PDR1* were present. Shorter chase times confirmed that this increase in overall yield was accompanied by an increase in the initial rate of import (data not shown). These results were not due to the stimulation of *ATP2* expression (which encodes pre-$F_1\beta$), since Northern blots of mRNA isolated from strains grown on media containing lactate as carbon source indicated that the steady state *ATP2* mRNA abundance was not increased in the strain overexpressing *PDR3* (Fig. 4B). Instead, our results indicate that the overexpression of *PDR3* or *PDR1* can partially restore $\Delta$1,2 pre-$F_1\beta$ import in a *tom70*$\Delta$ strain.

To further examine the effects of *PDR3* and *PDR1* overexpression, we next measured the steady-state level of $F_1\beta$ and ATPase activity in mitochondria isolated from strains grown on lactate as carbon source at 30°C. By Western blot analysis, we found that the steady-state level of $\Delta$1,2 pre-$F_1\beta$ in mitochondria isolated from the *tom70*$\Delta$ strain was decreased more than 10-fold compared to the WT strain (Fig. 5A), consistent with the poor import of $\Delta$1,2 pre-$F_1\beta$ in this strain. However, overexpression of *PDR3* or *PDR1* resulted in steady-state levels of $\Delta$1,2 pre-$F_1\beta$ that were 60 to 70% that of the WT, indicating that the overproduction of these transcription factors increased its import severalfold. We also examined whether the increased level of $F_1\beta$ was assembled into the mitochondrial ATPase complex. We found that overexpression of *PDR3* or *PDR1* resulted in a twofold increase in ATPase activity over the original mutant strain (Fig. 5B). This suggests that only a fraction of the imported $F_1\beta$ may be assembled into a functional ATPase complex. However, this level of assembly was apparently sufficient to increase mitochondrial ATP production enough to restore growth under the selective conditions.

***PDR3* overexpression increases *TOM72* mRNA abundance.** Since $\Delta$1,2 pre-$F_1\beta$ has been shown to rapidly fold into an import-incompetent conformation (5, 6, 21), we next asked whether the overproduction of these transcription factors stimulate $\Delta$1,2 pre-$F_1\beta$ import by increasing the expression of one or more cytosolic molecular chaperones. Recently, a microarray analysis identified 26 genes whose expression was stimulated by hyperactivating alleles of *PDR1* and *PDR3* (13). Most, but not all, of these genes contain a PDR element (PDRE) in their promoter region that has been shown to mediate positive control by both of these transcription factors. We performed a pattern analysis search on the *S. cerevisiae* genome with the PDRE sequence (5′-CCGCGG-3′) and identified two putative molecular chaperone genes, *XDJ1* and *PDR15*, that contained upstream PDRE elements. *PDR15* encodes a protein with limited homology to the Hsp70 family, while *XDJ1* is a hypothetical open reading frame whose product has 40% homology with Ydj1p, a chaperone previously shown to aid precursor binding to the outer mitochondrial membrane (11). However, Northern blot analysis indicated that the steady-state mRNA level of these genes was unaffected by *PDR3* overexpression (results not shown).

None of the genes encoding components of the TOM complex contained a PDRE in their promoter regions. However, since some genes whose transcription is controlled by *PDR1* and *PDR3* do not have an upstream PDRE, we examined the steady-state mRNA levels of most of the TOM subunits by Northern blot analysis. We found that there was little or no change in the levels of *TOM37*, *TOM22*, *TOM40*, and *TOM20* mRNA levels in response to the overexpression of *PDR3* (results not shown). However, the steady-state level of *TOM72* mRNA was reduced somewhat in the *tom70*$\Delta$ strain and sub-

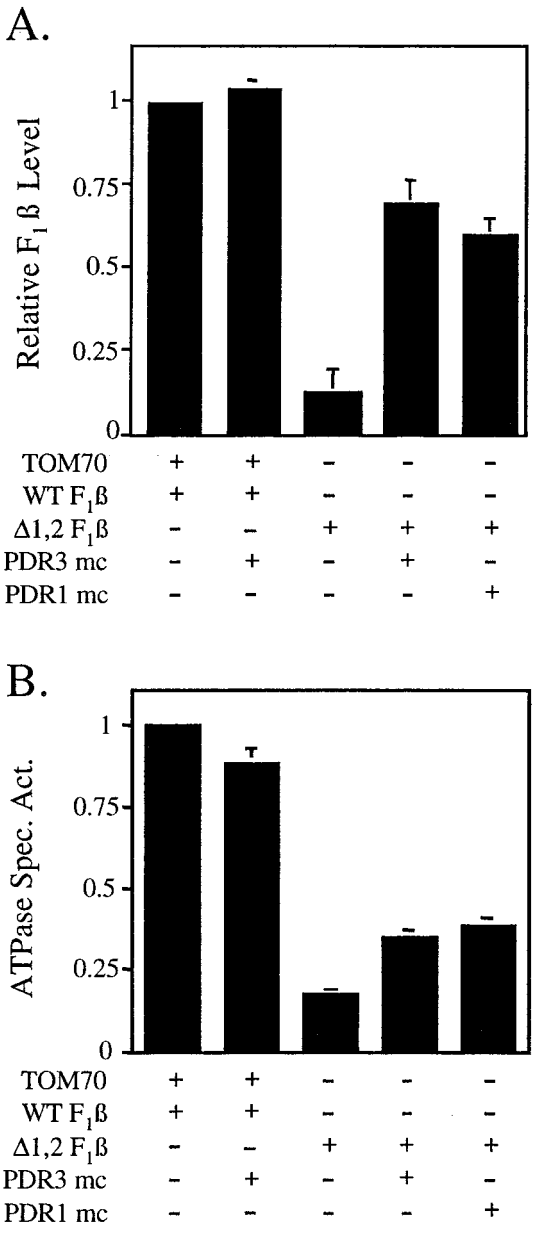


FIG. 5. Steady-state levels of $F_1\beta$ and ATPase activity following *PDR3* and *PDR1* overexpression in *tom70*$\Delta$ cells harboring $\Delta$1,2 pre-$F_1\beta$. (A) Mitochondria isolated from WT or mutant strains harboring control plasmids or plasmids containing *PDR3* or *PDR1* were subjected to immunoblot analysis with $F_1\beta$-specific monoclonal antibodies. The steady-state level of mature $F_1\beta$ in mitochondria from each strain was expressed relative to the level observed in WT mitochondria. In each case, samples were normalized to the level of porin measured on parallel blots ($n$ = 3). (B) Mitochondria freshly isolated from the strains described above were assayed for mitochondrial ATPase activity. The ATPase specific activity (Spec. Act.) (micromoles per minute per milligram of protein) in mitochondria from each strain was expressed relative to the specific activity measured in WT mitochondria ($n$ = 3).

sequently increased twofold as a result of *PDR3* overexpression. This result suggested that an up-regulation of *TOM72* may be responsible for the increased Δ1,2 pre-$F_1\beta$ import upon Pdr3p overproduction (Fig. 6A). To test this possibility, we introduced the *TOM72* gene into the *tom70*Δ strain on a multicopy plasmid. We found that Tom72p overproduction resulted in near WT levels of $F_1\beta$ and ATPase activity in our mutant strain (Fig. 6B and C). These results indicate that the overexpression of Tom72p, a little-characterized homologue of Tom70p, can compensate for the loss of Tom70p in the import of Δ1,2 pre-$F_1\beta$.

We next asked whether increased expression of *TOM72* is required for the stimulation of Δ1,2 pre-$F_1\beta$ import produced by *PDR1* and *PDR3* overexpression. To do this, we constructed a *tom70*Δ *tom72*Δ double mutant and examined the effect of *PDR3* overexpression on Δ1,2 pre-$F_1\beta$ import. We found that the loss of *TOM72* expression completely blocked the ability of *PDR3* overproduction to increase the amount of Δ1,2 pre-$F_1\beta$ in mitochondria (Fig. 7A). In addition, the stimulation of mitochondrial ATPase activity was also not observed upon *PDR3* overproduction in the absence of *TOM72* (Fig. 7B). We conclude from these results that *PDR3* (and presumably *PDR1*) overproduction increases Δ1,2 pre-$F_1\beta$ import by increasing the expression of the Tom72p import receptor.

## DISCUSSION

**Tom70p stimulates mitochondrial import of a precursor containing a defective amino-terminal signal.** Mitochondrial targeting signals are involved in several distinct steps of the mitochondrial protein import process. This includes interactions with cytosolic factors, import receptors, components of the TOM and translocase-of-the-inner-membrane complexes, and chaperones within the mitochondrial matrix. In our in vivo pulse-chase analysis, we found that Δ1,2 pre-$F_1\beta$, a precursor containing a minimal targeting signal, exhibited a reduced rate of import compared to the WT pre-$F_1\beta$ precursor. We found that the *tom70*Δ mutation greatly exacerbated this import defect, resulting in a severe decrease in the overall yield of imported precursor. One possible explanation for this result is that the increased dependence of the Δ1,2 targeting signal on the Tom70p receptor results from a reduced ability of the Tom20p receptor to efficiently recognize this minimal import signal. To test this possibility, we examined the ability of the Δ1,2 targeting signal to facilitate import into *tom70*Δ mitochondria. We previously reported that the presequence of pre-$F_1\beta$ acts as an intramolecular chaperone that maintains the precursor in a loosely folded, import-competent conformation (21). While the Δ1,2 pre-$F_1\beta$ import signal imported a DHFR fusion protein much less efficiently than the WT pre-$F_1\beta$ signal did, the minimal targeting signal could import a structurally destabilized form of DHFR (DHFR*) into isolated mitochondria as well as the WT pre-$F_1\beta$ import signal could. In the present study, we found that Δ1,2 pre-$F_1\beta$–DHFR* was also capable of import into *tom70*Δ mitochondria as efficiently as WT pre-$F_1\beta$–DHFR*. These results demonstrate that the Δ1,2 import signal retains the ability to mediate efficient import in the absence of the Tom70p receptor and suggest that the Δ1,2 pre-$F_1\beta$ targeting signal retains the ability to interact efficiently with the Tom20p receptor. Since the only difference between DHFR and DHFR* is the inability of the latter protein to fold into a stable conformation, these results suggest that Tom70p acts to maintain this precursor in a loosely folded conformation prior to import.

It has been suggested that Tom70p participates primarily in the import of mitochondrial proteins containing internal targeting signals (9, 10). However, several lines of evidence suggest that Tom70p also participates in the import of proteins containing amino-terminal signals. First, mitochondrial import defects associated with the deletion of *TOM20* can be partially suppressed by the overexpression of *TOM70*, indicating that these receptor subunits have overlapping functions (45). Second, the disruption of *TOM70* or the treatment of isolated WT mitochondria with antibodies directed against Tom70 inhibits the import of several mitochondrial proteins containing amino-terminal signals (24). Third, in vivo experiments show that *tom70*Δ cells import several precursor proteins with amino-terminal signals more slowly than WT cells (25). The presence of Tom70p has also been shown to accelerate the import of a wide variety of precursor proteins by enhancing the binding of precursors to the surface of the mitochondria (25). The results provided in our study are also consistent with Tom70p playing a general role of mitochondrial precursors containing amino-terminal signals.

**Tom72p functions as a mitochondrial import receptor.** Tom72p is a poorly characterized protein that is located on the outer mitochondrial membrane. It shares 53% amino acid identity with Tom70p and has been shown to be associated with other components of the outer membrane translocase complex (51). It has also been shown to cross-link with a 30- to 35-kDa protein that is distinct from the Tom70p binding partner, Tom37p. Despite its strong homology to Tom70p and similar subcellular localization, a knockout of the *TOM72* gene was not found to significantly affect the import of any of the mitochondrial proteins examined. It was also found that the weak mitochondrial protein import defects observed in a strain carrying disruptions of both *TOM70* and *TOM72* were similar to the defects observed in a strain carrying a *tom70*Δ mutation alone, other than a slightly increased temperature-sensitive growth defect on a nonfermentable carbon source (51). Our finding that the overexpression of *TOM72* suppresses the import defect of Δ1,2 pre-$F_1\beta$ in *tom70*Δ cells leads us to conclude that the functions of Tom70p and Tom72p overlap. We previously demonstrated that the Δ1,2 pre-$F_1\beta$ targeting signal was unable to maintain the precursor in an import-competent conformation (21), and data from our in vitro experiments described in this study further support that conclusion. This suggests that both Tom70p and Tom72p bind mitochondrial precursors and maintain them in an import-competent conformation prior to engaging the downstream components of the TOM complex, as previously suggested for Tom70p (25). The genome of *S. cerevisiae* has undergone a duplication in recent evolutionary time, and the *TOM70* gene (chromosome I) and *TOM72* gene (chromosome VIII) occur on one such duplicated region of the genome (32). It is presently unknown whether the duplicated copy of the gene encoding this mitochondrial protein import receptor is unique for this organism.

**The PDR pathway can influence the mitochondrial import machinery.** The ability of Pdr3p overexpression to suppress defects in mitochondrial protein import reveals a previously

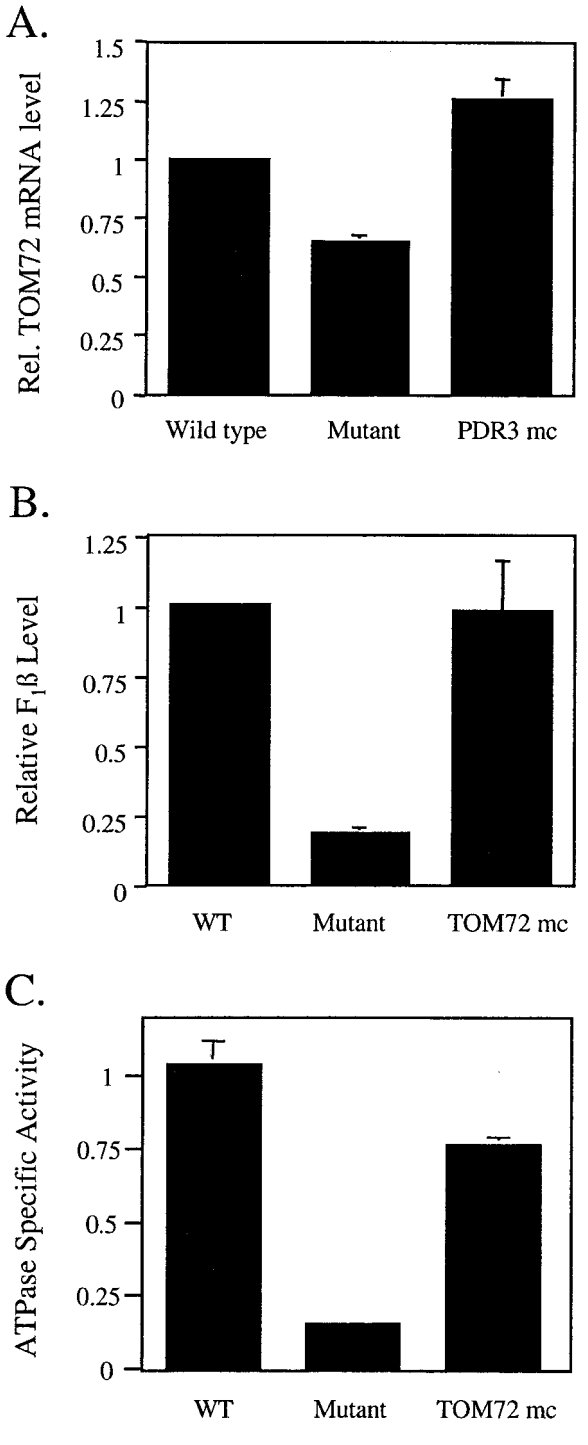


FIG. 6. Effects of *TOM72* overexpression in mutant cells. (A) Northern blots were performed on mRNA isolated from a WT or mutant strain harboring a control plasmid or a *PDR3* plasmid with DNA probes directed against *TOM72* as described in Materials and Methods. Strains were grown on lactate as carbon source at 30°C. The amount of mRNA was normalized to mRNA levels of actin ($n = 3$). (B) Mitochondria isolated from a WT or mutant strain harboring a control plasmid or *TOM72* on a 2μm plasmid were subjected to immunoblot analysis with monoclonal antibodies directed against pre-$F_1\beta$. Relative steady-state levels show the levels of mature $F_1\beta$ relative to that of the WT. The mitochondrial protein concentration was standardized for levels of porin ($n = 3$). (C) Mitochondria freshly isolated from the strains described above were monitored for mitochondrial ATPase specific activity. Relative activity expresses the ATPase specific activity of each strain relative to that of the WT ($n = 3$).

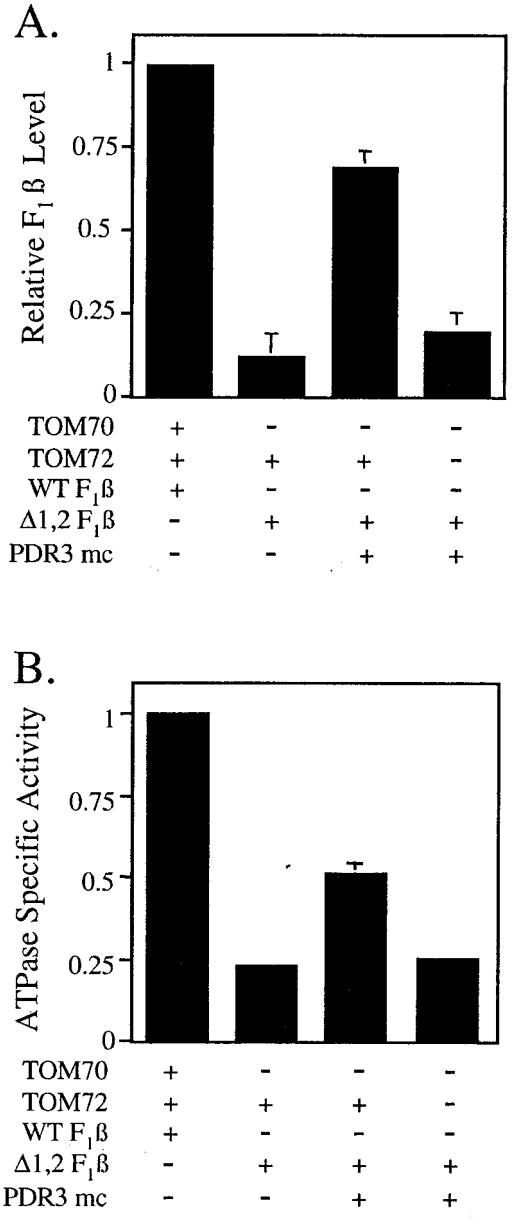


FIG. 7. Steady-state levels of $F_1\beta$ and ATPase activity following *PDR3* overexpression in *tom70*Δ or *tom70*Δ *tom72*Δ cells harboring Δ1,2 pre-$F_1\beta$. (A) Mitochondria isolated from WT and mutant strains and mutant strains harboring *PDR3* or *PDR1* on 2μm plasmids were subjected to immunoblot analysis with $F_1\beta$-specific monoclonal antibodies. The steady-state level of mature $F_1\beta$ in mitochondria isolated from each strain was expressed relative to the level observed in WT mitochondria. In each case, samples were normalized to the level of porin measured on parallel blots ($n = 3$). (B) Mitochondria freshly isolated from the strains described above were assayed for mitochondrial ATPase activity. The ATPase specific activity (micromoles per minute per milligram of protein) in mitochondria from each strain was expressed relative to the specific activity measured in WT mitochondria ($n = 3$).

unappreciated role for the PDR pathway in mitochondrial biogenesis. Our results show that the overexpression of Pdr3p or Pdr1p can restore the ability of this strain to grow on a nonfermentable carbon source at 15°C. This suppression was mediated by an increase in both the rate and yield of import of this defective precursor. Together, these effects raised the

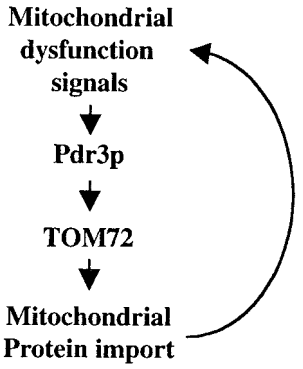


FIG. 8. Model showing that Pdr3p responds to mitochondrial dysfunction by stimulating mitochondrial protein import.

steady-state level of ATP synthase activity above the threshold required for growth under the selective conditions. These results clearly indicate that the PDR pathway can play a previously unrecognized role in mitochondrial biogenesis. A recent study found that this pathway is also activated in response to mitochondrial dysfunction. Hallstrom and Moye-Rowley reported that *PDR3* expression is up-regulated in strains containing mutations that compromise the electron transport chain or the maintenance of the mitochondrial genome (22). It was shown that the response to mitochondrial dysfunction by Pdr3p in some cases also requires activation by the retrograde signaling pathway, which modulates the expression of nuclear genes in response to changes in the mitochondrial status (31, 36). Our results demonstrate that Pdr3p overproduction can also stimulate the import of precursors that are incapable of efficient mitochondrial import when a mitochondrial stress is imposed. In this case, the inability to utilize a nonfermentable carbon source due to limiting ATP synthase activity did not induce *TOM72* transcription, suggesting that this stimulus alone cannot activate the PDR pathway. However, we found that *TOM72* transcription was induced under these conditions when the *PDR3* gene dosage was increased. It is possible that other forms of mitochondrial stress that provide a stronger activation of the PDR pathway may stimulate the import of some mitochondrial proteins by increasing the level of Tom72p. We propose that this mechanism represents a physiological response that optimizes mitochondrial biogenesis during mitochondrial stress or dysfunction (Fig. 8).

Our results indicate that the overproduction of Tom72p can suppress mitochondrial protein import defects associated with the absence of Tom70p. We also show that *TOM72* is partially regulated by Pdr3p, since *TOM72* mRNA abundance is increased when Pdr3p is overproduced. Other results presented in this study, such as a possible decrease in the ability to assemble the $F_1$-ATPase subunits into a functional complex in strains overproducing Pdr3p, also suggest that Pdr3p may affect mitochondrial function by other means. Additional studies will be required to further characterize this observation. When taken together, our results demonstrate that Pdr3p acts to regulate a broader set of genes than previously appreciated, including at least one gene involved in mitochondrial biogenesis. Unlike most genes previously shown to be regulated by Pdr3p, *TOM72* does not contain a strictly conserved PDRE in its promoter region; however, it possesses a degenerate PDRE containing a single mismatch (5′-CCGCGA-3′) almost 300 bp upstream of the initiating codon. This, however, does not negate the possibility that Pdr3p either directly or indirectly regulates *TOM72* expression. A genome microarray analysis of a yeast strain containing a hyperactivating allele of *PDR3* indicated that several genes regulated by Pdr3p do not contain PDREs in their promoters (13). Our results provide the first evidence that the PDR pathway can directly influence the expression of at least one component of the mitochondrial protein import machinery and provide further evidence that Pdr3p is involved in the regulation of a wider variety of genes than previously appreciated.

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