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The Yeast F_1-ATPase β Subunit Precursor Contains Functionally Redundant Mitochondrial Protein Import Information

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The NH_2 terminus of the yeast F_1-ATPase β subunit precursor directs the import of this protein into mitochondria. To define the functionally important components of this import signal, oligonucleotide-directed mutagenesis was used to introduce a series of deletion and missense mutations into the gene encoding the F_1-β subunit precursor. Among these mutations were three nonoverlapping deletions, two within the 19-amino-acid presequence (Δ5-12 and Δ16-19) and one within the mature protein (Δ28-34). Characterization of the mitochondrial import properties of various mutant F_1-β subunit proteins containing different combinations of these deletions showed that import was blocked only when all three deletions were combined. Mutant proteins containing all possible single and pairwise combinations of these deletions were found to retain the ability to direct mitochondrial import of the F_1-β subunit. These data suggest that the F_1-β subunit contains redundant import information at its NH_2 terminus. In fact, we found that deletion of the entire F_1-β subunit presequence did not prevent import, indicating that a functional mitochondrial import signal is present near the NH_2 terminus of the mature protein. Furthermore, by analyzing mitochondrial import of the various mutant proteins in [*rho*$^-$] yeast, we obtained evidence that different segments of the F_1-β subunit import signal may act in an additive or cooperative manner to optimize the import properties of this protein.

Mitochondrial protein import has served as a useful model system for studying the general phenomenon of protein movement across biological membranes. Over 90% of mitochondrial proteins are nucleus encoded and are initially synthesized on cytoplasmic ribosomes before being imported to their correct intramitochondrial locations (7). The import process is energy dependent (4, 9, 11, 30, 31) and requires an import signal within the mitochondrial protein, usually located within a transient presequence. The presequence is proteolytically removed when the NH_2 terminus of the protein arrives in the mitochondrial matrix (11, 24, 41). In addition, the existence of both cytoplasmic and mitochondrial (37, 52) *trans*-acting components of an import apparatus have been reported, but the characterization of such factors remains limited.

Gene fusion and deletion analyses have been used to demonstrate that the presequence (or in some cases an NH_2-terminal portion of the presequence) of several mitochondrial proteins is sufficient to direct heterologous "passenger" proteins into mitochondria. For example, it has been shown that the first 12 amino acids of the 70,000-molecular-weight (70K) outer membrane protein (15, 19), the first 9 amino acids of δ-aminolevulinate synthase (22), and the first 10 amino acids of the F_1-ATPase β subunit (46) are all sufficient to direct the mitochondrial import of heterologous proteins in vivo. No consensus primary amino acid sequence among the known mitochondrial presequences has been found, but most are rich in basic and hydroxylated residues and free of acidic residues. It has been proposed that presequences are able to form amphiphilic helices containing high hydrophobic moments which could contribute to their role as import signals (48). However, experimental data have indicated that presequences lacking a well-defined amphiphilic helical structure can also direct import (1). Other studies have shown that synthetic peptide presequences can disrupt lipid bilayers (38) and, in at least one case, can specifically compete with the import of intact precursor proteins (12). In spite of these studies, however, the precise sequence or structural requirements of a functional mitochondrial import signal have yet to be defined.

The yeast mitochondrial ATPase complex contains 10 defined subunits, 7 of which are encoded by nuclear genes, translated cytoplasmically, and imported into the mitochondrial matrix prior to assembly (8). The β subunit of the F_1-ATPase complex was one of the model proteins originally used to demonstrate that most mitochondrial proteins are synthesized as larger precursors in vivo and that the precursor but not the mature form of the protein is competent for mitochondrial import in vitro (11, 24). This represented the first strong evidence that presequences are required for mitochondrial import. The gene encoding the F_1-β subunit (*ATP2*) has been cloned and sequenced (39, 44), and gene fusion and deletion experiments have demonstrated that the mitochondrial import signal of the F_1-β subunit is located near the NH_2 terminus of the protein (6, 10, 46). The present wealth of information on the import properties of the F_1-β subunit both in vivo and in vitro as well as the availability of the gene encoding this protein make it an ideal candidate for detailed mutational analysis of the functional requirements of a mitochondrial import signal.

We report here the use of oligonucleotide-directed mutagenesis to extend the characterization of the mitochondrial import signal of this protein. By analyzing the import of a number of mutant F_1-β subunit proteins, we have found that removal of various portions of the leader does not significantly affect the steady-state level of the F_1-β subunit protein in its proper intramitochondrial location. In fact, by deleting the entire 19-amino-acid F_1-β subunit presequence (except for the initiating methionine residue), we demonstrated that the "mature" F_1-β subunit is competent for mitochondrial import in vivo. Based on a systematic analysis of various combinations of these mutations, we have defined three functionally redundant regions of the F_1-β subunit import signal that reside within the NH_2-terminal 34 amino acids of

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this precursor protein. These small regions appear to act in an additive or cooperative manner to optimize F_1-β subunit import into mitochondria.

MATERIALS AND METHODS

Strains and media. The *Saccharomyces cerevisiae* strain used in this study was SEY6215 (*MAT***a** *ura3-52 leu2-3,112 trp-Δ901 lys2-801 suc2-Δ9 GAL Δatp2::LEU2*). Construction of the *Δatp2::LEU2* disruption has been described (46), and it was introduced into this strain by standard yeast genetic techniques (42). Control experiments verified that this strain does not produce any antigen that can be recognized by F_1-β subunit-specific antisera. A cytoplasmically inherited petite ([*rho*$^-$]) derivative of SEY6215 (called SEY6215.1) was obtained by ethidium bromide enrichment as described (42). These strains are characterized by an absence of both mitochondrial DNA and protein synthesis, resulting in a respiration-deficient phenotype and a reduced membrane potential (13, 16). *Escherichia coli* strains used for plasmid maintenance and M13 growth were MC1061 [F$^-$ *hsdR hsdM*$^+$ *araD139* (*araABOIC-leu*)*7697 ΔlacX74 galU galK rpsL*] (3) and JM101 [Δ(*lac-pro*) *supE thi* F' *traD36 lacI*^{q}*ZΔM15 proAB*] (25).

Standard yeast (42) and *E. coli* (26) media were used, and tryptophan and lysine were added as needed. Wickerham minimal medium (50) containing 2% galactose was used for $^{35}SO_4^{2-}$ labeling experiments, with SO_4^{2-} salts replaced by chloride salts.

Plasmid constructions and oligonucleotide mutagenesis. pCβ1 was constructed as follows. A 2.2-kilobase (kb) *Eco*RI-*Bam*HI fragment was transferred from pCβ (10) to M13mp9 (25). By oligonucleotide-directed mutagenesis, an *Hin*dIII restriction site was created by a single T to G change 31 bases upstream of the initiation codon of the *ATP2* gene (AATCTT→AAGCTT). The *Eco*RI-*Bam*HI fragment containing the new *Hin*dIII site was returned to pCβ, and the resulting construction was named pCβ1. The 1.6-kb *Hin*dIII fragment containing the intact *ATP2* gene was transferred to M13mp8, and the resulting construction was used for all subsequent oligonucleotide mutagenesis procedures.

Oligonucleotide mutagenesis reactions were performed as described (21, 27). In all cases the DNA sequence of positive mutant candidates was confirmed by dideoxy sequencing analysis before subcloning (40). The 1.6-kb *Hin*dIII fragment was then subcloned into pCβ2, a derivative of the *ARS1 CEN4 URA3* vector pSEYC58 (10) carrying an *Eco*RI-*Hin*dIII fragment containing the *ATP2* promoter. The resulting constructions were identical to pCβ1 except for the specific site-directed mutations. Yeast cells were transformed to uracil independence with each of these plasmids by the lithium acetate method (20).

Radiolabeling and immunoprecipitations. Cultures were grown to mid-logarithmic phase (A_{600}, 0.5 to 1.0) in Wickerham minimal medium supplemented with 0.1 mM $(NH_4)_2SO_4$. Cells (2.5 OD_{600} units) were centrifuged, washed with minimal medium (minus SO_4^{2-}), and suspended in 1.25 ml of the same medium. After incubation for 30 min at 30°C, 625 μCi of carrier-free $Na_2{}^{35}SO_4$ (ICN) was added, and cultures were incubated for 5 min. Chase was initiated with the addition of 10 mM $(NH_4)_2SO_4$ and 100 μg of cycloheximide per ml, and 0.5-ml samples were harvested at the times indicated in the figure legends. Trichloroacetic acid (TCA) was immediately added to 5% final concentration to end the chase period. Precipitates were collected by centrifugation and washed twice with acetone. Dried pellets were suspended in 50 μl of lysis buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 1% sodium dodecyl sulfate [SDS]) and 2 OD_{600} units of unlabeled SEY6215 cells precipitated and washed in a similar way were added as cold competing antigen. Cells were lysed by vortexing for 1 min in the presence of glass beads (0.5 mm), followed by boiling for 3 min. The lysate was diluted by the addition of 1 ml of IP buffer (0.5% Tween 20, 50 mM Tris, pH 7.5, 150 mM NaCl, 0.1 mM EDTA), vortexed, and centrifuged for 10 min. The supernatant was carefully transferred to a clean tube, and 2 μl of F_1 ATPase β subunit antisera (a gift from M. Douglas) was added. Samples were incubated for 2 h on ice, followed by the addition of 50 μl of a 3.6% (wt/vol) suspension of protein A-Sepharose CL-4B beads (Sigma Chemical Co.). Incubation was continued for 30 min, and then the beads were collected by centrifuging for 30 s. The Sepharose beads were washed twice with IP buffer, twice with urea wash buffer (0.5% Tween 20, 100 mM Tris, pH 7.5, 200 mM NaCl, 2 M urea), and once each with 1% β-mercaptoethanol and 0.1% SDS. The beads were then suspended in 20 μl of 2× SDS sample buffer, boiled for 3 min, and centrifuged, and the supernatant was loaded onto an 8% polyacrylamide–SDS gel. Following electrophoresis, gels were treated with Autofluor (National Diagnostics) as recommended by the supplier, dried, and placed on film (Kodak XAR-5) at −70°C.

Fractionation and enzyme assays. Yeast cells carrying the indicated plasmids were grown on yeast nitrogen base medium (Difco Laboratories) with the appropriate supplements to an A_{600} of 0.5 to 1.0. Both spheroplasting and subcellular fractionation were done essentially as described (5) except that mitochondria were sedimented by centrifugation at 17,000 rpm for 10 min ([*rho*$^+$]) or 30 min ([*rho*$^-$]) in a Beckman JA-17 rotor. The efficiency of fractionation was monitored by assaying fractions for both the cytoplasmic marker α-glucosidase (14) and the mitochondrial marker cytochrome oxidase (49). In the experiments reported here, >99% of the total α-glucosidase activity was in the cytoplasmic fraction, while >90% of the total cytochrome oxidase activity was in the mitochondrial fraction. Published procedures were used to assay ATPase activity (33) and protein concentration (2).

For immunoblot analysis of subcellular fractions, cell equivalent amounts of cytoplasmic and mitochondrial protein (100 μg of cytoplasmic and ~5 to 6 μg of mitochondrial protein) were electrophoresed in adjacent lanes of an 8% polyacrylamide–SDS gel. Proteins were transferred to a Genescreen membrane (New England Nuclear Corp.) under the conditions recommended by the manufacturer. Immunoblotting was done with monoclonal F_1-ATPase β subunit antibodies and, as a mitochondrial control, outer membrane porin antisera (both gifts of G. Schatz) by a procedure supplied by New England Nuclear. Antibodies bound to specific antigens were detected by incubating the membrane with ^{125}I-labeled protein A (ICN), followed by washing and autoradiography.

RESULTS

Oligonucleotide-directed mutagenesis of the *ATP2* gene. Recent comparisons of a number of mitochondrial presequences have failed to reveal primary amino acid sequence homologies that potentially could be involved in an early step of import, such as binding to a receptor located in the cytoplasm or on the mitochondrial surface. However, it was found that most presequences are heavily biased toward basic and hydroxyl-carrying amino acid residues and against

	5 10 15	↓ 20 25 30 35	Generation Time (hr) Glucose	Glycerol-Lactate
wt β	M V L P R L Y T A T S R A A F K A A K	Q S A P L L S T S W K R C M A S A A Q S	3.0	5.6
R5-E	-----------E---------------------------------------	---	3.2	5.8
R12-E	----------------------------E--------------------	---	3.0	4.5
R5,12-E	-----------E----------------E--------------------	---	3.1	4.6
Δ1(5-12)	M V L P▨A A F K A A K	Q S A P L L S T S W K R C M A S A A Q S	2.9	5.4
Δ2(16-19)	M V L P R L Y T A T S R A A F▨	Q S A P L L S T S W K R C M A S A A Q S	3.1	5.2
Δ1,2	M V L P▨A A F▨	Q S A P L L S T S W K R C M A S A A Q S	3.2	5.9
Δ4(2-19)	M▨	Q S A P L L S T S W K R C M A S A A Q S	3.2	7.3
Δ3(28-34)	M V L P R L Y T A T S R A A F K A A K	Q S A P L L S T▨S A A Q S	3.2	6.4
Δ1,3	M V L P▨A A F K A A K	Q S A P L L S T▨S A A Q S	3.3	7.5
Δ2,3	M V L P R L Y T A T S R A A F▨	Q S A P L L S T▨S A A Q S	3.1	6.4
Δ1,2,3	M V L P▨A A F▨	Q S A P L L S T▨S A A Q S	3.5	N.G. (Gly⁻)
		SEY6215 (Δ*atp2* Parent)	3.0	N.G. (Gly⁻)

FIG. 1. Mutant F_1-β subunit proteins. NH_2-terminal residues of the wild-type F_1-β subunit precursor (44) and mutant derivatives as predicted from the nucleotide sequence. For missense mutants (R5-E, R12-E, and R5,12-E), dashed lines represent wild-type (wt) sequences. Cross-hatched boxes represent deletions. Basic residues are indicated with a plus sign; acidic residues are shown with a minus sign. The arrow above the sequence indicates the proteolytic processing site (46). After each of the centromere vectors containing the DNA coding sequences of these F_1-β subunit constructions was transformed into SEY6215, growth rates were determined in both YNB medium supplemented with 2% glucose (a fermentable carbon source) and YP medium supplemented with 3% glycerol and 3% DL-lactate (nonfermentable carbon sources). Two missense mutants (R12-E and R5,12-E) consistently grew 15 to 20% faster than the control strain on glycerol-lactate medium. The reason for this slight increase is unknown and was not pursued further. N.G., No growth.

acidic residues (34, 48). To test the general importance of these characteristics, oligonucleotide-directed mutagenesis was used to introduce a series of mutations into the gene encoding the F_1-β subunit precursor (*ATP2*). Initially, mutagenesis was directed toward the basic residues present within the 19-amino-acid presequence (46) and ranged from the substitution of acidic amino acid residues for the basic residues at positions 5 and 12 to deletions that removed various pairs of basic residues as well as the amino acids between them (Fig. 1). The mutated genes were moved into the single-copy *CEN4 ARS1* plasmid pSEYC58 (see Materials and Methods), and the resulting constructions were transformed into the Δ*atp2* yeast strain SEY6215 (which is unable to grow on a nonfermentable carbon source due to the inability to assemble a functional ATPase complex). Vassaroti et al. (47) have shown that yeast cells require near wild-type levels of functional mitochondrial ATPase to grow on nonfermentable carbon sources. Therefore, we initially tested import of the various mutant F_1-β subunits into mitochondria by determining whether any of the plasmids could restore growth of the Δ*atp2* strain on medium containing the nonfermentable carbon sources glycerol and lactate. Plasmids encoding various mutations within the F_1-β subunit presequence were able to restore growth of the Δ*atp2* strain to rates similar to those of the control strain (Fig. 1). Whether glutamic acid residues were substituted for one or both of the arginine residues at positions 5 and 12 (R5-E, R12-E, and R5,12-E) or different pairs of basic residues were deleted (Δ1 and Δ2), growth rates similar to that of the wild-type control strain were observed. Even when the deletions were combined in the Δ1,2 double mutant (which lost all four basic residues and 12 of the 19 total residues within the presequence), the growth rate of the mutant strain on glycerol-lactate medium was normal. This indicates that even in the absence of all four basic residues of the presequence, a level of F_1-β subunit import sufficient to support this growth could occur.

These results were unexpected because of the strong bias against acidic residues within most mitochondrial presequences and the importance generally placed on the basic residues found within these sequences. This led us to consider the possibility of additional import information residing within the NH_2 terminus of the mature F_1-β protein. To test this possibility, an additional mutant was constructed that deleted the coding sequence for amino acid residues 2 to 19 of the F_1-β precursor, the entire presequence beyond the initiation codon (Δ4 in Fig. 1). When introduced into the Δ*atp2* strain, growth on glycerol-lactate medium was again restored. We noted that two additional basic amino acids were present early in the mature F_1-β subunit at residues 11 and 12 of the mature protein (residues 30 and 31 of the precursor). To determine whether these residues were part of an import signal, amino acid residues 28 to 34 of the precursor protein (residues 9 to 15 of the mature protein) were deleted. While this deletion by itself (Δ3) still allowed growth on the nonfermentable carbon sources, the combination of deletions Δ1, Δ2, and Δ3 together (Δ1,2,3) completely abolished the ability of the plasmid to restore growth on glycerol-lactate medium. These results confirmed that a sequence within the mature F_1-β subunit can promote the import of this protein to a level sufficient to restore growth to near wild-type levels. Furthermore, we observed that any

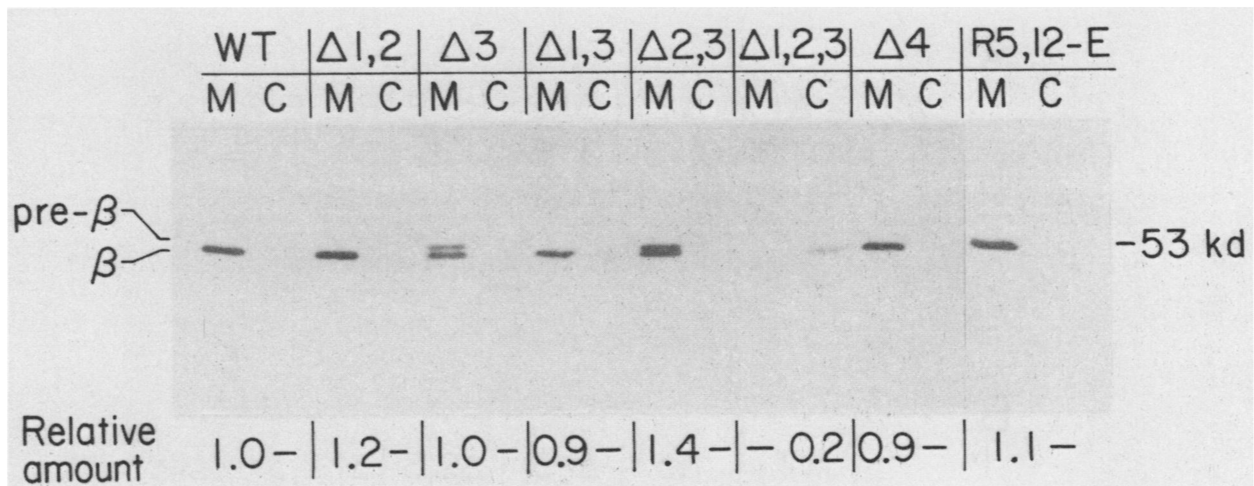

FIG. 2. Subcellular localization of mutant F_1-β subunit proteins. Cultures of SEY6215 expressing wild-type (WT) or mutant F_1-β subunit proteins were grown in YNB medium containing 2% galactose as the carbon source. Cells were enzymatically treated to form spheroplasts, fractionated into mitochondrial (M) and cytoplasmic (C) fractions (the cytoplasmic fraction is equivalent to the postmitochondrial supernatant of Daum et al. [5]), and cell equivalent amounts of these fractions were electrophoresed on SDS-polyacrylamide gels and immunoblotted as described in Materials and Methods. The position of the F_1-β subunit (53 kilodaltons [kd]) is shown.

pairwise combination of deletions Δ1, Δ2, and Δ3 (Δ1,2, Δ1,3, and Δ2,3) still allowed growth on glycerol-lactate medium, indicating that these deletions define three separate regions containing functionally redundant information capable of directing mitochondrial import of the F_1-β subunit precursor.

Quantitation of mutant F_1-β subunit localized to mitochondria. Although growth rates on a nonfermentable carbon source provide some indication of the import efficiency of the mutant F_1-β subunit proteins (47), more subtle differences in the import kinetics of the mutant proteins probably will not be detected. Therefore, to more carefully examine the level of targeting of the mutant F_1-β subunit proteins into mitochondria, a more quantitative assay was performed. Yeast cells harboring the mutant constructs were grown on galactose minimal medium and fractionated into mitochondrial and postmitochondrial fractions to quantitate both the amount of F_1-β subunit protein (determined by densitometry of immunoblots probed with F_1-β antisera) (5, 10) and the level of F_1-ATPase activity in the mitochondrial fractions isolated from these strains. It was found that both the amount of F_1-β subunit protein (Fig. 2) and the ATPase activity (Table 1) measured in the mitochondrial fractions from most of the strains were similar to those in the wild-type control. The only exceptions were the Δ1,3 and Δ1,2,3 mutants. The Δ1,3 mutant had wild-type amounts of the F_1-β subunit within mitochondria but only half the mitochondrial ATPase activity of the wild-type strain, suggesting that the combination of these mutations either impairs the assembly of a functional F_1-ATPase complex or reduces the activity of the assembled complex (although we have not excluded the possibility that some of the Δ1,3 protein detected may be mislocalized within the mitochondria). The Δ1,2,3 mutant, which was unable to grow on glycerol-lactate medium (Fig. 1), contained no detectable F_1-β subunit protein or F_1-ATPase activity (above background) in the mitochondrial fraction. In addition, the small amount of the Δ1,2,3 protein detected in the cytoplasmic fraction suggests that it is unstable in that subcellular compartment (pulse-labeling experiments have shown that the Δ1,2,3 F_1-β mutant protein is synthesized at normal levels; see Fig. 3B). These results demonstrate that in all cases other than the Δ1,2,3 mutant, mitochondrial accumulation of the mutant F_1-β subunits is similar to that in the wild-type control strain.

It was also apparent (Fig. 2) that two of the mutant proteins, Δ3 and Δ2,3, appeared to contain 30 to 50% of the mitochondrially localized F_1-β subunit in a precursor form. This indicates that the Δ3 deletion affects a region of the F_1-β subunit protein necessary for the efficient proteolytic processing of the precursor. (Δ1,3 may also be defective, but the precursor and mature forms apparently are not resolved in our gel system.) In spite of this processing defect, the mitochondrial ATPase levels in the Δ3 and Δ2,3 strains were similar to that in the wild-type control. This suggests that the precursor forms of these F_1-β subunit proteins have already been imported into the mitochondrial matrix and assembled into a functional ATPase complex. This is consistent with the results of Vassarotti et al. (46), who found that deletions extending from amino acid residue 36 of the F_1-β precursor (residue 17 in the mature portion of the protein) toward the

TABLE 1. Mitochondrial ATPase activity[a]

Plasmid	ATPase activity: Sp act (nmol/min per mg of protein)	ATPase activity: Relative activity
pCβ1	2.3	1.0
pCβΔ1,2	2.0	0.9
pCβΔ3	2.0	0.9
pCβΔ1,3	1.0	0.4
pCβΔ2,3	2.2	1.0
pCβΔ1,2,3	0.2	0.1
pCβΔ4	1.8	0.8
pCβR5,12-E	2.5	1.1

[a] Mitochondrial ATPase specific activity was not corrected for background (the mitochondrial ATPase specific activity of SEY6215 was ~0.1 nmol/min per mg of protein). Relative activity expresses the ATPase specific activity of each strain relative to that of the pCβ1 control strain (set at 1.0).

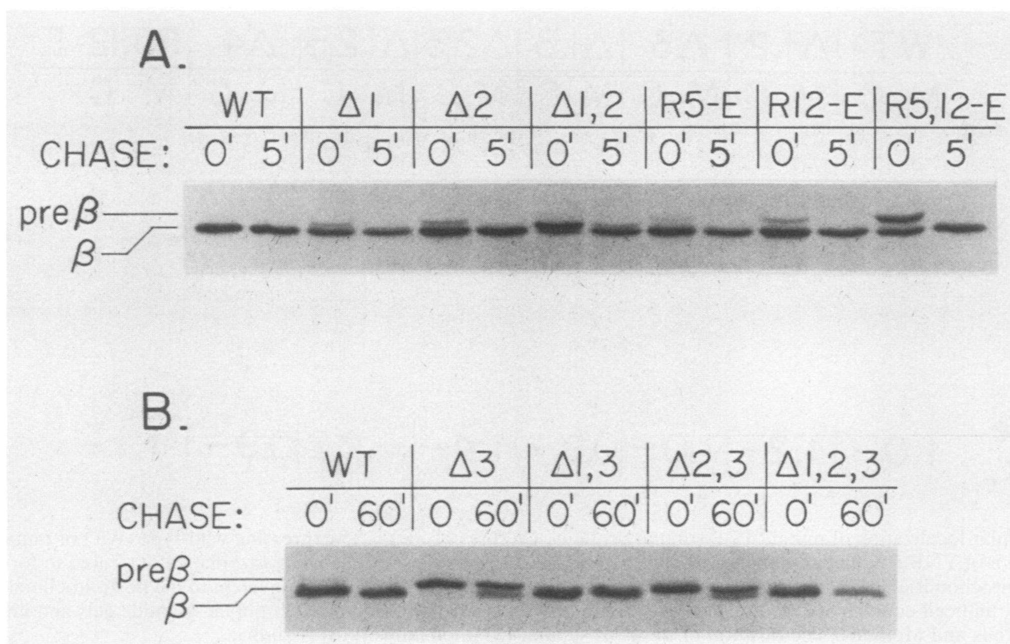

FIG. 3. Processing kinetics of mutant F_1-β subunit proteins. SEY6215 cells expressing wild-type (WT) or mutant F_1-β subunit proteins were labeled for 5 min with $Na_2{}^{35}SO_4$, followed by a chase in the presence of 10 mM $(NH_4)_2SO_4$ and 100 μg of cycloheximide per ml. The chase was terminated at the indicated times (0 or 5 min) by the addition of TCA. Immunoprecipitations were carried out as described in Materials and Methods. (A) Wild-type and F_1-β subunit leader sequence mutants; (B) wild-type and F_1-β subunit mutants carrying the Δ3 deletion.

NH_2 terminus disrupted the proteolytic processing but not the import of this protein.

Processing kinetics of mutant F_1-β subunit proteins. Most of the F_1-β subunit mutants not only grew normally on a nonfermentable carbon source but also contained normal mitochondrial levels of the F_1-β subunit protein and ATPase activity. However, since defects in the kinetics of mitochondrial import may not be reflected in the steady-state mitochondrial levels of the mutant F_1-β subunit proteins, we also examined the in vivo rate at which the mutant F_1-β precursor proteins were processed to the mature form. This provided an indication of the rate of import, since processing is normally rapid after import into mitochondria (35, 41). The wild-type β precursor protein, which has been reported to have an import half-time of 30 to 40 s (35), was barely detectable immediately after a 5-min labeling period with $^{35}SO_4{}^{2-}$ (consistent with the rapid import and maturation of this protein) (Fig. 3A). The mutant F_1-β subunit precursors, however, exhibited a range of processing delays. Mutants containing single amino acid substitutions (R5-E and R12-E) or deletions (Δ1 and Δ2) all showed slightly more precursor than the wild-type control immediately after labeling, indicating that these mutations reduced the rate of F_1-β subunit import to some extent. Two double mutants exhibited a more severe processing delay than the single mutants. The R5,12-E mutant protein was still roughly 50% precursor at the end of the 5-min labeling period, while the Δ1,2 mutant was approximately 80% precursor at that point. In spite of the large accumulation of precursor in these strains, essentially all of the R5,12-E precursor and most of the Δ1,2 precursor matured during a 5-min chase in the presence of excess unlabeled $SO_4{}^{2-}$ and cycloheximide, indicating that the import half-time of these proteins was still on the order of $\leq$2.5 min (only three- to fivefold longer than that of the wild-type F_1-β subunit precursor).

Since the previous fractionation experiments indicated that the F_1-β subunit mutants containing the Δ3 deletion were defective for processing (Fig. 2), the rate of conversion of the precursor to the mature form for these mutants does not represent an accurate measurement of their rate of import. When the Δ3 and Δ2,3 mutant strains were examined under 5-min pulse–5-min chase conditions, no mature protein could be detected (data not shown). Therefore, the experiment was repeated with a longer chase period (Fig. 3B). After a 60-min chase, it was found that only a small fraction of the precursor of these mutant proteins had matured. Because of this slow rate of processing of the Δ3 mutants, we could not accurately measure the rate of import of these proteins. However, this raised the concern that the processing of other mutants may not accurately reflect their rate of import into mitochondria but rather subtle effects on their processing rates within mitochondria. To exclude this possibility, an experiment was done with two F_1-β subunit mutants that showed a clear kinetic delay in processing

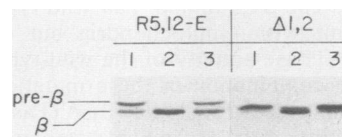

FIG. 4. Maturation of F_1-β subunit mutants is coupled to mitochondrial import. SEY6215 cells expressing the F_1-β subunit mutant R5,12-E or Δ1,2 were incubated for 5 min with $Na_2{}^{35}SO_4$, followed by a chase period in the presence of 10 mM $(NH_4)_2SO_4$ and cycloheximide (100 μg/ml). CCCP (40 μM) also was added to the indicated samples at the start of the chase period. The chase was continued for the indicated times and terminated by the addition of TCA. Chase conditions were 0 min in the absence of CCCP (lanes 1), 5 min in the absence of CCCP (lanes 2), and 5 min in the presence of CCCP (lanes 3).

TABLE 2. Growth rates of [rho^-] strains[a]

Class	Plasmid	Doubling time (h)
1	pCβ1 (wild type)	2.2
	pCβR5-E	2.1
	pCβR12-E	2.1
	pCβR5,12-E	3.2
	pCβΔ1	2.2
	pCβΔ2	2.2
	pCβΔ3	2.5
2	None	7.3
	pCβΔ1,2	7.0
	pCβΔ1,3	6.5
	pCβΔ2,3	6.3
	pCβΔ1,2,3	6.3
	pCβΔ4	6.3

[a] SEY6215.1 cells harboring the indicated plasmids were inoculated from plates that maintained a positive selection for the plasmid-encoded *URA3* marker and grown in YP medium containing 8% glucose at 30°C. Growth rates were determined by measuring the increase in cell density as determined by OD_{600} measurements.

(R5,12-E and Δ1,2). Strains expressing these proteins were again labeled for 5 min with $^{35}SO_4^{2-}$ and then chased in the presence of excess unlabeled SO_4^{2-} and cycloheximide with or without the uncoupling agent carbonyl cyanide *m*-chlorophenylhydrozone (CCCP; a compound that rapidly blocks mitochondrial protein import by eliminating the electrochemical potential across the inner mitochondrial membrane). If precursor processing is tightly coupled to import in these mutants, then the import block caused by CCCP should also block maturation (35). Alternatively, if import proceeds rapidly but intramitochondrial processing is delayed, maturation should proceed at a similar rate whether CCCP is present or not. It was found that the maturation of both mutant precursors was blocked in the presence of CCCP, indicating that these mutant proteins exhibit a defect in import and not in processing (Fig. 4). Therefore, maturation is a reasonable measure of the rate of import for mutant F_1-β subunit proteins other than those containing the Δ3 mutation. It was also found that the import block by CCCP could be overcome by the addition of β-mercaptoethanol and that maturation after this delayed import could be blocked by the addition of 1,10-phenanthroline (data not shown). These results demonstrate both the reversibility of the conditions used and the specificity of the processing by the matrix metalloprotease.

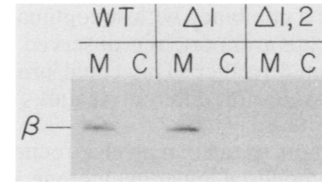

FIG. 6. Subcellular localization of mutant F_1-β subunit proteins in [rho^-] yeast cells. SEY6215.1 cells expressing wild-type (WT), mutant Δ1, or mutant Δ1,2 F_1-β subunits were grown in YNB medium containing 8% glucose as the carbon source. Fractionation and immunoblotting were done as described in the legend to Fig. 2 and Materials and Methods. M, Mitochondrial fraction; C, cytoplasmic fraction.

Mitochondrial import of the mutant F_1-β subunit proteins in [*rho*$^-$] yeast. The results presented in the previous sections show that import of most of the mutant F_1-β subunit proteins is compromised to only a small extent (at least in those cases where processing could be followed). However,

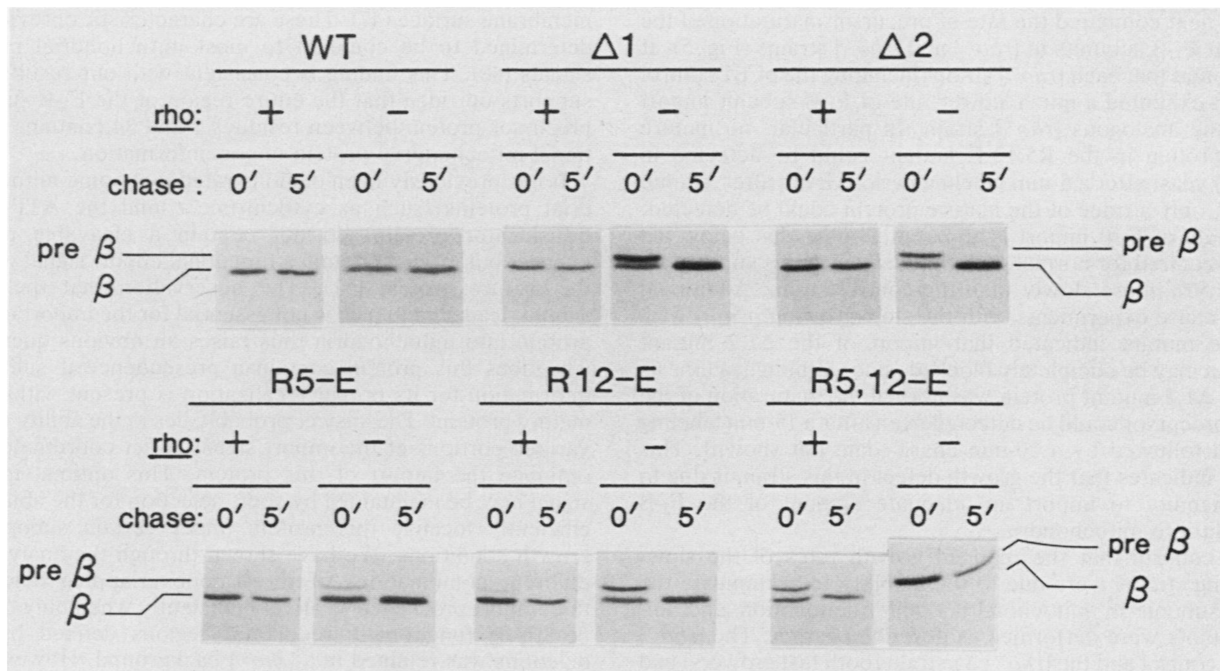

FIG. 5. Comparison of mutant F_1-β subunit protein processing kinetics in [rho^+] and [rho^-] yeast cells. SEY6215 and SEY6215.1 cells expressing wild-type (WT) or mutant F_1-β subunit precursors were grown in Wickerham minimal medium containing 2% galactose ([rho^+]) or 8% glucose ([rho^-]), labeled for 5 min with $Na_2{}^{35}SO_4$, and chased in the presence of 10 mM $(NH_4)_2SO_4$ and cycloheximide (100 μg/ml) for the indicated times (0 or 5 min). The chase was terminated by the addition of TCA. Immunoprecipitations were carried out as described in Materials and Methods.

we reasoned that if combined with suboptimal conditions for mitochondrial protein import, the observed import defects may be exaggerated further. This could provide additional insight into the roles of different features of the import signal.

It has been demonstrated that an electrochemical potential across the mitochondrial inner membrane is required for import (11, 30). Because respiration-deficient [*rho*$^-$] yeast lack both a functional proton-translocating ATPase and an electron transport chain, they probably are able to generate only a fraction of the normal membrane potential across the mitochondrial inner membrane (16). Although mitochondrial protein import still occurs in [*rho*$^-$] strains, we speculated that such suboptimal import conditions may exacerbate the import defects of at least some of the F_1-β subunit mutants, providing further information about the nature of these defects. To test this possibility, import of the mutant F_1-β subunit proteins was examined in the [*rho*$^-$] Δ*atp2* yeast strain SEY6215.1 (see Materials and Methods).

Examination of the growth rates of the [*rho*$^-$] F_1-β mutant strains in complex medium (containing 8% glucose as carbon source) revealed that they could be grouped into two classes (Table 2). The first class had growth rates similar to that of the [*rho*$^-$] pCβ1 (wild-type F_1-β) control strain, while the second class had growth rates about threefold slower, roughly comparable to that of the [*rho*$^-$] Δ*atp2* control strain (which encodes no F_1-β protein). The reduced growth rate of the [*rho*$^-$] Δ*atp2* control strain is presumably due to the absence of an intact F_1-ATPase complex, which together with the ADP-ATP translocator may function in the [*rho*$^-$] background to partially restore the electrochemical gradient across the inner mitochondrial membrane by hydrolyzing ATP in the mitochondrial matrix and then exchanging the ADP^{3-} for ATP^{4-} in the cytoplasm, generating a charge separation between the mitochondrial matrix and the cytoplasm (23, 29).

We next compared the rate of precursor maturation of the mutant F_1-β subunits in [*rho*$^+$] and [*rho*$^-$] strains (Fig. 5). It was found that each [*rho*$^-$] strain (including the pCβ1 control strain) exhibited a much slower rate of F_1-β subunit import than the analogous [*rho*$^+$] strain. In particular, no mature F_1-β protein in the R5,12-E mutant could be detected in [*rho*$^-$] yeast after a 5-min labeling period. Even after a 5-min chase, only a trace of the mature protein could be detected. This rate of F_1-β import is apparently somewhat below the level required for normal growth rates, since this strain grew about 50% more slowly than the control strain. Additional pulse-chase experiments with the slow-growing [*rho*$^-$] Δ1,2 double mutant indicated that import of the Δ1,2 mutant protein may be completely blocked, since although synthesis of the Δ1,2 mutant protein was normal, no maturation of the Δ1,2 precursor could be detected even after a 15-min labeling period followed by a 20-min chase (data not shown). This result indicates that the growth defect in this strain is due to the inability to import an adequate amount of the F_1-β subunit into mitochondria.

To confirm that the reduced growth rates of the slow-growing strains were due to the inability to accumulate the F_1-β subunit in mitochondria, cell fractionation and immunoblots were performed as described earlier. The [*rho*$^-$] pCβ1 control and the [*rho*$^-$] Δ1 strain (both fast growers) had normal mitochondrial levels of the F_1-β subunit, but this protein was not detected in the mitochondrial fraction of the [*rho*$^-$] Δ1,2 strain (a slow grower) (Fig. 6). This defect in the mitochondrial localization of the Δ1,2 mutant F_1-β subunit is not due to a general glucose growth medium effect, since pulse-chase experiments confirmed that the Δ1,2 mutant protein was imported efficiently when [*rho*$^+$] yeast were grown in glucose medium (data not shown). The absence of the Δ1,2 protein in the cytosolic fraction of the [*rho*$^-$] strain may be due to the apparent instability of the precursor F_1-β subunit within this fraction, as previously observed for the Δ1,2,3 mutant (Fig. 2), in conjunction with F_1-β subunit synthesis rates three-to fivefold lower in this experiment due to glucose catabolite repression (43).

DISCUSSION

Our results indicate that three distinct regions present within the amino-terminal 34 residues of the F_1-β subunit precursor are each capable of supplying a function necessary for the targeting and import of this protein into mitochondria. The simplest interpretation of these results is that various segments of the targeting signal can independently participate in directing functionally equivalent steps in the import process. Two observations in particular provide confirming evidence for functional redundancy in this mitochondrial import signal. First, it has been shown that a mutant F_1-β subunit precursor lacking amino acid residues 11 to 36 is still targeted and imported into mitochondria, demonstrating that amino acid residues 1 to 10 of the F_1-β subunit leader sequence are sufficient to direct mitochondrial import of this protein (46). Second, we have demonstrated in this study that the entire presequence can be deleted without blocking import of the F_1-β subunit protein, indicating that the mature protein also contains a functional import signal. Based on these results, we conclude that at least two of the three redundant regions we have defined contain separate, functional import signals. Recent structural modeling of the NH_2 terminus of the F_1-β subunit precursor by Fourier analysis indicated that the first 38 residues should maintain a strong amphipathic-helical character after interaction with a membrane surface (47). These are characteristics previously determined to be common to most mitochondrial import signals (48). This finding is consistent with our results and supports our idea that the entire region of the F_1-β subunit precursor protein between residues 1 and 34 contains functional mitochondrial protein import information.

It has previously been demonstrated that some mitochondrial proteins (such as cytochrome *c* and the ATP-ADP translocator protein) do not contain a cleavable presequence, but instead contain a functional import signal within the mature protein (51). The observation that the F_1-β subunit leader sequence is not essential for the import of this protein into mitochondria thus raises an obvious question: why does this protein contain a presequence if sufficient information for its proper localization is present within the mature protein? The answer probably lies in the ability of the various portions of the import signal to act coordinately to optimize the import of this protein. This optimal import signal may be maintained by some selection for the ability to efficiently localize this protein under certain suboptimal growth conditions. We have shown through the analysis of different combinations of three nonoverlapping deletions that import proceeded relatively efficiently when only one of the three functionally redundant regions defined by the deletions was retained in a [*rho*$^+$] background. However, it was found that in a [*rho*$^-$] background, little or no F_1-β subunit protein was imported in the mutants retaining only one of these domains. When two or more of these domains were present in the [*rho*$^-$] background, growth rates and mitochondrial F_1-β subunit levels were similar to those

observed in a [*rho*$^-$] strain expressing the wild-type F_1-β subunit protein. This conditional [*rho*]-dependent import phenotype suggests that these import domains act in an additive (or cooperative) manner to optimize F_1-β subunit import, enabling the process to proceed efficiently even under unfavorable conditions. The exaggerated import defects observed in the [*rho*$^-$] background can be attributed either directly to the absence of one or more proteins encoded by the mitochondrial genome or to a secondary effect caused by the loss of these proteins. Because [*rho*$^-$] yeast cells lack both a functional proton-translocating ATPase complex and an electron transport chain, it is likely that they are able to generate only a fraction of the normal membrane potential across the mitochondrial inner membrane (16). This reduced membrane potential may account for the exaggerated import defects exhibited by the mutant F_1-β subunit proteins.

We observed a strong correlation between the overall charge of the mutant F_1-β subunit import signals and their ability to direct import into mitochondria. Mutant F_1-β precursors containing deletions of several basic amino acid residues or containing acidic residues substituted for basic residues were found to be most impaired for import. It is tempting to speculate that this corresponds to an important role for the multiple basic residues generally found in mitochondrial presequences (34, 48). Mitochondria appear to represent the only intracellular organelle in eucaryotic cells with a membrane potential oriented to be electronegative inside. The positively charged import signals on mitochondrial proteins may be able to detect the uniquely oriented electrical field present in the mitochondrial inner membrane (or a close contact point between the inner and outer membranes) and use this distinguishing feature of the organelle (together with other components of the putative import machinery) to identify it as the correct delivery target. Indeed, it has been shown that this electrochemical potential contributes specifically to the membrane transfer of mitochondrial protein import signals (41), possibly by electrophoresis of the signal across the membrane. Consistent with such a model, the reduced charge associated with certain mutant F_1-β subunit import signals may only be sufficient to allow this process to occur in [*rho*$^+$], not in [*rho*$^-$] yeast cells. We intend to explore this model further by analyzing the import properties of additional mutants containing different numbers and distributions of charged amino acids in the import signal both in vivo and in vitro.

It previously was reported that the mature F_1-β subunit is not competent for import into mitochondria in vitro, while the F_1-β subunit precursor could be efficiently imported (24). However, we have shown here that the absence of the entire leader sequence (except the NH_2-terminal methionine) in the Δ4 mutant did not significantly affect accumulation of the F_1-β subunit in mitochondria in vivo (Fig. 2). Our preliminary experiments indicate that when synthesized in an in vitro transcription-translation system, the Δ4 mutant shown here to import into mitochondria in vivo did not import in vitro (unpublished results). This suggests that the conditions of in vitro import may represent suboptimal conditions compared with the in vivo conditions that facilitated the import of the Δ4 mutant F_1-β subunit protein. In this way, in vitro import may approximate import conditions such as those represented by our [*rho*$^-$] experiments, which were able to support import of the wild-type F_1-β subunit precursor but not of the Δ4 mutant (among others). Additional experiments are in progress to further examine import of each of the F_1-β subunit mutants in vitro. Another particularly interesting possibility is that loss of the presequence may restrict the import of the F_1-β subunit precursor to a strictly cotranslational mechanism, since this may be the optimal mode leading to efficient targeting and import of the precursor into mitochondria and would preclude mature F_1-β subunit import in vitro when added to the mitochondrial import system posttranslationally.

Vassarotti et al. (46) have recently shown that a series of deletions in the mature F_1-β subunit disrupt normal proteolytic processing of the F_1-β subunit precursor both in vitro and in vivo. Our results also indicate that deletion of the portion of the mature protein defined by the Δ3 mutation seriously impairs proteolytic cleavage of the presequence. This seven-amino-acid deletion removed residues 9 to 15 of the mature protein and was well separated from the cleavage site. Our results further indicate that the F_1-β subunit leader sequence probably contributes little direct information for processing, as none of the mutations within the presequence (including Δ2, which removed the four residues directly adjacent to the cleavage site) caused any observable kinetic processing defect detectable within the resolution of our experimental system. The rapid processing kinetics of these mutant proteins together with an observed sensitivity of the presequence mutants to processing inhibition by 1,10-phenanthroline suggests that processing is still being carried out by the correct matrix protease. This indicates that most or all of the processing information for the F_1-β subunit precursor lies within the mature sequence of the protein. This is in contrast to the results seen with pre-ornithine transcarbamylase (17), pre-alcohol dehydrogenase III (32, 45), and cytochrome oxidase subunit IV (18), in which regions of the leader sequence appear to be necessary for normal processing. An analysis of the processing signal of additional mitochondrial precursors is needed to determine the exact component(s) of a precursor protein necessary to facilitate specific recognition and cleavage by the matrix protease.

Mutagenesis procedures have been used to examine the functional targeting components of other mitochondrial protein import signals. Horwich et al. (17) introduced a series of both deletion and missense mutations into the leader sequence of pre-ornithine transcarbamylase and found that the central portion of the leader was essential for import into isolated rat liver mitochondria. Similarly, Hurt et al. (18) made a deletion analysis of the cytochrome oxidase subunit IV presequence and concluded that only the NH_2-terminal end of that presequence is capable of functioning as an import signal. This suggests that the import signal of these proteins may not contain redundant import information. However, Pilgrim and Young (32) found that after extensive bisulfite mutagenesis of the gene encoding the mitochondrial alcohol dehydrogenase III precursor of *S. cerevisiae*, no mutants were obtained that completely excluded this protein from mitochondria. This suggests that the alcohol dehydrogenase III precursor, like the F_1-ATPase β subunit precursor, may also contain redundant import information. Since redundant targeting information has also been observed in proteins imported into the nucleus (36; M. Hall, personal communication), redundant import signals may be characteristic not only of mitochondrial proteins, but also of many proteins destined for delivery to noncytoplasmic cellular locations.

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