The Amino Terminus of the Yeast F$_1$-ATPase β-Subunit Precursor Functions as a Mitochondrial Import Signal

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Abstract. The ATP2 gene of Saccharomyces cerevisiae codes for the cytoplasmically synthesized β-subunit protein of the mitochondrial F$_1$-ATPase. To define the amino acid sequence determinants necessary for the in vivo targeting and import of this protein into mitochondria, we have constructed gene fusions between the ATP2 gene and either the Escherichia coli lacZ gene or the S. cerevisiae SUC2 gene (which codes for invertase). The ATP2-lacZ and ATP2-SUC2 gene fusions code for hybrid proteins that are efficiently targeted to yeast mitochondria in vivo. The mitochondrially associated hybrid proteins fractionate with the inner mitochondrial membrane and are resistant to proteinase digestion in the isolated organelle. Results obtained with the gene fusions and with targeting-defective ATP2 deletion mutants provide evidence that the amino-terminal 27 amino acids of the β-subunit protein precursor are sufficient to direct both specific sorting of this protein to yeast mitochondria and its import into the organelle. Also, we have observed that certain of the mitochondrially associated Atp2-LacZ and Atp2-Suc2 hybrid proteins confer a novel respiration-defective phenotype to yeast cells.

ORGANELLE function in eucaryotes largely is determined by the unique set of proteins that reside within them. These proteins must be accurately targeted from their site of synthesis in the cytoplasm to their unique site of functional residence. Regulation of this intracellular protein traffic involves the participation of "sorting signals" within proteins that allow them to be specifically identified and then delivered to their correct organelle destination. We describe here an approach to define the sorting information present in a yeast mitochondrial protein.

Most mitochondrial proteins are coded for by nuclear genes. Many are synthesized as larger precursors with transient amino-terminal amino acid extensions (reviewed in references 14 and 28). These extensions tend to contain several basic amino acids and lack acidic amino acids. The pre-segments are processed from the protein after import into mitochondria by a chelator-sensitive protease in the matrix compartment (1, 22). Delivery to the matrix involves transport across both the outer and inner mitochondrial membranes. The electrochemical potential across the inner mitochondrial membrane is required for this transport to take place (13). In addition, the transient pre-segments on mitochondrial precursor proteins are required; processed precursors are not imported into mitochondria in vitro (13). Indeed, recent data indicate that the pre-segment alone is sufficient to deliver a protein into mitochondria. When the pre-segment of the yeast cytochrome c oxidase subunit IV was fused to the cytosolic protein dihydrofolate reductase, the resulting hybrid protein was transported into the mitochondrial matrix in vitro (15, 16). To analyze this problem in vivo, we have employed both gene fusion and deletion studies to look in detail at delivery to the mitochondrial matrix of the ATPase β-subunit protein.

The mitochondrial ATPase complex contains 10 defined subunits: 7 are encoded by nuclear genes and 3 are encoded by mitochondrial genes (10). Import of the F$_1$-ATPase β-subunit into the mitochondrial matrix has been well characterized. The nuclear ATP2 gene encodes the 509 amino acid β-subunit protein precursor. This precursor contains a transient amino-terminal extension of ~20 amino acids (19). Precursor but not mature β-subunit protein can be imported into mitochondria in vitro (13). Here, we show by gene fusion that the amino terminus of the precursor β-subunit protein contains a targeting signal that is sufficient to direct mitochondrial delivery of two proteins that normally do not reside in this organelle. Alteration of this amino-terminal sequence by deletion mutation blocks its ability to function as a mitochondrial delivery signal.

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Materials and Methods

Strains and Media

Saccharomyces cerevisiae strains used were SEY1201 (MATa ura3-52 leu2-3

112 sucl-2 ade2-l gal2) and SEY2102 (MATa ura3-52 leu2-3-112

sucl-2-d9 his4-519 gal2) (12). MDY2102 (MATa ura3-52 leu2-3-112 sucl-2-

19 hio-419 gal2 apt2::LEU2) was constructed by using a one-step gene

disruption technique (26). A 2.2-kb Eco RI-Bam HI fragment containing most

of the 3'-end of the 1.6-kb genomic fragment was cloned into pBR322 at the Eco RI

site at codon 34 in the gene. The yeast LEU2 gene isolated on a 2.0-kb Hpa I

fragment was ligated into this Pvu II site in the ATP2 structural gene.

The construction was confirmed by restriction endonuclease analysis. Digestion

of this construction with Bam HI generates a linear fragment of ATP2 DNA

disrupted at codon 34 with the LEU2 gene. Transformation of this linearized

dNA into SEY2102 yielded LEU2 transformants unable to grow on a non-

fermentable carbon source. The gene disruption at ATP2 was confirmed by both

genetic and physical methods.

The Escherichia coli strains were MC1061 [F' araD139 araB trpC-600

lacX74 galK galA rpsL thi] (41), MC1066 [F' lacZD PROXY74 galA galK

rpsL thi] (7), and MDY203 [F' (araD139 trpC-600 lacZD PROXY74 galA

rpsL thi) plasmid pSEY700 (gift from Bruce Duncan, University of Minne-

sota) and SE10 [F' (araD139 trpC-600 lacZD PROXY74 galA galK

rpsL thi)] (8). Appropriate digestion times were determined empirically by sizing the digested DNAs on agarose gels. The

fragments of pBR322 were separated by electrophoresis in agarose gels.

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fragments of pBR322 were separated by electrophoresis in agarose gels.

DNA Methods

All restriction endonuclease digestions, S1 nuclease digestions, and ligations with T4 DNA ligase were performed essentially according to instructions provided by the commercial supplier (New England Biolabs, Beverly, MA, or

Bethesda Research Laboratories, Gaithersburg, MD). Digestions with BaeI nuclease (Bethesda Research Laboratories) were performed at 23°C in buffer

recommended by the supplier except that 200 mM NaCl was used in place of

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but still had the Hind III site 5' of the SUC2 coding sequence. This plasmid was designated pSEY303 (Fig. 1). The plasmid contains a series of unique restriction enzyme sites useful for constructing fusions to the SUC2 gene. The DNA sequence and reading frame across these restriction sites is assumed based on the construction scheme employed.

Finally, plasmids pG and pCp8 were derived from the plasmid vectors pSEY8 and pSEYC58, respectively. Each was constructed by cloning a 2.6-kb Eco RI-Hind III fragment, containing the entire coding and regulatory sequences of ATP2, into the unique Eco RI, Hind III sites present in both pSEY8 and pSEYC58. Each codes for functional β-subunit protein in yeast.

**Isolation and Fractionation of Mitochondria**

Yeast cells harboring different plasmids were grown at 28-30°C to an A600 of 0.5 on yeast nitrogen base-2% dextrose medium (30) containing the appropriate amino acid supplements (7). 4 h before cell harvest, yeast extract was added.
to a final concentration of 0.5%. Mitochondria were prepared from yeast spheroplasts as previously described (5) and resuspended in 0.6M mannitol, 0.02M Tris-HCl, pH 7.4, 4 mM EDTA, 1 mM phenylmethylsulfonyl fluoride. Post-mitochondrial supernatant fractions (12,000 g supernatant) were further centrifuged at 100,000 g for 60 min. The recovery of mitochondria relative to cytosol was monitored by assaying the mitochondrial marker enzyme cytochrome oxidase and the cytosolic marker glyceraldehyde-3-phosphate dehydrogenase. In all fractions reported here, >90% of the total cytochrome oxidase activity in the crude cell extracts was recovered in the mitochondrial pellet fraction. Less than 3% of the total glyceraldehyde-3-phosphate dehydrogenase activity co-fractionated with the mitochondria. Samples for gel analysis were rapidly frozen in liquid nitrogen. Freshly prepared mitochondria were used for mitochondrial fractionation and digestion studies.

For mitochondrial fractionation analysis, organelles were resuspended at 10 mg/ml in 0.6 M sorbitol, 20 mM Hepes, pH 7.4. Intermembrane space material was released by dilution and a 30-min incubation in 5 vol 10 mM Tris-HCl, pH 8.0, 1 mM phenylmethylsulfonyl fluoride. A low speed pellet from the above dilution yielded a mitochondrial membrane and matrix fraction which was resuspended to 2 mg/ml in 1.5 M sorcute, 8 mM ATP, 8 mM MgCl2, pH 7.4. After brief sonication to vesiculizar the membranes and release the matrix protein, the samples were centrifuged at 200,000 g for 45 min. This procedure routinely yielded a matrix preparation containing 65-75% of the total fumarase activity (a soluble matrix enzyme) present in the starting mitochondrial preparation. Less than 10% of the starting fumarase activity was detected in the soluble inner mitochondrial space fraction. The pellet from this centrifugation containing total mitochondrial membrane was washed once with 10 mM Tris-HCl, pH 7.4, at 4°C (centrifugation at 200,000 g for 40 min) before either direct analysis on gels or membrane separation. The washed membranes in these studies were contaminated with <3% of the matrix enzyme fumarase present in the starting mitochondria. For resolution of mitochondrial membranes, this fraction (routinely containing >70% of the membrane bound cytochrome oxidase activity) was resuspended to 5 mg/ml in 50 mM Tris-HCl, pH 7.4, by brief sonication and then was loaded on a linear 20-70% sucrose gradient in the same buffer. Centrifugation in an SW 27.1 rotor (Beckman Instruments Inc., Palo Alto, CA) was for 16 h at 20,000 rpm. Fractions (0.7 ml) were collected from the bottom of the tube and assayed for enzyme activities.

Mitochondrial Digestion Studies

Freshly prepared mitochondria were resuspended at 5 mg/ml in 0.6 M mannitol, 20 mM Tris-HCl, pH 7.4, 2 mM EDTA (MTE). Proteinase K stock solutions were made fresh in MTE. 100-μl digestions contained 400 μg fresh mitochondrial in MTE plus the indicated amount of proteinase K. When included, Triton X-100 was added to 1% (wt/vol) stock solution to a final concentration of 0.3%. Digestions at 23°C for 30 min were terminated on ice by the addition of 0.5 mM phenylmethylsulfonyl fluoride from a fresh 10 mM ethanolic stock solution, and enzyme activities were analyzed immediately.

Immunological Studies

For whole cell immunoblot or immunoprecipitation analysis, ~5–6 ml of cells containing two ABA units were treated with trichloroacetic acid to a final concentration of 10% for 10 min and then processed for rapid lysis essentially as previously described (11). The trichloroacetic acid–treated cells were harvested and washed once with 1 ml 50% ethanol and then resuspended into 50 μl 1% SDS. Glass beads (0.5 mm) were added (0.15 g), and the samples were vortexed for 2 min and then heated in a boiling water bath for 3 min. For immunoprecipitation analysis, 1 ml of 2% Triton X-100/200 mM sodium phosphate, pH 7.0, 300 mM NaCl was added to the broken cells. This suspension was freed of cell debris and glass beads by centrifugation for 5 min at 10,000 g followed by the addition of the appropriate antiserum. For analysis of total cell homogenates by immunoblot gel electrophoresis, the broken cells were washed from the glass beads into SDS gel electrophoresis sample buffer (200 μl), giving an approximately final concentration of 0.5 mg/ml protein. SDS polyacrylamide gels were performed essentially as described (6). Electrophoretic transfer of gel resolved proteins to nitrocellulose was performed according to published procedures (34). Antigen bound by specific antibodies to nitrocellulose was detected by use of the commercially available horseradish peroxidase–goat–anti–rabbit antibody conjugate (Bio-Rad Laboratories, Richmond, CA). Autoradiography of dried SDS gels was performed as previously published (6). Standards used to determine the apparent molecular masses of hybrid protein were ferritin (220 kD), β-galactosidase (115 kD), phosphorylase b (94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), and carbonic anhydrase (29 kD).

Results

ATP2-lacZ Gene Fusions

Previously, we have shown by gene fusion that the amino-terminal 380 amino acids of the yeast ATP2 gene product can direct mitochondrial import of E. coli β-galactosidase (7). To define more precisely the ATP2 sequences directing mitochondrial import of the hybrid protein, we have constructed a series of shorter gene fusions between the ATP2 gene and the lacZ gene as described in Materials and Methods. Each gene fusion contains ATP2 regulatory and amino-terminal coding sequences fused in frame to a large carboxy-terminal coding segment of lacZ. All of the gene fusions direct the synthesis of active β-galactosidase in both E. coli and yeast. No β-galactosidase activity is expressed from the pSEY101 parent vector in these cells. Each of the gene fusions contains a unique Bam HI restriction site at the joint between ATP2 and lacZ sequences. Because of the approach used to construct the hybrid genes, the translational reading frame across this Bam HI site is the same in each of the fusions. Of 80 initially isolated ATP2-lacZ fusions, 15 were chosen based on DNA restriction analysis as a representative set of different sized classes of fusions. All of the analyses reported here were carried out with these 15 fusions (designated pS1ZI–pS1Z15, Fig. 2).

The location of each ATP2-lacZ fusion joint was determined by DNA sequence analysis (Table I). The DNA sequence results confirmed that in each gene fusion, the ATP2 coding sequence is in frame with the lacZ coding sequence. The levels of β-galactosidase expressed in crude extracts of the yeast strain SEY2102 from each of the 15 gene fusion constructs also was determined. The levels varied from ~200–400 U/mg total cell protein (pS1Z1–pS1Z5) to 1,000–2,000 U/mg total cell protein (pS1Z6–pS1Z15). Those gene fusions that contained a large amino-terminal coding segment of the ATP2 gene fused to lacZ expressed lower levels of β-galactosidase activity than the fusions that had only a short coding segment of ATP2 fused to lacZ. We suspected that this was related to plasmid stability as plasmids containing large ATP2-lacZ gene fusions (pS1Z1–pS1Z5) rapidly were lost in the absence of Ura selection. The pSEY101 plasmid, like other 2-μm DNA based plasmids, is normally maintained in multiple copies per yeast cell. However, the copy number per cell can vary. To stabilize plasmid copy number and its segregation properties, we transferred the ATP2-lacZ gene fusions into another plasmid, pSEY102, which contains the yeast centromere sequence of chromosome IV and the sequence ARS1 (Fig. 1). These sequences allow for the stable maintenance of this plasmid at approximately one copy per cell (reviewed in reference 2). The ATP2 segment from each of the 15 pS1Z plasmids was moved on an Eco RI-Bam HI DNA fragment into the Eco RI-Bam HI sites present in the vector pSEY102. This gave rise to a complementary set of ATP2-lacZ gene fusions.
designated pCβZ1-pCβZ15 (Table I). All maintain the correct translational reading frame between ATP2 and lacZ. β-Galactosidase expressed from these plasmids in the yeast strain SEY2102 varied from ~500 U/mg total cell protein for the larger gene fusions (pCβZ1-pCβZ6) to 1,000 U/mg total cell protein for the smaller fusions (pCβZ7-pCβZ15). In addition, the new ATP2-lacZ gene fusion constructs exhibited greater plasmid stability in the absence of Ura+ selection. For these reasons, most of our studies were carried out with the pCβZ constructs.

The levels of β-galactosidase activity expressed from each of the ATP2-lacZ gene fusions were shown to be regulated in a similar manner to that observed for wild type ATP2 gene expression (32). In 2% glucose media (repressing conditions), cells harboring the ATP2-lacZ gene fusions express β-galactosidase at a level fourfold lower than seen in the same cells transferred for 2 h to low (0.1%) glucose-containing media (derepressing conditions). The 1,100 bp of sequence 5' of the ATP2 structural gene contained in each of the fusion constructs apparently is sufficient for normal control of ATP2 gene expression.

That each of the pCβZ plasmids direct the synthesis of F1-β-subunit β-galactosidase hybrid proteins (also referred to as Atp2-LacZ hybrid proteins) was demonstrated by steady state 35SO4 labeling of yeast cells carrying these plasmids, followed by immunoprecipitation of the hybrid proteins with antisera against both the F1-β-subunit and β-galactosidase. The precipitates were resolved by SDS PAGE (Fig. 3). Unexpectedly, cells harboring ATP2-lacZ gene fusions that contain ATP2 coding sequences for <169 amino acids of the β-subunit protein did not express detectable hybrid proteins. Rather, the hybrid proteins appear to have been modified to a polypeptide with an apparent molecular weight similar to that of wild-type β-galactosidase. This result seems to relate to the fact that the smaller hybrid proteins are not targeted to mitochondria but rather accumulate in the cytoplasm (see below). Yeast cells that harbor these fusions (pCβZ10-15) still expressed β-galactosidase activity, indicating that this degradation product remains enzymatically active. The apparent molecular weight of the hybrid proteins detected in cells harboring the larger ATP2-lacZ gene fusions agrees well with the molecular weights predicted for these proteins from the DNA sequence results (Table I).

**Atp2-LacZ Hybrid Proteins Are Targeted to Mitochondria**

The subcellular location of the various Atp2-LacZ hybrid proteins was determined by isolating mitochondrial and cytosolic fractions from yeast strain SEY2102 that harbored each of the pCβZ plasmids. The cellular distribution of the hybrid proteins was determined both by β-galactosidase enzyme assays and by immunoblotting using antisera directed against β-galactosidase. Results of these fractionation studies are shown in Fig. 2. Clearly, ATP2 encoded sequences can direct β-galactosidase to mitochondria. We find that Atp2-LacZ hybrid proteins containing ≥169 amino acids of Atp2
species that co-migrates with wild-type (class III fusions; Fig. 2) are partially degraded to a proteinase K.

erase digestion conditions that inactivate a marker enzyme in amino-terminal amino acids of the a cause or a consequence of the observed lack of targeting is analyzing the accessibility of the hybrids in isolated intact mitochondria to externally added proteinase K (Fig. 4). The results show that β-galactosidase activity expressed from plasmids pCβZ1 and pCβZ7 co-migrates on sucrose gradients with the inner mitochondrial membrane.

The mitochondrial location of those Atp2-LacZ hybrid proteins that are protected from proteinase digestion was further analyzed by subfractionation of mitochondria isolated from cells harboring the fusion plasmids pCαZ1 and pCαZ7. As controls, the small amount of β-galactosidase expressed from plasmids pCβZ14 and pLG669-Z (which directs the synthesis of a cytoplasmic cytochrome c β-galactosidase hybrid protein [7]) that associates with mitochondria also was analyzed (Fig. 5). The results show that β-galactosidase activity expressed from plasmids pCβZ1 and pCβZ7 were as published.

The plasmids used to construct each gene fusion series and the determination of the fusion joints in ATP2 were as described in Materials and Methods. The predicted molecular weights of the β-galactosidase (17) and yeast invertase (33) were as published.

fused to LacZ are located in the mitochondrial fraction. Hybrid proteins with less β-subunit information than this are found in the cytosolic fraction. The cytosolic hybrid proteins (class III fusions; Fig. 2) are partially degraded to a protein species that co-migrates with wild-type β-galactosidase (Fig. 3). Whether the instability of these shorter hybrid proteins is a cause or a consequence of the observed lack of targeting is not yet clear. The results demonstrate, however, that 169 amino-terminal amino acids of the F1-β-subunit protein precursor are sufficient to direct mitochondrial delivery of the normally cytoplasmic E. coli enzyme β-galactosidase.

The nature of the association of the targeted Atp2-LacZ hybrid proteins with mitochondria was further probed by analyzing the accessibility of the hybrids in isolated intact mitochondria to externally added proteinase K (Fig. 4). The β-galactosidase activity associated with intact mitochondria isolated from cells harboring plasmids pCβZ1-pCβZ9 was found to be resistant to proteinase inactivation under proteinase digestion conditions that inactivate a marker enzyme in the outer mitochondrial membrane, anticycin-insensitive NADH cytochrome c reductase. However, in the presence of detergent, the β-galactosidase is readily accessible to proteinase K. On the other hand, the small but significant amount of β-galactosidase activity associated with mitochondria isolated from cells harboring plasmids pCβZ10-pCβZ15 was sensitive to proteinase K digestion even in the absence of the detergent Triton X-100 (Fig. 4). Apparently, hybrid β-subunit β-galactosidase proteins containing at least 169 amino acids of the β-subunit get delivered into mitochondria beyond the outer membrane proteinase barrier. Shorter hybrid proteins associate only weakly with the outer surface of the organelle. Indeed, most of the β-galactosidase associated with the surface of mitochondria in cells harboring short ATP2-lacZ gene fusions can be washed off the organelle with high salt (data not shown).

The mitochondrial location of those Atp2-LacZ hybrid proteins was as published. The nature of the association of the targeted Atp2-LacZ hybrid proteins with mitochondria was further probed by analyzing the accessibility of the hybrids in isolated intact mitochondria to externally added proteinase K (Fig. 4). The results show that β-galactosidase activity expressed from plasmids pCβZ1 and pCβZ7 were as published.

### Table 1. ATP2 Gene Fusions

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Figure 3. ATP2-lacZ-encoded hybrid proteins. Yeast whole cell extracts prepared from steady state 35S-labeled cells were immunoprecipitated with combined F1-β-subunit plus β-galactosidase antisera. Immunoprecipitates were resolved on an SDS 10-15% polyacrylamide gradient gel, which was then dried and autoradiographed. Cell extracts were prepared from yeast strain SEY2102 harboring plasmids pCαZ1, 6, 7, 9, 10, and 15. The positions of wild-type β-galactosidase (116 kD) and the F1-β-subunit protein (54 kD) are indicated. Each of the hybrid proteins exhibits some degree of proteolytic breakdown to a protein species with an apparent molecular weight similar to that of wild-type β-galactosidase.

Figure 4. β-subunit β-galactosidase hybrid proteins delivered to mitochondria are protected from digestion by externally added proteinase K. Mitochondria prepared from strain SEY2102 harboring the indicated ATP2-lacZ gene fusions on pCβZ plasmids were suspended at 4 mg/ml in isotonic buffer (see Materials and Methods). Digestions with proteinase K were performed at 37°C for 30 min in a total volume of 100 µl. Reactions were terminated on ice by the addition of 0.5 mM phenylmethylsulfonyl fluoride. O, β-galactosidase, digestion in the absence of detergent; K, β-galactosidase digestion in the presence of 0.3% Triton X-100; Δ, antimycin-insensitive NADH cytochrome c reductase, digestion in the absence of detergent.

Figure 5. β-subunit β-galactosidase hybrid proteins delivery to mitochondria was further probed by analyzing the accessibility of the hybrids in isolated intact mitochondria to externally added proteinase K (Fig. 4). The results show that β-galactosidase activity expressed from plasmids pCβZ1 and pCβZ7 were as published.
marker enzyme cytochrome oxidase. The small amount of β-galactosidase expressed from plasmids pCBZ14 and pLG669-Z that associates with mitochondria co-fractionates with the outer membrane NADH cytochrome c reductase marker enzyme. Further analysis of the precise nature of the association of this cytosolic protein into mitochondria in vitro. We decided to construct an additional series of gene fusions between the ATP2 gene and the SUC2 gene of yeast to determine if some feature about β-galactosidase might interfere with the mitochondrial delivery information presumably present early in the β-subunit protein. The SUC2 gene codes for the secreted enzyme invertase (3). This protein normally transits through the yeast secretory pathway to the cell surface (24). To construct these fusions, we used the SUC2 gene fusion vector pSEY303 (Fig. 1). As was done in the construction of the pCBZ plasmids, the various ATP2 DNA segments were isolated on Eco RI-Bam HI DNA fragments from plasmids pZ1-pZ15 and cloned directly into Eco RI-, Bam HI-digested pSEY303 plasmid DNA. The resulting set of ATP2-SUC2 gene fusion containing plasmids was designated pSH1-pSH15 (Fig. 2). Again, because the translational reading frame is the same across the Bam HI site in the plasmids pSEY101, pSEY102, and pSEY303, this simple DNA fragment exchange process gives rise to in-frame gene fusions. Hybrid proteins expressed from the ATP2-SUC2 gene fusions were identified in strains harboring these fusions by the immunoblotting technique using invertase-specific antisera (Fig. 6).

Unlike certain ATP2-lacZ gene fusions, all of the ATP2-SUC2 gene fusions direct the synthesis of stable hybrid proteins that migrate on SDS polyacrylamide gels with apparent molecular weights similar to those predicted based on the DNA sequence results (Table I). In addition, we observed approximately the same level of expression of each of the different sized ATP2-SUC2-encoded hybrid proteins in yeast.

Somewhat surprisingly, the β-subunit invertase hybrid proteins (also referred to as Atp2-Suc2 hybrid proteins) were found not to exhibit significant levels of the sucrose-cleaving enzyme activity of invertase. Detection of these hybrid pro-
teins therefore has been limited to immunologic techniques. We have previously demonstrated that a gene fusion constructed between the yeast MFa1 gene, which codes for the secreted pheromone α-factor, and the SUC2 gene does express a hybrid protein with invertase activity (12).

The subcellular distribution of the Atp2-Suc2 hybrid proteins was analyzed by using invertase-specific antisera to immunoblot the mitochondrial and cytosolic fractions isolated from yeast strain SEY2102 harboring the different pπl plasmids (Fig. 6). The Atp2-Suc2 hybrid proteins coded for by each of these plasmids was found to be located only in the mitochondrial cell fraction. No detectable cross-reacting invertase antigen was observed on the immunoblots in the cytosolic fraction (data not shown). Hybrid protein not delivered to mitochondria may be susceptible to degradation in the cytoplasm. However, because the level of hybrid protein recovered in the mitochondrial cell fraction was comparable for each of the ATP2-SUC2 gene fusions, it seems unlikely that a significant fraction of these hybrid proteins remains in the cytoplasm of the cell. 39 amino-terminal amino acids of the F1-β-subunit protein precursor therefore are sufficient to direct mitochondrial delivery of the normally secreted protein invertase.

Proteinase K protection experiments similar to those used to analyze the mitochondrial associated Atp2-LacZ hybrid proteins also were carried out with mitochondria isolated from cells carrying plasmids pπl1-pπl15. Each of the Atp2-Suc2 hybrid proteins encoded by these plasmids was found to be resistant to proteinase K digestion in intact mitochondria. The quantity and size of the hybrid proteins as detected by immunoblotting was the same with and without proteinase K treatment. After Triton X-100 solubilization of the mitochondria, all Atp2-Suc2 hybrid proteins were degraded by proteinase K (data not shown).

Mitochondria isolated from cells harboring plasmids pπl 1, 8, and 15 were subfractionated into a membrane fraction, a matrix fraction, and an intermembrane space fraction to determine the submitochondrial location of the Atp2-Suc2 hybrid proteins expressed by these plasmids (Fig. 7). In each case, the hybrid protein was found to co-fractionate quantitatively with the mitochondrial membranes. The hybrid proteins behaved similarly to the mitochondrial inner membrane ADP/ATP carrier protein used as a control membrane marker in these fractionation studies. As few as 39 amino-terminal amino acids of the β-subunit precursor could direct the import of invertase into a membrane location within the mitochondria.

**Effect of Internal Deletions in ATP2 on Mitochondrial Delivery of an Atp2-LacZ Hybrid Protein**

The minimal ATP2 sequence sufficient for mitochondrial delivery of ATP2-lacZ and ATP2-SUC2 gene fusion products is different (see above). The additional β-subunit sequences found to be required to direct β-galactosidase to mitochondria may not be part of the targeting signal but rather simply may act to separate this signal from the β-galactosidase protein, thereby making it available for proper recognition. To test this and, in addition, to map more accurately the targeting information present within ATP2, we constructed a number of deletions in the ATP2 sequences present in the pπl1 ATP2-lacZ hybrid gene. This was done both by deleting between unique DNA restriction sites present within the ATP2 gene and by limiting Bal31 exonuclease digestion at certain of the restriction sites. Deletions that maintain the normal reading frame of the ATP2 gene were identified by screening among the deleted plasmids for those that still direct expression of active β-galactosidase in yeast. The precise end points of the deletions were then determined by DNA sequencing. The location of the deleted forms of the ATP2-lacZ encoded hybrid proteins was determined by fractionating yeast cells that contained the deleted plasmids into mitochondrial and cytosolic fractions and then assaying each fraction for β-galactosidase activity (Fig. 8). We found only one deletion that prevented targeting of the Atp2-LacZ hybrid protein to mitochondria. This deletion removes the coding sequence for amino acids 4–34 of the β-subunit precursor protein. Other deletions that

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**Figure 7.** Co-fractionation of F1-β-subunit invertase hybrid proteins with the mitochondrial membrane fraction. Yeast strain SEY2102 harboring the indicated ATP2-SUC2 gene fusions was grown under Ura+ selection to an A_{600} of 1.5 then diluted 30-fold into semisynthetic salts media for 16 h before harvest. Mitochondria were prepared by a modified procedure of the published methods (5). Freshly prepared mitochondria in 0.6 M sorbitol, 20 mM Hepes, pH 7.4, at 10 mg/ml were diluted to 1.67 mg/ml with 10 mM Tris-HCl, pH 8.0. Fractions were prepared from each starting mitochondrial preparation essentially as described (5). 20 μg mitochondria (lane a), inner membrane space (lane b), matrix fraction (lane c), and membrane fraction (lane d) were subjected to electrophoresis on an SDS 7.5–15% polyacrylamide gradient gel and transferred to a nitrocellulose filter. Antisera prepared against yeast invertase or the Neurospora crassa ADP/ATP carrier protein of the mitochondrial inner membrane were used to detect the Atp3-Suc hybrid proteins (©) and the ADP/ATP translocator protein, respectively.
affected β-subunit protein sequences distal to amino acid 27 were found not to alter mitochondrial targeting of the Atp2-LacZ hybrid protein.

Import of Certain Atp2-LacZ and Atp2-Suc2 Hybrid Proteins Interferes with Normal Mitochondrial Functioning

We observed previously that yeast cells containing the pβZl ATP2-lacZ gene fusion cannot grow on nonfermentable carbon sources such as glycerol or lactate (7). Here we find that plasmids pβZ1-pβZ6, pCβZ1-pCβZ6, and pβI1-pβI6 (class I gene fusions; Fig. 2) all confer a respiration-negative (Gly-) phenotype. Yeast cells harboring the remaining gene fusion plasmids do not show this respiration defect. We have found that, when the β-subunit sequence and one lysine at position 16. No acidic amino acids of the β-subunit protein are sufficient to target E. coli β-galactosidase to mitochondria. With ATP2-SUC2 gene fusions, we found that even the smallest β-subunit invertase hybrid protein containing only 39 amino-terminal amino acids of the β-subunit precursor protein is delivered efficiently to mitochondria. The additional β-subunit information required for mitochondrial targeting of β-galactosidase does not appear to contain sequences necessary for mitochondrial delivery. We found that deletions in the pβZ1 ATP2-lacZ gene fusion that eliminate ATP2 coding sequences between codons 27 and codon 210 do not affect mitochondrial targeting of the β-subunit β-galactosidase hybrid protein (Fig. 8). When β-galactosidase is positioned close to the β-subunit targeting signal, it may alter the structure of this signal or mask it such that specific mitochondrial recognition cannot take place. More important, however, the results show that a short amino-terminal segment of the β-subunit protein is sufficient to direct mitochondrial delivery of both a normally cytoplasmic protein, β-galactosidase, and a secreted protein, invertase. A deletion within this targeting segment (pβZ1Δ4-34) prevents mitochondrial delivery of the pβZ1 ATP2-lacZ encoded hybrid protein. These in vivo observations are supported by recent in vitro studies of Hurt et al (15, 16). They found that when the amino-terminal pre-segment of the yeast cytochrome c oxidase subunit IV protein is fused to mouse dihydrofolate reductase, it can direct the import of this normally cytoplasmic protein into the mitochondrial matrix. Indeed, many nuclear-encoded mitochondrial proteins are made initially as larger precursors with transient amino-terminal pre-segments that target the proteins specifically to mitochondria.

We have employed gene fusion and deletion studies to map within the ATP2 gene of yeast the information that functions to target uniquely the product of this gene, the F1-ATPase β-subunit protein, to mitochondria. Our results indicate that a domain of this protein composed of 27 amino-terminal amino acids is sufficient in vivo to direct mitochondrial targeting and import of the protein.

Gene fusions provide a useful approach for defining the minimal sequence information necessary to direct protein delivery in cells. A series of gene fusions described here between the ATP2 gene and either the E. coli lacZ gene or the yeast SUC2 gene have permitted an in vivo study of mitochondrial protein import in yeast. Both sets of chimeric genes code for hybrid proteins that quantitatively co-fractionate with mitochondria. In our analysis of ATP2-lacZ gene fusions we found that at least 169 amino-terminal amino acids of the β-subunit protein are sufficient to target E. coli β-galactosidase to mitochondria. With ATP2-SUC2 gene fusions, we found that even the smallest β-subunit invertase hybrid protein containing only 39 amino-terminal amino acids of the β-subunit precursor protein is delivered efficiently to mitochondria. The additional β-subunit information required for mitochondrial targeting of β-galactosidase does not appear to contain sequences necessary for mitochondrial delivery. We found that deletions in the pβZ1 ATP2-lacZ gene fusion that eliminate ATP2 coding sequences between codons 27 and codon 210 do not affect mitochondrial targeting of the β-subunit β-galactosidase hybrid protein (Fig. 8). When β-galactosidase is positioned close to the β-subunit targeting signal, it may alter the structure of this signal or mask it such that specific mitochondrial recognition cannot take place. More important, however, the results show that a short amino-terminal segment of the β-subunit protein is sufficient to direct mitochondrial delivery of both a normally cytoplasmic protein, β-galactosidase, and a secreted protein, invertase. A deletion within this targeting segment (pβZ1Δ4-34) prevents mitochondrial delivery of the pβZ1 ATP2-lacZ encoded hybrid protein. These in vivo observations are supported by recent in vitro studies of Hurt et al (15, 16). They found that when the amino-terminal pre-segment of the yeast cytochrome c oxidase subunit IV protein is fused to mouse dihydrofolate reductase, it can direct the import of this normally cytoplasmic protein into the mitochondrial matrix. Indeed, many nuclear-encoded mitochondrial proteins are made initially as larger precursors with transient amino-terminal pre-segments that target the proteins specifically to mitochondria.

The sequence of a number of mitochondrial protein pre-segments recently has been determined (reviewed in reference 25). Though they share no clear primary sequence homologies, most contain several basic amino acids and lack acidic amino acids. Within the amino-terminal 27 amino acids of the β-subunit precursor, there are three basic amino acids. These include two arginine residues at position 5 and 12 of the sequence and one lysine at position 16. No acidic amino acids are present in this portion of the β-subunit protein. The amino-terminal signal peptides present on most secretory proteins also often contain basic amino acids and lack acidic amino acids (36). However, unlike the apparent random positioning of the basic amino acids seen in mitochondrial

Figure 8. Deletion of a portion of the β-subunit pre-segment blocks mitochondrial delivery of a β-subunit β-galactosidase hybrid protein. DNA restriction sites used to construct the indicated deletions (solid rectangles) are shown. The extent of each deletion as determined by DNA sequencing is indicated (e.g., pβZ1Δ4-34: codons for amino acids 4–34 of the pre-β-subunit protein have been deleted). The percentage of total cellular β-galactosidase activity that fractionates with mitochondria isolated from strain SEY2102 harboring each of the indicated constructs is shown. In addition, the effect these constructs have on the growth of strain SEY2102 on a nonfermentable carbon source also is indicated. GP, glycerol phenotype. M, mitochondrial.
pre-segments, the basic amino acids in secretory signal peptides are usually confined to the amino-terminal end of these peptides. Further experiments will be required to determine whether this simple sequence difference between these two sorting signals contributes to their unique targeting functions.

It is not at present clear why the mitochondrial targeting signals for the \( \beta \)-subunit protein and the cytochrome \( c \) oxidase subunit IV protein are positioned at the amino-terminal end of these proteins. Both of these proteins can be imported into mitochondria posttranslationally in vitro (15, 19). In addition, the gene fusion results indicate that these targeting signals can function independently of the sequences to which they are fused. This implies that sequences in the mature polypeptide do not actively participate in mitochondrial targeting of these proteins.

We have presented evidence that an amino-terminal domain of the \( \beta \)-subunit protein not only can direct cytoplasmic sorting of \( \beta \)-galactosidase or invertase to mitochondria but also is sufficient to direct the import of these proteins into the organelle. Atp2-LacZ and Atp2-Suc2 hybrid proteins associated with isolated intact mitochondria were found to be resistant to digestion with externally added protease K. Upon solubilization of the organelle with Triton X-100, the hybrid proteins are degraded by the protease. In addition, subfractionation of mitochondria has shown that the mitochondrially targeted Atp2-LacZ hybrid proteins are tightly associated with the inner mitochondrial membrane. We have found that the Atp2-Suc2 hybrid proteins also co-fractionate with isolated mitochondrial membranes. No Atp2-Suc2 hybrid protein was detected in either the matrix or intermembrane space compartments. We do not have direct biochemical evidence to demonstrate with which mitochondrial membrane the Atp2-Suc2 hybrid proteins are associated; however, based on our protease protection results and the similar respiration phenotype seen with both Atp2-LacZ and Atp2-Suc2 hybrid proteins we expect that both sets of hybrid proteins with the mitochondrial membrane is through interactions with the inner membrane ATPase complex. As few as 39 amino-terminal amino acids of the \( \beta \)-subunit precursor can cause an Atp2-Suc2 hybrid protein to become stably associated with the mitochondrial membrane. This short segment of the \( F_{1} \) ATPase \( \beta \)-subunit would not be expected to permit the Atp2-Suc2 hybrid protein to assemble together with the mitochondrial ATPase complex. Therefore, we presume that the Atp2-lacZ and Atp2-Suc2 hybrid proteins either become jammed in the mitochondrial inner membrane during transit through this membrane or fortuitously associate with the membrane because of some as yet unclear conformational property of these hybrid proteins.

An unexpected observation made in this work is the effect of certain mitochondrially targeted Atp2-LacZ and Atp2-Suc2 hybrid proteins on the functioning of this organelle. Yeast cells harboring the plasmids p12Z1-p12Z6, pCB21-pCBZ6, and pDF1-pDF6 (class I gene fusions; Fig. 2) cannot grow on mitochondrial-dependent carbon sources such as glycerol. A small deletion in the amino-terminal coding sequence of the ATP2-LacZ gene fusion present in plasmid p12Z1 eliminates mitochondrial targeting of the hybrid protein it codes for as well as the respiration-defective phenotype (Fig. 8). This suggests strongly that mitochondrial delivery of the hybrid protein and not simply the synthesis of this protein is required to observe this defect. We detect this phenotype with cells that harbor either high or low copy number plasmids, which carry these gene fusions, suggesting that overproduction of the hybrid proteins is probably not the cause of the phenotype. Also, we found that cells harboring either Atp2-LacZ or Atp2-Suc2 gene fusions exhibit this respiration-defective phenotype. This implies that it is not the result of effects caused by some unique sequence or structural feature present in the cytoplasmic protein \( \beta \)-galactosidase. Invertase, a protein that can traverse the endoplasmic reticulum membrane of yeast, also can produce the Gly\(^+\) growth defect. The fact that not all Atp2-LacZ and Atp2-Suc2 hybrid proteins that are delivered to mitochondria exhibit the respiration defect indicates that delivery alone is not the cause. The data suggest that larger hybrid proteins associate with mitochondria or some component within the organelle in a way different from smaller hybrid proteins. It is hoped that by isolating and characterizing mutants that overcome this respiration defect, we will be able to understand the mechanism of this hybrid protein-dependent phenotype.

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