

# The Amino Terminus of the Yeast F<sub>1</sub>-ATPase $\beta$ -Subunit Precursor Functions as a Mitochondrial Import Signal

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**Abstract.** The *ATP2* gene of *Saccharomyces cerevisiae* codes for the cytoplasmically synthesized  $\beta$ -subunit protein of the mitochondrial F<sub>1</sub>-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  $\beta$ -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.

**O**RGANELLE 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  $\beta$ -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<sub>1</sub>-ATPase  $\beta$ -subunit into the mitochondrial matrix has been well characterized. The nuclear *ATP2* gene encodes the 509 amino acid  $\beta$ -subunit protein precursor.<sup>1</sup> This precursor contains a transient amino-terminal extension of ~20 amino acids (19). Precursor but not mature  $\beta$ -subunit protein can be imported into mitochondria in vitro (13). Here, we show by gene fusion that the amino terminus of the precursor  $\beta$ -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.

<sup>1</sup> Takeda, M., A. Vassarotti, and M. Douglas. Manuscript submitted for publication.

## Materials and Methods

### Strains and Media

*Saccharomyces cerevisiae* strains used were SEY2101 (*MAT $\alpha$  ura3-52 leu2-3 leu2-112 suc2- $\Delta$ 9 ade2-1 gal2*) and SEY2102 (*MAT $\alpha$  ura3-52 leu2-3 leu2-112 suc2- $\Delta$ 9 his4-519 gal2*) (12). MDY2102 (*MAT $\alpha$  ura3-52 leu2-3 leu2-112 suc2- $\Delta$ 9 his4-519 gal2 atp2::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 *ATP2* gene in a modified pBR322 plasmid was opened at a unique PvuII 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*<sup>+</sup> transformants unable to grow on a nonfermentable carbon source. The gene disruption at *ATP2* was confirmed by both genetic and physical methods.

The *Escherichia coli* strains used were MC1061 [*F*<sup>-</sup> *araD139*  $\Delta$ (*ara* ABOIC-leu)7697  $\Delta$ *lacX74 galU galK rpsL hsdR*] (4), MC1066 [*F*<sup>-</sup>  $\Delta$ (*lacI*POZY)X74 *galU galK rpsL hsdR trpC9830 leuB600 pyrF74::Tn5*] (21), BD1528 [*ung1 nadB7 met supE supF hsdR*] (gift from Bruce Duncan, University of Minnesota) and SE10 [*F*<sup>-</sup>  $\Delta$ (*lac-pro*)*ara rpsL thi pyrF74::Tn5* ( $\phi$ 80d*lacZ* $\Delta$ M15)]. Strain SE10 was constructed by the standard technique of P1 transduction (23). P1 grown on strain MC1066 was used to transduce strain JM83 (35) to kanamycin resistance (conferred by the Tn5-encoded neomycin phosphotransferase II). A uracil-requiring kanamycin-resistant transductant (*pyrF::Tn5*) was isolated and designated SE10. The *E. coli pyrF* uracil auxotrophy can be complemented by the yeast *URA3* gene.

Standard yeast and *E. coli* media were used (23, 30).

### 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 Bal31 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 the recommended 600 mM NaCl. Other techniques for the isolation of plasmid DNA, agarose gel electrophoresis, and DNA transformation into *E. coli* and *S. cerevisiae* were performed with minor modification of previously published procedures (18, 20, 30). Dideoxy sequencing was performed with the modifications noted earlier (27).

### *ATP2-lacZ* Gene Fusion Constructions

The yeast *ATP2* gene has been cloned (27) and its entire nucleotide sequence has been determined.<sup>1</sup> A 2.2-kb Eco RI-Bam HI DNA fragment containing ~1,100 base pairs (bp) of DNA upstream of the *ATP2* translation start site and 1,100 bp of *ATP2* coding sequence was cloned into the Eco RI and Bam HI sites of plasmid pSEY101. This generated a plasmid, p $\beta$ Z1, that contains an *ATP2-lacZ* gene fusion in which 380 amino-terminal amino acids of the Atp2 protein are fused in frame to *E. coli*  $\beta$ -galactosidase (7). Additional *ATP2-lacZ* gene fusions were then constructed by first restricting plasmid p $\beta$ Z1 at its unique Bam HI site. The restricted plasmid was then subjected to treatment with the double-stranded exonuclease Bal31. Appropriate digestion times were determined empirically by sizing the digested DNAs on agarose gels. The deleted plasmids were restricted with Eco RI at a unique site mapping ~1,100 bp to the 5' side of the *ATP2* gene. The *ATP2* gene fragments obtained in this way were then ligated into the *lacZ* fusion vector pSEY101, which had been digested previously with Eco RI and Sma I. In-frame fusions between *ATP2* and *lacZ* were isolated after transformation into *E. coli* strain MC1066 on plates containing the  $\beta$ -galactosidase indicator X-gal.  $\beta$ -Galactosidase-positive (blue) *E. coli* transformants were picked, and plasmid DNA isolated from them was screened for *ATP2* DNA inserts by restriction endonuclease mapping. Such constructions were then transformed into yeast strain SEY2102 and tested for  $\beta$ -galactosidase expression. Yeast cells expressing  $\beta$ -galactosidase were further characterized for the presence of hybrid proteins of the predicted size, containing antigenic determinants of the F<sub>1</sub>- $\beta$ -subunit protein and *E. coli*  $\beta$ -galactosidase (see text). The precise fusion joints between *ATP2* and *lacZ* sequences for 15 of the ~80 *ATP2-lacZ* gene fusions isolated were determined by DNA sequencing.

### Construction of Plasmid Vectors

Plasmids used in this work are shown in Fig. 1. All of the plasmids can be shuttled between both *E. coli* and yeast. They contain selectable markers (*bla*

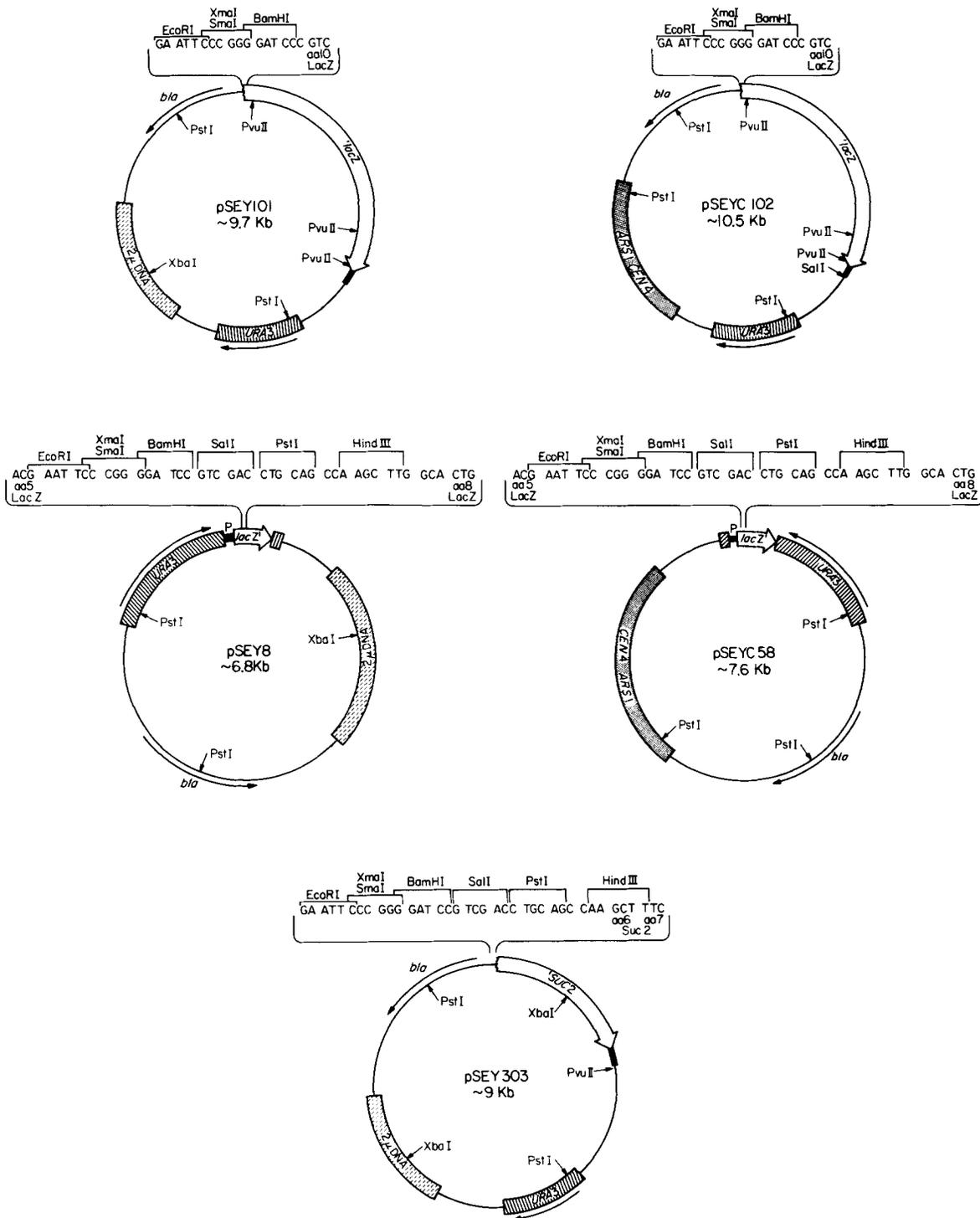
[*Amp*<sup>r</sup>] and *URA3*) and DNA segments (ColE1 *ori* of pBR322 and the *FLP* region of the 2- $\mu$ m circle DNA or the yeast chromosomal *ARS1 CEN4* segments) that permit maintenance of the plasmids in both *E. coli* and *S. cerevisiae*. The construction of plasmid pSEY101 has been described (7). Plasmid pSEY102 is a derivative of YCp50 (gift from R. Davis, Stanford University). The unique Sma I/Xma I site normally present in the *URA3* DNA segment (3' of the structural gene) contained in this plasmid was removed by digestion with Xma I followed by S1 nuclease treatment and ligation with T4 DNA ligase. This Sma I/Xma I-deleted derivative of YCp50 was then digested with Eco RI and Sal I and ligated with a 3.3-kb Eco RI-Sal I DNA fragment isolated from plasmid pCGS139 (gift from G. Vovis, Collaborative Research; 12) that carries the *E. coli lacZ* gene truncated at its 5' end. The resultant vector, pSEY102, contains unique Eco RI, Sma I, and Bam HI sites mapping to the 5' side of the truncated *lacZ* gene (Fig. 1).

The plasmids pSEY8 and pSEY58 both contain a 425-bp Hae II restriction fragment isolated from plasmid pUC8 (35). This Hae II fragment contains the promoter, operator, and coding region for the amino-terminal 59 amino acids of  $\beta$ -galactosidase (total protein has 1,024 amino acids) (17). In addition, it contains a number of restriction enzyme sites that are useful for DNA subcloning. The  $\beta$ -galactosidase peptide ( $\alpha$ -peptide) encoded by this DNA segment is expressed in *E. coli* and can restore  $\beta$ -galactosidase function to the large defective  $\beta$ -galactosidase protein fragment ( $\omega$ -peptide) coded for by the *lacZ* $\Delta$ M15 gene. This peptide complementation is referred to as  $\alpha$ -complementation (37). Cloning of DNA fragments into any of the restriction sites present in the coding sequence of this short  $\beta$ -galactosidase peptide disrupts the coding sequence and thereby prevents  $\alpha$ -complementation. This phenotype is easily detected on plates containing the  $\beta$ -galactosidase indicator X-gal. This permits rapid detection of plasmids containing DNA inserts.

Plasmid pSEY8 was derived from plasmid pCGS139. pCGS139 was digested with Eco RI and Sal I. This removed the 3.3-kb DNA fragment containing *lacZ*. The restricted plasmid was treated with S1 nuclease and then ligated together with T4 DNA ligase. The resulting plasmid was then restricted with Sma I at the unique site present in the *URA3* DNA segment contained in this plasmid. The restricted plasmid was ligated together with the 425-bp Hae II DNA fragment derived from the pUC8 vector. The ligated DNA was transformed into *E. coli* strain SE10. *Amp*<sup>r</sup>, *Ura*<sup>+</sup>, *Lac*<sup>+</sup> transformants were selected, and plasmid from such cells was subjected to DNA restriction analysis. Plasmid pSEY8 was isolated from among these transformants.

Plasmid pSEY58 is a derivative of plasmid YCp50. A 650-bp Eco RI-Sal I DNA fragment (pBR322 DNA sequences) was deleted from YCp50 as described above. Also, as above, the 425-bp Hae II DNA fragment obtained from the pUC8 plasmid was then cloned into the unique Sma I site present in the *URA3* region of this YCp50 derivative. This gave rise to the plasmid vector pSEY58 (Fig. 1).

Plasmid pSEY303 contains a truncated form of the yeast *SUC2* gene. The *SUC2* gene has been cloned (3) and its entire nucleotide sequence has been determined (34). *SUC2* contains a single Bam HI site at codon 263 in its coding sequence. This site was eliminated by sodium bisulfite mutagenesis. 2  $\mu$ g of a *SUC2*-containing plasmid, pRB58 (3), was restricted with Bam HI at its unique site in *SUC2*. The DNA was then treated with sodium bisulfite as described (31). The mutagenized plasmid was ligated with T4 DNA ligase and extensively redigested with Bam HI before transformation into *E. coli* strain BD1528. 10 ampicillin-resistant transformants were picked and plasmid DNA was isolated. Eight of the isolates contained plasmids that lacked the Bam HI site. Each of these eight Bam HI-defective plasmid isolates was transformed into the  $\Delta$ *SUC2* yeast strain SEY2102. Invertase activity as well as efficiency of invertase secretion was measured in these yeast transformants. Six plasmids directed expression and secretion of invertase that was indistinguishable from wild-type invertase. Two plasmids led to the expression of only low levels of invertase activity. One of the six Bam HI-defective plasmid isolates that makes normal levels of invertase activity was chosen as the source of the *SUC2* gene for constructing plasmid pSEY303. A 2.1-kb Hind III fragment was isolated from this pRB58 mutant plasmid and cloned into the unique Hind III site present in the pSEY8 vector. This DNA fragment carries the coding sequence for all of invertase except the first four amino acid codons of its 19 amino acid signal peptide. It also contains ~500 bp of DNA 3' of the *SUC2* structural gene. A pSEY8-*SUC2* plasmid in which the multiple restriction sites are positioned to the 5' side of the *SUC2* gene was identified by restriction analysis. The *SUC2* gene then was digested out of this pSEY8-*SUC2* plasmid with Eco RI and Pvu II (the Pvu II site maps just distal to the *SUC2* gene in the *lacZ* sequence of pSEY8). This 2.1-kb Eco RI-Pvu II fragment was ligated with plasmid pSEY101 that also had been digested with Eco RI and Pvu II. Plasmids that had replaced the *lacZ* gene present in pSEY101 with the *SUC2* gene were screened for by restriction analysis of ampicillin-resistant transformants obtained with this ligation mix. One such isolate then was digested partially with Hind III and treated with S1 nuclease before ligation. After transformation, a plasmid derivative was screened for that lacked the Hind III site 3' of the *SUC2* gene



**Figure 1.** Plasmid vectors employed in these studies. Construction of each of the plasmids shown is described in Materials and Methods. The approximate positions of DNA restriction enzyme sites for the indicated enzymes are as shown. Thin lines designate DNA derived from the plasmid pBR322. Heavy lines denote sequences associated with the 5' or 3' end of the gene that is adjacent to these sequences. Other regions of the plasmids are labeled and are described in Materials and Methods.

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 p $\beta$  and pC $\beta$  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  $\beta$ -subunit protein in yeast.

### Isolation and Fractionation of Mitochondria

Yeast cells harboring different plasmids were grown at 28–30°C to an  $A_{600}$  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 0.6 M mannitol, 0.02 M Tris-HCl, pH 7.4, 2 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 fractionations 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 1.8 M sucrose, 8 mM ATP, 8 mM MgCl<sub>2</sub>, pH 7.4. After brief sonication to vesicularize 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 membrane 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- $\mu$ l digestions contained 400  $\mu$ g fresh mitochondria in MTE plus the indicated amount of proteinase K. When included, Triton X-100 was added from a 10% (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 A<sub>600</sub> 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  $\mu$ l 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-100/20 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 antisera. 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  $\mu$ l), 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 on 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),  $\beta$ -galactosidase (115 kD), phosphorylase b (94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), and carbonic anhydrase (29 kD).

### Miscellaneous

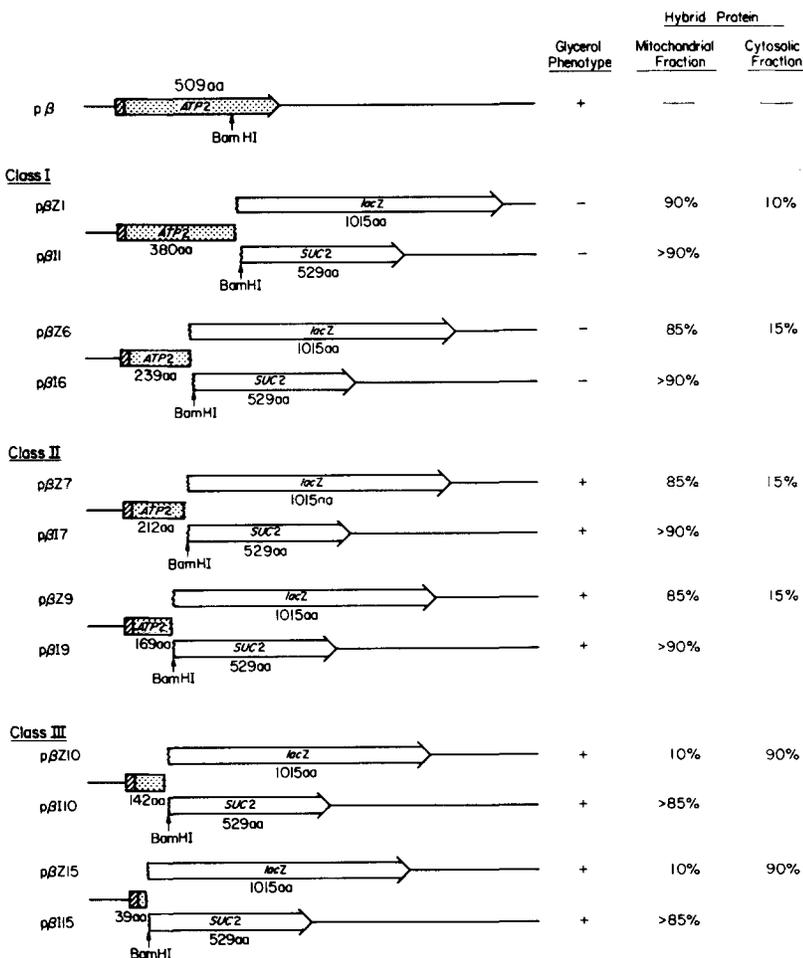
Mitochondrial enzyme activities and  $\beta$ -galactosidase activity were analyzed using minor modifications as previously published (7). Antisera to commercially available *E. coli*  $\beta$ -galactosidase (Bethesda Research Laboratories) was generated in rabbits as previously described (9). F<sub>1</sub>-ATPase  $\beta$ -subunit antisera was prepared in a similar manner from F<sub>1</sub>- $\beta$ -protein purified from the isolated ATPase as previously published (8). Invertase antisera was kindly provided by I. Schauer and R. Schekman (University of California, Berkeley) (29). Antiserum to the mitochondrial inner membrane ADP/ATP translocator protein was a gift from W. Neupert.

### 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*  $\beta$ -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  $\beta$ -galactosidase in both *E. coli* and yeast. No  $\beta$ -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 p $\beta$ Z1-p $\beta$ Z15, 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  $\beta$ -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 (p $\beta$ Z1-p $\beta$ Z5) to 1,000–2,000 U/mg total cell protein (p $\beta$ Z6-p $\beta$ Z15). Those gene fusions that contained a large amino-terminal coding segment of the *ATP2* gene fused to *lacZ* expressed lower levels of  $\beta$ -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 (p $\beta$ Z1-p $\beta$ Z5) rapidly were lost in the absence of Ura<sup>+</sup> selection. The pSEY101 plasmid, like other 2- $\mu$ m 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, pSEYC102, 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 p $\beta$ Z plasmids was moved on an Eco RI-Bam HI DNA fragment into the Eco RI-Bam HI sites present in the vector pSEYC102. This gave rise to a complementary set of *ATP2-lacZ* gene fusions,



**Figure 2.** *ATP2-lacZ* and *ATP2-SUC2* gene fusions. The relevant portion of plasmids encoding Atp2-LacZ and Atp2-Suc2 hybrid proteins are indicated. Only 6 of the 15 characterized gene fusions are shown. The cellular distribution of the hybrid proteins was determined as described in the text. We have divided the gene fusions into three classes based on the cell fractionation results and the glycerol growth properties that are exhibited by cells harboring these hybrid genes. Class I gene fusions ( $\beta Z1$ - $\beta Z6$  and  $\beta I1$ - $\beta I6$ ) code for hybrid proteins that cofractionate with mitochondria and confer a Gly<sup>-</sup> phenotype to yeast cells. Class II gene fusions ( $\beta Z1$ - $\beta Z9$  and  $\beta I7$ - $\beta I9$ ) code for hybrid proteins that cofractionate with mitochondria but do not confer a Gly<sup>-</sup> phenotype to yeast. Class III *ATP2-lacZ* gene fusions ( $\beta Z10$ - $\beta Z15$ ) code for hybrid proteins that are present in the cytosolic fraction of the cell whereas class III *ATP2-SUC2* gene fusions ( $\beta I10$ - $\beta I15$ ) code for hybrid proteins that cofractionate with mitochondria (see text). Class III *ATP2-lacZ* and *ATP2-SUC2* gene fusions do not cause a Gly<sup>-</sup> phenotype. The cytosolic fraction in the above fractionation studies contained <5% of the mitochondrial cytochrome oxidase activity and >95% of the cytosolic glyceraldehyde 3-phosphate dehydrogenase activity.

designated pC $\beta Z1$ -pC $\beta Z15$  (Table I). All maintain the correct translational reading frame between *ATP2* and *lacZ*.  $\beta$ -Galactosidase expressed from these plasmids in the yeast strain SEY2102 varied from ~500 U/mg total cell protein for the larger gene fusions (pC $\beta Z1$ -pC $\beta Z6$ ) to 1,000 U/mg total cell protein for the smaller fusions (pC $\beta Z7$ -pC $\beta Z15$ ). In addition, the new *ATP2-lacZ* gene fusion constructs exhibited greater plasmid stability in the absence of Ura<sup>+</sup> selection. For these reasons, most of our studies were carried out with the pC $\beta Z$  constructs.

The levels of  $\beta$ -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  $\beta$ -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 pC $\beta Z$  plasmids direct the synthesis of F<sub>1</sub>- $\beta$ -subunit  $\beta$ -galactosidase hybrid proteins (also referred to as Atp2-LacZ hybrid proteins) was demonstrated by steady state <sup>35</sup>SO<sub>4</sub> labeling of yeast cells carrying these plasmids, followed by immunoprecipitation of the hybrid proteins with antisera against both the F<sub>1</sub>- $\beta$ -subunit and  $\beta$ -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  $\beta$ -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  $\beta$ -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 (pC $\beta Z10$ -15) still expressed  $\beta$ -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 pC $\beta Z$  plasmids. The cellular distribution of the hybrid proteins was determined both by  $\beta$ -galactosidase enzyme assays and by immunoblotting using antisera directed against  $\beta$ -galactosidase. Results of these fractionation studies are shown in Fig. 2. Clearly, *ATP2* encoded sequences can direct  $\beta$ -galactosidase to mitochondria. We find that Atp2-LacZ hybrid proteins containing  $\geq 169$  amino acids of Atp2

Table I. *ATP2 Gene Fusions*

Position of fusion joint in <i>ATP2</i> ( <i>ATP2</i> codon at fusion joint)	<i>lacZ</i> fusion plasmids		<i>SUC2</i> fusion plasmids	
	Plasmid designation	Predicted size of hybrid proteins <i>kD</i>	Plasmid designation	Predicted size of hybrid proteins <i>kD</i>
380	p $\beta$ Z1 pC $\beta$ Z1	157	p $\beta$ I1	102
340	p $\beta$ Z2 pC $\beta$ Z2	153	p $\beta$ I2	98
307	p $\beta$ Z3 pC $\beta$ Z3	149	p $\beta$ I3	94
289	p $\beta$ Z4 pC $\beta$ Z4	147	p $\beta$ I4	92
267	p $\beta$ Z5 pC $\beta$ Z5	145	p $\beta$ I5	90
239	p $\beta$ Z6 pC $\beta$ Z6	142	p $\beta$ I6	87
212	p $\beta$ Z7 pC $\beta$ Z7	139	p $\beta$ I7	84
206	p $\beta$ Z8 pC $\beta$ Z8	138	p $\beta$ I8	83
169	p $\beta$ Z9 pC $\beta$ Z9	134	p $\beta$ I9	79
142	p $\beta$ Z10 pC $\beta$ Z10	131	p $\beta$ I10	76
120	p $\beta$ Z11 pC $\beta$ Z11	129	p $\beta$ I11	74
102	p $\beta$ Z12 pC $\beta$ Z12	127	p $\beta$ I12	72
90	p $\beta$ Z13 pC $\beta$ Z13	125	p $\beta$ I13	71
55	p $\beta$ Z14 pC $\beta$ Z14	122	p $\beta$ I14	67
39	p $\beta$ Z15 pC $\beta$ Z15	120	p $\beta$ I15	65

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  $\beta$ -galactosidase (17) and yeast invertase (33) were as published.

fused to LacZ are located in the mitochondrial fraction. Hybrid proteins with less  $\beta$ -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  $\beta$ -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 F<sub>1</sub>- $\beta$ -subunit protein precursor are sufficient to direct mitochondrial delivery of the normally cytoplasmic *E. coli* enzyme  $\beta$ -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  $\beta$ -galactosidase activity associated with intact mitochondria isolated from cells harboring plasmids pC $\beta$ Z1-pC $\beta$ Z9 was found to be resistant to proteinase digestion under proteinase digestion conditions that inactivate a marker enzyme in the outer mitochondrial membrane, antimycin-insensitive NADH cytochrome *c* reductase. However, in the presence of detergent, the  $\beta$ -galactosidase is readily accessible to proteinase K. On the other hand, the small but significant amount of  $\beta$ -galactosidase activity associated with mitochondria isolated from cells harboring plasmids pC $\beta$ Z10-pC $\beta$ Z15 was sensitive to proteinase K digestion even in the absence of the detergent Triton X-100 (Fig. 4). Apparently, hybrid  $\beta$ -subunit  $\beta$ -galactosidase proteins containing at least 169 amino acids of the  $\beta$ -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  $\beta$ -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 that are protected from proteinase digestion was further analyzed by subfractionation of mitochondria isolated from cells harboring the fusion plasmids pC $\beta$ Z1 and pC $\beta$ Z7. As controls, the small amount of  $\beta$ -galactosidase expressed from plasmids pC $\beta$ Z14 and pLG669-Z (which directs the synthesis of a cytoplasmic cytochrome *c*  $\beta$ -galactosidase hybrid protein [7]) that associates with mitochondria also was analyzed (Fig. 5). The results show that  $\beta$ -galactosidase activity expressed from plasmids pC $\beta$ Z1 and pC $\beta$ Z7 co-migrates on sucrose gradients with the inner mitochondrial membrane

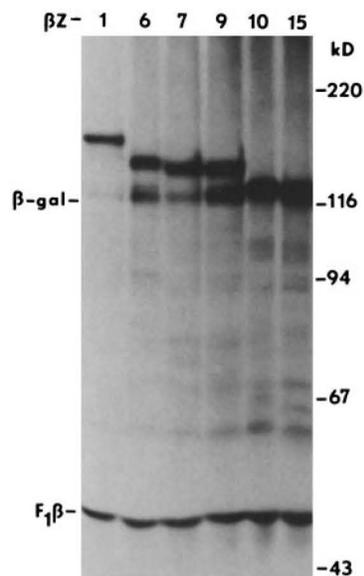


Figure 3. *ATP2-lacZ*-encoded hybrid proteins. Yeast whole cell extracts prepared from steady state <sup>35</sup>SO<sub>4</sub>-labeled cells were immunoprecipitated with combined F<sub>1</sub>- $\beta$ -subunit plus  $\beta$ -galactosidase antisera. Immunoprecipitates were resolved on an SDS 10–15% polyacrylamide gradient gel, which was then dried and autoradiographed. Cell extracts were prepared from yeast strain SEY2102 harboring plasmids pC $\beta$ Z1, 6, 7, 9, 10, and 15. The positions of wild-type  $\beta$ -galactosidase (116 kD) and the F<sub>1</sub>- $\beta$ -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  $\beta$ -galactosidase.

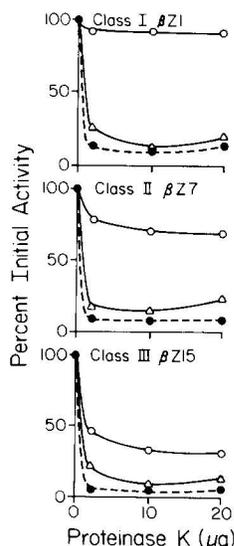
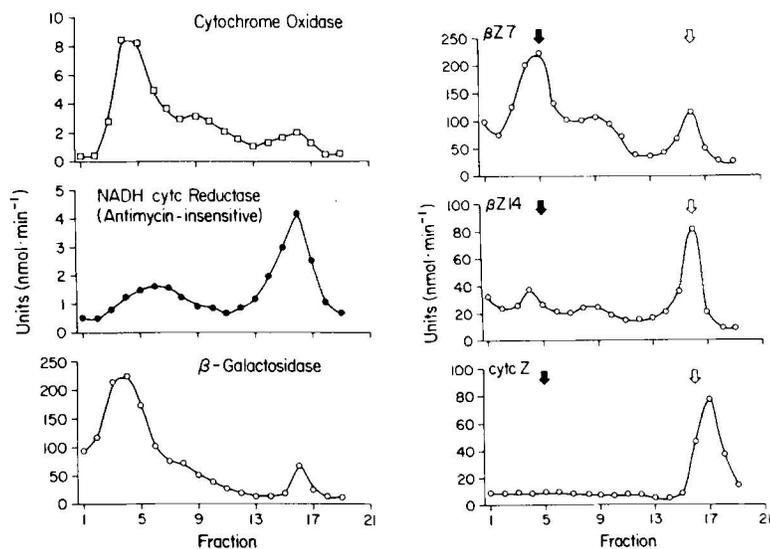


Figure 4.  $\beta$ -subunit  $\beta$ -galactosidase hybrid proteins delivered to mitochondria are protected from digestion by externally added proteinase. Mitochondria prepared from strain SEY2102 harboring the indicated *ATP2-lacZ* gene fusions on pC $\beta$ Z plasmids were suspended at 4 mg/ml in isotonic buffer (see Materials and Methods). Digestions with proteinase K were performed at 23°C for 30 min in a total volume of 100  $\mu$ l. Reactions were terminated on ice by the addition of 0.5 mM phenylmethylsulfonyl fluoride.  $\circ$ ,  $\beta$ -galactosidase, digestion in the absence of detergent;  $\bullet$ ,  $\beta$ -galactosidase, digestion in the presence of 0.3% Triton X-100;  $\Delta$ , antimycin-insensitive NADH cytochrome *c* reductase, digestion in the absence of detergent.



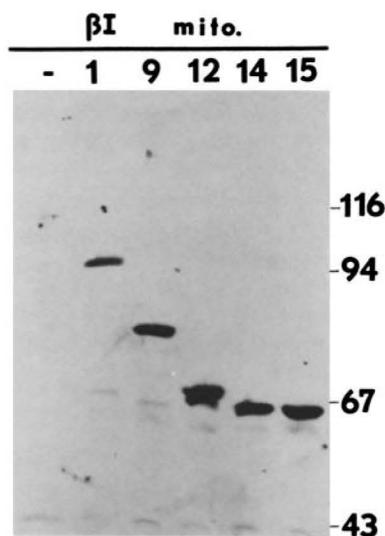
Recovery of the cytochrome oxidase and  $\beta$ -galactosidase enzyme activities for each of the different gradients was always >80%.

**Figure 5.**  $\beta$ -subunit  $\beta$ -galactosidase hybrid proteins delivered to mitochondria co-fractionate with mitochondrial inner membrane. Sonicated mitochondrial membranes (8–10 mg protein) prepared as described in Materials and Methods from strain SEY2102: harboring plasmids pC $\beta$ Z1, pC $\beta$ Z7, pC $\beta$ Z14, and pLG669-Z (labeled cytochrome Z) were resolved on linear 20–70% sucrose gradients and fractionated from the bottom of the tube. The left panel shows the complete fractionation results obtained with strain SEY2102 harboring the pC $\beta$ Z1 plasmid. Each fraction was assayed for the inner membrane marker enzyme cytochrome oxidase ( $\square$ ), outer membrane marker enzyme, antimycin-insensitive NADH cytochrome *c* reductase ( $\bullet$ ), and  $\beta$ -galactosidase ( $\circ$ ). The right panel shows the fractionation results obtained with strain SEY2102 harboring the remaining three gene fusion plasmids, as indicated. The level of  $\beta$ -galactosidase activity ( $\circ$ ) in each fraction is shown. Filled and unfilled arrows indicate where the peak enzyme activities for the inner and outer membrane marker enzymes were detected in each of the gradients.

marker enzyme cytochrome oxidase. The small amount of  $\beta$ -galactosidase expressed from plasmids pC $\beta$ Z14 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 the hybrid proteins with the mitochondrial inner membrane has not been possible, as mitochondria isolated from cells that express these Atp2-LacZ hybrid proteins have been found to be very fragile, preventing such studies.

### ATP2-SUC2 Gene Fusions

Our results indicate that at least 169 amino terminal acids of the  $\beta$ -subunit protein are required to direct mitochondrial import of *E. coli*  $\beta$ -galactosidase. Hurt et al. (16) have observed that a much smaller segment of cytochrome *c* oxidase subunit IV, when fused to dihydrofolate reductase, can direct 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  $\beta$ -galactosidase might interfere with the mitochondrial delivery information presumably present early in the  $\beta$ -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 pC $\beta$ Z plasmids, the various *ATP2* DNA segments were isolated on Eco RI-Bam HI DNA fragments from plasmids p $\beta$ Z1–p $\beta$ Z15 and cloned directly into Eco RI-, Bam HI-digested pSEY303 plasmid DNA. The resulting set of *ATP2-SUC2* gene fusion containing plasmids was designated p $\beta$ I1–p $\beta$ I15 (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).



**Figure 6.** Atp2-Suc2 hybrid proteins are efficiently targeted to yeast mitochondria. Mitochondria (40  $\mu$ g) prepared from strain SEY2102 harboring the indicated p $\beta$ I plasmids were resolved on a SDS 9% polyacrylamide gel. The gel fractionated proteins were transferred to nitrocellulose paper and immunoblotted with anti-invertase antisera plus a goat anti-rabbit horseradish peroxidase antibody conjugate. Control mitochondria were prepared from yeast strain SEY2102 harboring the *SUC2* plasmid vector pSEY303 (first lane).

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  $\beta$ -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 *MF $\alpha$ 1* gene, which codes for the secreted pheromone  $\alpha$ -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 p $\beta$ I 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  $F_1$ - $\beta$ -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 p $\beta$ I1-p $\beta$ I15. 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 treatment (a gel pattern identical to that shown in Fig. 6 was obtained even after the mitochondria had been subjected to proteinase 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 p $\beta$ I 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  $\beta$ -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  $\beta$ -subunit sequences found to be required to direct  $\beta$ -galactosidase to mitochondria may not be part of the targeting signal but rather simply may act to separate this signal from the  $\beta$ -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 p $\beta$ Z1 *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  $\beta$ -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  $\beta$ -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  $\beta$ -subunit precursor protein. Other deletions that

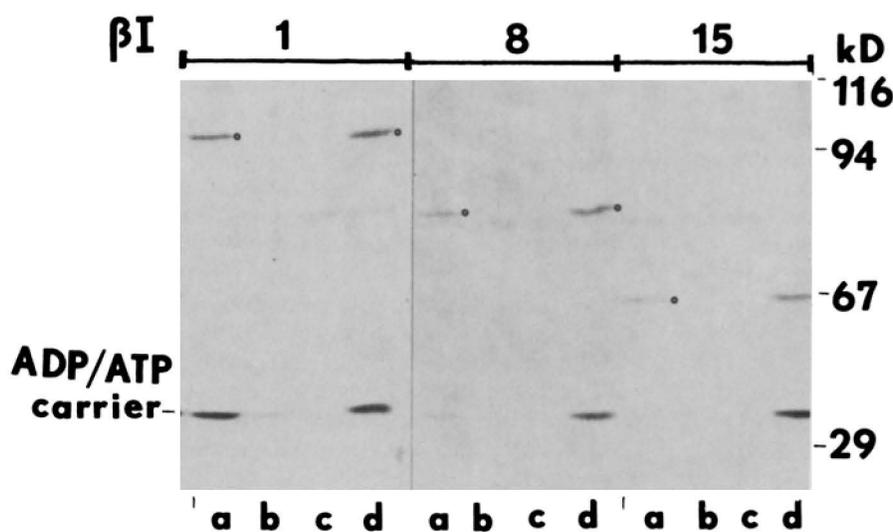
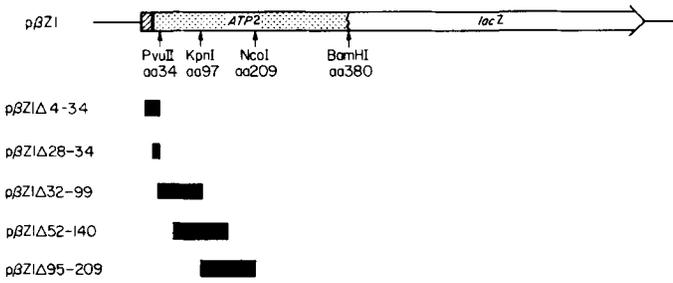


Figure 7. Co-fractionation of  $F_1$ - $\beta$ -subunit invertase hybrid proteins with the mitochondrial membrane fraction. Yeast strain SEY2102 harboring the indicated *ATP2-SUC2* gene fusions was grown under *Ura*<sup>+</sup> selection to an  $A_{600}$  of 1.5 then diluted 30-fold into semisynthetic 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  $\mu$ g 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 Atp2-Suc hybrid proteins (○) and the ADP/ATP translocator protein, respectively.



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.

affected  $\beta$ -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 p $\beta$ Z1 *ATP2-lacZ* gene fusion cannot grow on nonfermentable carbon sources such as glycerol or lactate (7). Here we find that plasmids p $\beta$ Z1-p $\beta$ Z6, pC $\beta$ Z1-pC $\beta$ Z6, and p $\beta$ I1-p $\beta$ I6 (class I gene fusions; Fig. 2) all confer a respiration-negative (Gly<sup>-</sup>) phenotype. Yeast cells harboring the remaining gene fusion plasmids do not show this respiration defect. We have found that a deletion within the *ATP2* coding sequence in the p $\beta$ Z1 plasmid (p $\beta$ Z1 $\Delta$ 4-34) eliminates mitochondrial delivery of the Atp2-LacZ hybrid protein coded for by this plasmid (Fig. 8). In addition, yeast cells harboring this mutant plasmid no longer exhibit a Gly<sup>-</sup> phenotype. Deletions of other *ATP2* sequences in plasmid p $\beta$ Z1 do not affect mitochondrial targeting of the Atp2-LacZ hybrid protein, nor do they eliminate the Gly<sup>-</sup> phenotype (Fig. 8). Consistent with these observations, *ATP2-lacZ* gene fusions that code for hybrid proteins detected in the cytoplasm do not show a defect in mitochondrial function (Fig. 2). However, many *ATP2-lacZ* and *ATP2-SUC2* gene fusions that code for hybrid proteins that are efficiently delivered to mitochondria also do not affect the growth of yeast cells on glycerol (class II gene fusions; Fig. 2). Targeting of the hybrid proteins to mitochondria alone therefore cannot explain the observed glycerol growth phenotype.

### Discussion

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 F<sub>1</sub>-ATPase  $\beta$ -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

GP	M
-	90%
+	10%
-	90%
-	80%
-	85%
-	85%

**Figure 8.** Deletion of a portion of the  $\beta$ -subunit presegment blocks mitochondrial delivery of a  $\beta$ -subunit  $\beta$ -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., p $\beta$ Z1 $\Delta$ 4-34: codons for amino acids 4-34 of the pre- $\beta$ -subunit protein have been deleted). The percentage of total cellular  $\beta$ -galactosidase activity that fractionates with mitochondria isolated from strain SEY2102

acids of the  $\beta$ -subunit protein are sufficient to target *E. coli*  $\beta$ -galactosidase to mitochondria. With *ATP2-SUC2* gene fusions, we found that even the smallest  $\beta$ -subunit invertase hybrid protein containing only 39 amino-terminal amino acids of the  $\beta$ -subunit precursor protein is delivered efficiently to mitochondria. The additional  $\beta$ -subunit information required for mitochondrial targeting of  $\beta$ -galactosidase does not appear to contain sequences necessary for mitochondrial delivery. We found that deletions in the p $\beta$ Z1 *ATP2-lacZ* gene fusion that eliminate *ATP2* coding sequences between codon 27 and codon 210 do not affect mitochondrial targeting of the  $\beta$ -subunit  $\beta$ -galactosidase hybrid protein (Fig. 8). When  $\beta$ -galactosidase is positioned close to the  $\beta$ -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  $\beta$ -subunit protein is sufficient to direct mitochondrial delivery of both a normally cytoplasmic protein,  $\beta$ -galactosidase, and a secreted protein, invertase. A deletion within this targeting segment (p $\beta$ Z1 $\Delta$ 4-34) prevents mitochondrial delivery of the p $\beta$ Z1 *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 peptide pre-segments. A mitochondrial matrix protease has been identified that will remove the pre-segments from a variety of mitochondrial precursor proteins, including the  $\beta$ -subunit protein (1, 22). It is tempting to speculate that each of these pre-segments contains the recognition determinant(s) that targets 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  $\beta$ -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  $\beta$ -subunit protein.<sup>1</sup> 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

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. It is not yet known, however, if targeting would still occur if the amino-terminal peptide signal were placed elsewhere in the protein such as at its carboxy-terminal end.

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 proteinase K. Upon solubilization of the organelle with Triton X-100, the hybrid proteins are degraded by the proteinase. 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 proteinase 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 reside in the mitochondrial inner membrane. We have been unable to biochemically discern the precise nature of this inner membrane association. However, it is clear that import of these hybrid proteins has, at least, been initiated.

We think it is unlikely that the association of the 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 p $\beta$ Z1-p $\beta$ Z6, pC $\beta$ Z1-pC $\beta$ Z6, and p $\beta$ I1-p $\beta$ I6 (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 p $\beta$ Z1 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<sup>-</sup> 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 better the mechanism of this hybrid protein-dependent phenotype.

We are grateful to Marjorie Britten, Mac Biggs, and Jane Goldsborough for expert technical assistance.

The work was supported by an award from the Searle Scholars Program (to S. D. Emr) and by National Institutes of Health Research Grants GM 32703 (to S. D. Emr) and GM 26713 (to M. G. Douglas). B. L. Geller was supported by a Postdoctoral Fellowship from the Robert A. Welch Foundation (I-814) Houston, Texas, and J. Garrett was supported by an E. S. Gosney Postdoctoral Research Fellowship, California Institute of Technology.

Received for publication 24 June 1985, and in revised form 29 October 1985.

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