

## Independent mutations at the amino terminus of a protein act as surrogate signals for mitochondrial import

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**Intracellular delivery of the mitochondrial F<sub>1</sub>-ATPase  $\beta$ -subunit precursor from the cytoplasm into the matrix of mitochondria is prevented by deletion of its mitochondrial import signal, a basic amphipathic  $\alpha$ -helix at its amino terminus. Using a complementation assay, we have selected spontaneous mutations which restore the correct *in vivo* localization of the protein containing the import signal deletion. Analysis of these mutations revealed that different functional surrogate mitochondrial targeting signals formed within a narrow region of the extreme amino terminus of the import signal deleted  $\beta$ -subunit. These modifications specifically replace different acidic residues with neutral or basic residues to generate a less acidic amphipathic helix within a region of the protein which is accessible for interaction with the membrane surface. The observations of this study confirm the requirement for amphipathicity as part of the mitochondrial import signal and suggest how mitochondrial targeting signals may have evolved within the extreme amino terminus of mitochondrial proteins.**

**Key words:** F<sub>1</sub>-ATPase/mitochondrial import protein/amino-terminal mutations

### Introduction

Intracellular delivery of proteins to different locations is initially directed by protein sequence determinants located in most cases at the amino-terminal end (Wickner and Lodish, 1985). In the best documented cases these involve the signal receptor particle (SRP)-mediated delivery of proteins into the membrane of the endoplasmic reticulum. This is proposed to occur by a mechanism in which insertion of hydrophobic signals or membrane-spanning elements occurs very soon after their synthesis in the cytoplasm. However, recent *in vitro* studies demonstrate that this secretion event for selected proteins can also occur after completion of protein synthesis by a mechanism which is independent of a membrane potential but requires hydrolysis of a high energy phosphate bond (Rothblatt and Meyer, 1986; Hansen *et al.*, 1986; Waters and Blobel, 1986). On the other hand, the post-translational sorting of proteins from the cytoplasm for delivery to other cellular organelles is defined by unique signals which are distinct from each other and those for SRP-mediated membrane insertion. The partitioning of proteins into the nucleus is defined by basic hydrophilic sequences which are shared among families of different nuclear proteins (Lanford and Butel, 1984; Kalderon *et al.*, 1984). Analysis of the determinants required for mitochon-

drial delivery have led to the suggestion that a consensus structure within the transient pre-sequence of precursor proteins may be important (Douglas *et al.*, 1986; Hurt and van Loon, 1986).

Translocation of proteins into mitochondria is catalyzed by short basic protein sequences which are located at the amino terminus of the cytoplasmic form of the protein. In all but a few mitochondrial proteins this targeting sequence is usually a transient species and is removed following delivery of the protein into the organelle. The special case of cytochrome *c* localization to the intermembrane space of mitochondria has been described (Pfanner and Neupert, 1987).

The signals which transport a protein across the bilayer are the same signals which direct the protein to mitochondria *in vivo*. Intracellular localization of non-mitochondrial proteins into mitochondria of yeast and animal cells, assayed by gene fusion experiments, is catalyzed by relatively small protein sequences (< 10 residues in some cases) which constitute less than half of the transient pre-sequence (Hurt *et al.*, 1985; Horwich *et al.*, 1985; Vassarotti *et al.*, 1987). Analysis of all the available mitochondrial import signals has revealed no primary sequence homology, although they exhibit a common set of properties which suggest their function in membrane transport (Von Heijne, 1986).

Mitochondrial targeting sequences in contrast to the signal utilized for SRP-mediated export are essentially basic hydrophilic sequences. They rarely contain acidic residues and exhibit a spacing of basic residues which can potentially form an amphipathic structure. Analysis of chemically synthesized peptides homologous to either mitochondrial (Roise *et al.*, 1986) or secretory signals (Briggs *et al.*, 1986) reveals that they will change their secondary structures depending on their environment. In a neutral aqueous buffer these peptides exhibit little ordered structure but assume a characteristic secondary structure under conditions which mimic interaction with a hydrophobic interface. Recent studies have demonstrated that a chemically synthesized pre-sequence peptide of the mitochondrial cytochrome oxidase subunit 4 can form an amphipathic helix when shifted to a solvent with reduced polarity (Roise *et al.*, 1986). Likewise, the pre-sequence for the mitochondrial F<sub>1</sub>-ATPase  $\beta$ -subunit is 95% random coil in neutral aqueous buffer but assumes a 75%  $\alpha$ -helical content in 60% trifluoroethanol (L.Gierasch and M.Douglas, unpublished). These observations indicate that the secondary structure formed within the pre-sequence and the extent to which an amphipathic structure is generated may define its efficiency in promoting import.

The chemically synthesized peptides can inhibit *in vitro* import by a mechanism that involves competition for the membrane potential that is required to drive pre-sequence insertion of the precursor into mitochondria. Current analyses indicate that the pre-sequence may act to destabilize or discharge sites which are formed on the mitochondrial membrane in an energy-dependent manner (Schleyer and Neupert, 1985).

In the present report we have examined the requirements for the delivery of a protein *in vivo* by asking a targeting sequence

to evolve at the end of a mitochondrial protein which has had its normal pre-sequence removed. The protein harboring the pre-sequence deletion is synthesized in the cytoplasm but is unable to be transported into mitochondria. We show that the correct mitochondrial localization of this protein is due to the formation of different surrogate import signals within a narrow region of its new amino terminus. These mutant signals have been selected directly by demanding function of the plasmid-encoded protein in a yeast host from which the resident copy of the wild-type gene (*ATP2*) has been deleted. Analysis of spontaneous, independent mutations which restore import of the pre-sequence deleted  $\beta$ -subunit indicates that the formation of an amphipathic structure with a reduced content of acidic residues within the new amino terminus is necessary for import competency.

## Results

### Deletions which reduce the extent of amphipathic structure formation at the amino terminus reduce the extent of mitochondrial delivery *in vivo*

The transient amino terminus of the  $F_1$ -ATPase  $\beta$ -subunit precursor contains the information required for its correct localization *in vivo* (Emr *et al.*, 1986). Fourier analysis of the hydrophobicities of the first 100 residues of the  $F_1$ -ATPase  $\beta$ -subunit precursor predicts regions of potential amphipathic second-

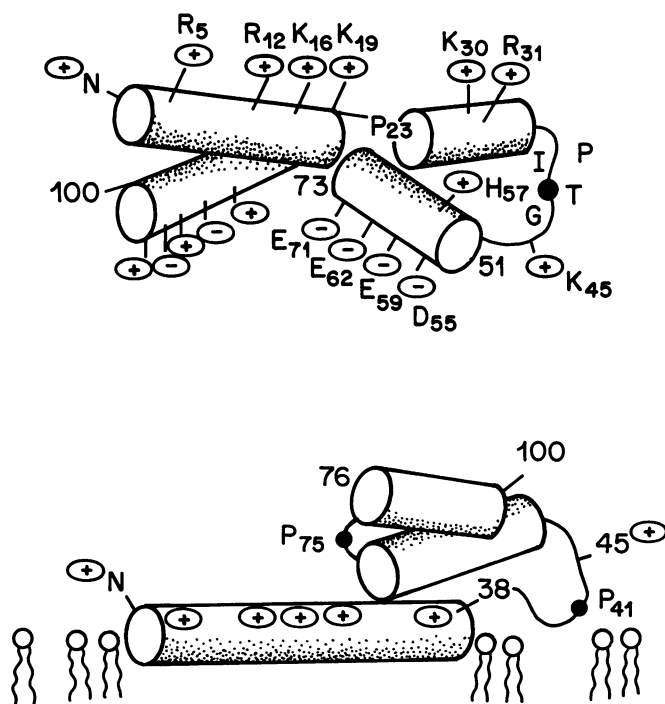
dary structure (Finer-Moore and Stroud, 1984). The extreme amino terminus consisting of the first 40 residues is basic (pI 10.26) and is proposed to exhibit a native fold in which apolar residues of this region associate with apolar residues distal to the extreme amino terminus (Figure 1A). Upon interaction with a hydrophobic or membrane surface the first 38 residues are proposed to maintain a strong amphipathic character (Figure 1B).

The first 19 residues of the  $F_1$ -ATPase  $\beta$ -subunit precursor make up the transient pre-sequence of the protein which is required for its correct mitochondrial localization *in vivo* and *in vitro* (Vassarotti *et al.*, 1987). Recent analysis of different imported proteins predicts a strong tendency for their pre-sequences to form amphipathic structures (Von Heijne, 1986). The pre-sequence of the cytochrome oxidase subunit IV precursor as well as the pre-sequence of the  $F_1$   $\beta$ -subunit precursor exhibit a high  $\alpha$ -helical content under conditions which mimic their interaction

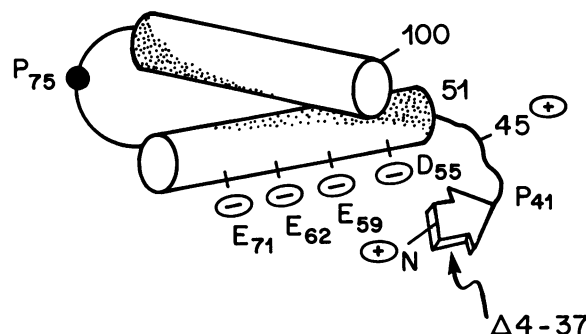
**Table I.** Deletions which progressively reduce the basic amphipathic sequence (residues 1–38) of the  $F_1$   $\beta$ -precursor progressively decrease its mitochondrial targeting

Basic residues present	% <i>ATP2-lacZ</i> hybrid in mitochondria
All Arg5 Arg12 Lys16 Lys19 Lys30 Arg31	95
Arg5 Arg12 Lys16 Lys19 – –	65
Arg5 Arg12 Lys16 – – –	45
Arg5 Arg12 – – – –	30
Arg5 – – – – –	15
None – – – – –	<2

Deletions within *ATP2* nested on codon 37 were generated as previously described (Vassarotti *et al.*, 1987). In-frame deletion joints between codon 37 and different amino-terminal lengths of *ATP2* were determined by DNA sequencing. The efficiency of mitochondrial targeting of the *ATP2-lacZ* hybrid was determined by measuring the percentage of total cell  $\beta$ -galactosidase present in the mitochondrial fraction. For this analysis cells harboring the respective constructs were grown on YNBD media maintaining a *Ura*<sup>+</sup> selection. At a cell density of  $1 \times 10^7$  cells/ml the cells were supplemented with 20% (v/v) of 1% yeast extract, 2% bactopectone, 1% dextrose and grown for an additional 4 h prior to harvest. Mitochondrial and post-mitochondrial supernatant fractions were prepared from yeast spheroplasts and enzymatic assays were performed. In each case the distribution of reporter hybrid was normalized to the mitochondrial cytochrome oxidase activity in each fraction (Emr *et al.*, 1986).



**Fig. 1.** Model of the first 100 residues of the  $F_1$ -ATPase  $\beta$ -subunit (*ATP2*). (A) Amphipathic analysis of the  $\beta$ -subunit precursor (Finer-Moore and Stroud, 1984) suggests the formation of a native fold in solution which contains four helical regions between residues 1 and 21 (I), 24 and 37 (II), 51 and 73 (III), 76 and 100 (IV). The first helix (I) exhibits a very amphipathic 'floating type' helix in which basic residues within the structure are segregated to a single 60° sector of the structure. The first 19 residues of the protein are removed following its import into the mitochondrial matrix space (Vassarotti *et al.*, 1987). Helix II is also basic and amphipathic. These helices are predicted to interact with helices in residues 50–100 of the precursor protein. Helix III, like helix I, exhibits a strong amphipathic character with acidic residues segregated to a narrow sector. (B) Interaction of the precursor with the membrane is proposed to yield a structure in which helices I and II would 'float' in the surface of the bilayer to straighten the bend due to a proline at residue 23.



**Fig. 2.** Proposed structure of the  $F_1$ -ATPase  $\beta$ -subunit from which helix I and II have been removed. Analysis of the  $\beta\Delta 4-37$  deletion in the same manner as described for Figure 1 indicates that the remaining residues of the 1–50 sequence revert to a  $\beta$ -structure whereas the sequence 51–100 will fold into a structure like the membrane-associated form of the wild-type sequence, Figure 1B. The region between residues 54 and 72 contains a segregation of acidic residues within the helix which is the most amino-terminal helix of the  $\Delta 4-37$  construct.

with the bilayer. One prediction from this is that changes which reduce the extent of amphipathic helix formed at the extreme amino terminus of the precursor protein will reduce its efficiency of import. Earlier studies from this laboratory have demonstrated that the first 39 residues comprising the membrane-associated helix (Figure 1B) of *ATP2* are necessary and sufficient to import non-mitochondrial proteins *in vivo* (Emr *et al.*, 1986). Nested deletions which begin at the carboxyl end of this amphipathic segment (residue 37) and systematically delete through the amphipathic structure, progressively reduce the steady-state level of fused reporter gene product ( $\beta$ -galactosidase) in mitochondria (Table I). The amphipathic sequence within the first 37 residues contains six basic residues which when progressively deleted reduced the amount of the reporter protein  $\beta$ -galactosidase fused to it into mitochondria (Table I). Thus reduction of the basic amphipathic stretch of sequence responsible for delivery into the organelle membrane progressively reduce the amount of total hybrid co-fractionating with mitochondria. Deletions which completely eliminate the amphipathic structure reduce intracellular delivery of the hybrid to a background level. The simplest interpretation of this observation is that a basic amphipathic structure defines mitochondrial delivery of the  $\beta$ -subunit precursor and that the strength of the targeting signal is determined in part by its length.

The complete *ATP2* target signal is more than sufficient to provide targeting of the  $F_1$   $\beta$ -subunit precursor. A nested deletion which retains only the first 10 residues of the precursor protein

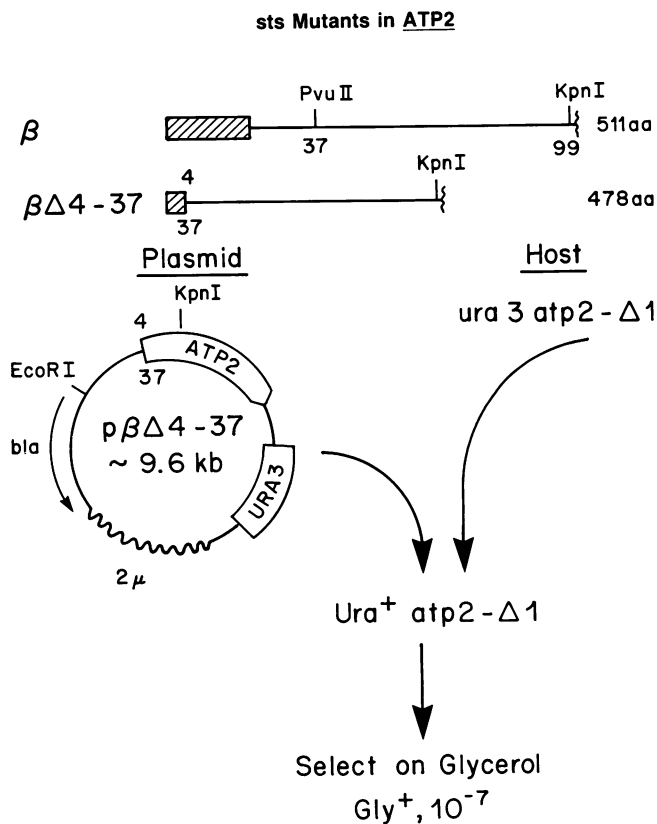
fused to residue 37 delivers only 15–20% of the hybrid reporter protein to mitochondria but will deliver an otherwise wild-type  $F_1$   $\beta$ -subunit to support growth of an *atp2- $\Delta 1$*  chromosomal deletion host on a non-fermentable carbon source (Vassarotti *et al.*, 1987). This construct retains only a single arginine at position 5 of the wild-type targeting sequence. A deletion which completely eliminates the potential for helix formation ( $\Delta 4$ -37), and which eliminates all of the charged residues at the amino terminus, is unable to target sufficient  $\beta$ -subunit into mitochondria to support growth on a non-fermentable carbon source. In all cases, the correct localization of different constructs was analyzed in the yeast host, AVY4-1, from which the resident *ATP2* gene has been deleted (Vassarotti *et al.*, 1987). This strain (*atp2- $\Delta 1$* ) is unable to grow on medium with glycerol, ethanol or lactate as the carbon source.

These observations are consistent with the proposal that the length of the basic amphipathic structure present at the amino terminus of the  $\beta$ -subunit precursor will define its efficiency of mitochondrial localization *in vivo*. Furthermore, deletions which reduce the mitochondrial delivery of the  $\beta$ -subunit protein by as much as 80–85% will provide sufficient functionally assembled subunit to support growth, albeit at rates slower than that due to the wild-type target signal.

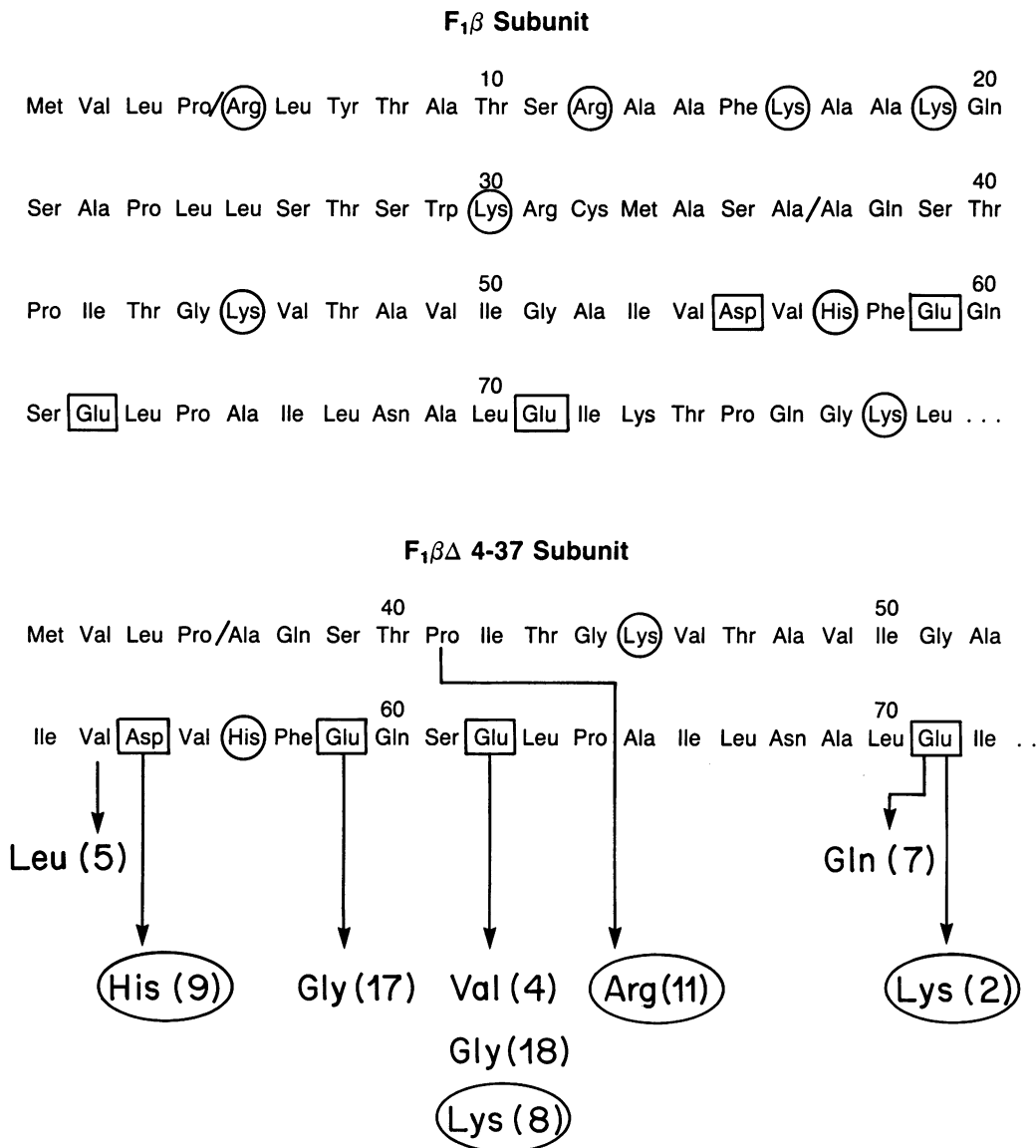
#### Independent mutations which restore import efficiency

Analysis of deletions through the  $\beta$ -subunit targeting sequence suggested that a reduction in the efficiency of mitochondrial import, presumably below a threshold level, prevented growth of the host on a carbon source requiring mitochondrial function. In order to further define the requirements for the delivery of the  $\beta$ -subunit protein, we took advantage of the observation that the 4-37 deletion in the  $\beta$ -subunit reduced its rate of mitochondrial targeting below that required to support growth on glycerol. Further analysis of the  $\beta\Delta 4$ -37 deletion suggests that the first 10 residues of the deletion construct may form a  $\beta$ -structure in the native fold. This protein is proposed to have little or no affinity for the membrane or a hydrophobic site. The structure predicted for the remainder of the amino terminus of the  $\beta\Delta 4$ -37 protein (Figure 2) is proposed to be the same for residues 50–100 of the wild-type  $\beta$ -subunit precursor associated with the bilayer (see Figure 1B). This protein is not recognized for import.

In preliminary studies, we observed that there was a small amount of  $\beta\Delta 4$ -37 protein associated with isolated mitochondria which was accessible to added proteinase. This small fraction of the protein presumably represented subunit associated with the surface of the organelle. No detectable growth of an *atp2- $\Delta 1$*  strain expressing the  $\beta\Delta 4$ -37 subunit was observed on glycerol after 20 days at 28°C. However, changes to the extreme amino terminus of the  $\beta\Delta 4$ -37 protein which restore some feature of the wild-type amphipathic target signal may be expected to increase the rate of import sufficiently to restore growth on glycerol. One prediction of this is that modifications to the amino terminus of the  $\beta\Delta 4$ -37 protein to either reduce the net negative charge of a helix or to extend the helix to include additional basic residues would increase its efficiency of mitochondrial targeting. To select for mutations in the protein which restored targeting, a yeast *Escherichia coli* shuttle plasmid harboring  $\Delta 4$ -37 *ATP2* yeast DNA (Figure 3) was transformed into the *atp1- $\Delta 1$*  mutant. *Ura<sup>+</sup>* yeast transformants harboring this plasmid express the same level of the  $\Delta 4$ -37  $\beta$ -subunit as the wild-type subunit except that it is not localized within mitochondria (not shown). This host vehicle combination fails to grow on glycerol; however, at a frequency of  $10^{-7}$ , independent colonies able to grow on



**Fig. 3.** Selection of surrogate targeting signal mutations (sts) in *ATP2*. A yeast  $2 \mu$ m plasmid containing the 4-37 deletion of the  $F_1$   $\beta$ -subunit ( $p\beta\Delta 4$ -37) was transformed into yeast strain AVY4/1 which contained the yeast *LEU2* gene in place of *ATP2* (see Materials and methods). This host harboring  $p\beta\Delta 4$ -37 was unable to grow on glycerol. At a frequency of  $10^{-7}$ , spontaneous mutants arose which were picked for further analysis.



**Fig. 4.** Determination of surrogate targeting signals within  $\beta\Delta 4-37$ . Plasmids harboring surrogate targeting signals in *ATP2*  $\Delta 4-37$  were sequenced. Nine independent point mutations, four transversions and five transitions were found (Table II). In all but three cases these mutations were picked up more than once in the initial mutant collection. In greater than half of the mutants selected these changes generated a non-preferred codon in yeast (see Table II). Shown (upper panel) is the primary sequence for the first 79 residues of *F<sub>1</sub>β* subunit precursor. The first 19 residues of this sequence is the transient pre-sequence which is removed by cleavage at Lys 19–Gln 20 by a metalloprotease located in the matrix (Vassarotti *et al.*, 1987). In the lower panel, *F<sub>1</sub>β*  $\Delta 4-37$  results from a fusion between Pro 4 and Ala 37 indicated by a slash. Basic amino acids are circled, acidic amino acids are indicated by squares. The numbering of the residues is that of the wild-type. The independent mutants generated replacements with one exception to either eliminate an acidic residue within helix III (residues 51–73) the first amphipathic helix of the  $\Delta 4-37$  construct (Figure 2) or expand the helix near the amino terminus (Pro 41 to Arg). The numbers in parentheses indicate allele number. Short arrows indicate changes which provide for slower growth of the *atp2Δ-1* host on glycerol. The longer arrows indicate changes which exhibit relatively faster growth (see Table II).

glycerol plates were selected for further analysis. These spontaneous revertants were able to grow on a non-fermentable carbon source rich in uracil and maintained the *URA3* plasmid. This indicated that the plasmid encoded copy was required for growth on the non-fermentable substrate.

Nineteen independent revertants were propagated on media maintaining selection for *Ura*<sup>+</sup>. The plasmid DNA in each case was recovered and used to re-transform a new *Ura3 atp2Δ-1* host. In each case, the re-transformed *atp2Δ-1* host was competent for growth on a non-fermentable carbon source, confirming that the mutation response for growth was located on the transforming plasmid. In addition, re-transformation of the 19 yeast host strains which had been cured of the original *URA3* plasmid with new

*pβΔ4-37* further confirmed that the original mutations were not due to changes in the host.

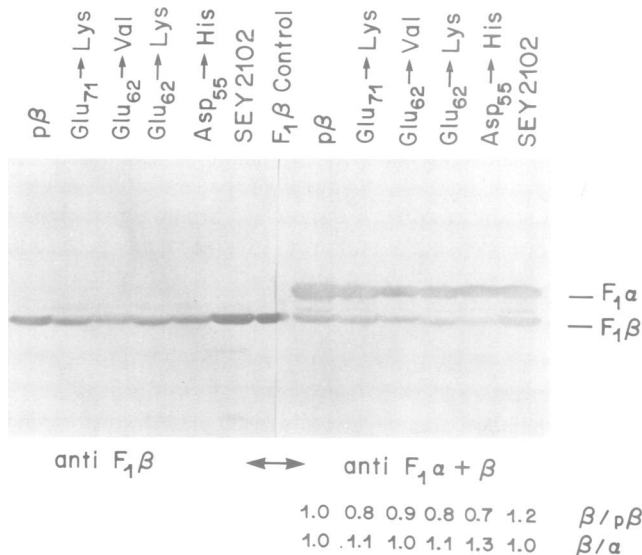
The plasmids which contained mutations to restore targeting of the  $\beta\Delta 4-37$  subunit were further characterized by restriction fragment exchange. Since the modifications were anticipated to reside within the amino terminus of the subunit, an *Eco*–*Kpn* fragment encoding the first 66 residues of the 4-37 deletion protein and 800 bp of 5' non-coding DNA were ligated in place of the same fragment in *pβΔ4-37* and assayed for complementation of the *atp2Δ-1* host. In every case the transferred *Eco*–*Kpn* fragment encoded information necessary to restore growth on glycerol.

The *Eco*–*Kpn* restriction fragments selected in this manner

**Table II.** Surrogate targeting signals support growth of yeast on glycerol at reduced rates

Allele	Change		Doubling time (*h)
	From	To	
sts 11	Pro41 (CCA)	Arg41 (CGA)	9.5
sts 5	Val54 (GTT)	Leu54 (CTT)	30.0
sts 9	Asp55 (GAC)	His55 (CAC)	10.5
sts 17	Glu59 (GAA)	Gly59 (GGA)	13.0
sts 4	Glu62 (GAG)	Val62 (GTG)	6.5
sts 18	Glu62 (GAG)	Gly62 (GGG)	15
sts 8	Glu62 (GAG)	Lys62 (AAG)	18
sts 2	Glu71 (GAA)	Lys71 (AAA)	10.5
sts 7	Glu71 (GAA)	Gln71 (CAA)	19
$\beta$ OK	—	—	4

With three exceptions, the alleles shown were selected more than once in this screen. Codon changes are shown in parenthesis. The numbering of residues is that of the wild-type  $F_1$   $\beta$ -subunit. Growth rates were measured at 30°C on YPglycerol.  $\beta$ OK is the wild-type subunit.



**Fig. 5.** Analysis of mitochondrial content of  $F_1$ -ATPase  $\beta$ -subunits harboring surrogate import signals. Yeast mutant AVY4/1 harboring  $p\beta\Delta 4-37$  with the indicated mutations were grown to midlog phase ( $2-5 \times 10^7$  cells/ml) on YP glycerol. Mitochondria were prepared from spheroplasts and 40  $\mu$ g in each case was resolved on an SDS gel. Following transfer of mitochondrial proteins to nitrocellulose the  $F_1$ -ATPase  $\alpha$ - and  $\beta$ -subunits were visualized with subunit-specific antiserum plus horseradish peroxidase-conjugated goat anti-rabbit antibody. In order to quantitate the amount of  $\alpha$ - and  $\beta$ -protein in each case the immunoblot was scanned with a recording densitometer and the areas under the peaks for each subunit were integrated. The numbers at the bottom of the figure indicate the content of  $F_1$   $\beta$ -subunit in each case relative to the wild-type control normalized to the content of the  $\alpha$ -subunit.

did not exhibit any apparent size difference when compared with the wild-type fragment. They were ligated into M13 and the coding region for the amino-terminal 66 residues of each mutant was determined by DNA sequence analysis. The results of this analysis are shown in Figure 4. First, independent, single base changes to the  $ATP2$   $\Delta 4-37$  gene, which yielded amino acid replacements within the first 40 residues of the resulting subunit, were sufficient in each case to promote import. We have called these new import signals surrogate targeting signals (sts). They all restored growth of  $atp2\Delta 1$  on glycerol. The changes in each case were confined to the sequence proposed to form the first amphipathic structure within the deletion construct (Figure 2).

We found no changes outside this region. These single base changes (Table II) in each case encoded amino acid replacements within the amino terminus which, with the exception of the weakest suppressor (sts 5), yielded an amphipathic helix with a reduced content of acidic residues. Thus, independent mutations within the  $\beta\Delta 4-37$  protein which either reduced the negative charge of the first available amphipathic helix or extended its length, restored sufficient import of the  $\beta$ -subunit protein to support growth on a non-fermentable carbon source.

#### Surrogate $ATP2$ import signals function with different efficiencies in yeast

Growth of the  $atp2\Delta 1$  mutant on a non-fermentable carbon source which was dependent upon the new import signals within the  $\beta\Delta 4-37$  occurred at different rates (Table II). The wild-type host or the  $atp2\Delta 1$  mutant containing a plasmid-encoded wild-type  $\beta$ -subunit grew at the same rate on the non-fermentable carbon source. Surrogate import signals in the plasmid encoded  $\beta\Delta 4-37$  supported growth on glycerol with doubling times that were 2–8 times longer than that of the wild-type subunit (Table II). The amount of subunit present in mitochondria prepared from representative mutants following growth on the non-fermentable carbon source was comparable to the level of either the wild-type strain or the  $atp2\Delta 1$  mutant harboring  $ATP2$  on the plasmid (Figure 5). Using subunit-specific antiserum against the mitochondrial  $F_1$ -ATPase  $\alpha$  or  $\beta$ -subunit we observed that mutants with doubling times up to five times that of the wild-type exhibited a level of the mitochondrial  $\beta$ -subunit which was 70–90% that of the plasmid-encoded wild-type subunit. In addition, the ratio of  $\alpha$ - to  $\beta$ -subunits within mitochondria harboring mutant  $\beta$ -subunits remained the same as that for the wild-type indicating that mutant subunits were assembled with the other major subunit within the ATPase complex. Integration of the different mutant constructs into the chromosome to reduce their copy number did not change the level of  $\beta$ -subunits or their stoichiometry relative to the  $\alpha$ -subunit within the organelle.

The wild-type level of mutant  $F_1$   $\beta$ -subunits in mitochondria harboring different surrogate import signals suggested that the reduced rate at which the cells grew could either be a function of their relative import efficiencies or their specific activities as part of the ATPase complex. To distinguish between these, the level of functional oligomycin-sensitive ATPase activity was determined in mitochondria containing the mutant  $\beta$ -subunits. When ATPase activities of isolated mitochondria were compared with controls, the specific activities were in fact very similar to that of the wild-type complex. The specific activities detected were 60–90% that of wild-type. Analysis of the oligomycin sensitivity of the energy transducing complexes containing the mutant  $\beta$ -subunits revealed that they exhibited a functional coupling between the  $F_1$ -ATPase and the membrane-bound  $F_0$  complex which was comparable to that of the wild-type complex (Table III). Further, the specific activity of the ATPase based on the amount of  $F_1$   $\beta$ -subunit protein was the same as that determined for the wild-type protein. The only difference between the mutant and wild-type complexes was the observation that the mutant complexes were more cold labile than the wild-type and exhibited a reduced activity as well as coupling efficiency following a single freeze–thaw cycle (not shown). The  $\beta$ -subunit of the mitochondria ATPase constitutes the active site of the energy-transducing enzyme. Studies in different laboratories to examine the function of this subunit using either mutations or covalent modifications within the highly conserved subunit from *E. coli* or from bovine mitochondria have revealed that the carboxy-

**Table III.** Mitochondrial ATPase activities of sts mutants are reduced but well coupled

Change	Mitochondrial ATPase		% Inhibition	Normalized activity
	-oligomycin	+oligomycin		
Pro41 → Arg	2.41	0.62	74	0.84
Asp55 → His	1.35	0.16	88	0.80
Glu59 → Gly	1.47	0.28	81	0.91
Glu62 → Val	1.60	0.94	63	0.74
Glu62 → Gly	1.44	0.30	79	0.79
Glu62 → Lys	1.82	0.29	84	0.95
Glu71 → Lys	1.71	0.12	93	0.89
Glu71 → Gln	2.02	0.34	83	0.77
$\beta$ OK	2.40	0.52	79	1.0
SEY2102	2.34	0.54	77	0.83

Analysis of ATPase activities in isolated mitochondria in the presence and absence of oligomycin was determined. The mitochondria were those analyzed in Figure 5 to quantitate the level of  $F_1 \beta$ -subunit present. In order to normalize ATPase activities to the amount of  $F_1 \beta$ -subunit present, the ATPase activities in each case were divided by the relative amount of subunit determined in immunoblots compared with the value for  $\beta$ OK which was set at one.

terminal region of the protein constitutes the active site (Noumi *et al.*, 1984a, 1984b, 1986). Thus, the specific activity of the  $\beta$ -subunit and its coupling to the other subunits in the complex is relatively insensitive to modifications within its extreme amino terminus. Taken together, these data indicate that the relative rates of growth observed reflect their efficiencies of *in situ* localization rather than their function within the assembled enzyme.

The profile of mitochondrial proteins present in hosts which grew at much reduced rates were the same as the wild-type strain. We propose that the reduced rate of  $\beta$ -subunit import and its assembly into the ATP-generating enzyme under growth conditions which demand its function, must influence in some manner the assembly of all other mitochondrial components. Although the mechanism for this control is unknown, it may reflect the fact that the ATP content of a cell containing the  $\beta$ -subunit with surrogate import signals may be limiting under these growth conditions. The cells all grow at the same rate when glucose is the carbon source. Thus, a minimum threshold level of mitochondrial ATP synthetase is necessary in the cell as the sole source of high energy phosphate required for cell growth on a non-fermentable carbon source. We propose that the growth of strains harboring surrogate import signals in *ATP2* is limited by the rate of  $F_1 \beta$ -subunit delivery to mitochondria.

## Discussion

### Implications for the role of secondary structure in mitochondrial targeting

Recent statistical analysis of >20 mitochondrial targeting sequences characterized to date has revealed that their most common feature is a strong amphipathicity (Von Heijne, 1986). Without exception these amphipathic structures for mitochondrial targeting appear to be localized at the amino-terminal end of the protein. The location and structure of the sequences probably reflect properties which are essential for their catalysis of the mitochondrial import event (Douglas *et al.*, 1986). It has recently been established that import into mitochondria requires a partial unfolding of the protein (Eilers and Schatz, 1986) and that the exposed amino-terminal end leads the rest of the molecule

(Schleyer and Neupert, 1985). Peptides with properties of an amphipathic helix destabilize lipid bilayers and disrupt membranes (Mollay and Kreil, 1983). The interaction of an amphipathic peptide like melitin with lipid bilayer is the best characterized example which serves as a model for the behavior expected of a mitochondrial import sequence. This mitochondrial signal in addition must be accessible within the precursor protein for interaction with its target. Independent studies have demonstrated that fusion of a mitochondrial target signal to the amino terminus of a variety of soluble cytoplasmic proteins is sufficient to promote their import *in vitro* and *in vivo*. To date there has been no demonstration that the location of these signals at sites distal to an exposed amino terminus will promote import. This accessibility is an essential property of the target signal as well as its ability to promote membrane destabilization as either a complex with membrane proteins or possibly as an aggregate with other mitochondrial target signal containing precursors on the membrane surface. Analysis of the amino-terminal 40 residues of 132 cytosolic proteins has shown that most can form helical structures which segregate charge in the same manner as the mitochondrial target sequence. However, in contrast to the mitochondrial target signal, the amphipathic structures located at the extreme amino terminus of cytosolic proteins are, with few exceptions, acidic (Von Heijne, 1986).

In the present study independent mutations within an accessible amphipathic helix on a cytoplasmic protein which reduce its content of acidic residues are shown to promote mitochondrial import. The independent amino acid replacements defined here have been uncovered by a sensitive genetic screen which demands correct localization of the protein. This analysis has revealed that the steady-state amount and activity of the mutant  $\beta$ -subunit protein in mitochondria is the same as that of the wild-type. Thus, the differences in growth rates observed are a function of the rate at which these mutant subunits reach their destination in the cell. The independent amino acid replacements defined in this study indicate that a reduction of acidic residues in the amino terminus is important for mitochondrial targeting. All of the surrogate import signals described here are, however, still quite acidic, and function with reduced efficiency *in vivo*. Complementation of the *atp2-Δ1* mutant is a sensitive screen which allows us to detect the function of these weak import signals. Some of the surrogate signals defined here direct inefficient but detectable *in vitro* import of the  $F_1 \beta$ -subunit into mitochondria. However, the extent of *in vitro* import mediated by these surrogate signals appears to be less than expected based on the analysis of growth rates. Specific deletions within the *ATP2* import signal, which cause small kinetic delays in the mitochondrial localization of the precursor in pulse-labeled cells, exhibit no detectable effect on growth rate. It would appear then that the wild-type import signal of the  $F_1 \beta$ -subunit is not the rate-limiting step for growth of yeast on a non-fermentable carbon source. Therefore, surrogate mitochondrial import signals which are rate limiting for growth are most likely weaker import signals than the reduced growth rates on glycerol would indicate.

We propose that an amphipathic structure at the amino terminus of the cytoplasmic protein must contain a minimum content of basic residues to initiate productive interaction with the energized mitochondrial membrane for the initiation of its transfer across the bilayer. The independent changes within  $F_1 \beta \Delta 4-37$  protein which yield an import-competent protein are consistent with this proposal. They further suggest a mechanism for the evolution of a more efficient mitochondrial import signal.

The ability of *ATP2* surrogate signals, which may resemble

the amino termini of some cytoplasmic proteins, to function as weak mitochondrial import determinants suggests that normally cytoplasmic proteins may be mislocalized into mitochondria at some low rate. On the other hand, this raises the intriguing possibility that some proteins with weak import signals might partition between mitochondria and other compartments of the cell in response to a given cellular condition. These dual localized proteins might be expected to contain a less than optimum mitochondrial import signal better able to sense subtle changes in the physiology of the cell to influence the distribution of the protein (Martin and Hopper, 1982; Natsoulis *et al.*, 1986; Dihanich *et al.*, 1987).

## Materials and methods

### Strains, strain constructions and media

*Saccharomyces cerevisiae* strains used in this study were SEY2102, MAT $\alpha$  *ura3-52 leu2-3 leu2-112 suc2- $\Delta$ 9 his4-519 gal2* (Emr *et al.*, 1983) and AVY4-1, an isogenic strain containing a 3.1-kb *Bgl*II fragment of *LEU2* in place of *ATP2* (Vassarotti *et al.*, 1987). AVY4-1 MAT $\alpha$  *ura3-52 leu2-3 leu2-112 suc2- $\Delta$ 9 his 4-519 atp2::LEU2* was constructed by the one-step gene replacement method (Rothstein, 1983).

The *E. coli* strain used was MC1066, F<sup>-</sup>  $\Delta$ (*lac*)X74 *galU galK rpsL hsdR trpC9830 leuB600 pyrF74::Tn5* (Martinez-Arias and Casadaban, 1983).

Yeast and *E. coli* media were utilized as published (Miller, 1974; Sherman *et al.*, 1979). Selection medium for growth of yeast transformants was as previously published (Emr *et al.*, 1986).

### DNA methods

Restriction endonuclease digestions and ligations with T4 DNA ligase were performed according to the instructions of the commercial supplier (New England Biolabs or Bethesda Research Laboratories, Inc.). Isolation of plasmid DNA, agarose gel electrophoresis and DNA transformation into *E. coli* and *S. cerevisiae* were performed as described (Ito *et al.*, 1983; Maniatis *et al.*, 1982; Birnboim and Doly, 1979). DNA sequence analysis was performed by the dideoxy chain termination method (Sanger *et al.*, 1980).

### Plasmids and DNA constructions

Plasmid p $\beta$  $\Delta$ 4-37 harboring *ATP2* with a pre-sequence deletion was constructed as follows. A 700-bp *Bam*HI fragment of *ATP2* containing 387 bp of coding DNA and ~300 bp of 3' non-coding DNA (Takeda *et al.*, 1985) was ligated into pSEY101 (Emr *et al.*, 1986). Restriction analysis confirmed that the fragment was oriented such that the sequence coding the terminal 129 codons of *ATP2* was proximal to the unique *Eco*RI site in pSEY101. This construct was opened at *Pvu*II sites within *lacZ* which removed all but 72 bp of DNA at the 5' end and 51 bp of DNA at the 3' end of *lacZ*. This was followed by brief treatment with exonuclease *Bal*31 (Silhavy *et al.*, 1984) sufficient to delete the *Bam*HI site distal to the *ATP2* coding DNA. This construct, ~7.1 kb, contains unique *Eco*RI and *Bam*HI sites for convenient insertion of an *Eco*-*Bam* fragment of *ATP2* DNA containing the  $\beta$  $\Delta$ 4-37 deletion (Vassarotti *et al.*, 1987). The 3' region in this construction retains DNA sufficient for correct transcription termination of *ATP2*.

### Miscellaneous

Mitochondria were prepared from spheroplasts as published (Daum *et al.*, 1982). Yeast cells harboring different plasmids were grown at 28–30°C to 2–4  $\times$  10<sup>7</sup> cells/ml on YNBD medium containing the required amino acid supplements (Douglas *et al.*, 1984). ATPase assays were performed at 30°C with an ATP regenerating system as previously described (Todd *et al.*, 1979). Published procedures were used for SDS–polyacrylamide gel electrophoresis (Douglas *et al.*, 1979). Immunodetection of proteins transferred to nitrocellulose with F<sub>1</sub>-ATPase  $\alpha$ -subunit and  $\beta$ -subunit antiserum (horseshoe peroxidase-conjugated goat anti-rabbit serum) was performed as previously described (Emr *et al.*, 1986). Protein was measured by a modification of the Lowry method (Lowry *et al.*, 1951). All nucleotides were purchased from PL laboratories. Oligomycin (Sigma) and X-gal (IBI) were used without further purification.

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