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Sequences Required for Delivery and Localization of the ADP/ATP Translocator to the Mitochondrial Inner Membrane

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The ADP/ATP translocator, a transmembrane protein of the mitochondrial inner membrane, is coded in *Saccharomyces cerevisiae* by the nuclear gene *PET9*. DNA sequence analysis of the *PET9* gene showed that it encoded a protein of 309 amino acids which exhibited a high degree of homology with mitochondrial translocator proteins from other sources. This mitochondrial precursor, in contrast to many others, does not contain a transient presequence which has been shown to direct the posttranslational localization of proteins in the organelle. Gene fusions between the *PET9* gene and the gene encoding β-galactosidase (*lacZ*) were constructed to define the location of sequences necessary for the mitochondrial delivery of the ADP/ATP translocator protein in vivo. These studies reveal that the information to target the hybrid molecule to the mitochondria is present within the first 115 residues of the protein. In addition, these studies suggest that the "import information" of the amino-terminal region of the ADP/ATP translocator precursor is twofold. In addition to providing targeting function of the precursor to the organelle, these amino-terminal sequences act to prevent membrane-anchoring sequences located between residues 78 and 98 from stopping import at the outer mitochondrial membrane. These results are discussed in light of the function of distinct protein elements at the amino terminus of mitochondrially destined precursors in both organelle delivery and correct membrane localization.

The ADP/ATP translocator protein is the most abundant protein in mitochondria and has served as a well-characterized model membrane protein for the analysis of both membrane transport and membrane biogenesis (17, 18). It is an integral protein localized in the mitochondrial inner membrane which catalyzes the exchange of adenine nucleotides across the bilayer. Early studies on the biogenesis of ADP/ATP translocator provided the first demonstration that mitochondrial precursor protein pools were present in the cytoplasm (12).

Subsequent analysis has revealed that the soluble precursor form of the translocator is not synthesized with an apparent transient presequence which is characteristic of most other mitochondrially imported proteins (14). The soluble precursor of the ADP/ATP translocator can be shown to exhibit structure and detergent-binding activity which distinguish it from the mature membrane-bound form (29). These observations have led to the proposal that the soluble cytoplasmic ADP/ATP carrier protein destined for mitochondria may be recognized and delivered as an oligomeric protein which inserts into the inner membrane after a specific binding event on the outer mitochondrial membrane (28).

Recent studies with *Neurospora Crassa* indicate that the cellular apparatus required for the mitochondrial import of the ADP/ATP translocator may be distinct from that required for the uptake of the presequence-containing (F_1-ATPase) β-subunit precursor (30). The F_1-ATPase is the soluble portion of the mitochondrial ATPase complex located on the matrix face of the mitochondrial inner membrane. Conditions which modify proteinaceous components on the outer mitochondrial membrane block the in vitro import of one but not the other. Thus, the nature of the precursors destined for mitochondria and the machinery required for their delivery strongly suggest that different classes and possibly unique components may participate in the mitochondrial import of different precursors (30).

Using a gene fusion approach, we recently defined the protein sequences necessary for the in vivo delivery of the *Saccharomyces cerevisiae* F_1-ATPase β-subunit to a positively charged hydrophilic sequence within the transient presequence (S. Emr, A. Vassarotti, J. Garrett, B. Geller, M. Takeda, and M. Douglas, J. Cell Biol., in press; A. Vassarotti, C. Smagula and M. Douglas, UCLA Symp. Mol. Cell. Biol., in press). To localize the noncleavable targeting signal which is required for the efficient delivery of the ADP/ATP translocator precursor, we sequenced the *PET9* gene encoding the protein (20) and utilized the gene to generate a limited set of gene fusion probes for defining its addressing signals. The data indicate that in vivo mitochondrial delivery of the translocator protein is determined by residues within the first 115 amino acids. Further, the delivery of the different hybrid gene products into a protease-protected space in the organelle occurred despite the expression of a well-documented membrane-anchoring or "stop transfer" hydrophobic region located near the amino terminus of the translocator protein sequence. Thus, unlike membrane-anchoring sequences at the immediate amino terminus which are proposed to stop further import of proteins at the outer membrane (13), the charged amino terminus of the ADP/ATP translocator protein most likely acts to bypass the outer membrane anchoring of a hydrophobic protein sequence 78 residues distal to the amino terminus.

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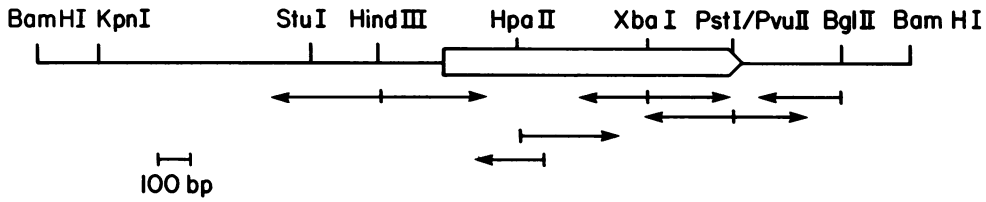

FIG. 1. Strategy for sequencing the *PET9* gene (309 amino acids) encoding the ADP/ATP translocator protein. Selected restriction sites within the 2.8-kb *Bam*HI fragment. *Hin*dIII and *Bgl*II were utilized to generate DNA sequences which when translated were homologous to the primary sequence available for other translocator proteins (1, 2). Completion of sequence analysis between *Hpa*II and *Xba*I required BAL 31 digestion from the *Pvu*II site prior to ligation into M13 mp8. The arrows indicate the overlapping regions, which were sequenced a minimum of three times each.

MATERIALS AND METHODS

Strains and media. The yeast strain used was *S. cerevisiae* SEY2102 (*MATα leu2-3 leu2-112 ura3-52 suc2-Δ9 his4-519 gal2*). Yeast cells were grown on minimal medium YNB supplemented with the appropriate nutritional requirements and 2% glucose (26). Mitochondria and postmitochondrial supernatant fractions were prepared from cells grown to a density of 1×10^7 to 2×10^7 cells per ml of YNB plus glucose while maintaining selection for *ura*$^+$ and then supplemented with one-third volume of YP medium (2% yeast extract, 2% Bacto-Peptone) 4 h prior to harvest. For transformations, yeast were grown to 1×10^7 to 2×10^7 cells per ml on YP plus 2% glucose (YPD). The *Escherichia coli* strains used were MC1066 (F$^-$ *lacX74 galU galK rpsL hsdR trpC9830 leuB600 pyrF*::Tn*5* (4) and JM101 (F$^-$ *lac pro supE traD36*). *E. coli* containing various plasmids and M13 phage were maintained on standard *E. coli* media.

DNA methods and transformations. Restriction endonuclease digestions and ligations with T_4 DNA ligase were performed according to the directions of the commercial supplier. DNA sequence analysis utilized a combination of chemical (19) and dideoxy chain termination-sequencing (24) methods. Techniques for the isolation of DNA, agarose gel electrophoresis, and *E. coli* transformations were performed with minor modification of published procedures (18). DNA transformation into *S. cerevisiae* utilized the lithium acetate-polyethylene glycol (BDH Chemicals PEG 4000) method (16). Digestions with BAL 31 nuclease (Bethesda Research Laboratories, Inc.) were performed at 30°C with a reduction in the final salt concentration to 200 mM.

Plasmid and gene fusions. The plasmid pBr6-19-28 was used as the source of DNA for the *PET9-lacZ* constructions (see Fig. 4). A 2.8-kilobase (kb) *Bam*HI fragment containing the complete ADP/ATP translocator gene in pBR322 was first cut with *Pvu*II, which restricted the plasmid at two sites, one within the vector and the other at codon 290 of the translocator gene. The 3,870-base pair (bp) *Pvu*II fragment containing the complete 5′ noncoding region of the gene plus 290 of 309 codons of *PET9* was digested with BAL 31 nuclease. Digestion times were adjusted empirically by sizing the digested DNAs on agarose gels. Sized fragments were ligated into *Sma*I-cut pSEY101. The details of this yeast-*E. coli* shuttle vector have been previously described (9). In-frame fusions were isolated after transformation into *E. coli* MC1066 on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside. The *PET9* inserts could be conveniently screened by digestion with *Bam*HI. Each construct retained a *Bam*HI site at the fusion joint between the *PET9* and *lacZ* coding sequences and a second *Bam*HI site in the yeast DNA insert 1,250 bp 5′ of the *PET9* start. This allowed for the easy transfer of *PET9* fragments selected as described above into *Bam*HI-cut pSEY101. These constructs were transformed into *S. cerevisiae* and examined for expression of β-galactosidase on the appropriate indicator plates (23) and in permeabilized whole cells (11).

Miscellaneous. Mitochondria were prepared from yeast spheroplasts as previously described (5). Mitochondrial subfractionation was done by the procedure previously defined (5) with minor modifications (10). Protease digestion of isolated fresh mitochondria was performed as previously described except 0.5% Triton X-100 was added when included (S. Emr et al., in press). Fumerase assays were performed as published (21). Labeling of yeast cells with $H_2{}^{35}SO_4$, immunoprecipitation, sodium dodecyl sulfide-polyacrylamide gel electrophoresis, and autoradiographic techniques were as defined in previous reports (6; S. Emr et al., in press). Polyclonal antiserum to commercially available *E. coli* β-galactosidase (Bethesda Research Laboratories) was prepared and characterized as described in earlier studies (8).

RESULTS

Genetic complementation of a yeast *pet9* mutant was used to select and characterize the plasmid encoding the ADP/ATP translocator protein (20). Genetic and physical studies have shown that the protein is encoded on a *Bam*HI fragment of 2.8 kb. In separate studies, this fragment was shown to hybridize to a relatively abundant RNA species which is coordinately regulated with other nuclear genes encoding mitochondrial proteins of the energy-transducing apparatus (27). Selected restriction sites within a 1,400-bp *Hin*dIII-*Bgl*II fragment were utilized to initiate the sequence analysis. Primary sequence comparison with the published ADP/ATP translocator protein of bovine and *Neurospora crassa* mitochondria (1, 2) was utilized to define the location of the gene on the fragment. To complete the sequence analysis, BAL 31 digestion from internal restriction sites was utilized to generate new endpoints within the gene for ligation into M13 (Fig. 1).

The sequence of the *PET9* gene is shown in Fig. 2. Within the 1,500 bp of DNA between *Hin*dIII and *Bgl*II is located a single open reading frame containing 309 codons. This open reading frame exhibits high homology with the primary sequence of the ADP/ATP carrier protein derived from other sources, further confirming the earlier assignment of the *PET9* gene product (20). The translational start of the *PET9* gene product is located 185 bp from the *Hin*dIII site. Unlike the ADP/ATP translocator gene characterized from *N. crassa* (2), which contains two introns beginning at codons 11 and 44, the *S. cerevisiae* gene exhibits a contiguous reading frame. The two genes exhibit excellent alignment of primary sequence beginning at residue 13.

The ADP/ATP translocator protein of *S. cerevisiae* con-

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                                                -516
                                                   CGCTTATAGAAGCAAGAGCTATAGGGCATGGCCTGCTTACAT  -475

                                                                    Stu I
-474 ACATATATATATATATATATATATATATATATAATCGCACTACATGCAGCGAGGCCTTGGCATACTCCCTCGAAGGATCGA -396

-395 CTGTTTGAGGTGCATCCTTCATTGTCTGTCGCAATTGGCGAGAATGAAGAGCACCTGCAAATGGGTGCACTTTTGAAGA -317

-316 TGGGCCTTGCCTGTTGGGTCTGCCTAAGTCAGCCTCGCCTAGCTCTAGGCGGAGGGTCTTTATCGGCGACTCAGCGTAC -238

                                                                    HinDIII
-237 TGTAGGGACCACCCCAGGCGCAACGGGCTGATTCGCACCCGGAAAGTAAAGCTTATTTCTGTCGGACGGAGAGTGTCCT -159

-158 TTTTCTCTCGCAGAAGACCATTTATTGGTCCTGAAACGGTATGAACATGTTATTATTCCTGTAACCGTGAAATAGGCAA -80

-79  GCCAAGGCATTATCGCAAAGAAGGCAGCACAGATTCTCGTATCTGTTATTCTTTTCTATTTTTCCTTTTTACAGCAGTA -1

1    ATG TCT CAC ACA GAA ACA CAG ACT CAG CAG TCA CAC TTC GGT GTG GAC TTC CTT ATG GGC  60
     met ser his thr glu thr gln thr gln gln ser his phe gly val asp phe leu met gly

61   GGC GTT TCT GCT GCC ATT GCG AAG ACG GGT GCC GCT CCC ATT GAA CGG GTG AAA CTG TTG  120
     gly val ser ala ala ile ala lys thr gly ala ala pro ile glu arg val lys leu leu

121  ATG CAG AAT CAA GAA GAG ATG CTT AAA CAG GGC TCG TTG GAT ACA CGG TAC AAG GGA ATT  180
     met gln asn gln glu glu met leu lys gln gly ser leu asp thr arg tyr lys gly ile

181  TTA GAT TGC TTC AAG AGG ACT GCG ACT CAT GAA GGT ATT GTG TCG TTC TGG AGG GGT AAC  240
     leu asp cys phe lys arg thr ala thr his glu gly ile val ser phe trp arg gly asn

                     HpaII
241  ACC GCC AAT GTT CTC CGG TAT TTC CCC ACG CAG GCG CTG AAT TTT GCC TTC AAA GAC AAA  300
     thr ala asn val leu arg tyr phe pro thr gln ala leu asn phe ala phe lys asp lys

301  ATT AAG TCG TTG TTG AGT TAC GAC AGA GAG CGC GAT GGG TAT GCC AAG TGG TTT GCT GGA  360
     ile lys ser leu leu ser tyr asp arg glu arg asp gly tyr ala lys trp phe ala gly

361  AAT CTT TTC TCT GGT GGA GCG GCT GGT GGT TTG TCG CTT CTA TTT GTA TAT TCC TTG GAC  420
     asn leu phe ser gly gly ala ala gly gly leu ser leu leu phe val tyr ser leu asp

421  TAC GCA AGG ACG CGG CTT GCA GCG GAT GCT AGG GGT TCT AAG TCA ACC TCG CAA AGA CAG  480
     tyr ala arg thr arg leu ala ala asp ala arg gly ser lys ser thr ser gln arg gln

481  TTT AAT GGA TTG CTA GAC GTG TAT AAG AAG ACA CTG AAA ACG GAC GGG TTG TTG GGT CTG  540
     phe asn gly leu leu asp val tyr lys lys thr leu lys thr asp gly leu leu gly leu

541  TAC CGT GGG TTT GTG CCC TCA GTT CTG GGT ATC ATT GTC TAC AGA GGT CTG TAC TTT GGC  600
     tyr arg gly phe val pro ser val leu gly ile ile val tyr arg gly leu tyr phe gly

                                                        Xba I
601  TTG TAC GAT TCT TTC AAG CCT GTG CTG TTG ACG GGG GCT CTA GAG GGG TCC TTT GTT GCC  660
     leu tyr asp ser phe lys pro val leu leu thr gly ala leu glu gly ser phe val ala

661  TCT TTC CTA TTA GGT TGG GTC ATT ACC ATG GGT GCT TCC ACT GCG TCG TAT CCC TTG GAT  720
     ser phe leu leu gly trp val ile thr met gly ala ser thr ala ser tyr pro leu asp

721  ACG GTA AGA AGA AGG ATG ATG ATG ACT TCG GGC CAG ACC ATC AAG TAC GAC GGT GCT CTG  780
     thr val arg arg arg met met met thr ser gly gln thr ile lys tyr asp gly ala leu

781  GAC TGT TTG AGA AAG ATT GTT CAG AAA GAG GGC GCG TAT TCC TTG TTC AAG GGC TGT GGT  840
     asp cys leu arg lys ile val gln lys glu gly ala tyr ser leu phe lys gly cys gly

                                  PstI   PvuII
841  GCC AAC ATA TTT AGA GGA GTC GCT GCA GCT GGT GTC ATC TCA TTG TAC GAT CAG TTG CAA  900
     ala asn ile phe arg gly val ala ala ala gly val ile ser leu tyr asp gln leu gln

901  CTC ATA ATG TTT GGC AAA AAA TTC AAG TGA AAAAAAAAGAAAACAACAAACGAATAAAATCTAAAAATT  969
     leu ile met phe gly lys lys phe lys  *

970  CTACATATTCTTGCTATTTATTTACATATTCATTCTTGCTACATACCTGTCTTATGCTATAATGACACTGGAGGTCTTC  1048

1049 TTCTCTTGAATTTATGTCCGACAATTCGTAAACGCCGTGCGGAAGGGAACCGTTTACGTACGCCAAGCTAAACATTGAA  1127

1128 AAAAAAAAAAAAAAAAAATGATAATAATAGTAATAATAGTAACGACGTAAAGGTAAAAGAAACAACAGACTTTTAAACTT  1206

                1223
1207 GTTAACTTTTGCATGAC
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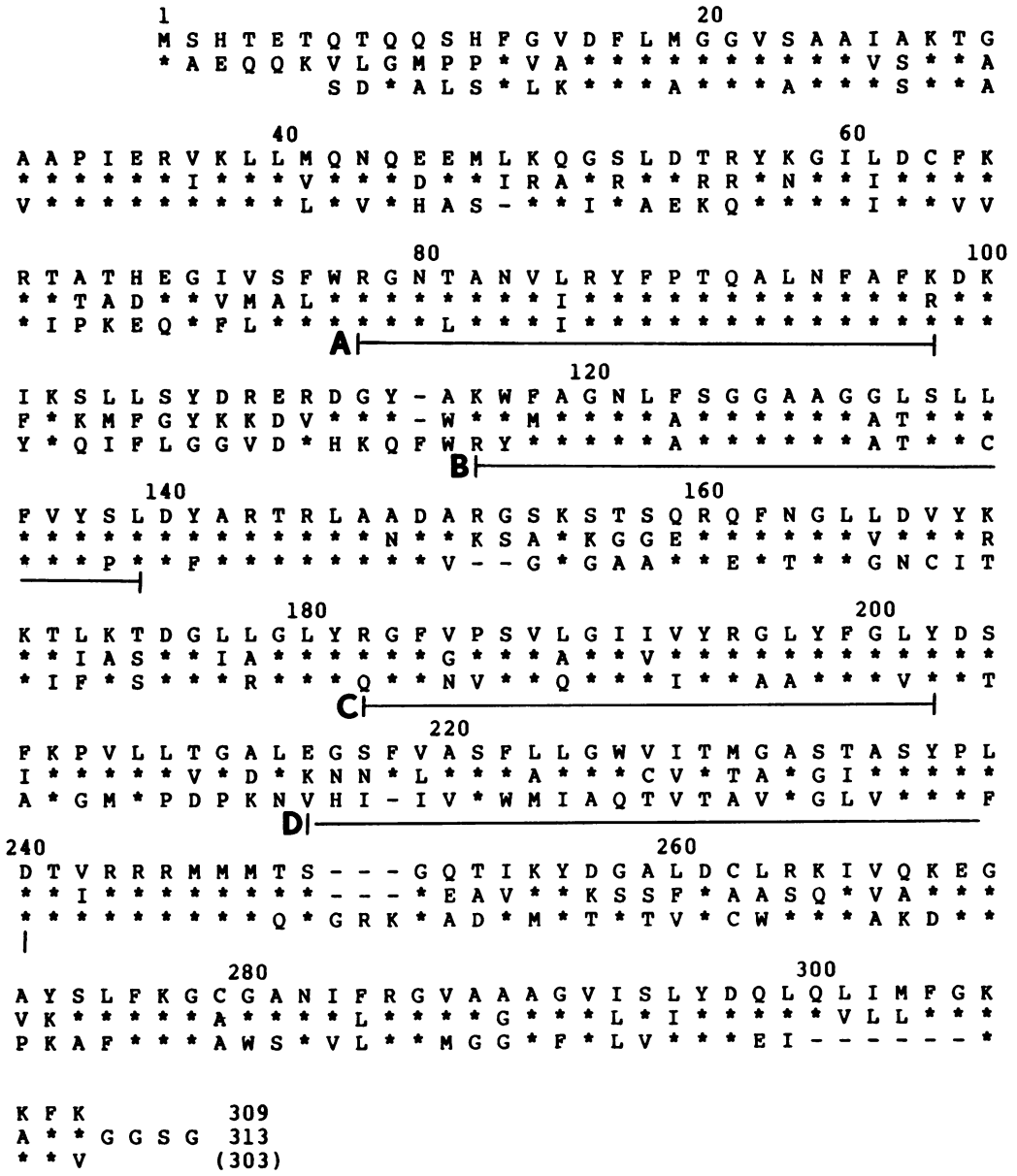

FIG. 3. Primary sequence comparison of different translocator proteins illustrates conservation within transmembrane segments. The primary sequences of the adenine nucleotide translocator proteins from *N. crassa* (2) and bovine (1) mitochondria were aligned with those from *S. cerevisiae* beginning at the initial methionine. Residues completely homologous to those of *S. cerevisiae* are noted with an asterisk (*). No conservative substitutions have been indicated. The regions underlined and designated A through D have been defined in previous biochemical (3, 17) and modeling (1, 2, 25) analyses as transmembrane hydrophobic sequences. The sequence derived from bovine mitochondria was determined by peptide sequence analysis (1) and lacks additional amino-terminal residues present in the primary translation product.

tains four membrane-spanning hydrophobic regions (Fig. 3) which have been defined in previous studies for the bovine and *Neurospora* proteins (1–3). These hydrophobic regions are predicted to form transmembrane helices (1, 3) and occur in the most highly conserved regions of the different protein sequences determined thus far. The unusual amino acid sequence RRRMMM adjacent to hydrophobic region D (Fig. 3, residues 243 to 248) in the yeast protein is conserved in all ADP/ATP translocator proteins characterized thus far and is in a region homologous with other ATP-binding proteins.

Gene fusions for analysis of targeting sequences. Studies from several laboratories have now demonstrated that the targeting and import of proteins into yeast mitochondria requires sequences which are present at the extreme amino

FIG. 2. Nucleotide sequence of the yeast *PET9* gene encoding the ADP/ATP translocator protein. A single open reading frame of 927 bp (309 codons) beginning at base pair 1 is shown. This sequence is flanked by 516 bp of DNA 5′ and 293 bp of DNA 3′ of the gene. The location of various restriction sites used in the sequence determination are shown.

terminus of the precursor protein (15, 22; S. Emr et al., in press). These targeting sequences are punctuated with basic amino acids and exhibit a net positive charge at physiological pH. In fact, the amino-terminal regions of almost all mitochondrially imported proteins characterized thus far lack acidic residues, with the notable exception of the ADP/ATP translocator protein. Therefore, it was of interest to determine whether this region of the translocator protein constituted part of the in vivo mitochondrial delivery signal.

Gene fusions were constructed between the *PET9* gene encoding different amino-terminal lengths of the carrier protein and the *E. coli lacZ* gene (Fig. 4). The yeast-*E. coli* shuttle plasmid pSEY101 was utilized for these constructions. This vehicle contains the yeast 2μm origin and *URA3* gene for efficient growth and selection in *S. cerevisiae* (9). In addition, it contains the gene *lacZ* in which the first nine amino acids have been replaced by a polylinker sequence. Expression of β-galactosidase in yeast in this vehicle requires the insertion of a yeast promoter and an in-frame translational start. For this the *PET9* gene in pBR322 (20) was cut with *Pvu*II. A 3.8-kb *Pvu*II fragment containing all the 5′ noncoding region and all but the final 19 codons of the gene was treated with BAL 31 for various lengths of time. Fragments of different lengths generated in this way were subsequently ligated into pSEY101 which had been cut with *Sma*I in the polylinker sequence. In-frame fusions were selected for the expression of β-galactosidase in *E. coli* and *S. cerevisiae* on the appropriate plates containing X-gal. Plasmids expressing the hybrid protein, which were selected in this manner, were subsequently characterized by DNA sequence analysis to determine the location of the fusion joints in the different constructs.

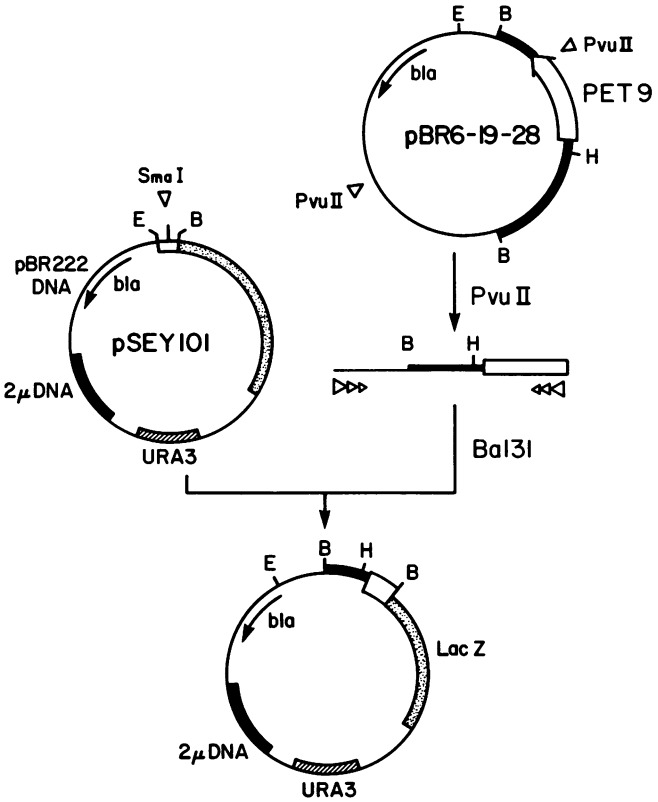

FIG. 4. Construction of *PET9-lacZ* gene fusions. The details of the source *PET9* DNA (pBr6-19-28) and yeast-*E. coli* gene fusion vehicle (pSEY101) have been described in previous studies (9, 20). The *PET9* gene on a 2.8-kb *Bam*HI fragment in pBR322 was cut with *Pvu*II. A 3.8-kb *Pvu*II fragment containing pBR322, all the 5′ noncoding region, and all but the final 19 codons of the *PET9* gene was treated with BAL 31 nuclease as described in Materials and Methods. Fragment populations of different lengths were subsequently ligated into pSEY101 which had been linearized at a unique *Sma*I site in the polylinker sequence. In-frame fusions were selected for expression of β-galactosidase in *E. coli* and *S. cerevisiae* on the appropriate plates containing the chromogenic indicator X-gal. E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII.

In the present study, three representative fusions were examined further to define the role of the hydrophobic regions in the mitochondrial delivery of the translocator (Fig. 5). For this analysis, fusion joints between *PET9* and *lacZ* were selected which contained one, three, or all of the hydrophobic regions (shown in Fig. 2). The *Bam*HI site retained in each of the fusions was used to determine the fusion joints by DNA sequencing.

Mitochondrial and cytoplasmic fractions were prepared from yeast transformants expressing each of the hybrids (Fig. 5). Quantitation of β-galactosidase activity in each of the mitochondrial fractions is summarized in Table 1. Gene fusions which expressed three (TZ222) or all (TZ281) hydrophobic regions of the translocator protein fused to *lacZ* were equally effective in the delivery of the hybrid protein to mitochondria. The translocator-β-galactosidase (TZ) fusion, TZ115, which contained only one membrane-spanning hydrophobic region (sequence A, Fig. 3) between residues 78 to 98, reduced slightly the percentage of total hybrid delivered to mitochondria. The TZ115 gene product consistently exhibited higher levels of β-galactosidase activity in the postmitochondrial supernatant fraction. This increased level of cytoplasmic β-galactosidase activity could result from removal of sequences of the translocator protein which improve the efficiency of the mitochondrial delivery. Additionally, the large β-galactosidase protein may interfere with the function of delivery signals near the amino terminus of the hybrid. We have observed in other studies that *lacZ* gene fusions to the yeast *ATP2* gene which express 112 amino-terminal residues of the F_1-ATPase β-subunit or fewer interfere with the function of targeting signals located within the first 28 residues of the protein (S. Emr et al., in press).

The amount of β-galactosidase gene product expressed in each of the TZ fusions was approximately the same, although the total activity of β-galactosidase in the cell increased as the amino-terminal length of the *PET9* gene product was reduced. Quantitative whole cell immunoprecipitation of the respective gene products (Fig. 6) indicated that approximately the same amount of hybrid protein was being expressed in each case. We propose that the small differences in total activity observed reflect the extent to which the different *PET9-lacZ* hybrids are associated with the mitochondrial membrane. The TZ281 and TZ222 proteins were efficiently targeted and firmly associated with membrane. The TZ115 protein which exhibited slightly reduced mitochondrial targeting yielded a small amount of soluble hybrid gene product. Based on the distribution of activities, the amount of the longer TZ hybrid proteins delivered to mitochondria was comparable with that of the control F_1-ATPase-β-galactosidase hybrid which has previously been shown to target to the organelle inner membrane (7). The specific activity of the *PET9-lacZ* hybrid gene products was approximately several orders of magnitude greater in the mitochondrial fraction than in the cytoplasmic fraction. Under these conditions, we could account for 70 to

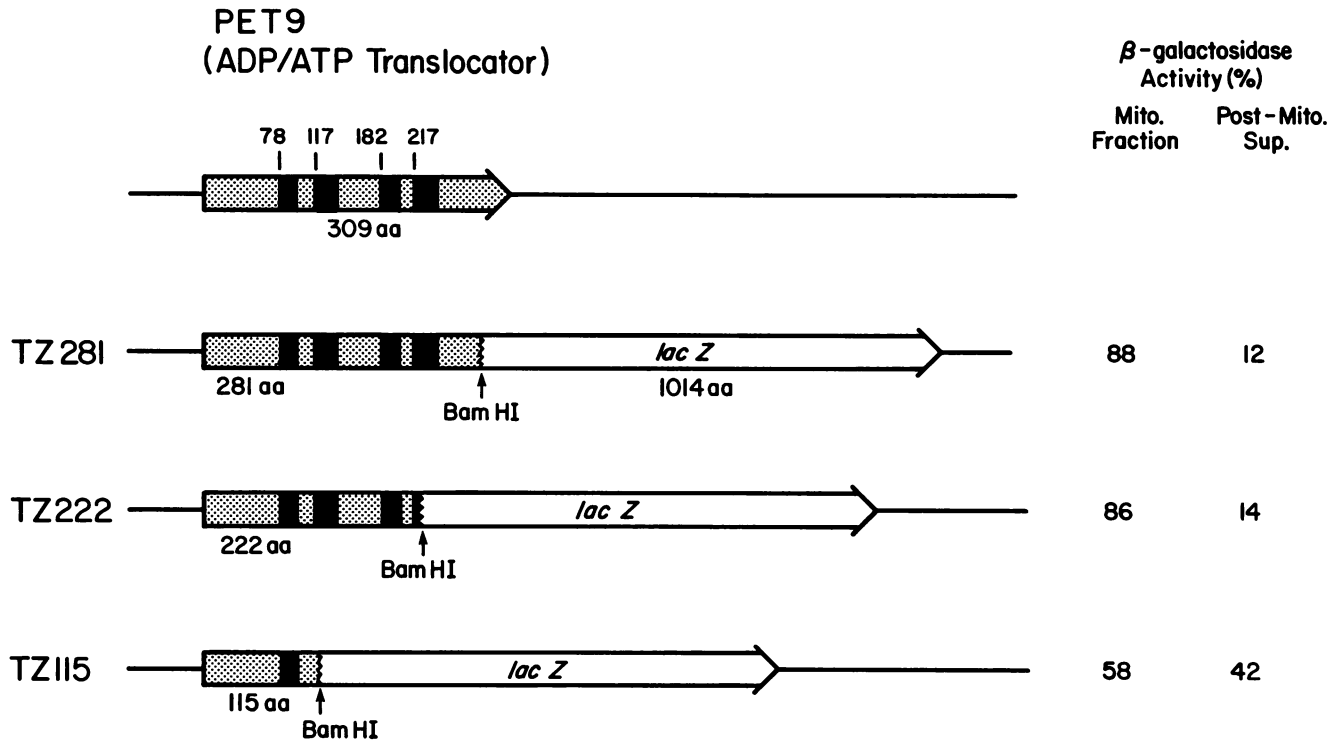

FIG. 5. Translocator sequences localize β-galactosidase hybrids to mitochondria. Plasmids constructed as defined in the legend to Fig. 4 were characterized by restriction endonuclease and DNA sequence analysis. In each case, a *Bam*HI fragment encoding the 5′ region and various lengths of the *PET9* gene product were moved to M13 mp8, and the DNA sequence of the fusion joint was determined. The constructs characterized in this manner are shown. In each case, the *Bam*HI site present at the fusion joint was contributed by pSEY101. The dark regions of the *PET9* gene product designate the transmembrane sequences defined in the legend to Fig. 3. To measure the distribution of β-galactosidase expressed from each of the translocator-*lacZ* (TZ) gene products, a mitochondrial (mito.) and a postmitochondrial supernatant (post-mito. sup.) fraction (maximum centrifugation at 100,000 × *g*) were prepared from log-phase yeast cells (see Materials and Methods). These fractions represented 9 and 84%, respectively, of the mitochondrial and postmitochondrial protein supernatant protein in the postnuclear homogenate. Recovery of whole cell β-galactosidase activity (11, 23) in each case was greater than 85%. aa, Amino acids.

90% of the total cellular β-galactoside activity. In addition, the mitochondrial specific activity of the TZ fusions was almost eightfold higher than control βZ380 4-36 deletion which cofractionated only 6% of the total cellular β-galactosidase activity in the washed mitochondrial pellet. Thus, analysis of in vivo delivery of *PET9-lacZ* hybrid proteins indicates that at least the first 115 residues of the translocator protein are sufficient for specific mitochondrial targeting. The hydrophobic segment (segment B, Fig. 3) between residues 117 to 139, however, may influence the efficiency of the delivery or binding event. Studies are currently in progress to further define the role of the 117 to 139 residue sequence in targeting efficiency.

To define the submitochondrial localization of the *PET9-lacZ* hybrid proteins being delivered to mitochondria, two sets of experiments were performed. Mitochondria prepared from strains harboring the TZ gene fusions (Table 2) were treated with protease to determine whether the translocator β-galactosidase protein was accessible to protease at a concentration which digested the protein in the presence of detergent. Like the *ATP2-lacZ* hybrid documented in earlier studies (7), the *PET9-lacZ* proteins appear to be localized within the organelle by a detergent-soluble barrier.

Further localization analysis utilized mitochondrial subfractionation techniques to determine whether the hybrid was being delivered to its correct target membrane. Mitochondria containing the different hybrid β-galactosidase proteins were subfractionated into matrix, inter membrane space, and total membrane fractions. The membrane fraction consisted of both inner and outer membrane. The enzymatic activity of fumerase, a mitochondrial matrix enzyme, was used to measure the matrix contamination

TABLE 1. Mitochondrial localization of adenine nucleotide carrier β-galactosidase hybrid protein[a]

Hybrid protein	Sp act (units/mg of protein)		Total units[b]	% Mitochondrial activity[c]
	Mito	PMS		
TZ115	205	12.9	2,082	61
TZ222	208	2.2	1,740	89
TZ281	194	2.3	1,584	89
βZ380[d]	284.8	1.74	3,335	94
βZ380 4-36[e]	27.1	40.2	3,570	6

[a] Mitochondria (Mito) and postmitochondrial supernatant (PMS) fractions were prepared from yeast spheroplasts. β-Galacosidase and protein measurements were performed as described in Materials and Methods.

[b] The total units are the units of β-galactosidase present in postnuclear fraction prior to separation of mitochondria. The total amount of protein processed for each sample was 60 to 80 mg.

[c] The distribution of total activity assumed that mitochondrial protein represents 9 to 10% of the total postnuclear protein of cells grown on a fermentable carbon source.

[d] βZ380 designates the *ATP2-lacZ* hybrid containing 380 residues of the F_1-ATPase β-subunit fused to *lacZ* (7).

[e] The βZ380 4-36 is the same construct but containing a deletion between codons 3 and 35 of *ATP2* (S. Emr et al., in press).

TABLE 2. Submitochondrial localization of hybrid proteins

Hybrid protein	% of total mitochondrial activity in[a]:			% Recovery[b]	Protein protection (% Activity remaining)[c]	
	Inner membrane space	Matrix	Membrane fraction		−Triton X-100	+Triton X-100
TZ115	4.6	9.1 (66)	57 (2.9)	70.7	86	8
TZ222	2.8	16.0 (58)	65 (0.01)	83.8	88	10
TZ281	3.5	11.5 (73)	77 (2.8)	92	97	10
βZ380	0.2	0.5 (70)	87 (1.8)	88	86	9

[a] Mitochondria prepared from log-phase cells expressing the indicated hybrid proteins were fractionated into membrane and soluble submitochondrial preparations. The total units of β-galactosidase activity in the starting mitochondrial preparation and in each fraction were determined. The numbers represent the percentage of total mitochondrial activity present in the respective fractions. Similar analysis was performed for the matrix marker enzyme fumerase (data shown in parentheses) to determine the extent of contamination of the membrane fraction with the soluble matrix fraction.

[b] The percentage of total mitochondrial activity accounted for in each case is indicated as percent recovery.

[c] To examine the accessibility of β-galactosidase protein to protease, freshly prepared mitochondria from the host strain harboring the respective hybrids were digested with proteinase K. Digestions were performed in 100 μl of isotonic buffer–0.6 M mannitol–10 mM Tris hydrochloride (pH 7.4) containing 0.4 mg of mitochondria and 10 μg of proteinase K with (0.5%) or without Triton X-100. After incubation at 23°C for 30 min, the samples were transferred to ice and phenylmethylsulphonyl fluoride was added to 1 mM. β-Galactosidase activities were immediately determined on duplicate samples. The values represent the percentage of activity remaining compared with samples incubated without proteinase K. Soluble *E. coli* β-galactosidase (Bethesda Research Laboratories) added with control mitochondria and treated with proteinase K under these conditions was completely digested in the absence of detergent.

of the membrane fraction. The control *ATP2-lacZ* hybrid protein fractionated with the mitochondrial membrane as previously demonstrated (7). Cofractionation of the TZ281 protein in the different mitochondrial fractions closely resembled that of the control *ATP2-lacZ* gene fusion product. This observation, coupled with the data that the hybrid protein is protected from a nonpenetrating membrane probe, strongly suggests that it is associated with the mitochondrial inner membrane; however, association with the inner surface of the outer membrane cannot be excluded at this time. The shortest fusion protein, TZ115, exhibited a slightly reduced copurification with the washed membrane fraction. This may be related to the observation that the recovery of this protein was consistently lower than that of the other hybrids (Table 2). We propose that the TZ115 hybrid protein is associated with the inner membrane but less firmly than the TZ281 hybrid. The intermediate *PET9-lacZ* gene fusion TZ222, like TZ281, expressed a hybrid which localized the β-galactosidase activity predominantly in the membrane fraction.

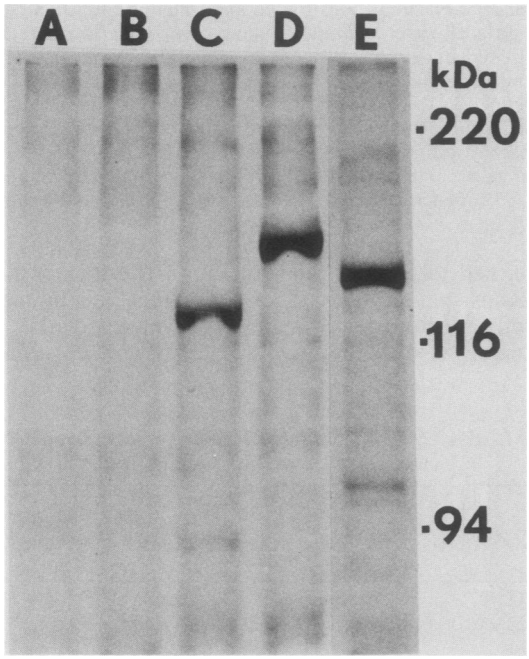

FIG. 6. Immunoprecipitation of hybrid translocator-*lacZ* hybrid proteins. *S. cerevisiae* SEY2102 cells harboring the indicated gene fusion plasmids were grown to mid-log phase (A_{600} of 0.5 to 0.7) on YNB medium plus glucose. In each case, the cells were harvested, washed, and labeled with 0.8 mCi of $^{35}SO_4$ as previously described. Whole cell extracts were prepared, and 15×10^6 cpm in each case was immunoprecipitated with either nonimmune serum or antiserum prepared against *E. coli* β-galactosidase. Antigen antibody complexes were solubilized in sodium dodecyl sulfate gel sample buffer. The samples were immunoprecipitated with either nonimmune serum (lane A) or anti β-galactosidase antisera (lanes B through E). Electrophoresis and autoradiography of the dried gel was as described previously (6). (A) and (B) pSEY101; (C) pTZ115; (D) pTZ281; (E) pTZ222.

All of the *PET9-lacZ* hybrid proteins exhibited a slightly higher β-galactosidase activity in the matrix fraction than the control *ATP2-lacZ* protein. This was not a function of the differences of matrix fractionation in the different preparations since the percentage of total mitochondrial fumerase activity in each case (Table 2) cofractionating with matrix remained relatively constant. The increased level of matrix-localized β-galactosidase activity defined in this study might represent proteolysis of the TZ hybrid protein attached to the matrix face of the inner membrane to release an active β-galactosidase fragment. To confirm that the hybrid proteins present in the cell were not modified in any significant way, immunoprecipitations were performed with antiserum prepared against β-galactosidase (Fig. 6). These data indicated that hybrid proteins of the predicted sizes constituted the majority of the β-galactosidase antigen present in labeled cells and that only small amounts of β-galactosidase-sized fragments (116 kilodaltons) were detectable. Thus, the small amount of activity detected in the matrix fraction may arise as the result of proteolytic release from the inner membrane during mitochondrial fractionation. The present data, however, do not rule out the intriguing possibility that a small population of the intact hybrid molecule may be transported into the mitochondrial matrix.

DISCUSSION

The ADP/ATP translocator protein is delivered to mitochondria by a pathway which initially utilizes a distinct

apparatus from that used for the import of proteins containing a transient presequence. Biochemical analysis of protein import into isolated mitochondria by Zwizinski et al. (30) has shown that conditions which block transport of the ADP/ATP translocator protein have little or no effect on the uptake of the F_1-ATPase β-subunit precursor. However, like the F_1-ATPase β-subunit precursor, a membrane potential is required for the proper insertion of the ADP/ATP translocator into the mitochondrial inner membrane. Since these proteins reside in distinct submitochondrial locations, it is of interest to define how information present in the primary sequence of these proteins is presented to the mitochondrial import apparatus to direct correct localization.

In the present study, we have examined the in vivo mitochondrial import of a transmembrane protein which must pass through the outer mitochondrial membrane during import yet stop in the inner membrane. Studies from several laboratories have now shown that the information present at the extreme amino terminus of mitochondrially imported proteins (to date presequences) is sufficient and necessary to direct their specific localization in mitochondria (22). These targeting elements with minor exception are nonhomologous sequences of 10 to 20 residues which exhibit a net positive charge at physiological pH. In the absence of membrane-anchoring sequences these targeting elements will direct a variety of nonmitochondrial proteins into the matrix (15; S. Emr et al., in press). However, the location of a transmembrane anchor sequence near the targeting element prevents the further import of a protein into the organelle. This was most clearly demonstrated in the case of the 70-kilodalton outer mitochondrial membrane protein of yeast, with the observation that modifications by partial deletion of a transmembrane anchor sequence located between amino acids 9 and 38 of the protein caused some mislocalization of the protein into the matrix (13). Thus, programming of localization within mitochondria will require protein sequences in addition to those defining mitochondrial delivery.

The mitochondrial ADP/ATP translocator represents a different precursor protein for mitochondrial import than those previously described. It is a major membrane-spanning protein localized in the mitochondrial inner membrane. A combination of biochemical and topographical studies have defined at least four transmembrane hydrophobic stretches of the protein (3, 25). In the present study, these occur at highly conserved regions of the protein between residues 78 to 98 (region A), 116 to 139 (region B), 181 to 202 (region C), and 215 to 240 (region D) (Fig. 3). Analysis of the localization of gene fusion products harboring one or all of these hydrophobic domains reveals that the protein in all cases is delivered in vivo to the mitochondrial inner membrane. This suggests that, in contrast to the delivery of proteins containing a transmembrane sequence for localization in the outer mitochondrial membrane, a mechanism exists for membrane hydrophobic sequences to bypass anchoring into the outer membrane during transit to the inner membrane. In the present study, the hybrid TZ gene products remained firmly membrane associated during subsequent submitochondrial fractionation analysis.

The present data also show the targeting and localization information is present in the first 115 residues of the translocator protein, although the efficiency with which these sequences function may be determined in part by distal regions of the protein. Analysis of fusions retaining shorter amino terminal segments of the translocator indicate that β-galactosidase does dramatically reduce the efficiency of mitochondrial targeting (G. Adrian and M. Douglas, unpublished observation). Thus, a refined analysis of delivery and import signals within the first 115 residues will require construction and analysis of deletions within this region. These studies are currently in progress.

Primary sequence analysis of the targeting region reveals that it retains a very well conserved transmembrane sequence between residues 78 and 98 which we propose may act as a stop sequence at the inner mitochondrial membrane. The topology of the residue 78 to 98 transmembrane sequence across the inner membrane is supported by surface labeling studies of mitochondria and inside-out submitochondrial particles. These studies reveal that a lysine group of the beef heart ADP/ATP translocator, which is conserved in the yeast protein at residue 49, is located on the matrix face, whereas a lysine corresponding to residue 113 is located on the cytoplasmic surface of the membrane (3). Thus, the final orientation of the first 115 residues of the protein is most likely defined by its targeting and anchoring information.

The ability of this membrane-anchoring domain to bypass the insertion into the outer membrane is probably a function of sequences more proximal to the amino terminus. It is envisioned that sequences located between the initiation methionine and residue 77 act in some manner to prevent an illegitimate outer membrane insertion event. The extreme amino terminus of the ADP/ATP translocator retains a net negative charge and exhibits an electrically neutral charge distribution up to the membrane-spanning region proposed here to stop transfer at the inner membrane. In the case of a transport stop of the 70-kilodalton outer membrane proteins at the outer membrane, the targeting and transmembrane sequences appear to be contiguous or partially overlapping (13). The present analysis would suggest that the transmembrane-anchoring sequences may be sufficiently distal from the import signal to allow the formation of a structure which can pass through the outer membrane. This model proposes that different conformations within the first 115 amino acids are presented to the outer versus inner membrane during import. Studies are currently in progress to further define the role of different residues within this region in determining mitochondrial membrane localization.

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