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Yeast mitochondrial ATPase subunit 8, normally a mitochondrial gene product, expressed *in vitro* and imported back into the organelle

David P.Gearing and Phillip Nagley

Department of Biochemistry and Centre for Molecular Biology and Medicine, Monash University, Clayton, Victoria 3168, Australia

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Subunit 8 of yeast mitochondrial F_1F_0-ATPase is a proteolipid made on mitochondrial ribosomes and inserted directly into the inner membrane for assembly with the other F_0 membrane-sector components. We have investigated the possibility of expressing this extremely hydrophobic, mitochondrially encoded protein outside the organelle and directing its import back into mitochondria using a suitable N-terminal targeting presequence. This report describes the successful import *in vitro* of ATPase subunit 8 proteolipid into yeast mitochondria when fused to the targeting sequence derived from the precursor of *Neurospora crassa* ATPase subunit 9. The predicted cleavage site of matrix protease was correctly recognized in the fusion protein. A targeting sequence from the precursor of yeast cytochrome oxidase subunit VI was unable to direct the subunit 8 proteolipid into mitochondria. The proteolipid subunit 8 exhibited a strong tendency to embed itself in mitochondrial membranes, which interfered with its ability to be properly imported when part of a synthetic precursor.

Key words: synthetic mitochondrial gene/mitochondrial import/ yeast/organelle evolution/ATPase

Introduction

Gene transfer during eukaryote evolution is thought to have relocated the majority of genes for mitochondrial proteins from the organelle into the nuclear compartment (Fox, 1983). Nevertheless, there are a small number of inner-membrane proteins which are consistently found to be encoded in the mitochondrial DNA (mtDNA) genome of different organisms; these include subunits 6 and 8 of the ATP synthase (ATPase) complex, subunits I, II and III of the cytochrome *c* oxidase complex and the cytochrome *b* apoprotein of the bc_1 complex (for a recent compendium, see Quagliariello *et al.*, 1985). The distribution of intensely hydrophobic domains throughout the polypeptides that comprise this group of inner-membrane proteins has been proposed to limit their site of synthesis to be within the mitochondrion (von Heijne, 1986a). There is, however, one notable exception of an extremely hydrophobic protein which is located in the inner membrane and yet which is found to be expressed in either the nucleus or mitochondrion depending on the organism. The ATPase subunit 9 proteolipid is encoded by the mitochondrial *oli1* gene in yeast (Sebald *et al.*, 1979a), but the homologous subunit 9 of many other organisms, including that of *Neurospora crassa* (Sebald *et al.*, 1979b) (denoted here N9) and mammals (Gay and Walker, 1985), is encoded in the nucleus. Concomitant with nuclear expression of subunit 9 has been the acquisition of a relatively long, hydrophilic leader sequence fused to its amino terminus (Viebrock *et al.*, 1982; Gay and Walker, 1985). In the case of *Neurospora* a 66 amino acid leader (N9-leader) precedes the 81 amino acid proteolipid (Viebrock *et al.*, 1982). This gives the precursor (pN9) an overall polar character and serves to solubilize N9 in the cytosol. The leader directs N9 to the mitochondria and facilitates its import (Schmidt *et al.*, 1983b) to the inner membrane *in vivo*. This import can be simulated *in vitro* with isolated mitochondria from *Neurospora*, rat liver or yeast (Schleyer *et al.*, 1982; Schmidt *et al.*, 1983a). Given this scenario for ATPase subunit 9, a significant question is raised concerning those proteins which in nature are always encoded in mtDNA: can such proteins be imported back into mitochondria following their expression outside the organelle?

The first of these proteins we have chosen to manipulate in this way is the ATPase subunit 8 (Macreadie *et al.*, 1983; Velours *et al.*, 1984) (denoted here Y8) encoded by the *aap1* gene in mtDNA of *Saccharomyces cerevisiae* (Macreadie *et al.*, 1983). The 48 amino acid subunit 8 (M_r 5815) is a proteolipid which can be extracted into organic solvents (Velours *et al.*, 1984) and is thus predicted to dissolve readily in lipid bilayers.

To enable the accurate and efficient production of Y8 by cytosolic translation systems, the yeast mitochondrial *aap1* gene has been redesigned and chemically synthesized (Gearing *et al.*, 1985). This was necessary because of extensive codon dictionary (Bonitz *et al.*, 1980) and codon usage (Bennetzen and Hall, 1982; Macreadie *et al.*, 1983) differences between the yeast mitochondrial and cytosolic translation systems. This synthetic gene (denoted *NAP1*) directed the expression *in vitro* of a polypeptide which was identical in properties to authentic Y8 (Nagley *et al.*, 1985).

In accord with the widespread requirement for imported mitochondrial proteins to be targeted into mitochondria by means of a cleavable N-terminal leader sequence (Hay *et al.*, 1984), it was considered necessary to furnish the Y8 with such a leader in order to facilitate the import. The 66 amino acid N9-leader was considered a suitable candidate for this role because of its demonstrated capacity (Schmidt *et al.*, 1983a) to deliver the very hydrophobic proteolipid N9 into yeast mitochondria. The 40 amino acid leader sequence of the precursor to yeast cytochrome oxidase subunit VI (Wright *et al.*, 1984) (denoted here Y*VI*) was similarly chosen to test its ability to direct import of Y8 into yeast mitochondria.

Here we report import experiments *in vitro* which demonstrate that Y8 can be specifically targeted into yeast mitochondria when fused to the N9-leader but not when fused to the Y*VI*-leader.

Results and discussion

Construction and expression of gene fusions

The fusion between a DNA segment encoding the N9-leader and the *NAP1* gene is depicted in Figure 1. The fused reading frame for pN9/Y8 (121 amino acids) encodes the full 66 amino N9-leader and the first five amino acids of the mature N9 proteolipid, the complete 48 amino acid sequence of Y8, plus two interposed serine residues resulting from the DNA sequence junction used to create the gene fusion. The pN9/Y8 fusion protein thus includes the same five amino acids downstream of the site at

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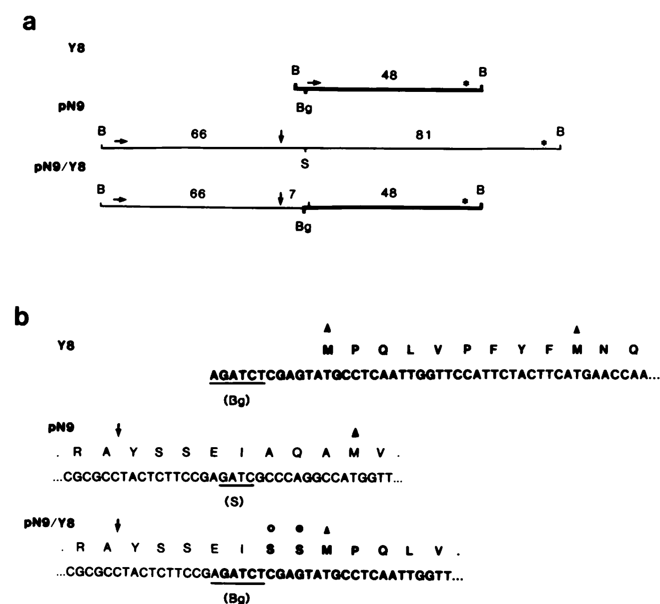

Fig. 1. Construction of pN9/Y8. **(a)** Relevant restriction sites of the gene constructs and predicted sizes of their encoded polypeptides. **(b)** Detail of sequence organization at the fusion point of pN9/Y8. Sequences derived from Y8 are shown bold. Other sequences are from pN9. Additional symbols indicate *Bam*HI (B), *Bgl*II (Bg) and *Sau*3AI (S) restriction sites, translational start (→) and stop (*) codons, methionine residues used in subsequent labelling experiments (▲) and the sites of predicted proteolytic cleavage (Schmidt *et al.*, 1983b) by matrix protease of mitochondria (↓). The serine codon marked (○) was introduced to make the *Bgl*II site in Y8 used for the gene fusion. The adjacent serine codon (●) is a consequence of the incorporation into the remodelled *NAP1* sequence [shown under **(b)** Y8] of the same sequence context before the ATG start codon (A at −3) as had been made in the original *NAP1* construction by Gearing *et al.* (1985).

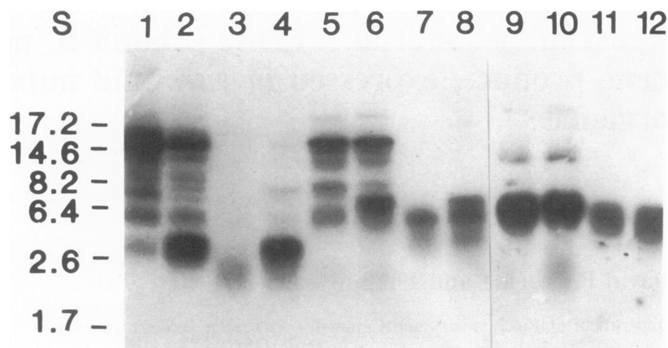

Fig. 2. Import of Y8 into mitochondria directed by N9-leader. Radiolabelled proteins resulting from transcription and translation *in vitro* were incubated with yeast mitochondria. **Lanes 1−4** show fluorograms of electrophoretically separated proteins in mitochondrial pellets following incubations with labelled pN9, **lanes 5−8** following incubations with pN9/Y8, and **lanes 9−12** following incubations with Y8. Odd and even numbered lanes: incubations with inactivated or active mitochondria, respectively. **lanes 1, 2, 5, 6, 9** and **10:** no further treatment; **lanes 3, 4, 7, 8, 11** and **12:** treatment with proteinase K. S, positions of CNBr fragments of myoglobin (Pharmacia) used as standards (sizes given in kd). The abnormal mobility of the mature N9 (apparent size 2.5 kd; **lane 4**) is characteristic of this proteolipid (M_r 8293; Sebald and Hoppe, 1981).

which the mitochondrial matrix protease cleaves the natural N9 precursor (Schmidt *et al.*, 1983b). The fusion between DNA encoding the Y*VI*-leader and *NAP1* was designed to achieve a similar outcome for the pY*VI*/Y8 construct (see below).

The genes were expressed *in vitro*, after insertion into the plasmid pSP64T (Krieg and Melton, 1984), by transcription using SP6 RNA polymerase. Flanking the inserted reading frame in the resulting capped transcripts were 5′- and 3′-untranslated sequences of a β-globin gene. The transcripts were translated in a rabbit reticulocyte extract to yield polypeptide products radiolabelled with [^{35}S]methionine. Import assays into isolated yeast mitochondria were performed (Schmidt *et al.*, 1983a) and the products analyzed by SDS−polyacrylamide gel electrophoresis.

Criteria for import into isolated yeast mitochondria

The criteria we adopted for successful import (Hay *et al.*, 1984; Douglas *et al.*, 1986) were checked under our conditions using the intact N9 precursor molecule expressed *in vitro* (Figure 2, lanes 1−4). The N9 precursor was cleaved in the presence of active mitochondria (lane 2) to yield mature *Neurospora* ATPase subunit 9 which was resistant to added proteinase K (lane 4). The specific cleavage of the N9 precursor did not occur with mitochondria treated with inhibitors and uncouplers to abolish the chemical potential across the inner membrane (lane 1), in which case there was also no resistance of the labelled proteins to added proteinase K (lane 3). These results confirm the previously reported ability of intact pN9 to direct *Neurospora* ATPase subunit 9 into isolated yeast mitochondria (Schmidt *et al.*, 1983a). The minor additional bands seen in Figure 2, lanes 1 and 2, may arise from inappropriate initiation at internal methionines within the N9-leader sequence or result from adventitious proteolysis, but they do not interfere with essential conclusions drawn in this study.

Import of yeast ATPase subunit 8 into mitochondria targeted by Neurospora ATPase subunit 9 leader

The results of import assays using the pN9/Y8 fusion protein are shown in Figure 2 (lanes 5−8). Comparison of lanes 5 and 6 in Figure 2 reveals that the major product of *in vitro* translation of the pN9/Y8 fusion protein (12 kd) was cleaved in the presence of active mitochondria to generate a major product of 5 kd which was resistant to externally added protease (lane 8). This satisfies the primary criteria for the successful import *in vitro* of Y8. The additional protease-resistant band of slightly higher mobility (~ 4 kd) apparent in lane 8 arose in the presence of inactivated mitochondria (lane 7) and is not a product of import (see following section).

The strong affinity of the proteolipid subunit 8 for mitochondrial membranes interferes with its import

The efficiency of import of Y8 directed by the N9-leader, assessed by specific cleavage of the precursors, was noticeably less than that of N9 itself (compare lanes 1, 2, 5 and 6 in Figure 2). Moreover, in parallel incubations using Y8 not furnished with a leader it was found that this proteolipid bound avidly to both activated and inactivated mitochondria (lanes 9 and 10) and was highly resistant to protease treatment, being only marginally reduced in size (lanes 11 and 12). Y8, when treated with proteinase K in the absence of membranes, undergoes extensive degradation (data not shown). It is apparent, therefore, that Y8 has a strong tendency to embed itself in mitochondrial membranes and thus be rendered protease-resistant.

We propose that the reduced import efficiency of the pN9/Y8 construct may be explained by the insertion into the membrane of some of these synthetic precursor molecules via the C terminus of the Y8 moiety. This explanation depends on the molecular characterization of material in the two bands observed following proteolytic digestion of the products of import depicted in Figure 2, lane 8. As shown by the sequence determination described below, the upper band represents Y8 imported by virtue of the

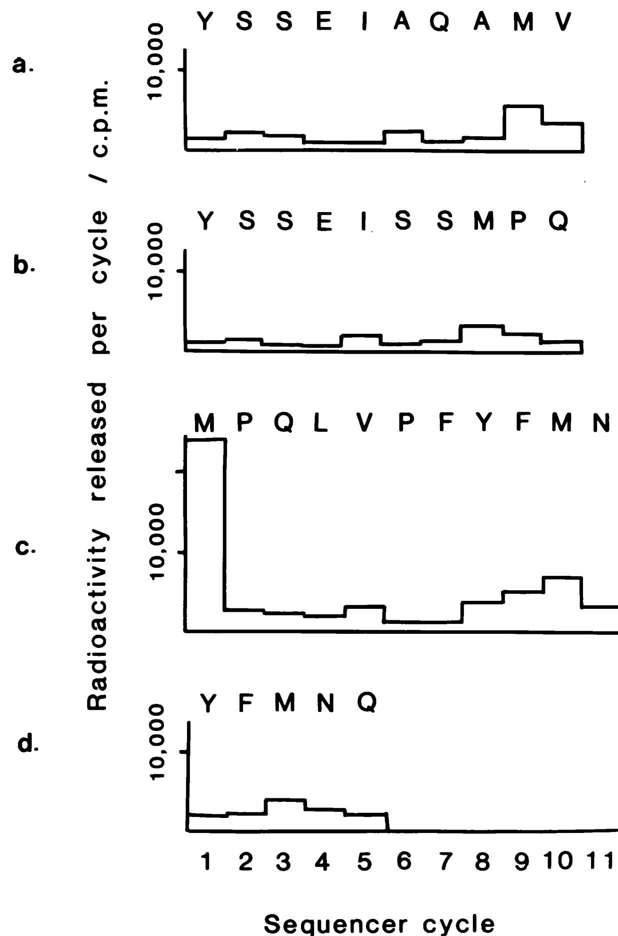

Fig. 3. Radio-sequencing of proteolipids labelled in methionine residues. Mitochondrial pellets were prepared from parallel experiments to those shown in Figure 2: **(a)** pN9 as in lane 2; **(b)** pN9/Y8 as in lane 6; **(c)** Y8 as in lane 10 and **(d)** Y8 as in lane 12. In each case proteolipids were extracted, subjected to Edman degradation and the radioactivity of the dried PTH-amino acids determined by liquid scintillation counting. Corresponding sequences inferred from the peak distribution are displayed in the single letter code.

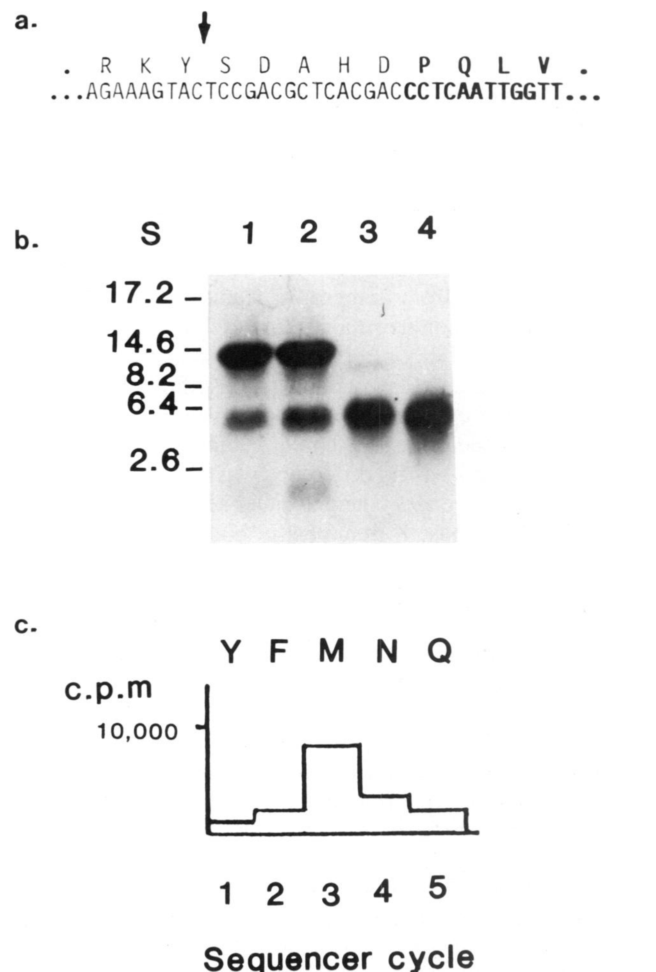

Fig. 4. Y*VI*-leader fails to direct import of Y8. **(a)** Detail of the construct pY*VI*/Y8 at the fusion point. Sequence derived from Y8 is shown bold. The predicted site of cleavage by matrix precursor is indicated (↓). **(b)** pY*VI*/Y8 expressed *in vitro* was assayed for import. **Lanes 1** and **3:** incubations with inactivated mitochondria; **lanes 2** and **4:** incubation with active mitochondria. **Lanes 1** and **2:** no further treatment; **lanes 3** and **4:** treatment with proteinase K. S, standards as in Figure 2. **(c)** Radio-sequencing of methionine-labelled proteolipids produced by proteinase K treatment of mitochondrially-adsorbed pY*VI*/Y8. A solvent extract was prepared of mitochondria from a parallel experiment to that in **(b) lane 4** and subjected to Edman degradation as in Figure 3.

fused transit peptide, processed by the matrix protease and protected from external protease. The lower band is considered to represent the product of externally digested fusion protein embedded in the outer membrane following penetration by the C-terminal region of Y8, formally equivalent to the protease-resistant band generated by leaderless Y8 bound to either active or inactivated mitochondria (Figure 2, lanes 11 and 12).

The Y8 derivatives were purified by solvent extraction of the mitochondria. Radiolabelled precursor proteins, where present, were quantitatively precipitated with the bulk of the proteins (data not shown) yielding an organic phase containing radiolabelled proteolipid in a form suitable for peptide sequencing. By monitoring the release of radioactivity (present in the methionine residues in the polypeptides) following successive cycles of automated Edman degradation, the N-terminal sequence could be deduced. In the case of the imported N9 (Figure 3a), the N terminus was ascertained to be at the precise point at which the mitochondrial matrix protease makes its final processing of N9 (Schmidt *et al.*, 1983b), since there is a peak of radioactivity in the ninth sequencing cycle. Significantly, this same matrix protease cleavage site was correctly recognized in the pN9/Y8 fusion protein, as demonstrated by the appearance of a radiolabelled peak in the eighth sequencer cycle (compare Figures 3b and 1b).

By contrast, Y8 synthesized without a leader, incubated with mitochondria and subjected to proteinase K treatment, yielded a radiolabelled peak at the third cycle (Figure 3d). This places the N terminus of the membrane-protected Y8 at residue 8. The previously noted marginal reduction in size on gel electrophoresis of this Y8 derivative relative to intact Y8 (Figure 2, lanes 9–12), which yielded the predicted radiolabelled peaks in the first and tenth cycles (Figure 3c), is consistent with a protection by the membrane of all but the seven N-terminal amino acids of this proteolipid. The proteinase K-resistant Y8 derivative generated by inactivated mitochondria exposed to pN9/Y8 (Figure 2, lane 7) has the same mobility as this membrane-protected truncated Y8 protein, and is inferred to have the same structure. There was insufficient radioactivity in the chloroform–methanol extract of the material corresponding to lane 7 to yield definitive sequence data. Nevertheless, an equivalent proteinase K-resistant band of much greater abundance was isolated from membrane-embedded Y8 derived from exposing mitochondria to pY*VI*/Y8; this material was shown to lack the first seven amino acids of Y8 (as described in the following section).

Cytochrome oxidase subunit VI leader does not promote import of ATPase subunit 8

The gene fusion between DNA coding for the Y*VI*-leader and the *NAP1* sequence results in a 92 amino acid fusion protein (Figure 4a) carrying the full 40 amino acid Y*VI*-leader, the first five amino acids of mature cytochrome oxidase subunit VI, and the last 47 amino acids of Y8. The first methionine residue of Y8 was deleted to prevent the appearance (Nagley *et al.*, 1985) of an abundant product of *in vitro* translation the same length as Y8, resulting from inappropriate initiation of translation at this internal methionine residue. Even so, a minor band was still encountered in addition to the major product of translation of pY*VI*/Y8 (Figure 4b, lanes 1 and 2); this minor band is inconsequential to the experiments described below.

The Y*VI*-leader was unable to direct the import of Y8 (Figure 4b, lanes 1 and 2) as no difference was observed in the pattern of labelled polypeptides bound to either active or inactivated mitochondria. Proteinase K treatment of both inactivated and active mitochondria incubated with pY*VI*/Y8 produced a resistant radiolabelled band ~4 kd in size (Figure 4b, lanes 3 and 4), equivalent to that observed for pN9/Y8 incubated with inactivated mitochondria and treated with proteinase K (Figure 2, lane 7). Radiosequencing of proteolipids derived from protease-treated pY*VI*/Y8 gave a major peak in cycle 3 (Figure 4c).

Given that the mobility of digested pY*VI*/Y8 is only marginally less than that of intact leaderless Y8 (data not shown) the peak of radioactivity corresponding to the third sequencer cycle is deduced to be met_{10} in the Y8 sequence (compare Figures 4c and 1b). Not only is this met_{10} residue the first internal methionine in the entire pY*VI*/Y8 fusion protein, but if cleavage by proteinase K had occurred two residues before the next methionine in the Y8 sequence (met_{20}) a 31 amino acid protein would have been produced, resulting in a significantly higher mobility than that observed in Figure 4b (lanes 3 and 4). It is concluded that pY*VI*/Y8 embeds into the mitochondrial membranes via the C-terminal region of Y8, the same interaction that was deduced for leaderless Y8 and also shown to be possible in the case of pN9/Y8 (see above). Positively charged residues found in the hydrophobic carboxy-terminal region of Y8 (Macreadie *et al.*, 1983; Velours *et al.*, 1984) may be presented as part of an amphiphilic helix to the mitochondrial membrane in the same manner proposed for the initial interaction of an authentic mitochondrial targeting sequence with the mitochondrion (Roise *et al.*, 1986; von Heijne, 1986b). Import of the pY*VI*/Y8 fusion protein was completely blocked by such a preferential insertion of the Y8 moiety into the membrane. Fortunately, this tendency could be significantly overcome by the N9-leader thus causing import of the pN9/Y8 fusion protein.

It is possible that the shorter length *per se* of the Y*VI*-leader (40 amino acids) compared with that of the N9-leader (66 amino acids) is the primary factor responsible for the relative inefficiency of the Y*VI*-leader in directing the import of Y8. More specifically, the Y*VI*-leader naturally functions to deliver to mitochondria a protein (cytochrome *c* oxidase subunit VI) whose hydrophobicity (Wright *et al.*, 1984) is certainly less than that of Y8; this leader may therefore be incapable of overcoming the tendency of Y8 to embed in a membrane. On the other hand, the N9-leader plays the natural role of solubilizing the hydrophobic N9 protein (Viebrock *et al.*, 1982) and delivering it to mitochondria (Schmidt *et al.*, 1983b), and thus can cope much better with the intensely hydrophobic Y8.

Prospects for expression and import of pN9/Y8 in vivo

The data presented above have demonstrated that import of the Y8 protein into mitochondria *in vitro* can be directed by the N9-leader, although we have not yet empirically determined the final submitochondrial destination of the imported Y8. Nevertheless, from its intensely hydrophobic character and its demonstrated strong affinity for mitochondrial membranes one can confidently expect that the imported Y8 will be found integrated in some measure into the inner mitochondrial membrane. Data presented here have shown that the pN9-leader directs the N-terminal portion of Y8 toward the matrix at least to the extent of allowing the matrix protease to cleave efficiently at its recognition site. What is not yet established is whether the Y8 moiety of the pN9/Y8 fusion protein acts as a 'stop-transfer' signal (Hurt and van Loon, 1986) and thus remains embedded in the inner membrane in this orientation (N terminus facing the matrix). An alternative possible outcome is that the pN9/Y8 fusion protein may be imported into the matrix in its entirety, then cleaved by the matrix protease; the Y8 could then reassociate with the inner membrane, perhaps with its N terminus facing the intermembrane space (as we would envisage for Y8 naturally synthesized on mitochondrial ribosomes and delivered directly to the inner mitochondrial membrane).

Whatever the topology of interaction of imported Y8 with the inner mitochondrial membrane, in general, the possibility that Y8 may eventually become oriented correctly can conveniently be tested by attempting functional restoration *in vivo* of the properties of an ATPase complex depleted of Y8. This can be monitored by the rescue of a mitochondrial *aap1* mutant (Macreadie *et al.*, 1983) to respiratory competence using the pN9/Y8 fusion protein expressed from a plasmid in the yeast nucleus. Such *in vivo* experiments would enable a test of the prediction made by von Heijne (1986a) that fusing a mitochondrial targeting sequence to a mitochondrially encoded protein might permit import of such a fusion protein *in vitro* but not *in vivo*. In preliminary experiments, we have inserted the DNA segment encoding pN9/Y8 into a yeast expression vector carrying the phosphoglycerokinase promoter. Thus far we have not observed rescue of the *aap1 mit*$^-$ mutation by the nuclear-encoded pN9/Y8 protein. A detailed analysis of the biosynthesis, transport and processing of the pN9/Y8 protein *in vivo* and its association, if any, with the ATPase complex, is required. Should conditions be found under which successful rescue is observed, then a strategy for the manipulation of the structure and function of mitochondrially encoded proteins is immediately presented.

Materials and methods

Gene constructions

DNA manipulations were performed using standard techniques as described by Maniatis *et al.* (1982). The *NAP1* sequence previously described (Gearing *et al.*, 1985) was engineered to produce a coding region for Y8, with a *Bgl*II site adjacent to its 5′ end, as follows: remodelled oligonucleotides 1 and 7 were synthesized chemically and then assembled by ligation with a mixture of oligonucleotides 2–6 and 8–12. pN9 was engineered by *in vitro* mutagenesis of the cDNA clone pAV48 (Viebrock *et al.*, 1982) to delete most of the 5′ and 3′ untranslated sequences and maintain the DNA segment from nucleotides 30 to 495 (Viebrock *et al.*, 1982). The Y8 coding region was fused to the N9-leader by ligation of their respective *Bgl*II and *Sau*3AI sites. The *COX6L*/*NAP1* construct described previously (Nagley *et al.*, 1985) was modified by site-directed *in vitro* mutagenesis in M13 (Nisbet and Beilharz, 1985) to delete both the first methionine codon in the *NAP1* coding region and the adjacent glycine codon (arising from the original *Sph*I site). The mutated version is denoted Y*VI*/Y8. All constructs were recovered as *Bam*HI fragments.

In vitro transcription, translation and import

Gene constructs were cloned into the *BglII* expression site of the *in vitro* transcription vector pSP64T (Krieg and Melton, 1984). Capped run-off transcripts were synthesized from linearized plasmids using a mixture (Krieg and Melton, 1984) including GpppG (Pharmacia) at 500 μM (ratio of GpppG:GTP was 10:1). Transcripts purified by phenol extraction and ethanol precipitation were incubated in a rabbit reticulocyte lysate (Promega) in the presence of [^{35}S]methionine (1250 Ci/mmol, Amersham) and post-ribosomal supernatants prepared (150 000 *g*, 20 min). Yeast mitochondria (Schmidt *et al.*, 1983a) prepared from *rho*$^+$ strain J69-1B (Macreadie *et al.*, 1983) were used for import assays in supplemented post-ribosomal supernatants prepared as described previously (Schleyer *et al.*, 1982). Inactivated mitochondria were prepared by 10 min pretreatment with 10 μM valinomycin, 10 μg/ml oligomycin and 10 mM KCN. Import was carried out at 25°C for 60 min, using 200 000 c.p.m. radiolabelled protein and 200 μg mitochondrial protein in a final volume of 100 μl. The reaction mixtures were chilled on ice then divided into two portions; one portion was digested with 500 μg/ml proteinase K (Boehringer-Mannheim) for 30 min at 0°C. Both portions were then made 1 mM with PMSF. Mitochondrial pellets were prepared by centrifugation and directly processed for electrophoresis in a 9–18% gradient polyacrylamide gel containing SDS (Shoeman and Schweiger, 1982). Gels were silver stained to locate protein standards then processed for fluorography.

Proteolipid extraction and radio-sequencing

Proteolipids were extracted from mitochondrial pellets with chloroform–methanol (2:1) and washed with water (Cattell *et al.*, 1971). Excess unincorporated [^{35}S]-methionine was removed by repeated washing of the organic phase with equal volumes of 50 mM methionine [pre-equilibrated with five volumes chloroform: methanol (2:1)]. When free label was reduced to <5% of that in the protein, the organic phase was loaded onto a glassfibre cartridge and subjected to Edman degradation in an Applied Biosystems gas-phase sequenator. Radioactivity of the dried PTH-amino acids was determined by liquid scintillation counting.

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