Volume 282, number 2, 425–430 FEBS 09717 May 1991

ADONIS 001457939100426I

Duplication of leader sequence for protein targeting to mitochondria leads to increased import efficiency

Maria Galanis, Rodney J. Devenish and Phillip Nagley

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

Received 31 January 1991; revised version received 13 March 1991

We describe a novel method for enhancing protein import into mitochondria, by tandemly duplicating the N-terminal cleavable leader peptide using a gene manipulation strategy. The import into isolated yeast mitochondria of passenger proteins (yeast mitochondrial ATP synthase subunits 8 and 9 and some mutagenised derivatives) that show little or no import when endowed with one such leader (that of *Neurospora crassa* mitochondrial ATP synthase subunit 9) is remarkably improved when the leader is tandemly duplicated. The import of these chimaeric proteins bearing a double leader is so rapid that a series of partially processed precursor intermediates accumulates inside the mitochondria before the final proteolytic release of leader sequences from the passenger proteins. It is considered that the duplicated leader greatly accelerates delivery of the import precursors to outer membrane receptor elements and the associated translocation systems, thereby enhancing precursor uptake into mitochondria.

Mitochondrial ATPase complex; Chimaeric fusion protein; Integral membrane protein; Leader peptide; Protein import; *Saccharomyces cerevisiae*

1. INTRODUCTION

The subcellular destination of many proteins can be systematically reprogrammed by use of suitable signal peptides that function as cleavable N-terminal leader sequences [1]. This approach has been especially well developed concerning protein translocation into mitochondria, where chimaeric fusions can be used to deliver passenger proteins into the organelle both in vitro and in vivo [2,3]. For example the soluble cytosolic protein dihydrofolate reductase can be readily targeted into mitochondria when fused to one of various leaders from naturally imported proteins (reviewed in [1–3]). Our laboratory has adopted such an approach for studying the structure and function of mitochondrially encoded proteins. The general strategy, denoted allotopic expression [4], involves restructuring an organellar gene such that it can be expressed in the nucleocytosolic system for efficient and manipulable expression both in vitro and in vivo, concomitant with delivery to mitochondria of the chimaeric fusion protein bearing a suitable signal peptide.

We have concentrated on two hydrophobic integral membrane subunits of the F_0 sector of the yeast mitochondrial ATP synthase (mtATPase) that are normally encoded in mtDNA of *Saccharomyces cerevisiae*. Subunit 8 (Y8; 48 amino acids) is involved in the assembly of F_0, and subunit 9 (Y9; 76 amino acids) is demonstrably a key component of the proton channel of mtATPase (reviewed in [5]). For the allotopic expression of Y8 and Y9 a series of chimaeric precursor proteins has been constructed that incorporate the 66 amino acid leader (N9L) from *Neurospora crassa* mtATPase subunit 9 (pN9). This long hydrophilic [6] leader has been initially fused via a 7-amino-acid bridge to either Y8 or Y9 passenger proteins (constructs denoted N9L/Y8-1 or N9L/Y9-1, respectively) [7–9]. Direct fusions between the N9L leader and Y8 or Y9 were also constructed (denoted N9L/Y8-2 or N9L/Y9-2, respectively) [9]. Both N9L/Y8-1 and N9L/Y8-2 are imported into mitochondria in vitro and, when expressed allotopically in vivo in a yeast host lacking mitochondrially encoded Y8, lead to the functional incorporation of imported Y8 into mtATPase (albeit at different efficiencies for the two fusion constructs) [9–12]. On the other hand, N9L/Y9-1 is imported relatively weakly into mitochondria [8] and N9L/Y9-2 does not import at all [9]; neither construct is able to rescue a subunit 9-deficient yeast following allotopic expression in vivo. Similar to N9L/Y9-2 and its import deficiencies [13], a series of C-terminally truncated derivatives of Y8 have been constructed which, when fused to N9L, are either poorly imported or totally import-incompetent [11].

It would clearly be desirable to introduce a general strategy for enhancing the effectiveness of the import functions in chimaeric precursor proteins that are dif-

Abbreviations: mtATPase, mitochondrial proton-translocating ATP synthase; pN9, precursor of *Neurospora crassa* mtATPase subunit 9; N9L, cleavable N-terminal leader from pN9; Y8 and Y9, subunits 8 and 9, respectively, of yeast mtATPase; bp, base pairs.

Correspondence address: P. Nagley, Department of Biochemistry, Monash University, Clayton, Victoria 3168, Australia. Fax: (61) (3) 565-4699.

Published by Elsevier Science Publishers B.V.

ficult to import. We report here that the tandem duplication of the leader presents an attractive solution to this problem.

2. MATERIALS AND METHODS

2.1. *Strains and gene constructs*

Saccharomyces cerevisiae strains J69-1B and YGL-1 have been described [14]. Gene constructs encoding the chimaeric precursors N9L/Y9-1 [8], N9L/Y9-2 [9], N9L/Y8-1 [7], N9L/Y8-2 [9] and its C-terminally truncated derivatives N9L/Y8-2(K47→STOP) and N9L/Y8-2(R42→STOP) [11] have been described previously.

2.2. *DNA manipulations*

For constructing genes encoding chimaeric precursors with duplicated N9L leaders (Fig. 1), the general strategy is shown in Fig. 2. Starting with a single stranded template in M13mp19 (kindly provided by L.B. Farrell) containing the coding region for pN9 flanked by *Bam*HI sites [7], the following oligonucleotide was used to introduce adjacent *Bss*HII and *Pst*I sites at the N-terminus of the N9L sequence: 5' CATACCCAACCATGCGCGCTGCAGACAAAAT-GGCC 3'. Site-directed mutagenesis was carried out using the Bio-Rad MutaGene in vitro mutagenesis kit. From a mutagenised double stranded M13 clone was excised, using *Bss*HII, a 207 bp DNA fragment containing a sequence encoding the first 64 residues of N9L plus a short N-terminal extension (see Fig. 2B). This fragment was introduced into *Bss*HII-cut pUC19 vectors containing inserts encoding N9L/Y9-1 or N9L/Y9-2 [8,9] or into *Bss*HII-cut pSP64T vectors containing N9L/Y8-1 or N9L/Y8-2 and its derivatives [7,9,11]. In each case, insertion in the appropriate orientation (confirmed by *Pst*I digestion) yielded a tandem duplication of N9L (Fig. 2). DNA encoding the double N9L leader constructs containing Y9 passenger proteins (Fig. 1) were then transferred as *Bam*HI fragments into *Bgl*II-cut pSP64T for expression in vitro [7,8]. General recombinant DNA methods were as described [15].

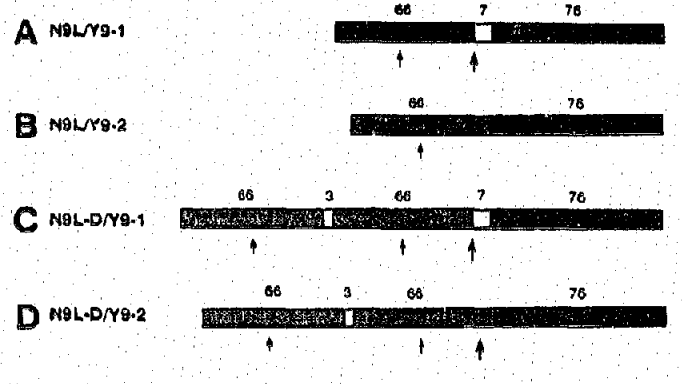

Fig. 1. Chimaeric fusion proteins and their double leader derivatives containing Y9 passenger protein. Single leader constructs N9L/Y9-1 (A) and N9L/Y9-2 (B) were respectively used to generate double leader constructs N9L-D/Y9-1 (C) and N9L-D/Y9-2 (D) (as outlined in Fig. 2). Hatched blocks, N9L; solid blocks, Y9; open blocks, spacer regions; 3 denotes a tripeptide introduced during the duplication of N9L (see Fig. 2); 7 denotes the bridge of the first 5 residues of mature *N. crassa* subunit 9 plus an additional 2 serine residues [7,8]; other numbers indicate amino acid sequence intervals; ↑, sites of matrix protease cleavage within N9L [16]; ↟, demonstrated cuts based on microsequencing of N-terminus of processed Y9 moiety [17]. Processing does not evidently occur between the first and second leaders (see text for details of cleavage sites).

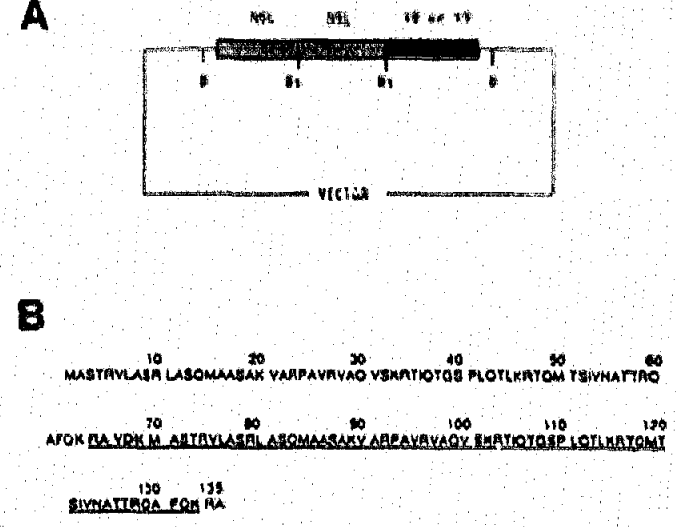

Fig. 2. Generic format of genes encoding chimaeric proteins carrying duplicated leaders. Panel A, gene organisation. The constructions are based on DNA encoding N9L/Y8 or N9L/Y9 fusions, which each have a *Bss*HII site just upstream of the 3' end of DNA encoding the N9L leader (enzymatically cleaved between codons for Lys^{64} and Arg^{65} [6]). This *Bss*HII site is the site of insertion of a *Bss*HII cassette encoding an additional N9L leader (underlined) which is essentially an entire second N9L coding region (see section 2.2). Panel B, amino acid sequence detail. Duplication of the N9L coding region (as in Panel A) generates two complete N9L leader sequences with the addition of 3 amino acids at their junction (resulting from the synthetic oligonucleotide containing *Bss*HII and *Pst*I sites). In the amino acid sequence shown here, the inserted N9L (corresponding to the *Bss*HII cassette) is underlined, whilst the 3 amino acid bridge is indicated in bold type.

2.3. *Import into isolated mitochondria*

Using ^{35}S-labelled chimaeric precursors expressed by transcription and translation in vitro, import experiments were carried out in vitro with mitochondria freshly isolated from yeast strains J69-1B or YGL-1 (partially depleted of endogenous subunit 8) [14]. In brief, import mixtures (100 μl) containing 10^6 dpm of ratiolabelled precursor and 100 μg mitochondrial protein were incubated at 27°C with vigorous shaking for the times indicated [8,14].

3. RESULTS

3.1. *Duplication of the N9L leader sequence in chimaeric fusion proteins*

To generate a tandemly repeated N9L leader it was necessary to design an N9L cassette which could be inserted into the existing gene segment encoding that leader. To achieve this a *Bss*HI site was introduced, by in vitro mutagenesis, into the N9L coding region just upstream of the N-terminus of this gene segment (Fig. 2). As the pN9 gene already possesses a *Bss*HII site at the C-terminus of N9L this manipulation results in the generation of an N9L-encoding *Bss*HII cassette. This cassette can then be conveniently inserted into the *Bss*HII site of a gene segment encoding an N9L leader fused to the synthetic Y8 or Y9 genes, to make the double leader expression cassettes for these genes (see Fig. 2A). These expression units can then be subcloned

into the in vitro expression vector pSP64T. Such an insertion results in the complete duplication of the N9L sequence with the addition of 3 amino acids at the junction of the two leaders (Fig. 2B). In practice, the only plasmid clones recovered containing the extra copy of the N9L sequence represented tandem duplications of N9L. Presumably, insertion of the 207 base pair *Bss*HII fragment in the opposite orientation leads to an inverted repeat structure at the DNA level (N9L sequences tail to tail) that is inhibitory to the replication of intact plasmids.

3.2. *Chimaeric double leader precursors for import of Y9*

Two chimaeric constructs have been made which carry a tandem repeat of the N9L leader fused to Y9 (Fig. 1). These are derived from the single leader constructs, N9L/Y9-1 and N9L/Y9-2, and are denoted N9L-D/Y9-1 and N9L-D/Y9-2, respectively. When the ^{35}S-labelled N9L-D/Y9-1 and N9L-D/Y9-2 precursors were imported into isolated wild-type J69-1B mitochondria the results were striking compared to the import behaviour of the corresponding single leader constructs (Fig. 3). Not only does the duplicated leader increase the efficiency of import of the previously poorly importing protein N9L/Y9-1 (panel A; compare lanes 2–6 with 7–9) but it is also capable of delivering a protein which was previously unimportable, namely N9L/Y8-2 (panel B; compare lanes 2–6 with 7–9). Approximately 30–50% of each radiolabelled precursor bearing a duplicated leader binds to the mitochondria in the import mix, compared to 20–30% for the single leader precursors (data not shown). Thus, a substantial proportion of the input N9L-D/Y9-1 and N9L-D/Y9-2 is imported into mitochondria.

The double leader N9L-D/Y9-1 precursor is imported very rapidly, with the mitochondrially bound precursor being almost completely processed, resulting in a protein of mature Y9 size (Fig. 3A, lane 6). In contrast, the single leader construct N9L/Y9-1 is imported to a much more restricted extent (Fig. 3A, lane 9) with only about half of the bound precursor being processed to the mature size Y9 protein. Note that a series of intermediate-sized partially processed import species are observed when N9L-D/Y9-1 is imported, especially prominent in lanes 2–4 of Fig. 3A. Presumably these species are generated by a relatively slow sequential processing at the matrix protease cleavage sites within

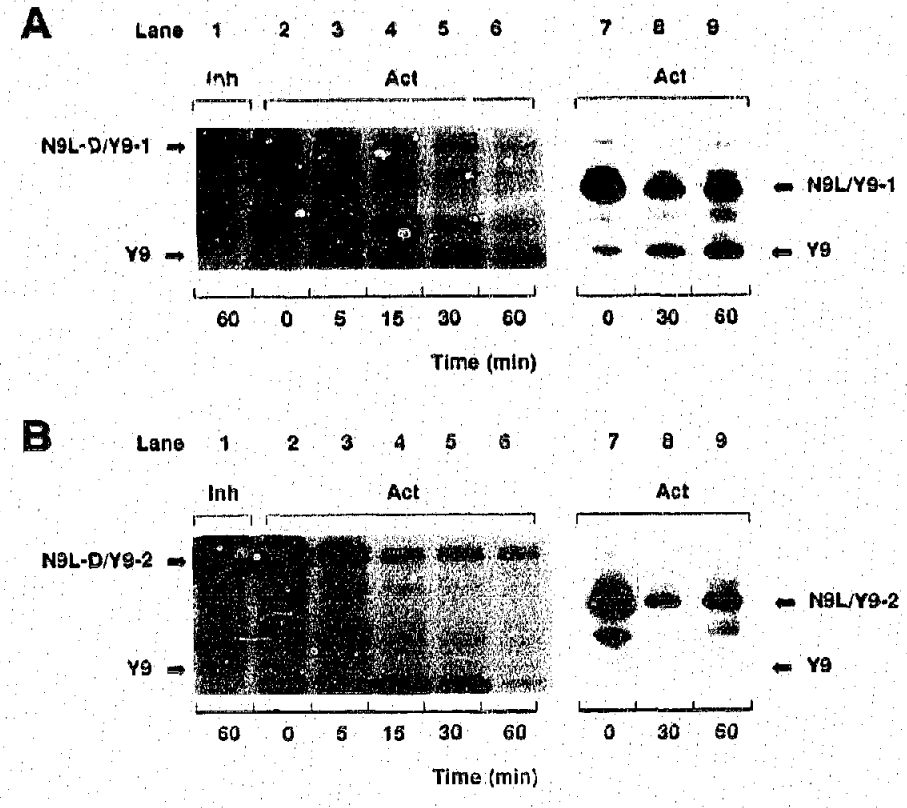

Fig. 3. Import properties of Y9 fusion proteins carrying double and single N9L leaders. From import mixtures (100 μl) containing J69-1B mitochondria and radiolabelled precursors, 20 μl samples were taken at the times indicated and processed for gel electrophoresis and fluorography. A, N9L-D/Y9-1 (lanes 1–6), N9L/Y9-1 (lanes 7–9); B, N9L-D/Y9-2 (lanes 1–6), N9L/Y9-2 (lanes 7–9). Where indicated mitochondria were pretreated (Inh) with an inhibitor cocktail [8]; Act indicates active import conditions. Positions on the gels of unprocessed fusion proteins and the Y9 standard are indicated.

N9L itself [16], between residues Thr[35] and Ile[36] within the first leader and between residues Thr[104] and Ile[105] within the second leader, and also at the end of the second leader to release Y9 with its N-terminal extension of 7 residues [8,17] (Fig. 1C). Note that for the more extensively processed forms, the abundance of radiolabel is correspondingly reduced. Thus, the number of ^{35}S-labelled methionine residues in the full length N9L-D/Y9-1 is 9, decreasing to 7, 4 and 3, respectively, with each of the inferred processing steps. The data in Fig. 3A (lanes 3–6) are therefore consistent with a sequential processing of N9L-D/Y9-1, although we have not formally demonstrated precursor-product relationships amongst the various species, particularly between the intermediate-sized species and the fully processed Y9. Nevertheless, the radioactivity in fully processed Y9 does not accumulate markedly after 5 min of import, suggesting a possible instability of imported Y9 inside the organelle. Finally, the potential processing site at the end of the first leader has evidently been inactivated by the fusion of the two leaders (Fig. 2B) and is no longer recognised by matrix protease, since no intermediate of N9L/Y9-1 size is generated in lanes 2–6.

Import of N9L-D/Y9-2 also results in very efficient uptake of the precursor. Here, the single leader construct N9L/Y9-2 does not import at all (Fig. 3B, lanes 7–9), but the corresponding double leader construct imports with extensive processing of the precursor to a Y9 protein (lanes 2–6). While intermediate-sized partially processed import products are observed as for N9L-D/Y9-1, a significant proportion of intact N9L-D/Y9-2 precursor remains bound to the mitochondria but not imported (lanes 4–6). Moreover, over the time course of the import experiment the intensity of the completely processed Y9 band appears to drop (lane 6), indicative of intramitochondrial degradation of this form of processed Y9. The single leader construct N9L/Y9-2 has, by contrast, never been observed by us to generate a Y9-sized product inside mitochondria, even after very short times of import (5 min or less) (R.H.P. Law, M. Galanis and P. Nagley, unpublished data).

Note that in this case the processed Y9 protein has a faster mobility on the SDS-PAGE system compared with authentic yeast subunit 9 (Fig. 3B, lanes 4–6). N-terminal sequencing of radiolabelled processed Y9 isolated from mitochondria after in vitro import has indicated the site of cleavage by the matrix protease in the N9L-D/Y9-2 precursor [17]. The processing site lies within the N9L sequence and generates an N-terminal extension of 8 residues upstream of the 76 amino acid sequence defining Y9. This N-terminal extension, containing 3 basic residues derived from the C-terminus of N9L, may account for the altered mobility of the Y9-2 protein observed on SDS-PAGE, and may contribute to the degradation of this processed Y9 derivative.

3.3. *Double leader import precursors of Y8 and derivatives*

The use of a duplicated N9L leader has also been demonstrated to have considerable utility for the analysis of Y8 and mutagenised derivatives. C-terminally truncated mutant forms of Y8 have been generated by site-directed mutagenesis in which codons specifying each of the positive charges near the C-terminus of Y8 have been individually converted to stop codons [11]. These mutants are either poor importers or totally import-incompetent. Factors predisposing to weak import are N9L/Y8-2 constructs, as opposed to N9L/Y8-1, and more extensive truncation of the C-terminal positively charged tail of Y8 [11].

When the N9L leader is duplicated in representative mutant constructs, efficient import of the precursors is now observed (Fig. 4). Full length Y8 constructs, N9L-

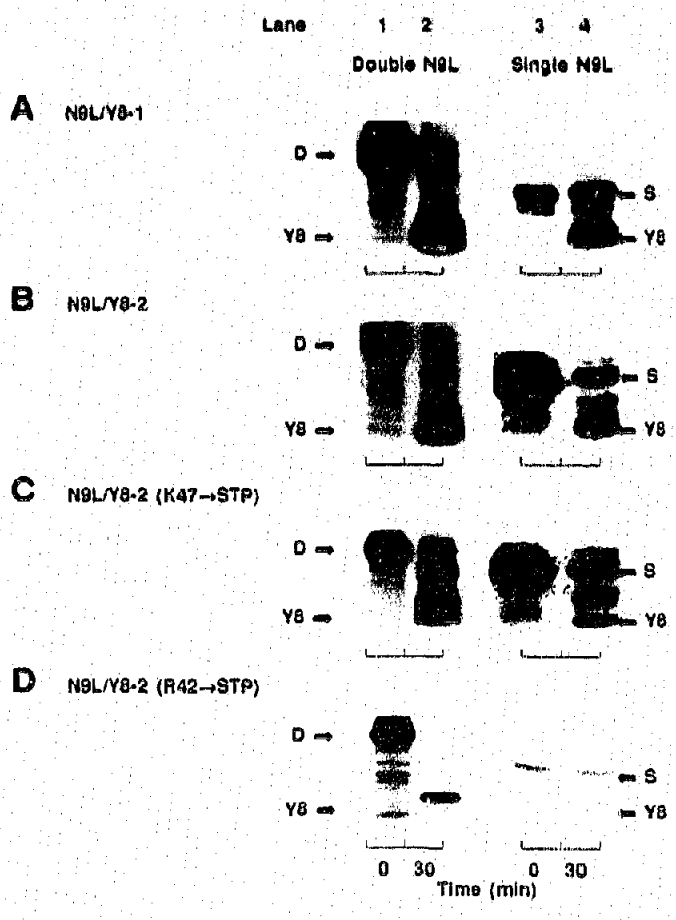

Fig. 4. Import properties of full length and truncated Y8 fused to double and single N9L leaders. Radiolabelled fusion proteins with either a double (lanes 1 and 2) or a single (lanes 3 and 4) N9L leader were incubated in import mixtures containing isolated YGL-1 mitochondria, partially depleted of Y8 [14], under active import conditions for the times indicated and samples were processed for gel electrophoresis and fluorography as in Fig. 3. For each panel, the parent single N9L leader construct is indicated. Positions on the gels of double leader precursors (D), single leader precursors (S) and the Y8 standard are indicated.

D/Y8-1 and N9L-D/Y8-2, were tested (panels A and B). The truncation mutants studied here include N9L-D/Y8-2(K47→STOP) which imports poorly with a single leader (Fig. 4C, lanes 3 and 4) and N9L-D/Y8-2(R42→STOP) which does not import at all with a single leader (Fig. 4D, lanes 3 and 4). All constructs were imported into isolated mitochondria of strain YGL-1 which have been partially depleted of endogenous subunit 8 [14], and which were chosen as the target mitochondria as they appear to be more efficient at import of these precursors [12]. When the import into these mitochondria of double leader versions of the above constructs is compared with the single leader versions, again the results are very striking (Fig. 4). This duplication results in extensive conversion of the precursor to a Y8 derivative protein after 30 min import (compare lane 2 with lane 4 for each individual construct). In general, duplication of the N9L leader in N9L/Y8 constructs results in little change in the amount of precursor which can bind to the isolated mitochondria for each Y8 variant, namely about 30% of input precursor (data not shown).

When using the double N9L leader to target the Y8 proteins to the mitochondrion the imported processed protein has approximately the same mobility as standard Y8 in each case except for N9L-D/Y8-2(R42→STOP). In this instance the Y8 derivative protein has an apparently slower mobility than reference mature Y8 (Fig. 4D, lane 2). Protease cleavage of the N9L/Y8-2 protein has been shown to occur within the Y8 sequence between the third and fourth residues [17]. Assuming the same cleavage site is used in N9L/Y8-2(R42→STOP) the product protein would be 38 residues long, mostly hydrophobic, with just one positive charge at Arg^{36} of the Y8 sequence. This may explain the reduced mobility on SDS-PAGE of this extensively truncated Y8 derivative (Fig. 4D, lane 2), instead of the anticipated accelerated mobility.

4. DISCUSSION

Here we have described the use of a tandemly duplicated N9L leader to enhance greatly the import of chimaeric fusion proteins that show little or no import when endowed with just one leader. The results have been obtained for various conformations of Y8 and Y9, both very hydrophobic passenger proteins, assessing import by use of isolated mitochondria and radioactively labelled precursors. The behaviour of the double leader constructs, on allotopic expression in vivo, is currently being assessed in our laboratory; preliminary data indicate that correct delivery of the passenger Y8 protein to produce functionally assembled mtATPase can occur at least with N9L-D/Y8-1 and N9L-D/Y8-2 (M. Galanis, unpublished). It will be important to assess assembly and function of truncated Y8 derivatives (cf. Fig. 4) whose import with one leader is very poor [11]. The delivery of Y9 to mtATPase in vivo, after allotopic expression of N9L-D/Y9-1 and N9L-D/Y9-2, is also under investigation using host strains unable to synthesize Y9 in mitochondria.

This leader duplication strategy could provide a general solution to the problem of inefficiently imported precursors. A similar strategy may not, however, be useful for proteins exported via the endoplasmic reticulum pathway. The yeast invertase leader was used to direct the secretion of human interferon α4 protein from yeast cells (M. Galanis, L.-F. Wang, R.J. Devenish, P. Nagley, unpublished). Yeast carrying single leader constructs expressed modest amounts of the interferon, with 40% secreted into the culture medium. In contrast, yeast carrying the double leader construct expressed lower levels of the interferon intracellularly and none of this interferon could be detected in the culture medium. There may be intrinsic differences between the mechanism of protein targeting [1] to the endoplasmic reticulum as opposed to mitochondria, such that a duplicated leader is very effective for mitochondrial import but is not useful for eukaryotic protein secretion.

What makes the duplicated N9L leader such an efficient mitochondrial targeting sequence for difficult passenger proteins such as Y8 or Y9 and their derivatives? Consideration of the recent detailed models for the import apparatus [18–20] indicates that, following delivery of the precursor to the mitochondrial surface by hsp^{70}-related proteins, there is interaction with the surface receptor elements that direct the precursor to a membrane-insertion element for subsequent translocation of the leader across the two mitochondrial membranes into the matrix. One key receptor that interacts with the presequence is likely to be the yeast equivalent of MOM19 [19]. Additional surface factors may be those which are involved in facilitatory interactions seen with passenger proteins containing hydrophobic domains [21]. Duplication of the leader probably results in accelerated delivery of the precursor to the outer membrane receptor and membrane insertion/translocation systems. Precursor translocation may be enhanced by improved interactions with intramitochondrial hsp^{70} proteins [19,20].

The Y8 and Y9 moieties of the chimaeric precursors bearing N9L constitute extremely hydrophobic domains; hence they tend to bind non-specifically to the mitochondrial outer membrane [13]. It could be envisaged that the increased overall hydrophilicity of the precursor, as a consequence of the extra leader, may decrease this non-specific binding. Thus, the precursor is now capable of more efficiently binding to the import receptor and import would not be so retarded due to non-productive membrane associations. Alternatively, the accessibility of receptor binding domains in the precursor may be important for successful import. If the precursor protein bearing a single leader is folded

tightly in such a way as to prevent interaction with the import receptor, the presence of a tandemly repeated leader may provide an extra, more mobile, N9L domain that can now interact with outer membrane elements leading to subsequent translocation into the organelle. It is furthermore possible that the translocation 'drive' generated by tandemly repeated N9L leaders mitigates against any tendency of the hydrophobic domains of the passenger proteins to be directed into the lipid bilayer of the outer membrane (or inner membrane) during translocation of the precursor towards the matrix [22].

Acknowledgements: This work was supported by the Australian Research Grants Scheme. We thank Dr Lin-Fa Wang and Professor Anthony Linnane for useful discussions and Dr Ruby Law and Mr David Grasso for their participation in control import experiments using single leader constructs.

REFERENCES

[1] Pugsley, A.P. (1989) Protein Targeting, Academic Press, San Diego.
[2] Hurt, E.C. and Van Loon, A.P.G.M. (1986) Trends Biochem. Sci. 11, 204–207.
[3] Hartl, F.-U., Pfanner, N., Nicholson, D.W. and Neupert, W. (1989) Biochim. Biophys. Acta 988, 1–45.
[4] Nagley, P. and Devenish, R.J. (1989) Trends Biochem. Sci. 14, 31–35.
[5] Nagley, P. (1988) Trends Genet. 4, 46–52.
[6] Viebrock, A., Perz, A. and Sebald, W. (1982) EMBO J. 1, 565–571.
[7] Gearing, D.P. and Nagley, P. (1986) EMBO J. 5, 3651–3656.
[8] Farrell, L.B., Gearing, D.P. and Nagley, P. (1988) Eur. J. Biochem. 173, 131–137.
[9] Law, R.H.P., Farrell, L.B., Nero, D., Devenish, R.J. and Nagley, P. (1988) FEBS Lett. 236, 501–505.
[10] Nagley, P., Farrell, L.B., Gearing, D.P., Nero, D., Meltzer, S. and Devenish, R.J. (1988) Proc. Natl. Acad. Sci. USA 85, 2091–2095.
[11] Nero, D., Ekkel, S.M., Wang, L.-F., Grasso, D.G. and Nagley, P. (1990) FEBS Lett. 270, 62–66.
[12] Grasso, D.G., Nero, D., Law, R.H.P., Devenish, R.J. and Nagley, P. (1991) Eur. J. Biochem., submitted.
[13] Law, R.H.P. and Nagley, P. (1990) Biochim. Biophys. Acta 1027, 141–148.
[14] Law, R.H.P., Devenish, R.J. and Nagley, P. (1990) Eur. J. Biochem. 188, 421–429.
[15] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn, Cold Spring Harbor Press, New York.
[16] Schmidt, B., Wachter, E., Sebald, W. and Neupert, W. (1984) Eur. J. Biochem. 144, 581–588.
[17] Galanis, M., Law, R.H.P., O'Keeffe, L.M., Devenish, R.J. and Nagley, P. (1990) Biochem. Int. 22, 1059–1066.
[18] Hartl, F.-U. and Neupert, W. (1990) Science 247, 930–938.
[19] Pfanner, N., Söllner, T. and Neupert, W. (1991) Trends Biochem. Sci. 16, 63–67.
[20] Baker, K.P. and Schatz, G. (1991) Nature 349, 205–208.
[21] Pfanner, N., Müller, H.K., Harmey, M.A. and Neupert, W. (1987) EMBO J. 6, 3449–3454.
[22] Singer, S.J. and Yaffe, M.P. (1990) Trends Biochem. Sci. 15, 369–373.