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Studies on the import into mitochondria of yeast ATP synthase subunits 8 and 9 encoded by artificial nuclear genes

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Direct fusions have been constructed between each of subunits 8 and 9 from mitochondrial ATPase of *Saccharomyces cerevisiae*, proteins normally encoded inside mitochondria, and the cleavable N-terminal transit peptide from the nuclearly encoded precursor to subunit 9 of *Neurospora crassa* mitochondrial ATPase. The subunit 8 construct was imported efficiently into isolated yeast mitochondria and was processed at or very near the fusion point. When expressed in vivo from its artificial nuclear gene, this cytoplasmically synthesized form of subunit 8 restored the growth defects of *aap1* mutants unable to produce subunit 8 inside the mitochondria. The subunit 9 construct was, however, unable to be imported into isolated mitochondria and could not, following nuclear expression in vivo, complement growth defects in mitochondrial *oli1* mutants. This behaviour is contrasted with the previously demonstrated import competence of another yeast subunit 9 fusion, bearing the first five residues of mature *N. crassa* subunit 9 interposed between its own transit peptide and the yeast subunit 9 moiety.

Import; Mitochondrial ATPase complex; Membrane assembly; Gene relocation; Site-directed mutagenesis; (*Saccharomyces cerevisiae*)

1. INTRODUCTION

The functional translocation of mitochondrial genes to the nucleus provides a powerful new strategy to study the assembly of membrane-associated enzyme complexes in mitochondria, such as the ATP synthase complex (mtATPase) [1]. In our laboratory, yeast mitochondrial genes encoding subunit 8 (*aap1*) and subunit 9 (*oli1*) have been redesigned for efficient expression in the yeast nucleus and constructed by complete chemical synthesis [2,3]. In order to achieve import into mitochondria, cytoplasmically synthesized versions of subunits 8 (Y8) and 9 (Y9) were furnished [3,4] with the N-terminal leader (N9L) from the precursor of *Neurospora crassa* mtATPase subunit 9, a mitochondrial protein naturally encoded by a nuclear gene. The respective constructs N9L/Y8 and N9L/Y9 (using our previous nomenclature [3]) each contain 66 residues of the N9L presequence plus the first 5 residues of mature *N. crassa* subunit 9, 2 serine residues resulting from DNA sequence at the relevant gene fusion, and the full amino acid sequence of Y8 (48 residues) or Y9 (76 residues). On import into isolated mitochondria of N9L/Y8 or N9L/Y9, the radiolabelled precursors were processed to yield derivatives of Y8 or Y9, respectively. Each derivative carries seven extra residues at the N-terminus generated through processing by matrix protease at the cleavage site defined by the *N. crassa* subunit 9 precursor sequence [3,4].

The functional properties of the genes encoding N9L/Y8 and N9L/Y9 in vivo have been tested by expressing these artificial genes in the nuclei of cells deficient in the relevant mitochondrially encoded

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Abbreviations: mtATPase, mitochondrial proton-translocating ATPase (ATP synthase); N9L, cleavable N-terminal leader from the presequence of *Neurospora crassa* mtATPase subunit 9; Y8 and Y9, subunits 8 and 9, respectively, of yeast mtATPase

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subunit. In the case of Y8, the defective respiratory growth properties of *aap1 mit*$^-$ mutant cells were complemented efficiently by N9L/Y8 expressed in the nucleus [5]. The mtATPase was shown to be functionally assembled, having incorporated cytoplasmically synthesized Y8 in spite of its seven extra N-terminal residues [5]. By contrast, the nuclear expression of N9L/Y9 has not thus far been demonstrated to complement defects in the mitochondrial *oli1* gene [3]. One possibility is that the extra seven N-terminal residues may compromise the assembly or function of the imported Y9.

In this paper we examine the properties of modified versions of these precursors from which the supernumerary seven residues have been removed by site-directed in vitro mutagenesis of the respective gene constructs. The data show that the modified version of N9L/Y8 (N9L directly fused to Y8) is imported into mitochondria to yield a properly processed Y8 which assembles functionally into mtATPase. On the other hand, we found that the modified N9L/Y9 precursor (N9L directly fused to Y9) can neither be imported into isolated mitochondria nor be used to complement *oli1 mit*$^-$ mutations after expression in vivo.

2. MATERIALS AND METHODS

2.1. *Strains and gene constructs*

Saccharomyces cerevisiae strains J69-1B [*rho*$^+$], the two derived *aap1 mit*$^-$ mutants M31 and M26-10, and the *oli1* mutants 3861 (*mit*$^-$) and h45 (temperature-sensitive *mit*$^-$) are as described [6–8]. Gene constructs encoding N9L/Y8 and N9L/Y9 (now denoted N9L/Y8-1 and N9L/Y9-1, respectively; see fig.1) are as described [3,4].

2.2. *Methods*

Site-directed in vitro mutagenesis was carried out according to Nisbet and Beilharz [9], using 26-mer oligonucleotides to mutagenize a single-stranded 'positive strand' template: 5′-CC AATTGAGGCATGGCGCGCTTCTGG-3′ for N9L/Y8-1 and 5′-GCACCAATTGCATGGCGCGCTTCTGG-3′ for N9L/Y9-1. The sequences of the mutagnized derivatives, N9L/Y8-2 and N9L/Y9-2, were confirmed [10].

Import experiments were carried out as described previously [3] using mitochondria isolated from strain J69-1B, and radiolabelled precursors prepared by in vitro transcription and translation of synthetic gene constructs [3]. Expression of synthetic gene constructs in vivo was carried out following their insertion into the expression vector pLF1 [5] and transformation [11] into suitable mutant yeast host cells. Growth media and genetic analysis were as described [5].

3. RESULTS AND DISCUSSION

3.1. *Modifications to import precursors of subunits 8 and 9*

The precursors previously used [3,4] for the successful import into isolated mitochondria of Y8 and Y9 have been renamed N9L/Y8-1 and N9L/Y9-1, respectively (fig.1, A and C). The seven supernumerary amino acids between the N9L transit peptide sequence and the N-terminal methionine of natural Y8 and Y9 were removed by in vitro mutagenesis to yield N9L/Y8-2 and N9L/Y9-2 (fig.1, B and D).

3.2. *Analysis of subunit 8*

The results of incubating the radiolabelled precursors N9L/Y8-1 and N9L/Y8-2 with isolated mitochondria under conditions conducive to protein import are shown in fig.2. Panels A and B show that both precursors are taken up and processed by active mitochondria (lane 3) to yield imported Y8 in a compartment protected from externally added protease (lane 5). Mitochondria inactivated by the collapse of the membrane potential do not allow import of the precursors (lane 2) which remain sensitive to added protease (lane 4).

The protease-resistant material in lane 4 of panels A and B shows an electrophoretic mobility slightly greater than that of the marker Y8 protein (lane 6). This material arises from the non-specific embedding into the mitochondrial membrane of the subunit 8 moiety of both N9L/Y8-1 and N9L/Y8-2 precursors. It was suggested previously [4] that this is due to penetration of the membrane by the C-terminal region of subunit 8.

The properly imported, processed versions of Y8 generated from N9L/Y8-1 (fig.2A, lane 5) and N9L/Y8-2 (fig.2B, lane 5) differ in their electrophoretic mobility. The slight retardation of the Y8 derivative of N9L/Y8-1, ascribed to the seven extra N-terminal residues [4], is not seen with the Y8 derived from N9L/Y8-2. Microsequencing of processed Y8 derived from N9L/Y8-2 radiolabelled with [^{35}S]methionine revealed a radioactive peak at the first and tenth sequencing cycles (not shown) as expected if N9L/Y8-2 were processed by matrix protease at the junction of N9L and Y8 sequences (see fig.1B). However, a further prominent peak of radioactivity was also recovered at the eighth cycle, suggesting an alternate cleavage reaction may be

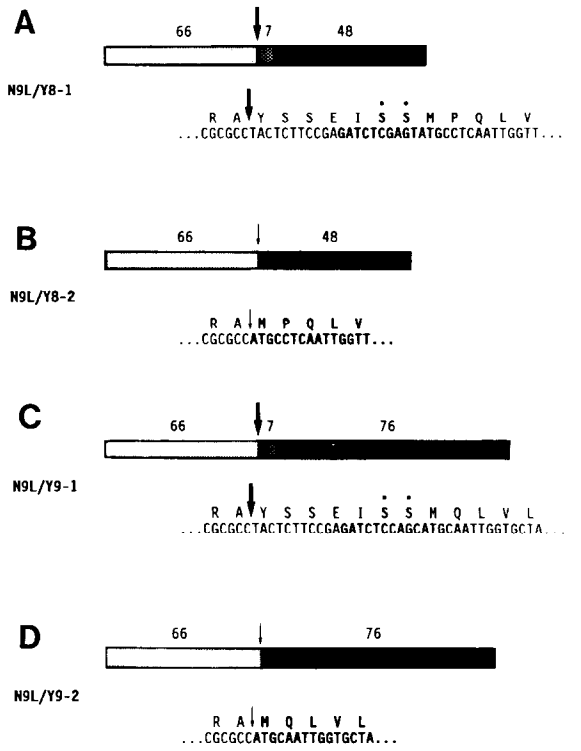

Fig.1. Details of gene fusions and the encoded protein constructs. Each panel contains an overview of the protein construct with the detail of sequence organization at the fusion point between yeast and *Neurospora* sequences. (Filled blocks) Y8 or Y9 protein sequences encoded by artificial yeast genes; (cross-hatched blocks) protein sequence derived from mature *N. crassa* subunit 9; (lightly stippled blocks) N9L leader sequence from the precursor of *N. crassa* subunit 9. Bold arrows indicate the site of cleavage by matrix protease of the precursor of *N. crassa* subunit 9; thin arrows represent the anticipated site of matrix protease cleavage of the direct fusion between N9L and Y8 or Y9. The amino acids are represented in single letter code, those encoded by synthetic DNA segments are shown bold; the serine residues marked by large dots arise from the DNA sequence at the fusion point [3,4].

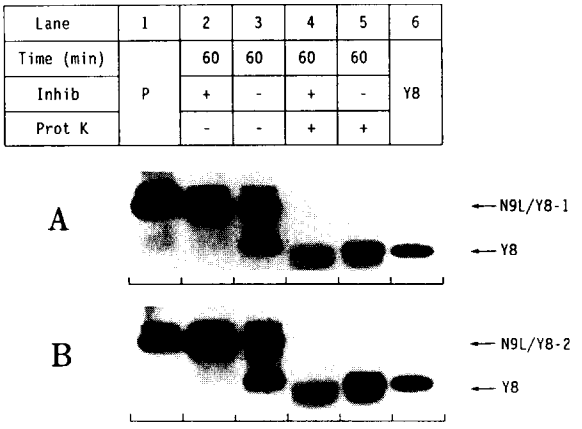

Fig.2. Import experiments into isolated mitochondria using fusions between N9L and Y8. Aliquots of the import mixture, containing radiolabelled N9L/Y8-1 or N9L/Y8-2 were taken at the times indicated and processed for gel electrophoresis. Where indicated, mitochondria were pretreated (Inhib) with an inhibitor cocktail, or treated with proteinase K following the import reaction [3]. The positions of the in vitro translation product Y8 from the *NAP8* gene [3] (lane 6) and the precursors (P) from the fusion genes (lane 1) are shown. Panel A, N9L/Y8-1; panel B, N9L/Y8-2. The contents of each lane are indicated schematically at the top of the figure.

occurring between residues Gln2 and Leu3 of the mature Y8 sequence.

The expression in vivo of N9L/Y8-2 in *aap1 mit*$^-$ mutant cells revealed that this modified Y8 precursor can be used to rescue the growth defects which result from the failure to synthesize Y8 inside mitochondria (fig.3). The *mit*$^-$ mutants M26-10 and M31, which both produce severely truncated forms of subunit 8 in mitochondria, were transformed with pLF1 expressing N9L/Y8-2. The Ade$^+$ transformants (fig.3B) regained the ability to grow on YEPE plates (non-fermentable carbon source ethanol) (fig.3C, sectors 4 and 6) unlike the non-transformed parent *mit*$^-$ cells (sectors 3 and 5). The growth on YEPE plates of M26-10 and M31 after transformation with pLF1-N9L/Y8-2 was comparable to the control wild type J69-1B (sector 1) and the previously studied T2-1 (M31 transformed with pLF1-N9L/Y8-1) (sector 2) [5]. When tested in liquid medium with ethanol as carbon source, the *aap1 mit*$^-$ mutants rescued with pLF1-N9L/Y8-2 showed similar growth rates to those of J69-1B and T2-1 (Braidotti, G., Nero, D. and Nagley, P., unpublished).

Finally, radiolabelling of proteins in vivo and immunoprecipitation of mtATPase [5] have shown that expression of N9L/Y8-2 in both *aap1* mutants M26-10 and M31 generates a Y8 protein which is imported and assembles into the functional mt ATPase complex (not shown). We have not yet been able to establish whether the functionally assembled Y8 represents only the full 48-amino acid polypeptide cleaved before Met1, or whether the putative 46 amino acid derivative formed by the alternate cleavage reaction (see above) is also functionally assembled.

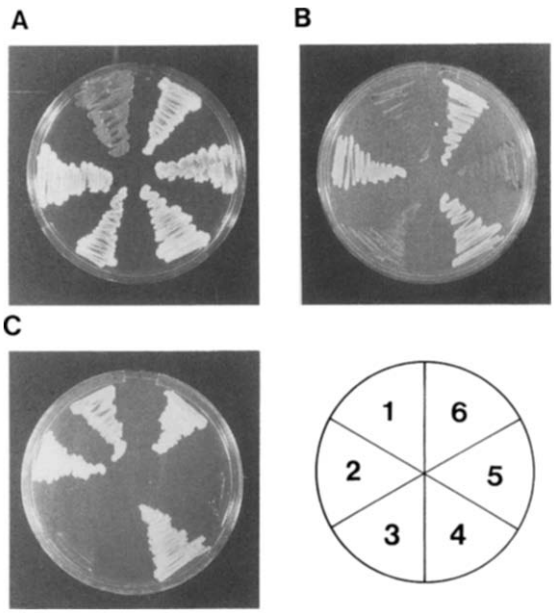

Fig.3. Growth properties of *aap1* mutants transformed with the vector pLF1 expressing fusions between N9L and Y8. Cells were streaked onto agar plates and incubated at 28° for 5 days. Sectors: 1, strain J69-1B; 2, strain T2-1 (M31 transformed with pLF1-N9L/Y8-1); 3, strain M26-10; 4, M26-10 transformed with pLF1-N9L/Y8-2; 5, strain M31; 6, M31 transformed with pLF1-N9L/Y8-2. Growth media [5] were YEPD (A), SD/His (B), YEPE (C). Cells of strain J69-1B (sector 1) on rich medium (A and C) were red and thus tend to lack contrast in these photographs. Note that the host strains all show an Ade^- His^- phenotype, but they become Ade^+ when transformed with pLF1 carrying the *ADE1* selectable marker [5].

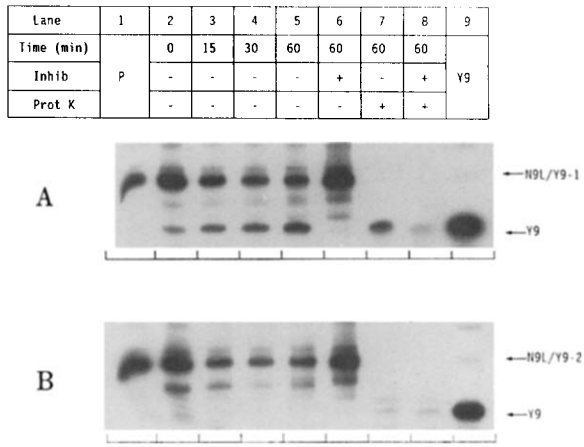

Fig.4. Import experiments into isolated mitochondria using fusions between N9L and Y9. All indications are as for fig.2 with the following exceptions. Markers are represented by the in vitro translation product Y9 from the *NAP9* gene [3] (lane 9) and the precursors (P) from the fusion genes (lane 1). Panel A, N9L/Y9-1; panel B, N9L/Y9-2.

3.3. *Analysis of subunit 9*

Radiolabelled protein constructs N9L/Y9-1 and N9L/Y9-2 were incubated with isolated mitochondria to compare their import properties in vitro (fig.4). N9L/Y9-1 (panel 4) behaves as previously reported [3], in that it is processed to yield a Y9 derivative (lanes 2–5) which is located in a compartment protected from externally added protease (lane 7). In contrast to the behaviour of Y8 constructs (fig.2), the non-specific embedding of Y9 is barely perceptible (fig.4A, lane 8).

Fig.4B shows that N9L/Y9-2 is not able to be imported. This construct is not processed by active mitochondria to yield material of the mobility of Y9 (lanes 2–5), even after 60 min of incubation. The faint band visible at the position of Y9 after proteinase K treatment (lane 7) is not considered to be an indication of import, because similar material is observed when inactive mitochondria are used with subsequent proteinase K treatment (lane 8). A series of import experiments which varied the experimental conditions yielded the same results as seen in fig.4B. Therefore, we conclude that N9L/Y9-2 is not imported at observable rates under these conditions. Presumably, an unanticipated structural feature of this construct severely impedes a critical step in the import process. For example, the N9L/Y9-2 protein may be folded or self-aggregated in such a way that the transit peptide component cannot be exposed effectively to the receptor apparatus on the outer mitochondrial membrane [12], or the construct may be unable to be unfolded to permit its translocation across the mitochondrial membranes [13].

The results of expressing N9L/Y9-2 in vivo are consistent with the above findings. The abnormal growth properties of mutants defective in the intramitochondrial expression of Y9 (e.g. the *oli1 mit*$^-$ strain 3861 or the temperature-sensitive *mit*$^-$ mutant h45) are not complemented by nuclear expression of genes encoding pLF1-N9L/Y9-2 (not shown). The failure of N9L/Y9-2 expressed in the nucleus to rescue *oli1* mutants in vivo is probably due to the inability of the putative precursor to be imported into mitochondria. On the other hand, whilst N9L/Y9-1 does not complement such mutations in vivo [3], this precursor is imported in vitro; the defect in vivo probably lies in the ability of Y9

bearing seven supernumerary N-terminal residues to assemble or to function in the mtATPase complex. One strategy we are exploring, in order to achieve the successful complementation of *oli1* mutations by Y9 expressed from an artificial nuclear gene, is to search for variants of N9L/Y9-2 arising in vivo which acquire, through some mutational event, the ability to be imported into mitochondria and to become functionally incorporated into the mtATPase complex.

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