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Interaction between mitochondria and the nucleus

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Summary

The interaction between the mitochondrial and the nuclear genome is in part mediated by proteins (and possibly also RNAs) which are encoded in the nucleus and imported into mitochondria. We are beginning to understand how proteins can penetrate across both mitochondrial membranes and how some of these proteins can regulate the expression of specific mitochondrial genes.

The problem

Mitochondria contain their own genetic system which manufactures most of the mitochondrial RNAs and a few (13 in humans) of the mitochondrial proteins. All the other hundreds of mitochondrial proteins, and probably also several mitochondrial RNAs, are encoded by nuclear genes and imported into the mitochondria. In spite of their genetic semi-autonomy, mitochondria are thus predominantly products of the nucleo-cytoplasmic system (Attardi & Schatz, 1988). In order to understand how mitochondria are made, we must first learn how the two genetic systems interact with each other. This is not a trivial problem since the two systems are separated by an array of membranes: the nuclear envelope, and the two mitochondrial membranes.

Macromolecular signals imported into mitochondria

The nuclear system can influence the mitochondrial system through informational macromolecules that are encoded in the nucleus and imported into the mitochondrial matrix (Fig. 1). Some of these signals affect expression of most, and perhaps all, mitochondrial genes (e.g. 2 and 3). Others control the expression of specific mitochondrial genes (e.g. 4, reviewed by Fox, 1986). Recent data suggest the possibility that mitochondria may also import a few of their RNAs (Chang & Clayton, 1987; Maréchal-Drouard *et al.* 1988).

At present there is no evidence that mitochondria export macromolecules. Thus, we know little of how mitochondria send signals to the nucleus. There is indirect evidence that such signals exist (Parikh *et al.* 1987) but their identity and mechanism of action are unknown.

Import of proteins into mitochondria

The import of nuclear-encoded proteins into mitochondria is a major mechanism by

Key words: mitochondria, biogenesis, regulation.

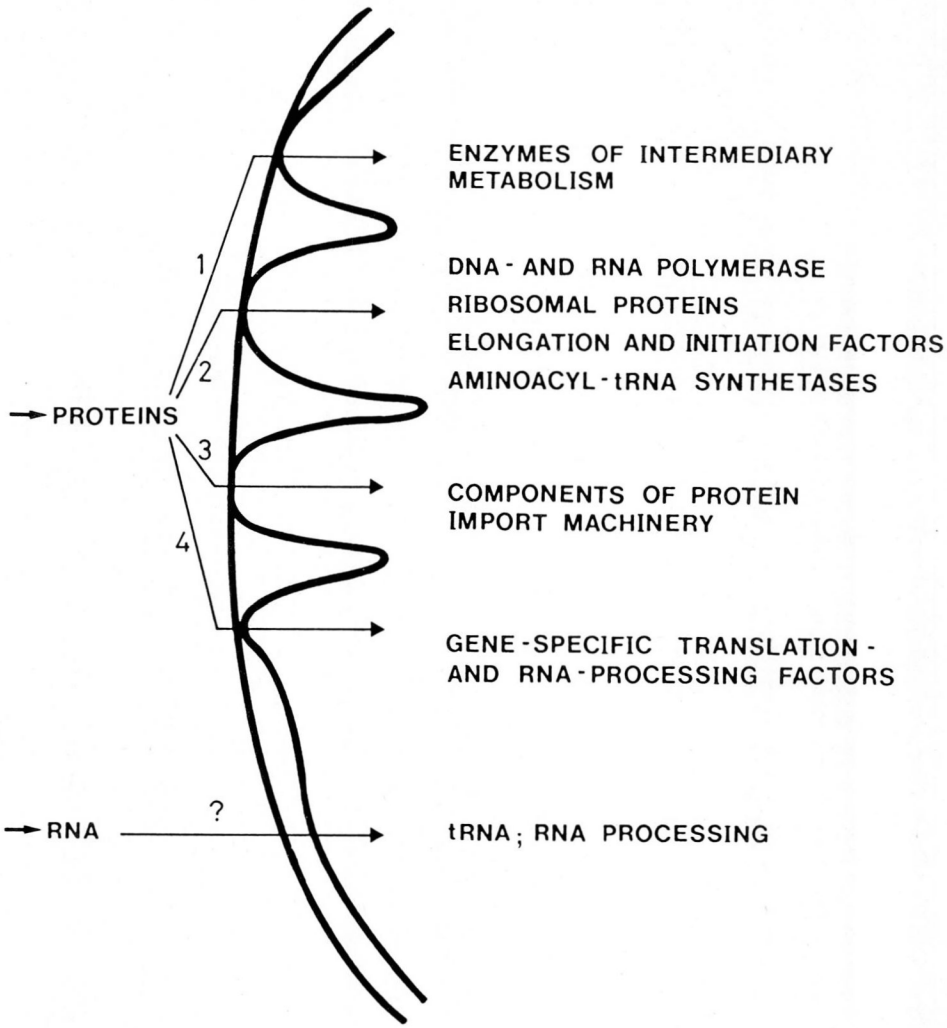

Fig. 1. Nuclear-encoded macromolecules imported into mitochondria. These macromolecules may function as signals controlling the interaction between the nuclear and the mitochondrial genome. Evidence for the import of RNA into mitochondria is still indirect. See text for explanation of numbers. (Reproduced with permission from Attardi & Schatz, 1988.)

which the nuclear genome controls mitochondrial biogenesis. This import process has been studied intensively in many laboratories; recent reviews by Douglas *et al.* (1986), Pfanner & Neupert (1987) and Attardi & Schatz (1988) summarize the field. Our own studies have frequently employed an artificial precursor protein which contains a mitochondrial targeting sequence (the presequence of yeast cytochrome oxidase subunit IV) fused to the N terminus of the cytosolic enzyme, mouse dihydrofolate reductase (DHFR). This fusion protein is readily imported and cleaved by mitochondria *in vitro* or *in vivo* (Hurt *et al.* 1984, 1985); it can be purified

STEPS	INHIBITORS
1. INSERTION OF PRESEQUENCE	UNCOUPLERS
2. UNFOLDING AND BINDING OF UNFOLDED CONFORMERS	FOLATE ANALOGS; LOW TEMPERATURE
3. RELEASE OF UNFOLDED CONFORMERS FROM BINDING SITE (S) OR FURTHER UNFOLDING	ATP-TRAPS
4. TRANSLOCATION OF UNFOLDED POLYPEPTIDE	
5. REFOLDING INSIDE	
6. REMOVAL OF PRESEQUENCE	MEMBRANE-PERMEANT CHELATORS; mas1 MUTATION

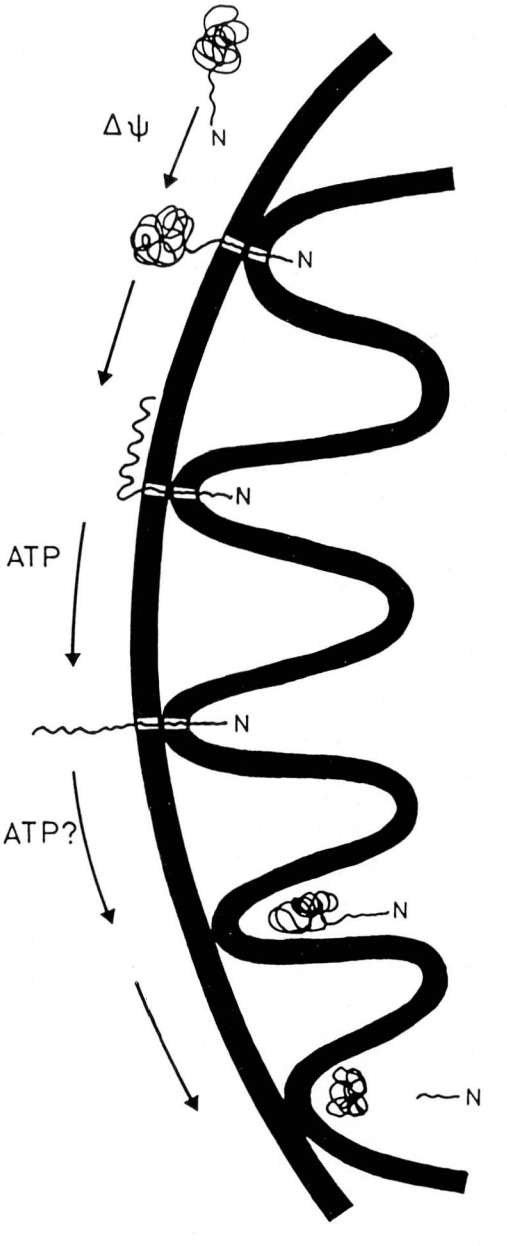

Fig. 2. Post-translational import of an artificial precursor protein into mitochondria. The precursor is a fusion protein containing the presequence of yeast cytochrome oxidase subunit IV attached to the N terminus of mouse dihydrofolate reductase (DHFR). Removal of the presequence may occur at any step following the potential ($\Delta\psi$)-dependent insertion across the inner membrane. (Reproduced with permission from Eilers *et al.* 1988.)

in milligram amounts (Eilers & Schatz, 1986; Endo & Schatz, 1988), and contains a 'mature' moiety (i.e. DHFR) whose three-dimensional structure is known (Volz *et al.* 1982). We have shown that the information for import of this protein into mitochondria only resides in the presequence, that import of the native (but not the completely unfolded) molecule requires ATP, and that import requires at least partial unfolding of the DHFR moiety (Hurt *et al.* 1984, 1985; Hurt & van Loon, 1986; Eilers & Schatz, 1986; Verner & Schatz, 1987; Eilers *et al.* 1987, 1988). Some of the import steps of this protein are depicted in Fig. 2.

Import of proteins occurs through sites of contact between the inner and outer mitochondrial membrane

Previous observations by others had already suggested that import of proteins into mitochondria might occur through regions in which the two membranes are in close apposition (Kellems *et al.* 1975; Schwaiger *et al.* 1987). However, these sites had not been separated from isolated inner and outer membranes. In order to selectively mark these import sites for subsequent isolation, we made use of the fact that addition of the purified fusion protein to isolated mitochondria in the absence of ATP generated a partly unfolded, surface-bound intermediate whose presequence was not yet cleaved off (Eilers *et al.* 1988). However, the intermediate could subsequently be 'chased' into the mitochondria upon addition of ATP; since this chase did not require a potential across the inner membrane (whereas generation of the intermediate did) this 'ATP-depletion intermediate' represented a true intermediate in the translocation process.

Mitochondria were allowed to accumulate the radiolabeled 'ATP-depletion intermediate', disrupted by sonication, and the sub-mitochondrial fractions were separated on a sucrose density gradient. If isolation of mitochondria and sonic disruption

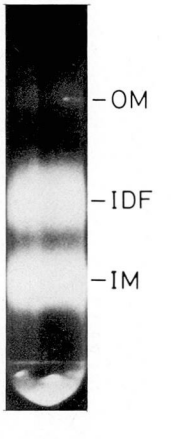

Fig. 3. Separation of submitochondrial fractions from yeast on a 0·85 M to 1·6 M-sucrose gradient. Yeast mitochondria were disrupted, in the presence of 10 mM-EDTA. OM, IDF and IM; positions of the outer membrane, 'intermediate density fraction', and inner membrane, respectively.

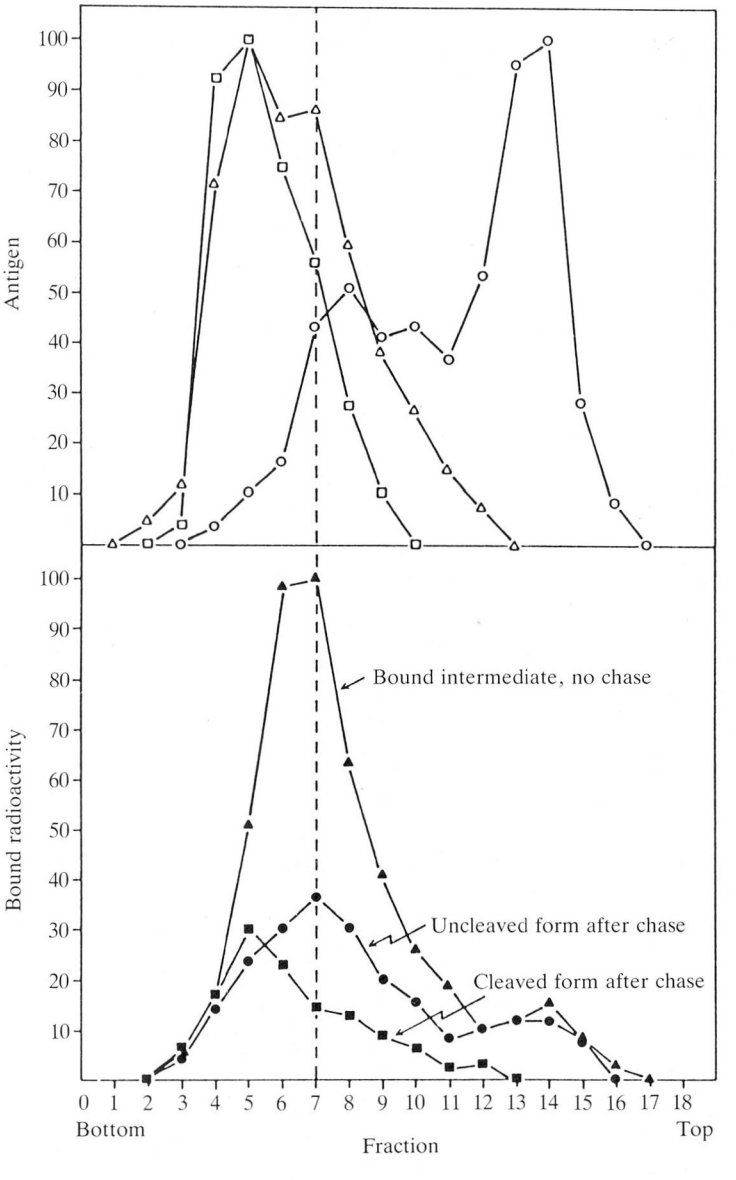

Fig. 4. The 'ATP-depletion' intermediate accumulates specifically in the 'intermediate density fraction' (cf. Fig. 3). Mitochondria were first allowed to form the radiolabeled 'ATP-depletion intermediate'; they were reisolated by centrifugation, and half of them were chased in the presence of ATP. 'Chased' and 'unchased' samples were then mixed with untreated carrier mitochondria, and converted to sub-mitochondrial particles by sonication. These particles were separated on a sucrose gradient (cf. Fig. 3) and each gradient fraction was analyzed for the following. Upper panel: membrane markers (□——□, citrate synthase; △——△, cytochrome oxidase subunit II; ○——○, 70K outer membrane protein). Lower panel: radiolabeled precursor (▲——▲, 'ATP-depletion intermediate' in fractions from unchased mitochondria; ●——● and ■——■, uncleaved and cleaved fusion protein, respectively, in fractions derived from ATP-chased mitochondria).

were carried out in the presence of EDTA, three distinct membrane fractions were obtained (Fig. 3). By testing for the presence of mitochondrial membrane markers, the lightest fraction was identified as outer membrane and the densest one as inner membrane. The 'intermediate density fraction' contained both types of membrane marker. However, the intermediate density fraction was unique in containing virtually all of the 'ATP-depletion intermediate' (Fig. 4). If the mitochondria were 'chased' in the presence of ATP before sonic disruption, the amount of radiolabeled intermediate associated with the intermediate density fraction was drastically reduced. This suggested that the 'ATP-depletion intermediate' had bound to discrete sites on the mitochondrial surface which, upon subfractionation, exhibited properties of both mitochondrial membranes.

A precursor protein jamming mitochondrial import sites identifies the intermediate density fraction as contact sites between the two membranes

In order to prove that the intermediate density fraction was indeed derived from sites of contact between the two mitochondrial membranes, we combined genetic engineering and chemical crosslinking techniques to produce a chimeric mitochondrial precursor protein which became stuck in the protein import machinery (Vestweber & Schatz, 1988*a*). To construct this chimeric protein, we first modified the above-mentioned fusion protein such that it contained a unique cysteine residue as its C-terminal amino acid (Vestweber & Schatz, 1988*b*). Using a bifunctional

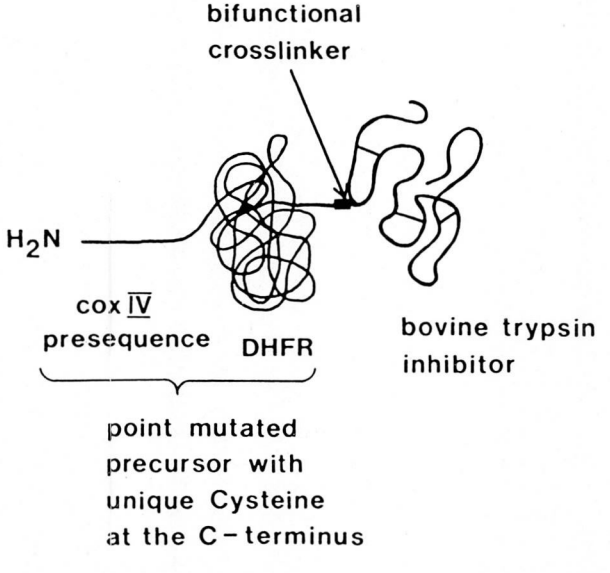

Fig. 5. A chimeric protein capable of 'jamming' import sites for proteins in isolated yeast mitochondria. The three internal disulfide bridges of bovine trypsin inhibitor are indicated by straight lines. Reproduced with permission from Vestweber & Schatz (1988*a*).

crosslinker, we then coupled this C-terminal cysteine to bovine trypsin inhibitor, a tightly folded, 6K ($K = 10^3 M_r$) protein with three internal disulfide bridges (Fig. 5). When this purified chimeric, radiolabeled precursor was presented to energized mitochondria, it was partly imported: its amino terminal presequence was cleaved off by the matrix localized protease, its radiolabeled DHFR moiety was located inside the mitochondrial membranes, but its bovine trypsin inhibitor moiety was still accessible on the mitochondrial surface. Inability to translocate completely across both mitochondrial membranes was probably caused by the inability of the bovine trypsin inhibitor moiety to unfold sufficiently to allow passage across membranes. The partly translocated chimeric protein did not collapse the potential across the mitochondrial membranes, but blocked import of several authentic mitochondrial precursor proteins. This is shown for the precursor to alcohol dehydrogenase III, a protein imported into the mitochondrial matrix of yeast (Fig. 6). Complete inhibition was obtained when 40 pmol of chimeric precursor become stuck per 1 mg of isolated mitochondria. This result indicated that the chimeric precursor and authentic precursor proteins share at least one component during their import. We also calculated that each isolated mitochondrial particle contains between 100 and 1000 'import sites' for proteins.

When mitochondria were first allowed to partly import the chimeric precursor and

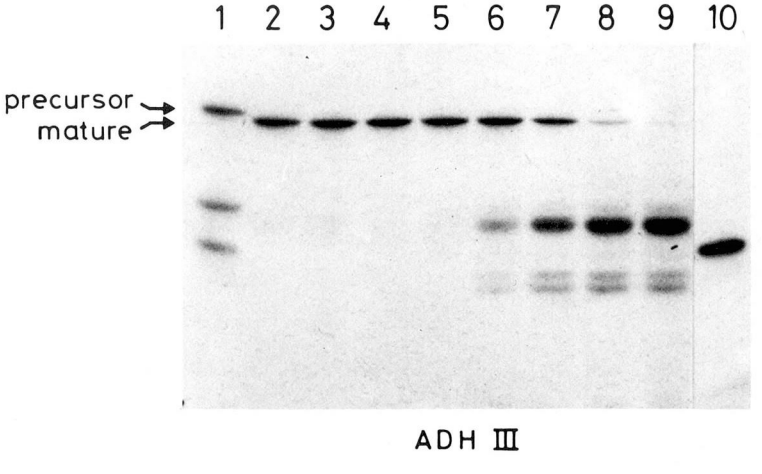

Fig. 6. The partly translocated chimeric precursor blocks import of authentic precursors into mitochondria. Isolated yeast mitochondria were allowed to import various levels of radiolabeled chimeric precursor, reisolated, and presented with *in vitro*-synthesized, radiolabeled precursor to the mitochondrial isozyme of alcohol dehydrogenase (ADH III). Lanes: 1, 20 % of the ADH III precursor added to each import assay; 2–5, import of ADH III precursor by four identical samples of control mitochondria; 6–9, import of ADH III precursor by mitochondria that had been preincubated with 125, 250, 375 and 500 ng, respectively, of chimeric precursor; 10, an aliquot (35 ng) of the bovine trypsin inhibitor-free fusion protein. Arrows on the left indicate the positions of the unprocessed and processed form of the ADH III precursor. Samples were treated with 250 $\mu g\,ml^{-1}$ proteinase K for 30 min at 30°C, followed by addition of 1 mM-phenylmethylsulfonyl fluoride, before being analyzed by SDS–polyacrylamide gel electrophoresis and fluorography.

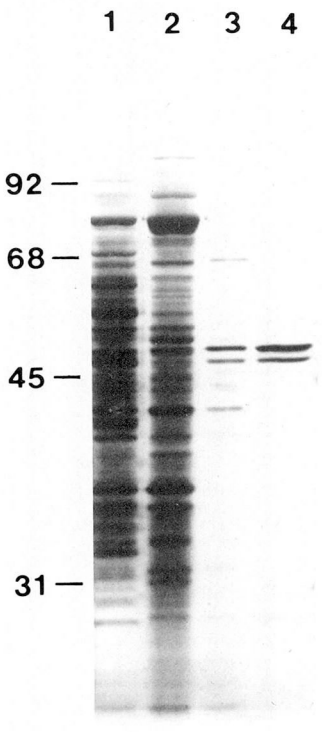

Fig. 7. Purification of the mitochondrial matrix protease. Shown is an SDS–polyacrylamide gel stained with silver. 1, total matrix fraction (6 μg); 2, Zn-chelate eluate (6 μg); 3, mono-*Q*-eluate (0·2 μg); 4, Superose 12 eluate (0·2 μg). Reproduced with permission from Yang *et al.* (1988).

then separated into the three submitochondrial fractions shown in Fig. 3, virtually all of the partly translocated, processed chimeric precursor was again associated with the 'intermediate density fraction'. This was strong evidence that this fraction was indeed derived from mitochondrial contact sites. Additional experiments revealed that this intermediate density fraction also contained binding sites for cytoplasmic ribosomes (Pon, L., unpublished). Detailed analysis of this fraction is in progress.

Isolation of components on the mitochondrial protein import machinery

Several years ago we isolated two yeast mutants that were temperature-sensitive for growth and for import of several mitochondrial precursor proteins (Yaffe & Schatz, 1984). The wild-type alleles of these two genes (termed *MAS1* and *MAS2)* were cloned and sequenced (Witte *et al.* 1988; Jensen & Yaffe, 1988). Recent experiments have shown (Witte *et al.* 1988; Yang *et al.* 1988; Jensen & Yaffe, 1988) that these two genes encode the two subunits of the matrix localized processing protease which had initially been identified in yeast by Böhni *et al.* (1980). Fig. 7 shows the purification of this enzyme from an isolated matrix fraction derived from yeast mitochondria. Purification from the matrix was about 320-fold, corresponding to an approximately

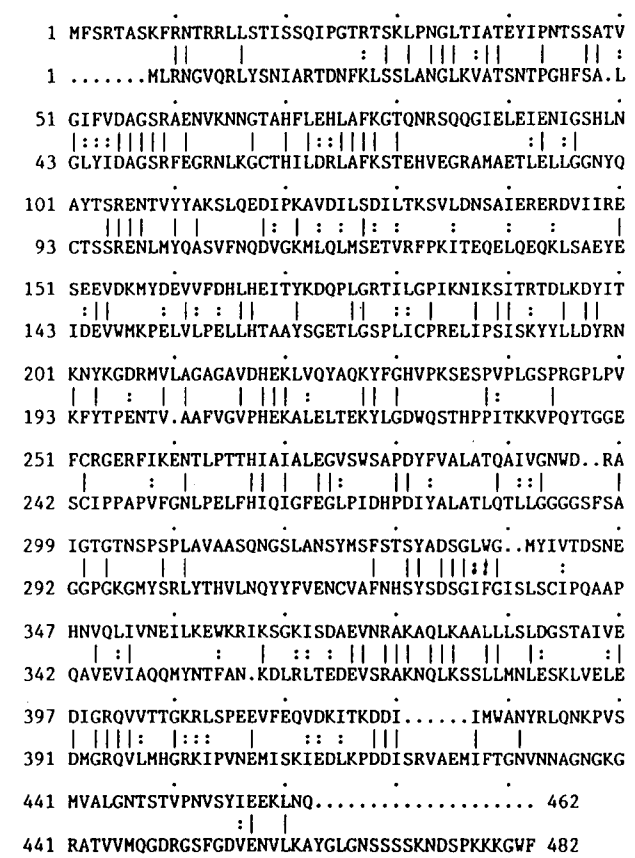

Fig. 8. The two subunits of the yeast mitochondrial matrix protease are homologous. The deduced amino acid sequences of the *MAS1* product (upper line) and the *MAS2* product (lower line) were aligned for maximal homology, using a program distributed by the Genetics Computer Group program package (University of Wisconsin, Madison, USA). Modified from Jensen & Yaffe (1988).

6000- to 8000-fold purification from total yeast cells. The enzyme exhibits an apparent size of 100K on sucrose gradients. An antibody generated against the *MAS1* gene product decorates the smaller of the two subunits whereas an antibody against the *MAS2* gene product specifically decorates the larger one. Jensen & Yaffe (1988) showed that these two subunits are highly homologous to each other (Fig. 8). The availability of the purified enzyme and of its two structural genes should now allow us to answer the interesting question of why mutations in any one of these two subunits block not only processing, but also import of precursor proteins *in vivo*. Most likely, the protease forms a labile complex with the mitochondrial protein import machinery.

Outlook

The past few years have witnessed several notable technical advances that should

allow us to dissect the interactions between mitochondria and nucleus at a new level of precision: mammalian mitochondria have been introduced into host cells by microinjection (King & Attardi, 1988); genes have been transformed into yeast mitochondria *in vivo* with the aid of a particle-gun (Johnston *et al.* 1988), and new yeast mutants defective in the import of proteins into mitochondria have become available (Horwich, A., personal communication). Recent experiments from our laboratory have also shown that mitochondria can import oligodeoxyribonucleotides if these are attached to the C terminus of a mitochondrial precursor protein (Vestweber & Schatz, 1989). This shows that the mitochondrial import machinery is surprisingly tolerant for the chemical nature of the macromolecule attached to a mitochondrial presequence. Although this is clearly a non-physiological process it suggests the possibility that mitochondria may, in fact, be capable of importing a much greater variety of macromolecules than has been suspected so far.

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