REVIEW ARTICLE

Targeting proteins to mitochondria: a current overview

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INTRODUCTION

Correct targeting of nuclear-encoded, cytoplasmically-synthesized proteins to mitochondria is one facet of a diverse pattern of intracellular traffic of nascent polypeptides that occurs within all eukaryotic cells. The complex internal organization of cells necessitates that organellar or secreted polypeptides, initially formed in the cytoplasm, must be delivered efficiently across one or more internal membranes before reaching their final intracellular or extracellular destinations. Specific diseases can arise when polypeptides are 'addressed' incorrectly and subsequently directed to the wrong compartment. In the case of lysosomal storage diseases, degradative enzymes are secreted erroneously into the bloodstream, resulting in the accumulation of undegraded products in the lysosomal compartment, e.g. glycosaminoglycans or glycolipids in the case of I-cell disease [1,2]. Recently, it has been recognised also that primary hyperoxaluria in some patients may be the result of a targeting defect in which the peroxisomal enzyme, L-alanine: glyoxylate aminotransferase aberrantly assumes a mitochondrial location. The molecular basis for this phenomenon appears to be a point mutation in the 5'-flanking region of the gene which contains a cryptic mitochondrial presequence. This mutation permits initiation of translation at a new upstream AUG site, resulting in the synthesis of an altered form of the enzyme with additional polypeptide sequence at the N-terminus, exhibiting the properties of a mitochondrial targeting signal [3].

The last decade has seen rapid progress in our understanding of how the cell achieves this remarkable feat of traffic management. Advances in recombinant DNA technology combined with elegant research from many major laboratories have been responsible for elucidating the basic mechanisms underlying these processes and have now begun to unravel the complex sequence of molecular events involved in the translocation of newly-formed proteins across membranes. A series of excellent, comprehensive reviews and three books have appeared from the major contributors to this research in recent years [4–10], so the purpose of this article is to provide a broad perspective on developments, with particular emphasis on mitochondrial targeting.

GENERAL MECHANISMS OF INTRACELLULAR TARGETING

The endoplasmic reticulum/Golgi route

A scheme illustrating in general terms how the organised distribution of specific classes of intracellular proteins is achieved is shown in Fig. 1. Primary sorting takes place during translation (with some exceptions for small polypeptides; see [11]) in which

newly-forming polypeptide chains containing the appropriate N-terminal signal sequences are rapidly sequestered onto the endoplasmic reticulum in a series of reactions involving a ribonucleoprotein complex, the signal recognition particle, a membrane-associated docking protein and possibly also ribosomal anchoring proteins. Thereafter this class of proteins, destined finally for locations in the endoplasmic reticulum, Golgi apparatus, lysosomes, plasma membrane or external space, is cotranslationally inserted into or translocated across the endoplasmic reticulum membrane where secondary sorting signals are responsible for directing these proteins to these various intracellular sites during their passage through a series of membrane-bound compartments leading from the lumen of the rough endoplasmic reticulum to the Golgi apparatus and eventually the plasma membrane [1,2].

Secreted proteins contain only the basic information in the form of a 15–30 amino acid 'signal' (usually located at the N-terminus) which effects their entry into the secretory pathway via translocation into the lumen of the endoplasmic reticulum. Signal peptides exhibit considerable sequence variability, although eukaryotic signals generally contain a central hydrophobic section with a minimal length of nine residues and a peptidase cleavage site loosely based on the sequence Ala-Xaa-Ala (see [8] and references therein). Current evidence suggests, therefore, that secondary structural characteristics are important in determining the functional efficiency of signal sequences. Proteins bound for other destinations en route to the plasma membrane are diverted from the main secretory pathway by the presence of additional structural features on these polypeptides.

For a plasma or organellar membrane protein, this requires the presence of a hydrophobic 'stop-transfer' sequence within the molecule, approximately 20 residues in length, which arrests its passage across the endoplasmic reticulum membrane, thus leading to eventual incorporation of the polypeptide into the external membrane. This is especially well illustrated in the case of membrane-bound and secreted forms of immunoglobulin heavy chain, which differ only in a short hydrophobic sequence at their C-termini [12]. Moreover, this segment can promote the membrane localization of a protein which is normally secreted in appropriate genetically-engineered constructs [13]. Soluble proteins in the lumen of the endoplasmic reticulum, many of which are involved in the maturation and assembly of nascent polypeptides entering this compartment, such as protein disulphide isomerase, BiP, a homologue of cytosolic hsp-70 (heat-shock proteins) required to mediate correct folding and subunit interaction, as well as various glycosyltransferases, are characterized by the presence of a conserved tetrapeptide sequence at their C-termini (KDEL in humans, HDEL in *Saccharomyces cerevisiae*, although there are variations on this

Abbreviations used: hsp 60 and hsp 70, heat-shock (stress-related) proteins of M_r values 60000 and 70000 respectively; COX III and IV, cytochrome c oxidase, subunits 3 and 4, respectively; OMM 70, MOM 19, MOM 38 and MOM 72, mitochondrial outer membrane proteins of M_r values 70000, 19000, 38000 and 72000 respectively; ISP 42, import site protein, M_r 42000; MAS 1/MIF 1 and MAS 2/MIF 2, mutants in non-identical subunits of the matrix processing protease; MIF 4, mutant in mitochondrial hsp 60.

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Fig. 1. General scheme for intracellular targeting of newly-synthesized polypeptides in eukaryotic cells

(a) In the presence of the appropriate signal sequence (—●—●—●—), the nascent protein emerging from the ribosome interacts with the RNA-containing signal recognition particle (▲), temporarily arresting translation and promoting the association of the peptide-ribosome complex with the endoplasmic reticulum (ER) via an initial interaction with a membrane-bound docking protein (■). On release of the signal recognition particle, translation is resumed coupled to the movement of the polypeptide into the lumen of the endoplasmic reticulum and cleavage of its signal sequence. After these primary sorting events, proteins are conveyed to their final destinations via the Golgi complex. In the absence of secondary sorting signals which divert this class of polypeptides to the appropriate intracellular compartment as described in the text, there is a 'default pathway' by which the remaining proteins which originally entered the endoplasmic reticulum are secreted from the cell. (b) The class of proteins synthesized on free cytoplasmic ribosomes are generally transported to the requisite organelle in a post-translational fashion. Their signal sequences (◆—◆—◆) are variable (and not always located at the *N*-terminus) to permit specific interaction with receptors on the surface of the organelle (secondary sorting). Their signal sequences do not share the general features of proteins synthesized on the membrane-bound ribosomes, so they do not interact with the signal recognition particle. See the text for further details.

theme in different species). This motif is recognised by a membrane-bound receptor located in a salvage compartment of the export pathway, thereby promoting the retrieval of this class of polypeptides into the endoplasmic reticulum [14].

Finally, for lysosomal enzymes, the sorting mechanism relies on the presence of a specific mannose 6-phosphate residue on the *N*-linked oligosaccharide chains of these glycoproteins which is recognised by a specific receptor, normally situated in the cisternal compartment of the Golgi complex. The phosphotransferase which is responsible for phosphorylation of specific mannose

residues on lysosomally-bound glycoproteins employs UDP-*N*-acetylglucosamine as a donor, presumably identifying an unknown three-dimensional motif on these polypeptides in the process [15]. Recent research in which segments of the lysosomal hydrolase cathepsin D are replaced with equivalent sequences from the related secretory protein glycopepsinogen have identified Lys-203 and the region from amino acids 265 to 292 as essential parts of the recognition site. Present indications are that a surface 'patch' involving multiple interacting sites is responsible for conferring the appropriate specificity [16].

The cytoplasmic route

The second major class of polypeptides is produced on free cytoplasmic polysomes prior to targeting to nuclei, mitochondria, peroxisomes and also to chloroplasts or glyoxisomes in higher plants, primarily in a post-translational fashion (see [4] and references therein). There is evidence, however, to indicate that uptake into mitochondria (and also chloroplasts) may also occur cotranslationally via ribosomes that are attached to the outer surface of the organelle [17]. Thus, there may be no obligatory route of entry into mitochondria and the two pathways may be employed to varying extents depending on the relative rates of protein synthesis and organelle biogenesis at different stages during growth and development.

In most cases, specific targeting to mitochondria or chloroplast membranes is achieved by the presence of an extra sequence of amino acids, often located at the *N*-terminus of the cytosolic precursor form, which can be cleaved to yield the mature protein during or immediately after import into the appropriate compartment. For nuclear and peroxisomal proteins, however, specific import sequences are generally incorporated into the structure of the mature protein, not necessarily at the *N*-terminus. Nuclear uptake signals, comprising short stretches of peptide chain abundant in basic amino acids (Lys is preferred to Arg) and a turn-inducing Pro or Gly residue, can be found in any region of the protein. Entry to the nuclear matrix is effected via distinctive pores in the double membrane of the organelle in an ATP-dependent step. Small proteins ($M_r < 66\,000$) may penetrate the nucleus passively in the absence of the usual targeting signals [18].

Recent work on peroxisomally-targeted proteins has identified a specific tripeptide, SKL, at their *C*-termini which is necessary and sufficient to direct import [19]. Site-directed mutagenesis of this SKL sequence in the luciferase gene has indicated that the first two residues could be substituted by A,C or H,R respectively although the final residue was found to be essential for functional import. A similar motif AKI and the variants GKI and AEI have recently been found to support correct targeting of the peroxisomal enzyme hydratase-dehydrogenase-epimerase in the yeast *Candida albicans* but not in *S. cerevisiae* [20]. As investigations on peroxisomal proteins are at an early stage and not all proteins from this organelle appear to contain these consensus sequences at their *C*-termini, other variations on this theme are possible [21].

TARGETING PROTEINS TO MITOCHONDRIA

Why is the import of mitochondrial proteins of particular interest? A major consideration is that in metabolically active cells, mitochondria are the most abundant organelles, constituting 10–20 % of intracellular protein. Thus a large volume of protein traffic consisting of several hundred different proteins must be directed to this compartment. Secondly, the mitochondrion itself is surrounded by a double membrane system, so imported polypeptides require to be targeted to a specific site within the organelle, namely matrix, intermembrane space, inner or outer membrane. Finally, the presence of a small mitochondrial genome, coding in mammals for a limited subset of 13 proteins which are produced on-site for local consumption as integral components of the multimeric complexes of the inner membrane involved in electron transport and ATP generation, requires the co-ordinated regulation of two separate genetic systems [22,23]. For example, the cytochrome *c* oxidase complex in eukaryotes, which catalyses transfer of reducing equivalents in the terminal segment of the respiratory chain between cytochrome *c* and oxygen, is composed of 12 or 13 distinct subunits where the

three largest proteins are synthesised on mitochondrial ribosomes while the remaining subunits are nuclear-encoded and imported from their site of synthesis in the cytoplasm. Recent evidence has demonstrated that many of the translational factors in yeast that control the level of expression of specific mitochondrial genes are nuclear-encoded and are therefore imported from the cytoplasm [24].

PRECURSOR STATES OF MITOCHONDRIAL PROTEINS

Essential features of targeting sequences

Precursor forms of mitochondrial polypeptides possess variable presequences of 15–70 amino acids in length which are usually present at the *N*-terminus of the protein. Although sequence comparison reveals no obvious homology, targeting sequences are generally rich in positively charged and hydroxylated amino acids and have the capability of forming amphiphilic α -helices (or possibly β -sheets) when in contact with the lipid bilayer, a characteristic which is thought to be crucial to their function in mitochondrial import [4–6].

A number of investigations have been conducted on the biophysical properties of chemically-synthesized peptides corresponding to mitochondrial targeting sequences and their modes of interaction with biological membranes or artificial lipid bilayers [25–28]. Three peptides representing the first 15, 25 and 33 amino acids of the yeast cytochrome *c* oxidase subunit IV (COX IV) precursor, which has a cleavable presequence of 25 amino acids, were found to have little secondary structure in aqueous solution but to insert spontaneously into lipid bilayers where the 25-mer was shown to assume an α -helical conformation [25]. Moreover, the longer peptides in particular were effective in disrupting unilamellar liposomes, an effect which was increased by imposition of a diffusion potential (negative inside). Uncoupling of respiratory control was also observed in yeast mitochondria and the surface activity of these peptides appeared to be related to their ability to form amphiphilic helices, i.e. with a highly asymmetric distribution of charged and apolar residues. In contrast, the binding of the 27-amino acid presequence of rat ornithine carbamoyltransferase to vesicles composed of neutral and anionic lipids was unaffected by the presence of a transbilayer potential of 80 mV (negative inside) [26].

In a computer modelling analysis of 23 mitochondrial targeting sequences, it was concluded that most if not all sequences could form α -helices with high hydrophobic moments, a convenient measure of their amphiphilicity in a suitable environment. In addition, the segments of maximal hydrophobic moment coincided closely with 'critical' regions of the presequences as defined by deletions and point mutations [27]. A model for mitochondrial signal peptide interaction with membranes has been proposed recently on the basis of two-dimensional n.m.r. studies on the interaction of a peptide closely related to the naturally-occurring 19-meric presequence of rat liver aldehyde dehydrogenase with dodecylphosphocholine micelles [28]. The presence of two amphiphilic helices was detected linked by a flexible 'hinge' region. As the *C*-terminal helix was the more stable, it was suggested that initial interaction with the lipid bilayer involved this helix while the *N*-terminal half of the presequence is folded back in a 'hairpin-like' structure which is not anchored within in the lipid bilayer. At this stage, lateral diffusion of the precursor permits the *N*-terminal region to bind specifically to the correct receptor. While such studies suggest that amphiphilic helices may promote binding of the precursor to the mitochondrial surface as a necessary prerequisite to recognition by the appropriate receptor, the sequence of events

Fig. 2. General routes of entry for mitochondrial precursors destined for the various subcompartments within the organelle

(a) **Outer membrane proteins.** Precursors do not possess a cleavable presequence. Insertion into the outer membrane (stage 1) is receptor-mediated (R) and may occur at or near contact sites but is independent of the mitochondrial membrane potential. Membrane-bound precursors become protease-resistant (stage 2) and assemble into their final oligomeric states (stage 3) within the confines of the lipid bilayer. Outer membrane proteins, as for all mitochondrial precursors, have the potential to be targeted to the mitochondrial matrix (default pathway) but are diverted to the outer membrane by suitably located, hydrophobic 'stop-transfer' sequences. (b) **Intermembrane space proteins.** The vast majority of precursors are translocated in a receptor-dependent (R), membrane potential ($\Delta\psi$)-dependent fashion via contact sites into the mitochondrial matrix initially prior to their re-export to the intermembrane space. Initial binding to receptor (stage 1) may be preceded by insertion of the amphiphilic helix into

occurring at this stage and the precise role of the membrane potential is still not well understood.

In gene fusion/deletion studies designed to test the minimal length of presequence required for successful import, it has been demonstrated that the first 12 amino acids of the cytochrome oxidase subunit IV (COX IV) presequence are sufficient to target the non-mitochondrial protein dihydrofolate reductase to this organelle in yeast [29]. In the case of β -galactosidase fusions with the presequence of δ -aminolaevulinic synthase, only the nine *N*-terminal residues appear critical for import *in vivo* [30]. Most naturally occurring presequences are 15–30 amino acids in length on average, providing the basic signal for translocation to the mitochondrial matrix.

Chloroplast targeting sequences display some similarities to their mitochondrial counterparts, especially in green algae such as *Chlamydomonas reinhardtii* [31]. However, in higher plants, transit peptides show greater differences, generally comprising an uncharged *N*-terminus, a central non-amphiphilic region and a *C*-terminal region which may form an amphiphilic β -strand [32]. Since both organelles coexist in plant cells, it is clear that they must possess distinctive features to ensure specificity of import. Surprisingly, *in vitro*, the presequence corresponding to the small subunit of the major chloroplast enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase is able to catalyse the uptake of the yeast COX IV subunit or mouse dihydrofolate reductase into yeast mitochondria although with low efficiency [33]. The physiological relevance of such *in vitro* studies is dubious, however, particularly since this sequence has been shown to direct the correct localisation of chloramphenicol acetyltransferase in transgenic tobacco plants [34].

Targeting to the various mitochondrial sublocations

It is not an essential feature of mitochondrial proteins that they possess a cleavable presequence, e.g. apocytochrome *c* of the intermembrane space, the adenine nucleotide translocase, an integral inner membrane protein, and the soluble matrix enzyme, isopropylmalate synthase. Interestingly, some inner membrane proteins, e.g. the adenine nucleotide translocase and the phosphate carrier, are synthesized as larger precursors which are processed in some species but not in others. For example, the bovine heart and rat liver phosphate transporters have presequences of 49 and 44 amino acids respectively while the equivalent polypeptide from *S. cerevisiae* is synthesized as the mature-sized protein [35–37].

There is no apparent requirement also for a cleavable presequence on polypeptides which insert specifically into the

mitochondrial outer membrane. In this group of proteins targeting information must be incorporated into the sequence of the mature protein. Surprisingly, in the case of the outer membrane protein OMM 70, targeting by the first 12 amino acids of its *N*-terminal sequence results in dihydrofolate reductase, the COX IV subunit or β -galactosidase accumulation in the mitochondrial matrix in appropriate constructs [38,39]. Thus it appears that all mitochondrial proteins are pre-programmed for entry into the interior of the organelle in the absence of additional diversionary signals which determine their final intramitochondrial location (see Fig. 2 and text below). In general, it is probably most convenient for interaction with mitochondrial receptors and least disruptive to the function of the mature protein to locate signalling information on an exposed region at the *N*-terminus. It is likely that many proteins contain sequences with the potential to act as mitochondrial presequences in inaccessible regions of their tertiary structure. For example, it has been shown that the non-mitochondrial protein dihydrofolate reductase contains a cryptic mitochondrial targeting sequence buried in the interior of the native protein [40]. In a remarkable study, Baker & Schatz (1986) were also able to demonstrate that approx. 100 bp fragments of *Escherichia coli* genomic DNA, cloned randomly onto the open reading frame of the plasmid-based COX IV gene, lead to restoration of cytochrome *c* oxidase function in approx. 3% of *S. cerevisiae* mutants in which the chromosomal gene for this subunit had been disrupted [41].

For mitochondrial precursors with extended presequences, it is clear that additional information is built into this region of the molecule, often associated with targeting to specific sublocations within the organelle. Thus all mitochondrial precursors are programmed to enter the matrix compartment in the absence of distally-located 'stop-transfer' or 'localization' signals or secondary targeting signals required for re-export to the intermembrane space. General routes of access for precursors entering the various mitochondrial sublocations are illustrated in Fig. 2. The disposition of hydrophobic sequences in relation to the matrix targeting signals at the extreme *N*-terminus plays a crucial role in delivering the precursor to the correct mitochondrial membrane. For example, the outer membrane protein OMM 70 is directed into the mitochondrial matrix in the absence of a short hydrophobic stretch of amino acids, residues 9–38, which is situated immediately adjacent to the *N*-terminal presequence [42]. Moreover, while a 12-residue presequence of this protein is sufficient to direct β -galactosidase to the matrix, a similar construct containing the first 29 amino acids of OMM 70 is inserted into the outer membrane with the β -galactosidase

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the lipid bilayer. Receptor migration to contact sites (stage 2) may be required prior to formation of translocation intermediate. In some cases, exposure of the precursors to the mitochondrial matrix (stages 3 and 4) may be extremely transient and there is recent unpublished evidence that re-export to the intermembrane space can begin before uptake into the matrix is complete (see [9]). Precursors usually possess a bipartite targeting signal (stage 3). The *N*-terminal matrix targeting signal is removed by the matrix processing protease (PP) (stage 4), revealing a secondary targeting signal, similar to bacterial export signal sequences, which promotes re-export to the intermembrane space (conservative sorting) (stage 5). The mature-sized proteins are generated during or shortly after entry into the intermembrane space by the action of distinct processing proteases located within this compartment. In some cases, e.g. for cytochrome *c*₁, this second proteolytic cleavage is dependent on the addition of cofactor. Cytochrome *c* is a notable exception in that apocytochrome *c* inserts itself partially across the outer membrane with no apparent energy requirement or involvement of a specific receptor. Access to the haem lyase situated in the intermembrane space allows the incorporation of haem, thereby inducing conformational changes which ensure completion of the translocation process and the functional maturation of the protein. (c) **Inner membrane proteins.** Precursors are imported in a receptor-mediated (R), energy-dependent ($\Delta\psi$) manner via contact sites. Stages 1 and 2 are similar to those for intermembrane space proteins. Not all precursors contain cleavable presequences, although the presence or absence of a presequence on a specific protein, e.g. the phosphate transporter, varies from species to species. Some hydrophobic integral membrane proteins, especially those with matrix targeting information located within the mature protein, e.g. the adenine nucleotide translocase, appear to use a different receptor (see the text for details) and may not become exposed to the matrix compartment to any appreciable extent, becoming embedded directly in the inner membrane (stage 3) for final assembly (stage 4). Processing, if it occurs, is catalysed by the standard matrix protease (PP). Intramembrane organisation is determined by the distribution of hydrophobic, stop-transfer sequences within these polypeptides. (d) **Mitochondrial matrix proteins.** All mitochondrial precursors containing the standard matrix targeting sequence are directed to this compartment in the absence of additional sorting 'signals' (default pathway). Uptake occurs via receptors (stage 1) and contact sites (stage 2) and is energy-requiring. Cleavage to the mature-sized polypeptide by the matrix protease (PP) (stage 3) occurs during or shortly after entry prior to assembly into the final oligomeric structure (stage 4). See the text, Fig. 3 and Table 1 for further details of the translocation and assembly process.

facing into the cytoplasm. Interestingly, with a presequence of intermediate size (19 amino acids), the enzyme is distributed to both locations in a manner which is dependent on its level of expression and the metabolic status of the cell [39].

For most proteins of the inner membrane, stop-transfer sequences often occur within the interior of the polypeptide located at some distance from the *N*-terminal targeting sequence and, as in the case of the ATP/ADP translocase, targeting information itself may be incorporated within the mature protein. As this polypeptide and related members of this family, e.g. the phosphate transporter and the brown fat uncoupling protein, all contain an internal three-fold repeat and multiple membrane-spanning elements, it is possible that each section of the molecule can be independently targeted to the organelle. In this context, a truncated form of the translocase from *Neurospora crassa* containing only the two *C*-terminal repeated sequence elements is apparently imported normally [43]. There is evidence for a 20-amino-acid stretch in the *C*-terminal region of each domain which has the properties of a mitochondrial targeting signal [44].

A group of proteins which are found in the intermembrane space or associated with the external face of the inner membrane, e.g. cytochrome *b₂* or the Rieske iron-sulphur protein, follow a particularly complex import pathway. This involves initial translocation of the precursor across both mitochondrial membranes into the matrix compartment, proteolytic processing to an intermediate state by the standard Mn^{2+} - or Zn^{2+} -requiring matrix-located protease and subsequent re-export across the inner membrane prior to the final maturation step catalysed by a separate membrane-bound protease exposed to the intermembrane space. Soluble forms of these precursors can be detected in the mitochondrial matrix if the import reaction is conducted at low temperatures (< 10 °C) or in the presence of chelating agents to inhibit the action of the first processing enzyme [45–49]. A yeast mutant has also been isolated which accumulates the intermediate-sized form of cytochrome *b₂* under appropriate conditions. Interestingly it also fails to process subunit II of cytochrome oxidase, a product of the mitochondrial genome, which is located largely on the cytoplasmic surface of the inner membrane in the intact complex [50]. Haem insertion, in the case of cytochrome *c₁*, appears to represent an obligatory step in the functional maturation of the protein, since this reaction is necessary for the intermediate-sized precursor to become a suitable substrate for an intermembrane space protease which appears to be distinct from the enzyme involved in maturation of cytochrome *b₂* [51]. Similarly, in the case of apocytochrome *c*, which is uniquely imported directly into the intermembrane space from the cytoplasm, translocation of the bulk of the apoprotein across the outer membrane and folding to the mature enzyme is induced by incorporation of the haem group catalysed by a haem lyase present in the intermembrane space [52].

The limited ability of mitochondria to re-export proteins from the matrix in this fashion is believed to reflect their prokaryotic origins and represents the conservation of export machinery present in the original endosymbiotic ancestors of this organelle; hence it is referred to as 'conservative sorting' for this reason (see [5] for a detailed review of the evidence). In this context it is clear that passage from the matrix to the intermembrane space is directed by the *C*-terminal segment of the extended presequences present on this class of precursors, i.e. they contain a bipartite signal sequence. Interestingly, analysis of this region of presequence indicates similarities with the export signals present on secreted bacterial proteins, e.g. *c*-type cytochromes from *Rhodobacter capsulata* and related species which are believed to be the closest living relatives of the early prokaryotic ancestors of mitochondria. A further variation of this theme is seen with the Rieske iron-sulphur protein which is exposed on the outer

surface of the cytochrome *bc₁* complex. Its presequence undergoes two-step processing with both events occurring in the matrix; moreover, there is no bacterial-like export sequence in the prepeptide, namely a hydrophobic stretch of 15–20 amino acids preceded by one or more positively charged residues. Presumably, in this case re-targeting information resides within the sequence of the mature protein.

Other types of information in mitochondrial presequences

In addition to providing the instructions for directing cytosolic precursors to specific sublocations within mitochondria, elongated presequences are implicated in a variety of secondary functions associated with the intracellular transport of nascent polypeptides. For example, the ATPase proteolipid subunit of *N. crassa*, a small hydrophobic inner membrane polypeptide of 81 amino acids, is synthesized initially with a 66-amino-acid presequence which is extremely basic and hydrophilic [53]. It is likely that this transit peptide is involved in maintaining the hydrophobic sequence of the mature protein in a soluble state en route to the organelle. In support of this idea, in yeast this subunit (ATPase subunit 9) is a product of the mitochondrial genome and is synthesized *in situ* as the mature 76-amino-acid hydrophobic peptide, i.e. without a hydrophilic presequence [54]. However, the extended hydrophilic presequence may have an additional function in facilitating the translocation of this hydrophobic integral protein across the lipid bilayer prior to its final insertion into the inner membrane from the matrix compartment.

The tendency of precursor forms to exist as high-*M_r* aggregates in the cytosol may represent an alternative mechanism for overcoming the problem of solubility, since it is known that precursors must be presented to the mitochondrial surface in a loosely folded, translocation-competent state for promoting efficient import. In the case of the three related mammalian mitochondrial 2-oxoacid dehydrogenase multienzyme complexes, including the pyruvate dehydrogenase complex, the cytosolic states of the distinct lipote acyltransferase (E2) core enzymes all contain extended targeting sequences (56–70 amino acids) which may prevent premature irreversible self-aggregation of these polypeptides into their characteristic 24-meric (octahedral) or 60-meric (icosahedral) intact assemblies prior to import [55].

RECOGNITION EVENTS AT THE MITOCHONDRIAL SURFACE

While it has been recognized for several years that the presence of an *N*-terminal targeting sequence is sufficient to promote mitochondrial uptake of polypeptides, the precise nature of the specific interaction of the targeting sequence with the mitochondrial membrane and subsequent events occurring during the passage of the precursor across the lipid bilayer are still under active investigation.

There is now also general agreement that proteinaceous receptors on the mitochondrial surface are required for the import process, since brief treatment with a variety of specific and non-specific proteases, e.g. trypsin, proteinase K and elastase, inactivates precursor uptake into the four mitochondrial sublocations although differences in sensitivity were also reported for particular groups of proteins, suggesting the involvement of specific import receptors (see [4] and references therein). Targeting of apocytochrome *c* to the intermembrane space proved extremely resistant to protease attack [56], reflecting the unique nature of the import pathway for this polypeptide which requires the participation of the haem lyase located in the intermembrane space.

Table 1. Current status of factors implicated in the import and assembly of nuclear-encoded mitochondrial precursors

Factor	Function	Status
Cytoplasmic factors		
Presequence binding factor (PBF)	Stabilization of precursors in translocation-competent state	Component of rabbit reticulocyte lysate required for import <i>in vitro</i>
hsp 70 subgroup of four homologous stress proteins SSA1p-4p	Maintain newly-synthesized precursors in a translocation-component state	Functionally redundant with overlapping specificities – essential for import <i>in vivo</i>
40 kDa protein	Unknown	Physiological significance <i>in vivo</i> to be established
N-Ethylmaleimide-sensitive component	Possible role in binding to presequences; presentation to receptor?	
Mitochondrial outer membrane factors		
MAS 70 (MOM 72?)	Import receptors: MOM 72, specific receptor for ATP/ADP translocase	MAS 70 disruption decreases rate of import of selective group of precursors <i>in vivo</i> by 50–80 %
MOM 17? (MOM 19)	Import receptors; MOM 19 promotes uptake of many, but not all, precursors <i>in vitro</i>	Physiological significance <i>in vivo</i> to be established
ISP 42 (MOM 38) or General Insertion Protein (GIP)	Component of import machinery located at contact sites	Essential for import <i>in vivo</i>
Mitochondrial matrix factors		
mt hsp 70	Role in transmembrane movement of precursors; transfer to hsp 60?	Essential for import <i>in vivo</i>
mt hsp 60	Role in ordered assembly of multimeric complexes	Essential for assembly <i>in vivo</i>
MAS 1/MIF 1(PEP) } MAS 2/MIF 2 (MPP) }	Removal of mitochondrial matrix targeting signal	Non identical subunits of matrix processing protease – essential for functional assembly <i>in vivo</i>
Mitochondrial inner membrane/intermembrane space factors		
Inner membrane protease I, IMP I	Specialised membrane-bound enzyme required for maturation of cytochrome <i>b</i> ₂ and mitochondrial gene product, subunit II of cytochrome <i>c</i> oxidase	Cells are respiration-deficient but viable
Unidentified second processing protease	Role in maturation of cytochrome <i>c</i> ₁ and other intermembrane space proteins	Physiological significance <i>in vivo</i> to be established
Haem lyase	Role in the import and functional maturation of cytochrome <i>c</i>	Cells are respiration-deficient but viable

These early studies indicated that several types of import receptors may be present on the mitochondrial surface which interacted specifically with particular subsets of polypeptides destined for the organelle. More recently, rapid progress has been achieved in identifying import receptors and other essential components of the import machinery through a combination of imaginative genetic, immunological and biochemical approaches.

Isolation of mutants with specific defects in receptor function and other elements of the import machinery has proved to be unexpectedly difficult. This appears to reflect the fact that many of the mitochondrial 'receptors' are functionally redundant in that a similar role may be performed by a secondary component of overlapping specificity or alternatively particular steps are not obligatory for import, permitting the precursor to enter at a later stage in the pathway. Establishing the physiological significance of such receptors is awkward, therefore, particularly in *in vitro* systems where isolated mitochondria are often present in vast excess such that they are importing essentially in a non-catalytic fashion.

The only mitochondrial membrane polypeptide to date which is known to be essential for protein import *in vivo* in yeast is termed import site protein 42 (ISP 42). It was identified originally as it can be cross-linked efficiently to a partially-translocated hybrid precursor of dihydrofolate reductase and bovine pancreatic trypsin inhibitor carrying a photo-activatable diazirin group at its C-terminus [57]. Surprisingly, its predicted protein sequence indicates the absence of obvious hydrophobic membrane-spanning regions. Current evidence would indicate that it is a key constituent of proteinaceous import channels located at contact sites between the inner and outer membranes,

which are believed to be of central importance in promoting the uptake of precursors into all mitochondrial sublocations [58]. In *N. crassa*, the equivalent polypeptide, termed MOM 38 (probably identical to the previously detected 'general insertion protein'), has been isolated as a multimeric complex containing several other outer membrane proteins including two import receptors, namely MOM 19 and MOM 72 [59]. These receptors were implicated in the import process after antibodies were prepared to a whole range of SDS/polyacrylamide gel-purified polypeptides of the outer mitochondrial membrane of *N. crassa* and tested for their ability to inhibit the import of specific precursors *in vitro*. At present MOM 72 appears to be involved specifically in the uptake of the adenine nucleotide translocase into the inner membrane [60] whereas MOM 19 is responsible for promoting the general uptake of a wide range of matrix-located proteins [61]. Interestingly, gene disruption of these proteins yields mutants which are viable and apparently retain the ability to transport the appropriate precursors *in vivo*. Thus these receptors may be functionally redundant or belong to a larger family of receptors with overlapping specificities. In this context, anti-idiotypic antibodies generated against primary antibodies to the peptide corresponding to the mitochondrial targeting sequence of subunit IV of cytochrome *c* oxidase have been employed to isolate a putative import receptor in yeast [62,63]. The isolated protein was found to be identical to the mitochondrial phosphate transporter which has been cloned and sequenced recently [37]. An interesting question arises as to whether this is a protein with dual functionality, although its status as an import receptor must be treated with caution, particularly as deletion of this gene does not affect mitochondrial

import *in vivo*. A summary of the status of the various factors, both mitochondrial and cytoplasmic, implicated to date in the uptake and assembly of mitochondrial precursors is presented in Table 1.

CYTOSOLIC FACTORS INVOLVED IN TRANSLOCATION AND IMPORT OF PRECURSORS

Not only must nascent, nuclear-encoded mitochondrial proteins be translocated specifically to the organelle but the precursor protein must be presented in the appropriate 'translocation-competent' state for ensuring efficient import. Initial indications to this effect arose from *in vitro* import studies using the mouse dihydrofolate reductase gene containing the yeast COX IV presequence. It was demonstrated that uptake of this specific chimaeric protein could be prevented by methotrexate, an inhibitor of dihydrofolate reductase that stabilizes the compact, tertiary structure of the native molecule; moreover, the urea-denatured construct could be imported much more rapidly than its native counterpart in a reaction which no longer exhibited a requirement for nucleoside triphosphates [65,66]. The general necessity for maintaining precursors in an open, 'loosely-folded' conformation prior to import has now been confirmed through studies on several proteins, including the Cu^{2+} -containing protein metallothionein [67], 5-enolpyruvyl-

shikimate-3-phosphate synthase, a chloroplast enzyme which binds the herbicide glyphosate [68] and the dihydrofolate reductase-trypsin inhibitor fusion protein described previously. This last construct becomes trapped as a transmembrane translocation intermediate at contact sites as a result of the compact disulphide-bonded structure of the bovine pancreatic trypsin inhibitor molecule [57]. Titration experiments have revealed that mitochondria of *N. crassa* are able to accommodate approx. 2000 precursor proteins at these specialised import sites [69].

It has now been recognised from genetic and biochemical evidence that the translocation competence of mitochondrial precursors *in vivo* is maintained via specific interaction with a highly-conserved group of cytoplasmic proteins with overlapping functions, the heat-shock or stress-related proteins (hsp 70s) which act as molecular chaperones to convey precursors to the surface of the organelle in a suitable conformational state [70,71]. These proteins or their homologues are expressed constitutively in all cells, although there is massive induction following exposure to a variety of stress factors, including heat. They are able to interact with aberrantly folded proteins probably on exposed hydrophobic surfaces and mediate their proper refolding via an ATP-dependent event which promotes the release of the bound proteins. Their constitutive expression in normal conditions now appears to reflect their primary function in the intracellular traffic of nascent polypeptides [71].

ATP (or GTP) at low levels is also reported to be required for

Fig. 3. Scheme illustrating involvement of transiently associated proteinaceous factors at various stages in the targeting, translocation and assembly of nuclear-encoded mitochondrial precursors in *S. cerevisiae*

(1) Initial interaction with cytoplasmic hsp 70 to maintain mitochondrial precursor in a 'translocation-competent' stage; (2) ATP-dependent release from cytoplasmic hsp 70 and interaction with mitochondrial receptor; (3) transfer of precursor to contact site between inner and outer membrane and formation of translocation intermediate, a process which is driven by the mitochondrial membrane potential; (4) association of translocation intermediate with mitochondrial hsp 70, required for import of the precursor; (5) transfer to mitochondrial hsp 60 for initiation of folding/assembly pathway on ATP-dependent release of hsp 60; (6) removal of cleavable presequences by matrix protease and final functional maturation. MAS I+II, hsp 60, hsp 70 and ISP 42 are factors in the import pathway which are known to be essential for functional import *in vivo* in yeast.

specific binding of many precursors to the mitochondrial surface while higher levels promote the initial insertion into the membrane in a protease-resistant state [72,73]. It is unclear at present whether these represent additional ATP-dependent steps or reflect the energy requirement associated with dissociation from cytosolic hsp 70. A membrane potential is also essential for driving the import process although this can be small (20–40 mV) since respiratory-deficient (ρ^-) mutants of yeast can still import cytosolic precursors normally, presumably generating a small membrane potential via the action of the electrogenic adenine nucleotide translocase [74]. The molecular basis for this requirement is still unknown; however, the translocation process can be arrested on formation of the membrane-spanning intermediate, e.g. by low temperatures or use of antibodies, permitting allocation of the energy-dependent event to an early stage in translocation, probably associated with initial insertion of the precursor [75,76].

Several additional cytosolic proteinaceous and RNA-containing species have also been reported to be necessary for efficient delivery and uptake of precursors, although their authenticity and possible physiological significance have not yet been fully established [77–79]. Recently, a presequence binding factor has been purified from a rabbit reticulocyte lysate which forms a translocation-competent intermediate with a purified mitochondrial precursor protein [80]. The possibility that mitochondrial targeting is mediated by an equivalent to the signal recognition particle, required for translocation of proteins into the endoplasmic reticulum, is an intriguing idea which merits further investigation, particularly as the involvement of an equivalent particle in bacterial secretion has only been established recently [81,82].

MITOCHONDRIAL FACTORS INVOLVED IN PRECURSOR MATURATION AND ASSEMBLY

Currently evidence has been obtained for the participation of at least four specific proteins of the mitochondrial matrix in processing and functional assembly of oligomeric complexes within the organelle. These include the processing protease (MAS 1/MIF 1), the protease enhancing protein (MAS 2/MIF 2), now believed to represent non-identical subunits [83–86] of the intact enzyme in yeast, and two recently described heat-shock proteins, designated hsp 60 and hsp 70 [6,87]. Yeast strains that were defective in processing were isolated by mass screening of mutants in which there was accumulation of higher- M_r mitochondrial precursors under normal growth conditions. The genes encoding these proteins have been cloned and sequenced and the purified enzymes characterized in some detail (see [6] for review).

Mitochondrial hsp 60 was identified in screens of temperature-sensitive yeast mutants for their ability to assemble active human ornithine transcarbamoylase trimers in the mitochondrial matrix. At the non-permissive temperature these MIF 4 (mitochondrial import function) mutants still import mitochondrial precursors but cannot assemble them properly. Thus the role of hsp 60 is analogous to chaperones reported in bacteria and chloroplasts which mediate the ordered assembly of multimeric complexes. Indeed its deduced amino acid sequences resembles that of the *groEL* gene product of *E. coli*. More recently, the involvement of an hsp 70-like protein related to the *E. coli dna k* gene product has been described which can be cross-linked to translocation intermediates trapped at contact sites in the mitochondrial membranes [88–90]. Thus interaction with mitochondrial hsp 70 occurs as soon as polypeptides undergoing translocation gain access to the mitochondrial matrix compartment. Release of the translocation intermediate from hsp 70 is an ATP-mediated

event which may be coupled to the final transfer of the imported polypeptide into the matrix. It has not been established if hsp 70 transfers incoming proteins directly to hsp 60; however, it is possible that hsp 70 may also maintain these proteins in the appropriate 'loosely-folded' state within the organelle prior to their final hsp 60-catalysed assembly into functional complexes. Precursors of cytochrome *b₂* and the Rieske iron-sulphur protein which enter the matrix only transiently en route to the inter-membrane space appear to interact with hsp 60 also in an ATP-dependent event. This again highlights similarities with the bacterial export pathway in view of the role of the *groEL* gene product in maintaining precursors in a suitable conformation for translocation across the bacterial plasma membrane see [91] for review). A diagrammatic representation of the involvement of heat-shock proteins in the translocation and assembly of mitochondrial precursors is provided in Fig. 3.

THE MITOCHONDRIAL GENOME

As indicated previously, the limited genetic capacity of mitochondrial DNA in eukaryotes, accounting for only about 5% of its constituent proteins, is responsible for the heavy reliance of the organelle on imported polypeptides. In most animal and higher plants, mtDNA accounts for less than 1% of total DNA whereas in yeast it can be as much as 15%. The mitochondrial genome exists in both linear and circular forms with typical sizes in mammals varying from 100 to 200 kbp although mtDNA can be as large as 2400 kbp in some higher plants. Variation in the organellar genome size may reflect the different evolutionary origins of these organelles and also the different evolutionary pressures acting on animal and plant cells [92]. In individual yeast strains, differences in mitochondrial DNA size are largely accounted for by the presence or absence of introns in particular genes (see [93] for a review).

Although the mitochondrial genome represents only a small fraction of total cellular DNA, its role is nevertheless crucial to successful organelle biogenesis which requires the co-ordinated expression of both nuclear and mitochondrial genetic systems. As indicated previously, most of the major respiratory complexes of the inner membrane including cytochrome *c* oxidase and the ATP synthase assemblies are formed from nuclear-encoded and mitochondrially-encoded subunits. Certain nuclear genes such as the yeast ATP synthase 4 gene (ATP 4) can influence the synthesis or assembly not only of the ATP synthase complex but also of other mitochondrial complexes such as cytochrome *c* oxidase. Disruption of the ATP 4 gene leads also to selective depletion in the levels of mitochondrially-encoded subunit 6. This subunit is transcribed normally but is unstable in the absence of its companion polypeptide [94]. In addition, however, to these effects on ATP synthase maturation there is also a 5-fold reduction in the cytochrome *c* oxidase activity of the mutant yeast. Interestingly, cytochrome *c* oxidase subunit III, a mitochondrial gene product, is found to be essential for the functional assembly of this complex *in vivo* whereas it can be removed from the purified enzyme without loss of activity [95]. Such studies indicate that some components of these complex oligomeric structures in which many of the polypeptides have no assigned function as yet may provide a structural framework for organisation of the catalytically-active subunits. Recently, it has also been established that the mitochondrial pyruvate dehydrogenase complex, a multimeric array of three separate enzymes, jointly responsible for catalysing the conversion of pyruvate to acetyl-CoA and NADH, contains an additional polypeptide, termed protein X, which exhibits a structural role in mediating the optimal interaction of dihydrolipoamide dehydrogenase, E3, with the E2 core assembly [96–98]. Similarly, structural proteins

may be necessary for the maturation of all the main respiratory assemblies which contain a mixture of products derived from both mitochondrial and nuclear genetic systems.

MECHANISMS FOR CO-ORDINATION OF NUCLEAR AND ORGANELLAR GENOME ACTIVITY

In HeLa cells nuclear protein factors have been detected that bind specifically to the 5'-flanking regions of the human nuclear-encoded genes for cytochrome c_1 and ubiquinone binding protein, both of which are constituents of the mitochondrial cytochrome bc_1 complex. These same transcriptional regulators also recognise specific sequence elements, termed mt3 and mt4, located in the promoter regions of the mammalian mitochondrial genome [99,100]. Thus co-ordinated transcriptional control of physically separated genomes may be mediated by the presence of identical or functionally equivalent regulatory effectors which interact with similar control elements in both nuclear and mitochondrial DNA. Clearly, this mechanism, if widespread, would permit the co-ordinated biogenesis of all the mitochondrial respiratory assemblies which contain the products of two physically separated genomes.

Co-regulation of mitochondrial and nuclear gene expression may be further refined via a common response to appropriate environmental stimuli, e.g. oxygen or carbon source. In yeast, the presence of oxygen regulates the level of mitochondrial haem which in turn influences the expression of a number of nuclear regulatory genes, controlling the synthesis of cytoplasmic subunits of the cytochrome c oxidase complex and also required for the transcriptional activation of the complementary mitochondrial genes. Such control mechanisms should lead to balanced production of both mitochondrial and nuclear-encoded subunits of the complex (see [101] for a review).

Elegant genetic evidence employing *S. cerevisiae* nuclear mutations which affect the production of individual mitochondrial gene products, e.g. cytochrome b or subunit II of cytochrome c oxidase, have shown that these genes code for gene-specific translation factors which bind to unique sites in the 5'-untranslated region of processed mitochondrial transcripts [23]. Thus it is apparent that the nuclear genome may exercise both transcriptional and translational control on the level of mitochondrial gene expression.

INVOLVEMENT OF MOBILE NUCLEIC ACIDS IN THE CO-ORDINATION OF GENOME EXPRESSION

In addition to utilizing common proteins, it is also possible for the mitochondrion and the nucleus to interact by commonly regulating either transcriptional or translation processes through the direct exchange of RNA and possibly DNA between the two compartments. There is now convincing evidence to indicate that small RNA molecules encoded by the nucleus play a role in the expression of the mitochondrial genome. In particular, some species of tRNA molecules, which have an important role in mitochondrial synthesis, have been shown to be nuclear-encoded and can be transported to the organelle where they can be acylated by mitochondrial aminoacyl-tRNA synthetase enzymes [102]. In the case of leucyl-tRNAs studied in the bean *Phaseolus vulgaris* the mitochondrial tRNA molecules are identical to their cytoplasmic counterparts apart from the presence of a methylated guanine at position 18. This methylation event may arise either as a consequence of import or act as a signal directing import. Interestingly, in yeast, lysyl-tRNA can be imported into the organelle and it too differs from the cytoplasmic form by methylation of a single base although in this case it is the cytoplasmic tRNA which contains the methylated residue. In contrast to the situation in bean, however, this tRNA could not

be acylated by a mitochondrial tRNA synthetase preparation. If translocation of specific tRNA molecules in this manner is established as a general phenomenon, it is clear that this could provide an important regulatory mechanism for co-ordinating the rates of protein synthesis in the two compartments. Other tRNA synthetases may be distributed similarly between these two locations, providing a potential mechanism for regulation of the relative rates of protein synthesis in the two compartments.

As well as transport of tRNA species, RNA has been shown to enter the organelle as part of a ribonucleoprotein complex which plays a role subsequently in mitochondrial RNA processing. Although no conclusive evidence exists to date, it is attractive to speculate this ribonucleoprotein is also active in nuclear RNA processing further linking nuclear and mitochondrial gene expression [103].

Mitochondrial RNA splicing is also a process that probably involves both genomes. Although mitochondrial introns have been shown to be self splicing *in vitro*, additional RNA maturase enzymes are necessary for efficient splicing [104]. This dependence on nuclear-encoded maturases effectively mediates the co-ordinated expression of the two genetic systems.

There is a tendency to consider that it is primarily mtDNA expression that is controlled by the nucleus and that there is little or no reciprocal control. However, there are reports of alterations in the levels of nuclear gene expression in response to impaired mitochondrial function. For example, in *Neurospora* and *Tetrahymena* treatment of wild-type cells with either ethidium bromide or chloramphenicol led to the increased synthesis of several nuclear-encoded mitochondrial components, a number of which were involved in mitochondrial protein synthesis [105]. One of these components has now been cloned and sequenced in *N. crassa*, leading to its identification as mitochondrial ribosomal protein S-24 [106]. Moreover, its mRNA levels are increased 5-fold on inhibition of mitochondrial protein synthesis. It is likely, therefore, that there is bidirectional communication between mitochondria and nucleus. Such a two-way flow of information would be the natural result of the endosymbiotic origins of mitochondria.

During the evolution of mitochondria, it seems certain that genetic information has passed to the nucleus, thereby increasing their interdependence and establishing the balanced integration of their respective functions. Thus, for example, ATPase subunit 9 is a mitochondrial product in yeast whereas the equivalent polypeptide is nuclear-encoded in *N. crassa*. There is evidence to suggest that DNA can move not only from the mitochondrion to the nucleus but also from the chloroplast to the nucleus and between mitochondria and chloroplasts in plants. It is possible that DNA does not move directly but as an RNA intermediate. This idea is supported by the observation that in *Oenothera* mitochondrial DNA there are sequences that bear striking homology to regions of reverse transcriptase and viral polyproteins [107]. As sequences which are known to have a mitochondrial genome location are always transcribed, this also argues strongly for informational exchange being mediated via an RNA intermediate. This view is also consistent with previously-discussed evidence for the movement of RNA across mitochondrial membranes.

At present it is not clear how RNA molecules might be directed to the mitochondrial matrix from the cytoplasm of the cell. In view of their net negative charge, it seems unlikely that free RNA molecules would be translocated via contact sites by a similar mechanism to precursor proteins, which require positively charged amphiphilic presequences. It is more likely that RNA uptake would be effected via a ribonucleoprotein as described earlier with proteins acting as carriers. Moreover, the ability of nucleic acids to move through contact sites in association with

protein has been demonstrated *in vitro* employing a modified dihydrofolate reductase construct in which a single- or double-stranded 24 bp segment of DNA is covalently attached via its 5' end to the C-terminus of the mitochondrial precursor [108].

FUTURE RESEARCH

An obvious task for the immediate future is to define more precisely the number and range of specificities of the various import receptors and nature of their interactions with precursor proteins. Analysis of the physiological importance of individual receptors may be hampered by the observation that several putative receptors do not appear to be essential for translocation, suggesting that they may perform equivalent or overlapping functions or that alternative mechanisms exist for entry of precursors into the import pathway. The presence of multiple species with similar roles appears to be a general feature of components involved in intracellular traffic. For example, the cytoplasmic hsp 70 proteins in yeast consist of a family of four polypeptides (the SSA genes) exhibiting a minimum of 80% sequence identity. It has been shown conclusively that disruption of three of these four genes is necessary to induce a phenotypic response, i.e. the accumulation of mitochondrial gene products in the cytoplasm. In this context, it is also of interest that mitochondria from *S. cerevisiae* from which the outer membrane has been selectively removed retain the ability to catalyse the uptake of precursors, consistent with the view that entry can be effected at a later stage in the import pathway *in vitro*.

The identification of ISP 42 in yeast (MOM 38 in *N. crassa*) as a key constituent of the import machinery involved in the transfer of intermediates across mitochondrial membranes is likely to provide a major stimulus to elucidation of other components of the translocation apparatus. Success in detecting the interaction of import receptors and the mitochondrial hsp 70 protein with this polypeptide suggests that it may be feasible to isolate the import machinery as an intact entity for reconstitution studies, leading to major advances in our understanding of the molecular details of the import process.

An additional area of importance, currently the focus of intensive research activity, is concerned with gaining a precise knowledge of the modes of action of the various chaperones (a) in mediating the intracellular transport of newly-synthesized polypeptides in a translocation-competent state and (b) in directing the ordered assembly of multimeric structures within specific cellular compartments. Advances in this area are clearly of considerable biotechnological significance in terms of achieving functional high-level expression of mammalian proteins in heterologous systems.

In the context of the mitochondrial genome, recent advances in the transformation of organelles employing particle gun accelerators has permitted the introduction of exogenous DNA into yeast mitochondria *in situ* [109]. This DNA can become stably integrated into the mitochondrial DNA by homologous recombination, providing an enabling technology to allow the insertion of foreign genes into mitochondria and chloroplasts. In one such study, the nuclear gene URA3 was inserted into the mitochondrial genome of a uracil-requiring (URA3⁻) host. The introduced gene was not expressed in the mitochondrion; however, the transforming DNA was spontaneously transferred to the nucleus at a relatively high frequency resulting in a URA3⁺ phenotype [110]. In parallel experiments no detectable movement of DNA from the nucleus to the mitochondrion could be demonstrated, indicating the possible unidirectional nature of this process. The mode of DNA transfer is not yet known but presumably reflects the mechanism by which the mitochondrial genome has markedly reduced in size during evolution.

An intriguing outstanding consideration relates to the absolute requirement for a functional mitochondrial genome in eukaryotes. Is the continual transfer of genes to the nucleus now complete and the residual complement of proteins expressed in the organelle the minimum necessary to ensure efficient integration with the nucleus? Artificial relocation of mitochondrial genes to the nucleus has been reported as has their expression and functional delivery back to the organelle [111]. In future, it may be possible to construct a eukaryotic cell in which the entire mitochondrial genome has been transferred to the nucleus. Analysis of an artificial cell of this type should provide important insights into the control of mitochondrial biogenesis.

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REFERENCES

- Kornfeld, R. & Kornfeld, S. (1985) *Annu. Rev. Biochem.* **54**, 631–664
- Pelham, H. R. B. (1989) *Annu. Rev. Cell Biol.* **5**, 1–23
- Purdue, P. E., Allsop, J., Isaya, G., Rosenberg, L. E. & Danpure, C. J. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 10900–10904
- Hartl, F.-U., Pfanner, N., Nicholson, D. W. & Neupert, W. (1989) *Biochim. Biophys. Acta* **988**, 1–45
- Hartl, F.-U. & Neupert, W. (1990) *Science* **247**, 930–938
- Baker, K. P. & Schatz, G. (1991) *Nature (London)* **349**, 205–208
- Das, R. C. & Robbins, P. W. (eds.) *Protein Transfer and Organelle Biogenesis* (1988) Academic Press, London
- Pugsley, A. P. (1989) *Protein Targeting*, Academic Press, London
- Wienhues, U. & Neupert, W. (1992) *Bioessays* **14**, 17–23
- Austen, B. M. & Westwood, O. M. R. (1991) in *Protein Targeting and Secretion* (Richwood, D., ed.), IRL Press, Oxford
- Rothblatt, J. A., Webb, J. R., Ammerer, G. & Meyer, D. I. (1987) *EMBO J.* **6**, 3455–3463
- Rogers, J., Early, P., Carter, C., Calame, K., Bond, M., Hood, L. & Wall, R. (1980) *Cell* **20**, 303–312
- Yost, C. S., Hedgpeth, J. & Lingappa, V. R. (1983) *Cell* **34**, 759–766
- Pelham, H. R. B. (1991) *Trends Biochem. Sci.* **15**, 483–486
- Pfeffer, S. R. & Rothman, J. E. (1987) *Annu. Rev. Biochem.* **56**, 829–852
- Baranski, T. J., Koelsch, G., Hartsuck, J. A. & Kornfeld, S. (1991) *J. Biol. Chem.* **266**, 23365–23372
- Ades, I. Z. & Butow, R. A. (1980) *J. Biol. Chem.* **255**, 9918–9924
- Silver, P. A. & Hall, M. N. (1988) in *Protein Transfer and Organelle Biogenesis* (Das, R. A. & Robbins, P. W., eds.), pp. 749–769. Academic Press, London
- Gould, S. J., Keller, G.-A., Hosken, N., Wilkinson, J. & Subramani, S. (1989) *J. Cell Biol.* **108**, 1657–1664
- Aitchison, J. D., Murray, W. W. & Rachubinski, R. A. (1991) *J. Biol. Chem.* **266**, 23197–23203
- Osumi, T. & Fujiki, Y. (1990) *Bioessays* **12**, 217–222
- Chomyn, A., Mariottini, P., Cleeter, M. W. J., Ragan, C. I., Matsuno-Yagi, A., Hatefi, Y., Doolittle, R. F. & Attardi, G. (1985) *Nature (London)* **314**, 592–597
- Chomyn, A., Cleeter, M. J. W., Ragan, C. I., Riley, M., Doolittle, R. F. & Attardi, G. (1986) *Science* **234**, 614–618
- Fox, T. D. (1986) *Trends Genet.* **2**, 97–100
- Roise, D., Horvath, S. J., Tomich, J. M., Richards, J. H. & Schatz, G. (1986) *EMBO J.* **5**, 1327–1334
- Skerjanc, I. S., Shore, G. C. & Silviu, J. R. (1987) *EMBO J.* **6**, 3117–3123
- von Heijne, G. (1986) *EMBO J.* **6**, 1335–1342
- Karslake, C., Piotto, M. E., Pak, Y. K., Weiner, H. & Gorenstein, D. G. (1990) *Biochemistry* **29**, 9872–9878
- Hurt, E. C., Pesold-Hurt, B., Suda, K., Oppliger, W. & Schatz, G. (1985) *EMBO J.* **4**, 2061–2068
- Keng, T., Alani, E. & Guarante, L. (1986) *Mol. Cell. Biol.* **6**, 355–364
- Franzen, L.-G., Rochaix, J.-D. & von Heijne, G. (1990) *FEBS Lett.* **260**, 165–168
- von Heijne, G., Steppuhn, J. & Herrmann, R. G. (1989) *Eur. J. Biochem.* **180**, 535–545

33. Hurt, E. C., Soltanifar, N., Goldschmidt-Clermont, M., Rochaix, J.-D. & Schatz, G. (1986) *EMBO J.* **5**, 1343–1350
34. Boutry, M., Nagy, F., Poulsen, C., Aoyasi, K. & Chua, N.-H. (1987) *Nature (London)* **328**, 340–342
35. Runswick, M. J., Powell, S. J., Nyran, P. & Walker, J. E. (1987) *EMBO J.* **6**, 1367–1373
36. Pratt, R. D., Ferraira, G. C. & Pedersen, P. L. (1991) *J. Biol. Chem.* **266**, 1276–1280
37. Phelps, A., Schobert, C. T. & Wohlrab, H. (1991) *Biochemistry* **30**, 248–252
38. Hurt, E. C., Muller, U. & Schatz, G. (1985) *EMBO J.* **4**, 3509–3518
39. Nakai, M., Hase, T. & Matsubara, H. (1989) *J. Biochem. (Tokyo)* **105**, 513–519
40. Hurt, E. C. & Schatz, G. (1987) *Nature (London)* **325**, 499–503
41. Baker, A. & Schatz, G. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 3117–3121
42. Hase, T., Muller, U., Riezman, H. & Schatz, G. (1984) *EMBO J.* **3**, 3157–3164
43. Pfanner, N., Hoeben, P., Tropschug, M. & Neupert, W. (1987) *J. Biol. Chem.* **262**, 14851–14854
44. Gavel, Y., Nilsson, L. & von Heijne, G. (1988) *FEBS Lett.* **235**, 173–177
45. Gasser, S. M., Ohashi, A., Daum, G., Bohni, P. C., Gibson, J., Reid, G. A., Yonetani, T. & Schatz, G. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 267–271
46. Hartl, F.-U., Schmidt, B., Wachter, E., Weiss, H. & Neupert, W. (1986) *Cell* **47**, 939–951
47. Hartl, F.-U., Ostermann, J., Guiard, B. & Neupert, W. (1987) *Cell* **51**, 1027–1037
48. Van Loon, A. P. G. M., Brandli, M. W. & Schatz, G. (1986) *Cell* **44**, 801–812
49. Van Loon, A. P. G. M. & Schatz, G. (1987) *EMBO J.* **6**, 2441–2448
50. Pratje, E. & Guiard, B. (1986) *EMBO J.* **5**, 1313–1317
51. Ohashi, A., Gibson, J., Gregor, I. & Schatz, G. (1982) *J. Biol. Chem.* **257**, 13042–13047
52. Nicholson, D. W., Kohler, H. & Neupert, W. (1987) *Eur. J. Biochem.* **164**, 147–157
53. Viebrock, A., Perz, A. & Sebald, W. (1982) *EMBO J.* **1**, 565–571
54. Macino, G. & Tzagoloff, A. (1979) *J. Biol. Chem.* **254**, 4617–4623
55. Lindsay, J. G. (1989) *Ann. N.Y. Acad. Sci.* **573**, 254–266
56. Nicholson, D. W., Hergersberg, C. & Neupert, W. (1988) *J. Biol. Chem.* **263**, 19034–19042
57. Vestweber, D., Brunner, J., Baker, A. & Schatz, G. (1988) *Nature (London)* **341**, 205–209
58. Baker, K. P., Schaniel, A., Vestweber, D. & Schatz, G. (1990) *Nature (London)* **348**, 605–609
59. Kiebler, M., Pfaller, R., Sollner, T., Griffiths, G., Horstmann, H., Pfanner, N. & Neupert, W. (1990) *Nature (London)* **348**, 610–616
60. Sollner, T., Pfaller, R., Griffiths, G., Pfanner, N. & Neupert, W. (1990) *Cell* **62**, 107–115
61. Sollner, T., Griffiths, G., Pfaller, R., Pfanner, N. & Neupert, W. (1989) *Cell* **59**, 1061–1070
62. Pain, D., Murakami, H. & Blobel, G. (1990) *Nature (London)* **347**, 444–449
63. Murakami, H., Blobel, G. & Pain, D. (1990) *Nature (London)* **347**, 488–491
64. Reference deleted
65. Eilers, M. & Schatz, G. (1986) *Nature (London)* **322**, 228–232
66. Eilers, M., Hwang, S. & Schatz, G. (1988) *EMBO J.* **7**, 1139–1149
67. Chen, W.-J. & Douglas, M. G. (1987) *J. Biol. Chem.* **262**, 15605–15609
68. della-Cioppa, G. & Kishore, G. M. (1988) *EMBO J.* **7**, 1299–1305
69. Rassow, J., Guiard, B., Wienhues, U., Herzog, V., Hartl, F.-U. & Neupert, W. (1989) *J. Cell Biol.* **109**, 1421–1428
70. Deshaies, R. J., Koch, B. D., Werner-Washburne, M., Craig, E. A. & Schekman, R. (1988) *Nature (London)* **332**, 800–805
71. Chirico, W. J., Waters, M. G. & Blobel, G. (1988) *Nature (London)* **332**, 805–810
72. Verner, K. & Schatz, G. (1987) *EMBO J.* **6**, 2449–2456
73. Pfanner, N., Tropschug, M. & Neupert, W. (1987) *Cell* **49**, 815–823
74. Reid, G. A. & Schatz, G. (1982) *J. Biol. Chem.* **257**, 13056–13061
75. Schleyer, M. & Neupert, W. (1985) *Cell* **43**, 339–350
76. Schwaiger, M., Herzog, V. & Neupert, W. (1987) *J. Cell. Biol.* **105**, 235–246
77. Ohta, S. & Schatz, G. (1984) *EMBO J.* **3**, 651–657
78. Ono, N. & Tuboi, S. (1988) *J. Biol. Chem.* **263**, 3188–3193
79. Firgaira, F. A., Hendrick, J. P., Kalousek, F., Kraus, J. P. & Rosenberg, L. E. (1984) *Science* **226**, 1319–1322
80. Murakami, K. & Mori, M. (1990) *EMBO J.* **9**, 3201–3208
81. Ribes, V., Romisch, K., Giner, A., Dobberstein, B. & Tollervey, D. (1990) *Cell* **63**, 591–600
82. Poritz, M. A., Bernstein, H., Strub, K., Zopf, D., Wilhelm, H. & Walter, P. (1990) *Science* **250**, 1111–1117
83. Pollock, R. A., Hartl, F.-U., Cheng, M. Y., Ostermann, J., Horwich, A. & Neupert, W. (1988) *EMBO J.* **7**, 3493–3500
84. Witte, C., Jensen, R. E., Yaffe, M. P. & Schatz, G. (1988) *EMBO J.* **7**, 1439–1447
85. Jensen, R. E. & Yaffe, M. P. (1988) *EMBO J.* **7**, 3863–3871
86. Yang, M., Jensen, R. E., Yaffe, M. P., Oppliger, W. & Schatz, G. (1988) *EMBO J.* **7**, 3857–3862
87. Cheng, M. Y., Hartl, F.-U., Martin, J., Pollock, R. A., Kalousek, F., Neupert, W., Hallberg, E. M., Hallberg, R. L. & Horwich, A. L. (1989) *Nature (London)* **337**, 620–625
88. Scherer, P. E., Kreig, U. C., Hwang, S. T., Vestweber, D. & Schatz, G. (1990) *EMBO J.* **9**, 4315–4322
89. Kang, P.-J., Ostermann, J., Shilling, J., Neupert, W., Craig, E. A. & Pfanner, N. (1990) *Nature (London)* **348**, 137–142
90. Craig, E. A., Kramer, J., Shilling, J., Werner-Washburne, M., Holmes, S., Kosic-Smithers, J. & Nicolet, C. M. (1989) *Mol. Cell. Biol.* **9**, 3000–3008
91. Gething, M.-J. & Sambrook, J. (1992) *Nature (London)* **355**, 33–45
92. Gray, M. W. (1989) *Trends Genet.* **5**, 294–299
93. Tabak, H. F. & Grivell, L. A. (1986) *Trends Genet.* **2**, 51–55
94. Paul, M. F., Velours, J., Arselin de Chateaubodeau, G., Aigle, M. & Guerin, B. (1989) *Eur. J. Biochem.* **185**, 163–171
95. Poyton, R. O., Trueblood, C. E., Wright, R. M. & Farrell, L. E. (1988) *Ann. N.Y. Acad. Sci.* **550**, 289–307
96. Rahmatullah, M., Gopalakrishnan, S., Radke, G. A. & Roche, T. E. (1989) *J. Biol. Chem.* **264**, 1245–1251
97. Lawson, J. E., Behal, R. H. & Reed, L. J. (1991) *Biochemistry* **30**, 2834–2839
98. Neagle, J. C. & Lindsay, J. G. (1991) *Biochem. J.* **278**, 423–427
99. Suzuki, H., Hosokawa, Y., Toda, H., Nishikimi, M. & Ozawa, T. (1990) *J. Biol. Chem.* **265**, 8159–8163
100. Suzuki, H., Hosokawa, Y., Nishikimi, M. & Ozawa, T. (1991) *J. Biol. Chem.* **266**, 2333–2338
101. Forsburg, S. L. & Guarente, L. (1989) *Annu. Rev. Cell Biol.* **5**, 153–158
102. Marechal-Drouard, L., Weil, J.-H. & Guillemant, P. (1988) *Nucleic Acids Res.* **16**, 4777–4788
103. Chang, D. D. & Clayton, D. A. (1989) *Cell* **56**, 131–139
104. Grivell, L. A. (1989) *Eur. J. Biochem.* **182**, 477–493
105. Barath, H. & Kuntzel, Z. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1371–1374
106. Kuiper, M. T. R., Akins, R. A., Holtrop, M., De Vries, H. & Lambowitz, A. M. (1988) *J. Biol. Chem.* **263**, 2840–2847
107. Schuster, W. & Brennicke, A. (1987) *EMBO J.* **6**, 2857–2863
108. Vestweber, D. & Schatz, G. (1989) *Nature (London)* **338**, 170–172
109. Johnston, S. A., Anziano, P. Q., Shark, K., Sandford, J. C. & Butow, R. A. (1988) *Science* **240**, 1538–1541
110. Thorsness, P. E. & Fox, T. D. (1990) *Nature (London)* **346**, 376–379
111. Nagley, P. & Devenish, R. J. (1989) *Trends, Biochem. Sci.* **14**, 31–35