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The first twelve amino acids (less than half of the pre-sequence) of an imported mitochondrial protein can direct mouse cytosolic dihydrofolate reductase into the yeast mitochondrial matrix

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Yeast cytochrome *c* oxidase subunit IV (an imported mitochondrial protein) is made as a larger precursor with a transient pre-sequence of 25 amino acids. If this pre-sequence is fused to the amino terminus of mouse dihydrofolate reductase (a cytosolic protein) the resulting fusion protein is imported into the matrix space, and cleaved to a smaller size, by isolated yeast mitochondria. We have now fused progressively shorter amino-terminal segments of the subunit IV pre-sequence to dihydrofolate reductase and tested each fusion protein for import into the matrix space and cleavage by the matrix-located processing protease. The first 12 amino acids of the subunit IV pre-sequence were sufficient to direct dihydrofolate reductase into the mitochondrial matrix, both *in vitro* and *in vivo*. However, import of the corresponding fusion protein into the matrix was no longer accompanied by proteolytic processing. Fusion proteins containing fewer than nine amino-terminal residues from the subunit IV pre-piece were not imported into isolated mitochondria. The information for transporting attached mouse dihydrofolate reductase into mitochondria is thus contained within the first 12 amino acids of the subunit IV presequence.

Key words: dihydrofolate reductase/cytochrome *c* oxidase/subunit IV/pre-sequence/yeast

Introduction

Sorting of proteins into their correct intracellular compartments generally involves 'signal' or 'leader' sequences which are either transiently or permanently attached to the transported polypeptide (Blobel, 1980). This is also true for proteins that are synthesized in the cytoplasm and then imported into mitochondria. Most of these nuclear-coded proteins have amino-terminal presequences which, upon import of the precursor polypeptide, are cleaved off by a chelator-sensitive protease in the mitochondrial matrix (Schatz and Butow, 1983; Hay *et al.*, 1984). The presequence of the nuclear-coded subunit IV of yeast cytochrome *c* oxidase contains 25 amino acids (Maarse *et al.*, 1984) and resembles other mitochondrial pre-sequences in its high content of serine, threonine and basic amino acids as well as its lack of acidic amino acids (e.g., Viebrock *et al.*, 1982; Kaput *et al.*, 1982; Nagata *et al.*, 1983; Sadler *et al.*, 1984; Horwich *et al.*, 1984; Morohashi *et al.*, 1984). By employing gene fusion, we have shown that this pre-sequence is sufficient to direct an attached cytosolic protein into the matrix of isolated yeast mitochondria (Hurt *et al.*, 1984b). Similar conclusions have been reported for the pre-sequence of ornithine transcarbamylase (a protein targeted to mitochondria; Horwich *et al.*, 1985) and for the presequence of the small subunit of ribulose-1,5-diphosphate carboxylase (a protein targeted to chloroplasts; van den Broeck *et al.*, 1985).

How can a mitochondrial presequence direct an attached polypeptide chain into mitochondria? Since the pre-sequences analyzed so far reveal no striking homologies, they may share some three-dimensional conformation that is not immediately obvious from amino acid sequence data. Detection of any common structural feature would be facilitated by defining the shortest possible sequence that can still direct an attached polypeptide into mitochondria. As shown here, the 12 amino-terminal residues of the subunit IV pre-sequence are sufficient to direct cytosolic dihydrofolate reductase into the mitochondrial matrix, both *in vitro* and *in vivo*.

Results

Construction of the fused genes

The gene fragment coding for the transient presequence (25 amino acids) of yeast cytochrome *c* oxidase subunit IV was shortened by limited exonuclease digestion from the end corresponding to the C terminus of the pre-sequence. The truncated gene fragments were then joined in-frame to the 5′ end of the gene coding for mouse dihydrofolate reductase and the fused genes were expressed either *in vitro* (by coupled transcription-translation) or *in vivo* (by transformation of yeast cells). The amino acid sequence of each fusion protein was determined by DNA sequencing of the gene around the fusion site. The derived amino acid sequences of the authentic subunit IV presequence and of the truncated presequences fused to mouse dihydrofolate reductase are shown in Figure 1.

Binding and import of the fusion proteins by isolated mitochondria

The various fusion proteins were synthesized *in vitro* in the presence of [^{35}S]methionine (Figure 2A, lane 9) and tested for binding to the surface of deenergized mitochondria (Figure 2A, lanes 1 and 2) and for import into energized mitochondria (Figure 2A, lanes 3-5). The approximate rate of import was also determined (lanes 6-8). For comparison, Figure 2A includes the corresponding results obtained with the authentic, *in vitro* synthesized subunit IV precursor. Fusion proteins containing at least 12 amino-terminal residues derived from the subunit IV pre-sequence were imported by energized mitochondria, as shown by the resistance against externally added protease in the absence (lane 4), but not the presence (lane 5) of detergent. The rates of import of these fusion proteins into mitochondria (mitochondria were limiting under these conditions; see also accompanying paper) were similar to that of authentic subunit IV precursor (lanes 6-8); however, the extent of import (~20-33% of the total precursor) appeared to be somewhat lower than that of the subunit IV precursor (~60%). These fusion proteins also bound efficiently to the surface of deenergized mitochondria (lane 1); in that case, they remained accessible to externally added protease (lane 2).

As reported earlier (Hurt *et al.*, 1984b), import of the fusion

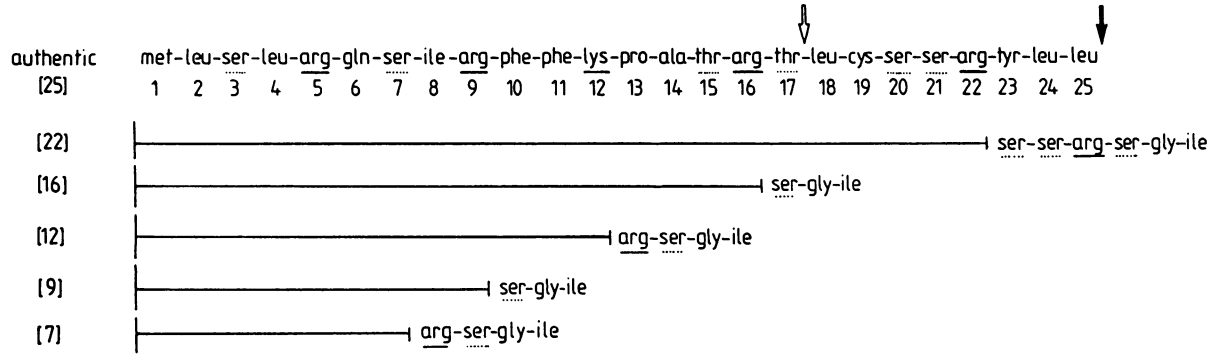

Fig. 1. Amino acid sequence of the authentic subunit IV pre-sequence (25 residues) and of the truncated subunit IV pre-sequences fused to the amino terminus of mouse dihydrofolate reductase. The bracketed numbers on the left denote the number of amino-terminal residues derived from the subunit IV pre-sequence. The amino acids following each bar are those introduced by gene fusion. The sequences shown end immediately before the first amino acid (methionine) of the attached mouse dihydrofolate reductase. Positively charged amino acids are underlined with solid lines, serine and threonine with dotted lines. The two sites cleaved by the mitochondrial processing protease(s) are indicated by the arrows (see Results for further details on these two sites).

protein containing 22 residues derived from the subunit IV pre-sequence was accompanied by cleavage to a shorter form (compare lanes 8 and 9); in contrast, import of the fusion proteins containing only 16 and 12 residues from the subunit IV pre-sequence was not accompanied by cleavage, at least within the detection limit of our gel system. This confirms the previous conclusion that, in experiments with trace amounts of radiolabeled precursors, import does not require proteolytic processing (Reid *et al.*, 1982; Zwizinski and Neupert, 1983). Similarly, fusion proteins containing 16 amino-terminal residues from the subunit IV pre-sequence or less were not cleaved upon incubation with solubilized matrix protease (Figure 2C). The imported, uncleaved precursor had crossed both mitochondrial membranes since it was protected against externally added protease even in mitoplasts (mitochondria whose outer membrane had been disrupted; data not shown). An amino-terminal region of the subunit IV pre-sequence shortened to seven residues no longer promoted import of dihydrofolate reductase by isolated mitochondria (Figure 2A and B); the corresponding fusion proteins thus resembled authentic mouse dihydrofolate reductase which is not imported by mitochondria (Hurt *et al.*, 1984a, Figure 2B; also cf. below). The fusion protein containing nine residues from the amino terminus of pre-subunit IV represented a border-line case; its import into mitochondria was detectable, but the extent of import was marginal (only ~2% of the total). This sudden drop in import suggests that the region between residues 9 and 12 of the subunit IV pre-sequence represents the border of a signal that is necessary for interaction of the precursor with the mitochondrial import machinery.

In general, the ability of the various fusion proteins to bind to the surface of deenergized mitochondria correlated with their ability to be imported. The only partial exception was the fusion protein containing seven residues derived from the subunit IV pre-sequence: while it could not be imported into mitochondria, its binding to deenergized mitochondria was several fold higher (Figure 2B) than that of authentic dihydrofolate reductase (Hurt *et al.*, 1984a). This increased binding could reflect either unspecific association of the positively charged truncated pre-sequence with the mitochondrial phospholipids or some residual targeting function.

Fusion proteins imported into isolated mitochondria are also imported into mitochondria in vivo

Our finding that the import of mouse dihydrofolate reductase into isolated mitochondria could be mediated by less than half of a cleavable authentic pre-sequence raised the question whether our *in vitro* import system was a legitimate test for the import process in living cells. To check this, the genes for authentic mouse dihydrofolate reductase and for the fusion proteins containing either 22 or 12 amino-terminal residues derived from the subunit IV pre-sequence were introduced into yeast cells by transformation and the steady-state subcellular distributions of the corresponding protein products were analyzed by subcellular fractionation and immune blotting with an antiserum against mouse dihydrofolate reductase. This antibody did not cross-react with yeast dihydrofolate reductase (see also Figure 3D lower panel).

Cross-contamination of mitochondria and cytosol was checked by immune blotting with antisera against hexokinase (a cytosolic protein) and citrate synthase (a mitochondrial matrix protein) and was found to be low (Figure 3, upper panels). As expected, authentic mouse dihydrofolate reductase behaved as a typical cytosolic protein; in contrast, the two fusion proteins co-fractionated with the mitochondrial particles and the mitochondrial marker, citrate synthase (Figure 3).

The two fusion proteins were transported across both mitochondrial membranes *in vivo*. First, both fusion proteins were inaccessible to externally added protease even in mitoplasts which had lost the outer membrane barrier (Figure 4, lower panels, lanes 2 and 3) and were not found in the intermembrane space fraction (Figure 4, lower panels, lanes 5). Second, dihydrofolate reductase activity of the shorter fusion protein (we only tested this fusion protein) was latent in mitochondria and mitoplasts, but could be stimulated >20-fold upon solubilization of the inner mitochondrial membrane; this indicated that externally added NADPH and dihydrofolate could only reach the enzyme upon disruption of the inner membrane (the outer membrane is freely permeable to these molecules) (Table I). In control experiments, mitochondria isolated from yeast cells expressing authentic dihydrofolate reductase from mouse had low levels of the dihydrofolate reductase activity which was not stimulated by detergents. Instead, these transformants had high levels of dihydrofolate reductase activity in the cytoplasm and this soluble activity was not affected by detergents (Table I).

The longer fusion protein was completely processed after import into mitochondria and became soluble within the mitochondrial matrix (i.e., it co-fractionated with matrix marker citrate synthase, data not shown). The shorter fusion protein was partially cleaved to a smaller form *in vivo* (Figures 3 and 4, lower

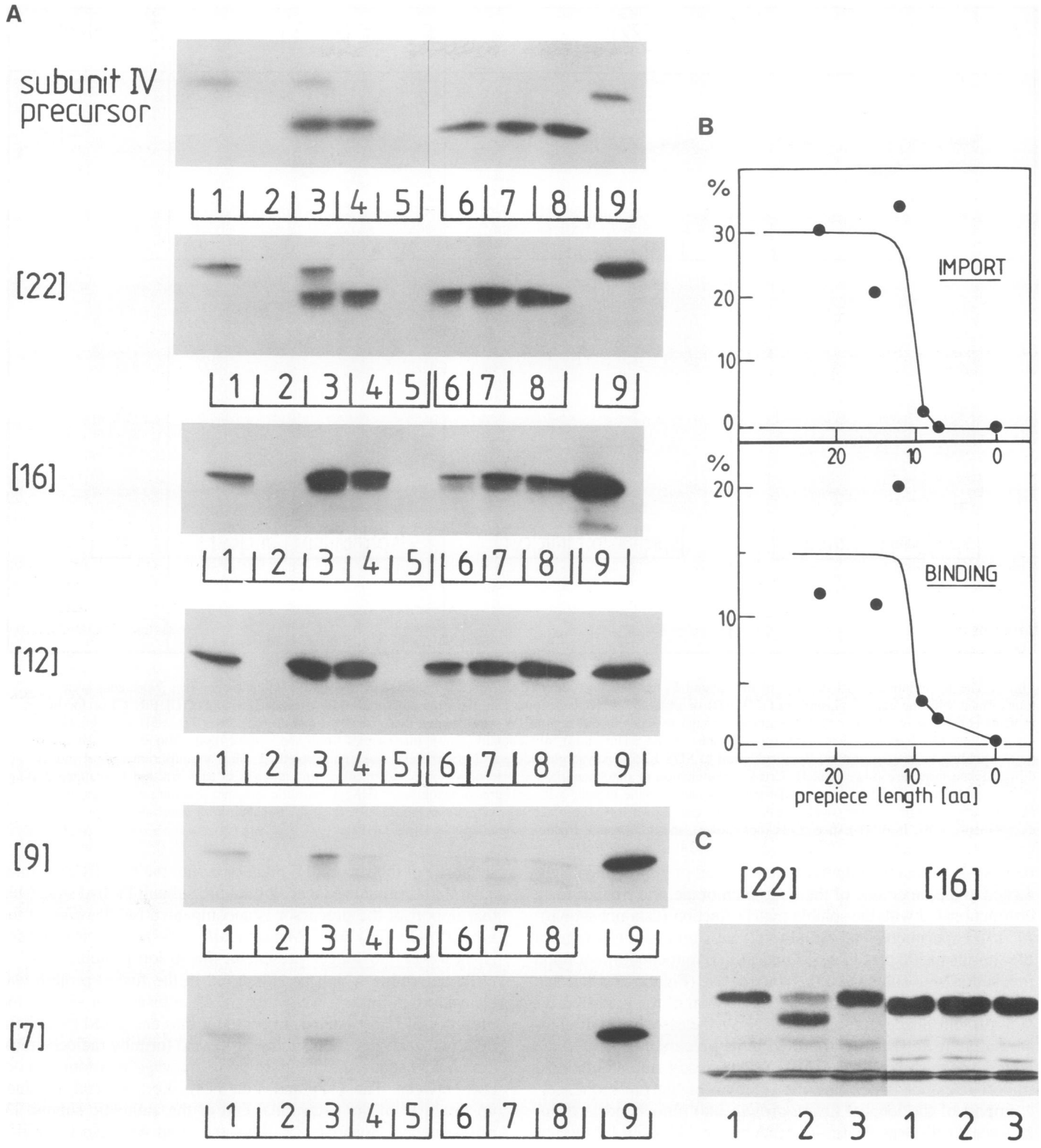

Fig. 2. The 12 amino-terminal residues of the subunit IV pre-sequence direct mouse dihydrofolate reductase into isolated mitochondria. **(A)** Authentic subunit IV precursor or fusion proteins containing different numbers (left margin) of amino-terminal residues of the subunit IV pre-sequence fused to dihydrofolate reductase were synthesized in the coupled transcription-translation system in the presence of [^{35}S]methionine and incubated with isolated yeast mitochondria for 30 min as indicated below and in Materials and methods. The products were analyzed by SDS-12.5% polyacrylamide gel electrophoresis and fluorography. **1**, deenergized mitochondria (binding assay); **2**, deenergized mitochondria, proteinase K; **3**, energized mitochondria (import assay); **4**, energized mitochondria, proteinase K; **5**, energized mitochondria, proteinase K, Triton X-100; **6,7,8**, same as **4** except that incubation was for 5, 10 and 20 min, respectively; **9**, 33% of the total translation product. **(B)** Import and binding of the various fusion proteins by isolated mitochondria. The labeled bands (shown in **A**) were quantified and expressed as % of the total fusion protein added to the mitochondria during each assay. **(C)** Processing of *in vitro* synthesized fusion proteins carrying 22 and 16 amino-terminal amino acids from the subunit IV pre-piece by the mitochondrial matrix fraction containing the processing enzyme. **1**, no protease; **2**, with processing enzyme; **3**, with processing enzyme and 2 mM 1,10-phenanthroline. The processing assay contained, in a final volume of 10 μl, 6 μg matrix protease (purified through step 2, cf. Böhni *et al.*, 1983) and 6 μl of radiolabeled transcription-translation mixture. After 30 min incubation at 30°C, the mixture was dissociated in SDS-containing sample buffer and analyzed as described in Hurt *et al.* (1984a).

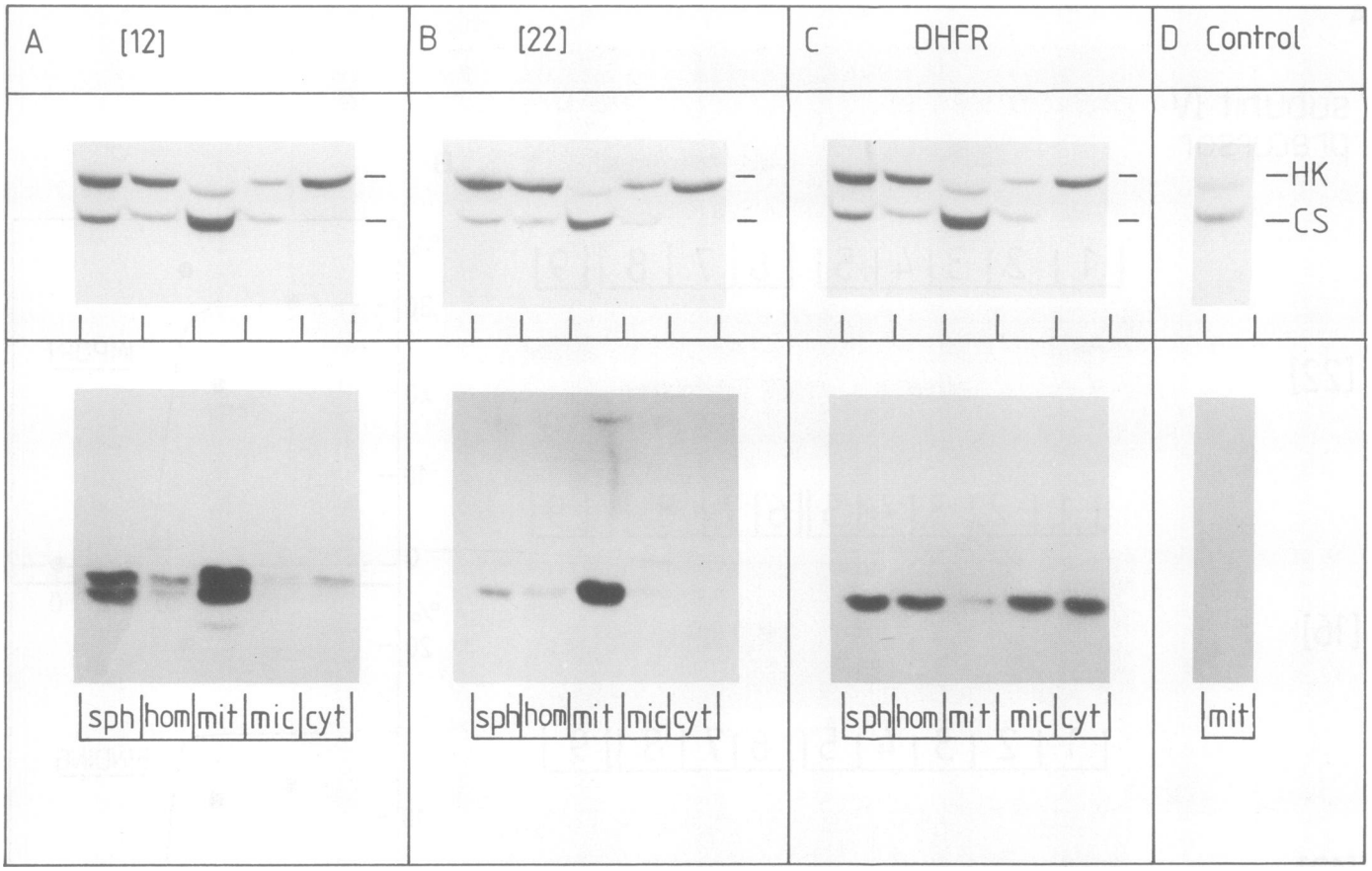

Fig. 3. The 12 amino-terminal residues of the subunit IV pre-sequence direct dihydrofolate reductase into yeast mitochondria *in vivo*. Yeast cells were transformed with the control plasmid pLGSD5 (**D**) or pLGSD5-derivatives containing the genes for mouse dihydrofolate reductase (DHFR; **C**) or for fusion proteins consisting of 12 (**A**) or 22 (**B**) amino-terminal residues of the subunit IV pre-sequence fused to dihydrofolate reductase. The cells were grown in liquid culture and fractionated into spheroplasts (sph), homogenate (hom), mitochondria (mit), microsomes (mic) and cytosol (cyt). Aliquots of each fraction (corresponding to 260 μg protein) were subjected to SDS-12.5% polyacrylamide gel electrophoresis and immunoblotting using an antiserum against mouse dihydrofolate reductase (lower panel). Cross-contamination of mitochondria and cytosol was checked by subjecting aliquots of each fraction (containing 260 μg protein) to SDS-polyacrylamide gel electrophoresis and immune blotting with antisera to hexokinases (HK; a cytosolic enzyme) and citrate synthase (CS; a mitochondrial enzyme; upper panel). In the experiment shown in **C** the percentage of cellular mouse DHFR co-fractionating with microsomes was exceptionally high. In other experiments (not shown), the microsomal fraction contained much less DHFR.

panels). Whereas the imported intact fusion protein remained attached to the inner side of the inner membrane, the smaller form co-fractionated with the soluble matrix fraction (data not shown). *In vitro* experiments had failed to reveal significant cleavage of the shorter fusion protein upon import into mitochondria or upon incubation with solubilized matrix protease (Figure 2). Cleavage *in vivo* may reflect non-specific degradation of the imported fusion protein inside mitochondria. Nevertheless, the shorter fusion protein was mainly imported in its precursor form into the matrix space showing that, even under steady-state conditions, import does not depend on the removal of the pre-sequence.

Import of the longest fusion protein into mitochondria of intact yeast cells was energy-dependent (i.e., blocked by the uncoupler CCCP) and rapid (i.e., most of the precursor was already processed after a pulse of 4 min) (Figure 5). The *in vivo* import of this fusion protein thus resembled that of authentic mitochondrial precursor polypeptides (Reid and Schatz, 1982; Yaffe and Schatz, 1984).

The subunit IV pre-sequence contains two potential sites for cleavage by mitochondrial processing protease(s)

It was not expected that the fusion protein containing 22 residues from the subunit IV pre-sequence would be cleaved upon import into mitochondria. A comparison of the deduced amino acid sequence of the subunit IV precursor with the directly determined amino-terminal sequence of mature subunit IV had suggested that import of the precursor is accompanied by cleavage of the Leu (25)-Gln (26) bond (Maarse *et al.*, 1984). This cleavage site is, however, no longer present in the fusion protein.

To determine which peptide bond in the fusion protein was cleaved upon import, we synthesized the protein *in vitro* in the presence of [^{35}S]cysteine, imported it into energized mitochondria, and analyzed the resulting processed form by radiochemical amino acid sequencing (Figure 6A). Cleavage was found to occur between Thr (17) and Leu (18), i.e., several residues upstream from the C-terminal end of the authentic subunit IV pre-sequence. Surprisingly, the same bond was also cleaved if authentic subunit IV precursor was cleaved by a mitochondrial matrix fraction (Figure 6B, open bars). However, when the authentic subunit IV precursor was imported into mitochondria, cleavage occurred between Leu (25) and Gln (26), (Figure 6B, filled bars), i.e., at the bond predicted earlier (Maarse *et al.*, 1984). When the N-distal cleavage site of subunit IV precursor was rendered non-functional by the introduction of five amino acids immediately upstream (two of them were acidic), the modified precursor was cleaved at the N-proximal Thr (17)-Leu (18) site, both by a matrix fraction and upon import into energized mitochondria (Figure 6C).

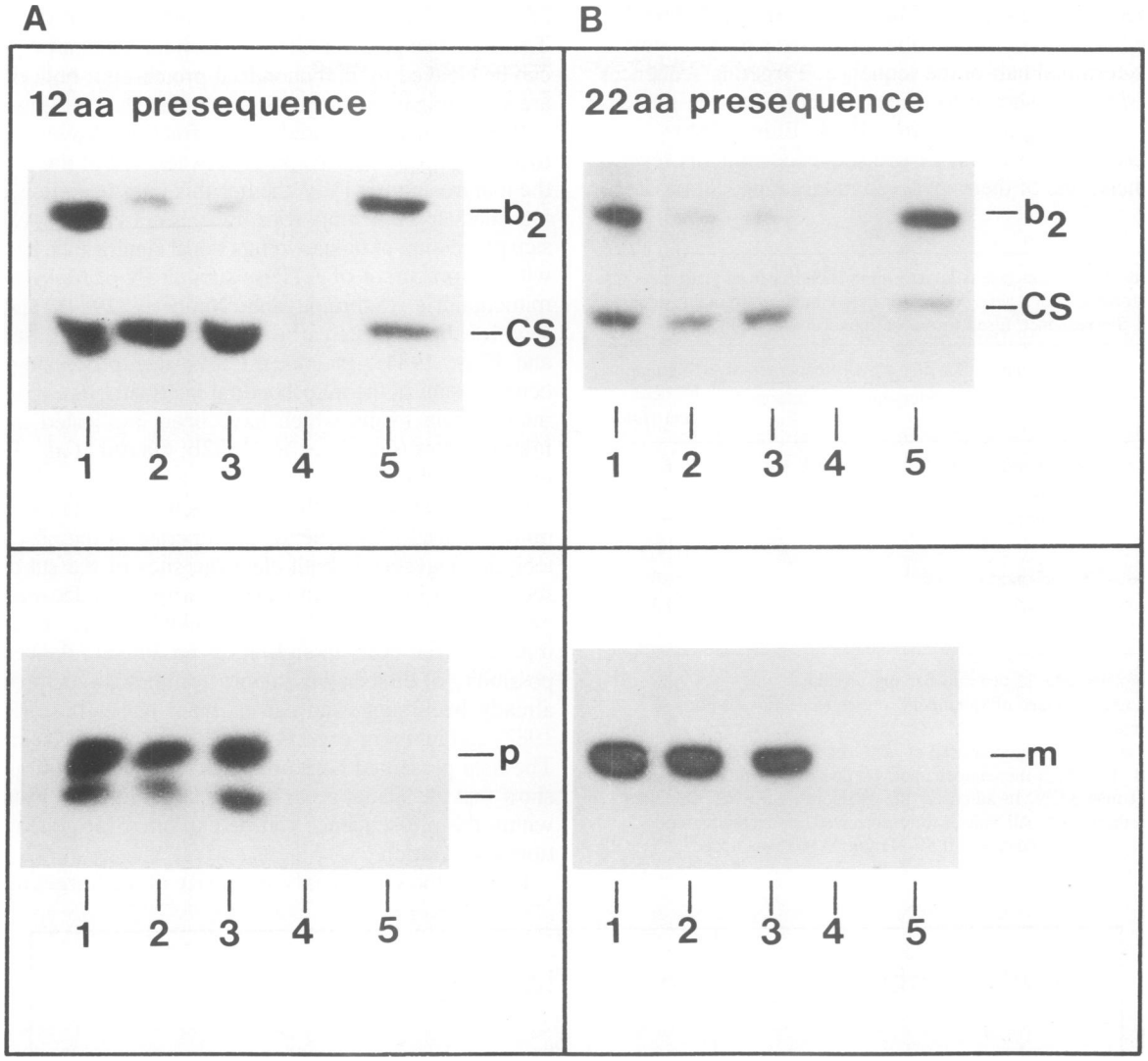

Fig. 4. The amino-terminal 22 or 12 amino acids of the subunit IV pre-sequence transport attached dihydrofolate reductase across both mitochondrial membranes *in vivo*. Mitochondria from yeast cells transformed with the appropriate fused genes were subfractionated into the various compartments. Fractions equivalent to 100 μg of mitochondrial protein were analyzed by SDS-12.5% polyacrylamide gel electrophoresis and immune blotting with antisera against the following proteins: upper panel: cytochrome b_2 (intermembrane space marker) and citrate synthase (matrix marker); lower panel: mouse dihydrofolate reductase. **1**, mitochondria; **2**, mitoplasts; **3**, mitoplasts treated with proteinase K; **4**, mitoplasts treated with proteinase K and Triton X-100; **5**, intermembrane space fraction. p and m, precursor and processed ('mature') form of the two fusion proteins. **A** and **B**, fusion proteins containing 12 or 22 amino-terminal residues of the subunit IV presequence fused to the amino terminus of dihydrofolate reductase.

The data of Figure 6 allow the following conclusions. (i) The subunit IV pre-sequence contains two potential cleavage sites for mitochondrial processing enzyme(s). (ii) Authentic subunit IV precursor is cleaved at the N-distal site during import into mitochondria and at the N-proximal site by soluble matrix protease. (iii) If the N-distal site is removed by gene fusion or inactivated by site-directed mutagenesis, the N-proximal site is cleaved both by soluble matrix protease and upon import into mitochondria. We are currently trying to identify the protease(s) catalyzing these two distinct cleavages within the subunit IV precursor.

Discussion

The present study shows that only part of the pre-sequence of cytochrome *c* oxidase subunit IV is required to transport an attached cytosolic enzyme into the mitochondrial matrix. This was shown both by *in vitro* and *in vivo* experiments. The shortest segment that proved to be active in our studies contained only the 12 amino-terminal residues of the 25-residue subunit IV presequence.

Does this dodecapeptide represent a 'minimal targeting' sequence? We cannot answer this for at least two reasons. First, our data leave open the possibility that even fewer than 12 amino acids might be sufficient since truncated subunit IV pre-sequences retaining 10 or 11 amino-terminal residues were not tested. It is also possible that the segment containing only nine residues might be at least partly active *in vivo* even though it was largely inactive with isolated mitochondria. Second, gene fusion experiments can always be criticized on the grounds that joining two polypeptide chains can have unexpected effects. For example, the amino terminus of mouse dihydrofolate reductase may either fortuitously contribute to the targeting function of an attached truncated pre-sequence or, conversely, interfere with the targeting function. Such effects would decrease or increase estimates of the length of a 'minimal targeting sequence'.

However, the major point of this paper is not the exact length

of a 'minimal targeting sequence' but the surprising finding that the targeting function of the subunit IV pre-sequence is located in the amino-terminal half of the sequence. Targeting sequences of similar size were shown to direct proteins into the nucleus (Hall *et al.*, 1984; Kalderon *et al.*, 1984; Ellis, 1985).

We suggest that the subunit IV pre-sequence contains several types of signals, one of them involved in targeting. Some of the additional signals appear to be related to proteolytic processing. There are two potential sites at which the subunit IV pre-sequence can be cleaved by mitochondrial protease(s); both of these sites are C-terminal from the targeting sequence (Figure 7).

We find that an isolated matrix fraction cleaves the precursor to a polypeptide whose size is between that of the precursor and the mature subunit IV. Whether this intermediate is a *bona fide* intermediate in the import pathway is not known. A similar two-step processing of unknown functional significance has been noted with the precursor of ATPase subunit IX of *Neurospora crassa* mitochondria (Schmidt and Neupert, 1984) and of ribulose-1,5-diphosphate carboxylase of pea chloroplasts (Robinson and Ellis, 1984). In contrast, two-step processing of several hemoproteins of the mitochondrial intermembrane space generates intermediate forms which have been implicated as functional intermediates (Gasser *et al.*, 1982b; Ohashi *et al.*, 1982; Daum *et al.*, 1982b).

Our data also show that the targeting function of a cleavable mitochondrial pre-sequence can operate in the absence of proteolytic cleavage. If both cleavage sites of the subunit IV pre-sequence are deleted, the amino-terminal dodecapeptide of the pre-sequence can still direct dihydrofolate reductase into mitochondria even though it is no longer cleaved off. The possibility of dissociating import from proteolytic processing had already been suggested earlier from pulse-chase (Reid *et al.*, 1982) and inhibitor experiments (Zwizinski and Neupert, 1983). The data presented here are particularly compelling since they show that the spatial separation of targeting and cleavage signals within the pre-sequence can be exploited for genetic and functional separation.

Finally, the surprisingly small size of the 'targeting sequence'

Table I. Dihydrofolate reductase is latent in mitochondria from yeast cells that had been transformed with a gene coding for the 12 amino-terminal residues of the subunit IV pre-sequence fused to mouse dihydrofolate reductase

Yeast transformed with	Subcellular fraction	Specific dihydrofolate activity[a]		Stimulation by detergent (fold)
		− detergent	+ detergent	
Subunit IV-dihydrofolate reductase fusion gene[b]	Mitochondria	1.35	29.2	21.6
	Mitoplasts	0.94	20.1	21.3
	Cytoplasm	4.25	3.8	0.9
Authentic mouse dihydrofolate reductase gene	Mitochondria	1.06	0.3	0.28
	Cytoplasm	11.6	9.7	0.83

[a]nmol dihydrofolate reduced per min per mg protein.
[b]12 amino-terminal residues of subunit IV presequence fused to mouse dihydrofolate reductase.
Dihydrofolate reductase was measured at 22°C in 50 mM Tris-HCl pH 8.0, 0.6 M sorbitol, 12 mM β-mercaptoethanol, 60 μM dihydrofolate, 50 μM NADPH and 1 mM KCN. In all cases, the reaction was >90% inhibited by 200 nM methotrexate. All values were corrected for methotrexate-resistant activity. Where indicated, 0.5% Triton X-100 was added ('+ detergent').

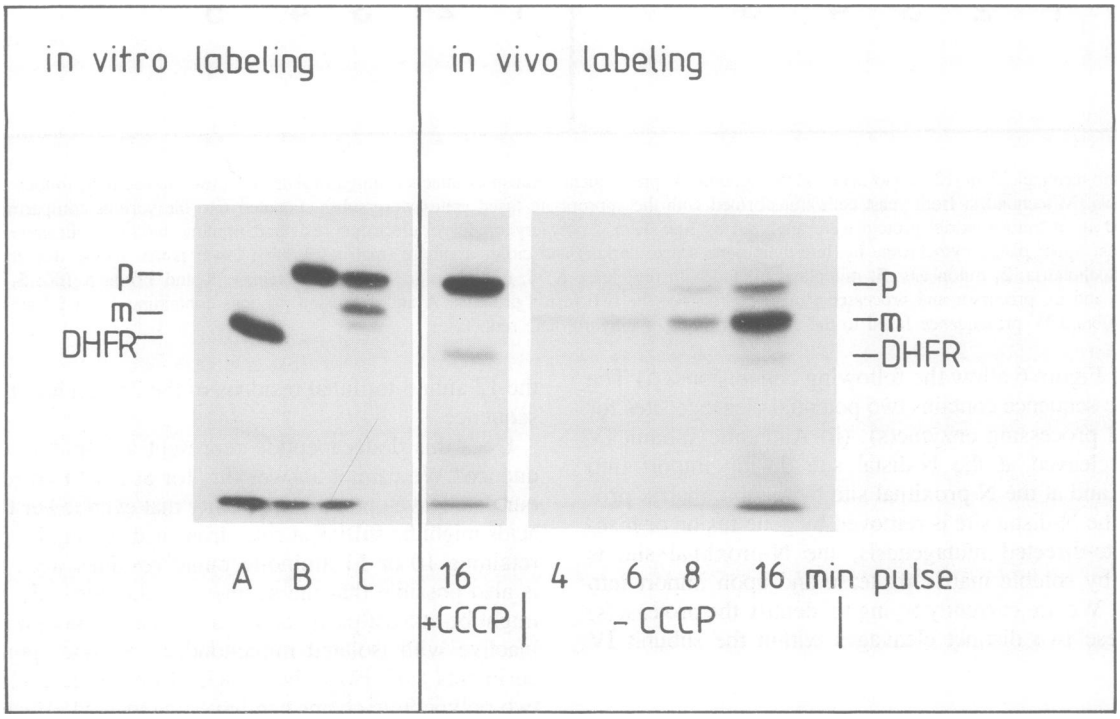

Fig. 5. The 22 amino-terminal residues of the subunit IV pre-sequence mediate the efficient, energy-dependent import of attached dihydrofolate reductase into yeast mitochondria *in vivo*. ***In vivo* labeling**: yeast cells transformed with a plasmid carrying the fused gene were either labeled with [^{35}S]methionine for 16 min in the presence of 20 μM CCCP or for 4, 6, 8 and 16 min in the absence of CCCP. They were then opened and analyzed by immunoprecipitation with an antiserum to mouse dihydrofolate reductase, SDS-12.5% polyacrylamide gel electrophoresis, and fluorography. ***In vitro* labeling**: for comparison, authentic dihydrofolate reductase **(A)** and the fusion protein **(B)** were synthesized *in vitro* in the presence of [^{35}S]methionine and the fusion protein was processed by partially purified matrix protease **(C)**. **Lanes A–C** are from the same gel slab as the lanes shown on the panel on the right. p, precursor; m, processed ('mature') form; DHFR, authentic dihydrofolate reductase.

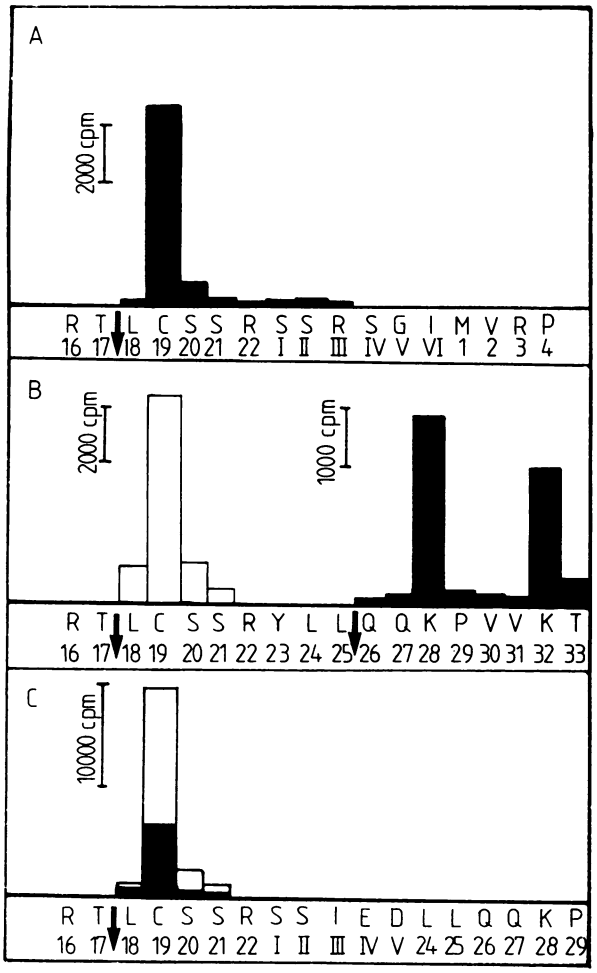

Fig. 6. The pre-sequence of cytochrome *c* oxidase subunit IV contains two cleavage sites for mitochondrial processing protease(s). The proteins mentioned below were synthesized in the coupled transcription-translation system with the labeled amino acid(s) indicated below and processed by either isolated yeast mitochondria or by incubation with a mitochondrial matrix fraction containing the processing enzyme. The processed products were resolved by SDS-polyacrylamide gel electrophoresis located by autoradiography, excised from the gel slab, eluted from the gel slices and radiosequenced by manual Edman degradation (Cerletti *et al.*, 1983). Recovery of radioactivity per radiolabeled amino acid varied between 30 and 50%. Filled bars: radioactivity released from processed products generated by import into intact mitochondria. Open bars: radioactivity released from processed products generated by isolated matrix fractions. (**A**) Fusion protein containing the 22 amino-terminal residues of the subunit IV pre-sequence fused to dihydrofolate reductase (Hurt *et al.*, 1984b); labeled with [^{35}S]cysteine. (**B**) Authentic subunit IV precursor labeled with [^{35}S]cysteine and [^{3}H]lysine. (**C**) Subunit IV precursor which had been altered by inserting five extra amino acids upstream of the second processing site (see text); labeled with [^{35}S]cysteine. Arabic numerals 16–33 denote amino acid residues of the subunit IV precursor, Arabic numerals 1–4 amino acid residues of dihydrofolate reductase, and Roman numerals amino acids introduced by gene manipulations. Arrows identify proteolytic cleavage sites.

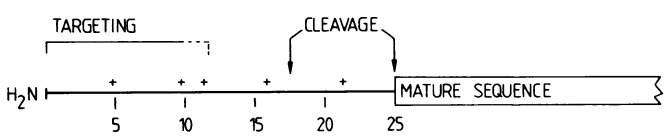

Fig. 7. Suggested distribution of targeting and cleavage sites in the pre-sequence of cytochrome *c* oxidase subunit IV. The numerals below the line indicate the number of amino acids counted from the amino terminus. The + signs on the top of the line identify positively charged amino acids.

found in this study should make it easier to analyze the structure and function of a mitochondrial pre-sequence that mediates transmembrane movement of an attached protein. We are now planning to synthesize large amounts of a dodecapeptide by chemical methods; the conformation of such a small oligopeptide could be analyzed much more easily than that of the intact pre-sequence or the entire subunit IV precursor molecule. The information obtained from such experiments should allow further insights into how mitochondrial pre-sequences function in the intracellular sorting of proteins.

Materials and methods

Strains and plasmids

The *Escherichia coli* strains used were HB 101 (Kedes *et al.*, 1975) and JM 101 (Messing, 1979). For expressing chimeric genes we used the *Saccharomyces cerevisiae* strain YNN 214 (*ura 3, lys 2, ade 2*) which was kindly provided by Mark Johnston and Ron Davis (Stanford, USA). Mitochondria for import studies were prepared from the *S. cerevisae* strain D 273-10B (ATCC 25657). Plasmids pDS 5/2 (Stueber *et al.*, 1984), pDS 5/2-1-Cox IV and pDS 5/2-1-PCOX IV-DHFR (Hurt *et al.*, 1984a, 1984b) and pLGSD5 (Guarente *et al.*, 1982) have been described.

DNA manipulations

Restriction, ligation, end-filling, *Bal*31 exonuclease digestion, agarose gel electrophoresis, etc., were done essentially as described by Maniatis *et al.* (1982). Small-scale and large-scale isolation of plasmids from transformed *E. coli* was done according to Birnboim and Doly (1979) and DNA sequencing by the dideoxy method as outlined by Hase *et al.* (1983).

Construction of fused genes

Fused genes coding for mouse dihydrofolate reductase containing various amino-terminal segments derived from the pre-sequence of yeast cytochrome *c* oxidase subunit IV were prepared from plasmid pDS 5/2-1-Cox IV as starting material. This plasmid carries the gene of the authentic subunit IV precursor under control of a phage T_5 promoter (Hurt *et al.*, 1984a). The plasmid was cut at its single *Eco*RV site which corresponds to amino acid 23 of the pre-sequence. The ends were digested for various time periods with *Bal*31 exonuclease, the shortened ends were filled in with the large fragment of *E. coli* DNA polymerase, and the linear plasmid was cut with *Hind*III and the large fragment (containing the truncated DNA fragments coding for the subunit IV pre-sequence) was isolated (Dretzen *et al.*, 1981). To obtain the gene for mouse dihydrofolate reductase, pDS 5/2 was cut with *Bam*HI, the ends were filled in as described above, the linear plasmid was cut with *Hind*III, and the smaller of the two fragments was isolated. This fragment (which contains the intact gene for dihydrofolate reductase) was ligated to the large DNA fragment (cf. above) carrying the truncated genes for the subunit IV pre-sequence. In order to screen for in-frame fusions, plasmids were used as templates for the *in vitro* transcription-translation system (Stueber *et al.*, 1984); plasmids carrying fusion genes coding for proteins of mol. wt. between 20 000 and 25 000 were used for DNA sequencing around the junction region.

Site-directed mutagenesis of the gene coding for the subunit IV precursor

A pDS 5/2-1-Cox IV derivative carrying an *Xho*I linker at the unique *Eco*RV site (Hurt *et al.*, 1984b) was cut with *Xho*I, the ends were filled in as described above, and the plasmid was recircularized. This introduced five additional amino acids without changing the reading frame (Figure 6C).

Expression of fused genes in yeast

The fused genes containing either 22 or 12 amino-terminal residues of the subunit IV pre-sequence linked to dihydrofolate reductase were recloned into the *E. coli*-yeast 'shuttle' vector pLGSD5, thereby placing them under control of a promoter inducible by galactose. pDS 5/2-1-PCOX IV-DHFR (containing the larger of the two above-mentioned fused genes) was cut at the unique *Pst*I site and ~870 nucleotides were removed from the 5'-non-coding region by digestion with *Bal*31. The plasmid coding for the shorter of the two above-mentioned fused genes (prepared as described in section 'Construction of fused genes') was cut with *Xho*I and ~170 nucleotides were removed by digestion with *Bal*31. These digestions removed interfering ATG codons in the 5'-non-coding region of the fused genes. Both plasmids were then cut with *Hind*III, the ends were filled in, and ligated to *Bam*HI linkers (New England Biolabs). After digestion with *Bam*HI, the small fragments containing the fused genes were isolated, recut with *Bam*HI, and inserted into the unique *Bam*HI site of pLGSD5. For control purposes, the gene for mouse dihydrofolate reductase (in the form of pDS 5/2 cut with *Bam*HI) was similarly inserted into the *Bam*HI site of pLGSD5. The pLGSD5-derived plasmids carrying the inserted genes in the correct orientation were introduced

into the yeast strain YNN 214 by transformation; transformants were selected on uracil-free plates and tested for expression of fusion genes or mouse dihydrofolate reductase by extraction with NaOH, SDS-polyacrylamide gel electrophoresis and immune blotting.

Yeast growth and subcellular fractionation

Yeast transformants were grown at 30°C to an OD 600 of ~2 on 0.67% Yeast Nitrogen Base (Difco), 0.05% glucose, 2% galactose, 30 μg lysine/ml and 20 μg adenine/ml. One generation before harvesting the cells, the growth medium was adjusted to 0.5% yeast extract and 2% galactose. The cells were converted to spheroplasts and subfractionated as described (Hase *et al.*, 1984). 1 g (wet weight) of cells yielded ~2.5 mg of mitochondrial protein, 1 mg of microsomal protein, and 25 mg of cytosolic protein (determined according to Lowry *et al.*, 1951). For subfractionation, mitochondria were resuspended to 10 mg/ml in 0.6 M mannitol, 20 mM Hepes pH 7.4, diluted 10-fold with 20 mM Hepes, pH 7.4 and kept on ice for 30 min. The resulting shocked mitochondria (mitoplasts) were separated from the contents of the soluble intermembrane space by centrifugation and further fractionated into soluble matrix and membranes (Daum *et al.*, 1982a).

Mitochondrial import experiments

Mitochondrial import assays were done essentially as described (Gasser *et al.*, 1982a), but using the coupled transcription-translation system (Stueber *et al.*, 1984; Hurt *et al.*, 1984a). Under these conditions, import of precursors is roughly proportional to the amount of mitochondria added. Mitochondria were deenergized with valinomycin (1 μg/ml). For protease digestion, the mixture was incubated for an additional 30 min at 0°C with 250 μg/ml proteinase K. Protease digestion was stopped with 1 mM phenyl methyl sulfonylfluoride and mitochondria were re-isolated through a 0.6 ml cushion of 20% sucrose. Where indicated, mitochondria or mitoplasts were first isolated by centrifugation and then incubated with proteinase K in the presence of 1% Triton X-100. (Essentially the same digestion protocol was also used to probe the submitochondrial location of unlabeled proteins by immune blotting.) The pelleted mitochondria were then analyzed for radiolabeled polypeptides by SDS-12.5% polyacrylamide gel electrophoresis and fluorography.

Miscellaneous

Published procedures were used for *in vitro* transcription/translation of genes cloned in pDS 5/2 (Stueber *et al.*, 1984; Hurt *et al.*, 1984a), for SDS-polyacrylamide gel electrophoresis, fluorography and immunoprecipitation (Gasser *et al.*, 1982a), for quantitative immune blotting (Suissa, 1983), for pulse-labeling of yeast cells (Yaffe and Schatz, 1984), for isolation of mitochondrial matrix and matrix processing enzyme (Böhni *et al.*, 1983), for radiosequencing of *in vitro*-synthesized and of processed, radiolabeled precursor polypeptides (Cerletti *et al.*, 1983) and for transformation of yeast with pLGSD5 derivatives (Ito *et al.*, 1983). Dihydrofolate reductase activity was measured as described by Hillcoat *et al.* (1967) except that 1 mM KCN and 0.6 M sorbitol were included in the assay medium. L-[^{35}S]methionine (>1000 Ci/mmol) and L-[^{3}H]lysine (>90 Ci/mmol) were purchased from Amersham, UK. L-[^{35}S]cysteine (>200 Ci/mmol) was prepared after Crawford (1973).

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