The EMBO Journal vol.4 no.13A pp.3509–3518, 1985

The first twelve amino acids of a yeast mitochondrial outer membrane protein can direct a nuclear-encoded cytochrome oxidase subunit to the mitochondrial inner membrane

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Communicated by G.Schatz

We have used an *in vivo* complementation assay to test whether a given polypeptide sequence can direct an attached protein to the mitochondrial inner membrane. The host is a previously described yeast deletion mutant that lacks cytochrome oxidase subunit IV (an imported protein) and, thus, neither assembles cytochrome oxidase in its mitochondrial inner membrane nor grows on the non-fermentable carbon source, glycerol. Growth on glycerol and cytochrome oxidase assembly are restored to the mutant if it is transformed with the gene encoding authentic subunit IV precursor, a protein carrying a 25-residue transient pre-sequence. No restoration is seen with a plasmid encoding a subunit IV precursor whose pre-sequence has been shortened to seven residues. Partial, but significant restoration is achieved by an artificial subunit IV precursor in which the authentic pre-sequence has been replaced by the first 12 amino acids of a 70-kd protein of the mitochondrial outer membrane. If this dodecapeptide is fused to the amino terminus of mouse dihydrofolate reductase (a cytosolic protein), the resulting fusion protein is imported into the matrix of yeast mitochondria *in vitro* and *in vivo*. Import *in vitro* requires an energized inner membrane. We conclude that the extreme amino terminus of the 70-kd outer membrane protein can direct an attached protein across the mitochondrial inner membrane.

Key words: mitochondrial outer membrane protein/cytochrome *c* oxidase subunit IV/dihydrofolate reductase/yeast

Introduction

Import of proteins from the cytoplasm into mitochondria seems to follow distinct routes (Neupert and Schatz, 1981). Polypeptides which are transported to intramitochondrial locations are generally made as larger precursors with amino-terminal pre-sequences. Import of these precursors is energy-dependent and followed by proteolytic removal of the pre-sequence (Schatz and Butow, 1983). Polypeptides of the mitochondrial outer membrane lack transient pre-sequences. Their insertion into the outer membrane is specific and does not require an energized mitochondrial inner membrane (Freitag *et al.*, 1982; Mihara *et al.*, 1982; Gasser and Schatz, 1983).

Where is the information which directs proteins into mitochondria? Strong evidence indicates that the cleavable pre-sequence of several mitochondrial precursor proteins is sufficient to direct an attached polypeptide into the mitochondrial interior (Hurt *et al.*, 1984a, 1985; Horwich *et al.*, 1985; van Loon *et al.*, 1985). This conclusion, however, cannot be directly applied to outer membrane proteins since these proteins lack cleavable pre-sequences. We had previously shown that the correct targeting and anchoring of a major 70-kd protein of the yeast mitochondrial outer membrane required only the first 41 amino acids of that protein (Riezman *et al.*, 1983; Hase *et al.*, 1984). This amino-terminal region includes two distinct sequences: an amino-terminal stretch of 11 residues which are predominantly hydrophilic and basic, and an immediately following stretch of 27 uncharged residues (Hase *et al.*, 1983). Deleting this uncharged stretch of the 70-kd protein (e.g., amino acid residues 12 – 106) appeared to misdirect a fraction of the mutated molecules into the mitochondrial matrix space (Hase *et al.*, 1984). Based on these observations, we had suggested the following: (i) import into a distinct intramitochondrial compartment is not mediated by compartment-specific 'import receptors'; (ii) the extreme amino terminus of the 70-kd protein functions as a targeting sequence whereas the subsequent uncharged region functions as an anchoring sequence which retains the protein in the outer membrane.

We now provide direct evidence that the first 12 amino acids of the 70-kd protein function as a mitochondrial targeting sequence. This dodecapeptide sequence resembles a cleavable mitochondrial pre-sequence in being hydrophilic, rich in basic and hydroxylated amino acids, and in being devoid of acidic amino acids (Figure 1). If the dodecapeptide is fused to two different polypeptides which by themselves cannot enter mitochondria, the resulting fusion proteins are imported into the mitochondrial interior, both *in vivo* and *in vitro*. Import into an internal mitochondrial location was verified by two independent criteria: energy-dependence of import, and restoration of an enzymic function associated with the mitochondrial inner membrane.

Results

The experimental approach: construction and testing of fusion genes

An oligonucleotide encoding the first 12 amino acids of the 70-kd protein was synthesized and joined in-frame to the 5′ end of the genes encoding (i) cytosolic mouse dihydrofolate reductase and (ii) a truncated subunit IV precursor lacking the first 19 amino acids of its transient pre-sequence. A gene encoding only the truncated subunit IV precursor ('pseudo-mature' subunit IV) lacking amino acids 2 – 19 of the pre-sequence was also constructed. The predicted amino acid sequences of the corresponding proteins around the fusion or deletion sites are shown in Figure 2. The modified genes were placed under the control of a yeast promoter and expressed *in vivo* after transformation of yeast cells. The subcellular distribution of the fusion proteins was then analyzed by cell fractionation and immunoblotting. Targeting of subunit IV-related proteins to the mitochondrial inner membrane was also ascertained by introducing the corresponding genes into a yeast mutant carrying an inactivated chromosomal gene for subunit IV (Dowhan *et al.*, 1985) and subsequent testing for restoration of cytochrome *c* oxidase activity and growth on glycerol.

The first 12 amino-terminal residues of a mitochondrial outer membrane protein can direct the cytosolic enzyme dihydrofolate reductase into the yeast mitochondrial matrix

Cytosolic dihydrofolate reductase from mouse is a suitable 'pas-

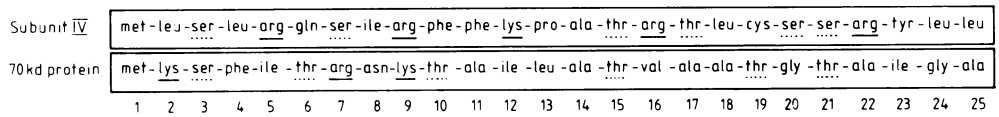

Fig. 1. The cleavable pre-sequence of subunit IV (an imported mitochondrial protein of the mitochondrial inner membrane) and the amino-terminal sequence of an imported 70-kd protein of the mitochondrial outer membrane. Positively charged amino acids are underlined with solid lines, serine and threonine residues with dotted lines. For the complete amino acid sequence of subunit IV and 70-kd protein see Maarse *et al.* (1984) and Hase *et al.* (1983).

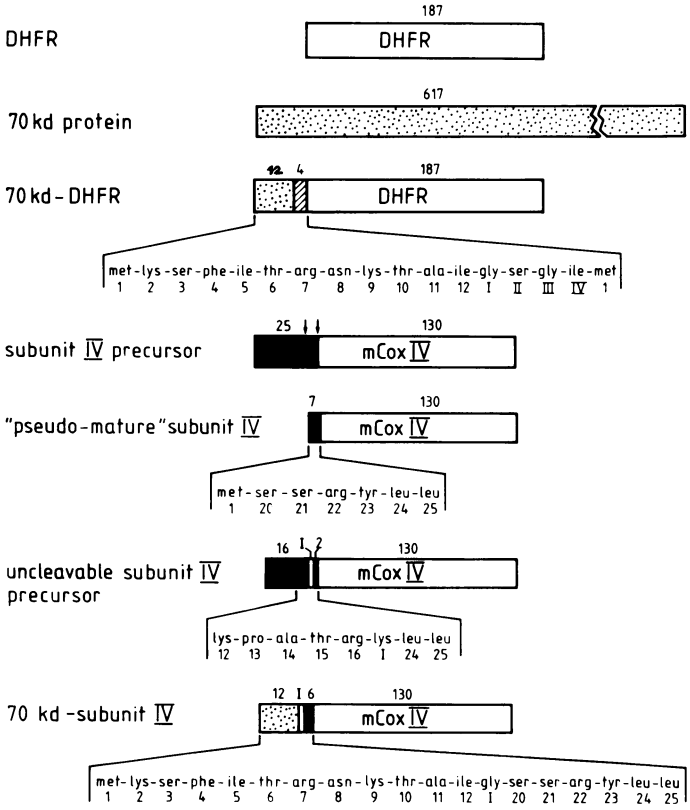

Fig. 2. Construction of fusion proteins. Boxes denote amino acid sequences, numerals on the top of each box the total number of amino acids. DHFR, dihydrofolate reductase; 70 kd protein, major 70-kd protein of the yeast mitochondrial outer membrane (dotted bar); 70 kd-DHFR, fusion protein containing the first 12 residues of the 70-kd protein (dotted bar), four residues introduced by the gene fusion (shaded bar) and the entire sequence of dihydrofolate reductase; subunit IV precursor, authentic subunit IV precursor containing a 25-residue pre-sequence (filled bar) and a 130-residue mature sequence (mCox IV; open bar); 'pseudo-mature' subunit IV, residues 2 – 19 of the subunit IV precursor were deleted; uncleavable subunit IV precursor, residues 17 – 23 of the subunit IV precursor were replaced by one lysine residue (Roman numeral); 70 kd-subunit IV, the first 19 residues of the subunit IV precursor were replaced by the first 12 residues of the 70-kd outer membrane protein plus one glycine residue (Roman numeral) introduced by gene fusion. Arrows, sites cleaved by the mitochondrial processing protease.

senger' which can be specifically transported into mitochondria if fused to mitochondrial pre-sequences (Hurt *et al.*, 1984a, 1984b, 1985; Horwich *et al.*, 1985; van Loon *et al.*, 1985). Can this 'passenger' also be transported into mitochondria if fused to a non-cleaved amino-terminal segment of a mitochondrial outer membrane protein which shows similarities to transient mitochondrial pre-sequences (see Figure 1)? When a fusion protein between the first 12 amino acids of the 70-kd outer membrane protein and dihydrofolate reductase (70 kd-DHFR; Figure 2) was synthesized by *in vitro* transcription/translation and then post-translationally incubated with de-energized yeast mitochondria, it bound to the organelles, but remained accessible to externally added protease (Figure 3A, lanes 2 and 3). Under the conditions of this experiment, 9% of the radiolabeled fusion protein bound to the mitochondrial surface; this level of binding is ~10-fold higher than that found with radiolabeled authentic dihydrofolate reductase under the same conditions (see also Hurt *et al.*, 1984b, 1985). When mitochondria were energized, part of the fusion protein became inaccessible to externally added protease (Figure 3A, lanes 4 and 5) suggesting that it had been transported into the mitochondrial interior. The authentic 70-kd protein bound to the surface of de-energized mitochondria but none was imported into the interior of energized mitochondria (Figure 3B). The rate of import of the 70 kd-DHFR fusion protein into isolated mitochondria was ~10-fold slower than that of a fusion protein containing the first 12 amino acids of the transient pre-sequence of subunit IV attached to the dihydrofolate reductase (Figure 3A, lanes 6 – 8; Figure 3C; see also Hurt *et al.*, 1985).

The inefficient transport of the 70 kd-DHFR fusion protein into isolated mitochondria prompted us to test whether targeting was more efficient *in vivo*. Therefore, yeast cells expressing authentic dihydrofolate reductase or the 70 kd-DHFR fusion protein were fractionated into mitochondria, microsomes and cytoplasm, and the steady-state distribution of both proteins was analyzed by immune blotting using a specific antiserum against mouse dihydrofolate reductase (Figure 4A). Cross-contamination of the subcellular and submitochondrial fractions was monitored by subjecting each fraction to immune blotting using antisera against the following marker proteins: hexokinase (HK, cytosol); citrate synthase (CS, mitochondrial matrix); cytochrome b_2 (Cytb_2, mitochondrial intermembrane space); cytochrome c_1 (Cytc_1, mitochondrial inner membrane). Authentic dihydrofolate reductase was exclusively found in the cytosol. The 70 kd-DHFR fusion protein fractionated with the mitochondrial (40%) and the cytosolic fraction (60%). Although dihydrofolate reductase seems quite resistant to proteases, we cannot exclude that the 70 kd-DHFR fusion protein is degraded with different rates within the various subcellular compartments. The fusion protein found in the mitochondrial fraction was inside the mitochondrial inner membrane as shown by the following observations. First, it was inaccessible to added protease in mitoplasts which had lost the intermembrane space marker cytochrome b_2, and, accordingly, the outer membrane barrier (Figure 4B, lanes 5 and 6). Second, the fusion protein co-fractionated with the matrix marker citrate synthase and the inner membrane marker cytochrome c_1 (Figure 4B, lanes 7 – 9); most likely, it weakly bound to the inner side of the mitochondrial inner membrane. Third, the dihydrofolate reductase activity of the fusion protein was latent in mitochondria, increasing 7-fold upon disruption of the mitochondrial inner membrane by detergents (data not shown; the inner membrane is not permeable for NADPH and dihydrofolate which are the substrates for the enzyme; see also Hurt *et al.*, 1985). These results show that the first 12 amino acids of the 70-kd outer membrane protein are sufficient to direct cytosolic dihydrofolate reductase to the matrix side of the mitochondrial inner membrane, although with lower efficiency than an authentic mitochondrial

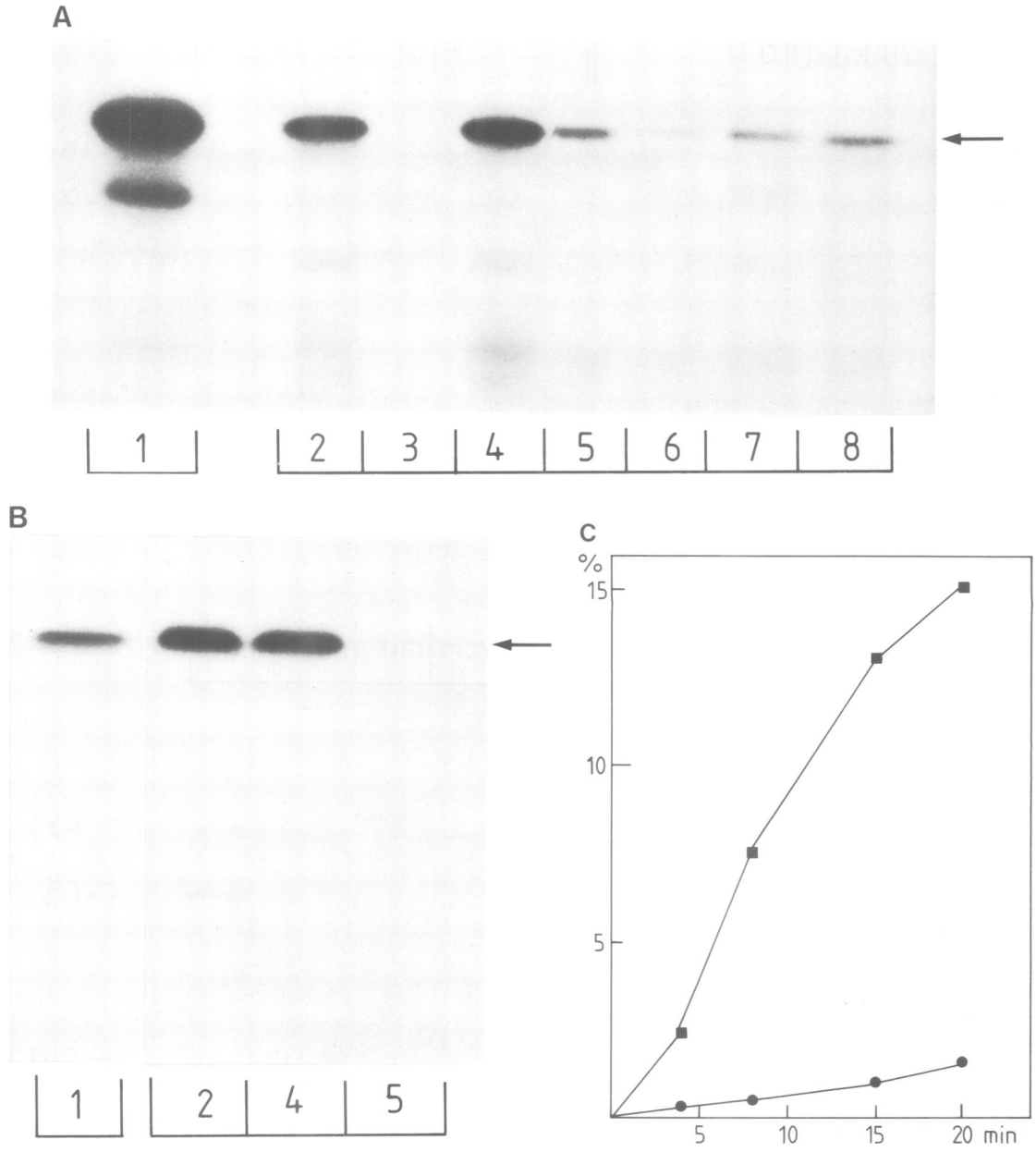

Fig. 3. Twelve amino-terminal residues of the 70-kd mitochondrial outer membrane protein direct mouse dihydrofolate reductase into isolated mitochondria. The 70 kd-DHFR fusion protein **(A)** and authentic 70-kd protein **(B)** were synthesized by *in vitro* transcription/translation in the presence of [^{35}S]methionine and incubated with isolated yeast mitochondria for 30 min at 30°C as indicated below and in Materials and methods. Mitochondria were re-isolated by centrifugation and analyzed by SDS-12.5% polyacrylamide gel electrophoresis and fluorography. **1**, 20% **(A)** and 40% **(B)** of total translation products; **2**, de-energized mitochondria (binding assay); **3**, de-energized mitochondria, proteinase K; **4**, energized mitochondria (import assay); **5**, energized mitochondria, proteinase K; **6,7** and **8**, same as **5** except that incubation was for 5, 10 and 20 min, respectively. The arrows indicate the 70 kd-DHFR fusion protein and authentic 70-kd protein. **(C)** Time-dependent uptake into isolated mitochondria of DHFR carrying at its amino terminus the first 12 residues of either the 70-kd protein (●—●) or of the subunit IV pre-sequence (■—■); the radiolabeled bands were quantified and expressed as % of the total fusion protein added to mitochondria.

pre-sequence. The authentic 70-kd protein probably stops in the outer membrane because the targeting sequence is immediately followed by an anchor consisting of a long uninterrupted stretch of uncharged amino acids (Hase *et al.*, 1984).

A complementation assay for testing whether cytochrome oxidase subunit IV is imported to the mitochondrial inner membrane

By disrupting the chromosomal gene for cytochrome oxidase subunit IV we have previously constructed a subunit IV-deficient yeast mutant. This mutant still synthesizes the remaining cytochrome oxidase subunits but lacks cytochrome oxidase activity, respiration and the ability to grow on a non-fermentable carbon source such as glycerol. All of these defects disappear if the mutant cells are transformed with the plasmid-borne gene for the authentic subunit IV precursor (Dowhan *et al.*, 1985). Thus, growth of the transformed mutant cells on glycerol proves that a functional subunit IV is being imported to the mitochondrial inner membrane.

When the mutant cells were transformed with a gene coding for a subunit IV precursor lacking most of its pre-sequence (Figure 2, 'pseudo-mature' subunit IV), they failed to grow on glycerol (Figure 5, A and B) and lacked cytochrome oxidase activity (Table I). However, they grew as rapidly as the wild-type parent on glucose, i.e., under fermentative conditions (Figure 5C). Im-

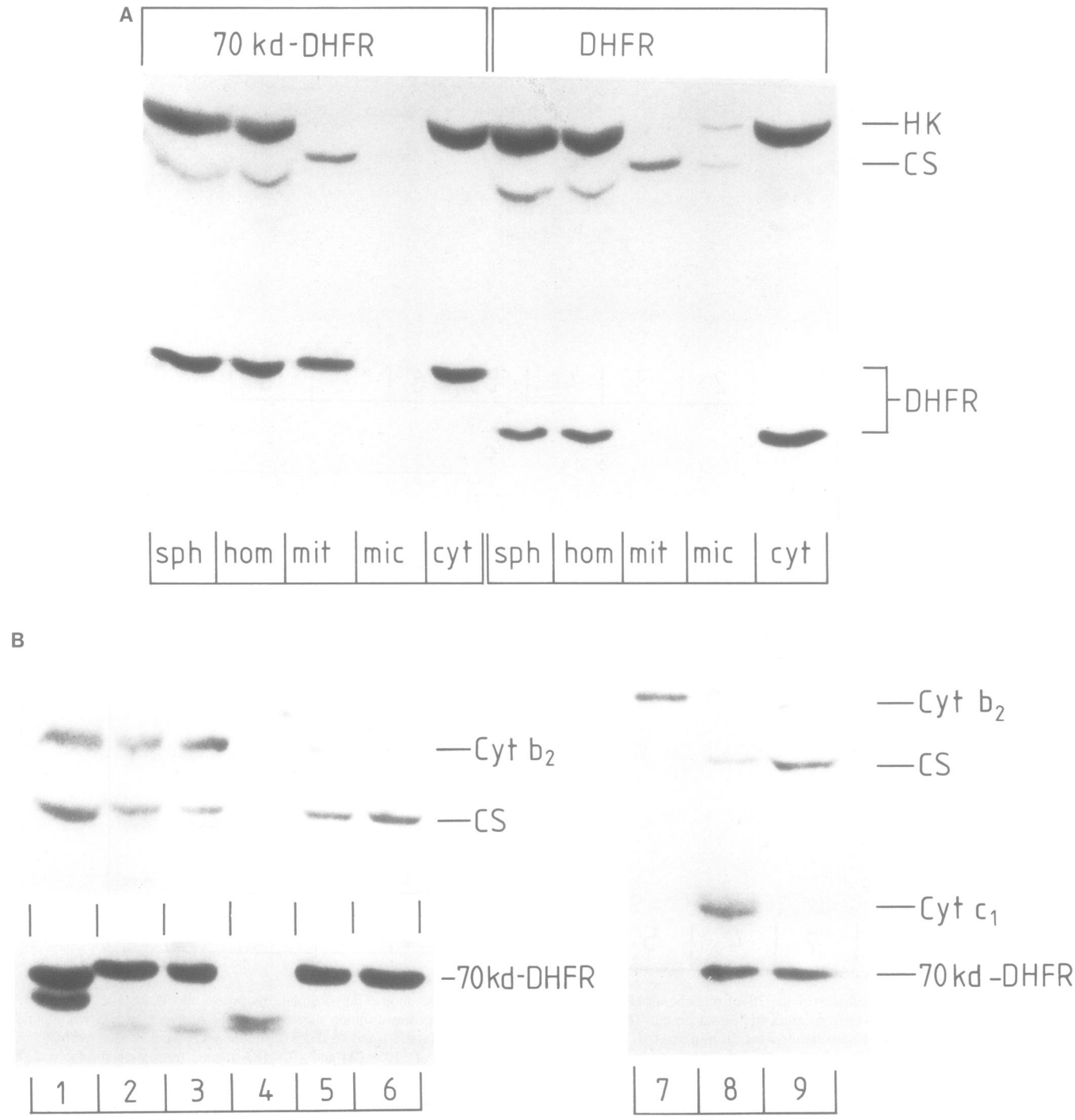

Fig. 4. The 12 amino-terminal residues of the 70-kd outer membrane protein direct attached mouse dihydrofolate reductase into the yeast mitochondrial matrix *in vivo*: subcellular fractionation studies. **(A)** Subcellular distribution of the 70 kd-DHFR fusion protein. Yeast cells transformed with the plasmid pLGSD5 containing the genes for mouse dihydrofolate reductase (DHFR) and for a fusion protein between the 12 amino-terminal residues of the 70-kd protein and DHFR (70 kd-DHFR) were grown in liquid galactose-containing medium and fractionated into spheroplasts (sph), homogenate (hom), mitochondria (mit), microsomes (mic) and cytosol (cyt) as described in Materials and methods. Aliquots of spheroplasts and homogenate (each equivalent to 8.7 mg wet weight of cells) and aliquots of mitochondria, microsomes and cytosol (each equivalent to 13 mg wet weight of cells) were analyzed by SDS-12.5% polyacrylamide gel electrophoresis and immune blotting using antisera against mouse dihydrofolate reductase (DHFR), hexokinase (HK, cytosolic marker) and citrate synthase (CS, mitochondrial marker). The labeled bands of the 70 kd-DHFR fusion protein were quantified as described in Materials and methods. The downward displacement of the CS antigen in the spheroplast and homogenate fractions is probably an artefact resulting from the larger amount of proteins co-electrophoresing with CS. **(B)** Submitochondrial distribution of the 70 kd-DHFR fusion protein. Mitochondria isolated from yeast cells expressing the 70 kd-DHFR fusion protein were subfractionated into submitochondrial compartments as described in Materials and methods. Fractions equivalent to 75 μg mitochondrial protein were analyzed by SDS-12.5% polyacrylamide gel electrophoresis and immune blotting using antisera against mouse dihydrofolate reductase, cytochrome b_2 (intermembrane space marker), citrate synthase (matrix marker) and cytochrome c_1 (inner membrane marker). **1**, mitochondria isolated from yeast cells expressing a fusion protein containing the first 12 amino acids of the subunit IV pre-sequence attached to dihydrofolate reductase (see Hurt *et al.*, 1985); **2–9**, mitochondrial fractions derived from cells expressing the 70 kd-DHFR fusion protein; **2**, mitochondria; **3**, mitochondria treated with proteinase K; **4**, mitochondria treated with proteinase K and Triton X-100; **5**, mitoplasts; **6**, mitoplasts treated with proteinase K; **7**, intermembrane space fraction; **8**, membrane fraction; **9**, matrix fraction.

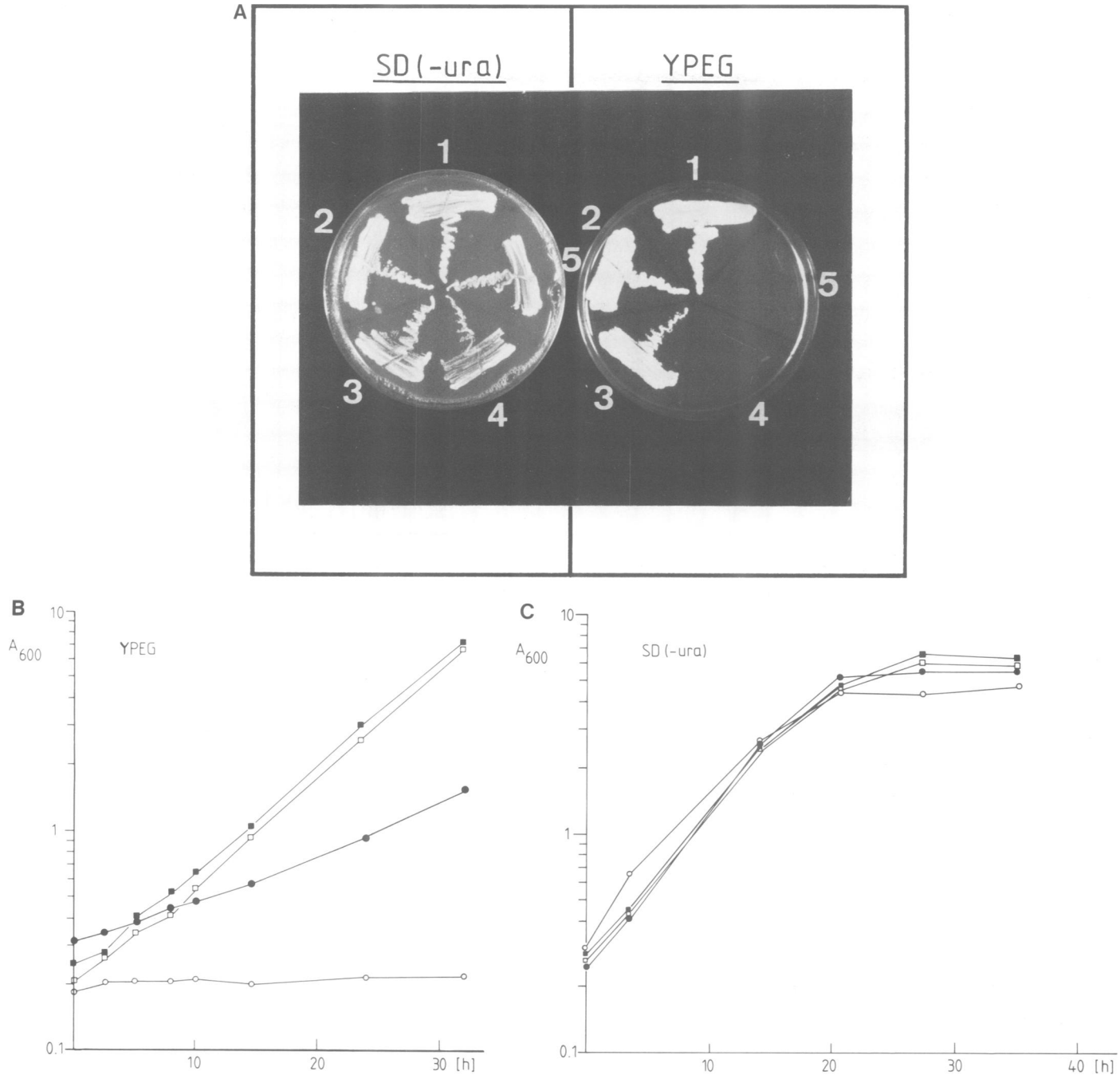

Fig. 5. The first 12 residues of the 70-kd outer membrane protein direct attached cytochrome oxidase subunit IV into the yeast mitochondrial inner membrane. Restoration of respiration in a subunit IV-deficient yeast mutant. A subunit IV-deficient yeast mutant was transformed with the autonomously replicating centromere yeast plasmid pAC1 in which the yeast alcohol dehydrogenase I promoter directed expression of genes encoding the following proteins: **(1)** authentic subunit IV precursor, **(2)** uncleavable subunit IV precursor, **(3)** the 70-kd-subunit IV fusion protein, and **(4)** 'pseudo-mature' subunit IV. As a control, plasmid pAC1 lacking any subunit IV-related gene was used for transformation **(5)**. **(A)** Growth on SD (−ura) and YPEG-plates: transformants were selected for growth on an uracil-free synthetic minimal medium containing 0.67% yeast nitrogen base, 2% glucose and 20 μg/ml L-histidine. Cells from single colonies were streaked either onto uracil-free SD-plates (SD−ura) or YPEG-plates containing 1% yeast extract, 2% peptone, 3% ethanol and 2% glycerol and were grown for 5 days at 30°C. **(B** and **C)** Growth of the transformants in liquid YPEG **(B)** and uracil-free SD-medium supplemented with 20 μg/ml L-histidine **(C)**. Cell growth was measured by absorbance at 600 nm. Authentic subunit IV precursor (■—■); uncleavable subunit IV precursor (□—□); 70-kd-subunit IV fusion protein (●—●); 'pseudo-mature' subunit IV (○—○).

mune blotting experiments and pulse-labeling showed that the subunit IV precursor lacking most of its pre-sequence was expressed in the transformant (Figure 6B, subpanel 4 and Figure 8D) but that it failed to accumulate in the mitochondria (Figure 6A, B). The truncated precursor was largely lost upon subfractionating the broken spheroplasts (Figure 6B, subpanel 4); either it was degraded or lost in the low-speed pellet. These *in vivo* studies are in line with the previous observation that a subunit IV precursor lacking most of its pre-sequence is not imported by isolated yeast mitochondria (Hurt *et al.*, 1984b).

Restoration of respiration by imported subunit IV does not require rapid removal of the pre-sequence by the matrix-located processing protease

Imported and processed subunit IV restores respiration by as-

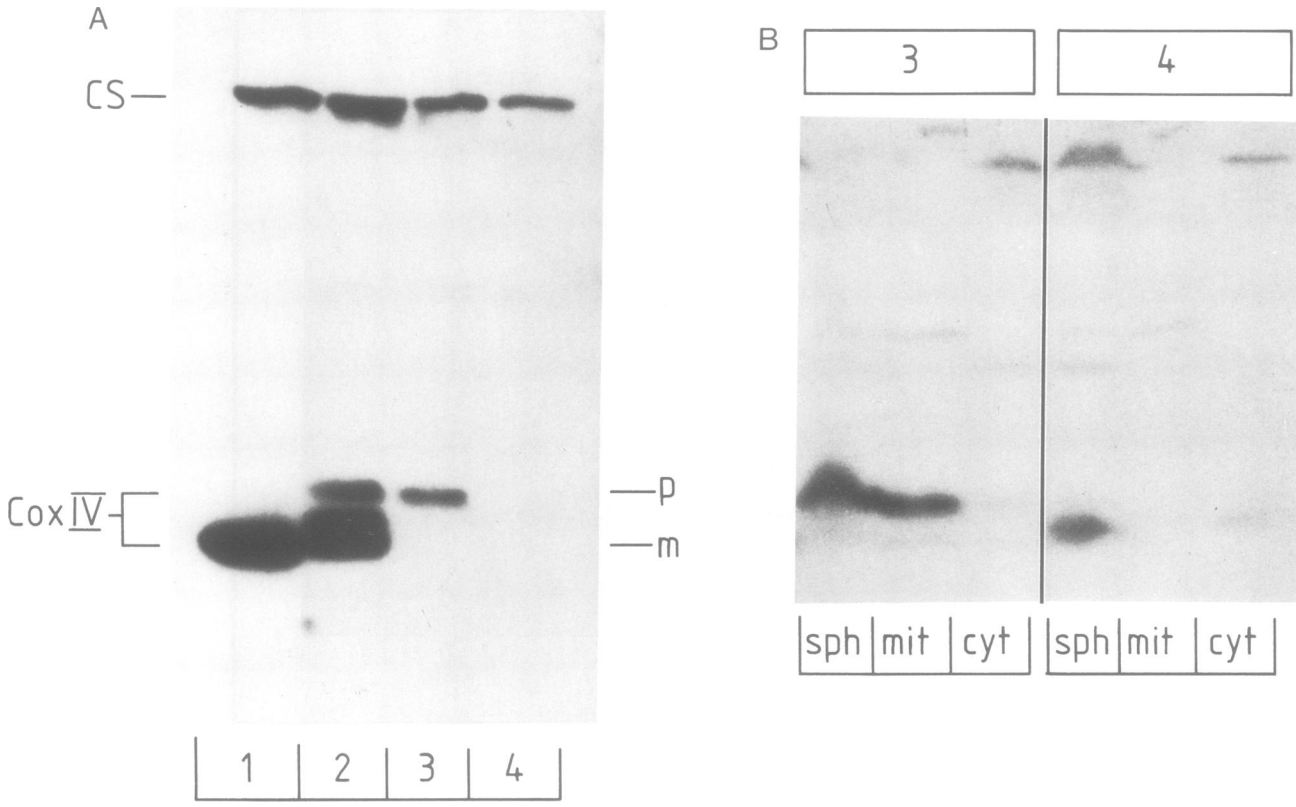

Fig. 6. The 70-kd-subunit IV fusion protein accumulates in mitochondria without significant proteolytic cleavage. Cells from the subunit IV-deficient mutant were transformed with the plasmid-borne genes coding for the authentic subunit IV precursor **(1)**, the *in vitro* uncleavable subunit IV precursor **(2)**, the 70-kd-subunit IV fusion protein **(3)** and the 'pseudo-mature' subunit IV **(4)**. The transformed cells were grown at 30°C to an OD 600 of ~1 in 1% yeast extract, 2% peptone and 0.1% glucose, converted into spheroplasts and subfractionated into mitochondria and post-mitochondrial supernatant (Hurt *et al.*, 1985). **(A)** Mitochondria from transformed cells equivalent to 120 μg protein were analyzed by SDS-14% polyacrylamide gel electrophoresis and immune blotting using antisera against subunit IV (Cox IV) and citrate synthase (CS, mitochondrial marker). p, precursor form; m, mature-sized form. **(B)** Subcellular fractionation of the subunit IV-deficient mutant transformed with genes encoding either the 70-kd-subunit IV fusion protein **(3)** or the 'pseudo-mature' subunit IV **(4)**. Spheroplasts (sph) and post-mitochondrial supernatant (cyt) (each equivalent to 15 mg wet weight of cells) and mitochondria (mit; equivalent to 30 mg wet weight of cells) were analyzed by SDS-14% polyacrylamide gel electrophoresis and immune blotting using an antiserum against subunit IV.

Table I. Mitochondria from the subunit IV-deficient yeast mutant expressing the 70-kd-subunit IV fusion protein have cytochrome *c* oxidase activity

Subunit IV-deficient yeast mutant transformed with the gene encoding	Cytochrome *c* oxidase activity				Succinate-cytochrome *c* reductase activity	
	Mitochondria		Spheroplasts		Mitochondria	
	(U/mg)	(%)	(U/g wet weight of cells)	(%)	(U/mg)	(%)
Authentic subunit IV precursor	1.65	(100)	38.6	(100)	0.19	(100)
Uncleavable subunit IV precursor	1.52	93	29.8	77	0.17	89
70-kd-subunit IV fusion protein	0.25	15	7.6	20	0.11	58
'Pseudo-mature' subunit IV	0.002	0.14	0.06	0.16	0.03	16

The subunit IV-deficient mutant transformed with the indicated genes was grown in 1% yeast extract, 2% peptone and 0.1% glucose to an OD 600 of ~1. Cells were converted to spheroplasts and fractionated into mitochondria and post-mitochondrial supernatant. Cytochrome *c* oxidase activity and succinate-cytochrome *c* reductase activity was measured as described in Materials and methods. Unit of activity is defined as that amount of enzyme which oxidizes or reduces 1 μmol of cytochrome *c* per min under the specified assay conditions. Activity present in spheroplasts was determined in homogenates (see Materials and methods).

sembling with the remaining eight cytochrome oxidase subunits into an active holoenzyme. This assembly is possible even with a modified subunit IV precursor (Figure 2; uncleaved subunit IV precursor) which is not cleaved *in vitro* by the matrix protease (data not shown) or upon import into isolated mitochondria (Figure 7). This *in vitro* uncleavable subunit IV precursor restores growth on glycerol (Figure 5, A and B) and cytochrome oxidase activity (Table I) to the subunit IV-deficient mutant. *In vivo*, however, the modified precursor accumulates in mitochondria largely in a cleaved form; this cleavage is much slower than that of the authentic subunit IV precursor (compare panels A and B of Figure 8). It is not clear whether the modified subunit IV precursor is slowly cleaved *in vivo* by the matrix-located processing enzyme or by other, unspecific, mitochondrial proteases. Since assembly of the modified subunit IV precursor into an active cytochrome *c* oxidase does not require fast cleavage by the chelator-sensitive mitochondrial matrix protease, the complementation assay should be generally useful in testing the targeting function of natural and artificial pre-sequences attached to 'pseudo-mature' subunit IV.

The extreme amino terminus of the 70-kd outer membrane protein can direct cytochrome oxidase subunit IV to the inner membrane

We constructed a fusion gene encoding the first 12 amino acids of the 70-kd outer membrane protein attached to the mature part of subunit IV (Figure 2). When this fusion gene was introduced into the subunit IV-deficient yeast mutant, it restored growth on

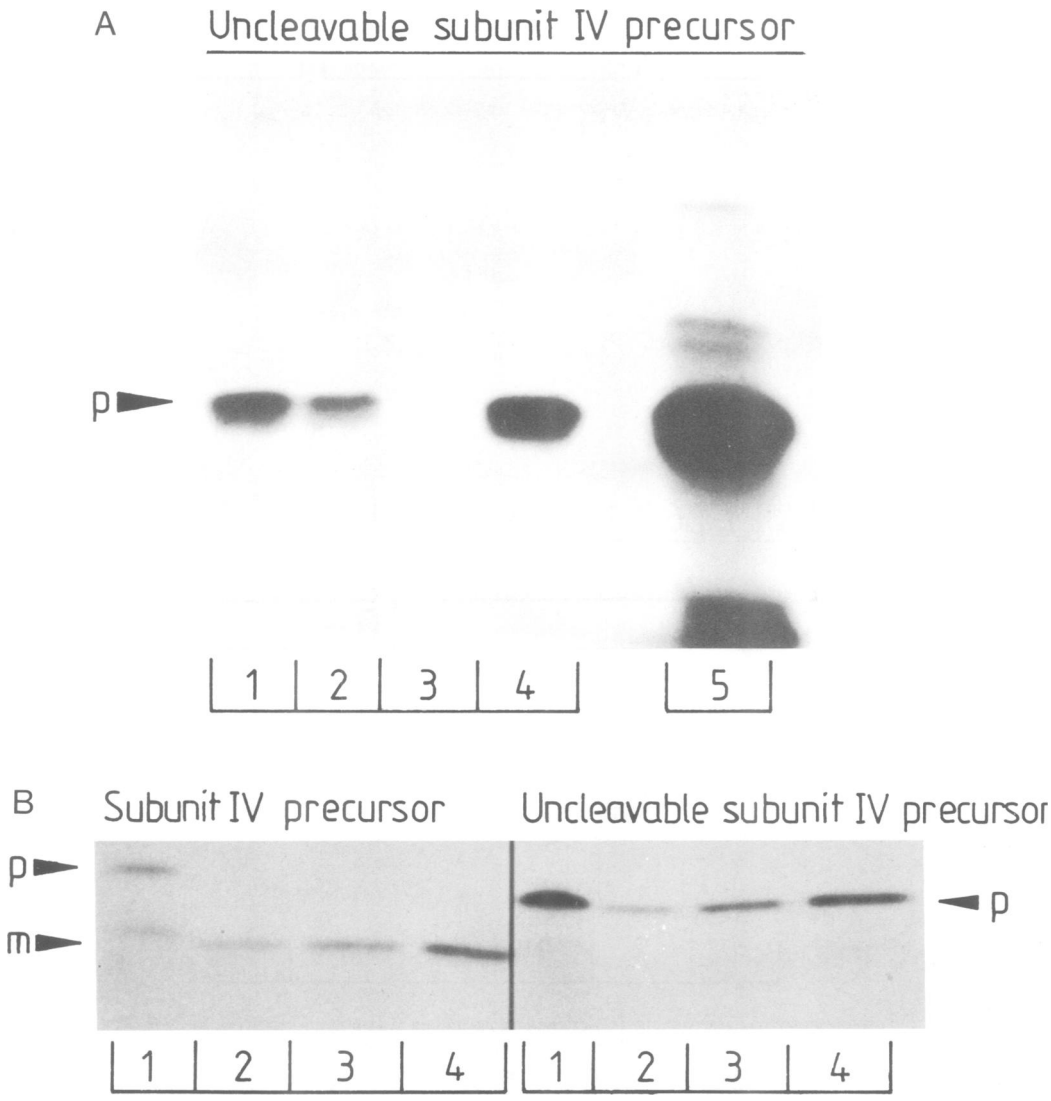

Fig. 7. A subunit IV precursor with a deletion in the C-terminal part of its pre-sequence is not cleaved upon import into isolated mitochondria. The gene coding for an uncleavable precursor of subunit IV was constructed as outlined in Materials and methods. Its deduced amino acid sequence around the deletion site is shown in Figure 2. Authentic and uncleavable subunit IV precursor were synthesized by *in vitro* transcription/translation in the presence of [^{35}S]methionine. **(A)** Binding and import of the uncleavable subunit IV precursor by isolated mitochondria (30 min); **1**, energized mitochondria, proteinase K; **2**, de-energized mitochondria; **3**, de-energized mitochondria, proteinase K; **4**, energized mitochondria; **5**, 100% of total translation products. **(B)** Time-dependent import of authentic and uncleavable subunit IV precursor into isolated mitochondria; **1**, 40% of total translation products; **2, 3** and **4**, energized mitochondria plus proteinase K; import was allowed for 3, 10 and 20 min, respectively. p and m, precursor and processed ('mature') form.

glycerol (Figure 5, A and B) and cytochrome oxidase activity (Table I) to the mutant cells. However, restoration was only partial: the transformants grew on glycerol/ethanol 2.6 times more slowly, and had 5 – 6 times less cytochrome oxidase activity than transformants expressing the authentic subunit IV precursor. Immune blotting and pulse-chase experiments revealed that the 70-kd-subunit IV fusion protein accumulated in the mitochondria without significant cleavage to mature-sized subunit IV (Figure 6A, panel 3 and Figure 8C). Since the fusion gene had been placed under control of the strong alcohol dehydrogenase promoter, the 70-kd-subunit IV protein was presumably synthesized in larger amounts than some of the other imported cytochrome oxidase subunits; it is possible, therefore, that cytochrome oxidase activity was restored by a small fraction of proteolytically cleaved fusion protein molecules that escaped detection under our conditions. However, the data shown in Figures 6 and 8 favor the view that the uncleaved fusion protein was assembled into the active holoenzyme. The less efficient complementation could either reflect less efficient import into mitochondria, less efficient assembly into cytochrome oxidase, lower activity of cytochrome oxidase holoenzyme containing the uncleaved fusion protein or a combination of these factors. In spite of these uncertainties, it is clear that the extreme amino terminus of the 70-kd outer membrane protein can direct subunit IV to the mitochondrial inner membrane in living yeast cells.

Discussion

In this study we have shown that the first 12 amino acids of a mitochondrial outer membrane protein can direct an attached protein to the mitochondrial matrix. This result is probably not an adventitious artefact of gene fusion since it was obtained with two different attached proteins – mouse dihydrofolate reductase and yeast cytochrome oxidase subunit IV lacking most of its own pre-sequence. Neither is the result an *in vitro* artefact since it was obtained with isolated mitochondria as well as with living

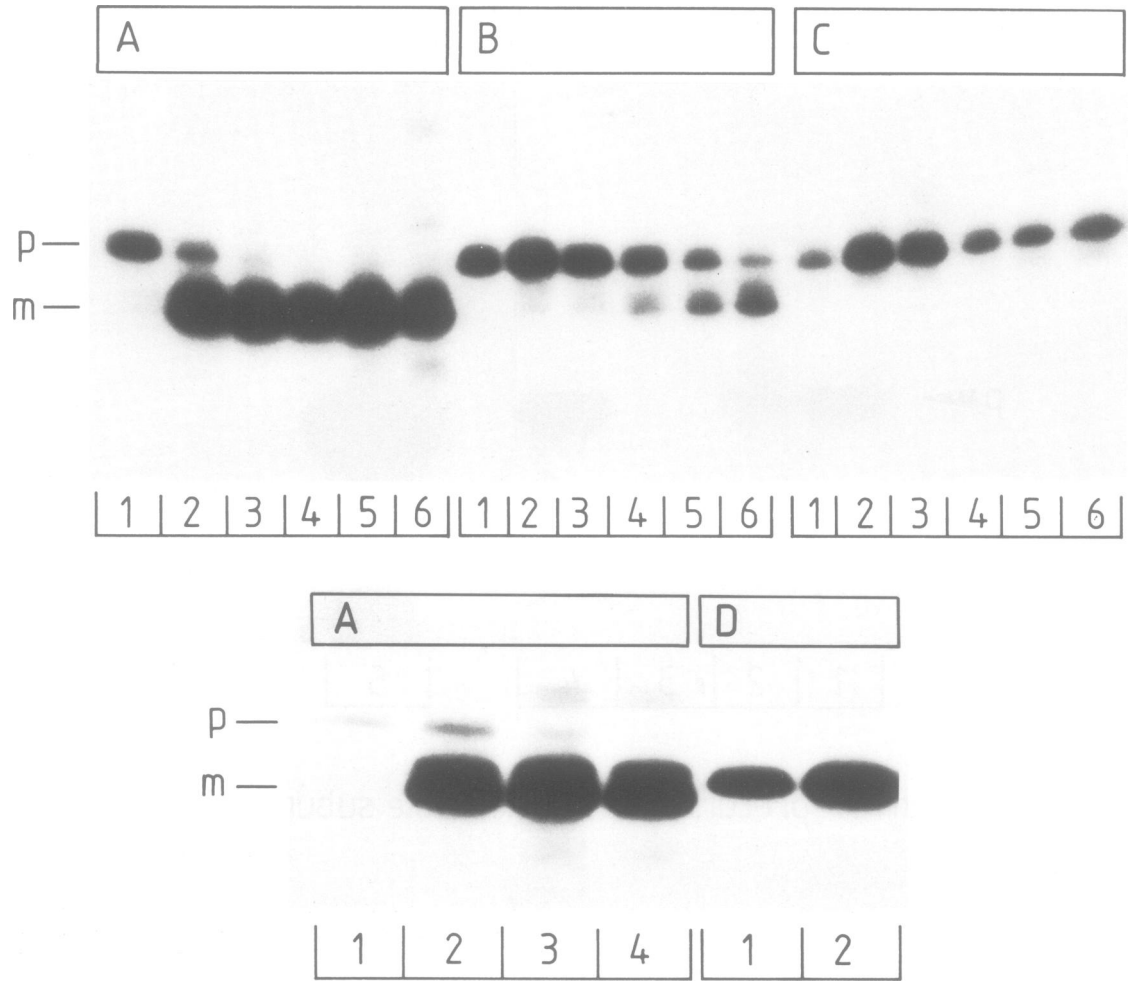

Fig. 8. Fate of the various subunit IV proteins *in vivo*. The subunit IV-deficient yeast mutant was transformed with the genes encoding wild-type subunit IV precursor **(A)**, uncleavable subunit IV precursor **(B)**, 70-kd-subunit IV fusion protein **(C)** and 'pseudo-mature' subunit IV **(D)**. The transformants were grown on SD (−ura) medium and pulse-labeled as described in Materials and methods with [^{35}S]methionine for 5 min in the presence **(1)** and in the absence **(2–6)** of 20 μM carbonyl cyanide m-chlorophenyl hydrazone (CCCP). Labeling was stopped by addition of 5 mM unlabeled methionine and the cells were chased in the absence of CCCP for 5 min **(3)**, 10 min **(4)**, 20 min **(5)** and 40 min **(6)**. p, precursor; m, processed ('mature') form. Pulse-labeling of the subunit IV-deficient mutant expressing wild-type subunit IV precursor (lower subpanel A) and the 'pseudo-mature' subunit IV (subpanel D) was done in a separate experiment.

yeast cells. Finally, targeting of the fusion proteins to an internal mitochondrial location (matrix/inner membrane) was ascertained by three separate methods: subfractionation of mitochondria, energy dependence of import, and restoration of a function tightly associated with the mitochondrial inner membrane.

These results provide firm evidence for the earlier conclusion that targeting of a major 70-kd protein to the yeast mitochondrial outer membrane involves an amino-terminal 'targeting sequence' which directs the protein to (and potentially into) the mitochondrion. Presumably, localization of the 70-kd protein to the outer membrane is ensured by a subsequent 'stop-transfer' or 'anchoring' sequence of uncharged residues that prevents translocation of the protein into the mitochondrial interior, thereby fixing it permanently into the outer membrane (Hase *et al.*, 1984). A similar amino-terminal two-domain structure has recently been found for the pore-forming protein ('porin') of the yeast mitochondrial outer membrane (Mihara and Sato, 1985). It is, thus, a distinct possibility that yet other mitochondrial outer membrane proteins share this amino-terminal motif.

The amino-terminal dodecapeptide of the 70-kd outer membrane protein is a less efficient 'matrix-targeting' sequence than the amino-terminal dodecapeptide of the subunit IV precursor, a protein normally targeted to the matrix space (Hurt *et al.*, 1985). This could simply reflect that fact that the amino-terminal peptide arbitrarily chosen by us (12 residues) was too short to comprise the complete matrix-targeting function residing in the 70-kd protein. Alternatively, the matrix-targeting sequence of the 70-kd protein could have evolved together with the adjacent putative stop-transfer sequence in order to optimize targeting to the outer membrane. Such co-evolution might well render the matrix-targeting sequence less effective by itself.

The present data argue against the view that intramitochondrial sorting of imported proteins is effected mainly by compartment-specific 'import receptors' on the mitochondrial surface. This view is also weakened by the finding that a protein of the outer membrane (Hase *et al.*, 1984) or the intermembrane space (van Loon *et al.*, 1985) can be re-routed to the matrix by deleting part or all of a putative 'stop-transfer' sequence close to, but not immediately at, the amino terminus. We suggest that intramitochondrial sorting of imported proteins is governed by a combination of matrix-targeting and stop-transfer sequences within the imported protein itself.

In this study we have introduced complementation of an *in vivo* function to assess the topological specificity of a mitochondrial

targeting sequence. Besides being sensitive and easy, this assay circumvents the uncertainties of determining the submitochondrial localization of an imported protein by submitochondrial fractionation or by measuring accessibility to externally added protease. Restoration of specific outer membrane functions in *Escherichia coli* has been a powerful aid in testing whether mutationally altered proteins of the *E. coli* outer membrane become properly localized *in vivo* (Benson and Silhavy, 1983). We believe that the *in vivo* test described here will be similarly helpful for studying how proteins are imported to specific intramitochondrial locations.

Materials and methods

Strains and plasmids

The *E. coli* strains used were HB 101 (Kedes *et al.*, 1975) and JM 101 (Messing, 1979). Dihydrofolate reductase from mouse and a fusion protein containing 12 amino-terminal residues of the 70-kd outer membrane protein attached to mouse dihydrofolate reductase were expressed in *Saccharomyces cerevisiae* strain YNN 214 (*α, ura 3, lys 2, ade 2*) which was kindly provided by Mark Johnston and Ron Davis (Stanford, CA). For assaying whether subunit IV was imported into mitochondria and assembled into active cytochrome *c* oxidase, the subunit IV-deficient yeast mutant Cox IV$^-$ (*α, his 3, ura 3*) was used (Dowhan *et al.*, 1985). Mitochondria for import studies were isolated from the *S. cerevisiae* strain D 273-10 B (ATCC 25657). Plasmids pDS 5/2 (Stueber *et al.*, 1984), pDS 5/2-1, pDS 5/2-1-Cox IV and pDS 5/2-1-Cox IV-DHFR (Hurt *et al.*, 1984b), pLGSD5 (Guarente *et al.*, 1982), pLGSD5-DHFR (Hurt *et al.*, 1985) and pFL-70k (Riezman *et al.*, 1983) have been described.

DNA manipulations

Published procedures were used for DNA restriction analysis, end-filling, *Bal*31 exonuclease digestion and agarose and polyacrylamide gel electrophoresis (Maniatis *et al.*, 1982). DNA sequencing was done by the dideoxy method (Sanger *et al.*, 1977) as outlined by Hase *et al.* (1983). Oligonucleotides were kindly synthesized by Dr J.Jiricny using a 380B DNA synthesizer (Applied Biosystems, USA) and were purified by polyacrylamide gel electrophoresis according to the manufacturer's instructions.

Gene constructions

Gene modifications and gene fusions are shown in Figure 2. Plasmid pDS 5/2-1-70 kd-DHFR was constructed as follows. The gene fragment encoding the first 12 amino-terminal residues of the 70-kd outer membrane protein was synthesized as the double-stranded oligonucleotide with *Eco*RI and *Bam*HI sticky ends at the 5′ and 3′ end, respectively. Both strands were synthesized as 43-mer oligonucleotides with the following sequences:

5′AATTCAATGAAGAGCTTCATTACAAGGAACAAGACAGCCATTG3′
3′GTTACTTCTCGAAGTAATGTTCCTTGTTCTGTCGGTAACCTAG5′

The strands were annealed and cloned into plasmids pDS 5/2-1 which had been cut with *Eco*RI and *Bam*HI. This insertion led to an in-frame fusion between the 3′ end of the 70-kd gene fragment and the 5′ end of the dihydrofolate reductase coding sequence. Plasmid pDS 5/2-1-70 kd-DHFR was used for *in vitro* transcription/translation yielding the 70 kd-DHFR fusion protein which was subsequently used for binding and import studies with isolated mitochondria. The 70 kd-DHFR gene was isolated from this plasmid as a 700-bp *Eco*RI/*Hind*III fragment; after end-filling with the large fragment of *E. coli* DNA polymerase, this fusion gene was cloned into the blunt-ended *Bam*HI site of plasmid pLGSD5. Plasmids carrying the 70 kd-DHFR fusion gene in the correct orientation were introduced into the yeast strain YNN 214 by transformation as described earlier (Hurt *et al.*, 1985). Plasmid pDS 5/2-1-pseudo-mature subunit IV was constructed in the following way: plasmid pDS 5/2-1 Cox-IV-DHFR containing the gene coding for the presequence plus 28 amino acids from the mature part of subunit IV attached to the DHFR gene (Hurt *et al.*, 1984b) was cut at its single *Eco*RI site 24 nucleotides upstream of the ATG start codon of the subunit IV pre-sequence. The 5′ non-coding region and most of the DNA sequence encoding the pre-sequence of subunit IV was removed by limited *Bal*31 exonuclease digestion. The shortened ends were filled in as described above and a *Cla*I linker (New England Biolabs) supplying an ATG start codon was attached. Subunit IV-DHFR fusion genes truncated at their 5′ ends were finally isolated as *Cla*I/*Hind*III fragments and inserted into a pDS 5/2-1 plasmid which had been modified by cutting at its single *Eco*RI site, end-filling and attachment of *Cla*I linker. This manipulation restored an *Eco*RI site 5′ to the *Cla*I site. This modified pDS 5/2-1 plasmid was cut with *Cla*I/*Hind*III and the large fragment containing the T5 phage promoter was isolated. This large DNA fragment was ligated with the 5′-truncated subunit IV-DHRF fusion genes isolated as *Cla*I/*Hind*III fragments (see above). In order to screen for in-frame fusions between the ATG start codon supplied by the *Cla*I linker and the 5′-deleted subunit IV-DHFR fusion genes, the resulting plasmids were used as templates for *in vitro* transcription/translation. Plasmids carrying truncated fusion genes encoding proteins of molecular size ~25 kd were used for DNA sequencing around the fusion region. A plasmid containing a 5′-truncated subunit IV-DHFR fusion gene encoding a fusion protein lacking amino acids 2 – 19 amino-terminal from the subunit IV pre-sequence was cut with *Pst*I/*Eco*RV and the smaller fragment containing the gene encoding the truncated subunit IV pre-sequence was isolated. Plasmid pDS 5/2-1-Cox IV was cut with *Pst*I/*Eco*RV and the larger fragment containing the pseudo-mature subunit IV gene was isolated. Both fragments were ligated, yielding plasmid pDS 5/2-1-pseudo-mature subunit IV encoding a 'pseudo-mature' subunit IV protein (Figure 2).

Plasmid pDS 5/2-1-uncleavable subunit IV precursor (Figure 2) was constructed as follows: pDS 5/2-1-Cox IV (Hurt *et al.*, 1984b) was cut with *Eco*RV at a site corresponding to amino acid 23 of the subunit IV pre-sequence. The ends were shortened by *Bal*31 exonuclease digestion, filled in and the plasmid was recircularized. Plasmids were tested in the *in vitro* transcription/translation system (Stueber *et al.*, 1984) for expression of subunit IV precursors which were no longer cleaved upon import into isolated mitochondria. The amino acid sequence of such an uncleavable precursor (as deduced from the DNA sequence) is shown in Figure 2.

Plasmid pDS 5/2-1-70 kd-subunit IV was constructed as follows. Plasmid pDS 5/2-1-70 kd-DHFR was cut at its single *Bam*HI site corresponding to amino acid 12 of the 70-kd sequence (see also Figure 2). 5′-overhanging ends were filled in by the large fragment of *E. coli* DNA polymerase and an *Xba*I linker (New England Biolabs) was attached to the 3′ end of the 70-kd gene fragment. It was cut with *Xba*I/*Pst*I and the smaller fragment containing the 70-kd gene fragment and the T5 phage promoter was isolated. Plasmid pDS 5/2-Cox IV was cut with *Pst*I followed by a partial *Xba*I digestion. A 4300-bp *Xba*I/*Pst*I fragment was isolated. The *Xba*I site of this large fragment corresponds to amino acid 21 within the subunit IV pre-sequence. Thus, most of the gene encoding the subunit IV pre-sequence had been deleted. Both isolated *Pst*I/*Xba*I fragments were ligated, yielding plasmid pDS 5/2-1-70 kd-subunit IV. This plasmid allowed *in vitro* expression of a fusion protein containing the first 12 amino-terminal amino acids of the 70-kd protein attached to 'pseudo-mature' subunit IV (see Figure 2, 70 kd-subunit IV). Finally, the genes encoding wild-type subunit IV precursor, uncleavable subunit IV precursor, 70-kd-subunit IV fusion protein and 'pseudo-mature' subunit IV were isolated from the *in vitro* expression plasmids as *Eco*RI/*Hind*III fragments and ligated with the large *Eco*RI/*Hind*III fragment of plasmid pAC1. pAC1 is a YCp50 derivative which carries the *Bam*HI/*Hind*III fragment from pMAC561 containing the yeast alcohol dehydrogenase I promoter (McKnight and McConaughy, 1983). By this insertion the various subunit IV genes were placed under the control of the yeast alcohol dehydrogenase I promoter, allowing efficient expression of these genes *in vivo*. Plasmid pAC1 containing the various subunit IV-related genes was used to transform the subunit IV-deficient mutant WD1. Transformants were selected on uracil-free plates supplemented with 20 μg histidine/ml and tested for expression of subunit IV and its derivatives by extraction with NaOH, SDS-polyacrylamide gel electrophoresis and immune blotting.

Yeast growth, subcellular and submitochondrial fractionation

The yeast strain YNN 214 transformed with plasmids pLGSD5-DHFR or pLGSD5-70 kd-DHFR was 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 time before harvesting the cells, the medium was supplemented with 0.5% yeast extract and 2% galactose. The subunit IV-deficient mutant transformed with the various pAC1 plasmids was grown in 1% yeast extract, 2% peptone and 0.1% glucose to an OD 600 of ~1. Cells were converted to spheroplasts and subfractionated into mitochondria, cytosol and microsomes as described earlier (Hase *et al.*, 1984; Hurt *et al.*, 1985). For mitochondrial 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 20 min. The resulting shocked mitochondria (mitoplasts) were separated from the soluble intermembrane space by centrifugation and further fractionated into soluble matrix and membranes (Daum *et al.*, 1982). For protease digestion, mitochondria or mitoplasts corresponding to 1 mg/ml protein were incubated for 30 min at 0°C with 250 μg/ml proteinase K. Protease-digestion was stopped by 1 mM phenylmethylsulfonyl fluoride and mitochondria or mitoplasts were re-isolated through a 0.6 ml cushion of 20% sucrose. Where indicated mitochondria were incubated with proteinase K plus 1% Triton X-100.

Miscellaneous

Published procedures were used for *in vitro* transcription/translation of cloned genes (Stueber *et al.*, 1984; Hurt *et al.*, 1984b) except that translation was performed only for 30 min, for mitochondrial *in vitro* import experiments (Hurt *et al.*, 1985), for *in vivo* pulse-labeling of yeast cells (Yaffe and Schatz, 1985), for SDS-polyacrylamide gel electrophoresis, fluorography and immunoprecipitation

(Gasser *et al.*, 1982), for quantitation of bands on X-ray films (Suissa, 1983) and for transformation of yeast with plasmids pLGSD5 and pAC1 (Ito *et al.*, 1983). DHFR activity was measured as outlined by Hurt *et al.* (1985). Published procedures were used to assay cytochrome *c* oxidase (Mason *et al.*, 1973) and succinate-cytochrome *c* reductase (Tisdale, 1967) activities. L-[^{35}S]methionine (>1000 Ci/mmol) was purchased from Amersham International, UK.

Acknowledgements

We wish to thank Dr J.Jiricny for oligonucleotide synthesis, Drs H.Bujard and D.Stueber for plasmid pDS 5/2, Dr L.Guarente for plasmid pLGSD5, Dr D. Allison for plasmid pAC1, N.Soltanifar for helping us with the experiment shown in Figure 5C, Dr W.Dowhan and C.Bibus for supplying the subunit IV-deficient yeast mutant, K.Suda, W.Oppliger and H.Brütsch for excellent technical assistance and M.Probst for typing the manuscript. Critical comments on the manuscript by Drs D.Allison, D.Roise, P.Srere, D.van Loon, K.Verner and C.Witte are gratefully acknowledged. This study was supported by grants 3.394-0.83 and 3.660-0.84 from the Swiss National Science Foundation and an EMBO long-term fellowship to E.C.H.

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Received on 7 October 1985

Note added in proof

After having submitted this manuscript we learned that a hybrid protein containing the first 21 residues of the 70 kd protein attached to a large C-terminal fragment of *E. coli* beta galactosidase is imported into the yeast mitochondrial matrix *in vivo* (Hase,T., Nakai,M. and Matsubara,H., submitted to *J. Biol. Chem.*).