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The amino-terminal region of an imported mitochondrial precursor polypeptide can direct cytoplasmic dihydrofolate reductase into the mitochondrial matrix

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Subunit IV of yeast cytochrome *c* oxidase is encoded by a nuclear gene, synthesized in the cytosol as a precursor with a transient amino-terminal extension of 25 amino acids, and imported into the mitochondria. By gene fusion, we have attached the amino-terminal 53 amino acids of the subunit IV precursor to the amino terminus of the mouse cytosolic enzyme dihydrofolate reductase. When the resulting fusion protein was synthesized in a transcription-translation system and then incubated with energized yeast mitochondria, it was imported into the mitochondrial matrix space and processed to a shorter form by the chelator-sensitive matrix protease. No evidence was obtained that the fusion protein became stuck across one of the two mitochondrial membranes. Thus, a non-mitochondrial protein can be transported into the mitochondrial matrix if it is fitted with a mitochondrial targeting sequence.

Key words: dihydrofolate reductase/subunit IV precursor/fusion protein/mitochondrial matrix

Introduction

Many nuclear-coded mitochondrial proteins are synthesized as larger precursors in the extramitochondrial cytoplasm (Hay *et al.*, 1984). Transport of these precursors into the mitochondria requires an electrochemical potential across the mitochondrial inner membrane and is followed by the removal of the amino-terminal precursor extensions by a chelator-sensitive protease in the matrix (Böhni *et al.*, 1980, 1983; Miura *et al.*, 1982; McAda and Douglas, 1982). Precursors whose prepiece has been removed *in vitro* by addition of the solubilized matrix protease can no longer be imported into mitochondria (Gasser *et al.*, 1982). This observation suggests that the transient prepiece has some function in the import pathway, but this function remains ill-defined. The following suggestions have been made: (i) the prepiece renders some hydrophobic polypeptides more soluble (Viebrock *et al.*, 1982); (ii) the prepiece stabilizes a unique folding of the precursor polypeptide (Wickner, 1979); (iii) the prepiece contains the information for targeting the attached 'mature' polypeptide to its correct intramitochondrial location (Schatz, 1979).

To test these possibilities we have studied the import of subunit IV of yeast cytochrome *c* oxidase. This enzyme contains at least nine non-identical subunits; three of these (I–III) are encoded by mitochondrial DNA and six (IV–VII, VIIa, VIII) are encoded by nuclear DNA (Schatz and Mason, 1974; Power *et al.*, 1984). Subunit IV is made as a larger precursor outside the mitochondria (Lewin *et al.*, 1980; Mihara and Blobel, 1980). The subunit IV precursor protein can now be manipulated by recombinant DNA methods since its gene has recently been cloned and sequenced (Maarse *et al.*, 1984). The deduced amino acid sequence of the precursor and the directly determined amino acid sequence of the mature subunit reveal that the subunit IV precursor contains a strongly basic transient presequence consisting of 25 amino acids and a slightly acidic 'mature' region consisting of 130 amino acids. By combining deletion- and gene fusion experiments we show that the amino-terminal region of the subunit IV precursor directs the precursor into the mitochondria. If this amino-terminal region is fused to a cytosolic protein, the resulting fusion protein is transported into the mitochondrial matrix.

Results

The experimental approach

The wild-type subunit IV gene and the various constructs derived from it were placed under control of a strong bacteriophage promoter (Stueber and Bujard, 1982; Bujard, 1980) and transcribed with purified *Escherichia coli* RNA polymerase. The resulting mRNA was capped and translated in a reticulocyte lysate in the presence of [^{35}S]-methionine (Figure 1). The radiolabeled polypeptide product was then tested for cleavage by purified matrix protease or for binding and import by isolated yeast mitochondria. As shown below and in the paper by Stueber *et al.* (1984), this method yields the desired gene products in high radiochemical yield and purity and thus makes tedious immunoprecipitations and long fluorographic exposures unnecessary.

The amino-terminal, but not a central region, of the subunit IV precursor is essential for import into mitochondria

To determine regions of subunit IV necessary for import we replaced the 25 amino acids of the transient presequence by

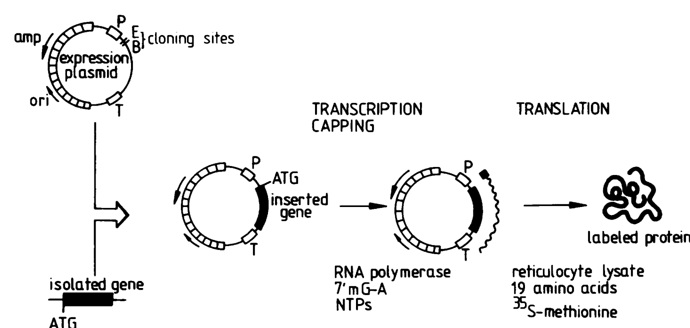

Fig. 1. *In vitro* expression of cloned genes in a transcription-translation system. P, bacteriophage T5 promotor; T, transcription termination sequences derived from bacteriophage lambda; amp, beta-lactamase gene; ori, origin of replication; 7′m G-A, 7′-methyl-guanosine adenosine; NTPs, nucleoside triphosphates. Open and shaded bars, denote sequences derived from bacteriophages and plasmid pBR322, respectively. Arrows indicate the direction of transcription, the wavy line with an attached filled-in box mRNA carrying a 5′ cap structure, B and E *Bam*HI and *Eco*RI restriction sites used for cloning. See Materials and methods for further details.

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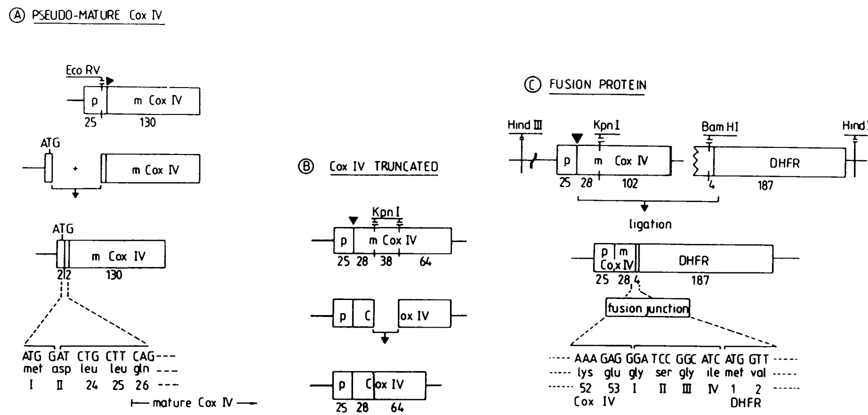

Fig. 2. Manipulation of the yeast gene for cytochrome *c* oxidase subunit IV. See Materials and methods for details. p and m deonote coding sequences specifying the transient prepiece (25 amino acids) and the 'mature' part (130 amino acids) of the subunit IV precursor. Cox IV, cytochrome *c* oxidase subunit IV; DHFR, dihydrofolate reductase. The numbers underneath the open bars give the numbers of amino acid residues in the prepiece and the 'mature' sequence. Residues in the 'mature' sequence are numbered such that the amino-terminal glutamine of mature subunit IV is the first residue. In **A** and **C**, the predicted nucleotide sequence and the derived numerals identify amino acids introduced by the gene manipulation procedure. The black arrow marks the site cleaved by the mitochondrial matrix protease.

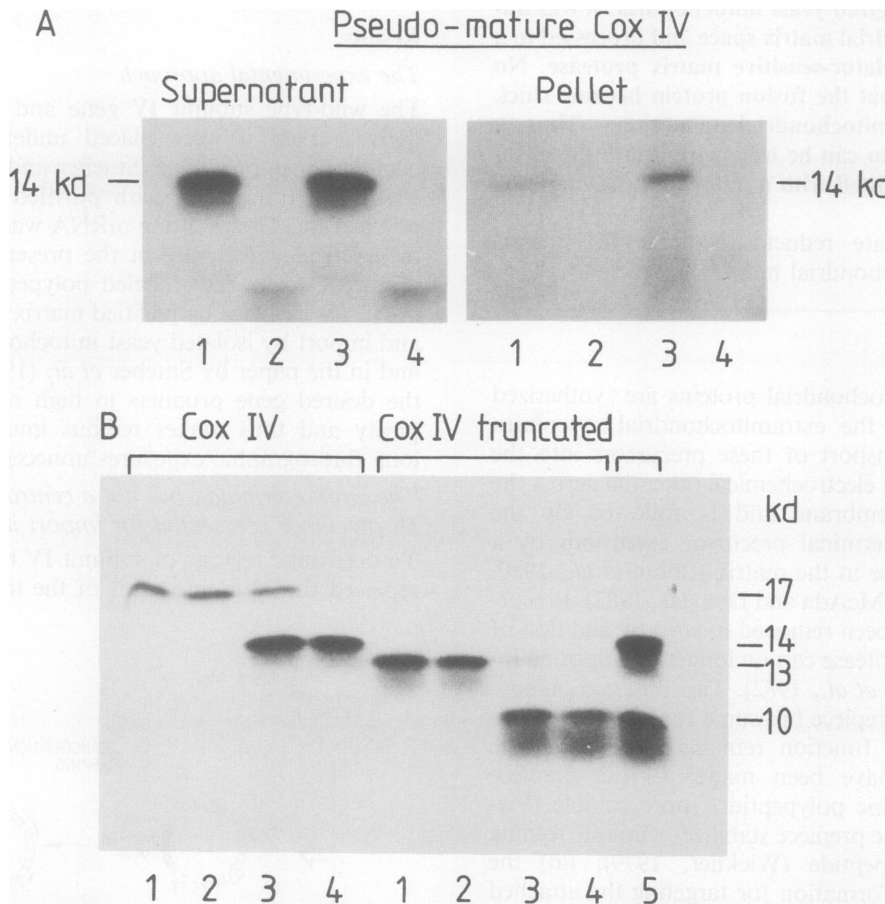

Fig. 3. The prepiece, but not an internal region, of the subunit IV precursor is required for binding and import by mitochondria. The following radiolabeled polypeptides were synthesized *in vitro*: wild-type subunit IV precursor (Cox IV; **panel B**); subunit IV precursor lacking 38 amino acids within the 'mature' part of the polypeptide chain (Cox IV truncated; **panel B**); and subunit IV precursor in which the prepiece had been replaced by the four amino acids Met Asp Leu Leu (Pseudo-mature Cox IV; **panel A**). **(A)** The polypeptides were incubated with isolated mitochondria at 30°C as described below. To measure binding, the mitochondria were de-energized; to measure import, the mitochondria were energized (See Materials and methods for details). In each instance, one aliquot was treated with trypsin to check whether a given polypeptide had entered the mitochondria and, as a result, had become inaccessible to the externally-added protease. The mitochondria were re-isolated and 100% of the mitochondrial pellet and 40% of the supernatant were analyzed by SDS-14% polyacrylamide gel electrophoresis and fluorography. **1**, mitochondria de-energized by K^+ and valinomycin; **2**, same as **1**, but treated with trypsin; **3**, energized mitochondria; **4**, same as **3**, but treated with trypsin. **(B)** The overall experiment was essentially the same as in **panel A** except that only the mitochondrial pellets were analyzed. **1**, mitochondria de-energized with K^+ and valinomycin and incubated at 0°C; **2**, same as **1**, but incubated at 30°C; **3**, energized mitochondria (30°C); **4**, same as **3**, but mitochondria treated with trypsin after incubation; **5**, energized mitochondria incubated at 30°C with both pre-subunit IV and the internally deleted subunit IV precursor. The molecular masses of pre-subunit IV (17 kd), mature subunit IV (14 kd), pseudo-mature subunit IV (14 kd), pre-subunit IV carrying an internal deletion (13 kd) and mature subunit IV carrying an internal deletion (10 kd) are indicated on the right.

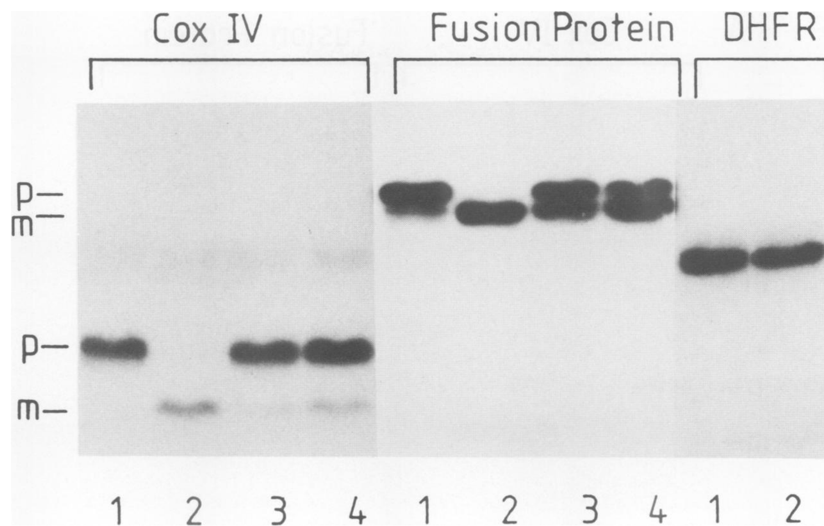

Fig. 4. The subunit IV-dihydrofolate reductase fusion protein is processed by partially purified matrix protease. The fusion protein, the subunit IV precursor (Cox IV) and dihydrofolate reductase (DHFR) were synthesized *in vitro* and incubated with partially purified processing protease from the yeast mitochondrial matrix (cf. Materials and methods). Fluorograph of a dried SDS-14% polyacrylamide gel is shown. 6 μg matrix protease carried through step 2 as described by Böhni *et al.* (1983) was used. **1**, no additions; **2**, incubated with processing protease for 30 min; **3**, incubated with processing enzyme and 1,10-phenanthroline for 30 min; **4**, incubated with processing enzyme for 2 min. p and m, precursor and mature form of subunit IV and the fusion protein.

the four amino acids Met Asp Leu Leu (Figure 2A). This 'pseudo-mature' subunit IV, unlike the authentic subunit IV precursor, did not significantly bind to, and was not imported by isolated yeast mitochondria (compare Figure 3A and B, Cox IV). Similarly, if the prepiece of radiolabeled subunit IV precursor was removed by adding purified matrix protease, the mature-sized subunit IV failed to bind to the mitochondrial surface (not shown). In contrast, removal of an internal stretch of 38 amino acids in the subunit IV precursor (Figure 2B, Cox IV truncated) did not abolish import: the shortened subunit IV precursor bound to the surface of de-energized mitochondria (Figure 3B, Cox IV truncated, lanes 1, 2) and was imported as well as processed to a shorter form by energized mitochondria (Figure 3B, Cox IV truncated, lanes 3–5).

A fusion protein containing the 53 amino-terminal amino acids of the yeast subunit IV precursor and mouse dihydrofolate reductase is processed by the mitochondrial matrix protease and imported into mitochondria

The *in vitro* transcription-translation experiments described here made use of a plasmid which also contained the mouse gene for dihydrofolate reductase. This enzyme is present in the cytosol of rodent cells (Wang *et al.*, 1967). We fused the 53 amino-terminal amino acids of the yeast subunit IV precursor to the entire dihydrofolate reductase protein (Figure 2C). This gene manipulation also introduced four additional amino acids at the junction of the two proteins. The apparent mol. wt. of the fusion protein (28 000) agreed well with the predicted value of 27 740 (dihydrofolate reductase = 21 600; 53 amino-terminal residues of the subunit IV precursor = 5700; four additional amino acids = 440; sum = 27 740).

The fusion protein still contains the cleavage site recognized by the chelator-sensitive matrix protease; indeed, when the radiolabeled protein was incubated with the partially purified protease, it was shortened by ~3 kd, corresponding to the size of the subunit IV prepiece (Figure 4, fusion protein, lanes 1,2,4). Processing was blocked by 1,10-phenanthroline, an inhibitor of the matrix protease (Figure 4, lane 3). Interestingly, the fusion protein was an even better substrate for the matrix protease than the authentic subunit IV precursor, at least under our *in vitro* conditions (Figure 4, Cox IV). As expected, *in vitro*-synthesized dihydrofolate reductase was not cleaved by the purified matrix protease (Figure 4, DHFR).

When the radiolabeled fusion protein was incubated with yeast mitochondria which had been de-energized by K^+ plus valinomycin, it bound to the organelles but remained outside since it was susceptible to externally-added protease (Figure 5A, lanes 1, 2). When the mitochondria were energized, the fusion protein was shortened by ~3 kd and the resultant shorter polypeptide was resistant to externally-added protease (Figure 5A, lanes 3, 4). This resistance was abolished by solubilizing the mitochondrial membranes with the detergent Triton X-100 (Figure 5A, lane 5). Production of a shortened, protease-inaccessible form of the fusion protein was also blocked by de-energizing mitochondria with KCN and the mitochondria-specific inhibitor carboxyatractylate (not shown). Exactly the same results were obtained with the authentic subunit IV precursor (Figure 5 and Maarse *et al*, 1984). Clearly, the fusion protein is imported into the mitochondria by an energy-dependent mechanism and cleaved to a shorter form in the mitochondrial matrix. Time-course experiments (not shown) suggested that the fusion protein was imported into mitochondria as efficiently as the subunit IV precursor. Generally, at least 50% of the *in vitro*-synthesized fusion protein was imported into mitochondria (cf. Figure 5B, fusion protein, lanes 2, mitochondrial pellet *versus* supernatant). In contrast, radiolabeled dihydrofolate reductase was not imported by energized mitochondria (Figure 5B).

The fusion protein is transported into the mitochondrial matrix space

Where inside the mitochondria does the processed fusion protein accumulate? Isolated mitochondria were allowed to import the radiolabeled fusion protein and the submitochondrial location of the processed fusion protein was determined by two approaches. In the first approach, the mitochondria

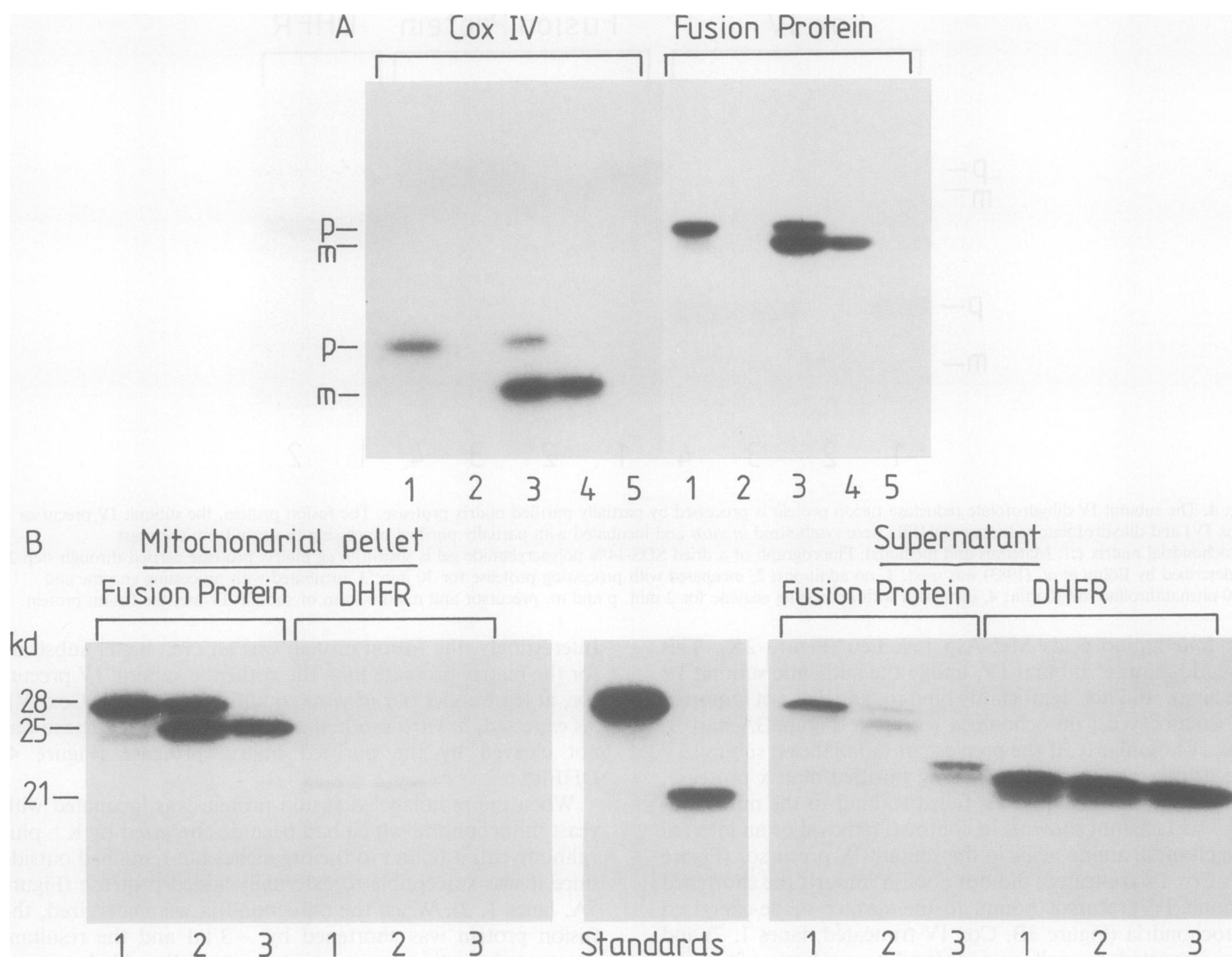

Fig. 5. The fusion protein, but not dihydrofolate reductase, is bound and imported by isolated mitochondria. Presubunit IV (Cox IV), the fusion protein and dihydrofolate reductase were synthesized *in vitro* and incubated with mitochondria de-energized with K^+ and valinomycin (to measure binding) or with energized mitochondria (to measure import). Fluorograms of dried SDS-14% polyacrylamide gels are shown. **(A)** 100% of the re-isolated mitochondria were analyzed. **1**, de-energized mitochondria; **2**, same as **1**, but mitochondria subsequently treated with proteinase K; **3**, energized mitochondria; **4**, same as **3**, but mitochondria subsequently treated with proteinase K; **5**, energized mitochondria but subsequently treated with proteinase K in presence of 1% Triton X-100. p and m, precursor and 'mature' forms of subunit IV or the fusion protein. **(B)** 100% of the reisolated mitochondria and 40% of the supernatants were analyzed. **1**, pellet and supernatant, respectively, from incubation with de-energized mitochondria; **2**, same as **1**, but with energized mitochondria; **3**, same as **2**, but mitochondria subsequently treated with trypsin. (Note that dihydrofolate reductase is highly resistant to trypsin, but not to proteinase K – cf. Fig. 5A) The middle panel shows 30% of the total translation products (upper band: fusion protein; lower band: dihydrofolate reductase) as reference standards. The molecular masses of the pre-fusion protein (28 kd), the processed fusion protein (25 kd) and dihydrofolate reductase (21 kd) are indicated on the left margin. The mature-sized fusion protein in the supernatant from the incubation with energized mitochondria probably reflects leakage from damaged mitochondria.

were subfractionated into their various compartments (intermembrane space, matrix and membranes) and each fraction was tested for the presence of radiolabeled fusion protein by SDS-polyacrylamide gel electrophoresis and fluorography. The purity of each submitochondrial fraction was assessed by immune blotting, using antibodies against the following marker enzymes: cytochrome b_2 (intermembrane space), citrate synthase (matrix) and cytochrome c_1 (inner membrane). As shown in Figure 6A, the processed fusion protein co-fractionated with the matrix marker, citrate synthase.

In the second approach, we checked whether the imported fusion protein was inaccessible to externally-added proteinase K even if the mitochondrial outer membrane had been ruptured. This proved to be the case (Figure 6B). As expected, the imported protein was degraded if the mitoplasts were exposed to proteinase K in the presence of Triton X-100.

Since the processed fusion protein found inside the mitochondria is water-soluble and protected from external proteinase K even in mitoplasts, it must have completely crossed both mitochondrial membranes. Had only part of the polypeptide chain crossed the inner membrane, treatment of mitoplasts with proteinase K should have generated a discrete shortened fragment, or fragments too small to be detected by our gel system.

Discussion

This study has yielded two main results: (i) the sequence targeting the subunit IV precursor to mitochondria is contain-

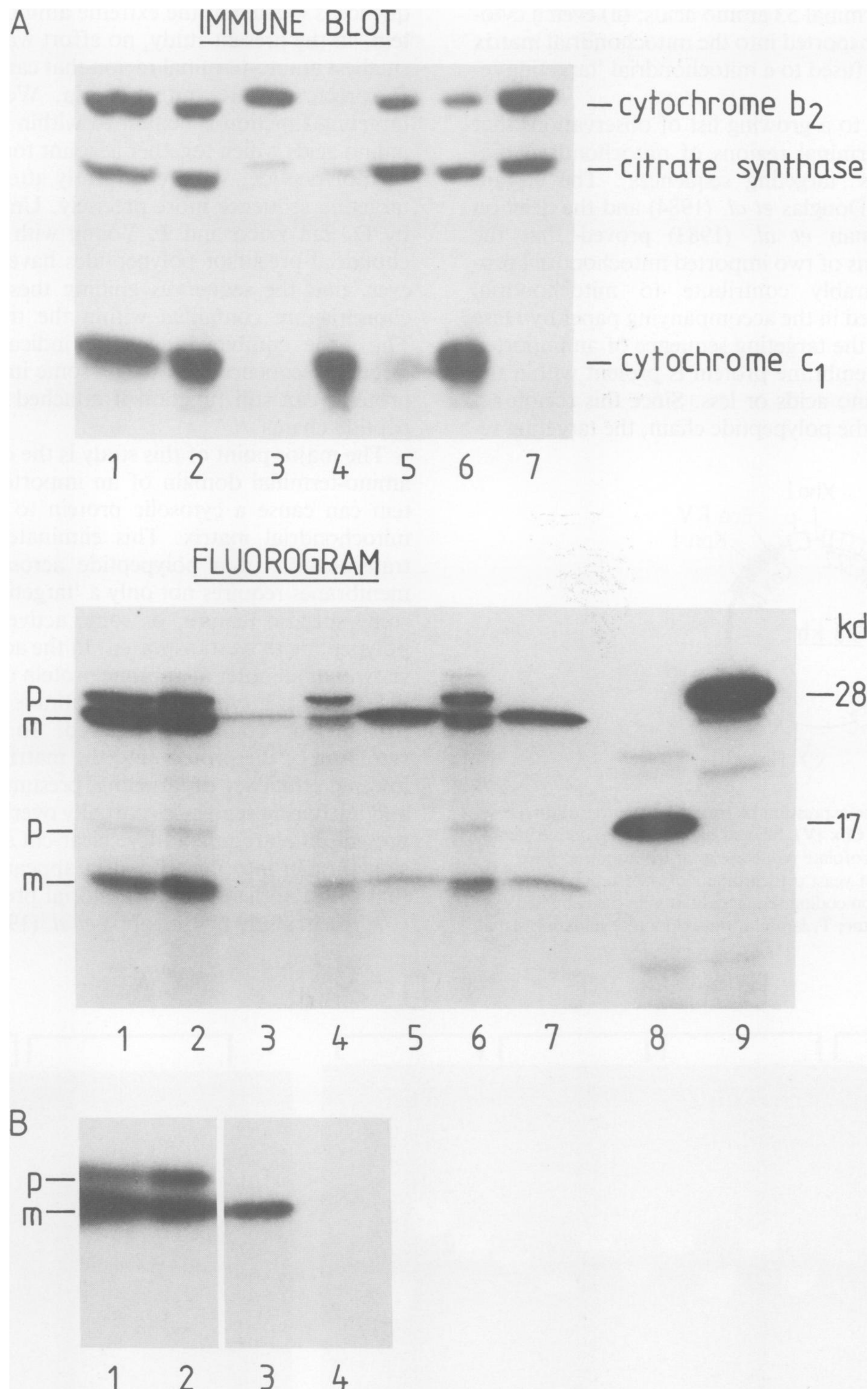

Fig. 6. The imported fusion protein is localized in the matrix. The subunit IV precursor and the fusion protein were synthesized *in vitro* and co-imported into isolated mitochondria. **(A)** The imported fusion protein co-fractionates with citrate synthase. After import, the mitochondria were re-isolated and fractionated into mitoplasts and intermembrane space; the mitoplasts were further fractionated into matrix and membrane fraction. Measured aliquots of each fraction were applied to a 12% SDS-polyacrylamide gel (**A, upper gel**; for immune blotting) or a 14% SDS-polyacrylamide gel (**A, lower gel**; and **B**; for direct fluorography). Immune blotting was performed with antibodies against the following intramitochondrial marker proteins: cytochrome b_2 (intermembrane space); citrate synthase (matrix); and cytochrome c_1 (outer surface of inner membrane). **1**, mitochondria; **2**, mitoplasts; **3**, intermembrane space fraction; **4**, membrane fraction obtained by sonication of mitoplasts; **5**, matrix; **6**, membranes obtained by repeated freezing and thawing of mitochondria (this procedure does not release soluble proteins as efficiently as sonication, cf. **lane 4**); **7**, combined matrix- and intermembrane space fraction (supernatant from frozen-thawed mitochondria); **8**, presubunit IV (35% of input); **9**, fusion protein (35% of input). The molecular masses of precursor (p) and mature (m) forms of both proteins are given on the right. **(B)** The imported fusion protein is inaccessible to proteinase K in mitoplasts. After import, part of the mitochondria were analyzed directly and part were converted to mitoplasts and aliquots of the mitoplasts were treated as described below. **1**, mitochondria; **2**, mitoplasts; **3**, mitoplasts treated with proteinase K; **4**, mitoplasts treated with proteinase K and 1% Triton X-100.

ed within the amino-terminal 53 amino acids; (ii) even a cytosolic protein can be transported into the mitochondrial matrix if its amino terminus is fused to a mitochondrial 'targeting sequence'.

The first result adds to a growing list of observations that implicate the amino-terminal regions of mitochondrial precursor polypeptides as 'targeting sequences'. The elegant gene-fusion studies of Douglas *et al.* (1984) and the deletion experiments by Riezman *et al.* (1983) proved that the carboxy-terminal regions of two imported mitochondrial proteins do not measurably contribute to mitochondrial targeting. Data presented in the accompanying paper by Hase *et al.* (1984) show that the targeting sequence of an imported mitochondrial outer membrane protein is present within the amino-terminal 41 amino acids or less. Since this region accounts for only 7% of the polypeptide chain, the targeting sequence is located at the extreme amino terminus of that protein. In the present study, no effort was made to identify the smallest amino-terminal region that can still guide the subunit IV precursor into mitochondria. We only show that the targeting function is contained within the amino-terminal 53 amino acids which together account for ~34% of the precursor polypeptide. We are currently attempting to identify the targeting sequence more precisely. Unpublished experiments by D. van Loon and T. Young with other imported mitochondrial precursor polypeptides have already shown, however, that the sequences guiding these precursors to mitochondria are contained within the transient presequences. Thus, the combined evidence indicates strongly that the targeting sequences of at least some imported mitochondrial proteins can still function if attached to heterologous polypeptide chains.

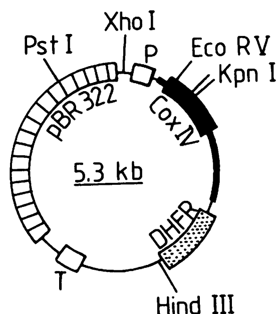

Fig. 7. Restriction map of the expression plasmid carrying the wild-type subunit IV gene (pDS 5/2-1 Cox IV). Shaded bar: sequences derived from pBR322; stippled bar: dihydrofolate reductase gene from mouse; filled thick bar: coding sequence of yeast cytochrome *c* oxidase subunit IV gene; filled thin bar: 5'- and 3'-non-coding sequences flanking the subunit IV coding region; P, T5 promotor; T, lambda transcription termination signal.

The major point of this study is the demonstration that the amino-terminal domain of an imported mitochondrial protein can cause a cytosolic protein to be imported into the mitochondrial matrix. This eliminates the possibility that translocation of a polypeptide across both mitochondrial membranes requires not only a 'targeting sequence', but also some specific feature, or some active participation, of the polypeptide to be transported. In the accompanying paper we show that an outer membrane protein can be transported into the matrix if a typical transmembrane anchoring sequence is partially or completely deleted. In that case, however, rerouting of the protein into the matrix is accompanied by a lowered efficiency of targeting, presumably because targeting and anchoring sequences partially overlap. In this respect, the present data are much more clear-cut since import of the fusion protein into mitochondria appears to be as efficient as that of an authentic mitochondrial precursor polypeptide.

A recent study by Lingappa *et al.* (1984) has shown that the

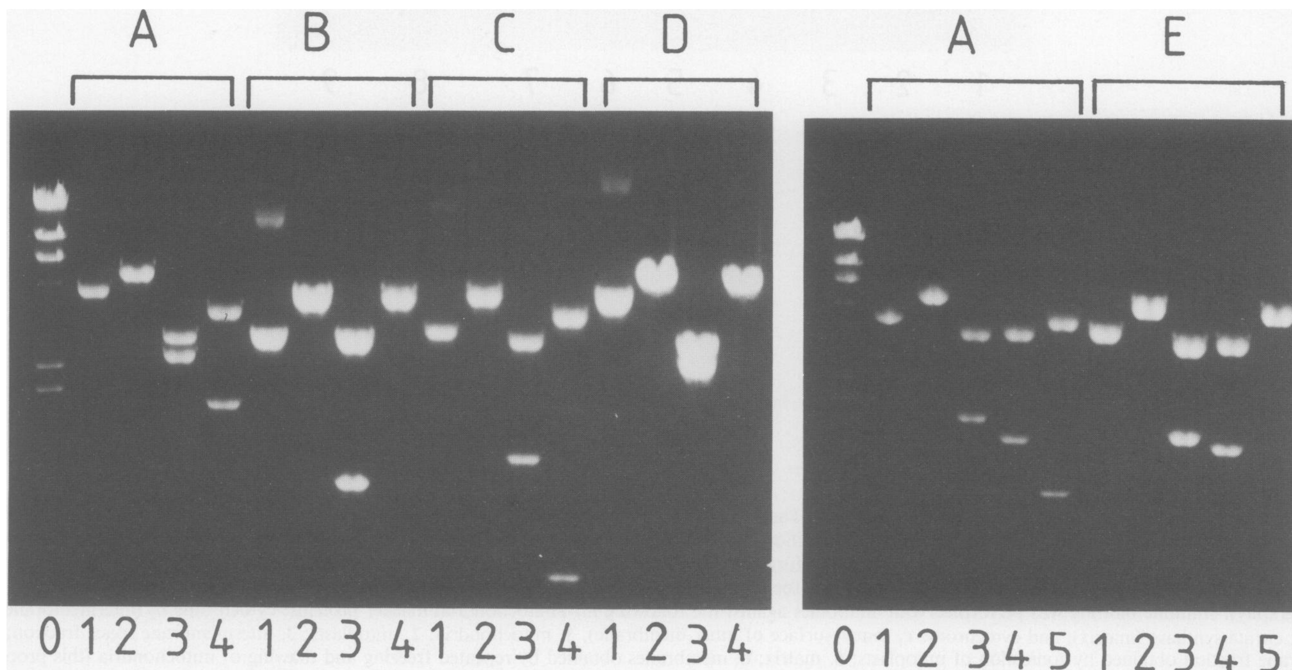

Fig. 8. Restriction endonuclease analysis of the various manipulated genes used in this study. The various plasmid DNAs were purified by CsCl-gradient centrifugation and analyzed by cutting with various restriction endonucleases followed by agarose gel electrophoresis and photography of the DNA fragments stained with ethidium. **A**, pDS 5/2-1-Cox IV (intact subunit IV gene); **B**, pDS 5/2 (dihydrofolate reductase gene); **C**, pDS 5/2-1 Cox IV-DHFR (fusion gene); **D**, pDS 5/2-1-mCox IV (gene coding for pseudo-mature subunit IV); **E**, pDS 5/2-1 tCox IV (gene coding for truncated subunit IV precursor). **Left panel: 0**, lambda DNA cut with *Hind*III; **1**, no restriction enzyme; **2**, *Xho*I; **3**, *Sac*I/*Pst*I; **4**, *Eco*RV/*Hind*III. **Right panel: 0**, lambda DNA cut with *Hind*III; **1**, no restriction enzyme; **2**, *Hind*III; **3**, *Eco*RV/*Hind*III; **4**, *Kpn*I/*Hind*III; **5**, *Cla*I/*Pst*I.

chimpanzee alpha globin chain can be translocated into the lumen of isolated dog pancreas microsomes if its amino terminus is fused to the signal sequence of bacterial beta-lactamase. In that case, translocation occurs co-translationally whereas in the case documented here translocation occurs post-translationally. We conclude that polypeptides can be translocated across biological membranes if they carry a properly positioned sequence functioning with the translocation machinery of that membrane.

Materials and methods

Strains, media and plasmids

E. coli strain HB 101 was grown in LB-medium (Kedes *et al.*, 1975); for selection of ampicillin-resistant transformants, the medium was supplemented with 100 μg ampicillin/ml. Plasmid pDS 5/2 (kindly provided by Drs. H. Bujard and D. Stueber, Hoffmann-La Roche Co., Basel, Switzerland) contains the *PvuII/EcoRI* fragment of pBR322, the bacteriophage T5 promoter P25, transcription termination signals from bacteriophage lambda, and the gene for dihydrofolate reductase from mouse (Stueber and Bujard, 1981; Bujard, 1980; Stone and Phillips, 1977). Plasmid pFL19-4 contains the entire gene for yeast cytochrome *c* oxidase subunit IV (Maarse *et al.*, 1984). The yeast strain used as a source of mitochondria was D273-10B (ATCC 25657); it was grown as described (Gasser *et al.*, 1982).

Recombinant DNA work

Published procedures were used for transformation of *E. coli* HB 101 (Mandel and Higa, 1970), small-scale and large-scale isolation of plasmid DNA from transformed *E. coli* cells (Birnboim and Doly, 1979), manipulations involving restriction endonucleases, T4 DNA polymerase, *Bal*31 exonuclease and the large ('Klenow') fragment of *E. coli* DNA polymerase (Maniatis *et al.*, 1982), electrophoresis in 0.7% agarose gels (Maniatis *et al.*, 1982) and recovery of DNA fragments from agarose gels using DEAE paper (Dretzen *et al.*, 1981).

Gene constructions

The entire gene for yeast cytochrome *c* oxidase subunit IV was excised from plasmid pFL19-4 with *Eco*RI, ATG codons in the 5'-non-coding region were removed by limited *Bal*31 digestion and the gene was then cut with *Bgl*II in the 3'-non-coding region. The promoter-distal *Eco*RI site of pDS 5/2 was removed by limited digestion with *Eco*RI, filling in with the large *E. coli* DNA polymerase fragment, and religation. The resulting plasmid pDS 5/2-1 was cut with *Eco*RI, blunt-ended as above, and cut with *Bam*HI. The large linear fragment was ligated with the manipulated subunit IV gene (cf. above). The resulting plasmid (pDS 5/2-1-Cox IV) was used for all subsequent gene manipulation. Its restriction map and pattern are given in Figures 7 and 8A.

To construct a gene for 'pseudo-mature' subunit IV, pDS 5/2-1--Cox IV was cut with *Eco*RV and an *Nde*I linker (New England Biolabs) supplying an ATG start codon was inserted (Figure 2A). The restriction pattern of the resulting plasmid is shown in Figure 8D.

To construct a gene for a subunit IV precursor carrying an internal deletion of 38 amino acids ('Cox IV truncated'), pDS 5/2-1-Cox IV was cut with *Kpn*I, the small *Kpn*I fragment was removed, and the large fragment was religated (Figure 2B). The restriction pattern of the resulting plasmid is shown in Figure 8E.

To construct a fused gene coding for the 53 amino-terminal amino acids of yeast cytochrome *c* oxidase subunit IV and the entire mouse dihydrofolate reductase gene (Figure 2C), plasmid pDS 5/2-1-Cox IV was cut with *Kpn*I and protruding 3' ends were removed with T4 DNA polymerase. The blunt-ended linear plasmid was cut with *Hind*III and the large fragment containing the phage T5 promoter and the 5'-proximal part of the subunit IV gene was fused to a *Bam*HI/*Hind*III fragment carrying the entire mouse gene for dihydrofolate reductase. (In order to fuse the two genes in-frame, the protruding end generated by *Bam*HI in the 5'-non-coding region of the dihydrofolate reductase gene was first filled in with the large fragment of *E. coli* DNA polymerase). The restriction pattern of the resulting plasmid is shown in Figure 8C.

In vitro transcription-translation

Genes which had been placed under control of the phage T5 promoter were transcribed according to Wiedmann *et al.* (1984). The incubation contained, in 10 μl: 20 mM Hepes pH 7.9, 10 mM Mg-acetate, 200 mM K-acetate, 0.2 mM spermidine, 0.5 mM each of GTP, CTP and UTP, 5 mM dithiothreitol, 100 μM 7'-methyl-guanosine-adenosine, 0.5 units of RNAse inhibitor from human placenta, 6 μg of plasmid DNA (purified in a CsCl gradient) containing the gene to be expressed, and 1 unit of *E. coli* RNA polymerase (New England Biolabs). To allow capping of the nascent mRNA, the mixture was incubated for 3 min at 37°C before ATP was added to 1 mM. After an additional 10 min, transcription was stopped by chilling. The entire mixture was directly added to 30 μl of nuclease-pre-treated reticulocyte lysate that had been supplemented with hemin, creatine phosphate, creatine kinase, the 19 amino acids except methionine, 130 mM KCl, 2 mM $MgCl_2$ (Pelham and Jackson, 1976) as well as with 250 μg/ml calf-liver tRNA, 50 units of human placenta RNAse inhibitor/ml and 1 mCi of [^{35}S]methionine (> 1000 Ci/mmol) per ml. The final volume was 50 μl. Translation was allowed to proceed for 60 min at 30°C. Aliquots were spotted on filter paper soaked with 5% trichloroacetic acid to measure incorporation of ^{35}S into protein. The entire mixture was then directly analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. As shown in Figures 3–6, most of the incorporated radioactivity was found in the polypeptide whose gene had been placed under the control of the strong phage T5 promotor. In a typical experiment, 7.5×10^5 c.p.m. were recovered in the subunit IV precursor (which contains three methionine residues), corresponding to at least 3 nmol protein (ignoring any radiochemical dilution of the added [^{35}S]methionine).

In vitro processing and import into mitochondria of radiolabeled polypeptides

Yeast mitochondria were isolated and incubated with radiolabeled precursor polypeptides as described by Gasser *et al.* (1982). The incubation (total volume 200 μl) usually contained 140 μg mitochondrial protein, 15 μl reticulocyte lysate containing the desired radiolabeled polypeptide, 40 mM KCl and, where indicated, 1 μg/ml valinomycin or 50 μg/ml carboxatractyloside and 1 mM KCN. After 30 min at 30°C, the mitochondria were re-isolated by centrifugation through a sucrose cushion. For digestion of externally-located polypeptides, they were then incubated for 30 min at 0°C with either 250 μg of trypsin/ml or 250 μg proteinase K/ml; the protease was inhibited with 1 mM phenylmethyl sulfonylfluoride and the mitochondria were re-isolated as mentioned above. Alternatively, mitochondria containing radiolabeled imported polypeptides were fractionated into mitoplasts, intermembrane space, matrix, and membranes (Gasser *et al.*, 1982) or directly dissociated in SDS-containing electrophoresis buffer. All samples were analyzed for radiolabeled polypeptides by SDS-polyacrylamide gel electrophoresis and fluorography. Precursors were post-translationally processed for 2 or 30 min at 30°C with the partially purified enzyme (6 μg of enzyme carried through step 2; Böhni *et al.*, 1983) from the yeast mitochondrial matrix. The total volume was 6 μl.

Miscellaneous

Published procedures were used for SDS-polyacrylamide gel electrophoresis and fluorography (Gasser *et al.*, 1982) except that polyacrylamide gels were heated for 5 min each at 100°C in 5% trichloroacetic acid water, and 10 mM Tris-Cl pH 7.4, before treatment with Na-salicylate. Immune blotting was done as described by Haid and Suissa (1983) and protein was measured according to Lowry *et al.* (1951). [^{35}S]methionine (> 1000 Ci/mmol) was purchased from Amersham Ltd., UK.

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Note added in proof

It was recently shown (Hurt *et al.*, 1984; *FEBS Lett.*, in press) that the cleavable prepiece of the subunit IV precursor is sufficient to direct cytosolic dihydrofolate reductase into the mitochondrial matrix.