The EMBO Journal vol.4 no.5 pp.1129-1135, 1985

A leader peptide is sufficient to direct mitochondrial import of a chimeric protein

Arthur L.Horwich[1], Frantisek Kalousek[1], Ira Mellman[2] and Leon E.Rosenberg[1]

Yale University School of Medicine, Departments of [1]Human Genetics and [2]Cell Biology, New Haven, CT 06510, USA

Communicated by G.Schatz

Most mitochondrial proteins are encoded in the nucleus and synthesized in the cytoplasm as larger precursors containing NH_2-terminal 'leader' peptides. To test whether a leader peptide is sufficient to direct mitochondrial import, we fused the cloned nucleotide sequence encoding the leader peptide of the mitochondrial matrix enzyme ornithine transcarbamylase (OTC) with the sequence encoding the cytosolic enzyme dihydrofolate reductase (DHFR). The fused sequence, joined with SV40 regulatory elements, was introduced along with a selectable marker into a mutant CHO cell line devoid of endogenous DHFR. In stable transformants, the predicted 26-K chimeric precursor protein and two additional proteins, 22 K and 20 K, were detected by immunoprecipitation with anti-DHFR antiserum. In the presence of rhodamine 6G, an inhibitor of mitochondrial import, only the chimeric precursor was detected. Immunofluorescent staining of stably transformed cells with anti-DHFR antiserum produced a pattern characteristic of mitochondrial localization of immunoreactive material. When the chimeric precursor was synthesized in a cell-free system and incubated post-translationally with isolated rat liver mitochondria, it was imported and converted to a major product of 20 K that associated with mitochondria and was resistant to proteolytic digestion by externally added trypsin. Thus, both in intact cells and *in vitro*, a leader sequence is sufficient to direct the post-translational import of a chimeric precursor protein by mitochondria.

Key words: leader peptide/mitochondrial import/gene fusion/ornithine transcarbamylase precursor

Introduction

Many cellular proteins reside and function in locations remote from their cytoplasmic site of synthesis. The systems of compartmentation which target and translocate these proteins to their particular destinations during or following synthesis are a current focus of investigation. The specificity of these systems is likely to reside both in the targeted proteins themselves and in cellular elements that recognize and compartmentalize them. Amino acid sequences which confer such specificity have been identified in newly synthesized polypeptides targeted to the endoplasmic reticulum (Walter *et al.*, 1984), the nucleus (Hall *et al.*, 1984; Kalderon *et al.*, 1984), and the mitochondria (Hay *et al.*, 1984).

Most mitochondrial proteins are encoded in the nucleus and synthesized in the cytosol on free polyribosomes as precursor polypeptides, larger than the corresponding mature forms by virtue of additional NH_2-terminal residues comprising leader peptides. These precursor proteins are post-translationally recognized by mitochondria, translocated across one or both mitochondrial membranes by an energy-dependent mechanism, and processed to their mature size by proteolytic removal of the NH_2-terminal leader peptides (Maccecchini *et al.*, 1979; Hay *et al.*, 1984). The leader peptides are required for mitochondrial import — precursors incubated with isolated mitochondria are imported, whereas the corresponding mature proteins fail either to be taken up or to interfere with uptake of the precursors (Kraus *et al.*, 1981; Hay *et al.*, 1984). Whether leader sequences act alone or in concert with sequences in the mature portions of the precursors is unknown. To address this question, we have asked whether a leader peptide can direct a non-mitochondrial protein to the mitochondria when the two are joined in a chimeric protein. To synthesize such a protein, we employed a strategy of gene fusion. A leader coding sequence was isolated from the cloned cDNA encoding the precursor of the mitochondrial matrix enzyme ornithine transcarbamylase (OTC); this was joined with the coding sequence for the enzyme dihydrofolate reductase (DHFR), normally found only in the soluble, cytosolic compartment. The targeting of the chimeric protein produced by this sequence was then studied in cultured cells and *in vitro*. In both cases, the chimeric protein was recognized by mitochondria, translocated by them, and proteolytically processed, demonstrating that the leader peptide is sufficient to direct mitochondrial import.

Results

Construction of plasmid encoding a chimeric protein containing the OTC leader sequence and DHFR

We constructed a plasmid to program synthesis of the chimeric protein by fusing cDNA segments encoding the two amino acid sequences in the proper frame for translation and joining them in turn with SV40 regulatory elements, as shown in Figure 1. First, a DNA fragment containing the SV40 origin of replication region joined with the human OTC cDNA sequence was isolated from the plasmid pSV2OTC (Horwich *et al.*, 1984). The SV40 sequence included an enhancer element and the early region promoter; the particular OTC cDNA segment encoded the 32 residues comprising the leader and the four contiguous NH_2-terminal residues of the mature subunit. A second DNA fragment containing the entire coding sequence for mouse DHFR, as well as five bases of 5'-untranslated sequence and 89 bases of 3'-untranslated sequence was isolated from the plasmid pDHF-R26 (Chang *et al.*, 1978). Each of the two DNA fragments was joined with *Eco*RI linkers and inserted into the *Eco*RI site of pBR328. Next, cloned sequences from SV40, including the small T intron and a polyadenylation signal, were inserted into the 3'-untranslated DHFR sequence of plasmid pR26. Finally, an *Eco*RI fragment from plasmid pRL1, containing the SV40 origin and OTC sequence, was inserted into the *Eco*RI site in plasmid pRS26 to produce the plasmid pLDHFR.

The DNA sequence of pLDHFR in the region joining the OTC and DHFR coding sequences, and the predicted amino acid sequence, is shown in Figure 1B. The OTC leader codons are followed by codons for the first four residues of the mature OTC

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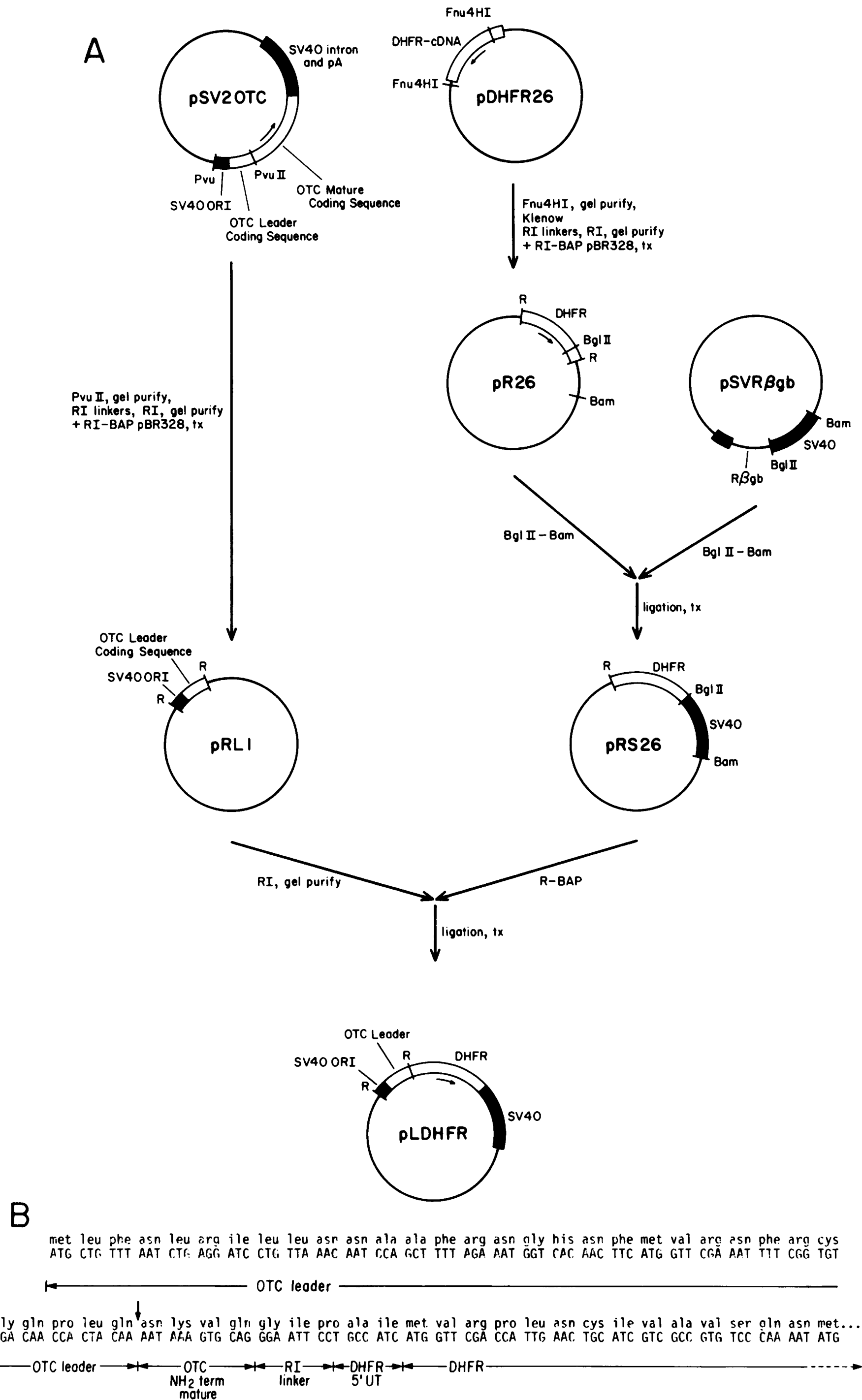

Fig. 1. **(A)** Steps in construction of pLDHFR. Plasmid pSV2OTC contains a human OTC cDNA sequence shown by the open bar, and adjoining SV40 regulatory elements, shown by dark bars. The *Pvu*II fragment containing the leader coding sequence and SV40 origin region was joined with *Eco*RI linkers, incubated with *Eco*RI and cloned in the *Eco*RI site of pBR328. Plasmid pDHFR26 contains a mouse DHFR cDNA sequence, shown by the open bar, inserted into the *Pst*I site of pBR322. The *Fnu*4HI fragment that includes the entire DHFR coding sequence was also treated to produce *Eco*RI termini and cloned in pBR328. SV40 sequences including the small T intron and polyadenylation signal were then introduced downstream of the DHFR coding sequence as a *Bgl*II-*Bam*HI fragment purified from the plasmid pSVRBgb, kindly provided by Peter Southern. The *Eco*RI fragment from pRL1 containing the OTC leader coding sequence and SV40 origin sequence was then inserted into the unique *Eco*RI site immediately upstream of the DHFR coding sequence in PRS26 to produce the plasmid pLDHFR. The arrow parallel to the open bar in pLDHFR, and in the other plasmids, designates the normal direction of transcription of the coding sequences. **(B)** Nucleotide sequence of the 5′ portion of the fusion sequence and predicted amino acid sequence. pLDHFR DNA was cleaved with *Bam*HI, which cleaves between bases 17 and 18 in the OTC leader coding sequence and at the junction of downstream SV40 sequences with pBR322. Following 5′ radiolabeling, the DNA was incubated with *Hinf*I and fragments containing the leader coding sequence and adjoining sequences were isolated and subjected to partial chemical cleavage reactions. The reaction products were displayed in a polyacrylamide/urea gel and the gel was autoradiographed. Sequence data are shown commencing with the OTC initiation codon and proceeding in an open reading frame that includes the OTC leader coding sequence, four contiguous codons from the mature OTC subunit, five codons from an *Eco*RI linker and DHFR 5′-untranslated sequence and codons for DHFR. The interrupted horizontal arrow serves to indicate that the remainder of the DHFR coding sequence is present as demonstrated by restriction analysis. The vertical arrow indicates the normal site of proteolytic cleavage of the OTC leader peptide from the mature OTC subunit. ORI, SV40 origin of replication; RI, *Eco*RI; BAP, bacterial alkaline phosphatase; tx, transformation.

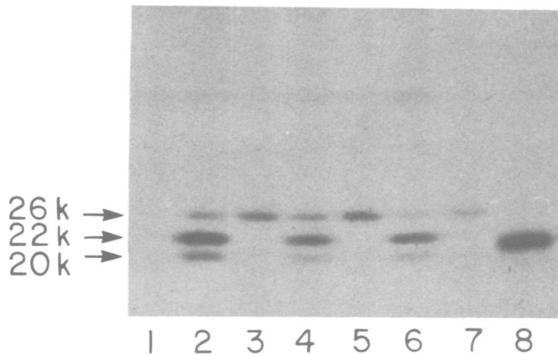

Fig. 2. Expression of OTC leader-DHFR chimeric protein in stable CHO cell transformants. A mutant CHO cell line, DG44, devoid of DHFR, was co-transfected with pLDHFR and pSV2Neo. DG44 and each of three stable G418-resistant transformants were grown to near confluence in 60 mm dishes in Ham's F12 medium. Cells were radiolabeled with [^{35}S]methionine in the absence or presence of 10 μg/ml rhodamine 6G (R6G). The monolayers were washed and harvested by detergent lysis, and the extracts were immunoprecipitated with anti-DHFR antiserum. The products were electrophoresed through an SDS-polyacrylamide gel, and the gel was fluorographed. Extract of DG44 (**track 1**); of transformant designated Line 1 (**track 2**), of Line 1 radiolabeled in the presence of R6G (**track 3**); of transformant Line 2 (**track 4**); of Line 2 in the presence of R6G (**track 5**); of transformant Line 3 (**track 6**); of Line 3 in the presence of R6G (**track 7**); of a HeLa cell line containing amplified mouse DHFR sequences (**track 8**).

subunit, two 2/3 codons from an *Eco*RI linker, two 1/3 codons from the 5'-untranslated DHFR sequence, and the codons for DHFR. The predicted amino acid sequence includes the site (arrow, Figure 1B) at which a mitochondrial matrix protease normally cleaves the leader from the OTC precursor (Horwich *et al.*, 1984). Thus we could assess mitochondrial entry of the chimeric protein by observing its proteolytic cleavage. The five residues predicted to follow the OTC sequence are Gly Ile Pro Ala Ile, followed by the initiator methionine of DHFR (which would normally be cleaved from the primary DHFR translation product). The predicted size of the chimeric protein is 26 K: 4 K from the OTC leader, 1 K from the 'connecting' residues and 21 K from native DHFR.

Expression of the chimeric protein in a CHO cell line

To facilitate detection of the chimeric protein in cultured cells through DHFR antigenic determinants, a cell line devoid of DHFR protein was used for transfection experiments. The cell line, DG44, kindly supplied by Larry Chasin, is a CHO-derived line deleted at both DHFR loci (Urlaub *et al.*, 1983). DG44 was co-transfected by $CaPO_4$ precipitation (Graham and Van der Eb, 1973; Wigler *et al.*, 1979) with pLDHFR and the plasmid pSV2Neo, encoding neomycin resistance, and selection was carried out with the neomycin analogue G418 (Southern and Berg, 1982). Three independent G418-resistant transformants were isolated, expanded and labeled with [^{35}S]methionine. Extracts of radiolabeled cells were immunoprecipitated with rabbit anti-mouse DHFR antiserum, and the products were analyzed by SDS-PAGE. Cells were labeled either in the absence or presence of rhodamine 6G (R6G), a fluorescent, mitochondria-specific dye which is also an inhibitor of mitochondrial protein import (Gear, 1974). The results are shown in Figure 2. The parental cell line DG44 contained no precipitable product, as expected (track 1). A cell line with amplified mouse DHFR sequences (track 8) contained a precipitable polypeptide with an apparent mol. wt. of 21 K, precisely the size of mouse DHFR. In the absence of R6G, each of the three G418-resistant transformants, designated LDHFR 1, 2, 3 (tracks 2,4,6) contained three precipitable products, with apparent sizes of 26 K, 22 K and 20 K (tracks 2,4,6). In the presence of R6G (tracks 3,5,7), only the 26-K protein was detected (tracks 3,5,7). These results suggest that, in the absence of inhibitor, the 26-K chimeric protein is translocated into the mitochondria and cleaved by mitochondrial protease(s) to produce 22-K and 20-K products; in the presence of inhibitor, however, the 26-K translation product remains in the cytosol.

Immunofluorescent analysis of a transformed cell line

To provide more direct evidence for the mitochondrial localization of the DHFR-precipitable products in the derived cell lines, analysis by indirect immune fluorescence was carried out. LDHFR 1 cells were grown on glass coverslips, fixed, permeabilized, and then incubated with anti-DHFR antiserum, followed by fluoresceinated goat anti-rabbit IgG antiserum. The staining pattern is shown in Figure 3, along with patterns displayed by two identically-prepared control lines, DG44 and wild-type CHO. DG44 cells, devoid of immunoprecipitable DHFR, failed to stain (Figure 3A). Wild-type CHO cells exhibited a pattern of uniform cytoplasmic staining (Figure 3B), as would be expected for endogenous DHFR. The LDHFR 1 cells displayed a punctate pattern of fluorescence (Figure 3C), distinctly different from that of either control, and characteristic of the distribution of mitochondria in CHO cells. An identical punctate pattern was observed when DG44, LDHFR 1 or wild-type CHO cells were stained with R6G (Figure 3D). These results indicate that the bulk of DHFR protein in LDHFR 1 is localized to mitochondria.

In vitro synthesis of the chimeric protein and import by isolated mitochondria

Import of nuclear-coded mitochondrial precursors, including the OTC precursor, can be reconstituted in a cell-free system by first synthesizing the precursors in a rabbit reticulocyte lysate translation system and then adding isolated rat liver mitochondria (Conboy and Rosenberg, 1981). To synthesize the OTC leader/DHFR chimeric protein *in vitro*, we joined its coding sequence with the *Salmonella* phage SP6 promoter (Krieg and Melton, 1984) in the manner shown in Figure 4. A *Bam*HI restriction fragment was excised from the plasmid pSPOTC1 (kindly provided by J.Hendrick), removing OTC sequences downstream from the *Bam*HI site in the leader coding sequence. A *Bam*HI-*Bgl*II fragment from the plasmid pLDHFR was inserted, reconstructing the chimeric coding sequence downstream from the SP6 promoter. The derived plasmid pSPLDHFR was cleaved downstream from the chimeric coding sequence with the enzyme *Sma*I, and an *in vitro* transcription reaction was performed using SP6 polymerase, GpppG, and nucleoside triphosphates. The reaction products were used to program a reticulocyte lysate translation reaction in the presence of [^{35}S]methionine. Upon analysis of the products by SDS-PAGE, a single major product was detected, with an apparent mol. wt. of 26 K (Figure 5, track 1). The product was precipitable with anti-DHFR antiserum and co-migrated with the species observed in the extracts of pulse-labeled LDHFR lines (not shown).

The translation mixture containing the 26-K product was incubated with isolated rat liver mitochondria for 1 h at 27°C, and the mixture was then separated by centrifugation into supernatant and mitochondrial pellet fractions. The fractions were solubilized, immunoprecipitated with anti-DHFR antiserum, and the products separated on SDS-PAGE as shown in Figure 5, tracks 2 and 3. The major product was a 20-K polypeptide found only

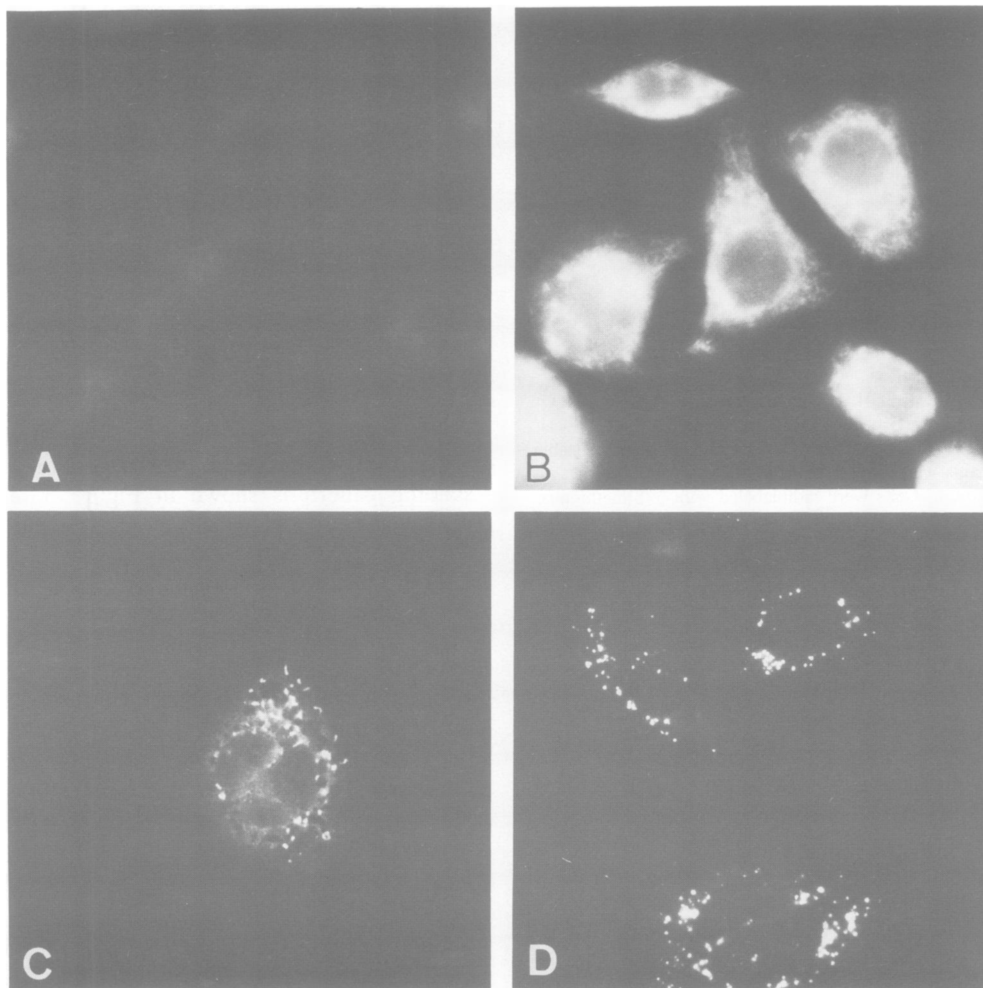

Fig. 3. Immunofluorescence of transformed and untransformed cell lines. Cells were grown on round glass coverslips, fixed, permeabilized with saponin and the intracellular distribution of DHFR visualized using a rabbit anti-human DHFR antibody followed by fluorescein-conjugated goat anti-rabbit $F(ab')_2$. **Panel A:** untransformed DG44 cells, which do not contain immunoprecipitable DHFR. **Panel B:** wild-type CHO cells, which contain endogenous immunoreactive DHFR. **Panel C:** LDHFR 1 cells, containing the chimeric protein and its products. **Panel D:** fixed but not permeabilized LDHFR 1 cells stained with rhodamine 6G to visualize mitochondria. Magnification: x1600.

in the mitochondrial fraction. A small amount of 26-K precursor polypeptide and several other minor species were detected in supernatant and pellet fractions. These results indicate clearly that the precursor is proteolytically processed by the addition of mitochondria and that the major proteolytic product becomes associated with the mitochondria. To demonstrate that the 20-K product was inside the mitochondria, two studies were conducted. First, mitochondrial subfractionations localized the 20-K protein to the matrix (not shown). Second, the incubated mixture of translation products and mitochondria was treated with trypsin before separation into supernatant and mitochondrial fractions. The results of this experiment are shown in Figure 5, tracks 4 and 5. No precipitable product could be detected in the supernatant, reflecting complete proteolysis of the material in track 2. Two major products were recovered in the mitochondrial fraction, one corresponding to the 20-K protein observed in the absence of trypsin (see track 3), and a second 21-K product that appeared to be generated by trypsin treatment.

Discussion

We have tested the hypothesis that the NH_2-terminal leader peptide of a nuclear-coded mitochondrial precursor contains sufficient information to direct the import of the precursor by mitochondria. We reasoned that, if a leader sequence is both necessary and sufficient for import, it might be capable of directing import of a polypeptide normally localized to the cytoplasm. For such an analysis, we selected the leader peptide of the human OTC precursor and the cytoplasmic enzyme DHFR. Cloned cDNA sequences for both of these polypeptides were available, enabling synthesis of a fused coding sequence producing the chimeric protein. The complete OTC cDNA sequence encodes an OTC precursor which contains the amino acid residues required for import; that is, when the OTC precursor is synthesized from the transfected cDNA in cultured HeLa cells, it is efficiently imported by their mitochondria (Horwich *et al.*, 1985). DHFR appeared attractive as the COOH-terminal 'passenger' in the chimeric protein because it is a small globular polypeptide with no known compartmentation signals. Furthermore, its small size (21 K) suggested that it might be translocated across membranes with greater facility than a larger protein such as β-galactosidase (Douglas *et al.*, 1984). X-Ray crystallographic analysis of DHFR has revealed that its NH_2 terminus lies at the surface of the polypeptide (Volz *et al.*, 1982); thus, an added NH_2-terminal leader peptide might be accessible for recognition and able, therefore, to function normally.

The chimeric protein predicted from the fused coding sequences

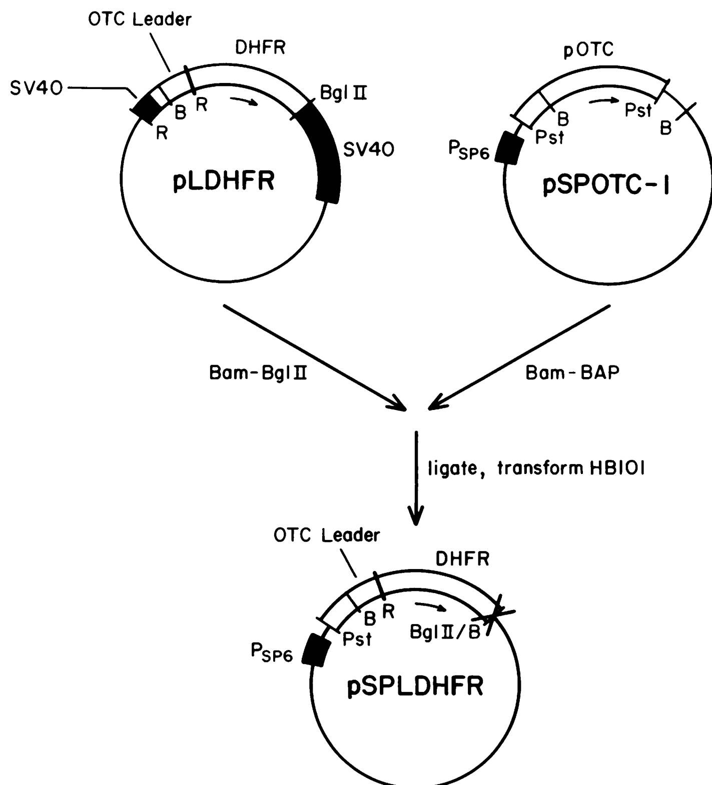

Fig. 4. Construction of plasmid containing OTC leader-DHFR fusion sequence joined with SP6 promoter. The plasmid pLDHFR was cleaved with *Bam*HI and *Bgl*II; a 739-bp fragment was isolated which contained the fusion sequences downstream from the *Bam*HI site in the OTC leader coding sequence. The fragment was joined with the ~3 kbp *Bam*HI fragment of pSPOTC-1 that contained the SP6 promoter joined with the OTC leader codons upstream from the *Bam*HI site, thus reconstructing the OTC leader-DHFR fusion sequence downstream from the SP6 promoter in the derived plasmid pSPLDHFR.

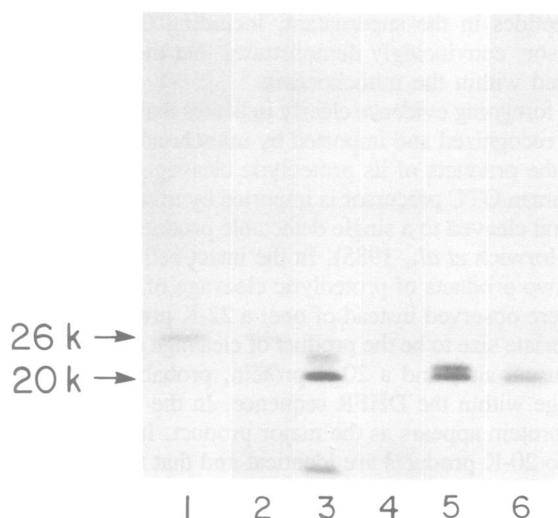

Fig. 5. Import of *in vitro*-synthesized chimeric protein by isolated mitochondria. The plasmid pSPLDHFR was transcribed *in vitro* and the products were used to direct a reticulocyte lysate translation reaction containing [^{35}S]methionine. Following translation, the mixture was incubated for 60 min at 27°C with isolated rat liver mitochondria. The mixture was then divided: one half was separated by centrifugation into supernatant and mitochondrial fractions; the other half was first incubated with trypsin and then so separated. The fractions were detergent-solubilized and immunoprecipitated with anti-DHFR antiserum. The products were electrophoresed through an SDS-polyacrylamide gel, and the gel was fluorographed. Aliquot of *in vitro* translation reaction, without immunoprecipitation **(track 1)**; supernatant following incubation with mitochondria **(track 2)**; mitochondrial pellet **(track 3)**; supernatant following trypsin treatment of mitochondrial incubation mixture **(track 4)**; mitochondrial pellet from trypsin-treated incubation mixture **(track 5)**; radiolabeled mouse DHFR marker **(track 6)**.

contains the OTC leader peptide at its NH_2 terminus, nine 'connecting' residues, and the entire amino acid sequence of DHFR at the COOH terminus. The fused sequence, programmed for expression in cultured cells, was co-transferred with a selectable *neo* marker into a DHFR$^-$ cell line. Selection with G418 was employed instead of selection with methotrexate because it was not known whether the chimeric protein would exhibit DHFR activity sufficient to confer methotrexate resistance, or whether even an active protein could confer methotrexate resistance when localized within the mitochondria.

The stable G418-resistant transformants contained a protein precipitable with anti-DHFR antiserum, with an apparent mol. wt. of 26 K, the same as that predicted for the chimeric protein. Two additional precipitable proteins with mol. wts. of 22 K and 20 K were also detected. When the transformants were incubated with an inhibitor of mitochondrial import, R6G, only the 26-K protein was observed. These results support the interpretation that the 26-K protein is a precursor protein which is imported by mitochondria and proteolytically cleaved by intramitochondrial proteases, generating 22-K and 20-K products. Inhibition of import of the chimeric precursor by R6G prevents it from reaching the intramitochondrial site of proteolytic cleavage. While the foregoing explanation seemed most likely, other interpretations could not be excluded, particularly because the mode of action of R6G is incompletely understood and may include effects on cellular systems other than those in mitochondria. It is possible, for instance, that inhibition of mitochondrial ATP synthesis might block an ATP-dependent proteolytic system operative outside the mitochondria, such as that mediated by ubiquitin (Hershko, 1983). If proteolytic cleavage of the 26-K protein was carried out by the ubiquitin system, extra-mitochondrial cleavage could be inhibited, indirectly, by R6G. To exclude such a possibility, direct demonstration of mitochondrial localization of anti-DHFR-precipitable proteins was carried out.

One direct analysis involved immunofluorescent staining in culture of a stable transformant containing the three DHFR-precipitable proteins. The cells exhibited a pattern of fluorescence clearly different from that of wild-type CHO cells which contain only cytoplasmic DHFR. This pattern almost assuredly represents staining localized to the mitochondria of the cells because it is indistinguishable from the patterns obtained when the transformant cells are incubated with the mitochondria-specific dyes R6G and rhodamine 123. Unfortunately, double fluorescent staining with both anti-DHFR and rhodamine on the same cells proved technically unfeasible.

As an additional direct demonstration that the chimeric precursor could be imported by mitochondria, an *in vitro* experiment was performed. Radiolabeled chimeric protein was synthesized *in vitro* by transcription of its cloned coding sequence using the SP6 system and translation of RNA products by rabbit reticulocyte lysate. The translation mixture contained the 26-K chimeric protein as the only radiolabeled product. When this mixture was incubated with isolated rat liver mitochondria, the major anti-DHFR-preciptable product was a 20-K protein that subfractionated with the mitochondrial matrix. The observation that treatment with externally added trypsin fails to proteolyze the 20-K product but completely proteolyzes anti-DHFR precipitable

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polypeptides in the supernatant, including the 26-K chimeric precursor, convincingly demonstrates that the 20-K product is localized within the mitochondria.

The foregoing evidence clearly indicates that the chimeric protein is recognized and imported by mitochondria, but the identity of the products of its proteolytic cleavage remains unclear. The human OTC precursor is imported by mitochondria of intact cells and cleaved to a single detectable product, the mature subunit (Horwich *et al.*, 1985). In the intact cell system described here, two products of proteolytic cleavage of the chimeric protein were observed instead of one: a 22-K protein, which is the appropriate size to be the product of cleavage of the OTC leader at its usual site, and a 20-K protein, probably the product of cleavage within the DHFR sequence. In the *in vitro* system, a 20-K protein appears as the major product. It seems likely that the two 20-K products are identical and that they are produced by the same mitochondrial protease. On the other hand, little of the 22-K protein observed in intact cells was found *in vitro*. An additional proteolytic product of 21-K was detected *in vitro*, but only following trypsin treatment of the mitochondrial mixture.

The conclusion that the OTC leader peptide contains sufficient information to direct mitochondrial import is likely to apply as well to other leader sequences. In this regard, Hurt and Schatz have recently demonstrated import by isolated yeast mitochondria of a chimeric protein containing the leader sequence of the precursor of yeast cytochrome oxidase subunit IV joined, by coincidence, with DHFR (Hurt *et al.*, 1984). Interestingly, proteolytic cleavage of leader residues from this chimeric precursor also occurs at an unusual site, although, in this case, the normal site is not present. Van den Broeck *et al.* (1985) have also recently demonstrated import by plant chloroplasts of a chimeric protein containing the leader peptide of the small subunit of RBPCase joined with bacterial neomycin phosphotransferase.

The question now arises as to how leader sequences target precursors to the mitochondria. Clearly, they must mediate specific recognition, because, in their absence, polypeptides neither bind to mitochondria nor are they imported. Recognition probably involves binding of a precursor through its leader sequence to the outer membrane of the mitochondria, most likely to specific receptor molecules (Hennig *et al.*, 1983). It will be of importance to determine whether the function of the leader in recognition is mediated through specific amino acid sequences, through charge, or through secondary or tertiary structure. We have recently demonstrated that substitution of the uncharged amino acid analogue, canavanine, for the positively-charged arginine residues in the OTC precursor results in nearly complete inhibition of its mitochondrial import and proteolytic processing (Horwich *et al.*, 1985). In combination with the conclusions presented here concerning leader sufficiency, we can infer that the arginine residues in the OTC leader sequence are involved with leader function. Such an involvement is not surprising, given the observation that the single feature shared by the mitochondrial leader sequences reported to date is their strikingly basic overall amino acid composition (Horwich *et al.*, 1984; Hay *et al.*, 1984). With the knowledge that the leader is sufficient to direct import, we can now focus on the importance of the basic residues and of others in the leader by studies involving site-directed mutagenesis.

Materials and methods

Materials

Restriction enzymes and other DNA-modifying enzymes were obtained from New England Biolabs. RNAsin was procured from Promega Biotech and SP6 RNA polymerase was from New England Nuclear. ^{32}P-Labeled nucleoside triphosphates and [^{35}S]methionine were obtained from Amersham. Formalin-fixed *Staphylococcus aureus* bacteria were from Bethesda Research Labs.

Plasmids

The cDNA plasmids pSV2OTC and pDHFR26 have been previously described (Horwich *et al.*, 1984; Chang *et al.*, 1979; see Figure 1). The fusion plasmids pLDHFR and pSPLDHFR were prepared using previously described methods of restriction endonuclease digestion, purification of DNA fragments, joining of fragments, bacterial transformation and colony screening (Maniatis *et al.*, 1982).

Cells, transfection and selection

Wild-type CHO cells and mutant CHO cells deficient in DHFR (line DG44, similar to line DG41 previously described in Urlaub *et al.*, 1983), generously provided by Larry Chasin, were grown in Ham's F-12 medium containing 10% fetal calf serum. The mutant cells were transfected using the $CaPO_4$ procedure of Wigler *et al.* (1979). Cells plated at a density of 5 x 10^4/100 mm dish were co-transfected with two plasmids: pLDHFR, encoding the chimeric protein, 15 μg/dish; and pSV2Neo, encoding the bacterial gene for neomycin resistance, 1 μg/dish. Selection was carried out by addition of G418 (Geneticin, Gibco), at a concentration of 400 μg/ml, to the medium 48 h following transfection.

Radiolabeling and immunoprecipitation

Procedures for radiolabeling cultured cells and for harvesting, immunoprecipitation and SDS-PAGE have been described previously (Fenton *et al.*, 1984). Where R6G was employed in metabolic labeling experiments, it was added to the radiolabeling medium at a final concentration of 10 μg/ml.

Immunofluorescence

Cells were grown on round glass coverslips at least 48 h and fixed using 3.5% paraformaldehyde (30 min; this and all other incubations were at room temperature). After washing in PBS, cells were permeabilized in fresh 0.05% saponin in PBS (10 min) and unreacted aldehyde groups quenched by incubation (10 min) in PBS-saponin containing 50 mM NH_4Cl. Coverslips were washed three times in PBS-saponin and incubated (10 min) in 10% pre-immune goat serum, 0.2% gelatin, 0.05% saponin in PBS (antibody buffer). Rabbit anti-DHFR or pre-immune serum was then added, at a dilution of 1:200 in antibody buffer and incubated for 30 min. After washing in PBS-saponin, cells were stained using fluorescein-labeled, affinity-purified goat anti-rabbit IgG (Tago, Burlingame, CA) diluted 1:40 in antibody buffer (30 min). Coverslips were viewed using a Zeiss fluorescence microscope using a 40x water immersion lens.

Mitochondria were stained with rhodamine dyes using cells fixed in 0.1% glutaraldehyde essentially as described (Johnson *et al.*, 1980). Cells were routinely incubated in 2.5 μg/ml rhodamine 6G for 15 min at room temperature, washed in PBS, and viewed immediately using a water immersion lens.

In vitro transcription

Plasmid DNA containing an SP6 promoter sequence joined with a cloned coding sequence was prepared for *in vitro* transcription by restriction cleavage at a unique *Sma*I site downstream from the coding sequence. The cleavage products were extracted with phenol and chloroform and ethanol precipitated. 1 μg of linearized plasmid DNA was then used in a transcription reaction (10 μl final volume) containing 10 mM GpppG, 500 μM ATP, 500 μM UTP, 500 μM CTP, 100 μM GTP, 5 μCi [α-^{32}P]GTP, 40 mM Tris pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 2 mM DTT, 10 units RNAsin, and 20 units SP6 polymerase. The reaction was carried out for 1 h at 40°C, and monitored by incorporation of radioactivity into TCA-insoluble material. The reaction products were extracted with phenol and chloroform and ethanol precipitated.

In vitro translation, mitochondrial processing and trypsin treatment

Cell free protein synthesis was carried out in a nuclease-treated rabbit reticulocyte lysate system prepared as described previously (Conboy and Rosenberg, 1981). The ethanol-precipitated products of an *in vitro* transcription reaction were re-suspended in 30 μl water; 3 μl were added to 20 μl of reticulocyte lysate containing 30 μCi [^{35}S]methionine, and the mixture was incubated for 1 h at 30°C, following which 9 μg cycloheximide was added. 1 μl of the products was added directly to SDS sample buffer. 5 μl of the translation products were combined with 5 μl ribosome-free reticulocyte lysate (prepared by a 1 h centrifugation of 100 000 *g*) and 5 μl of a suspension of freshly isolated rat liver mitochondria (Conboy *et al.*, 1982). The mitochondria had been suspended at a concentration of 6 mg protein/ml in a buffer (pH 7.4) containing 220 mM mannitol, 70 mM sucrose, 6 mM ADP, 6 mM $MgCl_2$, 20 mM potassium glutamate, 6 mM EGTA and 2 mM HEPES. The mixture was incubated at 27°C for 1 h and then either separated into supernatant and mitochondrial fractions by centrifugation at 10 000 *g* for 6 min or digested with TPCK-treated trypsin at a ratio of 1:500 trypsin:total mitochondrial protein for 10 min at 27°C. In some experiments, separation of mitochondria preceded treatment with trypsin. Supernatant and mitochondrial fractions were diluted with 1 ml of a solution containing 150 mM NaCl, 0.5% Triton X-100, 10 mM EDTA, 2% methionine, and 0.25% SDS, and immunoprecipi-

tation was carried out using 3 μl rabbit anti-mouse DHFR antiserum and formalin-fixed *S. aureus* cells.

Acknowledgements

We are grateful to Geoff Wahl and Mark Carman for helpful discussions; to Larry Chasin for providing the DG44 cell line; to Michelle Orsulak, Helen Plutner and Victoria Lewis for expert technical assistance; to Wayne Fenton and Tony Hunter for helpful comments during preparation of the manuscript; and to Connie Woznick for secretarial expertise. This work was supported by grants from the National Institutes of Health and a grant (to A.H.) from the Life and Health Insurance Medical Research Fund.

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Received on 22 February 1985