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The cleavable pre-sequence of an imported chloroplast protein directs attached polypeptides into yeast mitochondria

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The cleavable pre-sequences of imported chloroplast and mitochondrial proteins have several features in common. This structural similarity prompted us to test whether a chloroplast pre-sequence ('transit peptide') can also be decoded by the mitochondrial import machinery. In the green alga, *Chlamydomonas reinhardtii*, the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (a chloroplast protein) is nuclear-encoded and synthesized in the cytosol with a transient pre-sequence of 45 residues. The 31 amino-terminal residues of this chloroplast pre-sequence were fused to mouse dihydrofolate reductase (a cytosolic protein) and to yeast cytochrome oxidase subunit IV (an imported mitochondrial protein) from which the authentic pre-sequence had been removed. The chloroplast pre-sequence transported both attached proteins into the yeast mitochondrial matrix or inner membrane, although it functioned less efficiently than an authentic mitochondrial pre-sequence. We conclude that mitochondrial and chloroplast pre-sequences perform their function by a similar mechanism.

Key words: mitochondria/chloroplasts/pre-sequences/protein import/protein sorting

Introduction

Mitochondria and chloroplasts are formed by similar mechanisms (Schatz and Mason, 1974; Gillham *et al.*, 1978). Both organelles contain their own DNA which codes for only a limited number of organelle proteins; the majority of mitochondrial and chloroplast proteins are encoded by nuclear genes and imported by pathways exhibiting the following properties (Chua and Schmidt, 1979; Ellis, 1981; Schatz and Butow, 1983): (i) imported proteins are usually made as precursors with amino-terminal transient pre-sequences, (ii) import can occur post-translationally, (iii) import requires an energized organelle membrane, (iv) upon import, transient pre-sequences are removed by a chelator-sensitive protease located in the mitochondrial matrix (Böhni *et al.*, 1980, 1983); McAda and Douglas, 1982) or the chloroplast stroma (Robinson and Ellis, 1984). At least some of the mitochondrial and chloroplast pre-sequences carry sufficient information for directing attached 'passenger' proteins to their corresponding organelle (Hurt *et al.*, 1984a; Horwich *et al.*, 1985; van Loon *et al.*, 1986; van den Broeck *et al.*, 1985; Schreier *et al.*, 1985). Both types of pre-sequence are rich in basic and hydroxylated amino acids and usually lack acidic amino acids or extended hydrophobic stretches (for references, see von Heijne, 1986; Smeekens *et al.*, 1985). These features set them apart from the pre-sequences of proteins which are secreted across the bacterial plasma membrane or the endoplasmatic reticulum of eukaryotes (von Heijne, 1981).

The structural similarity of mitochondrial and chloroplast pre-sequences is at first sight surprising: one might assume that sorting of proteins between mitochondria and chloroplasts in a plant cell is effected by structurally different pre-sequences specific for only one of the two organelles. In this paper we show, however, that chloroplast pre-sequences can direct proteins into yeast mitochondria.

Results

A chloroplast pre-sequence directs attached mouse cytosolic dihydrofolate reductase into yeast mitochondria: in vitro and in vivo import studies

Gene fusion studies have recently shown that pre-sequences of imported mitochondrial and chloroplast proteins can direct attached 'passenger' proteins into the corresponding organelle (Hurt *et al.*, 1984a; Horwich *et al.*, 1985; van Loon *et al.*, 1986; van den Broeck *et al.*, 1985; Schreier *et al.*, 1985). Since both organelle pre-sequences have a few characteristics in common (see also Figure 1) we tested whether a chloroplast pre-sequence can 'mistarget' attached mouse cytosolic dihydrofolate reductase (DHFR) into yeast mitochondria. A fusion protein containing the first 31 amino acids of the *Chlamydomonas* ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco)-pre-sequence attached to mouse DHFR (Figure 2) was synthesized *in vitro* in the presence of [^{35}S]methionine and tested for its ability to be bound, and imported, by isolated yeast mitochondria (Figure 3A, pRubisco–DHFR). Binding and import were compared with that observed with DHFR attached to a mitochondrial pre-sequence (Figure 3A, pCoxIV–DHFR). Authentic mouse DHFR is neither bound nor imported by yeast mitochondria (Hurt *et al.*, 1984b, 1985a). With de-energized mitochondria, the chloroplast pre-sequence mediated binding of DHFR to the mitochondrial surface; no import occurred under these conditions since the fusion protein remained accessible to externally added protease (Figure 3A, lanes 2 and 3). With energized mitochondria, a significant percentage of the fusion protein molecules were imported: they became inaccessible to externally added protease (Figure 3A, lanes 4 and 5). The rest of the precursor remained still on the mitochondrial surface and was degraded by externally added protease (Figure 3A, lanes 4 and 5). The rate of import into intra-mitochondrial locations mediated by the chloroplast pre-sequence was approximately seven times slower than that mediated by an authentic mitochondrial pre-sequence (Figure 3B). The pRubisco–DHFR fusion protein was imported into mitochondria largely in its precursor form (Figure 3A, 'p'). Accordingly, the partially purified mitochondrial processing protease did not cleave the *in vitro* synthesized pRubisco–DHFR fusion protein (not shown). The attached chloroplast pre-sequence from *Chlamydomonas* no longer contained the region around the site cleaved by the authentic *Chlamydomonas* chloroplast processing enzyme (see Figure 2), but retained an intermediate processing site (between residue 26 and 27) which is used by the processing protease of vascular plant

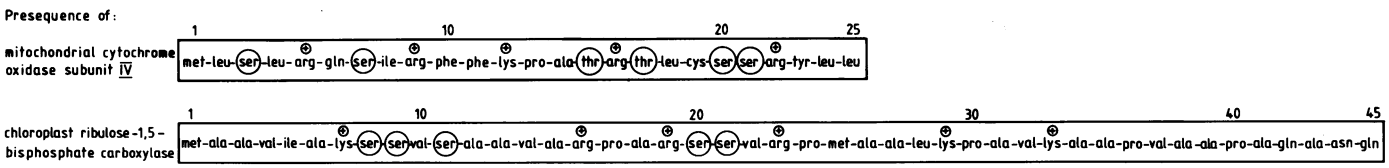

Fig. 1. Pre-sequences of imported mitochondrial and chloroplast proteins have similar features. The pre-sequence of yeast mitochondrial subunit IV of cytochrome *c* oxidase (Maarse *et al.*, 1984) and that of small subunit of ribulose-1,5-bisphosphate carboxylase from *Chlamydomonas* (Schmidt *et al.*, 1979; Goldschmidt-Clermont and Rahire, in preparation) are shown. Basic and hydroxylated amino acids are specially marked.

chloroplasts (Mishkind *et al.*, 1985). Since this intermediate cleavage site is located close to the fusion site to DHFR, it is not clear whether this site would have been recognized by the mitochondrial processing enzyme.

Similar results were obtained by fusing the entire 57-residue pre-sequence of ribulose-1,5-bisphosphate carboxylase small subunit from *Nicotiana sylvestris* to DHFR (for *Nicotiana* pre-sequence, see Pinck *et al.*, 1984). The overall extent of imported DHFR using the *Nicotiana* pre-sequence, however, was even lower than that mediated by the *Chlamydomonas* pre-sequence (data not shown).

In conclusion, chloroplast pre-sequences directed attached DHFR into isolated yeast mitochondria, although with distinctly lower efficiency than an authentic mitochondrial pre-sequence.

Targeting to and import into mitochondria of the pRubisco−DHFR fusion protein was also ascertained *in vivo*. Yeast cells expressing authentic mouse DHFR or the *Chlamydomonas* pRubisco−DHFR fusion protein under the control of a strong yeast promoter were subfractionated into mitochondria, microsomes and cytosol (Figure 4A). Under steady-state conditions, ~30% of the DHFR fusion protein was targeted to mitochondria by the attached chloroplast pre-sequence; 70% remained in the cytosol. Authentic mouse DHFR without an attached pre-sequence was only detected in the cytosol fraction.

Upon mitochondrial subfractionation, the mitochondria-associated pRubisco−DHFR fusion protein was almost completely recovered in the 'mitoplasts' (mitochondria whose outer membrane had been disrupted); only an insignificant percentage was found in the released intermembrane space fraction (Figure 4B). Since the fusion protein was largely inaccessible to externally-added protease in mitochondria (66% of the fusion protein was protease-protected) as well as in mitoplasts, it was located in the matrix space. The protease-sensitive fusion protein molecules were most likely bound to the mitochondrial surface. The matrix location of the fusion protein was further confirmed by the observation that the enzymic activity of the mitochondria-associated fusion protein was latent. NADPH and dihydrofolate, the substrates for DHFR, can freely diffuse into the mitochondrial intermembrane space, but not into the matrix; if these substrates are added to the outside of mitochondria, their interaction with matrix-located DHFR should be greatly stimulated by disruption of the inner membrane with detergents. This was indeed observed (Table I). The degree of detergent-induced unmasking was comparable with that seen with citrate synthase, a matrix enzyme. If assayed in the presence of detergent, 27% of the DHFR activity was located in mitochondria and the remaining 73% in the cytosol fraction, in good agreement with the results obtained by immune blotting (Figure 4A). Table I also shows that no significant latent DHFR activity was found with mitochondria from cells expressing authentic mouse DHFR without any

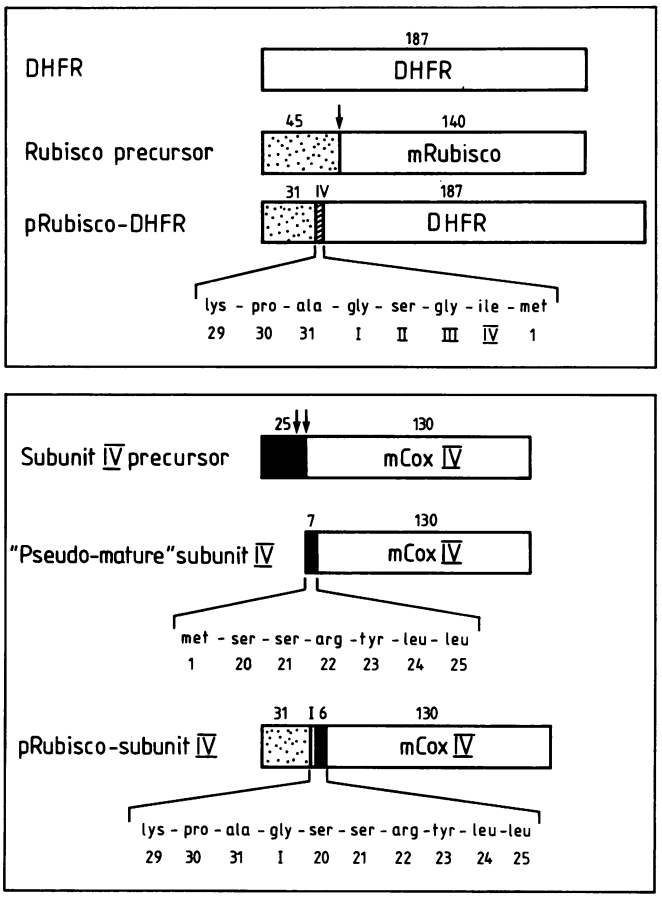

Fig. 2. Gene constructions and DNA restriction analysis. Boxes denote amino acid sequences, Arabic numerals on top of each box the total number of amino acid residues and Roman numerals amino acids introduced by gene fusion. DHFR, dihydrofolate reductase from mouse; Rubisco precursor, precursor to small subunit of ribulose-1,5-bisphosphate carboxylase from *C. reinhardtii* comprising a 45 amino acid long pre-sequence (dotted bar) and a 140 residue long 'mature' sequence (mRubisco; open bar); pRubisco−DHFR, fusion protein containing the amino-terminal 31 residues of pRubisco (dotted bar), four residues introduced by gene fusion (shaded bar) and the entire sequence of mouse DHFR. In the detailed representation of the fusion region, Arabic numerals preceding the Roman numerals denote the C-terminal amino acid residues of the attached pre-sequence. Subunit IV precursor, authentic precursor of subunit IV of cytochrome *c* oxidase comprising a 25 amino acid long pre-sequence (filled bar) and a 130 residue long mature sequence (open bar, 'mCoxIV'); 'pseudo-mature' subunit IV, residues 2−19 of the subunit IV pre-sequence were deleted; pRubisco−subunit IV, fusion protein containing the amino-terminal 31 residues of pRubisco (dotted bar), one glycine introduced by gene fusion (Roman numeral), residues 20−25 (Arabic numerals) of the pre-sequence (filled bar) and 130 residues of the mature sequence of subunit IV (open bar, mCoxIV). Arrows indicate sites cleaved by processing proteases.

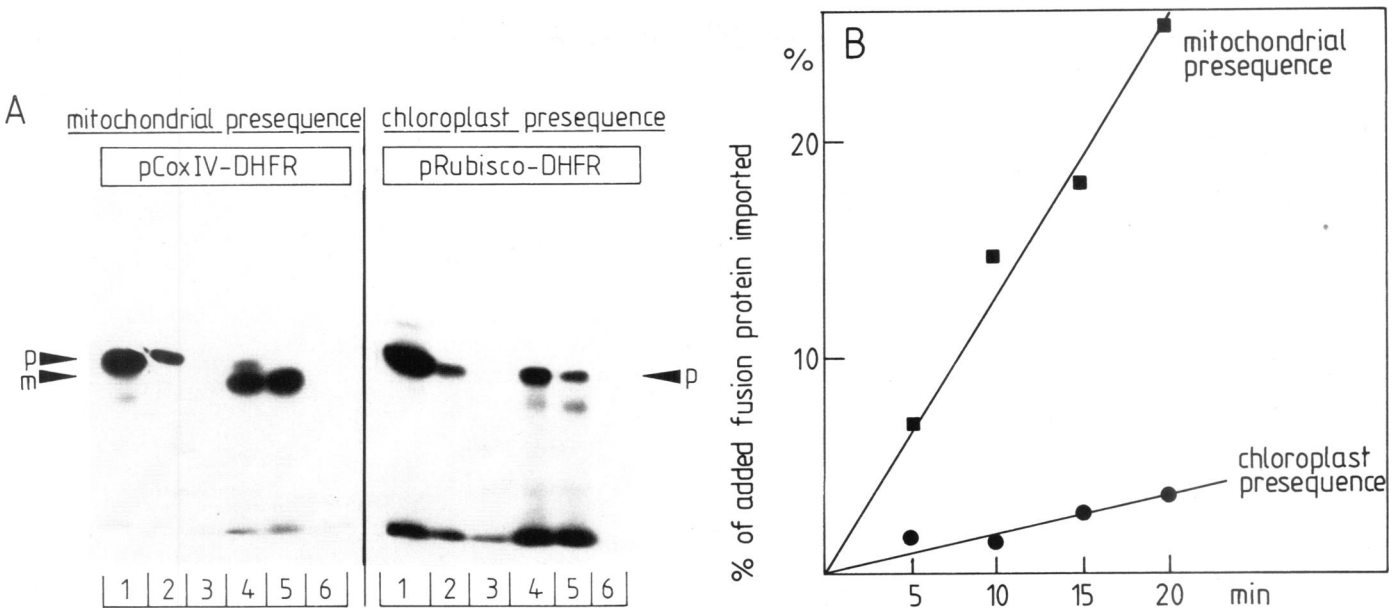

Fig. 3. A chloroplast pre-sequence directs the energy-dependent import of an attached cytosolic protein (mouse DHFR) into isolated yeast mitochondria. **(A)** Fusion proteins containing mouse DHFR attached to either the first 31 amino acids of the *Chlamydomonas* chloroplast pre-sequence (pRubisco – DHFR) or to the first 22 amino acids of a mitochondrial (subunit IV) pre-sequence (pCoxIV – DHFR) were synthesized *in vitro* in the presence of [^{35}S]methionine and incubated with isolated yeast mitochondria. After 20 min incubation at 30°C, mitochondria were re-isolated and analyzed by SDS – 12.5% polyacrylamide gel electrophoresis and fluorography. **1**, 30% of the radiolabeled fusion protein added to mitochondria; **2**, incubation with de-energized mitochondria (binding assay); **3**, de-energized mitochondria, proteinase K; **4**, energized mitochondria (import assay); **5**, energized mitochondria, proteinase K; **6**, energized mitochondria, proteinase K plus Triton X-100. The arrows indicate the precursor forms (p) of pCoxIV – DHFR and pRubisco – DHFR and the mature-sized (m) pCoxIV – DHFR. **(B)** Time-dependent import into isolated mitochondria of pCoxIV – DHFR ('mitochondrial pre-sequence') and pRubisco – DHFR ('chloroplast pre-sequence'). The radiolabeled bands obtained from an import experiment similar to that shown in **A** were quantified and expressed as % of the total fusion protein added to the mitochondria.

Table I. The enzymic activity of mouse DHFR is latent in mitochondria from yeast cells expressing the pRubisco – DHFR fusion protein

Yeast cells expressing the gene encoding	Subcellular fraction	DHFR activity measured in the presence of Triton X-100 (homogenate = 100%)	Unmasking of DHFR activity by Triton X-100	Unmasking of citrate synthase activity by Triton X-100
pRubisco – DHFR	mitochondria	27%[a]	21-fold	30-fold
fusion protein	cytosol	73%	0.88-fold	n.d.
Authentic mouse	mitochondria	4.0%	0.9-fold	24-fold
DHFR	cytosol	96%	1.0-fold	n.d.

DHFR activity was measured as described by Hurt *et al.* (1985a) in the absence or presence of 0.5% Triton X-100 at 22°C in 50 mM Tris-HCl, 7.4, 12 mM β-mercaptoethanol, 60 μM dihydrofolate, 50 μM NADPH and 1 mM KCN. All values were corrected for methotrexate-resistant activity.
[a]27% activity found inside mitochondria corresponds to 2.47 μmol NADPH oxidized/min/mg mitochondrial protein.
n.d., not determined.

attached pre-sequence. Taken together, these results show that the chloroplast pre-sequence directs *in vivo* import of DHFR into the yeast mitochondrial matrix, although less efficiently than an authentic mitochondrial pre-sequence.

A chloroplast pre-sequence directs attached cytochrome oxidase subunit IV into the mitochondrial inner membrane: in vivo restoration of cytochrome oxidase activity

We previously described a complementation assay for testing whether cytochrome oxidase subunit IV is imported to the mitochondrial inner membrane (Hurt *et al.*, 1985b; see also Figure 5A). The host is a yeast mutant in which the nuclear gene for the subunit IV precursor had been inactivated by 'gene disruption'. This mutant lacks functional cytochrome *c* oxidase and cannot grow on a non-fermentable carbon source such as glycerol (Dowhan *et al.*, 1985). Assembly of an active cytochrome *c* oxidase and growth on glycerol are restored if the mutant is transformed with the plasmid-borne gene for the wild-type subunit IV precursor. No restoration is observed if the mutant is transformed with the gene for an altered subunit IV precursor lacking most of its pre-sequence (Hurt *et al.*, 1985b; see also Figure 5B, C). When the subunit IV-deficient mutant was transformed with a fusion gene encoding the first 31 amino acids of the *Chlamydomonas* chloroplast pre-sequence attached to 'pseudo-mature' subunit IV (see also Figure 2), it regained growth on glycerol/ethanol (Figure 5B and C) and cytochrome *c* oxidase activity (Table II). Restoration of these functions is taken as proof that the chloroplast pre-sequence directed import of attached

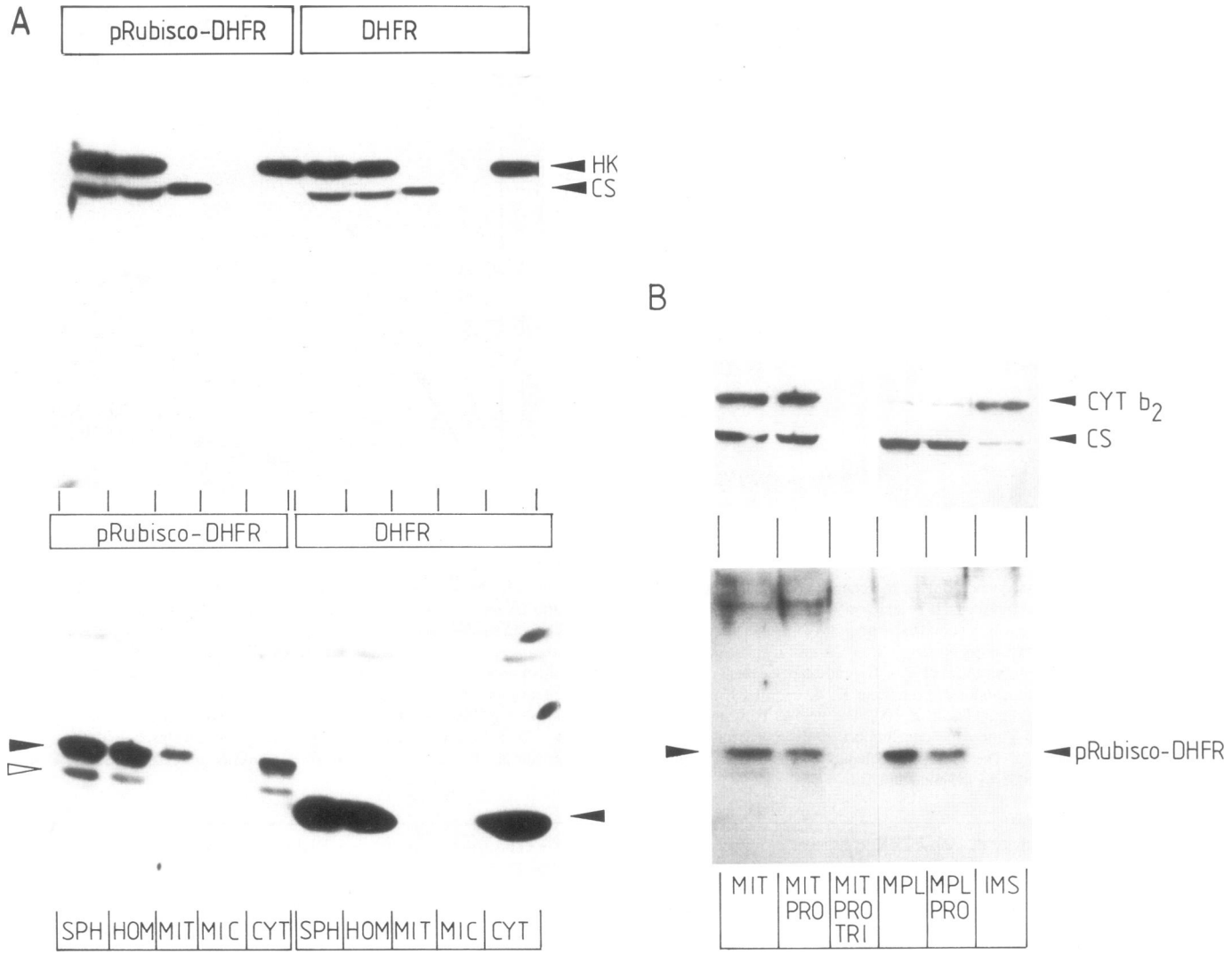

Fig. 4. A chloroplast pre-sequence directs attached mouse DHFR into yeast mitochondria *in vivo*. **(A)** Subcellular localization. Yeast cells expressing *Chlamydomonas* pRubisco – DHFR or authentic mouse DHFR were grown in galactose-containing minimal medium and fractionated into spheroplasts (SPH), homogenate (HOM), mitochondria (MIT), microsomes (MIC) and cytosol (CYT). Aliquots of each fraction corresponding to 10 mg (if probed with the DHFR antibody) or 3 mg wet weight of cells (if probed with the hexokinase and citrate synthase antibody) were analyzed by SDS – 12.5% polyacrylamide gel electrophoresis and immune blotting using antisera against hexokinase (HK, cytosolic marker) and citrate synthase (CS, mitochondrial marker; **upper panel**) and mouse DHFR (**lower panel**). The labeled bands were quantified as described in Materials and methods. The arrows on the left indicate the uncleaved fusion protein (closed arrow) and a proteolytic breakdown product (open arrow). The closed arrow on the right indicates mouse DHFR. **(B)** Submitochondrial localization. Mitochondria isolated from cells expressing the pRubisco – DHFR fusion protein were subfractionated into mitoplasts (mitochondria whose outer membrane had been disrupted) and intermembrane space. Fractions equivalent to 75 μg of mitochondrial protein were analyzed by SDS – 12.5% polyacrylamide gel electrophoresis and immune blotting using antisera against cytochrome b_2 (Cyt b_2, intermembrane space marker), citrate synthase (CS, matrix marker) and mouse DHFR. The upper panel shows only that part of the immune blot which contains the marker enzymes cytochrome b_2 and citrate synthase. The rest of the immune blot which is not illustrated here had the same low background as that shown in **A**. Accordingly, only the relevant part of the immune blots containing the marker enzyme bands citrate synthase, cytochrome b_2 and hexokinase are presented in Figure 6A and B (upper panel). MIT, mitochondria; MIT/PRO, mitochondria, proteinase K; MIT/PRO/TRI, mitochondria, proteinase K, Triton X-100: MPL, mitoplasts, MPL/PRO, mitoplasts, proteinase K; IMS, intermembrane space. The arrow shows the position of the pRubisco – DHFR fusion protein. The protease inaccessibility of citrate synthase in mitochondria and mitoplasts was 90% and 75%, that of the pRubisco – DHFR 66% and 55%, respectively.

subunit IV into the mitochondrial inner membrane and, in addition, allowed its correct assembly into a multienzyme complex of the mitochondrial inner membrane.

Restoration of cytochrome oxidase activity and of growth on a non-fermentable carbon source was only partial, however: the transformed cells expressing the pRubisco – subunit IV fusion protein grew 2.5 times more slowly and had 3 times less cytochrome oxidase activity than the transformants expressing authentic subunit IV precursor (Figure 5C and Table II). Mitochondria isolated from the yeast strain expressing the pRubisco – subunit IV fusion protein contained an immunodetectable subunit IV still carrying the chloroplast pre-sequence (Figure 6A; lane 1 and 3). Only a small fraction of the mitochondria-associated subunit IV was present as the mature-sized protein (Figure 6A, 'm'). The precursor form of the pRubisco – subunit IV fusion protein was inaccessible to externally-added protease in intact, but not in detergent-treated mitochondria (data not shown). At present it is not clear whether restoration of cytochrome oxidase is caused by the imported uncleaved fusion protein or only by the small amount of cleaved fusion protein. In contrast to the results obtained with pRubisco – DHFR (Figure 4A), virtually all of the pRubisco – subunit IV co-fractionated with the mitochondria

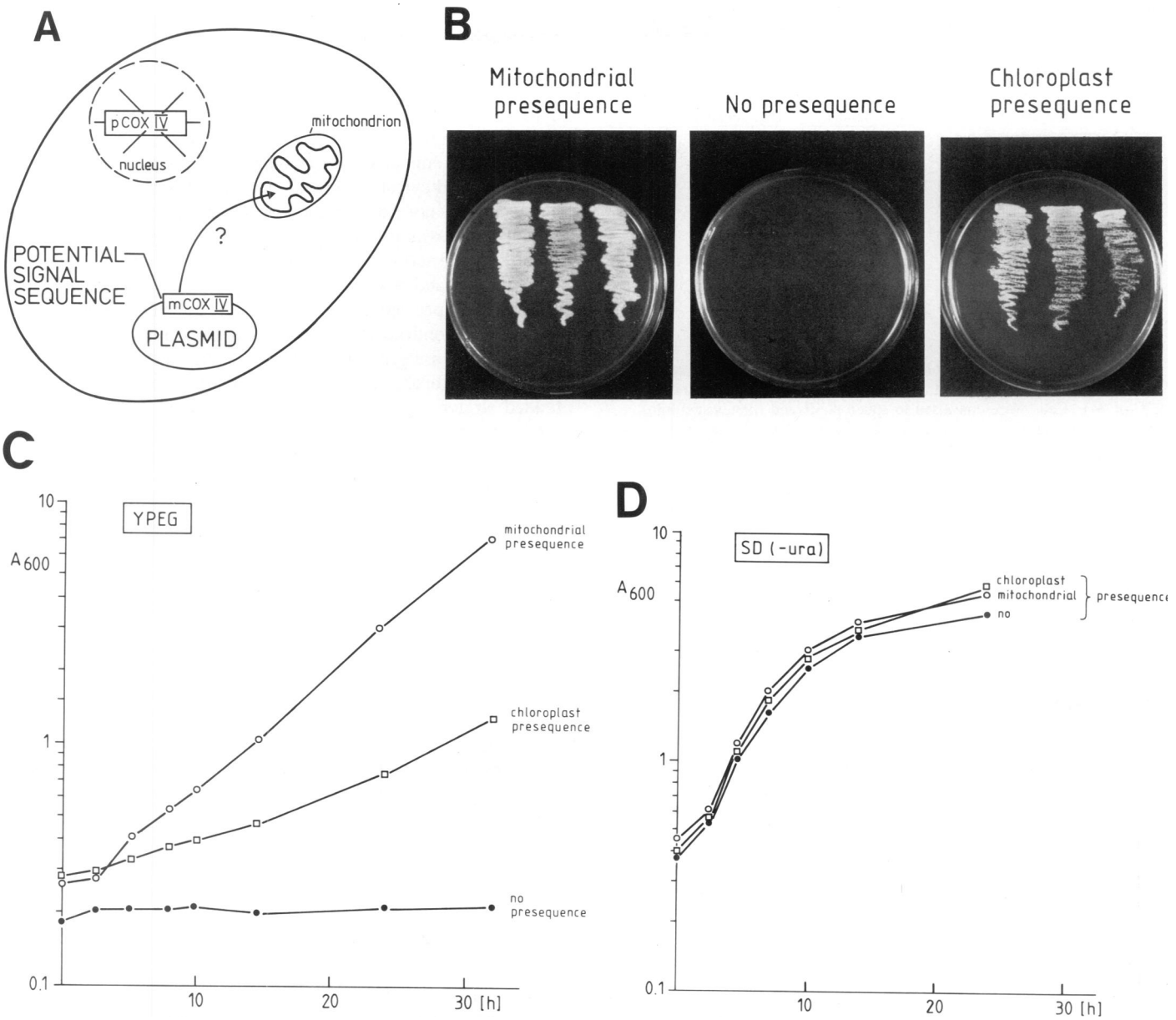

Fig. 5. A chloroplast pre-sequence directs attached cytochrome *c* oxidase subunit IV into the yeast mitochondrial inner membrane: Restoration of oxidative phosphorylation in a subunit IV-deficient yeast mutant. **(A)** Principle of the *in vivo* complementation assay. **(B)** The subunit IV-deficient mutant was transformed with the autonomously replicating centromere plasmid pAC1 on which the yeast alcohol dehydrogenase I promoter directed expression of genes encoding authentic subunit IV precursor ('mitochondrial pre-sequence'), 'pseudo-mature' subunit IV ('no pre-sequence') or pRubisco – subunit IV ('chloroplast pre-sequence'). The photograph shows growth on glycerol – ethanol of transformants carrying the indicated subunit IV genes. Transformants were first selected by growth on uracil-free minimal plates containing 0.67% yeast nitrogen base (Difco), 2% glucose and 20 μg L-histidine/ml. Three single colonies were streaked onto plates containing 1% yeast extract, 2% peptone, 3% ethanol and 2% glycerol (YPEG). Cells were grown for 5 days at 30°C and then photographed. **(C and D)** Growth of transformants in liquid YPEG **(C)** or uracil-free SD-medium supplemented with 20 μg L-histidine/ml **(D)**. Cell growth was measured by absorbance at 600 nm.

(Figure 6B). However, our inability to detect the fusion protein in the cytosolic fraction is probably caused by proteolytic degradation of subunit IV outside the mitochondria (cf. Hurt *et al.*, 1985b). The less efficient restoration of cytochrome oxidase activity by the pRubisco – subunit IV fusion protein could thus reflect one or more of the following: (i) the fusion protein is expressed less efficiently than the authentic subunit IV precursor; (ii) the uncleaved fusion protein restores cytochrome oxidase less efficiently than the authentic subunit, (iii) the chloroplast pre-sequence mediates protein import into yeast mitochondria less efficiently than the authentic subunit IV pre-sequence. In view of the results obtained with pRubisco – DHFR (cf. above) and the observation that upon short pulse-labeling of yeast cells comparable amounts of authentic subunit IV and the pRubisco – subunit IV fusion protein were made, the last two explanations appear as the most plausible ones. Despite these uncertainties, the observed restoration of cytochrome oxidase function convincingly proves that the chloroplast pre-sequence studied here directs subunit IV to the mitochondrial inner membrane *in vivo*.

Discussion

A considerable body of evidence suggests that the sorting of proteins between various intracellular organelles of a eukaryotic cell

Table II. Mitochondrial cytochrome oxidase activity is restored in the subunit IV-deficient yeast mutant by the fusion gene encoding a chloroplast pre-sequence attached to mature subunit IV

Subunit IV-deficient yeast mutant transformed with the gene encoding	Mitochondria (U/mg)	(%)	Spheroplasts (U/g wet weight of cells)	(%)
Authentic subunit IV precursor (mitochondrial pre-sequence)	1.19[a] 0.28[a]	(100)	10.2[a] 4.7[b]	(100)
'Pseudo-mature' subunit IV (no pre-sequence)	0	0	0	0
pRubisco – subunit IV (chloroplast pre-sequence)	0.36[a] 0.09[b]	30 32	5.6[a] 1.55[b]	55 33

The subunit IV-deficient yeast mutant transformed with the indicated genes was grown in 1% yeast extract, 2% peptone and 0.1% glucose to an O.D. 600 of ~1. Cells were converted to spheroplasts and fractionated into mitochondria and post-mitochondrial supernatant. Cytochrome *c* oxidase activity was measured in the presence of 0.2% sodium cholate in the spheroplasts and mitochondria. One unit of activity corresponds to the amount of enzyme which oxidizes 1 μmol of cytochrome *c* per min. Cytochrome *c* oxidase activity was measured in two independent experiments (a,b).

is mediated by specific 'transport sequences' on the sorted proteins themselves (Wickner and Lodish, 1985; Ellis, 1981; Hurt and van Loon, 1986). For example, amino-terminal 'pre-sequences' of imported mitochondrial proteins direct attached cytosolic proteins into mitochondria, but not the endoplasmic reticulum (Hurt *et al.*, 1985a; Horwich *et al.*, 1985; van Loon *et al.*, 1986); conversely, 'signal sequences of secretory proteins direct attached cytosolic proteins into the endoplasmic reticulum, but apparently not into mitochondria (Wickner and Lodish, 1985).

This study was prompted by the question whether organelle-specific signal sequences mediate the sorting of proteins between mitochondria and chloroplasts. This question is of interest because the cleavable pre-sequences (also called 'transit peptides') of imported mitochondrial and chloroplast proteins are in some respects similar. They are generally hydrophilic, rich in basic and hydroxylated amino acids and deficient in acidic amino acids and extended stretches of hydrophobic amino acids. Moreover, both types of pre-sequences can potentially form amphiphilic helices capable of interacting with phospholipid mono- and bilayers (Roise *et al.*, 1986).

Our results show that this similarity of structure is paralleled by a similarity of function: pre-sequences of proteins imported

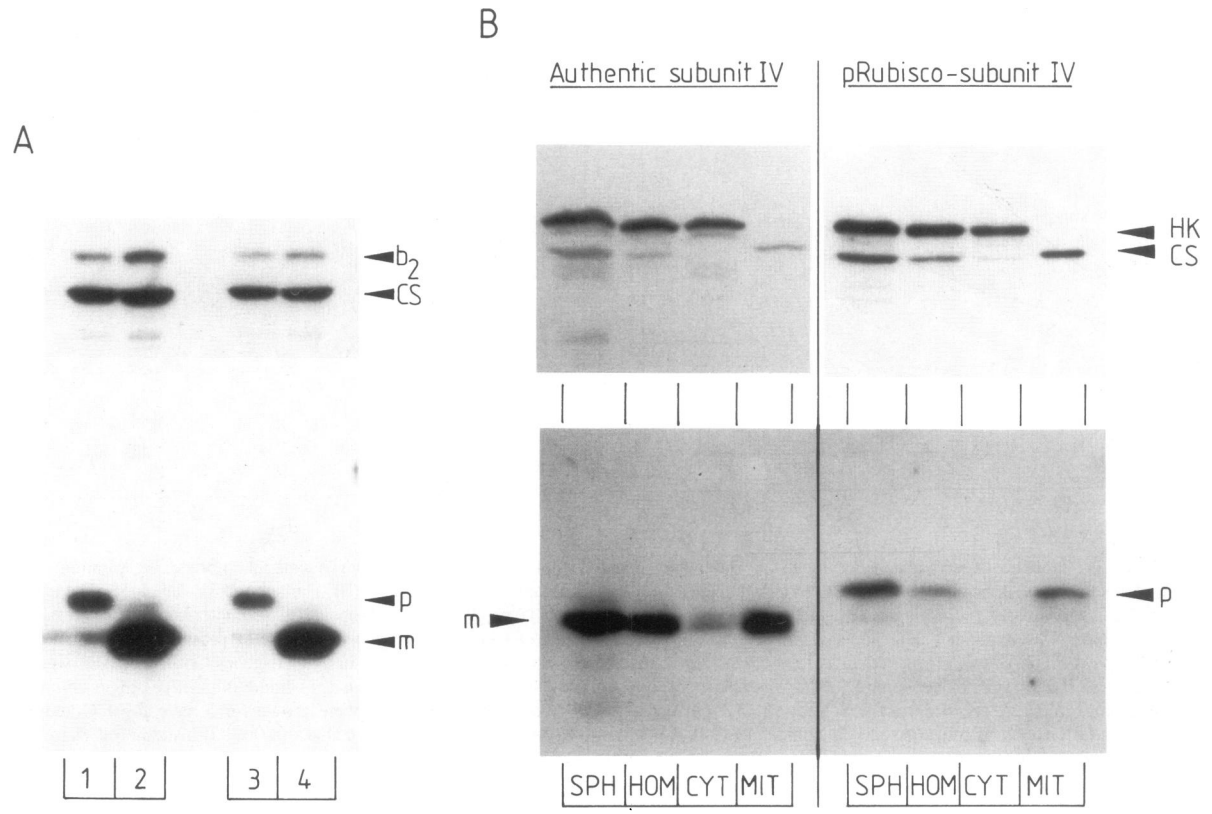

Fig. 6. The pRubisco – subunit IV fusion protein is imported into mitochondria largely in its precursor form. Cells from the subunit IV-deficient mutant were transformed with the genes encoding authentic subunit IV precursor or the pRubisco – subunit IV fusion protein (pRubisco – subunit IV) and grown at 30°C to an O.D. 600 of 1 in 1% yeast extract, 2% peptone and 0.1% glucose. Isolated cells were converted to spheroplasts and then homogenized; the homogenate was subfractionated into mitochondria and post-mitochondrial supernatant. **(A)** Mitochondria from the transformed cells expressing the pRubisco – subunit IV **(1,3)** and authentic subunit IV precursor **(2,4)** were analyzed by SDS – 14% polyacrylamide gel electrophoresis and immune blotting using antisera against yeast cytochrome oxidase subunit IV (lower panel) and against citrate synthase (CS) or cytochrome b_2 (b_2) (two mitochondrial markers). **1** and **2**, 100 μg mitochondrial protein; **3** and **4**, 50 μg mitochondrial protein. p and m, uncleaved pRubisco – subunit IV and mature subunit IV, respectively. The mature-sized form of pRubisco – subunit IV in **lanes 1** and **3** is not the result of overflow from **lanes 2** and **4**; this was confirmed in several other experiments (see also **B**, SPH). **(B)** Aliquots of spheroplasts (SPH), homogenate (HOM), post-mitochondrial supernatant (CYT) and mitochondria (MIT), each equivalent to 10 mg wet weight of cells, were analyzed by SDS – 14% polyacrylamide gel electrophoresis and immune blotting using antisera against cytochrome oxidase subunit IV (lower panel), hexokinase (HK, cytosolic marker) and citrate synthase (CS, mitochondrial marker) (upper panel). p and m, uncleaved pRubisco – subunit IV and mature subunit IV, respectively.

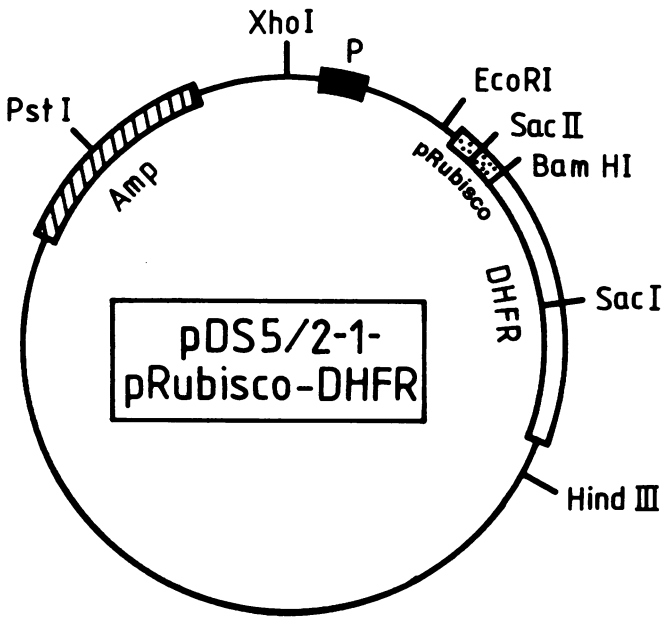

Fig. 7. Restriction map of the *Chlamydomonas* pRubisco – DHFR fusion gene inserted into the *in vitro* expression plasmid pDS5/2-1. For construction of the fusion gene, see Materials and methods; dotted bar, DNA encoding the first 31 amino acids of pRubisco; open bar, DHFR gene from mouse; shaded bar, β-lactamase gene (Amp); P, bacteriophage T5 promoter.

into chloroplasts can direct attached polypeptides into yeast mitochondria.

If the result is taken at face value, it raises the possibilities that protein sorting between mitochondria and chloroplasts in plant cells might not be absolutely precise and that the distinctive protein composition of these two organelles might not result from strictly separated import routes, but from selective degradation of 'misrouted' proteins inside each organelle. Some support for this view comes from immune electron micrographs which suggest that traces of a typical chloroplast protein (the small subunit of ribulose-1,5-bisphosphate carboxylase) are also present in mitochondria of the alga *Ochromonas danica* (Lacoste-Royal and Gibbs, 1985).

Upon more critical analysis, however, our present data do not yet prove protein 'missorting' between mitochondria and chloroplasts *in vivo*. First, these data are derived from a highly artificial system involving fusion proteins and heterologous systems for cellular expression and import. Second, the chloroplast pre-sequences used here mediate mitochondrial protein import less efficiently than authentic mitochondrial pre-sequences. Given a 'choice' between mitochondria and chloroplasts within a common cytoplasm, the chloroplast pre-sequences might well direct most, and perhaps all, of the attached protein molecules into chloroplasts. In order to study this, we are attempting to express some of these fusion genes in plant cells in order to determine the intracellular sorting (and missorting?) of the corresponding fusion proteins, but also of authentic organelle proteins, between mitochondria and chloroplasts.

However, the present data clearly establish a 'cross-reactivity' of chloroplast pre-sequences with yeast mitochondria. This striking finding could help to identify the translocation step(s) shared by mitochondria and chloroplasts and to unravel the mechanism by which these signal sequences perform their function. On the other hand, the chloroplast pre-sequences are rather poor mitochondrial targeting sequences. This suggests that pre-sequences of imported chloroplasts and mitochondrial proteins are somehow different. Identification of these differences should deepen our understanding of how proteins are sorted between mitochondria and chloroplast in a plant.

Materials and methods

Strains, plasmids and DNA manipulations

The *Escherichia coli* strain HB101 (Kedes *et al.*, 1975), the *Saccharomyces cerevisiae* strains YNN214 (α, *ura 3, lys 2, ade 2*; kindly provided by Mark Johnston and Ron Davis, Stanford, CA), D273-10B (ATCC 25657) and the subunit IV-deficient yeast mutant WD1 (α, *his 3, ura 3*; Dowhan *et al.*, 1985) were used. Plasmid pDS5/2 (Stueber *et al.*, 1984), pDS5/2-1, pDS5/2-1-COX IV-DHFR (Hurt *et al.*, 1984a), pDS5/2-1-'pseudo-mature' subunit IV, pDS5/2-1-70 kd subunit IV (Hurt *et al.*, 1985b), pLGSD5 (Guarente *et al.*, 1982), pLGSD5-DHFR (Hurt *et al.*, 1985a) and pAC1 (for reference, see Hurt *et al.*, 1985b) have been described. The coding region of the pre-sequence of ribulose-1,5-bisphosphate carboxylase from *C. reinhardtii* was isolated from plasmid 04.03 (Goldschmidt-Clermont and Rahire, in preparation). DNA restriction analysis, end-filling, ligations and agarose gel electrophoresis were performed according to published procedures (Maniatis *et al.*, 1982).

Construction of fusion genes

Most gene modifications and fusions are illustrated in Figure 2. Plasmid pDS5/2-1-pRubisco-DHFR was constructed in the following ways. A 579-bp *Sal*I fragment was isolated from plasmid 04.03 which encodes the gene for the pre-sequence of ribulose-1,5-bisphosphate carboxylase (pRubisco) from *C. reinhardtii*. This DNA piece was further digested with *Taq*I which cut 24 bp upstream of the ATG start codon of the pRubisco gene. After end-filling with a large fragment of *E. coli* DNA polymerase, *Eco*RI linkers (Biolabs) were attached to the ends and the DNA was further digested with *Bgl*I which cut at a position within the gene corresponding to amino acid 31 of the pRubisco pre-sequence. The 3'-protruding end generated by the *Bgl*I cut was removed by T4 DNA polymerase and the DNA was cut with *Eco*RI. The resulting 120-bp fragment which contained the 3'-shortened gene of the pRubisco pre-sequence was inserted into the *in vitro* expression plasmid pDS5/2-1 which had been opened at its single *Bam*HI site, blunt-ended as described above and re-cut with *Eco*RI. This ligation led to the in-frame fusion between the 3' end of the pRubisco pre-sequence and the 5' end of the DHFR coding sequence (Figure 2). A restriction map of plasmid pDS5/2-1-pRubisco-DHFR which allowed *in vitro* expression of this fusion gene is shown in Figure 7. The pRubisco – DHFR fusion gene was isolated from the *in vitro* expression plasmid as an *Eco*RI/*Hin*dIII fragment; after end-filling (see above) and attachment of *Bgl*II linkers (Biolabs), this fragment was inserted into the *Bam*HI site of plasmid pLGSD5. Plasmids carrying the pRubisco – DHFR in the correct orientation (under the control of a galactose-inducible yeast promoter) were introduced into the yeast strain YNN 214 by transformation. Plasmid pDS5/2-1-pRubisco-subunit IV was constructed as follows: plasmid pDS5/2-1-70 kd subunit IV (Hurt *et al.*, 1985b) was cut with *Bam*HI/*Pst*I and the large fragment encoding the pre-sequence-deleted subunit IV was isolated. Plasmid pDS5/2-1-pRubisco-DHFR was digested with *Bam*HI/*Pst*I and the small fragment encoding the first 31 residues of the pRubisco pre-sequence was isolated. The two purified *Bam*HI/*Pst*I fragments were ligated to each other yielding plasmid pDS5/2-1-pRubisco-subunit IV (see also Figure 2). Finally, the pRubisco – subunit IV fusion gene was isolated as an *Eco*RI/*Hin*dIII fragment and ligated with the large *Eco*RI/*Hin*dIII fragment of plasmid pAC1. By this insertion the fusion gene was placed under the control of the yeast alcohol dehydrogenase I promoter allowing *in vivo* expression of a fusion protein containing the first 31 amino acids of the pRubisco pre-sequence attached to subunit IV. Plasmid pAC1-Rubisco-subunit IV was used to transform the subunit IV-deficient yeast mutant WD1 (Dowhan *et al.*, 1985; Hurt *et al.*, 1985b).

Miscellaneous

The yeast strain YNN 214 transformed with plasmids pLGSD5-DHFR and pLGSD5-pRubisco-DHFR and the subunit IV-deficient mutant WD1 transformed with plasmid pAC1-wild-type subunit IV precursor, pAC1-'pseudo-mature' subunit IV and pAC1-pRubisco-subunit IV were grown as described earlier (Hurt *et al.*, 1984, 1985b). Spheroplasting of yeast cells and subcellular and submitochondrial fractionation was performed as described (Hase *et al.*, 1984; Hurt *et al.*, 1985b). Published procedures were used for *in vitro* transcription/translation of cloned genes (Stueber *et al.*, 1984; Hurt *et al.*, 1984b), for studying protein import into isolated mitochondria (Hurt *et al.*, 1985a), for SDS – polyacrylamide gel electrophoresis, immune blotting and fluorography (Gasser *et al.*, 1982), for quantitation of bands of X-ray films (Suissa, 1983) and for transformation of yeast with plasmids pLGSD5 and pAC1 (Ito *et al.*, 1983). DHFR and cytochrome *c* oxidase activities were determined as outlined (Hurt *et al.*, 1985a; Mason *et al.*, 1973). L-[^{35}S]methionine (> 1000 Ci/mmol) was purchased from Amersham International, UK.

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