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Transport of the yeast ATP synthase β-subunit into mitochondria

Effects of amino acid substitutions on targeting

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We have isolated the yeast *ATP2* gene encoding the β-subunit of mitochondrial ATP synthase and determined its nucleotide sequence. A fusion between the *N*-terminal 15 amino acid residues of β-subunit and the mouse cytosolic protein dihydrofolate reductase (DHFR) was transcribed and translated *in vitro* and found to be transported into isolated yeast mitochondria. A fusion with the first 35 amino acid residues of β-subunit attached to DHFR was not only transported but also proteolytically processed by a mitochondrial protease. Amino acid substitutions were introduced into the *N*-terminal presequence of the β-subunit by bisulphite mutagenesis of the corresponding DNA. The effects of these mutations on mitochondrial targeting were assessed by transport experiments *in vitro* using DHFR fusion proteins. All of the mutants, harbouring from one to six amino acid substitutions in the first 14 residues of the presequence, were transported into mitochondria, though at least one of them (I8) was transported and proteolytically processed at a much reduced rate. The I8 mutant β-subunit also exhibited poor transport and processing *in vivo*, and expression of this mutant polypeptide failed to complement the glycerol$^-$ phenotype of a yeast *ATP2* mutant. More remarkably, the expression of I8 β-subunit induced a more general growth defect in yeast, possibly due to interference with the transport of other, essential, mitochondrial proteins.

INTRODUCTION

Most mitochondrial proteins are encoded by nuclear genes and synthesized in the cytoplasm as larger precursors with *N*-terminal presequences [1,2]. Transport into mitochondria is mediated by receptors on the mitochondrial surface [3–5]. The recognition of mitochondrial precursor polypeptides by these receptors is poorly understood, but the requirement for specificity of recognition is clear, firstly since only a limited set of cytoplasmic polypeptides is transported into mitochondria, and secondly since these precursor polypeptides are targeted to the mitochondria and not to other cellular compartments. The *N*-terminal presequences of mitochondrial proteins show very low levels of sequence similarity but share a number of features including an abundance of basic amino acid residues, a high content of serine and threonine residues and a general absence of acidic residues [2,6]. The predicted ability of these presequences to form amphiphilic structures may also be important for targeting and transport [6–8].

The targeting information in several mitochondrial protein precursors has been localized to the *N*-terminal presequence by gene fusion studies. As few as 9–12 amino acid residues fused to a normally non-mitochondrial polypeptide have been shown to be sufficient for targeting to the mitochondrial matrix [9,10]. Presequences may also contain signals for intramitochondrial sorting [11], but it appears that each protein contains a matrix targeting sequence at the extreme *N*-terminus. Previous gene fusion experiments with the yeast *ATP2* gene have shown that the first 39 residues of the ATP synthase β-subunit are sufficient to direct invertase to the matrix [12]. Deletion studies have been used to localize further the targeting information within this presequence [13]. In this study we describe fusions of the ATP synthase β-subunit presequence with mouse dihydrofolate reductase (DHFR) which are efficiently targeted to mitochondria. We have attempted to identify particular features within the targeting sequence that are required for mitochondrial delivery by introducing amino acid substitutions and investigating the effects of such mutations on targeting and transport.

MATERIALS AND METHODS

Strains and plasmids

Escherichia coli strains MM294 [14] and HB101 were used for plasmid propagation, the ung$^-$ strain BD1528 [15] for propagation of bisulphite-treated DNA and NM522 for propagation of M13 recombinants.

Saccharomyces cerevisiae JQ1 was used as a recipient for transformation with plasmids containing various forms of the *ATP2* gene. Transformants were selected for growth in the absence of uracil on plates containing 0.17% yeast nitrogen base, 0.5% $(NH_4)_2SO_4$, 2% glucose, 2% agar and 50 μg of L-histidine/ml (SD+his medium). SD minimal medium was identical except for the absence of agar. YPD medium contained 1% yeast extract, 2% bactopeptone and 2% glucose. YPEG medium was identical, except that the glucose was substituted with 3% ethanol plus 2% glycerol. Mitochondria for import studies *in vitro* were isolated from *S. cerevisiae* D273-10B grown as described previously [6].

Plasmids pMC4 [17] and pDS6 [18] have been described

Abbreviation used: DHFR, dihydrofolate reductase.

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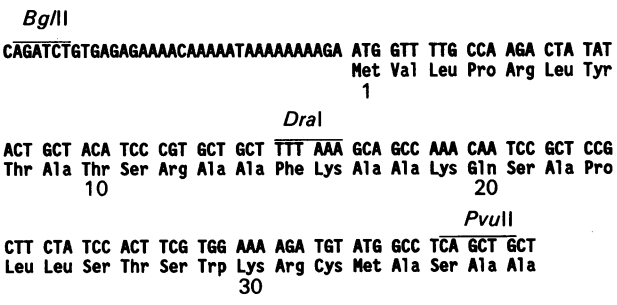

Fig. 1. Nucleotide sequence encoding the β-subunit presequence

The nucleotide sequence of the *ATP2* gene is shown from the engineered *Eco*RI and *Bgl*II sites as in pGR207 to the *Pvu*II site at codon 36 in the β-subunit coding region. The corresponding amino acid sequence is shown from the initiating methionine and restriction enzyme cleavage sites used in this study are indicated below the sequence.

in the literature; pDS7 is essentially the same as pDS5/2 [18]. The *ATP2* gene from *S. cerevisiae* was isolated from a genomic library on plasmid pFL1-11-3. The coding region was transferred to the transcription vector pDS6 in several steps *in vitro*. Since the efficiency of translation *in vitro* may be decreased when the initiation codon is preceded by a substantial length of untranslated RNA [19] and because these were no convenient restriction sites close to the *ATP2* initiation codon, we inserted a *Bgl*II site. pML4 was digested with *Nru*I, then with *Bal*31 exonuclease. This DNA was cut with *Eco*RI, made blunt-ended by the Klenow reaction and ligated in the presence of *Bgl*II linkers (pCAGATCTG). Recombinants were screened by restriction digests and by DNA sequence analysis. One plasmid was found with the linker inserted 27 bp upstream from the start codon; the DNA sequence of this region is shown in Fig. 1. The *Eco*RI–*Hin*dIII fragment from this plasmid was then transferred to pDS6 which had been cut with *Eco*RI plus *Hin*dIII so that the start codon was placed 64 nucleotides downstream from the transcription start site. The resulting plasmid was called pGR207.

To construct the β_{15}–DHFR fusion, pDS7 was cleaved with *Eco*RI, made blunt-ended by the Klenow filling-in reaction and then cleaved with *Xho*I. The large fragment was ligated with the 265 bp *Xho*I–*Dra*I fragment from pGR207. To construct β_{35}–DHFR fusions, pDS7 was first cut with *Bam*HI, the ends were filled in and the DNA was cleaved with *Xho*I. The large fragment was ligated with the 325 bp *Xho*I–*Pvu*II fragment from pGR207 or mutated derivatives.

ATP2 gene disruption

The *atp2* yeast strain JQ1 (*MATα his4 ura3 atp2::LEU2*) was constructed by the one-step gene disruption method [20]. The 1.8 kb *Nru*I–*Hin*dIII *ATP2* fragment from pFL1-11-3 was inserted into pSP65 [21] between the *Sma*I and *Hin*dIII sites of the polylinker, yielding plasmid pML4. The *Nru*I site was estimated by restriction mapping to lie 0.3 kb upstream of the *ATP2* initiation codon. The *ATP2* DNA was excised from pML4 as a 1.8 kb *Eco*RI–*Hin*dIII fragment and ligated with pAT153 [22] which had been cut with *Eco*RI and *Hin*dIII to yield plasmid pATP1. The 3.0 kb *Bgl*II fragment from YEp13 [23], containing the intact *LEU2* gene, was isolated and made blunt-ended by the filling-in reaction of DNA polymerase Klenow fragment, then inserted at the *Pvu*II site of pATP1, yielding the recombinant plasmid pJQ10. This plasmid was cut with *Sac*I plus *Hin*dIII and the digest was used directly to transform the haploid *S. cerevisiae* strain SF747-19D to LEU$^+$. SF747-19D (*MATα his4 ura3 leu2*) was provided by R. Schekman and C. Field, Berkeley, CA, U.S.A. The disruption of the chromosomal *ATP2* gene in one of the resulting transformants (JQ1) was confirmed by its inability to utilize non-fermentable carbon sources, by the absence of immunologically detectable ATP synthase β-subunit polypeptide and by Southern blot hybridization (results not shown).

Bisulphite mutagenesis

Plasmid pGR209 was derived from pGR207 as follows. pGR207 was linearized with *Bgl*II, made blunt-ended by filling in with Klenow fragment, then digested with *Sal*I. The larger of the two fragments, corresponding essentially to pDS6 DNA, was isolated and ligated with the 2.2 kb *Dra*I–*Sal*I fragment from pGR207 which contains most of the *ATP2* coding region. pGR209 thus lacks the first 15 codons of the ATP synthase β-subunit coding DNA plus 27 base pairs of 5′ non-coding DNA, but is otherwise identical to pGR207. Bisulphite mutagenesis was performed with gapped duplexes [24,25] formed between pGR207 which had been linearized with *Sma*I and pGR209 linearized with *Kpn*I. The digested plasmids (14 μg each) were extracted with phenol and precipitated with ethanol. The DNA was dissolved in 120 μl of water and then denatured by adding 19.2 μl of 1 M-NaOH and incubating at room temperature for 10 min. The separated strands were then re-annealed to form heteroduplex molecules by sequential addition of 1.12 ml of water, 160 μl of 0.5 M-Tris/HCl, pH 8.0, and 192 μl of 0.1 M-HCl, and then were incubated for 120 min at 63 °C. Bisulphite mutagenesis was performed essentially as described by Shortle & Nathans [26]. Gapped duplex DNA (1–2 μg) was mixed with 3 vol. of freshly prepared 4 M-sodium bisulphite, pH 6.0, to give a final DNA concentration of 20 μg/ml in 15 mM-NaCl/1.5 mM-Na citrate (pH 7.0)/2 mM-quinol, with all components at 0 °C when mixed. The solution was overlaid with liquid paraffin and incubated in the dark for 30–120 min at 37 °C. The reaction was terminated by dialysis according to Shortle & Nathans [26]. The DNA was precipitated with propan-2-ol, dissolved in 10 mM-Tris/HCl (pH 8.0)/0.1 mM-EDTA and used directly to transform *E. coli* BD1528. Plasmid DNA from transformants was screened by restriction digests and by sequencing of plasmid DNA [27,28].

Protein transport *in vitro*

Capped RNA was transcribed *in vitro* from plasmid DNA as described [9,18] and translated in a nuclease-treated rabbit reticulocyte lysate [29] in the presence of [^{35}S]methionine [9]. The translation products were incubated for 30 min at 28 °C with yeast mitochondria isolated as described previously [16] at a concentration of 210 μg of mitochondrial protein in 300 μl. Aliquots of 100 μl were then incubated at 0 °C for 15 min with (1) no additions, (2) proteinase K at 10 μg/ml or (3) proteinase K at 10 μg/ml plus 0.3% Triton X-100. Digestion was terminated by addition of phenylmethanesulphonyl fluoride to 1 mM. Mitochondria were re-isolated by centri-

fugation for 10 min at 4 °C in a microcentrifuge, washed once with 0.6 M-mannitol/20 mM-Hepes (pH 7.4) and resuspended in the same buffer. The mitochondrial pellet and the extramitochondrial supernatant fractions were precipitated with 10% trichloroacetic acid and resuspended in SDS sample buffer (50 mM-Tris/HCl (pH 6.8)/2 mM-EDTA/2% SDS/10% glycerol/0.01% Bromophenol Blue) for gel electrophoresis.

Miscellaneous

Published procedures were used for transformation of *E. coli* [30] and yeast [31], polyacrylamide-gel electrophoresis [32], protein blotting and immunochemical detection [33], pulse-chase labelling of yeast [34], immunoprecipitation [16], isolation of plasmid DNA [35], agarose gel electrophoresis [36] and DNA sequence analysis [37] using single-stranded M13 DNA [38] or double-stranded plasmid DNA [27].

Enzymes were purchased from a number of sources and were used according to the manufacturer's instructions. Messenger-dependent rabbit reticulocyte lysate, L-[^{35}S]methionine and [α-^{32}P]-dATP were purchased from Amersham International.

RESULTS

The yeast *ATP2* gene

The *ATP2* gene from *S. cerevisiae* was isolated from a genomic library in the plasmid pFL1 [39] by a two-step screening procedure [40] involving a differential hybridization with RNA from de-repressed and glucose-repressed yeast followed by identification of individual clones by hybrid-select translation. Since the gene was isolated independently [41], we have not extensively characterized the insert in this plasmid, designated pFL1-11-3, but have shown that it contains a functional gene as yeast transformants harbouring this plasmid over-express β-subunit several-fold. We have determined the nucleotide sequence of part of the *ATP2* gene and have found significant differences between our sequence and that reported by Takeda *et al.* [42] in the region encoding the β-subunit presequence. We have checked this sequence carefully and find six nucleotides more than did Takeda *et al.* [42] so that the β-subunit precursor contains 511 amino acid residues and not 509. The correct DNA sequence and corresponding amino acid sequence are shown in Fig. 1. The presequence has an amino acid composition similar to other mitochondrial targeting sequences [6] and in particular contains several basic amino acid residues and no acidic residues.

Transport of fusion proteins into isolated mitochondria

The location of targeting information in the β-subunit precursor was studied using the first 15 and the first 35 codons of the *ATP2* gene fused in frame with the entire DHFR coding region (β_{15}–DHFR and β_{35}–DHFR respectively). These fusions were transcribed and translated *in vitro* in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine. After incubation with isolated mitochondria, both of these radiolabelled fusion proteins were found associated with the organelles (Fig. 2), whereas DHFR lacking a targeting sequence remained in the suspending medium. It is clear that a substantial fraction of β_{15}–DHFR remained in the supernatant fraction after incubation with mitochondria and that

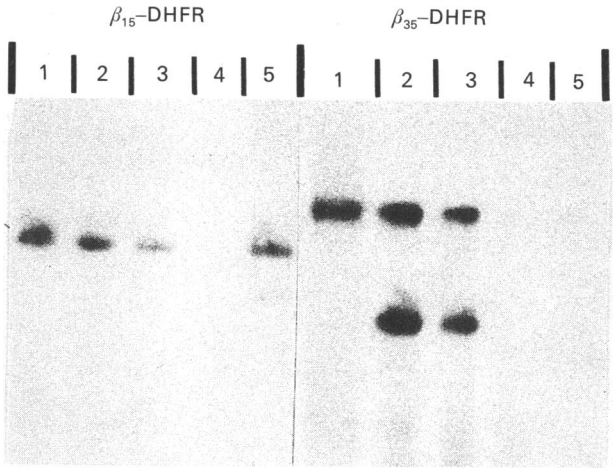

Fig. 2. *N*-terminal presequence of β-subunit directs mouse DHFR into isolated mitochondria

The β_{15}–DHFR fusion polypeptide and the β_{35}–DHFR fusion polypeptide were synthesized by transcription and translation *in vitro* in the presence of [^{35}S]methionine, and were incubated with isolated yeast mitochondria for 30 min at 28 °C. Aliquots were treated subsequently as described below and in the Materials and methods section. Proteins were analysed by SDS/polyacrylamide-gel electrophoresis and fluorography. Lane 1, total translation products; lane 2, mitochondria; lane 3, mitochondria after treatment with proteinase K; lane 4, mitochondria after treatment with proteinase K in the presence of Triton X-100; lane 5, supernatant.

much of the material associated with mitochondria was accessible to proteinase K and therefore was bound to the mitochondrial surface. However, a significant fraction of the β_{15}–DHFR was protected from externally added protease and had therefore been translocated across the mitochondrial membranes. β_{35}–DHFR was almost completely associated with mitochondria after the incubation period, indicating that the first 35 residues of the β-subunit constitute a more efficient targeting signal than the first 15. Nevertheless, these *N*-terminal 15 residues do indeed direct DHFR to the mitochondrion and allow transport across the mitochondrial membranes. The β_{35}–DHFR was partly converted to a lower molecular mass form, presumably due to processing by the mitochondrial matrix protease [43]. In this case, protease protection was observed with both the processed and unprocessed forms, indicating that each had been translocated across the mitochondrial membranes. It thus appears that proteolytic processing was relatively slow, possibly because the fusion is close to the site of cleavage.

Mutagenesis of the β-subunit targeting sequence

Having found mitochondrial targeting information in the *N*-terminal 15 residues of the β-subunit precursor, it was of interest to investigate which residues might be of particular importance for targeting. To achieve this, the DNA encoding the presequence was mutagenized with bisulphite, which leads to C to T transitions, using a gapped duplex approach [24,25] to restrict mutagenesis to the first 15 codons. Mutants were screened initially by restriction analysis of isolated plasmid DNA. We expected to find two types of plasmid, either containing the

Table 1. Amino acid substitutions in the β-subunit presequence

The wild-type nucleotide sequence of the first 15 codons and the corresponding amino acid sequence are shown. Only those amino acids which differ form the wild-type are shown for each mutant sequence.

		Amino acid sequence alterations														
	Codon...	ATG	GTT	TTG	CCA	AGA	CTA	TAT	ACT	GCT	ACA	TCC	CGT	GCT	GCT	TTT
Mutant	Amino acid...	M	V	L	P	R	L	Y	T	A	T	S	R	A	A	F
H5										V						
H19															V	
H64					S											
N1													C			
O4															V	
H6									I						V	
H63					S						I					
H13			I										H		T	
N7									I		I		C			
O35					S					V			C		V	
G5					L					V	I		C	V		
C1					L						I	F	C	V	V	
H2										V	I	F	C	V	V	
I8					S				I	V	I			V	V	

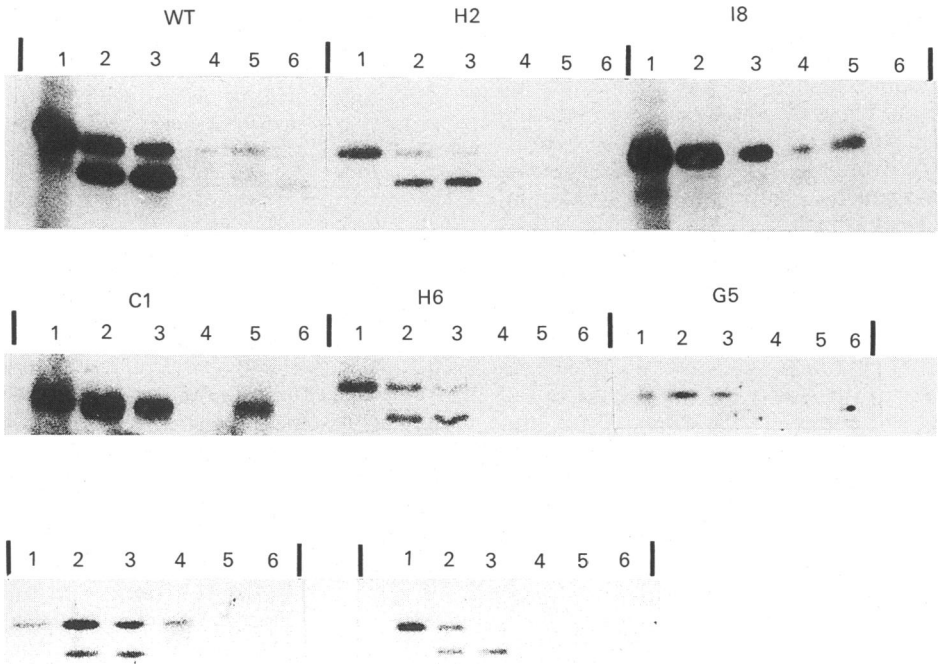

Fig. 3. Transport into isolated mitochondria directed by mutant presequences

Wild-type (WT) and mutant β_{35}–DHFR fusion polypeptides were synthesized *in vitro*, and transport was analysed as described in the legend to Fig. 2. Lane 1, total translation products; lane 2, mitochondria; lane 3, mitochondria after treatment with proteinase K; lane 4, mitochondria after treatment with proteinase K in the presence of Triton X-100; lane 5, supernatant; lane 6, supernatant after treatment with proteinase K.

entire *ATP2* coding region as in pGR207 or with the deletion as in pGR209, depending on which of the two strands of the original heteroduplex had been propagated. These two types of plasmid were readily distinguished by digests with *Dra*I and were found in approximately equal numbers with a few transformants harbouring both sizes of plasmid (results not shown).

Full-length plasmids were subjected to DNA sequence analysis using an oligodeoxyribonucleotide primer (GATTCAAATTGTGAGCGG) which hybridizes immediately 5′ to the insert in pDS6, thus allowing us to determine the nucleotide sequence of the presequence coding region. The mutant sequences were all of the expected type, either C to T or G to A transitions, the latter arising if the non-coding strand was mutated. In fact we observed a strong bias to mutations in the coding

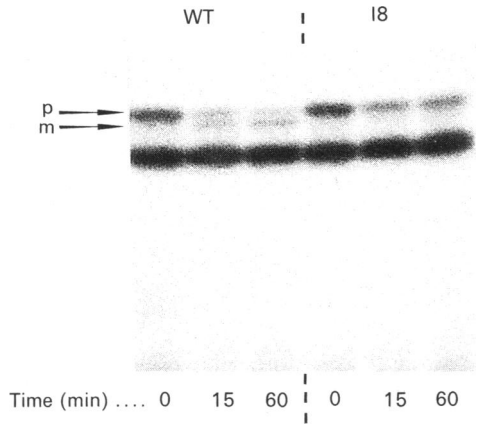

Fig. 4. Proteolytic processing of the β-subunit *in vitro*

Wild-type (WT) and I8 mutant β-subunit were synthesized *in vitro* and incubated at 28 °C with a mitochondrial matrix fraction containing 25 μg of protein. Samples taken at 0, 15 and 60 min were subjected to SDS/polyacrylamide-gel electrophoresis and fluorography. The positions of the precursor (p) and mature (m) β-subunit are indicated. The first three lanes contained samples with wild-type protein and the last three contained the I8 β-subunit. The heavy, lower band results from translation of an endogenous RNA in the reticulocyte lysate.

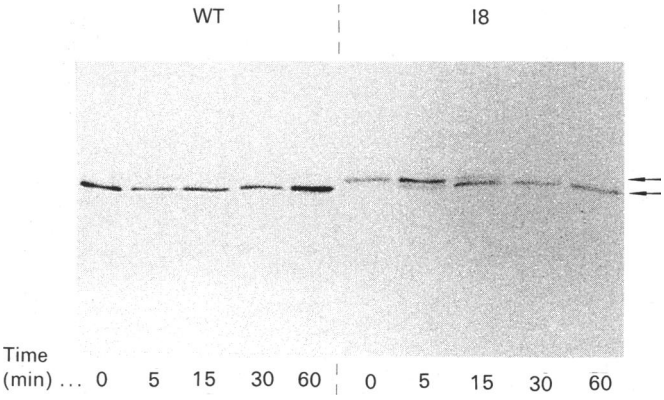

Fig. 5. Pulse–chase labelling of β-subunit

Transformants of the *atp2* yeast strain JQ1 harbouring pMC4-β (WT) and its derivative with the I8 mutation were grown to mid-exponential phase and pulse-labelled for 5 min with [^{35}S]methionine (5 μCi·ml^{-1}). Samples (0.1 ml) were taken immediately and after 5, 15, 30 and 60 min of a chase with unlabelled methionine. The ATP synthase β-subunit was immunoprecipitated and then subjected to SDS/polyacrylamide-gel electrophoresis and fluorography. The positions of the precursor (p) and mature (m) β-subunit are indicated.

strand, with only two mutants from the 18 sequenced having been mutated in the non-coding strand. The reasons for this bias are not clear. Several plasmids contained non-mutant *ATP2* DNA, whereas others containing from one to six substitutions were identified. Some substitutions were silent (e.g. CTA to TTA encoding Leu at position 6), but most mutants had altered amino acid sequences in the region of interest. The predicted mutant amino acid sequences are summarized in Table 1. The mutations introduced several amino acids which are less abundant in mitochondrial presequences than elsewhere [6], e.g. Val and Ile, and in some cases removed the positive charge due to Arg-12. The DNA sequence of the *Bgl*II–*Pvu*II fragment encoding each mutant presequence was determined, and to eliminate the possibility of spurious mutations outwith this region, these fragments were subcloned back into the original plasmid.

Mitochondrial targeting with mutant presequences

Plasmids containing the first 35 codons of the *ATP2* gene fused to DHFR were constructed for each of the mutants described above. This not only allowed targeting *in vitro* to be assessed, but also the ability of these β_{35}–DHFR fusions to be cleaved by the mitochondrial processing protease could be used as a marker for transport of the polypeptide to the mitochondrial matrix. Most of the mutant precursors were targeted to mitochondria, transported to a protease-resistant site and proteolytically cleaved in a manner indistinguishable from that found with the non-mutant β_{35}–DHFR fusion polypeptide (Fig. 3), showing that the mutations in these presequences had no markedly adverse effect on targeting. Three mutant precursor polypeptides (I8, C1 and G5) were found to be defective in transport. In each case, a substantial fraction of the precursor polypeptide was found associated with the mitochondria, but it remained largely susceptible to added proteinase K. Nevertheless, the finding that a significant fraction of the polypeptide had become protected from added protease indicates that transport had occurred to a small extent. None of these poorly transported mutant polypeptides underwent detectable processing, presumably because of a decreased rate of cleavage by the processing protease. When the full-length β-subunit with the I8 mutant presequence was synthesized *in vitro* and incubated with a mitochondrial matrix preparation containing proteolytic processing activity, no mature form was detected under conditions which led to extensive processing of the non-mutant β-subunit (Fig. 4). Similarly, proteolytic processing of I8 is very much slower *in vivo*. Pulse-chase experiments demonstrated that the I8 mutant β-subunit is matured over a period of several minutes, whereas the wild-type polypeptide was processed too quickly to allow detection of the precursor form after a 5 min labelling period (Fig. 5). Cell fractionation studies with these pulse-labelled yeast cells showed that not only processing but also transport of the I8 precursor polypeptide was impaired, consistent with this mutant's greater protease sensitivity after protein transport *in vitro* than is found with either wild-type or efficiently-transported mutant precursor polypeptides (Fig. 3).

Transport and assembly of mutant β-subunit precursors *in vivo*

The ability of the mutant presequences to support transport in a growing yeast cell was assessed by the formation of active enzyme required for growth on a non-fermentable carbon source. The yeast mutant JQ1 was constructed by disruption of the chromosomal *ATP2* gene of SF747-19D as described in the Materials and methods section. This strain fails to express β-subunit and is unable to utilize glycerol. Transformation of JQ1 with the plasmid pMC4-β, directing the expression of wild-type β-subunit, restored growth on glycerol. This

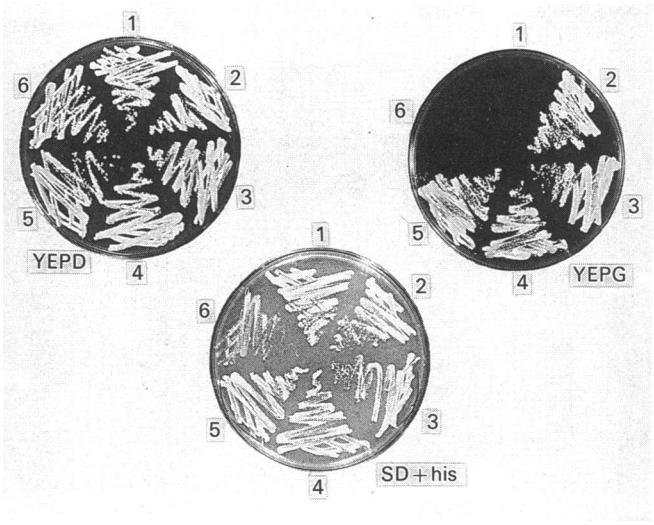

Fig. 6. Growth of yeast transformants expressing β-subunit with mutant presequences

Yeast transformants were streaked on solid media and grown at 30 °C for 3 days. The *atp*2 strain JQ1 harbouring pMC4 (1), pMC4-β (2) and pMC4-β derivatives encoding β-subunit with presequence mutations H6 (3), H13 (4), H63 (5) and I8 (6) were used. YEPD, YEPG and SD+his refer to media used (see the Materials and methods section).

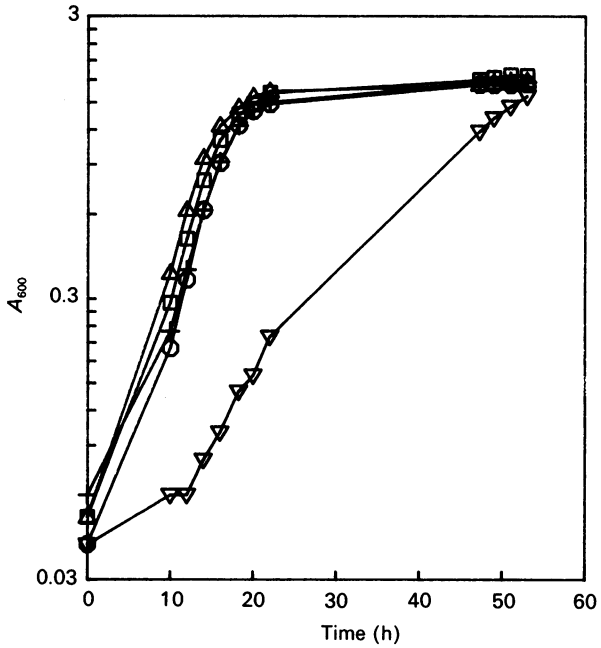

Fig. 7. Expression of a mutant β-subunit precursor inhibits growth

The *atp*2 mutant JQ1 (□) and transformants expressing β-subunit with wild-type (+) or mutant [Cl (△), H5 (○) and I8 (▽)] presequences were grown on minimal medium supplemented with histidine and, in the case of untransformed JQ1, uracil. Growth was at 30 °C in 100 ml cultures and was monitored by measuring the absorbance at 600 nm.

complementation assay was used to assess the behaviour of mutant precursor polypeptides. Since the mature β-subunit sequence was identical in each case, no difference was expected in the catalytic properties of these proteins, and failure to complement could be taken to indicate a defect in either transport or assembly of active enzyme. Most of the mutant precursors supported growth on glycerol (Fig. 6) and thus contained functional mitochondrial targeting sequences. The three mutant precursors which were defective in transport *in vitro* (I8, C1 and G5) also failed to support growth on glycerol. This inability to complement the *atp*2 mutation could not, at least with I8 and G5, be attributed to poor expression, as the levels of β-subunit protein in these mutants, assessed by Western blotting, were comparable with levels of wild-type β-subunit and of mutated precursors which did complement (results not shown). The level of β-subunit in C1 did, however, appear significantly lower. Much of the β-subunit polypeptide in the I8, C1 and G5 transformants had been proteolytically processed and was thus presumably located in the mitochondria.

The growth of the transformant expressing the I8 mutant polypeptide was not only defective on a non-fermentable carbon source, but surprisingly was also poor on minimal medium with glucose as carbon source (Fig. 6). Under these conditions, oxidative phosphorylation is not required for growth. This is seen clearly from the observation that untransformed JQ1, lacking β-subunit, grew at the same rate as did transformants expressing the functional, wild-type polypeptide. When expressing the I8 mutant polypeptide, growth was substantially slower (Fig. 7), so this mutant precursor appears not only to fail to complement a lack of β-subunit, but also has an inhibitory effect on growth. This inhibitory effect was not observed with another non-complementing mutant, C1 (Fig. 7).

DISCUSSION

We have shown here that the *N*-terminal presequence of the β-subunit of yeast ATP synthase is sufficient to direct a cytoplasmic protein, mouse DHFR, into isolated mitochondria. The first 15 residues of the presequence contain a functional targeting sequence but no cleavage site, whereas a fusion polypeptide with the first 35 residues was both transported and proteolytically processed. The precise extent of the presequence of ATP synthase β-subunit is not clear, since there is some uncertainty as to where cleavage occurs. Vassarotti *et al.* [13] suggested that cleavage takes place between Lys-19 and Gln-20, but the evidence for this is not complete. Amino acid sequence determination indicates that the mature β-subunit sequence begins at Ala-34 (B. Pevec & B. Hess, unpublished work), so the presequence may be between 19 and 33 residues in length. It is worth noting that the mitochondrial ATP synthase β-subunit has a high level of sequence similarity to the equivalent protein from *E. coli* [44] which lacks a presequence. The *E. coli* protein sequence begins at a position equivalent to residue 41 of the yeast polypeptide, close to the observed position of the *N*-terminus of isolated β-subunit.

We have investigated the nature of the targeting information in the β-subunit presequence by introducing a number of amino acid substitutions, and have shown that several mutations can be introduced with no discernible effect on transport. It thus appears that a functional mitochondrial targeting signal does not require a very specific amino acid sequence, but more probably depends on some special feature of three-

dimensional folding [6,7]. This conclusion accords with previous findings with mutated mitochondrial targeting sequences [45] and with the demonstration that a broad spectrum of essentially random amino acid sequences can restore mitochondrial targeting to a protein lacking its endogenous presequence [46]. Three mutant presequences were isolated which were clearly defective in transport and processing. Each of these was heavily mutated, with five or six amino acid substitutions, but there is no obvious, simple correlation between the nature of these mutations and the ability to direct transport into mitochondria *in vitro*. The molecular basis of the defect in these presequences is thus most likely to derive from an effect on the three-dimensional structure and behaviour of the presequence, as described above.

The presequence mutations described here were introduced by bisulphite mutagenesis as a pseudo-random method, but because of the nature of the mutations (C to T transitions) and because of the DNA sequence itself, the range of possible amino acid sequence changes was limited. Most of the mutations led to an increase in the hydrophobicity of the presequence. For example residues 10–14 in the parent presequence, TSRAA, were replaced by IFCVV in the mutant C1. Allison & Schatz [8] suggested on the basis of experiments with artificial presequences that a minimum overall hydrophobicity is required for targeting. The results reported here indicate that the hydrophobicity of the β-subunit presequence can be increased substantially without loss of function.

The results of transport studies with mutant presequences *in vitro* were qualitatively corroborated by experiments *in vivo*. β-Subunit precursors with mutant presequences but no alterations in the mature protein sequence were expressed in yeast, and their ability to complement the absence of wild-type β-subunit was used to indicate localization to the mitochondria. Only these mutant presequences which were defective *in vitro* (C1, G5 and I8) failed to support growth on a non-fermentable carbon source and thus failed to form active ATP synthase in the mitochondrion.

One of these non-complementing mutants (I8) exhibited an unexpected growth defect on minimal medium containing glucose. This defect is clearly not due to interference with oxidative phosphorylation, which is not required under these growth conditions. We have shown that the I8 β-subunit is defective both in its transport into mitochondria and as a substrate for the processing protease of the mitochondrial matrix, indicating that this mutant polypeptide, although able to pass along the transport pathway, apparently does so at a much reduced rate. This raises the possibility that the mutant precursor polypeptide may reside for a relatively long time in sites required for import not only of β-subunit but also of other mitochondrial proteins [47], and may thereby interfere with their transport. Since several mitochondrial proteins are essential during fermentative as well as oxidative metabolism, this rationale would account for the observed growth defect. Recent evidence suggests that the localization of a number of mitochondrial proteins is altered in yeast expressing the I8 β-subunit (E. Valentin & G. A. Reid, unpublished work). These findings suggest a basis for the identification of components of the mitochondrial targeting and transport machinery by isolation of extragenic suppressors of the above growth defect. A similar approach has been used successfully to investigate protein transport in *E. coli* [48].

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