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Assembly of functional proton-translocating ATPase complex in yeast mitochondria with cytoplasmically synthesized subunit 8, a polypeptide normally encoded within the organelle

(membrane assembly/mitochondrial biogenesis/artificial gene/yeast expression vector)

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ABSTRACT A mitochondrial gene from *Saccharomyces cerevisiae* encoding a hydrophobic membrane protein, subunit 8 of the F_0/F_1-type mitochondrial ATPase complex, has been functionally replaced by an artificial nuclear gene specifying an imported version of this protein. The experiments reported here utilized a multicopy expression vector (pLF1) that replicates in the nucleus of yeast cells and that carries an inserted DNA segment, specifying a precursor protein (N9/Y8) consisting of subunit 8 fused to an N-terminal cleavable transit peptide (the leader sequence from *Neurospora crassa* ATPase subunit 9). The successful incorporation of the imported subunit 8 into functional ATPase complexes after transformation with pLF1 expressing N9/Y8 was indicated by the efficient genetic complementation of respiratory growth defects of *aapl mit*$^-$ mutants, which lack endogenous subunit 8. The reconstitution of ATPase function was confirmed by biochemical assays of ATPase performance in mitochondria and by immunochemical analyses that demonstrated the assembly of the cytoplasmically synthesized subunit 8 into the ATPase complex. Reconstitution of ATPase function required the cytoplasmically synthesized subunit to have a transit peptide. The strategy for importation and reconstitution developed for subunit 8 leads to a systematic approach to the directed manipulation of mitochondrially encoded membrane-associated proteins that has general implications for exploring membrane biogenesis mechanistically and evolutionarily.

Considerable insight into the assembly and function of energy-transducing complexes of microorganisms has been derived from molecular genetic studies (1). The F_0/F_1-type mitochondrial ATPase (mtATPase) of *Saccharomyces cerevisiae* is such a complex, in which the three intensely hydrophobic proteins of the F_0 membrane sector have been subjected to extensive genetic and biochemical analyses (2, 3). The three proteins, subunits 6, 8, and 9, are encoded by yeast mtDNA. A multifaceted approach has been applied, including mutational analysis by gene sequencing and detailed investigation of the mutant phenotypes at physiological and molecular levels. The data obtained have begun to define the roles of individual amino acid residues and domains of these proteins in their interactive assembly into the membrane, in the function of the proton channel of F_0, and in energy-coupling leading to ATP synthesis catalyzed by the soluble F_1 sector of the complex. Thus it has been shown that both transmembrane stems (4) and a hydrophilic charged loop (5) of subunit 9 (76 amino acids), together with two of the five or more transmembrane stems (6) of subunit 6 (259 amino acids), participate in protonophoric functions and energy coupling (3). Studies on protein assembly (7) have shown that subunit 8, a 48-amino acid polypeptide (8) with a single transmembrane stem (9), is required for the assembly of subunit 6 into the mtATPase complex, while the assembly of subunit 8 is dependent upon the correct integration of subunit 9 into the membrane (2).

A powerful extension to these investigations of the mitochondrial F_0 sector would be the expression, under regulated conditions, of normal or specifically mutated versions of its constituent integral membrane proteins. Their specification within the organelle has, until now, prevented efforts in this direction. To overcome the lack of a suitable transformation system for returning manipulated mitochondrial genes to the organelle, we have devised (10) a strategy that introduces proteins into mitochondria that are normally gene products of mtDNA. This has been achieved with a chemically synthesized gene (11) for subunit 8 that was designed for optimal expression in the nucleo-cytosolic system of yeast and fused to a DNA segment encoding a leader sequence that successfully targeted subunit 8 into mitochondria *in vitro* (12).

An essential condition for the study of mitochondrial membrane proteins, such as subunit 8, by the manipulation of genes to be expressed in the yeast nucleus is that the re-routed proteins become functionally integrated into the relevant enzyme complex. As the critical test, we asked whether yeast *mit*$^-$ mutants that were unable to synthesize subunit 8 due to mutations in the mitochondrial *aapl* gene (8) could acquire the ability to grow on respiratory substrates when an artificial nuclear gene specifying an imported subunit 8 was inserted into the cell. In this paper we report that subunit 8 indeed was imported into mitochondria *in vivo* in such a way that it functionally assembled into the mtATPase complex. The restoration of mtATPase activity that occurs under these conditions not only opens the way to future directed manipulation of subunit 8—for example, by *in vitro* mutagenesis—but also has significant implications for the investigation of mitochondrial biogenesis and membrane assembly.

MATERIALS AND METHODS

Yeast Strains, Genetic Analyses, and Growth Media. The following *S. cerevisiae* strains were used (described in detail in ref. 8): the *mit*$^-$ mutants M31 and M26-10 carrying lesions in the *aapl* gene, both derived from strain J69-1B *MATα adel his5 cir*$^+$ [*rho*$^+$]; the petite strain G5 *MAT***a** *adel lys2 trpl* [*rho*$^-$], which contains a 681-base-pair segment of the

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Abbreviations: mtATPase, F_0/F_1-type mitochondrial ATPase; Chx, cycloheximide.

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wild-type mitochondrial genome encompassing the *aap1* gene. Genetic analyses were carried out (13) by using the following media: rich medium for yeast growth [1% yeast extract, 2% (wt/vol) peptone, and either 2% (wt/vol) glucose (YEPD) or 2% (vol/vol) ethanol (YEPE)]; synthetic medium (SD) [0.67% yeast nitrogen base (Difco) and 2% (wt/vol) glucose] and where required SD was supplemented with histidine (50 μg/ml) (SD/His) or histidine (50 μg/ml) plus adenine (100 μg/ml) (SD/His/Ade).

For subsequent preparation of mitochondria, cells were grown to the late logarithmic phase in batch culture in a yeast extract/salts medium (14) containing 1% glucose and supplemented with histidine plus adenine or with histidine alone, respectively, for J69-1B or Ade$^+$ transformants. Cultures were harvested at a cell density of 2.5–3.5 mg (dry weight)/ml, at a stage when cells were using ethanol for respiratory growth, released from glucose repression.

Yeast Expression Vector. The expression vector pLF1 (Fig. 1) was constructed by the sequential addition of three blocks of manipulated yeast DNA sequences to the plasmid YIp5 that consists of pBR322 carrying the yeast *URA3* gene (15). First, a transcription control unit with a *Bgl* II expression site was incorporated into YIp5. The construction of this transcriptional control unit, derived from the yeast *PGK* gene, was described for the expression vector pRJ55 (16). In pLF1 it comprises a 5′ promoter *PGK* segment of some 1330 base pairs (nucleotides −1350 to −17, relative to position +1, defined as the first base of *PGK* translational start ATG) that was joined (initially by a synthetic *Bam*HI site) to a 3′ transcriptional terminator *PGK* segment of 577 base pairs (nucleotides +955 to +1532). The *Bam*HI site of the *Sal* I–*Bam*HI 5′ promoter fragment was converted by site-directed *in vitro* mutagenesis in phage M13 derivatives (17) to a *Bgl* II site. This new *Sal* I–*Bgl* II *PGK* promoter fragment was inserted into a doubly digested (*Sal* I and *Bgl* II) YIp5 plasmid derivative (pRJ60) carrying the *Bam*HI–*Hin*dIII *PGK* transcription terminator fragment from pRJ55 (the unique *Bgl* II site in pRJ60 lies within the *PGK* terminator fragment), thereby generating pWD163.

Second, a 3.3-kilobase *Hin*dIII fragment from pJD219 (18) that spans the partially disabled *LEU2* gene (*leu2-d*) (19) and the 2-μm plasmid replication origin was ligated to pWD163 digested with *Hin*dIII, generating pWD172.

Third, a 1.9-kilobase *Xho* I fragment that carries the yeast *ADE1* gene (20, 21) was prepared as follows. Partial *Bam*HI digestion of plasmid pLF40 carrying a segment of yeast chromosomal DNA cloned into YRp7 (D. B. Kaback, personal communication) released a 1.9-kilobase fragment encompassing the *ADE1* gene and 276 base pairs of flanking pBR322 sequences at its 3′ side. This *Bam*HI fragment was cloned into the *Bgl* II site of a modified pUC19 plasmid (pLBF100) to produce pLBF101. In pLBF100 the original *Bam*HI site in the polylinker of pUC19 had been destroyed by insertion of a synthetic DNA adaptor (formed by the prior annealing of two synthetic oligonucleotides, 5′-GATCGGCTCGAGATCTCGAGC-3′ and 5′-GATCGCTCGAGATCTCGAGCC-3′) generating a *Bgl* II site flanked by *Xho* I sites. The 1.9-kilobase fragment released by *Xho* I digestion of pLBF101 was ligated into pWD172 cut with *Sal* I to yield pLF1 (Fig. 1).

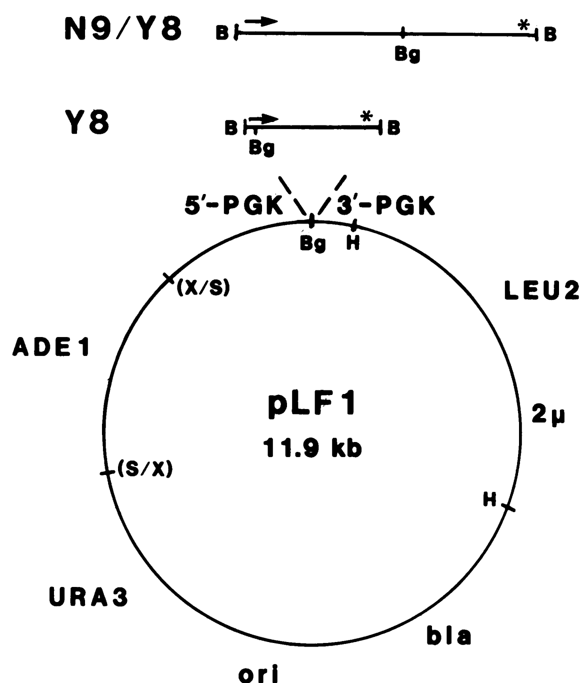

FIG. 1. Expression vector pLF1. Restriction sites relevant to the construction of this vector are shown (B, *Bam*HI; Bg, *Bgl* II; H, *Hin*dIII; S, *Sal* I; X, *Xho* I. S/X and X/S indicate junctions formed by ligation of cohesive ends produced by the relevant nonidentical restriction enzymes.). Relative positions are shown for the following DNA segments: *Escherichia coli* DNA specifying bacterial plasmid replication origin (*ori*) and ampicillin resistance (*bla*); yeast DNA including 2-μm plasmid replication sequences (2μ) and other designated chromosomal genes *ADE1*, *URA3*, and *LEU2* (in the *leu2-d* form) as well as transcriptional control sequences from the *PGK* gene. The direction of transcription of fragments inserted at the *Bgl* II expression site is clockwise from the 5′ promoter segment (5′-*PGK*) to the 3′ transcription terminator segment (3′-*PGK*). Into the expression vector were inserted (by ligation of *Bam*HI and *Bgl* II termini) the synthetic gene constructs (12) encoding Y8 or N9/Y8. The orientation of each insert was confirmed by digestion with *Bgl* II and *Hin*dIII. Additional symbols indicate translational start (→) and stop (*) codons.

Analysis of mtATPase Assembly and Function in Mitochondria. Labeling of cells with [^{35}S]sulfate in the presence or absence of cycloheximide (Chx), preparation of mitochondria, immunoprecipitation of assembled mtATPase complex with anti-β-subunit monoclonal antibody, gel electrophoresis, and fluorographic visualization of labeled polypeptides were as described (22), except that isolated mitochondria were initially lysed in 0.5% sodium cholate and 1% octyl β-D-glucopyranoside in place of 0.25% Triton X-100.

Methods for preparation of mitochondria and assays of respiration, ATPase activity, and ATP–P_i exchange activities were as described (14).

RESULTS

Artificial Gene Encoding the Imported Subunit 8. The chemically synthesized *aap1* gene, denoted *NAP1*, was fused to a DNA sequence encoding the N-terminal cleavable leader sequence (transit peptide) of the nuclearly encoded *Neurospora crassa* mtATPase subunit 9 (12). Expression of this composite gene resulted in a protein denoted N9/Y8 comprising the N-terminal transit peptide of 66 amino acids, the first 5 amino acids of *N. crassa* subunit 9, 2 additional serine residues resulting from the DNA sequence at the fusion point, and the entire 48 amino acids of *S. cerevisiae* subunit 8. The N9/Y8 protein was processed by isolated yeast mitochondria at the natural matrix protease cleavage site of the *N. crassa* subunit 9 precursor to generate an imported subunit 8 protein bearing an additional 7 N-terminal residues (12).

Host Yeast Strain. Strain M31 is a *mit*$^-$ mutant with a nonsense mutation in the *aap1* gene generating a 2-amino acid truncated derivative of subunit 8 (8). The spontaneous reversion rate of M31 to the *rho*$^+$ phenotype is exceedingly low ($<10^{-7}$), presumably because of a further frameshift mutation (base insertion) in the *aap1* coding region downstream of the nonsense mutation (8). Strain M26-10 is

another *aap1* mutant in which an 18-amino acid truncated derivative of subunit 8 is predicted. The nuclear *ade1* mutation in these strains provides a convenient color assay for the colony morphology of cells plated on YEPD medium. The irregularly shaped colonies produced by mit^- cells are pale pink to bronze, whereas the petite cells that are always present in cultures of *S. cerevisiae* (up to 70% in cultures of strain M31 and ≈50% in M26-10) produce smaller smooth white colonies.

Yeast Expression Vector. The expression vector pLF1 (Fig. 1) is an *E. coli–S. cerevisiae* shuttle vector (19) containing a *PGK* expression unit into which was inserted the fused DNA sequence coding for N9/Y8 or the *NAP1* gene encoding subunit 8 alone (Y8). The *ADE1* gene in pLF1 enabled direct selection of the vector in strains M31 and M26-10. This vector was propagated in the yeast nucleus in multiple copies because of its 2-μm plasmid replication sequences (19).

Genetic Properties of Transformants. Cells of strain M31 were transformed with pLF1 carrying the coding sequences for N9/Y8 (denoted pLF1-N9/Y8). Transformants showing Ade^+ phenotype developed after 4–7 days of growth on solid supplemented minimal medium (lacking adenine) with glucose as carbon source (SD/His). The colonies, of various sizes, were replica plated onto YEPE plates. Generally, the larger colonies grew well on YEPE plates ($EtOH^+$), while the smaller colonies failed to grow ($EtOH^-$). On the series of plates depicted in Fig. 2 one such $EtOH^+$ transformant, denoted T2-1, showed the expected auxotrophic requirements (Ade^+,His^-) as well as vigorous growth on YEPE comparable to that of the rho^+ strain J69-1B. The growth rates in liquid YEPE medium at 28°C of T2-1 and nine other independent transformants of M31 made with pLF1-N9/Y8 were not significantly different from that of J69-1B (data not shown). Strain M26-10 transformed with pLF1-N9/Y8 displayed similar $EtOH^+$ growth properties to those observed for T2-1 (Fig. 2). These data provided the primary evidence that the expression of a gene encoding N9/Y8 in the nucleus of yeast cells led to subunit 8 being imported into mitochondria and incorporated into a functional mtATPase complex.

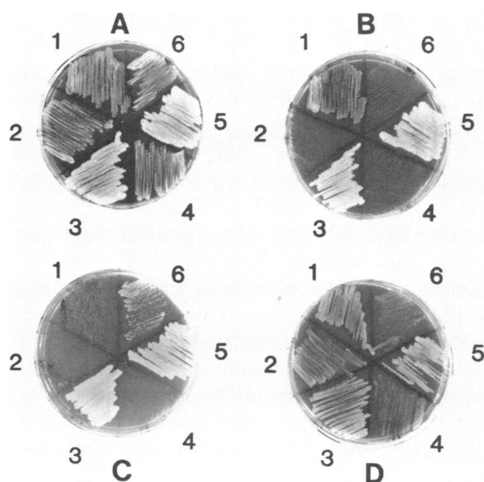

FIG. 2. Growth properties of *aap1* mutants and transformants. Cells were streaked onto agar plates and incubated at 28°C for 5 days. Sectors: 1, strain J69-1B; 2, strain M31; 3, strain T2-1 (M31 transformed with pLF1-N9/Y8); 4, strain M26-10; 5, strain T107-4 (M26-10 transformed with pLF1-N9/Y8); 6, strain T106-1 (M26-10 transformed with pLF1-Y8). Growth media were YEPD (*A*), YEPE (*B*), SD/His (*C*), SD/His/Ade (*D*). Cells of strain J69-1B (sector 1) on rich medium (*A* and *B*) were red and thus lack contrast in these photographs. None of the strains grew on unsupplemented SD medium (data not shown).

Further genetic analysis of transformants such as T2-1 showed that the presence of the pLF1-N9/Y8 plasmid was required for the $EtOH^+$ phenotype and that the original *aap1* mit^- mutation of M31 was retained in an otherwise complete mitochondrial genome. When strain T2-1 was grown under selective conditions (SD/His or YEPE medium) and then plated on a nonselective medium (YEPD), the great majority (>98%) of colonies were large and white (type I) and contained Ade^+,$EtOH^+$ cells. These were the genetically reconstituted transformants. The minority forms observed were medium-sized pink scalloped colonies (type II) and small white colonies (type III). In contrast, when strain T2-1 was propagated under nonselective conditions on YEPD medium, the proportions of type II and type III colonies increased substantially. When the cells present in a series of single type I colonies taken from YEPD agar were spread onto fresh YEPD plates, the three types of colony morphology were represented in the following proportions: type I at 45%; type II at 35%; type III at 20%.

The type II medium-sized pink scalloped colonies contained Ade^-,$EtOH^-$ cells and corresponded in morphological and genetic properties to the original M31 mit^- host strain. First, when mated to petite G5 carrying an intact *aap1* gene (8), type II colonies generated rho^+ diploids (data not shown). Second, they produced high frequencies (>50%) of type III colonies that contain only rho^- and rho^0 petite cells. Thus, loss of the pLF1-N9/Y8 plasmid from transformants such as T2-1 generated mit^- cells resembling those of strain M31 that were themselves unstable and produced petite cells. Whereas most of the type III colonies contained only Ade^- cells, a small proportion (<10%) contained Ade^+ cells and were presumed to have arisen by loss of mtDNA from transformants carrying pLF1-N9/Y8.

Complementation of *aap1* Mutations Requires the Nuclearly Expressed Subunit 8 to be Fused to a Transit Peptide. Transformation of strain M26-10 with the pLF1 vector carrying the *NAP1* gene coding for subunit 8 not fused to a transit peptide (pLF1-Y8) (Fig. 1) generated Ade^+,$EtOH^-$ transformants (Fig. 2). These pLF1-Y8 transformants produced only type II and type III colonies on YEPD plates. Many of these type II colonies showed pink sectoring on a white base, suggesting that the plasmid was unstable and readily lost. This plasmid instability was manifested in the poor growth of such transformants on the SD/His plate (Fig. 2). Significantly these Ade^+,$EtOH^-$ transformants produced rho^+ diploids after being mated to petite G5 (data not shown), thereby demonstrating the retention of the *aap1* mutant mit^- genome of strain M26-10.

It is concluded from these studies on strain M26-10 that the genetic reconstitution of the mtATPase complex by a nuclear encoded version of subunit 8 requires the presence of an N-terminal transit peptide to target the subunit 8 into mitochondria. Parallel genetic analyses of strain M31 transformed with pLF1-Y8 were carried out, but no stable Ade^+ transformants showing mit^- morphology and able to generate rho^+ diploids after mating to petite G5 could be detected.

Assembly of Imported Subunit 8 into the mtATPase Complex. The assembly of mtATPase was examined by an immunochemical approach as follows. Cells of the transformant T2-1 and of the rho^+ strain J69-1B were allowed to incorporate [^{35}S]sulfate to radiolabel proteins. One portion of each culture was treated with Chx during incorporation of [^{35}S]sulfate to suppress labeling of cytosolically synthesized proteins. Lysates of isolated mitochondria were incubated with an immobilized monoclonal antibody directed against the β subunit of the F_1 sector (7). The protein subunits of the immunoadsorbed mtATPase complex were resolved by gel electrophoresis (Fig. 3). Lane 1 shows the 10 subunits of the wild-type mtATPase complex in strain J69-1B, labeled in the absence of Chx (2, 7). A similar pattern was found for T2-1

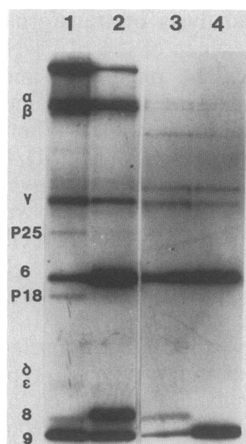

FIG. 3. Assembly of imported subunit 8 into mtATPase complex. Lysates of mitochondria from cells labeled with [^{35}S]sulfate were treated with an immobilized anti-β-subunit monoclonal antibody. Immunoadsorbed labeled polypeptides were separated by electrophoresis on a NaDodSO$_4$/12.5% polyacrylamide gel and visualized by fluorography. Lanes: 1, strain J69-1B with no Chx; 2, strain J69-1B labeled in presence of Chx; 3, strain T2-1 with no Chx; 4, strain T2-1 labeled in presence of Chx. The 10 subunits of the wild-type mtATPase complex visualized in lane 1 are indicated (2, 7).

(lane 2) except for a heavily labeled band of slightly reduced mobility compared to that of the wild-type subunit 8. Since the labeling of this band was completely suppressed by Chx (lane 4), this band was assigned to the imported version of subunit 8, over-produced from the multicopy pLF1-N9/Y8 vector and assembled with other mtATPase subunits. Its reduced mobility relative to natural subunit 8 is explained by the additional 7 amino acid residues at its N terminus resulting from matrix protease cleavage of the N9/Y8 precursor (12). The pattern in lane 4 also reveals that the only two mitochondrially synthesized components of the mtATPase complex in T2-1 are subunits 6 and 9; there is no band corresponding to the mitochondrially synthesized subunit 8 in strain J69-1B (lane 3). This confirms the genetic data presented above, indicating that in T2-1 the *aapl* gene is inactive and does not direct the production of subunit 8 inside mitochondria.

Functional Aspects of the Genetically Reconstituted mtATPase Complex. Measurements were made of respiratory functions and parameters of ATPase and ATP synthase activities in isolated T2-1 and J69-1B mitochondria. The results (Table 1) indicated that the genetically reconstituted transformant T2-1 was generally comparable in its activities to those of the reference *rho*$^+$ strain. The strains showed similar rates of respiration; the respiratory control ratio (the ratio of respiration rates in the presence or absence of ADP, respectively) in each case was ≈2, a typical value for yeast mitochondria. There was, however, a slightly reduced sensitivity of respiratory activity to inhibition by oligomycin in mitochondria of T2-1, relative to that of J69-1B. This reduced sensitivity to oligomycin was accentuated in the ATPase assays; the arithmetic difference was ≈25% inhibition comparing the values measured for J69-1B and T2-1. The inferred minor perturbation in proton channel function or energy-coupling to F_1 was borne out by the reduced ATP–P_i exchange activity of T2-1 mitochondria relative to that of J69-1B mitochondria (the transformant showing activities ≈70% of the wild type). Essentially the same data as in Table 1 have been obtained in three independent experiments of this type. For comparison, mitochondria of *aapl* mutants, with an improperly assembled F_0 sector lacking both subunits 8 and 6, showed no ADP-dependent respiration, no sensitivity to oligomycin for either respiration or ATPase activity, and a negligible ATP–P_i exchange activity (2, 23). The mild perturbation in F_0 function in T2-1 may arise either from the nonstoichiometric amounts of subunits 8 and 6 associated with the mtATPase complex (cf. Fig. 3) or from the extra 7 amino acid residues at the N terminus of the imported subunit 8.

DISCUSSION

Genetic and biochemical evidence has been presented here that a mitochondrial gene encoding a hydrophobic integral membrane protein can be functionally replaced by an artificial nuclear gene. This has considerable significance not only for the subunit 8 of mtATPase but also for wider aspects of mitochondrial membrane assembly.

It should now be possible to perturb in a controlled manner the composition of F_0 in the mtATPase complex by manipulating the quantity and nature of available subunit 8. For example, following modulation of the output of the N9/Y8-coding unit in the nucleus, either by use of a single-copy version of the artificial nuclear gene or by means of a regulated promoter, one could examine the properties of mtATPase complexes to which are delivered regulated quantities of imported subunit 8. This approach can be effectively complemented by systematic *in vitro* mutagenesis of N9/Y8 that allows direct tests of the significance of individual amino acids and domains in subunit 8. A major question is whether subunit 8 has a further role in the function of the proton channel itself, beyond that in the assembly of F_0, particularly with respect to subunit 6. Furthermore, the controlled expression of normal or mutant versions of subunit 8 should allow the development of genetic selection strategies to isolate more *aapl* mutants as well as cells with mitochondrial or nuclear mutations in genes coding for proteins that interact with subunit 8.

The ability to express mitochondrially encoded proteins in such a way that they can be imported into the organelle in functional forms has important implications for our appreciation of mitochondrial biogenesis in evolutionary and mechanistic terms. This is particularly interesting in the case of integral membrane proteins, whose incorporation into the membrane (24, 25) requires the initial targeting of the protein to the appropriate cellular membrane, followed by the correct folding of various transmembrane and exterior domains and their correct orientation in the membrane. It could be argued that the evolutionary persistence of mitochondrial

Table 1. Functional activities of mitochondria

Strain	Respiration, nmol of O_2 per min per mg: − ADP	+ ADP	Respiration inhibition by oligomycin, %	Specific ATPase activity, μmol/min per mg	ATPase inhibition by oligomycin, %	ATP–P_i exchange,* nmol/min per mg
J69-1B	138	282	49	0.42	74	356
T2-1	102	204	40	0.68	49	212

Activities indicated were determined in mitochondria isolated from catabolite-derepressed cells growing logarithmically in rich medium, with ethanol as the respiratory substrate. Oligomycin was used at a concentration of 100 μg/mg of mitochondrial protein.
*Activities were <10% sensitive to KCN (used at 2 mg/mg of protein) and >95% sensitive to both SF6847 (used at 20 μg/mg of protein) and oligomycin.

genes coding for particular proteins integral to the inner mitochondrial membrane (26) may reflect constraints on the ability of such proteins either to be successfully targeted from the cytosolic compartment (27) or to assume the correct orientation (24, 25) during their assembly into or transport across the mitochondrial membrane.

Such constraints evidently do not apply to yeast mt-ATPase subunit 8, whose mitochondrial *aap1* gene can be functionally replaced by an artificial nuclear gene. It is clear that imported subunit 8 is delivered into the mitochondria in such a way as to allow its proper assembly with the other F_0 subunits 9 and 6. We presume that the N-terminal transit peptide of the N9/Y8 precursor causes at least part of subunit 8 to effectively enter the matrix space (by its N terminus) thus allowing the matrix protease to cleave the leader. The imported subunit 8 is then freed to undergo its interactions with other F_0 subunits. We propose that it becomes integrated into the membrane leaving its C terminus facing the matrix, lying in the orientation suggested for subunit 8 normally produced inside mitochondria (28).

The present data do not allow us to distinguish the following two possibilities for the topogenesis of subunit 8 to its final membrane orientation. First, the N9/Y8 precursor may be exposed to the matrix space in its entirety (29), and the cleaved subunit 8 moiety then reinserts functionally into the membrane. Second, during import the hydrophobic subunit 8 moiety of N9/Y8 may remain embedded across the inner mitochondrial membrane, acting as a stop-transfer segment (30), oriented with the N terminus facing the matrix. However, one would have to evoke a subsequent reorientation, perhaps within the membrane, to allow the adoption of the functional configuration of subunit 8 (28).

The strategem described here thus provides a focal point for investigations into the topogenesis and assembly of hydrophobic proteins delivered to a membrane from the "non-natural" side. In this respect the present experiments add a significant extra dimension to other work (31, 32) on the expression of mitochondrial genes outside this organelle, which has been concerned with soluble enzymes active on mitochondrial nucleic acid substrates.

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