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Allotopic Expression of a Gene Encoding FLAG Tagged-subunit 8 of Yeast Mitochondrial ATP Synthase

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Subunit 8 of yeast mitochondrial ATP synthase is a polypeptide of 48 amino acids encoded by the mitochondrial *ATP8* gene. A nuclear version of subunit 8 gene has been designed to encode FLAG tagged-subunit 8 fused with a mitochondrial signal peptide. The gene has been cloned into a yeast expression vector and then expressed in a yeast strain lacking endogenous subunit 8. Results showed that the gene was successfully expressed and the synthesized FLAG tagged-subunit 8 protein was imported into mitochondria. Following import, the FLAG tagged-subunit 8 protein assembled into functional mitochondrial ATP synthase complex. Furthermore, the subunit 8 protein could be detected using anti-FLAG tag monoclonal antibody.

Key words: allotropic expression, ATP synthase, mitochondria, yeast

The adenosine triphosphate synthase (ATP synthase) also known as F_0F_1-ATPase is primarily responsible for production of energy used to drive cellular processes. The enzyme catalyses formation of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate (Pi) at the expense of the transmembrane electrochemical proton gradient generated by respiration chain. ATP synthase can also hydrolyse ATP and form a transmembrane proton gradient which can be used to facilitate ion transport and substrate uptake (Attardi & Schatz 1988).

The ATP synthase is a multisubunit complex. In yeast, the enzyme is composed of at least 17 subunits grouped into two components, F_1 and F_0 components. The F_1 component is comprised of subunits α, β, γ, δ, and ε, with stoichiometry of 3:3:1:1:1 (Cox *et al*. 1992). The F_0 component is composed of subunits b, oligomycin-sensitivity conferring protein (OSCP), d, e, f, g, h, i/j, k which are encoded by nuclear genes, and subunits 6, 8, and 9, which are encoded by mitochondrial genes (Stephens *et al*. 2000). The catalytic sites lay in the F_1 component while proton conductance takes place in the F_0 component. Based on studies carried out in *Escherichia coli*, it has been established that the catalytic mechanism of ATP synthase involves rotation of both subunits γ and ε (Noji *et al*. 1997). The enzyme therefore works as a molecular rotary motor (Senior & Weber 2004). It has recently been proven that the direction of subunit γ rotation during ATP synthesis is opposite to that of ATP hydrolysis (Diez *et al*. 2004).

The subunit 8 of yeast mitochondrial ATP synthase is a polypeptide of 48 amino acids with predicted molecular weight of 5.8 kDa (Macreadie *et al*. 1983). The protein is encoded by the mitochondrial *ATP8* gene. As a mitochondrially encoded protein, subunit 8 is transcribed and translated within the organelle. Subunit 8 of the yeast mitochondrial ATP synthase is considered to be the homologue of the mammalian subunit 8. Interestingly, however, no homologue of subunit 8 is found in *Escherichia coli* (Cox *et al*. 1992). It has been considered that subunit 8 has an essential structural role in the mitochondrial ATP synthase, being required for the assembly of a functional F_0 component (Marzuki *et al*. 1989).

Detail analysis of subunit 8 structure and function is still lacking. Although subunit 8 has been considered to participate in proton translocation, the exact role of subunit 8 in this function remains unclear.

A major problem in applying mutagenesis techniques to study structure and function of mitochondrially encoded protein such as subunit 8 is the lack of a reliable method for delivering mutagenised genes back into the mitochondria. To circumvent this problem, allotropic expression system (diagrammatically illustrated in Figure 1) has been developed for subunit 8 (Gearing & Nagley 1986). Allotopic expression is deliberate relocation of organellar genes to the nucleus and delivery of the gene products from the cytoplasm to the corresponding organelle (Nagley & Devenish 1989). For allotropic expression of subunit 8 gene, Gearing *et al*. (1985) designed a completely novel subunit 8 encoding DNA sequence to be expressed in the nucleocytosolic system. In this nuclear version of subunit 8 gene, as many as 31 codons out of 48 natural mitochondrial codons were altered. One codon (for Thr22) had to be changed to comply with the nuclear codon dictionary, otherwise the mitochondrial CTA codon for threonine would be translated to leucine on the cytosolic ribosomes. The other 30 codon changes were made essentially to optimize the expression of subunit 8 gene. To ensure that the cytoplasmically synthesized subunit 8 was imported into mitochondria, a DNA sequence encoding a mitochondrial signal peptide was fused to the N-terminus of the subunit 8 gene.

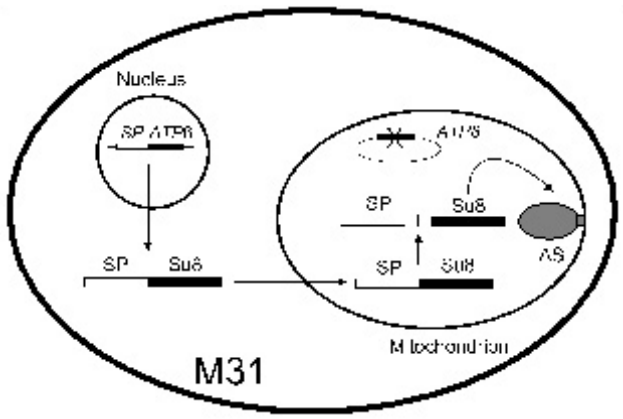

Figure 1. Schematic diagram of allotropic expression of subunit 8 gene. Allotopic expression of subunit 8 gene is carried out in yeast cells strain M31 where the natural *ATP8* gene is inactivated. Subunit 8 protein (black box) is synthesized from a plasmid borne copy of nuclear version of subunit 8 gene. Subunit 8 is expressed as a precursor protein fused with mitochondrial signal peptide (open box). Following synthesis subunit 8 is imported into mitochondria, processed and assembled into mitochondrial ATP synthase complex. Su8 = subunit 8, SP = mitochondrial signal peptide, *ATP8* = subunit 8 gene, *SP-ATP8* = gene construct encoding subunit 8 fused with mitochondrial signal peptide, AS = ATP synthase, M31 = yeast host cells strain M31.

By employing the allotropic expression system, Stephens *et al.* (2000) have successfully demonstrated that the subunit 8 has a single transmembrane domain which extends across the inner mitochondrial membrane. In addition, they have revealed that the C-terminal domain of subunit 8 is close to subunits d and f of the F_0 component. Further detail analysis of subunit 8 structure, topology, and proximity relationships with other subunits of the enzyme complex is required. In the present study, the subunit 8 is FLAG tagged in order to provide means for the use of immunochemistry methods for detecting subunit 8 protein. This will provide tool for probing subunit 8 topology and its interaction with other subunits.

In order to examine its functionality *in vivo*, the gene encoding the FLAG tagged-subunit 8 fused with mitochondrial signal peptide (see Figure 2) was cloned into a yeast expression. The resulted recombinant plasmid was then used to transformed yeast host cells lacking endogenous subunit 8, strain M31. The strain M31 is lack of endogenous subunit 8 due to mutation in the *ATP8* gene (Nagley *et al.* 1988). Since subunit 8 is an essential subunit of the mitochondrial ATP synthase complex, the strain M31 is therefore unable to form functional mitochondrial ATP synthase complex. Consequently, strain M31 cannot use respiratory substrates such as ethanol to support growth. Strain M31, however, can be grown on complete glucose medium.

Following transformation, the resulted transformants were platted on solid selective medium with glucose as a carbon source. Plates were incubated at 28 °C for 3 days. In order to examine whether the allotopically expressed FLAG tagged-subunit 8 rescued the ethanol negative phenotype of the strain M31, transformant colonies were transferred onto solid ethanol medium. Growth of transformants on ethanol medium observed upon four days of incubation (Figure 3) indicated that the

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                                                        10
          Met Pro Gln Leu Val Pro Phe Tyr Phe Met
N-ATP8 5'- ATG CCA CAA TTA GTT CCA TTT TAT TTT ATG
A-ATP8 5'- ATG CCT CAA TTG GTT CCA TTC TAC TTC ATG
                                                        20
          Asn Gln Leu Thr Tyr Gly Phe Leu Leu Met
          AAT CAA TTA ACA TAT GGT TTC TTA TTA ATG
          AAC CAA TTG ACC TAC GGT TTC TTG TTG ATG
                                                        30
          Ile Thr Leu Leu Ile Leu Phe Ser Gln Phe
          ATT CTA TTA TTA ATT TTA TTC TCA CAA TTC
          ATC ACC TTG TTG ATC TTG TTC TCC CAA TTC
                                                        40
          Phe Leu Pro Met le Leu Arg Leu Tyr Val
          TTT TTA CCT ATG ATC TTA AGA TTA TAT GTA
          TTC TTG CCA ATG ATC TTG AGA TTG TAC GTT
                                                48
          Ser Arg Leu Phe Ile Ser Lys Leu */Ser Ser
          TCT AGA TTA TTT ATT TCT AAA TTA TAA -3'
          TCC AGA TTG TTC ATC TCT AAG TTG AGC TCG

          Asp Tyr Lys Asp Asp Asp  *  *

          GAC TAC AAG GAC GAC GAT TAA TGA -3'
```

Figure 2. Comparison of natural mitochondrial *ATP8* gene and FLAG tagged-artificial (nuclear version) *ATP8* gene. The amino acid sequence of subunit 8 of yeast mitochondrial ATP synthase is shown in standard three letter amino acid code. Numbers refer to amino acid residues. The codon for natural mitochondrial *ATP8* gene and the artificial (nuclear version) *ATP8* gene are shown aligned. Different codons used in artificial gene are indicated by underline. Residues of the FLAG tag are indicated in bold. The subunit 8 and the FLAG tag are bridged by two serine residues. The nucleotide sequence of the mitochondrial signal peptide fused to the N-terminus of *A-ATP8* gene is not shown. *N-ATP8*: natural mitochondrial *ATP8* gene, *A-ATP8*: artificial (synthetic) *ATP8* gene, *: stop codon.

FLAG-tagged subunit 8 protein successfully assembled into functional enzyme complexes. The M31 strain expressing FLAG tagged-subunit 8 was then denoted strain FTC2.

The purpose of epitope tagging subunit 8 is to enable detection of subunit 8 protein using a specific antibody with high affinity to the tag. The ability to detect subunit 8 protein is important in analyzing subunit 8 topology and probing subunit 8 interaction with other subunits. The subunit 8 gene employed in the present study has been modified so as to incorporate coding sequences for a hexapeptide FLAG epitope tag (DYKDDD) at the C-terminus (see Figure 2). A commercially available anti-FLAG antibody (M2) is used to detect the tagged-subunit 8 protein. The FLAG tag system has been found to be useful in the studies of abundance, cellular location, posttranslational modifications, protein-protein interactions, and purification of particular tag proteins (Kolodziej & Young 1991). The small size of the FLAG epitope is proposed as having a minimal effect on protein conformation thereby reducing the possibility of disrupting the function of any tagged-protein.

Following mitochondrial isolation from strain FTC2 expressing the FLAG tagged-subunit 8, an aliquot of 100 µg mitochondrial protein was separated on SDS-PAGE. Mitochondrial proteins prepared from strain YM2 was also included as controls. Strain YM2 is strain M31 allotopically expressing non-tagged-subunit 8 gene (Roucou *et al.* 1999).

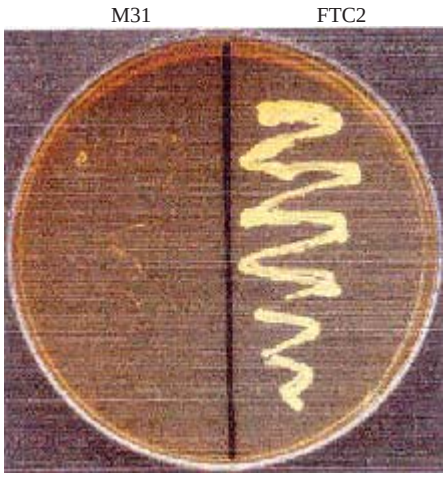

Figure 3. Functional assessment of allotopically expressed FLAG tagged-subunit 8. Host cell (M31) and transformants (FTC2) were grown on solid ethanol medium. The growth of FTC2 on ethanol medium indicated that the FLAG tagged-subunit 8 is successfully imported into mitochondria upon its translation in the nucleocytosolic system, and is assembled into functional mitochondrial ATP synthase complex.

The proteins were then transferred to PVDF membrane. The membrane was cut into two portions. One portion of the membrane containing subunit 8 protein was probed with anti-FLAG M2 antibody as a primary antibody. The second portion of the membrane containing subunit γ protein was probed with anti-subunit γ antibody. Detection of subunit γ was intended as a positive control.

Results (Figure 4) showed that the FLAG tagged-subunit 8 protein could be detected using anti-FLAG M2 monoclonal antibody. Subunit γ was also detectable in each sample. As expected, the non-tagged-subunit 8 protein isolated from the YM2 strain was not detectable.

The main goal of the present study is to examine as to whether the allotopically expressed FLAG tagged-subunit 8 protein is able to assemble into functional mitochondrial ATP synthase complexes as well as to ensure that the expressed FLAG tagged-subunit 8 can be detected using anti-FLAG tag monoclonal antibody. The present study has demonstrated that the allotopically expressed FLAG-tagged subunit 8 protein assembles into functional mitochondrial ATP synthase complexes. This indicates that the addition of FLAG tag at the C-terminus of subunit 8 protein does not abolish its ability to assemble and function in the enzyme complex. In addition, the FLAG tagged-subunit 8 protein can be detected using the anti-FLAG monoclonal antibody. The present study has therefore provided a further system for facilitating elucidation of subunit 8 structure and for probing subunit 8 interaction with other proteins in the mitochondrial membrane.

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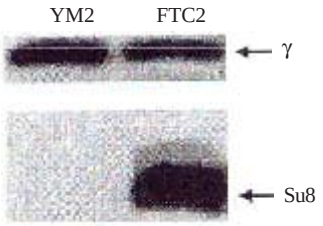

Figure 4. Detection of the FLAG tagged-subunit 8 protein. FLAG tagged-subunit 8 protein contained in mitochondrial lysate was detected in a Western blot analysis using the anti-FLAG M2 monoclonal antibody. Mitochondrial lysates were prepared from strains YM2 (strain M31 expressing subunit 8 without FLAG tag) and FTC2. The presence of subunit γ was also probed using anti-subunit γ antibody. γ = subunit γ of yeast mitochondrial ATP synthase, Su8 = subunit 8 of yeast mitochondrial ATP synthase.

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