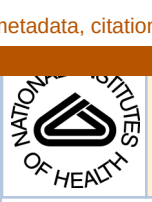

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Turnover of ATP synthase subunits in F_1-depleted HeLa and yeast cells

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Summary

Mitochondrial translation of the *Saccharomyces cerevisiae* Atp6p subunit of F_1-F_0 ATP synthase is regulated by the F_1 ATPase. Here we show normal expression of Atp6p in HeLa cells depleted of the F_1 β subunit. Instead of being translationally down-regulated, HeLa cells lacking F_1 degrade Atp6p, thereby preventing proton leakage across the inner membrane. Mammalian mitochondria also differ in the way they minimize the harmful effect of unassembled F_1 α subunit. While yeast mutants lacking β subunit have stable aggregated F_1 α subunit in the mitochondrial matrix, the human α subunit is completely degraded in cells deficient in F_1 β subunit. These results are discussed in light of the different properties of the proteins and environments in which yeast and human mitochondria exist.

Keywords

ATP synthase; F_1 ATPase; ATP6; HeLa cells; mitochondria; protein turnover

1. Introduction

Recent studies revealed that assembly of the F_O unit of the mitochondrial ATP synthase (F_1-F_O complex) in *Saccharomcyes cerevisiae* is modulated by F_1, which is required for translation of Atp6p and Atp8p [1]. This translational control, although differing in detail from the CES mechanism of chloroplasts [2], not unlike the latter, also ensures a balanced output of nuclear and mitochondrial gene products destined to assemble into hetero-oligomeric enzymes. The recently developed shβ-3 HeLa cell line, depleted in the β subunit of F_1 [3], made it possible to test if translation of the Atp6p and/or Atp8p, the only two endogenously expressed subunits of the mammalian complex, is regulated by F_1 in human mitochondria.

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F_1, when severed from its linkage to F_O, is a hydrophilic water soluble protein [4], while F_O is highly hydrophobic and in the absence of detergents is insoluble in water [5]. Mutations preventing assembly of the yeast F_1 oligomer, cause the α and β subunits to aggregate into inclusion bodies in the matrix [6, 7]. Mutants lacking α subunit have an aggregated form of the β subunit and *vice versa* [6]. We were, therefore, interested in comparing how yeast and mammalian mitochondria handle the potentially harmful effects of soluble and hydrophobic subunits that fail to normally assemble with their partners. We present evidence that, in contrast to yeast, translation of human Atp6p subunit is not regulated by F_1 and that mammalian and fungal mitochondria employ different means to dispose of unassembled F_1 subunits.

2. Materials and methods

2.1 In vivo labeling of HeLa cells

HeLa cells were grown to near confluency in a 6 wells plate [3]. Each well was washed two times with PBS, once with Dulbecco's modified eagle medium (DMEM) w/o methionine and cysteine and overlayed with 1 ml per well of the same medium. After 3 hours the cells were treated for an additional 10 min with 200 μg/ml emetine and were then pulsed for 20 min with ^{35}S-methionine plus ^{35}S-cysteine (S.A. > 1,000 Ci/mmol, MP Biochemicals, Solon, OH). The medium was removed and the cells were chased for different time periods by addition of 3 ml DMEM containing cold methionine and cysteine. The wells were washed with cold PBS and the cells lysed by addition of 200 μl 1X Laemmli sample buffer [8].

2.2 Isolation of mitochondria from HeLa cells

Cells were trypsinized, washed twice with PBS and incubated for 10 minutes at 4°C in 5 volumes of MSTE buffer (225mM mannitol, 75mM sucrose, 0.1mM EGTA, 30mM Tris-HCl, pH 7.4, and 1mM PMSF). Cells were homogenized in a glass-Teflon potter with 30 strokes with the pestle rotating at 690 rpm. The homogenate was centrifuged 500 × g for 5 minutes. The supernatant was centrifuged at the same speed for a second time. The clarified homogenate was then centrifuged at 21,000 × g for 15 minutes to pellet mitochondria. The pellet was suspended in MSTE buffer and the concentration of protein was determined by the Lowry method [9].

2.3 Purification of polysomes and analysis of ATP5a and actin mRNAs in HeLa cells

Mock-transfected and F_1-β-silenced shβ-3 HeLa cells [3] were grown to confluency in 15 cm diameter culture dishes. After addition of cycloheximide (100 μg/ml) to the growth medium cells were incubated for 5 min and washed two times with PBS plus cycloheximide. The cells were trypsinized, suspended in 14 ml of DMEM containing 100 μg/ml cycloheximide, centrifuged for 3 min at 450 × g_{av} and washed twice with the PBS – cycloheximide solution. The cell pellet was suspended in 300 μl of hypotonic buffer containing 10 mM Tris-HCl, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 2 mM DTT, 100 μg/ml cycloheximide, 7.5 μl RNasin, Complete Protease Inhibitor Cocktail w/o EDTA (Roche, Mannheim, Germany) and 0.5% NP40. The solubilized cells were centrifuged for 10 min at 5,000 × g_{av} and the supernatant was loaded on 3 ml of a linear 10–50% sucrose gradient containing the above concentrations of Tri-HCl, pH 7.5, KCl and $MgCl_2$ and 0.5% potassium deoxycholate, pH 8. The gradients were centrifuged in a Beckman SW60Ti rotor for 40 min at 369,000 × g_{av} and collected in 11 eleven equal fractions.

Total RNA was extracted from each gradient fraction by addition of 600 μl of Trizol reagent (Invitrogen, Carlsbad, CA) and 150 μl chloroform. Following centrifugation for 10 min at 11,000 × g_{av}, the supernatants were mixed with 450 μl of isopropanol and centrifuged as

above. The precipitated RNAs were rinsed with 75% ethanol, dried and dissolved in 10 μl water. The RNAs (one tenth of total) were converted to cDNAs with oligo(dT) and reverse transcriptase (Fermentas Inc., Glen Burnie, Maryland). The ATP5a and actin cDNAs were amplified by 20 cycles of PCR in the presence 20% of the cDNAs obtained from each fraction with 0.2 mM of each deoxynucleotide [α–^{32}P] dATP was added to a specific activity of 0.3 μCi/nmole. Primers 5′-aaggagatatagtgaagaggacag and 5′-ataagtcgtcatagatgatcaaagc were used for amplification of ATP5a and 5′-cattaaggagaagctgtgct 5′-tcttgatcttcattgtgctg of actin. The PCR products were separated on a 2.5% agarose gel that was dried and exposed to X-ray film. They were also quantified in a phosphorimager with ImageJ (http://rsbweb.nih.gov/ij/download.html).

2.4 Miscellaneous

Yeast, *atp2*, and double *atp1,atp2* [1] null mutants were transformed by the Li acetate method with p14bATP2, a pRS314-based plasmid containing bovine *ATP2* with the yeast *ATP2* promoter, leader and terminator sequence (kindly provided by Dr. David Mueller, University of Chicago Medical School). Mitochondria of wild type and mutant yeast were isolated [1] and proteins were separated by SDS-PAGE on 12% polyacrylamide gels [8]. The human F_1 α and β subunits were detected by Western blot analysis of HeLa cells or of yeast mitochondria with primary rabbit antibodies against the yeast proteins, and were visualized with Super Signal (Pierce Chemical Co., Rockford, IL).

3. Results

3.1 Mitochondrial translation of F_O subunits in HeLa cells lacking F_1 β subunit

Mammalian mitochondria encode Atp6p and Atp8p of the ATP synthase. Translation and turnover of these F_O subunits and their possible dependence on F_1 was assessed by a pulse-chase experiment with the recently described human shβ-3 HeLa cell line in which production of the F_1 ATPase is severely reduced by RNAi targeted to the ATP5b gene coding for the F_1 β subunit [3]. F_1-depeleted shβ-3 and mock HeLa cells, the latter expressing a scrambled RNAi sequence, were labeled *in vivo* for 20 minutes with a mixture of ^{35}S-methionine and cysteine in the presence of emetine to inhibit cytoplasmic protein synthesis. Turnover of the radiolabeled products was examined over a period of two hours after addition of cold methionine and cysteine (Fig. 1). Although incorporation of radiolabel into Atp6p was approximately 20% lower in the shβ-3 than in mock cells, this was also true of the cytochrome oxidase subunits Cox2p (COII) and Cox3p (COIII) (Figs. 1A, 1B). Atp8p was difficult to quantify because of a consistently high background in the shβ-3 cells. No significant differences were seen in the stability of the different translation products with the notable exception of Atp6p, which was stable in the mock cells but was reduced to 25% of the starting value after one hour and was nearly completely absent after two hours of chase in the shβ-3 cells (Fig. 1B). The almost complete loss of F_1 β subunit in shβ-3 cells [3] was confirmed by Western analysis with an antibody directed against the homologous yeast protein (lower panel in Fig. 1A).

3.2 Status of F_1 α subunit in shβ-3 HeLa cells

Depletion of the F_1 β subunit in the shβ-3 HeLa cells elicits a remarkable decrease of α subunit as evidenced by Western analysis of whole cells and of isolated mitochondria (Fig. 2A, B). Mitochondria of shβ-3 HeLa also displayed some reductions in subunits of the bc1 complex (Core1) and complex I (NDUFA9) but when compared to porin, these were not nearly as substantial as the almost complete depletion of F_1 α subunit (Fig. 2B). The absence of α subunit in the shβ-3 HeLa cells may be the result of transcriptional or translational down-regulation. Alternatively, the failure of the shβ-3 cells to assemble the F_1 oligomer could lead to proteolytic clearing of non-assembled α subunit. To distinguish between these

two possibilities, we measured polysome-associated α subunit mRNA (ATP5a) relative to the constitutively expressed actin mRNA in mock and shβ-3 HeLa cells. Following separation by sedimentation on sucrose gradients (Fig. 3A), total RNA was extracted from the fractions representing different size classes of polysomes. The RNA extracts were reverse-transcribed and the ATP5a and actin cDNAs amplified by PCR under template and number of PCR cycle-limiting conditions. Even though the distribution of the mRNAs across the polysomal region of the two gradients was different, the ratio of the ATP5a associated with polysomes was similar in the shβ-3 and mock cells, when normalized to actin mRNA (Fig. 3B). Since only mRNA in the process of being translated is associated with polysomes, this constitutes strong evidence that depletion of the β subunit does not affect transcription or translation of its partner F_1 α subunit and instead points to turnover as the explanation for the absence of the α subunit in the shβ-3 cells.

3.3 Solubility of unassembled bovine β subunit in yeast

The absence of F_1 α subunit in shβ-3 HeLa cells is in sharp contrast to the situation observed in yeast *atp2* mutants that have wild type levels of α subunit deposited as large aggregates in the matrix compartment of mitochondria [6, 7]. To ascertain if this difference is a property of the protein or of the internal environment of yeast and human mitochondria, we measured β subunit in a yeast *atp2* mutant transformed with a high copy plasmid containing a bovine *ATP2* gene fused to the import signal and 5′ and 3′ flanking sequences of yeast. This hybrid gene has been shown to complement a yeast *atp2* mutant [11]. The construct was introduced into the single *atp2* and the double *atp1* and *atp2* null mutants. As reported previously the bovine gene partially complemented the single yeast *atp2* [11] but did not complement the double *atp1* plus *atp2* mutant (Fig. 4A). All the yeast F_1-β subunit of wild type mitochondria, (associated with the fully assembled F_1-F_O complex), was extracted with lauryl maltoside (Fig. 4B). In MR6ΔATP1 lacking the F_1-α subunit, the F1-β subunit fails to assemble into the F_1 oligomer and forms aggregates that resist solubilization by non-denaturing detergents such as lauryl maltoside [6]. Bovine F_1-β subunit was detected in the mitochondria of yeast *atp2* mutant but at considerably lower levels than in wild type. Additionally, in this strain only 50% of the bovine β and yeast α subunits were recovered in the soluble fraction after extraction of mitochondria with lauryl maltoside (Fig. 4B). This fraction of bovine β subunit associated with F_1 and the F_1-F_O complex, represents substantially less of the complex than is present in the wild type mitochondria, which accounts for the considerably slower growth of the transformant on YPEG (Fig. 4A). The other half of the bovine β subunit in this strain was recovered in the insoluble fraction, presumably as an aggregated protein. The distribution of the endogenous F_1 α subunit in the soluble and insoluble fractions was approximately the same, indicating that unassembled α subunit had also aggregated (Fig. 4C). The double *atp1* and *atp2* mutant MR6ΔATP1,ΔATP2 transformed with *bATP2* had no detectable β subunit, indicating that in yeast, as in human cells, unassembled mammalian F_1 β subunit is degraded by proteolysis (Fig. 4).

4. Discussion

The dependence of Atp6p translation on F_1 in yeast provides a mechanism for a balanced output of nuclear and mitochondrial ATP synthase gene products and at the same time minimizes the accumulation of an Atp6p-Atp9p ring intermediate capable of dissipating the membrane potential of mitochondria [1]. Evidence presented here indicates that shβ-3 HeLa cells expressing 5% or less of the normal amount of F_1 β subunit, translate Atp6p and Atp8p at rates similar to control cells, when normalized to the other mitochondrial gene products. This argues against a role of F_1 in translational regulation of these F_O subunits in human mitochondria and implies either an alternate mechanism for coordinating their synthesis to

that of the nuclear products or, because of the homeostatically controlled environment of mammalian cells, constitutive expression of the endogenous products at rates proportionate to those of their nucleo-cytoplasmic partners may be a sufficient to ensure a stoichiometric output of the nuclear and mitochondrial genes products.

Pulse-chase experiments revealed newly translated Atp6p to be stable over a period of two hours in the control HeLa cells, but to be almost completely degraded in the F_1-depeleted cells. The susceptibility of Atp6p to proteolysis when it is not part of the fully assembled ATP synthase complex may be the principle means by which mammalian mitochondria protect themselves against the accumulation of a proton dissipating Atp6p-Atp9p ring intermediate.

The yeast α and β subunits form stable bulk aggregates when either or both subunits are prevented from assembling into native F_1 [6, 7]. Our evidence suggests that the mammalian subunits also tend to aggregate when are prevented from assembling into F_1. Only half of the bovine F_1 β subunit expressed in a yeast mutant lacking its own β subunit was extracted from mitochondria with a non-denaturing detergent, suggesting that the other half, recovered in the insoluble fraction, had failed to assemble into F_1 and had aggregated. Additionally, the mammalian F_1 subunits are prone to clearing by proteolysis. This is evidenced by the absence of the α subunit in the shβ-3 HeLa cells depleted of the β subunit as well as the undetectable levels of the bovine β subunit in the yeast *atp1* and *atp2* double mutant transformed with the bovine gene. This suggests that the mammalian β subunit contains proteolytically sensitive sites that are absent in the yeast counterpart. Knockdown in HeLa cells of DAPIT (diabetes-associated protein in insulin-sensitive tissue) has recently been shown to cause a decrease in ATP synthase [12]. In these cells there was a parallel decrease of F_1 α and β subunits, even though the levels of their mRNAs were not affected [12]. *S. cerevisiae* mutants lacking F_1 β subunit import α subunit normally and conversely mutants lacking α subunit are able to import the β subunit [6]. Assuming that this is also true of mammalian mitochondria, the observed proteolysis of α subunit in shβ-3 cells is likely to occur in the mitochondrial matrix as a result of its failure to be incorporated into F_1. Turnover of unassembled F_1 subunits is probably catalyzed in mitochondria by AAA-proteases that have been implicated in proteolysis of subunits of the respiratory complexes and of the ATP synthase [13]. It is also noteworthy that even though unassembled bovine β subunit is proteolyzed in both the single *atp2* and double *atp1* plus *atp2* null mutants, proteolysis is more efficient in the latter strain. This indicates that the presence of yeast F_1 α subunit in some way retards proteolysis of the bovine β subunit, thereby allowing some of it to aggregate.

Yeast mutants with aggregated α and/or β subunits accumulate a high percentage of secondary ρ^- and ρ^0 mutants that grow very slowly and appear as microcolonies on solid rich glucose medium (Fig. 4). Interestingly, the *atp1* and *atp2* double mutant transformed with the bovine β subunit gene and lacking aggregated F_1 subunits did not produce micro colonies. The correlation between the slow growth phenotype/mitochondrial DNA instability and the presence of aggregated F_1 subunits suggests that the inclusion bodies may be detrimental to the physical integrity of mitochondria. This could explain why efficient proteolytic removal of any unassembled or incorrectly folded subunits may be essential for the survival of mammalian mitochondria. For reasons that are not clear, this mechanism appears to be absent in yeast.

Acknowledgments

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Highlights

- Subunit Atp6p of HeLa cells ATP synthase is not translationally regulated by F_1.
- HeLa cells lacking F_1 clear mitochondria of newly translated Atp6p by proteolysis.
- Atp6p proteolysis in F_1-less cells safeguards against mitochondrial proton leakage.
- F_1 α subunit is rapidly degraded in HeLa cells depleted of its partner β subunit.

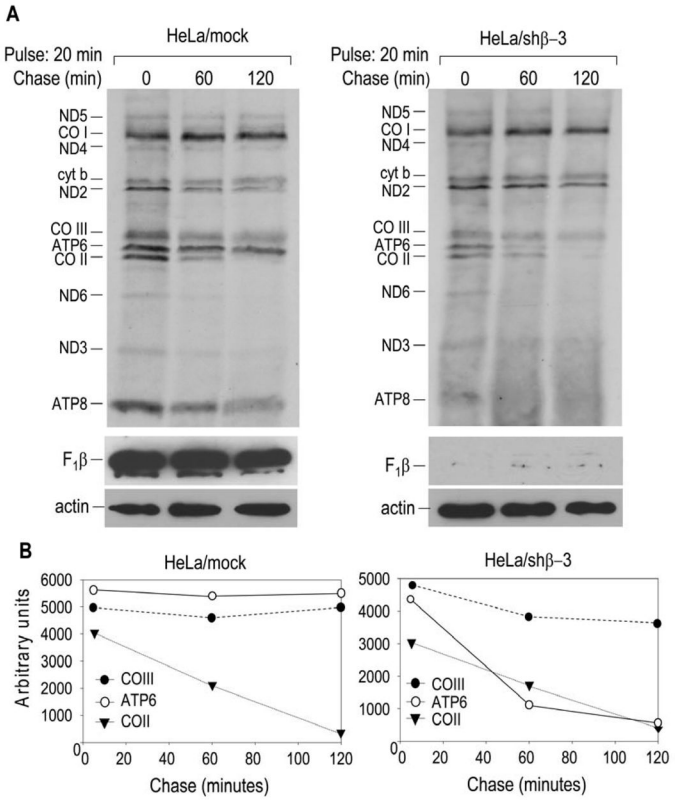

Fig. 1.

Pulse-chase labeling of HeLa cells. A. Mock and shβ-3 HeLa cells were grown and labeled for 20 min with ^{35}S-methionine and ^{35}S-cysteine as described under Materials and methods. The labeling medium was removed and replaced with fresh DMEM + 10% fetal bovine serum. After 5 minutes, cells were incubated for 1 and 2 hours in chase medium consisting of DMEM + 10% fetal bovine serum and cold methionine and cysteine. They were then washed once with cold PBS, solubilized in Laemmli sample buffer [8] and separated on a 12% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane and exposed to X-ray film for 2 days (upper panels). Subunits of NADH-coenzyme Q reductase (ND1, 3, 4–6) cytochrome oxidase (COI, II, and II), cytochrome b (cyt b) and ATP synthase (ATP6 and 8) were identified on the basis of previous reports of how these mitochondrial gene products migrate in a similar SDS-PAGE system [10]. The nitrocellulose membrane was also probed with a yeast antibody against the yeast β subunit (F_1-β) and human actin as a loading control (lower panels). B. The bands corresponding to Atp6p (ATP6), subunit 2 (COII) and 3 (COIII) of cytochrome oxidase were quantified with a phosphorimager.

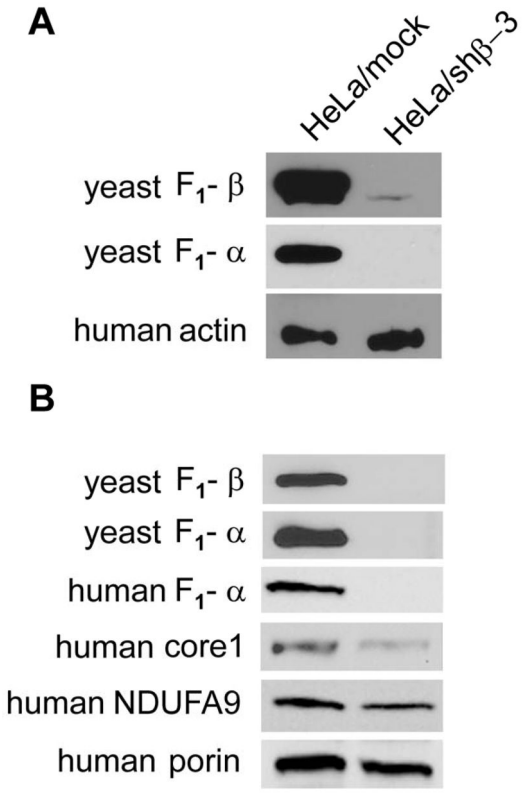

Fig. 2.

Absence of the F_1 α subunit in shβ-3 HeLa cells depleted of β subunit. A. Mock and shβ-3 HeLa cells grown in a 25 cm^2 falcon flask were dissolved in 200 μl of 1X Laemmli sample buffer [8] and 20 μl of each sample was separated by SDS-PAGE on a 12% polyacrylamide gel. Following transfer to nitrocellulose the blot was probed with antibodies against yeast F_1 α and β subunit and against human actin as a loading control. B. Mitochondria prepared from mock and shβ-3 HeLa cells, were loaded (10 μg protein/lane) and separated by SDS-PAGE on a 12% polyacrylamide gel. Proteins were transferred to nitrocellulose and probed with the indicated yeast and human antibodies.

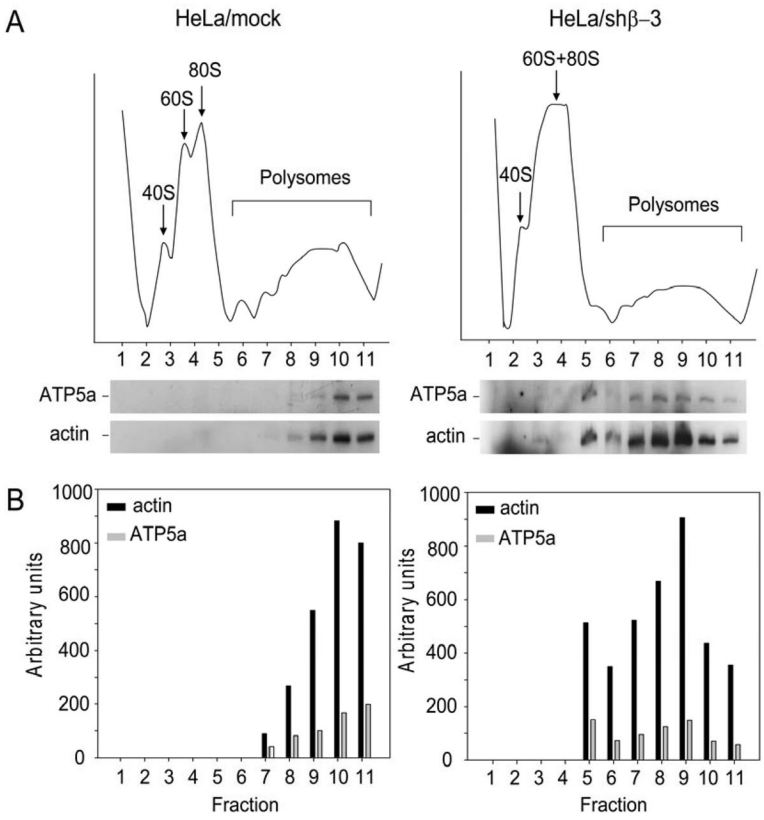

Fig. 3.

Quantification of F_1 α subunit mRNA in cytoplasmic polysomes of mock and shβ-3 HeLa cells. A. Mock and F_1 depleted shβ-3 HeLa cells grown to near confluency were extracted and polysomes separated by sedimentation in sucrose gradients. Each gradient was collected in eleven equal fractions that were analyzed for ATP5a (F_1 α subunit) and actin mRNA. B. The radiolabeled cDNAs shown in the lower panel of part A. were visualized with a phosphorimager and quantified.

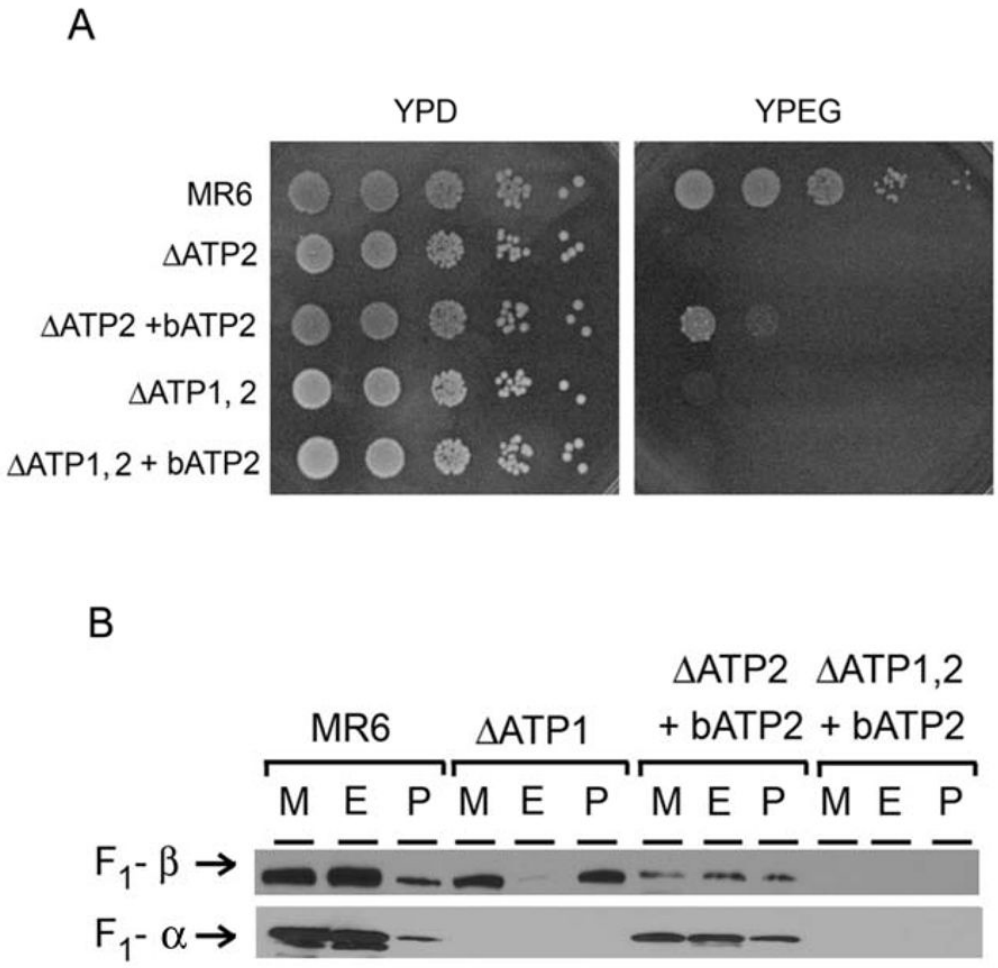

Fig. 4.

Solubility properties of yeast and bovine β subunit in different yeast mutants. A. The wild type MR6, the *atp1* null mutant MR6ΔATP2 (ΔATP2), and the single and double mutants MR6ΔATP2 (ΔATP2 + bATP2) and MR6ΔATP1,ΔATP2 (ΔATP1,2 + bATP2), respectively, each harboring bovine *ATP2* on a multicopy plasmid were grown overnight in liquid YPD. Serial dilutions were spotted on solid YPD and YPEG and incubated at 30°C for 2–3 days. The ρ^{-}/ρ^{0} microcolonies are not seen because they require a longer period of growth to be visible by the naked eye. B. Mitochondria were prepared from the indicated strains grown in rich galactose medium. The percentage of ρ^{-} and ρ^{0} cells was 93% in MR6ΔATP1 (these appeared as microcolonies after 1 week of incubation at 30°C), 93% in MR6ΔATP1, 63% in MR6ΔATP2 + bATP, and less than 1% in MR6ΔATP1,ATP2 + bATP. Mitochondria (M) at a protein concentration of 10 mg/ml and were adjusted to 1% lauryl maltoside and after incubation on ice for 10 min were centrifuged at 90,000 × g_{av} for 10 min. The supernatants (E) were removed and the pellets (P) suspended in the starting volume of buffer. Samples equivalent to 12.5 μg of starting mitochondrial protein were separated by SDS-PAGE and Western blots were probed with polyclonal rabbit antibodies against the yeast β and α subunits.