Expression of Bovine F$_1$-ATPase with Functional Complementation in Yeast Saccharomyces cerevisiae

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The mitochondrial F$_1$F$_0$-ATP synthase is a multimeric enzyme complex composed of at least 16 unique peptides with an overall molecular mass of \( \sim 600 \) kDa. F$_1$-ATPase is composed of \( \alpha_3 \beta_3 \gamma \delta \varepsilon \) with an overall molecular mass of 370 kDa. The genes encoding bovine F$_1$-ATPase have been expressed in a quintuple yeast Saccharomyces cerevisiae deletion mutant \( (\Delta \alpha \Delta \beta_2 \Delta \gamma \Delta \delta \Delta \varepsilon) \). This strain expressing bovine F$_1$ is unable to grow on medium containing a non-fermentable carbon source (YPG), indicating that the enzyme is non-functional. However, daughter strains were easily selected for growth on YPG medium and these were evolved for improved growth on YPG medium. The evolution of the strains was presumably due to mutations, but mutations in the genes encoding the subunits of the bovine F$_1$-ATPase were not required for the ability of the cell to grow on YPG medium. The bovine enzyme expressed in yeast was partially purified to a specific activity of about half of that of the purified enzyme from bovine heart mitochondria. These results indicate that the molecular machinery required for the assembly of the mitochondrial ATP synthase is conserved from bovine and yeast and suggest that yeast may be useful for the expression, mutagenesis, and analysis of the mammalian F$_1$- or F$_1$F$_0$- ATP synthase.

The mitochondrial F$_1$F$_0$-ATP synthase (EC 3.6.3.14) is a multimeric peptide complex composed of the water-soluble F$_1$-ATPase (EC 3.6.1.34), including a central stalk, the membrane bound F$_0$, and a second stalk, which bridges F$_0$ with F$_1$. The ATP synthase is composed of \( \sim 16 \) unique peptides, which range in stoichiometries from 1 to 10, and has an overall molecular mass of \( \sim 600 \) kDa. F$_1$-ATPase is composed of \( \alpha_3 \beta_3 \gamma \delta \varepsilon \) with an overall molecular mass of 370 kDa (1). F$_1$-ATPase is water-soluble and contains the active site of the ATP synthase, although by itself, it is only able to catalyze the hydrolysis of ATP. The active site is composed of the \( \alpha- \) and \( \beta- \) subunits, and the central stalk is composed of the \( \gamma-, \delta-, \) and \( \varepsilon- \) subunits. As such, there are three active sites in a single molecule (2). F$_0$ is a membrane-bound peptide complex that acts as a proton pore, which in the mitochondrion transports protons from the cytosol to the matrix space.

ATP is synthesized in the mitochondrial by the coupling of proton translocation from the cytosol through the F$_0$ in the mitochondrial with the phosphorylation of ADP at the F$_1$ portion of the enzyme (3). F$_1$F$_0$-ATP synthase is a molecular motor in which F$_0$ acts as a proton turbine that rotates the \( \gamma- \) subunit within the core of F$_1$ (4–7). The position of the \( \gamma- \) subunit determines conformation of the active site, thereby alternating the affinity of the active site for nucleotides from high to low (2, 8, 9). This change in the nucleotide affinity forms the basis for the binding change mechanism for ATP synthesis by the mitochondrial ATP synthase (10, 11).

The assembly of the mitochondrial ATP synthase is a very intricate process (12–16). The enzyme complex requires the expression and synthesis of nuclear-encoded precursor peptides followed by their import and processing in the mitochondrion. This import requires specific carrier proteins in the outer and inner mitochondrial membrane as well as general chaperones present in the cytosol and the matrix of the mitochondrion. The processing of the precursor peptides requires a specific protease, which cleaves the mitochondrial presequence once the precursor peptide enters the matrix space. The ATP synthase also requires the synthesis of peptides in F$_0$, which are encoded in the mitochondrial genome (three in yeast and two in mammals). After expression, the F$_0$ peptides must associate with nuclear-encoded subunits resulting in the holocomplex. The assembly of F$_1$ and F$_0$ portions of the enzymes require specific chaperones like proteins Atp10p, Atp11p, and Atp12p, which are described as “assembly factors” (17–20), as well as Oxa1p (21) and Yta10-12p (22–24). Human homologs for Atp11p and Atp12p have been identified and these genes are broadly expressed across eukaryotes (25). Thus, the synthesis, import, and assembly of the ATP synthase require a large number and a variety of proteins encoded in the nuclear and mitochondrial genome.

This study uses yeast Saccharomyces cerevisiae to study the assembly and structure/function of the bovine F$_1$-ATPase. The impetus for this study is to provide a basis for the biochemical and structural analysis of mutant variants of the mammalian enzyme. However, this study also provides insight into the evolutionary conservation of the proteins required for the assembly of the ATP synthase and the conservation of the ATP synthase from bovine to yeast.

**EXPERIMENTAL PROCEDURES**

**Media**—The yeast media and standard recipes are as described previously (26): YPD, 2% glucose, 1% yeast extract, and 2% peptone; YPGal, 2% galactose, 1% yeast extract, and 2% peptone; YPG, 3% glycerol, 1% yeast extract, and 2% peptone; YPAD, 2% glucose, 1% yeast extract, 2% peptone, and 20 mg liter$^{-1}$ adenine sulfate. Minimal me-

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### Yeast Strains and Genetics—

Yeast strains Relevant/added genotype Parent

| Yeast strains | Relevant/added genotype | Parent |
|---------------|-------------------------|--------|
| Δa | ade2–1, his3–1, 15, leu2–3, 112, trpl–1, ura3–1 | W303 |
| Δβ | ATP1Δ::loxP, KanMX ΔloxP | W303–1a |
| Δβgs | ATP2Δ::loxP, KanMX ΔloxP | Δβ |
| Δβ[pYATP2] | ATP3Δ::loxP, KanMX ΔloxP | Δβgs |
| Δαβ | ATP3Δ::loxP, KanMX ΔloxP | Δαβgs |
| Δαβγ | ATP3Δ::loxP, KanMX ΔloxP | Δαβγgs |
| Δαβγy | ATP3Δ::loxP, KanMX ΔloxP | Δαβγygs |
| ΔαβγyΔ | ATP3Δ::loxP, KanMX ΔloxP | ΔαβγyΔgs |
| ΔαβγyΔΔ | ATP3Δ::loxP, KanMX ΔloxP | ΔαβγyΔΔgs |
| ΔαβγyΔΔΔp | ATP3Δ::loxP, KanMX ΔloxP | ΔαβγyΔΔΔp |
| ΔαβγyΔΔΔpΔ | ATP3Δ::loxP, KanMX ΔloxP | ΔαβγyΔΔΔpΔgs |
| ΔαβγyΔΔΔpΔΔ | ATP3Δ::loxP, KanMX ΔloxP | ΔαβγyΔΔΔpΔΔgs |
| ΔαβγyΔΔΔpΔΔΔ | ATP3Δ::loxP, KanMX ΔloxP | ΔαβγyΔΔΔpΔΔΔgs |
| ΔαβγyΔΔΔpΔΔΔΔ | ATP3Δ::loxP, KanMX ΔloxP | ΔαβγyΔΔΔpΔΔΔΔgs |
| ΔαβγyΔΔΔpΔΔΔΔΔ | ATP3Δ::loxP, KanMX ΔloxP | ΔαβγyΔΔΔpΔΔΔΔΔgs |
| ΔαβγyΔΔΔpΔΔΔΔΔΔ | ATP3Δ::loxP, KanMX ΔloxP | ΔαβγyΔΔΔpΔΔΔΔΔΔgs |
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| ΔαβγyΔΔΔpΔΔΔΔΔΔΔΔΔΔΔΔΔ | ATP3Δ::loxP, KanMX ΔloxP | ΔαβγyΔΔΔpΔΔΔΔΔΔΔΔΔΔΔΔΔgs |
| ΔαβγyΔΔΔpΔΔΔΔΔΔΔΔΔΔΔΔΔΔ | ATP3Δ::loxP, KanMX ΔloxP | ΔαβγyΔΔΔpΔΔΔΔΔΔΔΔΔΔΔΔΔΔgs |
| ΔαβγyΔΔΔpΔΔΔΔΔΔΔΔΔΔΔΔΔΔΔ | ATP3Δ::loxP, KanMX ΔloxP | ΔαβγyΔΔΔpΔΔΔΔΔΔΔΔΔΔΔΔΔΔΔgs |
| ΔαβγyΔΔΔpΔΔΔΔΔΔΔΔΔΔΔΔΔΔΔΔ | ATP3Δ::loxP, KanMX ΔloxP | ΔαβγyΔΔΔpΔΔΔΔΔΔΔΔΔΔΔΔΔΔΔΔgs |
| ΔαβγyΔΔΔpΔΔΔΔΔΔΔΔΔΔΔΔΔΔΔΔΔ | ATP3Δ::loxP, KanMX ΔloxP | ΔαβγyΔΔΔpΔΔΔΔΔΔΔΔΔΔΔΔΔΔΔΔΔgs |

*See the "Results" for definition of "generation."

### Functional Expression of the Bovine F1-ATPase in Yeast—

The expression constructs were subcloned into four different plasmids to allow transformation of the genes into yeast. The yeast/bovine chimeric genes for the bovine α-, β-, γ-, δ-, and ε-subunits were cloned into pRS316, pRS313, pRS315, pRS314, respectively (32). The chimeric genes for the β- and ε-genes were grouped together on plasmid pRS314 because of the limitation of the number of auxotrophic markers in the yeast strain and the number of different plasmids available. The chimeric gene encoding the bovine ε-subunit was cloned into pRS314 for expression in ΔαβγyΔΔΔp.

### Evolution of the Strains—

The strains expressing bovine F1 and bovine F2, with the yeast δ-subunit, were evolved to grow on medium containing glycerol as the sole carbon source. Yeast (ΔαβγyΔΔΔp[Δαβγy]) containing the yeast δ-subunit but otherwise bovine F1 (and the entire bovine F1δ, ΔαβγyΔΔΔpΔp [Δαβγy]) were spread on YPG plates (estimated 5 × 10^7 cells) and incubated at 30 °C. The ΔpΔp mutation was added to the quintuple deletion strain in an attempt to reduce proteolysis during the purification of the bovine F1-ATPase. The PEP4 gene encodes the major protease present in the vacuole (33). The largest of the colonies (named first generation) were selected and grown in liquid YPG medium (50 ml) to stationary stage of growth. The cells were spread on YPG plates and incubated at 30 °C, and again the largest colony was selected (second generation). This scheme was repeated a third time to obtain the third generation strain.

The test tube evolution presumably selected for mutations that allowed for better assembly or function of the ATP synthase. To determine whether the mutations were in the genes encoding the bovine subunits, the plasmids were cured from the third generation yeast strain (Δf1-G3). This was done by first curing the plasmid containing the gene encoding the bovine α-subunit. The yeast were grown in YPD medium and then plated onto SD medium containing uracil and 5FOA. A single colony was selected that grew on 5FOA and was cultured again in YPD medium (3 ml). Cells were spread on plates containing YPD medium and replica-plated on SD drop-out medium lacking one of each
of the auxotrophic markers: adenine; leucine; histidine; tryptophan; or uracil. A colony was selected that was an auxotroph for each of the markers (bF1-G3). This strain was transformed with each of the plasmids containing the genes encoding the bovine subunits and was tested for growth on YPG medium. This strain (bF1-G3cp) was able to grow on YPG medium at a rate similar to that of the parent strain (bF1-G3). Isolation of Plasmids from Yeast and DNA Sequence Analysis—Yeast strain bF1-G3 was grown in YPG medium (1.5 liters) for 24 h, the yeast was harvested by centrifugation at 4,000 rpm for 10 min, and the cells were twice washed in cold distilled water. The cells (15 g) were incubated at 30 °C for 1.5 h. The cells were harvested by centrifugation and suspended in 1.2M sorbitol, 20 mM Tris-Cl, pH 7.3, and 50 mg of lyticase (Sigma) and incubated at 30 °C for 1.5 h. The cells were harvested by centrifugation and resuspended in 0.6 m sorbitol, 20 mM Tris-Cl (50 ml). A portion of this suspension (1.5 ml) was taken, and plasmid DNA was isolated using the Wizard Plus SV DNA purification system (Promega, Madison, WI). The DNA was dissolved in water (0.04 ml) and used to transform E. coli to B.etus. E. coli was transformed by electroporation using the manufacturer’s protocol (Bio-Rad). Bacteria were grown in LB medium with ampicillin (0.1 mg/ml) and the plasmid DNA was isolated with the Wizard Plus SV DNA purification system. The plasmid DNAs were tentatively identified by restriction map analysis, and then the entire coding regions encoding the bovine F1 subunits were determined by DNA sequence analysis. DNA sequence analysis was performed at the Cancer Research Center DNA Sequencing Facility at the University of Chicago.

Biochemical Studies—Mitochondria were isolated from yeast after growth in SC minus tryptophan or YPG after digestion of the cell wall with lyticase as described (34). The yeast cell wall was removed in 0.1 M Tris-Cl, pH 9.3, with 10 mM dithiothreitol at room temperature for 30 min. The cells were washed with cold water and suspended in spheroplasting buffer (1.2 M sorbitol, 20 mM KPi, pH 7.4) to a concentration of 0.1 g/ml. Lyticate (ICN Pharmaceuticals, Costa Mesa, CA) was added (2.5 mg/g yeast), and the cells were incubated for 30–45 min at 30 °C. The cells collected by centrifugation and gently washed with mitochondrial isolation buffer (0.6 M mannitol, 20 mM HEPES, pH 7.4, 0.5 mM phenylmethylsulfonyl fluoride) and then suspended in 5 volumes of mitochondrial isolation buffer. The cells were homogenized to break the mitochondria, and the debris was removed by centrifugation at 3,000 × g for 5 min. The mitochondria were collected by centrifugation at 18,000 rpm for 10 min in the Sorvall SS34 rotor. The mitochondrial pellet was washed twice with mitochondrial isolation buffer, frozen, and stored at −80 °C.

Partial Purification of the Bovine Mitochondrial ATPase—The mitochondria were suspended to a protein concentration of 10 mg/ml in mitochondrial isolation buffer, and chlorofor (one-half of volume) was added. The solution was rapidly mixed for 20 s, ADP was added to 2 mM, and the solution was centrifuged at 5,000 × g for 5 min at room temperature. The aqueous phase was taken and centrifuged at 27,000 rpm in an SW 28 rotor. The supernatant was loaded on a HighTrayQ (5 ml, Amersham Biosciences) at a flow rate of 3 ml/min. The column was equilibrated with TGE (20 mM Tris-Cl, 10% glycerol, 2 mM ATP, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, pH 7.8). The column was washed with TGE, and then a linear salt gradient (0–0.5 M KC1 in TGE, 35 ml) was used to elute the enzyme. The enzyme eluted at ~0.35 M KC1 in TGE. The fractions containing the enzyme were pooled and precipitated with the addition of a saturated solution of ammonium sulfate to a final concentration of 70% saturated ammonium sulfate and was stored at 4 °C.

The precipitated enzyme was collected by centrifugation and dissolved in SDX (0.25 ml, 0.25 M sucrose, 0.2 M NaCl, 0.05 M Tris-Cl, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, pH 8.0) and purified on a Superose 6 HR 10/30 column (Amersham Biosciences). The column was eluted in SDX at a flow rate of 0.5 ml/min. The sample eluted at the same position (15.6 ml) as yeast F1-ATPase. The fractions were pooled, concentrated, and precipitated with ammonium sulfate by the addition of solid ammonium sulfate to 70% saturation.

SDS gel electrophoresis was done by the method of Laemmli (35) using either a straight 12.5% acrylamide or linear gradient from 9 to 22% acrylamide. The gel was stained with Coomassie Blue or transferred to nitrocellulose for Western blot analysis (36). The Western blots were developed using the Vectastain kit (Vector Laboratories, Burlingame, CA) and detected by chemiluminescence using either the ECL reagent (Amersham Biosciences) or Supersignal (Pierce Biotechnology, Rockford, IL). Protein concentration was determined by modified methods of Bradford for water-soluble protein and by the method of Lowry for the membrane preparations with bovine serum albumin as the standard (37). ATPase activity was determined by a coupled enzyme reaction using 2 mM ATP and 3 mM MgCl2 at 30 °C (38). Efrapeptin and oligomycin were obtained from Sigma and used at a concentration of 5 μg/ml.

RESULTS

Construction of the Quintuple Deletion Mutant—To express bovine F1 in yeast S. cerevisiae, it was essential to delete the genes encoding the subunits of the yeast F1 ATPase. The genes encoding the α-β-γ-δ-ε subunits (ATP1, ATP2, ATP3, ATP16, and ATP15, respectively) were deleted sequentially in the order: ATP2; ATP1; ATP3; ATP15; and ATP16. The order of the deletions is important because deletions in ATP1 and ATP2 in the absence of a second mutation in either ATP1 or ATP2 result in the loss of mitochondrial DNA (31). This loss of mitochondrial DNA would preclude the ability for expression of a functional ATPase in the cell. The deletion mutations were made via homologous recombination using PCR products that targeted the site of disruption in the genome and contained a G418 resistance gene, KanMX, module (30, 31). The module was flanked by loxP sites, which are sites for recombination by the cre recombinase. Thus, the expression of the cre recombinase after deletion of the target gene with the KanMX module leaves only a single copy of the loxP site at the locus, which once contained the gene that was disrupted. Fig. 1 shows whole cell PCR verification of the disruptions in two strains: a quadruple deletion, ΔαΔβΔγΔεεεε, and the quintuple deletion, ΔαΔβΔγΔδΔεε. The PCR products at the deletion site reflect the complete removal of the gene with only a single loxP site remaining. Thus, the PCR product is smaller for the mutant site compared with the wild-type strain and the difference in size is almost equal to the size of the gene that was deleted. Note that ΔαΔβΔγΔεε is the parent of ΔαΔβΔγΔδΔεε.

Complementation of Bovine F1 with Yeast Deletion Mutants—In a previous study (30), it was demonstrated that the expression of the bovine α, β, γ, and ε subunits in the corresponding yeast deletion mutants could complement the mutation as evidenced by growth on a non-fermentable carbon source (YPG). Two questions were asked in the current study. First, can expression of the entire bovine F1-ATPase in yeast complement the quintuple deletion mutant, and second, can the yeast δ-subunit function in an F1-ATPase in which all of the F1 subunits with the exception of

| Table II | Plasmids used in this study |
| --- | --- |
| Plasmids | Expressed gene or coding region | Yeast promoter/leader peptide/terminator | Cloning vector |
| pRS313, pRS314, pRS315, pRS316 | None | AP1 | Ref. 32 |
| pYATP1 | Yeast gene coding for the α-subunit (ATP1) | AP1 | pRS316 |
| pYATP2 | Yeast gene coding for the β-subunit (ATP2) | AP2 | pRS316 |
| pbsa | Bovine α-subunit | AP1 | pRS316 |
| pbsb | Bovine β-subunit | AP2 | pRS313 |
| pbsc | Bovine γ-subunit | ATP2 | pRS315 |
| pbsd | Bovine δ-subunit | ATP2 | pRS314 |
| pbse | Bovine ε-subunit | ATP2 | pRS314 |
| pbsf | Bovine δ- and ε-subunits | ATP2 | pRS314 |

In a previous study (30), it was demonstrated that the expression of the bovine α-β-γ-δ-ε subunits in the corresponding yeast deletion mutants could complement the mutation as evidenced by growth on a non-fermentable carbon source (YPG). Two questions were asked in the current study. First, can expression of the entire bovine F1-ATPase in yeast complement the quintuple deletion mutant, and second, can the yeast δ-subunit function in an F1-ATPase in which all of the F1 subunits with the exception of...
the δ-subunit were from bovine? The second question was asked, because the previous study (30) indicates that the bovine δ-subunit was only bovine F₁ subunit that was unable to complement the corresponding yeast deletion mutation. Thus, it was of interest to determine whether the yeast δ-subunit would function in an otherwise bovine F₁. The first question was addressed by the expression of the bovine F₁-ATPase in a strain with a deletion mutation in the genes encoding the five peptides of the F₁-ATPase Δλδβγλδε. The strain used, ΔλδβγλδεΔpep4, also included the Δpep4 mutation. The Pep4 gene encodes a vacuolar protease, which is the major protease in yeast (33). The Δpep4 mutation was added to reduce the amount of proteolysis during purification of the bovine F₁, after expression in yeast but was inconsequential in relationship to forming a functional ATP synthase.

The second question was addressed by the expression of the bovine α-, β-, γ-, and ε-subunits in the strain with a deletion mutation in the corresponding genes, Δλδβγδε. The individual genes were cloned onto separate plasmids with different selectable markers. The chimeric genes for the δ- and ε-subunits were grouped on the same plasmid to limit the number of plasmids to four. As such, the four auxotrophic markers for leucine, histidine, tryptophan, and uracil were all complemented, leaving adenine as the sole auxotrophic marker in the strain. Fig. 2 shows that neither strain containing bovine F₁ in the deletion mutants with (Δλδβγδεgs [bo/βε]) or without (ΔλδβγδελδεΔpep4 [bF₁]) yeast δ-subunit was able to grow on YPG medium. This indicates that the chimeric ATP synthase containing bovine F₁ and yeast stalk and F₀ is not functional.

However, it was observed that, given sufficient time on YPG plates, colonies appeared from the cells expressing bovine F₁ that were able to grow on YPG medium. This happened at surprisingly high frequency estimated at ~10⁻⁶. A single colony was selected from both of the strains expressing bovine F₁ (with and without yeast δ) that were able to grow on YPG medium, and these strains are designated as y₀bF₁-G1 from Δλδβγδεgs [bo/βε] and bF₁-G1 from ΔλδβγδελδεΔpep4 [bF₁]. These strains are assumed to have obtained one or more mutations that allow better growth on YPG medium. These strains are designated as the “first generation.” The cells from the first generation were taken and grown in YPG medium (50 ml) to stationary stage. These cells were plated onto YPG plates, and the colonies that grew best were selected. It is thought that one or more mutations were again obtained in the strains, improving the function of the ATP synthase. As such, the strains were evolving to make a more functional chimeric ATP synthase containing yeast F₀ and stalk proteins and bovine F₁. The strains from this round are indicated as the “second generation” and are designated as y₀bF₁-G2 and bF₁-G2. This test-tube evolution was repeated again to obtain the “third generation” strains y₀bF₁-G3 and bF₁-G3. Fig. 2 shows that the progressive evolution of the strains produced strains that had progressively better ability to grow on YPG medium. Surprisingly, the strain containing the yeast δ-subunit, but otherwise bovine F₁ subunits, was as easily evolved as the strain containing the entire bovine F₁-ATPase.

The mutations that provided the cells the ability to grow on YPG could be in a number of different genes. It was tested whether the mutations occurred in any of the genes encoding

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**FIG. 1.** Confirmation of the deletion mutations. A, PCR reactions from the deletion mutant strains Δλδβγδεgs and Δλδβγδελδε. The primers for the PCR reaction flanked the regions encoding the α-subunit (ATP₁), β-subunit (ATP₂), γ-subunit (ATP₃), δ-subunit (ATP₁₆), and ε-subunit (ATP₁₅) as indicated. B, the predicted sizes of the wild type and the deletion mutations are shown. C, the general scheme of the PCR reaction and the relative position of the primers are shown. The loxp site is all that remains of the coding region of the deleted genes.
the bovine subunits for strain bF₁-G3 (Table I). Thus, the plasmids expressing the bovine subunits were cured from bF₁-G3 giving strain bF₁-G3c, and then the bovine expression plasmids were reintroduced into bF₁-G3c giving strain bF₁-G3cp. This strain grew apparently as well as the parent strain on YPG medium, indicating that the mutations in the genes encoding the bovine subunits were not required for growth on a non-fermentable carbon source (Fig. 2B). It is possible that mutations occurred in the genes encoding the bovine F₁ subunits, and this was not apparent by the growth studies of the cured strains. To determine whether the evolution resulted in any mutations in the coding region of the bovine subunits, the entire coding regions were sequenced for the five bovine genes. DNA sequence determination indicated that there were no mutations altering the coding region. However, it is possible that there are mutations in the regions outside the coding region that altered the expression of the bovine subunits. Regardless, mutations occurring outside the genes encoding the bovine F₁ subunits must be responsible for the ability of the cells to make a functional chimeric enzyme.

Biochemistry of the Bovine F₁-ATPase—Mitochondria were isolated from the bovine F₁ expression strains ybF₁-G3 and bF₁-G3, which contain the expression plasmids of the bovine peptides and evolved for growth on YPG medium. The cells were grown on SC medium minus tryptophan, and the mitochondria were isolated. Table III shows the resulting activity measurements of the isolated mitochondria and of the soluble enzyme after release with chloroform. Chloroform is able to release F₁ from the membrane, but only if it is properly assembled (39). The analysis indicates that the level of ATPase is considerably lower than the activity of the wild type strains. Furthermore, the ATPase is not as sensitive to oligomycin as the wild type yeast enzyme. This finding suggests that the ATPase activity is due to a mitochondrial F₁-ATPase.

Western blot analysis was performed to further demonstrate the expression of bovine F₁. In the analysis, antibodies directed against the bovine α- and β-subunits were used to detect the expressions of the corresponding subunits in yeast. Fig. 3 shows that the α- and β-subunits of the bovine F₁-ATPase are clearly present in the mitochondrial preparation and the chloroform-released enzyme. Furthermore, the yeast subunits are not as readily detected with the bovine antibodies, providing additional support for expression of the bovine enzyme in yeast. However, the antibodies directed against the yeast F₁ subunits were reactive toward the bovine peptide.

**Table III**

| Sample     | Mitochondria + Oligomycin | Chloroform Extract + Efrapeptin |
|------------|---------------------------|---------------------------------|
| W303-IA    | 1.1                       | 13.0                            |
| Δβ         | 0.02                      | 0.03                            |
| Δ[ΔpYATP2] | 0.31                      | 10.0                            |
| Δ[ΔpYATP1] | 0.40                      | 9.4                             |
| yF₁G₁-G₃  | 0.33                      | 4.1                             |
| bF₁-G₃     | 0.15                      | 2.4                             |

The bovine F₁-ATPase was purified after expression in yeast (with growth in YPG medium) as a final proof that the bovine F₁ enzyme was expressed and active. Table IV shows purification data on the bovine F₁-ATPase after expression in bF₁-G3. The final yield of enzyme is low, but this is due to a large loss in activity after release of the enzyme with chloroform. Evidence based on SDS-polyacrylamide gel electrophoresis suggests that proteolysis is a major problem and probably is responsible for this loss in activity. This proteolysis occurred despite the presence of the Δpep4 mutation in the strain, which was added to reduce the level of the major protease (see “Experimental Procedures”). The specific activity of the purified enzyme from the Superox 6 column (47 units·mg⁻¹) is ~50% that of the activity of the bovine enzyme purified from bovine heart mitochondria (40).

Fig. 4 shows an SDS-polyacrylamide gel of peptides contained with the bovine F₁-ATPase after purification by Superox 6 size-exclusion chromatography. The Coomassie Blue-staining pattern indicates that the enzyme is <50% pure, which accounts for the low specific activity. The band that appears to correspond to the α-subunit (marked) is partially degraded. This is confirmed by the Western blot analysis using the antibodies directed against the bovine α-subunit. In contrast, the β-subunit appears to be more resistant to proteolysis. Thus, the 50% decrease in the specific activity of the purified enzyme is due to the level of purification rather than to a decrease in the catalytic activity of the bovine enzyme purified from yeast.

**DISCUSSION**

The complementation of null mutations in genes encoding peptides of the ATP synthase with the expression of the bovine counterparts has been shown before. For instance, in the previous study (30) from this laboratory, the expression of bovine α-, β-, γ-, and ε-subunit complemented the corresponding mutations in yeast. Interestingly, the bovine δ-subunit was the only bovine F₁ subunit that was unable to complement the corresponding yeast mutation. However, the expression of rat subunit 5 (oligomycin sensitivity conferring protein, OSCP) (41) and bovine subunit h (42) also complemented the corresponding null mutations in yeast. Complementation by the expression of mammalian γ- and ε-subunits and subunits 5 and h are
of special interest, because these subunits are not highly conserved between yeast and these species (42, 33, 35, and 14.5% identical, respectively). Although the interactions between individual subunits within the ATP synthase may be important, the interactions are limited to just those within a single subunit. In the current study, we have expressed five different peptides contained within a multimeric enzyme complex, which contains numerous intersubunit interactions. In addition, the peptides must associate with many proteins involved in the synthesis and assembly of the enzyme.

The mitochondrial F$_1$-ATPase is composed of five subunits (α, β, γ, δ, and ε), all of which are important for the function of the ATP synthase. The α- and β-subunits are highly conserved (74% and 79% identical), whereas the remaining subunits are loosely conserved (42, 36, and 33% identical). The crystal structure of bovine (2, 43) and yeast F$_1$-ATPase (44) indicates that, in general, the non-conserved residues lie on the surface of F$_1$. Thus, whereas the conservation allows for conservation of the active site and the interactions of the γ-subunit within the core of F$_1$, it fails in the sites of interactions with other subunits of the ATP synthase, i.e. those of the stalks and F$_0$ subunits. F$_1$ is associated with a number of different peptides including at least subunits 6 and 8 of F$_0$, and subunits 5, h, and i of the stalks. There is little doubt that these interactions are compromised when bovine F$_1$ replaces the yeast F$_1$ in the yeast ATP synthase. This is indicated by the loss of the cells’ ability to grow on a non-fermentable carbon source (Fig. 2). The surprise came when it was observed that cells were easily selected and evolved that are able to grow on YPG medium. The high rate of appearance of cells that grow on YPG medium (estimated at $10^{-6}$) indicates that either there are many different mutations that will provide the functional complementation or that the genes being mutated are easily mutated. It is certain that mutations allowing functional complementation were not required in the genes encoding the bovine subunits of the F$_1$. Instead, the mutations either occurred in the nuclear chromosome or the mitochondrial genome. The rate of mutations in the mitochondrial genome is greater than that in the nuclear genome. This greater mutation rate is likely, because it is present in multiple copies and the mammalian DNA polymerase is more error prone than the DNA polymerase used in the nuclear genome (45), although this has been disputed in yeast (46). Thus, the high frequency of occurrence of the mutations is consistent with mutations occurring in the mitochondrial genome where F$_0$ subunits 6, 8, and 9 are encoded.

There are other possibilities that could explain the gain-of-function mutations. There are a large set of proteins required for the synthesis, import, and assembly of the ATP synthase. Any one of the genes encoding these proteins could be mutated to provide a more effective protein or to increase the expression of the protein. For example, the expression of the RAS2 gene was able to complement a mutation in the α-subunit of the yeast ATP synthase (47). A generalized increase in the expression of subunits of the ATP synthase or the assembly factors may occur with a large variety of mutations, thus explaining both the high frequency of occurrence and gain-of-function. An increase in the expression of a large numbers of genes, including many involved in oxidative-phosphorylation, occurred when yeast were evolved for growth on low concentration of glucose (48). Thus, the identity or location of the putative mutations responsible for the gain-of-function of bovine F$_1$ in yeast is not known.

Another surprise was the ability to obtain a functional chimeric enzyme containing the yeast δ-subunit with the remaining bovine F$_1$ subunits from bovine. This is in contrast to the previous study (30), which indicated that the expression of the bovine δ-subunit in yeast did not complement the deletion mutation in the gene encoding the yeast δ-subunit. The trivial explanation for this difference is that the yeast subunit works in the bovine enzyme but that the bovine subunit cannot work in the yeast enzyme. However, it is also possible that given the opportunity to mutate, as the yeast δ-subunit chimeric enzyme was allowed, the bovine δ-subunit would work in the yeast F$_1$-ATPase. There was no opportunity for the bovine δ-subunit to evolve in the yeast enzyme, because yeast strains with a null mutation in the gene encoding the δ-subunit lose their mitochondrial DNA, i.e. it becomes petite (31). The expression of the bovine δ-subunit in yeast did not prevent the petite formation, and thus any possibility of evolution to improve the function of the ATP synthase was lost with the loss of the mitochondrial DNA. In the current situation, the yeast strain containing the bovine α-, β-, γ-, and ε-subunits did not lose its mitochondrial DNA and thus could be evolved for growth on YPG medium.

The successful expression and purification of bovine F$_1$ from yeast suggest that it may be useful for biochemical and structural studies on the mammalian enzyme. This may become of greater significance if and when the human genome project identifies allelic differences in the genes encoding the ATP synthase. The cell has evolved a mechanism to uncouple the mitochondria with a number of uncoupler proteins, UCP1–3 (49–56). It would not be surprising if allelic differences in the genes encoding the ATP synthase were present and responsible for individual differences in traits. The identification of allelic differences would need to be tested for a functional relationship.
between the amino acid change and the structure and function of the enzyme before they could be concluded as significant.

The level of bovine F₁ after expression in yeast is still too low for many structural and biochemical studies. This is likely due to the unstable nature of the contacts of bovine F₁ with yeast F₉ and stalk proteins. An obvious solution to this problem is to express all of the peptides encoding the mammalian ATP synthase but might include one of many critical enzyme complexes. The use of yeast may be an important tool for the structure/function analysis of allelic differences contained within mammalian multimeric enzyme complexes.

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*Saccharomyces cerevisiae*

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