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Cloning and functional expression analysis of the alpha subunit of mouse ATP synthase[1]

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SUMMARY: The α subunit of the mitochondrial ATP synthase is part of the F_1 enzymatic complex known to bind ADP, phosphate and ATP and is at the heart of the mitochondrial energy-producing mechanism. The mouse embryonal carcinoma variant of the α subunit cDNA was cloned and the complete nucleotide sequences of two different lengths of clones were determined. Two distinct polyadenylation sites in the cDNA sequence were detected and two sizes of mRNAs were confirmed by Northern blot hybridization. Two putative ATP-binding motifs - A and B, have been hypothesized for this enzyme based on previous NMR work on another ATP-binding enzyme, adenylate kinase. We have constructed four deletion mutants of the α subunit of the mouse F_1-ATP synthase to examine the putative role of these domains. The resulting recombinant proteins were expressed and purified. Functional studies with the immobilized mutants proved the significance of both sites for ATP binding. © 1993 Academic Press, Inc.

Mitochondrial ATP synthase is an enzyme cluster built from two protein complexes, F_0 and F_1, engaged in the synthesis of the main cellular energy carrier, ATP (for reviews, see 1, 2). The hydrophobic F_0 complex is an integral part of the inner mitochondrial membrane, and is probably responsible for channeling protons across the inner membrane and coupling the H^+ gradient with the synthesis of ATP (3, 4). The synthesis itself is a function of the five-subunit F_1 complex, with stoichiometry $\alpha_3\beta_3\gamma\delta\varepsilon$. F_1 can be stripped off the membrane, and still conserve both its nucleotide-binding and ATPase hydrolytic activities; these properties are expressed by the $\alpha_3\beta_3$–subcomplex alone (5). It is believed that the α subunit binds ATP and exhibits no ATPase activity, while the β subunit is the site of ATP hydrolysis (6). Four ATP synthase α subunit sequences have been reported for three mammalian species (7-10); comparison between the bovine heart and liver α subunits implies the existence of two distinct coding genes. However, the bovine liver α subunit sequence is identical to the rat sequence even at the nucleotide level, which is highly improbable from an evolutionary point of view. We decided to address this problem by sequencing the mouse cDNA for the α subunit of ATP synthase and to compare our sequence with the previously published data. We also elected to make use of cells other than hepatocytes and cardiac myocytes as a source of mRNA.

[1] Sequence data from this article have been deposited with the GenBank Data Library under Accession No. L01062.

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Nuclear magnetic resonance studies on another ATP-binding enzyme, adenylate kinase, have identified two nucleotide binding sites in that molecule (6). Sequence comparisons have revealed the presence of the same consensus motifs in both the α and β subunits of ATP synthase and thus inferred the existence of two putative nucleotide binding sites, the so-called Walker homology motifs A and B (11), in these proteins (6, 9, 11, 12). Studies on bacterial ATP synthase have shown that mutations in these motifs affect nucleotide binding and functional activity of the α subunit (13-16). However, no data is available on the putative functional importance of these motifs in the mammalian enzyme. We therefore set out to construct expression mutants and use them to study the role of the two putative ATP-binding domains within the mouse α subunit.

We report here the complete nucleotide sequence of the α subunit of the mouse ATP synthase. Some conclusions are drawn from comparison of our sequence with the data bank. Our data are the first to show the existence of two transcript sizes deduced from the nucleotide sequence of the cDNA and confirmed by Northern blot analysis. Finally, various deletion mutants of ATP synthase were constructed and expressed in E.coli. The recombinant proteins were purified and tested for binding to [γ-^{32}P]ATP. Our results suggest that both putative nucleotide binding sites are functional for ATP binding.

MATERIALS AND METHODS

Cell cultures—Mouse P19 embryonal carcinoma (EC) cells were grown and induced to differentiate as described by Rudnicki and McBurney (17).

mRNA purification, cDNA library construction and screening, nucleotide sequencing, and Northern hybridization—All molecular biology techniques were carried out using standard procedures (18). cDNA libraries were constructed in the lambda-ZAP vector following the protocol of the manufacturer (Stratagene Corp., LaJolla, CA). Sequencing was carried out with the use of the Sequenase version 2.0 DNA sequencing kit (United States Biochemical, Cleveland, OH) on double-stranded DNA templates. Both strands were sequenced with the use of synthetic oligonucleotide primers. The probe for Northern hybridization was synthesized with the use of the Multiprime Labelling Kit (Amersham).

Deletion Mutagenesis—The following mutants were constructed (see also Fig.3): i) Clone 53K(A+B+): by double digestion of the library clone 2F3/5' with BamHI and EcoRI, formation of blunt ends and re-ligation of the ends 'in frame'; ii) Clone 41K(A-B+): by digestion of clone 2F3/5' with ClaI and re-ligation; iii) Clone 43K(A+B-): by double digestion of 53K(A+B+) with NruI and StuI and re-ligation of the larger fragment; iv) Clone 31K(A-B-): by double digestion of 53K(A+B+) with NarI and StuI, blunting the cohesive ends and re-ligating the larger fragment.

Expression and Purification of the Mutant Proteins—XL1-blue strain of E.coli was transformed with the recombinant pBluescript phagemid DNAs. SDS-PAGE was carried out with 9% acrylamide in the resolving gel. The bands corresponding to the recombinant ATP synthases were identified on the basis of their molecular weights. The proteins were electroeluted from the gel slices in dialysis bags at 300V for 3 hours; reverse electrical field was then applied for 40 seconds. The liquid was transferred into another dialysis bag and dialysed against distilled water overnight. The samples were finally frozen and lyophilized in a Speed Vac concentrator (Savant, Farmingdale, NY). Protein concentrations were measured using the Bio-Rad Protein Assay kit (Bio-Rad, Richmond, CA). The purity of the extracted proteins was assessed by SDS-PAGE and Coomassie staining. The identity of the recombinant proteins was ascertained by amino-acid sequencing using a 473A Protein Sequencer (Applied Biosystems, Foster City, CA).

ATP-binding Activity of the Recombinant Proteins—The ATP-binding activity of the purified recombinant mutant proteins was assessed as follows: equimolar amounts ($M_r \times 10^{-2}$ μg per drop) of the proteins were blotted onto nitrocellulose membranes. Pre-hybridization was performed in MG buffer (9) for 1-2 h with Denhardt's solution (18) as a blocking agent,

followed by a 1-6 h incubation with [γ-^{32}P]ATP at 42°C. The washings were made using MG buffer (without [γ-^{32}P]ATP and blocking agents) at 37-42°C for 40 minutes, with 4 changes of the buffer. Following the hybridization, [γ-^{32}P]ATP-binding by the four mutant proteins and the BSA control was assessed qualitatively by exposure of the nitrocellulose filters to photographic films.

RESULTS

The recombinant clones containing cDNA sequences for the mouse α subunit of mitochondrial F1-ATP synthase were obtained from our RA-treated P19 cells cDNA library by DNA-DNA colony hybridization. Three clones were sequenced, and two types of cDNAs were identified with different 3'-untranslated regions but otherwise identical primary structures. The nucleotide and deduced amino acid sequences of the longest clone are presented in Fig.1. The nucleotide sequence is 1852 bp, representing the longest animal α subunit sequence available until now. Based on comparison with other published sequences, Northern blot hybridization as well as the presence of a long open reading frame, we believe that our clones represent complete cDNAs.

Nucleotide sequence analysis of the mouse α subunit cDNA revealed two polyadenylation signals (AATAAA) in the clones with the longer 3'-untranslated region (positions 1811-1816 and 2159-2164); the upstream one is obviously silent. For the clones with the shorter 3'-end the upstream poly(A)-signal was apparently active, therefore their primary structure lacks the more distant signal. The downstream poly(A)-signal in the mRNA is preceded by a cytoplasmic polyadenylation element (CPE), also called adenylation control element (ACE), with the following consensus sequence for the mouse: UUUUUAU (19). Colony hybridization showed the presence of the two types of cDNAs in both the undifferentiated and RA-treated P19 libraries (not shown). Moreover, Northern blot hybridization confirmed the expression of the two distinct mRNAs in the DMSO-treated P19 derivatives (Fig. 2); their lengths corresponding to the lengths of the two types of cDNA clones.

At the level of the cDNA primary structure our sequence showed similarities with the previously published sequences for other mammalian α subunits of ATP synthase. The highest similarity (94%) was seen with the rat (9) and 'bovine' (8) liver α subunits (the two sequences are identical-see Discussion). Our clones also showed high similarity with the sequences for bovine heart (7) and human (10) α subunits (87 and 88%, respectively). At the amino acid level our sequence displayed from 64% similarity with the bovine protein (7) to 97.5% similarity with the published rat sequence (9).

The presence of two putative nucleotide-binding motifs (A and B) has been hypothesized for both the α and β subunits of ATP synthase. We constructed deletion mutants and used them to study the role of each domain. Four mutants (see diagram in Fig. 3) were constructed, expressed and purified as described in Material and Methods.

The results of the ATP-binding assay are shown in Fig.3. Comparable binding was observed with the wild-type protein (53K(A+B+)) and the two deletion mutants retaining either of the two putative binding sites (41K(A-B+) and 43K(A+B-), respectively). Both the 31K(A-B-) mutant, in which the A and B binding sites have been deleted, and the BSA negative control did not show any appreciable binding. These results establish the functional importance of both the A and B sites for nucleotide binding by the mammalian ATP synthase α subunit.

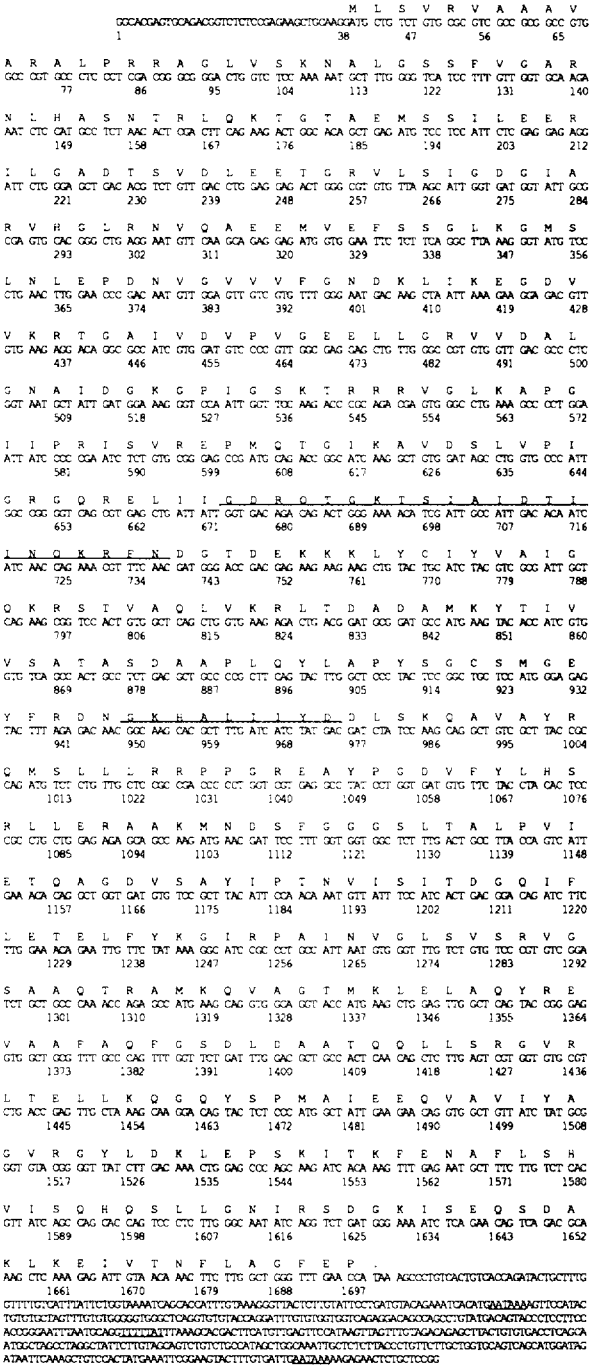

Fig. 1. Complete nucleotide cDNA and predicted amino-acid sequences of the α subunit of the mouse mitochondrial ATP synthase. The nucleotides are numbered from the first nucleotide in the 5'-untranslated region. The one-letter code for amino acids is used. The sequences for the putative ATP-binding motifs (A and B), the two polyadenylation signals (AATAAA) and the ACE (adenylation control element, TTTTTAT) are underlined. MacMolly software (Soft Gene Gmbh, Berlin, Germany) was used for processing the sequencing data, data bank searches and as an alignment tool.

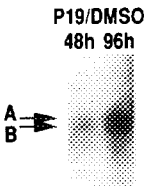

Fig. 2. Northern blot hybridization of DMSO-treated P19 cells mRNA with an ATP synthase α subunit probe. Poly(A)$^+$ RNA was extracted from cells treated with DMSO for 2 and 4 days, respectively. The arrows point to the two α subunit transcripts (approx. 2.0 and 2.4 kb).

DISCUSSION

The cloning of the mouse α subunit of the mitochondrial ATP synthase generated the first complete rodent sequence for this enzyme. Our experiments have also identified two different α subunit transcripts differing in their 3'-untranslated regions. Finally, our studies using deletion expression mutants of the enzyme have demonstrated the functional relevance of the two putative nucleotide binding sites on the molecule.

Our sequence is the longest available animal sequence. It helps to resolve an apparent discrepancy in the literature. Indeed, the published sequence for the bovine liver α subunit (8) is identical to the rat sequence down to the last nucleotide (9). Such similarity is highly improbable

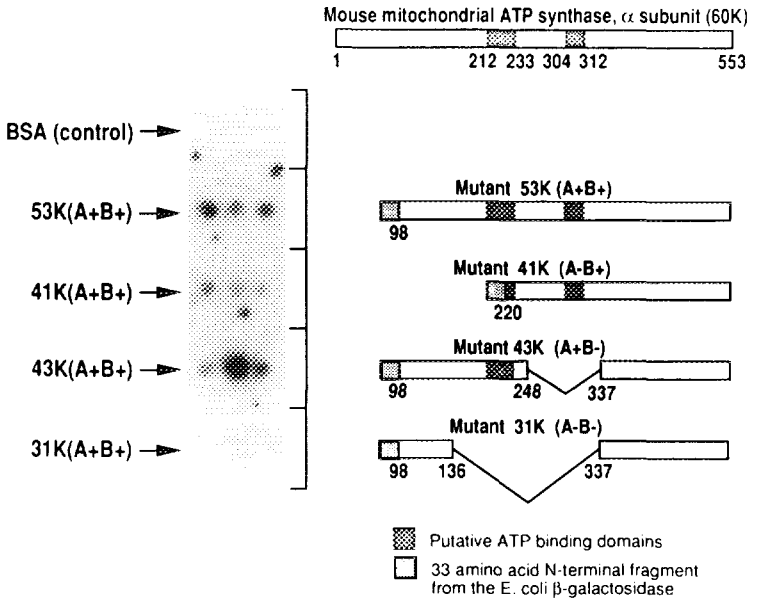

Fig. 3. Binding activity of the deletion mutants. [γ-^{32}P]ATP-binding of the purified recombinant proteins. Each of the mutant polypeptides was blotted in three parallel drops to avoid confusion with background signals. Equimolar amounts of the proteins were blotted as described in Materials and Methods. A schematic view of the α subunit deletion mutants is presented on the right-hand side. The top bar represents the complete amino acid stretch of the α subunit, with the two ATP-binding domains hatched. The lower bars show the different deletion mutants, constructed as described in Materials and Methods. The first 33 amino acids from the N-terminus are from the 5'-end of the E. coli lac Z gene derived from the pBluescript phagemids.

considering the mutation rate of DNA during evolution; at the very least, there are always silent mutations between species. Based on comparisons between the three sequences and the evolutionary proximity of mice and rats, the rat sequence appears correct, whereas the bovine liver sequence seems artifactual. The identification of only one correct bovine sequence, for the heart α-subunit (7), raises the question whether distinct non-allelic heart and liver genes for the α subunit actually exist.

We report here for the first time the existence of two types of mouse α-subunit mRNA, differing in their length. The following data support this conclusion: the two polyadenylation signals in the cDNA sequence; the two different types of clones found in the P19 cDNA libraries; and the two bands detected by Northern hybridization, whose sizes correspond to the cDNA types. The downstream polyadenylation signal in the mRNA is preceded by a cytoplasmic polyadenylation element (CPE or ACE) (19). Its presence enhances translation of stored messages during meiotic maturation (19). Since the cells that we have used are pluripotent cells (17), it would be interesting to investigate the changes in the equilibrium between the two species of mRNA during cell differentiation. Such studies could broaden our understanding of the mechanisms regulating mRNA half-life during cellular differentiation.

We have developed a different methodological approach for studying the ATP-binding activity. Studies performed to date relied on the use of NMR (6, 12) or TNP(trinitrophenyl)-ATP and ADP (9), [2-^{3}H]ADP (20), or 8-azido-[2-^{3}H]ATP (21) as tracers for assaying binding. [γ-^{32}P]ATP has the advantage of higher specific activity and is a good indicator of ATPase activity, since the label is removed *in situ* in the case of hydrolysis.

Our studies with the deletion mutants of the mammalian α subunit confirm the functional relevance of the Walker homology motifs for ATP binding, already established for the bacterial enzyme (13-16). Both regions individually confer the ability to bind ATP. Our findings are consistent with the previously formulated suggestion that the A domain binds ATP strongly and exhibits no ATPase activity (6). It could prove interesting to mutate the arginine-214 residue within the A domain of mutant 43K(A+B-) using site-directed mutagenesis. This residue is believed to be responsible for the strong binding to ATP and for the lack of hydrolytic activity (6). Indeed, the A domain of the β-subunit has an alanine residue in the corresponding position (6). Based on sequence comparison studies with adenylate kinase, this alanine residue is believed to confer hydrolytic activity (6). Our deletion mutants could prove useful as molecular tools to dissect the structure-function relationship of the various domains of the enzyme.

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