The δ -subunit of ATP synthase from bovine heart mitochondria

Complementary DNA sequence of its import precursor cloned with the aid of the polymerase chain reaction

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The δ -subunit of ATP synthase from bovine heart mitochondria is part of the extrinsic membrane domain, F_1 -ATPase. The mature protein is 146 amino acids in length and its function is obscure. It is encoded by a nuclear gene and is imported into the organelle. Two mixtures of oligonucleotides 17 bases long, designed on the basis of the known protein sequence, have been synthesized and employed as primers on bovine cDNA in the polymerase chain reaction. By this means a segment of bovine cDNA encoding part of the δ -subunit has been amplified, and this DNA segment has been employed to identify related cDNA clones in a library. These clones encode the mitochondrial import precursor of the δ -subunit; the protein sequence of the mature protein deduced from it is exactly the same as that determined earlier by direct sequence analysis. The clones have also been used to show that both the bovine and human genomes seem to contain a single gene for the δ -subunit.

INTRODUCTION

The δ -subunit of ATP synthase from bovine heart mitochondria is one of the five different polypeptides that comprise F_1 -ATPase, the extrinsic membrane sector of the enzyme. It is a globular protein of known sequence (Walker *et al.*, 1985), but its function in the enzyme is poorly defined. The membrane-bound ATP synthase complex appears to be an assembly of 14 different subunits (J. E. Walker, R. Lutter & M. J. Runswick, unpublished work), including those of F_1 -ATPase. All but two of them are encoded in nuclear genes (Fearnley & Walker, 1986) and the corresponding mRNAs are translated on cytoplasmic ribosomes, usually as precursors with *N*-terminal extensions that direct the proteins into the organelle (Schatz & Butow, 1983). From cDNA cloning experiments it is now evident that at least two of the subunits of ATP synthase are encoded by more than one expressed gene. For example, an intrinsic membrane component, the dicyclohexylcarbodi-imide-reactive subunit, has two different expressed genes, referred to as P1 and P2 (Gay & Walker, 1985a). The products of these two genes differ in their *N*-terminal import sequences, but removal of the pre-sequence produces the same mature protein. The genes are expressed differently in various bovine tissues. The second example is the α -subunit, another component of F_1 -ATPase, and the largest subunit in the ATP synthase complex. It also has two bovine genes; one is expressed in liver (Breen, 1988) and the second in heart (Walker *et al.*, 1985, 1989). Hybridization experiments conducted on restriction digests of bovine and human DNA with probes for other subunits of the enzyme (subunits b and d and the oligomycin conferral protein) detect multiple bands (Walker *et al.*, 1987a,b), and so suggest that they also may have multiple genes. However, these

experiments do not distinguish between expressed genes and pseudo-genes. Similar experiments indicate that the F_0 subunit, the ATPase inhibitor protein and the γ -subunit have single copy genes (Walker *et al.*, 1987b; Dyer *et al.*, 1989).

As discussed below we have characterized overlapping cDNAs encoding the mitochondrial import precursor of the δ -subunit of ATP synthase. The initial partial clone was produced from bovine cDNA by means of the polymerase chain reaction in which two degenerate synthetic oligonucleotides based upon the known protein sequence of the δ -subunit were employed as primers. This partial clone was then used to screen a bovine cDNA library, and thereby more extensive clones were isolated. These were shown by sequence analysis to code for the mitochondrial import precursor of the δ -subunit. They were used as hybridization probes to investigate the complexity of the gene family for the protein in cows and humans.

MATERIALS AND METHODS

cDNA synthesis

Total RNA was extracted from bovine heart muscle according to the method of Chirgwin *et al.* (1979), and mRNA was purified from it using mAP paper (Amersham International) as described before (Viñas *et al.*, 1990). Total cDNA was synthesized from poly(A)⁺ mRNA using a 'cDNA Synthesis System Plus' kit (Amersham International). First-strand cDNA was primed with oligo(dT) and synthesized at 42 °C for 1 h using 20 units of reverse transcriptase (Anglian Biotechnology)/ μ g of original mRNA. Second-strand DNA was synthesized using 23 units of *Escherichia coli* DNA polymerase I and 0.8 units of RNAase H/ μ g of original mRNA.

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The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X17019.

The polymerase chain reaction

Part of the sequence of the cDNA of the δ -subunit of ATP synthase was amplified from total cDNA by means of the polymerase chain reaction (Saiki *et al.*, 1988). The primers were 26 bases long and contained 9 bases of a linker sequence that included a restriction enzyme site, and 17 bases complementary to the target DNA. They were synthesized by phosphoramidite chemistry with the aid of an Applied Biosystems 380B oligonucleotide synthesizer. The forward primer (referred to as PCR-F) was made with the *EcoRI* linker, TAGGAATTC, and was followed by a degenerate sequence designed on the basis of the protein sequence TQVFFN, amino acid residues 24–29 of the bovine δ -subunit (Walker *et al.*, 1985). Therefore, the sequence of PCR-F was TAGGAATTCACNCARGTNTTYTTYAA and it contained 128 different sequences. The reverse primer, PCR-R, was a mixture of 96 different sequences. It contained the *HindIII* linker, CGAAGCTT, and was followed by a degenerate sequence GCYTCRTTNGCYTCDAT designed from the amino acid sequence, IEANEA, amino acids 135–140 of the bovine δ -subunit (Walker *et al.*, 1985). The amplification reactions were performed in a solution (100 μ l) containing 50 mM-KCl, 10 mM-Tris/HCl, pH 8.3, 1.5 mM-MgCl₂, each dNTP (200 μ M), both primers (2 μ M), cDNA (10 ng) and *Thermus aquaticus* DNA polymerase (3 units; Perkin Elmer Cetus). The reaction mixture was prepared without enzyme and preincubated at 94 °C for 2.5 min. Then the enzyme was added and the reaction mixture was covered with mineral oil (100 μ l; Sigma). For amplifications the following schedule was followed using a Techno Programmable Dri-Block PHC-1; 94 °C for 2.0 min (denaturation), 40 °C for 2 min (annealing), 70 °C for 2.0 min (synthesis) for 35 cycles, and finally a single incubation at 70 °C for 7 min. Then the reaction mixture was allowed to cool to 40 °C. The products of the polymerase chain reaction were fractionated by electrophoresis in a 1.2% agarose gel, transferred to a Hybond N membrane and hybridized with a radiolabelled mixture of synthetic oligonucleotides. This mixture contained 128 different 17mers and was based upon the protein sequence HAEDGT, amino acid residues 66–71 of the δ -subunit of bovine ATP synthase (Walker *et al.*, 1985). The conditions for radiolabelling and purification of synthetic oligonucleotides and conditions for hybridization have been described in earlier publications (Gay & Walker, 1985a; Powell *et al.*, 1989; Walker *et al.*, 1989). Radioautography revealed the presence of a single strongly hybridizing band corresponding to a product of about 350 bp in length. This product was recovered in a duplicate agarose gel fractionation and purified as described before. One-twentieth of it was amplified again by the polymerase chain reaction under the same experimental conditions as were used for the first amplification. One-tenth of the products of this reaction was fractionated by electrophoresis as above, and then stained with ethidium bromide. A doublet at about 350 bp could be seen. The upper band was recovered and half of it was digested for 2 h at 37 °C in a solution that contained 1 mM-dithiothreitol, 33 mM-Tris/acetate, pH 7.5, 66 mM-potassium acetate, pH 8.5, 10 mM-magnesium acetate, pH 7.6, 100 μ g of bovine serum albumin (fraction V)/ml and 3 mM-spermidine, and the restriction enzymes *EcoRI* (10 units) and *HindIII*

(10 units). Finally, the enzymes were inactivated by heating the solution at 70 °C for 20 min, and the products of the polymerase chain reaction were ligated to M13 mp18 and M13 mp19 vectors that had been digested with *HindIII* and *EcoRI*. These recombinants were transfected into *E. coli* TG1, the cells were grown up on agar plates in the presence of isopropyl β -D-thiogalactopyranoside and 5-bromo-4-chloroindol-3-yl β -D-galactopyranoside, and 15 white plaques from each transfection were selected for further characterization. DNA sequence analysis showed that they all coded for a segment of the δ -subunit of ATP synthase.

cDNA cloning

The bovine cDNA library and the experimental conditions that were used to screen it for a clone encoding the whole of the δ -subunit of ATP synthase have been described previously (Viñas *et al.*, 1990), except that in the present experiments the hybridization probe was a 'prime-cut' probe (Farrell *et al.*, 1983) prepared from the partial clone for the δ -subunit described above. About 5×10^5 recombinants were screened and 12 positively hybridizing clones were identified. They were rescreened with the same hybridization probe and eight of them still gave a positive signal. Plasmid DNA was prepared from six of them and their inserts were released by digestion with either the restriction enzymes *EcoRI* and *BamHI* or alternatively *EcoRI* and *HindIII*, both together and separately. The digestion products were analysed on a 1% agarose minigel and the restriction fragments that had arisen from the inserted DNAs were cloned into appropriately prepared M13 mp18 and M13 mp19 vectors.

DNA sequence analysis

DNA sequences were determined by the modified chain termination procedure (Sanger *et al.*, 1977; Biggin *et al.*, 1983). 'Compressions' in DNA sequences were resolved by the substitution of deoxy-7-deazaguanosine triphosphate in place of dGTP in the sequencing mixtures (Mizusawa *et al.*, 1986). Data were compiled and analysed with the aid of the computer programs DBUTIL (Staden, 1982) and ANALYSEQ (Staden, 1984).

Hybridization with genomic DNA

The preparation of human genomic DNA has been described previously (Walker *et al.*, 1987b). Samples of DNA (10 μ g) were digested with one of the restriction enzymes *BamHI*, *HindIII*, *KpnI* and *SacI*, and the digests were fractionated by electrophoresis in a 0.6% agarose gel. The fragments of DNA were transferred to Hybond N membranes by the method of Southern (1975) and were hybridized with a radioactively labelled 'prime-cut' probe (Farrell *et al.*, 1983). This probe was released with the restriction enzyme *HindIII* from a clone of the products of the polymerase chain reaction (see Figs. 1 and 2). Conditions for prehybridization, hybridization, washing and autoradiography have been given before (Walker *et al.*, 1987b).

RESULTS AND DISCUSSION

Isolation of complementary DNA clones encoding the δ -subunit of bovine ATP synthase

In experiments preceding those described in this paper, several unsuccessful attempts have been made to isolate

Fig. 1. Production of a partial cDNA clone for the δ -subunit of ATP synthase from bovine mitochondria by means of the polymerase chain reaction

Bovine cDNA was amplified with the degenerate primers PCR-F and PCR-R (see the Materials and methods section) and fractionated by electrophoresis in a 1.2% agarose mini-gel. Panel (a) shows an autoradiograph after 4 days exposure of the fractionated products of this amplification following hybridization with a radiolabelled degenerate oligonucleotide probe corresponding to amino acids 66–71 of the δ -subunit of ATP synthase. Material from this band was extracted from the gel, reamplified and fractionated as above, and stained with ethidium bromide (see panel b). The material in the upper band was recovered, cloned into M13 vectors and sequenced as described in the text. The positions of size markers are indicated (in kilobases).

Fig. 2. Sequence analysis of cDNA clones isolated from a bovine library encoding the mitochondrial import precursor of the δ -subunit of ATP synthase

The thick lines represent the inserted DNAs in the recombinant pBov $\delta.1$, pBov $\delta.2$ and pBov $\delta.3$. The inserts are flanked by *Eco*RI sites (∇), *Bam*HI sites ($\blacktriangledown$) and *Hind*III sites ($|$) as shown. In all clones, a *Bam*HI site is found in the polylinker and, in addition, a second site is found in clones pBov $\delta.2$ and pBov $\delta.4$ immediately before the poly(A) tail (see Fig. 3). In clone pBov $\delta.2$ this second site was overlapped by sequencing the *Eco*RI–*Hind*III fragment; the presence of the sequence A₈₈ at the 3' end of clone pBov $\delta.4$ prevented this *Bam*HI site from being overlapped in both senses of the DNA. The black boxes represent synthetic oligonucleotide primers that were used in the sequencing reactions, and the horizontal arrows the extents of the sequences that were determined.

cDNA clones for the δ -subunit of ATP synthase (S. J. Powell, M. J. Runswick & J. E. Walker, unpublished work) from a bovine library (Gay & Walker, 1985b). In these experiments degenerate synthetic oligonucleotides 17 nucleotides in length based upon the amino acid sequence of the protein were employed as hybridization probes. Three different segments of protein sequence were used for this purpose, TQVFFN, VHAEDG and IEANE, amino acid residues 24–29, 65–70 and 135–140 respectively of the bovine δ -subunit (Walker *et al.*, 1985), the degeneracies of the corresponding oligonucleotides being 128, 128 and 96. In contrast, similar experiments to isolate cDNAs for other subunits of ATP synthase (Walker *et al.*, 1985, 1987a,b, 1989; Gay & Walker, 1985a; Viñas *et al.*, 1990), for mitochondrial transport proteins (Runswick *et al.*, 1987; Powell *et al.*, 1989) and for components of mitochondrial complex I (Fearnley *et al.*, 1989; Pilkington & Walker, 1989; Runswick *et al.*,

1989) were conducted successfully on the same cDNA library. Many of the oligonucleotide probes employed in these experiments had comparable or even greater degeneracies than those unsuccessfully used for the δ -subunit, and so it appeared to be possible that cDNAs for this protein were poorly represented in, or were absent from, the library.

Therefore, it was decided to try a somewhat different approach and to use the polymerase chain reaction to amplify a region of bovine cDNA encoding part of the δ -subunit. For this purpose degenerate forward and reverse primers, PCR-F and PCR-R respectively, were synthesized using known amino acid sequences of the protein, and were used in amplification reactions (see the Materials and methods section and the legend to Fig. 1 for further details). These primers would be expected to give a product of about 350 bp in length. The products of the first set of amplifications were examined by agarose

Fig. 3. DNA sequence of cDNAs encoding the mitochondrial import precursor of the δ -subunit of bovine ATP synthase

The position of the *N*-terminal amino acid of the mature protein isolated from bovine hearts is shown. PCR-F and PCR-R are the positions of amino acid sequences that were used to design the forward and reverse mixed oligonucleotide primers respectively. They were employed to amplify with the polymerase chain reaction a segment of cDNA coding for the intervening region of the δ -subunit. The amino acid sequence used in the design of a third oligonucleotide mixture, employed as a hybridization probe, is also indicated. Two potential signals for polyadenylation of the mRNA are underlined twice, and 'Poly A' indicates an alternative site of polyadenylation observed in isolate pBov δ .1. A *Ban*I restriction site was used to produce 'prime cut' hybridization probes, and the position of a *Bam*HI site immediately adjacent to the poly(A) sequence is also marked.

gel electrophoresis, and a faint pair of bands about 350 nucleotides long could be discerned after staining with ethidium bromide. These bands were transferred to a Hybond N membrane and the upper band was found to hybridize strongly with a third degenerate oligonucleotide (see Fig. 1a; this oligonucleotide is designated as 'probe' in Fig. 3) based upon the amino acid sequence of residues 66–71 and found between those used to design PCR-F and PCR-R; the lower band also hybridized weakly. The upper band was recovered and was re-amplified in a further round of polymerase chain reactions. A doublet at about 350 bases was revealed by staining the fractionated products with ethidium bromide, but this time it stained strongly (see Fig. 1b). By cloning and sequencing the upper band it was shown to contain a sequence coding for amino acids 24–140 of the δ -subunit of ATP synthase. In other experiments (results not shown) the lower band was shown not to contain sequences coding for the δ -subunit, but the sequence that was determined

had regions that were closely related to both the primers and to the probe.

In order to extend the partial cDNA sequence to regions corresponding to the 3' and 5' extremities of the mRNA a number of possible strategies was considered. The simplest was to use the partial sequence as a hybridization probe to search for more extensive clones in a cDNA library. Alternatively, the sequence could be extended up the 3' poly(A) sequence by using a unique forward primer based on the known sequence and a reverse poly(T) primer in the polymerase chain reaction. Thirdly, both 3' and 5' sequences could be obtained in the same experiment by first circularizing the cDNA by ligating its 3' and 5' ends together, and by then linearizing this product by cleaving it at an appropriate restriction enzyme site. The segment containing the 3' and 5' extremities, which are ligated together, could then be amplified by the polymerase chain reaction. We have used this last approach successfully in investigations of

ovine cDNAs of the dicyclohexylcarbodi-imide-reactive subunit of ATP synthase (S. M. Medd & J. E. Walker, unpublished work) and of 6-phosphogluconate dehydrogenase (D. Somers, S. M. Medd & J. E. Walker, unpublished work). In the present case, it was decided to try the first approach. Therefore, the cDNA library that had failed to yield clones for the δ -subunit in the earlier experiments described above, was screened with a 'prime-cut' probe prepared from the partial clone. Among 5×10^5 recombinants, 12 gave a strong initial hybridization signal. Of these, eight rescreened with the same probe, six were shown by sequence analysis to contain sequences coding for parts of the δ -subunit and four of these were analysed completely (see Figs. 2 and 3). When the same number of recombinants from this library were screened with comparable prime-cut probes from cDNAs encoding other subunits of ATP synthase, considerably more positively hybridizing clones were detected (J. E. Walker and others, unpublished results). So it appears that cDNA clones for the δ -subunit are under-represented in the library (see below for further discussion of this point).

Complementary DNA sequence encoding the δ -subunit of bovine ATP synthase

The cDNA sequence, compiled from those of clones pBov δ .1–pBov δ .4 and presented in Fig. 3, is 985 nucleotides in length. It encodes a protein sequence that is identical with that of the mature δ -subunit of bovine ATP synthase, which was determined by direct sequence analysis of protein isolated from bovine hearts (Walker *et al.*, 1985). It also encodes a protein sequence of 22 amino acids before the *N*-terminus of the mature protein with characteristic features of mitochondrial import sequences. This *N*-terminal extension has a net basic charge, acidic amino acids are not present, it is not noticeably hydrophobic and an arginine residue is found in the import sequence close to the junction with the mature protein (von Heijne, 1986; Roise *et al.*, 1986). The majority, but by no means all, of the import sequences are about 25 amino acids long, and the presequence of the δ -subunit is 22 amino acids. The proposed translational initiator codon is not preceded by an in-phase termination codon, and so it is not possible to be certain that this proposal is correct, although it is quite likely to be so. The coding sequence is followed at its 3' end by a rather extensive non-coding sequence. This contains two potential signals for addition of poly(A) (see Fig. 3), and polyadenylation was observed to occur after nucleotides 774 (clone pBov δ .1) and 973 (clones pBov δ .2 and pBov δ .4).

A notable feature of the cDNA sequence is its unusually high content of G+C residues. The bovine genome has been estimated to have a G+C content of 42% (Chargaff & Lipschitz, 1953), whereas that of the cDNA for the δ -subunit of ATP synthase is 64%. The dinucleotide sequence CpG has been found to be clustered in mammalian genomes in 'CpG-rich' islands (Bird, 1986), which are usually associated with the 5' regions of genes and probably influence their expression. CpG is distributed throughout the cDNA sequence of the δ -subunit with the highest concentrations at the 5' and 3' ends, but without further experimentation it is not possible to know what the biological significance (if any) of this distribution may be. However, it could offer an explanation of our failure to clone cDNAs for the δ -

Fig. 4. Hybridization of restriction digests of DNA from cows and humans with a probe derived from the cDNA for the δ -subunit of ATP synthase

Samples of DNA from cow and man were digested with the restriction enzymes indicated. The fractionated digests were hybridized with the probe (nucleotides 182–531 in Fig. 3) under the conditions described in the Materials and methods section. Panels (a) and (b) show hybridizations with *SacI* (S), *KpnI* (K), *HindIII* (H) and *BamHI* (B) digests of bovine and human genomic DNA respectively.

subunit, for as a consequence of the high G+C content there are many regions in the cDNA (and in the mRNA) with the potential to form stable hairpin-loop structures, and particularly in the 3' region. During the first-strand cDNA synthesis this could cause the reverse transcriptase to pause or to stop, and thereby lead to an under-representation of clones for this protein in the library. Other explanations are also possible; for example, it is conceivable that the steady-state levels of mRNA for the δ -subunit are lower than those of other subunits of ATP synthase. Another cDNA encoding the 30 kDa subunit of mitochondrial complex I has been found to be over-represented in the same library (S. J. Pilkington & J. E. Walker, unpublished work). This appears to arise not from a superabundance of mRNA relative to other proteins in the same enzyme complex, but rather to be caused by a stable hairpin loop structure at the 5' end of the mRNA, which promotes second-strand cDNA synthesis when loop-back priming is employed, as it was in the present case.

Number of sequences encoding the δ -subunit of ATP synthase in humans

Hybridization of digests of bovine and human genomic DNA with a probe containing part of the cDNA sequence encoding amino acids 24–140 of the mature δ -subunit of ATP synthase produced simple patterns (see Fig. 4).

These are consistent with the presence of single genes for this protein in both genomes, although the possibility must also be considered that more distantly related DNA sequences that encode homologues of the δ -subunit were not detected in this hybridization experiment.

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