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Nucleotide sequence of the cloned mRNA and gene of the ADP/ATP carrier from *Neurospora crassa*

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A cDNA complementary to the mRNA of the ADP/ATP carrier from *Neurospora crassa* was identified among ordered cDNA clones by hybridizing total polyadenylated RNA to pools of 96 cDNA recombinant plasmids and subsequent cell-free translation of hybridization-selected mRNA. Further carrier cDNAs were found by colony filter hybridization at a frequency of 0.2 – 0.3%. The gene of the carrier was cloned and isolated on a 4.6-kbp *Eco*RI fragment of total *Neurospora* DNA, and the start of the mRNA was determined by S1 nuclease mapping. From the nucleotide sequence of the cDNA and the genomic DNA, the primary structure of the gene, of the mRNA and of the ADP/ATP carrier protein could be deduced. The gene occurs in a single copy in the genome and related genes are absent. It contains two short introns, and a pyrimidine-rich promoter region. The mRNA has a 46-bp 5′ end and a 219-bp 3′ end. There is an open reading frame coding for the 313 amino acid residues of the *Neurospora* carrier protein. The amino acid sequence is homologous in 148 positions with the established primary structure of the beef heart carrier.

Key words: mitochondrial ADP/ATP carrier/*Neurospora crassa*/mRNA and gene/nucleotide sequence/hybrid-selected translation

Introduction

The ADP/ATP carrier catalyses the exchange of ADP and ATP across the mitochondrial inner membrane (for review see Klingenberg, 1976, 1980; Vignais, 1976). The functional carrier is a dimer of two identical subunits of mol. wt. 30 000 – 33 000, depending on the species. The amino acid sequence of the beef heart carrier has been determined (Aquila *et al.*, 1982). The carrier protein is synthesized in the cytosol (Hackenberg *et al.*, 1978) and imported post-translationally into the mitochondria (Hallermayer *et al.*, 1977). The primary translation product has the same apparent mol. wt. as the mature protein (Zimmerman *et al.*, 1979). The isolation of the gene of the ADP/ATP carrier from yeast has been reported (O'Malley *et al.*, 1982), but so far no DNA sequence data for this gene have been published.

The present paper describes the isolation of a cloned cDNA corresponding to the mRNA of the ADP/ATP carrier from *Neurospora crassa*. The cDNA was identified by methods based entirely on the selection of mRNA by hybridization and the subsequent analysis of cell-free translation products by antibodies (Viebrock *et al.*, 1982). These procedures were adapted so that >1000 cDNA clones could be screened in one experiment. The amino acid sequence deduced from an almost full-length cDNA exhibits extensive homology with the corresponding protein from beef heart. The isolated cDNA could be used as a probe for the identification of the cloned gene.

Results

Identification of a cDNA clone encoding ADP/ATP carrier mRNA

Figure 1A, lanes a – m, shows a gel electrophoretic analysis of total cell-free translation products of the mRNAs selected by hybridization to 1152 cDNA plasmids. Each lane represents the cDNAs from 96 pooled clones (= 1 microtitre plate).

An aliquot of each translation assay was analysed by means of rabbit antibodies raised against a crude preparation of carrier protein from *N. crassa* (Figure 1B, lanes A – M). The mRNA selected by three cDNA pools (lane c = MTP 22, lane f = MTP 25, lane h = MTP 27) directed the synthesis of a polypeptide which is adsorbed by the antibodies, and which co-migrates with the mature carrier protein of mol. wt. 33 000 (Hackenberg *et al.*, 1978). The other immunoadsorbed proteins most likely originate from immunoglobulins raised against the impurities present in the crude carrier preparation.

The 96 cDNA plasmids of pool MTP 22 were investigated further, first in groups of eight and then individually. Finally, the cDNA plasmid of one clone (MTP 22/90) was identified which hybridized specifically with only the carrier mRNA (data not shown). The cDNA insert of this plasmid consists of 109 bp corresponding to nucleotides 201 – 309 of the coding sequence (see Figure 3). Short cDNA inserts occur frequently in the constructed clone bank, since the double-stranded cDNA has been cloned without sizing after S1 nuclease treatment. The small cDNA inserts often have the advantage that they are more specific in mRNA selection (W.Sebald, unpublished observation).

Screening for full-length cDNA

The short cDNA insert of clone MTP 22/90 was recloned into the *Pst*I site of vector pUR 222 (Rüther *et al.*, 1982), and then excised using the juxtaposed *Bam*HI and *Eco*RI sites of the vector. This fragment was labelled at the 5′ ends with [^{32}P]phosphate, and then used for sequencing and as a labelled probe for colony-filter hybridization. A screening of ~15 000 cDNA clones yielded 36 positive plasmids. Two of them contained cDNA inserts of 1136 bp (HA5) and 1177 bp (HA1), respectively. The cDNA from clone HA1 comprises the whole 3′ end of the mRNA including the poly(A) tail as well as the coding sequence up to the triplet coding for amino acid 5 (base pair 14) (Figure 3). The cDNA from clone HA5 is devoid of the poly(A) tail and contains the whole coding sequence up to the triplet encoding amino acid 4 (base pair 10).

Isolation of the gene of the ADP/ATP carrier

Total genomic DNA was cut with several restriction endonucleases, and the electrophoretically separated fragments

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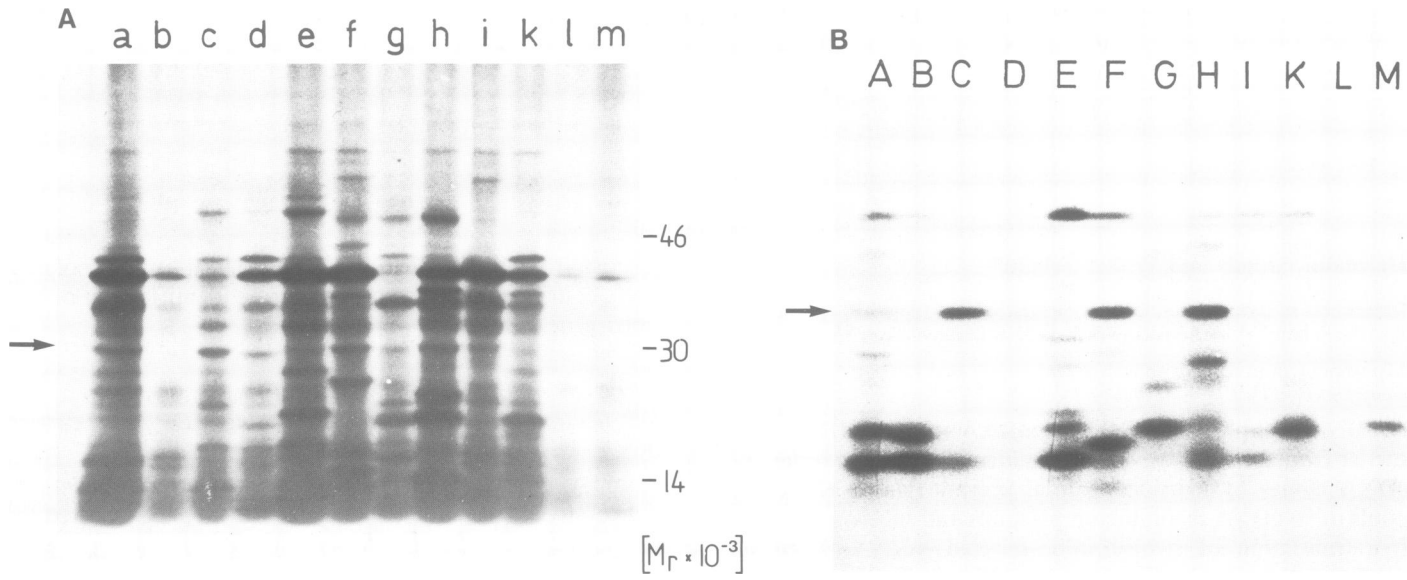

Fig. 1. Screening of 1152 cDNA plasmids for ADP/ATP carrier mRNA sequences. The cDNA plasmids from 96 pooled clones were covalently bound to paper and hybridized with total polyadenylated RNA. The selected mRNAs were translated in 15 μl of a cell-free wheat germ system with 15 μCi [^{35}S]methionine for 60 min. An aliquot (80%) of each assay was incubated with antiserum raised against a crude carrier protein from *N. crassa*. Total translation products **(A, lanes a – m)** and the immunoadsorbed proteins **(B, lanes A – M)** were separated by SDS-gel electrophoresis and visualized by fluorography. The functional ADP/ATP carrier protein migrates as indicated by the arrow.

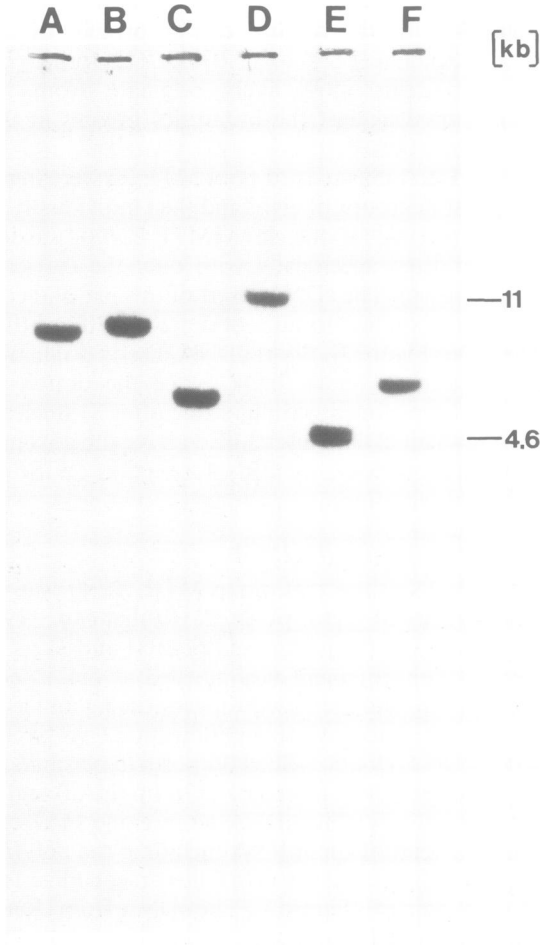

Fig. 2. Identification of the ADP/ATP carrier gene by Southern-blot analysis. Total DNA of *N. crassa* was cleaved with *Bam*HI **(A)**, *Eco*RI **(B)**, *Hind*III **(C)**, *Pst*I **(D)**, *Xba*I **(E)** and *Xho*I **(F)**, and electrophoretically separated on 1% agarose gels. After transfer to nitrocellulose, the fragments were hybridized (3 x SSC, 65°C, 1 x SSC = 150 mM NaCl, 15 mM trisodium citrate) with nick-translated insert of plasmid HA1.

were hybridized (Jeffreys and Flavell, 1977) with nick-translated cDNA from clone HA1. After hybridization, the filters were washed at various stringencies (65°C, 3 x SSC to 0.1 x SSC). Under all conditions a single labelled fragment was observed (Figure 2), indicating the presence of a single gene, and the absence of other related sequences in the genome. The hybridizing 4.6-kb *Eco*RI fragment was cloned and isolated. The sequence of ~2000 bp was determined which are located upstream from one of the *Eco*RI sites (Figure 3). This segment contains the whole sequence of the mRNA. A comparison of cDNA and genomic DNA revealed the presence of two short introns, which occur in the 5′-proximal end of the coding sequence.

Start of the mRNA

A *Sph*I-*Pvu*II fragment extending 320 nucleotides upstream from the presumptive ATG start codon was labelled at the 5′ end of the anti-sense strand, and hybridized with total polyadenylated RNA from *Neurospora*. The DNA protected from S1 nuclease digestion was analysed on a DNA sequencing gel in parallel with the *Sph*I-*Pvu*II fragment submitted to the four DNA sequencing reactions (Figure 4). The protected fragment starts at nucleotide –46 upstream from the ATG start codon.

Discussion

The cDNA representing a segment of the mRNA of the ADP/ATP carrier was isolated from an ordered cDNA clone bank which had been previously exploited for the isolation of a cDNA coding for the mRNA of the proteolipid subunit of the ATP synthase (Viebrock *et al.*, 1982). In the earlier experiments (Viebrock *et al.*, 1982) mRNA was selected by hybridization with pools of 12 cDNA plasmids. The present experiments demonstrate that this method is sufficiently sensitive so that pools of 96 plasmids may be used for the hybridization selection. Thousands of cDNA clones can therefore be analysed with a reasonable amount of work. The ADP/ATP carrier cDNA clones occur at a frequency range

Fig. 3. Nucleotide sequence of the gene of the ADP/ATP carrier. The mRNA start, as determined by S1 nuclease mapping, and the polyadenylation site are indicated by arrows. The conserved sequence before the translation start codon, as well as the conserved sequences at the boundaries and the interior of the introns are underlined.

of 1 per 300–400. A similar abundancy has been observed with the proteolipid clones (Viebrock *et al.*, 1982). Both polypeptides are on a molar basis the most abundant proteins of *Neurospora* mitochondria (Hackenberg *et al.*, 1978; Sebald *et al.*, 1979). Other mitochondrial membrane proteins of *Neurospora*, such as subunits of the ATP synthase and the respiratory complexes, exist in up to 6-fold lower molar amounts (Sebald *et al.*, 1979; von Jagow *et al.*,1973). If the abundancy of the mRNAs corresponds to that of the proteins, it should also be possible to isolate the cDNA clones of these less frequent proteins by the described procedures.

The sequence analysis of both the genomic DNA and a nearly full-length cDNA yields the structure of the ADP/ATP carrier mRNA. According to S1 nuclease mapping a major start of the mRNA is observed around nucleotide −46 upstream from the presumptive ATG initiation codon. The anti-sense strand at the transcription initiation site is very rich in purines. The same is true for the corresponding region of the proteolipid gene (Sebald, unpublished observation), of the *am* gene (Kinnaird and Fincham, 1983), and of the *qua3* gene (Alton *et al.*, 1982), but not for that of the *qua2* gene (Alton *et al.*, 1982) from *Neurospora*.

The first 5′-proximal AUG of the mRNA is assumed to determine the start of the protein coding sequence for the following reasons. (i) It represents the start of an open reading frame of 939 nucleotides, which codes for a protein sequence which is identical in 148 positions with the amino acid sequence of the beef heart carrier protein (Figures 5 and 6). (ii) The proposed *Neurospora* polypeptide is 16 amino acid residues longer than the beef heart polypeptide. This is in accordance with the observation, that the apparent mol. wt. of 33 000 of the *Neurospora* carrier is slightly higher than that of the beef heart carrier protein (mol. wt. 30 000). (iii) The bases preceding this ATG codon (ATATCACA) are similar to the corresponding segment of other *Neurospora* mRNAs (Viebrock *et al.*, 1982; Alton *et al.*, 1982; Kinnaird and Fincham, 1983; Wondt *et al.*, 1983; Schechtman and Yanofsky, 1983). This does not hold for all other in-phase ATGs of the sequence.

The carrier gene contains two introns, one after the 33rd and one after the 132nd base of the coding sequence. The nucleotides at the intron boundaries (GTAxGT----PyAG) correspond to intron-exon junction sequences of yeast and other *Neurospora* genes. The two introns contain a sequence TGCTPuACPu upstream of the 3′ splice site. These segments show some homology to sequences located at

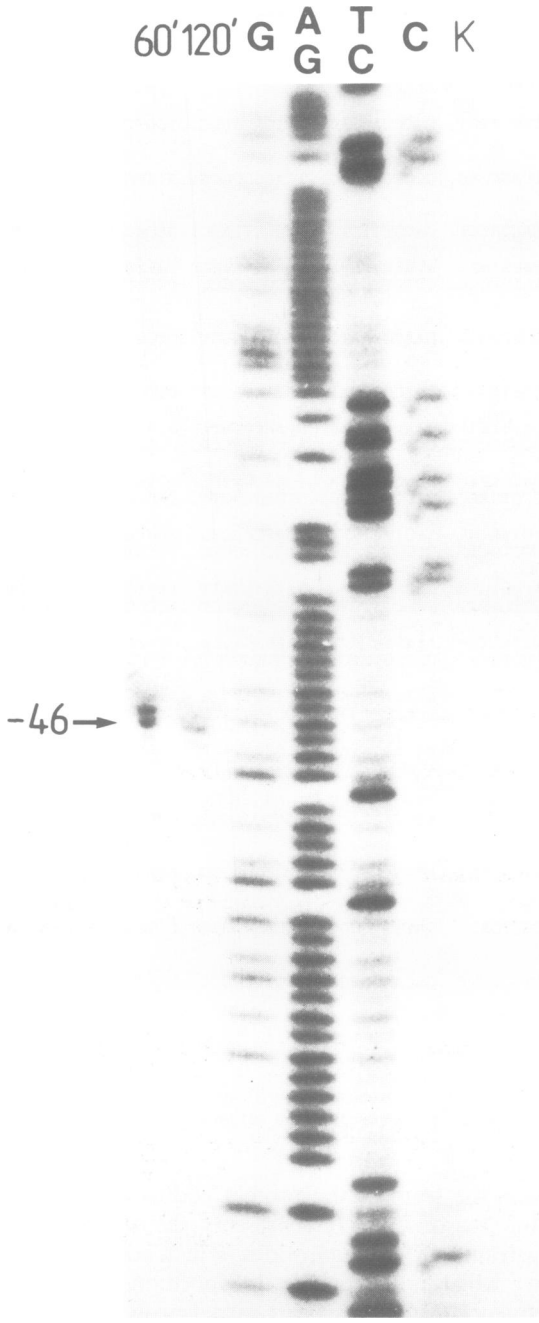

Fig. 4. Determination of the start of the mRNA by S1 nuclease mapping. S1 nuclease mapping was performed according to Weaver and Weissmann (1979). A double-stranded *Sph*I-*Pvu*II fragment, 5′ end-labelled at the *Sph*I site, was hybridized with total polyadenylated RNA. After hybridization, aliquots were digested with S1 nuclease (1000 U/ml) for 60 and 120 min. The RNA protected DNA was analysed on a sequencing gel in parallel with the same DNA fragment submitted to the four sequencing reactions and with the same DNA fragment hybridized with tRNA and treated with S1 nuclease as a control (K).

similar positions in the introns of other *Neurospora* genes (compiled by Kinnaird and Finach, 1983). They might correspond with the yeast intron sequence TACTAACA which Langford and Gallwitz (1983) have suggested to be essential for splicing. Remarkably, all *Neurospora* genes analysed up to now, with the exception of the *trp-1* gene, contain (usually two) short introns.

The primary translation product of the carrier is not shortened to a significant extent during the import into the mitochondria (Zimmerman *et al.*, 1979), in contrast to many other imported mitochondrial precursor proteins (for review see Hay *et al.*, 1983). Nevertheless, the import form of the precursor is released into the cytosol before the uptake into the organelle takes place, and apparently it has a conformation which differs from the functional protein (Zwizinski *et al.*, 1983). It is completely unclear at present which features of the carrier precursor determine the addressing for the mitochondria and the transport into the mitochondrial inner membrane. It has to be noted, however, that the carrier proteins from both *Neurospora* and beef heart have high positive charges (see Figures 5 and 6), as does the precursor of the proteolipid subunit of the *Neurospora* ATP synthase (Viebrock *et al.*, 1982). A specific pattern of positively charged surface residues could constitute the address label, and in principle a positive net charge could drive the protein across or into the mitochondrial inner membrane.

The amino acid sequence of the *Neurospora* ADP/ATP carrier derived from the nucleotide sequence of the mRNA exhibits a polarity profile as shown in Figure 5. Three domains of similar size and substructure (see Figure 6) can be discriminated, similar to but even more pronounced than those of the beef heart carrier (Aquila *et al.*, 1982). Each domain contains: (i) a lipophilic segment of 18–24 residues (I, III, V); (ii) a large polar segment of 31–36 residues (A, B, C); (iii) a 26-residue segment of middle polarity containing two or three basic residues but no acidic residues (II, IV, VI); and (iv) a short polar segment (a, b, c). There is marked homology between the amino acid sequences of the three domains (Figure 6), and it has been suggested that the whole polypeptide may have originated by triplication of an ancestral gene (Saraste and Walker, 1982). Interestingly enough, the homologies between the *Neurospora* and beef heart carrier are especially pronounced in certain segments of the polypeptide chain. (Identical positions are indicated in the middle part of Figure 5 by vertical bars, and they are underlined in Figure 6). The significance of these invariant features for the function of the ADP/ATP carrier remains to be established.

Materials and methods

Selection of mRNA by hybridization

cDNA clones were prepared and kept on microtitre plates at −20°C as described (Viebrock *et al.*, 1982). Individual clones were grown overnight at 37°C in 5 ml of LB medium plus tetracycline (5 μg/ml). 96 cultures were then combined for plasmid isolation. The procedures for binding of DNA to diazobenzyloxymethyl paper, for hybridization-selection of mRNA, for cell-free translation, for immunoadsorption, and for analysis of cell-free translation products by SDS-polyacrylamide gel electrophoreses are detailed elsewhere (Viebrock *et al.*, 1982).

Preparation of rabbit antisera

The ADP/ATP carrier was isolated from *Neurospora* mitochondria according to Hackenberg *et al.* (1978), except that the gel chromatography was performed with Sephadex G-100 (Pharmacia, Uppsala) in the presence of 2%

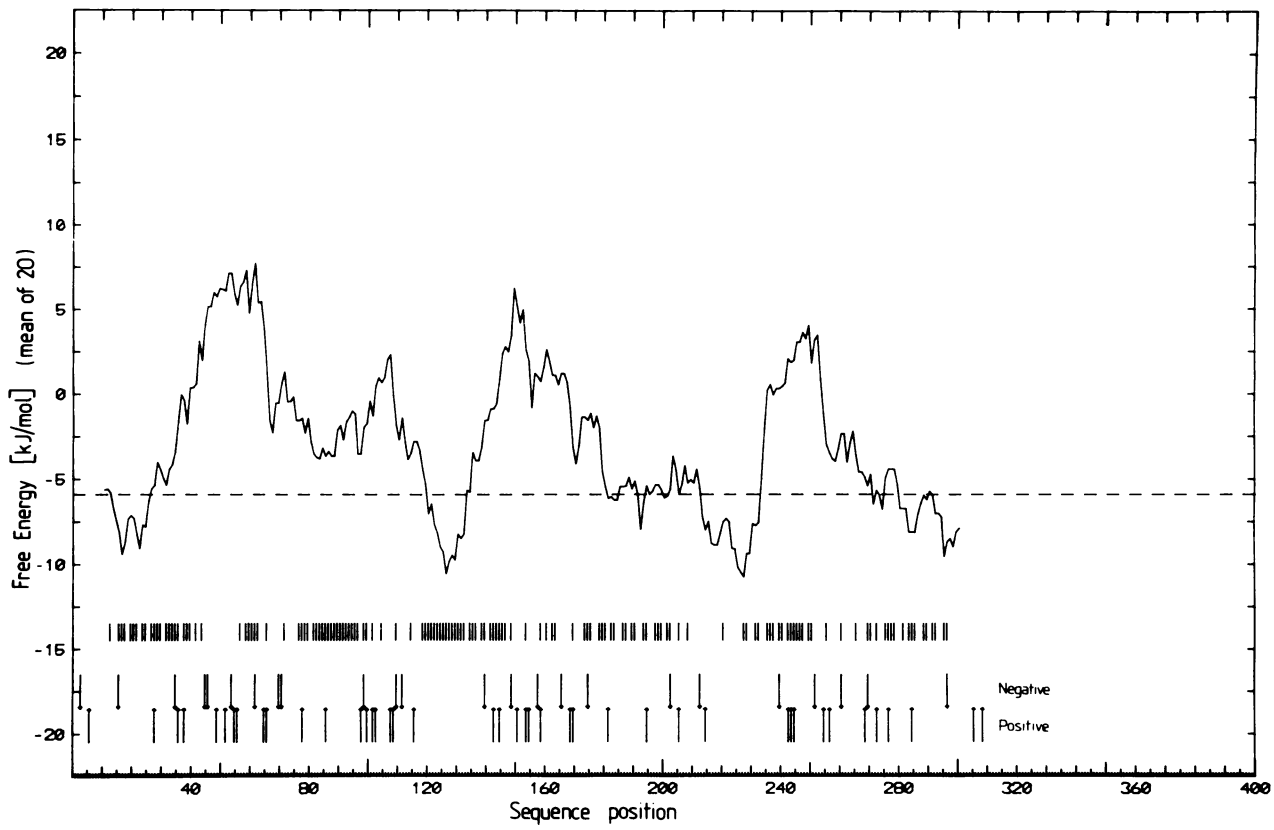

Fig. 5. Polarity profile and charged residues of the ADP/ATP carrier protein from *Neurospora*. **Top:** the gain of free energy during transition of a 20-residue segment from a random coil in water to an α-helix in the membrane is calculated for all sequence positions according to von Heijne (1981). **Middle:** positions occupied by identical amino acid residues in the *Neurospora* and beef heart carrier are indicated by a vertical bar. **Bottom:** the positions of basic residues (positive) and acidic residues (negative) are indicated by arrows.

Fig. 6. Triplicated structure of the carrier polypeptide from *Neurospora* and beef heart. B: Beef heart sequence; N: *Neurospora* sequence. Consensus: the alignment of the three segments is based on the positions which are occupied by identical or isofunctional residues. The amino acids are represented by the one-letter code, and postulated gaps by an asterisk. The lipophilic segments are designated I, III and V, and the segments of middle polarity II, IV and VI. The designation of the long polar segments in A, B and C, that of the short polar segments is a, b and c. Positions occupied by identical residues in the beef heart and *Neurospora* carrier are underlined. The basic residues in segments II, IV and VI are marked (+).

SDS. The eluted protein was precipitated with nine volumes of ethanol. Antisera were raised in rabbits with SDS-solubilized protein.

Isolation of the cloned gene

Genomic DNA of high mol. wt. was isolated from the cell-wall-less slime mutant (Perkins *et al.*, 1982) of *N. crassa* according to Gross-Bellard *et al.* (1973). The isolated DNA was cleaved with *Eco*RI, and then ligated with *Eco*RI-digested dephosphorylated vector pBR322. About 20 000 ampicillin-resistant clones were replicated on nitrocellulose filters, and submitted to colony-filter hybridization using nick-translated cDNA as probe.

DNA sequence analysis

Suitable DNA fragments were labelled either at the 5′ ends using [γ-^{32}P]ATP and T4 polynucleotide kinase, or at the 3′ ends using [α-^{32}P]dATP and the large fragment of *Escherichia coli* DNA polymerase I. Fragments labelled at single ends were obtained either by strand separation or after a second

cleavage. They were sequenced according to Maxam and Gilbert (1980), performing chemical modification at G, C plus T, C (Maxam and Gilbert, 1980), and A plus G (Burton, 1967). Both strands of the cloned cDNAs were analysed with the exception of a few short segments where one strand could be read unambiguously. The non-coding regions of the cloned genomic DNA were sequenced in both directions. The co-linearity of coding sequences in genomic DNA and cDNA was ascertained by establishing partial sequences and by mapping several restriction sites.

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