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PRIMARY STRUCTURE, *IN VITRO* EXPRESSION AND IMPORT INTO MITOCHONDRIA OF A 29/21-KDA SUBUNIT OF COMPLEX I FROM *NEUROSPORA CRASSA*

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A full-length cDNA clone coding for a cytoplasmically-synthesized subunit of complex I from *Neurospora crassa* (apparent molecular mass of 29 kDa) was isolated. DNA sequencing revealed an open reading frame coding for a protein containing 201 amino acids. A molecular mass of 21323 Da was calculated.

The precursor polypeptide was efficiently expressed *in vitro* and imported into isolated mitochondria. It is synthesized without a cleavable signal sequence and needs a membrane potential in order to bind to the mitochondrial membranes. © 1990 Academic Press, Inc.

Complex I (EC 1.6.5.3) seems to be the least studied constituent of the mitochondrial respiratory chain; this is probably due to its complicated composition of about 25 individual polypeptide subunits. The majority of the subunits of complex I are encoded by nuclear genes (for a recent review, see (1)). This applies also to most of the polypeptides that carry the 6-8 iron-sulfur clusters of the enzyme (2). Interestingly, a (rotenone-insensitive) isoform of complex I containing only nuclear-encoded subunits could be isolated from chloramphenicol-treated N. crassa cells (3). Most mitochondrial-imported proteins are synthesized in the cytoplasm with cleavable signal sequences that target them to the organelle (for review, see (4)).

In an attempt to elucidate the structure of cytoplasmically-synthesized subunits of complex I, we recently reported the molecular cloning of four nuclear-coded subunits of the *N. crassa* enzyme (5). Here, we describe the isolation and characterization of a full-length cDNA encoding a 29/21-kDa subunit. Efficient *in vitro* expression and import of the protein into isolated mitochondria was obtained. The primary structure of the polypeptide deduced from the DNA sequence is presented.

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MATERIALS AND METHODS

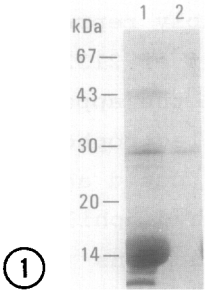
A lambda gt11 expression library containing sized cDNA inserts from *N. crassa* (300-900 base pairs) was screened with antiserum against the 29/21-kDa subunit of complex I. Several independent clones, whith proved to be related, were obtained (details will be published elsewhere,(5)). One of the isolated cDNA inserts (No. 51) was used to rescreen the library (containing cDNA inserts in sizes of 300-1500 base pairs) by hybridization. The largest cDNA insert found (No. 506) was subcloned into the pGEM4 transcription vector generating pGEM4.506. *In vitro* transcription and translation experiments were carried out as described (6, 7). Assays for import of precursor proteins into isolated *N. crassa* mitochondria were as reported (8).The isolation of the 29/21-kDa polypeptide and the production of antiserum against the protein (7), as well as immunoprecipitation and electrophoretic techniques (9), have been published. DNA sequencing was performed as detailed before (5). Both strands were sequenced at least twice.

RESULTS AND DISCUSSION

In vitro expression and nucleotide sequence of a cDNA clone encoding the 29/21-kDa subunit of complex I

pGEM4.506 was transcribed *in vitro* using SP6 RNA polymerase. The RNA was translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine, and the mixture was treated with an antiserum against the 29/21-kDa protein. Total translation products and the immunoprecipitated material were separately analyzed by SDS gel electrophoresis. A radioactively-labeled polypeptide could be specifically immunoprecipitated with the antiserum (Fig. 1). This result strongly suggests that cDNA insert 506 codes for the 29/21-kDa subunit of complex I.

The strategy used for sequencing the cDNA insert is outlined in Fig. 2. Fig. 3 displays the nucleotide sequence and the deduced



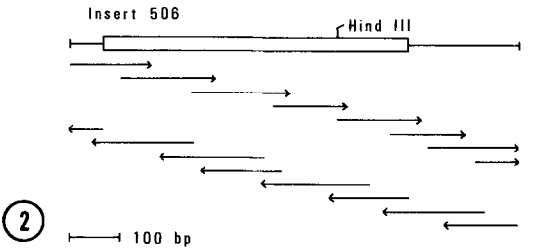


Fig.1. *In vitro* expression of the 29/21-kDa protein. The fluorograph shows: 1, total translation products; 2, material immunoprecipitated with antiserum against the 29/21-kDa protein.

Fig.2. Structure and sequencing strategy of the cDNA insert of pGEM4.506. The box represents the coding region. Arrows show the direction and extent of sequence determination.

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                                                                    GGAA
-60  TCCACACCTCGTCGTCGTTCACTTACACCACGATAACAAACGGGTTCTACAGCCGCCACC
  1  ATGGCTTCCAAAGTCGTCACCGGCGTGGTCAAGACCACCGCAGGCGGCGTTGTGCCCGTC
  1   M  A  S  K  V  V  T  G  V  V  K  T  T  A  G  G  V  V  P  V

 61  AGCCAGAAATACACTGTCCAATCCGTCGGCGTCTGGGAGCGCATCCGCCGCGCCTTCGCT
 21   S  Q  K  Y  T  V  Q  S  V  G  V  W  E  R  I  R  R  A  F  A

121  ATCGACCCTAACCGCTCCAACGGTGTTCCCTTGGTCCCCTACAATCGCAACCCATCCCCC
 41   I  D  P  N  R  S  N  G  V  P  L  V  P  Y  N  R  N  P  S  P

181  GGCTCCCTCGACCCCTTGGCCTACGACGATCCCGTCACCATTCCAGCCGGCGACATCGCC
 61   G  S  L  D  P  L  A  Y  D  D  P  V  T  I  P  A  G  D  I  A

241  GACAACCCGTACTGGAAGCGCGATGCCCGGCGCAACTACCCGCGCCTGAGCGTGGTGGGC
 81   D  N  P  Y  W  K  R  D  A  R  R  N  Y  P  R  L  S  V  V  G

301  CAGGCCGAGGCTGTTGCTCTGCTCAGTGTCGGCAGCGCGACGCACCCCCGTGTCGAGCTG
101   Q  A  E  A  V  A  L  L  S  V  G  S  A  T  H  P  R  V  E  L

361  GTGGGCGAGAACGGGAGCAAGCAGCTGGTTGCGGCGCAGGAGGCTGGCAAGACGGGTGGT
121   V  G  E  N  G  S  K  Q  L  V  A  A  Q  E  A  G  K  T  G  G
                                          Hind III
421  TTGGCCAAGTACTTTGAGGGAACCGGTGTGGAGGCTGGTAAGCTTGTGTTGGCGGAGACG
141   L  A  K  Y  F  E  G  T  G  V  E  A  G  K  L  V  L  A  E  T

481  GGAGGTCTGCCGCCGTTGCCTAGTGGTGAGAAGCTGGGGGAGGGCGGCAAGTGGGATGTT
161   G  G  L  P  P  L  P  S  G  E  K  L  G  E  G  G  K  W  D  V

541  TACAAGTATCAGTTGGCTGAGGAGCCTTCTTATTCTGAAGCCTACCCTTGCCGGTCGTTC
181   Y  K  Y  Q  L  A  E  E  P  S  Y  S  E  A  Y  P  C  R  S  F

601  TCTTAAATGGAAGAAGAGTGAGATGAGCTTGTGAGCAGGACACATGAGCAGAAAGGGACC
201   S

661  ACAGAACCTTTGATGGAGGGATAACAACAGACAGAACAAGACCACAGAGACCAGATACGT
721  TCGGGCTGGTGTACATAGCAGGCAAGCAAGCAAGCGGTAGACGGAACGTATAGACTTTGC
781  TTAGATTATGGCATTCAACGTCCAGAGGGAAATACGGTTTTTGAG
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Fig.3. Nucleotide sequence of the cDNA insert of pGEM4.506 and deduced primary structure of the 29/21-kDa protein.

protein sequence of the 29/21-kDa polypeptide. The cDNA insert has a length of 889 base pairs (we have estimated a size of 0.95 kilobases for the corresponding mRNA (5)), and contains an open reading frame coding for a protein of 201 amino acids. A TAA stop codon (indicated by a bar in Fig. 2) preceds, in frame, the first ATG triplet, thus indicating that it most probably represents the initiation codon. We calculated a molecular mass of 21323 Da for the protein, a value substantially smaller than the apparent molecular mass of 29 kDa estimated by SDS gel electrophoresis. Post-translational modifications of the protein could account for this discrepancy. The polypeptide is of rather hydrophilic nature and does not contain obvious membrane spanning domains (Fig. 4). Numberous β-turns are predicted in the secondary structure of the protein (Fig. 4), reflecting its high glycine content (11.4%). An extensively folded polypeptide chain would be thus expected.

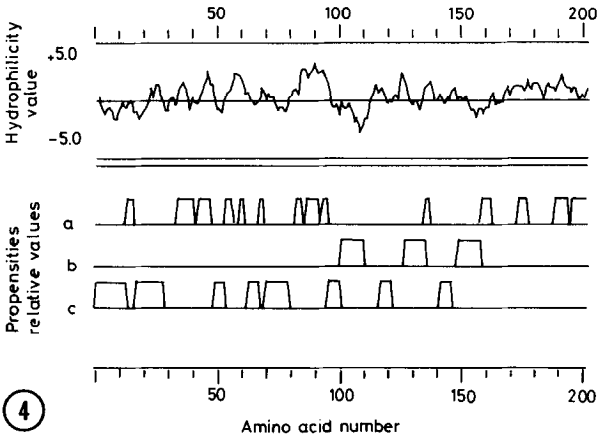
The *in vitro*-synthesized polypeptide (Fig. 1) and the mature subunit assembled into complex I (7) display the same apparent

molecular mass (29 kDa). This indicates that this subunit of complex I is not made as an extended precursor. In earlier experiments (translation of poly(A)$^+$ RNA in rabbit reticulocyte lysates in the presence of [^{35}S]methionine followed by immunoprecipitation with a specific antiserum) no radioactive precursor of the 29/21-kDa protein was detected (7). The amino acid sequence of the polypeptide may explain this (negative) result. Only one (amino-terminal) methionine is present and often this particular residue is removed in the reticulocyte lysates (R. Zimmermann, personal communication). In the experiments described above (see Fig.1), however, a weak labeling with [^{35}S]methionine of the *in vitro*-synthesized 29/21-kDa polypeptide was detected. This is probably due to incomplete removal of amino-terminal residues together with a more efficient synthesis of the protein (using specific RNA in this case).

No significant homology was found between the 29/21-kDa protein and sequences compiled in the latest versions of protein (MIPSX, NBRF) or DNA (MIPSY) data banks.

Import of the 29/21-kDa protein into isolated mitochondria

Using an *in vitro* transcription/translation system, as described above, we synthesized the 29/21-kDa polypeptide in the



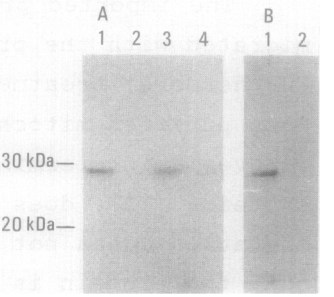


Fig.4. Predicted structural features of the 29/21-kDa polypeptide. Upper panel: polarity profile according to (10). Lower panel: potentials for α-helix (a), β-sheet (b) and β-turn (c) structures according to (11).

Fig.5. Import of the 29/21-kDa precursor into isolated mitochondria.All experiments were carried out in incubation buffer(8),containing 80 mM KCl, 5 mM $MgCl_2$ and 2 mM NADH. A fluorograph of the gel is shown. A) The import experiments were performed in the absence (1 and 3) or presence (2 and 4) of 1 µM valinomycin. Proteinase K was then added to the assays which results are depicted in lanes 3 and 4 (final conc. 80 µg/ml). B) After an import experiment, proteinase K was added to the assays in the absence (1) or presence (2) of 1% Triton X-100.

presence of [^{3}H]valine. Then, the lysates were incubated with isolated mitochondria either in the presence or absence of valinomycin, an inhibitor of mitochondrial membrane potential. Each experiment was performed in duplicate and only one of the samples was further treated with proteinase K. In the absence of valinomycin, the polypeptide was imported into mitochondria (proteinase-resistant location) (Fig. 5A). We analyzed fluorographs of three separate experiments by densitometry, and estimated that about 75% of the polypeptide had entered mitochondria. When the mitochondrial membrane potential is abolished by the action of valinomycin, neither import nor binding of the precursor to mitochondria occurred.

In order to confirm that the proteinase-resistant material observed in these experiments represented imported protein and was not merely due to resistence of the 29/21-kDa polypeptide to digestion by proteinase K under the conditions employed, an import experiment was performed, divided in two halves, and Triton X-100 was added to one of the aliquots. Then, both samples were treated with proteinase K. Fig. 5B shows that digestion of the 29/21-kDa protein occurred only in the presence of the detergent, indicating that the polypeptide had entered mitochondria. The proteinase K sensitivity of the polypeptide synthesised *in vitro* in reticulocyte lysates was also tested, revealing that the protein was sensitive to a 10-fold less concentration of proteinase than that used in the import experiments (result not shown).

The imported protein and the complex I-assembled subunit co-migrated with the precursor polypeptide upon electrophoresis. Furthermore, treatment of the *in vitro*-synthesized polypeptide with purified mitochondrial processing peptidase, the enzyme that removes amino-terminal sequences from mitochondrial-imported proteins (12), does not result in an altered electrophoretic migration (data not shown). These results support the conclusion that the protein is not synthesized with a cleavable presequence.

Mitochondrial targeting signals are usually positively charged, amphiphilic (13) and located at the amino-terminus of precursor proteins (4, 14). The amino-terminal region of the 29/21-kDa polypeptide clearly resembles a mitochondrial signal sequence, containing serine-, threonine- and basic (lysine) residues; the first acidic amino acid appearing is located at position 33 (glutamic acid, see Fig. 3).

Mitochondrial precursor proteins, which depend on a membrane potential for import, have been divided in two categories:

polypeptides that are able (class I) or unable (class II) to bind specifically to mitochondria in the absence of membrane potential (8). Due to its import characteristics, the 29/21-kDa subunit of complex I should be included in the class II precursors.

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