The 12.3 kDa subunit of complex I (respiratory-chain NADH dehydrogenase) from *Neurospora crassa*: cDNA cloning and chromosomal mapping of the gene

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The 12.3 kDa subunit of complex I (respiratory-chain NADH dehydrogenase) is a nuclear-coded protein of the hydrophobic fragment of the enzyme. We have isolated and sequenced a full-length cDNA clone coding for this polypeptide. The deduced protein is 104 amino acid residues long with a molecular mass of 12 305 Da. This particular subunit of complex I lacks a cleavable mitochondrial targeting sequence. In agreement with its localization within complex I, we have found that this subunit behaves like an intrinsic membrane protein. Nevertheless, the

deduced protein is rather hydrophilic, exhibiting no hydrophobic domain long enough to traverse a membrane in an α -helical conformation. The 12.3 kDa subunit shows a significant similarity to the hinge protein of complex III, suggesting that these two polypeptides may be involved in identical functions. This complex I subunit is coded for by a single gene. Applying restriction-fragment-length-polymorphism mapping, we located the gene on the right side of the centromere in linkage group I, linked to the *lys-4* locus.

INTRODUCTION

Respiratory-chain NADH dehydrogenase (complex I, EC 1.6.99.3) is one of the components of the inner mitochondrial membrane performing proton pumping coupled to electron transfer. It is the most complicated assembly among the oligomeric enzymes that participate in oxidative phosphorylation, both in terms of prosthetic groups and polypeptide subunits [for reviews, see Ragan (1987) and Weiss et al. (1991)]. In humans and *Neurospora*, seven complex I subunits are encoded and synthesized by mitochondria (Chomyn et al., 1986; Ise et al., 1985; Videira and Werner, 1989). However, the majority of the subunits, including most that bind prosthetic groups, are encoded by the nucleus (Chomyn et al., 1988). The DNA sequences of the human mitochondrial genes have been published for quite some time (Anderson et al., 1981). In the last years, we have participated in efforts to clone the nuclear-coded proteins.

The high similarity between complex I from fungi and mammals (Ragan, 1987; Weiss et al., 1991) makes *N. crassa* a good model for studying the enzyme. Since the cloning of a 20.8 kDa polypeptide of the fungal enzyme (Videira et al., 1990a), several other nuclear-coded subunits have also been cloned in this organism. In some cases, the existence of sequence similarities to proteins of known function provided good indications about the function of those subunits (see Weiss et al., 1991).

Still unknown at present is the organization of nuclear genes for complex I subunits. Thus we started to assign genes to specific linkage groups of the *N. crassa* genome. The gene encoding the protein described here was localized on chromosome I, close to the *lys-4* locus. Our interest in these experiments is not only to gain a general idea of the chromosomal localization of

genes coding for complex I subunits, but also because these assignments might be essential for gene-disruption experiments.

Here we describe the isolation and analysis of the cDNA encoding a 12.3 kDa subunit of the hydrophobic fraction of complex I. The similarity between this protein and the so-called hinge protein of the *b-c₁* complex may indicate that both polypeptides play a similar role in the respiratory chain.

MATERIALS AND METHODS

A mono-specific antiserum against the 12.3 kDa protein (Videira and Werner, 1989) was used to screen a *Neurospora* cDNA library cloned in the lambda gt11 expression vector. The procedure, as well as production of fusion proteins by recombinant phage and their use for affinity purification of antibodies, and the subcloning and sequencing of both strands of cDNA inserts, have been detailed previously (Videira et al., 1990a).

The techniques for the preparation of *N. crassa* mitochondria (Werner, 1977), carbonate extraction (Fujiki et al., 1982; Azevedo et al., 1992), protein gel electrophoresis (Laemmli, 1970; Videira and Werner, 1989) and Western blotting (Towbin et al., 1979), the detection of antigens bound to filters (Azevedo et al., 1992) as well as *in vitro* transcription and translation (Maniatis et al., 1982; Videira et al., 1990a), and methods for immunoprecipitation (Werner and Sebald, 1981; Zauner et al., 1985) have been published previously.

The 12.3 kDa protein, formerly referred to as the 14 kDa subunit, was isolated from complex I (Videira and Werner, 1989) and partially sequenced from the N-terminus by automated Edman degradation (Eckerskorn et al., 1988).

The 38 *N. crassa* strains representing the Multicent-2 cross kit

Abbreviation used: RFLP, restriction-fragment-length polymorphism.

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The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X68965.

(Metzenberg et al., 1984) were obtained from the Fungal Genetics Stock Center (FGSC # 4450-4487). Genomic DNA was isolated essentially as described by Borges et al. (1990), except that a final step of RNAase treatment, followed by organic extraction and ethanol precipitation, was included. Southern (1975) blotting on Hybond membranes, radioactive labelling of DNA using the multiprime labelling system and hybridization were performed according to the manufacturer's (Amersham) instructions.

RESULTS AND DISCUSSION

Cloning and characterization of cDNAs encoding the 12.3 kDa polypeptide (NUO-12.3)

An *N. crassa* cDNA expression library was screened with an antiserum against a nuclear-coded subunit of complex I (Videira and Werner, 1989). Several positive phages were isolated and shown, by Southern blotting, to belong to two unrelated groups (groups I and II; results not shown). In order to determine whether or not the relevant clone was isolated, *Escherichia coli* lysogens from the two groups of phage were prepared and induced for the production of the β -galactosidase fusion proteins. These were resolved by SDS/PAGE, blotted on to nitrocellulose membranes and used to affinity-purify immunoglobulins from the crude antiserum. Only the clones from group II were able to select immunoglobulins that recognize the relevant protein on Western blots of complex I (results not shown), indicating that group II phage indeed contain the correct cDNA insert.

Several cDNA inserts from phage of group II were subcloned in the pGEM4 transcription vector and sequenced at both termini. The sequences obtained were then searched for the N-terminus of the mature protein (see below). One particular cDNA insert (no. 14) was found to be full-length and was completely sequenced (Figure 1). A coding region of 312 bp, between 53 bp and 329 bp of 5' and 3' untranslated regions respectively, was found. The open reading frame codes for a protein of 104 amino acid residues, with a calculated molecular mass of 12305 Da. Strikingly, the predicted primary structure reveals a rather hydrophilic protein. No obvious membrane-spanning domain is found (Figure 2). The deduced primary structure of NUO-12.3 reveals the existence of only two cysteine residues. Thus this polypeptide is not a good candidate to bind iron-sulphur clusters unless other amino acids (e.g. histidine and tyrosine) serve as appropriate ligands.

In order to gain an insight into the function of this polypeptide, a sequence search in a protein data bank was performed. A significant similarity between NUO-12.3 and the so-called hinge protein of complex III from mammals was detected (see Figure 3). The *N. crassa* and bovine proteins display 47% similarity in a domain of 36 amino acids. The hinge protein mediates the interaction of cytochrome *c* with cytochrome *c*₁ (Wakabayashi et al., 1982), and may regulate the electron-transfer reaction between these polypeptides (Kim et al., 1987). It may be speculated that NUO-12.3 plays a similar role in complex I. In this respect it could allow the interaction between different complex I subunits or even between complex I and other proteins [for instance, with matrix dehydrogenases, a phenomenon that was reported in mammalian mitochondria (Fukushima et al., 1989)]. The last possibility is not so likely, however, if one assumes that NUO-12.3 is surrounded by other subunits of the hydrophobic arm of complex I (see below).

Protein data

The 12.3 kDa protein was isolated from complex I of *N. crassa*, applying chaotropic agents and hydrophobic-interaction

chromatography, as described previously (Videira and Werner, 1989). The purified polypeptide was subjected to automated Edman degradation, and the sequence PTPESAAFLAKKPT was obtained. This sequence matches the N-terminal sequence predicted from the clone pNUO-12.3 (amino acids 2–15; underlined in Figure 1), thus confirming unambiguously the identity of

Figure 1 Nucleotide sequence of full-length cDNA and deduced primary structure of the 12.3 kDa protein

(a) Structure and sequencing strategy of the cDNA. The box represents the translated region. Arrows show the direction and extent of sequence determination. (b) cDNA and protein sequences. The amino acid positions determined also by protein sequencing (residues 2–15) are underlined.

Figure 2 Hydrophilicity profile of the 12.3 kDa protein

Calculations were performed as described by Hopp and Woods (1981), using a window of 11 amino acids.

Figure 3 Alignment of the amino acid sequences of the 12.3 kDa protein (a) and the bovine hinge protein (b)

Numbers refer to positions of amino acid residues in the mature proteins. Identical residues are boxed, and conservative substitutions are indicated by asterisks. In the region shown, the human (Ohta et al., 1987) and bovine (Wakabayashi et al., 1982) hinge proteins are identical, except that the last position of the human polypeptide is occupied by aspartic acid instead of valine.

Figure 4 Localization of the 12.3 kDa protein after alkaline extraction of mitochondria

(a) Mitochondria were incubated with Na_2CO_3 solution, and half of the mixture was centrifuged to separate membranes (pellet) from solubilized material (supernatant). The total sample before centrifugation (T), the pellet (P) and the supernatant (S) were separated by SDS/PAGE. Then a Western blot was performed using antibodies against the 30.4 kDa and the 12.3 kDa subunits of complex I. (b) The 12.3 kDa polypeptide was synthesized *in vitro* in the presence of [^{35}S]methionine. The material was mixed with mitochondria and processed for Na_2CO_3 treatment as detailed in (a). After SDS/PAGE the gel was fluorographed. Abbreviation: M, molecular mass.

the isolated cDNA clone. This particular mitochondrial protein is synthesized without a cleavable targeting sequence (Videira and Werner, 1989). The initial methionine residue is, however, absent in the mature sequence.

As already mentioned, NUO-12.3 is a hydrophilic protein. Nevertheless, it is a component of the hydrophobic arm of complex I, a fragment of the enzyme that is believed to be embedded in the mitochondrial inner membrane (Tuschen et al., 1990). Thus the question is raised as to the kind of interaction through which NUO-12.3 is associated with the other subunits of the enzyme. On the basis of hydropathy data for the polypeptide one might predict that the 12.3 kDa subunit is in a hydrophilic environment. In order to test this possibility, we performed alkaline extraction of mitochondrial membranes. At the rather high pH used in this procedure (pH 11.5) protein-membrane interactions established through or with accessibility to the aqueous environment are readily disrupted. Hence, freshly isolated *N. crassa* mitochondria were incubated with 0.1 M Na_2CO_3 (pH 11.5) and separated into soluble and membrane fractions by centrifugation for 1 h at 165 000 g. The proteins in the pellet and supernatant were then resolved by SDS/PAGE and analysed by Western blotting with an antiserum against the 12.3 kDa subunit. As a positive control we also tested the behaviour of the 30.4 kDa subunit of complex I (Videira et al., 1990b), a protein that belongs to the peripheral arm of the enzyme (Weiss et al., 1991). This polypeptide is known to be extractable under these conditions (Azevedo et al., 1992). As shown in Figure 4(a), NUO-12.3 cannot be extracted from the mitochondrial inner membrane by applying this technique. This result strongly suggests that the interaction of NUO-12.3 with the membrane occurs in an environment not accessible to the aqueous medium. Because of this unexpected finding we still considered the possibility that the 12.3 kDa subunit *per se* would not be soluble under the conditions employed. Therefore, a parallel experiment was carried out. Immediately before treatment with the carbonate solution, mitochondria were mixed with radioactively labelled NUO-12.3 that was synthesized in a heterologous *in vitro* transcription/translation system. The samples were processed as described above, except that the gel was subjected to fluorography. As

Figure 5 Chromosomal mapping of *nuo-12.3*

(a) Genomic DNA from the indicated strains (Q2, Q4, R1 and R4) was digested with *EcoRI* and resolved on an agarose gel. Then a Southern blot was performed using radioactively labelled cDNA insert 14 as a probe. The autoradiograph is shown. (b) Segregation of the RFLP among progeny of the cross between polymorphic strains. The results were obtained from an experiment as described in (a). Restriction fragments (RF) of the Mauriceville type (M), of the Oak Ridge type (O) and unidentified (-) are indicated.

expected, NUO-12.3 alone does not precipitate under these conditions (Figure 4b), providing evidence that only the correctly folded/assembled subunit behaves as an intrinsic membrane protein. It should be noted that the *in vitro*-synthesized subunit co-migrates with the mature/endogenous subunit upon SDS/PAGE performed under reductive or non-reductive conditions (A. Videira and S. Werner, unpublished work). Thus it is unlikely that the assembled subunit undergoes some covalent modification (e.g., lipoylation or cysteine bridging with other intrinsic membrane protein) that could explain the resistance to the alkaline extraction.

We have described the molecular cloning of the 12.3 kDa subunit of the hydrophobic arm of complex I from *N. crassa*. Notwithstanding its hydrophilic characteristics and the absence of an obvious membrane spanning domain, NUO-12.3 behaves like an intrinsic membrane protein. On the bases of double immunodiffusion (Werner and Sebald, 1981), it was already known that the protein was not recognized by the specific antiserum using intact complex I; only when isolated complex I is exposed to a detergent like SDS or a combination of deoxycholate and 2-mercaptoethanol does a precipitation reaction occur (A. Videira and S. Werner, unpublished work). Thus at least the antigenic determinants of the polypeptide seem not to be accessible to the immunoglobulins in the intact enzyme. This result, as well as the resistance of NUO-12.3 to alkaline extraction from the mitochondrial inner membrane, suggest that the polypeptide is embedded within other complex I constituents.

Chromosomal assignment of the *nuo-12.3* gene

In order to localize the gene for NUO-12.3 in the genome of *N. crassa*, the restriction-fragment-length-polymorphism (RFLP) mapping approach was applied. Genomic DNA from the individual progeny of the Multicent-2 cross kit was isolated, treated with several restriction enzymes and separated by electrophoresis in agarose gels. Southern blots were then probed with ³²P-labelled cDNA encoding NUO-12.3. A RFLP was found when using the enzyme *Eco*RI. The results obtained are summarized in Figure 5. Comparison with known markers (Metzenberg and Grotelueschen, 1989), revealed that the segregation pattern observed is very similar to those displayed by several genes of linkage group I. To test the linkage, the χ -square statistical test was employed. The highest value, 28.2, (considering a limit value of 3.85 for one degree of freedom), was obtained between *nuo-12.3* and *lys-4*, clearly indicating that the two genes are linked.

Significant values were also obtained for other genes located in the vicinity of the *lys-4* locus.

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