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# Characterisation of the last Fe-S cluster-binding subunit of *Neurospora crassa* complex I[1]

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**Abstract**

We have cloned cDNAs encoding the last iron-sulphur protein of complex I from *Neurospora crassa*. The cDNA sequence contains an open reading frame that codes for a precursor polypeptide of 226 amino acid residues with a molecular mass of 24 972 Da. Our results indicate that the mature protein belongs probably to the peripheral arm of complex I and is rather unstable when not assembled into the enzyme. The protein is highly homologous to the PSST subunit of bovine complex I, the most likely candidate to bind iron-sulphur cluster N-2. All the amino acid residues proposed to bind such a cluster are conserved in the fungal protein. 

*Keywords:* Mitochondrion; NADH dehydrogenase; Complex I; Iron-sulfur protein; cDNA; (*Neurospora crassa*)

## 1. Introduction

The $H^+$-translocating NADH:ubiquinone oxidoreductase (complex I, EC 1.6.5.3) is the first enzyme of the mitochondrial respiratory chain. The mitochondrial enzyme contains about 40 polypeptides, seven encoded by mitochondria and the others of nuclear origin. Several prosthetic groups are involved in complex I activity, namely FMN and about six Fe-S clusters (for reviews see [1,2]). The findings that several bacteria have complex I (also called NDH-1) with a simpler polypeptide composition, but a full set of redox centres [3–5], supported the idea that a 'minimal complex I' would be formed by 14 subunits [1]. Of the 'minimal 14 subunits', only the homologue of the bovine PSST subunit [6] has not been identified in *Neurospora crassa*. The characteristics of PSST make it a likely candidate to bind iron-sulphur cluster N-2, the cluster of complex I with the highest midpoint potential and most probably the ubiquinone reducer [7,8]. It should be noted, however, that the TYKY subunit was also suggested to bind cluster N-2 [9]. The importance of cloning the PSST homologue in the fungus lies in the fact that, presently, this is the only organism that provides both an eukaryotic model for complex I study and the possibilities of genetic manipulation [2].

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[1] The sequence data have been submitted to the EMBL Data Library under the accession number AJ001520.

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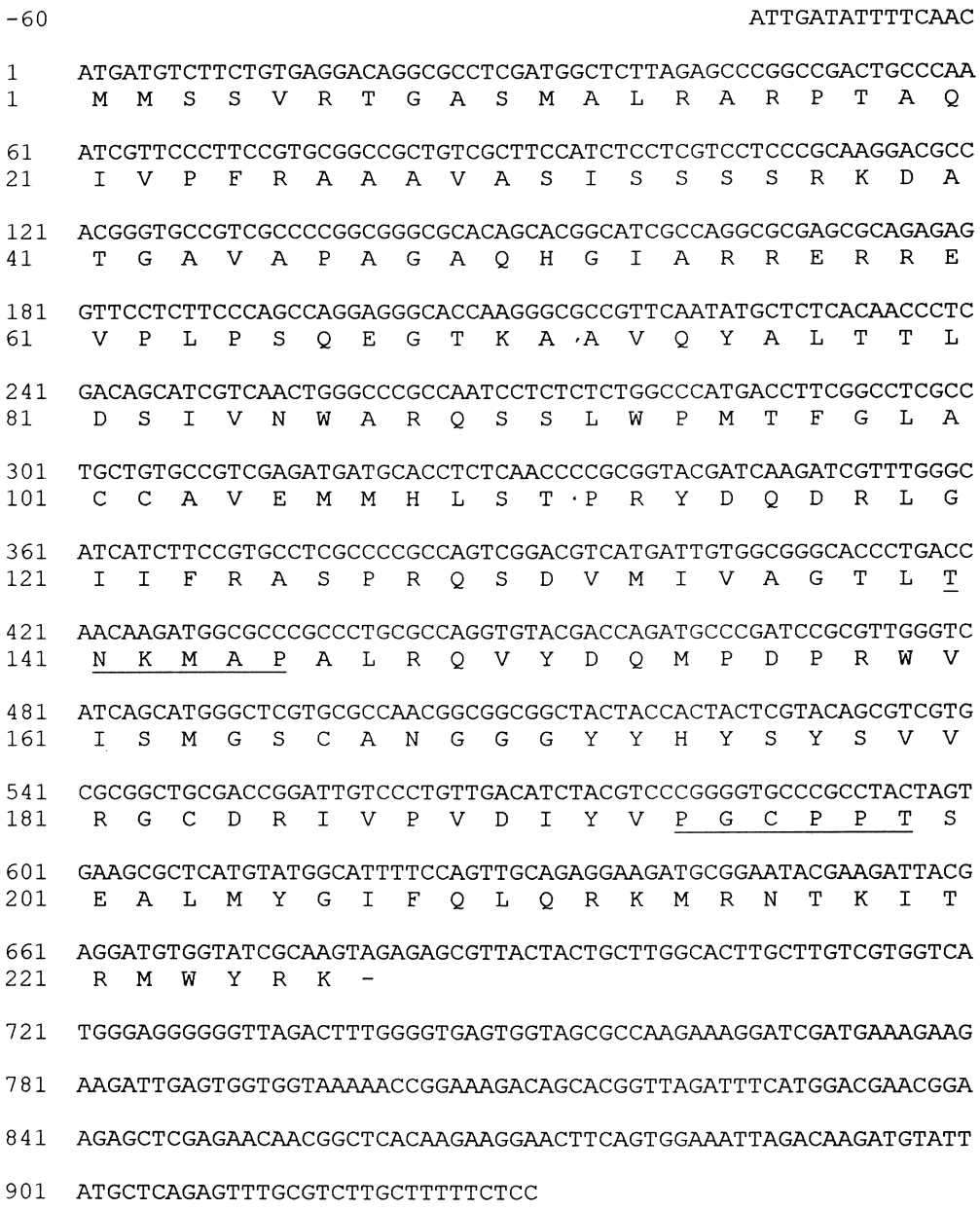


Fig. 1. cDNA sequence and deduced primary structure of the 19.3 kDa iron-sulphur protein of complex I from *N. crassa*. The conserved amino acid residues used to design degenerated primers are underlined.

## 2. Materials and methods

In order to clone the PSST homologue, we designed degenerated primers based on conserved amino acid sequences of the protein. Using these primers together with *N. crassa* genomic DNA in a PCR-amplification, we obtained a 190-bp fragment that was cloned in pUC19 and further subjected to an amplification/labelling reaction using the DIG DNA Labelling Kit (Boehringer Mannheim). $1\times10^6$ phages from a *N. crassa* cDNA library [10] were screened with this probe and seven positives were isolated. The cDNA inserts of three of them were then subcloned in the pGEM4 plasmid vector. The DNA sequence was obtained by sequencing both strands of the different cDNAs with ThermoSequenase (United States Biochemical), using pGEM4 as well as cDNA-specific primers, as described before

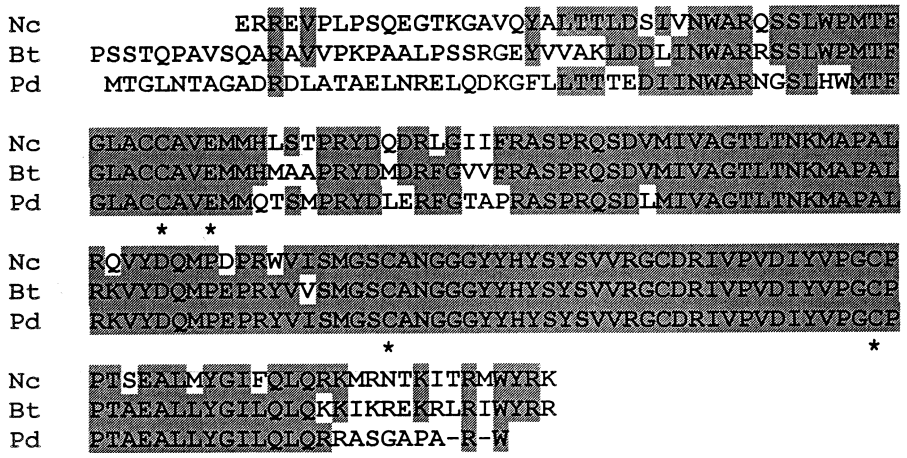


Fig. 2. Alignment of the protein sequences of the 19.3 kDa subunit of complex I from *N. crassa* (Nc, putative mature protein) with homologues from *B. taurus* (Bt) and *P. denitrificans* (Pd). Identical amino acids are shadowed and the four residues suggested to ligate Fe-S cluster N-2 are marked with asterisks.

[11]. In order to express the protein, the cDNA was cloned in the *Eco*RI site of plasmid pGEX-5X-2 (Pharmacia) and transformed into *Escherichia coli* strain BL21 [12]. Bacterial cells were induced for expression of the fusion protein during 3 h at 37°C by the addition of 1 mM IPTG to an exponentially growing culture ($A_{600}$ = 0.6–0.8). Inclusion bodies were washed twice with PBS containing 3 M urea, resolved by SDS-PAGE and blotted to nitrocellulose. Strips of nitrocellulose containing the relevant polypeptide were cut, solubilised with DMSO and used to raise rabbit antiserum [13]. The techniques for the preparation of *N. crassa* mitochondria [14], carbonate extraction [15], and Western blotting [13] have been published previously.

## 3. Results and discussion

The nucleotide sequence of the cloned cDNA contains an open reading frame that encodes a precursor protein of 226 amino acid residues with a molecular mass of 24 972 Da (Fig. 1). Although other initiation codons may lie further upstream, the indicated start codon is within a sequence with five matches to the seven-base *N. crassa* consensus sequence surrounding translation start codons [16], strongly suggesting that it represents the initiation codon. A computer analysis (based on the charge transition and arginine-2 rule) predicts that the first 56 amino acid residues represent a mitochondrial targeting peptide. This would yield a mature protein with a molecular mass of 19 337 Da, thus named the NUO-19.3 protein, close to its bovine and *Paracoccus denitrificans* homologues with 20.1 and 19.1 kDa, respectively [6,17].

Among other organisms, NUO-19.3 exhibits very high homologies with subunits from *Bos taurus* [6], *P. denitrificans* [17] (Fig. 2) and, slightly less, with *E. coli* complex [18]. This is probably the most conserved complex I protein and it has been suggested to house iron-sulphur cluster N-2 [19,20]. This Fe-S cluster would probably be coordinated by three cysteine residues that are conserved in all PSST homologues (a CysPro motif within one of them insinuates the presence of a [4Fe-4S] cluster), and a strictly conserved glutamate located three residues downstream the first conserved cysteine [6,7]. A cluster

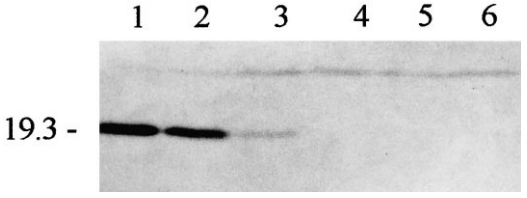


Fig. 3. Detection of NUO-19.3 in mitochondria from different *N. crassa* strains. Total mitochondrial proteins from the wild type strain (1), the complex I mutants nuo21 (2), nuo51 (3), nuo20.8 (4), and nuo21.3c (5), and wild type strain grown in the presence of chloramphenicol (6) were analysed in Western blots immunodecorated with antiserum against the 19.3 kDa protein.

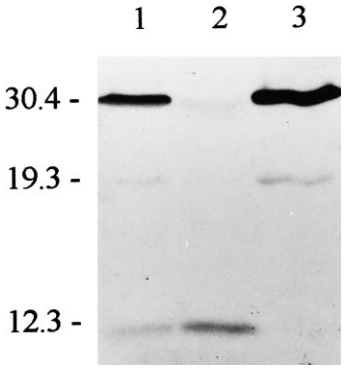


Fig. 4. Localisation of NUO-19.3 after alkaline extraction of mitochondria. Mitochondria from wild type *N. crassa* were subjected to an alkaline treatment and centrifuged. Untreated mitochondria (1) and the resulting membrane pellet (2) and soluble fractions (3) were analysed in Western blots immunodecorated with antiserum against the 30.4, 19.3 and 12.3 kDa subunits of complex I.

with such ligands would have unusual properties, in agreement with those of cluster N-2 [20]. It may be pointed out that several theories postulate that this centre is directly involved in proton translocation [20–22], meaning that both cluster N-2 and its harbouring subunit are of major importance to complex I activity.

We have also investigated the location of NUO-19.3 within complex I. Fig. 3 shows results of immunodetection of the protein in mitochondria from several strains of *N. crassa*. The mutants nuo51 [23], and nuo21 ([24]; unpublished data) are able to assemble an almost intact complex I except for the lack of the respective protein. Mutants nuo20.8 [25] and nuo21.3c ([11]; unpublished data) are blocked in complex I assembly: the former assembles the peripheral arm and intermediates of the membrane arm of the enzyme and the latter only forms the membrane arm of complex I. The peripheral arm of complex I accumulates in the wild type strain grown in the presence of chloramphenicol [26]. Interestingly, NUO-19.3 was clearly detected only when the peripheral and membrane domains of complex I are joined together (wild type strain and mutants nuo21 and nuo51, lanes 1–3 of Fig. 3). It is faintly visible or absent in mitochondria from strains forming unassembled peripheral arm (nuo20.8 and chloramphenicol-grown wild type, Fig. 3, lanes 4 and 6) or unassembled membrane arm (nuo21.3c, Fig. 3, lane 5). There are no predicted transmembrane α-helices in NUO-19.3. Nonetheless, the *E. coli* homologue of this subunit was reported to reside within the connecting fragment of the enzyme [19]. Due to this we have examined whether or not NUO-19.3 is an integral membrane protein and the results are shown in Fig. 4. NUO-19.3 was readily extracted from the mitochondrial membranes at high pH, indicating that it is not an intrinsic membrane protein. As controls in this experiment, we also analysed the behaviour of the (peripheral arm) 30.4 kDa and (membrane arm) 12.3 kDa subunits of complex I [27,28]. As expected, the 30.4 kDa protein can be solubilised by the alkaline treatment while the 12.3 kDa protein remains in the pellet fraction. The 12.3 kDa or the 20.8 kDa subunits of the membrane arm of complex I are not extracted from the membranes in the conditions employed, despite that both proteins are of a rather hydrophilic nature [10,28]. Taken together, our results suggest that NUO-19.3 belongs to the peripheral arm of complex I, even though it was not detected in this subcomplex (not shown). It was, however, found in subcomplexes Il and IlS of bovine complex I [29]. The reason for the absence of the fungal PSST from the isolated peripheral arm may be its instability when this domain of complex I is not assembled with the rest of the enzyme. This would be in agreement with the absence of cluster N-2 in the isolated peripheral arm of complex I from *N. crassa* [30], and the presence of this cluster in the bovine subcomplexes Iλ and IλS [29]. Interestingly, the TYKY-homologue of *N. crassa* was clearly identified in the peripheral arm of complex I [31], thus lending additional support to the suggestion that PSST, instead of TYKY, binds cluster N-2.

This work completes the identification and determination of the primary structure of all the 'minimal subunits' of complex I from *N. crassa*, and opens the way for further studies on this enzyme by means of site-directed mutagenesis.

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## References

[1] J.E. Walker, Q. Rev. Biophys. 25 (1992) 253–324.
[2] A. Videira, Biochim. Biophys. Acta 1364 (1998) 89–100.
[3] T. Yagi, T. Yano, S. Di Bernardo, A. Matsuno-Yagi, Biochim. Biophys. Acta 1364 (1998) 125–133.
[4] T. Friedrich, Biochim. Biophys. Acta 1364 (1998) 134–146.
[5] A. Dupuis, M. Chevallet, E. Darrouzet, H. Duborjal, J. Lunardi, J.P. Issartel, Biochim. Biophys. Acta 1364 (1998) 147–165.
[6] J.M. Arizmendi, M.J. Runswick, J.M. Skehel, J.E. Walker, FEBS Lett. 301 (1992) 237–242.
[7] T. Ohnishi, J. Bioenerg. Biomembr. 25 (1993) 325–330.
[8] U. Brandt, Biochim. Biophys. Acta 1318 (1997) 79–91.
[9] S.P.J. Albracht, A.M. de Jong, Biochim. Biophys. Acta 1318 (1997) 92–106.
[10] A. Videira, M. Tropschug, E. Wachter, H. Schneider, S. Werner, J. Biol. Chem. 265 (1990) 13060–13065.
[11] M. Duarte, M. Finel, A. Videira, Biochim. Biophys. Acta 1275 (1996) 151–153.
[12] F.W. Studier, B.A. Moffatt, J. Mol. Biol. 189 (1986) 113–130.
[13] A. Videira, S. Werner, Eur. J. Biochem. 181 (1989) 493–502.
[14] S. Werner, Eur. J. Biochem. 79 (1977) 103–110.
[15] J.E. Azevedo, U. Nehls, C. Eckerskorn, H. Heinrich, H. Rothe, H. Weiss, S. Werner, Biochem. J. 288 (1992) 29–34.
[16] T.L. Legerton, C. Yanofsky, Gene 39 (1985) 129–140.
[17] X. Xu, A. Matsuno-Yagi, T. Yagi, Biochemistry 31 (1992) 6925–6932.
[18] U. Weidner, S. Geier, A. Ptock, T. Friedrich, H. Leif, H. Weiss, J. Mol. Biol. 233 (1993) 109–122.
[19] H. Leif, V.D. Sled, T. Ohnishi, H. Weiss, T. Friedrich, Eur. J. Biochem. 230 (1995) 538–548.
[20] T. Ohnishi, Biochim. Biophys. Acta 1364 (1998) 186–206.
[21] A. Dupuis, J.M. Skehel, J.E. Walker, Biochemistry 30 (1991) 2954–2960.
[22] P.L. Dutton, C.C. Moser, V.D. Sled, F. Daldal, T. Ohnishi, Biochim. Biophys. Acta 1364 (1998) 245–257.
[23] W. Fecke, V.D. Sled, T. Ohnishi, H. Weiss, Eur. J. Biochem. 220 (1994) 551–558.
[24] J.E. Azevedo, M. Duarte, J.A. Belo, S. Werner, A. Videira, Biochim. Biophys. Acta 1188 (1994) 159–161.
[25] M.V. da Silva, P.C. Alves, M. Duarte, N. Mota, A. Loboda-Cunha, T.A.A. Harkness, F.E. Nargang, A. Videira, Mol. Gen. Genet. 252 (1996) 177–183.
[26] T. Friedrich, G. Hofhaus, W. Ise, U. Nehls, B. Schmitz, H. Weiss, Eur. J. Biochem. 180 (1989) 173–180.
[27] A. Videira, M. Tropschug, S. Werner, Biochem. Biophys. Res. Commun. 171 (1990) 1168–1174.
[28] A. Videira, J.E. Azevedo, S. Werner, P. Cabral, Biochem. J. 291 (1993) 729–732.
[29] M. Finel, A.S. Majander, J. Tyynelä, A.M.P. De Jong, S.P.J. Albracht, M. Wikström, Eur. J. Biochem. 226 (1994) 237–242.
[30] D.-C. Wang, S.W. Meinhardt, U. Sackmann, H. Weiss, T. Ohnishi, Eur. J. Biochem. 197 (1991) 257–264.
[31] M. Duarte, U. Schulte, A. Videira, Biochim. Biophys. Acta 1322 (1997) 237–241.