# Effects of disrupting the 21 kDa subunit of complex I from *Neurospora crassa*

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We have cloned and inactivated *in vivo*, by repeat-induced point mutations, the nuclear gene encoding a 21 kDa subunit of complex I from *Neurospora crassa*. Mitochondria from the nuo21 mutant lack this specific protein but retain other subunits of complex I in approximately normal amounts. In addition, this mutant is able to assemble an almost intact enzyme. The electron transfer activities from NADH to artificial acceptors of mitochondrial membranes from nuo21 differ from those of the wild-type strain, suggesting that the absence of the 21 kDa polypeptide results in conformational changes in complex I. Nevertheless, complex I of nuo21 is able to perform NADH:ubiquinone reductase activity, as judged by the observation that the respiration of mutant mitochondria is sensitive to inhibition by rotenone. We discuss these findings in relation to the involvement of complex I in mitochondrial diseases.

Key words: complex I, gene disruption, mitochondria, mitochondrial diseases.

## INTRODUCTION

The proton-pumping NADH dehydrogenase of the mitochondrial respiratory chain, complex I (EC 1.6.5.3), is composed of approx. 40 polypeptide subunits, 7 of which are encoded in mitochondrial DNA. Several protein-bound prosthetic groups, namely FMN and approximately six iron–sulphur clusters, are involved in its activity. Many bacteria also possess enzymes equivalent to eukaryotic complex I, with a similar constitution of prosthetic groups but with much fewer proteins. The prokaryotic enzymes contain homologues of the seven polypeptides encoded by mitochondria and homologues of seven proteins encoded by the nucleus in fungi or mammals (reviewed in [1–5]).

Different human mitochondrial diseases, including Parkinsonism, have been associated with deficiencies of complex I, especially concerning defects in the mitochondrial-DNA-encoded polypeptides [6,7]; however, the molecular mechanisms underlying disease are far from understood. Bacterial systems are useful models with which to study the role of mitochondrially synthesized subunits of complex I [8,9]. However, most of the nuclear-coded subunits of complex I are missing from bacterial genomes and it is expected that mutations in these proteins will be found as more human diseases are studied in detail. In fact, the first description of a human mutation affecting a nuclear-coded subunit of complex I has appeared recently [10]. The protein, called AQDQ, is phosphorylated by a mitochondrial cAMP-dependent kinase, although the role of this modification is unknown [11]. It belongs to the peripheral domain of complex I in *Neurospora crassa* [12] and is present in the iron–sulphur protein fragment of the bovine enzyme [13]. At present *N. crassa* is a superior system in which to investigate the utility of complex I proteins because it is the only eukaryotic organism in which the enzyme is quite well characterized and the disruption of the respective genes is routinely performed [5,14]. Furthermore, the fungal enzyme is wholly similar to that of mammals, in terms of both composition [5] and overall structure [15,16], and contains a 21 kDa polypeptide homologous to the mammalian AQDQ protein [12]. Here we describe the inactivation of the gene coding for the 21 kDa protein and the isolation of a specific mutant strain. The relationship between the phenotype of the mutant and mitochondrial disease is discussed.

## EXPERIMENTAL

Standard methods were used for cloning and DNA characterization [17,18]. The *nuo-21* gene was isolated from a *N. crassa* genomic library in phage J1 (from the Fungal Genetics Stock Center) by hybridization screening with the corresponding cDNA [12]. A *Sac*I fragment of genomic DNA (approx. 4 kb) was subcloned in plasmid pGEM4 [19] and characterized by restriction mapping (see Figure 1). A smaller *Eco*RV DNA fragment (1.3 kb), containing the entire coding region of *nuo-21*, was then ligated into pCSN44 [20] for the transformation of *N. crassa*. The protocols for the general manipulation of *N. crassa* wild-type strains 74-OR23-1A and 74-OR8-1a [21], fungal transformation and the selection and analysis of transformants [22–24] were used as published. The mutant strain nuo21 was identified by immunoblotting analysis of mitochondrial proteins with an antiserum that recognized the 21 kDa subunit of complex I [25,26].

The techniques for the crude preparation of mitochondria from *N. crassa* [27], antisera against complex I subunits and Western blotting [25] and sucrose-gradient centrifugation analysis of detergent-solubilized mitochondrial proteins [22] were used as described. The determination of NADH:ferricyanide [28] and NADH:hexa-ammineruthenium(III) [29] reductase activities were performed in the presence of 150 $\mu$M NADH and either 1.6 or 2 mM electron acceptor respectively; the assays with detergent-solubilized mitochondria also included rotenone and antimycin A, as specified below. Purified mitochondria and inside-out submitochondrial particles (SMP) were used for oxygen consumption experiments (A. M. P. Melo, M. Duarte and A. Videira, unpublished work). The quality of the preparations was assessed by the determination of cytochrome *c* oxidase (EC 1.9.3.1) and malate dehydrogenase (EC 1.1.1.37) activities in the presence and the absence of Triton X-100 [30] to calculate the latent

Abbreviation used: SMP, submitochondrial particles.

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activities of the enzymes. Oxygen consumption was measured polarographically at 25 °C in a Clark-type oxygen electrode (Hansatech). The assays of SMP were started by the addition of 1 mM NADH to the reaction medium containing 0.5 mg of protein, 0.3 M sucrose, 10 mM potassium phosphate, pH 7.2, 5 mM $MgCl_2$, 1 mM EGTA, 10 mM KCl, 4 $\mu$M carbonyl cyanide *m*-chlorophenylhydrazone and 0.02% BSA. The assays with mitochondria were started by the addition of 10 mM malate to reaction medium containing 1 mM $NAD^+$ and 5 mM pyruvate. Rotenone and antimycin A (Sigma) were added to final concentrations of 40 $\mu$M and 0.2 $\mu$g/ml respectively.

## RESULTS AND DISCUSSION

Genes can be duplicated in the genome of *N. crassa* by DNA transformation. When the duplicated copies of a gene are passed through a genetic cross, they are prone to suffer repeat-induced point mutations and become inactivated at a certain frequency [31]. Thus mutant strains can be recovered from the progeny of the cross. The 21 kDa subunit of complex I is encoded by a single-copy gene located on linkage group IV of the *N. crassa* genome [32]. Figure 1 shows a restriction-mapping analysis of the relevant DNA region. The 1.3 kb *Eco*RV fragment, containing the *nuo-21* gene, was cloned in pCSN44 and transformed back into *N. crassa* 74A. On the basis of a Southern analysis of genomic DNA of several transformants, with appropriate restriction enzymes and the relevant cDNA as a probe, we selected a strain carrying a single and complete extra copy of the *EcoRV* DNA fragment (results not shown). This strain was mated with *N. crassa* 74A. Late-ejected ascospore progeny from the cross [33] were grown individually; 38 of these were used in the preparation of mitochondria. An analysis of the organelle proteins by Western blotting led to the identification of the mutant strain nuo21, which specifically lacks the 21 kDa polypeptide (Figure 2).

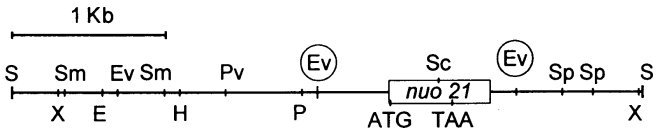


**Figure 1 Restriction map of *N. crassa* genomic DNA containing the *nuo-21* gene**

The enzymes used were as follows: E, *Eco*RI; Ev, *Eco*RV; H, *Hin*dIII; S, *Sac*I; Sc, *Sca*I; Sm, *Sma*I; Sp, *Sph*I; P, *Pst*I; Pv, *Pvu*II; X, *Xho*I. The extremities of the 1.3 kb fragment used for fungal transformation (encircled Ev) as well as the position and orientation of *nuo-21* are indicated.

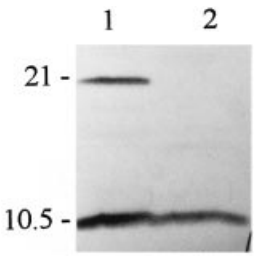


**Figure 2 Western blot analysis of mitochondrial proteins**

Electrophoretically resolved mitochondrial proteins from the wild-type strain (lane 1) and mutant nuo21 (lane 2) were immunodecorated with an antiserum against the 10.5 kDa subunit of complex I that also cross-reacted with the 21 kDa protein.

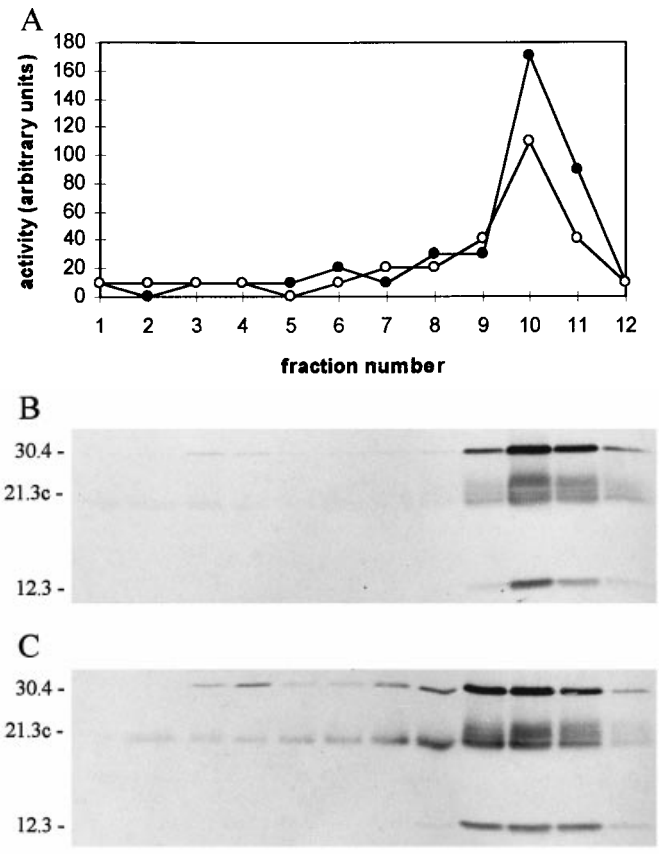


**Figure 3 Sucrose-gradient centrifugation analysis of Triton X-100-solubilized mitochondrial proteins**

(**A**) Fractions (1 ml) of the gradients (labelled 1–12 from top to bottom) were collected and assayed for NADH:hexa-ammineruthenium oxidoreductase activity (●, wild-type strain; ○, mutant nuo21). (**B**, **C**) Aliquots of the fractions obtained with the wild-type strain (**B**) and mutant nuo21 (**C**) were also analysed by Western blotting with a mixture of individual antisera against the subunits of complex I indicated at the left.

Similar experiments, conducted with antisera against other subunits of both the peripheral and membrane arms of complex I, revealed that these proteins are present in the mitochondria of nuo21 in amounts comparable with those found in the wild-type strain (results not shown). This is in slight contrast with other mutants of complex I, in which the absence of one protein has more or less drastic effects on the levels of other subunits of the enzyme.

An analysis of detergent-solubilized mitochondrial complexes from the wild-type and nuo21 strains by sucrose-gradient centrifugation indicated that the mutant assembled an almost intact complex I (Figure 3). As expected in these experiments [22], the wild-type complex I was eluted mostly in fractions 9–11 of the gradients (with a peak in fraction 9), as judged by the elution profile of the NADH:hexa-ammineruthenium reductase activity (Figure 3A), an artificial electron-transfer operation of complex I [34]. In support of this, the polypeptide subunits of complex I were also recovered mostly in fractions 9–11. Figure 3(B) shows the results obtained with the 30.4 kDa [35] and 21.3c kDa proteins [36] and with the 12.3 kDa protein [37], employed as markers for the peripheral and membrane arms of complex I respectively. The complex formed in nuo21 mitochondria behaved similarly to wild-type complex I (Figures 3A and 3C). The

**Table 1 Oxidation of NADH by detergent-solubilized mitochondria with the use of different electron acceptors**

Abbreviation: P.A., peripheral arm of complex I.

| Membrane source | Oxidoreductase activity (% of wild type) | | Ratio of hexa-ammineruthenium to ferricyanide |
|---|---|---|---|
| | NADH:ferricyanide | NADH:hexa-ammineruthenium | |
| Wild type | 100 | 100 | 1.2 |
| Mutants with P.A. | 20–40 | 40–60 | ~ 2 |
| Mutants without P.A. | 10–20 | 10–20 | – |
| nuo21 | 25 | 200 | 8.4 |

NADH:hexa-ammineruthenium reductase activities were lower than those of the wild type; we did not detect any significant NADH:ferricyanide reductase activity throughout the gradient of nuo21, even though some activity could be found in the mutant mitochondria (see below). It seems that the electron transfer activities of complex I of nuo21 (isolated in the gradients) are more labile than that of the wild type. Immunoprecipitation experiments were performed in mitochondria from the wild-type and nuo21 strains, with a subunit-specific antiserum that was able to co-precipitate the whole complex I [25], followed by electrophoresis and silver-staining of the gels. Except for the lack of the 21 kDa polypeptide in nuo21, the pattern of bands of the two strains was very similar (results not shown), thus corroborating our interpretation that an almost intact complex I was assembled in the mutant strain.

Complex I can oxidize NADH and reduce artificial electron acceptors such as ferricyanide and hexa-ammineruthenium. These redox reactions are insensitive to rotenone and occur in different reactive sites within the enzyme [29,34]. We have measured both activities in mitochondrial membranes and in Triton X-100-solubilized mitochondria from different complex I mutants. The mutants were divided into two categories comprising mutants assembling the peripheral arm of complex I and expected to perform these activities, and mutants lacking a functional peripheral arm of complex I (Table 1). In the first group we included strains nuo20.8 [38] and nuo12.3 [23], which accumulated this peripheral domain of complex I. In the second group we included strains nuo30.4 [24] and nuo21.3c (M. Duarte and A. Videira, unpublished work), in which the formation of the peripheral arm of complex I was disrupted, and nuo24 (M. Duarte and A. Videira, unpublished work), which assembles a non-functional complex. The NADH:ferricyanide and NADH:hexa-ammineruthenium oxidoreductase activities of mutants lacking the peripheral arm of complex I fell below 20% of the activities found in the wild-type strain, suggesting that they are mainly performed by complex I in the wild-type strain and their direct determination in solubilized mitochondria can be used to assess the 'functionality' of the enzyme in *N. crassa*. The NADH:ferricyanide oxidoreductase activity of nuo21, approx. 25% of the wild-type value, is within the range found for mutants assembling the peripheral arm of complex I. Surprisingly, the hexa-ammineruthenium reductase activity of nuo21 mitochondria was approximately double that in the wild-type strain (Table 1). The value of 8.4 obtained for the ratio of hexa-ammineruthenium to ferricyanide reduction activities of nuo21 mitochondria is very impressive when compared with the value of 1.2 determined in the wild-type strain. A plausible explanation for these observations is that the absence of the 21 kDa protein results in conformational changes in complex I, leading to an instability of the ferricyanide-reactive site and a greater accessibility of the reactive site to hexa-ammineruthenium. A stimulation of hexa-ammineruthenium reduction was previously observed in bovine SMP after treatment with *N*-bromosuccinimide [29,34].

The following experiments indicate that complex I of nuo21 is able to perform the more physiological NADH:ubiquinone oxidoreductase activity of the enzyme. We examined the rotenone-sensitive respiration of intact mitochondria or inside-out SMP of this strain. The specific activities of the oxygen uptake of mutant mitochondria respiring on pyruvate/malate or of SMP respiring directly on NADH as substrate were similar to the wild-type values. In the mutant, the complex I inhibitor rotenone partly blocked both activities to roughly the same extent as in the wild-type strain (Figure 4). The remaining activity probably arose from at least one internal alternative NADH dehydrogenase [39]. The addition of antimycin completely hindered any activity from both strains.

The inactivation of the gene encoding the 21 kDa polypeptide indicates that it is not essential for vegetative growth in *N. crassa*. This is not surprising because many (if not all) subunits of the fungal complex I can be disrupted [5,14]. The first (and yet unique) mutation in nuclear-coded subunits of complex I involved in mitochondrial diseases was found in the AQDQ human homologue of the 21 kDa protein [10]. The patient presented with a multisystemic disorder with a fatal progressive phenotype, owing to a pathological duplication of five base pairs in the gene that altered the C-terminal region and abolished the putative phosphorylation site of the protein [10,11]. The fact that the patient was homozygous for the mutation and originated from two heterozygous parents [10] suggests that a 'loss-of-function' phenotype is involved. Thus the nuo21 mutant of *N. crassa*, in which expression of the protein was abrogated, should mimic the human situation. One possibility, to explain why mutations in the AQDQ protein lead to a severe phenotype in humans, is that it affects an unknown function of complex I not directly related to the bioenergetic activity of the enzyme. Our results suggest that complex I lacking this protein is assembled and able to catalyse the rotenone-sensitive transfer of electrons from NADH to ubiquinone. In addition, there is no homologue of the 21 kDa polypeptide among the 14 protein constituents of prokaryotic complex I, which are considered to be the 'minimal structure' required for the coupling of electron transfer with proton translocation [1–4]. It should be noticed that an increased lactate concentration in body fluids, found in other complex I-deficient patients [40,41], was not seen in the AQDQ-defective patient [10]. Another strong possibility, however, is that a mutation in, or a lack of, the 21 kDa protein interferes with the efficiency of energy transduction by complex I. A decrease in NADH:ferricyanide reductase as well as in NADH:cytochrome *c* reductase was observed in both skeletal muscle and cultured skin fibroblast

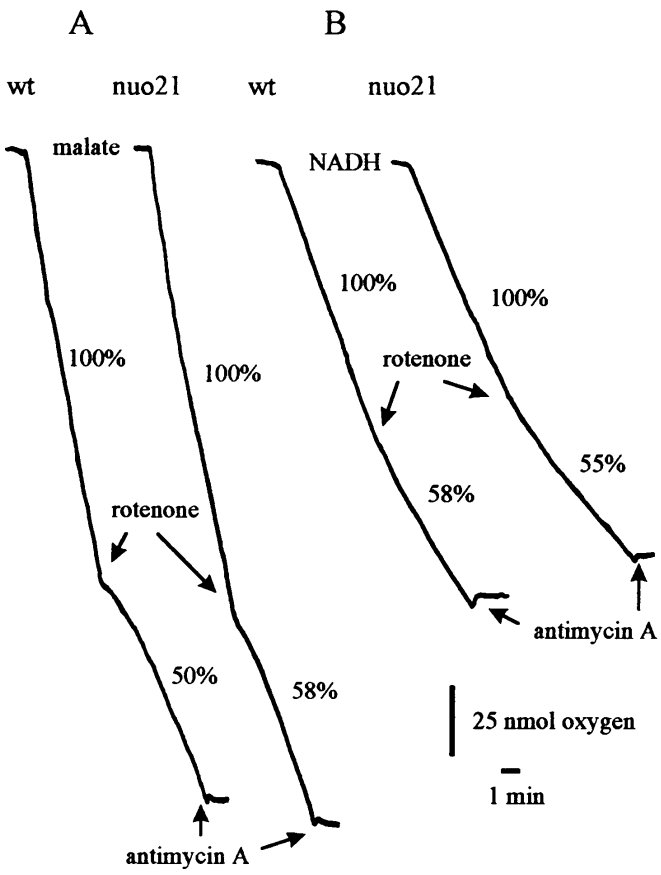


**Figure 4 Effects of inhibitors on the respiration of mitochondria (A) and SMP (B)**

The polarographic traces were obtained from the wild-type (wt) and nuo21 strains. The start of each reaction with either malate or NADH and the addition of respiratory chain inhibitors are indicated. The oxygen uptake rates are expressed as percentages of the initial rates.

mitochondria from the human patient [10]. We also found a decrease in NADH oxidation by ferricyanide in mutant nuo21, although we could not detect any significant decrease in the pyruvate/malate oxidation of intact mitochondria. It might be that the absence of the 21 kDa protein from the *N. crassa* mutant has a less pronounced effect on complex I than the presence of an altered form of the polypeptide, which might still assemble in the case of the human enzyme. The availability of the *N. crassa* nuo21 mutant permits further investigation of these issues.

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