Respiratory Chain Complex I Is Essential for Sexual Development in Neurospora and Binding of Iron Sulfur Clusters Are Required for Enzyme Assembly

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Manuscript received February 14, 2000
Accepted for publication June 19, 2000

ABSTRACT

We have cloned and disrupted *in vivo*, by repeat-induced point mutations, the nuclear gene coding for an iron sulfur subunit of complex I from *Neurospora crassa*, homologue of the mammalian TYKY protein. Analysis of the obtained mutant *nuo21.3c* revealed that complex I fails to assemble. The peripheral arm of the enzyme is disrupted while its membrane arm accumulates. Furthermore, mutated 21.3c-kD proteins, in which selected cysteine residues were substituted with alanines or serines, were expressed in mutant *nuo21.3c*. The phenotypes of these strains regarding the formation of complex I are similar to that of the original mutant, indicating that binding of iron sulfur centers to protein subunits is a prerequisite for complex I assembly. Homozygous crosses of *nuo21.3c* strain, and of other complex I mutants, are unable to complete sexual development. The crosses are blocked at an early developmental stage, before fusion of the nuclei of opposite mating types. This phenotype can be rescued only by transformation with the intact gene. Our results suggest that this might be due to the compromised capacity of complex I-defective strains in energy production.

THE life cycle of *Neurospora crassa*, a heterothallic filamentous fungus, includes a vegetative and a sexual phase. The latter is initiated when a protoperithecium of one mating type is fertilized by a male cell of the other mating type. Fertilized protoperithecia develop into perithecia within which asci are formed. A normal ascus produces eight black ascospores. Mutations in some genes may directly or indirectly interfere with the normal development of perithecia, asci, or ascospores, but little is known about the molecular structure of these genes or the functions of the encoded products (Raju 1992). The proton-pumping NADH:ubiquinone oxidoreductase commonly known as complex I (EC.1.6.5.3) couples electron transfer from NADH to ubiquinone with proton translocation across the inner mitochondrial membrane. Mitochondrial complex I consists of >30 polypeptide subunits of both nuclear and mitochondrial origin, as well as flavin mononucleotide and several iron-sulfur clusters. This enzyme is composed of two distinct subcomplexes, arranged perpendicularly to each other in an L-shaped structure, which undergo independent assembly. The membrane arm is embedded in the mitochondrial membrane, while the peripheral arm is mainly protruding into the mitochondrial matrix (Tuschen *et al.* 1990; Weiss *et al.* 1991; Walker 1992; Videira 1998). Enzymes equivalent to complex I (NDH1), but with fewer protein constituents, have also been identified in prokaryotes (Dupuis *et al.* 1998; Friedrich 1998; Yagi *et al.* 1998). Notably, despite its wide conservation, complex I is not present in yeast.

The expression of complex I may vary in different stages of the life cycle of an organism, as in *Trypanosoma brucei brucei* (Beattie and Howton 1996), or between different tissues, as in plants where an increased expression of complex I subunits in flowers was observed (Grohmann *et al.* 1996; Heiser *et al.* 1996). Thus, its importance is expected to depend on the organism, stage of development, or the specific tissue concerned. This enzyme is crucial for multiple processes in several organisms. For example, complex I mutants of *Salmonella typhimurium* cannot apparently activate ATP-dependent proteolysis under carbon starvation (Archer *et al.* 1993), development and sporulation of *Myxococcus xanthus* is prevented (Laval-Favre *et al.* 1997), and disruption of NDH1 genes of *Paracoccus denitrificans* seems to be a lethal event (Finel 1996). Complex I was associated with plant development, since a reduction in the expression of the NADH-binding subunit resulted in reduced male fertility due to disturbed pollen maturation (Heiser *et al.* 1997). Male sterility was also described in plants with mutations in the mitochondrial genome, including those affecting complex I genes (Marienfeld and Newton 1994; Pla *et al.* 1995; Gutierres *et al.* 1997). Furthermore, deficiencies in complex I activity appear to be responsible for the development of several pathogenic human conditions (Wallace 1992; Schapira 1998).

As an approach to understanding the relevance of

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specific proteins for the biogenesis and function of complex I, we are inactivating individual genes and investigating the phenotype of the resulting null mutants in the fungus *N. crassa*, which possesses an enzyme very similar to that of mammals (Videira 1998). The 21.3c-kD protein is a highly conserved nuclear-encoded subunit of complex I with homologues in many species, best documented in humans (Procaccio *et al.* 1997), *Bos taurus* (Dupuis *et al.* 1991), *Escherichia coli* (Weidner *et al.* 1993), *Rhodobacter capsulatus* (Chevallet *et al.* 1997), and *P. denitrificans* (Xu *et al.* 1993). It belongs to the peripheral domain of complex I in *N. crassa* (Duarte *et al.* 1997) and to the "connecting fragment" in *E. coli* (Leif *et al.* 1995). The bovine homologue (TYKY subunit) is present in the IλS subcomplex, a fragment of complex I containing all the prosthetic groups of the enzyme (Finel *et al.* 1994). The amino acid sequence contains two consensus motives of the ferredoxin type for binding of two [4Fe-4S] clusters (Walker 1992). Recently, mutations in the TYKY subunit of human complex I were found in a patient with Leigh syndrome, representing the first molecular genetic link between a nuclear-encoded subunit of complex I and the disease (Loeffen *et al.* 1998).

Herein we report the inactivation of the gene coding for the 21.3c-kD protein in *N. crassa* (the TYKY homologous subunit) and the characterization of the null mutant as well as of mutants obtained by site-directed mutagenesis. Homozygous crosses between *nuo21.3c* mutant strains were infertile and only the *nuo-21.3c* gene could complement this phenotype. We conclude that the 21.3c-kD iron-sulfur protein plays an important role in the assembly of complex I and that complex I is essential for normal development of the sexual cycle in *N. crassa.*

MATERIALS AND METHODS

Strains and plasmids: *N. crassa* strain 74-OR23-1A (wild type), the sterile helper strain (*a*^{m1}, *ad-3B, cyh-1*), and the Host VI strain (*a, Bml*^{r}, *pan-2, inl, inv, mei-2*; Fungal Genetics Stock Center (FGSC) no. 7256), designed for the disruption of essential genes located on linkage group VI (Metzenberg and Grotelueschen 1992), were obtained from the FGSC. In this article, we describe the mutant strain *nuo21.3c*, strain R18 in which the complex I phenotype of mutant *nuo21.3c* was rescued by transformation with pMYX2.21.3c, and strain G14 in which the fertility phenotype was rescued by transforming *nuo21.3c* with pNUO-21.3cPvH (see below). We also used the complex I repeat-induced point (RIP) mutations *nuo20.8* (da Silva *et al.* 1996), *nuo30.4* (Duarte *et al.* 1998), *nuo21* (Ferreirinha *et al.* 1999), and *nuo24* (Almeida *et al.* 1999), and two other mutants generated by homologous recombination, *nuo51* (Fecke *et al.* 1994) and *nuo78* (Preis *et al.* 1991), obtained from Dr. U. Schulte. The cDNAs coding for the 20.8- and 30.4-kD polypeptides were expressed in the corresponding mutant under the control of the quinic acid promoter of pMYX2 (Campbell *et al.* 1994). We used the plasmids pCSN44 (Staben *et al.* 1989) and pMYX2 for fungal transformation and the transcription vector pGEM4 (Melton *et al.* 1984), amplified in *E. coli* DH5α (Sambrook *et al.* 1989).

***N. crassa* manipulations:** Growth and crosses of *N. crassa* were carried out according to standard procedures (Davis and de Serres 1970). For expression of cDNA under the control of the quinic acid promoter of pMYX2, 10 mm quinic acid was added to the medium. Conidia from 7-day-old cultures were used to prepare spheroplasts, which were then transformed with recombinant pCSN44 or pMYX2 vectors and selected on plates containing hygromycin B or benomyl, respectively. Heterokaryon formation with the sterile helper strain was forced on Vogel's minimal medium (Davis and de Serres 1970).

Molecular cloning: Current protocols have been followed for molecular cloning and Southern blotting techniques (Sambrook *et al.* 1989). The *nuo-21.3c* gene, a single-copy gene located on linkage group VI of the fungus genome (Ferreirinha *et al.* 1998), was isolated from a *N. crassa* genomic library in phage J1 (obtained from FGSC) by hybridization with the corresponding cDNA. An *Eco*RI fragment of genomic DNA (~7 kb) containing the entire coding region of *nuo-21.3c*, as well as a 2.2-kb *Pst*I fragment, was subcloned in pGEM4. The latter recombinant plasmid was treated with *Hin*dIII (located in the polylinker region of pGEM4 and in the genomic DNA) and the relevant band (2.2 kb) was cloned into the *Hin*dIII site of pCSN44, generating pNUO-21.3cPH (see also Figure 1). The genomic DNA fragment PvH (2.9 kb) was ligated into pGEM4 previously digested with both *Sma*I and *Hin*dIII enzymes. The recombinant plasmid was then treated with *Sac*I and *Hin*dIII and the relevant fragment cloned into pCSN44 digested with the same restriction enzymes, creating plasmid pNUO-21.3cPvH (Figure 1). The cDNA coding for the 21.3c-kD subunit cloned in pGEM4 (Duarte *et al.* 1996) was cleaved with *Eco*RI, treated with Klenow to create blunt ends, and then ligated into the *Sma*I site of the expression vector pMYX2, downstream of the *qa-2* inducible promoter, giving rise to pMYX2.21.3c.

Mutant isolation: The recombinant plasmid pNUO-21.3cPH was introduced in *N. crassa* 74A by transformation. Several transformants were selected for growth on hygromycin B and purified by asexual transfers. Genomic DNA from these strains was analyzed by Southern blotting, using appropriate restriction enzymes and the cDNA as a probe, and four single-copy transformants were identified. One of these transformants carrying a duplication of the *nuo-21.3c* gene was crossed with strain FGSC no. 7256. This strain contains a mutant allele for the *pan-2* gene, located on LG VI, allowing selection for descendants carrying chromosome VI derived from the transformant, by plating ascospores on minimal medium, and thus mutant enrichment. Random progeny from the cross were germinated and their mitochondrial proteins were analyzed by Western blotting, leading to the identification of mutant *nuo21.3c*, which lacks the 21.3c-kD protein.

Protein analysis: The techniques for the preparation of *N. crassa* mitochondria (Werner 1977), protein determination (Bradford 1976), SDS-polyacrylamide gel electrophoresis (Zauner *et al.* 1985), Western blotting (Towbin *et al.* 1979; Blake *et al.* 1984), sucrose gradient centrifugation analysis of detergent-solubilized mitochondrial proteins (Alves and Videira 1994), and NADH:ferricyanide reductase activity of the sucrose gradient fractions (Hatefi and Stiggall 1978) have been published before. Rabbit antisera against complex I subunits were raised against purified proteins in the case of the 30.4-, 20.8-, and 12.3-kD polypeptides (Videira and Werner 1989), or against proteins expressed in bacteria in the case of the 78- (unpublished data), 51- (Almeida *et al.* 1999), and 21.3c-kD polypeptides (Duarte *et al.* 1997).

Site-directed mutagenesis: Site-directed mutagenesis was achieved using the Quik Change site-directed mutagenesis kit according to the manufacturer procedures (Stratagene, La

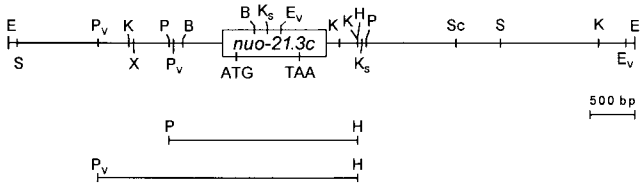

FIGURE 1.—Restriction map of *N. crassa* genomic DNA containing the *nuo-21.3c* gene. The 7-kb DNA fragment with *Eco*RI ends was isolated from a λJ1 phage and cloned in pGEM4. The position of *nuo-21.3c* and its initiation and stop codons is indicated. The enzymes shown are as follows: B, *Bam*HI; E, *Eco*RI; Ev, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; Ks, *Ksp*I; S, *Sal*I; Sc, *Sac*I; P, *Pst*I; Pv, *Pvu*II; and X, *Xho*I. The smaller 2.2-kb fragment (PH) was cloned into the unique *Hin*dIII site of pCSN44 (together with the *Pst*I/*Hin*dIII region of the polylinker of pGEM4). The PvH fragment was ligated into pCSN44 double digested with *Sac*I/*Hin*dIII.

Jolla, CA). Briefly, the full-length cDNA coding for the 21.3c-kD protein, cloned in the expression vector pMYX2, and two synthetic complementary oligonucleotide primers containing the desired mutation were used in a PCR reaction to create a mutated plasmid. The pairs of mutagenic oligonucleotide primers used were as follows:

C123A: 5′-TGCATCGCCGCCAAGCTCTGC-3′
C162A: 5′-TGCATTTACGCCGGATTCTGC-3′
C123S: 5′-TGCATCGCCTCCAAGCTCTGC-3′
C162S: 5′-TGCATTTACTCCGGATTCTGC-3′
C165S: 5′-TGCGGATTCTCCCAGGAGAGC-3′

and their complementary strands. The underlined nucleotides represent substitutions that change codons within the cDNA, resulting in the placement of alanine or serine residues instead of the cysteines present at positions 123, 162, and 165 in the protein sequence. A double C123A/C162A mutant gene was obtained by amplifying the C123A-altered plasmid with primers C162A. Mutagenesis was confirmed by complete sequencing (DUARTE *et al.* 1996) of all cDNA constructs. The mutated plasmids were transformed into the *nuo21.3c* mutant.

RESULTS

The RIP mutant *nuo21.3c* is defective in complex I assembly: A recombinant λJ1 phage, containing the nuclear gene encoding the 21.3c-kD subunit of the peripheral arm of complex I from *N. crassa*, was isolated and characterized. Figure 1 shows a restriction mapping analysis of the DNA region containing the *nuo-21.3c* gene. The recombinant plasmid pNUO-21.3cPH was transformed into *N. crassa* wild-type strain. A single-copy transformant was crossed with Host VI to generate *nuo21.3c* mutants by the RIP phenomenon, since duplicated DNA sequences in the genome of *N. crassa* are prone to permanent inactivation by G:C to A:T transitions when passed through a genetic cross (SELKER 1990). Late ejected ascospores from the cross (SINGER *et al.* 1995) were individually grown on minimal medium and 12 of them were used to prepare mitochondria. An analysis of the mitochondrial proteins by Western blotting led to the identification of the mutant strain *nuo21.3c* that specifically lacks the 21.3c-kD polypeptide

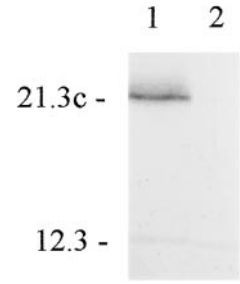

FIGURE 2.—Identification of mutant *nuo21.3c*. Electrophoretically resolved mitochondrial proteins from the wild-type strain (lane 1) and mutant *nuo21.3c* (lane 2) were analyzed by Western blotting with antisera against the 21.3c- and 12.3-kD subunits of complex I.

(Figure 2). Similar experiments using antisera against other subunits of complex I revealed that all proteins analyzed are present in the mitochondria of *nuo21.3c*, although with different stoichiometries as compared with the wild-type strain. For instance, the peripheral arm 30.4-kD subunit (VIDEIRA *et al.* 1990b) is reduced while roughly normal levels of the 12.3-kD polypeptide (VIDEIRA *et al.* 1993), a membrane arm component of the enzyme, were detected (Figure 2).

To evaluate the effect of the disruption of the *nuo-21.3c* gene, we investigated the state of complex I assembly. Mitochondria from the wild-type strain and mutant *nuo21.3c* were solubilized with Triton X-100 and centrifuged in linear sucrose gradients. The NADH:ferricyanide reductase activity as well as the distribution of several complex I subunits throughout the gradients was followed. Antisera against the 12.3- and 20.8-kD (VIDEIRA *et al.* 1990a) polypeptides as markers for the membrane arm, and against subunits 21.3c-kD (DUARTE *et al.* 1996) and 30.4-kD as markers for the peripheral arm of complex I, were used in this experiment (Figure 3). In the wild-type strain, the reductase activity elutes in fractions 9 and 10 of the gradient (Figure 3A), in agreement with the elution profile of the complex I proteins (Figure 3B). When mitochondria from the mutant *nuo21.3c* are analyzed, we do not detect significant NADH:ferricyanide activity in the gradient fractions, suggesting that neither complex I nor its peripheral arm are present. In fact, most of the 30.4-kD subunit elutes in fractions 3–5 (Figure 3C), representing a free subunit or a small unknown subcomplex. Other subunits of the peripheral arm of complex I were also detected, mainly in the top of the gradient (not shown), confirming that the peripheral arm is not assembled. The elution profile of the 12.3- and 20.8-kD proteins is somewhat broadened (Figure 3C) with a peak in fraction 9, which likely represents the membrane arm of complex I. In the absence of the peripheral arm, some aggregation of the membrane arm, due to the hydrophobic nature of this domain, might explain the migration of the 12.3- and 20.8-kD polypeptides throughout the gradient.

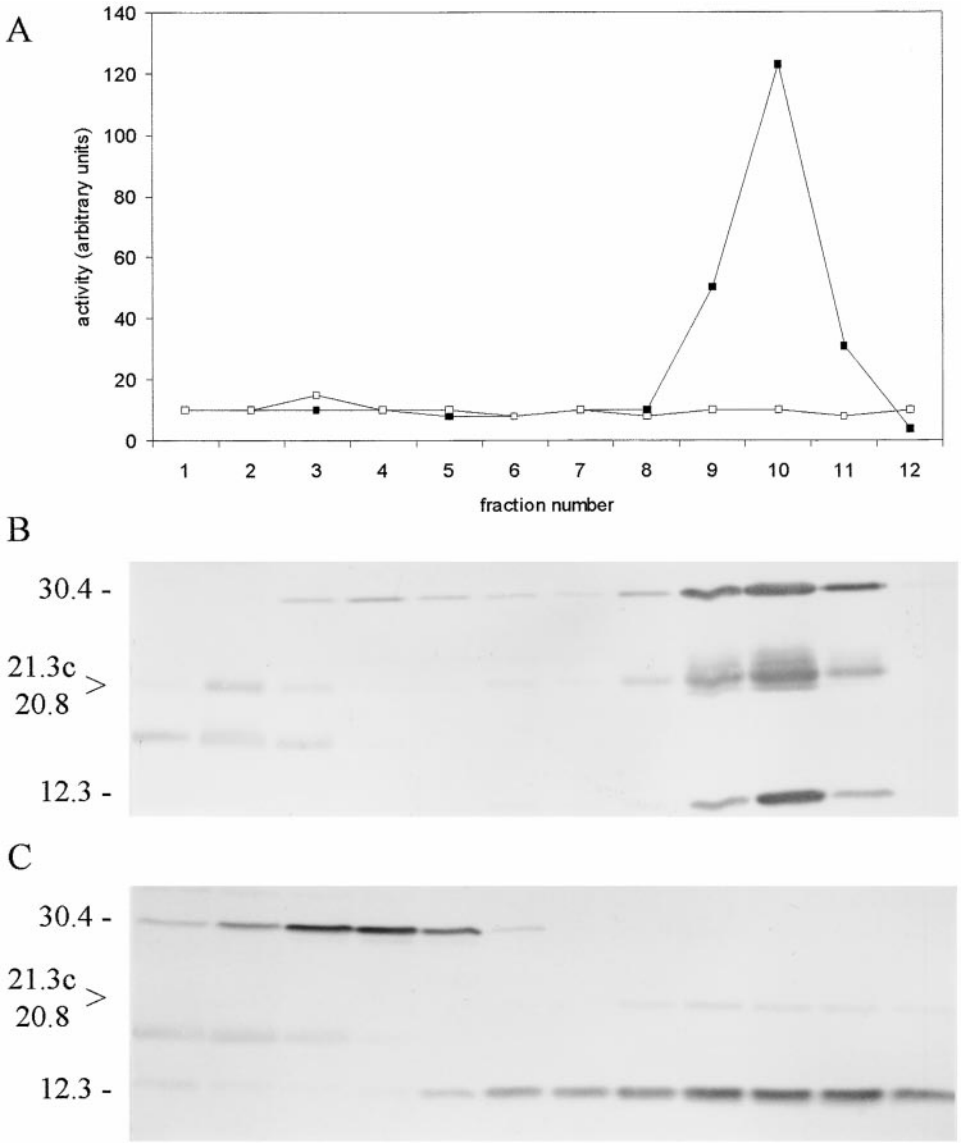

FIGURE 3.—Complex I is not assembled in *nuo21.3c*. Mitochondria were isolated, solubilized with the detergent Triton X-100, and centrifuged in 12-ml sucrose gradients. Fractions of the gradients (labeled 1–12 from left to right) were collected and assayed for NADH:ferricyanide oxidoreductase activity: (A) solid squares, wild type; open squares, *nuo21.3c*. Aliquots of the fractions obtained with material from the wild-type strain (B) and mutant *nuo21.3c* (C) were also resolved by SDS electrophoresis and blotted onto nitrocellulose. The membranes were immunodecorated with a mixture of individual antisera against the subunits of complex I indicated on the left. The 21.3c- and 20.8-kD polypeptides comigrate in this gel system. A band migrating with ~18 kD represents a nonspecific cross-reaction.

Protein binding of Fe-S clusters is required for complex I assembly: As with homologues in mammalian and bacterial enzymes, the *N. crassa* 21.3c-kD protein includes two sequence motifs CXXCXXCXXXCP for the binding of tetranuclear [Fe-S] clusters (WALKER 1992). In an attempt to get more information about the role that this subunit plays in complex I, we generated a set of mutant strains expressing altered forms of the cDNA encoding the polypeptide. Three putative cysteine ligands of the iron-sulfur clusters were mutated to either alanine or serine: C123 (the second cysteine in the first binding motif) and C162 and C165 (the second and third cysteines in the second motif, respectively). The following mutations were created: C123A, C123S, C162A, C162S, C165S, and a double mutation altering C123A/C162A. Each of these mutations was generated in plasmid pMYX2.21.3c by site-directed mutagenesis. The altered plasmids as well as pMYX2.21.3c vector were then introduced into the *nuo21.3c*RIP mutant and selected for benomyl resistance (CAMPBELL *et al.* 1994). Several transformants were isolated for each mutant and for the wild-type cDNA and tested for expression of the 21.3c-kD subunit on Western blots. Since quinic acid is not a good carbon source for Neurospora, we used both sucrose and quinic acid to grow the strains carrying a derivative of the pMYX2 plasmid. Fungal growth and expression of the cDNA-encoded gene product were maximal in these conditions. The protein levels showed considerable variation. Transformants with mutated cDNA had protein amounts comparable to each other but much lower than the amount observed in strains containing the wild-type cDNA. This difference is probably due to instability followed by degradation of the altered proteins. Transformants with the highest levels of expression of each protein were chosen for further analysis.

Mitochondria from strain R18, expressing the wild-type cDNA, were analyzed by sucrose gradient centrifu-

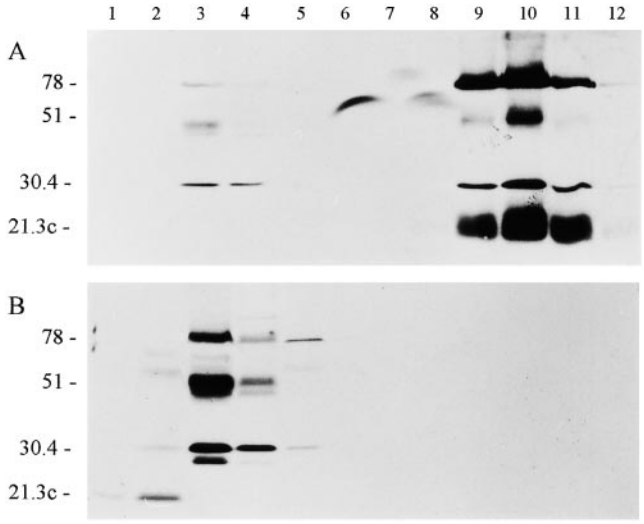

FIGURE 4.—Selected point mutations in *nuo21.3c* also prevent complex I assembly. Mitochondria from strains R18 (A) and the point mutant C162A (B) were analyzed by sucrose gradient centrifugation followed by Western blotting as described in the Figure 3 legend.

gation (as described above), revealing that the wild-type phenotype was rescued (Figure 4A). For each mutant carrying an altered cDNA, a similar analysis was performed. In all of the substitutions made, the mutated protein was detected in the top of the gradient (fractions 1 and 2), as exemplified for the C162A strain (Figure 4B), indicating that the modified subunits were not stably assembled into complex I. Furthermore, the analysis of other complex I subunits belonging either to the peripheral (Figure 4B) or the membrane arm of the enzyme (not shown) indicates that the phenotype of the point mutant strains resembles that of the *nuo21.3c* mutant. Only in the case of C162S could we detect a faint signal in fractions 10 and 11, suggesting some formation of complex I. If so, it represents a rather inefficient process. In fact, we could not measure any enzymatic activity in these fractions. These results stress the fact that the 21.3c-kD subunit plays a fundamental role in the assembly of complex I and highlights the requirement for prosthetic group binding in this process.

Sexual development is impaired in homozygous crosses of complex I (deficient) mutants: The mutant *nuo21.3c* grows reasonably well during the vegetative phase of the life cycle of *N. crassa*, despite producing less conidia than the wild-type strain. When grown in liquid minimal medium, ~80% of the mycelia wet weight was obtained than with wild type. No differences were found when the linear growth rate of both strains was compared in race tubes. We also did not observe differences in morphology and there are no special nutritional requirements of the mutant strain. We have presented preliminary evidence that complex I is essential during the sexual phase of the life cycle of the fungus (DUARTE *et al.* 1998). In this study, we performed homozygous crosses between *nuo21.3c* mutants, which resulted in the formation of barren perithecia with no ascospore progeny. We extended these observations and similar results were obtained with other complex I mutants where the enzyme is not functioning. For instance, homozygous crosses involving *nuo24* or *nuo51* mutants, strains able to assemble an almost intact complex I with no rotenone-sensitive NADH:ubiquinone oxidoreductase activity (FECKE *et al.* 1994; ALMEIDA *et al.* 1999), were infertile. The same result was obtained with the complex I mutant *nuo78* (HARKNESS *et al.* 1995), which, like *nuo21.3c*, completely lacks the peripheral arm of the enzyme.

Interestingly, we found that homozygous crosses between mutants lacking a 21-kD protein produced normal progeny. A Western blotting analysis of the progeny showed that all strains were *nuo21* and, thus, the result was not due to contamination with other strains. Similarly to *nuo24* and *nuo51*, these *nuo21* mutant strains assemble an almost intact complex I but, in contrast to them, their mitochondria display rotenone-sensitive respiration on NADH, indicating that complex I is active in electron transfer (FERREIRINHA *et al.* 1999). This suggests that a failure in energy production by complex I might be the cause for the impairment of sexual development in homozygous crosses.

The use of strains *nuo51* and *nuo78*, which were created by homologous recombination, excludes the possibility that the phenotype of crosses is due to a colateral effect of the RIP phenomenon used to generate mutants. As a control, heterozygous crosses between *nuo51* and *nuo78* were fully spore producing, indicating that the infertility phenotype was not associated with a particular strain but rather caused by the complex I deficiency. Homozygous crosses were barren in the early stages of the differentiation of ascogenous tissue, before karyogamy, since formation of croziers and young asci could not be observed. In homozygous crosses of complex I mutants, where one or both parents were present as forced heterokaryons with the sterile helper strain, sexual development was partially rescued to various stages, though not completed (N. B. RAJU, personal communication). Likely, the presence of mitochondria with functional complex I in the croziers (due to previous expression of wild-type genes in the nuclei from the sterile strain) allows the sexual process to proceed past the initial stages. Then, it might abort at different stages when these mitochondria become limiting. We suggest that continued expression and presence of a functional complex I is needed to complete a sexual cycle of *N. crassa*.

The fertility phenotype of *nuo21.3c* can be rescued by transformation with the intact gene: Since disruption of complex I genes causes sterility in homozygous crosses, it was expected that reinsertion of a wild-type copy of the cDNA at another location would restore fertility. However, crosses involving mutant *nuo21.3c*

and strain R18, carrying ectopic copies of the cDNA coding for this subunit, failed to sporulate. To exclude the possibility that the ectopic copy was integrated into a crucial gene for the development of the fungus, 20 different transformants were crossed separately with the mutant. The crosses were performed in medium containing only sucrose, sucrose plus quinic acid, and only quinic acid, to assure protein expression, but we could not observe ascospores in any of these matings. Similar experiments with two other complex I mutants, namely, *nuo20.8* (da Silva *et al.* 1996) and *nuo30.4* (Duarte *et al.* 1998), were also performed with the same end result. Furthermore, we mated two *nuo20.8* mutant strains, both expressing the cDNA coding for the 20.8-kD protein, and obtained many perithecia without ascospore progeny. These results might be caused by further RIPing of the ectopic cDNA copies. More likely, however, the quinic acid promoter used to support cDNA expression is either silent or not strong enough to provide the required amounts of protein during the sexual phase of the *N. crassa* life cycle. The inability of ectopic cDNAs to rescue mating phenotypes has been observed before (Ferreira *et al.* 1998).

To avoid these problems, we cloned the genomic DNA fragment PvH in pCSN44 (Figure 1). This fragment contains the coding region and a flanking sequence of ~1 kb upstream of *nuo-21.3c*, probably containing most of the promoter region (Nelson *et al.* 1997). The recombinant vector was transformed into a hygromycin-sensitive *nuo21.3c* mutant and 43 strains were isolated and separately crossed with a mutant strain of the opposite mating type. Figure 5 shows a genomic DNA analysis of one of these strains, named G14, and other relevant strains. When DNA from the wild-type strain is digested with *Bam*HI, bands of 8.5 kb, 8 kb, and 800 bp are observed (Figure 5A, lane 1). DNA from *nuo21.3c* mutants treated with the same enzyme reveals alterations of the restriction sites, shifting the 8.5-kb band to a 9.3-kb fragment (Figure 5A, lanes 2 and 4). The size of the new band is consistent with changes in the second *Bam*HI site in the coding region of the *nuo-21.3c* gene (see Figure 1). An alteration in the ectopic copy of the gene is also detected, giving rise to a 17-kb band in the hygromycin-resistant mutant (Figure 5A, lane 4). The *Eco*RV sites within both copies of the gene have also been modified as shown in Figure 5B (lanes 1, 2, and 4). Comparing the restriction pattern of DNA from the transformant G14 with that of the hygromycin-sensitive *nuo21.3c* mutant, extra bands of 4.4 kb, 2.5 kb, and 800 bp appear after digestion with *Bam*HI (Figure 5A, lanes 2 and 3), consistent with the introduction of ectopic DNA.

Among the 43 crosses, 7 were able to produce ascospores, including that of G14. The inability of the others to do so likely arises from further RIPing in the newly introduced DNA fragment, during the cross, or from its integration within a gene required for the sexual development or integration in a DNA region where it is not properly expressed. In all of the seven strains capable of reverting the "mating" phenotype, the 21.3c-kD subunit was detected after immunodecoration of mitochondrial proteins, indicating the requirement for gene expression. In addition, random progeny from the cross G14 × nuo21.3c were also analyzed by Western blot, revealing that protein expression segregated in 12 out of 17 spores analyzed (not shown). Thus, at least in this case, the exogenous *nuo-21.3c* gene had to pass the cross without RIP inactivation. These results confirm the requirement of a functional complex I for the completion of *N. crassa* sexual development.

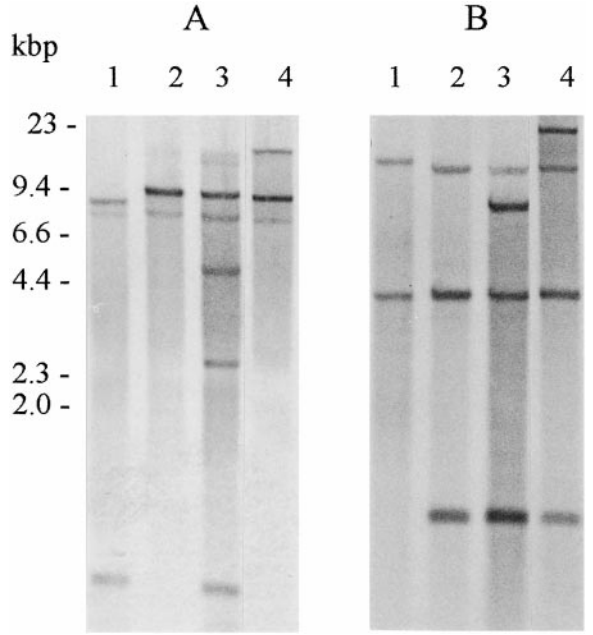

Figure 5.—Southern blot analysis of *N. crassa* strains used in this study. Genomic DNA from the wild-type strain (1), hygromycin-sensitive *nuo21.3c* mutant (2), complemented transformant G14 (3), and hygromycin-resistant *nuo21.3c* mutant (4) was prepared, digested with either *Bam*HI (A) or *Eco*RV (B), electrophoresed on agarose gels, and blotted onto nylon membranes. The hybridization probe was the 2.2-kb *Pst*I fragment carrying the *nuo-21.3c* gene.

DISCUSSION

The 21.3c-kD iron-sulfur subunit of complex I is highly conserved among different species, from bacteria to humans, and belongs to the 14 subunits that constitute the minimal structural unit for enzymatic activity (Walker 1992). These characteristics stress the physiological relevance of this polypeptide. We have generated a set of mutants in the protein. The *nuo21.3c* mutant, in which the *nuo-21.3c* gene was disrupted by RIPing, completely lacks the peripheral arm of complex I while it accumulates the membrane part of the enzyme. We conclude that the 21.3c-kD subunit has an important role in the assembly and/or stability of the peripheral

domain of complex I. Furthermore, the interference in enzyme assembly resulting from mutagenesis of specific amino acid residues of the polypeptide, potentially serving as ligands of iron-sulfur clusters, strongly suggests for the first time that binding of the prosthetic groups occurs before and is required for this process. In addition, we tend to speculate from our data that mutations in the amino acid residues that bind [Fe-S] clusters may have drastic effects in complex I assembly and/or activity, explaining why these types of mutations have not been found in association with human disease, since complete impairment of complex I function is probably incompatible with mammalian life and/or development.

The biogenesis and redox properties of membrane complexes containing bound iron-sulfur clusters are dependent upon the associated protein structure, which can be modified by mutating residues that bind prosthetic groups. The substitutions of cysteines with alanine residues, which contain an aliphatic side group, are not capable of providing a ligand to an iron in the modified site of the cluster. These substitutions are only capable of supporting [3Fe-4S] clusters (Mehari *et al.* 1995). The serine substitutions, which contain an hydroxyl side group, are potentially able to provide an oxygen ligand to an iron in the modified site of the cluster. Indeed, our cysteine to alanine substitutions gave rise to altered forms of the 21.3c-kD protein that do not associate with complex I. We also found that the majority of serine mutants are devoid of complex I, suggesting that serine is not an efficient ligand to the (appropriate) iron-sulfur centers. According to these results, the presence of two tetranuclear iron sulfur clusters in the 21.3c-kD protein is necessary for complex I assembly in *N. crassa*. Different results were obtained when analogous modifications were performed in the NuoI homologues of the 21.3c-kD subunit from *R. capsulatus* (Chevallet *et al.* 1997; Dupuis *et al.* 1998) and *E. coli* (Friedrich 1998). In *E. coli*, it seems that the individual replacement of all cysteines to alanine does not interfere with the assembly of complex I (Friedrich 1998). A similar result was suggested for the C67S mutant of *R. capsulatus* on the basis of the growth characteristics of the strain, whereas alteration of the fourth cysteine of the first binding motif, C74S, leads to the expression of an unstable protein with no complex I activity in the purple bacterium (Chevallet *et al.* 1997). The prokaryotic enzyme may be less sensitive to alterations in the structure of NuoI, since this polypeptide is expected to interact with fewer proteins than its mitochondrial homologue. On the other hand, the 21.3c-kD polypeptide displays a marked similarity with ferredoxin-like proteins that bear two [4Fe-4S] cluster-binding motifs (Duarte *et al.* 1996), making worthwhile a comparison with the mutagenesis experiments performed with PsaC, a component of photosystem I. Cysteine to serine mutations in PsaC result in an altered interaction of the protein with the photosystem I core, while alanine modifications lead to binding inability (Yu *et al.* 1997), as in the case of the 21.3-kD protein.

Another important finding of this work was the demonstration that complex I is essential for the sexual phase of the life cycle of *N. crassa*. Homozygous crosses between complex I-defective mutants fail to produce ascospores and this defect can be rescued by transformation with the wild-type gene. These findings were corroborated with the employment of mutant strains that were obtained by homologous recombination, in addition to those obtained by RIPing. On one hand, the need for complex I in the sexual development of the fungus could be due to some special function of the enzyme. It has been suggested that complex I inhibitors can induce apoptosis (Higuchi *et al.* 1998). The enzyme may be part of the mitochondrial permeability transition pore in rat (Fontaine *et al.* 1998) and involved in biosynthetic processes (Schneider *et al.* 1995). A subunit was identified as a G protein (Hegde 1998) and others are phosphorylated (Papa *et al.* 1996). On the other hand, the impairment of sexual development is observed in homozygous crosses of mutants in which the assembly of the whole enzyme is prevented or in mutants that assemble an almost intact enzyme without reductase activity, such as *nuo51* (Fecke *et al.* 1994) and *nuo24* (Almeida *et al.* 1999). In contrast, homozygous crosses of mutants that assemble an almost intact enzyme and display rotenone-sensitive oxidation of NADH, such as *nuo21* (Ferreirinha *et al.* 1999) and *nuo21.3a* (Alves and Videira 1994), are not blocked in sexual development. These results strongly suggest that the block in sporulation is due to a decrease in the total energy produced by mitochondria, a process that is probably tightly controlled during sexual development. The fact that we were only able to rescue the fertility phenotype of complex I mutants by transformation of the strains with genomic DNA pieces and not with cDNA is in agreement with this interpretation. It is likely that expression of cDNAs under the control of an unrelated promoter does not occur in the proper amounts (if any) during sporulation.

Complex I is responsible for the oxidation of NADH and thus for the redox state of the cell. In the fungus, absence of NADH oxidation by complex I might be compensated by alternative dehydrogenases (Weiss *et al.* 1970) and so the redox state of the cell is probably not affected. It is not known if these enzymes are active during sexual reproduction. In *Nicotiana sylvestris*, complex I defects have been associated with cytoplasmic male sterility (Gutierres *et al.* 1997). In these mutants, complex I activity decreases while functioning of other NADH dehydrogenases increases, but this compensatory effect is not sufficient to prevent the developmental anomalies observed. Several other mitochondrial mutations involving complex I genes have been described in plants, confirming that the functionality of

this enzyme is essential for normal cellular development, including chloroplast maturation and pollen development (Marienfeld and Newton 1994; Heiser *et al.* 1997). Increases in mitochondrial ATP levels and energy charge during embryogenesis of *Xenopus laevis* have recently been reported (Ammini and Hauswirth 1999). Also, deficiencies in complex I activity have been associated with human mitochondrial diseases (Schapira 1998). Mutations in nuclear and mitochondrial genes were described as the molecular cause of this enzyme deficiency in several patients (Loeffen *et al.* 1998). Our results further support the crucial role of complex I, a housekeeping function, in many cellular processes with certain energy demands. Complex I might be circumvented to a certain degree but is essential under special physiological conditions, such as sexual reproduction in *N. crassa*. Interestingly, other enzymes involved in basic metabolism are also required for developmental processes in different organisms. As recently shown for *Sordaria macrospora*, the cytosolic enzyme ATP citrate lyase is needed for fruiting body formation (Nowrousian *et al.* 1999). Antisense repression of mitochondrial citrate synthase leads to a specific disintegration of the ovary tissues during flower development in potato plants (Landschutze *et al.* 1995).

The suitability of *N. crassa* to generate mutants expressing altered proteins and the recent discovery of specific mutations in TYKY associated with Leigh syndrome (Loeffen *et al.* 1998) open the possibility of using the fungus as an excellent eukaryotic model to study human mitochondrial disease.

We thank Dr. U. Schulte and Dr. H. Weiss for supplying the nuo51 and nuo78 (homologous recombination) mutant strains, Dr. N. B. Raju for the cytological work, and Mrs. Laura Pinto for excellent technical assistance. This work was supported by the Portuguese Science Foundation through research grants to A.V. and a fellowship to M.D.

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Communicating editor: J. J. Loros