Inactivation of Genes Encoding Subunits of the Peripheral and Membrane Arms of Neurospora Mitochondrial Complex I and Effects on Enzyme Assembly

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Manuscript received September 19, 1994
Accepted for publication November 18, 1994

ABSTRACT

We have isolated and characterized the nuclear genes encoding the 12.3-kD subunit of the membrane arm and the 29.9-kD subunit of the peripheral arm of complex I from *Neurospora crassa*. The former gene was known to be located in linkage group I and the latter is now assigned to linkage group IV of the fungal genome. The genes were separately transformed into different *N. crassa* strains and transformants with duplicated DNA sequences were isolated. Selected transformants were then mated with other strains to generate repeat-induced point mutations in both copies of the genes present in the nucleus of the parental transformant. From the progeny of the crosses, we were then able to recover two individual mutants lacking the 12.3- and 29.9-kD proteins in their mitochondria, mutants nuo12.3 and nuo29.9, respectively. Several other subunits of complex I are present in the mutant organelles, although with altered stoichiometries as compared with those in the wild-type strain. Based on the analysis of Triton-solubilized mitochondrial complexes in sucrose gradients, neither mutant is able to fully assemble complex I. Our results indicate that mutant nuo12.3 separately assembles the peripheral arm and most of the membrane arm of the enzyme. Mutant nuo29.9 seems to accumulate the membrane arm of complex I and being devoid of the peripheral part. This implicates the 29.9-kD protein in an early step of complex I assembly.

RESPIRATORY chain NADH dehydrogenase or complex I is an oligomeric enzyme containing >30 polypeptide subunits of both nuclear and mitochondrial origin, as well as FMN and several iron-sulfur clusters as prosthetic groups. Complex I is connected with the inner mitochondrial membrane and is responsible for a rotenone-sensitive transfer of electrons from NADH to ubiquinone coupled with proton translocation from the matrix to the inter membrane space of the organelle (for review articles, see WEISS *et al.* 1991; WALKER 1992). The fact that enzymes of more simple composition from other organisms are able to perform similar activities is one of the hints suggesting that complex I may be involved in other functions. Studies in Neurospora indicated that the formation of complex I arises from the separate assembly of two parts that subsequently join together. These two parts are structurally arranged in a way that one of them, the peripheral arm, is greatly exposed in the mitochondrial matrix, whereas the other, the membrane arm, is mainly buried in the inner mitochondrial membrane (TUSCHEN *et al.* 1990; HOFHAUS *et al.* 1991; WEISS *et al.* 1991). One can roughly say that the prosthetic groups of complex I are located in the former arm, whereas the latter contains the mitochondrially synthesized together with some nuclear-coded subunits of the enzyme.

Because of the complexity of complex I, this has been a difficult enzyme to investigate. For instance, structural models of complex I organization and a clearer picture of its composition and the isolation of the enzyme from different organisms have been described much more recently as compared with other respiratory chain constituents. One of the reasons for the recent interest in complex I is certainly the relationship between enzyme deficiencies and human diseases (WEISS *et al.* 1991; WALKER 1992; WALLACE 1993). Particular questions concerning complex I are the contribution of specific subunits to the overall structure, features and assembly of the enzyme (including their topology and the binding of prosthetic groups). Several approaches, such as the isolation and characterization of individual proteins or groups of proteins, the application of immunologic and microscopic techniques, gene cloning and sequencing, have been used to deal with these questions. We are concentrating on another powerful strategy to study complex I, namely the inactivation of specific genes and analysis of the phenotype of the resulting mutants, using the fungus *Neurospora crassa* for two main reasons. First, the fungal enzyme is very similar to that of mammals [most subunits are conserved between the *N. crassa* and the bovine enzyme (AZEVEDO and VIDEIRA 1994)]. Second, it is now relatively easy to produce mu-

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tants in *N. crassa*, once a gene has been isolated, by creating repeat-induced point mutations in the relevant DNA. Briefly, in this method the cloned gene/DNA is duplicated in the genome of a strain and then passed through a genetic cross. During this process, both copies of the duplication may become affected by several GC to AT transitions, leading to gene inactivation (SELKER and GARRETT 1988; SELKER 1990).

Using this strategy, we recently produced an *N. crassa* complex I mutant by the specific disruption of the 21.3-kD subunit of the peripheral arm of the enzyme (ALVES and VIDEIRA 1994). In this report, we describe the separate inactivation of the nuclear genes *nuo-29.9* and *nuo-12.3*, coding, respectively, for the 29.9-kD subunit of the peripheral arm and the 12.3-kD subunit of the membrane arm of the fungal enzyme. We show that the proteins are not essential for fungal development, although they are necessary for the assembly of complex I and lead to the accumulation of subcomplexes of the enzyme. Apparently, although lack of the 12.3-kD subunit only prevents final assembly of preformed peripheral and membrane arms of complex I, absence of the 29.9-kD subunit seems to block formation of the peripheral arm of complex I.

MATERIALS AND METHODS

Growth and manipulation of *N. crassa*: Most fungal strains used are listed in Table 1. Growth media, conditions and general manipulation of *N. crassa*, including crosses, isolation of random ascospores and induction of forced heterokaryons, were carried out according to standard procedures (DAVIS and DE SERRES 1970; PERKINS 1986). For fungal transformation, spheroplasts were prepared from conidia of 7-day-old solid cultures and transformed with closed circular plasmid DNA essentially as described (VOLLMER and YANOFSKY 1986) using hygromicin B-containing plates for the selection of transformants (STABEN *et al.* 1989). Individual transformants were picked to slants of complete media containing 150 μg/ml of hygromycin B, and "purified" by conidial platings and asexual transfers before mating with the relevant strains. *N. crassa* genomic DNA was isolated from small mycelial cultures as reported (LEE *et al.* 1988) with minor modifications (VIDEIRA *et al.* 1993).

Molecular cloning: Current protocols have been followed for most molecular cloning techniques (SAMBROOK *et al.* 1989; VIDEIRA *et al.* 1990a). An *N. crassa* genomic library cloned in phage J1 (obtained from the Fungal Genetics Stock Center) was screened by hybridization (SAMBROOK *et al.* 1989) with cDNAs encoding the 12.3- (VIDEIRA *et al.* 1993) and 29.9-kD (VIDEIRA *et al.* 1990a) subunits of complex I. The cDNA for the 29.9-kD polypeptide previously obtained (refered as 33-kD protein in VIDEIRA and WERNER 1989; VIDEIRA *et al.* 1990a) was sequenced on both ends and corresponds to nucleotides 325–1054 of the published sequence (VAN DER PAS *et al.* 1991). Plasmids used for subcloning were pGEM3 and pGEM4 (MELTON *et al.* 1984) and pCSN44, which contains a hygromicin B-resistance gene, for fungal transformation (STABEN *et al.* 1989). A 4.4-kb *Kpn*I genomic DNA fragment containing the entire coding region of *nuo-29.9* from *N. crassa* was isolated from a recombinant J1 phage and cloned in pGEM3. The resulting recombinant plasmid was treated with *Hin*dIII (located in the polylinker region of pGEM3) and *Pvu*II (there is a site in the genomic DNA) and a relevant band was cloned in pCSN44 previously digested with *Hin*dIII and *Eco*RV. This procedure generated plasmid pNUO-29.9KP, which consists of pCSN44 containing *nuo-29.9* in a 2.0-kb *Kpn*I/*Pvu*II genomic fragment and the *Kpn*I/*Hin*dIII region of the polylinker of pGEM3 (Figure 1). A 1.9-kb *Bam*HI genomic DNA fragment containing the entire coding region of *nuo-12.3* from *N. crassa* was isolated from a recombinant J1 phage and cloned in pGEM4. The resulting recombinant plasmid was treated with *Hin*dIII (located in the polylinker region of pGEM4 and in the genomic DNA) and a relevant band was cloned in the *Hin*dIII site of pCSN44. This procedure generated plasmid pNUO-12.3BH, which consists of pCSN44 containing *nuo-12.3* in a 1.8-kb *Bam*HI/*Hin*dIII genomic fragment and the *Bam*HI/*Hin*dIII region of the polylinker of pGEM4 (see also Figure 1).

Genetic mapping: For gene mapping, the segregation of restriction fragment length polymorphisms were analyzed in the 38 strains (FGSC no. 4450–4487) of the Multicent-2 cross kit (METZENBERG *et al.* 1984). The gene for the 12.3-kD subunit was previously located on LG I (VIDEIRA *et al.* 1993). Analysis of the DNA of strains 4458–4461 digested with several restriction enzymes, using the cDNA for the 29.9-kD polypeptide as a probe in Southern blot experiments (SOUTHERN 1975), revealed a polymorphism when *Bam*HI was used. The segregation pattern of this polymorphism among the 38 strains [see no. 00034 in METZENBERG and GROTELUESCHEN (1992b)] shows that *nuo-29.9* is located on chromosome *IV*.

Protein analysis: The techniques for the preparation of *N. crassa* mitochondria (WERNER 1977; WERNER and SEBALD 1981), protein determination (BRADFORD 1976), SDS-polyacrylamide gel electrophoresis (WERNER and SEBALD 1981; ZAUNER *et al.* 1985), blotting and incubation of blots with antisera (TOWBIN *et al.* 1979; VIDEIRA and WERNER 1989), detection of alkaline phosphatase-conjugated second antibodies (BLAKE *et al.* 1984), sucrose gradient centrifugation analysis of detergent-solubilized mitochondrial proteins (ALVES and VIDEIRA 1994) and NADH:ferricyanide reductase activity of sucrose gradient fractions (HATEFI 1978) have been published before.

RESULTS

The mutation strategy: We used the RIP method (SELKER 1990) to inactivate specific complex I genes of *N. crassa*. We suspected that some subunits of the enzyme could be essential proteins because previous attempts to inactivate the respective genes were not successful. Also, many complex I polypeptides are well conserved and their similarity with other proteins suggests that they play important roles in the cell. Therefore, we decided to apply the technique of "sheltered RIP" (METZENBERG and GROTELUESCHEN 1992a; see also HARKNESS *et al.* 1994) that, by making use of particular *N. crassa* strains, allows the disruption of essential genes for which the chromosomal location is known. Briefly, there is a pair of strains (host and mate) carrying complementary auxotrophic markers on the particular chromosome. These strains also incorporate a mutant *mei-2* gene that leads to a high frequency of nondisjunction of chromosomes during meiosis when homozygous crosses are made (SMITH 1975). A trans-

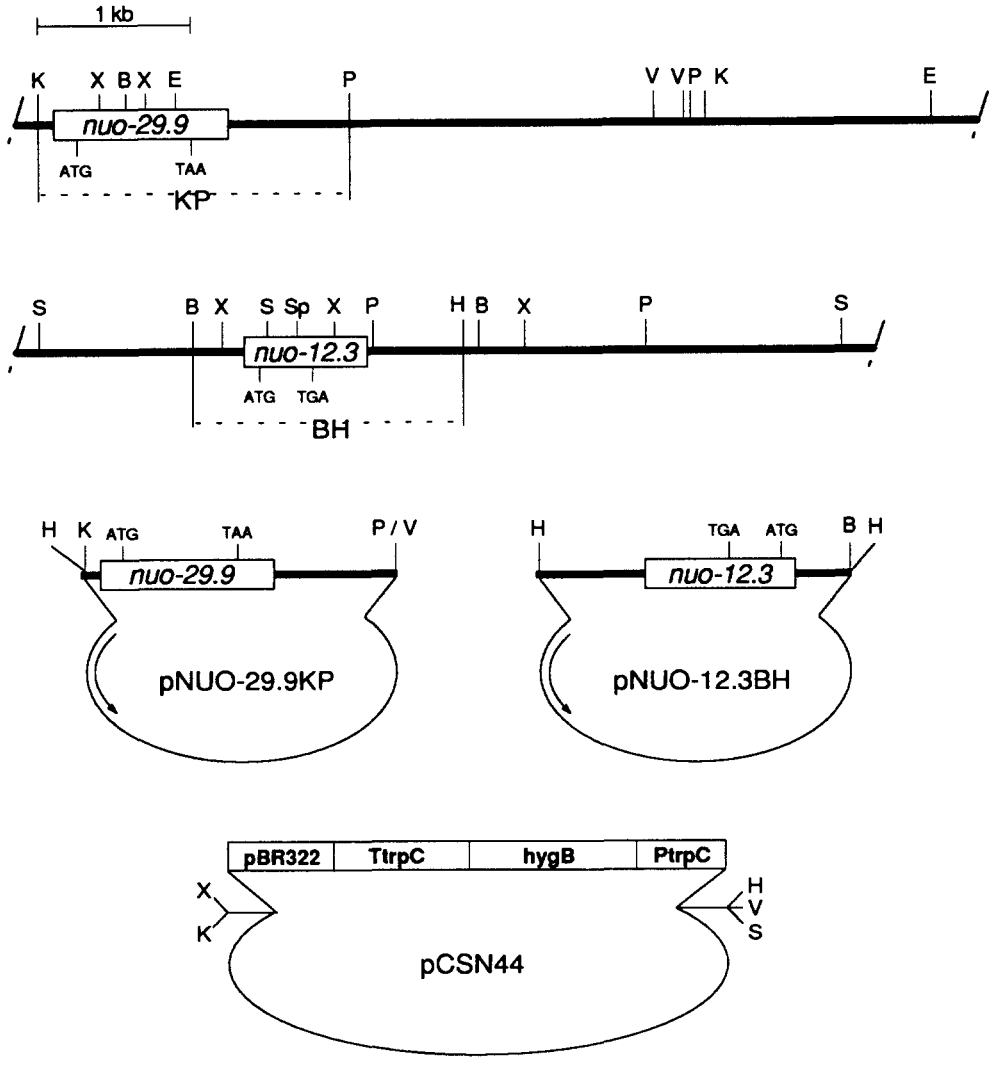

FIGURE 1.—Restriction map of *N. crassa* DNA regions containing *nuo-29.9* (upper) and *nuo-12.3* (lower) and construction of recombinant plasmids. The enzymes shown are as follows: B, *Bam*HI; H, *Hind*III; E, *Eco*RI; V, *Eco*RV; K, *Kpn*I; P, *Pvu*II; S, *Sal*I; Sp, *Sph*I; X, *Xho*I. The open boxes represent DNA sequences found in cDNA clones. Plasmid pNUO-29.9KP was obtained by cloning the 2.0-kb *Kpn*I/*Pvu*II fragment KP, together with the *Kpn*I/*Hin*dIII region of the polylinker of pGEM3, in pCSN44 double digested with *Hin*dIII and *Eco*RV. Plasmid pNUO-12.3BH was obtained by cloning the 1.8-kb *Bam*HI/*Hin*dIII fragment BH, together with the *Bam*HI/*Hin*dIII region of the polylinker of pGEM4, in the *Hin*dIII site of pCSN44 (see also MATERIALS AND METHODS for more details on plasmid construction). The arrows inside the constructs indicate the direction of transcription of the *hyg*B^r gene of pCSN44. The relevant features of pCSN44 are also diagrammed: unique restriction sites and a bacterial gene (*hyg*B^r) flanked by *Aspergillus nidulans* promoter and terminator sequences (PtrpC and TtrpC, respectively) that confers fungal resistance to hygromycin B.

formed host strain, carrying a duplicate copy of the gene of interest, is crossed with the mate strain. This results in the inactivation of the endogenous and ectopic copies of the gene of the transformant, at some frequency. By selecting ascospores in minimal medium, one is able to recover disomics for that particular chromosome that will rapidly decompose into heterokaryons during vegetative growth. If (enough) RIP has occurred during the cross, these cells will have a nucleus carrying the disrupted endogenous gene (from the transformant) and another nucleus carrying the wild-type copy of the gene (from the mate strain) that can complement the defect of the disrupted gene. It is possible later, using additional markers, to grow the heterokaryons in conditions where the nucleus with the disrupted gene is heavily favored, and, thus, a phenotype arising from the defective gene may be observed. We have applied this strategy to inactivate the *nuo-29.9* gene.

On the other hand, we realized that a mutant strain arising from RIP may or may not contain the copy of the gene introduced by transformation but will always have the inactivated endogenous gene derived from the transformed host. Knowing the chromosomal location

of a gene, it is possible to select directly for the endogenous host allele choosing an appropriate strain for breeding. The latter will have a marker (*e.g.*, an auxotrophic mutation) located as near the gene as possible to allow little or no recombination, and ascospores carrying this marker will be selected against (*e.g.*, by omitting the particular substance). This will of course only be possible when RIP is not a lethal event. The strains for sheltered RIP seemed appropriate because the mate strains carry an auxotrophic marker on the relevant chromosome and we successfully followed this approach to inactivate the *nuo-12.3* gene. However, selection for homokaryotic spores carrying only the potentially disrupted allele, derived from the host strain (adding the substance needed by the host and omitting the substance needed by the mate in the germinating medium), does not seem to be effective and one still obtains a high proportion of heterokaryons (see below and M. DUARTE and N. MOTA, unpublished results).

Isolation of mutant nuo29.9: A recombinant lambda J1 phage containing the nuclear gene encoding the 29.9-kD subunit of the peripheral arm of complex I from *N. crassa* was isolated and characterized. The gene was mapped to chromosome *IV* of the fungus. The 2.0-kb *Kpn*I/*Pvu*II DNA fragment KP, which contains the entire coding region of the gene and flanking sequences, was cloned in pCSN44 (Figure 1). The resulting plasmid pNUO-29.9KP was transformed into *N. crassa* strain Host IV. To isolate strains carrying a duplication of the gene, individual transformants were selected and their DNA was analyzed by Southern blotting, using the relevant cDNA as a probe. Figure 2 shows the results obtained with the single copy transformant T11. When wild-type DNA is digested with *Pst*I or *Eco*RV, single bands of 14 and 7.4 kb, respectively, are observed. When DNA from transformant T11 is treated with the same enzymes, extra bands of 2.0 and 13 kb, respectively, are detected. The 2.0-kb band obtained with *Pst*I indicates that DNA fragment KP introduced into T11 is intact, because this enzyme cuts on both sides of the fragment in plasmid pNUO-29.9KP (in pCSN44 in one side and in the small polylinker region derived from pGEM3 in the other side). Wild-type DNA digested with *Xho*I leads to the appearance of a 3.2-kb and a 224-bp (faintly visible) bands. With this enzyme, an extra band of 4.0 kb is seen in the transformant. These results indicate that the 29.9-kD protein is encoded by a single copy gene that has been duplicated in transformant T11.

Transformant T11 was crossed with Mate IV to inactivate by RIPing the two copies of the *nuo-29.9* gene in the transformant. Because both partners of the cross carry *mei-2*, a high frequency of spores with an abnormal number of chromosomes was expected. To select for chromosome *IV* disomics and taking advantage of the particular nutritional defects arising from their parents (LGs IV from T11 and Mate IV possess mutant alleles

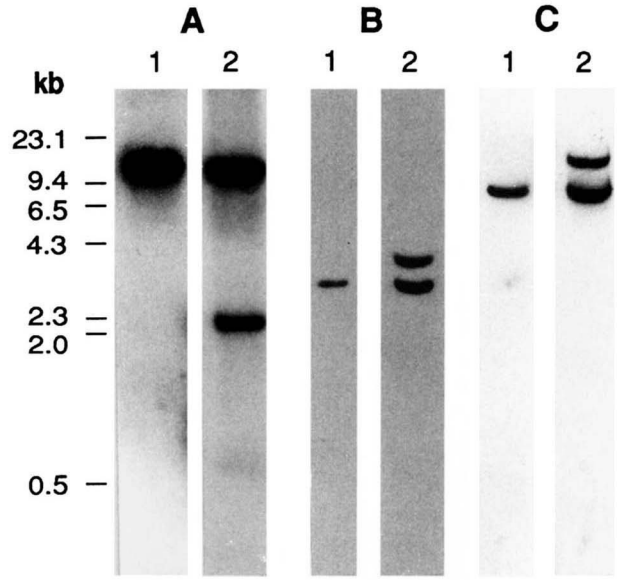

FIGURE 2.—Southern blotting analysis of a Neurospora strain transformed with plasmid pNUO-29.9KP. Genomic DNA from the wild-type strain (1) and transformant T11 (2) was prepared, separately digested with *Pst*I (A), *Xho*I (B), *Eco*RV (C), electrophoresed in agarose gels and blotted onto nylon membranes. The filters were probed with cDNA coding for the 29.9-kDa protein.

of the *pan-1* and *trp-4* genes, respectively), we germinated the spores on media lacking both pantothenate and tryptophan. We let 46 strains grow vegetatively to generate heterokaryons expected to contain a nucleus with the LG IV carrying the wild-type allele of *nuo-29.9* and *trp-4* (derived from Mate IV) and another nucleus type containing the LG IV carrying either *pan-1* and *nuo-29.9*$^{+}$ or *pan-1* and RIPed *nuo-29.9* (derived from T11). The latter also carries a marker conferring p-fluorophenylalanine resistance in LG IV (*mtr*^{r}), and it is expected that cells growing in the presence of this drug will have much higher proportions of this nucleus. We expected that this would result in reduced levels of the 29.9-kD protein in the case of heterokaryons where *nuo-29.9* had been RIPed.

We tested several concentrations of *p*-fluorophenylalanine in the growth media and found that 200 μM was enough to prevent growth of the wild-type strain but still allowed fairly good growth of most heterokaryotic progeny from the cross T11 × Mate IV. Therefore, we checked mitochondria from the 46 strains grown in the presence of 200 μM *p*-fluorophenylalanine for the presence of the 29.9-kD protein, using antiserum against this polypeptide in Western blot experiments. Using ~50 μg of mitochondrial protein, we found that three strains, named Mh11/13, Mh11/20 and Mh11/30 (Table 1), displayed very reduced or undetectable levels of the 29.9-kD protein (not shown). We believed that *nuo-29.9* could be an essential gene due to previous unsuccessful attempts to obtain mutants. Nevertheless,

TABLE 1

List of strains used in this work

Strain	Genotype or origin
Host IV	LG I: a; LG IV: *pan-1, mtr*^{r}, *trp-4*$^+$; LG V: *am*$^+$, *inl, inv, mei-2*
Mate IV	LG I: A; LG IV: *pan-1*$^+$, *mtr*^{s}, *trp-4*; LG V: *am*$_{132}$, *inl, inv, mei-2*
T11	A Host IV strain carrying an ectopic duplicated copy of the *nuo-29.9* gene
Mh11/13	An heterokaryotic strain carrying a mutant allele of *nuo-29.9*. Obtained from the progeny of a cross T11 × Mate IV selected on minimal media
Mh11/20	Same as Mh11/13
Mh11/30	Same as Mh11/13
M11/13	A *pan-1* homokaryon carrying a mutant allele of *nuo-29.9*, isolated from Mh11/13
M11/20	Same as M11/13 but isolated from Mh11/20. Also referred as mutant nuo29.9 in the text (contains the *nuo-29.9*^{RIP} gene)
M11/30	Same as M11/13 but isolated from Mh11/30
Host I	LG I: a^{m33}, *nic-2, ad-3*$^+$, *cyh-1*^{r}; LG V: *am*$^+$, *inl, inv, mei-2*
Mate I	LG I: A, *nic-2*$^+$, *ad-3, cyh-1*$^+$; LG V: *am*$_{132}$, *inl, inv, mei-2*
Helper strain	a^{m1}, *ad-3B, cyh-1*
T2h	A heterokaryon between the sterile helper strain and a Host I strain carrying an ectopic duplicated copy of the *nuo-12.3* gene
T3h	Same as T2h
T13h	Same as T2h
T26h	Same as T2h
T31h	Same as T2h
M2h/25	Also called mutant nuo12.3 in the text. An homokaryotic strain carrying a mutant allele of *nuo-12.3* (*nuo-12.3*^{RIP}). Obtained from the progeny of a cross T2h × Mate I, selected on minimal media containing glucose, inositol, nicotinamide and no adenine
M31h/25	Same as M2h/25 but obtained from a cross T31h × Mate I
M31h/13	A heterokaryotic strain carrying a mutant allele of *nuo-12.3*. Obtained as M31h/25

we tried to isolate *pan-1* homokaryons from these heterokaryons. We plated conidia from the heterokaryons on media containing pantothenate and no tryptophan, which should permit growth of both *pan-1* homokaryons and heterokaryons, and isolated several individual colonies. The homokaryotic nature of some of them was confirmed by their inability to grow on media without pantothenate. One homokaryotic isolate from each of the three heterokaryons, respectively called M11/13, M11/20 and M11/30, was further analyzed. Mitochondria from all these strains was shown by Western blotting to be devoid of the 29.9-kD subunit of complex I. Figure 3 illustrates the result obtained with strain M11/20, thereafter referred as mutant nuo29.9.

Isolation of mutant nuo12.3: As described above for *nuo-29.9*, we have isolated *N. crassa* genomic DNA containing the gene encoding the 12.3-kD subunit of the membrane arm of complex I. The gene was assigned previously to chromosome *I* of the fungus (VIDEIRA *et al.* 1993). We have cloned in pCSN44 a 1.8-kb *Bam*HI/*Hin*dIII DNA fragment (fragment BH of Figure 1), which contains the entire coding region of the gene and flanking sequences, generating plasmid pNUO-12.3BH. This plasmid was transformed into *N. crassa* strain Host I and individual transformants were isolated. To isolate strains carrying a duplication of the gene,

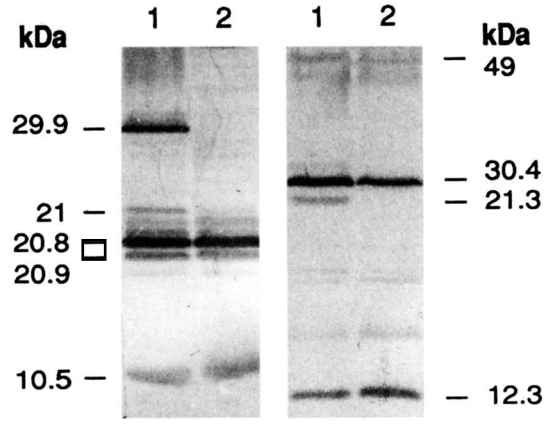

FIGURE 3.—Western blotting analysis of mitochondrial proteins from mutant nuo-29.9 with antisera against different complex I subunits. Total mitochondrial proteins from the wild-type strain (lanes 1) and strain M11/20 (lanes 2) were resolved by SDS gel electrophoresis and blotted onto nitrocellulose. The filters were incubated with mixtures of different antisera reacting with the subunits of complex I indicated by their molecular masses in the figure. Detection of antigen-antibody complexes was performed with alkaline phosphatase-conjugated second antibodies.

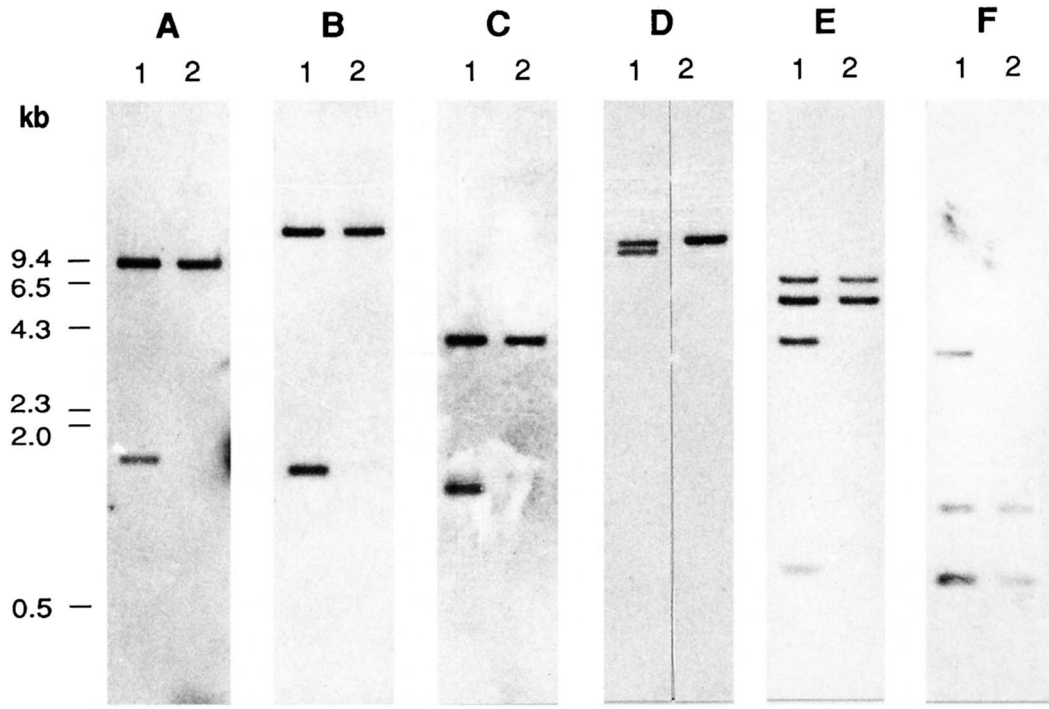

FIGURE 4.—Southern blotting analysis of a Neurospora strain transformed with plasmid pNUO-12.3BH. Genomic DNA from transformant T2 (1) and the wild-type strain (2) was prepared, separately digested with *Hin*dIII (A), *Pvu*II (B), *Sal*I (C), *Eco*RV (D), *Sph*I (E) and *Xho*I (F), electrophoresed in agarose gels and blotted onto nylon membranes. The filters were probed with cDNA coding for the 12.3-kDa protein.

DNA from 11 transformants was analyzed by Southern blotting using the relevant cDNA as a probe. Five single copy transformants (T2, T3, T13, T26 and T31) were used further in crosses with Mate I (see below). Figure 4 shows the results of Southern blots obtained with the single copy transformant T2. Using the enzymes *Hin*dIII, *Pvu*II and *Eco*RV , single bands of 8.4, 16 and 10 kb, respectively, can be detected in the wild-type DNA. The same enzymes lead to the appearance of extra bands of 1.8, 1.6 and 9.0 kb, respectively, in DNA from transformant T2. With *Sal*I, two bands of 3.7 and 1.6 kb in wild type and an extra band of 1.4 kb in T2 are visible. With *Sph*I, two bands of 7.0 and 5.6 kb in wild type and extra bands of 3.6 and 0.7 kb in T2 are seen. With *Xho*I, two bands of 1.3 and 0.7 kb in wild type and an extra band of 3.2 kb in T2 are detected. The 1.8-kb band visible in T2 when *Hin*dIII is used (the enzyme used for cloning in pCSN44) indicates that the ectopic DNA fragment BH is intact. Altogether, these results indicate that the 12.3-kD protein is encoded by a single copy gene that has been duplicated in transformant T2.

Forced heterokaryons between the sterile helper strain and transformants T2, T3, T13, T26 and T31 were prepared in minimal medium, generating strains T2h, T3h, T13h, T26h and T31h, respectively (see Table 1). These latter strains were separately crossed with Mate I to generate nuo12.3 mutants. As discussed above, one may select directly for ascospore progeny containing chromosome *I* derived from the Host I parent (and thus carrying mutations in *nuo-12.3* if RIP has occurred). From each of the five crosses, we have collected 27, 7, 39, 28 and 30 random germinated spores, respectively, that were selected on media containing nicotinamide and no adenine. Mitochondria from these strains were prepared and analyzed for the presence of the 12.3-kD protein, using antiserum against this polypeptide in Western blotting experiments. We could not detect this complex I subunit in one strain (M2h/25) from the cross T2h × Mate I and two strains from the cross T31h × Mate I (M31h/13 and M31h/25). Figure 5 shows the result obtained with strain M2h/25, thereafter referred to as mutant nuo12.3.

Although strains M2h/25 and M31h/25 are *nic-2* homokaryons, as confirmed by their inability to grow on media without nicotinamide, strain M31h/13 is still a heterokaryon (see also Table 1). Furthermore, only 16 of the 131 spores isolated from the five crosses were *nic-2* homokaryons. All others were heterokaryons despite being selected on media containing nicotinamide. This makes selection for the chromosome derived from the transformed host an inefficient process with this particular pair of strains. It is possible that more RIPed alleles of *nuo-12.3*, masked by the other nucleus type, existed among the heterokaryons analyzed.

Analysis of complex I assembly in the mutants: We

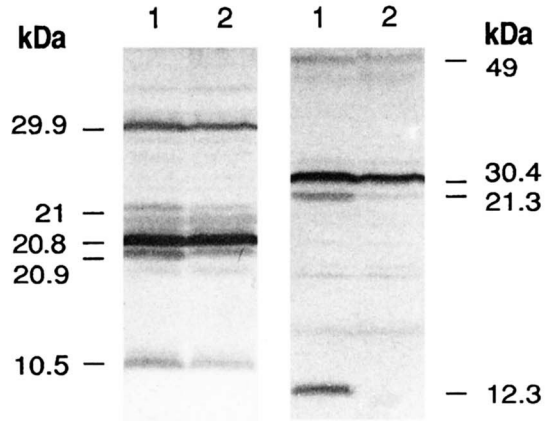

FIGURE 5.—Western blotting analysis of mitochondrial proteins from mutant nuo-12.3 with antisera against different complex I subunits. Total mitochondrial proteins from the wild type strain (lanes 1) and strain M2h/25 (lanes 2) were resolved by SDS gel electrophoresis and blotted onto nitrocellulose. The filters were incubated with mixtures of different antisera reacting with the subunits of complex I indicated in the figure. Detection of antigen-antibody complexes was performed with alkaline phosphatase-conjugated second antibodies.

have analyzed mitochondria of mutants nuo12.3 and nuo29.9 for the presence of other polypeptide components of complex I. The Western blots of Figures 3 and 5 reveal that all subunits of the enzyme analyzed are present, although with different stoichiometries as compared with the wild-type strain. In particular, the 21.3- (VIDEIRA *et al.* 1990b) and 21-kD (AZEVEDO *et al.* 1994) subunits of the peripheral arm of complex I are quite reduced in both mutants, as observed in complex I isolated from mutant nuo51 (FECKE *et al.* 1994) (in this article the 21-kD polypeptide is referred to as 18.3 kD). Because complex I seems to be formed from two domains (the peripheral and membrane arms) that are assembled independently and finally join together (TUSCHEN *et al.* 1990), we wanted to see the effect of inactivating genes coding for components of each arm. We have analyzed Triton-solubilized mitochondria from the two mutants by sucrose gradient centrifugation and followed the distribution of NADH:ferricyanide oxidoreductase activity in the fractions collected from the gradients. This activity can be performed by complex I or the peripheral arm of the enzyme alone (NEHLS *et al.* 1992). We also followed the distribution in the gradients of several polypeptide subunits of complex I. Aliquots of the gradient fractions were resolved by gel electrophoresis and blotted into nitrocellulose. After incubation with antibodies, the nitrocellulose filters were processed for the detection of antigen/antibody complexes. In these experiments, we have applied antisera against 75 (PREIS *et al.* 1991), 30.4 (VIDEIRA *et al.* 1990c), 29.9 (VAN DER PAS *et al.* 1991), 21 (AZEVEDO *et al.* 1994) and 10.5 kD (DUARTE *et al.* 1993) as markers for the peripheral arm and antisera against 20.9 (AZEVEDO *et al.* 1992), 20.8 (VIDEIRA *et al.* 1990a) and 12.3 kD (VIDEIRA *et al.* 1993) as markers for the membrane arm of the enzyme.

Figure 6 shows the results obtained with mutant nuo12.3. The NADH:ferricyanide activity from wild-type mitochondria elutes mostly at fractions 9 and 10 of the gradients (Figure 6A). In agreement with this, all complex I subunits analyzed are mainly detected in these two fractions (Figure 6B). This represents a typical behavior of the migration of complex I under the conditions used (see also ALVES and VIDEIRA 1994). When mitochondria from the mutant nuo12.3 are used, the NADH:ferricyanide activity elutes at fractions 7 and 8 (A) as well as most of the 75-, 29.9-, 21- and 10.5-kD subunits of the peripheral arm of complex I (Figure 6C). Thus, this material seems to represent the unassembled form of the peripheral arm of complex I. The elution profile of the 20.9- and 20.8-kD subunits of the membrane arm of the enzyme is somewhat different, with a peak in fraction 6 (C). This material of fraction 6 appears to represent an unassembled form of the membrane arm of complex I containing most or even all the polypeptide constituents (except for the 12.3-kD protein), because it should be about the same size of the peripheral arm of the enzyme.

Figure 7 shows the results of sucrose gradient centrifugation obtained with mutant nuo29.9. As described above in the experiment of Figure 6, wild-type complex I elutes mainly at fractions 9 and 10 of the gradients as deduced from the elution profiles of the NADH:ferricyanide activity (Figure 7A) and of the enzyme subunits (Figure 7B). In the case of the mutant nuo29.9, we do not detect significant NADH:ferricyanide activity in particular fractions of the gradients, suggesting that neither complex I nor its peripheral arm are present. In fact, most of the 30.4-kD subunit of the peripheral arm elutes in fraction 2 (Figure 7C) and should represent free subunit. Because small amounts of this subunit (and other peripheral arm subunits; not shown) can also be seen in the higher molecular weight region, we cannot totally exclude the possibility that some assembly of these proteins occurs but it may well represent unspecific protein aggregation. The 20.9-, 20.8- and 12.3-kD subunits of the membrane arm of complex I migrate practically throughout the gradient, although concentrating in the high molecular weight region. Our interpretation is that this material represents aggregates of the membrane arm of complex I, which thus accumulate in the mutant mitochondria. In fact, the more hydrophobic nature of the polypeptide constituents of this domain of the enzyme could explain this aggregation.

DISCUSSION

We have described the isolation and characterization of two *N. crassa* nuclear genes encoding complex I sub-

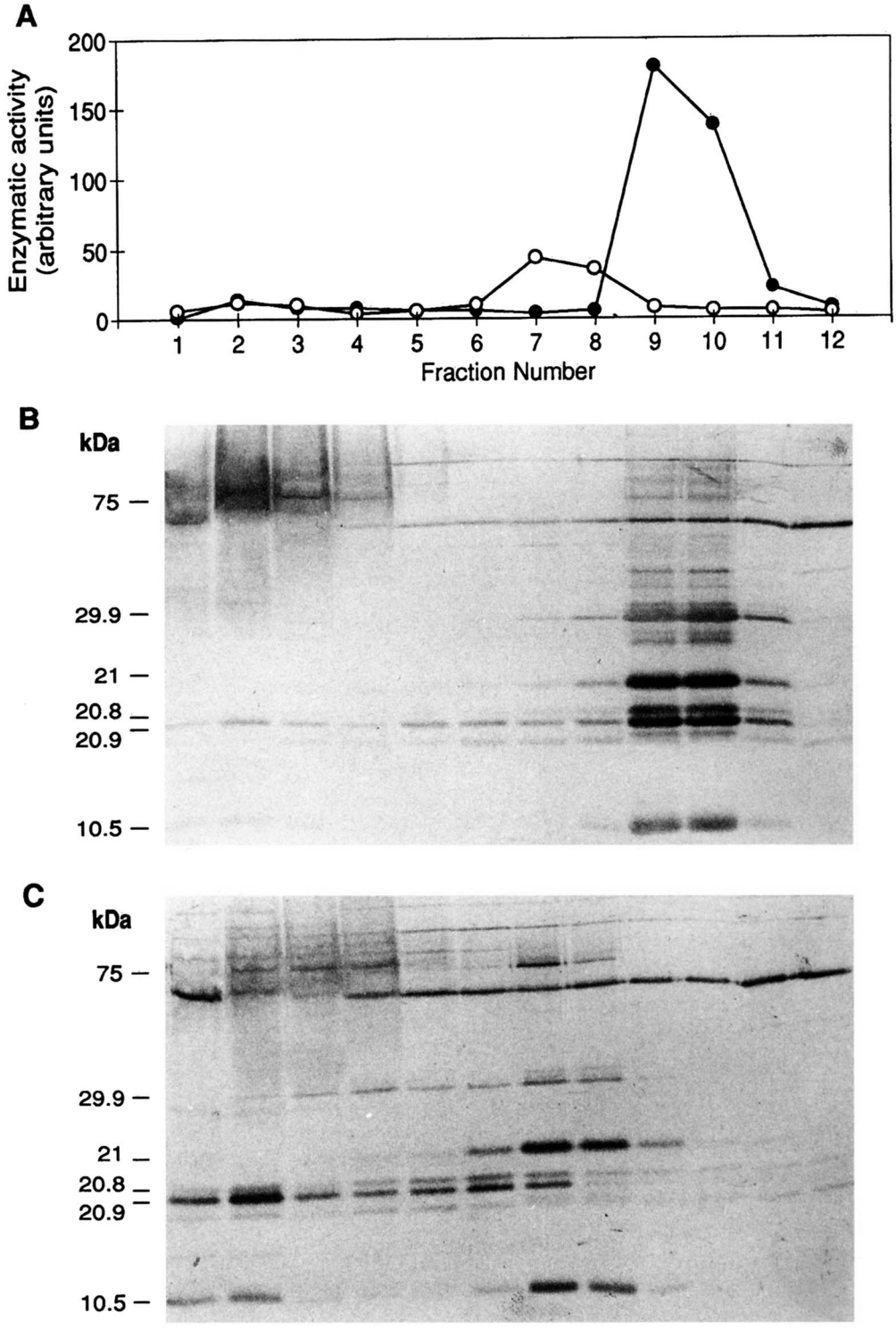

FIGURE 6.—Sucrose gradient centrifugation analysis of Triton-solubilized mitochondrial proteins from mutant nuo-12.3. Mitochondria were isolated, solubilized with Triton X-100 and centrifuged in sucrose gradients. Fractions (labeled 1–12 from top to bottom) of the gradients were collected. Aliquots of these fractions were assayed for NADH:ferricyanide reductase activity shown in panel A (●, wild type; ○, mutant nuo-12.3). Aliquots of the fractions obtained with material from the wild-type strain (B) and mutant nuo-12.3 (C) were also resolved by SDS gel electrophoresis and blotted onto nitrocellulose. The filters were incubated with a mixture of individual antisera against the subunits of complex I indicated in the left side of the figure. Detection was performed with alkaline phosphatase-conjugated second antibodies.

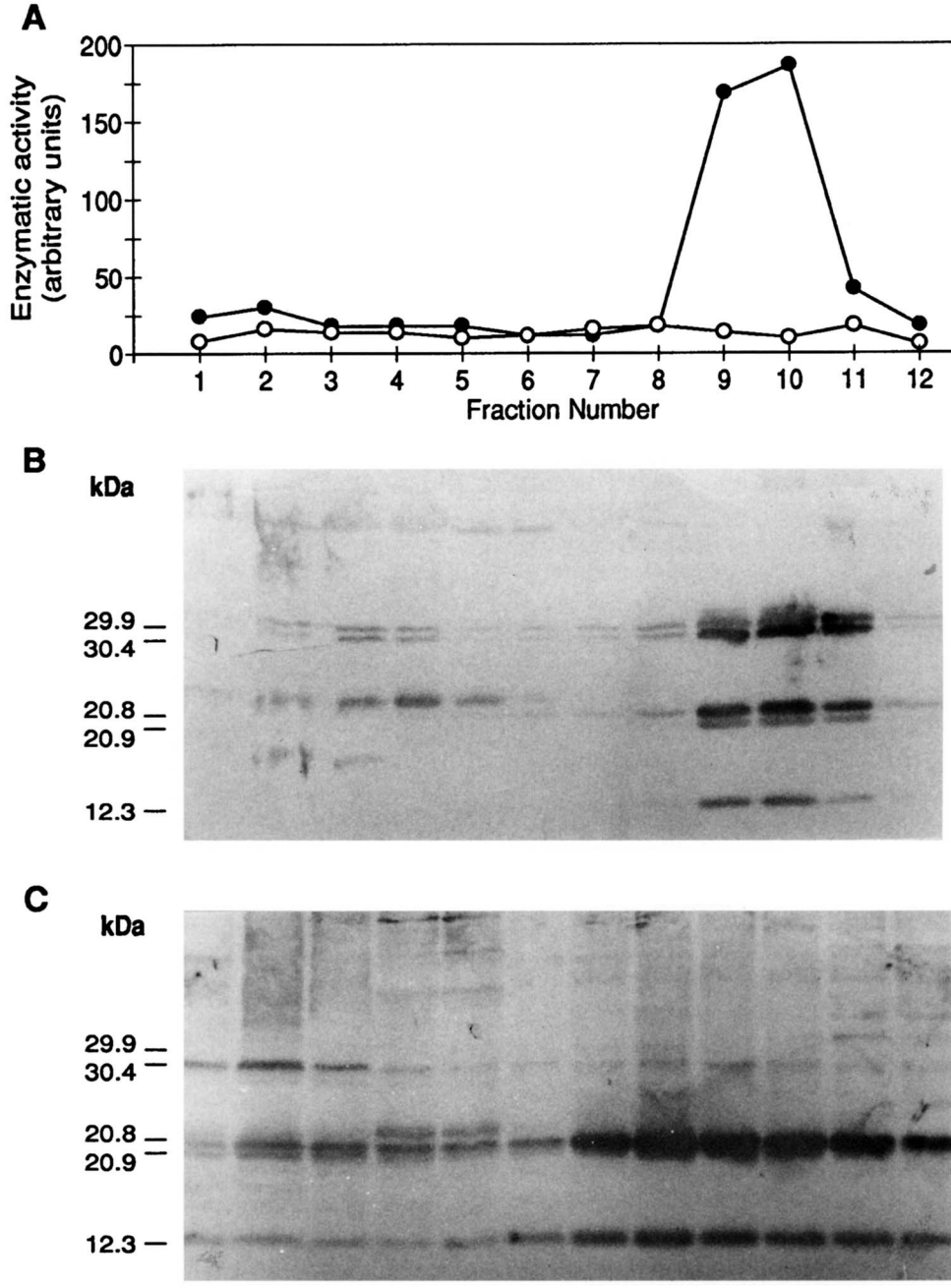

FIGURE 7.—Sucrose gradient centrifugation analysis of Triton-solubilized mitochondrial proteins from mutant nuo-29.9. Mitochondria were isolated, solubilized with Triton X-100 and centrifuged in sucrose gradients. Fractions (labeled 1–12 from top to bottom) of the gradients were collected. Aliquots of these fractions were assayed for NADH:ferricyanide reductase activity shown in panel A (●, wild type; ○, mutant nuo-29.9). Aliquots of the fractions obtained with material from the wild-type strain (B) and mutant nuo-29.9 (C) were also resolved by SDS gel electrophoresis and blotted onto nitrocellulose. The filters were incubated with a mixture of individual antisera against the subunits of complex I indicated in the left side of the figure. Detection was performed with alkaline phosphatase-conjugated second antibodies.

units, *nuo-12.3* and *nuo-29.9*, which code for the membrane arm 12.3-kD protein and the peripheral arm 29.9-kD protein, respectively. Using special strains designed for the disruption of essential genes by sheltered RIP (METZENBERG and GROTELUESCHEN 1992a), we have obtained mutants in both polypeptides. In the case of *nuo-12.3* disruption, we have applied a new procedure of direct selection of possible mutant ascospore progeny by selecting for the chromosome from the parental transformant, where RIPing had potentially occurred during the genetic cross. Consequently, it has been shown that neither complex I subunit is absolutely nec-

essary for fungal growth under standard conditions, although full assembly of the enzyme is prevented. Lack of the nuclear-coded components of complex I does not seem to prevent the expression of other subunits of the enzyme. These results, together with the isolation of other viable complex I mutants (see below), are leading to the idea that virtually any complex I subunit can be inactivated without the impairment of *N. crassa* development and that complex I may be a dispensable enzyme in this organism. Nevertheless, the mutants will certainly be useful for the understanding of the structure, function and biogenesis of complex I.

In fact, the absence of particular subunits of complex I results in quite different phenotypes regarding the formation of the enzyme. *N. crassa* strains disrupted for the peripheral arm 51- (FECKE *et al.* 1994) and 21.3-kD subunits (ALVES and VIDEIRA 1994) assemble an almost intact complex I together with the accumulation of intermediates of the enzyme in, at least, the latter strain. Disruption of the membrane arm 21.3-kD subunit (NEHLS *et al.* 1992) prevents complex I assembly and leads to the accumulation of the peripheral arm and two intermediates of the membrane arm of the enzyme. A similar situation seems to occur in a strain lacking the 20.8-kD subunit (VIDEIRA *et al.* 1990a) of the membrane arm of complex I (details will be published elsewhere). In human cells lacking the mitochondrial ND4 gene product, it appears that the other mitochondrially made subunits of complex I fail to assemble (HOFHAUS and ATTARDI 1993). Based on the analysis of detergent-solubilized mitochondrial complexes in sucrose gradients, we suggest that mutant nuo12.3 is able to form the peripheral and membrane domains of complex I, which are made separately (TUSCHEN *et al.* 1990), but not the intact enzyme. The 12.3-kD polypeptide shows some similarity with the hinge protein of complex III (VIDEIRA *et al.* 1993) and may be involved in direct interactions between the two parts of complex I. On the other hand, this protein seems to be unique to the fungal enzyme, as it has not been found in bovine complex I (FEARNLEY and WALKER 1992) nor in related bacterial NADH dehydrogenases (WEIDNER *et al.* 1993). Thus, lack of the protein may indirectly prevent complex I assembly by causing conformational changes in the membrane arm of the enzyme. The 29.9-kD subunit of complex I is conserved in the bovine enzyme (WALKER *et al.* 1992) but does not belong to a small subgroup of subunits related to the bacterial and other enzymes and, thus, considered as a "minimal" NADH:ubiquinone oxidoreductase (WALKER 1992; WEIDNER *et al.* 1993). Despite this, it seems to play an important role in the assembly of the mitochondrial complex I. Our results indicate that the peripheral arm is not assembled in mutant nuo29.9. Based on this phenotype, we propose that the 29.9-kD protein is involved in the earlier steps of complex I assembly. When the polypeptide is missing, the other proteins of the peripheral arm of the enzyme fail to assemble. In fact, this is the most severe defect found in complex I assembly among all Neurospora mutants obtained up to now. Lack of assembly of the peripheral arm might arise also from disruption of the 78-kD protein (T. A. A. HARKNESS, J. E. AZEVEDO, A. VIDEIRA and F. E. NARGANG, unpublished results) and seems to result from disruption of the 51-kD subunit in *Aspergillus niger* (PROEMPER *et al.* 1993), although the *N. crassa* nuo51 mutant is able to form an almost intact enzyme (FECKE *et al.* 1994). In *N. crassa*, it is also possible to disturb formation of the peripheral arm of complex I by growing cells under manganese deficiency (SCHMIDT *et al.* 1992).

Mutant nuo29.9 appears to be more severely defective in complex I than does mutant nuo12.3. Neither mutation appears to affect drastically the growth rate of the strains. We observed that they grow in liquid media at about two thirds of the wild-type rate. On the other hand, a very reduced capacity to produce conidia was observed in the nuo12.3 mutant alone. This phenomenon was also seen in a mutant lacking the 78-kD protein (T. A. A. HARKNESS, J. E. AZEVEDO, A. VIDEIRA and F. E. NARGANG, unpublished results). These observations suggest that other processes in the cell may be sensitive to the absence of particular complex I subunits alone rather than to complex I deficiencies.

We are greatly indebted to CLÁUDIA MACHADO, JOSÉ A. BELO, PAULO CASEIRO ALVES, NATÁLIA MOTA, MATILDE ROCHA, ANSELMO CARRAÇA, ABEL ROLDÃO, DR. JORGE E. AZEVEDO and Dr. ROBERT METZENBERG for their contribution to this work. The research was supported by Junta Nacional de Investigação Científica e Tecnológica from Portugal through research grants to A.V. and a fellowship to M.D.

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Communicating editor: R. H. DAVIS