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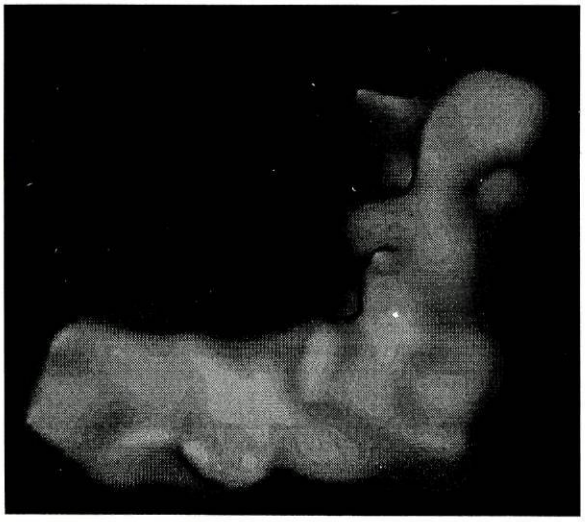
NADH:UBIQUINONA OXIDORREDUTASE (COMPLEXO I) DA CADEIA RESPIRATÓRIA: CLONAGEM E INACTIVAÇÃO DE GENES EM *Neurospora crassa*



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DISSERTAÇÃO DE CANDIDATURA AO GRAU DE DOUTOR EM CIÊNCIAS BIOMÉDICAS, ESPECIALIDADE DE BIOLOGIA MOLECULAR, APRESENTADA AO INSTITUTO DE CIÊNCIAS BIOMÉDICAS DE ABEL SALAZAR DA UNIVERSIDADE DO PORTO

ORIENTADOR: Professor Doutor Arnaldo Videira

PORTO
1999

Aos meus pais

De acordo com o Decreto-Lei 388/70 foram utilizados neste trabalho resultados dos artigos a seguir discriminados:

-Duarte, M., Sousa, R. & Videira, A. (1995). Inactivation of genes encoding subunits of the peripheral and membrane arms of *Neurospora* complex I and effects on enzyme assembly. **Genetics 139**, 1211-1221.

-Duarte, M., Finel, M. & Videira, A. (1996). Primary structure of a ferredoxin-like iron-sulfur subunit of complex I from *Neurospora crassa*. **Biochim. Biophys. Acta 1275**, 151-153.

-Duarte, M., Schulte, U. & Videira, A. (1997). Identification of the TYKY homologuos subunit of complex I from *Neurospora crassa*. **Biochim. Biophys. Acta 1322**, 237-241.

-Duarte, M., Mota, N., Pinto, L. & Videira, A. (1998). Inactivation of the gene coding for the 30.4 kDa subunit of complex I: is the enzyme essential for Neurospora? **Mol. Gen. Genet. 257**, 368-375.

-Sousa, R., Barquera, B., Duarte, M., Finel, M. & Videira, A. (1999). Characterisation of the last Fe-S cluster-binding subunit of *Neurospora crassa* complex I. **Biochim. Biophys. Acta 1411**, 142-146.

-Almeida, T., Duarte, M., Melo, A. M. P. & Videira, A. (1999). The 24 kDa iron-sulfur subunit of complex I is required for enzyme activity. **Eur. J. Biochem. 265**, 86-92.

-Ferreirinha, F., Duarte, M., Melo, A. M. P. & Videira, A. (1999). Effects of disrupting a 21 kDa subunit of complex I from *Neurospora crassa*. **Biochem. J. 342**, 551-554.

-Duarte, M. & Videira, A. (1999). Respiratory chain complex I is essential for sexual development in Neurospora and binding of iron-sulfur clusters are required for enzyme assembly. Submetido para publicação.

No cumprimento do disposto naquele Decreto-Lei, esclarece-se serem da nossa responsabilidade a execução das experiências apresentadas neste trabalho (excepto quando referido em contrário) assim como a sua interpretação e discussão.

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ABREVIATURAS

-ADP; difosfato de adenosina

-ATP; trifosfato de adenosina

-cAMP; monofosfato de adenosina cíclico

-cDNA; ácido desoxirribonucleico complementar

-EPR; ressonância paramagnética electrónica

-FAD; dinucleótido de flavina e adenina

-[FeS]; centro ferro-enxofre

-FMN; mononucleótido de flavina

-$FMNH_2$; forma reduzida do FMN

-GTP; trifosfato de guanosina

-MPP^+; 1-metil-4-fenilpiridina

-mRNA; ácido ribonucleico mensageiro

-mtDNA; DNA mitocondrial

-NAD(P)H; fosfato do dinucleótido de nicotinamida e adenina

-NADH; dinucleótido de nicotinamida e adenina

-NAD^+; forma oxidada do NADH

-nuo$\underline{x}$; mutante de *Neurospora crassa* para a subunidade de $\underline{x}$ kDa do complexo I

-tRNA; ácido ribonucleico de transferência

Nota: Alguns termos ingleses utilizados correntemente em Biologia Molecular não foram traduzidos, para evitar perda de clareza, e aparecem entre aspas no texto.

Capa: Modelo tridimensional da NADH:ubiquinona oxidoredutase de *Neurospora crassa* (de V. Guénebaut, California, EUA).

ÍNDICE

1-RESUMO

A enzima NADH:ubiquinona oxidorredutase (complexo I) da cadeia respiratória é uma unidade oligomérica, localizada na membrana interna da mitocôndria, que catalisa a transferência de electrões acoplada à translocação membranar de protões. Em *Neurospora crassa*, esta enzima é constituída por cerca de 35 subunidades diferentes (de origem nuclear e mitocondrial), um grupo FMN e vários centros [FeS]. A sua estrutura em forma de L, com o braço membranar embebido na membrana interna e o braço periférico projectado para a matriz, reflecte-se na organização funcional bem como no mecanismo de montagem da enzima. Dada a elevada complexidade desta enzima, questões como a localização dos diferentes grupos redox, a topografia dos diversos componentes e a sua função individual no complexo I, permanecem por responder. Com o intuito de contribuir para o esclarecimento de algumas destas questões, genes que codificam para subunidades do complexo I de *N. crassa* foram clonados, inactivados e os mutantes obtidos foram caracterizados.

Foram isolados vários cDNAs que codificam para a subunidade de 21.3c kDa do complexo I de *N. crassa*. Para isso, o gene *nqo9*, que codifica para uma subunidade de 23 kDa do complexo I de *Paracoccus denitrificans*, foi utilizado como sonda no rastreio de um banco de expressão de cDNAs de *N. crassa*, clonado no fago lambda gt11. As sequências destes cDNAs foram determinadas e a análise da estrutura primária da proteína revelou a existência duma extensa homologia com proteínas presentes desde procariotas (NQO9/NuoI) até mamíferos (TYKY). Tal como os seus homólogos, a proteína de 21.3c kDa possui sequências motivo para a ligação de dois centros [FeS] tetranucleares.

As subunidades de 21.3c kDa e de 19.3 kDa (homóloga da subunidade bovina PSST) foram expressas em *Escherichia coli*, como proteínas de fusão, purificadas e utilizadas para produzir anticorpos policlonais em coelho. Os anticorpos foram usados para localizar as respectivas subunidades no complexo I. A proteína de 21.3c kDa foi detectada na enzima madura e associada ao braço periférico. A subunidade de 19.3 kDa foi apenas imunologicamente visualizada na enzima madura, e nunca em qualquer dos intermediários de montagem formados nos diferentes mutantes. Estas observações

mostram que a subunidade de 21.3c kDa é um componente do braço periférico do complexo I de *N. crassa* e que a proteína de 19.3 kDa, uma proteína extrínseca de membrana, embora podendo pertencer ao domínio periférico, é muito instável quando este não se associa para formar a enzima. As implicações destes resultados na localização do centro [FeS] N2 são discutidas.

Os genes nucleares que codificam para 3 subunidades do complexo I (subunidades de 12.3 kDa, 30.4 kDa e 21.3c kDa) foram isolados, clonados num vector apropriado para transformação de *N. crassa* e inactivados pelo fenómeno de "RIPing". A influência das mutações produzidas, no processo de formação do complexo I, foi analisada em cada uma das estirpes mutantes (nuo12.3, nuo30.4 e nuo21.3c). A inactivação do gene que codifica para a subunidade do braço hidrofóbico de 12.3 kDa leva à acumulação de dois subcomplexos, um equivalente ao domínio periférico e o outro contendo subunidades do braço membranar, bloqueando o processo de formação da holoenzima. O fenótipo de montagem do complexo I nos mutantes nuo30.4 e nuo21.3c é muito similar. A disrupção de qualquer destas subunidades periféricas da enzima traduz-se na acumulação do braço membranar e na ausência de qualquer intermediário estável da parte periférica. Estes resultados sugerem que a inactivação de genes que codificam subunidades, quer da parte membranar quer do braço periférico, interfere com o processo de montagem do complexo I. Por outro lado, os intermediários presentes nos diferentes mutantes apoiam a formação independente dos dois domínios da enzima.

A actividade do complexo I foi determinada nas estirpes mutantes nuo24 e nuo21. No mutante nuo24 o consumo de oxigénio, utilizando substratos que produzem NADH, é completamente insensível à rotenona, o que indica que o complexo I formado não funciona na transferência de electrões. A oxidação do NADH é efectuada por NADH desidrogenases alternativas. A actividade NADH:ubiquinona redutase do complexo I presente no mutante nuo21 é sensível à rotenona e muito semelhante à da estirpe selvagem. A ausência deste polipéptido não altera a oxidação do NADH pela ubiquinona mas interfere com a redução de aceitadores artificiais.

O cDNA que codifica para a subunidade de 21.3c kDa foi clonado num vector de expressão contendo um promotor induzido por ácido quínico e introduzido no mutante nuo21.3c. A análise de proteínas mitocondriais das estirpes transformadas revelou a

expressão da proteína, permitindo restaurar a montagem do complexo I. Foram também expressas formas alteradas da subunidade de 21.3c kDa, em que resíduos de cisteína, envolvidos na ligação dos centros [4Fe4S], foram substituídos por alanina ou serina. As mutações produzidas resultaram numa disrupção na montagem do complexo I similar à observada no mutante nuo21.3c. Esta observação sugere que a ligação dos centros [FeS] é um pré-requisito para a formação do complexo I.

Foi estudado o comportamento dos diversos mutantes ao longo do ciclo de vida do fungo. Os resultados obtidos mostram que as estirpes mutantes, mesmo com defeitos acentuados no complexo I, apresentam um crescimento vegetativo não muito diferente do da estirpe selvagem, pelo que esta enzima não é essencial à propagação vegetativa do fungo. No entanto, em cruzamentos homozigóticos, muitas das estirpes mutantes são incapazes de completar a fase sexual do ciclo de vida. Apenas os mutantes com complexo I activo produzem ascósporos. Estas observações sugerem que o bloqueamento do desenvolvimento sexual está, provavelmente, associado a uma diminuição na produção de energia pela mitocôndria.

SUMMARY

NADH:ubiquinone oxidoreductase (complex I) of the respiratory chain is an oligomeric enzyme located in the inner mitochondrial membrane, where it catalyses electron transfer coupled to proton translocation. The *Neurospora crassa* enzyme is composed of around 35 dissimilar subunits (of both nuclear and mitochondrial origin), one FMN group and several [FeS] centres. The complex has an L-shaped structure, with the membrane arm embedded in the inner membrane and the peripheral arm protruding into the mitochondrial matrix, which is reflected in its functional organisation as well as in its mechanism of assembly. Due to the extreme complexity of the enzyme, questions concerning the localisation of the different redox centres, the topography of all the components of complex I and their individual role, remain unsolved. In order to contribute to the elucidation of some of these questions, genes encoding subunits of *N. crassa* complex I were cloned, inactivated and the mutant strains characterised.

Several cDNAs coding for the 21.3c kDa subunit of the *N. crassa* complex I were isolated. The lambda gt11 expression library was screened by hybridization with the *nqo9* gene of *Paracoccus denitrificans*, that codes for the 23 kDa subunit of complex I. The DNA sequences of the positive clones were determined. The deduced primary structure of the protein is highly homologous to an iron sulfur subunit of complex I from prokaryotes (NQO9/NuoI) to mammals (TYKY) and contains two cysteine-rich motives that may each bind a tetranuclear iron sulfur cluster.

The 21.3c kDa subunit and the 19.3 kDa polypeptide (homologous to the PSST subunit of bovine complex I) were expressed in *Escherichia coli* as fusion proteins, purified and used to produce rabbit antisera. These polyclonal antibodies were used to localise the proteins in one of the two arms of complex I. The 21.3c kDa subunit was detected in preparations of complex I and associated with the peripheral arm. The 19.3 kDa subunit was visualised in the assembled complex I and was never detected in mitochondria from strains forming only unassembled peripheral or membrane arms. These results show that the 21.3c kDa subunit belongs to the peripheral arm of complex I from *N. crassa*, and that the 19.3 kDa protein, an extrinsic membrane protein, is probably a peripheral component, that may be unstable when this domain is not assembled with the

rest of the enzyme. The implications of these results on the localisation of [FeS] cluster N2 are discussed.

The nuclear genes coding for three subunits of complex I (12.3 kDa, 30.4 kDa and 21.3c kDa subunits) were isolated, cloned in an appropriate vector for fungal transformation and inactivated by the RIP phenomenon. The effects of the mutations on the complex I assembly were analysed in each of the mutant strains (nuo12.3, nuo30.4 and nuo21.3c). The inactivation of the gene encoding the membrane arm 12.3 kDa subunit leads to the accumulation of two subcomplexes, one corresponding to the peripheral domain of the enzyme and the other containing most of the membrane arm subunits. Yet, the fully assembly of complex I is blocked. The state of complex I assembly in nuo30.4 and nuo21.3c mutants is very similar. Disruption of each of these peripheral subunits prevents the assembly of the enzyme, while its membrane arm accumulates. These results suggest that disruption of genes encoding subunits of the peripheral or membrane arm of complex I interfere with the enzyme assembly. On the other hand, the observation of different intermediates supports the independent formation of the two arms of the enzyme.

The complex I activity of the mutant strains nuo24 and nuo21 was determined. The oxygen uptake of nuo24 mitochondria, respiring on substrates producing NADH, was completely resistant to rotenone inhibition suggesting that the nuo24 complex I is not operating in electron transfer. The oxidation of NADH is carried out by alternative NADH dehydrogenase(s). The NADH:ubiquinone reductase activity of complex I from nuo21 is comparable to the wild type value and is inhibited by rotenone. The absence of this polypeptide does not change the NADH oxidation by ubiquinone but alters the reduction of artificial electron acceptors.

The cDNA coding for the 21.3c kDa subunit was cloned in a plasmid, under the control of an inducible promotor, and was then introduced in mutant nuo21.3c. An analysis of the mitochondrial proteins from several transformants revealed the expression of the 21.3c kDa protein, rescuing the complex I phenotype. Mutated 21.3c kDa proteins were also expressed in the nuo21.3c mutant. In these strains selected cysteine residues, which might be involved in the binding of the [4Fe4S] clusters, were substituted with alanines or serines. Regarding complex I assembly, the alterations resulted in a phenotype

similar to that of the null-mutant. This observation suggests that the binding of the iron sulfur centres is a prerequisite for the formation of complex I.

We have studied the behaviour of the different mutant strains through the life cycle of the fungus. The results obtained show that the mutant strains, even with gross defects in complex I, grow reasonably well during the vegetative phase, indicating that the enzyme is not essential for vegetative growth. However, in homozygous crosses several complex I mutants are unable to complete the sexual phase of the life cycle. Only the mutant strains with complex I activity produced ascospores, suggesting that a failure in energy production by mitochondria might be the cause for the impairment of sexual development during homozygous crosses.

RÉSUMÉ

L'enzyme NADH:ubiquinone oxydoréductase (complèxe I) de la chaîne respiratoire c'est une unité oligomérique située dans la membrane interne de la mitochondrie, qui catalyse le transfert d'électrons accouplée à la translocation de protons. Dans le cas de *Neurospora crassa*, cette enzyme est constituée par environ 35 sous-unités différentes (d'origine nucléaire et mitochondriale), un groupe FMN et plusieurs centres [FeS]. Sa structure en forme de L, avec le bras membranaire inseré dans la membrane interne et le bras périphérique projeté vers la matrice, se réflète dans l'organisation fonctionelle et aussi dans le mécanisme de montage de l'enzyme. À cause de la grande complexité de cette enzyme, des questions telles que la localisation de différents groupes redox, la topographie des plusieurs composants et leur fonction individuelle dans le complèxe I, restent sans réponse. Pour contribuer à l'eclaircissement de certaines questions mentionnées, des gènes qui codifient pour des sous-unités du complèxe I de *N. crassa* ont été clonés, rendus inactifs et les mutants obtenus ont été caractérises.

On a isolé des cDNAs qui codifient pour la sous-unité de 21.3c kDa du complèxe I du fongus. Le gène *nqo9*, qui codifie une sous-unité de 23 kDa du complèxe I de *Paracoccus denitrificans*, a été utilisé comme sonde pour chercher une banque d'expression de cDNAs de *N. crassa*. Les séquences de ces cDNAs ont été déterminées et l'analyse de la structure primaire de la protéine a révélé l'existence de protéines homologues présents depuis prokaryotes (NQO9/NuoI) jusqu'à des mammiféres (TYKY). Tel que ses homologues, la protéine du fongus a des séquences motif pour la liaison de deux centres [FeS] tétranucléaires.

Les polypéptides de 21.3c kDa et de 19.3 kDa (homologue de la protéine bovine PSST) ont été exprimés en *Escherichia coli* comme des protéines de fusion, qui ont été utilisées pour produire des anticorps policlonaux. Ces anticorps ont été utilisés pour localiser les sous-unités dans le complèxe I. La protéine de 21.3c kDa se trouve chez l'enzyme mûre et associé au bras périphérique. La sous-unité de 19.3 kDa a été immunologiquement visualisée dans l'holoenzyme et jamais dans les intermédiaires de montage qui se forment dans les différents mutants. Ces observations montrent que la sous-unité de 21.3c kDa est un composant du bras périphérique du complèxe I de *N.*

crassa et que la protéine de 19.3 kDa, une protéine extrinsèque à la membrane, quoiqu'elle puisse appartenir au domaine périphérique, elle est trés instable quand celui-ci ne s'associe pas pour former l'enzyme. Les implications de ces résultats dans la localisation du centre [FeS] N2 seront discutées.

Les gènes nucléaires qui codifient pour 3 sous-unités du complèxe I (sous-unité de 12.3 kDa, 30.4 kDa et 21.3c kDa) ont été isolés, clonés dans un vecteur adéquat pour la transformation de *N. crassa* et rendus inactifs par le phénomène de "RIPing". L'influence des mutations produites dans le processus de formation du complèxe I a été analysée pour chacun des mutants (nuo12.3, nuo30.4 et nuo21.3c). L'inactivation du gène qui codifie pour la sous-unité du bras hydrophobique de 12.3 kDa mène à l'accumulation de deux sous-complèxes, l'un équivalent au domaine périphérique et l'autre contenant des sous-unités du bras membranaire, ce qui bloque le processus de montage de l'holoenzyme. Le phénotype de montage du complèxe I dans les mutants nuo30.4 et nuo21.3c est très semblable. La disruption de n'importe quelle de ces sous-unités périphériques de l'enzyme se traduit par l'accumulation du bras membranaire et par l'absence d'intermédiaires stables de la partie périphérique. Ces résultats suggèrent que l'inactivation de gènes qui codifient des sous-unités, soit de la partie membraneuse, soit du bras périphérique, interfère avec le processus de montage du complèxe I. D'autre part, les intermédiaires présents dans les différents mutants confirment la formation indépendante des deux domaines de l'enzyme.

L'activité du complèxe I a été determinée dans les mutants nuo24 et nuo21. Dans le mutant nuo24, la consommation d'oxygène, en utilisant des substrats qui produisent NADH, est complètement insensible à la roténone, ce qui prouve que le complèxe I formé ne fonctionne pas dans le transfer d'électrons. L'oxydation du NADH est effectuée par des enzymes NADH déshydrogénases alternatives. L'activité NADH:ubiquinone réductase du complèxe I présente dans le mutant nuo21 est sensible à la roténone et très semblable à l'activité de la souche sauvage. L'absence de ce polypéptide ne trouble pas l'oxydation du NADH par l'ubiquinone, mais elle interfère avec la réduction d'accepteurs artificiels.

Le cDNA qui codifie pour la sous-unité de 21.3c kDa a été cloné dans un vecteur d'expression et ensuite introduit dans le mutant nuo21.3c. L'analyse des protéines

mitochondriales dans les transformants a révélé l'expression de la protéine, ce qui a restauré la montage du complèxe I. Des formes altérées de la sous-unité de 21.3c kDa dans lesquelles des résidus de cysteine, probablement engagés dans la liaison des centres [4Fe4S], ont été remplacés par alanine ou par sérine, ont été exprimés dans le mutant nuo21.3c. Les mutations produites ont eu comme résultat une disruption du montage du complèxe I semblable à l'observée dans le mutant nuo21.3c. Cette observation suggère que la liaison des centres [FeS] c'est une condition essentielle pour la formation du complèxe I.

On a etudié le comportement des différents mutants, pendant le cycle de vie du fongus. Les résultats obtenus suggèrent que les mutants, même avec des défauts accentués dans le complèxe I, présentent une croissance végétative qui n'est pas très différentte de celle de la souche sauvage, d'où l'on peut conclure que cette enzyme n'est pas essentielle à la propagation végétative du fongus. Cependant, quand les mutants sont en croisements homozygotiques, ils sont incapables de compléter la phase sexuelle du cycle de vie de *N. crassa*. Les mutants avec le complèxe I actif sont les seuls à produire des ascospores ce qui indique que le blocage du dévelopement sexuel est, probablement, associé à une diminution de la production d'énergie par la mitochondrie.

2-INTRODUÇÃO

As mitocôndrias são organelos presentes nas células eucariotas. Estes organelos estão envolvidos em vários processos celulares como a produção de energia ou a morte celular programada (apoptose). Deficiências nas funções mitocondriais têm sido associadas a doenças humanas degenerativas, cancro e envelhecimento, o que tem suscitado um interesse crescente no estudo da mitocôndria (ver Wallace, 1999). A fosforilação oxidativa, processo no qual se produz a maior parte da energia celular, tem sido um dos alvos desse interesse. Em mamíferos, esta via metabólica é composta por cinco complexos proteicos multiméricos denominados: NADH:ubiquinona oxidorredutase (complexo I); succinato:ubiquinona oxidorredutase (complexo II); ubiquinol:citocromo c oxidorredutase (complexo III); citocromo c oxidase (complexo IV) e ATP sintase (complexo V). Os quatro primeiros complexos, juntamente com ubiquinona e citocromo *c*, catalisam a transferência de electrões do NADH e succinato para o oxigénio. Ao nível dos complexos I, III e IV, a energia libertada nas reacções redox é acoplada à translocação de protões através da membrana interna mitocondrial, formando-se um gradiente protónico que é posteriormente utilizado, pelo complexo V, para sintetizar ATP (como artigo de revisão ver Hatefi, 1985).

Em plantas e fungos, a cadeia respiratória é bastante mais complexa do que a acima descrita. Nestes organismos existem várias NAD(P)H desidrogenases capazes de oxidar o NAD(P)H citoplasmático ou da matriz (Weiss *et al.*, 1970; Möller *et al.*, 1993; Rasmusson *et al.*, 1998) e ainda uma oxidase alternativa capaz de reduzir o oxigénio molecular (Moore & Siedow, 1991). No entanto, nenhuma destas enzimas tem a capacidade de bombear protões. O número de NAD(P)H desidrogenases varia de organismo para organismo podendo ir de uma (Kerscher *et al.*, 1999) a quatro (Melo *et al.*, 1996) enzimas diferentes. Contrariamente ao complexo I, estas NAD(P)H:ubiquinona oxidorredutases são constituídas por uma única cadeia polipeptídica e contêm FAD como cofactor (Yagi, 1991).

Apesar da enorme complexidade deste sistema, tem vindo a verificar-se um grande avanço no conhecimento, a nível molecular, dos componentes da cadeia respiratória. A estrutura atómica do domínio catalítico da ATP sintase, assim como dos

complexos III e IV, foi recentemente determinada por cristalografia de raios X (Abrahams *et al.*, 1994; Iwata *et al.*, 1995; Tsukihara *et al.*, 1996; Xia *et al.*, 1997). Há, no entanto, muitos aspectos que necessitam ser esclarecidos, nomeadamente ao nível do complexo I, o complexo mais complexo da cadeia respiratória mitocondrial e, talvez por isso, o mais desconhecido.

O trabalho aqui apresentado incide sobre a estrutura e biogénese deste primeiro componente da cadeia de fosforilação oxidativa. Ao longo deste capítulo vários aspectos do conhecimento actual sobre o complexo I serão detalhadamente expostos.

2.1-Distribuição filogenética do complexo I

A descoberta de que várias subunidades do complexo I são codificadas na mitocôndria (Chomyn *et al.*, 1985, 1986) permitiu estudar a distribuição filogenética desta enzima. Deste modo, a presença no DNA mitocondrial de "ORFs" que codificam proteínas homólogas das subunidades ND1-ND6 e ND4L (ver à frente) tem sido considerada como evidência para a existência de complexo I. Com base nesta característica, foi demonstrada a presença desta enzima em organismos eucariotas como fungos (Burger & Werner, 1986; Nelson & Macino, 1987), algas (Denovan-Wright & Lee, 1992), plantas (Stern *et al.*, 1986; Oda *et al.*, 1992), protozoários (Pritchard *et al.*, 1990), anfíbios (Roe *et al.*, 1985), peixes (Johansen *et al.*, 1990), mamíferos (Anderson *et al.*,1981) entre outros.

Uma observação surpreendente foi a da existência de 11 genes codificantes para proteínas homólogas de subunidades do complexo I no genoma de cloroplastos de várias plantas superiores (Ohyama *et al.*, 1986; Shinozaki *et al.*, 1986; Fearnley *et al.*, 1989; Videira *et al.*, 1990a). O papel fisiológico deste putativo complexo proteico não está, ainda, inteiramente esclarecido. Talvez esta enzima funcione como uma NAD(P)H:plastoquinona oxidorredutase participando no transporte cíclico de electrões à volta do fotossistema I, em condições de luz, e na cadeia respiratória do cloroplasto, na ausência de fotossíntese (Friedrich *et al.*, 1995; Burrows *et al.*, 1998; Shikanai *et al.*, 1998).

Dada a origem endossimbiótica da mitocôndria, é de prever a existência de proteínas homólogas de subunidades do complexo I em organismos procariotas. Assim, foi recentemente sequenciado o operão que codifica para uma NADH:ubiquinona oxidorredutase, homóloga do complexo I mitocondrial, em três bactérias, nomeadamente *P. denitrificans* (Xu *et al.*, 1991a,b, 1992a,b, 1993), *E. coli* (Weidner *et al.*, 1993) e *Thermus thermophilus* HB-8 (Yano *et al.*, 1997). Para além destes, há evidências da existência desta enzima em muitos outros organismos procariotas, incluindo a bactéria fotossintética *Rhodobacter capsulatus* (ver Dupuis *et al.*, 1998a).

A ubiquidade filogenética do complexo I tem, no entanto, uma excepção notável- algumas leveduras fermentativas, entre as quais a levedura *Saccharomyces cerevisiae*- não permitindo a utilização deste organismo no seu estudo (Nosek & Fukuhara, 1994).

2.2-Estrutura do complexo I

2.2.1-Componentes polipeptídicos

Foram já obtidas preparações relativamente puras de complexo I para vários mamíferos (Hatefi, 1985; ver Walker, 1992), fungos (Weiss *et al.*, 1991; Sackmann *et al.*, 1991), plantas (Leterme & Boutry, 1993; Herz *et al.*, 1994; Rasmusson *et al.*, 1994) e *E. coli* (Leif *et al.*, 1995). A análise electroforética destas preparações, em condições desnaturantes, sugere a presença de vários componentes polipeptídicos. Assim, é actualmente aceite que o complexo I procariota é constituído por 14 subunidades diferentes, estando os genes que codificam para estes polipéptidos organizados num operão. Da sequenciação completa do operão de *P. denitrificans*, *E. coli* e *T. thermophilus*, foi possível concluir que a ordem dos genes se encontra conservada. Em *E. coli*, dois dos genes presentes no operão que codifica para esta enzima encontram-se unidos produzindo uma única proteina (Blattner *et al.*, 1997), pelo que o complexo I é constituído por apenas 13 polipéptidos nesta bactéria.

Proteínas homólogas das 14 subunidades que formam o complexo I procariota foram encontradas em todos os organismos para os quais a enzima foi caracterizada (Friedrich *et al.*, 1995) e parecem constituir a unidade funcional mínima deste complexo.

No complexo I de *N. crassa*, um dos organismos eucariotas onde a enzima se encontra mais bem caracterizada, são detectadas cerca de 35 subunidades 27 das quais foram já caracterizadas ao nível da estrutura primária (ver Videira, 1998). No caso da enzima bovina, para além das 14 subunidades homólogas do complexo I procariota, Walker *et al.* (1992) encontraram evidência para a existência de, pelo menos, mais 28 subunidades polipeptídicas (ver tabela). A mesma complexidade foi descrita para o complexo I humano (ver Smeitink *et al.*, 1998). Algumas das subunidades acessórias encontram-se conservadas desde fungos até mamíferos, embora outras haja que parecem ser específicas duma determinada espécie (Walker, 1992; Videira, 1998).

Um aspecto interessante do complexo I mitocondrial é a sua dualidade genética. Como demonstrado por Chomyn *et al.* (1985, 1986), o mtDNA humano codifica para sete subunidades do complexo I. A informação genética para estas proteínas foi encontrada na mitocôndria de muitos outros organismos. Estes polipéptidos, denominados ND1-ND6 e ND4L, são extremamente hidrofóbicos e encontram-se embebidos na membrana interna mitocondrial através dos seus múltiplos domínios transmembranares (ver 2.2.5). Em plantas e protozoários, o número de subunidades do complexo I codificadas no genoma mitocondrial é variável, mas incluí sempre os sete polipéptidos altamente hidrofóbicos (Lang *et al.*, 1997; Rasmusson *et al.*, 1998).

É de salientar ainda a simplicidade do complexo I procariota quando comparado com a enzima mitocondrial. Assim, se em bactérias 13 ou 14 subunidades são suficientes para garantir uma actividade NADH:ubiquinona oxidorredutase associada a uma translocação de protões, qual é o papel das proteínas acessórias? É possível que muitas destas subunidades supranumerárias tenham como função optimizar ou regular a reacção catalisada pelo complexo I mitocondrial e/ou aumentar a estabilidade da enzima. O envolvimento de algumas subunidades como mediadores em fenómenos de "substrate chanelling" é também plausível (Sumegi & Srere, 1984). Por outro lado, é possível que o complexo I mitocondrial desempenhe funções bioquímicas adicionais, não executadas pela enzima procariota (ver adiante).

Tabela

N. crassa	*B. taurus*	*P. d./E. c.*	Grupos redox	Referências
78 kDa*	75 kDa	NQO3/NuoG	N4, N1b, N5?	1;2;3;4,40
51 kDa*	51 kDa	NQO1/NuoF	N3, FMN	1;5;6;4,40
49 kDa*	49 kDa	NQO4/NuoCD	Q	7;8;9;4,41
30.4 kDa*	30 kDa	NQO5/NuoCD	-	10;11;9;4
24 kDa*	24 kDa	NQO2/NuoE	N1a	12;13;14;4,40
19.3 kDa*	PSST	NQO6/NuoB	N2?	15;16;9;4,40
21.3c kDa*	TYKY	NQO9/NuoI	2xN2?	17;18;19;4,40
ND1	ND1	NQO8/NuoH	Q	20;20;19;4,40
ND2	ND2	NQO14/NuoN	-	20;20;19;4
ND3	ND3	NQO7/NuoA	-	20;20;19;4
ND4	ND4	NQO13/NuoM	-	20;20;19;4
ND4L	ND4L	NQO11/NuoK	-	20;20;19;4
ND5	ND5	NQO12/NuoL	-	20;20;19;4
ND6	ND6	NQO10/NuoJ	-	20;20;19;4
40 kDa*	39 kDa	-	-	21;22
29.9 kDa*	B13	-	-	23;24
21.3a kDa*	-	-	-	25
21.3b kDa*	-	-	-	26
21 kDa*	18 kDa	-	-	12;24
20.9 kDa*	-	-	-	27
20.8 kDa*	PGIV	-	-	28;29
17.8 kDa	-	-	-	30
14.8 kDa	B14	-	-	31;24
12.3 kDa*	PDSW	-	-	32;24
10.5 kDa	B8	-	-	33;24
9.3 kDa	B9	-	-	34;24
ACP*	SDAP	-	-	35;36
-	42 kDa	-	-	22
-	B22	-	-	24
-	ASHI	-	-	24
-	SGDH	-	-	24
-	B18	-	-	24
-	B17.2	-	-	37
-	B17	-	-	24
-	B15	-	-	24
-	B14.5a	-	-	38
-	B14.5b	-	-	38
-	15 kDa	-	-	24
-	B12	-	-	24
-	13 kDa	-	-	24
-	MLRQ	-	-	24
-	10 kDa	-	-	39
-	AGGG	-	-	24
-	MWFE	-	-	24
-	MNLL	-	-	24
-	KFYI	-	-	24

Tabela: Composição polipeptídica do complexo I de vários organismos.
As subunidades homólogas encontram-se alinhadas horizontalmente. As primeiras 14 subunidades constituem a unidade funcional mínima do complexo I. *P.d.*, *Paracoccus denitrificans*; *E.c.*, *Escherichia coli*; ND, subunidade codificada na mitocôndria; -, subunidade aparentemente inexistente na preparação enzimática; N<u>X</u>, centro [FeS]; Q, quinona; *, estirpe mutante existente. A localização mais provável dos diferentes grupos prostéticos é discutida em Ohnishi (1998). As referências para os mutantes de *N. crassa* encontram-se em Videira (1998). Referências: (1,7) Preis *et al.*, 1991, 1990; (2,36) Runswick *et al.*, 1989, 1991; (3,6,9,14,19) Xu *et al.*, 1992a, 1991a, 1992b, 1991b, 1993; (4) Weidner *et al.*, 1993; (5,11) Pilkington *et al.*, 1991a, 1991b; (8, 22) Fearnley *et al.*, 1989, 1991; (10,25,28,32) Videira *et al.*, 1990a, 1990b, 1990c, 1993; (12,27,30,31) Azevedo *et al.*, 1994a, 1992, 1993, 1994b; (13) Pilkington & Walker, 1989; (15) Sousa *et al.*, 1999; (16,38) Arizmendi *et al.*, 1992a,b; (17,33) Duarte *et al.*, 1996, 1993; (18,29) Dupuis *et al.*, 1991a,b; (20) Fearnley & Walker, 1992; (21) Röhlen *et al.*, 1991; (23) van der Pas *et al.*, 1991; (24) Walker *et al.*, 1992; (26) Nehls *et al.*, 1991; (34) Heinrich *et al.*, 1992; (35) Sackmann *et al.*, 1991; (37,39) Skehel *et al.*, 1998, 1991; (40) Ohnishi, 1998; (41) Darrouzet *et al.*, 1998.

2.2.2-Grupos prostéticos

À complexidade polipeptídica do complexo I está associada uma série de transportadores de electrões, nomeadamente uma molécula de FMN, ligada não covalentemente, e vários centros [FeS] (Hatefi *et al.*, 1962). Sete centros [FeS] diferentes foram detectados por EPR em preparações da enzima (Sled *et al.*, 1993). Destes, três são binucleares e foram designados por N1a, N1b e N1c e os restantes quatro- N2, N3, N4 e N5- são centros [FeS] tetranucleares (Ohnishi, 1979). Ohnishi refere que a concentração dos centros N1b, N2, N3 e N4 é equivalente à concentração de FMN (Ohnishi, 1979). No entanto, o grupo de Albracht tem contestado esta estequiometria sugerindo que a concentração do centro N1b é metade da do FMN (Albracht *et al.*, 1997). O centro N5, que até agora foi apenas detectado em complexo I bovino, existe em concentrações subestequiométricas relativamente à concentração de FMN (Ohnishi, 1993) daí que a sua existência, como verdadeiro componente do complexo I, seja largamente contestada. O centro binuclear N1a, devido ao seu potencial redox médio muito negativo, não foi ainda detectado por EPR no complexo I de vários organismos, nomeadamente no de *N. crassa* (Wang *et al.*, 1991). Quanto ao centro N1c, este foi apenas identificado em membranas de *E. coli* (Leif *et al.*, 1995), mas a sua presença é também esperada no complexo I de *T. thermophilus* (Yagi *et al.*, 1998).

Assim sendo, apenas os centros N1b, N2, N3 e N4 são unanimemente aceites como componentes intrínsecos do complexo I e apenas estes foram identificados em *N. crassa* (Wang *et al.*, 1991). No entanto, as sequências consenso para a ligação de centros [FeS], presentes em várias subunidades do complexo I, permitem a ligação de 8 centros (Ohnishi, 1998), o que parece sugerir que talvez os centros N1a e N5 sejam, também eles, componentes da enzima nos diferentes organismos. Por outro lado, a análise química da quantidade de ferro em preparações de complexo I -22 a 24 átomos- corrobora a hipótese da presença de mais centros [FeS] do que os visíveis por EPR (Ragan *et al.*, 1982).

Recentemente, experiências de espetroscopia permitiram detectar um novo grupo redox, de estrutura química ainda desconhecida, numa preparação de complexo I do fungo *N. crassa* (Friedrich *et al.*, 1998).

2.2.3-Locais de ligação do NADH, grupos prostéticos e ubiquinona

O local de ligação para o NADH foi identificado na subunidade de 51 kDa do complexo I bovino, por experiências de marcação por fotoafinidade (Chen & Guillory, 1981). Em experiências semelhantes realizadas com a enzima de *P. denitrificans* e de *T. thermophilus*, o homólogo da subunidade bovina de 51 kDa foi selectivamente marcado (Yagi & Dinh, 1990; Xu *et al.*, 1991a). A determinação da estrutura primária destes polipéptidos veio confirmar a existência de um domínio consenso para a ligação do ADP (ver Walker, 1992).

A labilidade da ligação do FMN ao complexo I tem dificultado a determinação experimental do seu local de ligação (Ragan, 1987). No entanto, uma série de observações apontam para a subunidade de 51 kDa como candidato mais provável. O facto do FMN ser o oxidante directo do NADH (Yano *et al.*, 1996), a ausência deste cofactor num mutante de *N. crassa*, que contém complexo I sem a subunidade de 51 kDa (Fecke *et al.*, 1994) e a interacção magnética entre o FMN e o centro [FeS] N3 que, como se discutirá adiante se encontra associado à subunidade de 51 kDa, pressupõe uma proximidade entre os dois grupos (Salerno *et al.*, 1977). Contudo, a possibilidade de outras subunidades contribuirem para a ligação do FMN, nomeadamente a subunidade de 24 kDa, não é de excluir completamente.

A determinação da estrutura primária das subunidades do complexo I forneceu informação sobre o possível número e localização dos centros [FeS] da enzima. Por exemplo, a existência do motivo $CXXCXXCX_nCP$ (em que X é qualquer aminoácido) nas subunidades de 75 kDa, 51 kDa e TYKY, esta última contendo duas cópias deste motivo, sugere a presença de centros tetranucleares associados a estes polipéptidos (Runswick *et al.*, 1989; Pilkington *et al.*, 1991a; Dupuis *et al.*, 1991a). Na subunidade PSST foi identificado um motivo com caracteristicas ligeiramente diferentes das do domínio anterior, mas também capaz de ligar um centro tetranuclear (Arizmendi *et al.*, 1992a). Os motivos de ligação para os centros binucleares foram encontrados nas subunidades de 75 kDa (Walker, 1992) e de 24 kDa (Pilkington & Walker, 1989).

Dado que o centro N1b foi detectado por EPR no fragmento FP do complexo I bovino (ver à frente; Ohnishi *et al.*, 1985), a ligação deste centro foi atribuída à subunidade de 24 kDa e, por exclusão de partes, o centro N1a estaria associado à subunidade de 75 kDa. No entanto, estudos de EPR realizados com a subunidade de *P. denitrificans* homóloga da subunidade de 75 kDa bovina, expressa em *E. coli*, sugerem que esta proteína liga o centro binuclear N1b, assim como o centro tetranuclear N4 (Yano *et al.*, 1995). Assim sendo, o centro N1a estaria localizado na subunidade de 24 kDa. De facto, os espectros de EPR obtidos para o heterodímero constituído pelas proteínas de *P. denitrificans* homólogas das subunidades de 24 kDa e 51 kDa bovinas, expressas em *E. coli*, vieram apoiar esta suposição (Yano *et al.*, 1996). A estrutura primária da subunidade de *E. coli* homóloga da subunidade de 75 kDa permite prever a existência de mais um motivo de ligação para um centro binuclear, provavelmente o centro N1c (Leif *et al.*, 1995). Este motivo foi também encontrado na subunidade homóloga de *T. thermophilus* (Yano *et al.*, 1997). No polipéptido de 75 kDa, é ainda identificado um motivo adicional para a ligação de um centro tetranuclear, que pode ser o centro N5 ou outro ainda não detectado por EPR (Fearnley & Walker, 1992). O centro N3 está associado à subunidade de 51 kDa como confirmado pela sua presença na fracção FP bovina, pela sua ausência num mutante de *N. crassa* sem esta proteína (Fecke *et al.*, 1994) e pela sua detecção por EPR no heterodímero atrás referido, após reconstituição (Yano *et al.*, 1996).

As restantes subunidades capazes de ligar centros [FeS] são a subunidade TYKY e a PSST e, portanto, ambas são candidatas para ligar o centro N2. A tentativa de atribuição

do local de ligação deste centro a uma destas subunidades tem sido assunto de grande controvérsia. A subunidade TYKY, que possui dois motivos para a ligação de dois centros [4Fe4S] (Dupuis *et al.*, 1991a), foi sugerida como contendo dois centros N2 (van Belzen *et al.*, 1992; Albracht *et al.*, 1997). Por outro lado, Ohnishi (1993) sugere a presença do centro N2 na subunidade PSST. O motivo de ligação para o centro [FeS] encontrado neste polipéptido contém apenas três cisteínas, podendo o outro ligando ser um glutamato (Arizmendi *et al.*, 1992a; Ohnishi, 1993). A distribuição dos ligandos, bem como a presença de ligandos mistos, podiam justificar o elevado potencial redox médio e a sua dependência com o pH, característicos deste centro (ver Ohnishi, 1998). Experiências apresentadas neste trabalho permitem localizar, indirectamente, o centro N2 na subunidade PSST (ver secção 4).

Todas as subunidades atrás mencionadas como possíveis locais de ligação para centros [FeS] fazem parte da unidade funcional mínima presente no complexo I de todos os organismos. Apenas uma das proteínas acessórias, a subunidade PGIV, foi considerada como potencial candidata para a ligação de um centro. Este polipéptido contém 8 cisteínas conservadas na proteína bovina (Dupuis *et al.*, 1991b) e de *N. crassa* (Videira *et al.*, 1990c).

O local de ligação para a ubiquinona tem sido atribuído à subunidade mitocondrial ND1. Esta subunidade foi marcada por análogos radioactivos e fotoactiváveis da rotenona (Earley & Ragan, 1984; Earley *et al.*, 1987) e é o componente, de todas as subunidades codificadas na mitocôndria, mais conservado filogeneticamente. A apoiar esta hipótese, estão os estudos realizados em células de doentes que possuem mutações pontuais no gene que codifica para a subunidade ND1 e que apresentam uma diminuição na actividade do complexo I e na sensibilidade à rotenona (Majander *et al.*, 1991). No entanto, estudos cinéticos posteriores revelaram que a rotenona não inibe a enzima de modo competitivo relativamente à ubiquinona, questionando as conclusões anteriores (Singer & Ramsay, 1992). Por outro lado, a utilização de um análogo biologicamente activo da ubiquinona, em experiências de marcação por fotoafinidade utilizando membranas de *N. crassa*, levou à identificação de um polipéptido de 9.3 kDa (Heinrich & Werner, 1992), homólogo da subunidade B9 do complexo I bovino (Heinrich *et al.*, 1992). A ausência de um homólogo desta proteína no complexo I procariota, torna

questionável a sua função na ligação da ubiquinona. Os resultados de marcação sugerem, no entanto, que esta subunidade se encontra próxima do local de redução da ubiquinona.

Uma outra abordagem permitiu identificar a subunidade de 49 kDa como fazendo parte do local de ligação da ubiquinona. Assim, mutantes de *R. capsulatus* resistentes à piericidina A foram isolados e caracterizados, verificando-se que a resistência ao antibiótico se devia à alteração de uma valina para metionina na subunidade homóloga da de 49 kDa bovina (Darrouzet *et al.*, 1998). Estes resultados e a natureza anfipática da ubiquinona levaram os autores a propor um modelo para a ligação do inibidor e, por extrapolação da ubiquinona, localizado na interface entre os domínios membranar e periférico do complexo I. Neste modelo, a cabeça polar da ubiquinona interactua com a subunidade de 49 kDa e a cauda hidrofóbica com a subunidade mitocondrial ND1.

2.2.4-Subunidades envolvidas numa via biossintética

A determinação da estrutura primária das subunidades do complexo I revelou a existência de duas subunidades homólogas de proteínas envolvidas em vias biossintéticas, as subunidades SDAP (ACP) e de 39 kDa. A subunidade SDAP é homóloga das "acyl-carrier proteins" procariotas (ACPs). As ACPs mitocondriais foram identificadas em bovinos (Runswick *et al.*, 1991), *N. crassa* (Sackmann *et al.*, 1991), humanos (Triepels *et al.*, 1999a) e outros organismos. *S. cerevisiae*, que não possui complexo I, contém uma ACP mitocondrial que se encontra solúvel na matriz (Chéret *et al.*, 1993). Estas proteínas possuem, covalentemente ligado, um grupo fosfopanteteína ao qual se liga um grupo acilo, através de uma ligação tio-éster.

A inactivação do gene que codifica para a ACP mitocondrial de *N. crassa* leva à disrupção do braço periférico da enzima e à montagem deficiente do domínio membranar (Schneider *et al.*, 1995). Neste mutante, o conteúdo em lisofosfolípidos está 4 vezes aumentado relativamente ao determinado para a estirpe selvagem e para outros mutantes em subunidades do complexo I. Na levedura *S. cerevisiae*, a inactivação do gene *acp* origina um mutante incapaz de crescer em substratos não fermentativos (Schneider *et al.*, 1995). Estes resultados não permitiram concluir acerca da função destas porteínas. No entanto, estudos recentes sugerem que a ACP mitocondrial transporta ácido lipóico que é,

posteriormente, transferido pelas lipoato transferases para as apoproteínas correspondentes (Jordan & Cronan Jr., 1997). Esta proteína está pois envolvida na síntese de ácidos gordos, sendo a principal função desta via a produção de ácido lipóico (Wada *et al.*, 1997).

A subunidade de 39 kDa apresenta uma homologia significativa com β-hidroxiesteróide desidrogenases, NAD(P)H dihidroflavonol redutases e isoflavona redutases e possui um local de ligação para o NAD(P)H (Fearnley & Walker, 1992). Estas enzimas catalizam reacções dependentes do NADPH em vias biossintéticas. As reacções redox catalizadas incluem a conversão dum grupo cetona em hidroxilo e *vice versa* ou a redução duma ligação dupla (Fearnley & Walker, 1992). A caracterização dum mutante de *N. crassa*, em que o gene codificante desta subunidade foi inactivado, não permitiu inferir acerca da função desta proteína. Esta estirpe, nuo40, monta um complexo I aparentemente intacto, mas incapaz de transferir electrões do NADH para a decilubiquinona (Schneider *et al.*, 1997). Embora especulativo, é possível que as subunidades SDAP e de 39 kDa cooperem na síntese de um grupo redox, ainda desconhecido, que se encontraria localizado no braço membranar (Schneider *et al.*, 1997).

2.2.5-Estrutura tridimensional do complexo I

Actualmente, existem protocolos estabelecidos para o isolamento de complexo I bovino, de *N. crassa* e de *E. coli* (Ise *et al.*, 1985; Finel *et al.*, 1992; Leif *et al.*, 1995) resultando em preparações da enzima num estado monodisperso. Uma massa molecular de mais de 900 kDa foi calculada para a enzima bovina, admitindo uma estequiometria unitária para cada subunidade e somando o peso de todos os 42-43 constituintes (Walker, 1992). A enzima de *N. crassa* parece ser ligeiramente mais pequena, dado o menor número de subunidades por que é composta-cerca de 35 (Weiss *et al.*, 1991). A massa molecular do complexo I de *E. coli* é de aproximadamente 550 kDa, como obtido por adição das diferentes subunidades e pela velocidade de sedimentação em gradientes de sacarose (Leif *et al.*, 1995).

A estrutura tridimensional destas três enzimas foi determinada por microscopia electrónica. A análise de cristais bidimensionais do complexo I de *N. crassa* revelou uma estrutura em forma de L (Hofhaus *et al.*, 1991), em que um dos braços se encontra embebido na membrana interna mitocondrial (braço membranar/hidrofóbico) e o outro, perpendicular ao primeiro, se projecta para a matriz (braço periférico/globular). Esta estrutura foi confirmada por análise de partículas isoladas (Guénebaut *et al.*, 1997). Estudos de microscopia electrónica com a enzima de *E. coli* permitiram verificar que, também neste organismo, a enzima possui uma estrutura em forma de L. A maior parte da diferença de massa entre os dois complexos encontra-se ao longo do braço membranar e na junção entre os dois braços (Guénebaut *et al.*, 1998). Apesar da diferença de massa, o comprimento do braço periférico é semelhante nos dois complexos e o braço membranar é apenas ligeiramente mais curto em *E. coli*. A estrutura do complexo I bovino, também em forma de L, apresenta um domínio globular extrínseco significativamente maior do que o da enzima fúngica, sugerindo que a massa adicional está associada com o braço globular. A junção entre os dois domínios do complexo I bovino, globular e membranar, é feita por uma parte mais fina que não foi descrita na enzima de *N. crassa*, mas parece estar presente no complexo I procariota (Grigorieff, 1998).

A corroborar este modelo estrutural encontram-se os estudos bioquímicos de fraccionamento da enzima em subcomplexos. O braço periférico do complexo I tem sido analisado, com algum detalhe, em vários organismos. Dois fragmentos, designados por fracção flavoproteica (FP) e fragmento IP "iron-protein", podem ser extraídos do complexo I bovino, por incubação com perclorato (Galante & Hatefi, 1979). A fracção flavoproteica é constituída pelas subunidades de 51 kDa, 24 kDa e 10 kDa e contém FMN e dois centros [FeS] (Ohnishi *et al.*, 1985). O fragmento IP engloba as subunidades de 75 kDa, 49 kDa, 30 kDa, 18 kDa (IP), 15 kDa (IP),13 kDa (IP) e B13 e, em algumas preparações, ainda as subunidades TYKY e PSST (ver Walker, 1992). Um subcomplexo equivalente ao FP foi expresso em *E. coli*, contendo as subunidades de *P. denitrificans* homólogas das subunidades de 51 kDa e 24 kDa (Yano *et al.*, 1996).

Finel *et al.* (1992, 1994), explorando a labilidade estrutural do complexo I bovino na presença de detergentes, isolaram e caracterizaram vários fragmentos que parecem

conter todos os grupos prostéticos conhecidos. Um destes fragmentos, o denominado subcomplexo Iα, contém cerca de 20 subunidades, entre as quais todas as que se encontram nas fracções FP e IP. Adicionalmente, este subcomplexo inclui ainda outras subunidades que possuem características de proteínas hidrofílicas. No entanto, a presença neste fragmento de 4 subunidades com potencial para atravessarem a membrana sugere que parte deste subcomplexo se encontra embebida na membrana. Os subcomplexos Iλ e IλS, obtidos por métodos ligeiramente diferentes do utilizado para isolar o subcomplexo Iα, são constituídos por 15 e 13 subunidades, respectivamente. De entre as subunidades presentes nestes três subcomplexos encontram-se 7 proteínas hidrofílicas, homólogas de subunidades do complexo I procariota, que parecem ser responsáveis pela ligação do FMN e de todos os centros [FeS] detectados por EPR (Finel *et al.*, 1994).

Quando o fungo *N. crassa* é crescido na presença de cloranfenicol (um inibidor da síntese proteica mitocondrial), um subcomplexo contendo cerca de 15 subunidades codificadas nuclearmente é acumulado na membrana interna da mitocondria (Friedrich *et al.*, 1989). Este subcomplexo difere dos subcomplexos bovinos atrás descritos porque não possui o centro [FeS] N2 (Wang *et al.*, 1991) nem a subunidade homóloga da PSST (Sousa *et al.*, 1999). A ausência simultânea destes dois componentes sugere que o centro se encontra ligado a esta proteína. A acumulação do braço periférico foi também observada em mutantes de *N. crassa*, em que foram inactivados genes que codificam para subunidades do domínio membranar (ver adiante).

O aumento do pH (6.0-7.5) cliva o domínio periférico do complexo I de *E. coli* em dois fragmentos (Leif *et al.*, 1995). Um dos fragmentos, denominado NADH desidrogenase, é constituído por três subunidades, homólogas das subunidades de 75 kDa, 51 kDa e 24 kDa, e contém FMN e, pelo menos, 4 centros [FeS] (N1b, N1c, N3 e N4). A presença neste fragmento do centro binuclear N1a e de um terceiro centro tetranuclear é sugerida pelos motivos de ligação existentes nas 3 subunidades e pela concentração de ferro. Este fragmento foi expresso em *E. coli*, purificado e analisado espectroscopicamente. Os resultados obtidos sugerem que esta preparação é, em tudo, semelhante ao fragmento correspondente no complexo I de *E. coli* (Braun *et al.*, 1998). O outro fragmento resultante do aumento do pH é composto pelos homólogos das

subunidades de 49 kDa, 30 kDa, TYKY e PSST e liga um centro tetranuclear com características similares às do centro N2 (Leif *et al.*, 1995).

A parte intrínseca, membranar, da enzima encontra-se menos caracterizada do que o domínio periférico. O fragmento hidrofóbico (HP), obtido por tratamento do complexo I bovino com perclorato, não é mais do que um aglomerado proteico insolúvel, contendo as restantes subunidades da enzima (ver Walker, 1992). O tratamento do complexo I bovino com detergentes origina um fragmento, denominado Iβ, contituído por cerca de 15 subunidades. Algumas destas proteínas possuem domínios de aminoácidos hidrofóbicos capazes de atravessar a membrana sugerindo que este fragmento se encontra embebido na membrana. No entanto, o fragmento Iβ não inclui todas as subunidades hidrofóbicas da enzima, não correspondendo portanto à totalidade do domínio membranar (Finel *et al.*, 1992).

Quando a enzima de *N. crassa* é incubada na presença de anião brometo, parte das suas subunidades dissociam-se perdendo qualquer estrutura definida. Contudo, cerca de metade das subunidades da enzima resiste, de forma aparentemente intacta, ao tratamento com agentes caotrópicos podendo ser isolada numa forma monodispersa. O fragmento resultante contém todas as subunidades codificadas na mitocôndria e muitas, se não todas, as de origem nuclear que não se encontram presentes no fragmento periférico (Friedrich *et al.*, 1989). Em termos de composição polipeptídica, este fragmento hidrofóbico é complementar do domínio periférico de *N. crassa* e o conjunto representa a totalidade da enzima do fungo.

O aumento do pH origina um subcomplexo hidrofóbico do complexo I de *E. coli*, constituído por sete polipéptidos hidrofóbicos, homólogos das subunidades mitocondriais (Leif *et al.*, 1995). Até agora, não foi identificado qualquer grupo prostético na parte membranar do complexo I.

2.3-Funções do complexo I

2.3.1-Transferência de electrões

A actividade do complexo I tem sido determinada, quer em preparações de complexo I isolado contendo detergentes, quer em membranas mitocondriais em que a enzima se encontra no seu ambiente membranar nativo, por medição da transferência de electrões do NADH para análogos da ubiquinona, sensível à rotenona. Outros agentes redutores podem ser oxidados pelo complexo I, designadamente NADPH e deamino-NADH (Friedrich *et al.*, 1994). Devido à sua natureza altamente hidrofóbica, a ubiquinona-10, o aceitador nativo presente na maior parte dos organismos, não pode ser utilizada em ensaios enzimáticos *in vitro* (Lenaz, 1998). Para contornar esta incapacidade, têm sido usados vários homólogos e análogos com cadeias laterais, na posição 6, mais pequenas, nomeadamente ubiquinona-1, ubiquinona-2 e decilubiquinona (ver Lenaz, 1998). No entanto, a menor hidrofobicidade destes aceitadores faz com que a sensibilidade da reacção a inibidores diminua. Também os subcomplexos Iα, Iλ e o braço periférico da enzima de *N. crassa* catalisam a transferência de electrões para quinonas de cadeia lateral pequena. Esta reacção é invariavelmente insensível à rotenona e à piericidina A (Friedrich *et al.*, 1989; Finel *et al.*, 1992, 1994) e parece envolver locais de ligação à quinona diferentes do local fisiológico. Por outro lado, o complexo I, assim como vários dos subcomplexos atrás mencionados, é capaz de transferir electrões do NADH para aceitadores artificiais, como hexacianoferrato ou hexaaminorutênio III (Hatefi, 1978; Sled & Vinogradov, 1993).

No entanto, a sequência de redução dos diferentes grupos redox não está completamente esclarecida. As principais dificuldades encontradas estão associadas à cinética de redução dos diferentes transportadores electrónicos pelo NADH, verificando-se a redução de todos os centros [FeS] da enzima em alguns milisegundos, e também à inexistência de inibidores que interrompam o fluxo de electrões em passos intermédios (Walker, 1992). A sequência actualmente aceite foi deduzida com base no potencial redox médio dos diferentes transportadores electrónicos da enzima e na sua possível localização espacial. Neste modelo, o FMN actua como oxidante directo do NADH. Do

$FMNH_2$, os electrões são transferidos, um de cada vez, para o grupo isopotencial dos centros N1b, N3 e N4 (Krishnamoorty & Hinkle, 1988). A apoiar esta sequência está a observação de que o complexo I existe em dois estados conformacionais interconvertíveis, um activo e o outro inactivo. A transformação da forma inactiva na forma activa é conseguida por incubação com quantidades sub-estequiométricas de NADH (ver Vinogradov, 1998), permitindo a redução dos centros N1b, N3 e N4 (Weiss & Friedrich, 1991). Por outro lado, a remoção do centro N4, por tratamento do complexo I com N-bromossuccinimida, não altera a cinética de transferência de electrões do NADH para a ubiquinona (Krishnamoorty & Hinkle, 1988). Estes factos parecem sugerir uma via ramificada para a transferência electrónica, em que o centro N4 tem como função o armazenamento temporário de electrões.

Do grupo isopotencial, os electrões são transferidos para o centro N2 localizado na interface entre o domínio periférico e a parte membranar. Dado o potencial redox médio deste centro ser o mais electropositivo da enzima, ele é considerado o redutor imediato da ubiquinona (Kotlyar *et al.*, 1990). A redução deste aceitador envolve a formação, rotenona-sensível, de duas espécies distintas de semiquinonas que são detectadas por EPR (Vinogradov *et al.*, 1995).

2.3.2-Mecanismo de transdução energética no complexo I

Um número considerável de modelos, sobre os mecanismos de transdução energética nesta enzima, tem sido elaborado ao longo do tempo não existindo, contudo, um modelo consensual teórico (ver Brandt, 1997). O número de protões translocados da matriz mitocondrial para o espaço intermembranar foi determinado em partículas submitocondriais acopladas. Os valores experimentais obtidos por vários grupos não são muito coerentes, variando de 3 a $5H^+$ por cada 2 electrões transferidos do NADH para a ubiquinona. No entanto, a razão de $4H^+/2e^-$ é em geral considerada a mais correcta (Weiss & Friedrich, 1991; Galkin *et al.*, 1999). Os diferentes modelos sobre a transdução energética no complexo I foram elaborados tentando explicar esta estequiometria.

Muitos destes modelos baseiam-se no acoplamento entre a transferência de protões e as diversas reacções de oxidação/redução que ocorrem na enzima. Atendendo ao

potencial redox médio dos vários transportadores electrónicos, poderia haver transdução energética na oxidação do $FMNH_2$ pelos centros isopotenciais e/ou na redução da ubiquinona pelo centro N2 (ver Brandt, 1997). Assim, ambos os transportadores de protões, FMN e ubiquinona, podem contribuir para a translocação protónica. Contudo, dada a localização periférica do FMN, o seu envolvimento na conservação de energia requer a existência de um longo canal protónico para o qual, até agora, não há evidência experimental. Por outro lado, a variação do potencial redox do centro N2, com o pH, levou alguns autores a sugerirem o envolvimento deste centro [FeS] na translocação de protões (ver Brandt, 1997).

Tentando integrar os novos conhecimentos obtidos ao nível da estrutura e função do complexo I, dois modelos para a conservação de energia nesta enzima foram descritos recentemente (Brandt, 1997; Dutton *et al.*, 1998). No modelo proposto por Brandt, o centro N2 e a ubiquinona contribuem, ambos, para a translocação protónica. A principal característica deste modelo é o acoplamento entre a redução do centro N2 e a desprotonação da ubihidroquinona. No outro mecanismo (Dutton *et al.*, 1998), apenas a ubiquinona está envolvida na transferência de protões. Ambos os modelos hipotéticos supõem a existência de 3 locais de ligação para a ubiquinona e a formação de semiquinonas. Recentemente, Brandt propôs uma alteração ao seu modelo em que dois dos locais de ligação para a ubiquinona foram substituídos por uma ubiquinona fortemente ligada. Esta ubiquinona funcionaria como grupo prostético capaz de se mover entre duas posições distintas (Brandt, 1999).

Uma outra hipótese a considerar baseia-se no acoplamento indirecto entre as duas actividades do complexo I. Por exemplo, a energia libertada na reacção redox poderá ser utilizada para provocar alterações conformacionais na enzima, alterações essas promotoras de translocação protónica. A apoiar esta hipótese, encontram-se experiências de "cross-linking" em que se verificou uma diferença no padrão de "cross-linking" das subunidades do complexo I, entre a enzima reduzida e não reduzida (Belogrudov & Hatefi, 1994). A redução do complexo I altera a susceptibilidade das subunidades da enzima à digestão pela tripsina (Yamaguchi *et al.*,1998), apoiando a ocorrência de alterações conformacionais extensas por ligação do substrato. Estes resultados levaram os autores a sugerirem que a energia libertada na reacção redox está acoplada a alterações

conformacionais na enzima, que resultam na alteração do pK_a de aminoácidos apropriados e, como consequência, na transferência de protões através da membrana interna.

2.3.3-Inibidores

Um grande número de compostos naturais e sintéticos, com estruturas químicas muito diversas, têm sido descritos como inibidores da actividade do complexo I. Poucos compostos inibem a oxidação do NADH, enquanto que a maior parte dos inibidores do complexo I impedem a redução da ubiquinona (Friedrich *et al.*, 1994; ver Degli Esposti, 1998). De entre os primeiros, é de referir o análogo do NADH, a ADP-ribose, um inibidor competitivo capaz de se ligar ao local de ligação deste substrato (Zharova & Vinogradov, 1997) e o difenilenoiodonium (DPI) que se liga irreversivelmente ao $FMNH_2$, impedindo a sua reoxidação (Majander *et al.*, 1994). De todos os inibidores que actuam ao nível da ubiquinona, os mais vulgarmente utilizados são a rotenona e a piericidina A. Estudos cinéticos, envolvendo vários destes inibidores do complexo I, sugerem que estes compostos podem ser agrupados em duas (Friedrich *et al.*, 1994) ou três (Degli Esposti, 1998) classes, representadas por piericidina A (classe I/tipo A), rotenona (classe II/tipo B) e capsaicina (tipo C). Contudo, não foi ainda esclarecido se estas classes correspondem a dois ou três locais distintos de ligação do inibidor e portanto da ubiquinona.

A classificação dos inibidores em classe I e classe II baseou-se no comportamento cinético dos diferentes compostos. Assim, os inibidores classe I actuam no complexo I de modo competitivo, enquanto que os representantes da classe II inibem a enzima de modo não competitivo relativamente à ubiquinona (Friedrich *et al.*, 1994). Esta classificação não engloba, contudo, alguns compostos cujo comportamento de inibição difere significativamente dos acima referidos. Isto levou Degli Esposti (1998) a propor o agrupamento em três classes. Os compostos pertencentes à classe tipo C funcionam como antagonistas do produto de reacção, quinol, e entre eles encontram-se os compostos neurotóxicos MPP^+ e seus derivados.

Mais recentemente, experiências de competição demonstraram que todos os inibidores do complexo I, que actuam ao nível da ubiquinona, parecem partilhar de um domínio único de ligação com locais de ligação parcialmente sobrepostos. Assim, o local de ligação da rotenona sobrepõe-se ao local de ligação da piericidina e ao local de interacção da capsaicina, ao passo que a ligação dos dois últimos inibidores não interfere uma com a outra (Okun *et al.*, 1999).

2.4-Biogénese do complexo I

2.4.1-Intermediários de montagem

Os mecanismos de montagem do complexo I foram estudados em *N. crassa*. Experiências de "pulse-labelling" permitiram observar a acumulação de um intermediário de montagem da enzima, constituído por todas as subunidades codificadas na mitocôndria e cerca de 10 de origem nuclear (Tuschen *et al.*, 1990). Curiosamente, este intermediário é em tudo idêntico ao fragmento hidrofóbico da enzima (ver 2.2.5) e foi, também, detectado na mitocôndria de células de *N. crassa* crescidas na ausência de manganês (Schmidt *et al.*, 1992). Por outro lado, foi já referido que, quando crescidas na presença de cloranfenicol, as células de *Neurospora* produzem apenas um subcomplexo da enzima (ver 2.2.5; Friedrich *et al.*, 1989). Estes resultados sugerem que os dois braços do complexo I são montados de forma independente. A confirmar estes dados estão experiências de inactivação de genes nucleares, que codificam para subunidades do complexo I de *Neurospora*, que bloqueiam passos específicos no processo de montagem da enzima (ver 2.4.2; Schulte *et al.*, 1994).

A análise dos mutantes permitiu verificar que a montagem do braço membranar é precedida pela formação de 2 intermediários, designados por pequeno e grande intermediário, que se associam posteriormente dando origem ao domínio membranar da enzima. O pequeno intermediário é constituído por 7 subunidades, duas das quais de origem mitocondrial. As restantes subunidades que se encontram presentes no braço membranar, incluindo as restantes 5 mitocondriais, associam-se para formar o grande intermediário. Este intermediário contém ainda duas proteínas de origem nuclear, que não

se encontram na enzima madura, e que parecem estar especificamente envolvidas na montagem do braço membranar, funcionando como "chaperones". Estas proteínas foram designadas por CIA80 e CIA35, para "complex I intermediate associated proteins", e a sua inactivação bloqueia a montagem do grande intermediário e, portanto, do complexo I (Küffner *et al.*, 1999). As proteínas CIA associam-se exclusivamente ao grande intermediário, sendo libertadas logo que o domínio membranar esteja formado e reutilizadas num novo ciclo de montagem.

Resumindo, num primeiro passo o pequeno e o grande intermediário associam-se para formar o braço membranar que, de seguida, se reúne com o domínio periférico para originar a enzima fúngica (Schulte *et al.*, 1994).

2.4.2-Inactivação de genes do complexo I

Como atrás mencionado, a inactivação de genes que codificam para subunidades específicas do complexo I de *N. crassa* bloqueia o processo de montagem da enzima, levando à acumulação de intermediários (ver Videira, 1998). O mesmo se verifica no caso de células humanas (Hofhaus & Attardi, 1993). Em organismos procariotas, a inactivação de genes do complexo I resulta, na maior parte dos casos, na ausência da enzima e de qualquer subcomplexo, o que pode estar relacionado com a maior instabilidade do complexo I procariota (Finel, 1996; Dupuis *et al.*, 1998b; Falk-Krzesinki & Wolfe, 1998).

Em *N. crassa*, a inactivação de genes que codificam para subunidades do braço periférico ou não interfere com a associação das outras subunidades, como no caso dos mutantes nuo51 (Fecke *et al.*, 1994), nuo21.3a (Alves & Videira, 1994), nuo40 (Schulte & Weiss, 1995), ou leva à formação do braço membranar sem qualquer vestígio da parte periférica, como se verifica nos mutantes nuo49 (Schulte *et al.*, 1994), nuo75 (resultados não publicados) e nuo19.3 (resultados não publicados). Um efeito surpreendente foi observado no mutante para a subunidade periférica de 9.6 kDa (ACP) (Schneider *et al.*, 1995). Nesta estirpe, não foi detectado qualquer intermediário da parte periférica e observou-se uma montagem deficiente do domínio membranar. Por outro lado, o braço periférico acumula em todos os mutantes para os quais os genes que codificam

subunidades da parte membranar foram inactivados. Estes mutantes acumulam, ainda, um ou ambos os intermediários do braço membranar. Com efeito, o pequeno intermediário é observado no mutante nuo20.9 (Schulte *et al.*, 1994) e ambos os intermediários estão presentes nos mutantes nuo21.3b (Nehls *et al.*, 1992) e nuo20.8 (da Silva *et al.*, 1996). Estes resultados vêm, mais uma vez, confirmar a montagem independente dos dois domínios do complexo I e verificar a presença, em todos os mutantes, de um fragmento membranar. No entanto, estirpes contendo delecções no mtDNA, que incluem os genes que codificam para as subunidades ND2 e ND3 do complexo I de *N. crassa*, acumulam o braço periférico e não possuem qualquer intermediário do domínio membranar (Alves & Videira, 1998). O facto das subunidades ND2 e ND3 pertencerem ao pequeno e grande intermediário, respectivamente, pode justificar a total ausência de fragmentos membranares neste mutante.

Alterações semelhantes foram constatadas em linhas celulares humanas que possuem mutações no mtDNA. Com efeito, a inserção de uma base no gene que codifica para a proteína ND4 leva à ausência desta subunidade e, como consequência, as restantes subunidades mitocondriais são incapazes de formar qualquer subcomplexo. Algumas das subunidades codificadas no núcleo associam-se formando um intermediário com actividade NADH:hexacianoferrato redutase (Hofhaus & Attardi, 1993). Uma situação, em tudo similar, foi descrita para uma linha celular de ratinho, em que foi detectada uma mutação "frameshift" no gene que codifica para a subunidade ND6 (Bai & Attardi, 1998). Contrariamente, a alteração da subunidade ND5, em células humanas, não parece interferir com a formação do braço membranar. Esta subunidade é, no entanto, essencial para a actividade do complexo I (Hofhaus & Attardi, 1995).

Em procariotas, a inactivação de genes que codificam para as subunidades homólogas das NDs não leva à formação de qualquer intermediário do braço periférico (Dupuis *et al.*, 1998b; Falk-Krzesinki & Wolfe, 1998). Há, no entanto, que ter em linha de conta a elevada instabilidade da enzima procariota, o que pode impedir a detecção dos intermediários formados. Em *R. capsulatus*, a inactivação do gene que codifica para a subunidade homóloga da TYKY não parece perturbar a montagem do braço membranar, abolindo completamente a formação do domínio periférico (Chevallet *et al.*, 1997).

Todos estes resultados parecem indicar que o processo de montagem do complexo I foi conservado no decurso da evolução, com a formação independente dos dois domínios e a sua reunião *en bloc* para formar a enzima madura.

2.5-Origem e evolução do complexo I

Actualmente, a evolução modular do complexo I é aceite como correcta e tem vindo a ser apoiada por algumas observações efectuadas em enzimas procariotas. Por exemplo, partes do complexo I apresentam homologia com subunidades da NAD^+-hidrogenase de bactérias (Pilkington *et al.*, 1991a), com subunidades da formato hidrogenoliase (Böhm *et al.*, 1990; Andrews *et al.*, 1997) e com transportadores de catiões (Friedrich & Weiss, 1997). Estas partes representam, provavelmente, módulos funcionais para a transferência de electrões e translocação de protões. No decurso da evolução, a fusão de complexos proteicos contendo os diferentes módulos levou à formação do complexo I ancestral (Friedrich & Weiss, 1997).

A NAD^+-hidrogenase de *Alcaligenes eutrophus* é uma enzima solúvel que cataliza a redução do NAD^+ pelo H_2. Funcional e estruturalmente, a enzima pode ser dividida em dois domínios (Tran-Betcke *et al.*, 1990): o primeiro, constituído pelas subunidades β e δ, retém a capacidade de redução de variadíssimas substâncias pelo H_2; o segundo, um heterodímero contendo as subunidades α e γ, possui actividade de NADH oxidorredutase e contém uma molécula de FMN e 3 a 4 centros [FeS]. A sequenciação do operão que codifica para esta enzima permitiu constatar uma elevada homologia entre o dímero α/γ e as subunidades de 75 kDa, 51 kDa e 24 kDa do complexo I. De facto, a subunidade α representa a fusão entre as subunidades de 51 kDa e 24 kDa; a subunidade γ apresenta homologia com os 200 aminoácidos N-terminais da subunidade de 75 kDa (Pilkington *et al.*, 1991a).

O complexo enzimático formato hidrogenoliase de *E. coli*, acopla a oxidação do formato, pela formato desidrogenase, à redução de protões pela hidrogenase. A sequenciação do operão codificante desta enzima (Böhm *et al.*, 1990) permitiu verificar a existência de uma surpreendente homologia entre várias subunidades da enzima procariota e subunidades do complexo I (ver Friedrich & Weiss, 1997). Assim, as

subunidades ND1, ND4, 49 kDa, 30 kDa, TYKY e PSST do complexo I bovino apresentam homologia com cinco subunidades da formato hidrogenoliase de *E. coli*. Uma observação curiosa foi a da subunidade HycE, da formato hidrogenoliase, representar uma fusão das subunidades de 30 kDa e 49 kDa do complexo I (Videira & Azevedo, 1994).

Um operão que codifica para uma segunda formato hidrogenoliase, em *E. coli*, foi recentemente caracterizado (Andrews *et al.*, 1997). Este operão contém todos os homólogos atrás referidos e, para além desses, mais 3 proteínas homólogas das subunidades ND4, ND4L e ND5 do complexo I. Transportadores bacterianos foram descritos como proteínas que apresentam homologia com as subunidades intrínsecas ND4 e ND5 (ver Friedrich & Weiss, 1997). A origem das outras subunidades membranares NDs é, ainda, desconhecida.

A constatação de todas estas homologias levou alguns autores a proporem um esquema hipotético de evolução para o complexo I mitocondrial (Friedrich & Weiss, 1997; Finel, 1998). Assim, dada a extensa homologia entre subunidades do complexo I e componentes da formato hidrogenoliase, é possível que estes dois complexos tenham evoluído a partir de um ancestral comum. Esta enzima ancestral teria já uma actividade de hidrogénio:quinona oxidorredutase translocadora de protões. O domínio membranar evoluiu, posteriormente, por duplicação de genes e aquisição de subunidades adicionais. Este complexo divergiu então para formar o complexo I procariota, por fusão com o módulo de NADH desidrogenase, e a enzima NAD(P)H:plastoquinona oxidorredutase presente em cianobactérias (ver 2.1). Posteriormente, estabeleceu-se uma relação de endossimbiose entre um organismo procariota e uma célula hospedeira, levando ao aparecimento da mitocôndria. A transferência dos genes, que codificam para as subunidades mais hidrofílicas do complexo I, do genoma mitocondrial para o nuclear e a adição de muitas outras subunidades que não se encontram na enzima procariota, levou ao aparecimento do complexo I mitocondrial (ver Finel, 1998).

2.6-Importância do complexo I

O complexo I tem como principais funções gerar um gradiente protónico e oxidar os equivalentes reductores regenerando o NAD^+. A importância desta enzima varia dependendo do organismo, estado de desenvolvimento e tecido considerado. De facto, a expressão do complexo I varia nos diferentes estados do ciclo de vida do *Trypanosoma brucei brucei* (Beattie & Howton, 1996), entre diferentes orgãos, como em plantas onde se verificou um aumento da expressão das subunidades do complexo I nas flores (Grohmann *et al.*, 1996; Heiser *et al.*, 1996) e ao longo do desenvolvimento embrionário de *Xenopus laevis* (Ammini & Hauswirth, 1999) e *Drosophila melanogaster* (Ragona *et al.*, 1999). Isto sugere que o complexo I é regulado de forma a responder a diferentes exigências energéticas e estados redutores.

Experiências de disrupção do complexo I, em diferentes organismos, permitiram concluir que esta enzima é essencial para uma série de processos celulares. Por exemplo, o complexo I é necessário para a transferência electrónica do NADH para o fumarato, em *E. coli* (Tran *et al.*, 1997). Em *Myxococcus xanthus*, a inactivação de um gene que codifica para uma subunidade do complexo I impede o desenvolvimento e a esporulação desta bactéria (Laval-Favre *et al.*, 1997). Em plantas, a redução da expressão da subunidade homóloga da de 51 kDa, por repressão "antisense", altera a maturação do pólen reduzindo a fertilidade masculina (Heiser *et al.*, 1997). O complexo I é essencial para o desenvolvimento sexual em *N. crassa* (este trabalho). Em humanos, uma alteração das propriedades catalíticas desta enzima tem também um efeito maléfico para a célula (ver 2.7). Todas estas deficiências parecem estar associadas a uma diminuição da eficiência do complexo I.

Por outro lado, é possível que o complexo I mitocondrial desempenhe funções bioquímicas adicionais, que estariam associadas a algumas das subunidades acessórias, ausentes na enzima procariota. Várias evidências têm sugerido esta hipótese como provável. Por exemplo, a subunidade de 18 kDa (IP), AQDQ, do complexo I bovino pode ser fosforilada por uma proteína cinase mitocondrial, dependente do cAMP (Papa *et al.*, 1996). A subunidade de 24 kDa foi identificada como sendo uma proteína G, capaz de ligar GTP (Hedge, 1998). O mRNA que codifica para a subunidade de 51 kDa humana

parece actuar como um supressor "antisense"da tradução do precursor de uma proteína induzida pelo interferão γ (IP-30) (Schuelke *et al.*, 1998). Este transcripto interfere com a via do interferão γ suprimindo a resposta imune em tecidos com necessidades energéticas elevadas. Por fim e como já referido, é provável que as subunidades de 39 kDa e SDAP estejam envolvidas numa via biossintética (ver 2.2.4).

2.7-Aspectos médicos do complexo I

No grupo das citopatias mitocondriais, são frequentemente encontradas deficiências na actividade do complexo I. Uma alteração, ainda que subtil, nas propriedades catalíticas e/ou estruturais da enzima pode ter efeitos catastróficos para a célula: diminuir a capacidade translocadora de protões da enzima, resultando num decréscimo na síntese de ATP; diminuir a capacidade de oxidação do NADH pela cadeia respiratória, o que é essencial para o funcionamento das enzimas do ciclo de Krebs e da via de oxidação dos ácidos gordos; e ainda, levar ao aumento da produção de radicais livres causando mutações no mtDNA, peroxidação lipídica e desnaturação das proteínas (ver Robinson, 1998).

Nos últimos anos, têm sido identificadas várias doenças humanas etiologicamente relacionadas com deficiências no complexo I. A grande maioria destas anomalias resulta de alterações ao nível do DNA mitocondrial ou nuclear. As consequências de uma cadeia respiratória deficiente são diferentes para os diversos tecidos, segundo a sua dependência da energia gerada na mitocôndria. Assim, os tecidos mais afectados são o sistema nervoso central, o músculo esquelético, o coração, o rim e o fígado. A localização mitocondrial de algumas das mutações causais confere características únicas a estas doenças (ver Wallace, 1992), sendo a sua transmissão genética efectuada de um modo não mendeliano, de mãe para filhos. Por outro lado, em grande parte dos transportadores destas mutações, verifica-se a co-existência de moléculas de mtDNA mutante e normal. Durante a divisão celular, a população mitocondrial pode oscilar para genótipos puros, mutantes ou normais, alterando a gravidade da doença.

Muitas das mutações pontuais no mtDNA dão origem a encefalopatias, das quais a neuropatia óptica hereditária de Leber (LHON) constitui um dos exemplos mais bem

caracterizados a nível molecular (ver Schapira, 1998). Nesta doença, foram identificadas mutações nas subunidades ND1, ND4 e ND6 contribuindo para uma melhor compreensão da função destes componentes. A substituição da arginina 340 por histidina na subunidade ND4, presente em 50% dos pacientes com LHON, não provoca qualquer alteração na actividade NADH:ubiquinona oxidorredutase do complexo I (Majander *et al.*, 1991). No entanto, em mitocôndrias isoladas, a taxa de oxidação de vários substratos (*e. g.* malato) está significativamente reduzida. Isto permitiu sugerir que este domínio da subunidade ND4 poderá estar envolvido na ligação de desidrogenases dependentes do NADH (Majander *et al.*, 1991). Recentemente, foi descrito um modelo bacteriano para esta mutação, em que foram reproduzidas as principais alterações bioquímicas observadas nas mitocôndrias dos doentes portadores de LHON (Lunardi *et al.*, 1998). Duas outras mutações associadas à LHON afectam a subunidade ND1 e originam substituições da leucina 285 por prolina e da alanina 52 por treonina. Estas alterações reduzem a actividade NADH:ubiquinona oxidorredutase do complexo I, provavelmente por interferirem com a ligação da ubiquinona (ver Walker, 1992). Um modelo bacteriano para a mutação na alanina 52 veio confirmar a importância deste resíduo e do domínio circundante na ligação e redução da ubiquinona (Zickermann *et al.*, 1998).

Um outro grupo de doenças, associado a deficiências na cadeia respiratória, afecta de modo pleiotrópico vários componentes mitocondriais. Estas doenças encontram-se bem caracterizadas ao nível molecular e envolvem mutações pontuais no mtDNA que codifica para tRNAs. O $tRNA^{Leu}$ e/ou o $tRNA^{Ile}$ encontra-se alterado nos pacientes com MELAS (encefalomiopatias mitocondriais com acidose láctica e "stroke-like episodes"). Os doentes afectados com MERRF (epilepsia mioclónica com "ragged red fibre") apresentam uma mutação pontual no $tRNA^{Lys}$ (ver Wallace, 1992), que causa uma diminuição na aminoacilação do tRNA e, como consequência, a terminação prematura da tradução (Enriquez *et al.*, 1995). Por outro lado, o síndrome de Kearns-Sayre é caracterizado pela existência de grandes delecções no mtDNA (delecções de 5.9 kb a 7 kb têm sido detectadas nestes pacientes), que provocam uma deficiência generalizada da cadeia respiratória (ver Brown & Wallace, 1994).

Apesar do grande número de mutações no mtDNA descritas até hoje, cerca de 50 (Chomyn, 1998), muitos dos doentes com deficiências no complexo I não possuem

qualquer destas alterações. Este facto e a dualidade genética das subunidades do complexo I pressupõem a existência de mutações em genes nucleares, que codificam para subunidades da enzima ou para proteínas envolvidas na biogénese da mitocôndria (Smeitink *et al.*, 1998). De facto, foram recentemente descritas mutações nos genes que codificam para as subunidades de 18 kDa (AQDQ) (van den Heuvel *et al.*, 1998), 51 kDa (Schuelke *et al.*, 1999), TYKY (Loeffen *et al.*, 1998) e PSST (Triepels, *et al.*, 1999b) associadas a doenças com sintomas clínicos diversos, desde encefalomiopatias até síndrome de Leigh. A transmissão mendeliana da deficiência no complexo I, bem como o ínicio dos sintomas durante a infância, são evidências de defeitos genéticos de origem nuclear.

Um outro estado patológico associado à deficiência na actividade do complexo I é a doença de Parkinson. No entanto, a relação entre as anomalias na enzima e o parkinsonismo não foi, ainda, determinada. Observações recentes sugerem que factores de ordem genética, mutações no DNA nuclear e/ou mitocondrial, assim como factores ambientais, contacto com toxinas, tenham um papel relevante na patologia (ver Schapira, 1998). A droga MPTP (1-metil-4-fenil-1,2,3,6-tetrahidropiridina) actua no sistema nervoso central, induzindo um estado clínico em tudo semelhante ao observado nos doentes com Parkinson. Este composto, uma vez no cérebro, é convertido em MPP^+, um catião lipofílico que é selectivamente captado pelo transportador de dopamina. Uma vez no citosol, o MPP^+ é acumulado nas mitocôndrias inibindo o complexo I, o que resulta numa diminuição da produção de ATP e num aumento de radicais livres. Estes efeitos podem levar à morte celular. Por outro lado, estudos com cíbridos permitiram verificar que, em alguns doentes, uma alteração no mtDNA parece ser responsável pelo defeito na actividade do complexo I. Nos restantes pacientes, a origem da deficiência nesta enzima continua por determinar, podendo estar associada a uma mutação no DNA nuclear (Schapira, 1998).

Por fim, estudos efectuados em membranas mitocondriais de fibroblastos, de um grupo heterogéneo de pacientes com deficiências no complexo I, revelaram um aumento na produção de radicais de oxigénio. Contudo, a quantidade de radicais superóxido, presente na mitocôndria, é modulada pela indução da enzima manganês superóxido dismutase (MnSOD). Esta indução é variável e parece depender, essencialmente, do

estado redox da célula e da quantidade de radical superóxido produzida (Pitkänen & Robinson, 1996; Robinson, 1998). Estes autores propuseram uma correlação entre o nível de indução da MnSOD e a gravidade dos sintomas apresentados pelos doentes. Assim, os pacientes que apresentavam sintomas mais graves eram aqueles em que se verificava uma maior indução da MnSOD (ver Robinson, 1998).

2.8-Características do organismo de estudo

2.8.1-Ciclo de vida do fungo *N. crassa*

N. crassa é um organismo eucariota pertencente à classe de fungos Ascomycetae. O seu ciclo de vida é bastante bem conhecido sendo capaz de se desenvolver por reprodução assexuada e sexuada. O esgotamento da fonte de carbono induz o processo de esporulação assexuada, com formação de macroconídias (esporos multinucleados) e microconídias (estruturas unicelulares). Quando, para além disso, a fonte de azoto se torna limitante, as hifas iniciam o processo de reprodução sexuada com formação das estruturas reprodutoras femininas, denominadas protoperitécias (Ricci *et al.*, 1991). As protoperitécias de um "mating type" (*e. g. A*) podem ser fertilizadas por macroconídias, microconídias ou micélio de "mating type" oposto (*a*). Após fertilização, estas estruturas desenvolvem-se dando origem às peritécias, nas quais o tecido "ascogenous" prolifera formando os asci. Nos asci imaturos, os dois núcleos haplóides de "mating type" oposto fundem-se e o núcleo diplóide entra, imediatamente, em meiose. Duas divisões meióticas, seguidas duma divisão mitótica resultam na formação de oito núcleos haplóides e, portanto, de oito ascósporos. Estes ascósporos são esporos dormentes, capazes de germinar e produzir micélio, após serem activados por acção do calor ou por exposição a certos produtos químicos (Raju, 1992; Springer, 1993).

2.8.2-Processos para a inactivação de genes em *N. crassa*

Actualmente, existem vários processos que permitem transformar, eficientemente, células de *N. crassa* (Vollmer & Yanofsky, 1986; Fincham, 1989). No entanto, e

contrariamente ao que acontece em leveduras onde a integração do DNA parece ocorrer exclusivamente por recombinação homóloga (Rine & Carlson, 1985), a transformação em *N. crassa* resulta numa complexa variedade de transformantes (Selker *et al.*, 1987). De entre eles, os transformantes em que o DNA se integra em locais ectópicos são os mais frequentes. A integração por recombinação homóloga é um acontecimento raro e pode resultar na duplicação em "tandem", separada por sequências de vector, ou na substituição directa do DNA endógeno (Selker *et al.*, 1987). Apesar de pouco frequente, o processo de recombinação homóloga tem sido utilizado para inactivar genes em *N. crassa*. Neste método, um fragmento de DNA, em que o gene de interesse foi total ou parcialmente removido e substituído por um gene que confere resistência a um antibiótico, é utilizado para transformar esferoplastos do fungo (Nehls *et al.*, 1992). Os transformantes são seleccionados em meio com antibiótico e analisados de modo a identificar a estirpe relevante.

Em *N. crassa*, o DNA transformado está sujeito a mutações e/ou metilações, durante a fase sexual do ciclo de vida do fungo, quando mais de uma cópia é introduzida no genoma ou quando o DNA transformado é homólogo de sequências endógenas. Qualquer sequência com tamanho superior a 500 bp é susceptível de inactivação quando duplicada. A inactivação é irreversivel envolvendo múltiplas transições de G:C para A:T. Este fenómeno, único em *Neurospora*, denomina-se RIP ("repeat-induced point mutation") e parece resultar da interacção directa DNA:DNA (Selker, 1990), como o sugerem várias observações. Por um lado, o RIP nunca inactiva apenas uma cópia duma duplicação, embora possa inactivar duas cópias de uma triplicação. Duplicações ligadas são inactivadas mais eficientemente por "RIPing" do que duplicações não ligadas. E por fim, o RIP está confinado ao núcleo. As células onde este fenómeno ocorre são heterocários com núcleos de ambos os pais e apenas são silenciadas as cópias presentes no núcleo em que ocorreu a duplicação (ver Selker, 1999). O mecanismo do RIP é desconhecido, mas o mais provável é envolver o emparelhamento de DNA, seguido de metilação e desaminação de algumas citosinas na região emparelhada. Os produtos do RIP estão normalmente metilados e podem desencadear metilação *de novo* (Selker, 1999).

Actualmente, o fenómeno de RIP é muito utilizado para inactivar genes específicos. Contudo, no caso de genes essenciais cuja inactivação é letal, utiliza-se uma técnica denominada por "sheltered RIP" (Metzenberg & Grotelueschen, 1992; Harkness *et al.*, 1994). Este método utiliza o fenómeno de RIP para inactivar o gene em causa e um conjunto de estirpes com características especiais. Assim, ambas as estirpes utilizadas no cruzamento contêm o alelo mutante *mei-2*, que leva a um elevado grau de não disjunção dos cromossomas homólogos (Smith, 1975) e, portanto, à formação de ascósporos dissómicos. Estes ascósporos, após germinarem, originam heterocários (Smith, 1974) compostos por dois núcleos diferentes, em que o núcleo que contém o gene mutado é complementado pelo núcleo normal. A presença de uma marca seleccionável no núcleo contendo o gene mutado permite, em condições de crescimento adequadas, obter heterocários em que o núcleo alterado é predominante. Nestas condições o produto do gene que foi alterado está presente em níveis subóptimos, permitindo estudar o fenótipo mutante.

3-OBJECTIVOS DO TRABALHO

O trabalho aqui apresentado visou o estudo da enzima NADH:ubiquinona oxidorredutase da cadeia respiratória do fungo *N. crassa*. Com o intuito de aumentar o conhecimento acerca da estrutura, função e biogénese deste complexo proteico, foi seguida uma abordagem para dissecar a enzima, utilizando mutantes de *N. crassa* nos quais genes que codificam para subunidades do complexo I foram inactivados. A caracterização destes mutantes teve como objectivo: (1) inferir o papel de um dado componente da enzima na estrutura e função da mesma; (2) obter informação acerca das vias de montagem do complexo I; (3) estudar a importância desta enzima ao longo do ciclo de vida do fungo.

Várias características tornam este organismo eucariota um modelo atractivo para estudos genéticos e bioquímicos: (1) a sua natureza haplóide; (2) a fácil manipulação genética e bioquímica, associada à existência de técnicas eficientes de transformação e inactivação de genes; (3) a semelhança entre o complexo I do fungo e a enzima de mamíferos permitindo a extrapolação de informação entre os dois sistemas. Esta semelhança é nítida quando se compara a estrutura primária das subunidades do complexo I de *N. crassa* com a da enzima bovina. Assim, das 27 subunidades da enzima fúngica cuja sequência foi já determinada, 85% têm homólogos no complexo I bovino. Por outro lado, como referido a estrutura tridimensional de ambos os complexos é também muito similar.

A primeira fase deste estudo consistiu no isolamento (cDNA e gene), caracterização e localização cromossómica de genes nucleares do complexo I. O interesse deste estudo foi determinar a estrutura primária de subunidades do complexo I e clonar fragmentos de DNA que contenham genes relevantes, tendo em vista a sua posterior inactivação. A segunda fase do trabalho incidiu sobre a obtenção e caracterização de estirpes mutantes em subunidades da enzima. Neste âmbito, os genes em estudo foram inactivados pelo fenómeno de "RIPing" e a influência das mutações, no processo de montagem do complexo I, foi analisada utilizando diversas técnicas bioquímicas e genéticas. Por fim, desenvolveu-se um sistema que permite fazer mutagénese dirigida de

proteínas em *N. crassa*. Com este sistema pretendeu-se estudar a influência de determinados aminoácidos na estrutura e função da respectiva proteína no complexo I.

4-TRABALHO EXPERIMENTAL

4.1-TRABALHO 1

-Duarte, M., Finel, M. & Videira, A. (1996). Primary structure of a ferredoxin-like iron-sulfur subunit of complex I from *Neurospora crassa*. **Biochim. Biophys. Acta 1275**, 151-153. Reprinted with permission from *Elsevier Science*.

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Short sequence-paper

Primary structure of a ferredoxin-like iron-sulfur subunit of complex I from *Neurospora crassa*

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Abstract

We have isolated cDNA clones encoding an iron-sulfur polypeptide subunit of the mitochondrial complex I of *Neurospora crassa*. The fungal cDNA library was screened by hybridisation with an heterologous probe from *Paracoccus denitrificans*. The DNA sequence of relevant isolates was determined and revealed an open reading frame encoding a precursor protein of 219 amino acid residues. The gene product is a ferredoxin-like protein that contains two cysteine-rich motives that may each bind a tetranuclear iron-sulfur cluster. The primary structure of the protein is highly homologous to the 23 kDa iron-sulfur subunit of complex I from bovine and *P. denitrificans*. Interestingly, an alanine residue within the second cluster-binding motif, which is conserved in complex I but replaced by tyrosine in similar chloroplast genes, is substituted for serine in *N. crassa*.

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

The mitochondrial H^+-translocating NADH dehydrogenase (Complex I, EC 1.6.5.3) is composed of about 40 polypeptide subunits, 7 of mitochondrial origin and the rest of nuclear origin. This oligomeric enzyme couples rotenone-sensitive transfer of electrons from NADH to ubiquinone to proton translocation across the inner mitochondrial membrane. Protein-bound prosthetic groups are involved in these reactions, a FMN molecule and several iron-sulfur clusters. The exact number of the redox centres is still under discussion, but there are at least one binuclear and three tetranuclear clusters that were characterised by EPR spectroscopy. In addition, there are indications for another cluster of each type, and perhaps even more [1,2]. Enzymes similar to mitochondrial complex I have been identified in prokaryotes and named NDH1. The bacterial operons encoding NDH1 of *Escherichia coli* and *P. denitrificans* were recently cloned and sequenced [3,4]. These enzymes are each composed of 14 proteins, seven of which are homologues of nuclear-coded subunits of bovine complex I, and the rest are homologous to subunits that are encoded in mtDNA. In spite of their presence in both mammalian and bacterial enzymes, two of the nuclear-coded subunits have not yet been identified in complex I of *N. crassa* nor in any of its assembly intermediates. One of these subunits is the 23 kDa iron-sulfur protein (bovine TYKY), and the other is a 20 kDa protein (bovine PSST) that might also bind a prosthetic group [5]. The TYKY subunit may bind two tetranuclear [Fe-S] clusters [6], and thus play a central role in the electron transfer reactions of complex I. On the protein level, there is 72% homology between the 23 kDa subunit of *P. denitrificans* and bovine TYKY [4]. This prompted us to undertake the cloning of the *N. crassa* homologue using the *P. denitrificans* gene as a probe, despite the high GC content of this bacterial DNA.

The *N. crassa* cDNA library [7] was screened by hybridisation, using a digoxygenin-labelled probe that corresponds to the nqo9 gene of *P. denitrificans* encoding the 23 kDa iron-sulfur subunit of NDH1 [4]. The probe was prepared by PCR using the cloned nqo9 gene as template, Vent DNA polymerase (New England Biolabs) and primers from both ends of the coding region. The nucleotide mixture in the reaction was composed of 70% unlabeled nucleotides and 30% DIG-labelling mix (Boehringer) and

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the PCR product was purified with Wizard PCR Preps (Promega). We have screened 5×10^5 library phage and detected 17 positives among them. The cDNA inserts of 6 independent phage were partially sequenced on both ends, and the longest one at the 5′ end was used to rescreen the library. The cDNA inserts of 2 out of 10 positive phage were further analysed, after subcloning in the pGEM4 plasmid vector [7]. The total DNA sequence was obtained by sequencing both strands of different cDNA inserts using Sequenase or Thermo-Sequenase (United States Biochemical) and cDNA-specific oligonucleotides as primers. The Sequenase recommended protocols were applied for sequencing with Thermo-Sequenase with the following modifications: labelling was performed at 42°C and the termination reactions were carried out at 72°C. Computer similarity searches in the EMBL databases were performed with FASTA [8] and other analysis of DNA and protein sequences were performed with the PC/GENE software package (IntelliGenetics).

Fig. 1 depicts the nucleotide sequence of the cDNA and the deduced amino acid sequence of the iron-sulfur subunit of complex I from *N. crassa*. The total DNA sequence is 1116 bp and encodes a precursor protein containing 219 amino acid residues, with a molecular mass of 24902 Da. We assume that the first methionine represents the initial amino acid residue, since no other ATG codon is found in the upstream sequence. The molecular weight of the resulting peptide is close to that of the bovine precursor protein and a mitochondrial targeting signal is predicted for the 34 N-terminal amino acid residues (see below). In addition, if the sequence 5′ to this ATG codon is translated, the resulting protein sequence does not have the characteristics of a mitochondrial presequence. The starting point of the mature polypeptide is less clear. A computer analysis predicts that either the first 34 or, less likely, 47 amino acids represent a presequence.

Another possibility is that the mature protein of *N. crassa* results from a two-step cleavage of the precursor polypeptide, because it contains two consensus sequences for such processing [9], RXIXXS (amino acids 20–25) and

```
-138                                                    GGCTTGCGACGACAACCA
-120 ACTACCTACCTAGCCACTCCTCTAATATCACACCTGGCGCAACGCATTTGCCTCACCTAC
-60  CCTGCCAGCCAACCTCAAAACCAACAATTAATCAACACCATCCACCGCAACCCGCCCATC

1    ATGCTCACCACCACTGCCTCTTCGGCTGCGGCCGTCGCGCGCCAGCTCACCACCCGCCGA
1     M  L  T  T  T  A  S  S  A  A  A  V  A  R  Q  L  T  T  R  R

61   GTCATCGCCCCCTCCTTCGTCTCTCAGGCCATCCGCACCTACGCAACCCCCGCCGGTCCC
21    V  I  A  P  S  F  V  S  Q  A  I  R  T  Y  A  T  P  A  G  P

121  CCGCCCAAGGGCTTCCGCATTCCGACGCCCAAGACGTGGGACCAAGAGGAGGAACACGTG
41    P  P  K  G  F  R  I  P  T  P  K  T  W  D  Q  E  E  E  H  V

181  CTGGATAAGAACGGACGGTACTTTCTTTTGACGGAGATGTTTAGGGGCATGTATGTGGCT
61    L  D  K  N  G  R  Y  F  L  L  T  E  M  F  R  G  M  Y  V  A

241  ATGGAGCAGTTTTTCAGGCCGCCGTACACAATCTATTACCCCTTCGAAAAGGGTCCCATC
81    M  E  Q  F  F  R  P  P  Y  T  I  Y  Y  P  F  E  K  G  P  I

301  TCCCCCCGCTTCCGCGGCGAGCACGCCCTTCGTCGTTACCCGTCGGGCGAAGAACGCTGC
101   S  P  R  F  R  G  E  H  A  L  R  R  Y  P  S  G  E  E  R  C

361  ATCGCCTGCAAGCTCTGCGAGGCCGTCTGCCCTGCTCAGGCCATCACCATCGAAGCTGAA
121   I  A  C  K  L  C  E  A  V  C  P  A  Q  A  I  T  I  E  A  E

421  GAGCGTGCCGATGGAAGCAGAAGGACGACCCGCTACGATATCGACATGACCAAGTGCATT
141   E  R  A  D  G  S  R  R  T  T  R  Y  D  I  D  M  T  K  C  I

481  TACTGCGGATTCTGCCAGGAGAGCTGTCCCGTGGATGCGATTGTGGAGAGTCCCAATGCG
161   Y  C  G  F  C  Q  E  S  C  P  V  D  A  I  V  E  S  P  N  A

541  GAGTACGCAACGGAGACGAGGGAGGAGTTGTTGTATAACAAGGAAAAGCTACTCTCTAAC
181   E  Y  A  T  E  T  R  E  E  L  L  Y  N  K  E  K  L  L  S  N

601  GGAGACAAGTGGGAGCCTGAGCTTGCGGCTGCTATTCGCGCCGATTCACCTTACAGATAA
201   G  D  K  W  E  P  E  L  A  A  A  I  R  A  D  S  P  Y  R  -

661  AGGGGTTGTGGCTTGAAGACCGGATAACAGGACGGACAATGAAGACAGAGATTCTTTTTT
721  TCGTTGTTGCTATGCTAGAGTTTCGGAACCAGCAGTTTCGGACGGGTTGCGGTATCAACA
781  GGACGCACCATTGTGTTATAGAAGGCAGGCAGGCAGGCAGGCGGACAGGCAAAAGAAGGC
841  TGTAGATACATTTGTGAATTGCGTCCCTCATCCAACTTCTTGTCTTTTTGAAGTCATCTC
901  GCCAGGGAAGATCCTATATGCGGCCAAAGGGACGCATCAAGAAACCTAAAGATGCTTGAA
961  AGGCTGAATGTACAATTA
```

Fig. 1. Nucleotide sequence of full-length cDNA and deduced primary structure of the iron-sulfur subunit of complex I from *N. crassa*. The consensus sequences for the binding of tetranuclear iron-sulfur clusters are underlined.

```
                                                    . .     .
NC  MLTTTASSAAAVARQLTTRRVIAPSFVSQAIRTYATPAGPPPKGFRIPTPKTWDQ -55
BT  ----------------------------------------TYKYVNLREPSM -12

    .      .     *  .  .*  . .        *. ** ****.**********
NC  EEEHVLDKNGRYFLLTEMFRGMYVAMEQFFRPPYTIYYPFEKGPISPRFRGEHAL -110
BT  DMKSVTDRAAQTLLWTELIRGLGMTLSYLFREPATINYPFEKGPLSPRFRGEHAL -67
PD   MAFDFARATKYFLMWDFIKGFGLGMRYFVSPKPTLNYPHEKGPLSPRFRGEHAL -54

    **** ********************.** * **********************
NC  RRYPSGEERCIACKLCEAVCPAQAITIEAEERADGSRRTTRYDIDMTKCIYCGFC -165
BT  RRYPSGEERCIACKLCEAVCPAQAITIEAEPRADGSRRTTRYDIDMTKCIYCGFC -122
PD  RRYPNGEERCIACKLCEAVCPAQAITIDAERREDGSRRTTRYDIDMTKCIYCGFC -109

    **.******** ** *..*** *** * * *** **..** *.*  .  *  **
NC  QESCPVDAIVESPNAEYATETREELLYNKEKLLSNGDKWEPELAAAIRADSPYR -219
BT  QEACPVDAIVEGPNFEFSTETHEELLYNKEKLLNNGDKWEAEIAANIQADYLYR -176
PD  QEACPVDAIVEGPNFEYATETREELFYDKQKLLANGERWEAEIARNLQLDAPYR -164
```

Fig. 2. Comparison of the protein sequences of the iron-sulfur subunit of complex I from *N. crassa* (NC), *Bos taurus* (BT) and *P. denitrificans* (PD). Only the mature sequence of bovine TYKY is shown. Identical residues (*) and conservative substitutions (·) are indicated.

RXFXXT (amino acids 66–71). This would yield a mature protein which is shorter than the precursor polypeptide by either 29 or 75 amino acid residues, respectively. It was previously suggested that the bovine homologue is cleaved twice to yield the mature TYKY protein [6].

As with homologues in bovine and bacterial enzymes, and similarly several ferredoxins and chloroplast gene products [5], the *N. crassa* protein includes two sequence motives CXXCXXCXXXCP that may bind tetranuclear [Fe-S] clusters. The 23 kDa subunit of complex I was suggested to bind the [Fe-S] cluster N-2 [6,10], a redox centre that might be directly involved in the proton-translocation activity of the enzyme. Interestingly, a highly conserved (from bacteria to mammals) alanine residue in the second [Fe-S] cluster-binding motif is replaced by serine (residue 168) in the fungal protein (Fig. 2).

In bovine complex I, this protein was suggested to be located at the interface between the 'core' subunits and the hydrophobic ones [10]. This is supported by recent results that locate the protein in the 'connecting fragment' of *E. coli* NDH1 [11]. Since the iron-sulfur centre N-2 was reported to be bound within membrane fragments of Neurospora complex I [12], it will be interesting to determine whether or not this protein is also located in the membrane domain of the enzyme.

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4.2-TRABALHO 2

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BBA

Identification of the TYKY homologous subunit of complex I from *Neurospora crassa*

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Abstract

A polypeptide subunit of complex I from *Neurospora crassa*, homologous to bovine TYKY, was expressed in *Escherichia coli*, purified and used for the production of rabbit antiserum. The mature mitochondrial protein displays a molecular mass of 21280 Da and results from cleavage of a presequence consisting of the first 34 N-terminal amino acids of the precursor. This protein was found closely associated with the peripheral arm of complex I. © 1997 Elsevier Science B.V.

Keywords: Complex I subunit; Iron–sulfur cluster N-2; Mitochondrion; (*Neurospora crassa*)

1. Introduction

Respiratory chain NADH dehydrogenase (complex I; EC 1.6.5.3) is a multimeric enzyme of the mitochondrial inner membrane. Mitochondrial complex I contains more than 30 polypeptide subunits, seven of which are encoded by mtDNA. It transfers electrons from NADH to ubiquinone through a series of protein-linked prosthetic groups (FMN and iron–sulfur clusters) and these reactions are coupled with proton translocation across the membrane. It is accepted that complex I contains at least one binuclear and three tetranuclear iron–sulfur clusters, but probably more [1,2]. Neurospora complex I was shown to be formed from two parts, the peripheral and membrane arms, that undergo independent assembly [3]. The peripheral arm appears to contain all prosthetic groups of the enzyme, with the exception of the tetranuclear iron–sulfur cluster N-2 [4], while several largely hydrophilic subcomplexes which contain all redox centres, including cluster N-2, were isolated from bovine complex I [5,6].

The assignment of the prosthetic groups to specific subunits of the enzyme is important to understand in more detail the structure and function of complex I. Based on different approaches, most of them have been tentatively assigned to specific subunits of the enzyme. Localization of iron–sulfur cluster N-2, however, is still under debate. It has been proposed that cluster N-2 can directly reduce ubiquinone and a

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proton pumping activity is associated with this electron transfer reaction [7–9], making this prosthetic group of complex I particularly important. Two proteins, called TYKY and PSST in bovine, appear as the most likely candidates to provide its binding site [6,10–12]. Here, we report that the *N. crassa* subunit homologous to TYKY is located in the peripheral arm of complex I and, therefore, most likely does not bind cluster N-2.

2. Material and methods

We expressed the putative mature form of the *N. crassa* TYKY-homologue in *E. coli* as a fusion protein containing a His-tag. The corresponding cDNA, cloned in pGEM4 [13], was amplified by PCR using *Pfu* DNA polymerase (Stratagene), the vector-specific T7 promotor primer and the cDNA-specific primer 5′-ccatccgccatatggcaacccccgccggtc-3′ (the underlined bases were altered from the *N. crassa* sequence to create a *Nde*I site). The resulting DNA was digested with *Nde*I and *Bam*HI and cloned into the pET19b plasmid vector (Novagen). In order to avoid sequencing the entire DNA obtained by PCR, a 3′-located *Sac*II/*Bam*HI fragment was replaced with the correspondent piece from the original cDNA. Both strands of the DNA located 5′ to the *Sac*II site were completely sequenced. Bacteria carrying the recombinant plasmid were grown to exponential phase ($A_{600} = 0.6$), induced for expression of the fusion protein for 3 h at 37°C using 1 mM IPTG and collected by centrifugation. Most of the protein was present in ''inclusion bodies'' and, after purification in nickel chelation columns under denaturing conditions, was used to produce rabbit antiserum [14]. A small portion of soluble protein was also purified by affinity chromatography under native conditions and used for digestion with Enterokinase (Sigma).

Techniques such as the preparation of mitochondria [15], isolation of complex I [16] and its peripheral arm [17], the analysis of Triton X-100 solubilised mitochondrial proteins in sucrose gradients [18], protein determination [19] and sequencing [20], SDS-polyacrylamide gel electrophoresis [21], blotting [22] and immunological detection of proteins using alkaline phosphatase-conjugated secondary antibodies [23], have been published before.

3. Results and discussion

We have expressed the mature form of an iron–sulfur subunit of complex I from *N. crassa* in an heterologous system and, after purification, used it to immunise rabbits. The protein is homologous to bovine TYKY [13] and, according to the size of the mature polypeptide, was named NUO-21.3c (there are two other subunits of complex I with the same molecular mass). In agreement with this, the antiserum readily recognises a protein displaying an apparent molecular mass of 22–24 kDa from extracts of *N. crassa* mitochondria (Fig. 1, lanes 1–4). The migration of this polypeptide is similar to the migration of the fusion protein produced in *E. coli* that was incubated with enterokinase in order to remove the His-tag (compare lanes 3 and 6 of Fig. 1). It should be noticed that the latter should contain two extra amino acids. Direct determination of the N-terminus of the mature mitochondrial protein revealed the sequence ATPAG, thus confirming the cleavage site of the precursor. Interestingly, this fits into a one-step cleavage of the precursor to yield the mature protein, while it was suggested that the homologous TYKY protein of bovine results from a two-step cleavage of its precursor [24]. Although this awaits experimental evidence, it obviously represents

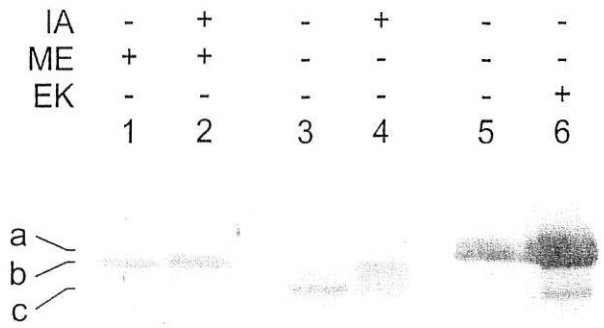

Fig. 1. Identification of the NUO-21.3c subunit of complex I. Total mitochondrial proteins (1–4) or NUO-21.3c protein that was expressed and purified from *E. coli* (5, 6) were analysed by Western blotting with antiserum against the latter polypeptide. The protein fused to a His-tag (a) as well as the mitochondrial reduced (b) and oxidised (c) forms of the protein are indicated. The experiments were performed in the presence (+) or absence (−) of iodoacetamide (IA), 2-mercaptoethanol (ME) and enterokinase (EK).

a further example where the same protein is processed in different ways in different organisms. The same has been observed with the Rieske iron–sulfur protein of complex III, which is cleaved in two steps in *N. crassa* [25], but undergoes one-step cleavage in mammals [26].

The mature NUO-21.3c protein has an electrophoretic migration that is faster in the absence than in the presence of 2-mercaptoethanol (compare lanes 1 and 3 of Fig. 1), suggesting the existence of intramolecular disulphide bridges that would prevent some of the cysteines from participating in the binding of iron–sulfur clusters. If this was true, there was not enough cysteines in the protein to bind the two iron–sulfur clusters that it is believed to contain. However, when mitochondria are dissociated with SDS in the presence of 10 mM iodoacetamide, that should prevent de novo formation of disulphide bridges, a slower migrating band can be seen in the absence of 2-mercaptoethanol (Fig. 1, lane 4). We suggest that intramolecular disulphide bridges readily form in NUO-21.3c only upon isolation of the protein from the membrane in denaturing conditions, presumably by release of the bound iron–sulfur cluster(s).

Fig. 2 shows that NUO-21.3c is associated with the peripheral arm of complex I. Using antiserum against the protein, it can be visualised in preparations of complex I or its peripheral arm alone. As controls, individual antisera against the 30.4 kDa subunit of the peripheral arm of complex I [27] and against the 20.8 kDa and 12.3 kDa subunits of the

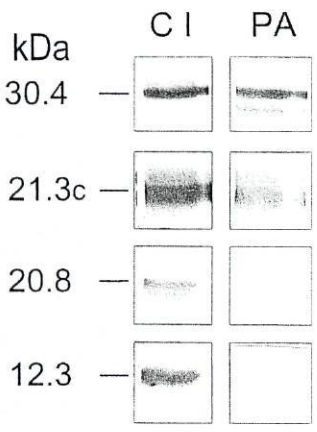

Fig. 2. Association of NUO-21.3c with the peripheral arm of complex I. Preparations of complex I (CI) or its peripheral arm (PA) were analysed by Western blotting with antisera against the subunits of complex I indicated in the left.

membrane arm of the enzyme [28,29] were included in this experiment. The 30.4 kDa protein can also be seen in both preparations, while the 20.8 kDa and 12.3 kDa are visible exclusively in the complex I preparation.

In order to further confirm these results, the behaviour of the NUO-21.3c protein was analysed in *N. crassa* mutants that bear different phenotypes regarding complex I. We used the stopper strain IAR 155A [30] that assembles the peripheral arm and lacks detectable membrane arm and mutant nuo30.4 that lacks the peripheral arm and accumulates the membrane arm of complex I (P.C. Alves, M. Duarte and A. Videira, unpublished observations). Mitochondrial proteins from these strains were solubilised with Triton X-100 and resolved by sucrose gradient centrifugation. Subsequently, fractions of the gradients were analysed by Western blotting with antisera against different subunits of complex I that can be used as markers for the peripheral and membrane arms of the enzyme [18,31,32]. Fig. 3 illustrates the results of this experiment. In the wild type strain, all proteins elute mostly in fractions 9–11 (panel A), the usual behaviour of complex I. In strain IAR 155A, NUO-21.3c co-elutes with the 30.4 kDa protein with a peak in fraction 8 and this material represents the peripheral arm of complex I (panel B). It can be seen that the membrane arm 12.3 kDa protein elutes mainly in fractions 2 and 3, thus representing "free" subunit. On the contrary, the NUO-21.3c protein behaves as "free" subunit in the nuo30.4 mutant, eluting in fractions 2 and 3 (panel C). In this strain, the membrane arm of complex I elutes mostly in fractions 8–10, as deduced by the behaviour of its 20.8 kDa and 12.3 kDa subunits. Furthermore, we have recently isolated a *N. crassa* mutant lacking NUO-21.3c and it appears that only the membrane arm and not the peripheral arm are assembled in this strain (details will be published elsewhere).

Our results clearly show that subunit NUO-21.3c is part of the peripheral arm of complex I from *N. crassa*. This domain holds most of the prosthetic groups of the enzyme, but has been shown to lack iron–sulfur cluster N-2 [4]. This is not due to a loss during the preparation of this subcomplex, since EPR analysis of membranes of mutant nuo21 did not detect cluster N-2 in the peripheral arm even in its native membrane surrounding [33]. Furthermore,

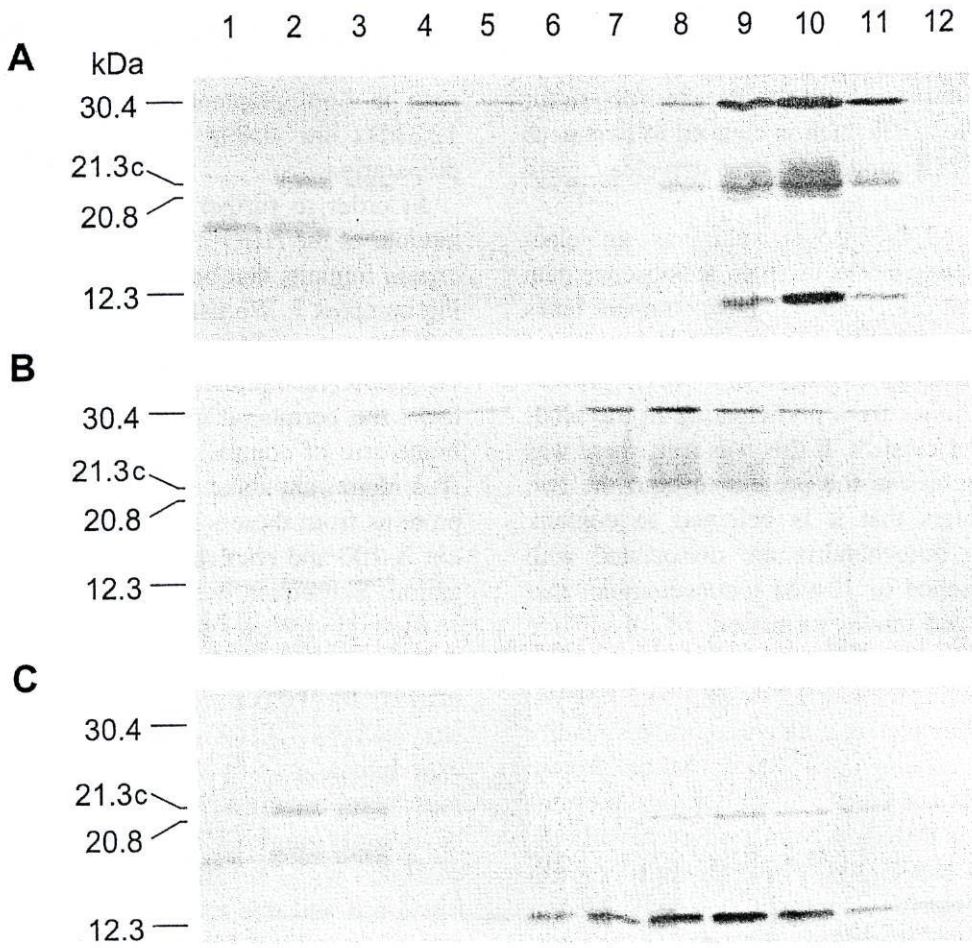

Fig. 3. Analysis of NUO-21.3c in different strains of *N. crassa*. Mitochondria from wild type (A), the stopper strain IAR 155A (B) and mutant nuo30.4 (C) were isolated and centrifuged in sucrose gradients in the presence of Triton X-100. Fractions of the gradients (labelled 1–12 from top to bottom) were collected and analysed by Western blotting with a mixture of antisera against the subunits of complex I indicated in the left.

cluster N-2 has been found in excess over the other clusters in mitochondrial membrane fragments of *N. crassa* grown under manganese depletion. Since the membrane arm is accumulated in these membranes, cluster N-2 was attributed to the membrane part of complex I [34,35]. Since all EPR-visible iron–sulfur clusters present in the peripheral arm have been attributed to the 78 kDa, 51 kDa and 24 kDa subunits, NUO-21.3c most likely binds iron–sulfur cluster(s), which have so far escaped detection by EPR. The conservation of cysteine motifs in this protein from a variety of organisms [10,13], strongly suggests that it binds two iron–sulfur clusters. Excluding TYKY and its homologues from providing the binding site for cluster N-2, leaves PSST [36] as the most likely candidate for this function. Its unusual binding motif, probably involving a glutamate residue [37], correlates to the particular features of cluster N-2 as its high pH-dependent midpoint-potential.

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4.3-TRABALHO 3

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

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Abstract

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

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

1. Introduction

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

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

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PII: S0005-2728(99)00014-6

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-60                                                          ATTGATATTTTCAAC

1    ATGATGTCTTCTGTGAGGACAGGCGCCTCGATGGCTCTTAGAGCCCGGCCGACTGCCCAA
1     M  M  S  S  V  R  T  G  A  S  M  A  L  R  A  R  P  T  A  Q

61   ATCGTTCCCTTCCGTGCGGCCGCTGTCGCTTCCATCTCCTCGTCCTCCCGCAAGGACGCC
21    I  V  P  F  R  A  A  A  V  A  S  I  S  S  S  S  R  K  D  A

121  ACGGGTGCCGTCGCCCCGGCGGGCGCACAGCACGGCATCGCCAGGCGCGAGCGCAGAGAG
41    T  G  A  V  A  P  A  G  A  Q  H  G  I  A  R  R  E  R  R  E

181  GTTCCTCTTCCCAGCCAGGAGGGCACCAAGGGCGCCGTTCAATATGCTCTCACAACCCTC
61    V  P  L  P  S  Q  E  G  T  K  A  A  V  Q  Y  A  L  T  T  L

241  GACAGCATCGTCAACTGGGCCCGCCAATCCTCTCTCTGGCCCATGACCTTCGGCCTCGCC
81    D  S  I  V  N  W  A  R  Q  S  S  L  W  P  M  T  F  G  L  A

301  TGCTGTGCCGTCGAGATGATGCACCTCTCAACCCCGCGGTACGATCAAGATCGTTTGGGC
101   C  C  A  V  E  M  M  H  L  S  T  P  R  Y  D  Q  D  R  L  G

361  ATCATCTTCCGTGCCTCGCCCCGCCAGTCGGACGTCATGATTGTGGCGGGCACCCTGACC
121   I  I  F  R  A  S  P  R  Q  S  D  V  M  I  V  A  G  T  L  T

421  AACAAGATGGCGCCCGCCCTGCGCCAGGTGTACGACCAGATGCCCGATCCGCGTTGGGTC
141   N  K  M  A  P  A  L  R  Q  V  Y  D  Q  M  P  D  P  R  W  V

481  ATCAGCATGGGCTCGTGCGCCAACGGCGGCGGCTACTACCACTACTCGTACAGCGTCGTG
161   I  S  M  G  S  C  A  N  G  G  G  Y  Y  H  Y  S  Y  S  V  V

541  CGCGGCTGCGACCGGATTGTCCCTGTTGACATCTACGTCCCGGGGTGCCCGCCTACTAGT
181   R  G  C  D  R  I  V  P  V  D  I  Y  V  P  G  C  P  P  T  S

601  GAAGCGCTCATGTATGGCATTTTCCAGTTGCAGAGGAAGATGCGGAATACGAAGATTACG
201   E  A  L  M  Y  G  I  F  Q  L  Q  R  K  M  R  N  T  K  I  T

661  AGGATGTGGTATCGCAAGTAGAGAGCGTTACTACTGCTTGGCACTTGCTTGTCGTGGTCA
221   R  M  W  Y  R  K  -

721  TGGGAGGGGGGTTAGACTTTGGGGTGAGTGGTAGCGCCAAGAAAGGATCGATGAAAGAAG

781  AAGATTGAGTGGTGGTAAAAACCGGAAAGACAGCACGGTTAGATTTCATGGACGAACGGA

841  AGAGCTCGAGAACAACGGCTCACAAGAAGGAACTTCAGTGGAAATTAGACAAGATGTATT

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

2. Materials and methods

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

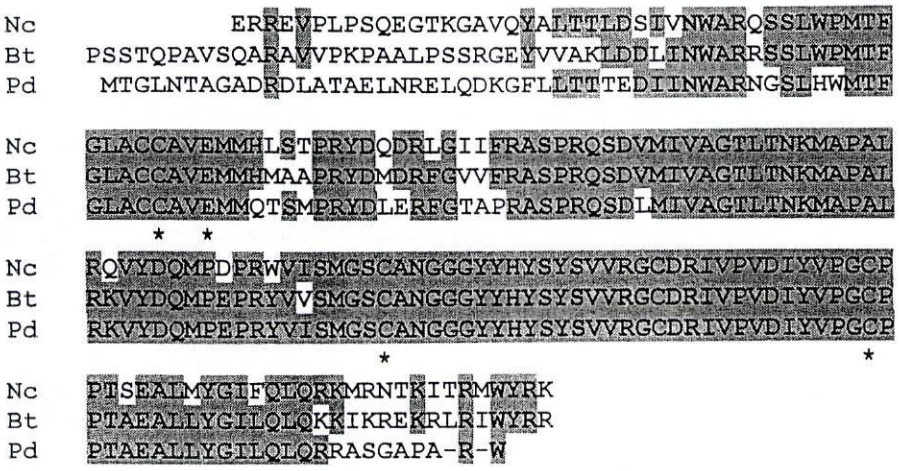

Fig. 2. Alignment of the protein sequences of the 19.3 kDa subunit of complex I from *N. crassa* (Nc, putative mature protein) with homologues from *B. taurus* (Bt) and *P. denitrificans* (Pd). Identical amino acids are shadowed and the four residues suggested to ligate Fe-S cluster N-2 are marked with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

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

3. Results and discussion

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

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

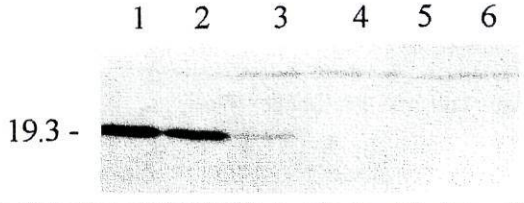

Fig. 3. Detection of NUO-19.3 in mitochondria from different *N. crassa* strains. Total mitochondrial proteins from the wild type strain (1), the complex I mutants nuo21 (2), nuo51 (3), nuo20.8 (4), and nuo21.3c (5), and wild type strain grown in the presence of chloramphenicol (6) were analysed in Western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

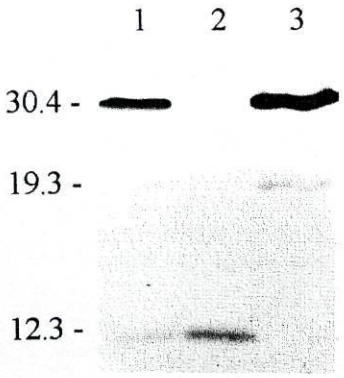

Fig. 4. Localisation of NUO-19.3 after alkaline extraction of mitochondria. Mitochondria from wild type *N. crassa* were subjected to an alkaline treatment and centrifuged. Untreated mitochondria (1) and the resulting membrane pellet (2) and soluble fractions (3) were analysed in Western blots immunodecorated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

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

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

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

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4.4-TRABALHO 4

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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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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-

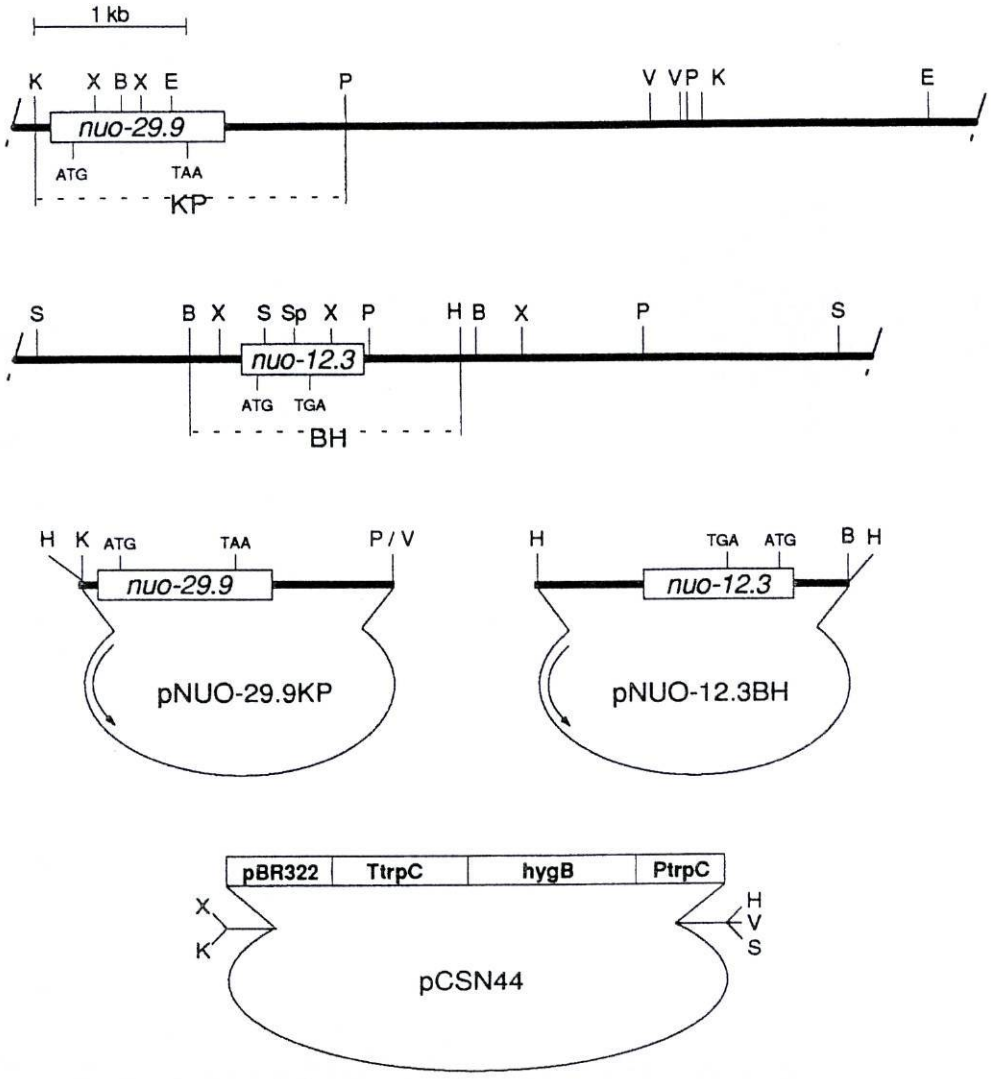

FIGURE 1.—Restriction map of *N. crassa* DNA regions containing *nuo-29.9* (upper) and *nuo-12.3* (lower) and construction of recombinant plasmids. The enzymes shown are as follows: B, *Bam*HI; H, *Hind*III; E, *Eco*RI; V, *Eco*RV; K, *Kpn*I; P, *Pvu*II; S, *Sal*I; Sp, *Sph*I; X, *Xho*I. The open boxes 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/*Hind*III region of the polylinker of pGEM3, in pCSN44 double digested with *Hind*III and *Eco*RV. Plasmid pNUO-12.3BH was obtained by cloning the 1.8-kb *Bam*HI/*Hind*III fragment BH, together with the *Bam*HI/*Hind*III region of the polylinker of pGEM4, in the *Hind*III 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 *hygB*^{r} gene of pCSN44. The relevant features of pCSN44 are also diagrammed: unique restriction sites and a bacterial gene (*hygB*^{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

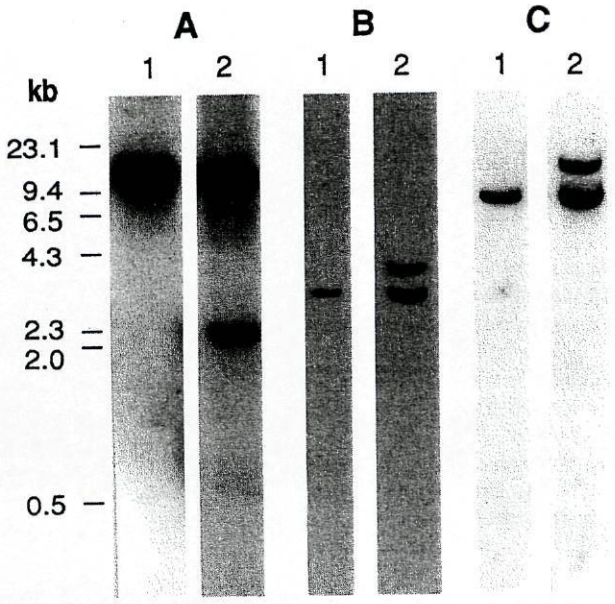

FIGURE 2.—Southern blotting analysis of a Neurospora strain transformed with plasmid pNUO-29.9KP. Genomic DNA from the wild-type strain (1) and transformant T11 (2) was prepared, separately digested with *Pst*I (A), *Xho*I (B), *Eco*RV (C), electrophoresed in agarose gels and blotted onto nylon membranes.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

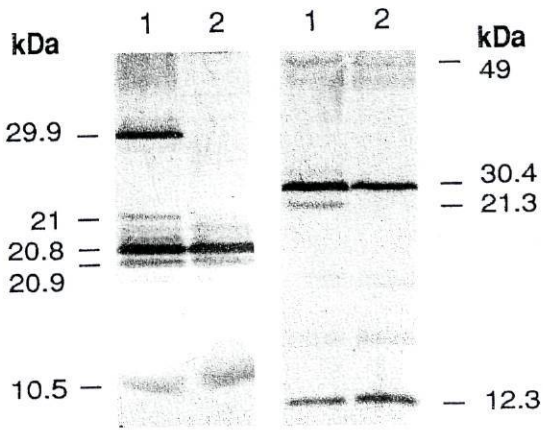

FIGURE 3.—Western blotting analysis of mitochondrial proteins from mutant nuo-29.9 with antisera against different complex I subunits. Total mitochondrial proteins from the wild-type strain (lanes 1) and strain M11/20 (lanes 2) were resolved by SDS gel electrophoresis and blotted onto nitrocellulose. 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.

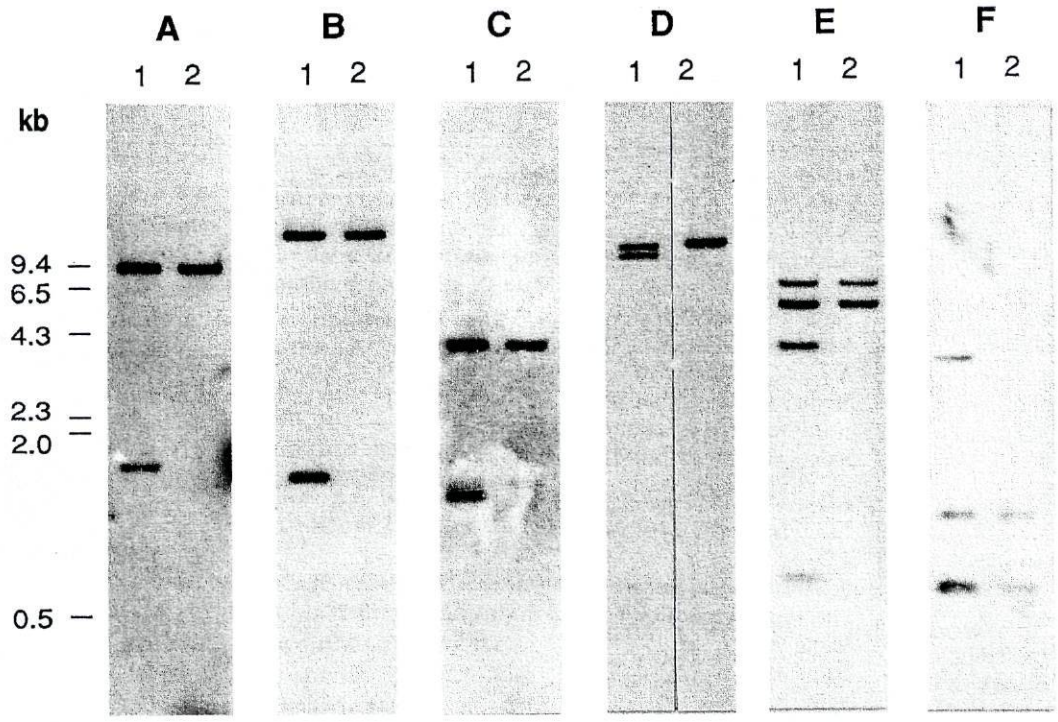

FIGURE 4.—Southern blotting analysis of a Neurospora strain transformed with plasmid pNUO-12.3BH. Genomic DNA from transformant T2 (1) and the wild-type strain (2) was prepared, separately digested with *Hin*dIII (A), *Pvu*II (B), *Sal*I (C), *Eco*RV (D), *Sph*I (E) and *Xho*I (F), electrophoresed in 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

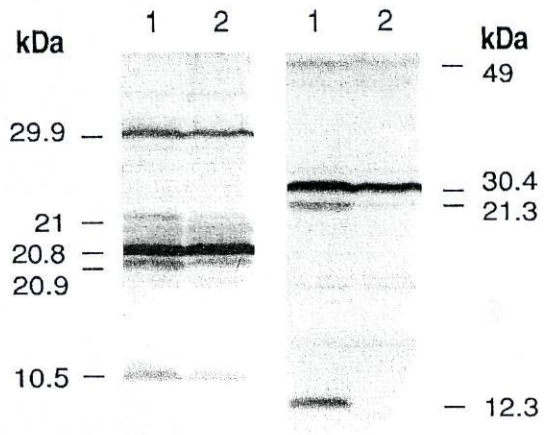

FIGURE 5.—Western blotting analysis of mitochondrial proteins from mutant nuo-12.3 with antisera against different complex I subunits. Total mitochondrial proteins from the wild type strain (lanes 1) and strain M2h/25 (lanes 2) were resolved by SDS gel electrophoresis and blotted onto nitrocellulose. 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-

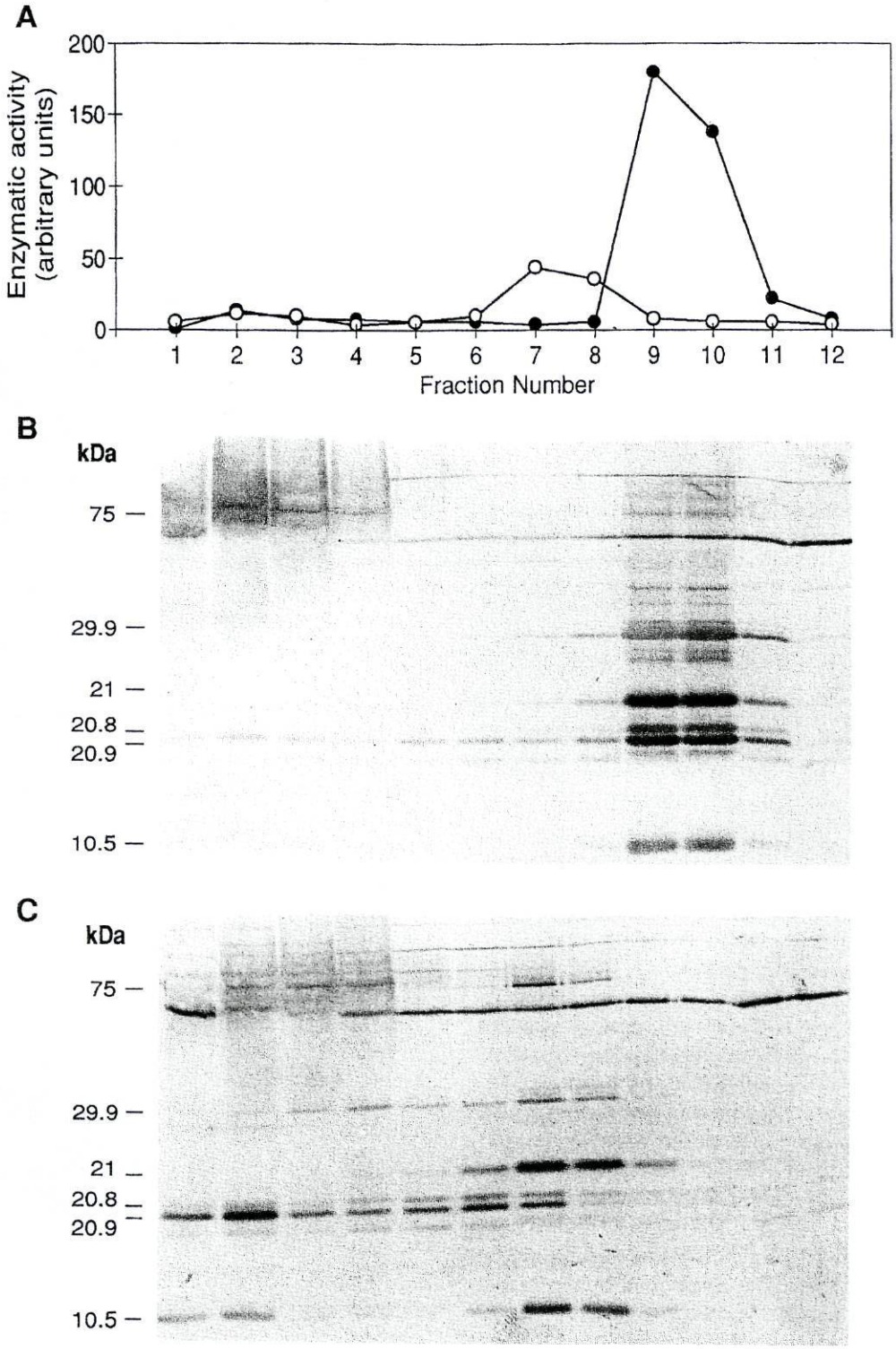

FIGURE 6.—Sucrose gradient centrifugation analysis of Triton-solubilized mitochondrial proteins from mutant nuo-12.3. Mitochondria were isolated, solubilized with Triton X-100 and centrifuged in sucrose gradients. Fractions (labeled 1–12 from top to bottom) of the gradients were collected. Aliquots of 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.

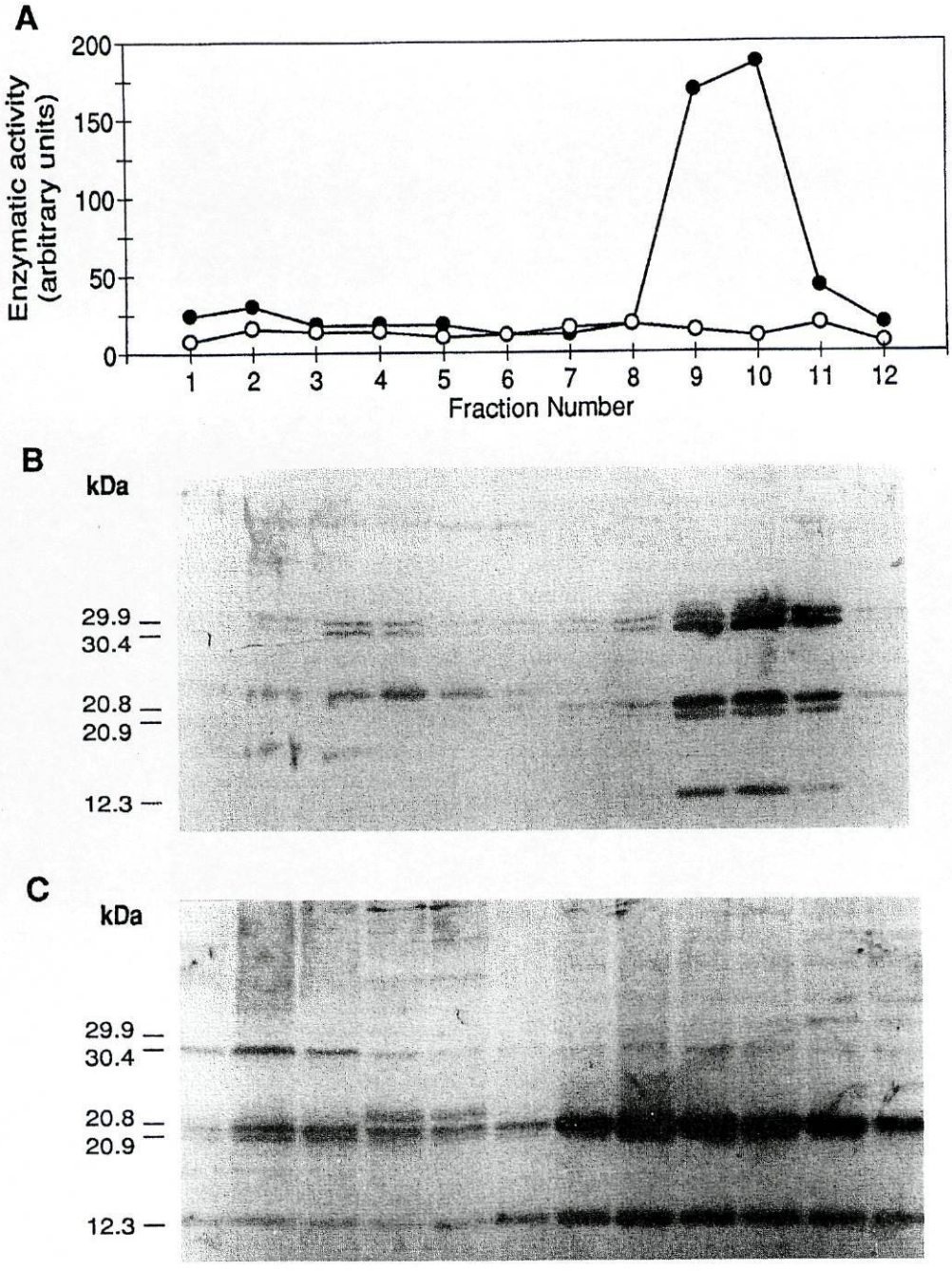

FIGURE 7.—Sucrose gradient centrifugation analysis of Triton-solubilized mitochondrial proteins from mutant nuo-29.9. Mitochondria were isolated, solubilized with Triton X-100 and centrifuged in sucrose gradients. Fractions (labeled 1–12 from top to bottom) of the gradients were collected. Aliquots of 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.

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4.5-TRABALHO 5

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ORIGINAL PAPER

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Inactivation of the gene coding for the 30.4-kDa subunit of respiratory chain NADH dehydrogenase: is the enzyme essential for Neurospora?

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Abstract We have isolated and characterised the nuclear gene that codes for the 30.4-kDa subunit of the peripheral arm of complex I from *Neurospora crassa*. The single-copy gene was localised on chromosome VI of the fungal genome by restriction fragment length polymorphism mapping. An extra copy of the gene was introduced into a strain of *N. crassa* by transformation. This strain was crossed with another strain in order to inactivate, by repeat-induced point mutations, both copies of the duplication carried by the parental transformant. Ascospore progeny from the cross were analysed and a mutant strain lacking the 30.4-kDa protein, nuo30.4, was isolated and further characterised. The mutant appears to assemble the membrane arm of complex I, while formation of the peripheral arm is prevented. Nevertheless, the mutant grows reasonably well – indicating that this well conserved protein is not essential for vegetative growth – and is able to mate with other strains both as male or female. Strains with multiple mutations are readily obtained from heterozygous crosses between different complex I mutants of *N. crassa*. On the other hand, homozygous crosses between several mutants, including nuo30.4, fail to produce ascospores. These results suggest that complex I plays an essential role during the sexual phase of the life cycle of the fungus.

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Introduction

The NADH dehydrogenase of the mitochondrial respiratory chain, commonly known as complex I (EC 1.6.5.3), is a very large multimeric enzyme located in the inner membrane of the organelle. It couples electron transfer from NADH to ubiquinone with proton translocation across the membrane, thereby contributing to the establishment of a transmembrane proton gradient needed for the synthesis of ATP (Hatefi 1985). Complex I contains more than 30 polypeptide subunits and protein-linked prosthetic groups, including FMN and at least four iron-sulphur clusters that participate in the activity of the enzyme. Studies in Neurospora have indicated that complex I has two domains, the peripheral and membrane arms, that are assembled independently (Tuschen et al. 1990). The former protrudes into the mitochondrial matrix and contains most of the prosthetic groups of the enzyme. Seven of the membrane arm polypeptides are encoded by mtDNA and their functions are largely unknown (for reviews see Weiss et al. 1991; Walker 1992). Complex I-like enzymes, named NDH-1, have also been identified in prokaryotes, e.g. *Escherichia coli* and *Paracoccus denitrificans* (Yagi et al. 1992; Weidner et al. 1993). These bacterial structures are composed of homologues of the seven mtDNA-encoded polypeptides and homologues of seven nucleus-encoded proteins of complex I from fungi or mammals. The fact that these enzymes are of a much simpler composition suggests that mitochondrial complex I might perform additional, unknown functions. Indeed, an acyl-carrier protein is a subunit of mitochondrial complex I (Brody and Mikolajczyk 1988; Runswick et al. 1991; Sackmann et al. 1991) and may be involved in lipid synthesis or repair (Schneider et al. 1995).

In order to investigate further the structure and function of complex I, we are using *Neurospora crassa* as a model system. The fungal enzyme is quite similar to that of mammals (Azevedo and Videira 1994; Duarte et al. 1996) and this organism allows the application of several genetic and biochemical methods. As an approach to the investigation of complex I, we inactivate specific genes of the enzyme and isolate and characterise the resulting mutants. Repeat-induced point mutations (RIP) are generated in the relevant genes in order to achieve inactivation. This technique is unique to *N. crassa* and is based on the fact that, with a certain frequency, genes that have been duplicated in the genome of the organism are inactivated by GC to AT transitions when passed through a genetic cross (Selker 1990; Selker and Garrett 1988). The inactivation of Neurospora complex I genes has also been achieved by replacement with disrupted copies (Nehls et al. 1992; Fecke et al. 1994; Schneider et al. 1995; Schulte and Weiss 1995). Using these strategies, the disruption of specific subunits of both the peripheral and membrane arms of complex I has allowed the recent isolation of several mutants with a variety of phenotypes (Alves and Videira 1994; Duarte et al. 1995; Harkness et al. 1995; Schulte and Weiss 1995; da Silva et al. 1996). In this report, we describe the inactivation of the nuclear gene coding for the 30.4 kDa subunit of the peripheral arm of complex I, a well conserved protein. Homologues of the gene encoding the protein of Neurospora and bovine have been found in bacteria, chloroplasts and plant mitochondria (Videira et al. 1990b; Pilkington et al. 1991; Lamattina et al. 1993; Weidner et al. 1993). Despite this, it appears that the protein is not essential for vegetative growth of Neurospora, although it is necessary for the assembly of complex I. A mutant lacking the protein seems to be unable to form the peripheral arm and accumulates only the membrane arm of the enzyme.

It comes as a surprise that Neurospora strains with gross defects in complex I, as a result of the disruption of particular subunits, appear to grow reasonably well (Duarte et al. 1995; Schulte and Weiss 1995). On the other hand, deletions or even point mutations in mitochondrial DNA encoding subunits of the enzyme (no mutation in a relevant nuclear gene has been found so far) seem to be involved in the development of severe human diseases (Wallace 1992). The fact that the fungus possesses alternative enzymes with NADH:ubiquinone reductase activity (Weiss et al. 1970) may provide an explanation for this. However, we found that homozygous crosses between many different strains carrying complex I deficiencies fail to complete the sexual phase of the life cycle of *N. crassa*. Thus, for the first time, we provide some evidence that complex I is essential for some cellular processes and demonstrate that it may not always be possible for other enzymes of the fungus to substitute for its constituent subunits.

Materials and methods

Strains, plasmids and cloning techniques

N. crassa strain 74-OR23-1A (wild type), the sterile helper strain (a^{m1}, *ad-3B*, *cyh-1*) and two strains designed for the disruption of essential genes located on linkage group VI (Metzenberg and Grotelueschen 1992), namely Host VI (*a*, Bml^r, *pan-2*, *inl*, *inv*, *mei-2*; FGSC #7256) and Mate VI (*A*, *ad-1*, am_{132}, *inl*, *inv*, *mei-2*; FGSC #7266) were obtained from the Fungal Genetics Stock Centre (Kansas City, Kan.). The original complex I mutants nuo12.3 (Duarte et al. 1995), nuo20.8 (da Silva et al. 1996), nuo21.3 (Alves and Videira 1994), nuo29.9 (Duarte et al. 1995) and nuo78 (Harkness et al. 1995) have been described previously. The triple complex I mutants TRP19 (*nuo-20.8*, *nuo-21.3*, *nuo78*) and TRP24 (*nuo-20.8*, *nuo-21.3*, *nuo29.9*) were obtained by sequentially crossing individual mutants. For the isolation of a genomic clone of *nuo-30.4*, a *N. crassa* genomic library cloned in phage J1 was screened with labelled cDNA encoding the 30.4-kDa subunit of complex I (Videira et al. 1990b). For gene mapping, the segregation of restriction fragment length polymorphisms was analysed in the 38 strains (FGSC #4450–4487) of the Multicent-2 cross kit (Metzenberg et al. 1984). Plasmid pCSN44 for fungal transformation (Staben et al. 1989) and the transcription vector pGEM4 (Melton et al. 1984) were maintained in *Escherichia coli* DH5α (Sambrook et al. 1989). Standard protocols were followed for restriction enzyme digestion, agarose gel electrophoresis, Southern blotting and subcloning (Sambrook et al. 1989).

N. crassa manipulations

Growth and crosses of *N. crassa* strains were carried out according to standard procedures (Davis and de Serres 1970; Perkins 1986). When a strain was used as a female in crosses, it was inoculated into the medium 5 days prior to the addition of the male parent. Conidia from 7-day-old cultures were used for the preparation of spheroplasts, which were then transformed with DNA and selected on plates containing hygromycin B, as described previously (Duarte et al. 1995). Individual transformants were "purified" by asexual transfers and conidial platings before further analysis. Heterokaryon formation with the sterile helper strain was forced on minimal Vogel's medium. *N. crassa* genomic DNA was isolated from small mycelial cultures (Lee et al. 1988; Videira et al. 1993). The linear growth rates of *N. crassa* strains were examined in race tubes (White and Woodward 1995).

Protein analysis

The techniques for protein determination (Bradford 1976), preparation of mitochondria (Werner 1977) and antibodies against complex I subunits (Videira and Werner 1989), SDS-polyacrylamide gel electrophoresis (Zauner et al. 1985), protein blotting (Towbin et al. 1979) and detection of antibody-antigen complexes with alkaline phosphatase-conjugated secondary antibodies (Blake et al. 1984) have been published. For analysis of mitochondrial protein complexes, the organelles were solubilised with Triton X-100 and fractionated by centrifugation in sucrose gradients (Alves and Videira 1994). NADH:ferricyanide reductase activity was measured according to Hatefi (1978).

Results

Isolation of mutant nuo30.4

In order to isolate mutants lacking particular subunits of complex I, we induce the generation of repeat-induced

point-mutations (RIP) in their structural genes. Briefly, the gene is introduced into a *N. crassa* strain by transformation. A transformant (host) carrying two copies of the gene (the endogenous and the ectopic) is isolated and crossed with a second strain (mate). During this process, the two copies of target DNA of the host may be affected by mutations leading to gene inactivation at a certain frequency. Thus, mutants can be recovered from progeny of the cross that carry the chromosome containing the endogenous gene from the parental transformant. We currently use pairs of strains that allow the inactivation of essential genes when its chromosomal location is known (Metzenberg and Grotelueschen 1992), even though we are inactivating non-essential genes. Since the strains carry markers on this particular chromosome, it is also possible after the cross to select against ascospore progeny that carry the chromosome derived from the mate, which results in enrichment for mutants (see Duarte et al. 1995 for a more detailed discussion).

We have screened a *N. crassa* genomic library and isolated a recombinant J1 phage that contains the gene encoding the 30.4-kDa subunit of complex I, *nuo-30.4*. In order to map this gene, we used the strains of the Multicent-2 cross kit. These strains represent the progeny of a cross between two strains (Oak Ridge and Mauriceville) that are highly polymorphic. The segregation of restriction fragment length polymorphisms among the progeny of the cross allows gene mapping by comparison with the segregation pattern of known genes. Therefore, we carried out Southern analysis of DNA from the strains, after digestion with different restriction enzymes, using total DNA from the recombinant J1 phage as a probe. We found a polymorphism when using the enzyme *Hin*dIII. The segregation pattern of this polymorphism among the 38 strains of the kit (Fig. 1) was compared with that of genes whose chromosomal location is known (see Metzenberg and Grotelueschen 1995), revealing that *nuo-30.4* is located on chromosome VI, closely linked to the *β*-tubulin gene (*Bml*).

We cloned a 1.9-kb genomic DNA fragment of *N. crassa*, containing the entire *nuo-30.4* gene, in plasmid pCSN44, creating plasmid pNUO30.4 (Fig. 2). This plasmid was then introduced into *N. crassa* Host VI

Strain	A A B B C C D D E E E E F F G G H H I I
	1 4 6 7 1 4 5 7 1 3 5 7 1 3 1 4 5 7 6 8
RF	M – M – M M M M O O M M M M O O O O O O
Strain	J J K K L L M M N N O O P P Q Q R R
	1 4 1 4 1 4 5 8 2 3 2 4 1 4 2 4 1 4
RF	O O O O M M O O O O M M M M O O M –

Fig. 1 Chromosomal mapping of *nuo-30.4*. The Figure shows the segregation pattern of restriction fragment length polymorphisms among the 38 strains of the Multicent-2 cross kit. Restriction fragments (RF) are of the Mauriceville (M) or the Oak Ridge (O) type or unidentified (–)

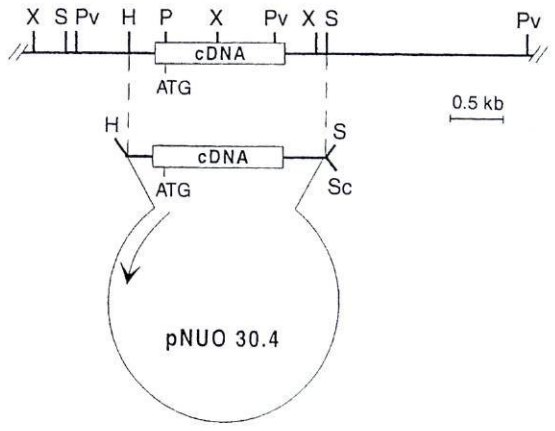

Fig. 2 Construction of plasmid pNUO30.4. Only the Neurospora genomic DNA is drawn to scale. A 1.9-kb genomic DNA fragment with *Hin*dIII and *Sal*I ends, containing the entire *nuo-30.4* gene, was cloned in plasmid pGEM4. This genomic DNA, together with a short *Sal*I-*Sac*I fragment of the pGEM4 multiple cloning site, was excised and cloned into pCSN44, double digested with *Hin*dIII and *Sac*I. The resulting plasmid was named pNUO30.4. The *open box* represents DNA sequences found in cDNA clones. The position of the start codon (ATG) is indicated. The *arrow* indicates the direction of transcription of the hygromycin B resistance gene of plasmid pCSN44. The enzymes shown are: H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*II; Sc, *Sac*I; S, *Sal*I; X, *Xho*I

and individual transformants were analysed by Southern blotting. Five single-copy transformants, named T15, T19, T23, T29 and T30 were retained and further used in genetic crosses. Fig. 3 shows the results of the analysis

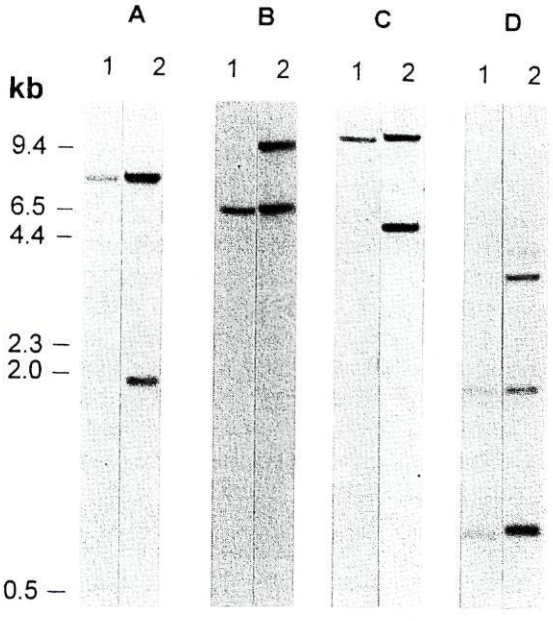

Fig. 3A–D Southern analysis of Neurospora strains transformed with plasmid pNUO30.4. Genomic DNA (2–3 μg) from wild type (lanes 1) and the transformant T29 (lanes 2) was isolated, separately digested with *Bam*HI and *Hin*dIII (**A**), *Eco*RV (**B**), *Hin*dIII (**C**) or *Xho*I (**D**), electrophoresed in agarose gels and blotted onto nylon membranes. The filters were probed with the cDNA coding for the 30.4-kDa protein

performed with T29. When wild-type DNA is double-digested with *Bam*HI and *Hin*dIII, a single band of 6.8 kb is observed. With DNA from T29, an extra band of 1.9 kb appears, indicating that the extra copy of genomic DNA introduced in this strain has remained intact. When wild-type DNA is digested with *Eco*RV and *Hin*dIII, single bands of 5.9 kb and 17 kb, respectively, are observed. With DNA from T29 digested with these enzymes, extra bands of 11 kb and 5.7 kb, respectively, are observed. When wild-type DNA is digested with *Xho*I, we detect two bands of 1.7 kb and 0.75 kb. Transformant T29 displays two extra DNA fragments of 3.7 kb and 0.75 kb, the latter being masked by a segment of the endogenous gene. A 4.3-kb band can also be seen and probably results from partial digestion of the DNA. Altogether, these results indicate that the *N. crassa* genome contains a single copy of *nuo-30.4* that has been duplicated in transformant T29.

In order to cross strains in minimal medium, heterokaryons were produced between the sterile helper strain and each of the single-copy transformants referred to above. These heterokaryons were then separately crossed with strain Mate VI. Random ascospore progeny from the different crosses were selected on media lacking adenine, which selects against those containing the chromosome VI derived from the Mate VI parent. Despite this selection, 174 out of the total of 217 spores recovered were disomics for chromosome VI, as a result of the *mei-2* mutation present in both of their parents (see also Duarte et al. 1995; da Silva et al. 1996), as judged by their ability to grow on medium lacking both pantothenic acid and adenine (chromosomes VI of Host VI and Mate VI are marked with *pan-2* and *ad-1*, respectively). Upon vegetative growth, disomic spores spontaneously break down, giving rise to heterokaryotic mycelia under appropriate conditions (Metzenberg and Grotelueschen 1992). The spores were then individually grown in the presence of pantothenic acid and used for the isolation of mitochondria. In order to identify mutants lacking the 30.4-kDa subunit of complex I, the organelle proteins were analysed by Western blotting using antiserum against the protein. No mutant was detected among 38, 30, 49 and 69 ascospores derived from the crosses involving strains T15, T19, T23 and T30, respectively. Two mutants, M1 and M14, out of 31 ascospores analysed were obtained from the cross between T29 and Mate VI. These mutants were also heterokaryons and the relevant homokaryons were isolated by conidial platings.

Figure 4 shows the results of the Western blotting analysis, using a mixture of different antisera, performed with strain M14, which was further characterised and renamed mutant nuo30.4. It can be seen that, apart from the absence of the 30.4-kDa protein, mitochondria from mutant nuo30.4 contain reduced levels of the 29.9-kDa (van der Pas et al. 1991) and 21.3-kDa (Videira et al. 1990c) subunits of the peripheral arm of complex I, and about normal levels of the 20.9-kDa (Azevedo et al. 1992), 20.8-kDa (Videira et al. 1990a) and 12.3-kDa

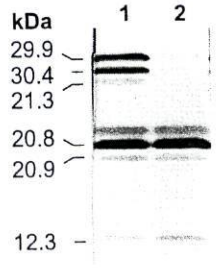

Fig. 4 Western analysis of mitochondrial proteins from mutant nuo30.4. Total mitochondrial proteins (100 μg) from the wild-type strain (lane 1) and mutant nuo30.4 (lane 2) were resolved by SDS gel electrophoresis and blotted onto nitrocellulose. The filters were incubated with a mixture of individual antisera against the indicated subunits of complex I

(Videira et al. 1993) components of the membrane arm of the enzyme. Other subunits of the peripheral arm of complex I, such as the 78-kDa (Preis et al. 1991), 49-kDa (Preis et al. 1990) and 21-kDa (Azevedo et al. 1994) polypeptides, are also less abundant (not shown). Presumably these proteins become more susceptible to degradation due to lack of assembly of the peripheral arm of complex I in mutant nuo30.4 mitochondria (see below).

Complex I assembly in the nuo30.4 strain

The state of assembly of complex I in mutant nuo30.4 was evaluated by gradient centrifugation analysis of Triton X-100-solubilised mitochondria prepared from the strain. Aliquots of fractions of the gradients were assayed for NADH:ferrycianide oxidoreductase activity, a marker for complex I or its peripheral arm alone (Nehls et al. 1992). This material was also analysed by Western blotting, using appropriate antisera, in order to follow the distribution of several complex I subunits (Fig. 5). We employed antisera against subunits that belong either to the peripheral arm or to the membrane arm of the enzyme. In the wild-type strain, most of the NADH:ferrycyanide oxidoreductase activity elutes in fractions 9–11 (Fig. 5A), in agreement with the elution profile of the proteins analysed on the Western blots (Fig. 5B). This material represents normal complex I, which typically migrates about two-thirds of the way down the gradients (Alves and Videira 1994). In the mutant strain nuo30.4, we could not detect significant NADH:ferrycyanide oxidoreductase activity above background in any fraction of the gradient (Fig. 5A). Furthermore, the 29.9-kDa subunit of the peripheral arm of complex I is only detected in fractions 1–3, probably representing a "free" form of the polypeptide (Fig. 5C). A 21.3c-kDa subunit of the peripheral arm of complex I (Duarte et al. 1996) is also detected only in fractions 1–3 (not shown). These results suggest that the mutant is unable to assemble the peripheral arm of complex I. Apparently, the membrane arm of the enzyme is formed in the mutant, as deduced from the

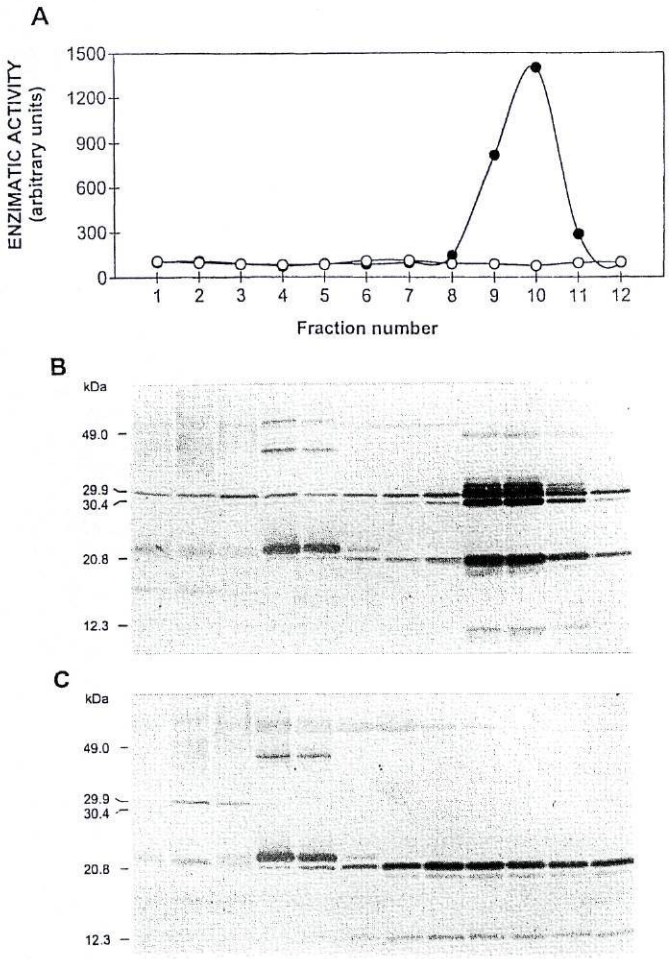

Fig. 5A–C Analysis by sucrose gradient centrifugation of Triton-solubilised mitochondrial proteins from mutant nuo30.4. Mitochondria (2.5 mg protein) were solubilised with Triton X-100 and centrifuged in 12-ml sucrose gradients. Fractions (labelled 1–12 from top to bottom) of the gradients were collected. **A** Aliquots (30 µl) of these fractions were assayed for NADH:ferricyanide reductase activity (*filled circles*, wild type; *open circles*, mutant nuo30.4). Aliquots of the fractions (250 µl) obtained with material from the wild type strain (**B**) and mutant nuo30.4 (**C**) were also resolved by SDS gel electrophoresis and blotted onto nitrocellulose. The filters were incubated with a mixture of individual antisera against the indicated subunits of complex I

elution profile of the 20.8-kDa and 12.3-kDa proteins, which peak in fraction 8, and in agreement with the demonstration that the two arms of complex I undergo independent assembly (Tuschen et al. 1990). The elution profile of the membrane arm is broadened somewhat (Fig. 5C); this presumably arises from some protein aggregation due to the more hydrophobic nature of this domain of complex I.

Mating of different complex I mutants

We have previously noted that mutations in complex I genes have some effect on conidia production by *N. crassa* (Duarte et al. 1995; Harkness et al. 1995).

Table 1 Linear growth rates of complex I mutants

Strain	Growth rate (cm/day)	Growth rate (%)
Wild type	9.02	100
nuo12.3	6.97	77
nuo20.8	6.25	69
nuo21.3	9.02	100
nuo29.9	7.48	83
nuo30.4	6.56	73
nuo78	6.98	77
TRP19	7.46	83
TRP24	9.08	100

Despite this, complex I mutants grow reasonably well during the vegetative phase of the life cycle of the fungus. We examined the linear growth rates of several mutants in race tubes and expressed them as a percentage of the wild-type rates (Table 1). The growth rates of the mutants vary from about 70–100%, as compared with wild type, and cannot be correlated with any particular phenotype reflecting the state of complex I assembly in these strains. The presence of auxotrophic markers in the mutants may have influenced the results. Nevertheless, even a triple complex I mutant, such as TRP24, can grow at wild-type rates. Due to the effect of the individual mutations, namely in the genes *nuo-20.8* (da Silva et al. 1996), *nuo-21.3* (Alves and Videira 1994) and *nuo-29.9* (Duarte et al. 1995), the TRP24 strain is expected to lack both the peripheral and membrane arms of complex I, containing only intermediary subcomplexes of the latter. Thus, severe defects in complex I structure have no concomitant impact on the linear growth rate of *N. crassa*.

In this work, we investigated the possible requirement for functional complex I genes during the sexual phase of the life cycle of *N. crassa*. Heterozygous crosses between different complex I mutants usually work well and double or triple mutants are readily obtained. However, homozygous crosses involving complex I mutants mostly fail to produce ascospores, although formation of perithecia usually takes place. We can conclude that this blockage of the life cycle of the fungus is not due to either male or female sterility of the mutants, because they seem to be able to behave both as male or female parents in crosses with wild-type strains. Furthermore, ascospore progeny are readily obtained from heterozygous crosses involving complex I mutants. Besides this, female-sterile mutants are usually recessive and, in heterokaryons with a helper genotype, they can function in mating, enabling homozygous crosses to be performed (Perkins 1984). We obtained heterokaryons between the sterile helper strain and nuo20.8 mutants. At least in this case, homozygous crosses also failed to sporulate.

Table 2 shows a summary of the main results obtained after crossing different complex I mutants of *N. crassa*. Many of the original complex I mutants also carry other genetic markers leading to particular nutritional requirements. Since the addition of these

Table 2 Production of ascospores in crosses involving complex I mutants

Strain[a]	nuo12.3A	nuo20.8A	nuo21.3A	nuo29.9A	nuo30.4A	nuo78A
nuo12.3a	–	+	+	+	+	nd
nuo20.8a	+	–	+	+	+	+
nuo21.3a	+	+	+	+	+	+
nuo29.9a	+	+	+	+	+	nd
nuo30.4a	+	+	+	+	–	nd
nuo78a	nd	+	+	nd	nd	–

[a] Mutants nuo12.3 and nuo20.8 are also nicotinamide deficient (*nic-2*), mutants nuo29.9 and nuo30.4 are also pantothenic acid deficient (*pan-1* and *pan-2*, respectively). Strains of the A mating type were used as the female parent in the crosses (the reciprocal crosses gave the same results)

substances to the media may inhibit the crossing of strains (Davis and de Serres 1970; Nelson and Metzenberg 1992), the mutants were back crossed with wild-type strains to eliminate most of those additional genetic markers. This backcrossing was also used to obtain strains of the A and a mating types. The resulting progeny were then selected on appropriate media and used for the experiments summarised in Table 2. Homozygous crosses involving mutants nuo12.3, nuo20.8, nuo30.4 and nuo78 failed to produce ascospore progeny. Homozygous crosses involving mutants nuo21.3 and nuo29.9 developed further and produced normal ascospores. To confirm that these spores did not arise from possible contamination of the crossing media with other strains, they were grown and checked for the presence of the particular complex I subunit by Western blotting. We analysed 18 ascospores from the homozygous cross involving the nuo21.3 strains and confirmed that all were mutants lacking the 21.3-kDa subunit of complex I. Likewise, we confirmed that all 51 ascospores isolated from the homozygous cross involving the nuo29.9 strains lacked the 29.9-kDa subunit of complex I.

Discussion

We have described the inactivation of a 30.4-kDa subunit of complex I from *N. crassa*. Lack of this protein prevents formation of the peripheral arm of the enzyme, and only the membrane portion accumulates. The fungal mutant is nevertheless viable. It should be noted that this polypeptide is one of the best conserved among the nucleus-encoded subunits of complex I, having homologues in the minimal NDH-1 enzyme of bacteria and in chloroplasts (Weiss et al. 1991; Walker 1992; Weidner et al. 1993). It is also similar to a subunit of a bacterial formate hydrogenlyase (Videira and Azevedo 1994). Five out of the seven nucleus-encoded proteins that have counterparts in bacterial NDH-1 (known as the bovine 75-kDa, 51-kDa, 24-kDa, TYKY and PSST polypeptides) are iron-sulphur proteins (Walker 1992). So far, the 51-kDa and 24-kDa components have not been found in chloroplasts. The function of the two other proteins (bovine 49 kDa and 30 kDa) is unclear. A preliminary description of a *N. crassa* strain mutant for the 49-kDa protein suggests that it has a nuo30.4-like phenotype in terms of complex I assembly (Schulte and Weiss 1995). The mutants isolated here will facilitate investigation of the role of these conserved proteins.

During the last few years, several complex I mutants of Neurospora have been isolated. These results have given rise to the idea that the enzyme might be dispensable in fungi (Nehls et al. 1992; Duarte et al. 1995; Schneider et al. 1995; Schulte and Weiss 1995; da Silva et al. 1996), in contrast to the importance that complex I appears to have in humans. Recently, it was shown that disruption of NDH-1 genes of *P. denitrificans* may be a lethal event (Finel 1996). In *E. coli*, strains lacking NDH-1 activity have a competitive disadvantage in stationary phase; despite this, they display growth rates similar to wild type during exponential phase (Zambrano and Kolter 1993). We now present evidence that complex I is essential in *N. crassa* for completion of the sexual phase of the life cycle. Homozygous crosses between complex I mutants, with the exception of mutants nuo21.3 and nuo29.9, fail to produce ascospores. The 21.3-kDa protein has been found only in *N. crassa* and apparently has no homology with known proteins (Videira et al. 1990c). The nuo21.3 mutant appears to be able to assemble complex I (without this protein) (Alves and Videira 1994). This may explain the results obtained. It is not so clear why crosses between nuo29.9 mutants produce ascospores. Based on the analysis of mitochondrial proteins from mutant nuo29.9 by sucrose gradient centrifugation, we suggested that the strain is unable to assemble the peripheral arm of complex I (Duarte et al. 1995). However, we also noticed that small amounts of peripheral arm subunits of complex I are present in the high-molecular-weight region of the gradients, and attributed this to unspecific protein aggregation (Duarte et al. 1995). It is therefore possible that some (labile) complex I is assembled in the nuo29.9 mutant. Nevertheless, the viability of the spores was strongly reduced under the standard activation and germination conditions employed. Preliminary results suggest that homozygous crosses involving two novel complex I mutants of *N. crassa* (T. Almeida, unpublished results), disrupted for the iron-sulphur 24-kDa (Azevedo et al. 1994) and 21.3c-kDa subunits (Duarte et al. 1996), are also blocked in the production of ascospores.

The life cycle of *N. crassa* includes an asexual phase, which results in the production of (macro- and micro-) conidia, and a sexual phase that results in the production of ascospores. The germination of conidia seems to depend upon respiratory chain activity (Stade and Brambl 1981), and a concomitant increase in NADH and NADPH content (Schmit and Brody 1976) suggests a specific role for complex I during this process. In sexual sporulation, many mutations interfere with the normal development of perithecia, asci or ascospores (Nelson and Metzenberg 1992; Raju 1992). Recently, it was found that mutations in the *car1* gene, the homologue of the mammalian gene that is defective in individuals with Zellweger syndrome, affected the sexual development of another ascomycete, *Podospora anserina* (Berteaux-Lecellier et al. 1995). Homozygous crosses of *car1* mutants do not sporulate because karyogamy is prevented. The protein is a component of peroxisomes and the mutants seem to lack these cellular organelles (Berteaux-Lecellier et al. 1995). In our case, the complex I mutants have apparently intact mitochondria, suggesting that the block in sporulation is due to the complex I deficiency. Electron microscopic analysis of mycelia did not reveal any structural alteration in mitochondria from mutant nuo20.8 (da Silva et al. 1996) or from mutants nuo78, nuo29.9 and nuo12.3 (R. Sousa, unpublished results). One possibility that might explain why homozygous crosses between complex I mutants fail to sporulate, is that some unknown function of the enzyme is needed for this complicated process. This would presumably require a full complex I, since the individual mutants have quite dissimilar phenotypes. They appear to assemble either only the membrane arm (nuo30.4 and nuo78) or only the peripheral arm and subcomplexes of the membrane arm of complex I (nuo20.8). In another case, both arms of the enzyme appear to be formed but are not combined to form the mature complex (nuo12.3). Another hypothesis to explain the block in sporulation is that the key factor is a relative lack of energy, since complex I contributes about one-third of the total energy produced by the mitochondrial respiratory chain. Defects in mitochondrial activities are known to be responsible for cytoplasmic male sterility in plants. It has been suggested that a threshold level of cytochrome oxidase activity is required for proper anther and pollen development in maize (Conley and Hanson 1995). Furthermore, the development of pathogenic human conditions has been attributed to a decrease in the capacity for oxidative phosphorylation below organ-specific energetic thresholds (Wallace 1992). The production and use of energy is probably more tightly controlled during the sexual phase than during the vegetative phase of fungal growth. It will be interesting to investigate if sexual development is also impaired in homozygous crosses involving mutants defective in other components of the respiratory chain.

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Respiratory chain complex I is essential for sexual development in Neurospora and binding of iron-sulphur clusters are required for enzyme assembly

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Summary

We have cloned and disrupted *in vivo*, by repeat-induced point mutations, the nuclear gene coding for an iron-sulphur subunit of complex I from *Neurospora crassa*, homologue of the mammalian TYKY protein. Analysis of the mutant nuo21.3c obtained revealed that complex I fails to assemble. The peripheral arm of the enzyme is totally disrupted while its membrane arm accumulates. Furthermore, mutated 21.3c kDa 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-sulphur 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.

Introduction

The life cycle of *N. crassa*, a heterothallic filamentous fungus, includes a vegetative and a sexual phase. The latter is initiated when a protoperithecium of one mating type is fertilised by a male cell of the other mating type. Fertilised 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 more than 30 polypeptide subunits of both nuclear and mitochondrial origin, as well as FMN and several iron-sulphur 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; Videira, 1998; Walker, 1992; Weiss *et al.*, 1991). 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).

The expression of complex I might vary in different stages of the life cycle of an organism, like 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 (Gutierres *et al.*, 1997; Marienfeld and Newton, 1994; Pla *et al.*, 1995). Furthermore, deficiencies in complex I activity appear to be responsible for the development of several pathogenic human conditions (Schapira, 1998; Wallace, 1992).

As an approach to understand the relevance of 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 kDa 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 kDa protein in *N. crassa* (the TYKY homologous subunit) and the characterisation 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 kDa iron-sulphur 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*.

Results

The RIP mutant nuo21.3c is defective in complex I assembly

A recombinant lambda J1 phage, containing the nuclear gene encoding the 21.3c kDa subunit of the peripheral arm of complex I from *N. crassa* was isolated and characterised. Fig. 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 twelve of them were used to prepare mitochondria. An analysis of the mitochondrial proteins by western blotting lead to the identification of the mutant strain nuo21.3c that specifically lacks the 21.3c kDa polypeptide (Fig. 2). Similar experiments using antisera against other subunits of complex I, revealed that all proteins analysed 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 kDa subunit (Videira *et al.*, 1990b) is reduced while roughly normal levels of the 12.3 kDa polypeptide (Videira *et al.*, 1993), a membrane arm component of the enzyme, were detected (Fig. 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 were followed. Antisera against the 12.3 kDa and 20.8 kDa (Videira *et al.*, 1990a) polypeptides as markers for the membrane arm, and against subunits 21.3c kDa (Duarte *et al.*, 1996) and 30.4 kDa as markers for the peripheral arm of complex I were used in this experiment (Fig. 3). In wild type strain the reductase activity elutes in fractions 9 and

10 of the gradient (Fig. 3A), in agreement with the elution profile of the complex I proteins (Fig. 3B). When mitochondria from the mutant nuo21.3c are analysed, 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 kDa subunit elutes in fractions 3 to 5 (Fig. 3C), representing 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 kDa and 20.8 kDa proteins is somewhat broadened (Fig. 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 kDa and 20.8 kDa polypeptides throughout the gradient. These results are also obtained in another complex I mutant where the peripheral arm of the enzyme is disrupted (Duarte *et al.*, 1998).

Protein binding of Fe-S clusters are required for complex I assembly

As with homologues in mammalian and bacterial enzymes, the *N. crassa* 21.3c kDa protein includes two sequence motives 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-sulphur 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 nuo21.3cRIP 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 kDa 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 analysed by sucrose gradient centrifugation (as described above) revealing that the wild type phenotype was rescued (Fig. 4A). Nonetheless, some of the complex I subunits were detected in the top of the gradient, suggesting that they are being produced in higher amounts than the 21.3c kDa protein. 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 (Fig. 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 (Fig. 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 we could 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 kDa 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 that it produces less conidia than the wild type 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). We now 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; unpublished data), were infertile. The same result was obtained with the complex I mutant nuo78 (Harkness *et al.*, 1995) which, as nuo21.3c, completely lacks the peripheral arm of the enzyme.

Interestingly, homozygous crosses between mutants lacking a 21 kDa protein (Azevedo *et al.*, 1994) 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 (unpublished data). This suggests that a failure in energy production by complex I might be the cause for the impairment of sexual development in homozygous crosses.

Homozygous crosses were barren in the early stages of the differentiation of ascogenous tissue, before karyogamy. Actually, the formation of croziers and young asci could not be observed (N. B. Raju, personal communication). Spore production could not be rescued in homozygous crosses of complex I mutants, where one or both parents were present as forced heterokaryons with the sterile helper strain. It should be noticed that these heterokaryons are expected to have a wild type complex I, due to complementation of any mutation with the nuclei derived from the sterile helper strain, but this nuclei do not participate in the cross. Cytological observations revealed that sexual development was partially rescued and proceeded to different phases of the process, after karyogamy (N. B. Raju, personal communication), likely due to the initial presence of mitochondria with intact complex I in the heterokaryotic cells. These results suggest that continued expression and presence of a functional complex I is needed to completion of the sexual cycle of *N. crassa*.

All evidence already indicated that the inability of homozygous crosses to complete sexual development is associated with the absence of a functional complex I. However, it was still possible that the phenotype arose as a co-lateral effect of the RIP phenomenon used to generate mutants, *e.g.*, ectopic gene copies could integrate inside and disrupt an essential gene or the RIP mutations could paste the desired genes and also affect nearby essential genes. In order to exclude these possibilities, two complex I mutants created by homologous recombination, nuo51 and nuo78, were included in our experiments. Mutant strains of opposite mating types were obtained by back-crossing with wild type strains. Homozygous crosses between nuo51 strains or between nuo78 strains also failed to sporulate. 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.

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, twenty 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 observed ascospores in any of these mates.

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. The cDNA coding for each of the proteins was expressed in the correspondent mutant strain and the complex I assembly was restored (not shown). Crosses between these strains and the respective mutants again failed to produce spores. Furthermore, we mated two nuo20.8 mutant strains, both expressing the cDNA coding for the 20.8 kDa 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).

In order to avoid these problems we cloned the genomic DNA fragment PvH in pCSN44 (Fig. 1). This fragment contains the coding region and a flanking sequence of about 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. Fig. 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 (Fig. 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 (Fig. 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 Fig. 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 (Fig. 5A, lane 4). The *Eco*RV sites within both copies of the gene have also been modified as shown in Fig. 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 (Fig. 5A, lanes 2 and 3), consistent with the introduction of ectopic DNA.

Among the 43 crosses, seven 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 kDa subunit was detected after immunodecoration of mitochondrial proteins, indicating the requirement for gene expression. In addition, random progeny from the cross G14 X nuo21.3c was also analysed by western blot. Fig. 6 shows that protein expression segregated in 12 out of 17 spores analysed and 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.

Discussion

The 21.3c kDa iron-sulphur subunit of complex I is highly conserved among different species, from bacteria to man, 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 kDa 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-sulphur clusters, strongly suggests that binding of the prosthetic groups occurs before and is required for this process.

The biogenesis and redox properties of membrane complexes containing bound iron-sulphur clusters are dependent upon the associated protein structure, which can be modified by incorporation of mutations to ligands of the prosthetic groups. The substitutions of cysteines with alanine residues, which contain an aliphatic side group, are not capable of providing ligands 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. Our *in vivo* mutants in which cysteines 123 and 162 were altered to alanine or serine and cysteine 165 was modified to serine give rise to altered forms of the 21.3c kDa protein that do not associate with complex I. The implication is that the

21.3c kDa subunit containing cysteine to alanine substitutions has a structure sufficiently different from that of the wild type protein to preclude its assembly. Although the serine substitutions are capable of supporting mixed ligand [4Fe-4S] clusters, we found that the majority of serine mutants are also devoid of complex I. It is likely that the C123S, C162S and C165S proteins also have quite altered structures, suggesting that serine is not an efficient ligand to the (appropriate) iron-sulphur centers. As an exception, the C162S substitution may lead to a low level of incorporation of the 21.3c kDa protein in complex I and, though inefficiently, the serine residue can replace cysteine as a [4Fe-4S] cluster ligand in this case.

According to our results, a stable three-dimensional structure of the 21.3c kDa protein, including the presence of two tetranuclear iron sulphur clusters, is necessary for complex I assembly in *N. crassa*. A careful assignment of any specific cluster to this protein was not possible with the isolated mutants. Different results were obtained when analogous modifications were performed in the NuoI homologues of the 21.3c kDa 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* based on 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 cyanobacterium (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 less proteins than its mitochondrial homologue, or the conformation of the mutated bacterial proteins is not significantly changed. On the other hand, the 21.3c kDa polypeptide displays a marked similarity with ferredoxin-like proteins that bear two [4Fe-4S] cluster binding motives (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 kDa protein. Preliminary work with the 24 kDa iron-sulphur subunit of complex I also indicate that alanine modifications prevent the assembly of the protein (unpublished data).

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. 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 induced 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 mutants that assemble an almost intact enzyme without reductase activity, such as nuo51 and nuo24. In contrast, homozygous crosses of mutants that assemble an almost intact enzyme and display rotenone-sensitive oxidation of NADH, such as nuo21 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 the normal cellular development, including chloroplast maturation and pollen development (Heiser *et al.*, 1997; Marienfeld and Newton, 1994). 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), opens the possibility of using the fungus as an excellent eukaryotic model to study human mitochondrial disease.

Experimental procedures

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*; FGSC#7256), designed for the disruption of essential genes located on linkage group VI (Metzenberg and Grotelueschen, 1992), were obtained from the Fungal Genetics Stock Center. In this work, 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 RIP mutants nuo20.8 (da Silva *et al.*, 1996), nuo30.4 (Duarte *et al.*, 1998), nuo21 (Azevedo *et al.*, 1994; unpublished data) and nuo24 (Azevedo *et al.*, 1994; unpublished data) 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 kDa and 30.4 kDa 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, 10mM 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 hybridisation with the corresponding cDNA. An *Eco*RI fragment of genomic DNA (about 7 kb) containing the entire coding region of *nuo-21.3c*, as well as a 2.2 kb *Pst*I fragment were subcloned in pGEM4. The later 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 Fig. 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 (Fig. 1). The cDNA coding for the 21.3c kDa 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 analysed 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#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 was germinated and their mitochondrial proteins

were analysed by western blotting leading to the identification of mutant nuo21.3c which lacks the 21.3c kDa protein.

Protein analysis

The techniques for the preparation of *N. crassa* mithocondria (Werner, 1977), protein determination (Bradford, 1976), SDS-polyacrylamide gel electrophoresis (Zauner *et al.*, 1985), western blotting (Blake *et al.*, 1984; Towbin *et al.*, 1979), 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.

Site-directed mutagenesis

Site-directed mutagenesis was achieved using the Quik Change™ Site-Directed Mutagenesis kit according to the manufacturer procedures (Stratagene). Briefly, the full-length cDNA coding for the 21.3c kDa 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:

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 amplifying the C123A altered plasmid with primers C162A. The mutagenesis was confirmed by complete sequencing (Duarte *et al.*, 1996) of all cDNA constructs. The mutated plasmids were transformed into the nuo21.3c mutant.

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Figure Legends

Fig. 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: 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.

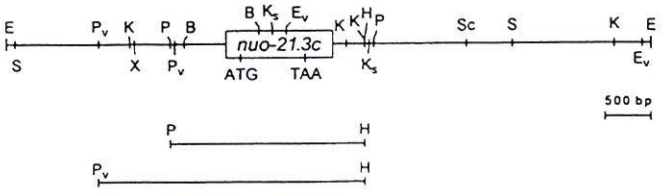
Fig. 2. Identification of mutant nuo21.3c.Electrophoretically resolved mitochondrial proteins from the wild type strain (lane 1) and mutant nuo21.3c (lane 2) were analysed by western blotting with antisera against the 21.3c kDa and 12.3 kDa subunits of complex I.

Fig. 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 (labelled 1-12 from top to bottom) were collected and assayed for NADH:ferricyanide oxidoreductase activity (panel A: closed 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 in the left side of the figure.

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

Fig. 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.

Fig. 6. The 21.3c kDa protein is expressed in the progeny of the cross between G14 and nuo21.3c. Mitochondrial proteins from 17 strains obtained from the progeny of the cross G14xnuo21.3c were separated by SDS PAGE and blotted onto a nitrocellulose membrane. The filter was immunodecorated with an antiserum directed against the 21.3c kDa polypeptide.


E
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Pv
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X
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B
Ks
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nuo-21.3c
ATG
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FIGURE 1

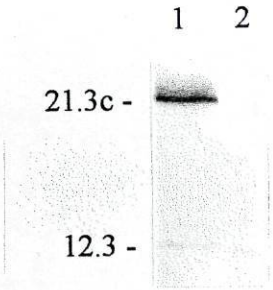
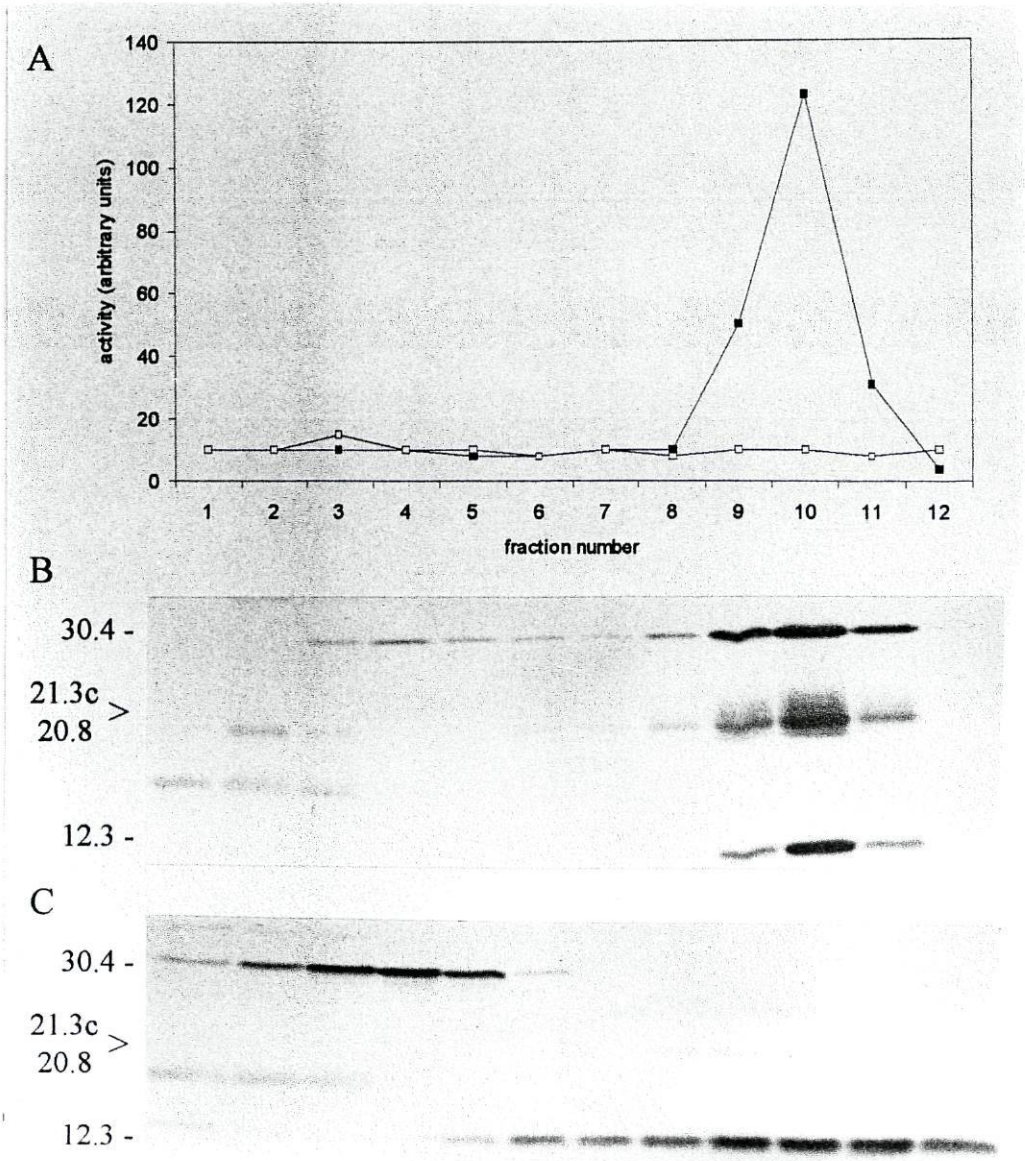


FIGURE 2


A
140
120
100
80
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40
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activity (arbitrary units)
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3
4
5
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7
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10
11
12
fraction number
B
30.4 -
21.3c
20.8
12.3 -
C
30.4 -
21.3c
20.8
12.3 -

FIGURE 3

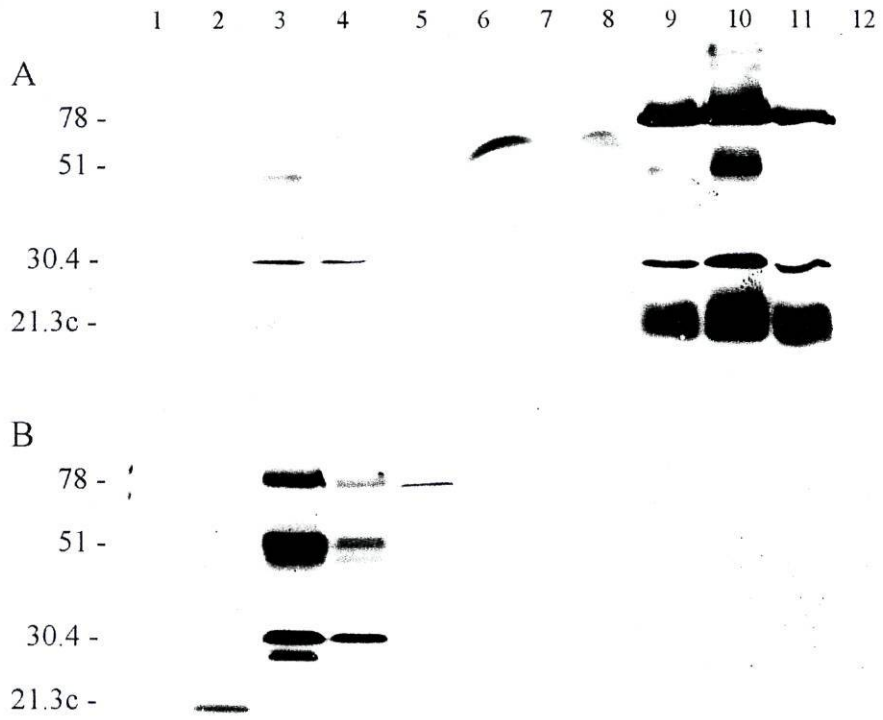

FIGURE 4

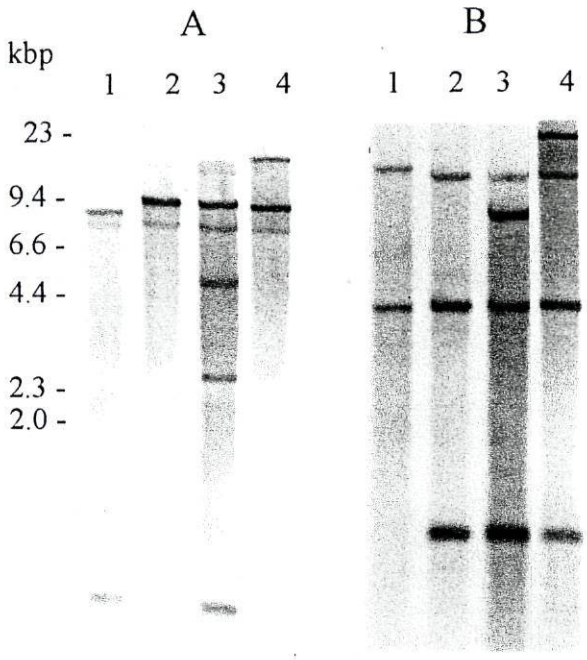

FIGURE 5

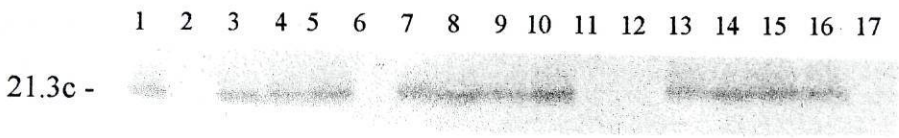

FIGURE 6

4.7-TRABALHO 7

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The 24-kDa iron–sulphur subunit of complex I is required for enzyme activity

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We have cloned the nuclear gene encoding the 24-kDa iron–sulphur subunit of complex I from *Neurospora crassa*. The gene was inactivated *in vivo* by repeat-induced point-mutations, and mutant strains lacking the 24-kDa protein were isolated. Mutant nuo24 appears to assemble an almost intact complex I only lacking the 24-kDa subunit. However, we also found reduced levels of the NADH-binding, 51-kDa subunit of the enzyme. Surprisingly, the complex I from the nuo24 strain lacks NADH:ferricyanide reductase activity. In agreement with this, the respiration of intact mitochondria or mitochondrial membranes from the mutant strain is insensitive to rotenone inhibition. These results suggest that the nuo24 complex is not functioning in electron transfer and the 24-kDa protein is absolutely required for complex I activity. This phenotype may explain the findings that the 24-kDa iron–sulphur protein is reduced or absent in human mitochondrial diseases. In addition, selected substitutions of cysteine to alanine residues in the 24-kDa protein suggest that binding of the iron–sulphur centre is a requisite for protein assembly.

Keywords: *Neurospora crassa*; mitochondria; complex I; gene disruption; mutagenesis.

The proton-pumping NADH dehydrogenase of the mitochondrial respiratory chain, or complex I (EC 1.6.5.3), catalyses the oxidation of NADH and the reduction of ubiquinone. This electron transfer reaction is coupled with proton translocation across the inner membrane of the organelle, thus leading to energy conservation. Enzymes equivalent to complex I, but with fewer protein constituents, have been identified in several prokaryotic organisms. These bacterial structures are composed of homologues of the seven mtDNA encoded polypeptides and homologues of seven nuclear encoded proteins of complex I from fungi or mammals. In terms of prosthetic group composition, the eukaryotic and homologous bacterial enzymes are very similar, containing FMN and about six iron–sulphur clusters. On the other hand, mitochondrial complex I contains around 40 polypeptide subunits and a particular function has not been ascribed for most of them (reviewed [1–5]).

Besides its important bioenergetic role, or because of this, deficiencies in complex I have been associated with several human mitochondrial diseases [6–8], thus raising the interest in the enzyme during the past years. A better knowledge on the structure and function of complex I and on the contribution of particular proteins to the biogenesis of the enzyme is needed to understand in more detail the mechanisms underlying disease. As an approach to study complex I, we are inactivating individual genes and investigating the phenotype of the resulting null-mutants, using *Neurospora crassa* as a model system. The fungal enzyme is quite similar to that of mammals and the fungus is presently the only eukaryotic organism where the disruption of complex I genes can be routinely performed [4,9].

A specially interesting subunit of complex I is the 24-kDa protein, encoded by the nuclear DNA, which is part of the flavoprotein fragment of the enzyme [3,10]. It is a well conserved protein, from bacteria to mammals, and contains four strictly conserved cysteine residues for the binding of an iron–sulphur cluster of the binuclear type [10], either N-1b [11] or more likely N-1a [12,13]. However, the role of the 24-kDa protein in electron transfer has not been clarified. On the other hand, recent work provided evidence that the 24-kDa polypeptide is a G protein [14]. In addition, studies on several human diseases with associated complex I defects revealed the absence or a particular reduction in the levels of the 24-kDa protein as compared to controls [15–18]. The protein is synthesized in the cytoplasm of *N. crassa* as an extended precursor polypeptide [19]. In this article, we describe the inactivation and site-directed mutagenesis of the gene coding for the 24-kDa protein and report on the characterization of the mutants obtained. We found that the protein is required for complex I activity and this phenotype may account for the symptoms found in mitochondrial disorders.

EXPERIMENTAL PROCEDURES

N. crassa strains and media

We used the wild type strain 74-OR23-1A and a strain carrying auxotrophic markers on linkage group V (FGSC #4455). The media and general procedures for manipulation of the fungus were done as described [20,21]. Spheroplasts prepared from 7-day-old wild type conidia were transformed with the recombinant plasmid pCSN-24SB that contains the *nuo-24* gene. The transformants were selected in plates containing hygromycin B (100 $\mu g \cdot mL^{-1}$) and purified by

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Abbreviations: SMP, submitochondrial particles.

Enzymes: NADH dehydrogenase (EC 1.6.5.3); cytochrome *c* oxidase (EC 1.9.3.1); malate dehydrogenase (EC 1.1.1.37).

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successive asexual transfers in slants of Vogel's minimal media containing 50 $\mu g \cdot mL^{-1}$ of the drug [22,23]. Genomic DNA of individual strains was isolated from 20 mL mycelial cultures [24] and analysed by Southern blotting (using selected restriction enzymes and the cDNA encoding the 24-kDa protein [19] as a probe), in order to identify single-copy transformants. These strains were separately crossed with strain FGSC #4455. The progeny of the genetic crosses were selected on minimal media, in order to enrich for mutants (see Results for the rationale), and used for the isolation of mitochondria. The mutant strain nuo24 was identified by immunoblotting analysis of the organelle proteins with antiserum against the 24-kDa subunit of complex I (see below).

Molecular cloning

We employed standard cloning techniques [25,26] and the plasmid vectors pGEM4 [27] and pCSN44 [28] for subcloning and fungal transformation, respectively. The *nuo-24* gene was isolated from an *N. crassa* genomic library in phage J1 (obtained from the Fungal Genetics Stock Center) by hybridization screening with cDNA coding for the 24-kDa protein. A *Sal*I fragment of genomic DNA (about 3 kb), containing the entire coding region of *nuo-24*, was then subcloned in pGEM4. A recombinant plasmid, containing the genomic DNA in the appropriate orientation, was digested with *Bam*HI, treated with Klenow to create blunt ends, and digested with *Hin*dIII (which cuts in the polylinker of pGEM4). The resulting DNA fragment, which contained the *Hin*dIII/*Sal*I region of the polylinker of pGEM4 connected to the *Sal*I/*Bam*HI (blunted) fragment of genomic DNA (about 2 kb), was isolated and ligated into pCSN44 previously digested with both *Hin*dIII and *Eco*RV, generating plasmid pCSN-24SB (see also Fig. 1).

Site-directed mutagenesis

Site-directed mutagenesis was performed in the cDNA encoding the 24-kDa protein with the QuickChange Site-Directed Mutagenesis Kit (Stratagene). The primers 5′-TTCAGGCTGCCACTACCACC-3′ and 5′-CCACCCCTGCCCAACTAGG-3′ (and their complementary primers) were used to achieve the C143A and C148A replacements, respectively (the underlined bases were modified from the cDNA sequence). A double C143A/C148A mutant gene was obtained by mutagenizing the C143A construct with the C148A primers. The alterations were

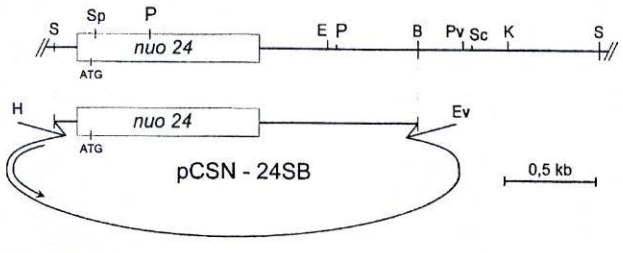

Fig. 1. Restriction map of an *N. crassa* genomic DNA piece containing the nuo-24 gene and construction of plasmid pCSN-24SB. The enzymes shown are as follows: B, *Bam*HI; E, *Eco*RI; Ev, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I; Sc, *Sac*I; Sp, *Sph*I. Plasmid pCSN-24SB, which contains *nuo-24*, was constructed by ligation of the depicted *Sal*I/*Bam*HI fragment of genomic DNA (together with the *Hin*dIII/*Sal*I region of the polylinker of pGEM4) into plasmid pCSN44 (see Experimental procedures for details). The arrow inside the construct indicates the direction of transcription of the *hyg*B^r gene of pCSN44.

confirmed by complete DNA sequencing of all cDNA constructs. The altered cDNAs were then cloned in the *Sma*I site of plasmid pMYX2, downstream of an inducible quinic acid promoter [29], and used to transform the nuo24 mutant.

Production of antibodies

The cDNA encoding the 24-kDa protein, previously cloned in plasmid pGEM4 [19], was amplified by PCR, cloned in the *Eco*RI site of expression vector pGEX-5X-1 (Pharmacia) and transformed into *Escherichia coli* BL21. We used primer 5′-CCGGAATTCGCGTATCAAGCA GC-3′, that hybridizes immediately upstream of the initial ATG codon and introduces an *Eco*RI site (underlined sequence), and the vector-specific T7 primer. The PCR product will thus include another *Eco*RI sequence downstream of the cDNA coding sequence, from the polylinker of the vector. A crude preparation of the protein was obtained from disrupted bacterial cells, previously induced for expression as described by the manufacturer, and was further purified by SDS gel electrophoresis due to lack of solubility of the protein in conditions compatible with affinity chromatography. A partial cDNA encoding the 51-kDa protein was amplified by PCR from a λgt11 expression library [26], double-digested with *Sph*I and *Nsi*I, cloned in the expression vector pQE-31 (Qiagen) digested with *Sph*I and *Sal*I, and transformed into *Escherichia coli* M15. We designed the primers 5′-CGG-CCGTTACCCGCCCGAC-3′ and 5′-GTTCCATTATTCACTT-GAAAACC-3′, hybridizing just upstream of a *Sph*I site and downstream of a *Nsi*I site of the cDNA sequence, respectively [30], which leads to the expression of the C-terminal 430 amino acid residues of the protein including its predicted highest antigenic determinants. Expression and purification of the protein under denaturing conditions was performed as described by the manufacturer. Rabbit immunization and collection of antisera against the 24-kDa protein, the 51-kDa protein and other subunits of complex I was carried out as before [31].

Protein analysis and enzymatic activities

The techniques used for the preparation of crude mitochondria [32], protein determination [33], SDS/PAGE [32,34], blotting and incubation of blots with antisera [31,35], detection of alkaline phosphatase-conjugated second antibodies [36], sucrose gradient centrifugation analysis of detergent-solubilized mitochondrial proteins [37], NADH: ferricyanide reductase activity [38] and NADH:hexaammineruthenium(III) reductase activity [39] have been published before. Determination of the latter was done with a 2-mM concentration of hexaammineruthenium in the same reaction media used in the NADH:ferricyanide assay.

Oxygen consumption

The procedures for the purification of mitochondria on Percoll gradients [40] and the preparation of inside-out submitochondrial particles (SMP) were given [41]. To assess the quality of the preparations, the activities of cytochrome *c* oxidase (EC 1.9.3.1) and malate dehydrogenase (EC 1.1.1.37) were measured in the presence and in the absence of Triton X-100 [42] to calculate the latent activities of the enzymes. Oxygen consumption at 25°C was measured polarographically in a Clark-type oxygen electrode (Hansatech). The SMP assays were started with the addition of 1 mM NADH to the reaction medium (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, 8 μM CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) and 0.02% of bovine serum albumin). The assays with mitochondria were started with 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 μM and 0.2 $\mu g \cdot mL^{-1}$, respectively.

RESULTS

Isolation of a nuo24 mutant strain

Gene inactivation can be achieved *in vivo* in *N. crassa*, taking advantage of a unique phenomenon, that results in the generation of repeat-induced point-mutations in the target gene and is called RIP [43]. Briefly, a (transformed) host strain carrying a duplication of the target gene (endogenous and ectopic copies) is crossed with a mate strain. During this process, the duplicated genes of the host DNA may be inactivated by RIP and the correspondent mutants can be isolated from the progeny of the cross. Knowing the chromosomal location of the gene, it is possible to enrich for mutant progeny by using a mate strain carrying genetic markers on this chromosome (e.g. auxotrophic markers) and selecting against them (e.g. omitting the substances from the media), because the mutants will carry the chromosome derived from the host strain (see [23] for a more detailed discussion).

The 24 kDa iron–sulphur subunit of complex I from *N. crassa* is encoded by a single-copy gene located on linkage group V of the fungal genome [44]. We isolated a genomic clone and further characterized about 3 kb of DNA that contains the entire coding sequence of the gene (Fig. 1). A smaller DNA fragment of about 2 kb was cloned in pCSN44 and the resulting pCSN-24SB plasmid was introduced in wild type *N. crassa* by transformation. We isolated and analysed the DNA from several individual transformants by Southern blotting, using selected restriction enzymes and the relevant cDNA as a probe, in order to distinguish single-copy transformants. A transformant called T6, carrying a single and complete extra-copy of the *nuo-24* gene, was identified (not shown) and then crossed with strain FGSC #4455, which has the mutant alleles *lys-1* and *inl* in linkage group V. Late ejected ascospores (in which RIP should be more efficient than in early spores [45]) were selected on growth media lacking lysine and inositol. Mitochondria were isolated from the individual strains and analysed by Western blotting with an antiserum against the 24-kDa protein. Absence of the polypeptide was found in three out of 40 strains analysed. One of the mutants was used in further experiments and named mutant nuo24 (Fig. 2). Several other subunits of complex I are present in the mutant mitochondria, although at slightly different stoichiometries as compared to the wild type strain. However, an exceptionally significant reduction in the levels of the 51-kDa subunit was observed (Fig. 2).

Complex I assembly and function in nuo24

We wanted now to know whether the different subunits of complex I, present in the mutant mitochondria, were assembled into the enzyme. Therefore, we analysed the mobility of solubilized mitochondrial complexes in sucrose gradients. Mitochondria from the wild type and mutant strains were isolated, solubilized with the nondenaturing detergent Triton, and centrifuged in 12 mL sucrose gradients. The gradients were fractionated into 1 mL portions and aliquots were analysed for

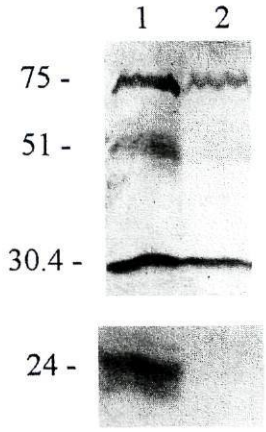

Fig. 2. Western blot analysis of mitochondrial proteins. Total mitochondrial proteins (100 μg) from the wild type strain (lane 1) and nuo24 (lane 2) were resolved by SDS gel electrophoresis and blotted onto nitrocellulose membranes. The membranes were immunodecorated with a mixture of antisera against different subunits of complex I or an antiserum against the 24-kDa protein, as indicated on the left side of the figure.

the presence of complex I subunits and for enzyme activity. We measured the NADH:ferricyanide reductase activity, an artificial electron transfer process that is characteristic of complex I or just the peripheral domain of the enzyme [46]. To see the distribution of complex I proteins in the gradient, we used a Western blotting technique with antisera recognizing specific subunits of both the peripheral and membrane arms of the enzyme. These two domains of complex I are formed independently of each other and often fall apart as a result of mutations [4,9]. The results of these experiments are shown in Fig. 3. As expected, most of the wild type complex I can be recovered in fractions 9 and 10, as deduced from the elution profile of the NADH:ferricyanide reductase activity (Fig. 3A). In agreement with this, the 75, 51, and 30.4 kDa subunits of the peripheral arm and the 20.8 and 12.3 kDa subunits of the membrane arm of complex I (references in [4]) also elute mainly in these two fractions (Fig. 3B). Regarding the nuo24 mutant, the distribution of the same polypeptides also displays a peak in fraction 9, indicating that an almost intact complex I is formed in this strain (Fig. 3C). A severe reduction of the 51-kDa protein in fractions 9 and 10 of the mutant mitochondria can be observed. As compared to wild type, we also observe larger amounts of different complex I subunits in the low molecular mass region of the gradients, suggesting that their assembly into the nuo24 enzyme is not as efficient. Surprisingly, we did not detect significant NADH:ferricyanide reductase activity in any fraction of the gradients from mutant nuo24, suggesting that the 24-kDa subunit is essential for this complex I activity. An analysis with another artificial electron acceptor, hexaammineruthenium, which receives electrons from a site different from that of ferricyanide [39], gave again no indication of NADH oxidation activity in the nuo24 gradients (not shown). In agreement with this, we found that the levels of either of these two activities measured directly in mitochondrial membranes or Triton-solubilized mitochondria from nuo24 represent less that 20% of the wild type levels. These values are within the range found with other mutants of complex I that fail to assemble the peripheral arm of the enzyme, suggesting also that these activities can be used as a good assessment of complex I function in *N. crassa* when performed directly with mitochondrial membranes or Triton-solubilized mitochondrial complexes (unpublished data). These oxidoreductase activities

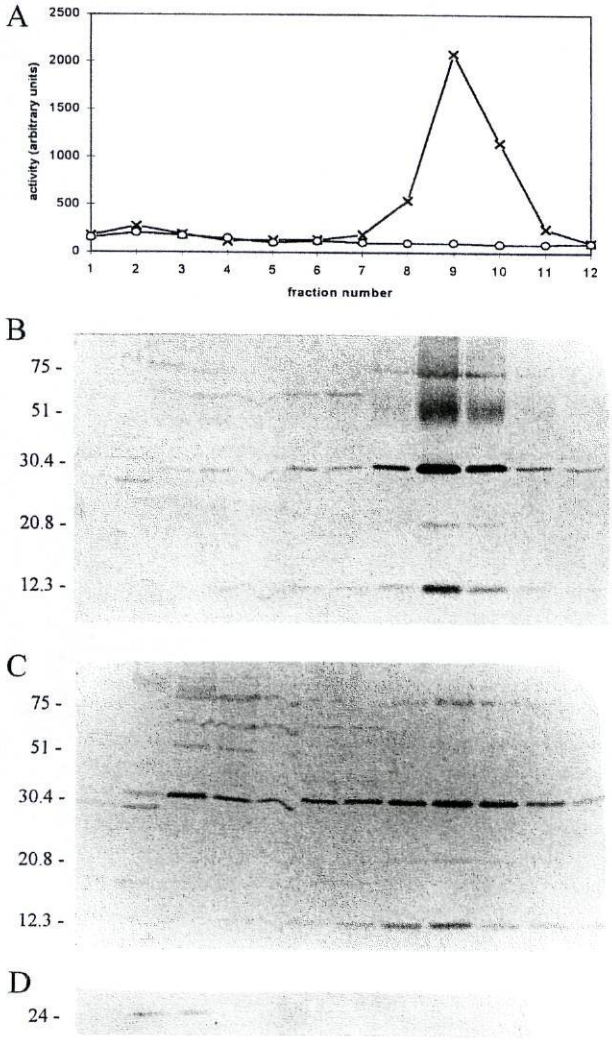

Fig. 3. Sucrose gradient centrifugation analysis of Triton-solubilized mitochondrial proteins. Mitochondria (5 mg) were isolated, solubilized with Triton X-100 and centrifuged in sucrose gradients. Fractions of the gradients (numbered 1–12 from top to bottom) were collected and assayed for NADH:ferricyanide oxidoreductase activity (panel A: ×, wild type; ○, mutant nuo24). Aliquots of the gradient fractions were also precipitated with trichloroacetic acid, resolved by SDS gel electrophoresis and blotted onto nitrocellulose. The membranes were immunodecorated with antisera against different subunits of complex I, as indicated on the left side of panel B (wild type strain), panel C (mutant nuo24) and panel D (mutant C148A).

do not require the participation of most redox centres of complex I [47].

Electrons could still be accepted from NADH by the nuo24 complex, used to reduce other iron–sulphur centres of the enzyme and passed on to ubiquinone and the rest of the respiratory chain. To investigate this issue, we analysed the rotenone-sensitive respiration of intact mitochondria or inside-out SMP obtained from wild type and nuo24 strains. To assess the quality of the preparations, we determined the latent activities of marker enzymes. Purified mitochondria from wild type and nuo24 displayed about 95% latent activity for cytochrome *c* oxidase and 88% and 90%, respectively, latent activity for malate dehydrogenase. The SMP preparations from wild type and nuo24 showed 89% and 86%, respectively, latent activity for cytochrome *c* oxidase and 17% and 12%, respectively, latent activity for malate dehydrogenase. Figure 4 illustrates the results obtained with this kind of experiment. The

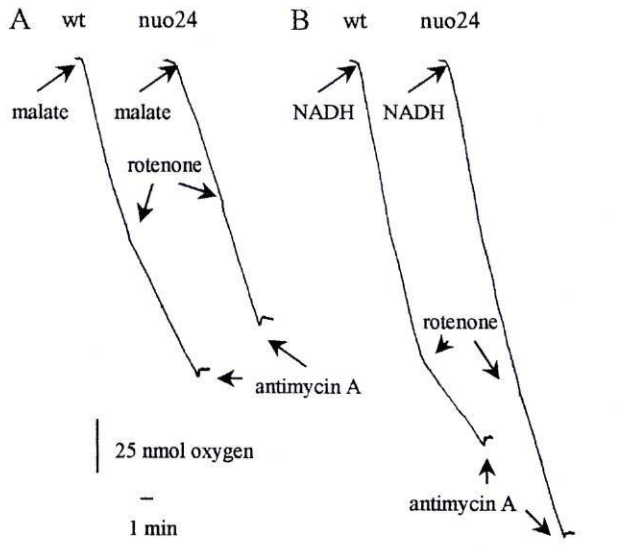

Fig. 4. Illustration of O_2 consumption experiments. The figure depicts polarographic traces of respiration performed by mitochondria (A) or SMP (B), obtained from the wild type and nuo24 strains. The start of the reactions with either malate or NADH and the addition of respiratory chain inhibitors are indicated.

oxygen uptake of wild type mitochondria respiring on pyruvate/malate could be inhibited by about 40% with the complex I inhibitor rotenone (see also Table 1). In conformity with this, 47% of the oxygen consumption of wild type SMP respiring directly on NADH as substrate could be inhibited by rotenone. In sharp contrast, similar experiments performed with mitochondria or SMP derived from mutant nuo24 showed that respiration of this strain is resistant to rotenone inhibition (Fig. 4 and Table 1). Antimycin A completely inhibits the respiratory activities of both the wild type and nuo24 strains. Taken together, these results indicate that the complex I formed in nuo24 mitochondria is not operating in electron transfer, and the oxidation of NADH is carried out by alternative enzymes.

Site-directed mutagenesis of the 24-kDa protein

In order to investigate in more detail the role of the iron–sulphur cluster of the 24-kDa protein for complex I assembly

Table 1. Measurements of the respiratory activities of mitochondria and SMP. The activities are expressed as nmol of O_2 per mg of protein per min. Values in parentheses indicate percentage inhibition. The data was obtained from at least three independent experiments performed as described in Fig. 4.

Preparation	Wild-type	nuo24
Mitochondria		
+ Malate	49.6	69.2
+ Rotenone	30.4	69.2
	(39)	(0)
+ Antimycin A	0	0
	(100)	(100)
SMP		
+ NADH	51.7	43.4
+ Rotenone	27.5	42.7
	(47)	(1.6)
+ Antimycin A	0	0
	(100)	(100)

and function, we started the mutagenesis of selected cysteine residues of the polypeptide. The *N. crassa* protein contains eight cysteines but only four of them, namely C143, C148, C186 and C190, are well conserved [19]. The corresponding cysteine residues were shown to serve as ligands for the iron–sulphur cluster in *Paracoccus denitrificans* [48]. We separately substituted C143 and C148 with alanine residues and also constructed the double mutated cDNA C143A/C148A. The three obtained cDNAs were cloned in an appropriate vector and transformed into the mutant strain nuo24. After induction of the respective proteins in the resulting transformants, mitochondria were prepared from the strains and analysed by sucrose gradient centrifugation as described above. The phenotype of all strains was similar to the phenotype of mutant nuo24, except that the mutated 24 kDa proteins could be detected in the low molecular mass region of the gradients only, fractions 2–3, as depicted for strain C148A (Fig. 3D). We conclude that the mutated 24 kDa proteins are unable to assemble into complex I. It appears that binding of the iron–sulphur cluster to the 24-kDa protein occurs before (and is required for) protein assembly.

DISCUSSION

In this report, we described the inactivation of the nuclear gene encoding the 24-kDa iron–sulphur subunit of complex I from *N. crassa*. The resulting nuo24 mutant is able to grow vegetatively, as found so far with all fungal strains in which complex I subunits have been disrupted [4,9]. In the fungus, absence of NADH oxidation by complex I might be compensated by alternative NADH dehydrogenases [49], as in the case of plants [50] and in contrast to the situation in mammals, as currently believed. Although the 24-kDa protein may play a physiologically relevant role as a G protein *in vivo* [14], this is not an essential function. In addition, we presented evidence for the first time that binding of the iron–sulphur cluster to the 24-kDa protein is essential for its assembly into complex I. We independently substituted two cysteine residues of the polypeptide involved in cluster binding [48] with alanine residues, which cannot function as ligands for the prosthetic group [13]. These altered proteins could be expressed in *N. crassa* mitochondria but failed to assemble into complex I.

Upon chaotropic resolution of bovine complex I, it is possible to isolate a flavoprotein fragment capable of electron transfer from NADH to artificial electron acceptors, such as ferricyanide, and composed of only three proteins: the 51-kDa polypeptide, which harbours FMN and the tetranuclear iron–sulphur N-3 and provides a binding site for NADH, the 24-kDa iron–sulphur polypeptide and a 10-kDa polypeptide [3]. Complex I is thought to have evolved from the association of different enzymatic modules, one of which, the NADH dehydrogenase module containing the 51 and 24 kDa polypeptides, was added at a later stage [51,52]. A mutant lacking the 24-kDa protein is apparently able to assemble an almost intact complex I in *N. crassa*. Similarly, a nuo51 mutant also assembles a complex I structure lacking only the 51-kDa polypeptide [53]. These results suggest that the NADH dehydrogenase module of complex I is located peripherally and does not contribute significantly to the overall structure of the enzyme. In this context, it is worth noting that the chloroplast homologue of complex I appears to have a different electron input device [51]. The other complex I subunits (called 49 kDa, 30 kDa, TYKY and PSST in bovine), that are conserved in both chloroplast and bacterial complex I and in bacterial formate hydrogenlyase, probably form a core moiety with a more important structural function. In bacteria, they were shown to connect the NADH dehydrogenase module with the membrane-located domain of complex I [54]. In fact, disruption of the genes encoding the mitochondrial 49 kDa [9], 30 kDa [55] and TYKY proteins ([56]; unpublished data) completely disturbs the formation of the peripheral domain of complex I.

The fact that complex I from nuo24 is not functional in electron transfer is puzzling. The 24 kDa protein probably binds iron–sulphur cluster N-1a [12,13], a redox centre that is not reducible with NADH and displays a very low midpoint redox potential [13] which is difficult to reconcile with electron transfer from NADH to ubiquinone. While still limited information on electron transfer within complex I is available, it is believed that a branched electron transfer occurs before reduction of the tetranuclear iron–sulphur cluster N-2, the electron-donor to ubiquinone. Thus, even in the absence of the iron–sulphur cluster bound by the 24-kDa protein, electron transfer should be possible through FMN, the isopotential clusters N-1b, N-3 and N-4 and then cluster N-2. Based on the fact that we found reduced levels of the 51-kDa subunit in the nuo24 complex, we strongly suggest that the 24-kDa protein is needed for a proper assembly of the 51-kDa subunit. Without the NADH-binding 51 kDa subunit, complex I does not work in NADH oxidation, as shown with a mutant enzyme that only lacks this protein [53]. Because we still observe small amounts of the 51-kDa polypeptide associated with the nuo24 complex, we speculate that it is not in a proper conformation. These explanations are consistent with the findings that, when expressed in *E. coli*, the (51-kDa homologous) NQO1 subunit of *P. denitrificans* complex I was present in an insoluble fraction unless it was coexpressed with the (24 kDa homologous) NQO2 subunit [57]. When both proteins were coexpressed, they formed a soluble complex with 1 : 1 stoichiometry [57]. Our interpretation explains why electron transfer by the nuo24 complex to acceptors binding at different sites within complex I, such as ferricyanide and hexaammineruthenium [39], is also affected. An alternative explanation, although unlikely, is that the iron–sulphur cluster bound by the 24-kDa protein is an obligatory route of the electron transfer through complex I or that conformational changes in complex I due to absence of the 24-kDa polypeptide could prevent electron transfer. Anyway, an important finding is that absence of the 24-kDa protein compromises the electron transfer function of complex I and will eventually lead to a reduction in the total energy production by the mitochondrial respiratory chain.

It has been hypothesized that reduction of the energy production in mitochondria below threshold levels is implicated in mitochondrial human disorders [6]. A reduction in the relative amounts of the 24-kDa polypeptide or even undetectable levels of the protein have been described in some of these diseases [15–18], despite the fact that no mutation affecting the mature protein has yet been found. The phenotype of the nuo24 mutant strain provides an 'energetic' explanation for these observations. When the levels of the protein are diminished, the contribution of complex I to energy conservation is accordingly lowered, possibly due to improper assembly of the 51-kDa protein, and this may cause the disease symptoms. In agreement with this, an increased susceptibility to Parkinson's disease was recently found associated with mutations in the mitochondrial signal sequence of the human 24 kDa polypeptide, which presumably affects the levels of the mature protein inside mitochondria [58]. The mutant described here represents a useful eukaryotic model for further work on the role of the 24-kDa iron–sulphur subunit of complex I.

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4.8-TRABALHO 8

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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 μM 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 μM 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 μM and 0.2 μg/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).

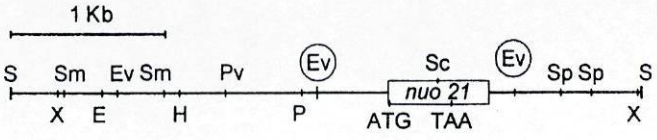

Figure 1 Restriction map of *N. crassa* genomic DNA containing the *nuo-21* gene

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.

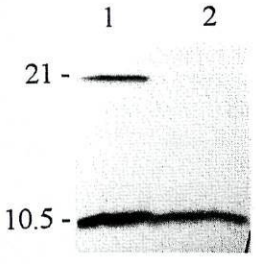

Figure 2 Western blot analysis of mitochondrial proteins

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.

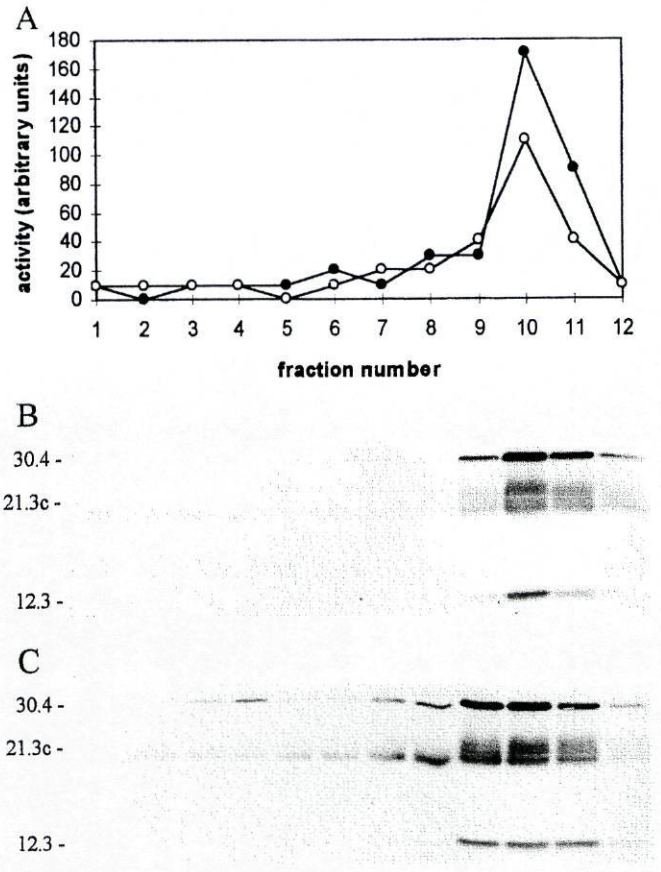

Figure 3 Sucrose-gradient centrifugation analysis of Triton X-100-solubilized mitochondrial proteins

(**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

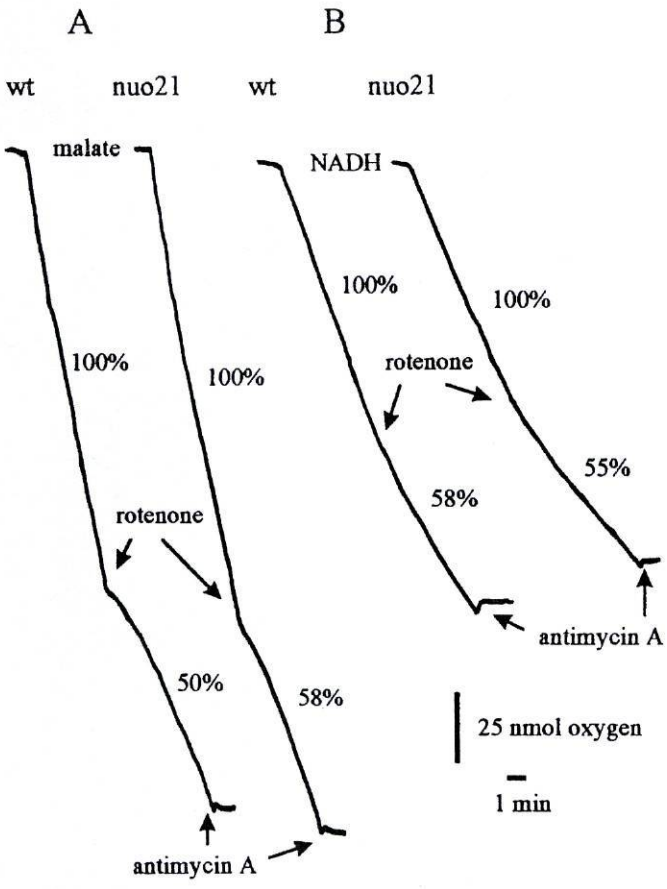

Figure 4 Effects of inhibitors on the respiration of mitochondria (A) and SMP (B)

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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5-CONCLUSÕES FINAIS E PERSPECTIVAS

Com o trabalho aqui apresentado pretendeu-se contribuir para uma melhor compreensão da estrutura e função de determinados componentes do complexo I, dos mecanismos de biogénese desta enzima no fungo *N. crassa* e dos fenómenos associados a deficiências na enzima.

A determinação da estrutura primária da subunidade de 21.3c kDa veio confirmar a presença de dois domínios de ligação para centros tetranucleares, bem como a elevada homologia entre esta subunidade e proteínas de procariotas e eucariotas (trabalho 1). A localização deste polipéptido no domínio periférico do complexo I, associada à ausência do centro tetranuclear N2 neste domínio (Wang *et al.*, 1991), permitiu-nos sugerir que esta proteína não é responsável pela ligação do respectivo centro. Talvez os motivos consenso, presentes no polipéptido de 21.3c kDa, sejam responsáveis pela coordenação de centros tetranucleares que ainda não foram detectados por EPR (trabalho 2). A determinação da estrutura primária e localização da última subunidade do complexo I de *N. crassa*, presente na unidade funcional mínima desta enzima, veio apoiar a sugestão anterior. Assim, a subunidade de 19.3 kDa não se encontra nem no domínio membranar nem na parte periférica do complexo I, sendo apenas imunologicamente detectada em proteínas mitocondriais de estirpes que montam a holoenzima. A subunidade de 19.3 kDa é provavelmente um componente da parte periférica da enzima, muito instável quando este domínio não se encontra associado com a parte membranar (trabalho 3). Esta proteína de 19.3 kDa possui apenas três resíduos de cisteína conservados, pelo que os ligandos do centro tetranuclear a ela associado terão que ser mistos incluindo, provavelmente, um glutamato (Ohnishi, 1998). Um centro com estes ligandos deverá apresentar características espectroscópicas diferentes, o que estaria de acordo com as características do centro N2. Por outro lado, o facto deste centro ser apenas detectado na enzima madura vem corroborar a atribuição da sua coordenação à subunidade de 19.3 kDa. Com a determinação da estrutura primária destas duas subunidades do complexo I, 27 dos cerca de 35 componentes da enzima do fungo foram já identificados. A caracterização das restantes subunidades poderá constituir um dos objectivos futuros, permitindo esclarecer o quão diferentes são as enzimas de *N. crassa* e bovina.

A montagem do complexo I envolve a formação de intermediários relativamente estáveis. A quantidade destes intermediários em células de *N. crassa* crescendo em condições padrão é pequena. No entanto, verificou-se a acumulação destes subcomplexos aplicando condições de crescimento que perturbam o processo de montagem da enzima. Assim, a deficiência em manganês retarda a formação da parte periférica levando à acumulação do braço membranar (Schmidt *et al.*, 1992) e a presença de cloranfenicol no meio de cultura induz a acumulação do domínio periférico (Friedrich *et al.*, 1989). A inactivação de genes mitocondriais ou nucleares que codificam para subunidades do complexo I também interfere, de forma mais ou menos drástica, com a formação da enzima, permitindo o estudo dos diferentes intermediários que se acumulam nas estirpes mutantes. A análise de uma estirpe de *Neurospora*, contendo uma delecção no mtDNA que inclui as subunidades ND2 e ND3, revelou a acumulação do braço periférico e a ausência de qualquer intermediário do domínio membranar (Alves & Videira, 1998). Também os mutantes descritos neste trabalho apresentam fenótipos de montagem do complexo I bastante diversos, verificando-se a acumulação de dois ou três intermediários distintos. A ausência da subunidade membranar de 12.3 kDa não afecta a formação do braço periférico levando à acumulação de um intermediário contendo parte, ou a totalidade, das subunidades constituintes do domínio membranar (trabalho 4). Em oposição, os mutantes nuo30.4 e nuo21.3c apenas possuem o braço membranar, pelo que a inactivação dos genes que codificam para estas subunidades periféricas impede a formação da parte hidrofílica da enzima (trabalhos 5 e 6). Quanto à subunidade de 29.9 kDa, a sua presença parece ser necessária para a estabilidade do domínio periférico e/ou da enzima madura (trabalho 4).

O mutante nuo24 não apresenta qualquer actividade NADH:ubiquinona redutase sensível à rotenona. A oxidação do NADH é executada por enzimas NADH desidrogenases alternativas. O complexo I observado nesta estirpe é enzimaticamente inactivo, não contém a subunidade de 24 kDa e apresenta quantidades subestequiométricas da subunidade que liga o NADH (51 kDa). Esta proteína de 24 kDa parece ter um papel importante na montagem e/ou estabilidade da subunidade de 51 kDa (trabalho 7). Contrariamente, a ausência da proteína acessória de 21 kDa não afecta a actividade NADH:ubiquinona redutase do complexo I presente no mutante nuo21. No

entanto, a redução de aceitadores artificiais, como hexacianoferrato ou hexaaminoruténio III, encontra-se alterada no mutante nuo21, o que sugere que o complexo I formado nesta estirpe apresenta alterações conformacionais provocadas pela falta da subunidade (trabalho 8).

Juntamente com os aqui referidos, existem actualmente 16 mutantes para subunidades do complexo I de *N. crassa* codificadas no núcleo, englobando 12 subunidades periféricas (7 pertencentes à unidade funcional mínima e 5 acessórias) e 4 subunidades do braço membranar (ver Videira, 1998). A caracterização destes 16 mutantes permitiu sugerir uma via de montagem para o complexo I do fungo, em que intermediários pré-formados se associam sequencialmente para originar a enzima madura. O pequeno e o grande intermediário formam-se independentemente e a ausência de qualquer dos seus constituintes impede a associação dos subcomplexos formados para originar o braço membranar, não interferindo com a montagem do domínio periférico. A inactivação de genes que codificam para subunidades acessórias do braço periférico não impede a formação da enzima aumentando, no entanto, a sua instabilidade. Uma excepção foi descrita para a subunidade ACP. A ausência desta proteína traduz-se na completa disrupção do complexo I (Schneider *et al.*, 1995). O domínio hidrofílico associa-se com o braço membranar mesmo quando alguma das subunidades acessórias anteriores não se encontra presente, ou quando as subunidades de 51 kDa ou 24 kDa estão ausentes. A inactivação dos genes que codificam para as subunidades de 78 kDa, 49 kDa, 30.4 kDa, 21.3c kDa e 19.3 kDa, pertencentes à unidade funcional mínima, impede a formação de qualquer intermediário estável, contendo subunidades do braço periférico. No que respeita aos intermediários acumulados nos diferentes mutantes, torna-se agora necessário proceder à sua caracterização mais detalhada, determinando a composição polipeptídica exacta, os centros [FeS] e a actividade enzimática.

Para além do papel estrutural, a função de muitos dos componentes do complexo I, mesmo aqueles cujo gene foi inactivado, continua por definir. Um dos problemas da ausência de uma determinada subunidade é o efeito pleiotrópico na montagem dos restantes polipéptidos do complexo I. Contudo, outro tipo de estratégia pode agora ser utilizado para descortinar a função destas subunidades, como por exemplo, a realização de experiências de mutagénese dirigida. As mutações efectuadas devem alterar a função

da subunidade, mas promover a montagem estável do complexo I. Esta abordagem foi utilizada para determinar qual, ou quais, os centros [FeS] associados à subunidade de 21.3c kDa e qual o seu papel no complexo I. No entanto, a alteração de resíduos supostamente envolvidos na ligação dos centros conduziu a um fenótipo muito semelhante ao observado no mutante nuo21.3c. Com efeito, a substituição de resíduos de cisteína por alanina ou serina impede a ligação dos centros [FeS] e, consequentemente, a formação do complexo I, não sendo assim possível determinar qual o(s) centro(s) [FeS] coordenado(s) por esta subunidade (trabalho 6). Para tentar contornar esta dificuldade, poder-se-á alterar aminoácidos localizados próximo dos ligandos, mas não os directamente envolvidos na coordenação do centro. A escolha desses resíduos pode basear-se em pesquisas de homologia entre proteínas com motivos semelhantes. Por outro lado, a capacidade de expressar proteínas alteradas em *N. crassa* vem permitir utilizar este eucariota como modelo no estudo de doenças mitocondriais humanas. Recentemente, foram identificadas duas mutações na subunidade TYKY, do complexo I humano, associadas a estados patológicos (ver 2.7). Estas mutações foram reproduzidas na subunidade de 21.3c kDa e expressas no mutante nuo21.3c. Resultados preliminares indicam que ambos os mutantes possuem complexo I, pelo que as subunidades mutadas são capazes de se associar aos restantes componentes para formar a enzima (resultados não mostrados).

Um outro aspecto importante deste trabalho foi a demonstração de que o complexo I é uma enzima essencial para a fase sexual do ciclo de vida da *N. crassa*, embora seja dispensável à propagação vegetativa do fungo. Cruzamentos homozigóticos entre diversos mutantes sugerem a diminuição na produção de energia como o factor responsável pela interrupção do processo de esporulação (trabalho 5 e 6). No entanto, a existência de outras funções associadas ao complexo I, necessárias para o desenvolvimento sexual do fungo, não é de excluir. A determinação dessas putativas funções é uma das perspectivas mais aliciantes para trabalhos futuros, podendo ajudar a elucidar alguns dos aspectos característicos da enzima. Por outro lado, o facto das NADH desidrogenases alternativas não serem capazes de substituir o complexo I durante a fase sexual do desenvolvimento do fungo, levanta algumas questões pertinentes. Por exemplo, será que estas enzimas se encontram activas durante este período? Como são regulados os

seus genes e os genes do complexo I durante as diferentes fases de diferenciação da *N. crassa*? É interessante referir que outras enzimas do metabolismo são também essenciais para a diferenciação celular de diversos organismos. Por exemplo, a enzima ATP citrato liase é necessária para a formação das estruturas reprodutoras em *Sordaria macrospora* (Nowrousian *et al.*, 1999). Na batateira, a redução da expressão da citrato sintase resulta na diminuição do número de flores formadas e na desintegração do tecido dos ovários (Landschütze *et al.*, 1995). Isto sugere que talvez outras enzimas, envolvidas em vias metabólicas diversas, tenham um papel relevante na diferenciação celular. Será que os restantes complexos da cadeia respiratória são essenciais para a reprodução sexual em *N. crassa*?

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