A Nuclear Mutant of Neurospora crassa Lacking Subunit 1 of Cytochrome c Oxidase*

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A new, nuclear, cytochrome-deficient mutant of Neurospora crassa has been isolated. The mutant is characterized by its slow growth rate, high dependence on the alternate oxidase pathway of respiration, absence of spectrally detectable cytochrome aa₃, and low levels of cytochrome c oxidase activity. The amount of cytochrome b appears to be normal in the mutant whereas the amount of cytochrome c is elevated approximately 2-fold, compared to the amounts found in wild type.

Sodium dodecyl sulfate-gel electrophoretic analysis of immunoprecipitates obtained from the mitochondria of the mutant using antibody directed against holocytochrome c oxidase revealed that only subunits 5 and 6 of the enzyme could be detected by this method. Mitochondria isolated from cells labeled in the presence of cycloheximide were examined using antibodies specific to each of the mitochondrial synthesized subunits of the enzyme. This analysis revealed a complete absence of immunoprecipitable subunit 1 in the mitochondria of the mutant. Subunits 2 and 3 were present, although the amount of subunit 2 was somewhat lower than the amount found in mitochondria isolated from wild type grown under similar conditions. The amount of subunit 3 in the mutant was comparable to the amount found in wild type.

Since there is no direct evidence which shows that the gene for subunit 1 is found on mtDNA, the possibility that the nuclear mutation directly affects the polypeptide sequence of the subunit cannot be completely eliminated. However, if it is assumed that mitochondrial translation products are encoded by mtDNA, then the fact that a nuclear mutation gives rise to a deficiency of a mitochondrially synthesized polypeptide suggests that a regulatory entity necessary for the production of subunit 1 of cytochrome c oxidase may be missing or altered in the mutant described here.

The cytochrome c oxidase of Neurospora crassa and of yeast is composed of seven polypeptides (1-3). The three largest subunits are synthesized on mitochondrial ribosomes (1, 3, 4) while the four smaller polypeptides are translated on cytosolic ribosomes (3, 5). As discussed previously (6-12), it is probable that the mitochondrially synthesized polypeptides are encoded by mitochondrial genes and that the genetic information for the cytoplasmically translated subunits is found on nuclear DNA.

Evidence has also been presented which suggests that a complex system of control involving both nuclear and mitochondrial genes governs the production of the enzyme. For example, Ono et al. (13) have shown that the production of subunit 3, or its integration into a functional enzyme, is influenced by a nuclear gene in yeast. Bertrand et al. (14, 15) have suggested that a system of regulation for the production of the oxidase also exists in Neurospora crassa. These suggestions have recently been strengthened by the finding that the cytochrome aa₃ deficiency in the mi-3 cytoplasmic mutant of the organism is likely due to an altered regulatory gene affecting the production of subunit 2 of cytochrome c oxidase (16).

This paper describes a new, nuclear, cytochrome aa₃-deficient mutant of Neurospora crassa which completely lacks an immunoprecipitable subunit 1 of cytochrome c oxidase. No other nuclear mutant of any organism deficient only in cytochrome aa₃ is known to lack specifically this mitochondrially synthesized subunit of the enzyme. Since the defect is the result of a mutation in nuclear DNA, it is possible that a regulatory gene is affected.

MATERIALS AND METHODS

Strains—All strains used in this study were derived from 74-OR23-1A or 74-OR8-1A wild types. The mutation was induced by N-methyl-N-nitro-N-nitrosoguanidine in the strain nic-1 pan-2 al-2 IC-7A, which possesses a wild type cytochrome system. The mutant was selected by a procedure to be described elsewhere utilizing a tetrazolium reduction test originally described by Edwards et al. (17) and modified slightly by Bertrand et al. (15). The original isolate was crossed as the conidial (male) parent to a wild type strain (50a) commonly used in our laboratory. (It is of note that the 50a strain and nic-1 pan-2 al-2 IC-7A are virtually identical with respect to growth, spectral, and respiratory properties.) One of the cytochrome aa₃-deficient, slow growing progeny from this cross, namely KF1-94, was used in the experiments described here.

Media, Growth Conditions, and Crossing—All cultures were grown on solid or in liquid Vogel's medium (18) at room temperature with the exception of cultures used to measure growth rate. These were grown at a constant temperature of 25°C. The composition of

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2 Unpublished observations.
standard media and procedures for crossing have been described previously (19).

Growth Rates—5 x 10^6 conidia were added to 250-ml flasks containing 50 ml of Vogel's medium. The flasks were shaken on an Eberbach shaker at 140 cycles per min at 25°C. Following different periods of growth, the material in the flask was collected by filtration, washed with distilled water, dried for 48 h at 75°C, and weighed.

Respiration—Oxygen utilization by whole mycelium was measured polarographically with a Clark oxygen electrode (Yellow Springs Instruments, Cleveland, Ohio). The respiration medium was Vogel's minimal medium containing 1% glucose as substrate. The procedure has been described previously (20, 21). Salicylhydroxamic acid was added from a 0.25 M solution in ethanol. KCN was added from a 0.1 M solution in Buffer 1 (0.01 M Tris-HCl, pH 7.2; 0.005 M EDTA).

Isolation of Mitochondria and Cytochrome Spectra—In order to obtain mitochondria for cytochrome spectra and cytochrome c oxidase assays, mycelium was grown at room temperature in liquid Vogel's medium. Wild type strains produced enough mycelium for spectra (from an inoculum of approximately 10^6 conidia per ml in 250 ml of medium) in 16 to 18 h, whereas the mutant required 40 to 50 h of growth. The cells were broken by grinding with acid-washed sand. The smooth paste was suspended in Buffer 1 containing 0.44 M sucrose. Cell debris, nuclei, and sand were removed by centrifuging once at 1100 x g for 15 min. The supernantant was carefully layered onto a step gradient consisting of 10 ml of 2.0 M sucrose and 10 ml of 0.8 M sucrose. Each step was made up in Buffer 1. The mitochondria were removed from the interface of the gradient after centrifuging for 1 h at 28000 x g. The purified mitochondria were diluted with 5 volumes of Buffer 1 and pelleted by centrifugation at 28000 x g for 45 min. The pelleted mitochondria were resuspended in Buffer 1 and used for cytochrome spectra or cytochrome c oxidase assays. Cytochrome spectra were obtained as described by Bertrand and Pittenger (22) using a Beckman Acta V double beam recording spectrophotometer.

The absolute mitochondrial cytochrome concentrations were calculated by the method of Williams (23) using the absorption coefficients given by Lambowitz et al. (24). A correction factor for the contributions of cytochromes b and c was employed in the calculations (25).

Mitochondria were isolated from labelled cells by the method of Bertrand and Werner (16). In all cases, phenylmethylsulfonyl fluoride, a protease inhibitor, was added immediately after harvesting the cells to a concentration of 1 mM.

Cytochrome c Oxidase Assay—Cytochrome c oxidase activity was determined spectrophotometrically by following the oxidation of reduced cytochrome c at 550 nm at 25°C. The reaction mixture (final volume 3.0 ml) contained 0.03 M NaPO_4, buffer (pH 7.2), 0.5% Tween 80, and 1.0 mg of reduced cytochrome c (Sigma, horse heart, type III). The reaction was started by addition of 0.1 ml of a diluted mitochondrial suspension. Reduced cytochrome c was prepared by adding a few crystals of sodium dithionite to a 5 mg/ml solution of cytochrome c. Excess reducing agent was removed by bubbling air through the solution for 60 s. Enzyme activity is expressed as the first order rate constant for the oxidation (26).

Protein Determination—Protein was determined by the biuret method (27).

Labeling of Cells—The procedures for uniform labeling of cells and for selective labeling of mitochondrial translation products have been described previously (19).

Immunological Procedures, Gel Electrophoresis, and Determination of Radioactivity—The purification of Neurospora crassa cytochrome c oxidase (28-30), the large scale preparation of the subunits of the enzyme (29-31), the immunization of rabbits and sheep (31), the immunoprecipitation of cytochrome oxidase and its subunits (31), and the procedures for polyacrylamide gel electrophoresis (28) and determination of radioactivity (16) have been described. Gel electrophoresis was done using 15% acrylamide gels.

RESULTS

Segregation and Growth Characteristics—The mutant was originally isolated as a slow growing, tetrazolium nonreducing strain. Examination of the cytochrome spectrum of the mutant revealed that it had lower amounts of cytochrome aa₃ than wild type strains. In order to obtain pure, single ascospore isolates containing the mutation, the mutant was crossed as the conidial (male) parent to the wild type strain 50a. The ascospores produced from this cross were found to grow at one of two different rates. Most of the spores produced were scored as "fast," according to the system described by Bertrand et al. (15). However, about 10% of the spores were classified as "slow." Cytochrome spectra of mitochondria isolated from the mycelium produced by the different types of ascospore isolates revealed that the slow growing isolates had a deficiency of cytochrome aa₃. The results of this cross indicate that the mutation is located on the nuclear genome, since crossing the original isolate 50a as a male produced progeny with the defect. The fact that only about 10% of the spores isolated carried the defect indicated that some ascospore lethality or incomplete penetrance may be associated with the mutation. The low percentage of recovery of ascospores carrying the mutation has persisted in subsequent crosses. We are attempting to map the mutation.

The mutant strain also exhibits a slow growth character in liquid culture as exemplified by Fig. 1. It can be seen that the growth rate of KTU-34 never reaches the rates of the 50a wild type shown in the figure. In addition, the lag period before the onset of rapid growth is 10 to 15 h longer in the mutant than in wild type.

Spectral Properties—The mitochondria of KTU-34 contain no spectrally detectable cytochrome aa₃ (Fig. 2A). Comparison with the 50a wild type spectrum, shown in Fig. 2B, indicates that the amount of cytochrome b in the mutant appears to be normal while the amount of cytochrome c is elevated about 2-fold. The absolute mitochondrial cytochrome concentrations (nanomoles/mg of mitochondrial protein) are 0.28 (S.D. ± 0.02), 0.45 (S.D. ± 0.02), and 0.74 (S.D. ± 0.08) for cytochromes aa₃, b, and c, respectively, in wild type (three determinations). In KTU-34, cytochrome aa₃ is not detectable. The concentrations of cytochromes b and c in the mutant are 0.32 (S.D. ± 0.01) and 1.25 (S.D. ± 0.12) nanomoles/mg of mitochondrial protein, respectively (four determinations).

Respiration and Cytochrome c Oxidase Activity—The mycelium of KTU-34 is highly dependent on the alternate (salicylhydroxamic acid-sensitive) oxidase system, which was described by Lambowitz and Slayman (20). The tracings in Fig. 3 represent typical patterns of oxygen uptake by KTU 34 and...
wild type 50α mycelium. In all trials, oxygen consumption in both strains was inhibited by greater than 99% when both KCN and salicylhydroxamic acid were present. However, the average respiration rate of wild type mycelium after the addition of KCN alone was only 0.6% of the initial rate (S.D. ± 0.8%, eight determinations), while the mutant respirates at an average of 8% of its original rate (S.D. ± 10%, eight determinations) following the addition of KCN. In contrast, when salicylhydroxamic acid is added to respiring mycelium before the addition of KCN, oxygen consumption in KTU-34 is decreased to an average of 18% of the original rate (S.D. ± 4%, eight determinations), while wild type continues to respire at 92% of its original rate (S.D. ± 8%, eight determinations).

In agreement with the respiration data and with the fact that cytochrome aa3 cannot be detected in the cytochrome spectrum of the mutant, the cytochrome c oxidase activity in isolated mitochondria of KTU-34 is very low. The specific rate constant for cytochrome c oxidase activity in mitochondria isolated from wild type was found to be 0.651 (S.D. ± 0.064) units/mg of mitochondrial protein, whereas the rate in KTU-34 mitochondria was only 0.025 (S.D. ± 0.005) units/mg of mitochondrial protein.

Holocytochrome Oxidase Immunoprecipitation—Since the mitochondria of KTU-34 contained no spectrally detectable cytochrome aa3, it was of interest to discern whether or not the polypeptides of the oxidase were present in the mutant. Fig. 4 shows the gel electrophoretic pattern of cytochrome oxidase immunoprecipitated from a mixture of wild type and KTU-34 mitochondria, labeled uniformly with L-[14C]leucine and L-[3H]leucine, respectively. The subunits (1 to 7) of the oxidase correspond to molecular weights of 41,000, 28,000, 21,000, 16,000, 14,000, 12,000, and 10,000, respectively (1, 32). The 14C label appears in seven distinct peaks as expected (31). However, the 3H label is found only in the peaks representing subunits 5 and 6 of the oxidase. The amount of these subunits seems to be somewhat less than the amount found in wild type. There is virtually no 3H label in the molecular weight regions of the other subunits.

It has been shown that the antibody produced against holoenzyme reacts predominantly with antigenic sites on sub-
units 5 and 6 (16). Therefore, it was still possible (despite the results shown in Fig. 4) that the remaining subunits of the oxidase were present, but perhaps not associated with subunits 5 and 6. Alternatively, one or more of the subunits could have been missing, thus preventing assembly of the remaining polypeptides. In order to distinguish between these possibilities, mitochondria from KTU-34 were examined using antibody specific to each of subunits 1, 2, and 3.

**Subunit Specific Immunoprecipitation**—Fig. 5, A, B, and C shows the electrophoretic pattern obtained from immunoprecipitates of subunits 1, 2, and 3, respectively. In each case, wild type mitochondria obtained from mycelium labeled with L-[3H]leucine in the presence of cycloheximide and KTU-34 mitochondria labeled with L-[14C]leucine in a similar fashion were mixed prior to solubilization and immunoprecipitation. Fig. 5A shows very clearly that no immunoprecipitable subunit 1 is present in the mitochondria of the mutant. However, subunits 2 and 3 are present (Fig. 5, B and C, respectively), although the amount of subunit 2 seems to be somewhat reduced in the mutant. It is doubtful that the lack of subunit 1 can be attributed to proteolytic breakdown, since phenylmethylsulfonyl fluoride was present during the isolation procedure.

**Mitochondrial Translation Products**—The above results indicated that the mitochondria of the mutant contain no immunoprecipitable subunit 1. However, it was possible that a form of subunit 1 which was not recognized by the antibody was present. For this reason, the gel electrophoretic pattern of the mitochondrial translation products of KTU-34 labeled with L-[3H]leucine in the presence of cycloheximide was examined (Fig. 6). Wild type mitochondria were prepared from cells labeled with L-[14C]leucine and mixed with the mutant mitochondria prior to solubilization and electrophoresis. The [3H] tracing in Fig. 6 shows at least seven distinct peaks corresponding to molecular weights of 41,000, 31,000, 28,000, 21,000 to 19,000, 15,000, 11,000, and a low molecular weight peak of less than 10,000. These peaks correspond to the molecular weights of subunit 1 of cytochrome c oxidase, the cytochrome b apoprotein, subunit 2 of cytochrome c oxidase, an unseparated peak containing subunit 3 of cytochrome c oxidase and a mitochondrial ATPase component, an unknown polypeptide, a second ATPase component, and another unknown polypeptide (1, 33, 34).

The amount of [3H]-labeled material in the $M_r = 41,000$ region is exceedingly small as compared to the amount of [14C]-labeled material. If the small amount of [3H]-labeled material...
which remains does represent a polypeptide, it may be a nonimmunoprecipitable form of subunit 1. On the other hand, the small peak could represent a previously unknown mitochondrial translation product. The amount of $^3H$ label in the $M_1 = 28,000$ region is also small. This is in agreement with the result obtained using subunit 2 specific immunoprecipitation (Fig. 5B).

**DISCUSSION**

The fact that the mitochondria of the KT11-34 mutant of *Neurospora crassa* are deficient in cytochrome $a_3$ indicates that the mutant belongs in the cya category of Bertrand et al. (1b). A tentative designation of cya-U-34 is suggested, since the map position of the mutation is not yet established. The mutant is also characterized by its slow growth rate in liquid culture and from ascospores. Preliminary indications are that the mutant is female sterile.

The studies on the respiratory properties of cya-U-34 indicate that the normal electron transferring pathway is almost nonfunctional, and that the mutant must rely heavily on the alternate oxidase system of respiration. Furthermore, the assays of cytochrome c oxidase activity suggest that the altered mode of respiration is due to a deficiency of the oxidase, since cya-U-34 mitochondria have less than $4\%$ of the specific activity of wild type mitochondria. Supporting this conclusion is the fact that no holocytochrome oxidase can be immunoprecipitated from the solubilized mitochondria of the mutant using antibody directed against the enzyme (Fig. 4).

The deficiency of cytochrome $c$ oxidase in cya-U-34 can be more specifically related to a deficiency of immunoprecipitable subunit 1 in the mitochondria of the mutant (Fig. 5A). Analysis of the mitochondrial translation products confirms this finding for the most part, although a very small amount of material is present in the molecular weight range of subunit 1. However, since this material is not recognized by the antibody to wild type subunit 1 (Fig. 5A), it is unlikely that it serves the normal function of the subunit. The amount of subunit 2 is also reduced in cya-U-34, although it is definitely present. The results obtained from both subunit 2 specific immunoprecipitation (Fig. 5B) and the examination of mitochondrial translation products confirm the presence of this subunit, although it seems to be present in low amounts as compared to wild type. Subunits 3, 5, and 6 are present in the mutant as well. Unfortunately, it was not possible to test for the presence of subunits 4 and 7 due to lack of a suitable antibody.

There are many possible explanations for the absence of subunit 1 in cya-U-34, the most simplistic being that it is lost during the isolation procedure. However, this seems unlikely since subunits 2, 3, 5, and 6 are not lost. Furthermore, mitochondria isolated from other mutants, which do not have an assembled cytochrome $c$ oxidase, do not lose the subunit $16$. It is possible that the mutation in cya-U-34 results in the formation of an altered subunit 1. Such an alteration could lead to rapid degradation, incorrect processing, or loss of antigenicity of the subunit. However, a large body of circumstantial evidence suggests that polypeptides which are translated in the mitochondria are encoded by mtDNA (6-12). This evidence is further supported by recent site of synthesis experiments on the proteolipid of the mitochondrial ATPase in yeast and *Neurospora crassa*. In yeast, the proteolipid is made on mitochondrial ribosomes (35) and is encoded by mtDNA (36). However, in *Neurospora crassa* it is a nuclear gene product (37) and is synthesized on cytosolic ribosomes (38). These observations suggest that the site of synthesis of a protein gives a good indication as to the location of the gene for that protein. Furthermore, the recent work of Cabral et al. (39) has provided direct evidence that subunit 2 of cytochrome $c$ oxidase in yeast is encoded by mtDNA. Nevertheless, since it has not been definitely shown that subunit 1 of *Neurospora crassa* is encoded on mtDNA, it is possible that mRNA coding for subunit 1 is transcribed in the nucleus and transported into the mitochondrion for translation. Thus, the mutation in cya-U-34 could directly affect the amino acid sequence of the subunit, although it is unlikely.

Alternatively, the nuclear gene could encode an enzyme which is involved in processing a precursor polypeptide(s). Although there is no evidence for the existence of a larger precursor of the subunit, it has been suggested that high molecular weight mitochondrial translation products (such as subunit 1 of cytochrome $c$ oxidase) are the result of associations between smaller polypeptides (40-44). If this were the case, the mutation in cya-U-34 might affect an "organizer molecule" or enzyme necessary to achieve the proper association or linking of these peptides. However, if such an entity was defective in the mutant it might be expected that anti-subunit 1 antibody would recognize antigenic sites on these hypothetical smaller unassociated polypeptides. This is obviously not the case, as no material is precipitated from the mutant using the subunit 1 specific antibody (Fig. 5A). The small peak representing the polypeptide(s) of less than 10,000 molecular weight (Fig. 6) could represent these small "unlinked" polypeptides. However, the amount of $^3H$ labeled material present in this peak is by no means sufficient to account for the amount of subunit 1 observed in the $^{14}C$-labeled wild type mitochondria. Nevertheless, the above suggestion for the effect of the cya-U-34 mutation cannot be eliminated completely, as it is possible that only a fully assembled subunit would possess the correct antigenic sites, while the smaller unassociated polypeptides might be rapidly degraded in vivo.

The GL1-38 mutant of yeast also lacks subunit 1 of cytochrome $c$ oxidase (45). However, GL1-38 is quite different from cya-U-34 in that the absence of subunit 1 is only apparent if the mutant is grown on glucose medium in the absence of $\delta$-aminolevulinic acid (45). Furthermore, the loss of subunit 1 is not specific in GL1-38. Subunits 5 and 7 of the oxidase, as well as all spectrally detectable cytochromes, are also missing in this mutant when grown under the above conditions (46). GL1-38 has been shown to be deficient in $\delta$-aminolevulinic acid synthetase (46). Therefore, the phenotype of the mutant is due to a deficiency of heme synthesis. Although cya-U-34 lacks only cytochrome $a_3$, and is thus obviously not deficient in heme synthesis per se, it does not exclude the possibility that a step in the synthesis of heme $a$ itself is blocked. It may be that a normal heme $a$ is required for the production of subunit 1 in *Neurospora crassa*. However, the results of Werner et al. (32) argue against this explanation, since wild type cells grown in copper-deficient medium contain no heme $a$ but do contain the seven polypeptides of cytochrome $c$ oxidase in assembled form.

The possibility that the cya-U-34 mutation affects a regulatory gene is perhaps the most attractive hypothesis. It is conceivable that the product of one or more nuclear gene(s) is normally required for the production of subunit 1. In the mutant, the postulated gene product(s) may be rendered ineffective and thus prevent either transcription or translation of the subunit 1 mitochondrial gene. Such a regulatory molecule(s) might take the form of a small polypeptide or an effector molecule. Evidence has already been provided for the individual regulation of subunit 2 production in *Neurospora crassa* (16) and for subunit 3 in yeast (13). Therefore, it might
be expected that subunit 1 production is also regulated independently.

In *mi-3*, the extremely low level of subunit 2 seems to be accompanied by a concomitant reduction in the amounts of subunits 1 and 3 (16). Similarly, the absence of subunit 1 in the mutant.

A number of cytochrome *aa₃*-deficient mutants which lack subunit 1 of cytochrome *c* oxidase have also been described in yeast (12, 47). However, these mutants are maternally inherited and may, therefore, directly affect the gene encoding the subunit, or a regulatory site on mtDNA.

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