

Cloning and Analysis of the Alternative Oxidase Gene of *Neurospora crassa*

Qihong Li,* R. Gary Ritzel,* Lesley L. T. McLean,* Lee McIntosh,[†] Tak Ko,[‡]
Helmut Bertrand[‡] and Frank E. Nargang*

*Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2E9, [†]Department of Energy Plant Research Laboratory and Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824-1312 and [‡]Department of Microbiology, Michigan State University, East Lansing, Michigan 48824-1101

Manuscript received August 28, 1995
Accepted for publication October 13, 1995

ABSTRACT

Mitochondria of *Neurospora crassa* contain a cyanide-resistant alternative respiratory pathway in addition to the cytochrome pathway. The alternative oxidase is present only when electron flow through the cytochrome chain is restricted. Both genomic and cDNA copies for the alternative oxidase gene have been isolated and analyzed. The sequence of the predicted protein is homologous to that of other species. The mRNA for the alternative oxidase is scarce in wild-type cultures grown under normal conditions, but it is abundant in cultures grown in the presence of chloramphenicol, an inhibitor of mitochondrial protein synthesis, or in mutants deficient in mitochondrial cytochromes. Thus, induction of alternative oxidase appears to be at the transcriptional level. Restriction fragment length polymorphism mapping of the isolated gene demonstrated that it is located in a position corresponding to the *aod-1* locus. Sequence analysis of mutant *aod-1* alleles reveals mutations affecting the coding sequence of the alternative oxidase. The level of *aod-1* mRNA in an *aod-2* mutant strain that had been grown in the presence of chloramphenicol was reduced several fold relative to wild-type, supporting the hypothesis that the product of *aod-2* is required for optimal expression of *aod-1*.

THE mitochondria of many organisms contain a second terminal oxidase in addition to cytochrome *c* oxidase. This alternative oxidase is most easily characterized by its resistance to classical inhibitors of the electron transport chain, such as cyanide or antimycin A, and its sensitivity to hydroxamic acids, *n*-propyl galate, and disulfiram. The branchpoint of the alternative oxidase from the main respiratory chain is at the ubiquinol pool. Electrons from reduced ubiquinone are donated directly to oxygen to form water so that two energy-conservation sites of the standard mitochondrial electron transport chain are bypassed (HENRY and NYNS 1975; LAMBERS 1982; MOORE and SIEDOW 1991; MCINTOSH 1994). The alternative pathway appears naturally in various tissues and/or developmental stages of plants. The regulation of its activity is complex and appears to involve both transcriptional and posttranscriptional mechanisms (MOORE and SIEDOW 1991; UMBACH and SIEDOW 1993; MILLAR *et al.* 1993; MCINTOSH 1994; UMBACH *et al.* 1994). Although the physiological significance of the alternative pathway is in most cases unclear, one confirmed role is in blooms of the Araceae (*e.g.*, Voodoo lily), where a high rate of respiration through the alternative oxidase results in the release of energy as heat leading to the volatilization of compounds that attract pollinating insects (MEEUSE 1975; RASKIN *et al.* 1987).

In *Neurospora crassa* there is little or no alternative oxidase activity under normal growth conditions. However, activity is induced by inhibitors of mitochondrial transcription, translation, and electron transport (LAMBOWITZ and SLAYMAN 1971; LAMBOWITZ *et al.* 1972; HANSENS *et al.* 1974; HENRY and NYNS 1975). It is also induced in mutants that are deficient in components of the cytochrome-mediated electron transport chain (LAMBOWITZ *et al.* 1972; BRIDGE and BERTRAND 1983) or by growth of wild-type strains in the absence of a factor essential for the formation of the cytochrome system, such as copper (SCHWAB 1973).

At least two genes, *aod-1* and *aod-2*, are required for alternative oxidase activity in *N. crassa* (BERTRAND *et al.* 1983). By *in vivo* labeling of mitochondrial proteins synthesized under both inducing and normal conditions, it was found that, upon induction, wild-type strains and 19 of 20 *aod-1* mutants accumulated a polypeptide that was tentatively identified as being associated with the alternative oxidase. All four *aod-2* mutants tested and a single *aod-1* mutant (*aod-1-4*, originally named "ANT1") (EDWARDS *et al.* 1978) did not accumulate the polypeptide (BERTRAND *et al.* 1983). Similar results were obtained in an immunological study (LAMBOWITZ *et al.* 1989) using monoclonal antibodies raised against the *Sauromatum guttatum* enzyme (ELTHON and MCINTOSH 1987; ELTHON *et al.* 1989). The antibody reacted with *N. crassa* polypeptides of 37 and 36.5 kD, which were present at a high concentration in mitochondria isolated from wild-type cells grown in the pres-

Corresponding author: Frank E. Nargang, Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2E9. E-mail: frank_nargang@biology.ualberta.ca

ence of chloramphenicol, but were barely detectable in mitochondria isolated from wild-type cells grown under standard conditions. These proteins were also present in the cytochrome-deficient mutant, [*poky*], grown without chloramphenicol (LAMBOWITZ *et al.* 1989). Examination of *aod* mutant strains showed that *aod-2-4* contained very low levels of either band, even following growth in chloramphenicol. Mutant *aod-1-1* did synthesize the proteins when grown in the presence of chloramphenicol but not under normal growth conditions. The *aod-1-4* mutant did not contain either of the two bands regardless of the presence or absence of chloramphenicol in the growth medium (LAMBOWITZ *et al.* 1989). Based on these findings, it was suggested that *aod-1* was the structural gene for the alternative oxidase while the *aod-2* gene encoded either a component that regulates the induction of alternative oxidase activity or a protein required for stable accumulation of the *aod-1* polypeptide (BERTRAND *et al.* 1983; LAMBOWITZ *et al.* 1989).

To begin a study of the *N. crassa* alternative oxidase and its regulation, we have isolated and characterized the gene encoding the protein responsible for alternative oxidase activity. The gene was identified using conserved regions of the protein identified from other species to design degenerate primers for PCR amplification. Our results provide direct evidence that the *aod-1* gene encodes the alternative oxidase. Reduced levels of the *aod-1* transcript in an *aod-2* mutant strain favor the hypothesis that *aod-2* is involved in the transcriptional regulation of the *aod-1* gene.

MATERIALS AND METHODS

Strains and growth conditions: *N. crassa* strains used in this study are listed in Table 1 (FGSC, Fungal Genetics Stock Center). Growth of *N. crassa* and other routine manipulations of the organism have been described previously (DAVIS and DESERRES 1970). Where stated in the text, strains were grown in medium containing chloramphenicol at a concentration of 2 mg/ml. *Escherichia coli* strain XL-1 Blue, carrying various recombinant plasmids, was grown under standard conditions (AUSUBEL *et al.* 1992).

PCR with degenerate primers or specific primers: The degenerate primers for amplification of a segment of the alternative oxidase gene from *N. crassa* genomic DNA were derived from two highly conserved regions of alternative oxidase from other species. The degenerate primer specific to the 5' conserved region was 5'-AA(TC)GA(AG)(CA)GIATGCA(TC)-(TC)T-3' (I = inosine) and corresponds to the conserved amino acid sequence NERMHL. The degenerate primer specific to the 3' end of the gene was 5'-GC(TC)TC(TC)TC(TC)-TCIA(GA)(GA)TA-3'. Its complementary strand encodes YLEEEA. (The position of these regions is shown in Figures 1 and 2, see RESULTS.) The reaction yielded an alternative oxidase-specific product of ~150 bp.

The PCR reaction was carried out in a Robocycler 40 (Stratagene, LaJolla, CA) in a 20- μ l volume containing 50 mM Tris-HCl (pH 9.0), 1.5 mM MgCl₂, 0.4 mM 2-mercaptoethanol, 0.1 mg/ml bovine serum albumin, 200 μ M of each dNTP, each primer at 0.5 mM, and 2 units of Taq DNA polymerase. The reaction was initiated by denaturation at 95° for 4 min. Thirty

subsequent cycles were carried out at 91° (denaturation) for 1 min, 53° (annealing) for 2 min, 73° (extension) for 2 min. Conditions for subsequent PCRs with other gene-specific primers varied according to the specific primers used.

Screening of a *N. crassa* genomic library: A *N. crassa* genomic cosmid DNA library from strain NCN53 was constructed previously (GESSERT *et al.* 1994), using the cosmid vector pSV50. This vector contains a benomyl resistance gene to provide selection in *N. crassa* and an ampicillin resistance gene for selection in *E. coli* (VOLLMER and YANOFSKY 1986). A colony filter representation of this library was screened by standard techniques (AUSUBEL *et al.* 1992). The probe was the 150-bp fragment of the alternative oxidase gene obtained by PCR amplification of *N. crassa* genomic DNA using the degenerate primers described above. The PCR product was purified from an agarose gel using glassmilk (VOGELSTEIN and GILLESPIE 1979) and labeled using an oligo-labeling kit according to the supplier's instructions (Pharmacia, Baie D'Urfe, PQ).

Isolation of the cDNA clone of the alternative oxidase gene: A cDNA library constructed from cells induced for the alternative oxidase was not available so we attempted to isolate a clone from a standard library. As the alternative oxidase is expressed at an extremely low level under normal growth conditions, a standard cDNA library would be expected to include very few clones containing alternative oxidase cDNA. Therefore, to maximize the likelihood of success, a PCR approach was taken using pools containing large numbers of clones. A portion of a wild-type cDNA library, constructed in the ZAP vector (provided by M. SACHS), was divided into 12 pools (each containing ~10⁵ pfu). The pools were individually amplified at 37° overnight in a 1.5 ml microcentrifuge tube containing 500 μ l of L-broth and 10 μ l of saturated XL-1 Blue host cells. Following overnight growth, one drop of chloroform was added and 1 μ l of phage suspension from each tube was added to a separate fresh tube containing 20 μ l of the degenerate primer PCR mix described above. The PCR was performed in a Robocycler as described above except that the initial denaturation step was for 8 min to insure disruption of phage particles. Electrophoresis of the reaction products revealed one positive pool. An aliquot of this pool was plated for individual plaques and screened using the 150-bp PCR-amplified fragment of the alternative oxidase gene as a probe. *In vivo* excision of the phage clone generated plasmid pGR-1 that contained a 1.3-kb insert. Its identity as an alternative oxidase cDNA was confirmed by complete sequencing of both strands.

DNA sequencing and analysis: DNA sequencing of plasmid DNA was performed using a Sequenase kit from Amersham (Oakville, ON) according to the supplier's instructions. In some cases, PCR products were sequenced directly. The PCR products were separated on an agarose gel to remove unincorporated primers and nucleotides, and the appropriate band was purified using glassmilk (VOGELSTEIN and GILLESPIE 1979). One to two micrograms of purified DNA was placed in a 1.5-ml microcentrifuge tube, combined with 1 μ l of sequencing primer (100 ng/ μ l), boiled in a waterbath for 5–8 min, and then quickly chilled in an ethanol/dry ice bath. The contents were spun to the bottom of the tube by brief centrifugation. The subsequent sequencing reaction steps were essentially the same as the standard sequencing method with Sequenase, except that 2 μ l of MnSO₄-containing buffer (provided in Amersham Sequenase kit) was used instead of the standard Sequenase reaction buffer and the labeling mix was 2.5-fold more concentrated than recommended for the standard sequencing reaction. Sequencing gels were dried and exposed to Kodak X-AR film. Analysis of DNA and protein sequences was performed with the programs of PCGENE or

TABLE 1
Strains used in this study

Strain name (alternate name)	Genotype	Origin, source, and/or reference
NSA-95 (7001)	<i>aod-1-1 a</i>	H. BERTRAND
NSK-1 (7021)	<i>aod-1-2 a</i>	H. BERTRAND
ANT-1-4 (7202)	<i>aod-1-4 A</i>	EDWARDS <i>et al.</i> (1978)
QDED-6-59 (7216)	<i>aod-1-6 A</i>	H. BERTRAND
LT-9	<i>aod-1-6 pan-2 a</i>	NCN235 × QDED-6-59
FDED-7-7 (7207)	<i>aod-1-7</i>	H. BERTRAND
NSBAN-4 (7064)	<i>aod-2-4 nic-1 al-2 a</i>	H. BERTRAND
NCN7 (Emerson)	<i>A</i>	FGSC #424
NCN10	<i>nic-1 al-2 A</i>	H. BERTRAND
NCN20	<i>A</i>	F. E. NARGANG
NCN27 (Mauriceville-1c)	<i>A</i>	FGSC #2225
NCN34 (Costa Rica)	<i>A</i>	FGSC #851
NCN35	<i>cot-1 A</i>	FGSC #4065
NCN39 (Lein 7)	<i>A</i>	A. LAMBOWITZ
NCN53	<i>[mi-3] su-1</i>	F. E. NARGANG
NCN57 (50a)	<i>a</i>	H. BERTRAND
NCN69	<i>cyt-2-1 pan-2 a</i>	F. E. NARGANG
NCN109	<i>cyb-1-1 nic-1 pan-2 al-2 a</i>	H. BERTRAND
NCN184	<i>[poky] nic-1 al-2 a</i>	H. BERTRAND
NCN235	<i>pan-2 a</i>	F. E. NARGANG
NCN251 (74A)	<i>A</i>	FGSC #2489

DNASTAR Megalign. The amino acid sequences of the alternative oxidases from different species (Figure 2) were aligned using the CLUSTAL program of PCGENE. Some minor adjustments in the alignment were made by inspection. Membrane spanning domains were predicted by the RAOARGOS program.

Isolation of total RNA from *N. crassa* mycelia: A procedure modified from CHIRGWIN *et al.* (1979) was used to isolate total cellular RNA from mycelium of *N. crassa*. All reagents were prepared in distilled water that had been treated with diethylpyrocarbonate. The mycelium was harvested by filtering through Whatman paper, washed with water and immediately frozen in liquid nitrogen. The frozen cells were ground to homogeneity using a mortar and pestle with acid-washed sea sand and a solution of 4 M guanidinium isothiocyanate, 20 mM sodium acetate, 0.1 mM dithiothreitol, 0.5% N-lauroylsarcosine (final pH 5.5). The suspension was centrifuged in a Sorvall SS-34 rotor at 9000 rpm for 20 min at 4°. The supernatant was overlaid gently onto 5 ml of a CsCl solution (5.7 M CsCl, 0.1 mM EDTA) in a centrifuge tube and centrifuged in a Beckman fixed angle 50Ti rotor at 44,000 rpm overnight at 20°. The total RNA pelleted at the bottom of the tubes was resuspended in 0.1% SDS, 50 mM EDTA (pH 7.8), extracted once with water-saturated phenol, once with chloroform, and recovered by ethanol precipitation. The total RNA was resuspended in diethylpyrocarbonate-treated deionized water or deionized formamide and stored at -80°.

Southern and Northern blot analysis: Southern blots were analyzed by standard techniques (AUSUBEL *et al.* 1992). Genomic DNA from *N. crassa* was isolated by the procedure of SCHECHTMAN (1986). RNA samples were electrophoresed and blotted as described (FOURNEY *et al.* 1988; AUSUBEL *et al.* 1992). Labeled probes were prepared using an oligolabeling kit (Pharmacia, Baie D'Urfe, PQ).

Restriction fragment length polymorphism (RFLP) mapping of the alternative oxidase gene: The location of the alternative oxidase gene on *N. crassa* chromosomes was determined by RFLP mapping (METZENBERG *et al.* 1984, 1985).

DNA from each of the RFLP mapping strains (Fungal Genetics Stock Center strains #4450 through #4488) was isolated (SCHECHTMAN 1986), and ~5 µg from each was digested separately with appropriate restriction enzymes. Restriction fragments were separated by electrophoresis through 0.8% agarose gels and transferred to nylon membrane for Southern blot analysis. The segregation of the restriction fragment patterns, detected using a labeled cosmid containing the alternative oxidase gene as the probe, was compared with the segregation of known genetic and other RFLP markers (METZENBERG *et al.* 1984, 1985; METZENBERG and GROTELUESCHEN 1993) to determine the chromosomal location of the cloned gene.

Primer extension and reverse transcriptase (RT)-PCR: The primer extension reaction was performed using SUPERSCRIPT-RT (Gibco-BRL, Burlington, ON), a modified reverse transcriptase lacking RNaseH activity. Use of this enzyme prevents the degradation of mRNA during the first strand DNA synthesis reaction. Total RNA (1 µg) isolated from wild-type strain NCN10 mycelium grown in the presence of chloramphenicol was combined with 1 µl of [γ -³²P]-ATP end-labeled primer (MANIATIS *et al.* 1982) in a total volume of 14 µl, heated to 70° for 10 min, and then quick chilled on ice. After a brief centrifugation, 2 µl of 10× synthesis buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl, 25 mM MgCl₂), 1 µl of 10 mM dNTP mix, 2 µl of 0.1 M dithiothreitol and 1 µl of Superscript RT (200 U/µl) were added to the tube. The reaction was incubated at room temperature for 10 min, then transferred to a 42° water bath for 50 min, and terminated by incubating the tube at 90° for 5 min. The RNA was hydrolyzed by adding NaOH to a final concentration of 0.4 M and incubating at 65° for 30 min. The NaOH was neutralized by an equimolar concentration of HCl. The cDNA was recovered by ethanol precipitation and washed three times with 70% ethanol. The DNA pellet was briefly dried and resuspended in 50 µl of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA. Before loading onto the sequencing gel, 1 µl of the above cDNA solution was combined with 3 µl of sequencing stop solution and placed in a boiling water bath for 2 min. Proper dilutions were made

-1354 ctgatctcaatatacaggaatcgccccattatccctcccgtttatgttttaccaagcactgacttccctcgtacgtcttgctgtaccatc -1260
 ccttctgaataaaccacttccctcccacatcttgggtatatacaatgcctccctccagaaggettattgcggggtcagtgctgtgcgacgggcccctcagtcagggtcggtgctgagtc -1140
 ctgacaaaaccatcagttcgccaacatgcccgtcttatttccctttttcttttctctctttaggacatcttgatatacgacgatgcacacatataatgtacgg -1020
 cctcggtcaagcagcagtgctaggtgcagcaggcctagaaggatggcttttggagaagctgttaacgcagtggtggagagaatgattcataaaacaactagggtggatgctaaagctctgc -900
 tctttcaccaaaatggtctggaccaggagtcacccgaaaatgccctcgtcgtcaatatttgtacaccactccaccccgttccgacacgggataaatattggacacttgcattgttc -780
 cttcgatctacactctacgaataactaccactgacgtcacagcaaaagttagaagtttaacatgaagccattcgcaacttggccccgcacattgttltgatatttgaggttcggaa -660
 gcaaccaagccattgctcagtcagtgctgagtgctctatgagaacaagtgcgactggtgtccgtttccctccgctcgctattactgtcagaaaaatctccaacacataaactcgccaagt -540
 gccctgtcaaccaaccaactatcaatgatctcgaatctcctgtggctgcttctgtgtgagtcacaatccccagctcttagtgtacttgcctcaggggctcgctgttctctggat -420
 atgtacaaggctaatcccagaaatggtgcggaagtggaactgcgcctatggctgtcaccocgggaggtcccgcacccgcaaacacagacatccagctgaccaccacaatagatgccg -300
 gttgccactttgaggattcaaaatgagattttgctcgtgtagaatctggagcttccgggttcccttgcgtagcggcctatttgcctgttccctggattgtcttgatgttaaaaaat -180
 ggagattgcttggcagtgctggaactctattgctcctttgagaccagcagcagcaaacctcggtgtttcagtcagctctcgtattcctcctgaaaggagttgcaactggg -60

 +1 A012> | <A021
 ggcagaaaggacgataaaacgtcccgtgctagtgctgtccgacacataggaccatcaacaaacctcaagcaggttccattacaacttcacatcactccctaaactctcg ATG 59

 <A05
 AAC ACC CCC AAA GTA AAC ATA CTC CAC GCT CCA GGA CAA GCG GCC CAA CTA AGC CGT GCC CTG ATA TCA ACC TGC CAT ACT CGG CCT CTC 149
 N T P K V N I L H A P G Q A A Q L S R A L I S T C H T R P L 31
 G C
 CTG CTC GCG GGC TCT CGA GTG GCC ACT TCC TTA CAT CCA ACA CAG ACA AAC CTC TCT TCC CCA TCA CCT CGC AAT TTC TCG ACA ACA AGC 239
 L L A G S R V A T S L H P T Q T N L S S P S P R N F-L S T T S 61
 G T T A C T C G A C T G A A G A T T T C T T C C G G C A A A G A G A C C G C C T A T A T C C G G C A G A C C A C C C G C G T G G C C T C A T C A T G G A T G G A C A G A G 329
 V T R L K D F F P A K E T A Y I R Q T P P A W P H H G W T E 91
 G A A G A G A T G A C C T C G G T T C C C G A G C A C C G G A A A C C C G A G A C T G T G G C G A T T G G C T C G A T G G A A A C T C G T A C G A A T C T G T C G G t a g g t 421
 E E M T S V V P E H R K P E T V G D W L A W K L V R I C R 120
 A g
 aaattccaaggagggttcccatattgcccctcaaggtatcttgcggaagcgggaagcagacgtagattcaaggtgttetaatatcaaacagA TGG GCC ACT GAT ATA GCG ACG 533
 W A T D I A T 127
 G G C A T A C G T C C A G A G C A A G T T G A T A A A C A C C C G A C C C G A C C A C C A C C A C C A C C C A A T G G g t a t g t t c 625
 G I R P E Q Q V D K H H P T T A T S A D K P L T E A Q W 155
 a
 t a t g g a g c c c t a c g a g g a c g g a a t g g c a a c a a g c t a a c c a a a t g t c g c g a a t c c a t a t a g C T C G T C G C T T C A T C T T C G A A T C C G C G G C G T T C C C 729
 L V R F I F L E S I A G V P 169
 C
 G G C A T G T A G C C G G C A T G C T C G C C A C C T C C G T C G G C T C A A C G A G A C A A C G G C T G G A T C G A G A C T T T A C T T G A A G A A T C G 819
 G M V A G M L R H L H S L R R L K R D N G W I E T L L E E S 199
 d e g e n e r a t e 5 ' p r i m e r >
 T A C A A C G A G C G C A T G C A C C T C C A C C T T T A T G A A G A T G T G C G A A C C C G G C C T C T C A T G A A G A C G C T C A T C T T G G A G C G C A G G C G T C 909
 Y N E R M H L L T F M K M C E P G L L M K T L I L G A Q G V 229
 <A01
 T T C T T C A A C G C C A T G T T T C T C A G C T A C C T G A T C T C C C A A A T C A C C C A C C G G T T T G T C G G T T A C C T C G A G G A G G C C G T A C A T A C C 999
 F F N A M F L S Y L I S P K I T H R F V G Y L E E E A V H T 259
 <degenerate 3' primer
 T A C A C G C G G T G C A T C A G G G A G A T T G A G G A A G G T C A C T T G C C A A A G T G G A G C G A C G A A A A G T T T G A G A T C C C G A G A T G C G G T G A G G T A T 1089
 Y T R C I R E I E E G H L P K W S D E K F E I P E M A V R Y 289
 C
 T G G C G C A T G C C G A G G G A A G C G G A C G A T G A A G A C T T G A T C A T C A T C G C G C G A C G A G G C A G T G C A T A G G G C G T T A A T C A T A C A 1179
 W R M P E G K R T M K D L I H Y I R A D E A V H R G V N H T 319
 T
 C T G A C C A A T T T G A C C A G A A G G A G G A T C C G A A T C C G T T T G T G A G C G A C T A T A A G G A G G C G A G G C G G G A G G A G A C C G G T C A A T C C G G C T 1269
 L S N L D Q K E D P N P F V S D Y K E G E G G R R P V N P A 349
 C
 T T G A A G C C G A C G G A T T G A A A G G C G G A G G T C A T C G G T T G A t g t t g g g c g g a a g g t c t t g a c a g a t g g t t g t g t t t g g g t t c a t g a a g c c a g g c g t t t t t g g a c 1375
 L K P T G F E R A E V I G 362
 | polyadenylation site
 c a a g t t g t g t a t t a t g c g t g t a c a c t a g a t a c c c c c c t c t c t t t g c t g t t t t g c g g t t t t g t a a a a a g a t a c t g g g t t c t t c t c t c t g t g c a g c a g t t c a c t g t t c a a g g t g t g t 1495
 a c t t g t t t c g g a t c t t c a c t c c g a a g t c g t t g a t c t c a t g a t c a c t a t a t a g c a a t a a c a a a c c t g a t a t g t c a a g c c t t t t c g a t c c g t t c c c c g g g t a a c t a t c c g g c t c g a g 1615
 t t c a c a t t t g c a g c t t c t a c c c a a c g c a t a g a a a c c a a g g t a g t g a a c g t g c t c a c t t g a a t t c g a t c a g t g a t t a a a t t a t g t g c t c a g c c a t g a t c a a c c g a a t a t a t c g t g a c g g 1735
 c g a g t c a t g g a c c t t c c c g t g g t t t g c t g a t a t a c c t a g g a t g a t a g c t c a a g a c g t c c a t t a g a g t g t c a t a t t t a c a c g c g t a g t g t a g g c a c c c t c g a a a t g a a g c g g g g a a 1855
 t g a t g t g a a a c a a t g g t a t t a t g a g c g a t a t a c a a a g t c a a a t a a t g g c t c g a a g a a g a a a t g t t g g t c g a a c g a a a t a c a g g g c t g t g g c t g t c a a g c c a c t g a a g t g c a a t t 1975
 t g a t a g c g t g g t g a a c t t t g g g t a a c a t a t t t g c c g g a g a t c t 2020

FIGURE 1.—Nucleotide sequence of the *N. crassa* alternative oxidase gene, derived from strain NCN53, and deduced amino acid sequence. Coding sequences are shown in uppercase. Introns and other untranslated sequences are indicated in lowercase. Numerals in normal font designate nucleotides and italicized numerals indicate amino acids. The C determined to be the major transcription start site is designated as +1. Putative regulatory elements including a TATA box, a CAAT box, and a *N. crassa* transcription start consensus sequence (TCATCANC) (BRUCHEZ *et al.* 1993) are indicated with asterisks below the sequence. The cAMP-responsive element (CRE) sequence is indicated by carets. The polyadenylation site found in the cDNA is marked by a vertical line above position 1469. Polymorphisms observed in all sequences determined from genetic backgrounds different

CAAT and TATA boxes as well as near matches to the consensus sequences for *N. crassa* transcription and translation start sites (FIGURE 1) (BRUCHEZ *et al.* 1993). We also identified a perfectly conserved (TGACGTCA) cAMP-responsive element (CRE) at position -746 to -739. This sequence element was originally demonstrated to be involved in cAMP-mediated regulation of eukaryotic gene transcription and has been shown to bind specific transcription factors in a variety of systems (ROESLER *et al.* 1988; HABENER 1990; MEYER and HABENER 1993; LALLI and SASSONE-CORSI 1994).

A cDNA clone of the alternative oxidase gene was isolated from a preexisting wild-type cDNA library, constructed from a culture grown under standard conditions, as described in MATERIALS AND METHODS. The clone represents a nearly full-length cDNA, except that ~100 bp at the 5' end do not match the genomic sequence, and it lacks the predicted AUG start codon. Thus, this clone proved to be useful for defining the sites of introns in the genomic sequence, but inappropriate for determining the 5' end of the transcript (see below). Comparison of the cDNA and genomic sequences revealed introns of 96 and 70 bp in the genomic sequence. The sequences at the boundaries of both introns and their putative splice sites match the consensus sequences deduced from other *N. crassa* nuclear genes (BRUCHEZ *et al.* 1993). Translation of the sequence with introns removed demonstrated that the alternative oxidase gene encodes an open reading frame of 362 amino acids, predicting a polypeptide with a molecular mass of 41,436 Da (Figure 1). The codon usage (not shown) of the alternative oxidase gene most closely resembles that of genes expressed at low levels in *N. crassa*, such as *cyt-2* (DRYGAS *et al.* 1989), in that a high proportion of codons (58 of 361) contain A in the third position.

Seven genetic polymorphisms, relative to the original genomic sequence from plasmid pAOGB-11 (derived from strain NCN53), were observed in sequences derived from both the cDNA (74A genetic background) and the genomic sequence of the *aod* mutant strains examined (see below, 74A or unknown genetic backgrounds). The polymorphisms are indicated on Figure 1. Only one leads to an amino acid change, Leu in the 74A background *vs.* Phe in the NCN53 background at codon position 57. This position is poorly conserved in other alternative oxidases and occurs within the predicted mitochondrial targeting sequence of the *N. crassa* protein (see below).

Computer analysis predicted a mitochondrial targeting peptide that is rich in positively charged amino acids, lacks acidic amino acid residues, and has a high content of hydroxylated residues, all features characteristic of mitochondrial targeting signals (HARTL *et al.* 1989) and consistent with the mitochondrial location of the enzyme. Using the "R at position -10" pattern (HENDRICK *et al.* 1989; GAVEL and VON HEIJNE 1990) to

predict the most likely cleavage site of the presequence suggested an intermediate cleavage preceding the Phe residue at position 57 and a second cleavage following the Arg residue at position 64. Thus, the mature protein would start with a Leu residue (Figure 1) and have a molecular mass of 34,682 Da. When the deduced protein sequence was compared to the alternative oxidases from the yeast *Hansenula anomala* (SAKAJO *et al.* 1993) and two plants, *Sauromatum guttatum* (RHOADS and MCINTOSH 1991) and *Arabidopsis thaliana* (KUMAR and SOLL 1992), several regions of close similarity were found (Figure 2). The most highly conserved regions are clustered in the central regions of the protein. Membrane spanning domains predicted in all four proteins show good positional alignment. Pairwise alignments of the *N. crassa* alternative oxidase protein with that from each of the other species revealed that the greatest degree of identity occurs with the protein from *H. anomala*. The use of both the *N. crassa* and *H. anomala* sequences for alignment to the plant sequences produced a somewhat different alignment in the N-terminal one-third of the protein than previous alignments employing only *H. anomala* (SAKAJO *et al.* 1993; VANLERBERGHE and MCINTOSH 1994). There are several amino acids that are completely conserved among all the sequences. These have been suggested as potential metal binding sites (VANLERBERGHE and MCINTOSH 1994) and include the Cys at 119 and His at positions 178, 205, 246, 313 and 318 (numbering based on the *N. crassa* protein sequence shown in Figure 2). The completely conserved Cys (position 119) may also be a candidate for the residue involved in the disulfide linkage that results in the formation of homodimers of the alternative oxidase (UMBACH and SIEDOW 1993; UMBACH *et al.* 1994; VANLERBERGHE *et al.* 1995).

Mapping the transcription start site of the alternative oxidase gene: Since the isolated cDNA clone contained a 5' end that did not match any region of the 1.4-kb genomic sequence that has been obtained upstream of the alternative oxidase translation start site, it seemed likely that this was an artifact that arose during the construction of the library. Therefore, we wished to determine if a transcript that contained the genomic sequence immediately upstream of the translation start existed in cells induced to contain alternative oxidase. RT-PCR was performed on total RNA isolated from a culture induced for alternative oxidase by growth in chloramphenicol, using primers specific for the alternative oxidase gene designed from the genomic sequence. Primer AO1 is complementary to a region of the coding strand within the structural gene, while AO12 begins in a region that matches the consensus sequence for a transcription start site in *N. crassa*, ~50 bp upstream of the translation start codon (Figure 1). The position of primers chosen allowed us to distinguish between authentic RT-PCR products, produced by synthesis of cDNA from mRNA, and any product

that might be formed from genomic DNA contamination in the RNA preparation. That is, a PCR product formed from genomic DNA should contain the two introns (Figure 1) and would be 166-bp longer than the predicted mRNA/cDNA product. A single PCR product of the expected size was detected and its sequence was determined by direct sequencing of the PCR product (not shown). The sequence is identical to the genomic sequence except for the absence of the two predicted introns proving the existence of a contiguous mRNA containing the predicted leader region joined to the structural gene. We conclude that the 5' end sequence in the original cDNA clone is most likely an artifact derived by an unknown mechanism during the creation of the library. Although the possibility that the cDNA represents a splicing product whose 5' end is derived from a distant upstream exon cannot be excluded, the observation of a single band on Northern blots (see below) favors the notion that a single transcript is produced from the gene.

To map the authentic 5' end of the transcript, primer extension was performed using two different primers. AO5 is an 18-base oligonucleotide corresponding to the region ~30 bp downstream of the AUG start codon and ~90 bp downstream of the predicted +1 transcription start site. AO21 is a 22-base oligonucleotide from the region immediately upstream of the predicted AUG start codon and 21 bp downstream of the predicted transcription start site. Figure 3 shows the primer extension products obtained with these two primers together with sequencing reactions using the same primers. The extension reaction using the AO21 primer gave one major band ending at the position designated as +1 in Figure 1, as well as several weaker and shorter bands. The latter are likely due to either the premature termination of RT or the presence of shorter incomplete primers in the synthetic oligonucleotide preparation. The AO5 primer extension reaction gave several bands. One of the major bands also matches the transcription start site deduced using primer AO21 (Figure 3). Given the lack of bands from the AO21 reaction that correspond to those in the AO5 reaction, it seems likely that the extra bands from AO5 are due to mispriming on unrelated transcripts. Based on these results, we conclude that the major 5' end of the alternative oxidase transcript is at the site indicated as +1 in Figure 1. The position of this site is in good agreement with the location of the transcription start site predicted by analysis of the DNA sequence. However, it is possible that there are other minor sites of initiation adjacent to the +1 position, since additional bands are present below the major initiation site in both extension reactions.

Genomic Southern hybridization of the alternative oxidase gene: To determine the number of copies of the alternative oxidase gene in *N. crassa*, genomic DNA was isolated from strain NCN53, digested with different restriction enzymes, and probed with the alternative

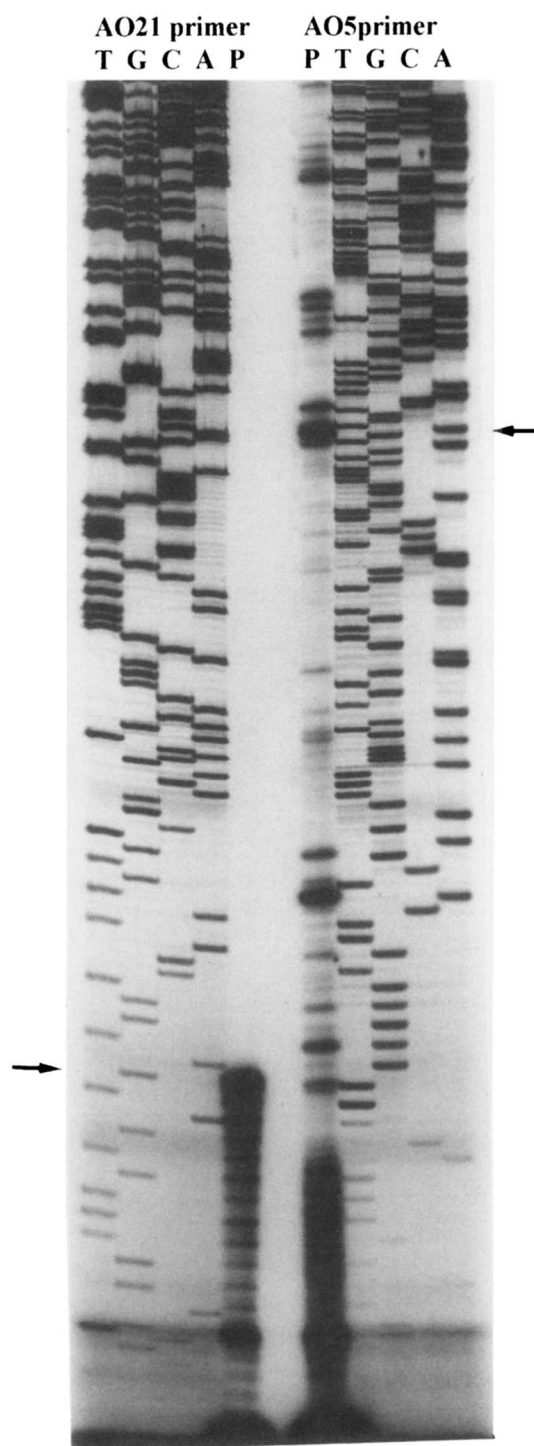


FIGURE 3.—Primer extension mapping of the 5' end of the alternative oxidase transcript. Primer extension was performed using oligonucleotides AO5 and AO21 (see Figure 1), both of which are complementary to the *aod-1* transcript and correspond to bases (bottom strand, 5' to 3') +111 to +93 for AO5 and +43 to +22 for AO21. The primer extension lane is indicated as P while A, C, G, and T indicate sequencing reactions performed using the same primer as in the extension reaction. The products of the reactions were analyzed by electrophoresis on a 10% polyacrylamide sequencing gel. The primer extension products determined to represent the most likely transcription start site are indicated by arrows.

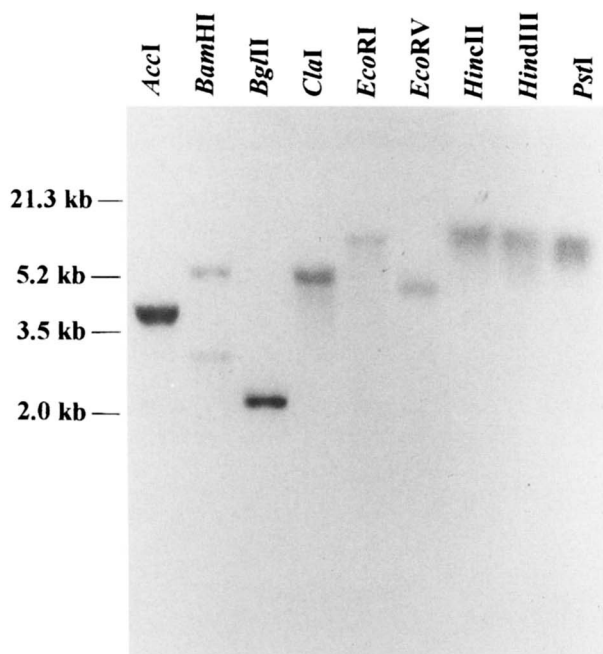


FIGURE 4.—Determination of the copy number of *aod-1*-like sequences in the *N. crassa* genome by Southern blot analysis. Genomic DNA (5 μ g) was digested with individual restriction enzymes, electrophoresed through a 0.8% agarose gel and transferred to a nylon membrane. The blot was probed with a labeled cDNA fragment containing the alternative oxidase structural gene. Because the cDNA contains a short illegitimate 5'-end, the hybridizing sequence begins immediately following the AUG start codon of the gene. Hybridization was detected by autoradiography. The restriction enzyme used for digestion of the genomic DNA is indicated on the top of each lane. DNA marker sizes in kb are indicated at the side. Within the region encompassed by the cDNA probe, there are no recognition sites for the restriction enzymes used with the exception of *Bam*HI and *Eco*RV. For *Eco*RV, the presence of a single hybridizing band is explained by the fact that one of the expected fragments contains only ~60 bp of sequence that would hybridize to the probe.

oxidase cDNA. The results shown in Figure 4 indicate that there is one copy of the alternative oxidase gene in *N. crassa*.

Localization of the alternative oxidase gene to a *N. crassa* chromosome: Two loci required for alternative oxidase function have been identified by genetic analysis of alternative oxidase deficient (*aod*) mutants in *N. crassa* (BERTRAND *et al.* 1983). The *aod-1* gene was mapped to LG IV, 23 map units to the left of the *trp-4* gene and close to the centromere (PERKINS *et al.* 1982; BERTRAND *et al.* 1983). The *aod-2* gene was genetically mapped to LG II, 7 map units to the left of *arg-5*, close to the centromere and *thr-3* (PERKINS *et al.* 1982; BERTRAND *et al.* 1983). To correlate the identity of the gene cloned in this study with one of the known genetic loci affecting alternative oxidase activity in *N. crassa*, RFLP mapping was performed using the method developed by METZENBERG *et al.* (1984, 1985) with cosmid 23F7 as the probe for the RFLP blots. Comparison of the segregation pattern of the cloned gene with that of

TABLE 2
Mutations in *aod-1* alleles

Mutant allele	Description
<i>aod-1-1</i>	C to T transition at codon 82, changes CCC (Pro) to CTC (Leu)
<i>aod-1-2</i>	As <i>aod-1-1</i>
<i>aod-1-4</i>	Single base pair deletion of the T in the third position of codon 130 causes frameshift. Mutated gene encodes a truncated protein of 249 amino acids.
<i>aod-1-6</i>	C to A transversion at codon 173, changes GCC (Ala) to GAC (Asp)
<i>aod-1-7</i>	Multiple changes that alter the sequence TCC (starting at bp 177, codon 41) to TTAA causes frameshift. Mutated gene encodes a truncated protein of 223 amino acids.

the published location of markers on *N. crassa* linkage groups revealed that the cloned alternative oxidase gene is near the centromere of LG IV (not shown) and corresponds well to the genetically determined location of the *aod-1* locus. During the course of this study, it was discovered that markers previously thought to be located on LG II (X17:10G and R64-3) (METZENBERG *et al.* 1993) are actually located on LG IV (R. L. METZENBERG, personal communication).

Characterization of *aod* mutant gene sequences: *N. crassa* mutants deficient in alternative oxidase activity do not have a detectable phenotype under normal growth conditions but have much reduced cyanide-resistant alternative oxidase activity when grown in the presence of chloramphenicol or antimycin A (EDWARDS *et al.* 1974, 1978; BERTRAND *et al.* 1983). To further examine the nature of the alternative oxidase mutants, we determined the sequence of the *aod-1* gene in five *aod-1* mutants and one *aod-2* mutant. All five *aod-1* mutants were found to have alterations in the coding sequence of the gene (Table 2). No alterations were detected in the *aod-2-4* mutant. The *aod-1-4* and *aod-1-7* mutants contain frameshift mutations, while *aod-1-6* contains a single missense mutation. The latter is a relatively severe change (Ala to Asp) that occurs in a position conserved as either an Ala or Gly (Figure 2, *N. crassa* amino acid 173) in all other alternative oxidases sequenced to date. Furthermore, this alteration affects a hydrophobic region that may form part of a membrane-spanning domain in all four of the proteins compared in Figure 2. The *aod-1-1* and *aod-1-2* mutants both contain the same mutation resulting in a single amino acid change (Pro changed to Leu, amino acid 82, Figure 2). Since this change was detected in two separate mutant strains, and because the alignment of alternative oxidases from various species does not show strong conservation in this region, we were concerned that the change at codon 82 might

simply reflect a natural polymorphism. Ideally, this could be resolved by determining the sequence of the gene in the strains involved in the lineage of these mutants. However, the strains involved could not be identified with certainty. To determine indirectly if the change represented a polymorphism, we determined the sequence of PCR-amplified DNA of the *aod-1* gene in a number of standard laboratory strains and natural isolates including NCN7, NCN20, NCN27, NCN34, NCN35, NCN39 and NCN57 (Table 1). None of these strains contained the change observed in the *aod-1-1* and *aod-1-2* mutants and we conclude that the change observed is the cause of the alternative oxidase deficiency. This conclusion is supported by the finding that the mutant respiratory phenotype in both strains can be rescued by transformation with the cloned *aod-1*⁺ gene (not shown). Transformation with the *aod-1*⁺ gene also rescues the phenotype of *aod-1-4*, *aod-1-6*, and *aod-1-7* but does not restore alternative oxidase activity to the *aod-2-4* mutant.

Northern analysis of the alternative oxidase transcript: It was of interest to determine directly if the induction of *aod-1* gene expression was at the level of transcription. Figure 5 shows the hybridization of an alternative oxidase probe to RNA isolated from wild-type strain NCN251 grown in either the absence or presence of chloramphenicol and three cytochrome-deficient mutants, *cyt-2-1*, *cyb-1-1*, and [*poky*] grown in the absence of chloramphenicol. In wild-type, the presence of chloramphenicol resulted in a highly induced level of the transcript whereas in cultures grown without chloramphenicol, the transcript was barely detectable, even following longer exposure times. The transcript was also present in the three cytochrome-deficient mutants, without chloramphenicol induction. Cytochrome-deficient mutants have been shown to possess the alternative respiratory pathway under normal growth conditions (BERTRAND *et al.* 1983; LAMBOWITZ *et al.* 1989). The results shown in Figure 5 strongly suggest that the induction of alternative oxidase gene expression is at the transcriptional level and are in agreement with earlier findings that the transcriptional inhibitor actinomycin D impaired the induction of the alternative pathway (EDWARDS and UNGER 1978). However, the present data do not exclude the possibility that mRNA stability (SURDEJ *et al.* 1994) may also be involved in alternative oxidase regulation.

The mRNA level of the alternative oxidase was also examined in several *aod* mutants (Figure 6). In all cases, the mRNA of the alternative oxidase was barely detectable in cultures grown under normal conditions but was induced in the presence of chloramphenicol. However, even allowing for unequal loading of samples on the gel, the level of the induced mRNA in *aod-2-4*, *aod-1-4*, *aod-1-6* and *aod-1-7* appears to be lower than in wild type and the *aod-1-1* and *aod-1-2* mutants (Figure 6). The decreased mRNA levels in *aod-1-4*, *aod-1-6*, and *aod-*

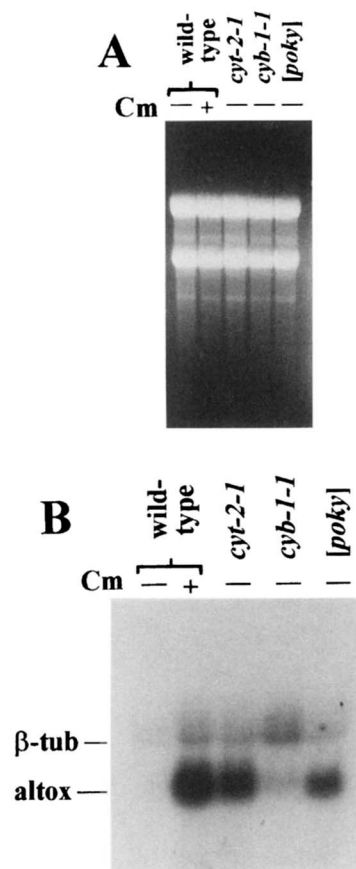


FIGURE 5.—Northern blot analysis of alternative oxidase mRNA in wild-type strain NCN251 and the cytochrome-deficient mutants *cyt-2-1*, *cyb-1-1*, and [*poky*]. Each lane contains 15 μ g of total RNA isolated from the strain indicated on the top of the figure. Cultures were either induced for alternative oxidase by growth in the presence of chloramphenicol (Cm+) or not induced for the enzyme by growth in the absence of chloramphenicol (Cm-). RNAs were electrophoresed through a formaldehyde-agarose gel and transferred to a nylon membrane as described in MATERIALS AND METHODS. The membrane was hybridized to probes for the alternative oxidase (indicated as altox in the figure) and β -tubulin (indicated as β -tub). The alternative oxidase probe was a 2.3-kb *Bgl*II fragment containing the entire structural gene. The β -tubulin probe was the *Sal*I fragment from plasmid pSV50 (VOLLMER and YANOFSKY 1986). The identity of the transcripts was determined by knowledge of their predicted sizes and comparison to RNA size standards run on the same gel (not shown). (A) Ethidium bromide stained gel. (B) Autoradiograph following hybridization. The β -tubulin gene was chosen as an internal control for RNA loading. However, as shown in B, β -tubulin appears to be inappropriate for these studies as its expression is elevated by the presence of chloramphenicol in the growth medium. Ethidium bromide staining (A) suggests that RNA loading in each lane is fairly uniform.

1-7 under induced conditions could be due to enhanced degradation of mRNA as a consequence of the particular mutations in each mutant. The decreased mRNA level seen in the *aod-2-4* mutant strain grown in the presence of chloramphenicol is presumably due to a mutation at the *aod-2* locus that results in decreased expression of the *aod-1* gene.

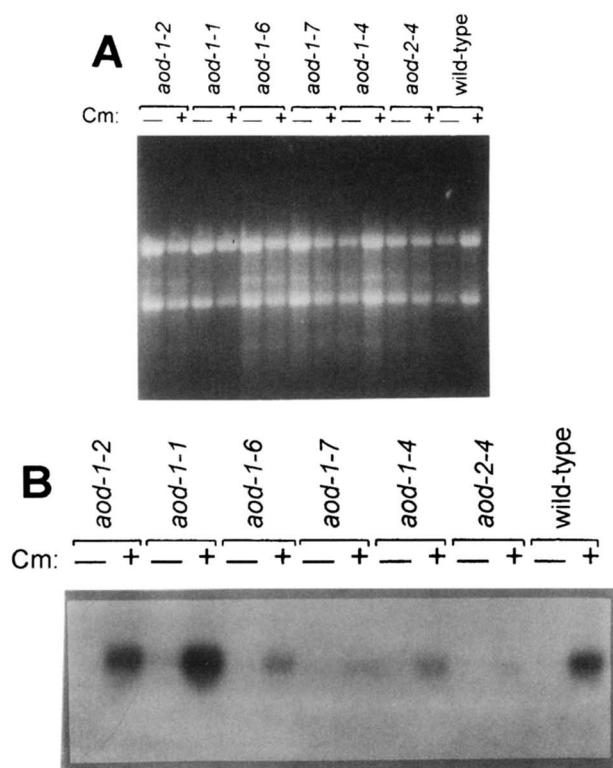


FIGURE 6.—Northern blot analysis of the alternative oxidase mRNA in *aod* mutants. Each lane contained $\sim 5 \mu\text{g}$ total RNA isolated from cultures grown under conditions that were either noninducing (no chloramphenicol in the medium, Cm $-$) or inducing (presence of chloramphenicol, Cm $+$) for alternative oxidase expression. The alternative oxidase probe was the same as described for Figure 5. The β -tubulin probe used in Figure 5 was omitted. (A) Ethidium bromide-stained gel. (B) Autoradiograph following hybridization.

DISCUSSION

Using degenerate PCR primers designed from the most conserved regions of alternative oxidase proteins from other species, we have isolated and determined the sequence of both genomic and cDNAs for the alternative oxidase of *N. crassa*. The isolated gene encodes a protein homologous to the *A. thaliana* protein that has been shown to impart KCN-insensitive, salicylhydroxamic acid-sensitive respiration on *E. coli hemA* mutant cells that lack cytochromes (KUMAR and SOLL 1992). RFLP mapping of the cloned DNA and rescue of mutants with the cloned gene define it as the *aod-1* gene of *N. crassa*, previously described in genetic studies of alternative oxidase mutants. Furthermore, *N. crassa* mutants previously mapped to the *aod-1* locus were found to have mutations affecting the coding sequence of the cloned gene. Taken together, these data provide direct evidence that *aod-1* is the structural gene of the alternative oxidase in *N. crassa*. However, these observations do not rule out the possibility that the alternative pathway may require other proteins for full activity. Such proteins might be involved in assembly or maintenance of active alternative oxidase.

In *N. crassa* alternative oxidase activity is only detectable in cultures where mitochondrial function has been impaired by inhibitors or mutations (LAMBOWITZ *et al.* 1972; EDWARDS *et al.* 1978; BERTRAND *et al.* 1983). In this study we have shown that the mRNA of the *aod-1* gene is rare in wild-type cultures grown under normal conditions, but it is highly prevalent in cultures grown in the presence of chloramphenicol, an inhibitor of mitochondrial protein synthesis. It is also present in three cytochrome-deficient mutants examined, *cyt-2-1*, *cyb-1-1* and [*poky*]. It is conceivable that an increase in *aod-1* mRNA stability, under conditions in which normal respiration is compromised, plays a role in the expression of the alternative oxidase (SURDEJ *et al.* 1994). However, the simplest interpretation would be that induction occurs at the transcriptional level as previously suggested (EDWARDS *et al.* 1974; EDWARDS and ROSENBERG 1976). Consensus sequences for CAAT and TATA boxes are observed in the appropriate locations immediately upstream of the *aod-1* gene, and it seems likely that sites required for controlling the expression of the gene reside upstream of these sequences. In this regard, the CRE found in the -740 region seems an obvious potential site of regulation. CREs have been shown to bind transcription factors whose activity is influenced by a variety of conditions that are communicated via signal transduction pathways (ROESLER *et al.* 1988; HABENER 1990; MEYER and HABENER 1993; LALLI and SASSONE-CORSI 1994). A CRE has been found in another *N. crassa* gene that was characterized separately as a glucose-repressible gene (MCNALLY and FREE 1988; WANG *et al.* 1994) and as a clock-controlled gene (LOROS *et al.* 1989). We are currently investigating the functional role of the CRE and other regions upstream of the *aod-1* coding sequence. Preliminary results from gel retardation assays have shown that a DNA fragment (-775 to -557) including the CRE is capable of binding a protein from *N. crassa* cell extracts. A related fragment (-718 to -504) that does not include the CRE shows no evidence of binding (Q. LI and F. E. NAR-GANG, unpublished results).

The mRNA level of the alternative oxidase in the *cyb-1-1* mutant is not as high as in the other two cytochrome-deficient mutants, *cyt-2-1* and [*poky*]. The latter two grow extremely slowly, while *cyb-1-1* grows only slightly slower than wild type. The cytochrome deficiencies in *cyt-2-1* and [*poky*] are severe (BERTRAND and PITTENGER 1972; BERTRAND *et al.* 1977), while *cyb-1-1* is only partially deficient in cytochrome *b* (BERTRAND and COLLINS 1978). These observations suggest that the induction of the alternative respiratory pathway is regulated in response to the flux of electrons through the cytochrome-mediated respiratory pathway.

Mutants in the *aod-2* gene are also deficient in alternative oxidase activity (BERTRAND *et al.* 1983). It was previously suggested that the function of *aod-2* was to encode either a component that regulates induction of

alternative oxidase activity or a protein required for stable accumulation of the *aod-1* polypeptide (BERTRAND *et al.* 1983; LAMBOWITZ *et al.* 1989). The low level of *aod-1* mRNA observed in the *aod-2-4* mutant grown under inducing conditions favors the notion that the *aod-2* gene product is a factor involved in transcriptional induction of the *aod-1* gene. Presumably the product of the *aod-2* gene responds to, interprets, or transmits to the *aod-1* gene, a signal generated in mitochondria due to inefficient cytochrome-mediated respiration.

A number of models have been proposed to explain alternative oxidase induction. EDWARDS *et al.* (1974) suggested a scheme that was based on a model originally devised by BARATH and KÜNTZEL (1972) for the regulation of nuclear genes specifying the mitochondrial genetic apparatus of *N. crassa*. The model assumes that the regulation is achieved by the direct control of a repressor-like mitochondrial gene product(s) that binds to the controlling elements of relevant nuclear genes. As long as mitochondrial protein synthesis continues to function normally, repressor protein is synthesized and the nuclear genes are repressed. Treatment with chloramphenicol results in inhibition of mitochondrial protein synthesis with the consequent derepression of the repressed nuclear genes. Although this model is somewhat oversimplified, it remains a formal possibility that may be involved in controlling the expression of *aod-1*. Other models incorporate various mechanisms for sensing changes in the level of cytochrome-mediated electron transport (ALLEN 1993; MINAGAWA *et al.* 1993). The presence of the CRE upstream of the *aod-1* coding sequence suggests that signals might be transmitted from the mitochondria to the nucleus via a signal transduction pathway.

Alternative oxidase regulation may also be influenced by more global mechanisms. Earlier experiments (SLAYMAN 1977) showed that growth of *N. crassa* cultures on medium containing ethanol as the sole carbon source led to a moderate induction of cyanide-resistant respiration, though cytochrome chain-mediated respiration still predominated. Similar effects have been observed in *H. anomala* (MINAGAWA and YOSHIMOTO 1987) and *Moniliella tomentosa* (VANDERLEYDEN *et al.* 1978). In the present study we observed that *N. crassa* cultures grown in medium containing either citrate or pyruvate as the sole carbon source contained cyanide-resistant respiratory activity (not shown). Thus, it is possible that alternative oxidase gene expression in *N. crassa* is subject to metabolite-mediated regulation, possibly by a mechanism related to the retrograde regulation proposed for the CIT2 gene in yeast (LIAO and BUTOW 1993). Certain intermediates could play a key role in connecting respiratory function with other metabolic activities whose level reflects the functional state of mitochondria. The stimulation of alternative oxidase activity in plant mitochondria by pyruvate (UMBACH *et al.* 1994; VANLERBERGHE *et al.* 1995) suggests that metabolites may also have a direct

effect on respiratory activity. Future studies, including defining the minimal sequence element(s) required for the transcriptional induction of the alternative oxidase gene and identifying regulatory proteins/effector molecules that activate or derepress its expression, should provide insight on the mechanism of the communication between mitochondria and nucleus.

This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada to F.E.N., the American Heart Association and Muscular Dystrophy Association to H.B., and by Department of Energy, National Science Foundation, and U.S. Department of Agriculture-CSRS grants to L.M.

LITERATURE CITED

- AKINS, R. A., and A. M. LAMBOWITZ, 1985 General method for cloning *Neurospora crassa* nuclear genes by complementation of mutants. *Mol. Cell. Biol.* **5**: 2272–2278.
- ALLEN, J. F., 1993 Redox control of transcription: sensors, response regulators, activators and repressors. *FEBS Lett.* **332**: 203–207.
- AUSUBEL R. A., R. BRENT, R. E. KINGSTON, D. D. MOORE, J. G. SEIDMAN, *et al.*, 1992 *Current Protocols in Molecular Biology*. Grene and Wiley Interscience, New York.
- BARATH, Z., and H. KÜNTZEL, 1972 Induction of mitochondrial RNA polymerase in *Neurospora crassa*. *Nature New Biol.* **240**: 195–197.
- BERTRAND, H., and R. A. COLLINS, 1978 A regulatory system controlling the production of cytochrome *aa₃* in *Neurospora crassa*. *Mol. Gen. Genet.* **166**: 1–13.
- BERTRAND, H., and T. H. PITTENGER, 1972 Isolation and classification of extranuclear mutants of *Neurospora crassa*. *Genetics* **71**: 521–533.
- BERTRAND, H., F. E. NARGANG, R. A. COLLINS and C. A. ZAGOZESKI, 1977 Nuclear cytochrome-deficient mutants of *Neurospora crassa*: isolation, characteristics and genetic mapping. *Mol. Gen. Genet.* **153**: 247–257.
- BERTRAND, H., C. A. ARGAN and N. A. SZAKACS, 1983 Genetic control of the biogenesis of cyanide insensitive respiration in *Neurospora crassa*, pp. 495–507 in *Mitochondria*, edited by R. J. SCHWEYEN, K. WOLF and F. KAUDEWITZ. Walter de Gruyter Co., Berlin.
- BRIDGE, P., and H. BERTRAND, 1983 The cytochrome oxidase inhibitor azide also inhibits the alternative pathway of *Neurospora crassa*. *Can. J. Biochem. Cell. Biol.* **62**: 129–136.
- BRUCHEZ, J. J. P., J. EBERLE and V. E. A. RUSSO, 1993 Regulatory sequences in the transcription of *Neurospora crassa* genes: CAAT box, TATA box, introns, poly (A) tail formation sequences. *Fungal Genet. Newsletter* **40**: 89–96.
- CHIRGWIN, J. M., A. E. PRZYBYLA, R. J. MACDONALD and W. RUFFER, 1979 Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**: 5294–5299.
- DAVIS, R. H., and F. J. DE SERRES, 1970 Genetic and microbiological research techniques for *Neurospora crassa*. *Methods Enzymol.* **17A**: 79–143.
- DRYGAS, M., A. M. LAMBOWITZ and F. E. NARGANG, 1989 Cloning and analysis of the *Neurospora crassa* gene for cytochrome *c* heme lyase. *J. Biol. Chem.* **264**: 17897–17906.
- EDWARDS, D. L., and E. ROSENBERG, 1976 Regulation of cyanide-insensitive respiration in *Neurospora*. *Eur. J. Biochem.* **62**: 217–221.
- EDWARDS, D. L., and B. W. UNGER, 1978 Induction of hydroxamate-sensitive respiration in *Neurospora* mitochondria. Transcription of nuclear DNA is required. *FEBS Lett.* **85**: 40–42.
- EDWARDS, D. L., E. ROSENBERG and P. A. MARONEY, 1974 Induction of cyanide-insensitive respiration in *Neurospora crassa*. *J. Biol. Chem.* **249**: 3551–3556.
- EDWARDS, D. L., J. H. CHALMERS, H. J. GUZIK and J. T. WARDEN, 1978 Assembly of the cyanide-insensitive respiratory pathway in *Neurospora crassa*, pp. 865–872 in *Genetics and Biogenesis of Chloroplasts and Mitochondria*, edited by T. BÜCHER, W. NEUPERT, W. SEBALD and S. WERNER. North-Holland Publishing Co., New York.
- ELTHON, T. E., and L. MCINTOSH, 1987 Identification of the alterna-

- tive terminal oxidase of higher plant mitochondria. *Proc. Natl. Acad. Sci. USA* **84**: 8399–8403.
- ELTHON, T. E., R. L. NICKELS and L. MCINTOSH, 1989 Monoclonal antibodies to the alternative oxidase of higher plant mitochondrial. *Plant Physiol.* **89**: 1311–1317.
- FOURNEY, R. M., J. MIVAKOSHI, R. S. DAY III and M. C. PATERSON, 1988 Northern blotting: efficient RNA staining and transfer. *Focus* **10**: 5–7.
- GAVEL, Y., and G. VON HEIJNE, 1990 Cleavage-site motifs in mitochondrial targeting peptides. *Protein Eng.* **4**: 33–37.
- GESSERT, S. F., J. H. KIM, F. E. NARGANG and R. WEISS, 1994 A polyprotein precursor of two mitochondrial enzymes in *Neurospora crassa*: gene structure and precursor processing. *J. Biol. Chem.* **269**: 8189–8203.
- HABENER, J. F., 1990 Cyclic AMP response element binding proteins: a cornucopia of transcription factors. *Mol. Endocrinol.* **4**: 1087–1094.
- HANSENS, L., E. D'HONDT and H. VERACHTERT, 1974 Cyanide-insensitive respiration in *Moniliella tomentosa* and effect of drugs on respiration and polyol biosynthesis. *Arch. Microbiol.* **98**: 339–349.
- HARTL, F.-U., N. PFANNER, D. W. NICHOLSON and W. NEUPERT, 1989 Mitochondrial protein import. *Biochim. Biophys. Acta* **988**: 1–45.
- HENDRICK, J. P., P. E. HODGES and L. E. ROSENBERG, 1989 Survey of amino-terminal proteolytic cleavage sites in mitochondrial precursor proteins: leader peptides cleaved by two matrix proteases share a three-amino acid motif. *Proc. Natl. Acad. Sci. USA* **86**: 4056–4060.
- HENRY, M. F., and E. J. NYNS, 1975 Cyanide-insensitive respiration: an alternative mitochondrial pathway. *Sub-Cell. Biochem.* **4**: 1–65.
- KUMAR, A. M., and D. SOLL, 1992 *Arabidopsis* alternative oxidase sustains *Escherichia coli* respiration. *Proc. Natl. Acad. Sci. USA* **89**: 10842–10846.
- LALLI, E., and P. SASSONE-CORSI, 1994 Signal transduction and gene regulation: the nuclear response to cAMP. *J. Biol. Chem.* **269**: 17359–17362.
- LAMBERS, H. 1982 Cyanide-resistant respiration: a non-phosphorylating electron transport pathway acting as an energy overflow. *Physiol. Plant.* **55**: 478–485.
- LAMBOWITZ, A. M., and C. W. SLAYMAN, 1971 Cyanide-resistant respiration in *Neurospora crassa*. *J. Bacteriol.* **108**: 1087–1093.
- LAMBOWITZ, A. M., E. W. SMITH and C. W. SLAYMAN, 1972 Electron transport in *Neurospora* mitochondria: studies on wild type and *poky*. *J. Biol. Chem.* **247**: 4850–4858.
- LAMBOWITZ, A. M., J. R. SABOUTIN, H. BERTRAND, R. L. NICKELS and L. MCINTOSH, 1989 Immunological identification of the alternative oxidase of *Neurospora crassa* mitochondria. *Mol. Cell. Biol.* **9**: 1362–1364.
- LIAO, X., and R. A. BUTOW, 1993 *RTG1* and *RTG2*: two yeast genes required for a novel path of communication from mitochondria to the nucleus. *Cell* **72**: 61–71.
- LOROS, J. J., S. A. DENOME and J. C. DUNLAP, 1989 Molecular cloning of genes under control of the circadian clock in *Neurospora*. *Science* **243**: 385–388.
- MANIATIS, T., E. F. FRITSCH and J. SAMBROOK, 1982 *Molecular Cloning Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- MCINTOSH, L., 1994 Molecular biology of the alternative oxidase. *Plant Physiol.* **105**: 781–786.
- MEEUSE, B. J. D., 1975 Thermogenic respiration in aroids. *Annu. Rev. Plant Physiol.* **26**: 117–126.
- METZENBERG, R. L., and J. GROTELUESCHEN, 1993 Restriction polymorphism maps of *Neurospora crassa*: update. *Fungal Genet. Newsl.* **40**: 130–133.
- METZENBERG, R. L., J. N. STEVENS, E. U. SELKER and E. MORZYCKA-WROBLEWSKA, 1984 A method for finding the genetic map position of cloned DNA fragments. *Fungal Genet. Newsl.* **31**: 35–39.
- METZENBERG, R. L., J. N. STEVENS, E. U. SELKER and E. MORZYCKA-WROBLEWSKA, 1985 Identification and chromosomal distribution of 5S rRNA genes in *Neurospora crassa*. *Proc. Natl. Acad. Sci. USA* **82**: 2067–2071.
- MEYER, T. E., and J. F. HABENER, 1993 Cyclic adenosine 3',5'-monophosphate response element binding protein (CREB) and related transcription-activating deoxyribonucleic acid-binding proteins. *Endocrine Rev.* **14**: 269–290.
- MILLAR, A. H., J. T. WISKICH and D. A. DAY, 1993 Organic acid activation of the alternative oxidase of plant mitochondria. *FEBS Lett.* **329**: 259–262.
- MINAGAWA, N., and A. YOSHIMOTO, 1987 The induction of cyanide-resistant respiration in the absence of respiratory inhibitors in *Hansenula anomala*. *Agric. Biol. Chem.* **51**: 2263–2265.
- MINAGAWA, N., S. KOGA, M. NAKANO, S. SAKAJO and A. YOSHIMOTO, 1992 Possible involvement of superoxide anion in the induction of cyanide-resistant respiration in *Hansenula anomala*. *FEBS Lett.* **302**: 217–219.
- MOORE, A. L., and J. N. SIEDOW, 1991 The regulation and nature of the cyanide-resistant alternative oxidase of plant mitochondria. *Biochim. Biophys. Acta* **1059**: 121–140.
- PERKINS, D. D., A. RADFORD, D. NEWMAYER and M. BJORKMAN, 1982 Chromosomal loci of *Neurospora crassa*. *Microbiol. Rev.* **46**: 2444–2448.
- RASKIN, I., A. EHMAMN, W. R. MELANDER and B. J. D. MEEUSE, 1987 Salicylic acid: a natural inducer of heat production in *Arum lilies*. *Science* **237**: 1601–1602.
- RHOADS, D. M., and L. MCINTOSH, 1991 Isolation and characterization of a cDNA clone encoding an alternative oxidase protein of *Sauromatum guttatum* (Schott). *Proc. Natl. Acad. Sci. USA* **88**: 2122–2126.
- ROESLER, W. J., G. R. VANDENBARK and R. W. HANSON, 1988 Cyclic AMP and the induction of eukaryotic gene transcription. *J. Biol. Chem.* **263**: 9063–9066.
- SAKAJO, S., N. MINAGAWA and A. YOSHIMOTO, 1993 Characterization of the alternative oxidase protein in the yeast *Hansenula anomala*. *FEBS Lett.* **318**: 310–312.
- SCHECHTMAN, M., 1986 A moderate scale DNA preparation for *Neurospora*. *Fungal Genet. Newsl.* **33**: 45–46.
- SCHWAB, A. J., 1973 Mitochondrial protein synthesis and cyanide-resistant respiration in copper-depleted, cytochrome oxidase deficient *Neurospora crassa*. *FEBS Lett.* **35**: 63–66.
- SCHWEIZER, M., M. E. CASE, C. C. DYKSTRA, N. H. GILES and S. R. KUSHNER, 1981 Identification and characterization of recombinant plasmids carrying the complete *qa* gene cluster from *Neurospora crassa* including the *qa-1⁺* regulatory gene. *Proc. Natl. Acad. Sci. USA* **78**: 5086–5090.
- SLAYMAN, C. W., 1977 The function of an alternative terminal oxidase in *Neurospora*, pp. 159–168 in *Functions of Alternative Terminal Oxidase*, edited by H. DEGN, D. LLOYD and G. C. HILL. Pergamon Press, Oxford.
- SURDEJ, P., A. RIEDL and M. JACOBS-LORENA, 1994 Regulation of mRNA stability in development. *Annu. Rev. Genet.* **28**: 263–282.
- UMBACH, A. L., and J. N. SIEDOW, 1993 Covalent and noncovalent dimers of the cyanide-resistant alternative oxidase protein in higher plant mitochondria and their relationship to enzyme activity. *Plant Physiol.* **103**: 845–854.
- UMBACH, A. L., J. T. WISKICH, J. N. SIEDOW, 1994 Regulation of alternative oxidase kinetics by pyruvate and intermolecular disulfide bond redox status in soybean seedling mitochondria. *FEBS Lett.* **348**: 181–184.
- VANDERLEYDEN, J., L. HANSENS and H. VERACHTERT, 1978 Induction of cyanide-insensitive respiration in *Moniliella tomentosa* by the use of n-propanol. *J. Gen. Micro.* **105**: 63–68.
- VANLERBERGHE, G. C., and L. MCINTOSH, 1994 Mitochondrial electron transport regulation of nuclear gene expression: studies with the alternative oxidase gene of tobacco. *Plant Physiol.* **105**: 867–874.
- VANLERBERGHE, G. C., D. D. DAY, J. T. WISKICH, A. E. VANLERBERGHE and L. MCINTOSH, 1995 Alternative oxidase activity in tobacco leaf mitochondria. Dependence on tricarboxylic acid cycle-mediated redox regulation and pyruvate activation. *Plant Physiol.* **109**: 353–361.
- VOGELSTEIN, B., and D. GILLESPIE, 1979 Preparative and analytical purification of DNA from agarose. *Proc. Natl. Acad. Sci. USA* **76**: 615–619.
- VOLLMER, S. J., and C. YANOFKY, 1986 Efficient cloning of genes of *Neurospora crassa*. *Proc. Natl. Acad. Sci. USA* **83**: 4869–4873.
- WANG, Z., M. DEAK and S. J. FREE, 1994 A cis-acting region required for the regulated expression of *grg-1*, a *Neurospora* glucose-repressible gene. Two regulatory sites (CRE and NRS) are required to repress *grg-1* expression. *J. Mol. Biol.* **237**: 65–74.