University of Alberta

Alternative oxidase expression in *Neurospora crassa*

by

Lesley Lorraine Tanton 

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To my family, without whose support this would not have been possible

Abstract

The fungus *Neurospora crassa* contains two respiration pathways. The standard cytochrome-mediated electron transport is present under normal growth conditions, but when the cytochrome path is disrupted, an alternative, cyanide-insensitive pathway becomes active. The alternative oxidase transfers four electrons directly from reduced ubiquinone to oxygen, bypassing two of three proton-pumping sites. The *N. crassa* alternative oxidase is encoded by the nuclear gene *aod-1*, while a second gene *aod-2* plays an undetermined role in regulating the expression of *aod-1*. The regulation of *aod-1* is largely unknown. The sequences of the upstream region of the *aod-1* gene were compared with the corresponding region of *aod-1* from the fungi *Gelasinospora* and *Aspergillus nidulans*. *N. crassa* and *Gelasinospora aod-1* shared a canonical cyclic-AMP response element (CRE), flanked by inverted repeats, approximately 800 base pairs from the start of the coding sequence. The *A. nidulans* sequence contained a CRE-related element, with seven of eight of the base pairs conserved, also flanked by short inverted repeats, about 730 bases from the start codon. Electrophoretic mobility shift assays revealed that *N. crassa* protein(s) bind specifically to the CRE, and that the binding factor is enriched in the 25% ammonium sulfate fraction. However, transformation of an *aod-1* mutant strain with constructs containing *aod-1*$^{+}$ and upstream sequence lacking the CRE produced transformants with normal regulation of KCN-insensitive respiration. An open reading frame (ORF) was identified upstream of *aod-1*, which was in opposite orientation to *aod-1* and expressed an approximately 1 kb transcript. The CRE is not likely to be involved in the regulation of this ORF, as the gene order of this region is not conserved between *N. crassa* and *A. nidulans*. Analysis

of *aod-1* expression patterns revealed variations in the amount of *aod-1* mRNA present in uninduced cultures, contrary to early reports suggesting that, upon induction, transcription was required for alternative oxidase expression in *N. crassa.* No alternative oxidase protein was ever found in these cultures. Nuclear run-on assays revealed that there is a basal level of transcription in uninduced cultures that is up-regulated upon inhibition of the cytochrome pathway. These data suggested that *aod-1* regulation involves both transcriptional and post-transcriptional mechanisms.

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Abbreviations

ADP	adenosine 5'-diphosphate
A. nidulans	*Aspergillus nidulans*
A. niger	*Aspergillus niger*
A. thaliana	*Arabidopsis thaliana*
AA	antimycin A
amp	ampicillin
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
bisacrylamide	N,N'-methylenebisacrylamide
bHLH/Zip	basic helix-loop-helix-leucine zipper
BLAST	basic local alignment search tool
bleo	bleomycin
bp	base pair
bromophenol blue	3',3",5',5"-tetrabromophenol sulfonphthalein
BSA	bovine serum albumin
Ca^{2+}	calcium ion
CaMKIV	calcium/calmodulin kinase IV
cDNA	complementary DNA
cm	centimeter
Cm	chloramphenicol
CNBr-Sepharose	cyanogen bromide Sepharose
cpm	counts per minute
CRE	cyclic-AMP response element
CREB	cyclic-AMP response element binding protein
CREM	cyclic-AMP response element modulator
CTP	cytidine 5'-triphosphate
°C	degrees Celsius
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
ddNTP	2',3'-dideoxynucleotide 5'-triphosphate
DEPC	diethylpyrocarbonate
dGTP	2'-deoxyguanosine 5'-triphosphate
DH_2O	distilled water
DMSO	dimethlysulfoxide
DMQ_9	demethoxy-Q_9
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
dTTP	2'deoxythymidine 5'-triphosphate
dUTP	2'deoxyuridine 5'-triphosphate
E. coli	*Eschericiha coli*
EDTA	ethylenediaminetetraacetic acid

EMSA	electrophoretic mobility shift assay
EST	expressed sequence tag
FGSC	Fungal Genetics Stock Center
g	gram
gDNA	genomic DNA
GMP	guanosine 5'-monophosphate
GTP	guanosine 5'-triphosphate
H_2O_2	hydrogen peroxide
H. anamola	*Hansenula anomola*
HEPES	N-[2-hydroxyethyl] piperazine-N'-2-ethanesulfonic acid
hr	hour
ICER	inducible cyclic-AMP early repressor
IMP	inosine 5'-monophosphate
IPTG	isopropyl-D-thiogalactoside
kan	kanamycin
kb	kilobase pair
KCN	potassium cyanide
kDa	kiloDaltons
l	liter
M	Mole/Molar
MAPK	mitogen activated protein kinase
M. grisea	*Magnaporthe grisea*
µCi	microCurie
mCi	milliCurie
µg	microgram
mg	milligram
min	minute
µl	microliter
ml	milliliter
mM	milliMole/milliMolar
mm	millimeter
mtDNA	mitochondrial DNA
mtHSP70	mitochondrial heat shock protein 70
MMO	methane monoxygenase
MOPS	3-(N-morpholino) propanesulfonic acid
MPP	matrix processing peptidase
mRNA	messenger RNA
MW	molecular weight
N_2	nitrogen
N. crassa	*Neurospora crassa*
N. intermedia	*Neurospora intermedia*
N. tetrasperma	*Neurospora tetrasperma*
ng	nanogram
O_2	oxygen
OD	optical density
ORF	open reading frame

PAGE	polyacrylamide gel electrophoresis
P. anserina	*Podospora anserina*
PCR	polymerase chain reaction
pDNA	plasmid DNA
PEG	polyethylene glycol
P_i	inorganic phosphate
PKA	protein kinase A
PMSF	phenylmethylsulfonyl fluoride
RNA	ribonucleic acid
RNase	ribonuclease
RNR R2	ribonucleotide reductase subunit 2
ROS	reactive oxygen species
rpm	revolutions per minute
SA	salicylic acid
S. guttatum	*Sauromatum guttatum*
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulfate
sec	second
SHAM	salicylhydroxamic acid
S. pombe	*Schizosaccharomyces pombe*
TAO	trypanosome alternative oxidase
T. brucei	*Trypanosoma brucei*
TCA	tri-carboxylic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TFB	TATA binding factor
TIM	translocase of the inner mitochondrial membrane
TOM	translocase of the outer mitochondrial membrane
Tris	tris (hydroxymethyl) aminomethane
Triton X-100	octylphenoxypolyethanol
tRNA	transfer RNA
Tween20	polyoxyethylenesorbitan monolaurate
UAMH	University of Alberta Microfungus Collection and Herbarium
UAS	upstream activating site
UAS_r	$UAS_{retrograde}$
uORF	upstream open reading frame
UTP	uridine 5'-triphosphate
UTR	untranslated region
V	volt
W	watt
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactoside

1 Introduction

1.1 Mitochondrial structure and function

Mitochondria are essential organelles found in all eukaryotes. They have four distinct subcompartments: the outer membrane, intermembrane space, inner membrane and matrix, each of which is involved in distinct biological functions. The inner membrane of the mitochondrion is organized into structures called cristae, which provide a larger surface area to contain the respiratory protein complexes that reside within the inner membrane. Early two-dimensional microscopy of mitochondrial substructure led to a model that described cristae as baffles, with large regions of intermembrane space surrounding the baffles; however, recent advances in three-dimensional microscopy have challenged this view (FREY and MANNELLA 2000). Analysis of rat liver mitochondria demonstrated that cristae are tubular, with discrete connections to the inner membrane called cristae junctions, which seemed to restrict the openings of the cristae to the intermembrane space. In rat neural mitochondria, the tubular cristae were observed to merge and form sheets that run parallel to each other, but the cristae still contain cristae junctions with the inner membrane (FREY and MANNELLA 2000). *Neurospora crassa* cristae seem to have sheets of parallel cristae as well (FREY and MANNELLA 2000). Recent observations support the theory suggesting that cristae are not rigid, static entities, but rather have a dynamic quality that allows them to adapt to changing cellular conditions. For example, the mitochondria of osteosarcoma cells form a reticulum in G1 phase that fragments into smaller tube-like structures during S phase (CAPALDI 2000). The presence of cristae junctions suggests that there may be less diffusion of materials throughout the mitochondria than the baffle model implies, which may allow mitochondria to regulate the rates of various metabolic reactions, including those of oxidative phosphorylation by regulating the localization of various metabolic components.

Recent advances in microscopy also provide evidence that the textbook view of isolated, bean-shaped mitochondria functioning independently was not entirely accurate. Much evidence has accumulated from a variety of organisms that mitochondria actually form a large dynamic reticulum that is present throughout the

cell. The reticulum may divide by fission and later join by fusion. These events appear to be related to the type of cell and stage of growth (NUNNARI et al. 1997). Recent micrographs of HeLa cells and rat liver cells also show long tube-like mitochondria and sheets of the endoplasmic reticulum interlaced with each other (MANNELLA 2000). The functional significance of the interaction is not yet known, although it has been suggested that Ca^{2+} storage and/or intracellular Ca^{2+} signaling may be involved (MANNELLA 2000).

The mitochondrial subcompartments carry out specialized functions. For example, the outer membrane contains the transport machinery involved in the import of cytosolically translated mitochondrial proteins into the mitochondria, which consist of receptors that recognize preproteins with mitochondrial targeting signals as well as a general import pore that the proteins pass through during import (NEUPERT 1997); (REHLING et al. 2001). The most common protein in the outer membrane is porin, which forms a pore for metabolites (PFANNER and GEISSLER 2001). The intermembrane space contains protein complexes that are involved in shuttling proteins targeted to the mitochondrial inner membrane or matrix to the appropriate inner membrane import machinery. Cytochrome *c*, a member of the electron transport chain, and cytochrome *c* heme lyase (CCHL), which covalently attaches a heme group to apocytochrome *c* (DUMONT et al. 1988; NICHOLSON et al. 1988), are also present within the intermembrane space. The inner membrane contains many components of the respiratory pathway involved in oxidative phosphorylation, the ATP synthase machinery as well as the inner membrane protein translocases needed for protein import into the inner membrane or matrix. In addition, the inner membrane contains the proteins required to transport ATP, ADP and phosphate between the matrix and the intermembrane space. The matrix contains the enzymes of Kreb's cycle and fatty acid oxidation. The matrix is also where the mitochondrial DNA (mtDNA) and its transcription and translation machinery are located (GRIFFITHS et al. 1995; KENNELL et al. 2002).

Mitochondria have their own genomes, encoding a small number of the proteins involved in oxidative phosphorylation as well as some of the proteins and specialized RNA molecules responsible for transcribing and translating the mtDNA.

Mitochondrial genome organization varies widely among species. In all species examined to date, mtDNA is circular. In metazoans, the mitochondrial genome is compact with few nucleotides that are not directly involved in coding for gene products or required for replication. For example, the *Caenorhabditis elegans* mitochondrial genome is 14 kb and the human mitochondrial genome is 16.5 kb. Within metazoan mitochondrial genomes most of the genes are conserved, but the gene order may differ, and the DNA sequence of the genes has diverged rapidly during vertebrate evolution. Plant mitochondrial genomes are much larger. The *Arabidopsis thaliana* mitochondrial genome is a mid-sized plant mtDNA at about 370 kb. The mtDNA of plants undergoes frequent recombination, but the DNA sequence divergence is quite low compared to animals. There are also large introns present in plant mtDNA, some of which contain open reading frames. Why plant mtDNA is so large is unclear, 63% of *A. thaliana* mtDNA has no known function. An unusual form of mitochondrial genome organization is seen in trypanosomes, where the mitochondria contain huge amounts of mtDNA organized as concatamers of 40 to 50 maxicircles, of about 30 kb each, and 5000 to 10 000 minicircles of 645 to 2500 base pairs each. The maxicircles encode 10 respiratory subunits and two ribosomal RNAs while the minicircles encode several guide RNAs used during extensive mtRNA editing (SCHEFFLER 1999). Fungal mtDNA is intermediate in size between plants and animals, and contains introns. *Neurospora crassa* has a 62 kb mitochondrial genome that encodes seven polypeptides of the NADH dehydrogenase (complex I), the apocytochrome *b* polypeptide of complex III, the three largest subunits of cytochrome oxidase (complex IV), the ATP6 and ATP8 polypeptides of mitochondrial ATP synthase, the large and small ribosomal RNAs, 27 tRNAs and ribosomal protein S5 as well as several unassigned open reading frames (GRIFFITHS et al. 1995; KENNELL et al. 2002). All of the remaining proteins needed for proper mitochondrial function are encoded in the nucleus, translated in the cytoplasm and imported into the mitochondria by translocases found in the outer and inner membranes.

Proper import and sorting of nuclear-encoded, cytosolically translated proteins into mitochondria are crucial to the functioning of the organelle. *Saccharomyces cerevisiae* and *N. crassa* have been the most extensively studied organisms with

respect to the import of proteins into mitochondria. Nuclear encoded messages for mitochondrial proteins are translated to form mitochondrial protein precursors on cytosolic ribosomes. Most mitochondrial precursor proteins contain presequences that have no consensus sequence but which are generally capable of forming an amphipathic helix that presents positively charged residues on one face and hydrophobic residues on the other (VON HEIJNE 1986; VON HEIJNE 1996). Presequences are removed in the matrix by the matrix processing peptidase (MPP). For precursors without presesequences, the targeting information is contained within the mature protein sequence. Precursors are recognized and bound by specific receptors of the multisubunit TOM complex (Translocase of the Outer mitochondrial Membrane) and are routed through the TOM complex translocation channel (MAYER et al. 1995; RAPPAPORT et al. 1997).

The *N. crassa* TOM holo complex contains at least seven different proteins: Tom70, Tom40, Tom22, Tom20, Tom7, Tom6 and Tom5 (AHTING et al. 1999; SCHMITT et al. submitted). Tom70 is a non-essential receptor that stimulates the import of a small number of precursors, including members of the ATP/ADP carrier family, and cytochrome c_1 (SCLOSSMANN et al. 1994; STAN et al. 2000). Tom20 is the receptor for the majority of precursors, including those bearing presequences (SOLLNER et al. 1989) and may act with Tom22 and Tom 40 to form the *cis* site to which the precursors bind prior to being translocated (RAPPAPORT et al. 1997). Regardless of which receptor precursors interact with, they enter the mitochondria through the GIP (General Import Pore), which consists of the tightly associated subunits Tom40, Tom22, Tom7, Tom6 and Tom5 (HONLINGER et al. 1995; KURZ et al. 1999; SCHMITT et al. submitted). Tom22 is an essential protein in *N. crassa* (NARGANG et al. 1995), but its role is controversial. It was proposed that negative charges in its cytosolic domain interact with the positively charged presequences of precursors (SCHATZ 1997), however removal of many of these charges from the *N. crassa* protein has little effect on import (NARGANG et al. 1998). The membrane spanning domain of Tom22 may be involved in organizing the TOM complex while its cytosolic domain is involved as a docking point for Tom70 and Tom20 (VAN WILPE et al. 1999). Tom40 is an essential protein in *N. crassa* (TAYLOR et al. 2002)

and in yeast (BAKER et al. 1990). It is the major component of the TOM complex and the translocation pore (HILL et al. 1998), and it may form the major portion of the *trans* binding site (RAPPAPORT et al. 1997). The *trans* site is located on the inner membrane side of the TOM complex and interacts with precursors as they are being translocated (RAPPAPORT et al. 1997).

Once precursors have entered the TOM complex channel and bound to the *trans* site, they interact with one of two multisubunit TIM complexes (Translocase of the Inner mitochondrial Membrane). Movement of precursors into or through the inner membrane requires an electrochemical gradient (GASSER et al. 1982). Precursors containing a presequence interact with the TIM23 complex, which is composed of Tim23, Tim17 and Tim44 (BERTHOLD et al. 1995). Tim44 attaches to the complex on the matrix side of the inner membrane. In the presence of membrane potential, Tim23 exists as a dimer and its N-terminal region integrates into the outer membrane (BAUER et al. 1996; DONZEAU et al. 2000). Once a presequence is encountered, the Tim23 dimer dissociates and opens a channel through the complex. The precursor proteins bind to mitochondrial Hsp70, which aids in its correct folding and ultimately requires ATP to release the precursor (VOOS et al. 1999). Subsequent mtHSP70 molecules binding the precursor brings it further into the matrix, but it is not known if the precursor is pulled through the pore by mtHSP70 or if transport is driven by Brownian motion (BAUER et al. 2000; VOISINE et al. 1999). Presequences are removed in the matrix by the matrix processing peptidase (BRUNNER et al. 1994). Many of the hydrophobic proteins of the inner membrane are imported by the TIM22 complex, which is composed of Tim22, Tim54 and Tim18 (KERSCHER et al. 1997; KERSHCER et al. 2000). The TIM22 complex interacts with intermembrane space proteins Tim9, Tim10, and Tim12 which form a complex that interacts with proteins to be imported by the TIM22 complex (ADAM et al. 1999) and prevent exposing the hydrophobic proteins to the hydrophilic intermembrane space (ENDRES et al. 1999). The Tim23 protein is imported into the inner membrane by the TIM22 complex. For the highest efficiency of Tim23 import, another intermembrane space complex, composed of Tim8 and Tim13 is required (DAVIS et al. 2000; KOEHLER et al. 1999; PASCHEN et al. 2000). Another inner membrane protein, Oxa1, is required for the

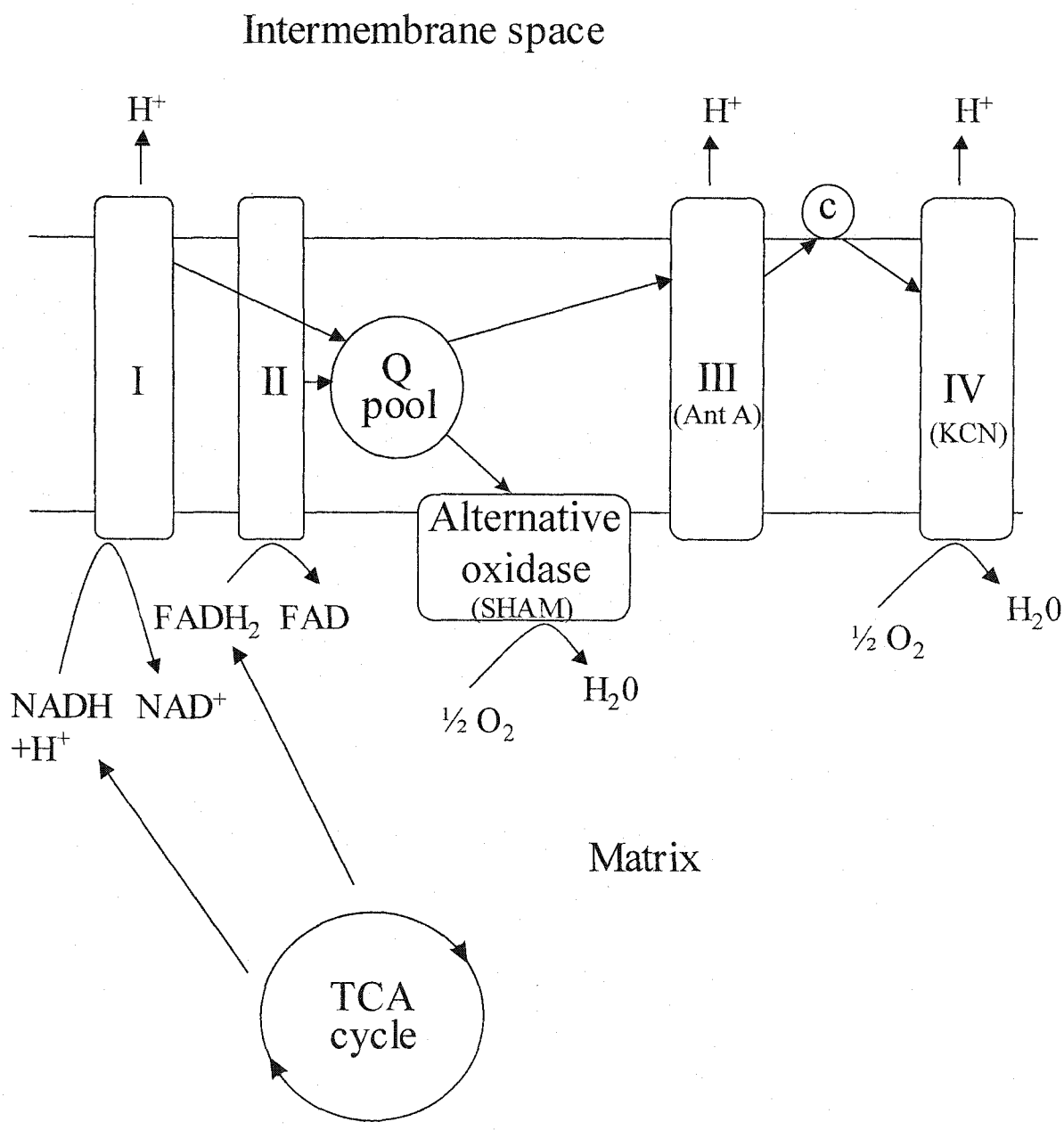
insertion of a number of mitochondrially synthesized subunits of cytochrome oxidase and ATP synthase into the inner membrane (HELL et al. 1997; HELL et al. 1998; HELL et al. 2001; KERMORGANT et al. 1997), as well as a small number of nuclear-encoded inner membrane proteins that are fully imported into the matrix before insertion into the membrane (HELL et al. 1997).

1.2 Mitochondrial respiration, standard and alternative components

Mitochondria are responsible for capturing the energy released during cellular respiration. Electrons are transferred to NAD^+ to form NADH $+H^+$ by enzymes of the tri-carboxylic acid (TCA) cycle. As pyruvate from glycolysis is converted to acetyl CoA and proceeds through the cycle, reduced electron carriers $NADH+H^+$ and $FADH_2$ are produced. These are re-oxidized and the electrons are passed to the electron transport chain. There are four protein complexes in the standard electron transport chain of the mitochondrial inner membrane (Fig. 1). These complexes are NADH:ubiquinone oxidoreductase (Complex I), succinate:coenzyme Q reductase (Complex II), coenzyme Q:cytochrome *c* oxidoreductase (Complex III) and the terminal oxidase cytochrome *c* oxidase (Complex IV), which transfers electrons directly to oxygen to form water (JOSEPH-HORNE 2001). As the electrons pass down the electron transport chain through complexes I, III and IV, each pumps protons across the inner membrane, from matrix to intermembrane space, to generate a protonmotive force. Succinate dehydrogenase (Complex II) transfers electrons from succinate via $FADH_2$, generated by the TCA cycle, to coenzyme Q without associated proton pumping. All fungi share this general pathway, with the exception of some yeasts (including *Saccharomyces cerevisiae*), which lack a true Complex I but have a simple, non proton pumping NADH dehydrogenase (JOSEPH-HORNE 2001).

The protonmotive force created by proton pumping is used by ATP synthase (sometimes called Complex V) to generate ATP (JOSEPH-HORNE 2001). ATP synthase consists of a matrix component, which includes the peripheral membrane protein, the catalytic F_1 subunit plus the associated F_O subunit, a transmembrane protein in the mitochondrial inner membrane that functions as a proton channel. The ATP synthase carries out the reaction "$ADP+P_i \rightarrow ATP$" in the mitochondrial matrix

Figure 1. Standard cytochrome and alternative oxidase electron transport systems. Complexes associated with proton pumping across the mitochondrial inner membrane are indicated by arrows above each complex. Antimycin A (Ant A) inhibits complex III, cyanide (KCN) inhibits complex IV, and salicylhydroxamic acid (SHAM) inhibits the alternative oxidase.



(FUTAI et al. 2000). During ATP synthesis, protons flow through the enzyme from the intermembrane space back to the matrix causing a rotation in the γ subunit of F_1, which causes a change in the catalytic site and allows release of ATP (FUTAI et al. 2000).

Interestingly, standard cytochrome respiration may shorten the potential life span of organisms. In the nematode *C. elegans*, research into extended life span has revealed a link between the amount of coenzyme Q present, reactive oxygen species (ROS) and lifespan. The *C. elegans clk-1* mutant, which lacks the ability to synthesize coenzyme Q and gains its coenzyme Q from its diet of *E. coli*, has a life span of up to 10% longer than wild type worms. The extended life span was suggested to be from a decreased level of coenzyme Q. This was tested directly by providing *C. elegans* with a diet lacking coenzyme Q. Wild type worms had a life-span of 59% longer than those fed coenzyme Q. The *clk-1* mutants also had an extended life span with this diet, although these mutants did not live as long as the wild type worms, likely because the *clk-1* worms do not synthesize any of their own coenzyme Q. The *clk-1* mutant animals accumulate demethoxy-Q_9 (DMQ_9), an intermediate of Q biosyntheisis. DMQ_9 can receive electrons from complex I, but not complex II. Another gene in *C. elegans* that increases life span when mutated is *daf-2*, whose product shares identity with mammalian insulin-like signaling molecules; *daf-2* is believed to be involved in regulating the transcription of nuclear genes involved in respiration. In the long lived mutants, there is less respiration and thus decreased production of ROS, as well as a yet uncharacterized increase in ROS scavenging. In the absence of dietary coenzyme Q, *daf-2* mutants had a longer life span than wild type worms grown under the same conditions. It was suggested that lowered levels of coenzyme Q present within mitochondria, as well as reduced transcription of genes required for full respiration would reduce the amount of ROS produced. This, coupled with an increase in ROS scavenging, as seen in the *daf*-2 mutants, would result in a significantly extended life span (LARSEN and CLARKE 2002).

In addition to the cytochrome-mediated respiratory pathway, an alternative pathway with a second terminal oxidase exists in a variety of organisms, including

green algae and most higher plants (HENRY and NYNS 1975), as well as fungi (LAMBOWITZ and SLAYMAN 1971), some yeasts, including *Candida albicans*, (HUH and KANG 1999), and some protists such as *Trypanosoma brucei*, (CLARKSON et al. 1989). The yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* do not have an alternative oxidase, nor do any animal species analyzed to date. Alternative oxidase is specifically inhibited by various compounds that do not interfere with standard cytochrome respiration, including hydroxamic acids such as salicylhydroxamic acid (SHAM) (SCHONBAUM et al. 1971), disulfram (GROVER and LATIES 1978) and n-propyl gallate (PARRISH and LEOPOLD 1978), but is resistant to inhibitors of the standard cytochrome chain, such as cyanide (KCN) and antimycin A (LAMBOWITZ and SLAYMAN 1971). Using respiration inhibitors specific to certain points in the respiratory pathway, it was determined that the alternative oxidase forms a branch point from the main cytochrome respiratory chain on the substrate side of cytochrome *b*, a component of Complex III (LAMBOWITZ et al. 1972; SLAYMAN 1977). Later work demonstrated that ubiquinol donates electrons to both the alternative pathway and the cytochrome pathway. Thus, ubiquinol was proposed to be the branch point (VON JAGOW and BOHRER 1975). Alternative oxidase is now known to function as a second terminal oxidase in the mitochondrial electron transport chain, donating electrons directly from reduced ubiquinol to oxygen (MOORE and SIEDOW 1991) (Fig. 1).

The alternative oxidase has been shown to increase the life span of the filamentous fungus *Podospora anserina*. Under wild type conditions, as *P. anserina* ages, there is an accumulation of defective mtDNA, possibly due to the presence of ROS due to standard respiration (DUFOUR et al. 2000), which leads to senescence. Inactivation of the nuclear gene *COX5*, which encodes a subunit of cytochrome *c* oxidase, induces the alternative oxidase pathway and a decrease in the production of ROS (DUFOUR et al. 2000). Copper depletion also induces alternative oxidase activity (BOURGHOUTS et al. 2001). The expression of alternative oxidase causes a marked increase in the lifespan of *P. anserina*. The inactivation of *COX5* produced cultures that grew seven- to eight-times further in race-tubes, as measured in centimeters of mycelial growth, than wild type strains before senescing, with some isolates not

reaching senescence after one and a half years (DUFOUR et al. 2000). Consistent with these ovservations, cultures of *P. anserina* grown on standard medium will senesce after approximately 50 days, whereas cultures grown in the presence of a copper chelator can survive for up to 130 days before undergoing senescence (BOURGHOUTS et al. 2001).

1.3 Alternative oxidase in plants

There were several early studies that described a second respiratory pathway in plants. A historical overview of these early reports records forty papers published from 1919 to 1969, but many of the initial discoveries were not pursued (LLOYD and EDWARDS 1977). However, in the early 1970s these alternative pathways of electron transport began to receive increased attention, and concerted efforts were begun to try to explain the system. While studying oxidative phosphorylation in *Euglena gracilis*, Sharpless and Butow found that when grown in the presence of glutamate and malate the organism exhibited oxidative phosphorylation consistent with the presence of three sites coupled to electron transport and proton pumping, as found in mammalian mitochondria. However, they also discovered an alternative pathway that could bypass one of these complexes in a specific manner (SHARPLESS and BUTOW 1970b). They noted that this alternate pathway bypassed the antimycin-sensitive site (Complex III of the standard cytochrome pathway), though it was thought that the alternate pathway involved the terminal oxidase of the standard cytochrome pathway (SHARPLESS and BUTOW 1970b). Further investigation revealed that *Euglena* could grow in the presence of antimycin. Addition of AMP, GMP or IMP stimulated respiration significantly, and this stimulation was unusual because it was not inhibited by KCN, as is standard mitochondrial respiration. It was determined that the alternative oxidase reduced oxygen to water independently of the terminal oxidase of the standard pathway. The pathway reached a maximum activity in cultures by the time the cells had divided twice in the presence of antimycin, and, after a temporary persistence of activity, the pathway was lost after transfer to media that did not contain antimycin. The presence of this activity was not dependent upon antimycin and was seen transiently upon transfer of cells between media with different carbon

sources, but that did not contain antimycin. Even though the alternative oxidase system did not appear to use the cytochrome system past cytochrome *b*, the cytochrome complexes were still present within mitochondria with alternative oxidase activity (SHARPLESS and BUTOW 1970a).

Studies of species of the algal genus *Chlorella* also revealed a respiratory pathway that was insensitive to the standard respiration inhibitors KCN and carbon monoxide, indicating that there were at least two separate oxidase systems present in this genus (SARGENT and TAYLOR 1972). Investigators demonstrated that the flow of electrons through the alternative oxidase pathway and the standard pathway occurred at the same time, but partitioning of the electrons varied depending upon conditions encountered by the organism. In fresh cultures of *Chlorella*, 35% of total oxygen uptake involved the second (KCN-insensitive) oxidase (SARGENT and TAYLOR 1972).

Standard electron transport generates protonmotive force, which is used to synthesize ATP (section 1.2). Many of the early studies of alternative oxidase were designed to determine whether the enzyme was coupled to ATP production in a similar manner. Moore and colleagues studied the mitochondria of several species of plants after growth in antimycin A and calculated the protonmotive force generated. They discovered that the addition of ADP failed to increase the respiratory rate and did not reduce the protonmotive force as it did for standard cytochrome respiration. As well, the addition of hydroxamic acids reduced oxygen consumption by the alternative oxidase but did not affect the protonmotive force (MOORE et al. 1978). Studies revealed that, in the presence of an uncoupler, the protonmotive force was reduced to zero but oxygen consumption by the alternative oxidase was unaffected. This demonstrated that the alternative oxidase does not pump protons across the inner membrane (MOORE et al. 1978). Thus, alternative oxidase is energetically wasteful, as much potential energy is not harvested, but is instead lost as heat.

The terminal oxidase protein was first identified and purified from *Sauromatum guttatum* (voodoo lily) thermogenic tissues. The protein was used to generate polyclonal antibodies that recognized a cluster of proteins of molecular weights 37, 36 and 35 kilodaltons (kDa) (ELTHON and MCINTOSH 1987). This cluster of proteins was seen in all tissues and monoclonal antibodies prepared against

individual proteins within the cluster cross-reacted with the other proteins. Thus, the individual bands were proposed to be translationally modified versions of the protein of a single gene (ELTHON et al. 1989). The first cDNA encoding an alternative oxidase protein was isolated from an *S. guttatum* cDNA expression library. The previously described antibodies recognized a larger protein with an apparent molecular mass of 48 kDa expressed from the cDNA containing the alternative oxidase coding sequence, generated from poly(A)$^+$ RNA of *S. guttatum* (RHOADS and MCINTOSH 1991). The additional mass of the protein was due to an N-terminal, 63 amino acid putative mitochondrial targeting sequence. Rhoads and McIntosh concluded that this cDNA represented a nuclear gene, designated *aox1*, which encoded an alternative oxidase protein. The genomic copy of *aox1* was found to be a single, nuclear-encoded gene with four exons in *S. guttatum* (RHOADS and MCINTOSH 1993). The *aox1* gene can confer KCN-insensitive respiration to *E. coli* as well as *S. pombe*, if the gene product is targeted to the mitochondria of the yeast, indicating that only one polypeptide is required for alternative oxidase activity (ALBURY et al. 1996; KUMAR and SOLL 1992).

Early experiments in other plants, such as *Arabidopsis thaliana,* soybean, and tobacco also suggested that alternative oxidase was encoded by single genes. However, later work revealed that alternative oxidase genes are present in multiple copies in several plants. *A. thaliana* has at least four copies of the AOX gene (SAISHO et al. 1997), soybean has at least three copies (WHELAN 1996), tobacco at least two (WHELAN et al. 1995) and rice at least two (ITO et al. 1997). These different genes are expressed in tissue specific and developmentally specific manners (FINNEGAN et al. 1997; SAISHO et al. 1997). Why some plants, such as *S. guttatum*, have only one gene for alternative oxidase and others have multigene families is not known, but having different copies of the gene expressed in distinct places or at distinct times may allow finer control of alternative oxidase expression in these plants.

1.4 Alternative oxidase in *Neurospora* and other fungi

Early studies of alternative oxidase in *Neurospora* involved the slow growing [*poky*] strain. Haskins *et al.* (HASKINS et al. 1953) investigated the cytochromes in the

mitochondria of this strain and discovered an excess of cytochrome *c* and deficiencies in cytochromes aa_3 and *b* compared to wild type, indicating that normal cytochrome respiration was greatly reduced in this strain. Further investigation revealed that [*poky*] possessed a second, KCN-insensitive, terminal oxidase (TISSIERES et al. 1953). It was eventually shown that KCN-resistant respiration could be artificially induced in wild type cells by disrupting the standard mitochondrial cytochrome chain with inhibitors of mitochondrial function (such as antimycin A or KCN), by the addition of chloramphenicol (which impedes mitochondrial protein synthesis and inhibits the formation of several respiratory complexes), by mutations in genes encoding subunits of the cytochromes or by copper deprivation (BERTRAND et al. 1983; EDWARDS et al. 1974; LAMBOWITZ and SLAYMAN 1971). Wild type *Neurospora* cells contained little alternative oxidase activity but growth in the presence of inducing agents produced a 20-fold increase in alternative oxidase activity (LAMBOWITZ and SLAYMAN 1971).

To study the alternative oxidase and its regulation in *Neurospora*, mutants in alternative oxidase were created. Using a strain that could not survive in media lacking the sugar inositol, conidia were mutagenized and inoculated into growth media lacking inositol and containing antimycin A. Under such conditions, any conidia that contained mutation(s) in the gene(s) required for producing alternative oxidase would not grow because the alternative oxidase needed for growth in the presence of antimycin A was non-functional. Conidia that contained mutations that did not affect alternative oxidase could germinate and begin to grow in the presence of antimycin A because the alternative oxidase would function in these conidia, but these germinated conidia would then die due to the lack of inositol, which is required for cell wall synthesis. Non-growing conidia (including alternative oxidase mutants) were harvested and replica plated onto plates containing inositol without antimycin A and plates containing inositol with antimycin A. Colonies that grew in the absence but not presence of antimycin A were presumed to have a deficiency in some element of the alternative oxidase pathway (BERTRAND et al. 1983; EDWARDS et al. 1976). Bertrand *et al.* analyzed all 21 of the alternative oxidase deficient strains arising from their screen and three of those identified by Edwards et al. (EDWARDS et al. 1976) and identified two complementation groups (BERTRAND et al. 1983). The genes

comprising these complementation groups were named *aod-1* and *aod-2* and were found to be nuclear genes that mapped to linkage group IV and linkage group II, respectively. To determine if any genes required for the production of alternative oxidase existed on mtDNA, strains of both *N. crassa* and the closely related species *N. intermedia* that contained different deletions of portions of the mitochondrial genome were tested for the presence of alternative oxidase. Regardless of what portion of the mitochondrial DNA was deleted, alternative oxidase was present at high levels, indicating that no mitochondrial genes are involved in coding for any portion of the alternative oxidase enzyme (BERTRAND et al. 1983).

Seventeen of the mutants isolated by Bertrand et al. and the three from Edwards et al. (EDWARDS et al. 1976) contained a mutation in *aod-1*, while the remainder were *aod-2* mutants (BERTRAND et al. 1983). In an attempt to identify the alternative oxidase protein, wild type and alternative oxidase mutant strains were grown in non-inducing media and then transferred to inducing medium containing ^{35}S-methionine, to label newly synthesized proteins. The cultures were incubated for 40 to 60 minutes and their mitochondrial proteins were compared. Wild type *Neurospora* displayed a putative alternative oxidase polypeptide upon induction, as did 19 of 20 *aod-1* mutant strains. The polypeptide was not observed in any of the four *aod-2* mutant strains. These observations were consistent with the interpretation that *aod-1* is the structural gene. The function of the *aod-2* gene remains unknown, but it seems to be involved in the regulation of *aod-1* (BERTRAND et al. 1983).

These conclusions were strengthened by analysis of western blots of total mitochondrial proteins using the monoclonal antibody against *S. guttatum* alternative oxidase (ELTHON et al. 1989). Little to no alternative oxidase protein was present in uninduced wild type *N. crassa* cultures, but the protein was highly induced upon growth in chloramphenicol. The antibody reacts to two proteins of 36.5 and 37 kDa in *N. crassa*. The [*poky*] mutant constitutively expressed alternative oxidase and generated proteins of 36.5 and 37 kDa. These proteins were also seen in the induced cultures of the *aod-1-1* mutant, but as no KCN-insensitive respiration is seen in this mutant the protein is likely non-functional. The *aod-1-4* mutant did not express any protein under either non-inducing or inducing conditions. The induced *aod-2-4*

mutant contained no 36.5 kDa band, and expressed the 37 kDa band at a level much lower than the induced wild type strain (LAMBOWITZ et al. 1989). The presence of inactive protein at near wild type levels in induced *aod-1-1* and the absence of such bands in induced *aod-2-4* supported the hypothesis that *aod-1* is the structural gene and that *aod-2* regulates the expression of *aod-1* in a yet unidentified manner (LAMBOWITZ et al. 1989).

Subsequently, the *N. crassa aod-1* gene was cloned, sequenced, and the amino acid sequence deduced ((LI et al. 1996) and Fig. 6 of this thesis). By comparison to known alternative oxidase structural genes, *aod-1* was established to encode the *N. crassa* alternative oxidase protein. Sequence analysis of mutants confirmed that *aod*-1 mutations affected the structural gene. The putative regulatory gene product encoded by *aod-2* has not been identified at this point.

Many other fungal species contain alternative oxidases, including *Moniliella tomentosa* (HANSSENS et al. 1974), *Aspergillus niger* (KIRIMURA et al. 1999), *Magnaporthe grisea* (YUKIOKA et al. 1998a), *Candida parapsilosis* (GUERIN and CAMOUGRAND 1986) and *Candida albicans* (HUH and KANG 1999). In most of these fungi, alternative oxidase is encoded by a single gene. However, when the *AOX1* gene of *Candida albicans* was disrupted, a second alternative oxidase protein was induced upon growth in antimycin A, suggesting this organism possessed more than one alternative oxidase gene. Further investigation revealed a second alternative oxidase gene 1.3 kb upstream of AOX1 in the same transcriptional direction. The genes were named *AOX1a* and *AOX1b* (HUH and KANG 2001). Both genes can confer KCN insensitive respiration when transformed into *S. cerevisiae*, but in *C. albicans AOX1a* appears to be constitutively expressed while *AOX1b* expression is induced upon growth in media with inducing agents, as seen in *N. crassa* (HUH and KANG 2001). Multigene families have also been seen in some plants (section 1.3).

1.5 Physiological role of alternative oxidase

The most well defined role for alternative oxidase is found in the voodoo lily *S. guttatum*. This species uses the heat generated by the alternative oxidase to volatilize aromatic compounds in thermogenic blooms, for the purpose of attracting

pollinating insects (MEEUSE 1975). In general, there are several conditions that seem to increase the activity levels of alternative oxidase. In plants, alternative oxidase is increased at low temperatures, where survival may require the plant to exist in cold temperatures for periods of development. In wheat, barley and other plants that are susceptible to chilling, exposure to low temperatures increased alternative oxidase activity by over 40%, suggesting a thermogenic role for alternative oxidase in these plants (BREIDENBACH et al. 1997; ITO et al. 1997). Increased levels of alternative oxidase activity were also seen after wounding or pathogen attack, and during distinct developmental stages (MCINTOSH 1994).

It is also proposed that alternative oxidase acts as an "overflow pathway" in cases where the normal cytochrome pathway is either overloaded or limited. In plants, it has been shown that the alternative oxidase pathway is regulated by the activity of the cytochrome pathway, suggesting that alternative oxidase only becomes active when the proportion of the ubiquinone pool is in reduced form is extremely high, on the order of 90% (BAHR and BONNER 1973). With advances in measurement techniques, it was discovered that electron flow through the alternative pathway does indeed depend upon the reduction level of the ubiquinone pool but alternative oxidase becomes active when the reduced ubiquinone pool is at 35 to 40%. When the reduced ubiquinone pool is small, the cytochrome bc_1 complex oxidizes the reduced ubiquinone in a linear fashion and the amount of oxygen consumed is directly proportional to the oxidation of ubiquinone. However, if for any reason the pool is highly reduced, the alternative pathway becomes active, but in a non-linear fashion. It does not replace cytochrome bc_1 function, so that the energetically wasteful oxidation by alternative oxidase is limited (DRY et al. 1989).

The alternative oxidase and standard cytochrome pathways can operate at the same time, and it is sometimes necessary to examine the level of functioning of each pathway individually. The "capacity" of one pathway refers to the amount of oxygen consumed in the presence of an inhibitor of the other pathway. For example the amount of oxygen consumed in the presence of KCN is the capacity of the alternative oxidase. The "engagement" of the alternative oxidase pathway describes how much of the total respiration rate of the cell is contributed by the alternative oxidase

pathway. The difference between capacity and engagement values in a given situation indicates that electrons are portioned to each pathway in specific amounts, depending on the status of the cell. If the alternative oxidase capacity is higher than its engagement, the cell's ability to restrict the energetically wasteful pathway when it is not needed is demonstrated. The "activity" of a pathway describes which electrons in the system flow through the pathway in the absence of any inhibitors (McINTOSH 1994; MOORE and SIEDOW 1991).

The level of alternative oxidase functioning in a cell can also be related to carbon skeleton proliferation in the mitochondria, combined with the cell's metabolic requirements. It was found in tobacco that an increase in the TCA cycle intermediate citrate caused a rapid increase in both the *Aox1* mRNA levels and AOX1 protein (VANLERBERGHE and McINTOSH 1996). In the presence of an increase in citrate, the first organic acid in the TCA cycle, the mitochondria may transmit a signal to the nucleus that alternative oxidase is required due to a backlog of respiration intermediates. In radish seedlings, which are unable to store sugars in their roots, alternative oxidase activity accounted for 70% of the respiration. However, in older plants that were actively storing sugars in their roots, alternative oxidase activity was much less active, though not totally abolished. This was also seen in carrot and beet roots (LAMBERS 1980). This suggested that the alternative oxidase oxidized excess sugars when there was too much carbohydrate in the roots and it could not be stored or used for structural growth. This would prevent a build up of a large pool of sugars in the cells (LAMBERS 1980; LAMBERS 1982).

Reduced ubiquinone has been implicated in the production of reactive oxygen species (ROS) via auto-oxidation by molecular oxygen. The production of ROS increased when terminal steps in respiration are blocked (POYTON and McEWEN 1996). ROS such as superoxide ($^{\cdot}O_2^-$), hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2) are produced as a normal consequence of aerobic respiration. These molecules are also produced in larger amounts than normal after inhibitors of the respiratory chain are added (WAGNER 1995). ROS molecules are highly damaging to many cellular components and can oxidize proteins, damage membranes and mutate DNA.

Under extreme conditions, ROS can cause enough damage to result in cell death (POYTON and MCEWEN 1996).

All aerobic organisms have developed mechanisms to deal with ROS, including the actions of superoxide dismutase and peroxidase, but plants seem to be able to reduce the production of ROS under some circumstances, thus preventing damage. Using tobacco strains that constitutively overexpressed or underexpressed the alternative oxidase gene *Aox1*, it was found that an inverse relationship exists between the abundance of alternative oxidase in mitochondria and the cellular levels of ROS, suggesting that alternative oxidase does indeed prevent the generation of ROS by lessening the amount of ubiquinol and other reduced respiratory intermediates present in the mitochondria (MAXWELL et al. 1999).

Another physiological function for alternative oxidase activity is suggested by the observation that some plant parasitic fungi may encounter KCN-based compounds as a host defence mechanism. Alternative oxidase would be useful to evade such a response (MCINTOSH 1994). *N. crassa* does not fall into the category of a plant pathogen.

1.6 Alternative oxidase protein structure and regulation

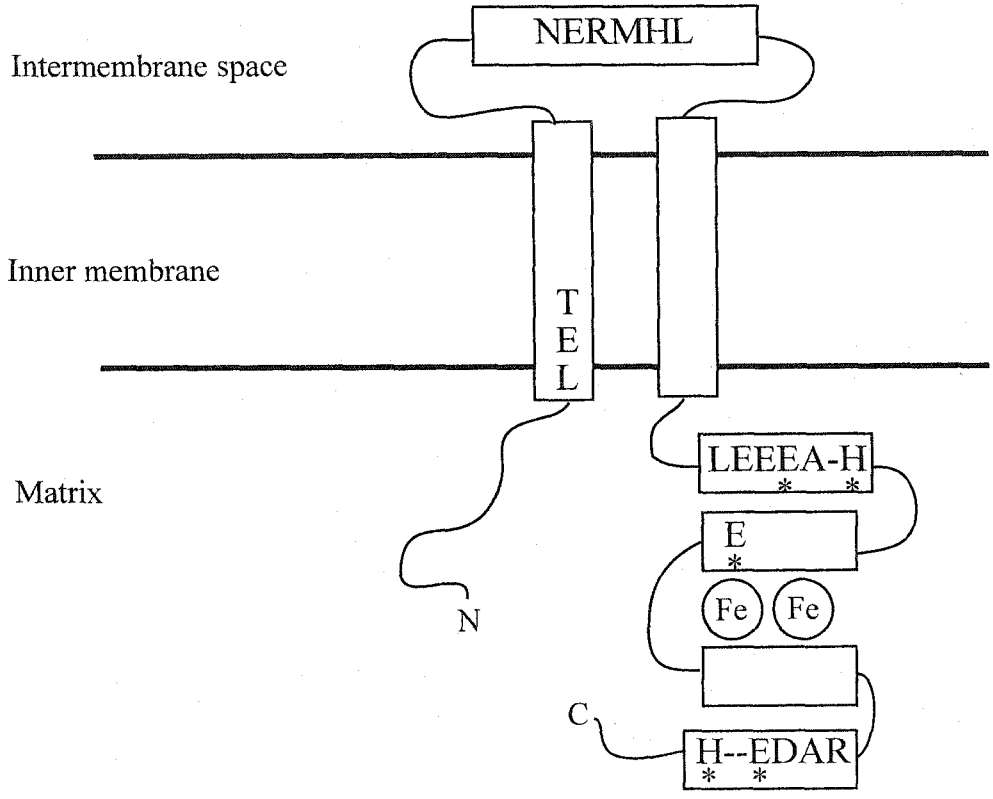
The mature plant alternative oxidase (without the mitochondrial targeting sequence) contains approximately 280 amino acids depending on the species (SIEDOW 2000) while the *N. crassa* mature protein is 297 amino acids (LI et al. 1996). There are many conserved features between plant and fungal alternative oxidase proteins including regions that are proposed to form alpha helixes as well as N- and C-terminal hydrophilic regions. Experiments with mitoplasts exposed to proteases indicated that both termini are exposed to the matrix of the mitochondria (VANLERBERGHE et al. 1997). The highest regions of similarity between plant and fungal alternative oxidase amino acid sequences occur in the central portion of the protein (residues 158 to 318 of the 362 amino acids of unprocessed *N. crassa* AOD1), while the C- and N-termini are much more variable (ANDERSSON and NORDLUND 1999; CHANDEL et al. 1998; KIRIMURA et al. 1999; LI et al. 1996).

Alternative oxidase activity in both plants and fungi is inhibited by the presence of iron chelating agents; furthermore, the growth of the protozoan *Trypanosoma brucei* in media lacking iron produces a non-functional protein, suggesting that the protein uses iron to transfer electrons (CHAUDHURI et al. 1998; EDWARDS et al. 1976; MINAGAWA et al. 1990). Once the genes encoding alternative oxidase had been discovered in several species of plant and fungi, comparison of the amino acid sequences led to the discovery of several conserved amino acid domains between the alternative oxidase and other iron-binding proteins (MOORE et al. 1995). Analysis of the amino acid sequence of the alternative oxidase protein suggested it was highly similar to methane monoxygenase (MMO), a member of the class II type of di-iron proteins, which are characterized by possessing two (D/E-X-X-H) motifs that are contained within two of four α-helices present in the protein. Class I and Class III di-iron proteins also contain four alpha-helical bundles, but the iron binding ligands are different from those of Class II, consisting of five or eight conserved histidine residues, respectively (FOX et al. 1994; MOORE et al. 1995). The alternative oxidase proteins analyzed contain three copies of conserved (D/E-X-X-H) motifs, two of which occur within proposed alpha-helical bundles that are exposed to the matrix. The similarity with class II di-iron proteins, in addition to secondary structure predictions of the alternative oxidase, suggested it was an integral inner membrane protein with two membrane spanning alpha helices. However, differences between the MMO and alternative oxidase protein sequences required that the latter be modeled with shorter alpha-helical regions and without glutamate or aspartate residues in one of the helices predicted to be involved in binding the iron atoms. The di-iron site and both the N and C termini would reside within the matrix in this model (MOORE et al. 1995) (Fig 2A). Moore et al. also speculated that there might be a ubiquinol binding pocket formed by an interaction between the matrix-oriented portions of the membrane spanning helices, which would be close enough to the di-iron site for it to undergo reduction by ubiquinol (MOORE et al. 1995).

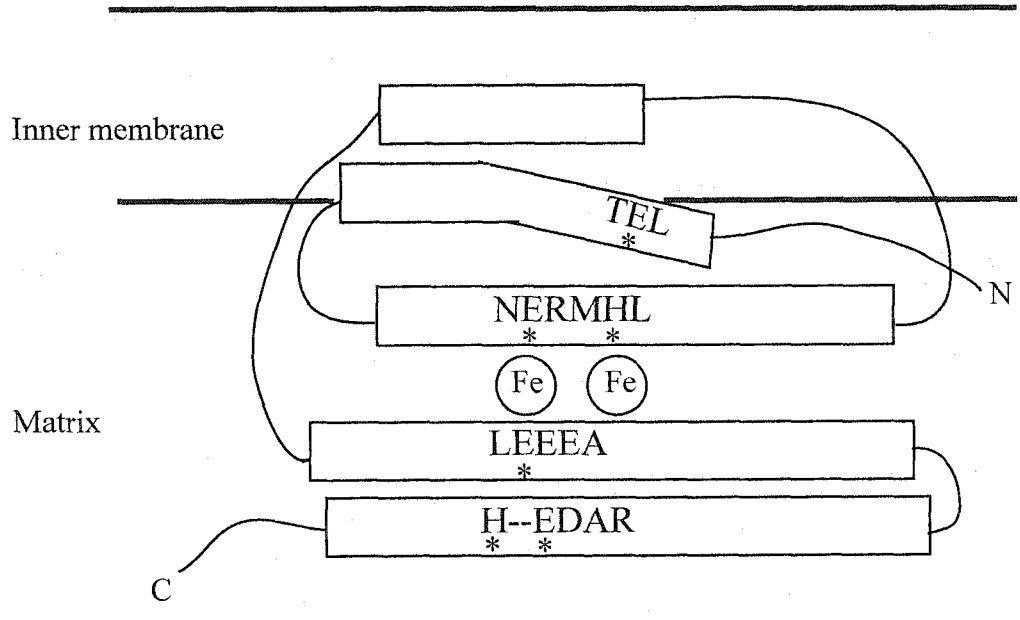
A later analysis using 20 alternative oxidase sequences from 13 species, as well as a distantly related plastid terminal oxidase (IMMUTANS, *Im* gene) from *Arabidopsis* (WU et al. 1999), suggested another possible model of the enzyme's

Figure 2. Two models of alternative oxidase conformation. A. Proposed conformation of alternative oxidase in the Moore and Siedow model (Moore et al., 1995). The model is based on analysis of protein sequence from six species. Conserved amino acid residues believed to be involved in binding iron atoms are indicated with asterisks. B. The model proposed by Andersson and Nordlund (ANDERSSON and NORDLUND 1999; BERTHOLD et al. 2000). The model is based on alternative oxidase alignments of 20 alternative oxidase proteins from 13 species. Amino acid residues predicted to be involved in binding the iron atoms are indicated with asterisks. Rectangles represent α-helices predicted in the models.

A Moore and Siedow model



B Andersson and Nordlund model



topology in the inner membrane. In one of the putative iron-binding motifs (LEEEA-H) of the original model, the histidine residue was found not to be conserved in all of the alternative oxidase sequences, and one of the most highly conserved motifs in the larger analysis (NERMHL) was present within the intermembrane space in the original model, where it did not function in binding the iron atoms (ANDERSSON and NORDLUND 1999). The conserved NERMHL region is an E-X-X-H motif, known to be important for di-iron binding in the class II di-iron proteins (MOORE et al. 1995). Andersson and Nordlund proposed a new model that suggested there were no membrane-spanning helices, but that one and a half of the five proposed alpha-helices were embedded within the membrane, with the rest of the protein being in the matrix (ANDERSSON and NORDLUND 1999); (BERTHOLD et al. 2000) (Fig. 2B). Comparisons of the new model with class II di-iron proteins show that the new model is more consistent with respect to both the length of the proposed iron-binding helices as well as the spacing of linker regions between the helices (ANDERSSON and NORDLUND 1999; BERTHOLD et al. 2000). Evidence supporting this model was provided by site-directed mutagenesis of trypanosomes alternative oxidase, which is capable of conferring SHAM-sensitive respiration to an *E. coli* heme mutant (AJAYI et al. 2002; CLARKSON et al. 1989). Mutagenesis of the conserved EAEH motifs (His-269 to lysine, or Glu-266 to alanine—*T. brucei* numbering) or of the conserved NERMHL motif (His-165 to alanine—*T. brucei* numbering) eliminated the ability to confer SHAM-sensitive respiration to *E. coli*, while several non-conserved residues were mutagenized without affecting alternative oxidase activity (AJAYI et al. 2002). Site directed mutagenesis of *S. guttatum* alternative oxidase, expressed in the mitochondria of *S. pombe*, also supported the important functional identifications found for the *T. brucei* enzyme. When Glu-217 (*S. guttatum* numbering) of the conserved NERMHL motif was mutated to alanine, the alternative oxidase produced was inactive (ALBURY et al. 2002). This supports the Andersson/Nordland model (ANDERSSON and NORDLUND 1999), as this motif is important in their active site, but was proposed to be within the intermembrane space in the original model (MOORE et al. 1995).

Although the newer model seems more accurate, Andersson and Nordlund may have misidentified one of the amino acid residues involved in iron binding. The Andersson/Nordlund model proposes that the iron-binding residue in the LEEEA motif is the Glu-268 (the first of the three in this motif—*S. guttatum* numbering), due to the inclusion of the IMMUTANS sequence in the model (BERTHOLD et al. 2000). The *S. guttatum* mutation of Glu-270 (the third of the three in the motif) to alanine produced a non-functional enzyme, suggesting that it was involved in iron binding in at least some species (ALBURY et al. 2002). The Andersson/Nordlund model also includes a slightly hydrophobic crevice lined with several conserved amino acids. This crevice is proposed to be the ubiquinol binding site (ANDERSSON and NORDLUND 1999; BERTHOLD et al. 2000). The cleft is close to two conserved amino acids that were shown to be involved in ubiquinol binding during a mutagenesis screen in *A. thaliana* (ANDERSSON and NORDLUND 1999; BERTHOLD 1998).

There are also two conserved tyrosine residues in the proposed cleft, Tyr-253 and Tyr-280 (*A. thaliana* numbering) (BERTHOLD et al. 2000). Tyrosine radicals are known to be generated at the di-iron site in the Class II di-iron protein ribonucleotide reductase (RNR), subunit 2, and have been proposed to be involved in reduction and oxidation of bound oxygen (ANDERSSON and NORDLUND 1999; BERTHOLD et al. 2000). More direct evidence of the role of the conserved tyrosine residues in alternative oxidase function came from mutagenesis studies of the *S. guttatum* alternative oxidase that was targeted to the mitochondria of *S. pombe*. Tyr-253 mutated to phenylalanine did not reduce antimycin-insensitive respiration in the mitochondria of *S. pombe* cells expressing the mutated copy of alternative oxidase (ALBURY et al. 2002). However, mutagenesis of Tyr-275 (equivalent to Tyr-280 in (BERTHOLD et al. 2000)) to phenylalanine did eliminate antimycin-insensitive respiration, confirming a role in alternative oxidase function, although it has not been determined conclusively that this residue is involved in ubiquinol binding (ALBURY et al. 2002).

One of the fundamental differences between the plant and fungal alternative oxidases is a conserved cysteine residue found in the N-terminal region of all plant alternative oxidase sequences to date, but not in any of the fungal sequences (SIEDOW

2000). Alternative oxidase has been found to exist in soybean mitochondria as a dimer. When isolated mitochondria were treated to poise the alternative oxidase in either fully oxidized or fully reduced state, it was found that the fully reduced form of the protein was significantly more active than the oxidized form (SIEDOW 2000). In the presence of an excess of reduced ubiquinol, the disulfide bond is reduced but the monomers remain in close proximity to each other. An α-keto acid, such as pyruvate, reacts with the reduced enzyme and forms a charged residue, a thiohemiacetal (-S-COH(R)CO_2^-), which activates the enzyme (RHOADS et al. 1998). It was found that there are two highly conserved cysteine residues among the plant, but not the fungal, alternative oxidases, both of which are proposed to be located within the matrix. Either or both of these cysteines could have been involved in disulfide linkage and/or activation by α-keto acids (VANLERBERGHE and MCINTOSH 1997). Site-directed mutagenesis of *A. thaliana* alternative oxidase expressed in *E. coli* revealed that replacing Cys-128 with alanine did not have an effect on either disulfide linkage of alternative oxidase dimers or activation by α-keto acids (RHOADS et al. 1998). However, replacing Cys-78 with alanine eliminated the disulfide linkage, though the proteins were still present as dimers within the membrane, indicating that other domains may be involved in dimerization (RHOADS et al. 1998). A conserved region of about 40 amino acids that surrounds the regulatory cysteine, which is present in all plant alternative oxidases studied to date, has not been observed in any fungal proteins (UMBACH and SIEDOW 2000). It could represent a domain responsible for dimerization. Finally, Cys-78 also appears to be involved in α-keto acid stimulation of the enzyme. When the residue is replaced with an alanine, the protein is unresponsive to pyruvate activation. Further evidence that Cys-78 is involved in activation through a charged intermediate was obtained by replacing Cys-78 with a glutamate residue. This amino acid mimics the charged moiety of a thiohemiacetal, and resulted in a constitutively active enzyme that is incapable of being further activated by pyruvate (RHOADS et al. 1998).

Due to the absence of a conserved cysteine, there is no evidence that fungal alternative oxidase protein ever forms dimers (UMBACH and SIEDOW 2000). This is supported by experiments that examined protein extracts from mitochondria of the

fungi *Neurospora* and *Pichia* prepared in the absence of any reducing agents. Immunoblotting revealed no dimeric alternative oxidase. Even in the presence of chemical cross-linkers, only the monomeric form of *Pichia* alternative oxidase was ever seen, consistent with the lack of any fungal specific dimerization domains (UMBACH and SIEDOW 2000). The cysteine conserved in plants is also involved in activation of the alternative oxidase by pyruvate (RHOADS et al. 1998) and this activity is not seen in the fungal enzyme. However, the activity of the fungal enzyme can be stimulated by nucleotides ADP, AMP and GMP by an unknown mechanism (SAKAJO et al. 1997; VANDERLEYDEN et al. 1980a; VANDERLEYDEN et al. 1980b). Purine nucleotides had no effect on *A. thaliana* alternative oxidase activity (UMBACH and SIEDOW 2000). It thus seems likely that there should be specific region(s) in fungal alternative oxidase that are required for purine nucleotide activation. Two fungal specific sequence domains are known. The first is 20 to 25 amino acids in length and occurs just N-terminal to the first hydrophobic region and is the primary content of the second exon in most fungal alternative oxidase genes (UMBACH and SIEDOW 2000). The second fungal specific domain is a short region at the C-terminus that is present in fungi but not in plants (UMBACH and SIEDOW 2000). It is not known if either of these fungal specific regions is responsible for activation by purine nucleotides.

There are also conditions that cause an increase in either the transcription of alternative oxidase or an increase in the stability of the mRNA, both of which are potential mechanisms for increasing alternative oxidase activity. The mechanisms have been shown to be used under different circumstances to increase alternative oxidase activity. When mangoes ripen, an increase in alternative oxidase activity is accompanied by an increase in transcription and protein levels (VANLERBERGHE and MCINTOSH 1997). Microarray analysis comparing induced and uninduced isolates of *A. thaliana* suggested that transcriptional regulation may play a role in induction of alternative oxidase by antimycin A (YU et al. 2001). However, the trypanosome alternative oxidase (TAO) is regulated at the level of mRNA stability. The TAO transcript has a much longer half-life in the bloodstream form versus the procyclic form, and the transcript levels were higher in the bloodstream form as well

(CHAUDHURI et al. 2002). Transcript degradation was inhibited if cells were incubated with cycloheximide (CHAUDHURI et al. 2002).

1.7 Reactive oxygen signaling and alternative oxidase expression

There is evidence of alternative oxidase induction by reactive oxygen species (ROS), as discussed briefly in section 1.5. Salicylic acid (SA) is a potent inducer of alternative oxidase in plants, including *S. guttatum*, tobacco, and potato (WAGNER 1995). Salicylic acid inhibits catalase activity, which in turn causes an increase in intracellular H_2O_2 (WAGNER 1995). Introduction of SA into tobacco cultures causes inhibition of ATP synthesis and O_2 uptake, but this response was inhibited by inclusion of an antioxidant, suggesting that ROS may be involved in causing standard cytochrome respiration dysfunction as well as inducing alternative oxidase.

Incubation of isolated tobacco mitochondria with SA had no effect on mitochondrial function, indicating that SA effects require other components that are not found in isolated mitochondria (XIE and CHEN 1999). Analysis of the promoter sequence of the *S. guttatum aox-1* gene, revealed sequences similar to genes that are up-regulated upon pathogen attack (RHOADS and MCINTOSH 1993). This finding is in accord with research showing that SA is also directly involved in plant disease resistance and resistance to pathogen attack (XIE and CHEN 1999). SA induces alternative oxidase in this plant by causing an increase in the accumulation of the *aox1* transcript (RHOADS and MCINTOSH 1993). A similar increase in transcript accumulation was seen in the rice blast fungus *M. grisea* upon exposure to H_2O_2, in this case due to an increase in the level of transcription of the gene. Northern blots and nuclear run off assays revealed that the transcript was produced constitutively, but was undetectable under non-inducing conditions, suggesting degradation of the mRNA under normal growth conditions. Upon induction with H_2O_2 or the fungicide SSF-126, transcript accumulation occurred and the level of transcription rose markedly as well (YUKIOKA et al. 1998b).

In experiments that did not involve SA, such as the incubation of *Petunia hybrida* cells directly in H_2O_2, an increase in the presence and activity of alternative oxidase was seen, though the manner of activation was unidentified (WAGNER 1995).

In the yeast *Hansenula anomala*, it was found that antimycin A inhibited electron transport, which caused an increase in O_2^- and an increase in alternative oxidase activity (MINAGAWA et al. 1992). If antimycin A and flavone, a free radical scavenger, were added together, both the generation of O_2^- and induction of KCN-insensitive respiration were inhibited, and antimycin-induced synthesis of the putative alternative oxidase protein was completely abolished. This suggested that O_2^- might be involved directly in inducing KCN-insensitive respiration (MINAGAWA et al. 1992). ROS levels within the cell cause a disruption of normal mitochondrial function, and may explain how H_2O_2 or SA indirectly activates nuclear stress-induced genes. When the drug bongkrekic acid, which blocks mitochondrial permeability transition pores involved in mitochondrial signaling during apoptosis in animal cells, was used prior to addition of antimycin A, SA or H_2O_2, it completely inhibited stress gene activation in tobacco cells (MAXWELL et al. 2002). It was suggested that mitochondria may actively function to communicate intracellular stress signals to the nucleus. This may explain why diverse stress induced genes, including pathogen response genes and alternative oxidase, have regulatory regions that are similar (RHOADS and MCINTOSH 1993). All these genes have different inducers, but it may be that all are activated by similar mitochondrial signals.

1.8 Communication between the mitochondria and nucleus

There must be communication between mitochondria and nucleus as the proper functioning of the mitochondria depends upon many nuclear gene products, some of which may be up-regulated or down-regulated depending upon mitochondrial status. The cell must be able to interpret the signal and alter transcription or use some form of post-transcriptional regulation to generate the required response. Several mitochondrial signals have been discovered, and it is entirely possible that more than one may be utilized under a given set of circumstances or that different signals may elicit the same cellular response depending upon the situation of the cell.

1.8.1 Retrograde regulation in *S. cerevisiae*

One of the most studied mitochondria to nucleus communication systems occurs in the yeast *S. cerevisiae*. It has been shown that the state of mitochondria can

influence nuclear gene expression in a process termed "retrograde regulation". Cells lacking mitochondrial DNA (called ρ^0 petite mutants), or cells in which mitochondrial respiration is compromised, contain 6- to 30-fold higher levels of Cit2p compared to respiratory competent ρ^+ cells. The nuclear gene *CIT2* encodes a peroxisomal isoform of citrate synthase, which catalyzes the first step of the glyoxylate cycle (LIAO and BUTOW 1993). The upregulation of this gene generates metabolic intermediates such as succinate that can enter the TCA cycle. This allows the bypass of steps in the TCA cycle that release CO_2, allowing these carbon molecules to be used in biosynthetic pathways (SEKITO et al. 2000). The region upstream of the *CIT2* gene was found to contain an upstream activating site (UAS) involved in both basal and retrograde up-regulation that was termed $UAS_{retrograde}$ (UAS_r). Two nuclear genes were found to be involved in the retrograde regulation of *CIT2*. These were named *RTG1* and *RTG2* (for retrograde regulation mutants 1 and 2, respectively). Both single mutants eliminate *CIT2* retrograde regulation, but are not lethal. RTG1 encodes a 19 kDa basic helix-loop-helix-leucine zipper (bHLH/Zip) transcription factor that has been found to interact with UAS_r as a heterodimer with the product of a third *RTG* gene, *RTG3* (JIA et al. 1997). The biochemical function of Rtg2p is not currently known, but when it is absent, Rtg3p is hyperphosphorylated and constitutively cytoplasmic. The signal(s) that Rtg2p responds to also remain undetermined (SEKITO et al. 2000). The Rtg1p/Rtg3p dimer binds to a sequence in the UAS_r termed the R box (JIA et al. 1997). The *RTG* genes influence *CIT2*, which provides a supply of metabolites, such as succinate, to the TCA cycle that participate in anabolic pathways involved in the synthesis of α-ketoglutarate, which helps to ensure there is sufficient glutamate for biosynthetic pathways (SEKITO et al. 2000).

1.8.2 Reactive oxygen species signaling

As discussed in sections 1.5 and 1.7, reactive oxygen species (ROS) are produced during respiration in mitochondria and can cause significant cellular damage, which may induce alternative oxidase expression. In addition to the proposed role of ROS signaling in alternative oxidase induction, ROS are involved in several different types of signaling. The responses to extracellular ROS include activation of protein kinases that are involved in regulation of transcription. The

direct targets are not the kinases themselves, but rather the redox sensitive cysteine residues found in the phosphatases that activate the kinases, including tyrosine kinases, that are involved in a diverse array of signalling pathways (FINKEL 2000). In mammals, the transcription factor NF-κB, is activated in response to conditions that lead to the generation of ROS, including certain viral infections or oxidative stress. The ROS physically interact with an inactive NF-κB in the cytoplasm, releasing an inhibitory subunit. The active NF-κB travels to the nucleus, and binds to the promoters of genes containing NF-κB enhancer elements (POYTON and MCEWEN 1996). As discussed in section 1.7, there are several species of plants and fungi that induce alternative oxidase upon exposure to extracellular H_2O_2 as well as ROS that are created intracellularly. In *Arabidopsis*, there is also evidence for a MAPK kinase that is activated by the presence of H_2O_2, which then establishes a stress signaling phosphorylation cascade (KOVTUN et al. 2000).

1.8.3 Calcium ion signaling

Another potential mechanism of signaling from the mitochondria has to do with the function of the organelle in calcium storage. Mitochondria that are undergoing distress could release Ca^{2+} ions to signal the nucleus. There are precedents for a role for Ca^{2+} in respiratory gene regulation. In rice, under conditions of low oxygen (hypoxia), Ca^{2+} was found to be involved in signaling the presence of low oxygen conditions to the nucleus. In wild type rice, anaerobic growth conditions repress the expression of *AOX1a* as well as nuclear-encoded cytochrome pathway genes, but mitochondria-encoded cytochrome pathway genes are not repressed. In hypoxic conditions, the addition of ruthenium red, which inhibits Ca^{2+} release from mitochondria, caused an increase in *AOX1a*, but showed no effect on the levels of cytochrome pathway nuclear genes *COX5b*, *COX5c*, and *ATP2* (TSUJI et al. 2000). This indicated that the mitochondrial signal of low oxygen is Ca^{2+} based, and is responsible for the hypoxic repression of alternative oxidase. However, there must also be a calcium independent pathway to regulate other nuclear-encoded respiratory genes (TSUJI et al. 2000). In mammals, when rat myoblasts were treated with mitochondrial inhibitors that resulted in a disruption of membrane potential, increased levels of cytosolic Ca^{2+} were seen. This in turn activated Ca^{2+}-responsive transcription

factors such as Ca^{2+}-dependent protein kinase C and Ca^{2+}-calcinurin (AMUTHAN et al. 2001). A similar release of Ca^{2+} is seen in mice cells with decreased mitochondrial DNA, or in the presence of mitochondrial poisons (BISWAS et al. 1999). In rat neural cells, an influx of Ca^{2+} can activate Ca^{2+}/calmodulin kinase II (Cam kinase). Cam kinase can phosphorylate transcription factors, including the cyclic-AMP response element binding protein (CREB), which binds to a cyclic-AMP response element (CRE). CREB that was phorphorylated by Cam kinase was able to activate *in vitro* transcription of the *c-fos* gene, which has several CRE sites in its promoter. This provided evidence that the use of Ca^{2+} as a signal from dysfunctional mitochondria to the nucleus may be able to activate transcription of genes containing a CRE (DASH et al. 1991; SHENG et al. 1991).

1.9 Objective of this study

The major topic of this thesis was to study the upstream sequence and regulation of the alternative oxidase structural gene *aod-1* in *N. crassa*. The presence of a CRE upstream of *aod-1* was of interest because of the role the CRE is known to play in the transcriptional regulation of genes in many species, including the *N. crassa grg-1* gene (WANG et al. 1994). CREs bind proteins, which respond to signals received by the cell, so the protein binding capacity of the CRE upstream of *aod-1* was investigated. Recently, transcriptional activation of a gene containing a CRE was observed in response to mitochondrial dysfunction. In human cell lines with the mitochondrial MERRF (mitochondrial encephalopathy with ragged-red muscle fibers) disease, in cell lines treated with ethidium bromide to cause a severe reduction in the amount of mtDNA present and in wild type cells with mitochondria impaired by addition of respiration inhibitors, CREB was activated by phosphorylation and increased transcription of a reporter construct under the control of a CRE (ARNOULD et al. 2002). As alternative oxidase is induced in *N. crassa* in response to mitochondrial dysfunction, the possibility of the CRE playing a role in this induction was investigated. During the course of these studies, the involvement of post-transcriptional regulation of *aod-1* mRNA and AOD-1 protein accumulation became evident.

2 Methods and Materials

2.1 Strains and growth conditions

N. crassa strains used in this study are listed in Table 1. Vegetative growth of all fungal strains was on Vogel's medium (DAVIS and DESERRES 1970), either liquid or solid (containing 1.5% agar). 1.5% sucrose, in the form of table sugar, was included when extensive hyphal growth was desired, while 1.5% sorbose was used if colonial growth was needed. All media was supplemented with nutrients as necessary. For long term storage, isolates of all fungal species and strains that formed conidia on slants were placed at –80°C.

2.2 Conditions of growth for *N. crassa*

General procedures for growth and handling of *N. crassa* were as described by Davis and Serres (DAVIS and DE SERRES 1970). To generate large amounts of conidia to serve as inocula for liquid cultures, *N. crassa* was grown on 50 ml of solidified Vogel's media in a 250 ml Erlenmeyer flask for 36 to 48 hr at 30°C, then allowed to conidiate at room temperature in constant light for at least 5 to 7 days. These are referred to as "conidia flasks".

Samples to be tested for only oxygen consumption were grown in 50 ml of liquid Vogel's medium that was inoculated at a concentration of 10^6 conidia/ml of media using conidia harvested in dH_2O. Cultures were grown with shaking (150 revolutions per minute) at 30°C, unless otherwise noted. In the absence of inhibitors, cultures were grown for 12 to 14 hr. In the presence of chloramphenicol (2 mg/ml), cultures were grown for 18 to 25 hr, and in the presence of antimycin A (0.5 μg/ml) growth was for 47 to 49 hr. In all cases, inhibitors were added at the start of the growth of the culture.

Cultures used for RNA isolation alone or RNA isolation and mitochondrial isolation were grown in 250 ml volumes. Growth of cultures for large-scale protein isolation (section 2.33) was in 8 L of Vogel's medium in 9 L glass carboys, placed in a 30°C water bath for 12 to 16 hr with aeration from filtered air bubbled through

Table 1
N. crassa strains used in this study.

Strain name (previous name)	Genotype	Source
74-OR23-IV*A*	*A*	FGSC #2489
NCN 10	*nic-1 al-2 A*	A. Lambowitz
NCN 235	*pan-2 a*	Nargang lab
7001 (NSA-95*a*)	*aod-1-1 a*	H. Bertrand
7021 (NSK-1*a*)	*aod-1-2 a*	H. Bertrand
7202	*aod-1-4 pan-2 a*	H. Bertrand
7216 (QDED-6-59*A*)	*aod-1-6 A*	H. Bertand
7064 (NSBAN-4*a*)	*aod-2-4 nic-1 al-2 a*	H. Bertrand
7207	*aod-1-7 pan-2 a*	H. Bertrand
H1	*pan-2 a*	7207 transformed with pMAX*
35-4	*pan-2 a*	7207 transformed with pMAX*
J6	*pan-2 a*	7207 transformed with pΔMAX*
8-30	*pan-2 a*	7207 transformed with pΔMAX*
K5	*pan-2 a*	7207 transformed with pMMAX*
K6	*pan-2 a*	7207 transformed with pMMAX*
39-1	*pan-2 a*	7207 transformed with pMMAX*
L11	*pan-2 a*	7207 transformed with pMCMAX*
L18	*pan-2 a*	7207 transformed with pMCMAX*

* Plasmids are described in Table 3 and section 2.39 of this thesis

sterile tubing. These cultures were inoculated with the conidia harvested from 5 fresh (less than 2 weeks old) conidia flasks, without calculating the inoculum in conidia/ml.

2.3 Conditions of growth for *Gelasinospora*

Growth of *Gelasinospora* on solid Vogel's medium was at 30°C for 48 hr. *Gelasinospora* does not conidiate; therefore growth for the purpose of inoculating liquid media was on standard Vogel's solid medium in Petri plates. Small regions (approximately 1 cm x 1 cm) of mycelia were scraped off the agar with a sterile spatula and added directly to 50 ml liquid media. *Gelasinospora* liquid cultures were grown in standard Vogel's medium at 30°C for 36 to 48 hr with shaking (150 rpm).

2.4 Conditions of growth for *Aspergillus nidulans*

Aspergillus nidulans was grown on solid Vogel's medium in Erlenmeyer flasks at 37°C for 24 to 36 hr and then shifted to constant light at room temperature for 5 to 7 days for conidiation. *A. nidulans* liquid cultures were grown in Vogel's medium at 37°C with shaking (150 rpm) for 23 to 25 hr.

2.5 Cytochrome spectra

Cytochrome spectra were obtained according to Bertrand and Pittenger (BERTRAND and PITTENGER 1969) with a Shimadzu UV-265 spectrophotometer (Guelph, ON), using mitochondria isolated as described in section 2.48.

2.6 Preparation of *N. crassa* spheroplasts

Spheroplasts were prepared as described (AKINS and LAMBOWITZ 1985; SCHWEIZER et al. 1981). Fresh conidia were harvested in dH_2O, counted and inoculated into 500 ml Vogel's medium at a concentration of 5 X 10^6 to 1.5 X 10^7 conidia/ml of medium. These were grown at 30°C with shaking (50 rpm) for 4 to 7 hours and aliquots of conidia were examined for evidence of germination at 30 min intervals, using a light microscope (length of time for germination is strain dependent). A conidium was considered germinated when a small protuberance was seen. The ideal time for spheroplasting was when approximately 80% of the conidia

had germinated. The conidia were then harvested by centrifuging for 10 min at 5000 rpm in the Sorvall GS-3 rotor. The supernatant was discarded and the bottles containing the germinated conidia were kept on ice at all times. The conidia were washed once with 100 ml cold sterile dH_2O and twice with 100 ml cold sterile 1M sorbitol (Sigma, Oakville, ON). The final pellet was resuspended to a total of 9 ml in 1M sorbitol and the concentration of conidia was determined using a haemocytometer. The concentration was adjusted to between 10^8 and 10^9 conidia/ml using sterile 1M sorbitol to dilute as needed. The conidia were transferred to a sterile 250 ml Erlenmeyer flask and 5 mg Lysing Enzyme (Sigma, Oakville, ON) was added per ml of suspension. The mixture was incubated at 30°C with gentle shaking (100 rpm). Periodically, 10 µl aliquots were removed and placed on a microscope slide to assess the spheroplast formation. Addition of 10 µl 10% sodium dodecyl sulfate (SDS) caused conidia with digested cell walls to burst, giving rise to "ghosts" that were visible upon microscopic examination. When ghosts comprised 90% of the sample, spheroplasting was considered to be optimal. At this point, the flask contents were poured into a sterile 50 ml screw-tap tube and centrifuged at the lowest setting in a clinical centrifuge for 10 min. The supernatant was removed by aspiration and the spheroplasts were washed twice with 45 ml 1M sorbitol and once with sterile MCS (see appendix). Finally, the spheroplasts were resuspended in 6 to 8 ml sterile MCS and their concentration adjusted to 2.5 to 5 X 10^8 spheroplasts/ml. For each ml of spheroplast solution, 13 µl dimethlysulfoxide (DMSO), 65 µl Heparin (5 mg/ml) and 275 µl PMC (see appendix) were added. After gentle mixing the spheroplasts were aliquoted in volumes of 50 µl to 1 ml and stored at –80°C.

2.7 Transformation of *N. crassa* using spheroplasts

Frozen spheroplasts were thawed on ice. Plasmid DNA (5 µg) to be transformed was linearized with a suitable restriction enzyme, mixed with sterile dH_2O to a final volume of 60 µl, added to 100 µl spheroplasts and gently mixed. The suspension was incubated on ice for 30 min and then mixed with 1.6 ml sterile PMC (see appendix) in a 50 ml sterile tube. Following incubation at room temperature for 20 min, top agar containing the appropriate selection agent (see appendix), at

approximately 45°C, was added to fill the tube. The tube was mixed by gentle inversion and 10 ml was poured onto each of 5 viability plates containing the appropriate selection agent (see appendix). Plates were placed at 30°C until transformed colonies were visible. If bleomycin was used as the selection agent, three days incubation was required; if benomyl was used, two to three days incubation was required; if hygromycin was used two days of incubation was required.

2.8 Transformation of *N. crassa* by electroporation

Electroporation was done with modification of a previously described method (MARGOLIN et al. 1997; MARGOLIN et al. 2000). Sterile dH_2O (50 ml) was used to harvest conidia from two to four fresh (one week old) conidia flasks (section 2.2). The conidia were washed 3 times with 50 ml 1M sorbitol by centrifugation at top speed for 1 min in a clinical centrifuge and resuspended in 10 to 15 ml of 1M sorbitol. The conidia were counted and resuspended in 1M sorbitol, to give a final concentration of 2 to 2.5 X 10^9 conidia/ml. The conidia could be used at this point or stored in 40 µl aliquots at –80°C for later use. Frozen aliquots were pelleted with a 30 sec centrifugation in a microcentrifuge and washed twice with 1M sorbitol before use.

Linearized plasmid DNA (5 µg, in a final volume of 5 µl) was mixed with 45 µl of fresh or frozen conidia, prepared as above. This mixture was placed in a pre-chilled electroporation cuvette and kept on ice until electroporation. A BioRad Gene Pulser (BioRad, Mississauga, ON) was used with settings of 2.1 kV, 475 Ohms, 25 µF. Immediately after the pulse (time constant 11 to 12 millisec), 1 ml of ice-cold 1M sorbitol was added and the conidia were allowed to recover for 1 hr at 30°C before being plated on viability plates in top agar (see appendix) containing the appropriate selection agent. The plates were placed at 30°C for 3 days to allow transformed colonies to form. Transformants were purified as described in section 2.9.

2.9 Purification of single *N. crassa* colonies

Individual transformed colonies were picked from transformation plates using sterile Pasteur pipettes and transferred to slants containing 1 to 2 ml solid Vogel's medium and 0.5X concentration of the selective agent. The slants were placed at

30°C for two to three days to allow mycelia to spread over the surface of the medium and then were placed in the light at room temperature for 2 to 3 days to allow the formation of conidia. Conidia were then spread on viability plates (see appendix) containing 1X concentration of the selection agent to obtain single colony isolates of each transformant. These plates were placed at 30°C for several days and colonies were picked as above and transferred to 5 ml slants of Vogel's media with no selection agent. These were allowed to grow for two nights at 30°C then placed in the light to conidiate at room temperature. A round of purification of single colonies was used to increase the likelihood of obtaining homokaryotic isolates of each transformant.

2.10 Measurement of oxygen consumption in *N. crassa*

Oxygen consumption of *N. crassa* grown in liquid culture was measured in a Yellow Springs Instruments (Model 530) oxygen monitor (YSI, Yellow Springs, OH) equipped with a Clark-type oxygen electrode. Approximately 1 ml of fresh mycelia, removed directly from a growing culture, was added to 2 to 3 ml of Vogel's medium that had been bubbled with air for approximately 30 sec. Oxygen consumption was measured in the closed chamber. Stock solutions of the inhibitors KCN (0.1M dissolved in 10 mM Tris-Cl, pH 7.2; 5 mM EDTA) and SHAM (0.33M, dissolved in 95% ethanol) were made daily, just prior to use. 50 µl of the KCN solution and 100 µl of the SHAM solution were added to mycelia and the effect on oxygen consumption was recorded on a Pharmacia chart recorder. Addition of SHAM to the chamber resulted in an increase in $[O_2]$ of the sample due to the ethanol, which contains more dissolved oxygen than does the KCN solvent.

2.11 DNA sequencing

For the early stages of this work DNA was sequenced by the $[^{33}P]$-ddNTP PCR based sequencing method using an Amersham Pharmacia thermosequenase kit (Amersham-Pharmacia, Baie d'Urfe, QB) in accordance with the instructions of the manufacturer. The products of the reaction were separated by electrophoresis using polyacrylamide gels (see appendix). Gels were made and run with 1X glycerol

tolerant gel buffer (see appendix). Gels were poured between glass plates with 0.25 mm spacers and pre-run for 30 min to 1 hr at 30 watts (W). Samples were loaded using a shark's tooth comb and electrophoresed at 30 W until the bromophenol blue dye ran to the bottom of the gel (short run) or until the xylene cyanol ran to the bottom of the gel (long run). Following electrophoresis, the gel was dried and exposed to Kodak MR film overnight at room temperature.

In the later stages of this work, DNA sequence was obtained using a PCR-based fluorescent sequencing kit, the DYEnamic ET system (Amersham Pharmacia, Baie d'Urfe, QB). Sequencing reactions were prepared according to the supplier's instructions, electrophoresed on a 373 stretch sequencer (Applied Biosystems, Foster City, CA) and sequence profiles were produced using Applied Biosystems sequencing analysis software version 3.4.1 (Applied Biosystems, Foster City, CA). The electrophoresis and production of sequence was performed by the Molecular Biology Service Unit (Department of Biological Sciences, University of Alberta). All DNA sequences were determined entirely on both strands.

2.12 DNA sequence analysis

The programs of DNAMAN (Version 4.13, Lynnon Biosoft) were used to analyze sequences. Sequences were also subjected to Basic Local Alignment Search Tool (BLAST) searches against various genomic and expressed sequence tag (EST) databases.

2.13 RNA isolation

All reagents used in RNA work were made with diethylpyrocarbonate (DEPC) treated H_2O prepared by stirring dH_2O containing 0.1% DEPC overnight and then autoclaving for 45 min. Equipment used for RNA work was washed in the detergent RBS35 (Pierce, Rockford, IL), then rinsed with DEPC treated H_2O. *N. crassa* cultures grown for RNA isolation (section 2.2 for growth conditions) were harvested in a Buchner funnel lined with Whatman #4 filter paper. Between 50 and 100 mg of the harvested mycelium was wrapped in foil and immediately frozen in liquid N_2. The sample was ground in liquid N_2 using a mortar and pestle. RNA was isolated using a

Qiagen RNEasy Plant RNA isolation kit as described by the supplier (Qiagen, Mississauga, ON) The RNA was eluted with two 50 µl volumes of RNase-free H_2O. The samples were stored at –80°C.

2.14 RNA gels and blotting

The RNA gel apparatus was treated with H_2O_2 (3%) for at least 1 hr before use. RNA gels (15 cm x 21 cm x 1cm) were made by mixing 20 ml 10X MOPS-EDTA buffer (see appendix), 170 ml of DEPC treated H_2O and 2.4 g agarose. The agarose was melted by heating it in a microwave oven and 10 ml 37% formaldehyde was added just before pouring the gel. The gels were poured and electrophoresed in a fume hood.

The RNA sample (5 µg) to be run was suspended in freshly prepared RNase free loading buffer (to a total volume of 50 µl), containing formamide to denature the RNA (see appendix). Samples were incubated at 65°C for 15 min to further denature the RNA. Immediately prior to loading, 5 µl loading dye (see appendix) was added. The gels were electrophoresed at 150 V for 2 hr or at 25 V for 18 hr.

Prior to blotting the RNA to nylon membrane (ICN Biotrans membrane, Montreal, QB), the gel was rinsed three times for 20 min each in DEPC dH_2O to remove the formaldehyde. The gel was photographed and transferred to nylon membrane using 10X SSC (see appendix) as the wicking buffer. The RNA was allowed to transfer overnight. The membrane was then rinsed for 5 min in 2X SSC and baked under vacuum at 80°C for one hr (or dried at room temperature overnight) before pre-hybridization.

2.15 Northern pre-hybridization and hybridization

Pre-hybridization buffer (see appendix) was made fresh each time a northern hybridization was performed. Membranes (cut to the size of the gel, typically 8 cm x 12 cm) were incubated in 50 ml pre-hybridization solution at 42°C for at least 3 hr in rotating bottles in a hybridization oven (Tyler Research, Edmonton, AB). Hybridization was performed with 25 ml of pre-hybridization buffer containing denatured probe (see below) for 18 to 24 hr at 42°C. Following hybridization, the

membranes were washed twice with 2X SSC, 0.1% SDS, at room temperature for 30 min. The third wash consisted of 1X SSC, 0.1% SDS and was for 30 min at room temperature. The fourth wash was at 55°C, using pre-warmed buffer (0.2X SSC, 0.1% SDS), for 45 min. The blots were wrapped in Saran-wrap and exposed to Kodak XAR film at –80°C for an appropriate time, usually overnight.

2.16 Radioactive labeling of DNA

Plasmid DNA (5 μg) or a specific restriction fragment (generated from 5 μg plasmid) was denatured by boiling for 5 min, followed by 3 min in an ice/salt water bath. The denatured DNA was combined with 5 μl of α-[^{32}P] dCTP (10 mCi/ml), 12 μl dNTP mix (0.5 mM each dATP, dGTP and dTTP in dH_2O), 8 μl random hexanucleotide mix (Roche Scientific, Laval, QB), 5 μl React 2 buffer (Gibco BRL, Burlington, ON) and 3 μl Klenow enzyme. The mixture was incubated at 37°C for 1 to 4 hr. The labeling reaction was passed through a Sephadex G50 spin column (section 2.17) to remove unincorporated nucleotides. Probes were denatured by boiling for 5 min and incubated in an ice/salt water bath for 3 min before they were added to hybridization bottles. Usually, the entire labeled probe was used without determining the counts/minute (cpm).

2.17 Preparation and use of spin columns

Sephadex G50 powder (2 g) was mixed with 50 ml sterile TE (see appendix), to form a slurry. This was autoclaved for 15 min to allow full swelling of the beads. To create a spin column, a 1 ml syringe was plugged with silanized glass wool and the Sephadex slurry was pipetted carefully into the syringe, avoiding air bubbles. The syringe was placed inside a 13 ml polycarbonate tube and this was centrifuged for 30 sec at top speed in a clinical centrifuge. A second addition of Sephadex slurry and a second centrifugation were usually required to allow the Sephadex bed to reach the 0.9 ml mark of the syringe. To remove unincorporated nucleotides from labeling reactions, the reaction was placed on the surface of the Sephadex bed, and a 1.5 ml microcentrifuge tube, with the cap removed, was placed in the bottom of the centrifuge tube to collect the flow through. The column was centrifuged as above, 25

to 50 µl more buffer was added, and a second centrifugation was performed to remove any DNA remaining on the column.

2.18 Oligonucleotides used in this study

Oligonucleotide primers were used for PCR amplification of DNA and for sequencing the upstream region of *N. crassa aod-1* as well as the alternative oxidase genes and surrounding sequences of *Gelasinospora* and *A. nidulans*. The oligonucleotides were synthesized by the Molecular Biology Service Unit of the Department of Biological Sciences at the University of Alberta. The sequence of the oligonucleotides, their use, and their position within the genes are listed in Table 2.

2.19 Polymerase Chain Reaction

Typical PCR amplification reactions consisted of 1 µl (10 ng/µl) each of two 20 to 25 base oligonucleotide primers, 1 µl plasmid (1 ng/µl) or 1 µl genomic DNA (10 to 100 ng/µl), 40 µl 1.1X PCR mix (see appendix) and 10 units of *Taq* DNA polymerase. The initial step of the PCR reaction was 5 min incubation at 95°C to ensure complete denaturation of the template. This was followed by 30 rounds of thermal cycling. Annealing of primers to the template DNA was done at 58°C to 60°C for 30 sec to 1 min, depending on G-C content of the primers (following the guidelines in (SAMBROOK and RUSSELL 2001)). DNA polymerization was done at 72°C for 1 min per kb of DNA to be synthesized, followed by denaturation at 92°C for 1 min.

2.20 Restriction analysis and agarose gel electrophoresis

Restriction endonucleases and other DNA and RNA modifying enzymes including T4 DNA ligase, T4 kinase, RNase A and Klenow fragment were purchased from either Gibco BRL (Burlington, ON), Pharmacia Biotech (Baie d'Urfe, QB), New England Biolabs (Mississauga, ON) or Roche Scientific (Laval, QB). All were used according to the manufacturer's instructions.

For standard plasmid and genomic work, gels were typically 0.8% agarose dissolved in 1X TAE (see appendix) containing ethidium bromide (final

Table 2
Primers used during this study

Primer name	Sequence (5' to 3')	Use of primer	5' primer end **	3' primer end **
aoa-1*	CTCGAGGCCTCTCTTCCTCAC	*aod-1* upstream sequencing	-3004	-2983
aoa-3*	GGTCGAGATGTCGATGGTGTT	*aod-1* upstream sequencing	-2530	-2509
aoa-4*	CCTTCTTCGCCTTCTCGCGC	*aod-1* upstream sequencing	-2228	-2208
aoa-5*	CCACGAGTCGAGAACGTCGC	*aod-1* upstream sequencing	-2182	-2162
aoa-6*	TGGGTTTCGAACAGGTTGTG	*aod-1* upstream sequencing	-2049	-2029
aoa-6A*	GCGCAACAGGAAAGTCGATG	*aod-1* upstream sequencing	-2026	-2006
aoa-6b*	AGGAGAGCGAGGAAGACTTA	*aod-1* upstream sequencing	-1791	-1771
aoa-7*	GCGCAAAGAGGGAAAAGGCA	*aod-1* upstream sequencing	-1811	-1791
aoa-8a*	GAGTGTGGTTCCGTTCGCAG	*aod-1* upstream sequencing	-1581	-1561
aob-1*	GATCAGTGCCCCAAGAGGCCA	*aod-1* upstream sequencing	-3291	-3270
aob-2*	CCAATGCTTCCCGGGTAGG	*aod-1* upstream sequencing	-3068	-3049
aob-3*	TGCCAAGACAAAGACCTCGC	*aod-1* upstream sequencing	-2777	-2797
aob-3a*	GCGAGGTCTTTGTCTTGGCA	*aod-1* upstream sequencing	-2795	-2775
aob-4*	TTTACCGGGTATAATCCTCAT	*aod-1* upstream sequencing	-3040	-3061
aob-5*	AGTCGCGACTTGGGGGTGA	*aod-1* upstream sequencing	-2993	-3012
aoc-1*	GCCGAGGACTCCGAGGTAGAG	*aod-1* upstream sequencing	-1288	-1309
aoc-2*	GCGACGTTCTCGACTCGTG	*aod-1* upstream sequencing	-1311	-1330
aoc-3a*	TTAAACACGCTGAGGACCTG	*aod-1* upstream sequencing	-2409	-2429
aoc-4*	GCTCGCCAAGAGCCTCAAGA	*aod-1* upstream sequencing	-2655	-2675
FNA192*	AACGGGAGGAATAAATGGGGAC	*aod-1* upstream sequencing	-1309	-1331
FNA193*	CAAGGTGCCTGACCCCTGCTC	*aod-1* upstream sequencing	-1581	-1602
FNA198*	CCTGATGCCTGCGCCGGCTTGC	*aod-1* upstream sequencing	-1866	-1888
AOORF-1*	GTGTCGTGGGCCTCAGCAAT GGCA	*N. crassa* ORF PCR	-2841	-2817
AOORF-2*	CGAGGAAGAGGAGGAGTCTT CCGCC	*N. crassa* ORF PCR	-2076	-2101
AO9	CGCCTGGCTTCATGAACCC	*N. crassa aod-1* gene PCR	1363	1344
AO12	TCACAAACCTCAAGCGAGT TCCA	*N. crassa aod-1* gene PCR	2	25
AO26*	TTTTCAGTCAGCTCTCGTATT	PCR of *aod-1-7* mutant region	-112	-91
AO27*	GAAATCCTTCAGTCGAGT	PCR of *aod-1-7* mutant region		
AO32*	ACTGTGGGCGATTGGCTCGC ATGG	*Neurospora aod-1* intron PCR	371	394
AO33*	CATGCCGGCTACCATGCCGG GAAC	*Neurospora aod-1* intron PCR		
AO40*	TTGAATTCGACCACTTGCAA TTGTTCCTTCGATC	PCR of *aod-1* CRE region	-795	-769
AO41*	TTGAATTCGTCTCGATGTCG GGGGCCATAAGTTGCGAATG	PCR of *aod-1* CRE region	-685	-717
AO42*	TTGAATTCCCTTCGATCCTA CACTCTACGAATAACTA	PCR of *aod-1* CRE region	-781	-755
AO43*	TTGAATTCGCTCGAATGGCTT CATGTTAAACTTCTAACTTT	PCR of *aod-1* CRE region	-705	-736
AnORF3*	TTGCTTCCCTTATCAGCTG CCCTCT	*A. nidulans* ORF PCR		
AnORF4*	AAGTCGAAGTCGAAACCGA AACCGA	*A. nidulans* ORF PCR		

* Indicates oligonucleotides created during this study
** Numbering according to Fig. 6 for *N. crassa*, Fig. 11 for *Gelasinospora*, and Fig. 14 for *A. nidulans*

Table 2 continued

Primer name	Sequence (5' to 3')	Use of primer	5' primer end **	3' primer end **
AnAO4*	TTTCATCTGCGCTCATAATTG	*A. nidulans* altox gene sequence	1367	1338
AnAO5*	TATGGACCTGGTCGACTGGCTA	*A. nidulans* altox gene sequence	415	435
AnAO9*	TAACCATTCCTTTTCCGTCAT	*A. nidulans* altox gene sequence	479	499
AnAO16*	GACATCGAATTCATAATGCTG	*A. nidulans* altox gene sequence	-7	14
AnAO17*	CAGCATTATGAATTCGATGTC	*A. nidulans* altox gene sequence	14	-7
AnAO18*	TGGCGCAGATCGAGACGCTCC	*A. nidulans* altox gene sequence	658	677
AnAO19*	TTCGTGGCTCAACTAATAATC	*A. nidulans* altox gene sequence	655	636
AnAO20*	GAAGCCGGGACGGGACCACGAA	*A. nidulans* altox gene sequence	-654	-633
AnAO21*	AGAACGTATTCCGCAGCACGC	*A. nidulans* altox gene sequence	-1233	-1212
AnAO22*	GGGAGCAAATGGGAGAACATT	*A. nidulans* altox gene sequence	1212	1231
GAX1*	TTCTTCAACGCCATGTTCC TCAGCTACCTG	*Gelasinospora* altox gene sequencing	894	924
GAX3*	AAAATCACCCACCCGGTTC GTCGGCTACTTCG	*Gelasinospora* altox gene sequencing	935	966
GAX7*	CAACATAGGATGCACTTTGAA	*Gelasinospora* altox gene sequencing	-1164	-1143
GAX8*	TGATGCACCGCGTGTAGGTAT	*Gelasinospora* altox gene sequencing	1002	981
GAX9*	AAGTGTGTATGTACTTCGGA	*Gelasinospora* altox gene sequencing	1459	1479
GAX10*	ACTGGTGTTCCGTTTCCCTCCG	*Gelasinospora* altox gene sequencing	-620	-599
GAX11*	CAAGATCGTTGAGGGACAATAT	*Gelasinospora* altox gene sequencing	460	440

* Indicates oligonucleotides created during this study

** Numbering according to Fig. 6 for *N. crassa*, Fig. 11 for *Gelasinospora* and Fig. 14 for *A. nidulans*

concentration of 0.5 μg/ml). The gels were electrophoresed in 1X TAE buffer. Gels were typically run at 150 V for 1 to 2 hr. When smaller DNA fragments (<500 base pairs) were being analyzed, 2% agarose gels were used.

2.21 Isolation of DNA from agarose gels

DNA was isolated from standard agarose gels using the glassmilk method (Geneclean II kit, from Q Biogene, Carlsbad, CA). Small DNA fragments (<100 base pairs) were purified from low temperature gelling agarose using a protocol modified from (SAMBROOK and RUSSELL 2001). The desired region of the gel was excised and placed into a 1.5 ml microcentrifuge tube. TE buffer (see appendix) was added so that the agarose was present at a final concentration of <0.4%. The tube was placed at 65°C until the agarose was melted. An equal volume of phenol was added and the tube was placed in a microcentrifuge tube mixer for 10 min. The tube was then centrifuged for 5 min and the aqueous phase was placed in a new tube. If there was a large white interface, the phenol phase, including the interface, was re-extracted with an equal volume of TE and the two aqueous phases were combined. The DNA was precipitated with ethanol (section 2.22) and the DNA pellet was dissolved in 50 μl to 100 μl dH_2O.

2.22 Ethanol precipitation of DNA

DNA in an aqueous solution was precipitated by the addition of 3M sodium acetate (1/10 of the volume of the DNA solution) and 2.5 volumes of ice cold 95%. If the concentration of the DNA was low, 5 μg of tRNA as a carrier was also added. This was mixed well and incubated at -20°C for at least 1 hr, then centrifuged for 5 min. The pellet was rinsed with 70% ethanol, air-dried for 10 to 30 minutes, and then dissolved in a suitable amount of dH_2O.

2.23 Southern blotting

DNA was electrophoresed on an agarose gel and photographed alongside a ruler. The gel was processed to prepare the DNA for transfer by incubating for 10 min in 0.2 N HCl which randomly breaks the phosphate backbone of the DNA, creating

smaller, more easily transferred, fragments. The acid incubation was followed by three 5 min washes with dH_2O. The DNA was then denatured by two 20 min treatments in 1.5M NaCl, 0.5N NaOH. The gel was rinsed with dH_2O and neutralized by a 30 min incubation in 3M sodium acetate. The gel was rinsed again in dH_2O, and placed face down on Saran Wrap on the bench top. Nylon membrane, pre-wetted in dH_2O was placed on top of the gel, followed by 4 pieces Whatman #4 3MM paper and paper towels cut to the size of the gel. A glass plate and flask containing about 300 ml of dH_2O was placed on top of the towels. This allowed capillary action to draw the DNA out of the gel onto the membrane during the overnight transfer. The membrane was rinsed in 2X SSC and baked for one hour at 80°C under vacuum prior to pre-hybridization.

2.24 Southern hybridization

Membranes were pre-hybridized for at least 3 hr at 65°C in pre-hybridization solution (see appendix). Denatured probe was added to 25 to 50 ml pre-hybridization solution and the membranes were hybridized overnight at 65°C in a hybridization oven (Tyler Research, Edmonton, AB). The blot was washed twice with 2X SSC, 0.1% SDS at room temperature for 5 min, followed by two 15 min washes at 65°C with pre-warmed 0.1X SSC, 0.1% SDS. The membranes were wrapped in Saran Wrap and exposed to Kodak XAR film at –80°C for appropriate times, usually overnight.

2.25 Fungal genomic DNA isolation

Genomic DNA was isolated from 50 to 200 ml liquid cultures of fungal cells grown to saturation using the method of Schechtman (SCHECTMAN 1986) with some modifications. The cultures were harvested by vacuum filtration through Whatman #4 filter paper. For each gram of mycelium, 1.5 g acid washed sea sand and 2 ml new isolation buffer (see appendix) were added. The mycelium was ground to an even slurry using a mortar and pestle. The suspension was placed in a Sorvall SS-34 tube, and the volume was adjusted to 10 ml with new isolation buffer. The sample was shaken vigorously for 15 to 30 sec and then mixed by gentle inversion for 1 min. The

tube was placed at 70°C for one hr and chilled on ice for at least 10 min prior to adding 0.64 ml 8M potassium acetate pH 4.3. The tube was inverted several times to mix the contents, placed on ice for one hr, and then centrifuged at 14 000 rpm for 15 min at 4°C in a Sorvall SS-34 rotor. The supernatant was transferred to a clean tube and an equal volume of ice-cold isopropanol was added. The sample was mixed and centrifuged at 10 000 rpm in the SS-34 rotor for 10 min to pellet the DNA. The pellet was rinsed in 70% ethanol, air dried for 10 min, dissolved in 400 µl 1mM ethylenediaminetetraacetic acid (EDTA) pH 8.0, and 200 µl High Salt Buffer (see appendix) and 15 µl Ribonuclease A (RNase A) (10mg/ml, see appendix for recipe) were added. The mixture was transferred to a 1.5 ml microcentrifuge tube and incubated at 37°C for 30 min. An equal volume of phenol was used to extract the DNA solution. Sodium acetate (1/10 of the sample volume) was added to the aqueous phase and the DNA was precipitated with 2 volumes of cold 95% ethanol. Following centrifugation at 14 000 rpm in a microcentrifuge for 5 min, the supernatant was removed and the pellet was washed with 70% ethanol. The DNA was dissolved in 100 to 500 µl of sterile dH_2O, depending on the size of the pellet, and stored at –20°C.

2.26 Growth conditions of *Eschericia coli*

E. coli cells were grown in LB media (see appendix), unless otherwise mentioned, at 37°C. Liquid cultures were shaken at 200 rpm for aeration. Antibiotics for selection of cells transformed with plasmids were added after autoclaving. Ampicillin (amp) was used at a final concentration of 100 µg/ml, kanamycin (kan) at a final concentration of 50 µg/ml.

2.27 Preparation of competent *E. coli* for transformation

E. coli cells of strain XL2 Blue or XL10 Blue (Stratagene, LaJolla CA) were prepared by the method of Inoue (INOUE et al. 1990), with cells grown at 18°C to maximize competence. Small aliquots of the competent cells (250 µl) were flash frozen in liquid nitrogen (N_2) and stored at –80°C until needed.

2.28 *E. coli* transformation

E. coli cells were transformed by placing 250 µl thawed competent cells mixed with DNA (in a volume less than 1/10 than that of the cells) on ice for 30 min. The mixture was heat shocked at 42°C for 30 sec and 800µl LB media (see appendix) was added. The tube was gently mixed, and then incubated at 37°C for 20 min. Aliquots were spread on LB plates containing amp (see appendix) and incubated at 37°C overnight.

2.29 Colony lift and screen

Transformed *E. coli* cells were plated on 100 mm plates, containing LB and ampicillin, (see appendix) and grown overnight at 37°C, the plates were removed when the colonies were large enough to be seen, but not larger than 1 mm. Nitrocellulose filters were placed on the colonies and allowed to remain there until thoroughly wetted. Orientation marks were made, using a needle dipped in India ink, before removing the membrane from the plate. The plates were placed back at 37°C for 1 to 2 hr to regenerate the colonies and then stored at 4°C. The membranes were placed colony side up onto filter paper saturated with 0.5 N NaOH and allowed to incubate for 5 min. The filter was then transferred to filter paper saturated with 1M Tris-HCl, pH 7.6, for 3 min and finally to filter paper saturated in 0.5M Tris-HCl pH 7.6, 1.5M NaCl, for 5 min. The filters were rinsed by swirling gently in the third buffer to remove bacterial debris, then air-dried on filter paper and baked under vacuum at -80°C for 2 hr.

The hybridization and washes were performed in sealed hybridization bags containing 8 to 12 filters in a 65°C water bath with gentle shaking. Pre-hybridization and hybridization solutions were the same as those used in Southern blots, with 100 ml used for pre-hybridization and 50 ml used for hybridization (section 2.23). The hybridization conditions used were the same as used as for a standard Southern blot (section 2.23). The membranes were washed in a Tupperware container using the same wash solutions as for Southern blots. The membranes were wrapped in Saran Wrap, taped to Whatman #4 filter paper with orientation marks made with ink

containing ^{32}P and exposed to film overnight at -80°C. Individual positive colonies from the plates were chosen. Plasmid DNA from each colony was isolated (section 2.30) and examined for the presence of the desired DNA.

2.30 Small-scale plasmid DNA isolation

E. coli cells containing plasmid DNA were grown in 5 to 10 ml cultures of LB plus ampicillin (see appendix) overnight at 37°C. Cells were pelleted by centrifuging in 1.5 ml microcentrifuge tubes. The alkaline lysis "miniprep" procedure of Birnboim and Doly (BIRNBOIM and DOLY 1979) was used to isolate small amounts of plasmid DNA.

2.31 Large-scale plasmid DNA isolation

Plasmid DNA was isolated from 500 ml of overnight culture using Qiagen Maxi-prep kits (Qiagen, Mississauga, ON), following the manufacturer's instructions.

2.32 Determination of nucleic acid concentration

Absorbance of nucleic acid solutions was measured at 260 nm using a spectrophotometer (Shimadzu UV-265, Guelph, ON) and quartz cuvettes. 1 unit of absorbance at OD_{260} was taken to represent 40 μg/ml RNA. For double stranded DNA, 1 unit of absorbance at OD_{260} was taken to represent 50 μg/ml (SAMBROOK and RUSSELL 2001).

2.33 Large-scale protein isolation

To obtain large amounts of protein for the attempts to purify the cyclic-AMP response element (CRE) binding protein, cultures of NCN 251 (also known as 74-OR23-IV*A*) were grown in 8 l of Vogel's media for 12 to 14 hr then harvested through a sintered glass funnel. All of the remaining steps were performed on ice, with chilled equipment and reagents. Mycelium, acid-washed sea sand and HEPES grinding buffer (see appendix), in a 1:1:2.5 ratio, were homogenized in a household blender until a smooth slurry was obtained. This slurry was then passed through a grind mill to completely break open cells and centrifuged in a Sorvall GS-3 rotor at

5000 rpm for 5 min to remove the sand and cellular debris. The supernatant was centrifuged in a Sorvall SS-34 rotor for 60 min at 15000 rpm. The resulting supernatant was the crude protein extract of the cells.

2.34 Determination of protein concentration

Protein concentration was determined by the Bradford method, using BioRad Protein Assay reagent (Mississauga, ON), following the manufacturer's instructions, with bovine serum albumin as a standard.

2.35 Construction of target DNA molecules for protein binding

2.35.1 Restriction enzyme method

The plasmid pAOPFGuB (50 μg) (Table 3) was digested with *Nco*I and *Apa*I, the reaction was run on a 0.8% agarose gel and the 1.2 kb fragment was purified. The fragment was then digested with *Taq*I and the reaction was run on a 2% agarose gel and fragments of 496 nucleotides, 272 nucleotides and 402 nucleotides were purified (section 2.21). These fragments were called Fragments I, II and III, respectively (illustrated in Fig. 15).

2.35.2 PCR method

PCR primers (Table 2) were designed to amplify DNA fragments of 76 base pairs (primers AO42 and AO43) and 110 base pairs (primers AO40 and AO41), containing the CRE and adjacent inverted repeats found upstream of the *aod-1* transcription start site (illustrated in Fig. 15). Typically 40 PCR reactions were run, all of the reactions were pooled and the DNA was ethanol precipitated (section 2.22). The resulting DNA pellet was resuspended in 50 μl dH_2O and digested with *Taq*I, the digests were run on a 2% low melt agarose gel and purified from the gel (section 2.21). The digestion with TaqI generated DNA fragments of 58 base pairs and 89 base pairs with 5' sticky ends to allow ^{32}P labeling of the target DNA (section 2.36).

Table 3
Plasmids and cosmids used during this study.

Vector	Source	Description
pBluescript KS	Stratagene	cloning vector
23F7 (cosmid)	Q. Li	*aod-1* (from NCN53) plus flanking sequences in pSV50 (Gessert et al., 1994; Li et al., 1996)
pGUSN358-S	Clontech	β-glucoronidase in pUC119, reporter construct
pAOGE-1	Q. Li	8 kb *Eco* R1 fragment containing *aod-1* cloned in pBluescript (Li et al., 1996)
pAOPB	This study	6 kb of *aod-1* upstream sequence in pBluescript
pAOPFGuB	This study	3.3 kb *aod-1* upstream sequence, β-glucoronidase gene, and bleomycin resistance gene. Created using pGUS (purchased from Clontech Palo Alto CA) and pAOPB
pMAX	C. Nargang	*aod-1* 3 kb of upstream sequence, 0.36 kb down-stream sequence, bleomycin resistance gene in pUC119. Created using pAOPFGuB and pAOGE-1
pΔMAX	C. Nargang	*aod-1*, 3kb of upstream sequence with the CRE deleted, 0.36 kb downstream sequence, bleomycin resistance. Created using pCM4 and pAOGE-1
pMMAX	This study	*aod-1*, 255 bp of upstream sequence, 0.36 kb downstream sequence, bleomycin resistance gene. Created by modifying pMAX
pMCMAX	This study	*aod-1*, 10 bp of upstream sequence, 0.36 kb downstream sequence, bleomycin resistance gene. Created by modifying pMAX
pCM1	C. Nargang	Single base mutation of the CRE in pAOPFGuB
pCM2	C. Nargang	Single base mutation of the CRE in pAOPFGuB
pCM3	C. Nargang	Single base mutation of the CRE in pAOPFGuB
pCM4	C. Nargang	Deletion of 8 base pair CRE in pAOPFGuB
pGAX-27	This study	5 kb *Gelasinospora* alternative oxidase gene cloned into pBluescript
M10B5 (cosmid)	FGSC *A. nidulans* compressed library	*Aspergillus nidulans* sequence homologous to the ORF upstream of *aod-1* in *N. crassa*
51B6 (cosmid)	FGSC: *A nidulans* chromosome specific libaray	*Aspergillus nidulans* sequence homologous to *N. crassa* ORF upstream of *aod-1*

Table 3 continued

Vector	Source	Description
71G12 (cosmid)	FGSC: *A nidulans* chromosome specific libaray	*Aspergillus nidulans* alternative oxidase gene and flanking regions
72C5 (cosmid)	FGSC: *A nidulans* chromosome specific libaray	*Aspergillus nidulans* alternative oxidase gene and flanking regions
p8a2A	C. Nargang	*aod-1* cDNA in pBluescript
pSV50	FGSC	cosmid cloning vector, containing benomyl resistance gene and ampicillin resistance gene (Vollmer and Yanofsky, 1986)
pH3H4	FGSC	*N. crassa* histone 3 and histone 4 genes (Woudt et al., 1994)

2.36 Radioactive labeling of DNA for the Electrophoretic Mobility Shift Assay (EMSA)

A DNA fragment purified from an agarose gel (section 2.21) was suspended in 43 µl dH_2O and mixed with 3 µl α-[^{32}P] dCTP (10 mCi/ml), 5 µl Klenow reaction buffer (from supplier of enzyme), and 1 µl Klenow enzyme (10 units). This was incubated at room temperature for 20 min, and passed through a Sephadex G50 spin column (section 2.17). A 1 µl aliquot of the labeled DNA was counted in a scintillation counter in 5 ml of water (Cerenkov counting). The volume of the labeled DNA was adjusted with TE (see appendix) to obtain a concentration of 8000 counts per minute (cpm) per µl.

2.37 EMSA binding reaction

For a standard 10 µl binding reaction, 1 µl of labeled target DNA (8000 cpm/reaction) 20 to 30 µg of protein (variable volume, usually 2 to 7 µl), 1 µl 10X binding buffer (see appendix), 5 µg poly (d[I-C]), and dH_2O, to a total volume of 10 µl, were incubated at room temperature for 20 min. If the protein solution was too dilute to contain 20 to 30 µg protein in a 10 µl reaction volume, a 20 µl total reaction was used with 2 µl 10X binding buffer and dH_2O to 20 µl. The amount of target DNA, poly (d[I-C]) and incubation time were as in the 10 µl reactions. One µl (2 µl for the larger volume reactions) of 10X loading dye prepared in TBE (see appendix) buffer was added after incubation. The reactions were then immediately loaded onto a 6% non-denaturing polyacrylamide gel (see below).

2.38 EMSA gel preparation and electrophoresis

The EMSA is based on the observation that DNA with protein bound to it will exhibit a mobility shift in a non-denaturing polyacrylamide gel relative to the same DNA with no protein bound (CARTHEW et al. 1985). 20 cm x 20 cm x 2 mm non-denaturing 6% polyacrylamide gels (see appendix) were used for EMSAs. The electrophoresis buffer was 0.5X TBE (see appendix). Gels were pre-run at 4°C for 1

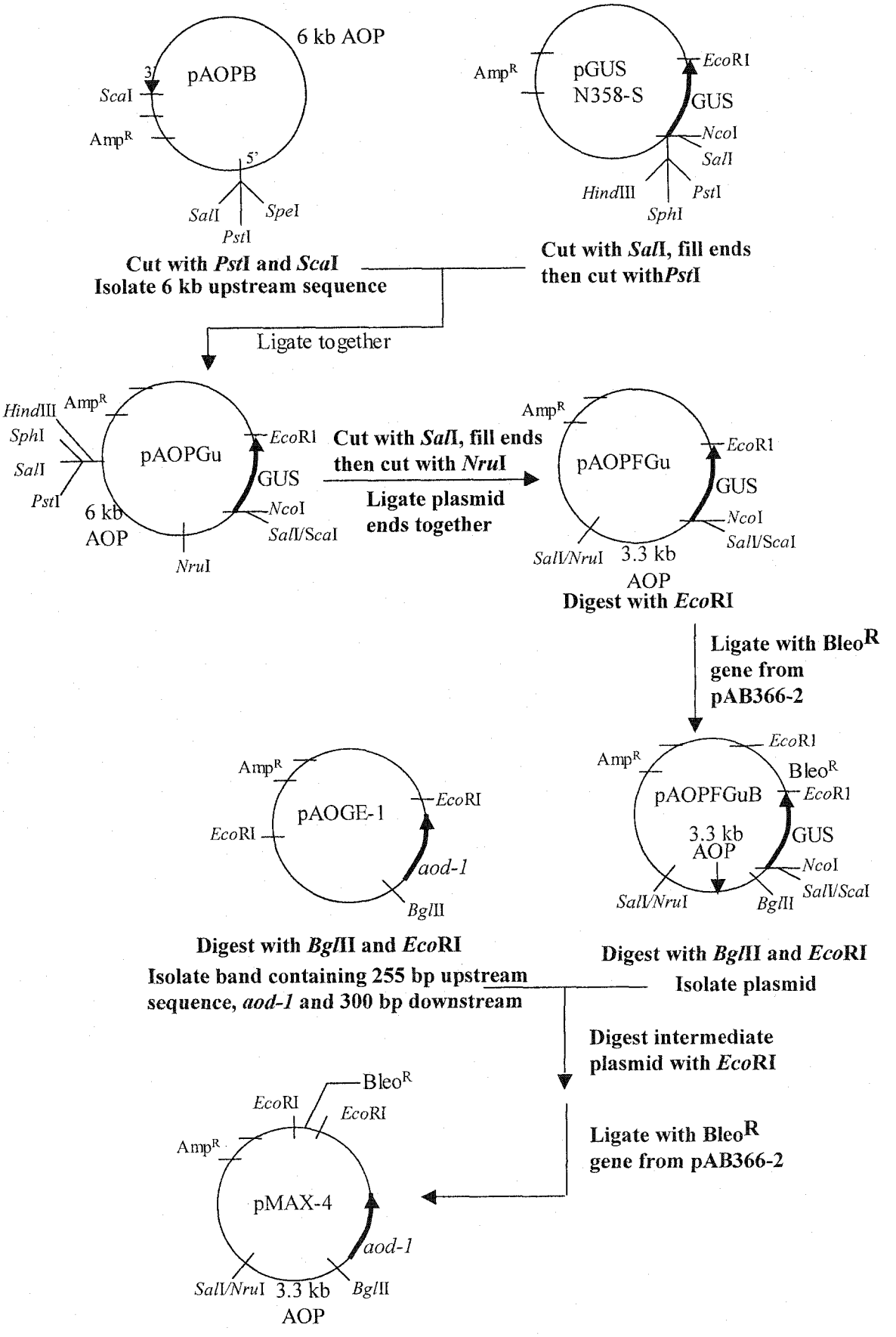
hr at 150 volts (V) and electrophoresis of samples was at 200 V for 2.5 to 3 hr at 4°C. The gel was dried and exposed to Kodak XAR film at –80°C for 12 to 14 hr.

2.39 Construction of plasmids for functional promoter analysis

In an effort to determine the minimal upstream region that could rescue an *aod-1* mutant phenotype and control the expression of KCN-insensitive respiration in the manner seen in wild type cultures, a series of plasmids was constructed. Each contained a different amount of upstream sequence, *aod-1*$^+$ and 0.7 kb of 3' untranslated region. The plasmids also contained a bleomycin resistance gene as a selectable marker. Some of the steps in plasmid construction included the use of plasmids constructed in an unsuccessful attempt to develop a reporter gene construct under the control of *aod-1* upstream sequence. To this end, 6 kb of upstream sequence from pAOPB (Table 3) was removed upon digestion with *Pst*I and *Sca*I and ligated into pGUSN358-S (a β-glucuronidase reporter vector from Clontech, Palo Alto, CA) to create pAOPGu (Fig. 3). The upstream region in this vector was reduced in size to 3.3 kb by digestion with *Sal*I and *Nru*I, and a bleomycin resistance gene from pAB366-2 was added as a selectable marker. This plasmid was called pAOPFGuB (Fig. 3) and was initially used to monitor the expression of the β-glucuronidase gene by alternative oxidase promoter sequences. When it was discovered that the reporter construct was not working as well as hoped, pAOPFGuB was used as a base plasmid to create plasmids with *aod-1* under control of various lengths of upstream sequences. pAOPFGuB was digested with *Bgl*II and *Eco*RI leaving in place 3 kb of *aod-1* upstream sequence, with the 5' most 255 base pairs, the β-glucuronidase gene and the bleomycin resistance gene removed. The digested vector was then ligated with the *aod-1* gene and 255 base pairs of upstream sequence that had been removed from pAOGE-1 (Table 3) with *Bgl*II and *Eco*RI. A bleomycin resistance gene from pAB366-2 was ligated in to create pMAX (Table 3).

Four plasmids containing *aod-1* upstream regions with mutated CRE sequence were created by Cheryl Nargang, using site directed mutagenesis of pAOPFGuB, which were called pCM1, pCM2, pCM3 and pCM4. The first three had single base pair mutations in the CRE, while pCM4 had the entire 8 base pair element deleted.

Figure 3. Construction of plasmids used in this study. Originally constructed to serve as a reporter construct for *aod-1* upstream sequences, pAOPFGuB was modified to test the expression of *aod-1* under the control of different amounts of promoter sequence. Plasmid construction is discussed in detail in section 2.39.



Using the same procedure as to generate pMAX (above, and Fig. 3), pCM4 was digested with *Bgl*II and *Eco*RI and ligated with *aod-1* from pAOGE-1. This plasmid was ligated with a bleomycin resistance gene, and was named pΔMAX. A plasmid containing 255 base pairs of sequence upstream of the +1 transcription start site, pMMAX (Table 3), was created by digesting pMAX with *Hind*III and *Bgl*II, separating the fragments using agarose gel electrophoresis, gene cleaning the plasmid fragment, filling the overhangs left by the restriction enzymes and ligating the ends of the plasmid together. A plasmid with only 10 base pairs of upstream sequence, pMCMAX (Table 3), was created by cutting pMAX with *Hind*III and *Nde*I, separating the fragments by agarose gel electrophoresis, purifying the plasmid fragment using glass milk (section section 2.21), filling the overhangs left by the restriction enzymes and ligating the ends of the plasmid together.

2.40 Electrophoretic analysis of proteins

Proteins (30 μg per lane) were electrophoresed on 12.5% denaturing polyacrylamide gels (see appendix) according to Sambrook and Russell (SAMBROOK and RUSSELL 2001).

2.41 Ammonium sulfate precipitation of proteins

Ammonium sulfate fractionation of crude protein extract was done using solid ammonium sulfate. The amount to be added per unit volume to achieve the required concentration was determined previously (SCOPES 1982). The samples were placed on a rocking table at 4°C for 30 min, and then centrifuged at 15 000 rpm for 45 min in a Sorvall GSA rotor. The pellet was resuspended in HEPES grinding buffer (see appendix) and dialyzed at 4°C overnight against 1.5 l of HEPES grinding buffer, which was changed once. If supernatant aliquots were to be analyzed as well, aliquots of these were dialyzed in the same manner. All dialyzed samples were centrifuged for 5 min at 5 000 rpm in a Sorvall SS-34 rotor to remove insoluble material and the supernatant was aliquoted and stored at –80°C until needed.

2.42 Preparation and use of a DNA affinity column

The column was prepared as described previously (BOLLAG et al. 1996). 1.5 g cyanogen bromide-Sepharose 4B (CNBr-Sepharose) (Sigma, Oakville, ON) was suspended in dH_2O to generate a final volume of 5 ml. The Sepharose was washed on a sintered glass funnel with 200 ml cold 1mM HCl, 200 ml dH_2O and 200 ml 10 mM potassium phosphate (pH 8.0). The slurry was placed in a 15 ml screw cap tube with 2 ml of 10 mM potassium phosphate (pH 8.0). *Eco*RI-digested 58 base pair PCR product (total from forty PCR reactions, as described in section 2.35.2) was added and the tube was placed on a rocking table at room temperature for 16 hr. The liquid was then removed on a sintered glass funnel and the resin was washed with 200 ml of water and 100 ml of 1M ethanolamine-HCl (pH 8.0). To inactivate the CN-Br, the resin was resuspended in 7 ml of 1M ethanolamine-HCl (pH 8.0) in a 15 ml screw cap tube. This tube was placed on a rocking table for 6 hr. The Sepharose was then washed with 100 ml of 10 mM potassium phosphate (pH 8.0), 100 ml of 1M potassium phosphate (pH 8.0), 100 ml of 1M KCl, 100 ml of dH_2O and finally 100 ml of storage buffer (see appendix). The resin was resuspended in 7 ml of this buffer and stored at 4°C.

Before use, the resin was poured into a disposable column (BioRad, Mississauga, ON) fitted with a stop-cock and equilibrated with 10 column volumes (approximately 50 ml) affinity chromatography buffer (see appendix). To reduce non-specific DNA/protein interactions, the protein was mixed with 1 mg poly (d[I-C]) per 30 mg total protein, incubated for 20 min on ice and centrifuged at 10 000 rpm for 10 min to remove any aggregates. This protein solution was passed over the column at 15 ml/hr (gravity flow) and the flow through was collected and passed over the column again. The column was washed twice with 2 column volumes of affinity chromatography buffer, in 0.15M KCl (see appendix), and eluted successively with one column volume each of 0.2M KCl, 0.3M KCl, 0.6M KCl and 1.0M KCl, each made up in affinity chromatography buffer. Each eluate was collected successively in two equal volumes. The washes and elution fractions were dialyzed at 4°C overnight in 1.5 l HEPES dialysis buffer (see appendix) with one change of buffer. The solutions were concentrated using centrifugal filter devices (Millipore, Nepean, ON),

with a molecular weight cutoff of 30 kDa, to a final volume of less than 1 ml each. The protein concentration was measured (section 2.34) and the samples were tested for enrichment of the putative CRE binding protein using EMSA (sections 2.37-2.38). Samples were stored at –80°C.

2.43 Biotinylation and ^{32}P double labeling of PCR products

The 58 base pair PCR product (section 2.35.2) was obtained by pooling forty 40μl PCR reactions. The primers designed to amplify this product (Table 2) were designed to generate *Eco*RI sites at each end of the product upon amplification. The DNA was ethanol precipitated and digested with *Eco*RI to generate ends that could be labeled with biotin and/or α-[^{32}P]-dNTP. The digested DNA was isolated from a 2% low melting point agarose gel (section 2.20) and resuspended in 50 μl dH_2O. The DNA (5 μl) was mixed with 10 μl each of 1mM dCTP and dGTP, 2 μl of 1mM biotin dUTP, 3 μl of α- [^{32}P]-dATP (10 mCi/ml), 5 μl of 1mM dATP, 5 μl Klenow reaction buffer, 1 μl (10 units) Klenow fragment and 10 μl dH_2O. The reaction was incubated at room temperature for 30 min. Unincorporated nucleotides and biotin were removed using a Sephadex G50 spin column that had been equilibrated by washing with four column volumes of SDS column buffer (see appendix). In some cases, DNA was labeled only with biotin UTP. In these cases, the procedure was as described above, except 10 μl of a 1mM concentration of dATP was used with no α- [^{32}P]- dATP.

2.44 Biotin-streptavidin magnetic bead pull down experiments

The binding of protein from cell extracts to biotinylated CRE-containing DNA fragments was designed as a scaled up EMSA reaction consisting of 510 μl of the resuspended and dialyzed 25% ammonium sulfate fraction of cellular protein (3 to 5 mg), 100 μl poly (d[I-C]) (80 to 100 μg), 70 μl of 10 X EMSA buffer (see appendix) 20 μl of the CRE containing PCR product labeled with ^{32}P and biotin, or only biotin. This was incubated at 4°C for 1 hr on a rocking table. The magnetic streptavidin beads (1 ml from Promega's mRNA purification kit) (Promega, Nepean, ON) were washed 3 times with 500 μl 0.5 X TBE, resuspended in 500 μl 0.5 X TBE, and added

to the above EMSA mixture after the incubation step. The mixture was placed on the rocking table at 4°C for an additional 30 min. A magnetic microcentrifuge tube holder (Promega, Nepean, ON) was used to attract the streptavidin beads and any biotin-DNA/protein complexes to the side of the tube. The beads were washed twice with 500 µl buffer (20 mM HEPES (pH 7.9), 50 mM KCl, 1mM EDTA), by mixing with a pipettor and rebinding of the beads to the magnet. The washes were followed by elutions using 500 µl each of buffers with increasing salt concentration: first, 20 mM HEPES (pH 7.9), 300 mM KCl, 1 mM EDTA; second, 20 mM HEPES (pH 7.9), 600 mM KCl, 1mM EDTA; and third, 20 mM HEPES (pH 7.9), 1M KCl, 1mM EDTA. The solutions were concentrated using centrifugal filter devices (Millipore, Nepean, ON), with a molecular weight cutoff of 30 kDa, to a final volume of less than 1 ml each. The protein concentration was determined (section 2.34) and the samples were tested for enrichment of the putative CRE binding protein, using EMSA (sections 2.37-2.38).

2.45 Coomassie blue staining of proteins

If gels were to be stained directly for protein content, the gels were trimmed and placed in 1X Coomassie stain (see appendix) with gentle shaking overnight. The next morning, several changes of destain (see appendix), warmed to approximately 50°C in a microwave oven, were applied. Destaining was continued until the protein bands were clearly visible with little blue background in the rest of the gel. The gels were dried between pieces of cellulose film (Biorad, Mississauga, ON).

2.46 Western blots

If proteins were to be detected with antiserum to specific mitochondrial proteins, the gels were electro-blotted using a liquid transfer apparatus (Biorad, Mississauga, ON). The gels were trimmed, and soaked in transfer buffer (0.025M Tris base, 0.19M glycine, 20% methanol) for 30 min. The nitrocellulose membrane (Osmonics Inc., Westborough, MA) was also soaked in transfer buffer for 30 min. The gel and membrane were sandwiched between 12 layers of Whatman #4 3MM

paper and brillo pads in the transfer apparatus. Transfer was at 60 V for 1.5 hr. Following transfer, the membranes were used for immunodetection (section 2.47).

2.47 Detection of proteins using antisera

The membrane was blocked with milk buffer (see appendix), for 45 min with gentle shaking. Primary antiserum to the protein of interest was suspended in milk buffer (containing 0.02% sodium azide) at dilutions (usually 1/500 to 1/10,000) specific for each antiserum (raised in rabbits), and incubated with the membrane with gentle shaking for 60 min. The membrane was washed 3 times for 5 min with milk buffer and then exposed to secondary antibody (1:3000 dilution of goat anti-rabbit antibody (Biorad, Mississauga, ON) suspended in milk buffer for 45 min. The membrane was washed three times for 5 min with TBS/Tween (see appendix) and once for 5 min with TBS (see appendix). The bound antibody was detected using a LumiGLO chemiluminescent detection kit (Mandel, Guelph, ON), following the manufacturer's instructions. The membrane was exposed to Kodak XAR film for times varying from 5 sec to several min.

2.48 Isolation of *N. crassa* mitochondria

Mitochondria were harvested from the same cultures that RNA was isolated from (section 2.2 for growth conditions). The portion of mycelium to be used for mitochondria isolation was weighed, placed in a mortar and an equal weight of sand was added. 2.5 ml SEMP (see appendix) was added per gram of mycelium and this was ground into a slurry with a pestle. After grinding, the samples were placed in a Sorvall SS-34 rotor and centrifuged for 5 min at 5000 rpm. The supernatant was transferred to a new tube and the centrifugation was repeated. The supernatant was again transferred to a new tube and centrifuged for 20 min at 12000 rpm. The mitochondrial pellets were resuspended in 100 to 500 µl of SEMP and stored at -80°C until needed.

2.49 Isolation of *N. crassa* nuclei

Three 2 L flasks each containing 500 to 600 ml of Vogel's medium were inoculated with 10^6 conidia/ml and grown with 150 rpm shaking for 12 to 13 hr (uninduced) or 24 hr (2 mg/ml Cm added). The cultures were harvested by filtration, and, when pooled, generated approximately 10 g mycelium. The procedure for nuclear isolation was modified from a previously described procedure (YUKIOKA et al. 1998b). All equipment and reagents used to isolate nuclei were RNase free (section 2.13). The harvested mycelia (10 g) were soaked in 40 ml ice-cold nuclear grinding buffer (see appendix) for 10 min. In a large mortar, the mycelium and buffer were ground together for a few sec to homogenize the mixture. Liquid N_2 was added and grinding continued until the mycelia were powdery. The mixture was allowed to melt into a slurry and was then filtered through two layers of pre-wetted Miracloth (BioRad, Mississauga, ON). The flow through was centrifuged for 20 min in a Sorvall SS-34 rotor at 6500 rpm. The pellets were resuspended in 3 ml grinding buffer. The nuclei were then further purified by passage through a sucrose gradient. Centrifuge tubes (3 ml capacity) were filled with a layer of 1.75 ml of a sucrose cushion containing 72% sucrose, dissolved in grinding buffer, and 0.75 ml of resuspended nuclei were carefully layered on top. The samples were centrifuged in an Optimax benchtop ultracentrifuge (Beckman, Toronto, ON) at 37 000 rpm for 45 min using a TLA 110 rotor. The pellets were resuspended in 500 µl nuclear suspension buffer (see appendix) and stored at –80°C in 200 µl aliquots.

2.50 Nuclear run-on assays

All equipment and reagents were RNase free. 200 µl thawed nuclei were mixed with 60 µl of 5X nuclear run-on buffer (see appendix) and 100 µCi α-[^{32}P]UTP (10 mCi/ml). The contents of the reaction were mixed by inverting the tube and flicking it gently. The reaction was incubated at 30°C for 30 min with mixing every 10 min. At the end of the incubation period, 2µl (approximately 12 500 units) FPLC pure (RNase free) DNase I (Roche, Laval, QB) was added and the tube was incubated at 30°C for 5 min. The DNA digestion step was repeated with an additional 2 µl of DNase I. Following this digestion, 28 µl 10X SET (see appendix) and 10 µl

Proteinase K (10 mg/ml) were added, followed by a 45 min digestion at 45°C. The samples were then extracted with 360 µl phenol: chloroform: isoamyl alcohol (25:24:1). The tube contents were mixed and centrifuged for 5 min at top speed in a microcentrifuge. A repeat extraction of the lower phase with 100 µl 1X SET was performed and the aqueous phases were pooled.

To precipitate the RNA, 200 µl of 7.5M ammonium acetate, 5 µg of tRNA (as a carrier), and 600 µl of cold isopropanol were added. The tube was placed in a dry ice/ethanol bath for 20 min and then centrifuged at top speed in a microcentrifuge for 20 min. The pellet was resuspended in 100 µl TE and dissolved by incubation at 37°C for 5 min. The labeled RNA was passed through a Sephadex G-50 spin column (section 2.17).

Cerenkov counting was performed on 1 µl of the labeled RNA, used to probe the membrane bound DNA (section 2.51). The counts were equalized among all of the probes to be used in a given experiment (usually 2 X 10^6 cpm per probe). The volume of each was made up to 1 ml with TES solution (see appendix).

2.51 Preparation of target DNA for nuclear run-on assays

Plasmid DNA (of varying amounts, usually 5 µg, 2.5 µg, 1 µg and 0.5 µg) was linearized with an appropriate restriction enzyme in a volume of 40 µl and denatured by the addition of 4µl of 3M NaOH. Denaturation was promoted by incubation at 65°C for 30 min. The sample was then cooled slowly to room temperature and 1 volume (44 µl) 2 M ammonium acetate was added to neutralize the sample. A drop of 1X TAE (see appendix) containing 0.4% w/v bromophenol blue was added for visualization of blotting. A dot blot apparatus was used to blot 5 µg, 2.5 µg, 1 µg and 0.5 µg amounts of each target DNA to a nylon Biotrans membrane (ICN, Montreal, QB) following the directions that came with the apparatus (Tyler Research, Edmonton, AB).

2.52 Hybridization and washing of nuclear run-on membranes

The membranes with target DNA were baked for 1 hr at 80°C under vacuum and then pre-hybridized for 1 hr or more at 65°C in 1 ml TES (see appendix) plus 1 ml TES containing 0.6M NaCl. A 15 ml screw cap tube taped to the inside of a standard hybridization oven bottle was used for all of the hybridization steps. The probe was adjusted to approximately 2 X 10^6 cpm/ml in a total of 1 ml TES solution. The hybridization solution was the 1 ml of probe RNA in TES plus 1 ml TES/NaCl solution. The hybridization was performed at 65°C for 36 to 48 hr.

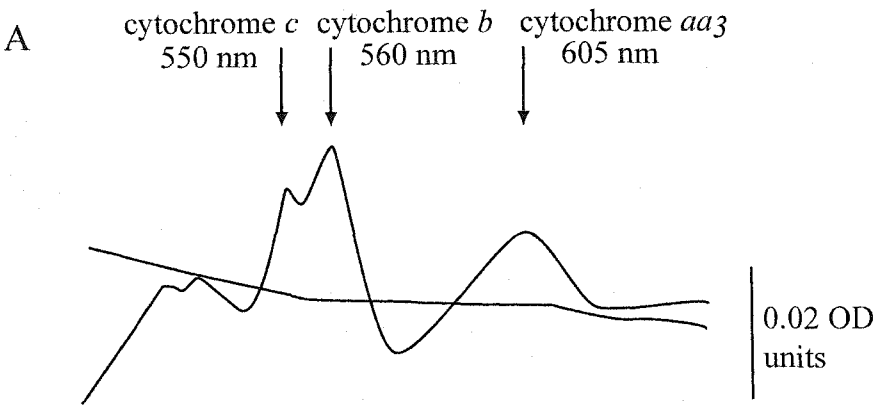
The membranes were washed twice for 1 hr each at 65°C in 10 ml 2X SSC. They were then washed at 37°C for 30 min in 8 ml 2X SSC plus 8 µl RNase A to digest non-hybridized RNA and once again for 1 hr at 37°C in 2X SSC with no RNase. The membranes were wrapped in Saran-Wrap and exposed to Kodak XAR film overnight at –80°C.

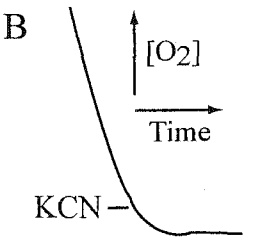
3 Results

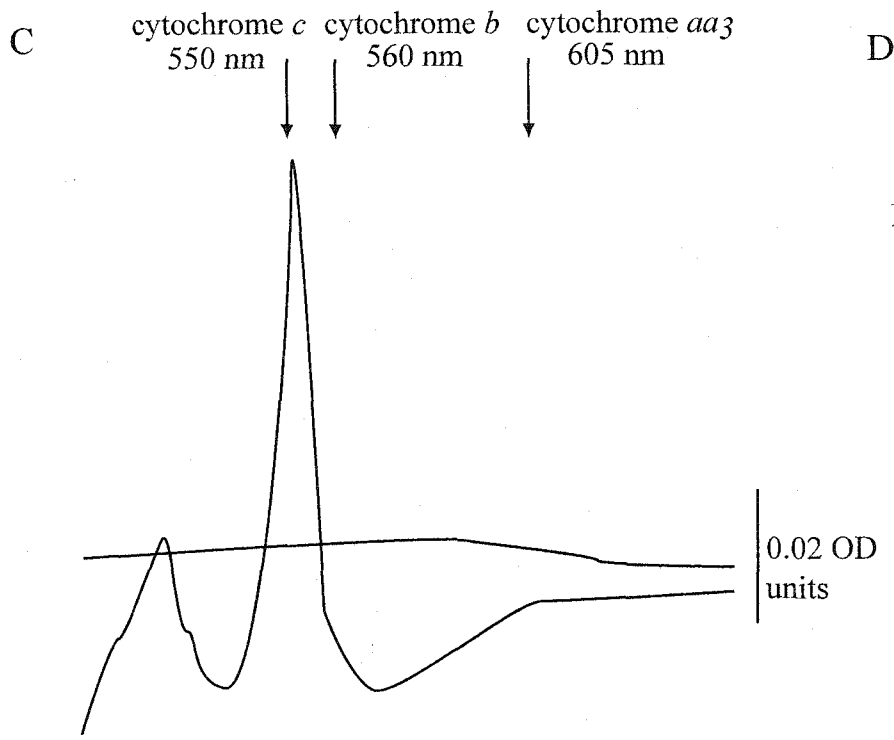
3.1 Assay of alternative oxidase

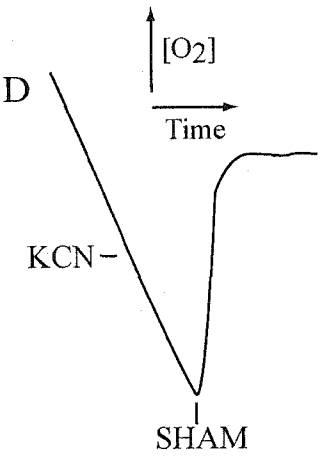
Early studies on *N. crassa* alternative oxidase (EDWARDS et al. 1974; LAMBOWITZ and SLAYMAN 1971) demonstrated that when agents that directly (e.g., antimycin A) or indirectly (e.g., chloramphenicol) inhibit electron transport are included in the growth medium, they result in the induction of KCN-insensitive respiration (alternative oxidase). Antimycin A acts by inhibiting complex III of the standard electron transport chain (SCHEFFLER 1999) (Fig. 1). The action of chloramphenicol is to inhibit mitochondrial protein synthesis by interacting with the large subunit of the mitochondrial ribosome (SCHEFFLER 1999). This reduces the amount of mitochondrial translation products resulting in decreased formation of the complexes containing these proteins. The result of growth in chloramphenicol can be easily monitored by examining mitochondria isolated from cultures grown in the inhibitor, as the levels of cytochrome aa_3 (Complex IV) and cytochrome *b* (Complex III) are reduced under these conditions (Fig. 4). Throughout most of this thesis, the

Figure 4. Cytochrome spectra of wild type and chloramphenicol inhibited cultures. A. Cytochrome content of mitochondria from cultures grown in standard Vogel's medium. B. Respiration of cells grown in standard medium, as in panel A. Oxygen consumption is measured over time and is inhibited by KCN. C. Cytochrome content of mitochondria from cultures grown in Vogel's medium supplemented with chloramphenicol (Cm, 2 mg/ml). D. Respiration of cells grown as in panel C. Oxygen consumption continues after addition of KCN but is shut off upon the addition of SHAM.









method used to induce KCN-insensitive respiration was by growth of cells in chloramphenicol (Fig. 4).

3.2 Assignment of *aod-1* and *aod-2* mutant alleles

The original studies of *N. crassa* alternative oxidase defined two genes involved in the expression of the enzyme (BERTRAND et al. 1983). The *aod-1* gene (chromosome IV) was thought to encode the structural gene while *aod-2* (chromosome II) was believed to encode a regulatory factor required for *aod-1* expression. I first became involved in the study of alternative oxidase while the Nargang laboratory was characterizing mutants originally isolated in the Bertrand lab (BERTRAND et al. 1983). An ongoing study by Qiuhong Li involved the cloning and sequencing of the alternative oxidase structural gene from wild type as well as the *aod-1* alleles from the *aod-1* and *aod-2* mutant strains isolated in the original study of Bertrand *et al* (1983). The structural gene was cloned using degenerate primers and the entire gene was eventually isolated and sequenced from both cDNA and cosmid libraries (LI et al. 1996). As expected, mutant strains *aod-1-1*, *aod-1-2* and *aod-1-4* were found to contain mutations in the structural gene (LI et al. 1996). However, other sequencing results conflicted with the gene designations that had been assigned to the strains obtained from the Bertrand laboratory. Specifically, a strain designated *aod-2-6* was found to contain a transversion of C to A in the *aod-1* gene, at codon 173 of the alternative oxidase preprotein coding sequence, which would result in an amino acid change of alanine to aspartatic acid. In addition, the *aod-2-7* strain was found to contain a +1 frame shift mutation in the *aod-1* gene, at codon 41 of the preprotein. It was possible that the missense mutation in the strain designated *aod-2-6* was a genetic polymorphism that did not affect *aod-1* function. However, the frameshift in the strain designated *aod-2-7* would obviously affect *aod-1* function and it seemed likely that this strain was actually an *aod-1* mutant. To ascertain whether the lack of alternative oxidase in these two strains was due to mutations in *aod-1* or *aod-2*, I wished to determine if the *aod-1*$^{+}$ gene could rescue alternative oxidase expression in each mutant strain. Therefore, I transformed each of these strains, as well as all other alternative oxidase deficient strains in the lab (*aod-1-1*, *aod-1-2* and *aod-1-4*), with

cosmid 23F7 (Table 3). This cosmid contains the entire *aod-1* coding sequence plus flanking regions. The vector also contains a benomyl resistance gene for selection of transformants on benomyl containing media, since there is no effective selection for alternative oxidase activity. Transformants were isolated, purified, and assayed for the presence of alternative oxidase in uninduced and induced cultures (grown in the absence or presence of chloramphenicol, respectively). The *aod-1*$^+$ cosmid was found to restore inducible alternative oxidase activity to transformed strains *aod-1-1, aod-1-2,* and *aod-1-4* as well as the strains designated *aod-2-6* and *aod-2-7* (Table 4). Transformants of these *aod-1* mutants that did not contain alternative oxidase activity (Table 4) are most likely the result of integration of the benomyl marker but not the *aod-1*$^+$ gene. As a negative control, strain 7001 (*aod-1-1*) was transformed with the benomyl resistant vector pSV50 (VOLLMER and YANOFSKY 1983). Seven transformants were analyzed and none contained alternative oxidase activity. The data in Table 4 corroborated the sequencing data and showed that the strains designated "*aod-2-6*" and "*aod-2-7*" actually contained mutant alleles of the structural gene, *aod-1*. The incorrectly designated mutants are now called *aod-1-6* and *aod-1-7* respectively. The *aod-2-4* mutant was not rescued by the *aod-1*$^+$ gene nor did it contain a mutation in the *aod-1* gene. Thus, this strain appears to be a true *aod-2* mutant (Table 4).

3.3 Upstream sequence of *aod-1,* and analysis of open reading frames

The original sequencing of *aod-1* and flanking regions included 1.3 kb of sequence upstream of the structural gene (LI et al. 1996). This revealed potential TATA and CAAT boxes upstream of *aod-1* and a polyadenylation site downstream. The +1 transcription start site was identified by primer extension (LI et al. 1996). A CRE element 746 base pairs upstream of the transcription start point of the gene was also identified (Fig 5). Since the CRE was a potential regulatory element, I wished to obtain additional sequence in the region so that the existence of other genes that might be influenced by this element could be assessed. Therefore, I sequenced 2.3 kb of additional upstream sequence (Fig. 6). Before I finished laboratory work on this thesis, the *Neurospora* genome sequence was released. Comparison of my sequence

Table 4
Assignment of *aod-1* and *aod-2* mutant alleles.

Laboratory Strain name	Initial allele designation	Transformants tested	Transformants rescued	Final allele designation
7001	*aod-1-1*	21	7	*aod-1-1*
7001*	*aod-1-1*	7	0	*N/A*
7021	*aod-1-2*	22	6	*aod-1-2*
7202	*aod-1-4*	8	2	*aod-1-4*
7064	*aod-2-4*	28	0	*aod-2-4*
7216	*aod-2-6*	13	4	*aod-1-6*
7207	*aod-2-7*	13	9	*aod-1-7*

* 7001 was transformed with empty vector pSV50 as a negative control.

Figure 5. Linear representation of *N. crassa* alternative oxidase gene, *aod-1*, and surrounding sequence. Upper line: start and stop codons, CRE, CAAT box, TATA box and polyadenylation site are indicated. +1 indicates transcription start site as determined by primer extension (Li et al. 1996). Arrow indicates direction of transcription. Lower line: enlargement of coding sequence, showing size of exons and introns.

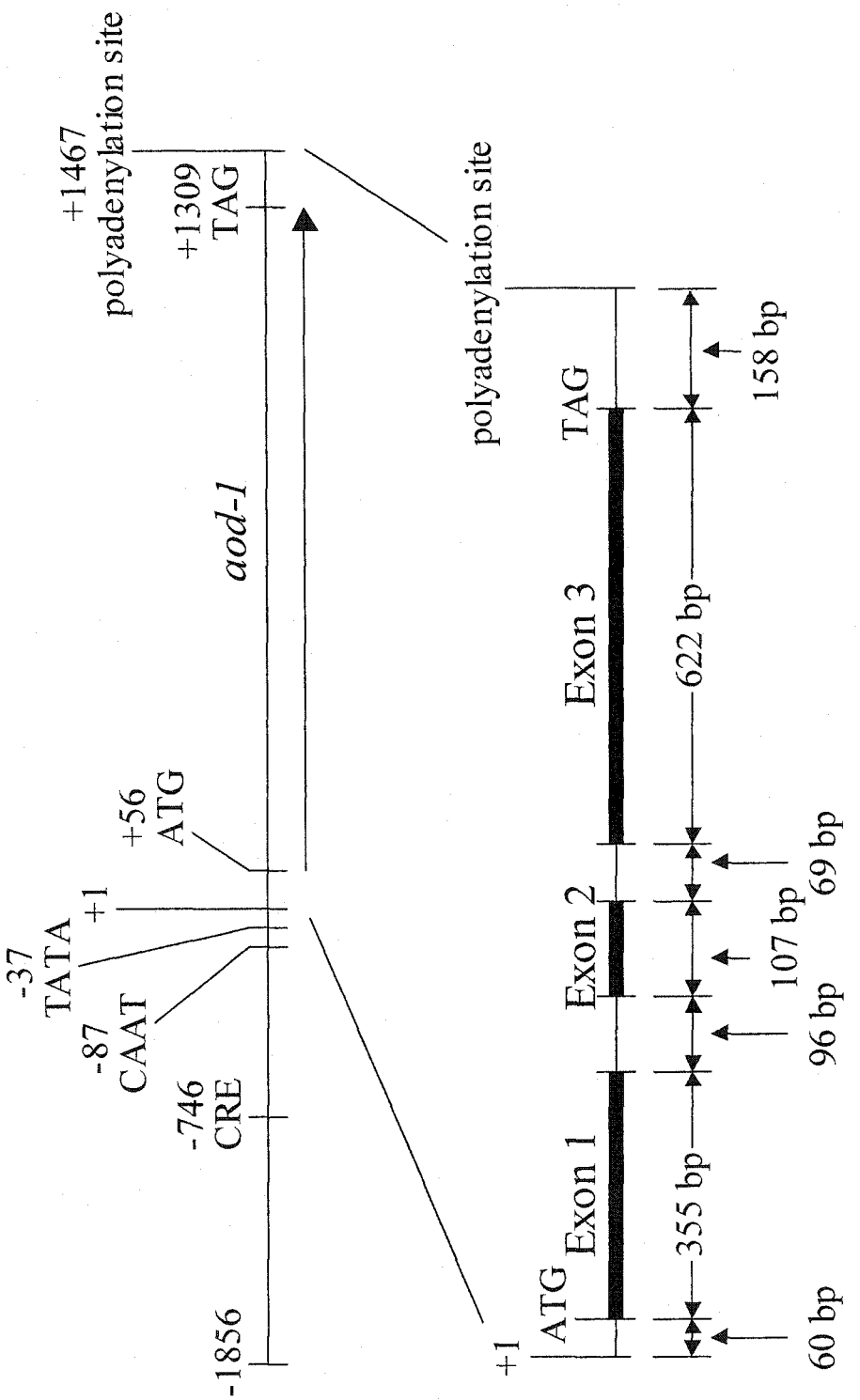


Figure 6. Sequence of the *aod-1* gene and surrounding sequence in *N. crassa*. Introns and other untranslated regions are in lower case letters, the amino acid coding sequences are in upper case letters. The CRE as well as the TATA and CAAT boxes are indicated in bold type and underlined, the inverted repeats on either side of the CRE are underlined. The +1 transcription start site is indicated in the sequence and the asterisks below indicate an *N. crassa* transcription start consensus signal (BRUCHEZ 1993; LI et al. 1996). Numerals in standard font indicate nucleotide number while numerals in italics designate amino acids. The putative coding sequence of the upstream ORF (see text) is in upper case letters and underlined, with predicted intron sequences in lower case. Transcription of the ORF is in the opposite orientation to *aod-1*.

ggtgtcatggtcgggaaatacacctgttcactggtgtaagtttctgagagggttctgaga -3482
tggtgactgaaatagtgttggttgtaattttagccatggcatcgataagagaacctcaca -3422
ccaggatgatatgcagcctggactttcgaaacgtgatgctcgccttcattattgaatgca -3362
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gtcgcgactcgatcagtgccccaagaggccatcctccttttttcgtccagtctggcactg -3242
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AAGTCATCActagagcgaaacgttagcaatcgttccccacgcgtatcttttctcactcca -2882
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GCTAGCAATCTTCTTGATCTCGTCGCTCTTGAGGCTCTTGGCGAGCTGCTTGGAGAACTC -2542
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CGAATCCTCAGCCAGCTGGCGGGCGCGCTCGGCCTGACGCTCGGCGATGCGCTGCGCCTT -2282
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ggggcaggaaaggacgatataaacgtcccgtgtctagtgctgtccgacacatatggacca -2

 +1
tcatcacaaacctcaagcgagttccattacaacttcacatcactccctaaactctcg ATG 59
 ********                                                    M   1

AAC ACC CCC AAA GTA AAC ATA CTC CAC GCT CCA GGA CAA GCG 101
N   T   P   K   V   N   I   L   H   A   P   G   Q   A   15

GCC CAA CTA AGC CGT GCC CTG ATA TCA ACC TGC CAT ACT CGG 143
A   Q   L   S   R   A   L   I   S   T   C   H   T   R   29

CCT CTC CTG CTC GCG GGC TCT CGA GTG GCC ACT TCC TTA CAT 185
P   L   L   L   A   G   S   R   V   A   T   S   L   H   43

CCA ACA CAG ACA AAC CTC TCT TCC CCA TCA CCT CGC AAT TTC 227
P   T   Q   T   N   L   S   S   P   S   P   R   N   F   57

TCG ACA ACA AGC GTT ACT CGA CTG AAG GAT TTC TTC CCG GCC 269
S   T   T   S   V   T   R   L   K   D   F   F   P   A   71

AAA GAG ACC GCC TAT ATC CGG CAG ACA CCA CCC GCG TGG CCT 311
K   E   T   A   Y   I   R   Q   T   P   P   A   W   P   85

CAT CAT GGA TGG ACA GAG GAA GAG ATG ACC TCG GTT GTT CCC 353
H   H   G   W   T   E   E   E   M   T   S   V   V   P   99

GAG CAC CGG AAA CCC GAG ACT GTG GGC GAT TGG CTC GCA TGG 395
E   H   R   K   P   E   T   V   G   D   W   L   A   W   113

AAA CTC GTA CGA ATC TGT CG gtaggtaaattccaaggagggttccca  442
K   L   V   R   I   C   R                               120

cattgcccctcaaggtatcttgcggacgcggaagcagacgtagattcaaagtg   495

ttctaatatcaaacag A TGG GCC ACT GAT ATA GCG ACG GGC ATA  539
                  W   A   T   D   I   A   T   G   I    129

CGT CCA GAG CAG CAA GTT GAT AAA CAC CAC CCG ACG ACC GCC 581
R   P   E   Q   Q   V   D   K   H   H   P   T   T   A   143

ACC AGC GCG GAC AAA CCT CTG ACC GAA GCC CAA TGG gtatgtt 624
T   S   A   D   K   P   L   T   E   A   Q   W          155

ctatggagccctacgaggacggaatggcaacacaagctaaccaaatgtcgcgaat 679

ccatatag CTC GTC CGC TTC ATC TTC CTC GAA TCC ATC GCC GGC 723
         L   V   R   F   I   F   L   E   S   I   A   G   167

GTT CCC GGC ATG GTA GCC GGC ATG CTC CGC CAC CTG CAC TCC 765
V   P   G   M   V   A   G   M   L   R   H   L   H   S   181

CTC CGT CGG CTC AAA CGA GAC AAC GGC TGG ATC GAG ACT TTA 807
L   R   R   L   K   R   D   N   G   W   I   E   T   L   195
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CTT GAA GAA TCG TAC AAC GAG CGC ATG CAC CTC CTC ACC TTT 849
L   E   E   S   Y   N   E   R   M   H   L   L   T   F   209

ATG AAG ATG TGC GAA CCC GGC CTC CTC ATG AAG ACG CTC ATC 891
M   K   M   C   E   P   G   L   L   M   K   T   L   I   223

TTG GGA GCG CAG GGC GTC TTC TTC AAC GCC ATG TTT CTC AGC 933
L   G   A   Q   G   V   F   F   N   A   M   F   L   S   237

TAC CTG ATC TCC CCC AAA ATC ACC CAC CGG TTT GTC GGT TAC 975
Y   L   I   S   P   K   I   T   H   R   F   V   G   Y   251

CTC GAG GAG GAG GCC GTA CAT ACC TAC ACG CGG TGC ATC AGG 1017
L   E   E   E   A   V   H   T   Y   T   R   C   I   R   265

GAG ATT GAG GAA GGT CAC TTG CCA AAG TGG AGC GAC GAA AAG 1059
E   I   E   E   G   H   L   P   K   W   S   D   E   K   279

TTT GAG ATC CCG GAG ATG GCG GTG AGG TAT TGG CGC ATG CCG 1101
F   E   I   P   E   M   A   V   R   Y   W   R   M   P   293

GAG GGG AAG CGG ACG ATG AAG GAC TTG ATC CAT TAT ATC CGC 1143
E   G   K   R   T   M   K   D   L   I   G   Y   I   R   307

GCG GAC GAG GCA GTG CAT AGG GGC GTT AAT CAT ACA CTG AGC 1185
A   D   E   A   V   H   R   G   V   N   H   T   L   S   321

AAT TTG GAC CAG AAG GAG GAT CCG AAT CCG TTT GTG AGC GAC 1227
N   L   D   Q   K   E   D   P   N   P   F   V   S   D   335

TAT AAG GAG GGC GAG GGC GGG AGG AGA CCG GTC AAT CCG GCT 1269
Y   K   E   G   E   G   G   R   R   P   V   N   P   A   349

TTG AAG CCG ACG GGA TTT GAA AGG GCG GAG GTC ATC GGT TGA 1311
L   K   P   T   G   F   E   R   A   E   V   I   G   *   362

tgttgggcggaaggtcttgacagatggttgtggtttgggttcatgaagccaggcgttttt 1371
ggaccaagttgttgtattatgcgtgttacactagataccccctctctttgctgtttctg 1431
gcgttttggtaaaaagatactgggttcttctctctgtgcagcagttcactgtttcaaggt 1491
gtgtacttgtttcggatcttcactccgaagtcgttgatctctcatgatcaactatatcta 1551
gcaataacaaacctgatatgtcaagccttttcgatccgttccccgggtaactatccggct 1611
cgagttcacatttgcagcttctacccaacgcatagaaacccaaggtagtcgaacgtgctc 1671
acttgaattcgatcagtgattaaattatgtgctcagccatgatcaaccgaatatatcgtg 1731
acggcgagtcatggaccttcccgtggtttgctgatgtatacctaggatgatagctcaaga 1791
cgtccattagagtgtctatatttacacgcgtctagtgtaggcaccctcgaaatgaagcgg 1851
ggaatgatgtgaaacaatggtattatgcagcgatatatcaaagtcaaataatggctcgaa 1911
gaagaaatgttggtcgaacgaaattacagggctgtggctgtgtacaagccactgaagtgc 1971
aatttgattagcgtggtgaactttgggtaacatattttgccggagatct            2020
```

of the *aod-1* region to this sequence revealed that there were some small differences between the sequences. Re-examination of my sequence did not reveal any ambiguity in the data for the discrepancies in question. Therefore, I assume the differences are due to strain specific genetic polymorphisms. The *aod-1* region sequenced in this study and LI et al. (1996) was from strain *su[mi-3]* (BERTAND and PITTENGER 1972). The strain sequenced in the genome project was 74-OR23-IVA (*Neurospora* Sequencing Project. Whitehead Institute/MIT Center for Genome Research, www-genome.wi.mit.edu). The majority of nucleotide differences were in non-coding sequences, and none occurred in any of the conserved, putative regulatory elements discussed above. Nucleotide changes that occurred within exons did not change the amino acid coded for, with one exception: Codon 57 changes from phenylalanine to leucine, but this amino acid is within the putative mitochondrial signaling region of the protein (see Table 5).

Visual inspection and computer analysis (using the DNAMAN computer programs) of the total upstream sequence revealed several upstream open reading frames (ORFs). To determine if any of the open reading frames identified in the sequence (Fig. 7) were expressed, tBLASTx searches were performed comparing the upstream region with the *N. crassa* morning, evening and perithecial expressed sequence tag (EST) libraries, the *A. nidulans* EST library, as well as the non-redundant EST library at GENBANK (ALTSCHUL et al. 1997). The single ORF in reading frame -1 (Fig. 7) was found to have high identity with one *A. nidulans* EST of unknown function, j4d05a1. The 5' end of ESTs in the A. nidulans library is designated "r1" and the 3' end is designated "f1". j4d05a1.r1 has 56% identity and 68% similarity over 48 amino acids predicted from the sequence obtained with reverse primer; j4d05a1.f1 has 63% identity and 76% similarity over 62 amino acids predicted from the sequence obtained with forward primer) (Fig. 8). The *Neurospora* Sequencing Project, Whitehead Institute/MIT Center for Genome Research site (www-genome.wi.mit.edu), which uses FGENESH, FGENESH+ and GENEWISE programs to predict potential proteins, predicted that this ORF is the only one in the region that might encode a protein. Small exons at both the 5' and 3' end of the ORF were also predicted, resulting in a 275 amino acid polypeptide (Figs. 6 and 7).

Table 5
Sequence mismatch between *aod-1* sequence in thesis and *N. crassa* genome database.

Mismatch location*	Thesis sequence	*N. crassa* genome sequence	Amino acid change
-3521	Gap inserted	T	N/A
-3064	A	Gap inserted	N/A
-3062	G	A	N/A
-3055	G	T	N/A
-3046	C	T	N/A
-3042	A	G	N/A
-3035	T	A	N/A
-3034	A	T	N/A
-2667	C	T	N/A
-2666	T	C	N/A
-2500	T	C	N/A
-1989	C	T	N/A
-1942	T	C	N/A
-1912	G	A	N/A
-1902	A	T	N/A
-1588	G	C	N/A
-1547	Start of four bp deletion	N/A	N/A
-1400	Start of four bp deletion	N/A	N/A
-1341	T	C	N/A
-691	C	A	N/A
-690	C	A	N/A
-631	A	T	N/A
-308	A	C	N/A
-272	G	A	N/A
-130	C	G	N/A
197 (Codon 47)**	A	G	No
225 (Codon 57)**^	T	C	Phe to Leu
456	G	Gap inserted	N/A
676	G	A	N/A
771 (Codon 183)**	T	C	No
1161 (Codon 313)**	T	C	No
1180 (Codon 320)**	C	T	No
1313	G	C	N/A
1412	Start of four bp deletion	N/A	N/A
1628	G	A	N/A
1766	T	C	N/A
1787	A	G	N/A
1858	Start of three bp insertion	N/A	N/A
1986	G	C	N/A

*Numbering as in Figure 6 of thesis
**If a nucleotide within an exon was altered, the amino acid codon affected is in brackets
^ The only codon to specifiy different amino acids in the two sequences represents an amino acid in the predicted mitochondrial targeting presequence of the protein
N/A= not applicable

Figure 7. Open reading frames upstream of *aod-1* coding sequence. Arrows indicate scale representation of all open reading frames larger than 74 amino acids in the 3.6 kb upstream from the *oad-1* start site. The number above each indicates the number of amino acids (aa) predicted in each ORF. The predicted amino acid sequences of these ORFs were tested against predicted amino acid sequences in several databases: *N. crassa* morning, evening and perithecial EST libraries, *A. nidulans* EST libraries and the non-redundant EST libraries of GenBank. The only homology identified was between an *A. nidulans* EST of unknown function (j4d05a1) and the ORF in the –1 reading frame. A 275 amino acid polypeptide with two introns that includes this ORF was predicted at the *Neurospora* Sequencing Project web site (Whitehead Institute/MIT Center for Genome Research www-genome.wi.mit.edu) using the FGENESH, FGENESH+ and GENEWISE programs to predict potential coding sequences. The introns in the sequence are shown as wavy lines.

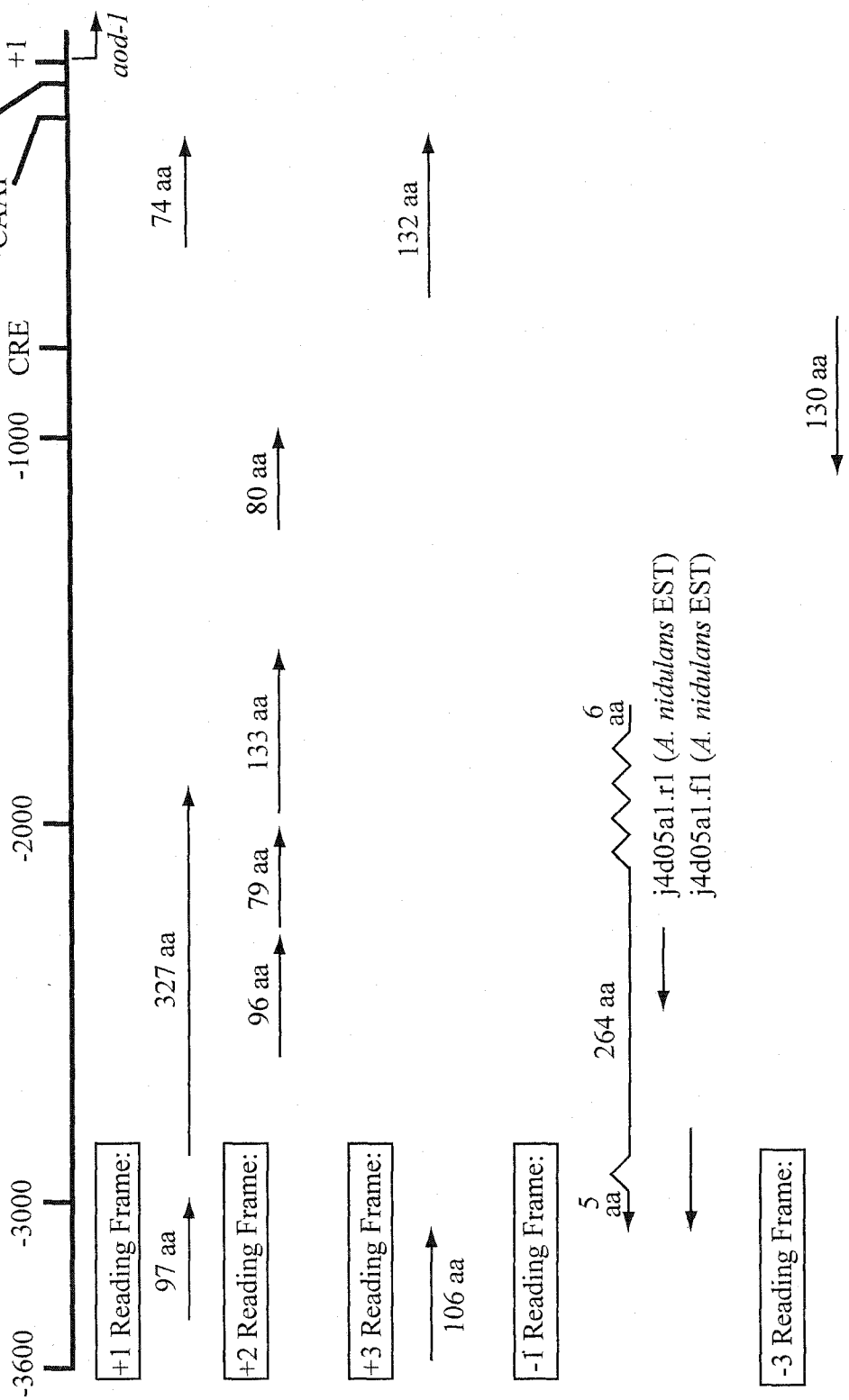
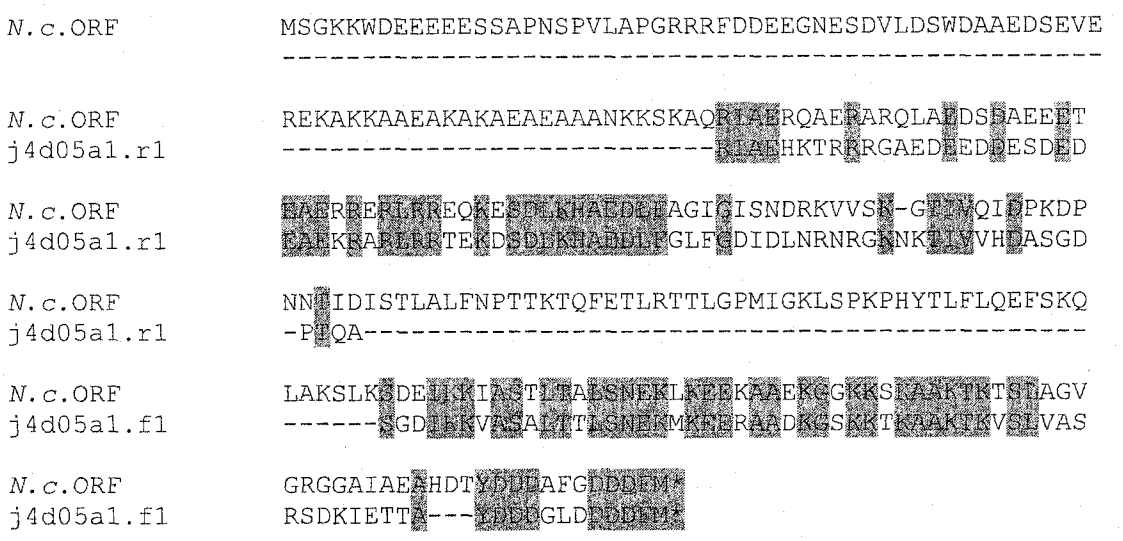


Figure 8. Amino acid alignment of *N. crassa* ORF upstream of *aod-1* and the *A. nidulans* EST j4d05a1. The designations ".f1" and ".r1" refer to the terminus of the EST sequenced. j4d04a1.f1 was sequenced with forward primer and represents the 5' terminus of the EST, while j4d05a1.r1 was sequenced with reverse primer and represents the 3' terminus of the EST.



To determine if this ORF was expressed in *N. crassa*, I performed a northern transfer of RNA isolated from cultures grown under normal conditions or in the presence of alternative oxidase inducing compounds. The membrane was hybridized with a PCR product containing the 3' end of the ORF (primers aob-1 and aoc-3a, Table 2). A transcript of approximately 1 kb was seen under all conditions tested, though the level of the transcript was somewhat reduced in the culture grown in antimycin A (Fig. 9). Thus, the ORF is clearly expressed in *N. crassa* with a relatively abundant transcript. It is unclear why it is not present in any of the *N. crassa* EST libraries. It is conceivable that the expression of this ORF is influenced by the CRE. The distance from the predicted start codon of the ORF to the CRE is 1006 nucleotides, while the distance between the CRE and the start codon of *aod-1* is 803 nucleotides.

3.4 Alternative oxidases and upstream regions in other fungal species

To determine if any potential regulatory elements have been conserved upstream of the correlate of the *aod-1* gene in different species, I analyzed alternative oxidase genes and upstream regions in different species. I chose *Gelasinospora*, which is a member of the same order as *N. crassa*, Sordariales (CARLILE and WATKINSON 1994), and a more distantly related species, *Aspergillus nidulans*, which is from a different order, Eurotiales (CARLILE and WATKINSON 1994). The orders Eurotiales and Sordariales diverged approximately 280 million years ago (BERBEE and TAYLOR 1993). The species of *Gelasinospora* are morphologically very difficult to distinguish from one another so that the exact species I studied could not be determined.

I performed a Southern blot of various restriction endonuclease digestions of *Gelasinospora* genomic DNA and hybridized this with a ^{32}P labeled 1.2 kb PCR amplification product of *N. crassa aod-1* produced from plasmid pAOGE-1 (Table 3) using the primers AO9 and AO12 (Table 2). This revealed an approximately 5 kb fragment in the DNA digested with *Xba*I and *Eco*RI that hybridized with the

Figure 9. Northern blot hybridized with probe specific for the predicted 275 amino acid *N. crassa* ORF upstream of the *aod-1* gene. Total RNA was isolated from cultures grown in standard Vogel's medium, Vogel's medium supplemented with antimycin A (AA, 0.5 μg/ml) or Vogel's medium supplemented with Cm (2 mg/ml), electrophoresed on an RNA agarose gel, and transferred to a nylon membrane. The membrane was hybridized with ^{32}P labeled PCR product containing the 3' end of the ORF. A. Ethidium bromide stained gel, with molecular weight standards indicated on the right. B. Northern blot, with the size of the ORF RNA indicated on the right.

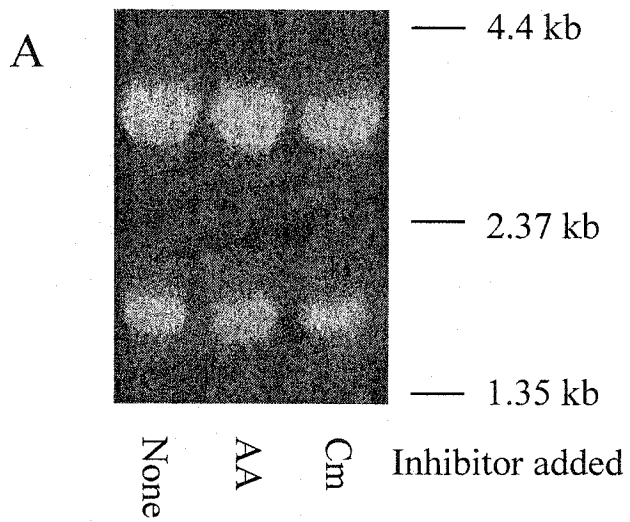
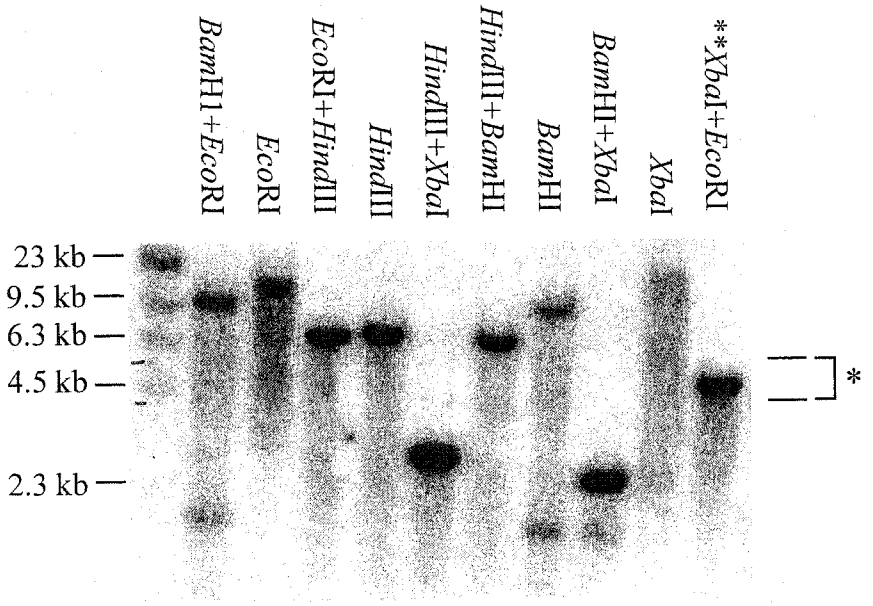


Figure 10. *Gelasinospora* Southern blot probed with *N. crassa aod-1* sequence. *Gelasinospora* genomic DNA (5 μg per lane) was digested with the enzymes shown, electrophoresed on an agarose gel, blotted to a nylon membrane and hybridized with a ^{32}P labeled PCR amplification of *aod-1* from plasmid pAOGE-1 using the primers AO9 and AO12. This revealed an approximately 5 kb fragment in the DNA digested with *Xba*I and *Eco*RI. A large amount (200 μg) of *Gelasinospora* genomic DNA was digested with *Xba*I and *Eco*RI, electrophoresed on an agarose gel and a region encompassing 4.4 to 6 kb, indicated by the asterisk, was purified from the gel, ligated into pBluescript and transformed into *E. coli*. The double asterisks indicate the enzyme combination chosen to cleave the gDNA in an attempt to clone the alternative oxidase gene and surrounding sequences contained within a 4.4 to 6 kb fragment. A colony screen was performed to isolate colonies containing *Gelasinospora* alternative oxidase coding sequences. Molecular weight size standards are shown on the left.



alternative oxidase coding sequence (Fig. 10). *Gelasinospora* genomic DNA (200 μg) was digested with these enzymes and electrophoresed on a gel. A region encompassing 4.4 kb to 6 kb was removed from the gel, the DNA was purified from the agarose, ligated to pBluescript, and transformed into *E. coli.* A colony screen was then performed, using the same probe as for the Southern blot, to isolate colonies containing *Gelasinospora* alternative oxidase sequences. Several potential positives were isolated and pGAX-27 was discovered to contain alternative oxidase coding sequence, by PCR analysis with the primers GAX1 and GAX7 (Table 2). Three kb of alternative oxidase coding and flanking sequence from this plasmid was sequenced (Fig. 11).

The high level of identity made it possible to use the *N. crassa* sequence to predict the positions of introns and the start codon. The predicted *Gelasinospora* alternative oxidase precursor protein was found to be 95% identical to the *N. crassa* protein (Fig. 12; *A. nidulans* sequence discussed below). The upstream sequence of *Gelasinospora* was also found to be highly similar to that of *N. crassa* for most of the region sequenced. This included the TATA box as well as a CRE at approximately the same distance upstream from the start of the coding sequence as in *N. crassa.* There are also conserved five base pair inverted repeats on either side of the CRE. (Fig.11). Due to the restriction sites involved in cloning, only 1.5 kb of upstream sequence were present. Therefore, no information about the presence of the expressed ORF seen in *N. crassa* (section 3.3) was available.

To aid in the cloning of the *A. nidulans* alternative oxidase gene, I obtained a portion of the *A. nidulans* genomic sequence homologous to *N. crassa aod-1* from Monsanto's Cereon Microbial Sequence Database. Using this sequence, I designed PCR primers to amplify a 1 kb alternative oxidase product from *A. nidulans* genomic DNA. This was labeled with ^{32}P and used to probe *A. nidulans* cosmid libraries (from the FGSC). Thirty-six potential positives were discovered. Using the *A. nidulans* PCR primers designed to amplify the alternative oxidase gene, I tested the 36 cosmids and found two from which the expected 1 kb alternative oxidase band could be amplified (71G12 and 72C5, Table 3). These cosmids were purified and the alternative oxidase structural gene and surrounding region was sequenced directly. As the cosmid

Figure 11. Sequence of the alternative oxidase region of *Gelasinospora* DNA. Introns and other untranslated regions are in lower case letters, the predicted amino acid coding sequences are in upper case letters. A CRE and a putative TATA box are indicated in bold type and underlined, the inverted repeats on either side of the CRE are underlined. Numerals in standard font indicate nucleotide number, while numerals in italics designate amino acids. The +1 site is predicted based upon sequence similarity in the region with *N. crassa aod-1*.

```
aaaaccgaacgggtctttgcgacaacgatgtctggcgcgacgaggaaaagtttgcgtgct -1375
gtggttgtgtccaactgcaaaagagaagccgatcgctgtctgagaagtcgaaaaagcgag -1315
caggggtcaggcaccttgtggttcaggcagtgggcaaaataattgttagcggggtgagaa -1255
gaccagcagtgaaacagagacagcgtttcacaggttcttcaaggttcttcgacgtctagg -1195
attttaataccatgccaagaccttcagtctcaacataggatgcactttgaagactggcat -1135
acaagtatgcccatcccgttcagtttttagttttttgtgtttttggacatcttgtata -1075
tgcacgatatatgtacggcttcggtcaagagaatatccgttggagccctctgtcatgtac -1015
caggtgcagcctagaaggacggcggtttgtaaaagtttgttaacgcatgtgaagagaggg -955
attcataaacaactaggcagacgctaaaactcctagacttcaacaaaatagccaatcctg -895
gagccacccaaaaatgctctgcgccgtcaatacttcgtatcccacttacgctccgcttcc -835
gacgcgggataatagttggatgcttgcaattgttcctgtcgctcctcgatcctaaactct -775
accgataactatcactgacgtcacaacgaagttggaagtctaacatgaagccattcgcaa -715
ttgatggccgaagaaatttctttgaatatatgcaggttcggaatcaatcaagccaatgct -655
caatcatgctgagtgtcctatgacacaagtttcgactggtgttccgtttccctccgttcg -595
ctattattgtcagcagaatctccaatacacaattcaagaaagtgccatttcgaccaacca -535
accaaccatcactgttccgagtcttgtgtggttgtctttgttctgagtcacaatcgccca -475
gtctgactgctcaggggctcgctgtactttaggatacaaggctagtaagtagtcccgaaa -415
atgttgcggaactgcgccctatggcttgtcacccgggaggtcccgacaccagaaaacaac -355
gacatccagctgaccatcacaatagatgcccggttgccgtttcgagaactcaaaatgaga -295
tctctatcctggttgaagatctggacctttccgggttcccttcacgagcggccgctattt -235
gcttgttactgagcacgaggcggtatttaaatttgtctcgatgctaaaaaatggggatcg -175
cttgggcagtgtcggaactctattgctcctttgagacaaggggacggacaaactcggtga -115
tttcagtcagctctcgtattccaagttctccttgggaatgagttgcaactggcggtaaga -55
                                                          +1
aaggacgatataaacgtcctgtgtctcttgtgctgccttacacatatggatcatcatcac +6
aaacctcaagcgagtttatatctttacatcactcttcatactctcg  ATG AAC ACC  61
                                                   M   N   T   3

CCC AAA GTA AAC ATA CTC TAC TCT CCA GGA CAA GCG GCC CAA CTA  105
P   K   V   N   I   L   Y   S   P   G   Q   A   A   Q   L   18

AGC CGT ACC CTG ATA TCG ACC TGC CAC ACC CGG CCT TTC TTA CTC  150
S   R   T   L   I   S   T   C   H   T   R   P   F   L   L   33

GGT GGT CTT CGA GTG GCC ACT TCT TTG CAT CCA ACA CAG ACA AAC  195
G   G   L   R   V   A   T   S   L   H   P   T   Q   T   N   48

CTC TCT TCA TCA CCA CCT CGA GGC TTC ACG ACA ACA AGT GTT GTT  240
L   S   S   S   P   P   R   G   F   T   T   T   S   V   V   63

CGA CTG AAG GAC TTC TTC CCA GCC AAG GAG ACC GCC TAC ATC CGA  285
R   L   K   D   F   F   P   A   K   E   T   A   Y   I   R   78

CAG ACA CCA CCT GCA TGG CCT CAT CAT GGA TGG ACT GAG GAA GAG  330
Q   T   P   P   A   W   P   H   H   G   W   T   E   E   E   93

ATG ATC TCG GTT GTT CCC GAG CAC CGG AAA CCC GAG ACT GTG GGC  375
M   I   S   V   V   P   E   H   R   K   P   E   T   V   G   108

GAC TGG CTC GCA TGG AAA CTC GTT CGA ATT TGT CG gtaggtaattc   421
D   W   L   A   W   K   L   V   R   I   C   R               120

cccaaaggaattttcccatattgtccctcaagtatcttgcagacgcggaagcagacgc   479

agaatcaaagtgatctaacatcaagcag A TGG GGC ACC GAT ATA GCG ACG   521
                              W   G   T   D   I   A   T     127
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```
GGC ATA CGT CCA GAG CAG CAA GTT GAC AAA AAC CAC CCG ACG ACC  566
G   I   R   P   E   Q   Q   V   D   K   N   H   P   T   T   142

GCC ACC AGT GCG GAT AAA CCT CTA ACA GAA GCC CAA TGG gtacgtc  612
A   T   S   A   D   K   P   L   T   E   A   Q   W           155

ctatgaagccctacgagtacggaatggcagcatatgctaaccaaatatcacaatccata  670

tag CTA GTC CGC TTC ATC TTC CTC GAA TCG ATC GCC GGC GTT CCC  715
    L   V   R   F   I   F   L   E   S   I   A   G   V   P   170

GGC ATG GTA GCC GGC ATG CTC CGG CAC CTG CAC TCT CTC CGG CGC  760
G   M   V   A   G   M   L   R   H   L   H   S   L   R   R   185

CTC AAA CGA GAC AAC GGC TGG ATC GAG ACT TTA CTC GAA GAG TCC  805
L   K   R   D   N   G   W   I   E   T   L   L   E   E   S   200

TAC AAC GAG CGC ATG CAC CTC CTC ACC TTC ATG AAG ATG TGC GAA  850
Y   N   E   R   M   H   L   L   T   F   M   K   M   C   E   215

CCA GGT CTT CTC ATG AAG ACG CTC ATC TTG GGA GCG CAG GGC GTC  895
P   G   L   L   M   K   T   L   I   L   G   A   Q   G   V   230

TTC TTC AAC GCC ATG TTC CTC AGC TAC CTG GTC TCC CCC AAA ATC  940
F   F   N   A   M   F   L   S   Y   L   V   S   P   K   I   245

ACC CAC CGG TTC GTC GGC TAC CTC GAG GAG GAG GCC GTG CAT ACC  985
T   H   R   F   V   G   Y   L   E   E   E   A   V   H   T   260

TAC ACG CGG TGC ATC AGG GAG ATT GAG GAA GGG CAC TTG CCA AAG 1030
Y   T   R   C   I   R   E   I   E   E   G   H   L   P   K   275

TGG AGC GAT GAA AGA TTT GAG ATC CCG GAG ATG GCG GTG AGG TAT 1075
W   S   D   E   R   F   E   I   P   E   M   A   V   R   Y   290

TGG CGC ATG CCG GAG GGG AAG AGG ACG ATG AAG GAC TTG ATT TAT 1120
W   R   M   P   E   G   K   R   T   M   K   D   L   I   Y   305

TAT ATC CGG GCG GAC GAG GCA GTG CAT AGG GGC GTT AAT CAT ACG 1165
Y   I   R   A   D   E   A   V   H   R   G   V   N   H   T   320

CTG AGT AAT TTG GAT CAG AAG GAG GAT CCG AAT CCG TTT GTG AAC 1210
L   S   N   L   D   Q   K   E   D   P   N   P   F   V   N   335

GAC TAT AAG GAG GGT GAA GGC GGG AGG AGA CCG GTT AAT CCG GCT 1255
D   Y   K   E   G   E   G   G   R   R   P   V   N   P   A   350

TTG AAG CCG ACG GGG TTT GAA AGG GCG GAG GTC ATC CGT TGA tct 1300
L   K   P   T   G   F   E   R   A   E   V   I   R   *       363

tgggtggaaggttttgacagatgattgtggtttgggttcatgaaactaggcgtttttgga 1360
ccaagttgtattgggcgtgttaatatcccactctctttgctgtttgtggcgttctggtaa 1420
aagatactgggttctatctgtgcaactgttcattgttccaagtgtgtatgtacttcggat 1480
cttcatcccgaaagtcgttggtatcttatgaagcggdatatccgcaacaaaactgatatg 1540
tcaagctttctcctttcgcccccgaattattatcttggtccagttccacatggcaacttc 1600
taccaaatg                                                    1609
```

Figure 12. Sequence of *A. nidulans* alternative oxidase gene and surrounding region. Introns and other untranslated regions are in lower case letters, the amino acid coding sequences are in upper case letters. A CRE-like element and a putative TATA box are indicated in bold type and underlined, the inverted repeats flanking the CRE-like element are underlined. Numerals in standard font indicated nucleotide number while numerals in italics designate amino acids.

```
cttgcaattctcggtggaagtctctgcaatgtgatttagatcacatgtggcgaatgaag  -1715
tatccaactctcgaaagcaagagcatattaggaacgagatgttaatcccaaattcaaggt -1655
tgaggcgggttatatgcagctcgcttgaagccccgcagtgggccctggtcaacctcagct -1595
tcaaccgctctagactccttcggatcctacacctaccgaccaggatgacatgttaacact -1535
cctctgcttgatcgtcggcgagaatatcacaaaataaacatggcaactcaaagggcatgt -1475
ttggctgtcagatttattgctacaagcttatacttacgactgttgtcgctgacttttctc -1465
agattgcgttcaaagtccatggcaccgttcagggtacgtcttcagttctaactagtttca -1355
ttcttagccagctatgtttaacaagtacctctacaggcgtgggtttccggtaggcacagt -1295
ttatacaggtcaaccattagacatttctgatcatgtcgttacaccagtgatttcaccatg -1235
aagcgtgctgcggaatacgttctcagcggctgggtgaagaacactgattgcggaagggta -1175
cgtttgtagtccctattaatgctaacaatcttgcaatgtcttgcatcgctcgtcagagtc -1115
actaaatgcatatgaaggtcgaaggcgaagcacaaggctcagaagagggtatccaaaaat -1055
ttctcaaggatatcaataatggccctcgacttgcacatgtagtcaagctggagaagaaga  -995
cactcgatccgaaagacggagagagtcattttgggcttaggaagacatcggcgactgttt  -935
ttgattcactttgatggtcgactatacagtgtatactttggttcaagcagaataaagtcc  -875
atccgctcggcctcccagtttccatgaaaagtagtctcagcgtagccttttcatccggtc  -815
ctctgcttacgtggtagccaatttttgaggggcaagcaggaacgcattgccccagaatct  -755
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gggtactcagccctgagagttccgattcctgcctcagattgcctctggttgaatttttttt -155
tataggtctttgtatttagttggcatatataaatacaaaaaaagttgcagtcccaggtca  -95
actccttatgtatgtcaaagcaacatcttatcttgactaagtacagttcgtcagaatgga  -35
cagcacctttcaacaataaacgatatcagcatt ATG AAT TCG ATG TCA ACA     +18
                                   M   N   S   M   S   T      6

ACG GGG CCT ATT AGG GGT GGC GCT ATA CCG AAA CAT TAC CTG  60
 T   G   P   I   R   G   G   A   I   P   K   H   Y   L  20

CAA TTC ACA GTG CGG ACC TAT ACG AGG AGT ATG GCG AGC GCT 102
 Q   F   T   V   R   T   Y   T   R   S   M   A   S   A  34

GGG TTA CGA TAC AGC AAT CCG CCC CTC GTC AAA AAA TGC TAC 144
 G   L   R   Y   S   N   P   P   L   V   K   K   C   Y  48

GAC CAA CCG ACA GGG AAA AGG TTC ATT TCC TCC ACG CCA CAA 186
 D   Q   P   T   G   K   R   F   I   S   S   T   P   Q  62

TCA CAG ATC AAA GAC TAT TTC CCT CCT CCT GAT GCA CCG AAA  228
 S   Q   I   K   D   Y   F   P   P   P   D   A   P   K  76

ATA GTC GAG GTG AAA ACA GCG TGG GCT CAT CCT GT gtaagttat  272
 I   V   E   V   K   T   A   W   A   H   P   V           88

ccaatcttcaagagaggcgacagatgctgatcaagaacag C TAC AGT GAA GAA 325
                                          Y   S   E   E  92

GAA ATG CGT GCA GTC ACT GTT GGC CAT CGC GAG GCA AAG AAT  367
 E   M   R   A   V   T   V   G   H   R   E   A   K   N  106

TGG TCT GAC TGG GTA GCG CTT GGG AGT GTC CGC CTG CTC CGA  409
 W   S   D   W   V   A   L   G   S   V   R   L   L   R  120

TGG GGT ATG GAC CTG GTC ACT GGC TAT AAA CAC CCT GCG CCG  451
 W   G   M   D   L   V   T   G   Y   K   H   P   A   P  134
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GGC CAA GAA GAC ATC AAG AAG TTT CAG ATG ACG GAA AAG GAA  493
 G   Q   E   D   I   K   K   F   Q   M   T   E   K   E  148

TGG TTA AGA AGA TTT GTC TTC TTG GAG AGC GTC GCG GGT GTA  535
 W   L   R   R   F   V   F   L   E   S   V   A   G   V  162

CCT GGA ATG GTT GGT GGT ATG CTA AGG CAT TTG AGG AGT CTC  577
 P   G   M   V   G   G   M   L   R   H   L   R   S   L  176


AGA CGT ATG AAG CGA GAT AAC GGA TGG gtatgtcgagatttctttcat 625
 R   R   M   K   R   D   N   G   W                       185

cttatacatttcgtggctcaactaataatcaatgcgcag ATC GAG ACG CTC CTT 679
                                         I   E   T   L   L 190

GAG GAG GCA TAC AAT GAG CGT ATG CAC TTG CTC ACA TTC CTC  721
 E   E   A   Y   N   E   R   M   H   L   L   T   F   L  204

AAG ATG GCC GGA CCT GGG TGG TTC ATG CGC TTA ATG GTC CTT  763
 K   M   A   G   P   G   W   F   M   R   L   M   V   L  218

GGA GCG CAA GGA GTG TTT TTC AAC GGC TTC TTC CTC TCT TAT  805
 G   A   Q   G   V   F   F   N   G   F   F   L   S   Y  232

CTC ATC TCG CCA CGT ACC TGT CAT CGT TTC GTC GGC TAT CTC  847
 L   I   S   P   R   T   C   H   R   F   V   G   Y   L  246

GAG GAG GAA GCC GTG CTC ACT TAC ACT CGG GCC ATC AAA GAC  889
 E   E   E   A   V   L   T   Y   T   R   A   I   K   D  260

CTC GAA AGC GGC AGG CTG CCG CAC TGG GAA AAG CTG GAG GCT  931
 L   E   S   G   R   L   P   H   W   E   K   L   E   A  274

CCA GAG ATC GCT GTC AAG TAC TGG AAA ATG CCT GAG GGT AAC  973
 P   E   I   A   V   K   Y   W   K   M   P   E   G   N  288

CGG ACC ATG AAG GAT CTG TTG CTG TAT GTC CGA GCG GAC GAG 1015
 R   T   M   K   D   L   L   L   Y   V   R   A   D   E  302

GCC AAA CAT CGC GAG GTC AAC CAC ACG CTA GGG AAC CTG AAG 1057
 A   K   H   R   E   V   N   H   T   L   G   N   L   K  316

CAA GCG GTC GAC GTC AAC CCT TTC GCC GTT GAA TGG AAG GAT 1099
 Q   A   V   D   V   N   P   F   A   V   E   W   K   D  330

CCG TCT AAA CCG CAT CCT GGC AAA GGG ATC AAA CAC TTA AAG 1141
 P   S   K   P   H   P   G   K   G   I   K   H   L   K  344

ACC ACC GGC TGG GAA CGA GAG GAG GTT GTT TGA gcatttatgcat 1186
 T   T   G   W   E   R   E   E   V   V   *               354

ttataattgtggttttgtcattcgcgggagcaaatgggagaacatgcatatatgattaaa 1246
gcggccgcaaattggttggacttgagcatagcatcgcggatatctcctctccttagtctt 1306
tattggtaatagaatgttcttgaacctgttcagattaattgatatggaccggccatgcaa 1366
ttatgagcgcagatgaaacatacactaaactatattgggatagcaatatcc          1357
```

libraries were chromosome specific, I was able to assign the alternative oxidase gene to Linkage Group VII of *A. nidulans*. I used published amino acid sequence for the *Aspergillus niger* alternative oxidase cDNA (KIRIMURA et al. 1999) to determine the start codon and intron positions (Fig. 12). Since the +1 transcription start site was not known, the first base in the predicted start codon of the coding sequence was defined as "+1" for further discussion. Alignment of the alternative oxidase protein from *A. nidulans* with those of *N. crassa* and *Gelasinospora* revealed that the N-terminal portions of the *A. nidulans* and *N. crassa* proteins are more divergent than the central more conserved region (Fig. 13). This divergent region includes the N-terminal mitochondrial targeting signal, which would be expected to diverge relatively rapidly, as well as approximately 40 amino acids of the mature protein. The extreme C-terminal region is also fairly divergent between *A. nidulans* and *N. crassa*. The overall identity between the amino acid sequences of *N. crassa* and *A. nidulans*, including the mitochondrial targeting sequence, is 50%, which is similar to the 49% identity between *N. crassa* and the yeast *H. anomala* (LI et al. 1996). The amino acids proposed to be involved in binding iron at the active site are conserved and marked by asterisks in Fig. 12 (ANDERSSON and NORDLUND 1999; BERTHOLD et al. 2000), (section 1.6). The intron positions of the alternative oxidase genes are conserved between *Gelasinospora* and *N. crassa*, but not in *A. nidulans* (Fig. 13).

The region upstream of the *A. nidulans* alternative oxidase gene has little direct sequence homology to the *N. crassa* region; however there is an identical TATA box at approximately the same distance upstream of the start codon in both species. Strikingly, a sequence about 750 nucleotides upstream of the ATG was identified that has seven of the eight consensus CRE bases (TGACGTCG versus the consensus TGACGTCA). There are also 6 base pair inverted repeats flanking the element (Fig. 14). The fact that the CRE does not exactly match the consensus does not preclude a role for it in regulation as there are many cases in which non-consensus CRE elements are involved in binding transcription factors (HABENER et al. 1995).

Figure 13. Alignment of the predicted precursor alternative oxidase proteins of *N. crassa* (*N.c.*), *Gelasinospora species* (*G.s.*) *and A. nidulans* (*A.n.*). Identical amino acids are highlighted in black. Similar amino acids are indicated with grey highlighting. Asterisks represent conserved amino acids predicted to be involved in binding the iron atoms that form the enzyme's di-iron catalytic core, based upon residue conservation across species (ANDERSSON and NORDLUND 1999; BERTHOLD et al. 2000). The asterisk in parentheses indicates a conserved amino acid found to be essential for alternative oxidase function in *A. thaliana* (ALBURY et al. 2002), but not predicted to bind iron by Andersson and Nordlund. Arrowheads indicated the position of introns in *N. crassa* and *Gelasinospora*, and diamonds indicate the position of *A. nidulans* introns. The plus sign represents the predicted start of the mature *N. crassa* alternative oxidase protein.

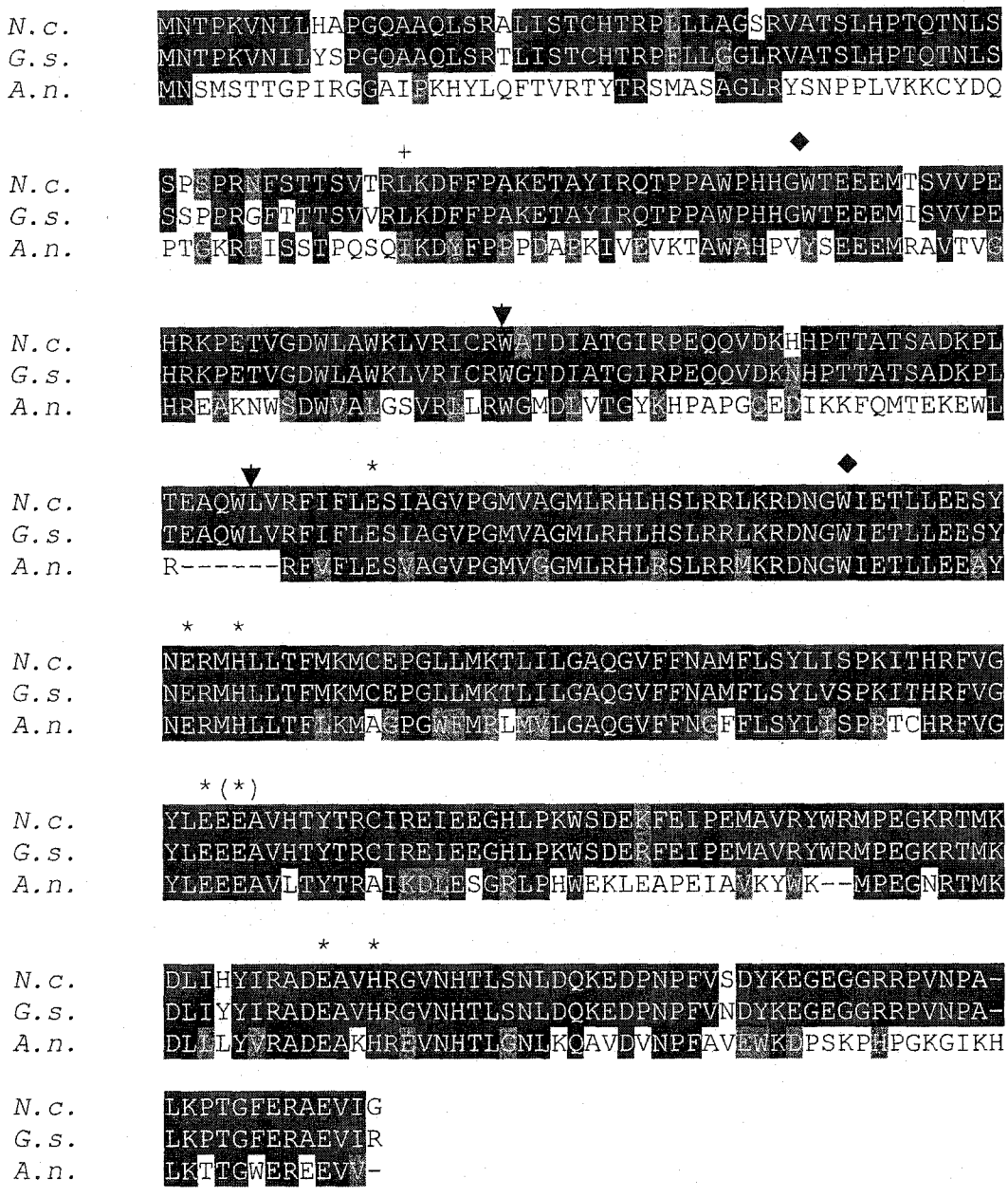
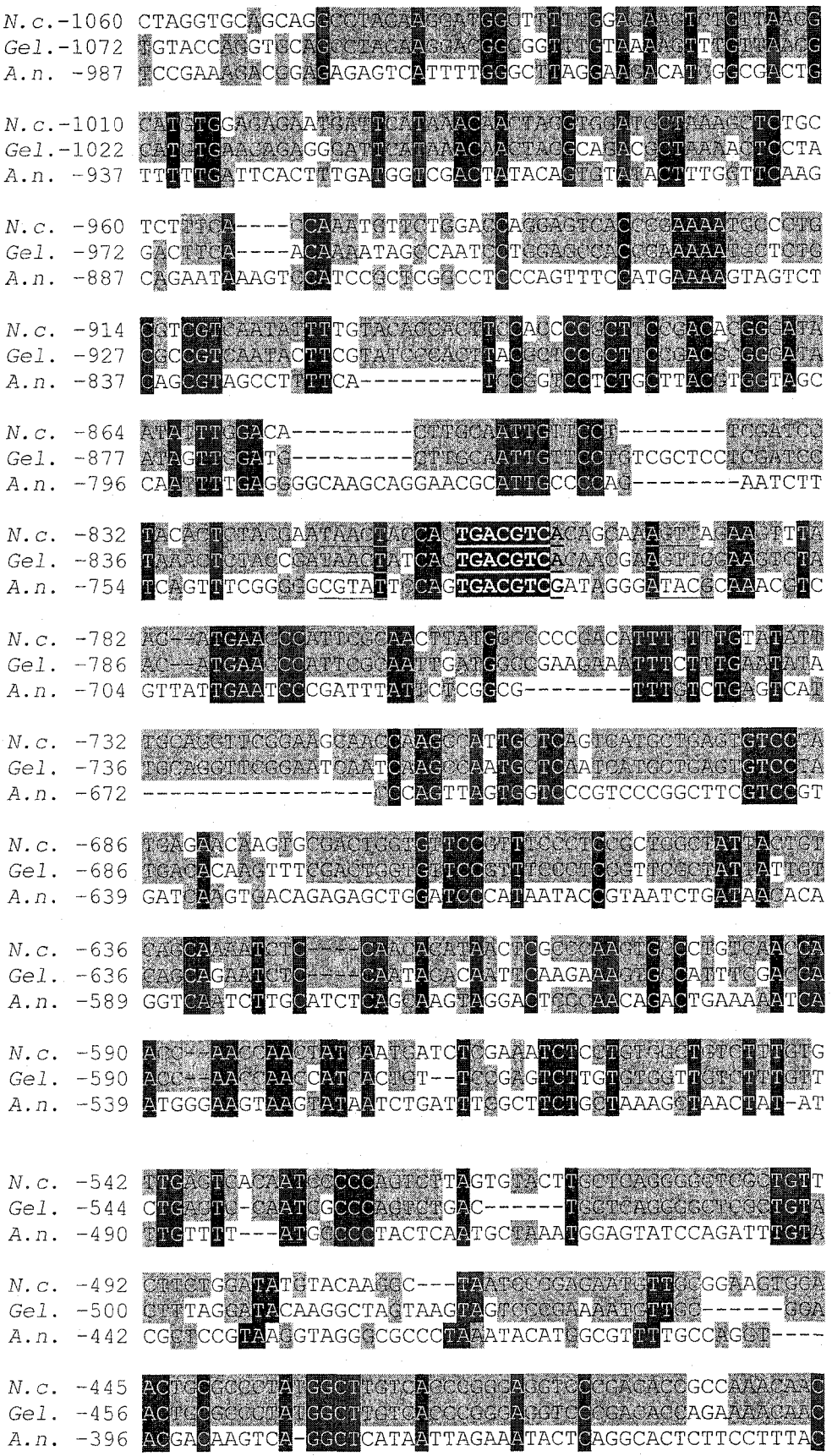
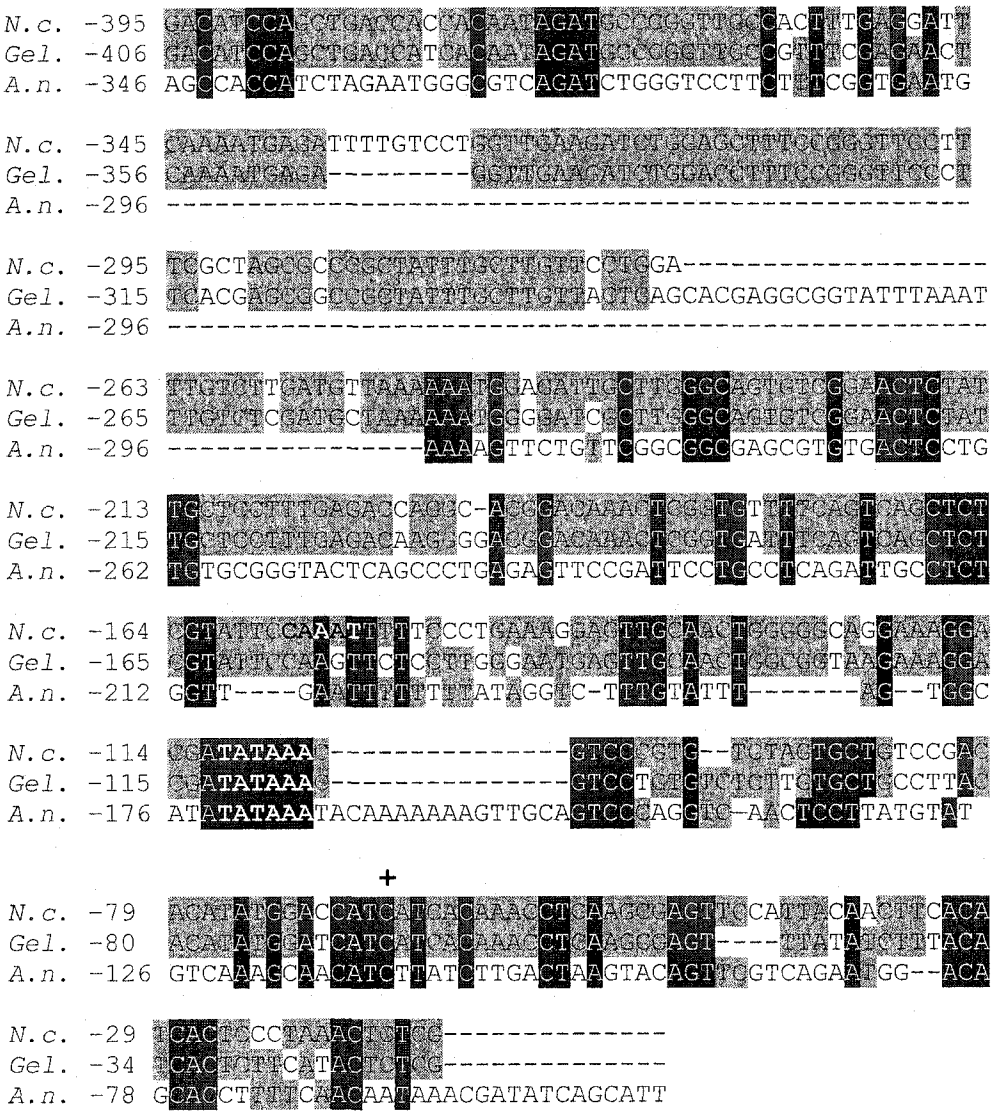


Figure 14. Alignment of 1 kb of sequence upstream of the alternative oxidase genes of *N. crassa* (*N.c.*), *Gelasinospora species* (*G.s.*) *and A. nidulans* (*A.n.*). Numbering is from the ATG of all species. The TATA box is indicated in bold type, the CRE is indicated by bold underlined type and the inverted repeats flanking the CREs are underlined. The plus sign indicates the start of transcription for *N. crassa* and likely *Gelasinospora* due to the similarity of their sequences. The transcription start site of *A. nidulans* is not known. Dashes indicate gaps generated by the DNAMAN computer program to optimize alignment in other regions.





3.5 Homologous ORF in *A. nidulans*

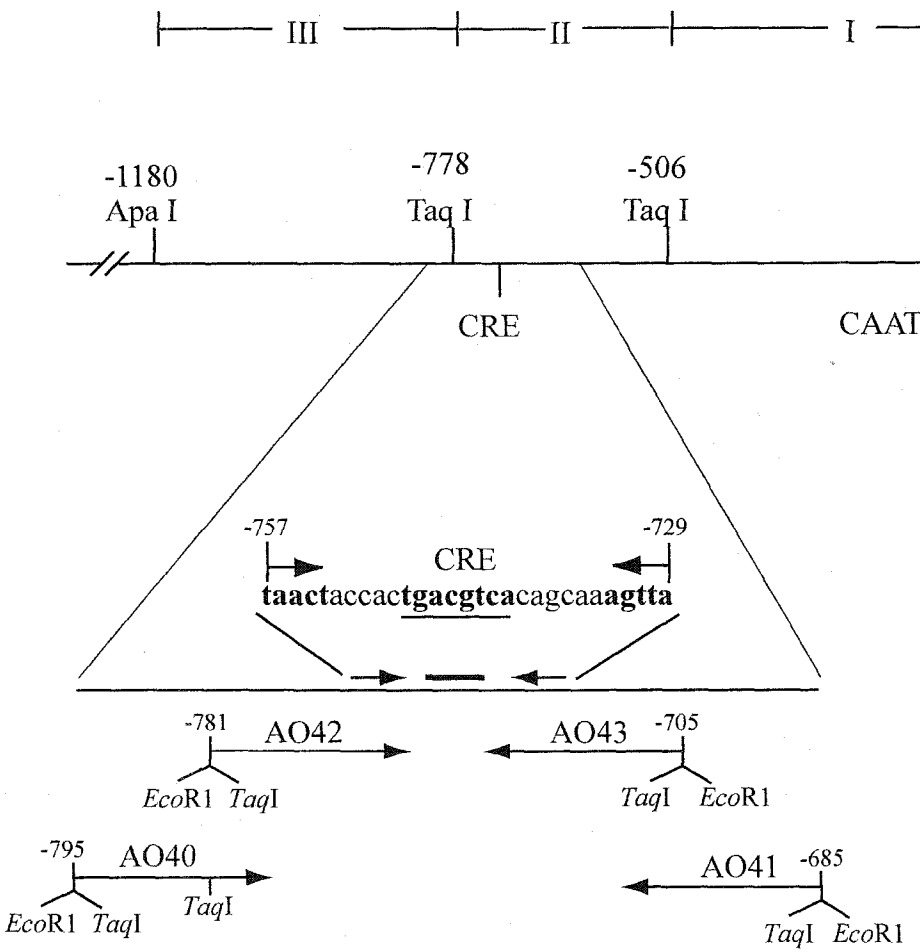
To determine if the expressed 275 amino acid ORF found upstream of *aod-1* in *N. crassa* (section 3.3) was in the same position relative to the alternative oxidase gene in *A. nidulans*, I obtained sequence from Monsanto's Cereon Microbial Sequence Database of the region of the *A. nidulans* EST that was shown to be homologous to the *N. crassa* ORF. This sequence was used to design primers to amplify the *A. nidulans* ORF by PCR (Table 2). The PCR product was labeled with ^{32}P and used to probe two cosmid libraries of *A. nidulans* DNA (obtained from the FGSC). Eighteen potential positive cosmids were identified. These were subjected to PCR screening using the primers designed to amplify the ORF. Two of the cosmids were found to contain the ORF (M10B5 and 51B6, Table 3). The *A. nidulans* ORF was not sequenced fully.

The *A. nidulans* cosmid libraries were chromosome specific, so that I was able to determine that the *A. nidulans* ORF was located on Linkage Group V. Thus, as the alternative oxidase gene of *A. nidulans* is found on Linkage Group VII, the gene order in the two species has not been conserved. This observation suggests that the expressed ORF does not utilize the CRE sequences found upstream of the alternative oxidase gene in both species for its expression, and supports the view that the CRE is involved with alternative oxidase expression.

3.6 Binding of *N. crassa* protein to the CRE

CREs are known to be involved in the regulation of a wide range of genes (SHAYWITZ and GREENBERG 1999) including the *grg-1* gene (glucose repressible gene) of *N. crassa* (WANG et al. 1994). To determine if the CRE upstream of the *aod-1* gene bound protein(s) and if it played a role in regulating *aod-1* expression, electrophoretic mobility shift assays (EMSAs) were performed. As described in section 2.35.2, restriction enzymes were used to generate three fragments from the 1.2 kb immediately upstream of the *aod-1* translation start site (Fig. 15). Each fragment was isolated, purified, end labeled with ^{32}P, incubated with total cellular protein extract and examined using EMSA. Fragment I, closest to the *aod-1* structural gene, and Fragment II, containing the CRE, each showed mobility shifts (Fig. 16A).

Figure 15. Generation of target DNA for EMSAs. 1.2 kb of sequence upstream of *aod-1*, from plasmid pAOPFGuB, was used to generate restriction fragments and PCR products for use as target DNA molecules for EMSA. The restriction fragments used and their position in the sequence are shown. The numbers used are relative to the transcription start site +1. The CRE sequence is underlined and the 5 base pair inverted repeats are indicated in bold lettering with arrows above the inverted repeats. The primers AO40 and AO41 generated a PCR fragment of 110 base pairs, which became a target DNA molecule of 89 base pairs after digestion with *Taq*I. The primers AO42 and AO43 generated a PCR product of 76 base pairs, which became a target DNA molecule of 58 base pairs after digestion with *Taq*I. Digestion with *Taq*I allowed ^{32}P labeling of the fragments. Digestion with *Eco*RI allowed labeling with ^{32}P and biotin, or with biotin alone.

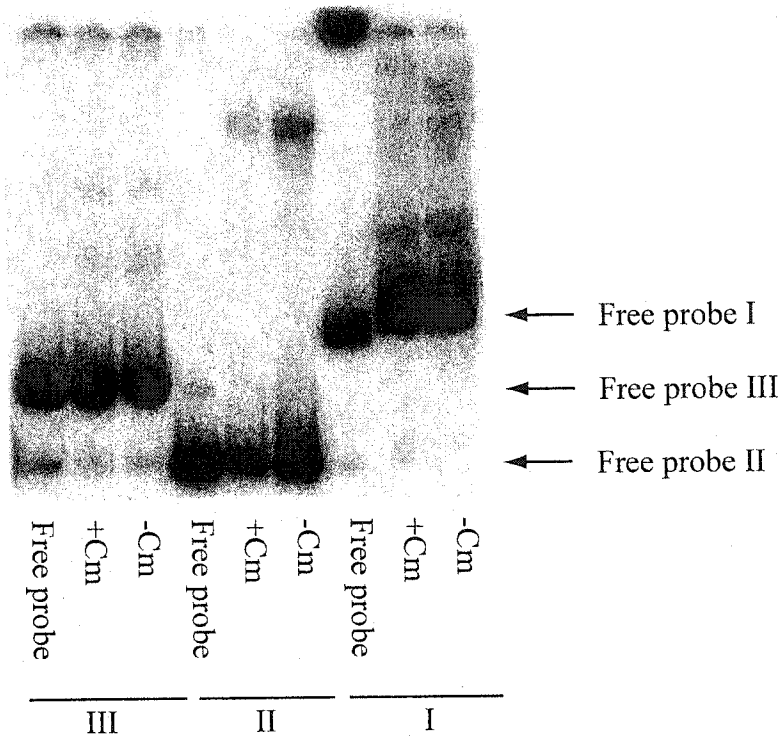


Original EMSA target DNA Fragments

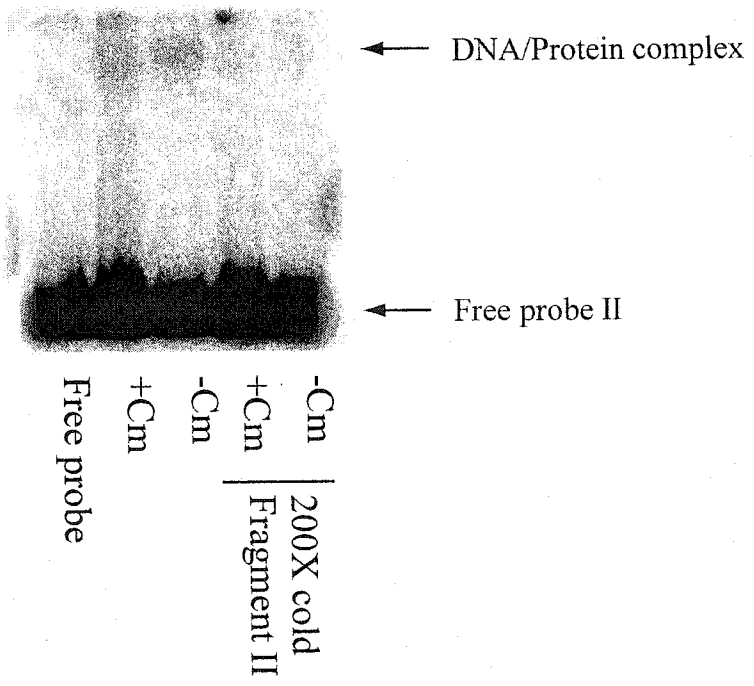
Upstream region of *aod-1,* from plasmid pAOPFGuB

Figure 16. EMSA demonstrating binding of protein(s) to sequences upstream of *aod-1*. Each lane contains 8000 counts per minute (cpm) of ^{32}P labeled target DNA in 1X EMSA buffer (see appendix). Lanes labeled "-Cm" indicate that the binding reaction was performed with protein extracts from cells grown without Cm in the media, while binding in those labeled "+Cm" was done with protein extracts from cultures grown in the presence of Cm (2 mg/ml). All lanes with protein also contain a 50 000 fold excess of poly[d(I-C)] (based on estimated weight of target DNA). A. EMSA pattern seen with Fragments I, II (contains the CRE), and III (Fig. 15) comprising 1.2 kb upstream of *aod-1*, as target DNA. B. Binding to Fragment II in the absence and presence of a 200 fold excess of unlabeled Fragment II.

A



B



Weakly visible shifted bands were also seen with Fragment III. I decided to concentrate on Fragment II since it contained the CRE, an element known to be involved in genetic regulation, and also because the pattern of binding within Fragment I seemed very complex.

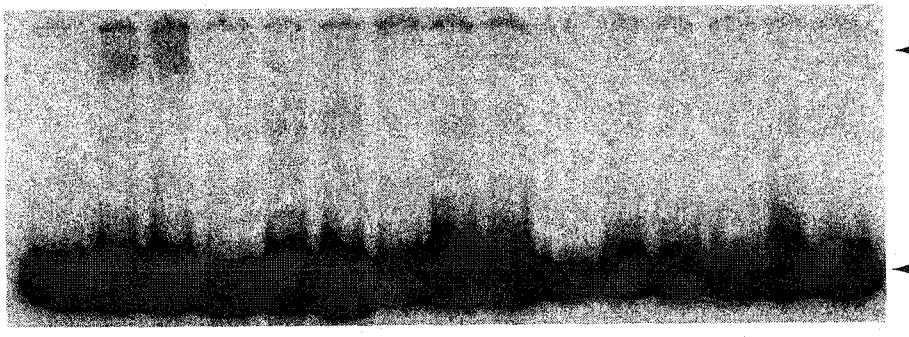
The data in Fig. 16A showed that the CRE containing fragment was shifted by extracts from both induced (+Cm) and uninduced (-Cm) cultures. Many CRE binding proteins (CREBs) in other species are known to bind to the CRE under both inducing and non-inducing conditions. Regulation of gene expression occurs by differential phosphorylation of the CREB in response to different cellular conditions (HABENER et al. 1995; MEYER and HABENER 1993).

To demonstrate that the binding observed was not an artefact, I tested for competition with excess cold Fragment II. In the presence of 200-fold excess cold Fragment II binding to the labeled Fragment II was greatly reduced, while the presence of an excess of approximately 50 000 times non-specific poly [d(I-C)] did not affect binding. Thus, binding appeared to be due to a specific sequence on Fragment II (Fig. 16B). I also examined binding to Fragment II that was lacking the CRE. Plasmids containing mutated CRE sequences were created that contained individual base changes or lacked the entire eight base pair CRE (Fig. 17). The different Fragment II DNAs containing these mutated sequences were labeled with ^{32}P and incubated with protein extracts. No specific binding was seen with any of the fragments that contained an altered or deleted CRE (Fig. 17). Taken together, these data demonstrate that a *N. crassa* protein or multicomponent factor binds specifically to the CRE upstream of *aod-1*.

3.7 Regulatory effects of upstream region

Cosmid 23F7 (Table 3) contains the *aod-1* gene, and surrounding sequences. It was known from previous work (LI et al. 1996) that this cosmid could restore inducible alternative oxidase activity to *aod-1* mutant strains. In an effort to determine if plasmids with a smaller upstream region could control the expression of *aod-1*, plasmid pMAX, which contains 3.3 kb of sequence upstream of the structural gene, was created. The plasmid also contains a bleomycin resistance gene as a

Figure 17. EMSA using Fragment II containing wild type CRE or one of four altered CRE sequences. Each lane contains 8000 counts per minute (cpm) of ^{32}P labeled target DNA in 1X EMSA buffer (see appendix). Lanes labeled "-Cm" indicate that the binding reaction was performed with protein extracts from cells grown without Cm in the media, while binding in those labeled "+Cm" was done with protein extracts from cultures grown in the presence of Cm (2 mg/ml). All lanes with protein also contain 50 000 fold excess of poly[d(I-C)]. The nucleotides in pCM1, pCM2 and pCM3 altered from wild type are indicated in lower case letters, while the CRE in pCM4 is deleted entirely.



selectable marker (Table 3; Fig. 18). The plasmid was linearized and transformed into spheroplasts of strain 7207, containing the frameshift allele *aod-1-7* (Li et al. 1996), (Table 1). Transformants were purified and then examined for alternative oxidase activity by assessing sensitivity or resistance to KCN in respiration assays following growth in both standard Vogel's medium and Vogel's medium containing 2 mg/ml chloramphenicol. Assuming that regulation of alternative oxidase in *N. crassa* occurred at the transcriptional level, there were three possible outcomes of these transformation experiments. First, if the upstream region within the plasmid contained all of the sequences required for normal induction of alternative oxidase there would be KCN-resistant respiration in cultures grown in the presence of the inducing agent chloramphenicol. Second, if the promoter was unable to express alternative oxidase, then no KCN-resistant respiration would be seen in either growth condition. The third possibility was that the 3.3 kb upstream region would be unable to respond to a negative regulatory signal and there would be constitutive expression of alternative oxidase in any growth condition.

In *N. crassa,* the vast majority of DNA integrations during transformation are non-homologous and occur at ectopic sites within the genome. Furthermore, in all transformation experiments, some bleomycin resistant transformants would be expected not to have integrated critical regions of the upstream sequence and/or the *aod-1* structural gene. Such transformants would have no alternative oxidase activity under any circumstances. Therefore, several different isolates from each transformation were tested to maximize the chances of finding rescued isolates. No attempt was made to determine the number or position of insertions in the transformants.

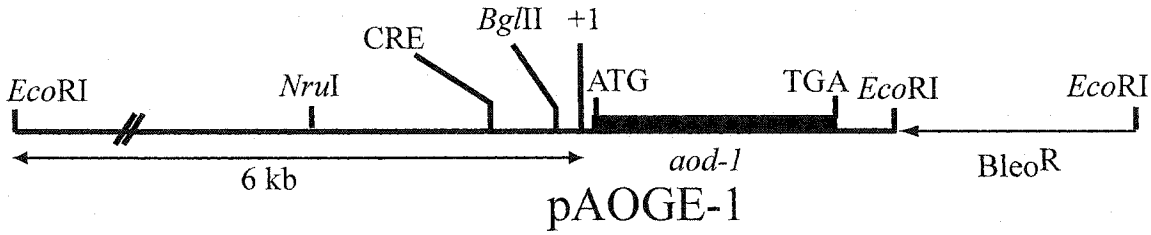
As discussed above, cosmid 23F7 was able to successfully rescue the *aod-1-7* mutant as judged by the presence of KCN-insensitive respiration in the transformants grown in the presence of chloramphenicol (Table 6). Plasmid pMAX (Fig. 18), which contains 3.3 kb of upstream sequence, was also shown to be capable of restoring normal induction patterns, since 17 of 21 transformants showed inducible alternative oxidase activity (Table 6). To determine if removal of the CRE would affect the expression of *aod-1*, plasmid pΔMAX was constructed. This plasmid is identical to

Table 6
Plasmids with or without the CRE can restore alternative oxidase activity with normal control to the *aod-1* frameshift mutant 7207.

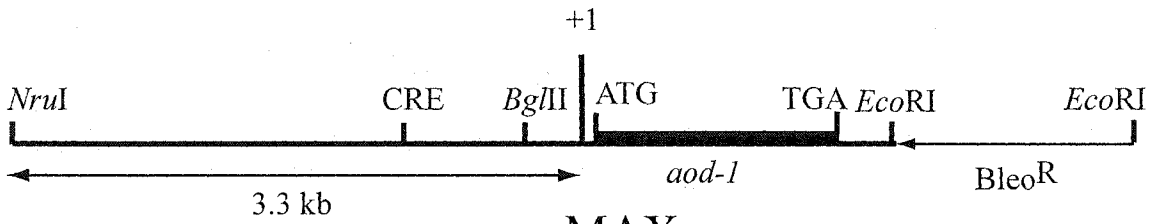
Transforming plasmid	Upstream sequence	Total transformants	Rescued Transformants*
23F7 (cosmid)	At least 6 kb	13	9
pMAX	3.3 kb	21	17
pΔMAX	3.3 kb, deleted CRE	32	22

* Transformants behave like wild type cells in that they contain no KCN-resistant respiration when grown without chloramphenicol, but do contain KCN-resistant, SHAM sensitive respiration following growth in chloramphenicol.

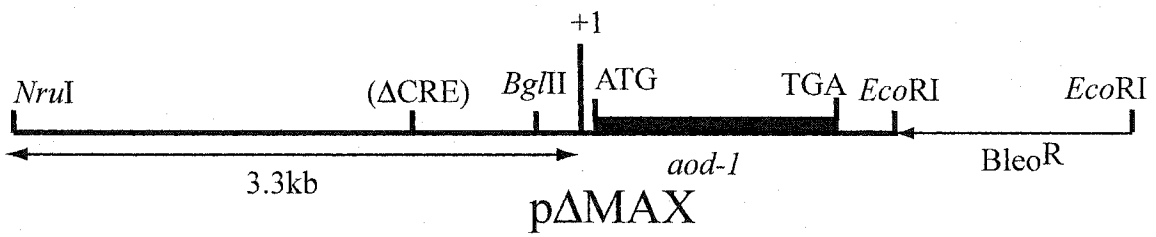
Figure 18. Composition of plasmids containing varying amounts of sequence upstream of *aod-1*. The amount of sequence upstream of the +1 transcription start site of the *aod-1* structural gene in each plasmid is indicated. The coding sequence is indicated by the thick line. The plasmids also carry a bleomycin resistance ($Bleo^R$) gene.



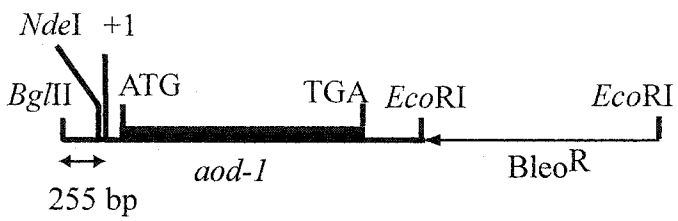
pAOGE-1



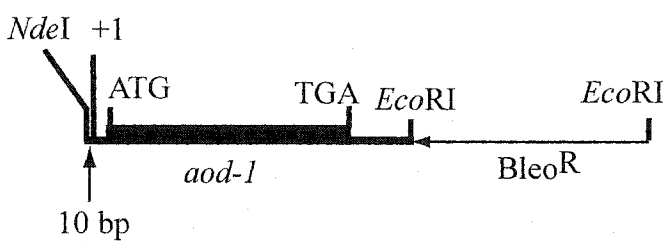
pMAX



pΔMAX



pMMAX



pMCMAX

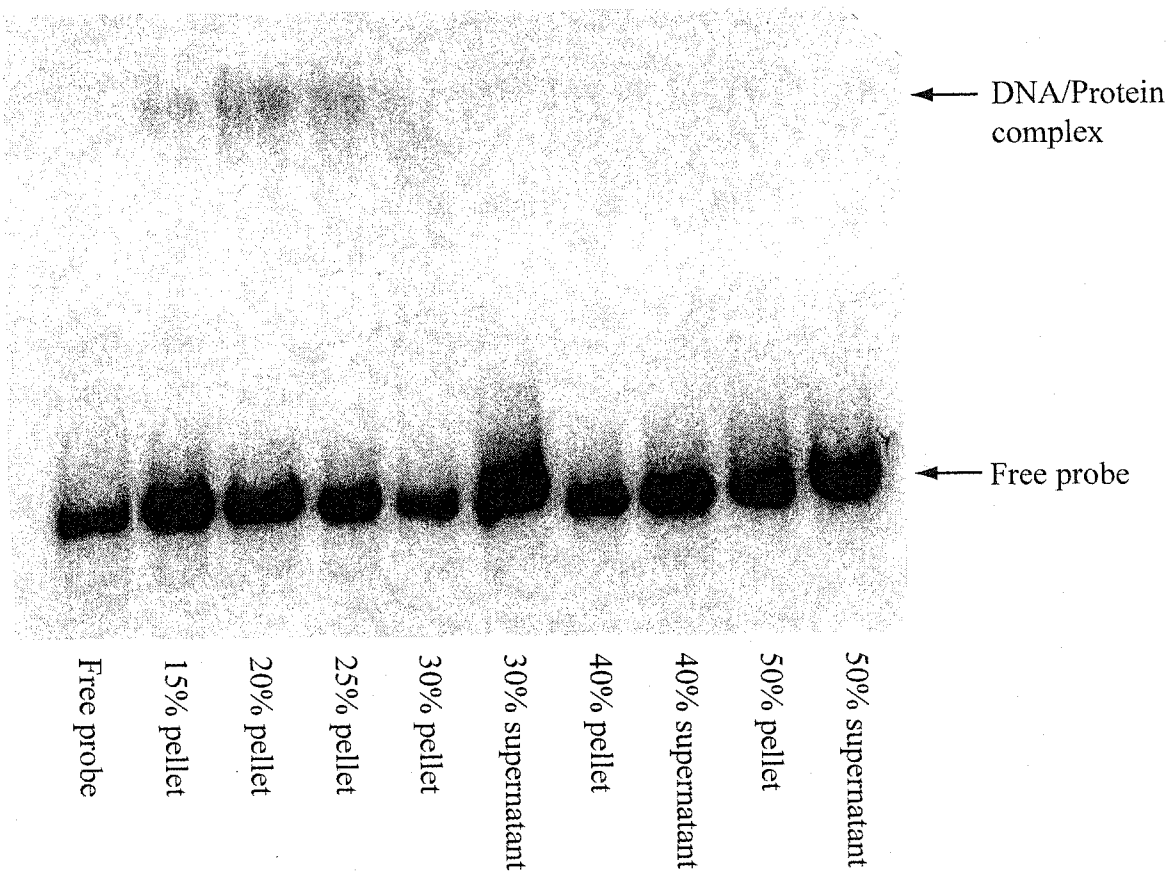
pMAX except that it lacks the eight base pair CRE sequence (Fig. 18). Of 32 CRE deletion transformants tested, 22 showed normal induction of alternative oxidase, suggesting that there is no role for the CRE in induction of the enzyme upon growth in chloramphenicol (Table 6). The observation that the CRE was not required for expression of alternative oxidase was confirmed in further experiments with truncated promoters (section 3.13).

3.8 Purification of the CRE-binding proteins

The data presented above suggested that the CRE element was not involved in induction of alternative oxidase during growth in chloramphenicol, although it was conceivable that growth in other conditions not tested, might require the element for induction of the enzyme. Since there have been no CRE binding proteins purified from *N. crassa*, I decided to attempt to purify the CRE binding protein(s). Characterization of the protein and determination of its role(s) in the growth and genetic regulation of the organism would be of interest in any case. If purification was successful the protein could be sequenced and, through a reverse genetics approach, the gene could be mutated allowing the study of mutant phenotypes. Given the circumstantial evidence for involvement of the CRE in alternative oxidase regulation, a mutant might reveal a role in the expression of the gene. Development of an antibody to the protein would also allow localization studies and the determination of protein binding partners in co-immunoprecipitation experiments.

As an initial step in the purification, I determined that the putative CRE binding protein(s) was precipitated with 25% ammonium sulfate using EMSA as an assay for presence of the binding activity (Fig. 19). This result also suggested that the binding activity was quite stable, able to withstand multiple rounds of ammonium sulfate treatments and dialysis while maintaining binding capacity. For these experiments I used only extracts from uninduced cultures since it was already shown that protein(s) from both induced and uninduced cultures bind to the CRE region of Fragment II (Figs. 16 and 17).

Figure 19. EMSA with Fragment II (Fig. 15) and proteins precipitated out of crude protein extract by ammonium sulfate. Each lane contains 8000 cpm of ^{32}P labeled target DNA. Lanes with protein contain 30 μg of protein from extracts isolated from cultures grown under conditions that do not induce alternative oxidase activity. The extract was mixed with increasing amounts of ammonium sulfate. The resolubilized pellets and supernatants were used for binding following dialysis to remove ammonium sulfate. All lanes with protein also contain 50 000 fold excess of poly[d(I-C)].



3.9 DNA affinity column

The CRE containing Fragment II was 272 base pairs in length, and while it worked well to test basic binding of protein to the CRE, a smaller piece of DNA for use in attempts to isolate the binding protein using an affinity column was preferred. Bollag et al. (BOLLAG et al. 1996) suggested that fragments of 14 to 50 nucleotides are optimal for DNA affinity columns. I designed four PCR primers to generate fragments smaller than the 272 base pair Fragment II (Fig. 15). Target DNA molecules of 58 base pairs and 89 base pairs (section 2.35.2) were tested for binding capability, and both were found to bind protein (Fig. 20). I chose the smaller 58 base pair fragment for constructing an affinity column for the putative CRE binding protein.

The column was created by attaching the 58 base pair PCR fragment containing the CRE to cyanogen-bromide sepharose resin (BOLLAG et al. 1996) (section 2.42). The 25% ammonium sulfate fraction containing the putative CRE binding protein was passed over the column. Although I attempted this several times, no protein could ever be shown to have bound the resin, as all of the binding activity came out in the flow through of the column (Fig. 21). I was unable to determine the reason for this lack of protein retention, although several possibilities were examined. It was possible that the CRE binding activity was destabilized by high KCl concentrations and/or dialysis to remove the KCl. However, different salt concentrations were tested and ruled out as a cause for lack of binding during the subsequent attempt at purification using streptavidin-biotin (section 3.15). It was also possible that the DNA fragment did not bind the CN-Br resin, but when the DNA fragment was ^{32}P labeled and bound to the resin, Cerenkov counting before and after CN-Br resin binding and washing demonstrated that roughly 67% of the applied counts were retained on the column. It was also possible that the CRE was inaccessible to the protein(s) once the fragment was attached to the resin.

Figure 20. EMSA of PCR products containing the CRE upstream of *aod-1*. Design of 58 base pair and 89 base pair target DNA molecules is shown in Fig. 15. Each lane with protein contains 30 μg of crude protein extract or 30 μg from the precipitated proteins or the proteins in the supernatant after treatment with 25% ammonium sulfate. Each lane contains 8000 cpm of ^{32}P labeled target DNA in 1X EMSA buffer (see appendix). All lanes with protein also contain 50 000 fold excess of poly[d(I-C)].

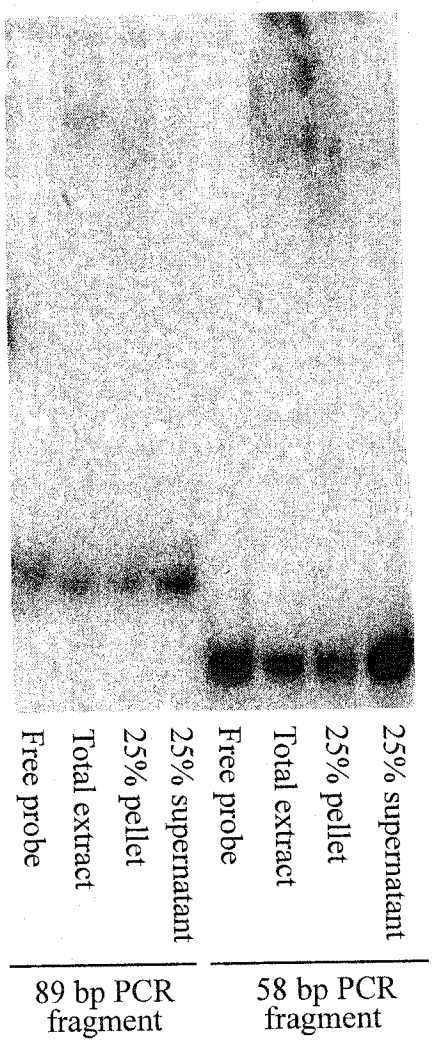
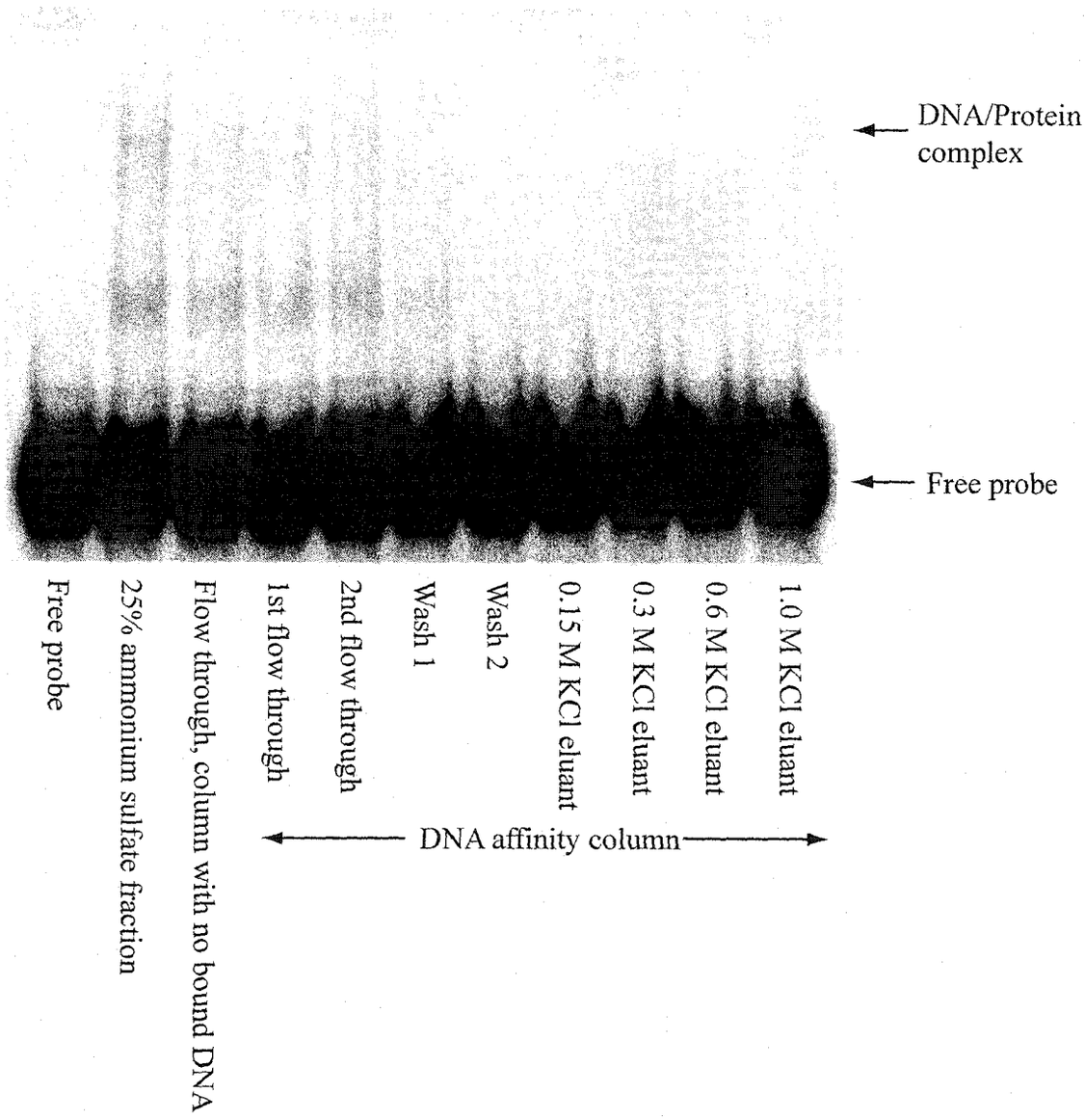


Figure 21. EMSA of DNA affinity column fractions. Each lane contains 8000 cpm of ^{32}P labeled target DNA (58 base pair PCR product). Each lane contains 30 μg of protein or the amount contained in 16 μl of protein solution, if the centrifugal concentration did not concentrate the proteins enough to allow determination of protein concentration. All lanes with protein also contain 50 000 fold excess of poly[d(I-C)].



3.10 Streptavidin-assisted purification of proteins bound to biotinylated target DNA

Due to the failure of the DNA affinity column, described above, I attempted to isolate the putative CRE binding protein using magnetic streptavidin beads and CRE containing DNA that had been biotinylated. The PCR primers AO42 and AO43 (Table 2) used to generate the CRE containing target DNA products were designed with *Eco*RI and *Taq*I restriction sites at each end so that the ends of the target DNA fragment remaining after digestion with *Eco*RI was 64 base pairs in length and could be labeled with biotin and/or ^{32}P. When a CRE containing fragment, labeled with both ^{32}P and biotin, was incubated with the 25% ammonium sulfate fraction normal binding was seen, showing that the biotin did not inhibit protein binding to the DNA fragment (Fig. 22). However, similar to the situation using the DNA affinity column in section 3.9, the CRE binding protein always did not remain bound to the biotin-labeled DNA molecules. The proteins were thus not associated with the streptavidin/ magnetic bead pellet, but rather remained in the supernatant portion of the reaction. I eliminated obvious problems that might have explained the lack of success, such as non-specific binding of radioactivity to streptavidin, and the possibility of increased salt concentrations followed by dialysis destabilizing the protein or hindering its binding ability.

3.11 Ion exchange column purification

A final effort to purify the CRE-binding protein by more traditional means was made. The 25% ammonium sulfate fraction, suspended in a 5 mM KCl buffer (see appendix), was passed over the anion exchanger Q sepharose and the cation exchanger SP sepharose. After the proteins were bound to either column, columns were washed with 5 mM KCl buffer followed by step wise elution with 0.25 M KCl, 0.5 M KCl and 1M KCl buffer (see appendix). The eluates were dialyzed against 5 mM KCl buffer and tested for binding ability with the 58 base pair target DNA fragment. The putative CRE binding protein did not bind to the SP sepharose under the conditions used but did bind to the Q sepharose and was efficiently eluted with 0.5 M KCl (Fig. 23). A biotin-streptavidin purification was attempted using the

Figure 22. EMSA of biotinylated and radioactively labeled DNA. Target DNA is the 76 base pair PCR product digested with *Eco*RI to generate a 64 base pair target DNA molecule. Each lane except "free probe" contains 8000 cpm of ^{32}P labeled target DNA. Each lane contains 30 μg of protein or the amount contained in 16 μl of protein solution, if the centrifugal concentration did not concentrate the proteins enough to allow determination of protein concentration. All lanes with protein also contain 50 000 fold excess of poly[d(I-C)].

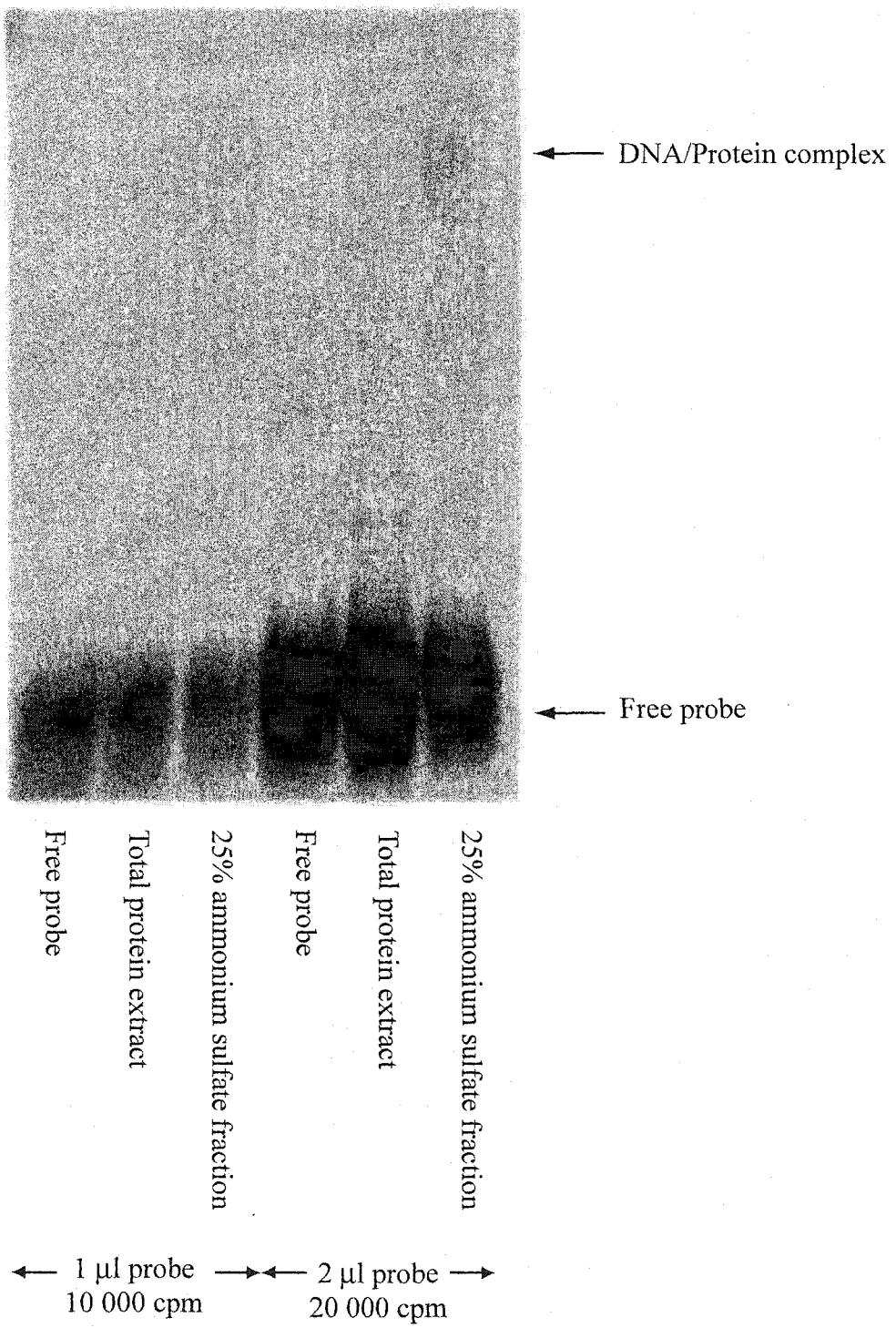


Figure 23. EMSA of proteins following ion exchange chromatography. Each lane contains 8000 cpm of ^{32}P labeled target DNA (58 base pair PCR product). Each lane except "free probe" contains 30 μg of protein or the amount contained in 16 μl of protein solution, if the centrifugal concentration did not concentrate the proteins enough to allow determination of protein concentration. All lanes with protein also contain 50 000 fold excess of poly[d(I-C)].

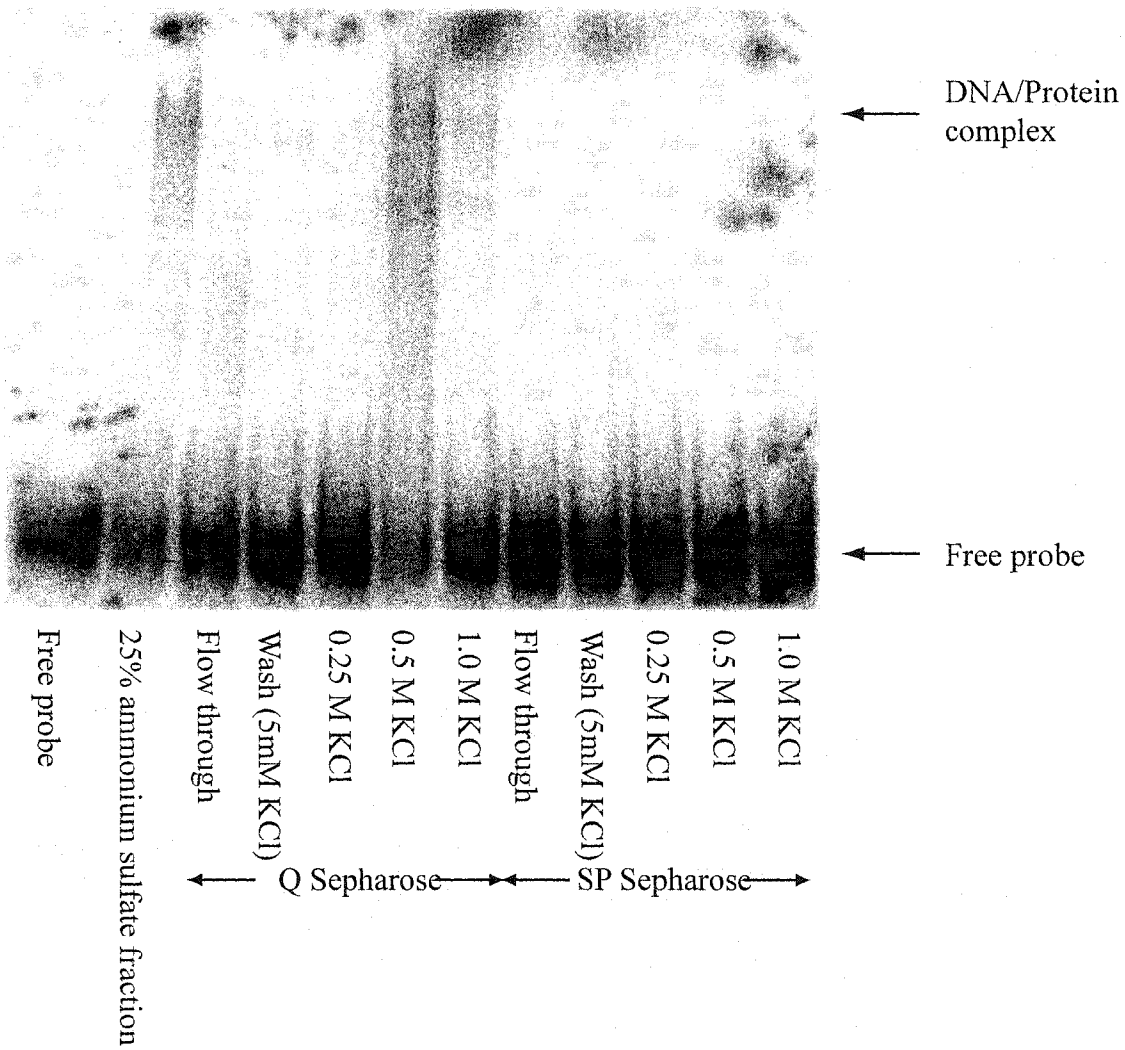
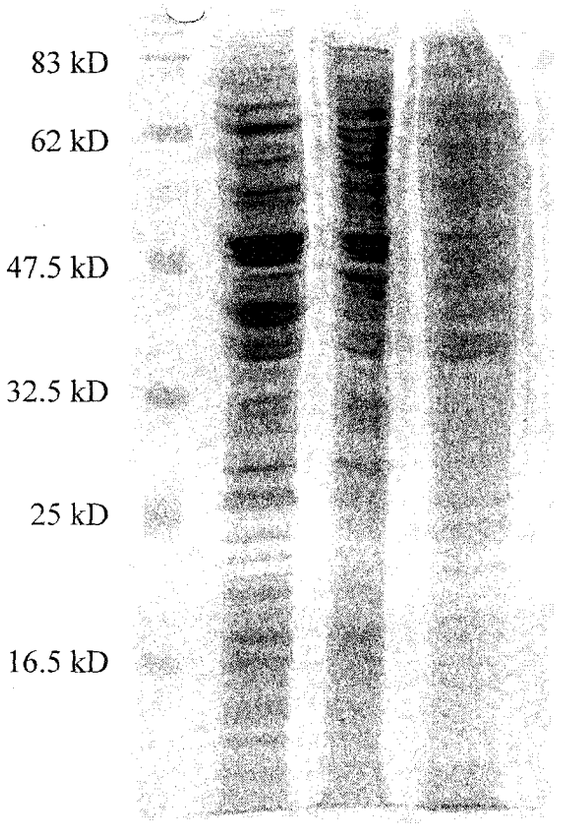


Figure 24. Coomassie blue stained gel of protein purification steps. Each lane contains 30 μg of protein run on a 12% SDS-PAGE gel. The position of molecular weight markers is shown on the left.



Q-sepharose eluate to bind the biotinylated DNA. However, the target DNA was unable to pull down the binding activity using the magnetic streptavidin beads.

SDS-PAGE was used to evaluate the level of protein purification following elution from Q sepharose. The gel was stained with Coomassie blue, and the proteins present in total cellular extract, 25% ammonium sulfate fractionation and 0.5 M KCL elution from Q sepharose were compared. Although some purification is visible at each stage, there are still many proteins in the Q sepharose fraction (Fig. 24). I was unable to continue the purification due to time constraints.

3.12 Conditions affecting accumulation of *N. crassa aod-1* transcript

Early studies suggested that there was little to no KCN-resistant respiration in untreated wild type *N. crassa* (LAMBOWITZ and SLAYMAN 1971) and it was shown, using actinomycin D, that transcription was required for the appearance of KCN-insensitive respiration following addition of chloramphenicol to *N. crassa* cultures (EDWARDS and UNGER 1978). I performed a blot of RNA isolated from wild type, *aod-1* mutant strains, and the *aod-2-4* mutant strain grown under non-inducing or inducing conditions and the results supported these earlier findings (Fig. 25). In these initial studies, the uninduced cultures of all the strains appeared to contain very low levels of *aod-1* RNA detectable only upon long exposures of the blots. Wild type strains showed large increases in alternative oxidase mRNA when grown in chloramphenicol. Different mutant strains showed variation with regard to the amount of *aod-1* message present in cells grown under inducing conditions. For example, *aod-1-4, aod-1-6, aod-1-7* and *aod-2-4*, have little *aod-1* mRNA following growth in inducing conditions. *aod-1-4* and *aod-1-7* contain frameshift mutations leading to premature stop codons, and their mRNAs are likely removed by nonsense-mediated decay (MAQUAT 2000). *aod-2-4* is likely deficient in regulation of *aod-1*, preventing expression under inducing conditions (LAMBOWITZ et al. 1989; LI et al. 1996). *aod-1-1* and *aod-1-2*, which do accumulate mRNA upon induction, have identical missense mutations (LI et al. 1996) that do not appear to affect message stability, while *aod-1-6* has a different missense mutation (LI et al. 1996) that appears to affect stability since the mRNA does not accumulate.

Figure 25. Presence of *aod-1* mRNA in wild type and mutant strains of *N. crassa* grown in the presence and absence of chloramphenicol. The top panel shows the ethidium bromide gel of total RNA (5 μg per lane) isolated from each strain grown under either non-inducing (-Cm) or in the presence of Cm (2 mg/ml) (+Cm) to induce the alternative oxidase. Molecular weight markers are indicated on the right. The RNA was transferred to a nylon membrane and hybridized with ^{32}P labeled *aod-1* and 255 base pairs of promoter removed from pAOGB-11. The lower panel shows the result of the hybridization. The position of the 1.3 kb *aod-1* mRNA is indicated.

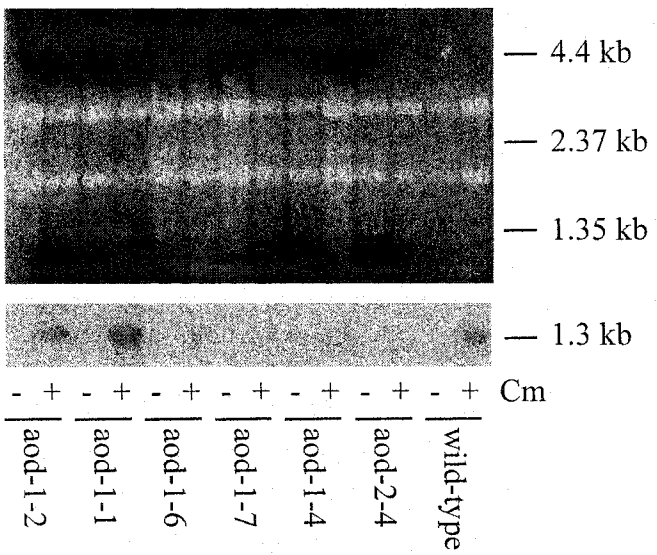
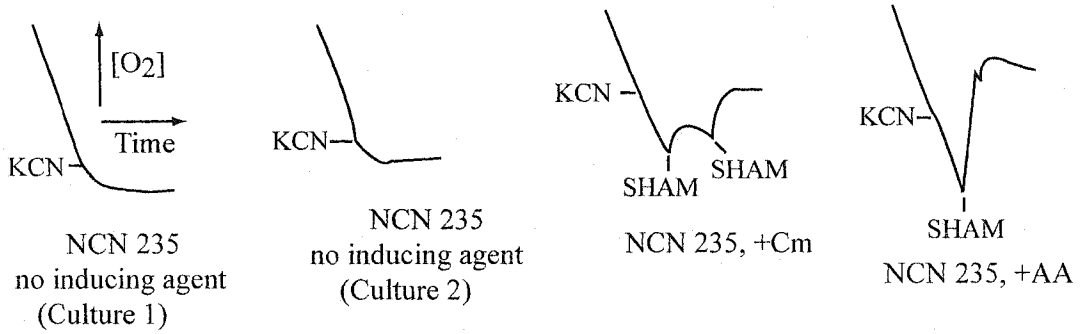


Figure 26. *aod-1* mRNA accumulates in some uninduced cultures. Cultures were grown in either standard Vogel's medium, Vogel's medium supplemented with Cm (2 mg/ml) or AA (0.5 μg/ml). A. Respirometer tracings, the vertical axis represents oxygen concentration in solution and the horizontal axis represents time. The point at which KCN or SHAM were added to the mycelium is indicated. B. Ethidium bromide stained gel of total RNA and exposure of northern blot hybridized with ^{32}P labeled p8A2a, which contains *aod-1* cDNA.

A



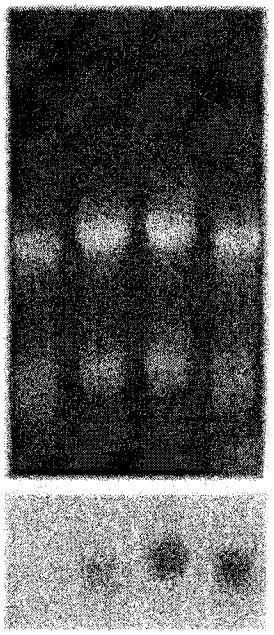
NCN 235
no inducing agent
(Culture 1)

NCN 235
no inducing agent
(Culture 2)

NCN 235, +Cm

NCN 235, +AA

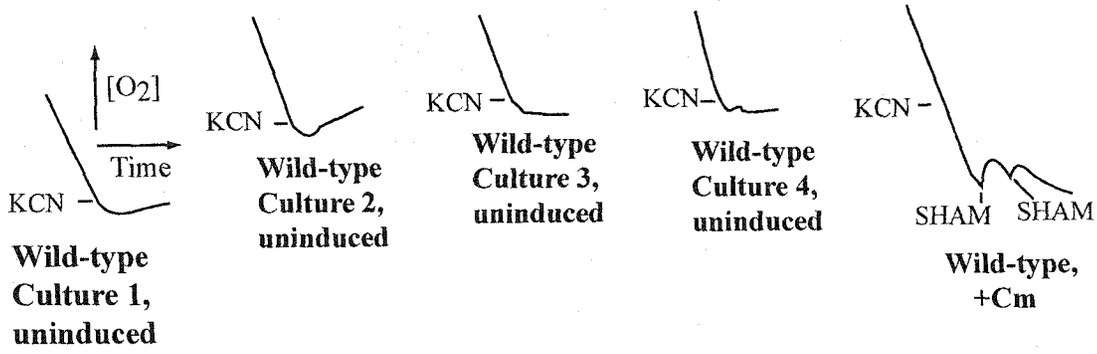
B



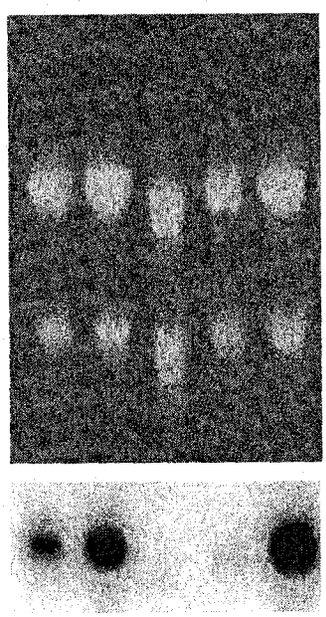
1	2	n/a	n/a	Culture number
-	-	+	-	Cm
-	-	-	+	AA

Figure 27. Uninduced cultures containing *aod-1* mRNA do not contain the alternative oxidase protein. Uninduced cultures were grown in standard Vogel's medium (-Cm). Induced cultures were grown in Vogel's medium supplemented with Cm (2 mg/ml) (+Cm). A. Respirometer tracings, the vertical axis represents oxygen concentration in solution and the horizontal axis represents time. The point at which KCN or SHAM were added to the mycelium is indicated. B. The top portion of the panel shows the ethidium bromide stained gel of total RNA (5 μg/lane). The positions of the molecular weight markers are indicated on the right. The lower portion of the panel shows the northern blot following hybridization with ^{32}P labeled plasmid p8A2a, which contains *aod-1* cDNA. The position of the 1.3 kb *aod-1* mRNA is indicated. C. Mitochondria were isolated from the same cultures as the RNA, and 30 μg of mitochondrial protein from each culture were subjected to SDS-PAGE and electroblotted onto nitrocellulose membrane. The blots were decorated with antibodies to *N. crassa* alternative oxidase protein (AOD) or Tom70, a mitochondrial outer membrane protein (GRAD et al. 1999), used as a loading control.

A

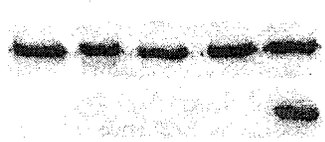


B



WT1	WT2	WT3	WT4	WT+	Culture number
-	-	-	-	+	Cm

C



WT1	WT2	WT3	WT4	WT+	Culture number
-	-	-	-	+	Cm

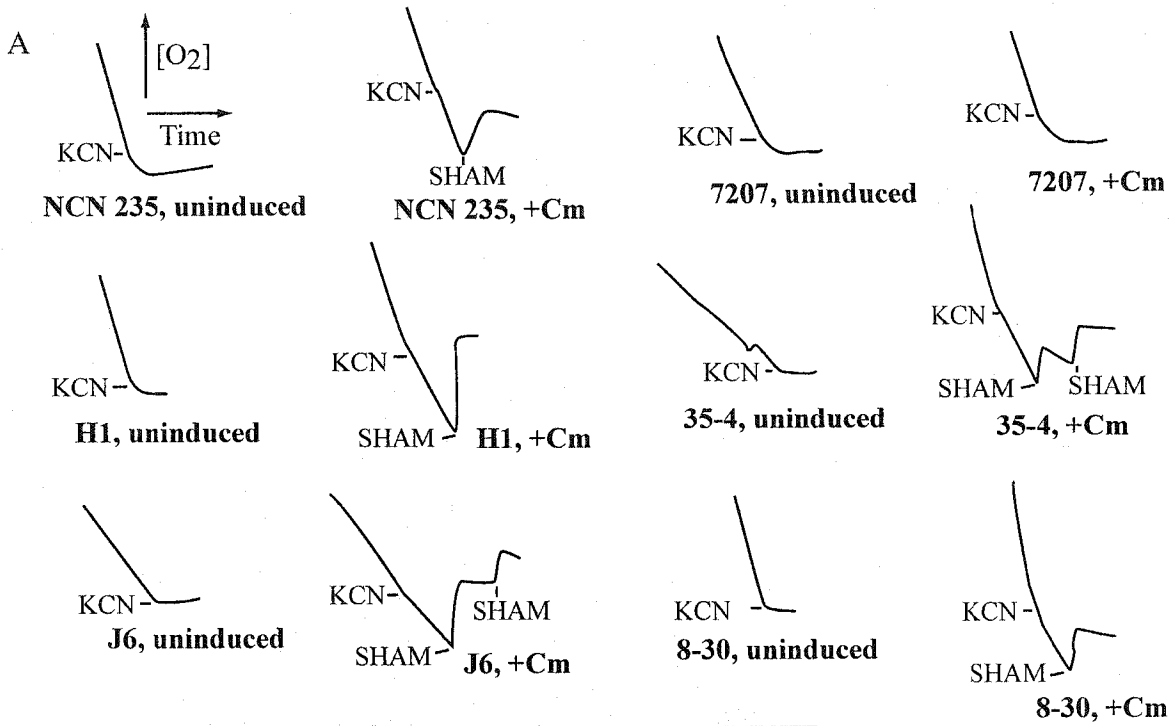
In later experiments with wild type cultures, respiration results confirmed that the cultures grown in the absence of an inducing agent contained no discernable KCN-insensitive respiration, while those grown in the presence of either chloramphenicol or antimycin A showed clear induction of KCN-insensitive respiration (Figs. 26A and 27A). However, northern analysis of these cultures revealed that, occasionally, considerable amounts of *aod-1* transcript were present in cultures grown in standard Vogel's media without inducers, despite the lack of observable KCN-insensitive respiration (Figs. 26B and 27B). Since the phenomenon appeared in occasional cultures with no apparent difference from other cultures (compare cultures 1 and 2 in Fig. 26B and cultures 1 through 4 in 27B), I more carefully investigated different growth conditions, which may not have been carefully controlled in previous experiments. Although growth at 25°C seemed to increase the frequency of finding *aod-1* mRNA in uninduced cultures compared to growth at 30°C, the results were inconsistent. Furthermore, others in the laboratory have since grown uninduced cultures at 20°C and saw no *aod-1* mRNA. Similarly growth of uninduced cultures in constant darkness seemed to increase the frequency of finding *aod-1* mRNA in uninduced cultures, but these results were also inconsistent.

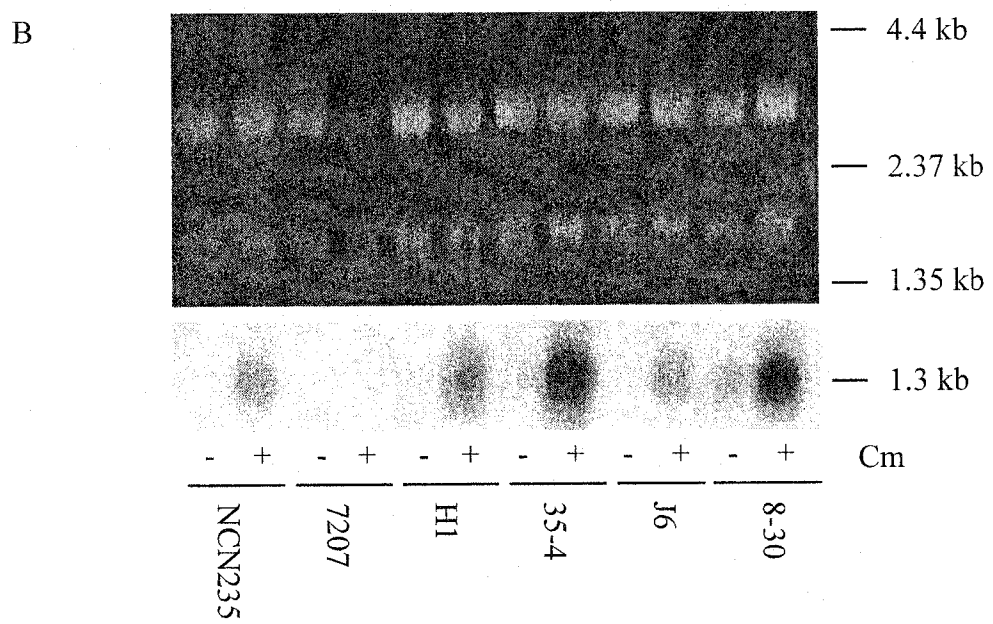
Due to these unexpected results, I isolated mitochondria from uninduced cultures containing *aod-1* mRNA but not KCN-insensitive respiration, to determine whether AOD1 protein was present but not functional. Mitochondrial proteins were subjected to SDS-PAGE and blotted onto nitrocellulose membranes. Decoration of the blots with antibody against *N. crassa* alternative oxidase revealed no alternative oxidase protein from any of the mitochondria of cultures grown in uninducing cultures conditions (Fig. 27C). Therefore, despite occasional variability in the expression of *aod-1* mRNA, AOD-1 protein is not expressed under non-inducing conditions.

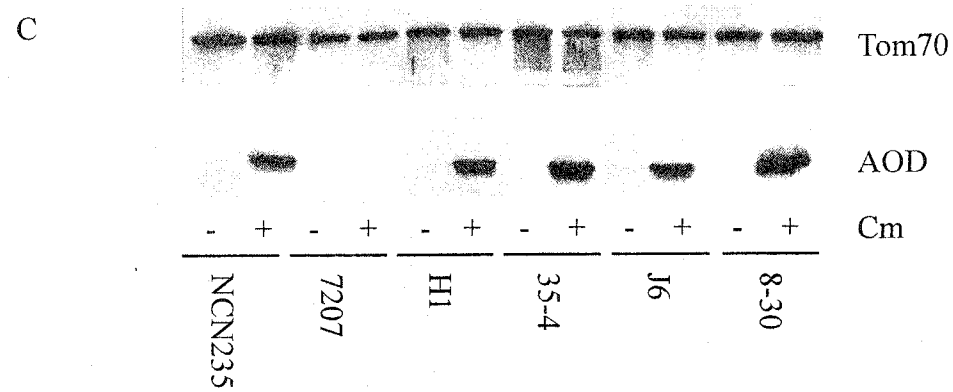
3.13 Effect of amount of upstream sequence on *aod-1* expression

As discussed in section 3.7, plasmids containing either 6 kb or 3.3 kb of upstream sequence were capable of rescuing an *aod-1-7* frameshift mutant, strain 7207. It was also shown that the deletion of the CRE sequence had no effect on the

Figure 28. The CRE is not necessary for control of *aod-1* expression. The *aod-1-7* frameshift mutant strain (7207) was transformed with either plasmid pMAX or pΔMAX. Transformants were purified and grown in the absence of Cm (uninduced) or presence of Cm (2 mg/ml) (+Cm). Panels A, B and C are as described for Fig. 27. H1 and 35-4 are pMAX transformants. J6 and 8-30 are pΔMAX transformants.







induction of KCN-insensitive respiration (Table 6). Two pMAX transformants (H1 and 35-4) and two pΔMAX transformants (J6 and 8-30) were further analyzed for *aod-1* mRNA and protein levels. As expected, all induced cultures contained KCN-insensitive respiration while uninduced cultures did not (Fig. 28A). Similarly, all induced cultures contained *aod-1* mRNA while uninduced cultures did not (Fig. 28B). The exception was the uninduced strain 8-30 culture, which did contain a small amount of mRNA. Despite considerable variation in the amount of mRNA present, all induced cultures contained a similar level of AOD1 protein while none of the uninduced cultures contained any detectable alternative oxidase protein (Fig 28C). The negative control samples of untransformed strain 7207 contained no detectable mRNA or protein. In an attempt to determine if smaller fragments of upstream sequence could function as promoters, plasmids pMMAX and pMCMAX were created. These plasmids contained 255 base pairs and 10 base pairs of sequence upstream from the +1 transcription start site, respectively (Fig. 18). These plasmids were transformed into *aod-1-7* mutant strain 7207, selected on bleomycin containing media and were purified as described in section 2.9. The purified transformants were tested for KCN-insensitive respiration after growth in inducing and uninducing conditions. As described in section 3.7, some transformants may have integrated only the bleomycin resistance gene and will not express alternative oxidase activity under any circumstances. For pMMAX, 18 of 22 transformants tested showed fully inducible alternative oxidase expression and 1 showed partial induction (Table 7). For pMCMAX, 36 transformants were tested and six of these initially showed a high level of KCN-insensitive respiration when grown in media containing chloramphenicol and two showed lower levels. However, retesting of these positive transformants revealed only partial induction of KCN-insensitive respiration in any of the isolates (Table 7).

Two pMCMAX transformed strains (L11 and L18) and three pMMAX transformed strains (K5, K6 and 39-1) were analyzed with respect to *aod-1* mRNA and protein content. As seen previously, in the absence of inducing agents, respiration of the cultures ceased when KCN was introduced. However, in the presence of chloramphenicol, KCN-resistant alternative oxidase was seen in all cultures (Fig. 29A). However, cultures of strains L11 and L18, derived from transformation with

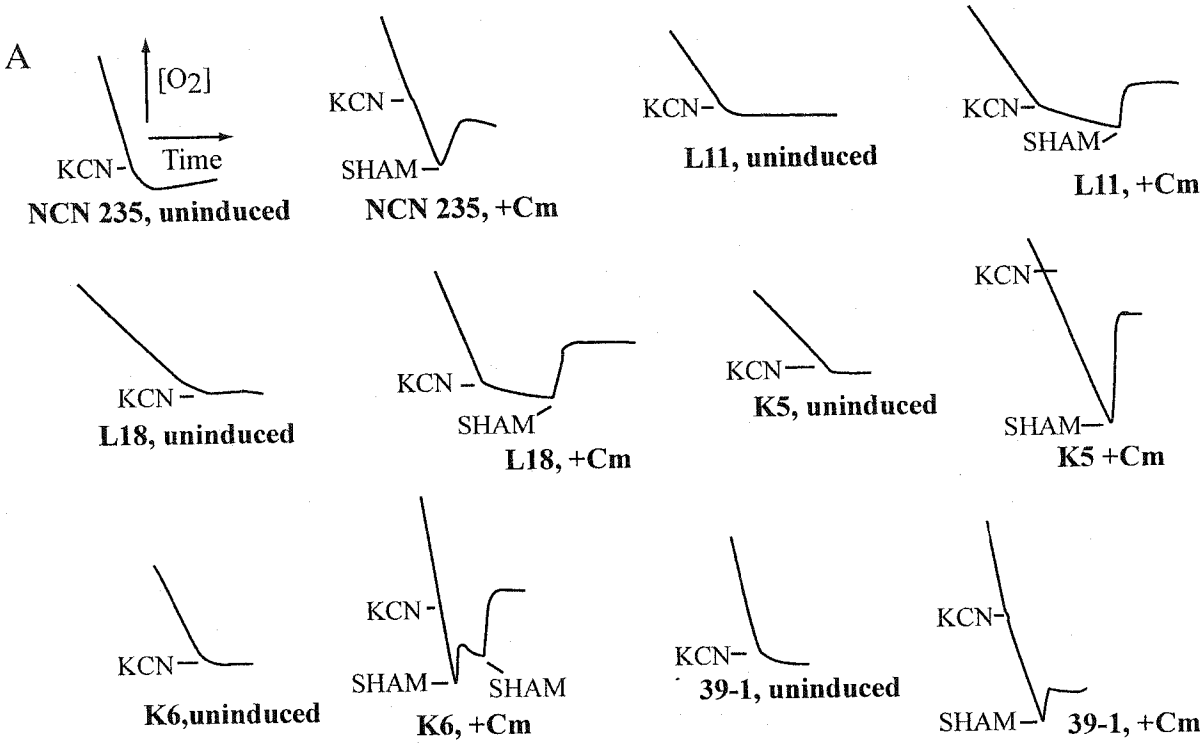
Table 7

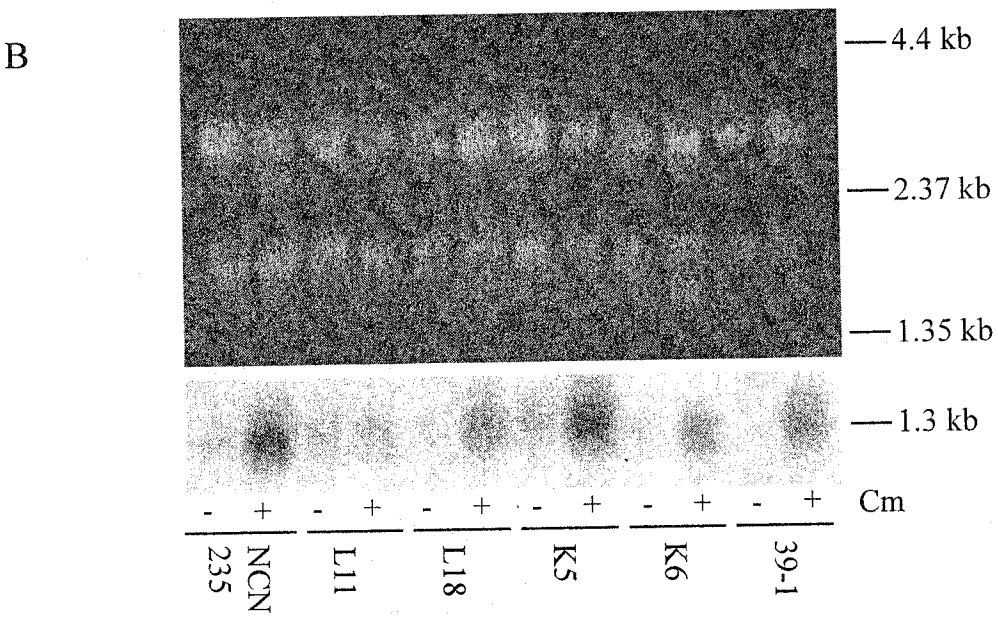
Plasmids containing regions of sequence upstream of *aod-1* can restore full or partial alternative oxidase activity to the *aod-1* frameshift mutant 7207.

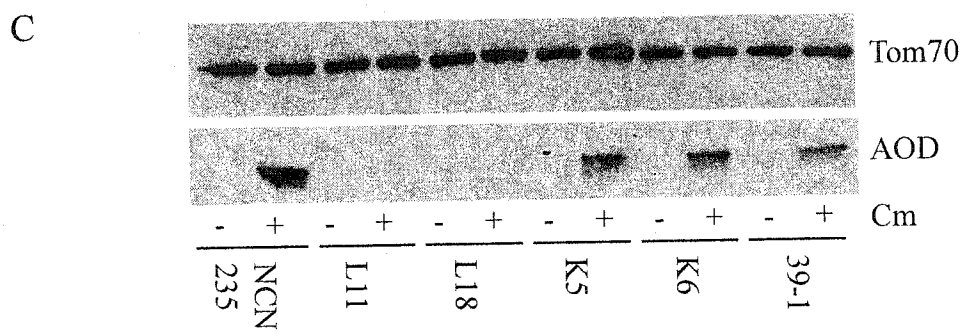
Transforming plasmid	Upstream sequence	Total transformants	Rescued transformants	
			Partial induction	Full induction
PMMAX	250 bp	22	1	18
PMCMAX	10 bp	36	2	6*

*Isolates initially showing full induction showed partial induction during subsequent analyses

Figure 29. A 255 base pair upstream sequence is sufficient to control *aod-1* expression. Panels as described in Figure 27. K5, K6 and 39-1 are pMMAX transformants. L11 and L18 are pMCMAX transformants. Transformants were purified and grown in the absence of Cm (uninduced) or presence of Cm (2 mg/ml) (+Cm).







pMCMAX, contain only a small amount of KCN-insensitive respiration when grown in chloramphenicol (Fig. 29A). Homologous integration of pMCMAX into the *aod-1* locus of 7207 was ruled out as an explanation for the presence of the low level of inducible KCN-insensitive respiration in these isolates. Primers downstream of the *aod-1-7* mutation and upstream of the *Nde*I site, which were used to create pMCMAX were used to amplify the region. If homologous integration did not occur, the PCR product should still have contained the *aod-1-7* mutation, as pMCMAX, with the *aod-1*$^{+}$, sequence would have integrated elsewhere in the genome. This was seen in L11 and L4, two pMCMAX isolates with inducible KCN-insensitive respiration, as well as in a transformant lacking KCN-insensitive respiration. Thus, the low level of inducible

alternative oxidase activity seen in pMCMAX transformants was not due to homologous integration into 7207.

Analysis of the RNA isolated from these cultures demonstrates that while there is a small amount of RNA in a few of the uninduced cultures, specifically L11 and K5, there is an increase of *aod-1* RNA in all cultures when grown in the presence of chloramphenicol, except L11 (Fig. 29B). It appears that the 255 base pairs of upstream sequence in the pMMAX transformants is capable of regulating the *aod-1* gene in a normal fashion, upon induction by chloramphenicol. The alternative oxidase protein was found in all induced cultures except for L11 and L18 (Fig. 29C). Thus, even though there appears to be a slight induction of KCN-insensitive respiration in both L11 and L18, and an apparent induction of *aod-1* RNA in L18 when grown in the presence of chloramphenicol, this does not correlate to the presence of measurable alternative oxidase protein.

3.14 Intron sequences in other species

Regulatory elements have been found in introns of genes from species as diverse as *C. elegans* (ZHANG and EMMONS 1999), and mice (COULON et al. 1999). There are two introns present within the *N. crassa aod-1* gene, and I wished to look at introns in the corresponding gene of related fungal species for regions of conserved sequence that might have a regulatory role, even though the introns in *aod-1* were less

than 100 nucleotides long. To compare *aod-1* intron sequences in various species, I PCR amplified the *aod-1* introns of two close relatives of *N. crassa*: *N. intermedia* and *N. tetrasperma*, using *N. crassa aod-1* primers AO32 and AO33 (Table 2). In addition, the sequenced introns from *Gelasinospora* and *A. nidulans* (section 3.4) were included in the analysis (Figs 11 and 13). *N. intermedia* and *N. crassa* are extremely closely related, with *N. tetrasperma* being a slightly more distant relative (DAVIS 2000). *Gelasinospora* is evolutionarily more distant from *N. crassa*, though they are part of the same taxonomic family. A comparison of the position of the introns in each species is shown in Fig. 12. For all of the *Neurospora* species sequences, both exon and intron sequences analyzed were nearly identical (Fig. 30A). While it is possible to interpret this as conservation of intron sequences and infer their functional importance from this analysis, it seems more likely that the species are too closely related to have undergone significant sequence divergence, even in nonessential regions. *Gelasinospora* is slightly more distantly related to *N. crassa*, but both the nucleotide and amino acid sequences are still highly conserved (Figs. 12 and 14). The intron sequences of the *Gelasinospora* alternative oxidase gene were also very similar to the introns of *N. crassa aod-1* (Fig. 30A).

Comparison of the alternative oxidase coding genes from *N. crassa* and *A. nidulans* revealed the presence of two introns in *A. nidulans* that differ in both position and sequence between the species (Figs. 12 and 30B). The second *A. nidulans* intron shows no sequence similarity with either of the *N. crassa* introns (Fig. 30B). The first *A. nidulans* intron showed slight sequence similarity with the second intron of *N. crassa*, but it is largely in the sequences, required for intron splicing (EDELMAN and STABEN 1994) (Fig. 30B). This comparison suggests that there are no conserved regulatory functions within the introns of these alternative oxidase genes, at least in these fungal species. This conclusion was recently confirmed by others in the lab who made constructs of 3.3 kb of upstream sequence with *aod-1* cDNAs. When these were transformed into strain 7207, no change in the regulation of alternative oxidase compared to 7207 transformed with pMAX (3.3 kb of upstream sequence and genomic *aod-1*, Fig. 18) was observed in purified transformants (C. Nargang and F. Nargang, personal communication).

Figure 30. Comparison of introns in the alternative oxidase genes of three *Neurospora* species: *N. crassa* (*N.c.*), *N. intermedia* (*N.i.*) *and N. tetrasperma* (*N.t.*); *Gelasinospora species* (*G.s.*); *and A. nidulans* (*A.n.*). *N. crassa* introns had been identified and sequenced prior to this study (Li et al., 1996). *N. intermedia* and *N. tetrasperma* were PCR amplified using primers designed for *N. crassa*, AO32 and AO33. Introns of *Gelasinospora* and *A. nidulans* were identified in this study (Figs. 11 and 14, respectively). Sequences required for intron splicing are underlined. A. Introns of *Neurospora* species and *G. spp*. Dark grey highlight shows nucleotides conserved among all of the species, light grey highlighting shows nucleotides that are conserved among only two or three sequences. B. Introns of *N. crassa* and *A. nidulans*, with conserved nucleotides highlighted in grey.

A

Intron 1

```
N.c.  GTAGGTAAATTCC--AAGGAGGCTTCCCATATTGCCCCTCAAGGTATCTTGC
N.i.  GTAGGTAAATCCC--AAGGAGTGTCCACATATTGCCCCTCAAG-TATGTTGC
N.t.  GTAGGTAAATCCC--AAGGAGTGTTCCCATATTGCTCCTCAAG-TATCTTGC
G.s.  GTAGGTAATTCCCCAAAGGAATTTTCCCATATTGTCCCTCAAG-TATCTTGC

N.c.  GGACGCGGAAGCAGACGTAGATTCAAAGTGTTCTAATATCAAACAG
N.i.  GGATCGGGAACGAGACGTAGAATCAAAGTGTTCTAATATCAAACAG
N.t.  GGACGCGGAAACATACGTAGAATCAAAGTGTTCTAATATTAAACAG
G.s.  AGACGCGGAAGCACACGCAGAATCAAAGTGATCTAACATCAAGCAG
```

Intron 2

```
N.c.  GTATGTTCTATGGAGCCCTACGAGGACGGAATGGCAACACAAGCTAACCAA
N.i.  GTAGGTTATATAGAGCCCTGCGAGGACGGAATGGCAACACAAGCTAACCAA
N.t.  GTATGTTTCATGGAGCCCTACGATGACGGAATGGCAACACATGCTAACCAA
G.s.  GTACGTCCTATGAACGCCTACGAGTACGGAATGGCAGCATATGCTAACCAA

N.c.  ATGTCGCGAATCCATATAG
N.i.  ATGTCGCGAATCCATATAG
N.t.  ATGTCGCGAATCCATATAG
G.s.  ATATCAC-AATCCATATAG
```

B

Intron 2 of *N. crassa* and intron 1 of *A. nidulans*

```
N.c.  GTATGTTCTATGGAGCCCTACGAGGACGGAATGGCAACACAAGCTAACCAA
A.n.  GTAAGTT--ATCCAATCTTCAA------GAGAGGCGACAGATGCTGATCAA

N.c.    ATGTCGCGAATCCATATAG
A.n.    -------GAA------CAG
```

Intron 2 of *A. nidulans*

GTATGTCGAGATTTCTTTCAGCTTATACATTTCGTGGCTCAACTAATAATCAATGC
GCAG

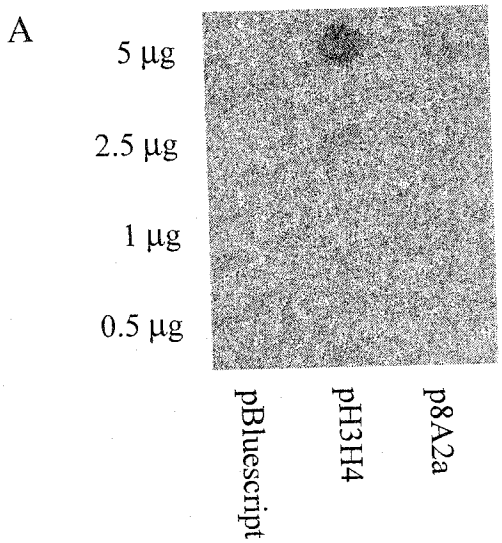
3.15 Direct measurement of *aod-1* transcription

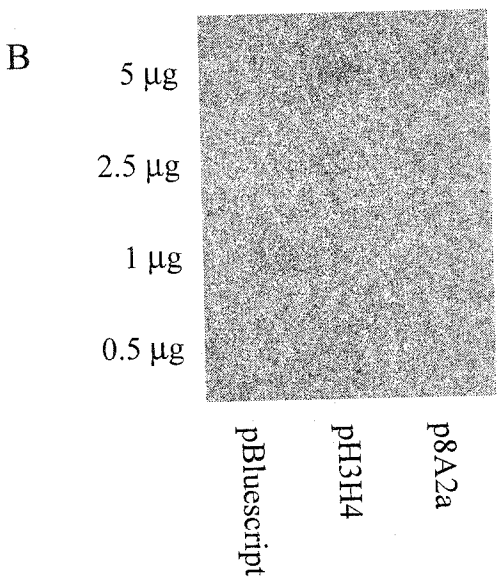
A comparison of the *aod-1* mRNA levels in uninduced and induced cultures quite clearly demonstrates that there are differences in accumulation of *aod-1* mRNA in different uninduced cultures (Figs. 26B and 27B), suggesting that post-transcriptional mechanism(s) may influence *aod-1* mRNA. Therefore, it was conceivable that post-transcriptional events entirely controlled *aod-1* expression, and I wished to determine if transcriptional activation played any role. Nuclear run-on assays (FARRELL 1998) were used to examine differences in transcription rates of *aod-1* in induced and uninduced cultures.

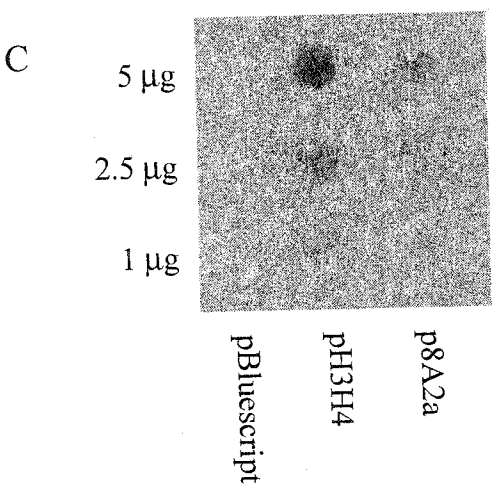
Nuclei were isolated from cultures grown in the presence and absence of chloramphenicol. Nuclei were incubated in the presence of ATP, CTP, GTP and ^{32}P-UTP to label transcripts that were arrested in the process of being transcribed when the nuclei were isolated. Labeled transcripts were isolated and hybridized to membranes bound with target DNA containing *aod-1*. The positive control was *N. crassa* histone genes, which are transcribed constitutively (WOUDT et al. 1983); they are not affected by the presence of chloramphenicol in the media, demonstrated by RNA blots (not shown). Empty pBluescript vector was used as a negative control.

Hybridization to pH3H4 with the labeled transcripts confirmed that the procedure was successful in isolating intact nuclei with functioning transcriptional machinery (Fig. 31, all panels). I consistently observed that the nuclei isolated from cultures grown in non-inducing conditions were actively transcribing *aod-1* RNA at the point of nuclei isolation as can be seen in a comparison of pBluescript and p8A2a lanes (Fig. 31A). Under non-inducing conditions, there is consistently more transcript of the histone genes, compared to transcript seen for *aod-1*. Unfortunately, the transcripts seen in nuclei isolated from induced cultures gave variable results. In some cases, the levels of histone and *aod-1* mRNA were approximately equal, suggesting an induction of *aod-1* transcription (Fig 31B). However, in other cases (Fig 31C), there was no obvious change in the ratio of histone and *aod-1* mRNA between uninduced and induced cultures. Thus, these results are not able to determine if *aod-1* mRNA production is constitutive or inducible.

Figure 31. Nuclear run-on assay. Nuclei were isolated from wild type cultures grown in either Vogel's medium (non-inducing conditions, Panel A) or in standard Vogel's medium supplemented with Cm (2 mg/ml) (inducing conditions, Panels B and C). Nascent transcripts present upon harvesting nuclei were labeled by the addition of ATP, CTP, GTP and [^{32}P] α-UTP. The transcripts were purified, hybridized to membranes bound with target DNA: pBluescript as a negative control, p8A2a, which contains *aod-1* cDNA, and as a positive control, pH3H4 which contains *N. crassa* histone genes 3 and 4. 0.5 μg to 5 μg of target DNA was used for hybridization, as indicated. The histone genes are actively transcribed and should not be influenced by the presence of Cm. Panels B and C show the inconsistent amounts of *aod-1* transcription observed in nuclei isolated from cells grown in Cm.







4 Discussion

Alternative oxidase is a nuclear gene coding for a second terminal oxidase found in the mitochondria of higher plants as well as some fungi and protists. In fungi such as *N. crassa*, alternative oxidase activity is present only when the standard mitochondrial cytochrome chain is disrupted (BERTRAND et al. 1983; EDWARDS and UNGER 1978; LAMBOWITZ et al. 1972). Alternative oxidase donates four electrons directly from reduced ubiquinol to oxygen, forming water. This bypasses two of three proton pumping sites of the standard cytochrome chain, so that the energy normally conserved in ATP molecules is lost as heat (LAMBERS 1982; MOORE and SIEDOW 1991). The enzyme is not sensitive to the classic inhibitors of cytrochrome mediated respiration such as KCN and antimycin A (LAMBOWITZ and SLAYMAN 1971), but the enzyme is sensitive to specific inhibitors such as SHAM (SCHONBAUM et al. 1971), and n-propyl gallate (PARRISH and LEOPOLD 1978). Comparisons of conserved sequences of alternative oxidase proteins suggest that it is a Class II di-iron protein, similar to methane monooxygenase and ribonucleotide reductase subunit 2, which are characterized by two iron binding motifs (D/E-X-X-H) contained within four alpha helical bundles. The current model, supported by experimental evidence, is that alternative oxidase is embedded in the inner mitochondrial membrane but does not contain any membrane spanning domains, and both the N- and C-termini are exposed to the matrix (ANDERSSON and NORDLUND 1999; BERTHOLD et al. 2000) (Fig 2B).

The structural gene encoding alternative oxidase in *N. crassa, aod-1*, has been cloned and is localized to linkage group IV (LI et al. 1996). *aod*-1 genes in mutant strains deficient for alternative oxidase activity, obtained from the Bertrand laboratory, were sequenced. The results were contradictory as two mutants originally named *aod-2-6* and *aod-2-7* were found to have mutations in *aod-1*. Other strains, *aod-1-1, aod-1-2* and *aod-1-4* also showed mutations in *aod-1*, as expected (LI et al. 1996). The cosmid 23F7, containing the *aod-1*$^+$ gene and surrounding sequences (Table 3), was transformed into each of the mutant strains and the transformants were analyzed for alternative oxidase activity. The *aod-1*$^+$ cosmid was found to restore inducible KCN-insensitive respiration to transformants of strains *aod-1-1, aod-1-2*

and *aod-1-4* as well as the strains that had been designated *aod-2-6* and *aod-2-7* (Table 4), thus confirming the sequencing results. Therefore, the "*aod-2-6*" and "*aod-2-7*" strains were renamed *aod-1-6* and *aod-1-7*, respectively. There is a second gene involved in the expression of alternative oxidase in *N. crassa*, *aod-2*, which is localized to linkage group II. It has not been cloned but is believed to be involved in the expression of *aod-1* (BERTRAND et al. 1983; EDWARDS and UNGER 1978). The mechanism of regulation of *aod-1* remains to be elucidated.

Recently, another alternative oxidase gene was discovered during BLAST searches of the *Neurospora* genome (F. Nargang, personal communication). This gene has been named *aod-3*. The predicted protein sequence is roughly as similar to *aod-1* as it is to the alternative oxidase of *A. nidulans*. The yeast *C. albicans* has two alternative oxidase genes, one of which is expressed constitutively while the other is induced by typical inducing agents such as antimycin A (HUH and KANG 2001). Differential regulation of alternative oxidase has also been described in plants (FINNEGAN et al. 1997; SAISHO et al. 1997). However no evidence of an *aod-3* transcript in either uninduced cells or cells grown in the presence of chloramphenicol could be seen (C. Nargang and F. Nargang, personal communication). BLAST searches of the *N. crassa* morning evening and perithecial libraries did not find an EST for *aod-3,* while *aod-1* was found in four different evening library ESTs. The data showing lack of expression of *aod-3* are in agreement with the observation that *aod-1* mutant cells contain no detectable alternative oxidase activity in cells induced by chloramphenicol or antimycin A, though it is possible that unknown conditions may result in *aod-3* expression.

In an effort to further understand the control of *aod-1* expression, 3.6 kb of upstream DNA was sequenced and analyzed for regions that might affect regulation. Several potential regulatory sites upstream of *aod-1* have been identified, including elements potentially involved in recruiting RNA polymerase II to the promoter: a CAAT box, TATA box and transcription start site (LI et al. 1996) (Figs. 5 and 6 of this thesis). There are many elements that modulate the transcription of genes, elements that can increase, decrease, or maintain levels of transcription depending upon the regulatory signals received. RNA polymerase II interaction with the DNA is

either enhanced or impeded by elements in the region surrounding the gene in question. Some nuclear genes contain elements such as the TATA and CAAT boxes that encourage binding by RNA polymerase II. The TATA binding factor (TBF) and related proteins in fact orchestrate the assembly of active RNA polymerase II on DNA (BURLEY and ROEDER 1996). *Neurospora* genes contain many other upstream activation and/or repression elements that regulate gene expression via specific protein binding and not all *Neurospora* genes contain TATA or CAAT boxes (DAVIS 2000).

A consensus CRE was identified further upstream of *aod-1*, with 5 base pairs inverted repeats six nucleotides to either side of the CRE (LI et al. 1996) (Fig. 6 of this thesis). The CRE is a cis-acting regulator of gene transcription. The cyclic-AMP response element (CRE) is responsive to transcription factors activated in response to several signalling pathways, including the well-studied cyclic-AMP signaling pathway, which is a system used by many eukaryotic systems to regulate gene transcription in response to intracellular and/or extracellular signals. In the case of regulating transcription via hormonal signals, adenylyl cyclase, a membrane associated protein, is stimulated by the binding of specific ligands to receptors that are coupled to G-proteins. Upon activation, adenylyl cyclase catalyzes an increase in intracellular levels of cAMP, which in turn binds to protein kinase A (PKA), releasing an inhibitory subunit. The activated PKA affects gene regulation by phosphorylating several nuclear and cytoplasmic transcription factors. There is a family of proteins that are phosphorylated by PKA and that bind to CREs or related sequences. The first of these to be identified was called CRE-binding protein (CREB). CREB is a member of a family of basic region/leucine zipper (b-zip) transcription factors (LALLI and SASSONE-CORSI 1994). These proteins can also be phosphorylated by a kinase other than cAMP-responsive PKA. For example, in mammalian brain cells, an influx of calcium ions activates the Ca^{2+}/calmodulin-dependent kinase II, which phosporylates CREB and thus activates transcription of CRE regulated genes in these cells (DASH et al. 1991; SHENG et al. 1991). CRE binding proteins all share many features including a conserved basic DNA binding domain, and a region with a heptad leucine repeat capable of forming an alpha helix

with the seven leucines aligned along one edge, immediately C-terminal to the DNA binding domain. The leucine residues of two such proteins can interact forming homo- or hetero-dimeric DNA-binding complexes, with the DNA binding and activation domains forming the "arms" of a Y shaped structure with the leucine zipper structure forming the "stem" (SHAYWITZ and GREENBERG 1999). Phosphorylation of CREB occurs within a phosphorylation domain called the P-box at a conserved serine residue, Ser133, which is indispensable for transcriptional activation. Other residues in the P-box are phosphorylated as well, and this is required for full transcriptional activation. The actual recruitment of transcriptional machinery involves two conserved glutamine rich domains called Q1 and Q2 boxes, on either side of the P-box (SASSONE-CORSI 1995; SHAYWITZ and GREENBERG 1999).

Mammalian CREs can be bound by several combinations of homo- or hetero-dimers of the b-zip family discussed above. These proteins were first described as transcription activators, but later work has revealed that they can also repress transcription as well. One CRE binding transcriptional repressor, called the cAMP response element modulator (CREM) is a close relative of CREB. CREM exons encode domains that are involved in activation; alternative splicing can create isoforms that function as transcriptional repressors lacking one or the other of the Q boxes. It has been proposed that CREM modulates gene expression by two possible mechanisms. As a homodimer, it may directly compete with CREB for CRE binding sites. Alternatively, CREM may form heterodimers with CREB, thereby reducing the ability of CREB to fully activate transcription. An alternatively spliced form of CREM, called ICER (inducible c-AMP early repressor), contains the DNA binding and leucine zipper dimerization domains, but lacks both transactivating domains. ICER is a potent transcriptional repressor. In certain tissues, alternative splicing can generate $CREM_{\tau}$ which is highly similar in sequence to CREB and is a transcriptional activator (SHAYWITZ and GREENBERG 1999).

So far, none of these genes or proteins have been identified in filamentous fungi. However, the genes for two CRE binding proteins have been discovered in *S. cerevisiae*, *SKO1* and *HAC1*. The products of both genes bind to CRE sequences and function to turn off gene expression (NEHLING et al. 1992; NOJIMA et al. 1994). The

gene for a CRE binding protein, *pcr1*$^{+}$, has been identified in *S. pombe*, and was implicated in the activation of a sexual development gene (WATANABE and YAMAMOTO 1996).

Recently, CREB has been found to be activated by mitochondrial dysfunction in human cells, and several nuclear genes encoding mitochondrial proteins have been found to contain CRE sequences (ARNOULD et al. 2002). In human cell lines that have mitochondrial dysfunction, as well as in wild type cells with mitochondria impaired by oligomycin or antimycin A, CREB was found to be phosphorylated at Ser133 and localized to the nucleus. A luciferase reporter construct, driven by a promoter that contained several CRE sites, demonstrated that the phosphorylated CREB was active and able to increase transcription in response to mitochondria dysfunction (ARNOULD et al. 2002). Several kinases are able to phosphorylate CREB, depending upon the signal(s) received by the cell that need to be converted into transcriptional regulation by CREB. In this instance of mitochondrial signaling, there seemed to be a role for calcium/calmodulin kinase IV (CaMKIV), because both a Ca^{2+} chelator and a dominant-negative form of CaMKIV inhibited CREB phosphorylation and luciferase expression (ARNOULD et al. 2002).

In *N. crassa*, a CRE is known to regulate the *grg-1* gene, which is a highly expressed glucose-repressible gene. In *grg-1*, a CRE binding protein acts to turn off gene expression when glucose concentrations are high. The CRE upstream of *grg-1* was shown to bind proteins in extracts isolated from cells grown under conditions of either glucose sufficiency or starvation (WANG et al. 1994). In this instance, the CRE works in concert with another regulatory element, the *Neurospora* Repressor Site (NRS) and both are located 450 to 500 nucleotides upstream of *grg-1*. The *grg-1* gene is allelic to an *N. crassa* clock gene, *ccg-1*, which is regulated by developmental stage and light levels, which requires interaction with photoreceptor proteins. It is not known if the CRE plays a role in the circadian regulation in this aspect of the expression of the gene.

To determine if the CRE upstream of *aod-1* played a role in the regulation of the gene, the presence of the element in the region upstream of the alternative oxidase gene of other fungal species was examined. In addition to the CRE type regulatory

sequences, coding sequences and other features of the region were also compared. I cloned the *Gelasinospora* alternative oxidase into pBluescript, creating pGAX-27 (Table 3) and sequenced the gene and surrounding regions (Fig. 11) Nucleotide sequence was obtained for a region of the *A. nidulans* alternative oxidase gene, from Monsanto's Cereon Microbial Sequence Database. This was used to develop PCR primers for the synthesis of a product to probe a cosmid library. Two cosmids containing alternative oxidase, 71G12 and 72C5 were identified (Table 3). The alternative oxidase coding region and surrounding regions were sequenced from these cosmids (Fig. 12). As expected based on their phylogeny, the *Gelasinospora* protein is much more similar to the *N. crassa* alternative oxidase than is the *A. nidulans* protein (Fig 12). However, high conservation in the middle portion of the sequences of all three alternative oxidases was evident. Regions of similarity include the predicted iron-binding ligands (marked with asterisks in Fig 12), proposed by Andersson and Nordlund's model of alternative oxidase structure (ANDERSSON and NORDLUND 1999; BERTHOLD et al. 2000). The position of introns in the *A. nidulans* gene differs from that of *N. crassa* and *Gelasinospora* (discussed below).

Canonical CREs (TGACGTCA) with flanking inverted repeats were found 747 nucleotides and 760 nucleotides upstream of the transcription start sites in the alternative oxidase genes of *N. crassa* and *Gelasinospora*, respectively. A CRE-like element, with seven of eight bases conserved (TGACGTCG), also with flanking inverted repeats, was found 729 nucleotides upstream of the start codon of the alternative oxidase gene in *A. nidulans*. A putative CRE (TGACGTCT) 230 nucleotides upstream of the start codon of the alternative oxidase gene in *M. grisea* has also been described, but its protein binding capacity and role in regulation of alternative oxidase expression are not known (YUKIOKA et al. 1998a).

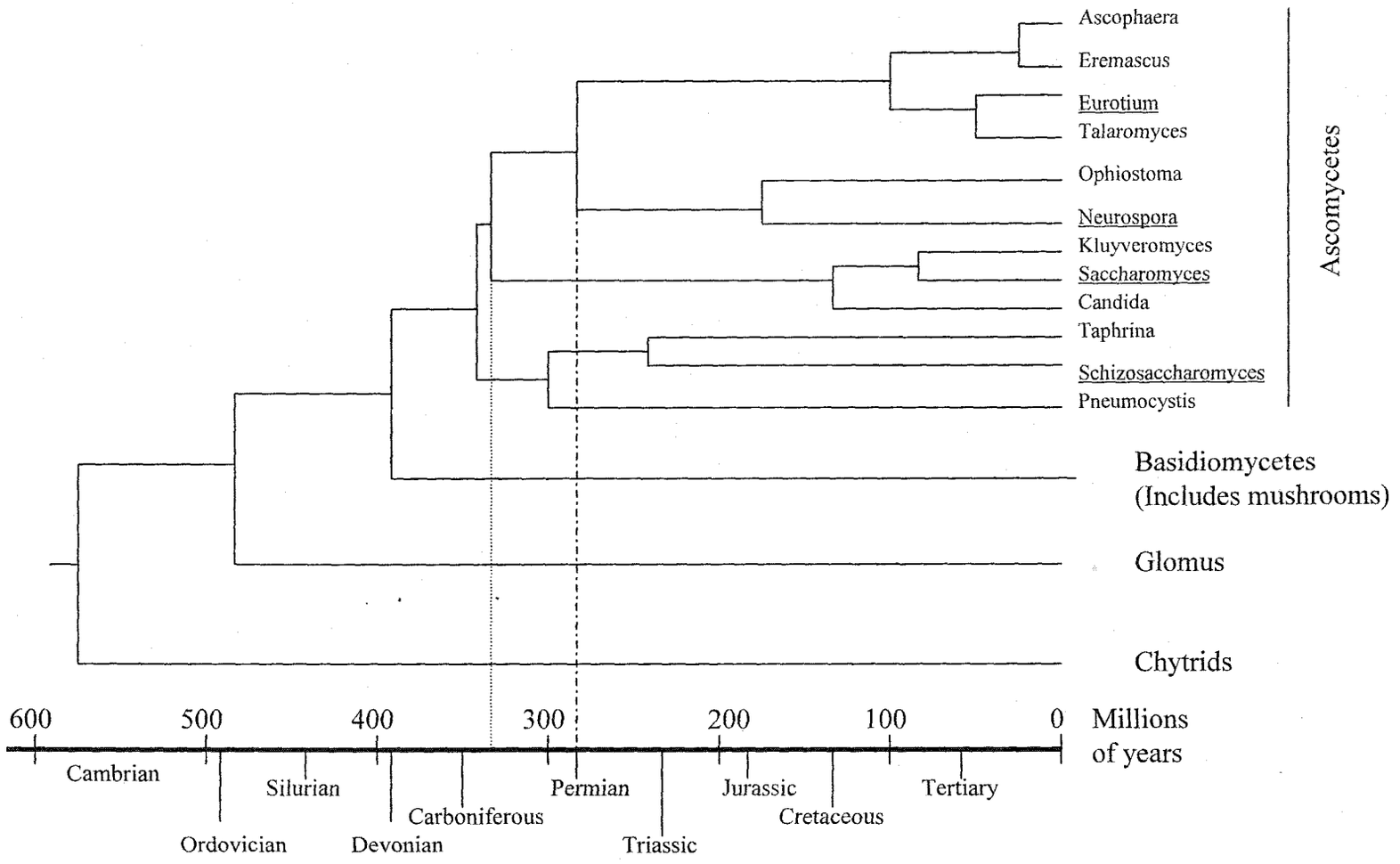
Fragments of DNA containing the alternative oxidase CRE were shown to bind protein when they were incubated with *N. crassa* total protein extract and examined by EMSA. Initially a 272 base pair fragment containing the CRE (Fig. 15) was used as a target DNA to demonstrate binding to the CRE, and it was found that the protein(s) that bind to this CRE exist in cells grown under either inducing or non-inducing conditions (Fig.16), as seen with the *grg-1* gene. It was later discovered that

a 58 base pair target DNA fragment containing the CRE (Fig. 15) was also able to bind the protein (Fig. 20). Deletion of the CRE or mutation of single nucleotides within the CRE abolished the mobility shift, confirming the specificity of binding (Fig. 17). The binding protein was enriched in a 25% ammonium sulfate fraction (Fig. 19). Attempts to isolate and purify the CRE binding protein using a DNA affinity column (Fig. 21) and a biotin-streptavidin approach (Fig. 22) were unsuccessful for unknown reasons. Attempts to isolate the protein in the future will likely focus on traditional protein purification methods. Initial results presented in this work have shown that the CRE binding protein can be further purified by binding to a sepharose Q column and elution with a 0.5 M KCl buffer (Fig. 23). Isolation of the protein would allow the amino acid sequence to be determined which could be used to identify the gene encoding the protein. Disruption of this gene in *N. crassa* and analysis of the mutant phenotype will aid in determining the role of the CRE, if any, in regulating *aod-1*expression.

Mobility shift assays using the restriction fragment nearest to the start site of *aod-1* (Fragment I, Fig. 15) showed complicated protein binding patterns when incubated with protein extract (Fig. 16). In order to purify the proteins involved in binding to elements within this region, smaller target DNA fragments must be generated, incubated with protein extracts and tested individually for mobility shifts. This may identify the components of basic transcription machinery, as well as any other proteins specifically involved in regulating the expression of *aod-1*. Given the findings from transformation experiments using pMMAX, this approach might be useful in the isolation of factors specifically involved in the transcriptional regulation of *aod-1* in response to induction by chloramphenicol.

The CRE upstream of *aod-1* has been shown to bind protein, but it does not appear to effect *aod-1* regulation under the conditions used to induce alternative oxidase in this thesis. Five plasmids were constructed containing *aod-1*$^{+}$, with varying lengths of upstream sequence, 360 base pairs of downstream sequence and a selectable marker for bleomycin resistance (Fig 18). Each of these was transformed into strain 7207, which contains the *aod-1-7* frameshift mutation (Table 1). The presence of KCN-insensitive respiration in transformants grown in the presence of

Figure 32. Evolutionary tree representing fungal divergence times. Adapted from another source (BERBEE and TAYLOR 1993). *Aspergillus* species are members of the order Eurotiales. Eurotiales and the order Sordaria, containing *Neurospora* and *Gelasinospora* species, shared a common ancestor approximately 280 million years ago. The filamentous fungi and the yeasts *S. cerevisiae* and *S. pombe* shared a common ancestor approximately 340 million years ago.



Represents approximate time of divergence of *Neurospora* and *Aspergillus*

Represents approximate time of divergence of yeasts and filamentous fungi

chloramphenicol, but not in the absence of chloramphenicol, would indicate that the upstream sequence present was sufficient to control induction of *aod-1*. Cosmid 23F7, with an undetermined amount of upstream sequence, and pMAX, containing 3.3 kb of upstream sequence, both rescued the *aod-1-7* mutation effectively, as did plasmid pΔMAX, containing 3 kb of upstream sequence with the 8 base pair CRE deleted (Table 6). This indicated that the CRE did not play a role as an activating element in *aod-1* expression upon growth in chloramphenicol. Further evidence against a role for the CRE was the observation that plasmid pMMAX (Fig.18), which contains the *aod-1* gene under the control of 255 base pairs of upstream sequence, also rescued the *aod-1-7* phenotype (Table 7). A small number of pMCMAX transformants, 8 of 36, containing only 10 base pairs of upstream sequence, initially showed fully induced KCN-insensitive respiration (Table 7). The induction of alternative oxidase generated by this plasmid will be discussed below.

Since the CRE did not seem to have an effect upon *aod*-1 induction, it was possible that it was regulating another gene in the region. To determine if there was another gene in the vicinity of the CRE, the sequence surrounding the element was subjected to tBLASTx searches with the *N. crassa* morning, evening and perithecial EST libraries, the *A. nidulans* EST library and the non-redundant EST library at GENBANK. One 275 amino acid ORF (Fig. 6) was discovered to have high identity with an *A. nidulans* EST of unknown function (Figs. 7 and 8). This *N. crassa* ORF is in opposite orientation to *aod-1* and expressed a transcript in *N. crassa* (Fig 7).

I wished to determine if the gene order of the ORF and alternative oxidase gene was conserved between *N. crassa* and *A. nidulans*, a species that is evolutionarily distant from *N. crassa.* The fungal species that were used in this thesis, *N. crassa, N. intermedia, N. tetrasperma, Gelasinospora,* and *A. nidulans,* are members of the division *Eumycota,* subdivision *Ascomycotina.* The *Neurospora* species and *Gelasinospora* belong to the order *Sordariales*, while *A. nidulans* belongs to the order *Eurotiales* (CARLILE and WATKINSON 1994). A comparison of the order *Sordariales* (representative species in Fig. 32, *N. crassa*) and the order *Eurotiales*, which includes *A. nidulans* (representative species in Fig. 32, *Talaromyces flavus* and

Eurotium rubrum), suggested a common ancestor of the orders approximately 280 million years ago (BERBEE and TAYLOR 1993) (Fig. 32).

A. nidulans cosmids containing the homologue of the *N. crassa* upstream ORF were assigned to linkage group V, while cosmids containing the *A. nidulans* alternative oxidase gene, were localized to linkage group VII. Thus, the order of the ORF and alternative oxidase genes has not been conserved between *A. nidulans* and *N. crassa*, but CRE (or CRE-like) elements, are present upstream of the alternative oxidase genes in both species (Fig. 14). These data suggest that the CRE is specifically associated with the alternative oxidase gene rather than the ORF. As discussed earlier, a CRE or CRE-like element is present upstream of the alternative oxidase genes of *Gelasinospora* and *M. grisea* as well. This conservation of the element suggests that it may play an as yet unidentified role in the regulation of alternative oxidase expression in fungi. It is possible that some pathways of alternative oxidase induction respond to different inducing agents or stresses while the response to inducing agents such as chloramphenicol that result in reduced electron flow through the cytochrome pathway utilizes a non-CRE dependent induction mechanism. It would be useful to define other conditions for alternative oxidase induction that might depend on the CRE. This could include induction at different times in the life cycle, response to different carbon sources, and stresses such as altered pH and nutrient limitation, etc. It should be noted that the data presented here do not rule out the possibility that the CRE plays a role in negatively regulating transcription under non-inducing conditions. If a post-transcriptional mechanism of mRNA degradation exists in uninduced cells, any increase or decrease in transcription caused by the presence or absence of the CRE would not be detected.

As the CRE seemed not to affect induction of alternative oxidase during growth in chloramphenicol, other conserved regulatory regions among *N. crassa*, *Gelasinospora* and *A. nidulans* were sought, as non-coding sequences conserved between evolutionarily distant species could indicate sequences important for regulation. Analysis of upstream sequences in *N. crassa, Gelasinospora and A. nidulans* manually, did not reveal the presence of any putative regulatory sequences other than the CRE and TATA box (Fig 14). Another potential region for

conservation of regulatory elements in these species was within the introns of the genes. This form of regulation has been described in organisms as varied as *C. elegans* and mice. In *C. elegans* lineage specification, the transcription factor *pal-1* is active only in the male tail. A mediator protein binds to a regulatory element in the fifth intron of *pal-1* in non-male tail lineages to prevent transcription of the gene (ZHANG and EMMONS 1999). In mice fibroblasts, the *c-fos* gene is upregulated by an increase in intracellular calcium, which activates several transcription factors, one of which interacts with a regulatory element in the first intron, this in turn disrupts a transcription pause site allowing transcription to proceed (COULON et al. 1999). This upregulation is the first step in a cycle of *c-fos* expression and activity, which can influence the transcription of other downstream genes that are not directly signaled by Ca^{2+}.

The introns present in the alternative oxidase genes of the *Neurospora* species, *N. crassa*, *N. intermedia* and *N. tetrasperma*, as well as *Gelasinospora* were nearly identical in both sequence and position within the gene (Fig. 30A), thus no information about conserved regions that may be involved in gene regulation could be inferred. *A. nidulans* introns were significantly different in position within the alternative oxidase gene (Fig. 13). The sequence of the first intron in the *A. nidulans* gene was somewhat similar to that of the second intron in the *N. crassa* gene, however, the sequence represents the 5' splicing region and putative lariat structure. The second intron of *A. nidulans* showed no similarity with either intron of *aod-1* (Fig. 30B). Furthermore, the size of these fungal introns is significantly smaller that those known to be involved in genetic regulation. The intron in *C. elegans pal-1* that contains the regulatory element is 1.4 kb long (ZHANG and EMMONS 1999), and the intron of mouse *c-fos* that responds to calcium signals is nearly 1 kb in length (COULON et al. 1999). In contrast, the introns found in fungal alternative oxidase genes are less than 100 base pairs in length (Fig 30). The small size of the *aod-1* introns, the lack of conservation of intron sequences among fungal species and the observation that *aod-1* cDNA under the control of 3.3 kb of upstream sequence show wild type KCN-insensitive respiration (section 3.14) strongly suggest that the introns of alternative oxidase genes do not play a role in regulating its expression.

The initial northern hybridization experiments with wild type and alternative oxidase mutants, grown in the presence and absence of chloramphenicol indicated that there was virtually no *aod-1* mRNA in uninduced cultures, with a large increase upon growth in chloramphenicol (Fig. 25). Different *aod-1* mutant strains varied in the amount of mRNA that was present upon induction, but there was always an increase in mRNA upon growth in chloramphenicol. *Aod-2-4* had no detectable mRNA in either growth condition, presumably because there was a deficiency in its ability to respond to the induction. These results were consistent with earlier results where the inhibitor actinomycin D was used to show that transcription was required for induction of alternative oxidase in *N. crassa* (EDWARDS and UNGER 1978).

As work on this thesis progressed, northern hybridizations demonstrated that *aod-1* mRNA was present in some uninduced cultures, in varying amounts, even though these cultures never contained KCN-insensitive respiration (Figs. 26, 27). Furthermore, long exposures of northern hybridizations revealed that most uninduced cells contain at least small amounts of *aod-1* mRNA. However, western blot analysis of mitochondrial proteins isolated from these cultures indicated that there was no alternative oxidase protein present regardless of the amount of mRNA present (Fig. 27B and C). In an effort to determine what conditions may be influencing this accumulation of RNA in uninduced cultures, I experimented with carefully controlled temperature and light levels. There appeared to be a small correlation with increased levels of RNA in cultures grown at 25°C versus 30°C and those grown in the dark versus the light, but these were not reproducible from experiment to experiment. Others in the laboratory have also been unable to find conditions that consistently result in high level of *aod-1* mRNA in uninduced cultures. In fact, one experiment in which cells were grown at 20°C failed to reveal in increase in *aod-1* message (K. Kessler and F. Nargang, personal communication).

Transcript and protein levels were also measured in cells of strain 7207 (*aod-1-7* frameshift mutation) transformed with the plasmid pMAX, pΔMAX, pMMAX or pMCMAX (Fig. 18). None of the transformants demonstrated any KCN-insensitive respiration in uninduced cultures. In the presence of chloramphenicol, all cultures except the mutant strain 7207 exhibited KCN-resistant respiration (Figs. 28A and

29A). However, the strains L11 and L18, transformed with pMCMAX showed only very slight induction of KCN-insensitive respiration. Northern hybridizations of RNA from these cultures show that a few of the transformants have RNA present in the uninduced cultures, but there is an increase in *aod-1* mRNA in all cultures grown in chloramphenicol, with the exception of L11 (Figs. 28B and 29B). These results are complicated by the fact that small amounts of RNA have sometimes been observed in 7207 cells (K. Kessler and F. Nargang, personal communication). Thus, the levels of mRNA in the L11 and L18 cultures, which produce no detectable AOD1 protein under any circumstances, may be explained as having been derived from *aod-1-7*. Western blot analysis of mitochondrial proteins isolated from these cultures demonstrated that none of the uninduced cultures contained detectable alternative oxidase protein. However, in the transformants grown in the presence of chloramphenicol, all contained alternative oxidase protein, with two exceptions. Strains L11 and L18, even though they appear to have partial induction of KCN-insensitive respiration, do not contain AOD1 protein. The presence of significant amounts of *aod-1* mRNA in the induced culture of pMCMAX transformant L18 may be explained if the complete *aod-1* structural gene inserted at an ectopic location and is transcribed constitutively from another promoter in the transformant. In uninduced cultures, the mRNA may be degraded and it may persist in induced cultures. The lack of AOD1 protein produced in induced cells may be due to the context near the 5' end of this transcript. If there were another AUG it could initiate translation out of frame, or a chimeric mRNA may be inefficiently translated. Due to the variation of *aod-1* mRNA levels seen in wild type cultures, the presence of small amounts of mRNA in uninduced cultures of 8-30, K5 and L11 is unlikely to be due to constitutive transcription of *aod-1* under the control of an ectopic promoter.

Due to the northern results showing the presence of at least low levels of *aod-1* mRNA in uninduced cultures on long exposures and the occasional uninduced culture containing large amounts of *aod-1* mRNA, I wanted to determine if there was constitutive transcription of *aod-1* occurring in uninduced cultures. Nuclear run-on assays were used to label transcripts that were arrested at the point of nuclear isolation. The run-ons did indicate that there was active transcription of *aod-1* mRNA

in uninduced cultures since some hybridization was observed to *aod-1* DNA as compared to the empty vector control (Fig. 31A). These data suggest some form of post-transcriptional control is involved in the expression of alternative oxidase activity. This has also been described in the rice blast fungus *M. grisea*, in which there was a basal level of transcription of alternative oxidase, with active degradation of the mRNA by factor(s) sensitive to cycloheximide (YUKIOKA et al. 1998b). RNA stability as well as the basal level of transcription increased several fold when the fungus was exposed to the respiration inhibiting fungicide SSF-126 or to H_2O_2 (YUKIOKA et al. 1998b).

There are several ways that nuclear gene expression can be regulated other than via transcriptional mechanisms. For example, alteration in intron splicing can give rise to different proteins (CONLON et al. 2001) and stability of the mRNA can influence the amount of protein produced. The trypanosome alternative oxidase (TAO) is regulated at the level of mRNA stability (CHAUDHURI et al. 2002). Trypanosomes exist in two forms, the bloodstream form and the procyclic form. The bloodstream form does not have cytochromes and respires exclusively using TAO, while the procyclic form has a normal cytochrome pathway. Many genes are transcribed polycistronically and regulated post-transcriptionally in trypanosomes, including TAO. The TAO transcript has a much longer half-life in the bloodstream form versus the procyclic form, and the actual transcript level was about 4 times higher in the bloodstream form (CHAUDHURI et al. 2002). Transcript degradation was inhibited if the cells were incubated with cycloheximide, as was seen in *M. grisea*. The method of TAO mRNA stabilization is not known, but the TAO transcript has a long 3' UTR (CHAUDHURI et al. 2002). The 3' UTR has been implicated in regulation via mRNA stability in a variety of organisms, from yeast to human. In fact, one regulatory element called the AU-rich element is seen in *S. cerevisiae* as well as human genes. A yeast transcript called *TIF51A*, whose product is involved in mRNA capping, possesses an AU-rich element in its 3' UTR, which regulated the turnover of the mRNA by stabilizing the mRNA in the presence of glucose and destabilizing the mRNA in media lacking glucose. Transposition of the yeast "AU-rich element" from the human *c-fos* gene regulates *TIF51A* in a similar fashion, demonstrating the

conservation of this mRNA turnover mechanism from yeast to humans (VASUDEVAN and PELTZ 2001).

Both the 5' and 3' UTRs can play important roles in post-transcriptional regulation. One example of the involvement of 5' UTRs is the upstream open reading frame (uORF) found in the 5' UTRs of several fungal genes involved in amino acid biosynthesis. Single conserved uORFs are found in the *S. cerevisiae CPA1* and the *N. crassa arg-2* mRNAs, whose products are involved in the biosynthesis of arginine (GEBALLE and SACHS 2000). In the presence of low levels of arginine, ribosomes scanning for initiation codons from the 5' end of the mRNA cause initiation at either the uORF or the *arg-2* start codon in an unrestricted fashion. In the presence of excess arginine, ribosomes stall at the uORF, causing a reduction in translation initiation at the *arg-2* AUG codon. The stalled ribosomes prevent other ribosomes from scanning past the uORF to reach the downstream initiation site (WANG and SACHS 1997).

The results of run-on assays using nuclei isolated from cultures grown in the presence of chloramphenicol were somewhat variable, with some experiments showing a large increase in transcription levels, while others showed virtually no increase (Fig. 31B and C). Nuclear run-on assays that have been performed by others since I left the lab support my data showing a low level of constitutive transcription of *aod-1*. In addition, convincing data for transcriptional induction has also been obtained (C. Nargang and F. Nargang, personal communication). While discussing my results during the preparation of this thesis, we realized that cells grown in the presence of chloramphenicol from the start of the culture, as I had used for the isolation of nuclei for my run-on assays, might no longer be transcribing the gene at an enhanced rate. It was deemed more reasonable to grow cells to early log phase and then add antimycin A for one to two hours before harvesting the cells for the isolation of nuclei. Antimycin A should immediately prevent respiration through complex III and thus provide a strong signal for induction of alternative oxidase. Such experiments revealed an approximately 7.5 fold increase in transcription of *aod-1* (C. Nargang and F. Nargang, personal communication).

The available data suggest that the regulation of *N. crassa* alternative oxidase production is a synthesis of transcriptional and post-transcriptional regulatory

mechanisms. The *aod-1* gene appears to be constitutively transcribed, but the transcript may not be translated in uninduced cells. Given the fact that there is usually little *aod-1* mRNA observed in uninduced cells, it is conceivable that the mRNA might be unstable in the absence of an induction signal. Factors preventing translation might also be present only in uninduced cells. Another possibility is that existing mRNA is translated, but the AOD-1 protein may be quickly degraded in uninduced cells. Upon induction, there is an increase in transcription of *aod-1*, and efficient translation of the mRNA to give the characteristic cyanide-insensitive respiration and accumulation of the AOD-1 protein.

Future work will attempt to define the regions in the upstream sequence required for transcriptional control of *aod-1* in response to various inducing agents and growth conditions, using promoter deletions. To determine factors required for post-transcriptional control, both the upstream and downstream untranslated regions of *aod-1* mRNA will be analyzed. Deletions of these sequences may reveal regions important for regulation of expression of alternative oxidase. Identification of proteins, if any, that interact with the mRNA will also be required for a more complete picture of alternative oxidase regulation.

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Appendix

1. Bacterial media

Antibiotics added to bacterial media

Antibiotic	Stock	Final concentration
Ampicillin	50mg/ml in dH_2O	100μg/ml
Kanamycin	50mg/ml in dH_2O	50μg/ml

Stocks were filter sterilized and stored at -20°C in small aliquots.

L-broth

10 g Bacto-tryptone (Difco)
5 g yeast extract (Difco)
5 g NaCl
The solution was made up to 1 liter with dH_2O and sterilized by autoclaving for 20 min at 121°C. For LB plates, 15g agar (Difco) was added prior to autocalaving.

X-gal AMP plates

After autoclaved LB media was cooled to 60°C to 65°C, 1 ml of filter sterilized IPTG (25 mg/ml stock in dH2O), 2 ml of X-gal (25 mg/ml in dimethyl formamide) and 2 ml ampicillin (50 mg/ml stock) per liter of media were added prior to pouring. The plates were stored in the dark at 4°C.

SOB media

20% w/v tryptone
5% w/v yeast extract, salts at 10mM concentration each
10 mM NaCl
10 mM KCl
Made to 1 liter, autoclaved, added sterile $MgCl_2$, $MgSO_4$ to a final concentration of 10mM each.

2. Fungal growth media

Antibiotics added to media

Antibiotic	Stock	Final concentration
Antimycin A	1 mg/ml in ethanol	0.5 μg/ml
Benomyl	1 mg/ml in ethanol	0.5 μg/ ml
Bleomycin*	1mg/ml in ethanol	1 μg/ml viability plates
Bleomycin*	1 mg/ml in ethanol	0.5 μg/ml top agar and slants
Chloramphenicol	200 mg/ml in ethanol	2 mg/ml

* When using bleomycin selection, 0.5 g caffeine was added per liter of media, to enhance the effectiveness of the bleomycin.

Biotin solution

5 mg of biotin was dissolved in a final volume of 400 ml of 50% (v/v) ethanol and stored at 4°C.

10X sugar solution
200 g sorbose
5 g fructose
5 g glucose
2 g myo-inositol
This was dissolved in dH_2O, adjusted to 1 l, and sterilized by autoclaving.

Supplements to media
Nicotinamide 10 mg/ml dH_2O
Pantothenate 10 mg/ml dH_2O
1 ml of each was added to 1 l of media, as needed

Top Agar
20 ml 50X Vogel's salts
182 g sorbitol
1 ml 1X trace elements
15 g agar
Supplements were added as required before the solution was made up to 850 ml. After autoclaving, 100 ml of 10X sugars solution was added. Antibiotics were added as required after autoclaving and cooling to 60°C to 65°C.

4X Trace elements stock
50 g citric acid
50 g $ZnSO_4 \cdot 7H_2O$
10 g $Fe(NH_4)_2(SO_4) \cdot 6\ H_2O$
2.5 g $CuSO_4 \cdot 5\ H_2O$
0.5 g $MnSO_4 \cdot H_2O$
0.5 g H_3BO_3
0.5 g $Na_2MoO_4 \cdot 2\ H_2O$
20 mg $CoCl_2$
The solution was made up to 1 l. A 1X stock was made as a working solution. Both were stored at 4°C.

Viability plates
20 ml 50X Vogel's salts
1 ml biotin solution
1 ml 1X trace elements
15 g agar
Supplements were added as required and the solution was made up to 900 ml with dH2O. After autoclaving, 100 ml of 10X sugar solution was added before pouring into plates.

Vogel's medium
20 ml 50X Vogel's salts
1 ml 1X trace elements
1 ml biotin
15 g table sugar (sucrose)
Supplements were added as necessary and the solution was made up to 1 liter with dH_2O and autoclaved.

50X Vogel's salts
125 g $Na_3citrate{\cdot}2\ H_2O$
250 g KH_2PO_4
100 g NH_4NO_3
10 g $MgSO_4{\cdot}7\ H_2O$
5 g $CaCl_2{\cdot}2\ H_2O$
The first three ingredients were dissolved sequentially in 650 ml of H_2O. The $MgSO_4$ and the $CaCl_2$ were dissolved in 35 ml and 100 ml H_2O respectively. These two solutions were added slowly and sequentially to the first. When completely dissolved, it was adjusted to 1 liter with H_2O. Chloroform (5 ml) was added as a preservative.

Westergaard's salts (20X stock)
20 g KNO_3
20 g KH_2PO_4
10 g $MgSO_4{\cdot}7H_2O$
2 g NaCl*
2.3 g $CaCl_2{\cdot}2H_2O$*
*These were dissolved in dH_2O and added separately once the other salts had dissolved.
dH_2O was added to 1 l, and 10 ml chloroform was added as a preservative.

Westergaard's crossing plates (1 liter)
50 ml 20X Westergaard's salts
10 g sorbose
0.1 g glucose
1 ml biotin stock
5 ml trace elements
20 g agar
Supplements were added if needed and the solution was made up to 1 liter with dH_2O and autoclaved.

3. Other reagents and solutions
Acrylamide gel (denaturing, for DNA sequencing)
6% w/v acrylamide (29:1 acrylamide:bis-acrylamide)
8M urea
The acrylamide and urea were dissolved in 1X glycerol tolerant buffer then filtered through Whatman #4 filter paper and degassed. It was stored in dark bottles at room temperature.

Acrylamide gel (non-denaturing, for EMSAs)
6% w/v acrylamide (29:1 acrylamide:bis-acrylamide)
The acrylamide was dissolved in 0.5X TBE then filtered through Whatman #4 filter paper and degassed. It was stored in dark bottles at room temperature.

Acrylamide resolving gel (for proteins)
20.8 ml12.5% 29:1 acrylamide:bis-acrylamide
6.3 ml resolving buffer
0.5 ml 10% SDS
22.5 ml dH_2O
This was made just prior to use, and was polymerized with the addition of 40µl TEMED and 1.6 ml 10% APS.

Acrylamide stacking gel (for proteins)
2.5 ml 29:1 acrylamide: bis-acrylamide
5 ml stacking buffer
200 µl 10% SDS
12.3 ml dH_2O
This was made just prior to use, and was polymerized with the addition of 40 µl TEMED and 150 µl 10% APS.

DNA affinity chromatography buffer, various salt concentrations (Section 2.42)
20 mM Tris-Cl, pH 8.0
0.15 M KCl
1 mM EDTA

10% APS
10% w/v ammonium persulfate in dH_2O

10X binding buffer for EMSA reactions
100 mM HEPES-KOH pH 7.9
500 mM KCl
10 mM EDTA
50 mM DTT
50% v/v glycerol

1X Coomassie stain
0.25% Coomassie brilliant blue w/v in (50% methanol, 10% glacial acetic acid)
After stirring, this was poured through a fluted filter paper to remove particulates.

Destain
40% v/v methanol
10% v/v glacial acetic acid

100X Denhardt's solution
2% w/v ficoll
2% w/v BSA Fraction V
2% w/v polyvinylpyrrolidone

Ethidium bromide
Ethidium bromide powder was dissolved at a concentration of 5 μg/ml in dH_2O and stored in dark bottles at room temperature. It was used at a final concentration of 0.5 μg/ml agarose gel.

1X glycerol tolerant gel buffer
90 mM Tris-base
30 mM taurine
0.5 mM EDTA

Glucose buffer
50 mM glucose
2.5 mM Tris-HCl pH 7.5
2 mM EDTA

Grinding buffer for protein isolation
20 mM HEPES-KOH pH 7.9
100 mM KCl
5 mM DTT
2 mM EDTA
1 mM PMSF
Dissolve PMSF in ethanol to generate a 100 mM solution immediately before use.

HEPES protein grinding/dialysis buffer
20 mM HEPES-KOH pH 7.9
100 mM KCl
5 mM DTT
2 mM EDTA
1 mM PMSF
Make solution of everything but PMSF.Make fresh PMSF just before use by dissolving in ethanol, to generate a 200 mM PMSF solution. Add to buffer slowly with stirring to ensure it enters into solution.

High Salt Buffer (fungal genomic DNA isolation)
100 mM NaCl
25 mM Tris-HCl pH 7.4
2 mM EDTA

1X Laemlli buffer
0.19 M glycine
0.025M Tris-base
0.1% w/v SDS

Loading dye (DNA gel)
0.4% w/v Bromophenol blue
1X TAE (or TBE)
1 mM EDTA
50% v/v glycerol

Loading dye (RNA gel)
0.4% w/v Bromophenol blue
1X MOPS-EDTA
50% v/v glycerol

MCS (also called SMC)
10 mM 1 M MOPS-NaOH pH 6.3
50 ml 1 M $CaCl_2$
1 M Sorbitol

Milk buffer
5% w/v skim milk powder in TBS/Tween
0.02 M Tris-base
0.15 M NaCl
2.5% v/v Tween 20
If the milk buffer was to be used to store primary antibody, 0.02% (w/v) sodium azide was added as a preservative.

10X MOPS-EDTA
0.2 M MOPS
50 mM sodium acetate pH 5.2
10 mM EDTA
pH was adjusted to 7.0 with NaOH.

Nuclear grinding buffer
10mM Tris-HCl pH 7.0
10 mM EDTA
4 mM spermidine
1 mM spermine
100mM KCl
0.1% v/v β-mercaptoethanol
0.5% v/v Triton X-100
17% sucrose

Nuclear suspension buffer
10mM Tris-HCl pH 8.3
40% v/v glycerol
5 mM $MgCl_2$
0.1 M EDTA

Nuclear run-on 5X buffer
25 mM Tris-HCl pH 8.0
12.5 mM $MgCl_2$
740 mM KCl
1.25 mM each ATP, CTP, GTP

New Isolation Buffer
10 mM EDTA
1% w/v SDS
100 mM Tris-HCl pH 8.0

New Wash
50% v/v (10 mM Tris-Cl pH 7.5, 1 mM EDTA, 100 mM NaCl)
50% v/v 95% ethanol

PEG + MOPS
42% w/v PEG-4000
10 mM MOPS-NaOH pH 6.3

PMC
50 mM $CaCl_2$
95% v/v PEG + MOPS
Autoclave each solution separately before mixing

Cyanide solution
0.1 M KCN dissolved in 10mM Tris-HCl pH 7.2, 5 mM EDTA. Wear gloves when handling KCN, dissolve it in the fume hood, and always ensure the pH of the solvent is over 7.0 as an acidic pH will generate toxic cyanide gas.

1.1X PCR mix
50 mM Tris-Cl pH 9.0
1.5 mM $MgCl_2$
0.4 mM β-mercaptoethanol
0.1 mg/ml BSA
0.2 mM each dNTP
This was aliquoted to 1.5 ml microcentrifuge tubes and stored at -20°C.

Pre-hybridization buffer for RNA blots, per 200 ml:
50% v/v formamide
5X SSPE
10X Denhardt's solution
50 μg/ml denatured salmon sperm DNA
1.5% w/v SDS

Pre-hybridization solution for DNA blots
10X Denhardt's solution
2X SSC
1% w/v SDS
100 μg/ml denatured salmon sperm DNA

RNase A
10mg/ml in 0.1M sodium acetate, pH 5.2
The RNaseA solution was aliquoted into 1.5 ml microcentrifuge tubes and boiled for 15 min to remove contaminating DNase activity. Aliquots were stored at -20°C until needed.

RNA gel loading buffer, per ml:
50% v/v deionized formamide
6.5% v/v formaldehyde
1X MOPS-EDTA buffer
50 μg/ml ethidium bromide

Sephadex G 50 spin column SDS buffer
10 mM Tris-HCl pH 8.0
1 mM EDTA
0.1% w/v SDS
Filter sterilize before use.

10X SET
1.5M NaCl
0.2M Tris-HCl pH 8.0
10 mM EDTA

SEMP
0.25 M sucrose
10 mM MOPS-NaOH pH 6.3
1 mM EDTA
1 mM PMSF
Store the SEM (no PMSF) at 4°C and make fresh PMSF just before use by dissolving 35 mg/ml in ethanol, which is a 200 mM solution. Add 5 μl of this per ml of SEM used.

SHAM solution
0.33 M SHAM (salicyl hydroxamic acid)dissolved in ethanol

10X SSC
0.15 M sodium citrate pH 7.0 (pH with HCl)
1.5 M NaCl

20X SSPE
3.6M NaCl
200mM sodium phosphate pH 7.4
20 mM EDTA

Storage buffer (CNBr-Sepharose)
10 mM Tris-HCl, pH 7.6
0.3 M NaCl
1 mM EDTA
0.02% w/v NaN_3 (sodium azide)

50X TAE
2 M Tris-acetate
0.05 M EDTA
Tris base (242 g), 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA (pH 8.0) were dissolved in dH_2O and the final volume was adjusted to 1 liter.

TB
10 mM PIPES-HCl pH 6.7
55 mM $MnCl_2$
15mM $CaCl_2$
250mM KCl

10X TBE
0.89 M Tris-borate
0.89 M boric acid
0.02 M EDTA
Tris base (108 g), 55 g boric acid and 40 ml of 0.5 M EDTA (pH8.0) were dissolved in dH_2O for a final volume of 1 liter.

TBS (for westerns), per liter
20 mM Tris-HCl, pH 7.5
0.5 M NaCl

TBS/Tween
TBS as above, with 5 ml of Tween 20 added per liter

TE
10 mM Tris-HCl (of any desired pH)
1 mM EDTA

TES solution
10 mM Tris-HCl pH 7.5
10 mM EDTA
0.1 % w/v SDS

Transfer buffer
25 mM Tris base
190 mM glycine
20% v/v methanol

Washes 1 and 2, Southern (DNA) blots
2X SSC
0.1% w/v SDS

Washes 3 and 4, Southern (DNA) blots
0.1X SSC
0.1% w/v SDS

Washes 1 and 2, Northern (RNA) blots
2X SSC
0.1% w/v SDS

Wash 3, Northern (RNA) blots
1X SSC
0.1% w/v SDS

Wash 4, Northern (RNA) blots
0.2X SSC
0.1% w/v SDS