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THE UNIVERSITY OF ALBERTA

Characterization of Two Extranuclear Cytochrome aa_3-Deficient Mutants of *Neurospora crassa*

by

Edmond George Lemire

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF Doctor of Philosophy

Genetics

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Characterization of Two Extranuclear Cytochrome aa_3-Deficient Mutants of *Neurospora crassa* submitted by Edmond George Lemire in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Frank Nargang
Supervisor

R B Hodgetts

P E Morgan

External Examiner

Date April 19, 1988

Cette thèse est dédiée à ma famille : mes parents, Donat et Cécile, mes frères, Lionel, Bernard et Luc, et ma soeur, Annette.

Abstract

The [*mi-3*] and [*exn-5*] extranuclear mutants of *Neurospora crassa* are both characterized by their deficiency in cytochrome aa_3 levels. Various approaches were used to more fully characterize these two mutants.

DNA sequence analysis of the [*mi-3*] *oxi-3* gene revealed a missense mutation in codon 448 of the mature subunit 1 polypeptide of cytochrome *c* oxidase. The G/C to T/A transversion results in a tyrosine residue at this position instead of the normally present aspartic acid. Three lines of evidence suggest that this mutation confers the [*mi-3*] phenotype. Firstly, the amino acid substitution is a relatively severe change in that an amino acid with a negatively charged side chain is replaced by one that carries a polar, but uncharged aromatic side chain. Secondly, the amino acid residue at this position appears to be conserved as either an aspartic acid or a glutamic acid in diverse species. This implies that an acidic residue may be functionally important at that position. Finally, the transversion mutation appears to segregate with the mutant phenotype in forced heterokaryons.

The DNA sequence of the [*exn-5*] *oxi-1* gene revealed a missense mutation in subunit 2 of cytochrome *c* oxidase. A C/G to T/A transition in the 15th codon of the mature subunit 2 coding region specifies an isoleucine residue in the [*exn-5*] polypeptide, while the wildtype has a threonine at this position. No genetic evidence is available at this time to confirm that this mutation confers the cytochrome aa_3-deficient phenotype to [*exn-5*] strains. The severity of the amino acid substitution, a polar amino acid is replaced by a non-polar residue, and the observation that hydroxylated amino acids are found exclusively at this position in the subunit 2 polypeptides of various organisms is good circumstantial evidence that this substitution gives rise to the [*exn-5*] phenotype.

In light of the above findings, a model is proposed to explain the manner in which the mutations manifest the respiratory-deficient phenotype in these mutants and their interactions with other genes.

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I would first like to thank my supervisor and friend, Dr. Frank Nargang, for his encouragement, guidance and support. He had to spend many hours listening to my ideas and models, most of which were not very good. Somehow, he managed to be patient with me. He never discouraged me from asking more questions. I really appreciate the great times at the Faculty Club, Power Plant and at the many Lab parties as well as the many chats about hockey.

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Abbreviations

A. nidulans	*Aspergillus nidulans*
ant^R	antibiotic resistance
ant^S	antibiotic sensitive
Ap^R	ampicillin resistance
ATPase	adenosine triphosphatase
Bn^R	benomyl resistance
bisacrylamide	*N,N'*-methylenebisacrylamide
bp	base pair
bromphenol blue	3',3'',5',5''-tetrabromophenolsulfonphthalein
BSA	bovine serum albumin
Cm^R	chloramphenicol resistance
Ci	Curie
coI	cytochrome oxidase subunit 1
coII	cytochrome oxidase subunit 2
coIII	cytochrome oxidase subunit 3
coxII	cytochrome oxidase subunit 2
coxIII	cytochrome oxidase subunit 3
cpm	counts per minute
C. reinhardtii	*Chlamydomonas reinhardtii*
c^{7}dGTP	7-deaza-2'-deoxyguanosine 5'-triphosphate
Da	Daltons
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
ddATP	2',3'-dideoxyadenosine 5'-triphosphate
ddCTP	2',3'-dideoxycytidine 5'-triphosphate
ddGTP	2',3'-dideoxyguanosine 5'-triphosphate
DEAE	diethylaminoethyl

dGTP	2'-deoxyguanosine 5'-triphosphate
dH,O	distilled water
dITP	2'-deoxyinosine 5'-triphosphate
D. melanogaster	*Drosophila melanogaster*
DNA	deoxyribonucleic acid
dTTP	3'-deoxythymidine 5'-triphosphate
D. yakuba	*Drosophila yakuba*
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid, disodium salt
ELISA	enzyme-linked immunosorbent assay
FGSC	Fungal Genetics Stock Center
Hepes	*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid
HNQO	2-heptyl-4-hydroxyquinoline-*N*-oxide
IPTG	isopropyl-β-D-thiogalactoside
kbp	kilobase pairs
kDa	kiloDaltons
KLH	keyhole limpet hemocyanin
Km^R	kanamycin resistance
LiDS	lithium dodecyl sulfate
mA	milliAmperes
MNNG	*N*-methyl-*N*'-nitro-*N*-nitrosoguanidine
MOPS	3-(*N*-morpholino)propanesulfonic acid
Mr	relative molecular weight
mRNA	messenger ribonucleic acid
MSP	mitochondrial structural protein
MW	molecular weight
NADH	nicotinamide adenine dinucleotide, reduced form
N. crassa	*Neurospora crassa*

P. anserina	*Podospora anserina*
PEG	polyethylene glycol
PMSF	phenylmethylsulfonyl fluoride
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
rpm	revolutions per minute
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulfate
SHAM	salicylhydroxamate
Sm^R	streptomycin resistance
S. pombe	*Schizosaccharomyces pombe*
Tc^R	tetracycline resistance
Tris	Tris(hydroxmethyl)aminomethane
triton X-100	octyl phenoxy polyethoxyethanol
TTP	thymidine 5'-triphosphate
tween 20	polyoxyethylenesorbitan monolaurate
UV	ultraviolet
URF	unidentified reading frame
X-GAL	5-bromo-4-chloro-3-indolyl-β-D- galactoside
X. laevis	*Xenopus laevis*

I. Introduction

Mitochondrial Genetics

The advent of extranuclear genetics began with the discovery of cytoplasmic respiratory-deficient mutants in yeast (Ephrussi and Hottinguer, 1951) and in *Neurospora* (Mitchell and Mitchell, 1952; Mitchell *et al.*, 1953). Evidence that mitochondria contained DNA (Luck and Reich, 1964) and that these organelles were transmitted exclusively by the protoperithecial parent in *Neurospora* (Reich and Luck, 1966) lead to the conclusion that maternally inherited characteristics were encoded by the mitochondria. Direct evidence supporting this theory was obtained for *Neurospora* in 1965. Diacumakos *et al.* (1965) microinjected purified mitochondria from the cytoplasmic [*abn-1*] mutant (Garnjobst *et al.*, 1965) into a wildtype strain. The microinjected wildtype cultures acquired the maternally inherited mutant characteristics of slow-growth as well as deficiencies in cytochrome aa_3 and *b* (Diacumakos *et al.*, 1965; Garnjobst *et al.*, 1965).

Today, much more is known about the nature of the mitochondrial genome. The complete mitochondrial DNA sequences from various eukaryotes have been published. (Anderson *et al.*, 1981; Bibb *et al.*, 1981; Anderson *et al.*, 1982; Clary and Wolstenholme, 1985; Roe *et al.*, 1985). The size of the mitochondrial genomes vary from *ca.* 16 kbp in animals to up to 2500 kbp in plants (Sederoff, 1984; Mulligan and Walbot, 1986). Fungal mitochondrial genomes are intermediate in size (*ca.* 17 kbp to >100 kbp)(Clark-Walker and Sriprakash, 1982; Sederoff, 1984). The size differences, at least in fungal organelle genomes, can be accounted for by the presence of optional introns and the length of intergenic spacer regions (Sederoff, 1984; Breitenberger and RajBhandary, 1985).

Despite the size variations, mitochondrial genomes are functionally conserved and essentially encode the same number of proteins (Sederoff, 1984; Breitenberger and RajBhandary, 1985; Mulligan and Walbot, 1986). The three largest subunits of cytochrome *c* oxidase, apocytochrome *b*, and some components of both the NADH dehydrogenase complex and the mitochondrial ATPase are specified by genes in the mitochondrial genome (Sederoff,

1984; Breitenberger and RajBhandary, 1985). The remainder of mitochondrial structural genes encode components of the mitochondrial translation apparatus such as the large and small rRNA subunits, a ribosomal protein and tRNA molecules (Sederoff, 1984; Breitenberger and RajBhandary, 1985).

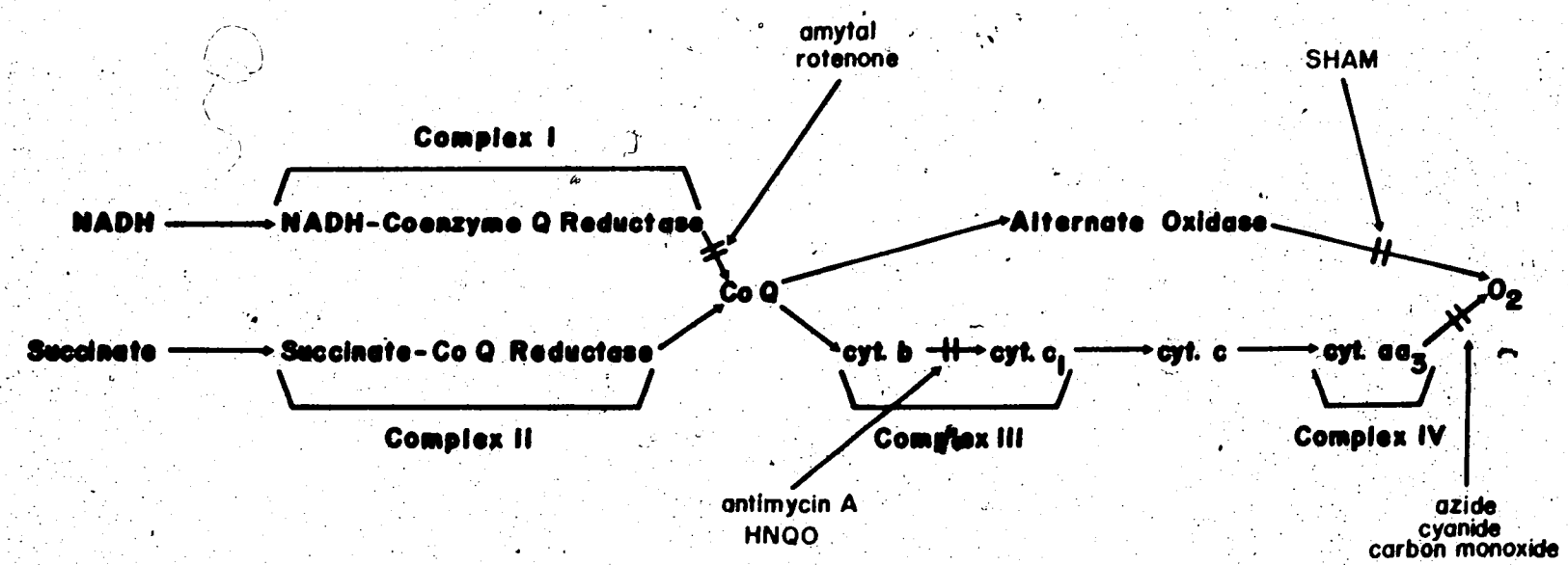
A novel feature characteristic of organelle genomes is the use of a genetic code different from the universal genetic code (Barrell *et al.*, 1979; Fox, 1979; Anderson *et al.*, 1981; Fox and Leaver, 1981; Anderson *et al.*, 1982; Bibb *et al.*, 1981; Wallace, 1982; de Bruijn, 1983; Clary and Wolstenholme, 1983b; Clary and Wolstenholme, 1985; Roe *et al.*, 1985). For example, in *Neurospora*, the UGA codon specifies a tryptophan residue instead of a termination codon (Heckman *et al.*, 1980).

Cytochrome c Oxidase

Cytochrome *c* oxidase, the terminal enzyme in the respiratory chain, catalyzes the transfer of electrons from cytochrome *c* to oxygen in the mitochondria of eukaryotes (Kadenbach and Merle, 1981; Tzagoloff, 1982; Capaldi *et al.*, 1983; Denis, 1986). The transfer of electrons from cytochrome oxidase to oxygen can be prevented by azides, cyanides and carbon monoxide (Figure 1). In *Neurospora*, cytochrome oxidase is composed of at least seven subunits, only three of which are mitochondrially encoded (Sebald *et al.*, 1973; Schatz and Mason, 1974). Cytochrome oxidases from higher eukaryotes like bovine and rat may consist of as many as 12 or 13 subunits (Kadenbach and Merle, 1981; Capaldi *et al.*, 1983; Denis, 1986) or as few as 5 subunits are observed in some plants (Denis, 1986).

The holoenzyme, in addition to the polypeptide subunits, contains four electron acceptors, two heme groups and two copper atoms (Capaldi *et al.*, 1983; Denis, 1986). Subunit 1 is thought to be the site of attachment of hemes *a* and a_3 and one of the copper atoms (Denis, 1986). Subunit 2 is the site of attachment of the second copper atom and is the subunit that interacts with cytochrome *c* (Capaldi *et al.*, 1983; Denis, 1986).

Figure 1. Electron transport chain of *Neurospora* mitochondria. (References: Jackson and Lightbown, 1958; Lambowitz and Slayman, 1971; Stryer, 1975; Bertrand *et al.*, 1976; Tzagoloff, 1982).


amytal
rotenone
SHAM
Complex I
NADH
NADH-Coenzyme Q Reductase
Alternate Oxidase
Co Q
O_2
Succinate
Succinate-Co Q Reductase
cyt. b
cyt. c_1
cyt. c
cyt. aa_3
Complex II
Complex III
Complex IV
antimycin A
HNQO
azide
cyanide
carbon monoxide

Mitochondrial Import of Proteins

Pulse-labeling experiments in the presence of inhibitors like cycloheximide and chloramphenicol was the initial major source of information concerning the origin of mitochondrial polypeptides (Sebald *et al.*, 1973; Schatz and Mason, 1974). As little as 5% of the mitochondrial protein mass is synthesized on mitochondrial ribosomes. The bulk of mitochondrial proteins are encoded by the nucleus and transported from the cytoplasm into the mitochondria (Schatz and Mason, 1974; Schatz and Butow, 1983; Schatz, 1987). Cytoplasmically translated proteins are imported into the mitochondria and guided to one of the four major mitochondrial locations: the matrix, the inner membrane, the intermembrane space or the outer membrane (Schatz, 1987). The import of proteins into the mitochondria can be divided into four discrete steps: 1) mitochondrial recognition of precursor polypeptides, 2) translocation of the precursors across the membrane(s), 3) processing of the precursor proteins and, 4) their assembly into multimeric complexes (Harmey and Neupert, 1984).

The mitochondrial recognition of precursors is mediated by receptors located on the cytoplasmic surface of the outer membrane (Schatz and Butow, 1983; Harmey and Neupert, 1984). Protein receptors are specific for precursors; they do not bind mature polypeptides (Harmey and Neupert, 1984). Some receptors are specific for particular proteins, for example apocytochrome *c* has its own receptor (Zimmermann *et al.*, 1981; Hennig *et al.*, 1983). But most receptors are not so specific and they bind and translocate a number of different polypeptides (Harmey and Neupert, 1984; Hay *et al.*, 1984).

Proteins that bind to mitochondrial receptor molecules contain information necessary for intracellular targeting. This information is contained within amino-terminal presequences (Douglas *et al.*, 1986; Hurt and van Loon, 1986). These presequences function in intra-mitochondrial sorting and determine the final destination of the protein (Douglas *et al.*, 1986; Hurt and van Loon, 1986; Schatz, 1987). Matrix and inner membrane proteins have presequences that are rich in positively charged and hydroxylated amino acids and devoid of acidic residues (Hurt and van Loon, 1986). The presequences of intermembrane space

proteins, in addition to being rich in basic and hydroxylated amino acids, have a long stretch of uncharged amino acids (Hurt and van Loon, 1986). Proteins destined to the outer membrane lack cleavable presequences, but their amino-terminal sequences resemble the presequences of proteins destined for the intermembrane space (Hurt and van Loon, 1986).

Translocation of proteins to the outer membrane does not require a membrane potential, but the transport of polypeptides across the inner membrane is energy dependent (Schatz and Butow, 1983; Harmey and Neupert, 1984; Reid, 1984; Douglas *et al.*, 1986). Some precursors, like the yeast cytochrome b_2 and cytochrome c_1 polypeptides, are transported by a two step process to the intermembrane space, but most appear to be translocated in a single step. (Schatz and Butow, 1983; Reid, 1984).

Concomitant with translocation into the mitochondria, or shortly thereafter, the precursor polypeptides undergo some form of post-translational modification (Hay *et al.*, 1984). Many precursor proteins have their amino-terminal presequences cleaved by a protease (Böhni *et al.*, 1983; Cerletti *et al.*, 1983; Yaffe *et al.*, 1985; Pratje and Guiard, 1986). Other proteins undergo such post-translational modifications as heme attachment before assembly into protein complexes (Zimmermann *et al.*, 1979; Schatz and Butow, 1983; Hay *et al.*, 1984; Tzagoloff *et al.*, 1986).

Nuclear-Mitochondrial Interactions

Mitochondria are composed of hundreds of proteins, most of which are encoded by the nucleus (Schatz and Mason, 1974; Schatz, 1987). Nuclear mutants of yeast that carry defects affecting mitochondrial gene expression are referred to as *pet* mutants (Tzagoloff, 1982). Over 200 complementation groups have been identified for the collection of about 4000 different *pet* mutants (Michaelis *et al.*, 1982). The complementation groups were organized into three classes. Most mutants exhibited no detectable differences relative to the wildtype when the ^{35}S-labeled mitochondrial translation products were separated on LiDS polyacrylamide gels, while about 10% of the *pet* mutants had defects in mitochondrial protein synthesis (Michaelis *et al.*, 1982; Fox, 1986). A third class of *pet* mutants had either reduced

amounts or the complete absence of specific mitochondrially encoded proteins (Michaelis *et al.*, 1982; Fox, 1986). This latter class of *pet* mutants encodes proteins that regulate the expression of mitochondrial genes by specifically affecting a post-transcriptional step (Fox, 1986).

Nuclear mutations that prevent the translation of particular mitochondrial transcripts include the following: *cbp6*, *cbs1*, *cbs2*, *pet54*, *pet111* and *pet494*. The three mutants, *cbp6*, *cbs1* and *cbs2*, have defects in nuclear-encoded functions essential for the translation of cytochrome *b* transcripts (Dieckmann and Tzagoloff, 1985; Rödel *et al.*, 1985; Fox, 1986; Rödel, 1986; Rödel *et al.*, 1986). Both *PET54* and *PET494* genes encode products that permit translation of the coxIII message, while *PET111* specifically affects translation of the coxII mRNA (Costanzo and Fox, 1986; Costanzo *et al.*, 1986; Fox, 1986; Poutre and Fox, 1987).

A second class of nuclear genes encode products not required for the translation of mitochondrial messages, but are necessary for the post-translational processing of mitochondrial and nuclear precursor proteins. The *COR1* gene encodes a 44 kDa protein that processes apocytochrome *b* to mature cytochrome *b*, possibly through the addition of heme (Tzagoloff *et al.*, 1986). The *mas1* and *ts*2858 mutations prevent processing of precursor polypeptides by inhibiting, either directly or indirectly, the removal of transient presequences (Pratje *et al.*, 1983; Yaffe *et al.*, 1985; Pratje and Guiard, 1986). The *TS*2858 gene product appears to be required for the removal of presequences from the mitochondrially encoded cytochrome *c* oxidase subunit 2 polypeptide and the nuclear derived protein, cytochrome b_2 (Pratje *et al.*, 1983; Pratje and Guiard, 1986). The matrix protease, encoded by the *MAS1* gene, cleaves off amino-terminal extensions from imported mitochondrial precursor polypeptides (Böhni *et al.*, 1983; Yaffe *et al.*, 1985).

Other *pet* mutants have pleiotropic deficiencies in the respiratory chain, characteristic of defects in the mitochondrial transcriptional or translational apparatus (Michaelis *et al.*, 1982; Fox, 1986). Nuclear genes code for the majority of the components necessary for mitochondrial protein synthesis including most ribosomal proteins, and all aminoacyl-tRNA synthetases, initiation, elongation and termination factors (Schatz and Mason, 1974). A defect

in any one of these genes is expected to adversely affect mitochondrial gene expression, consequently leading to a respiratory-deficient phenotype. Recently, *pet* mutants with lesions in mitochondrial tRNA synthetases (Myers and Tzagoloff, 1985; Pape *et al.*, 1985; Natsoulis *et al.*, 1986; Chatton *et al.*, 1988) have been characterized.

Of particular interest was the finding that the *HTS1* gene in yeast encodes both the mitochondrial and cytoplasmic histidine-tRNA synthetases (Natsoulis *et al.*, 1986). The *HTS1* gene is transcribed from two alternate promoters to produce transcripts of differing length with different translational in-frame start codons. The longer message apparently encodes the mitochondrial synthetase, while the shorter transcripts specify the cytoplasmic histidine-tRNA synthetase function (Natsoulis *et al.*, 1986). Mutations affecting the upstream AUG translation initiation codon eliminate the mitochondrial synthetase function. This leads to a respiratory-deficient phenotype because of the adverse effects on mitochondrial translation, but the levels of cytoplasmic histidine-tRNA synthetase remain unaltered (Natsoulis *et al.*, 1986). A similar transcriptional pattern is observed for the yeast valyl-tRNA synthetases (Chatton *et al.*, 1988).

Recently, more nuclear mutants affecting the biogenesis of cytochrome oxidase in yeast were isolated. One class of mutants, consisting of 14 complementation groups, appears to be defective in the assembly of holocytochrome oxidase (McEwen *et al.*, 1986). Mutants in this class are deficient in cytochrome *c* oxidase activity, yet they contain apparently normal amounts of all cytochrome oxidase subunits. It has been proposed that the the primary defects in these mutants may affect heme biosynthesis, the presence of cardiolipin or some other function necessary for the assembly of holocytochrome oxidase (McEwen *et al.*, 1986).

Despite the overwhelming number of examples of nuclear genes regulating mitochondrial gene expression, as evident by the large number of *pet* complementation groups, there are indications that the converse may also be true. Mitochondria may export proteins that influence nuclear gene expression (Edwards and Rosenberg, 1976; Yaffe and Schatz, 1984; Fischer Lindahl, 1985; Parikh *et al.*, 1987). In mice, it is well documented that a class I cell surface antigen, *mta*, is maternally inherited (Fischer Lindahl and Bürki, 1982; Fischer

Lindahl *et al.*, 1983; Fischer Lindahl, 1985). The nuclear gene, *hmt*, is the structural gene for *mta* and it maps to the mouse major histocompatibility complex (Fischer Lindahl *et al.*, 1983; Fischer Lindahl, 1985). Its expression on the cell surface is regulated by a cytoplasmic factor, *mtf*. (Fischer Lindahl *et al.*, 1983; Fischer Lindahl, 1985). Specific *mtf* types are associated with mitochondrial DNA restriction fragment length polymorphisms (RFLP), thus supporting the view that *mtf* is encoded by the mitochondrial genome (Fischer Lindahl, 1985). The nature of *mtf* and its mode of action is not understood at present.

Edwards and Rosenberg (1976) concluded from their experiments studying the regulation of the alternate oxidase in *Neurospora* that a mitochondrial gene product regulated cyanide-insensitive respiration in a negative manner by acting at a site external to the organelle. There was no mention of the mechanism of action of the regulatory protein.

A final example, whereby the mitochondrial genotype influences nuclear gene expression, was observed in yeast. Specific nuclear mRNAs from different mitochondrial respiratory-deficient strains were differentially expressed among isonuclear yeast strains (Parikh *et al.*, 1987). Two classes of transcripts influenced by the mitochondrial genotype were detected. Class I transcripts were expressed at high levels in all respiratory-deficient strains examined, regardless of the nature of the mitochondrial mutation, while class II mRNAs were only abundantly expressed in ρ^- and ρ^0 strains, but not in *mit*$^-$ and ρ^+ strains. (Parikh *et al.*, 1987). This suggests that the type of mitochondrial lesion influences the levels of expression of class II genes. Cytochrome oxidase subunit 6 was found to be an example of a class II gene (Parikh *et al.*, 1987).

Evidence from mouse, *Neurospora* and yeast suggests that a mitochondrial function exerts its influence outside the organelle to regulate the expression of nuclear genes. Presently, there is no firm evidence to support this view. However, there are indications that genetic material has moved from the mitochondria to the nucleus (Timmis and Scott, 1984; Yaffe and Schatz, 1984). Examples of homologous mitochondrial DNA sequences found in the nuclear genome has been demonstrated for such diverse organisms as sea urchin (Jacobs *et al.*, 1983), locust (Gellissen *et al.*, 1983), human (Tsuzuki *et al.*, 1983), yeast (Hudson *et al.*, 1985),

Podospora (Wright and Cummings, 1983) and *Neurospora* (van den Boogaart *et al.*, 1982a).

In *Podospora*, mitochondrial DNA sequences (a-sen DNA) associated with senescence have been observed to transpose to the nucleus (Wright and Cummings, 1983; Timmis and Scott, 1984). The *mex1* strain, a *Podospora* mitochondrial mutant, lacks the mitochondrial DNA restriction fragment encoding the *oxi-3* gene and from which the a-sen DNA sequences are derived (Vierny *et al.*, 1982; Wright and Cummings, 1983). The excision and/or amplification of the a-sen DNA appears to play a role in senescence (Vierny *et al.*, 1982). The *mex1* mutant has transferred its subunit 1-encoding *oxi-3* gene to the nucleus and in the process has escaped senescence (Vierny *et al.*, 1982; Wright and Cummings, 1983). The nuclear *oxi-3* gene apparently provides the normally mitochondrially specified function (Wright and Cummings, 1983; Timmis and Scott, 1984).

The transfer of DNA between genomes may have also occurred in *Neurospora* (van den Boogaart *et al.*, 1982a; Yaffe and Schatz, 1984). Two copies of the ATPase 9 gene are present in *Neurospora* (van den Boogaart *et al.*, 1982a). The functional gene is located in the nuclear genome, while the mitochondrial version is apparently not expressed (van den Boogaart *et al.*, 1982a; Yaffe and Schatz, 1984).

Yeast Cytoplasmic Mutants

The field of extranuclear genetics in yeast thrived with the discovery of numerous cytoplasmic mutants. The mitochondrial variants of yeast have been classified into four families: the cytoplasmic or vegetative petites, the antibiotic-resistant mutants, the *mit* mutants and the *syn* mutants (Tzagoloff *et al.*, 1979; Tzagoloff, 1982).

The cytoplasmic petites consist of a heterogeneous group of extranuclear mutants characterized by respiratory deficiencies due to large deletions in the mitochondrial genome. The retained segment of mitochondrial DNA is amplified tandemly to produce a genome size similar to that of wildtype strains. Vegetative petites are often also deficient in mitochondrial protein synthesis and oxidative phosphorylation because the large deletions usually result in the loss of *syn* and *mit* genes, respectively (Schatz and Mason, 1974; Tzagoloff, 1982). Petites

are induced by such mutagens as ethidium bromide and acridine dyes (Tzagoloff, 1982).

The antibiotic-resistant (ant^R) mutants are resistant to various drugs and antibiotics, while wildtype cells are normally sensitive (ant^S) to these antibiotics. Thus, the ant^R mutants are able to grow on nonfermentable substrates in the presence of inhibitors. Different types of inhibitors are readily distinguishable by their modes of action. Certain drugs like antimycin A inhibit electron transport by interacting with specific cytochromes (Tzagoloff, 1982; Figure 1). Oligomycin, another drug, interferes with the ATPase complex and directly inhibits oxidative phosphorylation, while other inhibitors like chloramphenicol eliminate mitochondrial protein synthesis by interacting with ribosomal subunits (Tzagoloff, 1982). Ant^R mutants are easily obtainable by plating mutagenized yeast cells on nonfermentable media supplemented with an inhibitor (Tzagoloff, 1982).

Mit mutants are unable to grow on nonfermentable substrates because of point mutations in genes necessary for electron transport and oxidative phosphorylation. (Tzagoloff *et al.*, 1979; Tzagoloff, 1982). Phenotypically, *mit* mutants appear to be similar to vegetative petites, but further analysis reveals the fact that *mit* mutants are revertable and that they have retained their capacity for mitochondrial translation because their lesions are restricted to single loci (Tzagoloff, 1982).

The final class of yeast cytoplasmic variants consist of the *syn* mutants. These harbour point mutations in genes required for mitochondrial protein synthesis (Tzagoloff *et al.*, 1979). While both cytoplasmic petites and *syn* mutants have an impaired mitochondrial translation system, only *syn* mutants are revertable. This test readily distinguishes this class of mutants from the vegetative petites (Tzagoloff, 1982).

Neurospora Extranuclear Genetics

There is a distinct lack of respiratory mutants in *Neurospora* when compared with yeast. This is because of the powerful selection scheme that exists for finding respiratory mutants in yeast because yeast is a facultative anaerobe and Neurospora is an obligate aerobe. Mutations that completely eliminate mitochondrial functions in yeast are not lethal. Yeast

respiratory mutants are readily apparent on medium containing low levels of glucose and a high glycerol concentration (Tzagoloff, 1982). The mitochondrial mutants, unable to respire, stop growing when the supply of glucose is exhausted, while wildtype cells are not affected by the depletion of glucose. Respiratory-competent cells continue to grow to form large colonies. In contrast, *Neurospora* completely lacks the ability to ferment. Lesions that completely eliminate mitochondrial functions are therefore lethal. It is evident then, that any collection of *Neurospora* respiratory mutants consists of those having a reduction rather than a complete loss of mitochondrial function (Bertrand and Pittenger, 1972).

Attempts have been made to induce specifically extranuclear mutations in *Neurospora*. Such mutagens as acriflavine (Srb, 1958), UV (McDougall and Pittenger, 1966), MNNG (Bertrand and Pittenger, 1972) and 2-aminopurine (Rosenberg *et al.*, 197[illegible] have been used, but the lack of a suitable selection scheme has made it difficult to recover extranuclear mutants. Many techniques have been used either solely or in combinations to select for respiratory mutants of *Neurospora*: filtration concentration (Nargang and Bertrand, 1978; Pittenger and West, 1979), inositol-less death method (Edwards *et al.*, 1973; Rosenberg *et al.*, 1976; Pittenger and West, 1979), screening for cyanide-insensitive or salicylhydroxamate (SHAM)-sensitive respiration (Edwards *et al.*, 1973; Nargang and Bertrand, 1978; Pittenger and West, 1979; see Figure 1), reduction of dyes such as tetrazolium or sodium tellurite (Gillie, 1970; Edwards *et al.*, 1973; Rosenberg *et al.*, 1976; Nargang and Bertrand, 1978), addition of cycloheximide prior to mutagenesis (Rosenberg *et al.*, 1976), periods of growth interspersed with successive UV irradiation treatments (McDougall and Pittenger, 1966)and the continuous growth of *Neurospora* cultures (Bertrand and Pittenger, 1969).

None of these methods were very efficient at recovering respiratory-deficient mutants of any kind, but extranuclear mutants were still particularly rare. A number of factors, in addition to being an obligate aerobe, may account for the lack of cytoplasmic mutants in *Neurospora*. The larger genome size of the nucleus and the observation that most proteins found in the mitochondria are imported from the cytoplasm makes it difficult to specifically induce cytoplasmic variants. The majority of respiratory-deficient mutants recovered have

acquired mutations in the nuclear rather than mitochondrial genome.

Another important factor is the large number of mitochondria in coenocytic hyphae and conidia and the observation that each mitochondrion contains more than one molecule of DNA (Lewin, 1987). Consequently, in order for an extranuclear mutation to be expressed, it must be suppressive so as to produce a detectable mutant. This may not be compatible with the need to be a leaky mutant. Other factors such as the permeability of mitochondria to mutagens may also play a role.

Backer and Birky (1985) have concluded that in the absence of selection, random drift and random partitioning are the primary mechanisms in determining the fate of a new ant^R mutation in yeast. It is conceivable that similar mechanisms exist in *Neurospora* for fixing extranuclear gene mutations. Thus, it is likely that most mitochondrial mutations are lost rather than fixed. With such constraints regulating the fixation of cytoplasmic mutations, it is not surprising that there are few mitochondrial mutants in *Neurospora*, and that of the 16 extranuclear mutants classified by Bertrand and Pittenger (1972), 10 arose spontaneously. Because of the very low frequency of recovery of cytoplasmic mutants in their MNNG mutagenesis experiment, Bertrand and Pittenger (1972) could not exclude the possibility that the four mitochondrial variants recovered were also spontaneous rather than induced. Thus, as many as 14 of 16 cytoplasmic mutants may have arisen spontaneously.

Classification of Neurospora Extranuclear Mutants

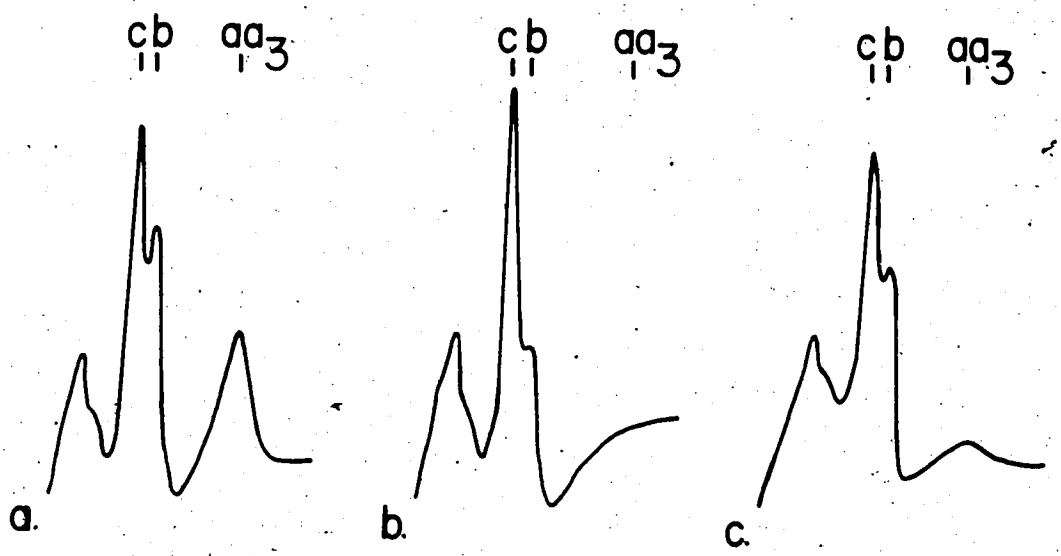
Bertrand and Pittenger (1972) and Bertrand *et al.* (1976) have proposed a scheme to classify *Neurospora* cytoplasmic mutants. The classification system proposes the establishment of four groups. Members of a group have identical or similar phenotypes and are suppressed by the same nuclear suppressor genes. Group I mutants are deficient in cytochrome aa_3 and *b*. The original *N. crassa* extranuclear mutant, [*poky*], is the best known member of this group, but eight other mutants are also included in this group (Mitchell and Mitchell, 1952; Bertrand and Pittenger, 1972; Bertrand *et al.*, 1976). The nature of the primary defect in [*poky*] and other group I mutants has been determined by Akins and Lambowitz (1984). A 4 bp deletion

at the 5' end of the 19S rRNA gene is present in [*poky*] and all other group I mutants examined. The lesion promotes the synthesis of aberrant 19S rRNA molecules and is sufficient to account for the pleiotropic effects observed in group I mutants. It is likely the primary defect affecting all group I mutants (Akins and Lambowitz, 1984). Variations in the phenotype of group I mutants are likely due to differences in nuclear background and/or to secondary lesions in the mitochondrial DNA since all are suppressed by the nuclear suppressor *f* gene (Mitchell and Mitchell, 1956; Bertrand *et al.*, 1976; Akins and Lambowitz, 1984). It has been suggested that all group I mutants may simply be reisolates of the original [*poky*] mutant (Akins and Lambowitz, 1984).

The extranuclear mutants, [*mi-3*] and [*exn-5*], are the only members of group II. Both of these mutants have deficiencies in cytochrome aa_3 (Figure 2). The [*mi-3*] mutant also has been shown to have reduced cytochrome *c* oxidase activity (Bertrand and Pittenger, 1972; Bertrand *et al.*, 1976). It is likely that [*exn-5*] is also deficient in this activity, but this has never been examined. The mutant phenotype of group II mutants is suppressible by *su-1*, a nuclear gene linked to *al-2* on linkage group I (Gillie, 1970; Bertrand and Pittenger, 1972; Bertrand *et al.*, 1976; Perkins *et al.*, 1982). However, differences between the two mutants are known to exist. For example, the induction of cytochrome aa_3 by the nuclear gene *cyb-1* or by antimycin A is characteristic of [*mi-3*] and not [*exn-5*] cultures. (Bertrand *et al.*, 1976; Bertrand and Collins, 1978; Bertrand, 1980).

Group III mutants have the "stopper" growth phenotype and are deficient in cytochromes aa_3 and *b* (Bertrand and Pittenger, 1972; Bertrand *et al.*, 1976). Stopper mutants contain insertions or deletions in their mitochondrial genome (Bertrand *et al.*, 1980). It has been suggested that the "stop-start" growth phenotype is a consequence of the competition between defective and less defective mitochondrial DNA species. The defective mitochondrial DNAs tend to predominate, but the less defective molecules must be retained to sustain growth (Bertrand *et al.*, 1980). These characteristics differentiate the group III mutants from the [*poky*] and [*poky*]-like variants.

Figure 2. Cytochrome spectra obtained from the mitochondria of a) wildtype b) [*mi-3*] and c) [*exn-5*] *Neurospora crassa* strains.


c b
11
aa3
1
a.
c b
11
aa3
1
b.
c b
11
aa3
1
c.

The sole representative of group IV is [*cni-3*]. This mutant has a full complement of mitochondrial cytochromes, but has a mutation that has affected the alternate oxidase system in a manner that allows it to be constitutively expressed (Lambowitz and Slayman, 1971; Edwards and Rosenberg, 1976; Rosenberg *et al.*, 1976; see also Figure 1). The experimental evidence suggested that a mitochondrially encoded protein regulates the alternate oxidase pathway by acting at a site external to the mitochondrion (Edwards and Rosenberg, 1976).

The [mi-3] Extranuclear Mutant

Since it was originally described (Mitchell *et al.*, 1953), the [*mi-3*] mutant has been studied sporadically. The mutant arose spontaneously from ascospores produced from a cross of wildtype Abbott 12*a* protoperithecia to wild 1400-4*A* and has a maternally inherited abnormal phenotype distinct from [*poky*] (Mitchell *et al.*, 1953). The [*mi-3*] mutant strains are deficient in both cytochrome *aa*$_3$ levels (Figure 2b) and in cytochrome *c* oxidase activity and have a growth rate intermediate between [*poky*] and wildtype strains (Mitchell *et al.*, 1953; Tissieres and Mitchell, 1954; Bertrand and Pittenger, 1972; Bertrand *et al.*, 1976).

Gowdridge (1956) forced heterokaryons between [*mi-3*] and [*poky*] and between [*mi-3*] and a wildtype strain. Of the 12 heterokaryons formed between the two cytoplasmic mutants, all but 2 acquired the [*mi-3*] phenotype suggesting that [*mi-3*] was suppressive over [*poky*]. When a wildtype strain was used instead of [*poky*], only 50% (5/10) of the heterokaryon cultures acquired the mutant phenotype. This indicated that [*mi-3*] mitochondria did not have the same advantage over wildtype mitochondria that they did over [*poky*]. From her results, Gowdridge (1956) concluded that [*poky*] and [*mi-3*] cytoplasms cannot interact in a heterokaryon to form a wildtype strain or one that has an intermediate phenotype.

A decade passed before further studies on [*mi-3*] appeared in the literature. Woodward and Munkres (1966) isolated mitochondrial structural protein (MSP) which had an apparent MW of 23,000 as judged by sedimentation equilibrium studies. When MSP from an [*mi-3*] strain was compared to wildtype MSP preparations in experiments measuring dissociation constants, it was found that the mutant MSP-NADH and MSP-malic dehydrogenase

complexes dissociated more rapidly. In addition, amino acid analyses revealed that [*mi-3*] strains contained one less tryptophan residue per mole of MSP when compared to four other strains. Fingerprinting of tryptic peptides revealed the absence of a tryptophan-containing peptide. The conclusion drawn was that [*mi-3*] had acquired a missense mutation in the MSP structural gene changing a normally specified tryptophan residue to some undetermined amino acid (Woodward and Munkres, 1966). Subsequent experiments questioned the validity of this conclusion by demonstrating that the MSP preparations consisted of a mixture of proteins most probably specified by both the nuclear and mitochondrial genomes (Sebald *et al.*, 1968). In 1972, a retraction was published (Zollinger and Woodward, 1972).

Lambowitz and Slayman (1971) showed the existence of an alternate oxidase system in [*poky*]. The [*mi-3*] mutant also respires by means of the alternate oxidase because it shows antimycin and cyanide-insensitive respiration (von Jagow *et al.*, 1973). The alternate oxidase system is also functional, albeit at a low level, in wildtype cells. (Lambowitz and Slayman, 1971; Figure 1). This level may be increased substanstially by supplementing the media with antimycin A, cyanide, oligomycin, ethidium bromide, chloramphenicol or starving the culture for copper (Lambowitz and Slayman, 1971; Schwab, 1973; Edwards *et al.*, 1974; Bertrand *et al.*, 1976; Edwards and Rosenberg, 1976; Szakacs and Bertrand, 1976). Juretić (1976) showed that a mutant blocked for the synthesis of phosphatidyl choline, *chol-1* (Luck, 1965), induces its mitochondrial alternate oxidase when starved for choline.

Preliminary experiments by Bertrand *et al.* (1976) indicated that [*mi-3*] and the more recently isolated group II mutant, [*exn-5*], have mutations which affect the regulation of cytochrome oxidase biosynthesis rather than structural components of cytochrome aa_3. This conclusion was based on the observation that a nuclear gene mutation, *cyb-1* (Bertrand *et al.*, 1977), that confers a cytochrome *b* deficiency, suppresses the [*mi-3*] cytochrome aa_3 deficiency. Suppression is not observed in *cyb-1* [*exn-5*] double mutants even though the cytochrome aa_3 deficiency of both group II mutants is efficiently suppressed by *su-1*. The *cyb-1* mutation also suppresses the cytochrome oxidase deficiency of *cyt-2-1*, a nuclear mutant deficient in cytochromes aa_3 and *c* (Mitchell *et al.*, 1953; Bertrand *et al.*, 1977; Bertrand and

Collins, 1978). The *cyt-2-1* mutant is now known to have a mutation in the gene encoding cytochrome *c* heme lyase (Drygas, M.E., R.A. Akins, A.M. Lambowitz and F.E. Nargang, in preparation).

Further experiments revealed that the induction of cytochrome aa_3 involves an indirect gene interaction since *cyb-2*[1] [*mi-3*] double mutants also have higher levels of cytochrome aa_3 than [*mi-3*] strains (Bertrand and Collins, 1978; Bertrand, 1980). It was shown that the suppressive interaction was likely related to the blockage of electron transport in complex III (cytochrome bc_1 segment) since antimycin A, an inhibitor of the electron transport chain in the cytochrome bc_1 region of the transport chain (Figure 1), also markedly stimulated the growth and partially relieved the cytochrome aa_3 deficiency of [*mi-3*] (Bertrand and Collins, 1978; Bertrand, 1980). Oligomycin, an inhibitor of the ATPase (Tzagoloff *et al.*, 1979; Tzagoloff, 1982) and 2-heptyl-4-hydroxyquinoline-*N*-oxide (HNQO) (Jackson and Lightbown, 1958) also induced the production of cytochrome aa_3 (Bertrand, 1980). Antimycin A, oligomycin and HNQO likewise suppressed the cytochrome aa_3 deficiency in *cyt-2-1* and the nuclear cytochrome aa_3-deficient mutant, *cya-3-16* (Bertrand *et al.*, 1977), but it did not stimulate their growth rates (Bertrand and Collins, 1978). The suppression not only resulted in the induction of cytochrome aa_3, but also reduced the excess levels of cytochrome *c* in [*mi-3*] and *cya-3-16* (Bertrand and Collins, 1978). The [*exn-5*] mutant and other nuclear cytochrome oxidase-deficient mutants were not suppressed by growth in media supplemented with these inhibitors, indicating that this is a locus-specific phenomenon (Bertrand and Collins, 1978; Bertrand, 1980). At about the same time, it was shown that the [*mi-3*] mutant had a deficiency of immunoprecipitable cytochrome oxidase subunit 2 (Bertrand and Werner, 1977) and accumulated a larger cytochrome oxidase subunit 1 polypeptide (Bertrand and Collins, 1978; Bertrand and Werner, 1979). The subunit 2 deficiency was alleviated in *cyb-1* [*mi-3*] double mutants (Bertrand and Collins, 1978). It was concluded that the [*mi-3*] mutant may have sustained a lesion in a locus regulating the biosynthesis of cytochrome oxidase.

[1]*Cyb-2* mutants have a nuclear mutation that leads to a complete absence of cytochrome *b* and reduced amounts of cytochrome aa_3 (Bertrand *et al.*, 1977).

These observations, the suppression by inhibitors of electron transport and the two *cyb* loci, the subunit 2 deficiency and the larger subunit 1 polypeptide, were taken as indications of a complex regulatory system that specifically controls the production of cytochrome oxidase in *Neurospora*. A basic model was formulated to account for these results (Bertrand and Collins, 1978). The model postulated the existence of at least two control circuits that regulate the production of cytochrome oxidase. The first circuit was thought to be involved in the constitutive production of cytochrome oxidase. Lesions in loci affecting this control circuit (*i.e.* [*mi-3*], *cyt-2-1*, *cya-3-16*) lead to a cytochrome oxidase deficiency (Bertrand and Collins, 1978). The second controlling element appears to increase cytochrome oxidase levels when there is an impairment of electron flow in the cytochrome bc_1 segment of the electron transport chain (Bertrand and Collins, 1978; Figure 1). This second circuit is responsible for the induction of cytochrome oxidase in antimycin A-supplemented cultures of [*mi-3*], *cyt-2-1* and *cya-3-16*. The same mechanism is thought to be involved in the suppression of the cytochrome oxidase deficiency by *cyb-1* and *cyb-2* loci (Bertrand and Collins, 1978). Other control circuits must exist in order to account for the other nuclear and cytoplasmic mutants and the observation that mutants having an abnormal complement of cytochromes have excess levels of cytochrome *c* (Bertrand and Collins, 1978; Bertrand, 1980). The latter indicates that some common parameter like electron transport is affected (Bertrand and Pittenger, 1972). Further evidence supporting this view comes from the observation that wildtype cultures grown in the presence of antimycin A and chloramphenicol have excess cytochrome *c* levels (Woodward *et al.*, 1970; Bertrand and Pittenger, 1972).

The [*mi-3*] mutant, as well as two nuclear mutants, *299-1* and *cyt-2-1*, accumulate a larger cytochrome oxidase subunit 1 polypeptide having a molecular weight of 45 kDa compared to the normal 41 kDa polypeptide (Bertrand and Collins, 1978; Bertrand and Werner, 1979). This larger polypeptide, demonstrated to be a precursor of mature subunit 1, was processed upon induction of cytochrome *aa*$_3$ with antimycin A in the [*mi-3*] mutant (Werner and Bertrand, 1979; Werner *et al.*, 1980). The 45 kDa precursor did not assemble into the cytochrome oxidase holoenzyme complex, but the 41 kDa mature subunit 1

polypeptide did associate with the other subunits (Bertrand and Werner, 1979; Werner and Bertrand, 1979). Pulse-labeling experiments by others demonstrated the immunodetectability of the subunit 1 precursor in wildtype cells (Van't Sant *et al.*, 1981; Van't Sant and Kroon, 1983). By altering the growth conditions of the wildtype (labeling experiments performed at 9°C or in the presence of chloramphenicol), they noticed an increase in the amount of precursor relative to the amount in control cells. They concluded that these conditions prevented the processing of mitochondrial precursors (Van't Sant and Kroon, 1983).

Edman degradation of the precursor and mature polypeptides isolated from wildtype cultures revealed that the 45 kDa precursor was blocked at its amino terminus with *N*-formylmethionine, while serine was the amino-terminal residue of the 41 kDa polypeptide (Werner *et al.*, 1980). These observations lead to the conclusion that the precursor polypeptide bore an amino-terminal extension of 25 to 35 amino acids which would account for the apparent 4000 MW difference (Werner *et al.*, 1980). Therefore, it was quite unexpected that only a 2 amino acid N-terminal prepiece was predicted on the basis of DNA sequence analysis of the subunit 1-encoding *oxi-3* gene (Burger *et al.*, 1982; de Jonge and de Vries, 1983; Figure 3). Additionally, the DNA sequence data revealed an unusual C-terminal extension of about 20 amino acids relative to the human and yeast subunit 1 polypeptides (Burger *et al.*, 1982; de Jonge and de Vries, 1983; Figure 4).

Finally, recent work has demonstrated that the *Neurospora* mitochondrial genome is probably transcribed from only a few promoters (Breitenberger *et al.*, 1985; Burger *et al.*, 1985). The *cob-oxi-3-URF1*-region is transcribed together on a single transcript that can be processed by the precise excision of tRNA molecules or at sites other than tRNA sequences (Burger *et al.*, 1985). The fact that there are so few *Neurospora* mitochondrial transcripts (Breitenberger *et al.*, 1985; Burger *et al.*, 1985), no introns in the [*mi-3*] *oxi-3* gene (Lemire and Nargang, 1986) coupled with the genetic and immunological studies on [*mi-3*], probably rules out transcriptional regulation as the affected process in either [*mi-3*] or [*exn-5*].

Figure 3. Amino acid sequence of the N-termini of cytochrome oxidase subunit 1 polypeptides from various species. The asterisks (*) denote the position of in-frame AUN codons. The filled square (■) denotes the position of the sole in-frame AUAA codon. The filled circle (●) denotes the methionine residue at -2 that is thought to be the N-terminal residue of the subunit 1 precursor polypeptide. The vertical arrow (↓) indicates the proteolytic cleavage site that gives rise to the mature subunit 1 polypeptide. The underlined amino acids indicate the homology to the subunit 2 presequence. (References: *N. crassa*: Burger *et al.*, 1982; de Jonge and de Vries, 1983; *A. nidulans*: Waring *et al.*, 1984; *C. reinhardtii*: Vahrenholz *et al.*, 1985; Boer and Gray, 1986; Kück and Neuhaus, 1986; *D. melanogaster*: de Bruijn, 1983; *D. yakuba*: Clary and Wolstenholme, 1983b, 1985; Bovine: Anderson *et al.*, 1982; Human: Anderson *et al.*, 1981; Maize: Isaac *et al.*, 1985; Mouse: Bibb *et al.*, 1981; Rat: Grosskopf and Feldmann, 1981; *S. cerevisiae*: Bonitz *et al.*, 1980; Wheat: Bonen *et al.*, 1987; *X. laevis*: Roe *et al.*, 1985).

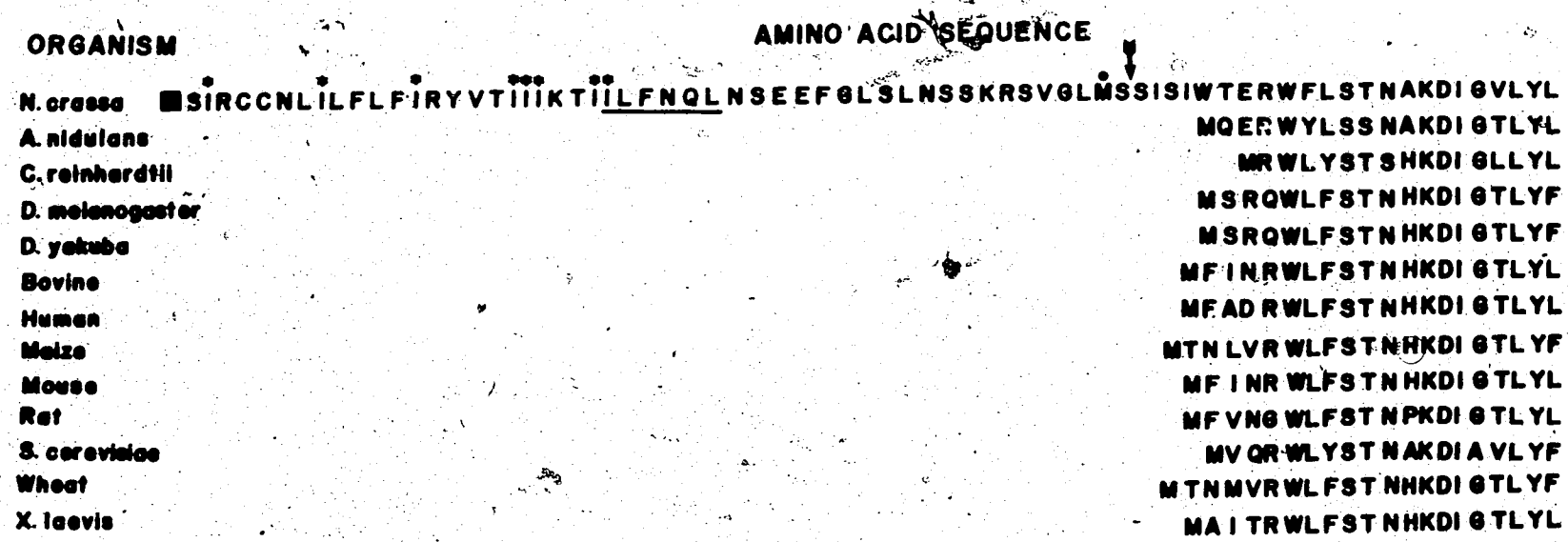

ORGANISM
AMINO ACID SEQUENCE
N. crassa ■SIRCCNLILFLFIRYVTIIIKTIILFNQLNSEEFGLSLNSSKRSVGLMSSISIWTERWFLSTNAKDIGVLYL
A. nidulans MQERWYLSSNAKDIGTLYL
C. reinhardtii MRWLYSTSHKDIGLLYL
D. melanogaster MSRQWLFSTNHKDIGTLYF
D. yakuba MSRQWLFSTNHKDIGTLYF
Bovine MFINRWLFSTNHKDIGTLYL
Human MFADRWLFSTNHKDIGTLYL
Maize MTNLVRWLFSTNHKDIGTLYF
Mouse MFINRWLFSTNHKDIGTLYL
Rat MFVNGWLFSTNPKDIGTLYL
S. cerevisiae MVQRWLYSTNAKDIAVLYF
Wheat MTNMVRWLFSTNHKDIGTLYF
X. laevis MAITRWLFSTNHKDIGTLYL

Figure 4. Amino acid sequence of the C-termini of various eukaryotic cytochrome oxidase subunit 1 polypeptides. The numbers at the end of each amino acid sequence denotes the length of each polypeptide. The exact length of the *S. pombe* and *P. anserina* subunit 1 polypeptides has not been determined because the genes have only been partially sequenced (References: *N. crassa*: Burger *et al.*, 1982; de Jonge and de Vries, 1983; *A. nidulans*: Waring *et al.*, 1984; Bovine: Anderson *et al.*, 1982; *C. reinhardtii*: Vahrenholz *et al.*, 1985; Boer and Gray, 1986; Kück and Neuhaus, 1986; *D. melanogaster*: de Bruijn, 1983; *D. yakuba*: Clary and Wolstenholme, 1983b, 1985; Human: Anderson *et al.*, 1981; Maize: Isaac *et al.*, 1985; Mouse: Bibb *et al.*, 1981; *P. anserina*: Jamet-Vierny *et al.*, 1984; Rat: Grosskopf and Feldmann, 1981; *S. cerevisiae*: Bonitz *et al.*, 1980; *S. pombe*: Lang, 1984; Wheat: Bonen *et al.*, 1987; *X. laevis*: Roe *et al.*, 1985).

ORGANISM	AMINO ACID SEQUENCE
N. crassa	EWSISSPPKPHSFASLPLQSSSFFLSFFRLSSYGEQKEISGRQN (555)
A. nidulans	EWCLTSPPKPHAFASLPLQS (523)
Bovine	EWLNGCPPPYHTFEEPTYVNLK (514)
C. reinhardtii	EWVLLATPAHHALSQVLRTASSH (505)
D. melanogaster	EWYQNTPPAEHSYSELPLLTN (511)
D. yakuba	EWYQNTPPAEHSYSELPLLTN (512)
Human	EWLYGCPPPYHTFEEPVYMKS (513)
Maize	EWLVQSPPAFHTFGELPTIKETRNQSSC (528)
Mouse	EWLHGCPPPYHTFEEPTYVKVK (514)
P. anserina	EWALSSPPKPHAFVSLPLQSNILRSLF
Rat	EWLHGCPPPYHTFEEPSYVKVK (514)
S. cerevisiae	EPLLTSPPAVHSFNTPAVQS (510)
S. pombe	EWLLHSPVHEHAFNTLPTKSI
Wheat	EWLVQSPPAFHTFGELPAVKETKS (524)
X. laevis	EWLQGCPTPYHTLKTSLVQINHQMIKS (519)

The Present Study

The studies undertaken in this thesis were designed to understand and characterize the nature of the [*mi-3*] and [*exn-5*] mutations. Examination of the [*mi-3*] *oxi-3* gene and its 5' flanking region was initiated on the basis that the mutant accumulated a subunit 1 precursor polypeptide that bore an amino-terminal extension (Bertrand and Werner, 1979; Werner and Bertrand, 1979; Werner *et al.*, 1980). This region was analyzed by examining restriction fragments on polyacrylamide gradient gels and by DNA sequence analysis. These approaches revealed the presence of an RFLP upstream of the coding region as well as a transversion mutation in the subunit 1-encoding *oxi-3* gene. Genetic studies with forced heterokaryons should reveal whether or not the subunit 1 missense mutation or the RFLP are related to the mutant phenotype.

The uncertainty about the primary structure of the subunit 1 polypeptide prompted efforts to resolve this problem through Western blot analysis of mitochondrial translation products using antisera raised against conjugated peptides homologous to specific regions of the presumed precursor polypeptide. Such experiments should permit determination of the processing steps required for maturation of the subunit 1 precursor polypeptide.

Previous studies on the [*exn-5*] mutant failed to reveal any clues as to which cytochrome oxidase component was affected in the [*exn-5*] mutant (Bertrand and Werner, 1979). Since the phenotypes of both [*mi-3*] and [*exn-5*] have several common aspects, and the [*mi-3*] mutant had acquired a mutation in the subunit 1 gene (Lemire and Nargang, 1986), it was conceivable that [*exn-5*] also had a mutation in the *oxi-3* gene. DNA sequence analysis of the [*exn-5*] col gene was undertaken as an initial step to characterize this mutant.

An attempt to clone and characterize the nuclear suppressor, *su-1* (Gillie, 1970), was also included in this study. This gene suppresses the group II extranuclear mutants, [*mi-3*] and [*exn-5*] (Bertrand *et al.*, 1976). Knowledge of the nature of the suppressive activity should give insight into some of the complex interactions important in the biogenesis of cytochrome oxidase. In addition, characterization of this gene may reveal clues as to the location of the [*exn-5*] lesion. Steps were taken to clone this gene using standard *Neurospora* transformation

procedures (Akins and Lambowitz, 1985; Vollmer and Yanofsky, 1986).

II. Materials and Methods

Media and Buffers

The composition of all media and buffers is given in the appendix.

E. coli Strains and Culture Conditions

Strains of *E. coli* used are described in Table 1. Bacterial strains were grown in L-broth (Lennox, 1955; see appendix) at 37°C in a shaker-incubator. Overnight cultures of

Table 1. Bacterial Strains.

Strain	Genotype	Reference
E. coli HB101	F⁻ *,hsdS20,recA13, ara-14,proA2,lacY1, galK2,xyl-5,rpsL20, mtl-1,supE44,λ⁻*	Maniatis *et al.*, 1982
E. coli JM103	*Δ(lac-proAB),supE,thi, strA,sbcB15,endA, [F'traD36,proAB, lacI^{q}ZΔM15]*	Messing, 1983; Yanisch-Perron *et al.*, 1985
E. coli JM83	*ara,Δ(lac-proAB),rpsL, ϕ80,lacZΔM15*	Yanisch-Perron *et al.*, 1985

JM103 were grown in DM Salts (Davis and Mingioli, 1950) containing thiamine and glucose (see appendix). Bacto-agar (Difco) was added to 1.5% (w/v) for solid media and to 0.7% (w/v) for soft agar. X-GAL (dissolved in *N,N*-dimethylformamide) and IPTG were added to a final concentration of 50 μg/mℓ and 25 μg/mℓ, respectively. Antibiotics, for selective purposes, were added as required (Table 2). For amplification of plasmids in liquid cultures,

chloramphenicol (dissolved in 95% ethanol) or spectinomycin were added to a final concentration of 170 $\mu g/m\ell$ and 300 $\mu g/m\ell$, respectively, when the culture's absorbance reached 0.6 A_{600} (Maniatis *et al.*, 1982). For long term storage of *E. coli* strains, glycerol stocks were prepared as described in Maniatis *et al.* (1982). An 850 $\mu\ell$ aliquot from a saturated overnight culture was added to a sterile microcentrifuge tube containing 150 $\mu\ell$ of sterile glycerol. Glycerol stocks were stored at -20°C or at -70°C.

Table 2. Antibiotics added to bacterial media.

Antibiotics	Stock Concentration ($mg/m\ell$)	Final Concentration ($\mu g/m\ell$)
ampicillin[1]	10	100
streptomycin[1]	100	100
tetracycline-HCl[1,2]	2	20
chloramphenicol[3]	34	20
kanamycin[4]	25	50

1. Dissolved in dH_2O, filter-sterilized and stored at 4°C.
2. Solution was light sensitive. Stored in the dark.
3. Dissolved in 95% ethanol and stored at -20°C.
4. Dissolved in dH_2O, filter-sterilized and stored at -20°C.

Plasmid and Cosmid DNA Vectors

Plasmids pBR322 (Bolivar *et al.*, 1977), pBR325 (Bolivar, 1978), pUC18 or pUC19 (Yanisch-Perron *et al.*, 1985), pKGS (Kuhn *et al.*, 1986) and the cosmid vector, pSV50 (Vollmer and Yanofsky, 1986) were used as cloning vectors (Table 3). Table 4 lists the recombinant plasmids obtained from others or constructed for use in this study.

Table 3. Plasmid and Cosmid Cloning Vectors.

Vector	Antibiotic Resistances	References
pBR322	Ap^R, Tc^R	Bolivar *et al.*, 1977
pBR325	Ap^R, Tc^R, Cm^R	Bolivar, 1978
pUC18	Ap^R	Yanisch-Perron *et al.*, 1985
pUC19	Ap^R	Yanisch-Perron *et al.*, 1985
pKGS	Km^R	Kuhn *et al.*, 1986
pSV50	Ap^R, Bn^R	Vollmer and Yanofsky, 1986

E. coli Transformation Procedure

E. coli strains were transformed by the procedure of Norgard *et al.* (1978). A 250 $\mu\ell$ inoculum from a fresh overnight culture was added to 25 mℓ of L-broth and incubated at 37°C with shaking until the absorbance of the culture reached 0.5 A_{650}. At this time, the cells were harvested in sterile Oak Ridge centrifuge tubes (7,000 rpm for 5 minutes)[2] and washed once with 10 mℓ of transformation mix #1 (100 mM NaCl, 5 mM Tris·HCl, pH 7.1[3], 5 mM $MgCl_2$). The cell pellet was resuspended in 10 mℓ of cold transformation mix #2 (100 mM $CaCl_2$, 250 mM KCl, 5 mM Tris·HCl, pH 7.1, 5 mM $MgCl_2$) and left on ice for 30 minutes to several hours. The cells were pelleted as before and resuspended in a small volume of transformation mix #2 (*ca.* 250 to 1000 $\mu\ell$). DNA (*ca.* 0.1 to 0.5 μg) was added to 250 $\mu\ell$ of competent cells and left on ice for 30 minutes. After heat-shocking the transformation mixture in a 45°C water-bath for 2 minutes, the transformation mixture was incubated at 37°C for 30 to 60 minutes after the addition of 1 mℓ of L-broth. Aliquots (25 to 125 $\mu\ell$) were plated on selective media.

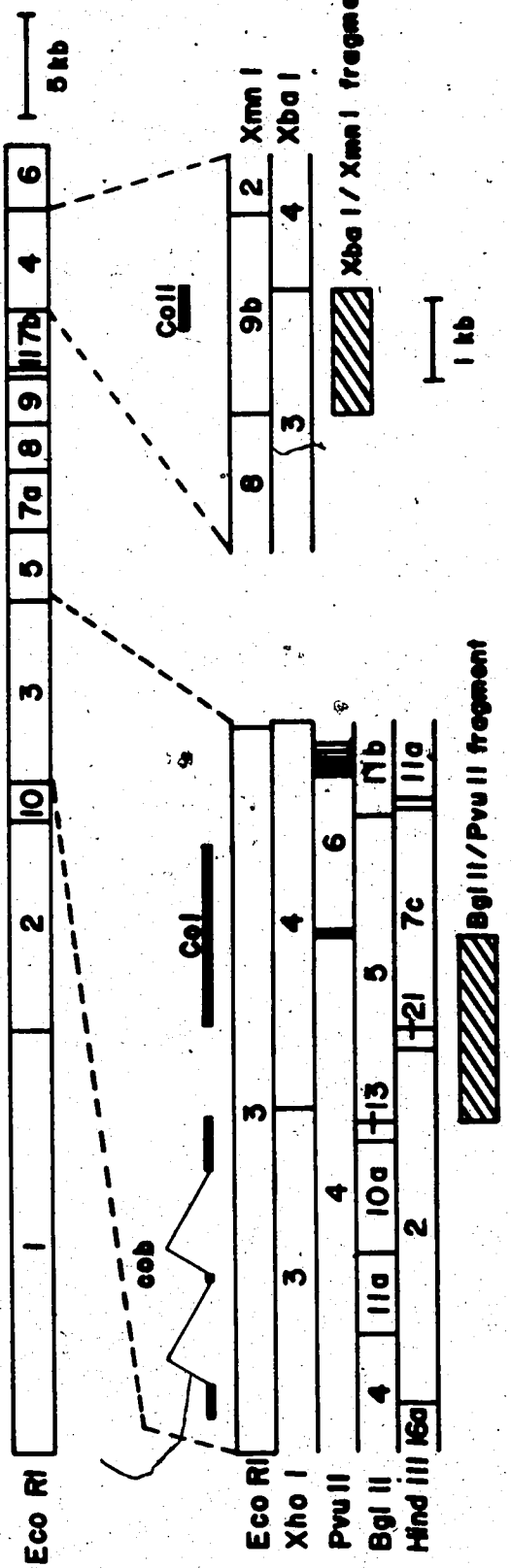
[2]Unless otherwise stated, centrifugation was in an SS-34 rotor (Sorvall) at 4°C.
[3]The pH of Tris buffers was adjusted at room temperature.

Table 4. Recombinant Plasmids.[1]

Plasmid	Description
pDH1	[*mi-3*] EcoRI-4 fragment in pBR325
pEL1	Abbott 12*a* EcoRI-4 fragment in pBR322
pEL2	[*mi-3*] BglII/PvuII fragment in pBR322
pEL3	Abbott 12*a* BglII/PvuII fragment in pBR322
pEL5	[*mi-3*] EcoRI/XhoI fragment in pBR322
pEL6	[*mi-3*] HindIII-7c fragment in pUC19
pEL7	10-9-1[2] EcoRI/XhoI fragment in pUC19
pEL8	[*exn-5*] EcoRI-4 fragment in pUC19
pEL9	[*exn-5*] XbaI/XmnI fragment in pKGS
pEL10	*nic 240* XbaI/XmnI fragment in pKGS
pEL11	10-45-3[2] XhoI-4 fragment in pUC19
pEL12	10-45-4[2] XhoI-4 fragment in pUC19
pEL13	10-45-1[2] XhoI-4 fragment in pUC19
pEL14	10-45-5[2] XhoI-4 fragment in pUC19
pEL16[3]	[*exn-5*] EcoRI-4 fragment in pUC19
pEL17	45-10-3[4] XhoI-4 fragment in pUC19
pEL164	pSV50 cosmid clone containing *AL-2* gene
pFN1	[*mi-3*] EcoRI-4 fragment in pBR322
pFN2[5]	[*mi-3*] EcoRI-3 fragment in pBR322
pFN3	10-45-7[2] HindIII-7c fragment in pUC19
pHBE3[6]	74*A* EcoRI-3 fragment in pBR322
pHBE4[6]	74*A* EcoRI-4 fragment in pBR322
pLN1	Abbott 12*a* EcoRI-3 fragment in pBR322
pLN2	[*mi-3*] EcoRI-3 fragment in pBR322
pPK1	[*exn-5*] EcoRI-3 fragment in pUC18

1. See Figure 5 for a description of the cloned fragments.
2. Slow-growing heterokaryon formed when [*mi-3*] and *nic* 240 were superimposed on minimal media.
3. Independently cloned from pEL8.
4. Fast-growing heterokaryon formed when [*mi-3*] and *nic* 240 were superimposed on minimal media.
5. An extra EcoRI fragment was inadvertently cloned along with the EcoRI-3 fragment.
6. A generous gift from H. Bertrand, University of Guelph.

Figure 5. Partial restriction map of the regions of the mitochondrial genome encoding the *oxi-3* (coI) and *oxi-1* (coII) genes. (References: Agsteribbe *et al.*, 1980; Macino, 1980; Burger *et al.*, 1982; Citterich *et al.*, 1983; de Jonge and de Vries, 1983; Macino and Morelli, 1983; de Vries *et al.*, 1985; Taylor and Smolich, 1985; Burger and Werner, 1986).


5 kb
1 kb
Eco RI
Xho I
Pvu II
Bgl II
Hind III
Eco RI
Xmn I
Xba I
Col I
Col II
Xba I / Xmn I fragment
Bgl II / Pvu II fragment
eob

Rapid Plasmid DNA Isolation

For analytical purposes, plasmid DNA was isolated by a modification of the alkaline-lysis procedure of Birnboim and Doly (1979). The cells from 5 mℓ of a plasmid-harboring *E. coli* overnight culture grown in L-broth containing the appropriate antibiotic were pelleted (7,000 rpm for 5 minutes). The supernatant was discarded, the pellet was suspended in 200 μℓ of cold 50 mM glucose, 25 mM Tris·HCl, pH 7.5, 10 mM EDTA and transferred to a microcentrifuge tube. A 400 μℓ aliquot of a freshly-prepared solution of alkaline-SDS (0.2 M NaOH, 1% (w/v) SDS) was added. The tube was mixed well by inversion and left on ice for 5 minutes. The addition of 300 μℓ of cold 3 M sodium acetate, pH 4.8, precipitated the proteinaceous material and non-supercoiled DNA. After 10 minutes on ice, the tube was centrifuged in a microcentrifuge[4] for 5 minutes. The supernatant (750 μℓ) was transferred to a fresh tube and the DNA was precipitated by the addition of 450 μℓ of cold isopropanol. After 5 minutes at -20°C, the DNA was pelleted by centrifugation (5 minutes in a microcentrifuge). The supernatant was removed by aspiration and the pellet was resuspended in 200 μℓ of water. The DNA was reprecipitated by filling the tube with cold 95% ethanol following the addition of 100 μℓ of cold 7.5 M ammonium acetate, pH 7.5. The tube was mixed by inversion and centrifuged for 5 minutes. The supernatant was removed by aspiration and the pellet was dried in a vacuum dessicator. The nucleic acid pellet was suspended in 100 μℓ of water and 5 to 10 μℓ was used in a 50 μℓ digest containing RNase A (2 μℓ of a 10 mg/mℓ solution; Maniatis *et al.*, 1982).

When the DNA was to be used in *Neurospora* transformation experiments, the rapid plasmid procedure was modified slightly. The procedure was followed exactly as described above except that the nucleic acid pellet at the end of the procedure was resuspended in 100 μℓ of cold 50 mM glucose, 25 mM Tris·HCl, pH 7.5, 10 mM EDTA instead of 100 μℓ of dH_2O. A second extraction was performed on the nucleic acid pellet similar to the procedure described by Ahmed (1987). To this solution was added 200 μℓ of alkaline-SDS. Following a

[4]Unless otherwise indicated, centrifugation in the microcentrifuge was carried out at room temperature.

5 minute incubation at room temperature, 150 $\mu\ell$ of cold 3 M sodium acetate, pH 4.8 was added. The resulting solution was mixed and allowed to sit for 5 minutes at room temperature. The contents of the tube were centrifuged in a microcentrifuge for 5 minutes. The supernatant was transferred to another tube and the nucleic acids were precipitated by the addition of 2 volumes of cold 95% ethanol. Following a 5 minute incubation step on ice, the nucleic acids were recovered by centrifugation in a microcentrifuge for 10 minutes. The ethanol was decanted and the pellet was dried briefly under vacuum. The pellet was resuspended in 250 $\mu\ell$ of dH_2O. An equal volume of cold 5 M LiCl, 50 mM Tris·HCl, pH 7.5 was added. The tube was mixed well by inversion and left for 5 minutes on ice. The tube was centrifuged for minutes in a microcentrifuge and the RNA pellet was discarded. The DNA was recovered after the addition of two volumes of cold 95% ethanol to the supernatant and centrifugation in a microcentrifuge for 10 minutes. The pellet was washed with cold 70% ethanol, resuspended in dH_2O and precipitated again with 95% ethanol. The pellet was resuspended in 100 $\mu\ell$ of dH_2O and used in *Neurospora* transformation experiments.

Large-Scale Plasmid and Cosmid DNA Isolations

Plasmid DNA was isolated by a modification of the procedure of Kahn *et al.* (1979). Cells from 1 ℓ of an amplified culture were pelleted at 4°C in either a GSA or GS-3 rotor (5,000 rpm for 5 minutes) and resuspended in 2 mℓ of 25% (w/v) sucrose, 50 mM Tris·HCl, pH 7.5. A small amount of lysozyme (*ca.* 30 mg) was dissolved in the cell suspension and left for 10 minutes on ice. A solution of EDTA, pH 8.0 (2 mℓ) was added and the mixture was left on ice for 10 minutes. Cells were lysed by the addition of 4 mℓ of 4% (v/v) triton X-100. After incubating on ice for 10 minutes, the visibly viscous solution was centrifuged (18,000 rpm for 30 minutes). The supernatant was decanted to a fresh centrifuge tube. Cesium chloride (1 g for every mℓ of solution) and ethidium bromide (600 $\mu\ell$ of a 10 mg/mℓ solution) were added to the supernatant and mixed until the cesium chloride was thoroughly dissolved. The solution was left in the dark at room temperature for 15 minutes. This solution

was then centrifuged (15,000 rpm for 15 minutes) to pellet the insoluble material. The supernatant was transferred to 13 X 51 mm Quick-Seal™ tubes (Beckman) and the plasmid DNA was banded by equilibrium-density centrifugation in a VTi65 rotor (Beckman) for 6 hours at 54,000 rpm (at 20°C).

Cosmid DNA or plasmid DNA was also isolated by the SDS-lysis or alkaline-lysis procedures (Maniatis *et al.*, 1982) with minor modifications. In the SDS-lysis procedure, the cells from a 1 ℓ saturated culture were washed once with 150 mM NaCl, 10 mM Tris·HCl, pH 7.3, 1 mM EDTA and resuspended in 20 mℓ of 10% (w/v) sucrose, 50 mM Tris·HCl, pH 7.5. A 4 mℓ aliquot of a freshly-prepared lysozyme solution (10 mg/ml in 0.25 M Tris·HCl, pH 7.5) and 16 mℓ of 0.25 M EDTA, pH 8.0 were added and mixed well by inversion. After 10 to 30 minutes on ice, 8 mℓ of 10% (w/v) SDS was added. After a quick, but gentle mixing, 12 mℓ of 5 M NaCl was added. The solution was mixed gently and placed in an ice-water bath for 1 hour. The high molecular weight DNA was pelleted by centrifuging the mixture (18,000 rpm for 30 minutes). The supernatant was decanted to a clean tube and the DNA was precipitated by the addition of 0.6 volumes of cold isopropanol. After incubating on ice for 10 minutes, the DNA was recovered by centrifugation (12,000 rpm for 10 minutes). The supernatant was discarded and the pellet was resuspended in 7 mℓ of distilled water. The DNA was banded in CsCl-ethidium bromide gradients as described previously.

In the alkaline-SDS procedure, cells from a 1 ℓ saturated culture were suspended in 20 mℓ of 50 mM glucose, 25 mM Tris·HCl, pH 7.5, 10 mM EDTA. Lysis was achieved by mixing in 40 mℓ of a freshly-prepared solution of 0.2 M NaOH, 1% (w/v) SDS. After 10 minutes on ice, the solution was neutralized by the addition of 30 mℓ of cold 3 M sodium acetate, pH 4.8, mixing by inversion and incubating on ice for 10 minutes. The high molecular weight DNA was pelleted by centrifugation at 4°C in an SW-28 rotor (Beckman) at 28,000 rpm for 30 minutes. The supercoiled DNA was precipitated from the supernatant by the addition of 0.6 volumes of cold isopropanol. The DNA was recovered by centrifugation, resuspended in water and banded in a CsCl-ethidium bromide gradient as described above.

DNA collected from CsCl-ethidium bromide gradients was extracted several times with salt-saturated isopropanol (see appendix) to remove the ethidium bromide. CsCl was removed by dialysis against water. The DNA solution was extracted twice with water-saturated phenol and once with chloroform/iso-amyl alcohol (24/1) (v/v) before recovering the DNA by ethanol precipitation.

M13 Transfection

The M13 replicative form or M13 single-stranded DNA was transfected into JM103 as previously described in the *E. coli* transformation protocol (Norgard *et al.*, 1978). After the addition of DNA to the competent cells and incubating on ice for 30 minutes, aliquots of the transfection mixture were added to 3 mℓ of soft agar supplemented with 250 μℓ of a saturated JM103 culture, 10 μℓ of sterile IPTG solution (25 mg/mℓ) and 50 μℓ of X-GAL solution (25 mg/mℓ in *N,N*-dimethylformamide) and overlayed onto L-broth plates. The plates were incubated at 37°C for at least 6 hours.

Isolation of M13 Single-Stranded DNA

M13 single-stranded DNA was isolated by a modified version of the following procedure (M13 Cloning and Sequencing System: A Laboratory Manual, New England BioLabs, Inc.). Individual plaques were picked using sterile pasteur pipets and blown into separate 250 mℓ Erlenmeyer flasks containing 25 mℓ of L-broth inoculated with 100 μℓ of cells from a JM103 overnight culture. The culture was incubated at 37°C with vigorous shaking for 8 to 11 hours. The cells were pelleted by centrifugation (15,000 rpm for 30 minutes). The supernatant was carefully decanted to a fresh tube, taking care to avoid disturbing the pellet. To the supernatant was added 6 mℓ of 10% (w/v) PEG-8000, 2.5 M NaCl. After mixing, the solution was incubated on ice for at least 30 minutes. The precipitated phage was pelleted (10,000 rpm for 20 minutes) and the supernatant was discarded. The pellet was resuspended in 650 μℓ of 0.3 M NaCl, 0.1 M Tris·HCl, pH 7.3, 1 mM EDTA. To this was added 10 μℓ of

a 10% (w/v) SDS-Solution and 10 $\mu\ell$ of protease K Solution (see appendix) followed by a 30 minute incubation at 37°C. The solution was extracted twice with water-saturated phenol and once with chloroform/iso-amyl alcohol (24/1) (v/v). The single-stranded DNA was precipitated by the addition of 1 mℓ of 95% ethanol. The DNA was recovered by centrifuging in a microcentrifuge for 5 minutes. The pellet was dried under vacuum and resuspended in 200 $\mu\ell$ of water. The amount of single-stranded DNA to be used in a hybridization reaction was estimated by running 2 $\mu\ell$ aliquots on a 0.8% agarose gel along with standards whose DNA concentration were known.

Isolation of the M13 Replicative Form

A single plaque was added to 25 mℓ of L-broth containing 100 $\mu\ell$ of inoculum from a fresh JM103 saturated culture. After 2 to 3 hours at 37°C with vigorous shaking, this was used to inoculate 500 mℓ of L-broth. A 1 mℓ inoculum from a JM103 saturated culture was also added and the culture was incubated at 37°C for 10 hours with vigorous shaking. The M13 replicative form was isolated from the cells by the SDS-lysis or alkaline-SDS procedures as described previously.

Neurospora crassa Strains and Culture Conditions

Strains of *N. crassa* used are described in Table 5. The Abbott 12*a* strain was obtained from the Fungal Genetics Stock Center (FGSC). It is the wildtype protoperithecial parent of the original [*mi-3*] mutant (Mitchell *et al.*, 1953). It became apparent during the course of the study that this was not a *bona fide* Abbott 12*a* strain, but this strain was used as a type I mitochondrial DNA standard since it appears to be wildtype in all respects (Lemire and Nargang, 1986; Newmeyer *et al.*, 1987).

Vegetative propagation of *Neurospora* was on modified Vogel's medium (Davis and de Serres, 1970; see appendix) containing 1.5% (w/v) agar (Sigma). The culture was incubated at 30°C until the mycelial growth was deemed sufficient, at which time they were

Table 5. *Neurospora crassa* Strains.

Strain	Genotype	Source
74-OR23-1*A* (74*A*)	*A*	H. Bertrand
UC-40 ([*mi-3*])	*A*, *pan-2*, [*mi-3*]	H. Bertrand
nic 240	*A*, *nic-1*, *al-2*	H. Bertrand
Abbott 12*a*	*A*	FGSC #351
[*exn-5*]	*a*, *nic-1*, *al-2*, [*exn-5*]	H. Bertrand
su-1, [*mi-3*]	*a*, *pan-2*, su^{mi-3}-*1*, [*mi-3*]	H. Bertrand
nic 237	*a*, *nic-1*, *al-2*	H. Bertrand
arg-6	*A*, *arg-6*	FGSC #266
4003-HU-2*a*	*a*, *pan-1*, *al-1*	H. Bertrand
al-1	*A*, *al-1*	FGSC #901
cyh-1	*a*, *cyh-1*	FGSC #4013
hom	*a*, *hom*	FGSC #282
un-7	*a*, *un-7*	FGSC #2176

taken out and exposed to light to induce conidiation. Liquid Vogel's medium was inoculated with conidia (*ca.* 10^6/mℓ) and incubated at 25 to 30°C in a shaker-incubator until the culture reached saturation.

Silica Gel Preservation of Neurospora Strains

Neurospora strains were preserved on silica gel as described by Davis and de Serres (1970). Two dram screw-cap vials half-filled with silica gel (grade H, type II, 6-12 mesh) were placed in an oven at 180°C with the caps loosened to activate the silica gel. After 90 minutes the vials were removed from the oven and allowed to cool with the caps tightened. Conidia from fresh slants (slants < 10 days old) were suspended in 1 to 2 mℓ of a sterilized 10% (w/v) solution of reconstituted skim milk. The conidial suspension was added to the

activated silica gel; enough suspension was added to be fully absorbed by the silica gel. After adding the conidial suspension, the vial was quickly transferred to an ice-water bath for 10 minutes. The vial was vortexed vigorously and stored at room temperature. After one week, if the grains of silica did not appear dry or if growth was evident, the vials were discarded. If these criteria were met, a grain or two of the silica stock was transferred to solid Vogel's medium to check viability. Stocks were stored at -20°C in a sealed container containing silica gel.

Heterokaryon Construction

Heterokaryons were forced by superimposing conidia from two different auxotrophs onto unsupplemented media (Bertrand and Pittenger, 1969). When strains containing differences in their mitochondrial DNA were forced as heterokaryons, they were subcultured for a number of generations to allow them to become homoplasmic (Lemire and Nargang, 1986).

Neurospora Genetic Crosses

The protoperithecial parent was inoculated on modified crossing media (Davis and de Serres, 1970; see appendix) and stored in the dark at room temperature for 1 to 2 weeks. Mutant strains were incubated at 30°C to accelerate their growth and then removed to room temperature to permit protoperithecial development. Fresh conidia from the prospective male parent was spread over the protoperithecia. Fertile matings showed significant development and darkening of perithecia as soon as 12 hours after fertilization. Approximately 2 weeks after fertilization, the asci matured and ejected their ascospores. Ascospores were collected using a sterile wooden applicator stick and transferred to sterile water. They were activated by heat-shocking at 55°C for 1 hour. Aliquots were plated on supplemented solid Vogel's media and incubated at 30°C for about 12 hours. Germinated spores were transferred individually to supplemented Vogel's slants and then checked for nutritional requirements.

Mitochondrial DNA Isolation

The mycelia from an early stationary or late log phase liquid culture of *N. crassa* was harvested by vacuum filtration in a Buchner funnel. All subsequent steps were performed at 4°C unless stated otherwise. The mycelia was ground using a mortar and pestle, acid-washed sand (see appendix) and a minimal volume of grinding buffer (15% sucrose, 50 mM Tris·HCl, pH 7.3, 0.25 mM EDTA) until a smooth paste was formed. The ground mycelia was suspended in grinding buffer and centrifuged at 3,000 rpm for 10 minutes to pellet the sand and cellular debris. The supernatant was layered onto a step gradient consisting of layers of 2.0 M sucrose and 0.8 M sucrose, each made in 50 mM Tris·HCl, pH 7.3, 0.25 mM EDTA (Nargang and Bertrand, 1978). The gradient was centrifuged at 18,000 rpm for 1 hour and the mitochondria were collected at the interface of the 0.8 M and 2.0 M sucrose layers. The mitochondria were diluted with 100 mM Tris·HCl, pH 7.1, 5 mM EDTA and pelleted by centrifugation (12,000 rpm for 30 minutes).

Alternatively, the mitochondria were purified by flotation-gradient centrifugation as described by Lambowitz (1979) with minor modifications. After the initial centrifugation to pellet the sand and debris, the supernatant was centrifuged at 15,000 rpm for 40 minutes to pellet the mitochondria. The crude mitochondrial pellet was suspended in 60% (w/v) sucrose, 50 mM Tris·HCl, pH 7.3, 0.25 mM EDTA, ensuring that the mitochondrial suspension was denser than the 55% (w/v) sucrose layer. If required, the density of the mitochondrial suspension was increased by the addition of 80% (w/v) sucrose, 50 mM Tris·HCl, pH 7.3, 0.25 mM EDTA. Aliquots (*ca.* 10 ml) of 55% (w/v) Sucrose, 50 mM Tris·HCl, pH 7.3, 0.25 mM EDTA and 44% (w/v) Sucrose, 50 mM Tris·HCl, pH 7.3, 0.25 mM EDTA were the other components of the gradient. Flotation gradient centrifugation was performed in either an SW-28 rotor (Beckman) at 28,000 rpm for 150 minutes or in an SW-40 rotor (Beckman) at 40,000 rpm for 90 minutes. Gradient-purified mitochondria were diluted with excess 100 mM Tris·HCl, pH 7.1, 5 mM EDTA and pelleted (12,000 rpm for 30 minutes).

Mitochondrial DNA was isolated as described (Lemire and Nargang, 1986). The mitochondrial pellet was resuspended in 3 mℓ of 100 mM Tris·HCl, pH 7.1, 5 mM EDTA. SDS was added to a final concentration of 1% (w/v). The sample was extracted twice with water-saturated phenol and once with chloroform/iso-amyl alcohol (24/1)(v/v). Cesium chloride (1 g for every mℓ of supernatant) and ethidium bromide (30 μℓ of a 10 mg/mℓ solution) were added and the DNA was banded by equilibrium-density centrifugation as described previously. Both bands (corresponding to supercoiled and linear/nicked DNA) were collected. The ethidium bromide and cesium chloride were removed as previously mentioned.

Cytochrome Spectral Analysis

Cytochrome spectra were obtained by a modified version of the Bertrand and Pittenger (1969) procedure using a Perkin-Elmer Model 559 or a Shimadzu UV-265 recording spectrophotometer. Crude or gradient-purified mitochondria were suspended in 3 mℓ of 2.5% (w/v) sodium deoxycholate, 10 mM Tris·HCl, pH 7.5, 5 mM EDTA. The dissolved mitochondria were centrifuged for 5 minutes in a microcentrifuge. The supernatant was transferred to spectrophotometer cuvettes. The baseline was obtained by scanning the air-oxidized samples from 650 nm to 500 nm. A few crystals of potassium ferricyanide was added to the reference cuvette to ensure complete oxidation of its contents and the solution in the sample cuvette was reduced with a few grains of sodium dithionite. The scan from 650 nm to 500 nm was repeated. The α absorption peaks for cytochrome aa_3, cytochrome *b* cytochrome *c* are at 608 nm, 560 nm and 550 nm, respectively (Bertrand and Pittenger, 1972).

Preparation of Neurospora Sphaeroplasts

Sphaeroplasts were prepared essentially as described by Akins and Lambowitz (1985). A conidial suspension in sterile water was prepared using fresh conidia (< 2 weeks old) and its concentration was determined using a haemocytometer. A 2 ℓ flask containing 1 ℓ of 0.5X Vogel's (see appendix) was inoculated with the conidial suspension to give a final

concentration of 5 X 10^6 to 1.5 X 10^7 conidia/mℓ. The culture was incubated at 25 to 30°C with gentle shaking (*ca.* 200 rpm). Once greater than 80% germination was achieved as judged by counting in a haemocytometer the number of germinated conidia as a fraction of total conidia, the conidia were harvested in sterile GS-3 centrifuge bottles. (5,000 rpm for 10 minutes). The conidia were washed once with sterile dH_2O and twice with sterile 1 M sorbitol. After the final wash the conidia were resuspended in about 9 mℓ of sterile 1 M sorbitol. The concentration of the conidial suspension was determined and adjusted, if necessary with sterile 1 M sorbitol, to reduce the concentration to below 1 X 10^9 conidia/mℓ. The conidial suspension was transferred to a sterile 250 mℓ Erlenmeyer flask and Novozym™ 234 (Novo Laboratories Inc.) was added to 15 mg/mℓ. The solution was incubated at 30°C with gentle agitation (*ca.* 100 rpm) for 1 to 2 hours. The extent of sphaeroplasting was monitored microscopically by determining the ratio of ghosts to total conidia plus ghosts in an SDS-treated sample. The desired level of sphaeroplasting was ≥ 90%. After the sphaeroplasting step was completed, the sphaeroplasts were pelleted in a clinical centrifuge (10 minutes at the lowest speed) and washed twice with sterile 1 M sorbitol and once with sterile 20 mM MOPS, pH 6.3, 50 mM $CaCl_2$, 1 M sorbitol (MCS). The sphaeroplasts were suspended in 6 to 8 mℓ of sterile MCS and the sphaeroplast concentration was adjusted to between 2.5 to 5 X 10^8 sphaeroplasts/mℓ. For each mℓ of sphaeroplast solution the following was added: 275 $\mu\ell$ of sterile 40% (w/v) PEG-4000, 20 mM MOPS, pH 6.3, 50 mM $CaCl_2$ (PMC), 13 $\mu\ell$ of dimethylsulfoxide and 65 $\mu\ell$ of a sterile heparin solution (5 mg/mℓ). The mixture was gently mixed and the sphaeroplasts were transferred to sterile microcentrifuge tubes and stored at -70°C. The sphaeroplast viability was determined by plating dilutions onto viability plates (see appendix) using top agar lacking benomyl (see appendix). Viability frequencies of greater than 10^7/mℓ were expected.

Neurospora Transformation Procedure

Neurospora transformations followed the sib-selection procedure of Akins and Lambowitz (1985) using a *Neurospora* genomic library constructed in the cosmid vector, pSV50 (Vollmer and Yanofsky, 1986). The frozen sphaeroplasts (6×10^6 to 3.5×10^7 viable sphaeroplasts/μg DNA) were thawed on ice. About 2.5 μg of DNA per reaction was used in the first two rounds of transformation. The amount of DNA used decreased in subsequent rounds of transformation because of its decreasing complexity. The DNA, in a volume equal to 60% of the sphaeroplast volume, was added to the thawed sphaeroplast solution and incubated on ice for 30 minutes after mixing gently. Sterile PMC (10X the sphaeroplast volume) was added to the transformation mixture and left on ice for 20 minutes at room temperature. The transformation mixture was added to top agar (*ca.* 10 mℓ/plate; see appendix). Plates were incubated at 30 to 42°C.

Alternatively, if the conidiation of the transformants was important, the following procedure was used. The transformation mixture was added to 30 mℓ of regeneration media (see appendix) and incubated at 25°C for 4 hours with gentle agitation (*ca.* 100 rpm) The cells were harvested in a sterile Oak Ridge centrifuge tube (7,000 rpm for 10 minutes) and washed once with 0.5X Vogel's. The supernatant was removed by aspiration and the pellet was suspended in about 1 mℓ of 0.5X Vogel's medium. Aliquots were spread on benomyl plates and the plates were incubated at 30°C. After several days the plates were removed to the light for conidiation.

Conjugated Peptides

Peptides homologous to three regions of the presumed cytochrome oxidase subunit 1 precursor were conjugated to keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) (Figure 6). The KLH conjugates were used to elicit an immune response in rabbits, while the BSA conjugates were used as antigens in the indirect ELISA assays. The conjugates were synthesized by the Alberta Peptide Institute (Edmonton). was greatly appreciated for

this aspect of the project. Protein hydrophilicity and hydropathy profiles were used to select the optimal antigenic regions (Hopp and Woods, 1981; Kyte and Doolittle, 1982; Figure 7).

Figure 6. Synthetic peptides homologous to different regions of cytochrome oxidase subunit 1 and conjugated to KLH and BSA.

1) --Glu-Glu-Phe-Gly-Leu-Ser-Leu-Asn-Ser-Ser-Lys-amide

2) --Pro-Arg-Arg-Ile-Ser-Asp-Tyr-Pro-Asp-Ala-Phe-amide

3) --Glu-Gln-Lys-Glu-Ile-Ser-Gly-Arg-Gln-Gln-Asn-amide[5]

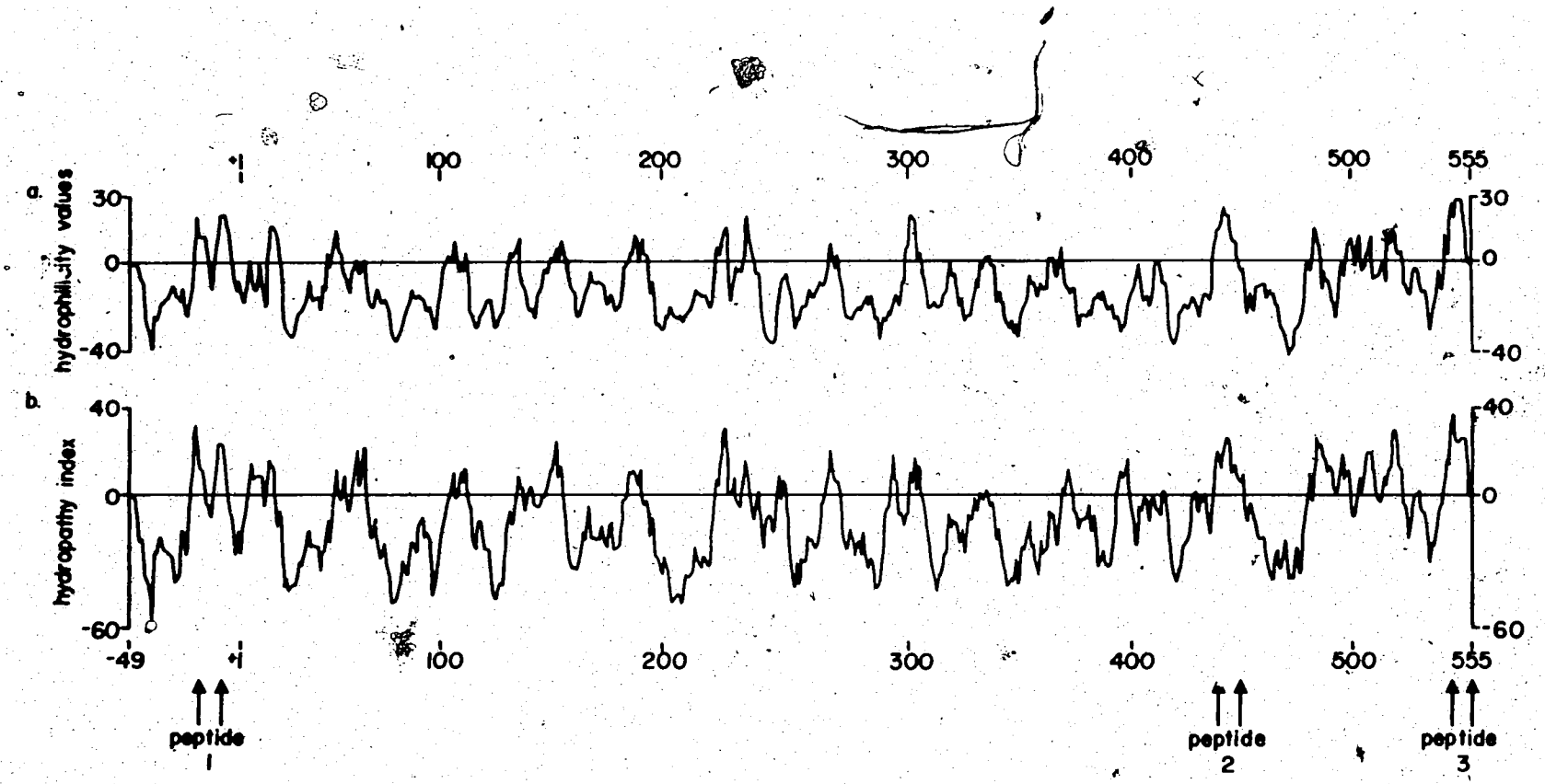
Antibody Production

The KLH-conjugated peptide (*ca.* 400 μg) was dissolved in 0.5 mℓ of sterile 0.9% (w/v) NaCl and mixed with 0.5 mℓ of Freund's complete adjuvant (Difco Laboratories) until the consistency was that of mayonnaise. Prior to the first injection, the rabbit was bled to obtain pre-immune sera. Equal volumes (0.25 mℓ) of the antigen-adjuvant mixture was injected under each shoulder blade and in each hip muscle. Two weeks later the rabbits were injected again, but Freund's incomplete adjuvant (Difco Laboratories) was used instead of complete adjuvant. Two weeks after the second injection, 10 mℓ of blood was collected. If the antibody titer in the antisera, determined by indirect ELISAs, was sufficiently high (colour reaction at 10^{-4} dilution), the rabbits were sacrificed and the blood was used to obtain large quantities of antisera; otherwise the rabbit was again injected with an incomplete adjuvant/antigen mixture and the antibody titer was checked two weeks later.

To obtain sera, the blood was allowed to clot overnight at 4°C. The sera was separated from the blood clot by centrifugation at top speed in a clinical centrifuge. The blood clot was discarded and the supernatant (antisera) was stored at -20°C after adding NaN_3 to 0.02% (w/v).

[5] An extra glutamine residue was accidentally incorporated into this peptide at position 9.

Figure 7. Heptapeptide polarity profiles of the cytochrome oxidase subunit 1 open reading frame using the programs of a) Hopp and Woods, 1981 and, b) Kyte and Doolittle, 1982.


a.
hydrophilicity values
30
0
-40
+1
100
200
300
400
500
555
b.
hydropathy index
40
0
-60
-49
+1
100
200
300
400
500
555
peptide 1
peptide 2
peptide 3

Indirect ELISA

The indirect ELISA procedure of Voller (1980) was used. Aliquots (100 $\mu\ell$) of antigen stock solution (5 $\mu g/m\ell$ of BSA-conjugated peptide in coating buffer; see appendix) were added to the wells of a Dynatech immulen flat bottom plate (Fisher Scientific), placed in an airtight container lined with wet paper towels and left overnight at 4°C. The next morning the wells were emptied by aspiration and washed three times with PBST (see appendix). Any remaining liquid in the wells was removed by aspiration. A 100 $\mu\ell$ aliquot of antisera (diluted 1/100 using PBST-BSA (2% (w/v) BSA in PBST), was added to the top row. 10 $\mu\ell$ from the 1/100 dilution was added to 90 $\mu\ell$ of PBST-BSA in the the adjacent well in the same column. This was continued until all the wells in a column contained increasing 10 fold dilutions of antisera. The plate was again placed in an airtight container lined with wet paper towels, but this time left at room temperature for 2 hours. The wells were emptied by aspiration and washed three times with PBST as before. A 100 $\mu\ell$ aliquot of alkaline phosphatase-conjugated goat anti-rabbit IgG (Boehringer Mannheim; diluted 1000 fold in PBST-BSA) was added to each well. Following a 2.5 hour room temperature incubation (same conditions as before), the wells were emptied and washed with PBST as before. 100 $\mu\ell$ of substrate solution (see appendix) was added to each well. After 30 minutes, the absorbance at 450 nm was determined using a Titertek Multiscan Microplate Reader (Flow Laboratories, Inc.).

Isolation of Mitochondrial Translation Products

Mitochondrial translation products were labeled by the procedure of Bertrand and Werner (1977). Briefly, 500 $m\ell$ of liquid Vogel's medium was inoculated with 1 X 10^9 conidia and incubated at 30°C with shaking until the culture reached log phase (12 to 24 hours depending on the strain). To 100 $m\ell$ of the exponentially growing culture, cycloheximide (0.1 mg/$m\ell$) was added and mixed for 2.5 minutes before the addition of the radioisotope. Either 1 mCi of L-[4,5-^{3}H]leucine (45-70 Ci/mmol) or 0.5 mCi of L-[^{35}S]methionine (>800

Ci/mmol) or 100 μCi of L-[^{14}C(U)]leucine (>300 mCi/mmol) (Amersham) was added and incorporation of the label proceeded for 1 hour at 30°C with shaking. The culture was harvested by vacuum filtration in a Buchner funnel and frozen immediately with liquid nitrogen. All subsequent steps were performed at 4°C. The frozen mycelia was ground in a mortar after the addition of grinding buffer (500 $\mu\ell$), PMSF (5 $\mu\ell$ of a 200 mM solution in ethyl acetate) and acid-washed sand. Once ground to a paste, the mixture was suspended in grinding buffer and centrifuged (3,000 rpm for 10 minutes) to pellet the debris. The supernatant was decanted to a clean tube and the mitochondria were pelleted (10,000 rpm for 30 minutes). The mitochondria were washed twice with 0.1 M Tris·HCl, pH 7.5 and stored frozen at -20°C. Unlabeled mitochondrial proteins used in the immunoblot experiments were also obtained from log phase cultures. The cycloheximide and labeling steps were omitted.

Immunoprecipitations

Labeled mitochondrial translation products (*ca.* 200,000 cpm of ^{3}H and/or *ca.* 50,000 cpm of ^{14}C-labeled proteins) were dissolved in 1 mℓ of triton buffer (2% (v/v) triton X-100, 0.3 M NaCl, 50 mM Tris·HCl, pH 7.0, 1 mM EDTA). The solution was centrifuged in a microcentrifuge for 10 minutes and the supernatant was transferred to a clean tube.

The protein A/antibody complex was prepared as follows: protein A sepharose (Pharmacia) was hydrated in triton buffer containing 0.02% (w/v) NaN_3 by mixing at 4°C for at least 1 hour. Hydrated protein A was stored indefinitely at 4°C. Hydrated protein A (*ca.* 8 mg) was added to an aliquot of antiserum (*ca.* 50 to 100 $\mu\ell$) and mixed for 1 hour at 4°C. The protein A/antibody complex was pelleted by centrifugation (5 minutes in a microcentrifuge). The pellet was resuspended in 100 $\mu\ell$ of triton buffer.

The solution of mitochondrial proteins in triton buffer was added to the protein A/antibody suspension and mixed for 1 hour at 4°C. The sample was centrifuged for 5 minutes in a microcentrifuge and the pellet was washed three times with triton buffer and twice with 0.3 M NaCl, 50 mM Tris·HCl, pH 7.0, 1 mM EDTA. The pellet was denatured by

adding a buffered solution of SDS and β-mercaptoethanol as described in the section on polyacrylamide gel electrophoresis.

Immunoblots

The BRL Immunodetection Kit was used for immunoblots and the manufacturer's instructions were followed (Bethesda Research Laboratories, Inc.). Protein samples, separated on either Laemmli gels (Laemmli, 1970) or SDS slab gels (Bertrand and Werner, 1977; Bertrand and Werner, 1979) were electrophoretically transferred to nitrocellulose paper (pore size = 0.45 μm; Schleicher & Schuell, Inc.) using a Trans Blot Cell (Bio-Rad). The transfer buffer used was 25 mM Tris (pH 8.3), 192 mM glycine, 20% (v/v) methanol, 0.1% (v/v) SDS. Transfers were carried out on a constant voltage power supply at 40 Volts for 40 minutes. Following the transfer, the nitrocellulose filter was sealed in a hybridization bag with excess 1% (w/v) BSA, 50 mM Tris·HCl, pH 7.5, 0.2 M NaCl (TBS) for 30 minutes at room temperature using gentle agitation. The blocked filters were incubated with dilutions of antisera (1/100 to 1/1000) in TBS (5 to 10 mℓ per 100 cm^2). After an overnight incubation at 4°C with gentle agitation, the filters were washed three times for 15 minutes with excess wash buffer (0.5% (w/v)BSA, 50 mM Tris·HCl, pH 7.5, 0.2 M NaCl, 0.1% (v/v) tween 20). The filter was then incubated with the secondary antibody (biotinylated goat anti-rabbit IgG diluted 1/1000 in TBS). Filters were washed three times as before. The streptavidin-alkaline phosphatase conjugate (1/3000 dilution in TBS) was added and the filters were gently agitated for 30 minutes at room temperature. The filters were washed three times with 50 mM Tris·HCl, pH 7.5, 0.2 M NaCl, 0.1% (v/v) tween 20.

In order to visualize the biotin-labeled protein bands, the following procedure was used. Filters were washed with excess 0.1 M Tris·HCl, pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$. The substrate solution was prepared by adding 44 $\mu\ell$ of nitroblue tetrazolium chloride to 10 mℓ of 0.1 M Tris·HCl, pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$, mixing and then adding 33 $\mu\ell$ of 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt. After mixing gently, the filter was

immersed in substrate solution for 10 to 30 minutes in the dark with periodic agitation. The color development was terminated by washing the filters in excess water.

Polyacrylamide Gel Electrophoresis

Electrophoresis of mitochondrial proteins was on 10% (w/v) acrylamide (diluted from a 30% (w/v) acrylamide/bisacrylamide (29/1)(w/w) stock), 0.1 M Tris·HCl, pH 8.0, 1% (w/v) SDS vertical slab gels (20 cm X 20 cm X 3 mm (or 1.5 mm)) (Bertrand and Werner, 1979)[6]. Gels were run at 75 to 100 Volts (constant voltage) with 0.1 M Tris·HCl, pH 8.0, 1% (w/v) SDS as the reservoir buffer. Before loading, protein samples (100,000 cpm of ^{3}H or 10,000 cpm of ^{14}C or 20,000 cpm of ^{35}S-labeled proteins) were denatured by adding an equal volume of 10% (w/v) SDS, 4% (v/v) β-mercaptoethanol, 0.2 M Tris·HCl, pH 8.0 and leaving at room temperature for at least 4 hours. The denatured protein samples were centrifuged for 5 minutes in a microcentrifuge and the supernatant was transferred to a clean tube. Prior to loading, 1/10 volume of loading dye (0.1 M Tris·HCl, pH 8.0, 1% (w/v) SDS, 50% (v/v) glycerol, 0.1% (w/v) bromphenol blue) was added.

Electrophoresis of mitochondrial proteins was also carried out on Laemmli (1970) gels. Gels (20 cm X 20 cm X 3 mm (or 1.5 mm)) consisted of a stacking gel (3% (w/v) acrylamide (prepared from a 30% (w/v) acrylamide/bisacrylamide (29/1)(w/w) stock, 0.125 M Tris·HCl, pH 6.8, 0.1% (w/v) SDS) and a separating gel (10% (w/v) acrylamide, 0.375 M Tris·HCl, pH 8.8, 0.1% (w/v) SDS). The reservoir buffer used was 25 mM Tris, pH 8.3, 192 mM glycine, 0.1% (w/v) SDS. Proteins were denatured by the addition of an equal volume of 4% (w/v) SDS, 0.125 M Tris·HCl, pH 6.8, 10% (v/v) β-mercaptoethanol, 20% (v/v) glycerol, 0.1% (w/v) bromphenol blue. The samples were left at room temperature for at least 4 hours and centrifuged for 5 minutes in a microcentrifuge before loading. Gels were run at 15 mA

[6]Werner and Bertrand (1979) did not find any significant difference in the resolution of precursor and mature subunit 1 polypeptides when separated on 7.5% to 20% SDS polyacrylamide gels. We did not detect the subunit 1 precursor using 15% gels and tried 10% gels since they resolve proteins better in the 40,000 to 50,000 MW range than do 15% gels (Hames, 1981).

during the stacking phase and increased to 25 mA when the proteins had entered the separating gel.

Slicing Protein Gels

Lanes from Laemmli gels or SDS slab gels were sliced using a manual gel slicer (Tyler Research). The 1 mm slices were transferred individually to scintillation vials containing 0.75 mℓ of 0.1 M Tris·HCl, pH 8.0, 1% (w/v) SDS. The vials were incubated at 65 to 70°C for at least 6 hours. 5 mℓ of Aqueous Counting Scintillant (Amersham) was added to each vial and mixed vigorously. Samples were counted in a Beckman LS7500 Scintillation Counter.

DNA Sequencing

DNA sequencing was by the method of Sanger *et al.* (1977) on M13 single-stranded DNA templates (Messing, 1983). Briefly, single-stranded DNA clones were constructed using M13mp7 (Messing *et al.*, 1981), M13mp8, M13mp9 (Messing and Vieira, 1982), M13mp10, M13mp11 (Norrander *et al.*, 1983), M13mp18 or M13mp19 (Yanisch-Perron *et al.*, 1985). The single-stranded DNA template (*ca.* 0.5 to 1.0 μg) was annealed to the appropriate M13 primers (New England BioLabs, Inc. and Regional DNA Synthesis Laboratory) in 7 mM Tris·HCl, pH 8.0, 7 mM $MgCl_2$, 50 mM NaCl (total volume of hybridization mixture was 12 μℓ) in a microcentrifuge tube. Annealing of the primer to the template was achieved by placing the sample in boiling water or by heating at 70°C for 15 minutes and allowing it to cool slowly to at least 37°C. Once cooled, the hybridization mixture was transferred to a Nunc Microwell Plate (60 wells with lid). To this was added 1 μℓ of 0.1 M dithiothreitol and 1 μℓ of radioisotope. Either α^{32}P-dATP (>600 Ci/mmol) (NEN) or α^{35}S-dATP (>1000 Ci/mmol)(Amersham) was used as the radioactive label. In preparation for the elongation reaction 1 μℓ of Klenow Fragment (1 Unit/μℓ) was mixed in. Aliquots (3 μℓ) were dispensed to four adjacent wells. To initiate the elongation reaction an equal volume of the A, C, G and T mixes (see appendix) was added to the appropriate wells, mixed and incubated in an air

incubator (43 to 55°C). After 10 to 20 minutes, 1.5 $\mu\ell$ of chase (see appendix) was added to the 4 wells and placed in the air incubator for 10 to 20 minutes. The reactions were stopped by adding 14 $\mu\ell$ of dye-formamide mix (see appendix) to each well. The samples were transferred to microcentrifuge tubes, denatured by placing in boiling water for 5 minutes and loaded onto 6% (w/v) acrylamide (prepared from a 40% (w/v) acrylamide/bisacrylamide (19/1)(w/w) stock), 8.33 M urea, 0.1 M Tris-borate, pH 8.3, 2 mM EDTA. The gels (40 cm X 20 cm X 0.25 mm and 80 cm X 20 cm X 0.25 mm) were run at 25 and 50 Watts (constant power setting), respectively. When problems with secondary structure were encountered, either dITP (Sanger *et al.*, 1982) or c⁷dGTP (Barr *et al.*, 1986) replaced dGTP in the sequencing mixes (see appendix).

In some cases, supercoiled double-stranded DNA templates were used in the sequencing reactions as described by Ahmed (1987), except that CsCl-banded plasmid DNA was used. Supercoiled plasmid DNA (2 μg in 20 $\mu\ell$ of water) was denatured by the addition of 2 $\mu\ell$ of 2 M NaOH, 2 mM EDTA. After 7 minutes at room temperature, neutralization was achieved by adding 3 $\mu\ell$ of 3 M sodium acetate, pH 5.0 and 7 $\mu\ell$ of dH_2O. The DNA was precipitated by the addition of cold 95% ethanol, pelleted in a microcentrifuge (10 minutes at 4°C), washed with cold 70% ethanol and dried under vacuum. The DNA pellet was dissolved in 9 $\mu\ell$ of distilled water. Primer (60 ng) and 1.5 $\mu\ell$ of 10X sequencing buffer (100 mM Tris-HCl, pH 8.0, 50 mM $MgCl_2$, 75 mM dithiothreitol) was added to the template DNA. Annealing was achieved by incubating the template/primer mixture for 20 minutes at 37°C. The DNA sequencing reactions were performed exactly as described above except that 5 [illegible] of Klenow fragment (5 Units/$\mu\ell$) was used in each reaction.

When α^{35}S-dATP was used, gels were dried at 80°C in a Model 483 Slab Dryer (Bio-Rad). Autoradiography was performed at room temperature for 20 to 30 hours before the XAR-5 X-ray film (Kodak) was developed according to the manufacturer's instructions. ^{32}P-labeled gels were not dried; autoradiography was performed at -20°C and the X-ray film was developed after 12 to 16 hours.

Polyacrylamide Gradient Gel Electrophoresis.

DNA was also separated on non-denaturing polyacrylamide gradient gels (40 cm X 20 cm X 3 mm). Gels consisted of a 5 to 15% polyacrylamide gradient (made from a 40% (w/v) acrylamide/bisacrylamide stock (19/1)(w/w)) in 0.1 M Tris-borate, pH 8.3, 5% (v/v) glycerol, 2 mM EDTA poured onto a 25 mℓ plug of 20% acrylamide in the same buffer. The 15% acrylamide solution contained 20% sucrose to stabilize the gradient during the construction of the gel. The slots were poured separately using the 5% acrylamide solution, after the gel had polymerized. All acrylamide solutions were degassed prior to use. Electrophoresis was performed for 48 hours at 400 V (constant voltage) with 0.1 M Tris-borate, pH 8.3, 2 mM EDTA as the running buffer. The gels were stained for 30 minutes in 0.5 μg/mℓ of ethidium bromide followed by 30 minutes of destaining in distilled water. The bands were visualized and photographed on a UV transilluminator.

Agarose Gel Electrophoresis

Gels were made to 0.8% (w/v) agarose in 0.1 M Tris-borate, pH 8.3, 2 mM EDTA and contained ethidium bromide (0.5 μg/mℓ). DNA samples were made to 5% (v/v) glycerol containing bromphenol blue just prior to loading. Electrophoresis was carried out at 25 to 100 Volts on a constant voltage power supply.

Purification of DNA from Agarose Gels

DNA was isolated from agarose gels by three different methods: 1) from low-melting point agarose (Bethesda Research Laboratories, Inc.), 2) from DEAE paper (Whatman) and 3) from NA-45 membrane (Schleicher & Schuell, Inc.). When isolated from low-melting point agarose the DNA was electrophoresed on a 0.8% gel. The desired fragment was excised from the gel and melted at 65°C after adding an equal volume of 50 mM Tris-HCl, pH 7.3, 0.5 mM EDTA. An equal volume of water-saturated phenol was added and the sample was placed at 65°C for 10 minutes, mixing occasionally. The sample was mixed thoroughly and the phases

were separated by centrifugation (10,000 rpm for 5 minutes). The aqueous phase was transferred to a clean tube and the phenol phase was extracted with an equal volume of 50 mM Tris·HCl, pH 7.3, 0.5 mM EDTA (heated to 65°C). The phases were separated by centrifugation and the aqueous phases were combined. This was extracted twice with water-saturated phenol and once with chloroform/iso-amyl alcohol (24/1)(v/v). The DNA was recovered by ethanol precipitation after the addition of 3 M sodium acetate, pH 7.0 to a final concentration of 0.3 M.

DNA was also isolated from agarose gels using DEAE paper (DE-81 filter paper, Whatman). After the DNA bands were separated on an agarose gel, the band of interest was located and a slit was made immediately in front of it. The DEAE paper was inserted into the slit and electrophoresis was continued. Once the band disappeared into the paper, it was removed from the slit and packed into a P-1000 blue tip (Type BR-40, Bio-Rad). The paper was washed once with water (1 mℓ) and three times with 400 μℓ aliquots of 0.1 M NaCl, 10 mM Tris·HCl, pH 7.3, 1 mM EDTA. The bottom of the tip was sealed with Parafilm™ and 200 μℓ of 1 M NaCl, 10 mM Tris·HCl, pH 7.3, 1 mM EDTA was added, ensuring that it covered the paper. This was left for at least 2 hours at 4°C. The liquid was forced out using a P-1000 pipetman (Gilson) into a microcentrifuge tube. Another 200 μℓ of 1 M NaCl, 10 mM Tris·HCl, pH 7.3, 1 mM EDTA was added and forced out. The eluted liquid was extracted twice with water-saturated phenol and once with chloroform/iso-amyl alcohol (24/1)(v/v). The DNA was recovered by ethanol precipitation. tRNA (4 to 5 μg) was sometimes added as a carrier.

NA-45 membrane (Schleicher & Schuell, Inc.) was also used to isolate DNA from agarose gels. To increase the binding capacity of the membrane, it was first washed with 10 mM EDTA, pH 8.0 for 10 minutes then 5 minutes in 0.5 M NaOH followed by several rapid washes in dH_2O. The membranes were stored at 4°C in dH_2O. After the DNA fragments were separated on an agarose gel, a strip of NA-45 membrane was placed in an incision immediately ahead of the band of interest. Electrophoresis was continued until the DNA band

disappeared into the membrane. The membrane was removed from the slit and freed of agarose by thoroughly shaking in a microcentrifuge tube containing 0.15 M NaCl, 20 mM Tris·HCl, pH 7.3, 0.1 mM EDTA. The DNA was eluted by submerging the membrane in 450 $\mu\ell$ of 1.0 M NaCl, 20 mM Tris·HCl, pH 7.3, 0.1 mM EDTA and incubating at 65°C for 45 minutes with occasional swirling. The liquid was extracted twice with water-saturated phenol and once with chloroform/iso-amyl alcohol (24/1)(v/v). The DNA was recovered by ethanol precipitation.

Filter-Colony Hybridization

Colonies were grown directly on Bio Trans™ nylon membranes (ICN Biomedicals, Inc.) overlayed on selective media or patched onto the nylon membrane overlayed on selective media. Filter-colony hybridizations were performed as recommended by the manufacturer. Cells were lysed by placing the membrane on 3MM paper (Whatman) saturated with 1.5 M NaCl, 0.5 M NaOH for 5 minutes. Neutralization was achieved by placing the membrane for 5 minutes on 3MM paper saturated with 3 M sodium acetate, pH 5.5. The excess liquid was blotted with 3MM paper and the membrane was air-dried for 30 minutes before baking at 80°C for 1 hour.

Membranes were pre-hybridized for at least 1 hour at 65°C with hybridization solution (5X Denhardt's (see appendix), 5X SSPE (see appendix), 0.2% (w/v) SDS, 500 μg/mℓ of heat-denatured salmon sperm DNA). About 4 mℓ of solution was used per 100 cm^2 of membrane. In the hybridization reaction, 2 X 10^6 cpm of ^{32}P-labeled probe was used per 500 cm^2 of membrane and about 2 mℓ of hybridization solution 100 cm^2 of membrane. Hybridization was allowed to take place overnight at 65°C. Membranes were washed three times for 30 minutes at room temperature with excess 5 mM sodium phosphate, pH 7.0, 1 mM EDTA, 0.2% (w/v) SDS (250 mℓ per 100 cm^2) and vigorous agitation (*ca.* 200 rpm). The membranes were wrapped with Saran Wrap™ in preparation for autoradiography.

Radiolabeling DNA Restriction Fragments

DNA fragments were radioactively labeled by the oligo-labeling method of Feinberg and Vogelstein (1983, 1984). Linear DNA (25 to 50 ng in dH_20) was purified from agarose and mixed with 10 $\mu\ell$ of OLB buffer (see appendix) and dH_20 to 41 $\mu\ell$. The DNA/primer mixture was placed in boiling water for 3 minutes and allowed to cool slowly to 37°C. BSA (2 $\mu\ell$ of a 10 mg/mℓ solution) and 5 $\mu\ell$ of α^{32}P-dCTP (3000 to 4000 Ci/mmol) were added. The labeling reaction was initiated by the addition of 2 $\mu\ell$ of Klenow fragment (1 Unit/$\mu\ell$). The reaction was left overnight at room temperature. The reaction was stopped by adding 200 $\mu\ell$ of stop buffer (20 mM NaCl, 20 mM Tris·HCl, pH 7.5, 2 mM EDTA, 0.25% SDS, 1 μM dCTP). Purification of the unincorporated label from the DNA probe was not necessary. The number of cpm incorporated into the probe was determined by the following procedure: an aliquot (5 $\mu\ell$ of the terminated labeling reaction) was spotted onto a DE-81 filter (Whatman) and washed with 0.3 M ammonium formate (5 mℓ). The filter was transferred to a scintillation vial containing 5 mℓ of Aqueous Counting Scintillant (Amersham) and counted in a Beckman LS7500 Scintillation Counter to determine the cpm/$\mu\ell$.

Enzymes

Restriction endonucleases, T4 DNA Ligase and Klenow fragment were obtained from Bethesda Research Laboratories, Inc. or New England BioLabs, Inc. and were used in accordance with the suppliers' instructions.

III. Results and Discussion

Nature of the Precursor and Mature Subunit 1 Polypeptides

The nucleotide sequence of the *oxi-3* gene did not resolve the problem concerning the nature of the amino-terminal presequence in the coI precursor polypeptide as discussed in the introduction on page 21 (Burger *et al.*, 1982; de Jonge and de Vries, 1983). The only in-frame ATG initiation codon is just two codons upstream from the start of the mature subunit 1 polypeptide (Figures 3 and 8). It did not seem likely that 2 amino acids could account for the 4000 MW difference between the precursor and mature polypeptides. A number of proposals were put forth.

The first model proposed the existence of an intron in the coI gene that would fuse the out-of-frame ATG codon at position -450 to the mature coding sequence (Figure 8). This would extend the reading frame upstream of the mature coding sequence and thus, would encode the N-terminal prepiece. Northern analyses of the coI transcript has ruled out this possibility (Burger *et al.*, 1982; Burger and Werner, 1983).

A second model suggested that only 2 amino acids were proteolytically removed from the N-terminus and this led to the observed molecular weight difference between precursor and mature subunit 1 (de Jonge and de Vries, 1983; Figure 3). It is has been established that hydrophobic proteins run anomalously on SDS gels thereby leading to a significant discrepancy between the actual and apparent molecular weights of such proteins (Burger *et al.*, 1982; de Jonge and de Vries, 1983). On SDS gels, mature subunit 1 has an apparent molecular weight of 41 kDa, but its true molecular weight, deduced from translation of the DNA sequence, is *ca.* 60 kDa (Burger *et al.*, 1982; de Jonge and de Vries, 1983). This observation gives credence to the suggestion that amino-terminal processing involves the removal of only 2 amino acids (de Jonge and de Vries, 1983).

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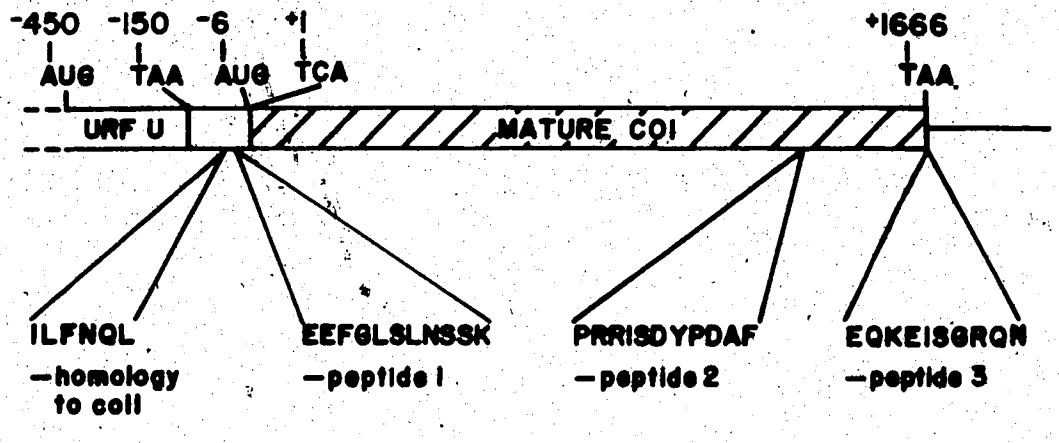
[1]Portions of this chapter have been published elsewhere (Lemire, E.G. and F.E. Nargang. 1986. A missense mutation in the *oxi-3* gene of the [*mi-3*] extranuclear mutant of *Neurospora crassa*. J. Biol. Chem. 261:5610-5615) and are included in this thesis with the publisher's permission.

Figure 8. The position of the start (AUG) and stop (TAA) codons, the regions homologous to the conjugated peptides and the homology to the subunit 2 presequence are indicated in relation to the mature subunit 1 coding region.


-450
-150
-6
+1
+1666
AUG
TAA
AUG
TCA
TAA
URF U
MATURE COI
ILFNQL
—homology to coII
EEFGLSLNSSK
—peptide 1
PRRISDYPDAF
—peptide 2
EQKEISGRQN
—peptide 3

A third alternative suggested that the *N. crassa* subunit 1 polypeptide had a C-terminal extension which was removed to give the mature subunit. This possibility was based entirely on the observation that the *N. crassa* protein is *ca.* 20 amino acids longer at the C-terminus relative to the subunit 1 sequences from plants, fungi and other higher eukaryotes[1] (Burger *et al.*, 1982; de Jonge and de Vries, 1983; Figure 4). Conceivably, proteolytic cleavage of amino acid residues could occur at both the C- and N-termini to yield the mature polypeptide (Burger *et al.*, 1982; de Jonge and de Vries, 1983). There is a precedence for similar processing. The *N. crassa* laccase has residues removed from both the N- and C-termini (Germann *et al.*, 1988). The alpha chains of pea seed isolectins and the variant surface glycoprotein (VSG) from trypanosomes are both post-translationally modified by C-terminal processing (Borst, 1983; Rini *et al.*, 1987).

The use of alternative codons to initiate translation of the coI message is a fourth possibility (Burger *et al.*, 1982; de Jonge and de Vries, 1983). Unorthodox codons like AUAA (Clary and Wolstenholme, 1983b; de Bruijn, 1983; Clary and Wolstenholme, 1985), AUGA (Waring *et al.*, 1984) or AUN (Bibb *et al.*, 1981; Wallace, 1982) are used as translation initiation codons in other mitochondrial systems. Although there is no evidence to support the use of alternate initiation codons in *Neurospora*, such an occurrence would permit an amino-terminal extension of up to 50 amino acid residues, depending on which initiation codon is used (Figure 3). Cleavage of a presequence of this length would easily account for the observed molecular weight difference between the precursor and mature subunit 1 polypeptides. Adding support to this hypothesis is the observation that a block of 6 amino acids upstream of the mature coding sequence shows considerable homology to a region of the subunit 2 amino-terminal presequence (van den Boogaart, 1982b; Macino and Morelli, 1983; Table 6 and Figures 3 and 8).

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[1]The subunit 1 amino acid sequences from the protozoans were not included in this analysis because they differ significantly and have limited homology to the coI polypeptides of other eukaryotes (de la Cruz *et al.*, 1984; Hensgens *et al.*, 1984; Pritchard *et al.*, 1986; Ziaie and Suyama, 1987).

Table 6. Homology between an in-frame amino acid sequence preceding the mature subunit 1 coding region and a region of the subunit 2 amino-terminal presequence.

subunit 1: --Ile-Leu-Phe-Asn-Gln-Leu-20 amino acids-mature protein
subunit 2: --Leu-Leu-Phe-Asn-Asn-Leu-5 amino acids-mature protein

Conceivably, this homology might reflect a functional relatedness of presequences such as a requirement for insertion into the membrane or a protease recognition site.

A final model hypothesizes that the removal of the two amino acid N-terminal prepiece is coupled to some other post-translational modification (*i.e.* phosphorylation) which would affect its mobility in SDS gels.

The five models are refuted by the observations that no serine or isoleucine residue is found within 10 steps of the *N*-formylmethionine at the N-terminus (Burger *et al.*, 1982) and that the prepiece consists of at least 12 amino acids (Burger and Werner, 1983). The latter information concerning the length of the prepiece excludes all the models that postulate the existence of a 2 amino acid presequence. Furthermore, any use of the unorthodox initiation codons AUN, AUAA or AUGA leads to the presence of either a serine and/or an isoleucine residue within 10 steps of the initiating methionine (Figure 3). Consequently, all models contain some aspect which conflicts experimental observations.

Unless an unprecedented mechanism for synthesis of the mature polypeptide is invoked, one or both of the observations concerning the nature of the prepiece must be incorrect. In an effort to resolve this dilemma and to differentiate among the models presented above, peptides homologous to specific regions of the coI polypeptide were synthesized and conjugated to carrier proteins (Figures 6 and 8). The synthetic peptides were used to elicit an immune response in rabbits in order to obtain antigen-specific antisera (Shinnick *et al.*, 1983). The choice of peptides was based on more or less established parameters (Shinnick *et al.*, 1983): 1) the peptide should contain mainly polar or charged amino acids, 2) peptides containing proline tend to be good antigens, 3) amino acids at the N- or C-terminus often induce protein-reactive antibodies, and 4) peptides should be 10 or

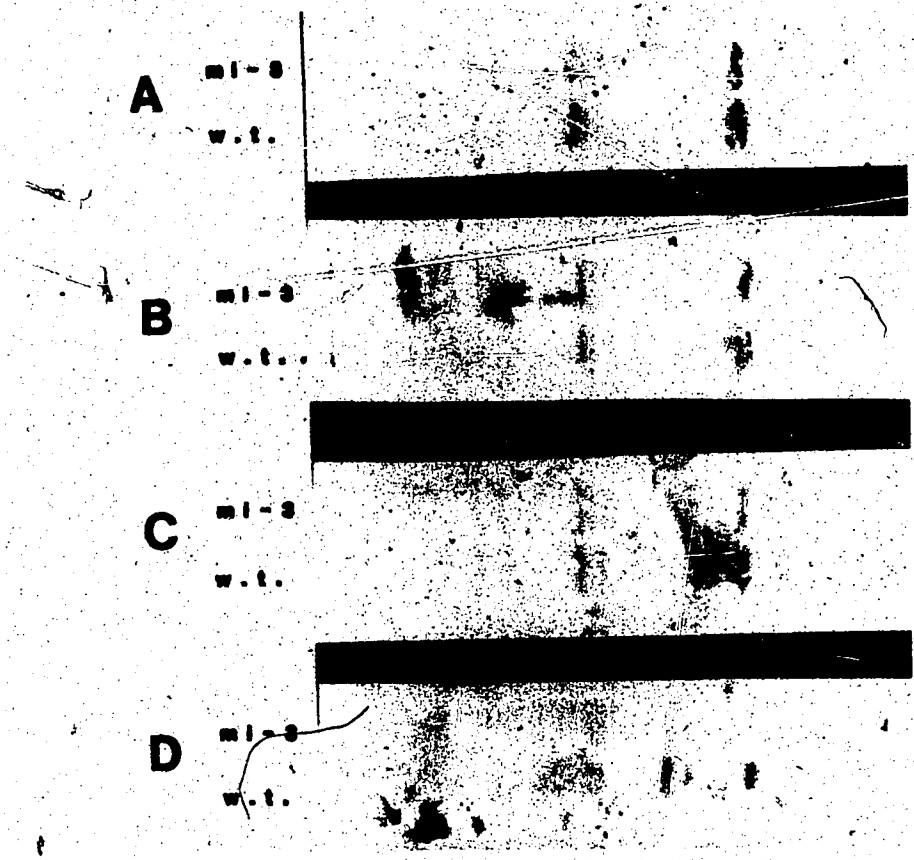
more amino acids in length. With these parameters taken into consideration, the hydropathy and hydrophilicity analysis programs (Hopp and Woods, 1981; Kyte and Doolittle, 1982; Figure 7) were run, and the choice of peptides was made. The first synthetic peptide was homologous to amino acids -18 to -8 (Figures 6 and 8). This sequence was chosen to determine whether or not the N-terminal presequence extended upstream of the methionine residue at -2 (Figures 3 and 8). If the homology to the subunit 2 presequence was functionally important, the antigenic site mimicked by peptide 1 should be detectable.

The sequence of peptide 2 was homologous to amino acids 440 to 450 of mature subunit 1 (Figures 6 and 8). Antibodies raised against this immunogen should react with both the precursor and mature polypeptides and function as a control in immunoblot experiments. Subsequently, it was discovered that [*mi-3*] harboured a missense mutation in codon 448 leading to an asp to tyr substitution (Table 9; Lemire and Nargang, 1986). It was hoped antisera against this peptide would only react with the wildtype polypeptide, thereby substantiating the nucleic acid sequence data.

The sequence of peptide 3 was derived from the last 10 amino acid residues (546 to 555) at the subunit 1 C-terminus (Figures 6 and 8). Antibody obtained from an immune response against this synthetic antigen should determine whether or not proteolytic cleavage of C-terminal residues is involved in the maturation of the this precursor (Figure 4).

As can be seen in Figure 9D, subunit 1 appears as a broad band when subunit 1-specific antibody is used as the primary antibody. No subunit 1 band is visible in the other blots where antisera against the synthetic antigens was used, but other bands of unknown origin (some may be degradation products) are apparent (Figure 9A-C). The ineffectiveness of the antisera was unexpected since a colour reaction was present at the 10^{-4} dilution in indirect ELISA experiments (Table 7). No further immunoblot experiments were performed because of these unsatisfactory results. Therefore, the exact nature of the post-translational steps required to produce the mature subunit 1 polypeptide from the precursor remains a mystery.

Figure 9. Immunoblots of [*mi-3*] and wildtype mitochondrial translation products using different primary antibodies: A) peptide 1-specific antisera, B) peptide 2-specific antisera, C) peptide 3-specific antisera and D) subunit 1-specific antisera. An adjacent lane containing ^{3}H wildtype mitochondrial translation products (•—•) was sliced and the profile is shown below the immunoblots. The arrows indicate the position of the protein markers having the approximate molecular weight of 43,000 Da and 25,700 Da. The subunit 1 peak is located between these markers.


A
m1-8
w.t.
B
m1-8
w.t.
C
m1-8
w.t.
D
m1-8
w.t.

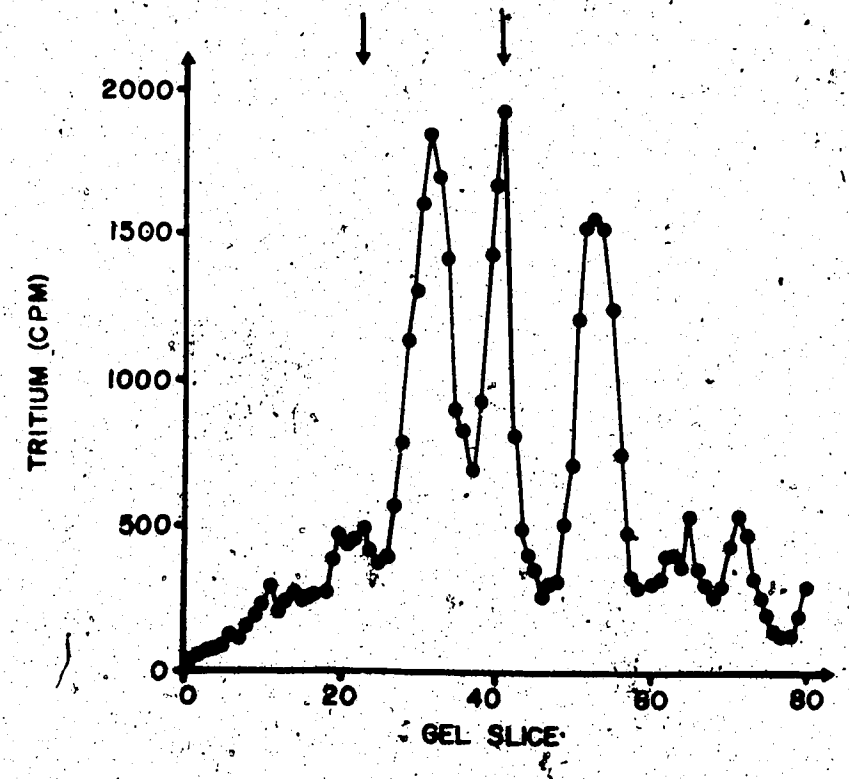

TRITIUM (CPM)
2000
1500
1000
500
0
0
20
40
60
80
GEL SLICE

Table 7. Results of ELISA assays.

Dilution	Absorbance at 450 nm		
	1[1]	2	3
10^{-2}	>1.5	>1.5	>1.5
10^{-3}	0.36	0.63	1.0
10^{-4}	0.06	0.11	0.26
10^{-5}	n.d.[2]	0.02	0.06

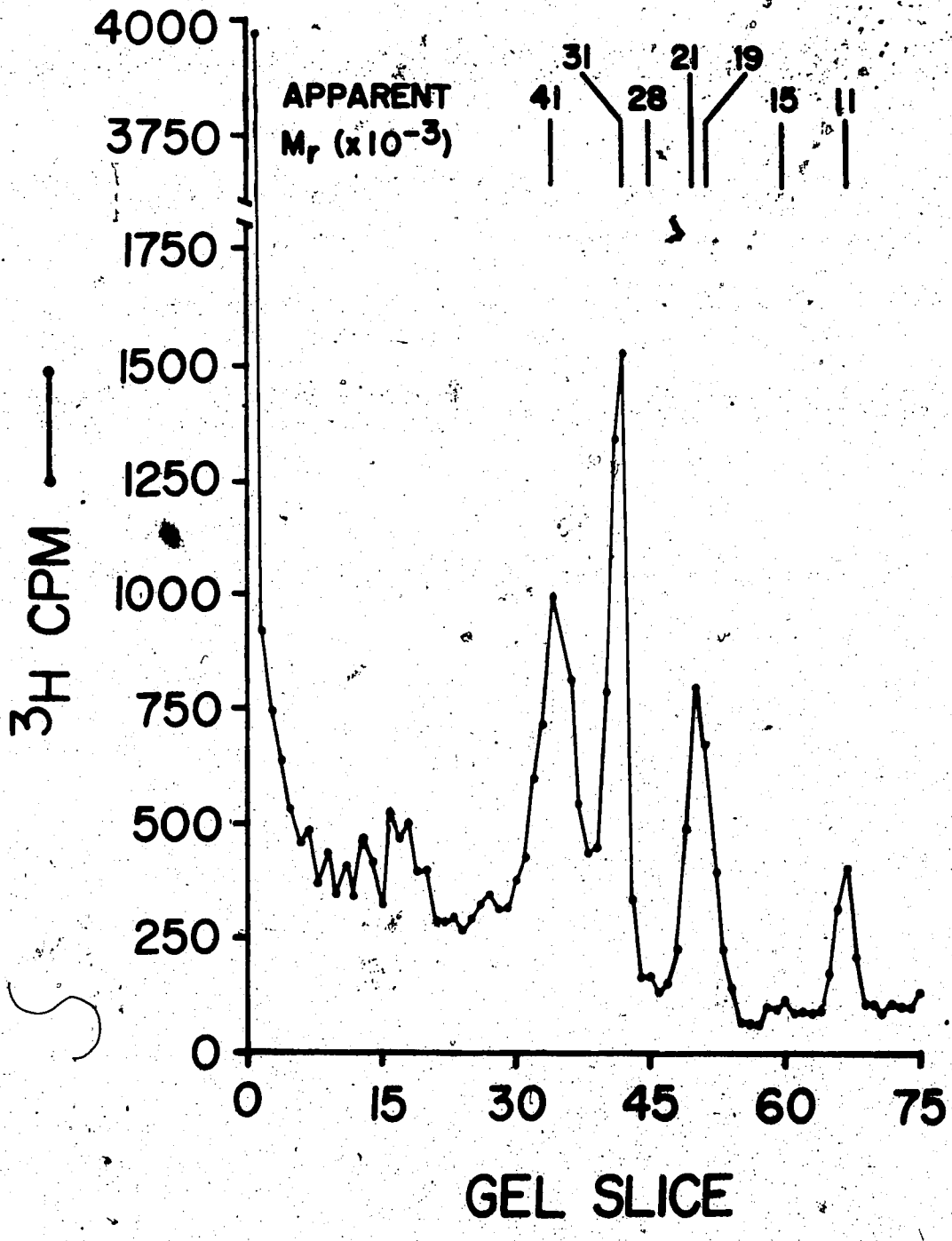
1. Denotes the antisera raised against the appropriate peptide (i.e. 1 corresponds to the antisera obtained when peptide 1 was used as the antigen, and so on).
2. no detectable colour reaction.

Analysis of [mi-3] mitochondrial translation products

Previous studies demonstrated that the [*mi-3*] mutant accumulated a larger cytochrome oxidase subunit 1 polypeptide (Bertrand and Werner, 1979) that was converted to the mature form upon induction of cytochrome *aa*$_3$ with antimycin A (Werner and Bertrand, 1979; Werner *et al.*, 1980). Evidence from amino acid sequencing indicated that the precursor bore an N-terminal extension relative to mature subunit 1 (Werner *et al.*, 1980). With this knowledge, the idea of developing an *in vitro* assay for the processing of the subunit 1 precursor from [*mi-3*] using wildtype mitochondrial extracts was pursued. Such an assay would allow us to determine whether suppressed [*mi-3*] (*su-1*, [*mi-3*]) or antimycin A-supplemented [*mi-3*] cultures had more processing activity than [*mi-3*] strains. Additionally, purification of the processing activity could be achieved through this assay.

The entire approach was contingent upon being able to distinguish between the precursor and mature forms of cytochrome oxidase subunit 1 on 10% SDS polyacrylamide gels as described by Bertrand and Werner (1979). Figure 10 shows a profile of the mitochondrial translation products from a wildtype culture labeled with ^{3}H-leucine in the presence of cycloheximide. A peak with a molecular weight of *ca.* 50 kDa is often seen in preparations. It is thought to correspond to the ribosomal protein *S5*, though it has not been demonstrated

Figure 10. A profile of ^{3}H mitochondrial translation products from a wildtype strain (*nic* 240). The peaks correspond to coI (41 kDa), cytochrome *b* (31 kDa), coII (28 kDa), coIII (21 kDa), ATPase subunit 6 (19 kDa), an unknown polypeptide (15 kDa) and ATPase subunit 8 (11 kDa). The cytochrome *b* and coII peaks and the coIII and ATPase subunit 6 peaks did not separate and appear as one. The 15 kDa polypeptide is almost completely absent in this profile. References: Sebald *et al.*, 1973; Weiss and Ziganke, 1974; Jackl and Sebald, 1975; Bertrand and Werner, 1977; Nargang *et al.*, 1979.


4000
3750
1750
1500
1250
1000
750
500
250
0
APPARENT
M_r (x10^{-3})
31
21
19
41
28
15
11
^{3}H CPM
0
15
30
45
60
75
GEL SLICE

conclusively (Burke and RajBhandary, 1982; Breitenberger and RajBhandary, 1985).

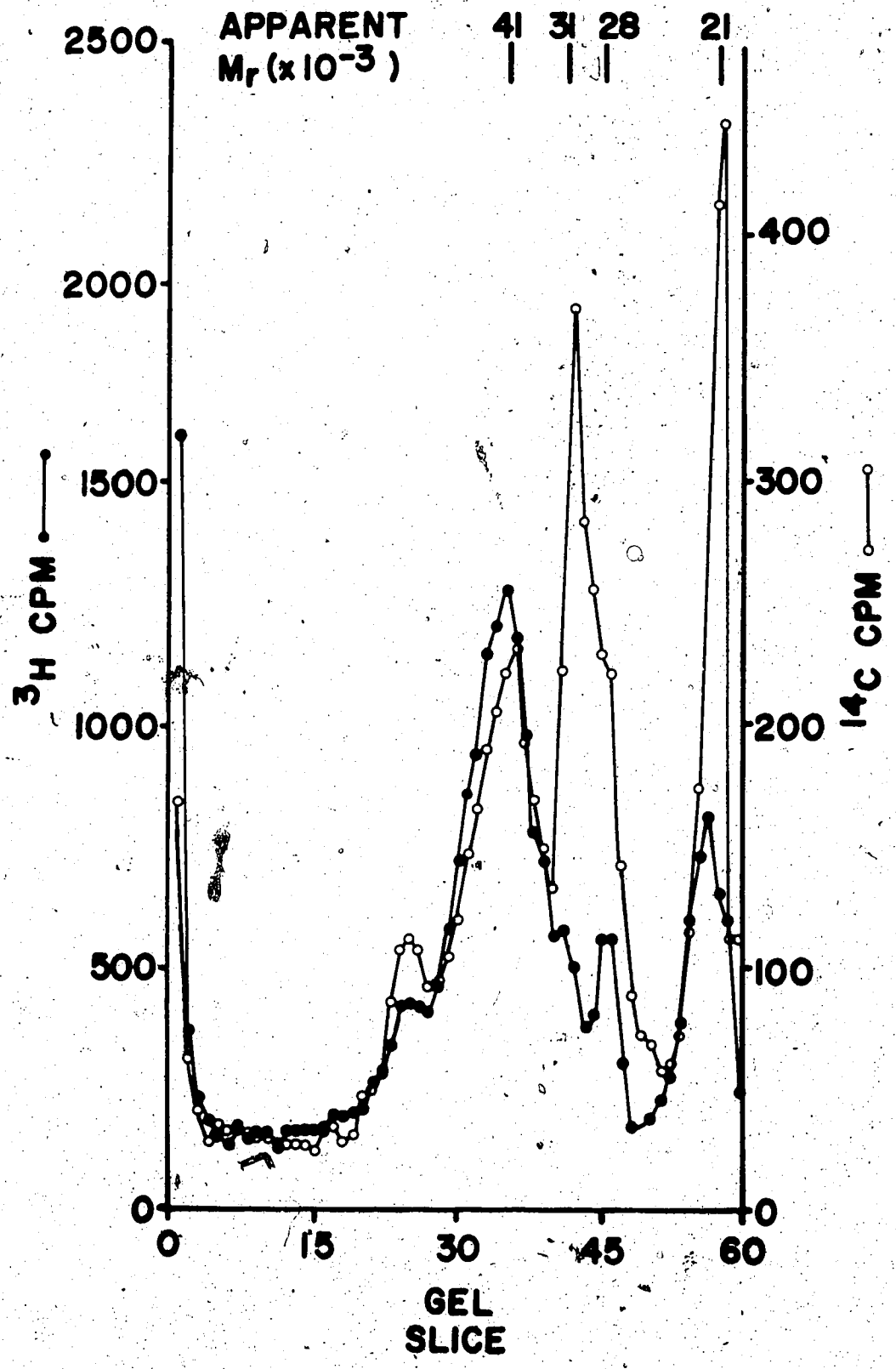
Differentially-labeled mitochondrial translation products from [*mi-3*] and wildtype were isolated, mixed and separated on a 10% SDS polyacrylamide gel. Figure 11 shows that the subunit 1 peaks were not separated. Subunit 1 specific antisera (generous gift from Dr. S. Werner, U. of Munich) was used to separate the subunit 1 polypeptides from the other mitochondrial translation products since this should enhance the detection of the molecular weight difference between subunit 1 precursor and mature polypeptides. The subunit 1 immunoprecipitates from ^{3}H-labeled [*mi-3*] and ^{14}C-labeled wildtype mitochondrial proteins were electrophoresed (Figure 12). The molecular weight difference detected by others (Bertrand and Werner, 1979) was not apparent.

Since the two forms of subunit 1 could not be separated on 10% SDS gels as described earlier, the decision was made to use a different gel system. Others have resolved the two forms using the Laemmli (1970) gel system (Van't Sant *et al.*, 1981; Van't Sant and Kroon, 1983). Figure 13 shows the profile of subunit 1 immunoprecipitates separated on a 10% Laemmli gel. The mutant subunit 1 peak does appear to be shifted slightly towards the cathode indicating a higher molecular weight form, but this result could not be obtained consistently. Based on these results, no definite conclusion on the presence of a higher molecular weight subunit 1 polypeptide could be made.

Others researchers have observed that the source of SDS used in the preparation and running of SDS polyacrylamide gels affected the mobility of mitochondrial proteins (Chomyn *et al.*, 1986). Two other commercial sources of SDS were used in the preparation and electrophoresing of protein samples on 10% SDS slab gels. Neither source of SDS resolved the precursor from the mature form. The results from one such experiment using Ultrapure™ SDS (Bethesda Research Laboratories, Inc.) are profiled in Figure 14.

It had been demonstrated previously that mitochondrial precursor polypeptides accumulated in wildtype when labeling was performed at 9°C (Van't Sant *et al.*, 1981; Van't Sant and Kroon, 1983). It was hoped that labeling the mutant mitochondrial translation

Figure 11. Differentially-labeled mitochondrial translation products from *nic* 240 (●—●) and [*mi-3*] (o—o) separated on a 10% SDS gel.


APPARENT
M_r (x 10^{-3})
41
31
28
21
2500
2000
1500
1000
500
0
400
300
200
100
0
^3H CPM
^{14}C CPM
0
15
30
45
60
GEL
SLICE

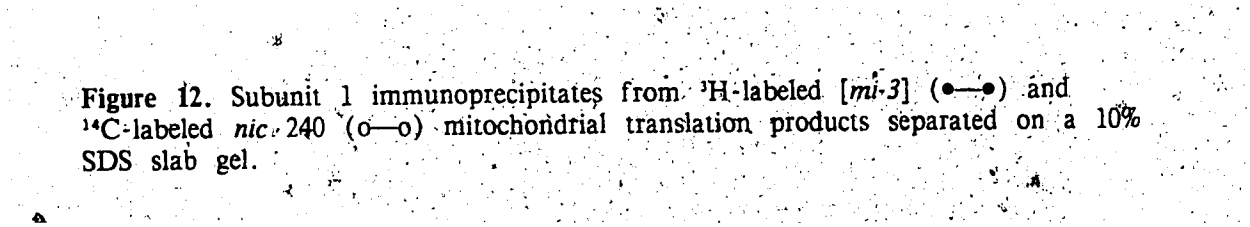
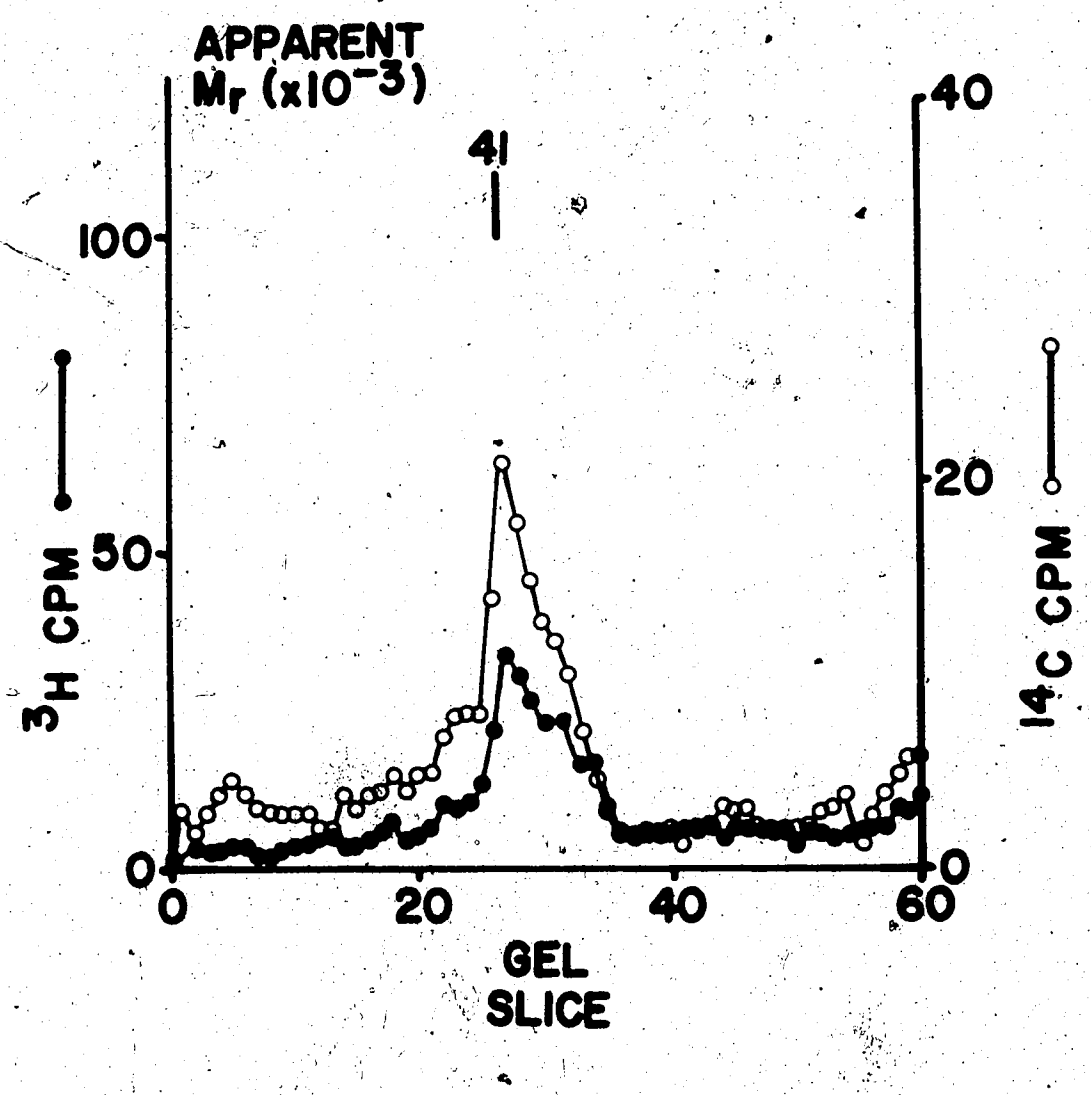


Figure 12. Subunit 1 immunoprecipitates from ^{3}H-labeled [*mi-3*] (•—•) and ^{14}C-labeled *nic-240* (o—o) mitochondrial translation products separated on a 10% SDS slab gel.


APPARENT
M_r (x10^{-3})
41
100
50
0
^{3}H CPM
40
20
0
^{14}C CPM
0
20
40
60
GEL
SLICE

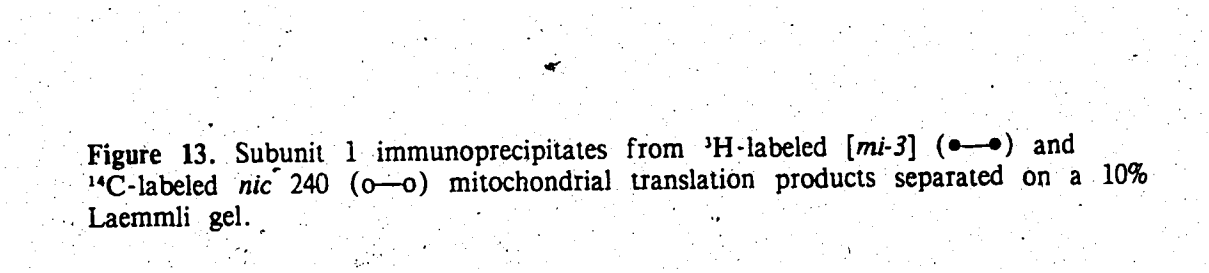


Figure 13. Subunit 1 immunoprecipitates from ^{3}H-labeled [*mi-3*] (•—•) and ^{14}C-labeled *nic* 240 (o—o) mitochondrial translation products separated on a 10% Laemmli gel.

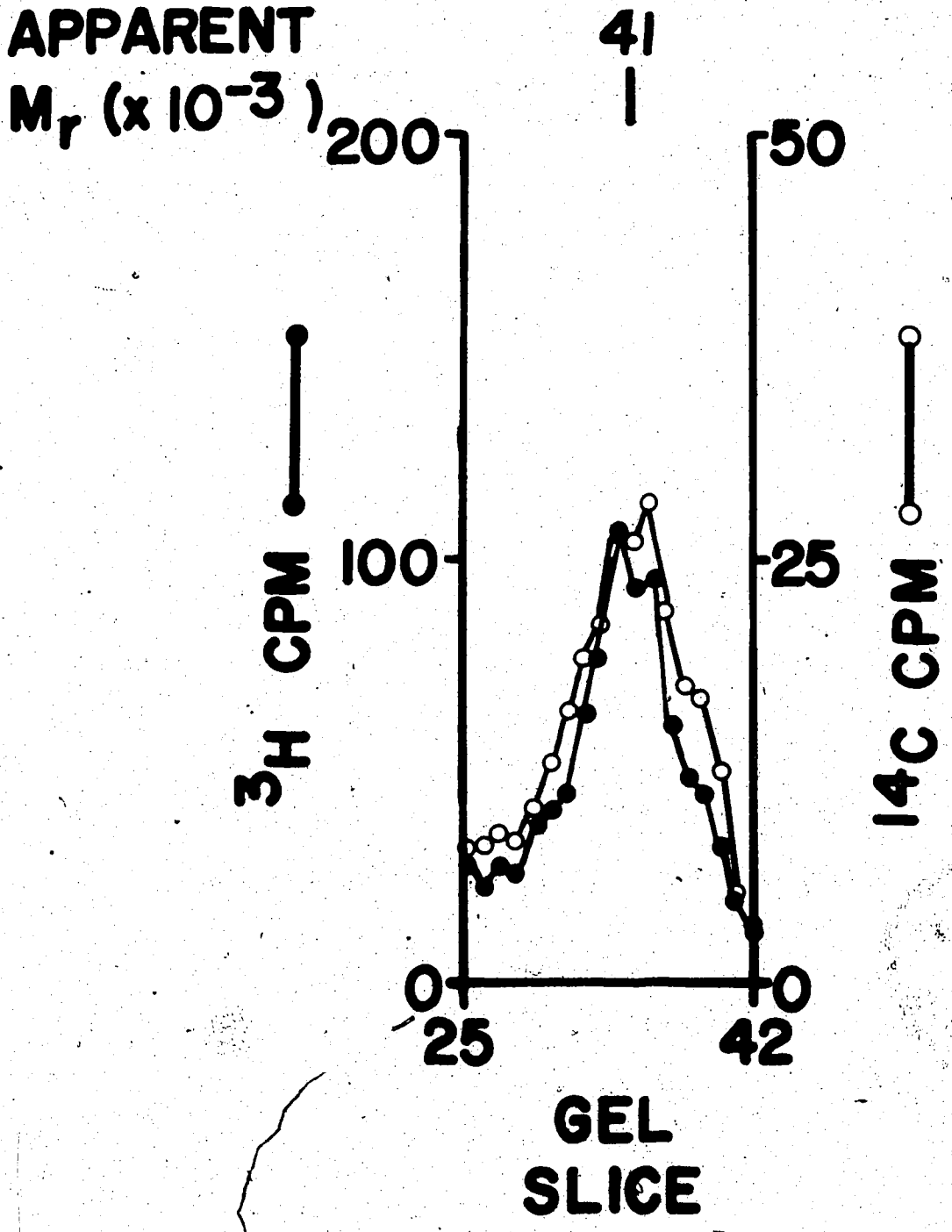
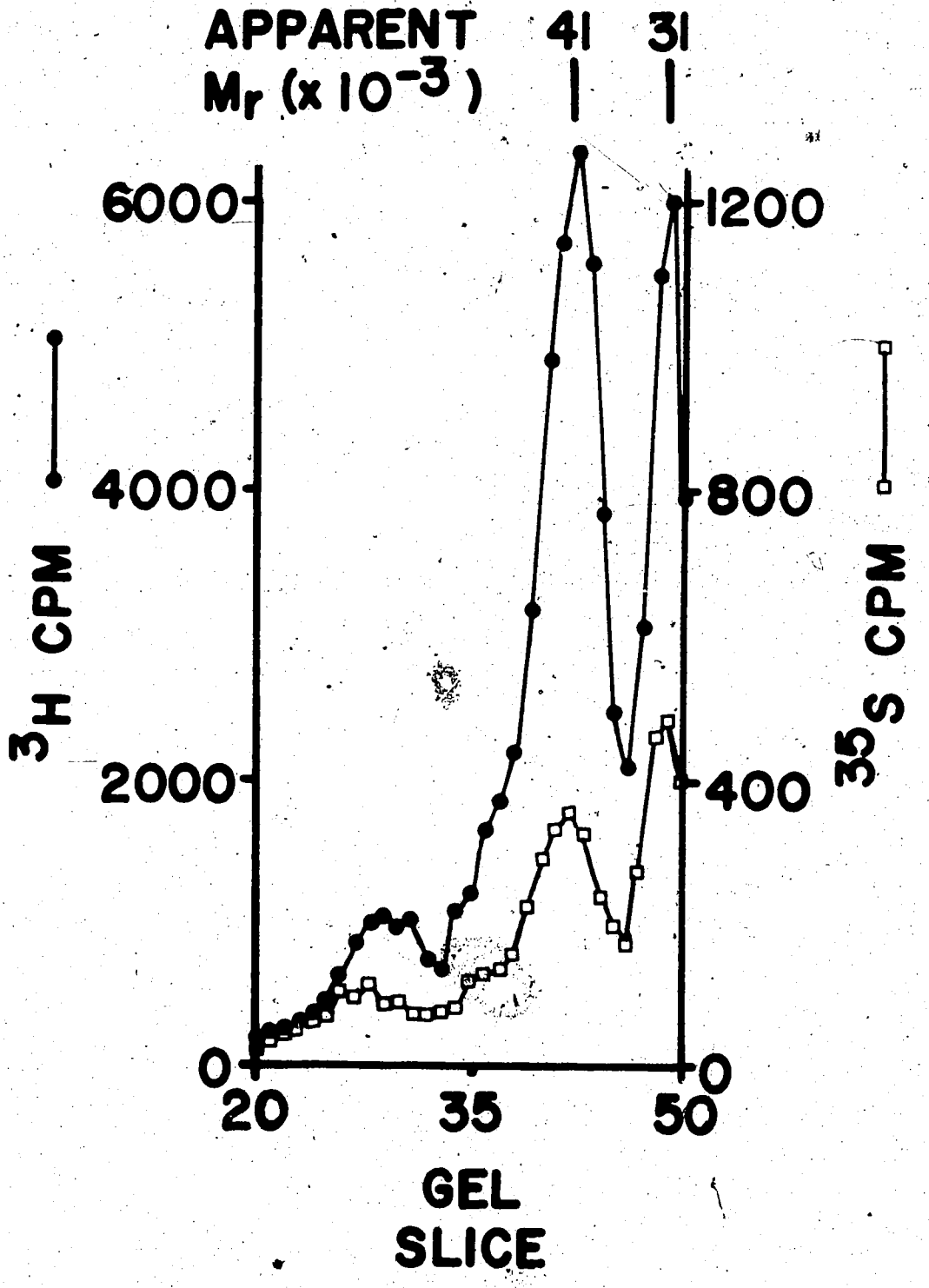

APPARENT
M_r (x 10^{-3})
200
100
0
41
50
25
0
^3H CPM
^{14}C CPM
25
42
GEL
SLICE

Figure 14. Differentially-labeled mitochondrial translation products from *nic* 240 (●—●) and [*mi-3*] (□—□) separated on a 10% SDS gel using Ultrapure™ SDS (Bethesda Research Laboratories, Inc.).


APPARENT
Mr (x 10⁻³)
41
31
6000
4000
2000
0
1200
800
400
0
3H CPM
35S CPM
20
35
50
GEL
SLICE

products at the lower temperature might be sufficient to distinguish between the two forms. However, this approach also proved unsuccessful. No differences could be detected between subunit 1 polypeptides of [*mi-3*] and wildtype in either whole mitochondrial translation products (Figure 15) or in immunoprecipitation profiles (Figure 16).

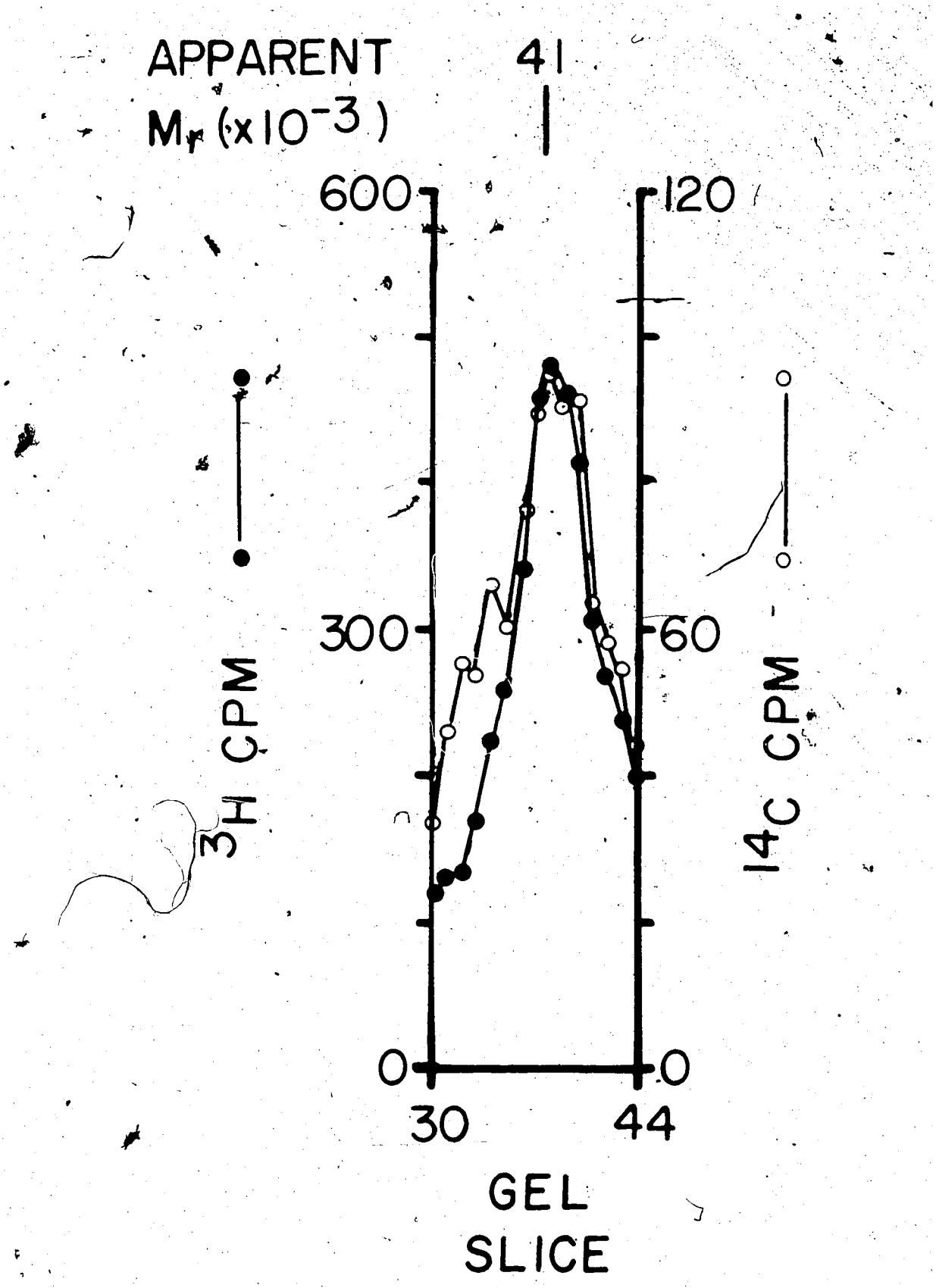
Failure to detect the subunit 1 precursor in our [*mi-3*] strain using two different gel systems and different commercial preparations of SDS led us to consider the possibility that this strain was not [*mi-3*] IL-40. Closer examination of this strain confirmed that it had the correct nutritional requirement (*pan-2*), the correct mating type (*A*) and a deficiency in cytochrome aa_3 (Figure 17a). Induction of cytochrome aa_3 did occur in antimycin A-supplemented cultures (Figure 17b), characteristic of [*mi-3*] strains (Bertrand and Collins, 1978; Bertrand, 1980). Furthermore, the slow-growth and cytochrome aa_3-deficient phenotype was found to be inherited in a maternal fashion (Table 8). These data confirm that the strain is in fact [*mi-3*] since there are no other *Neurospora* strains known to possess all of these characteristics.

Table 8. Non-Mendelian segregation of the [*mi-3*] phenotype.

Genetic Cross		F1 Progeny	
♀	♂	[*mi-3*]	wildtype
[*mi-3*]	X *nic 237*	24/24	0/24
[*mi-3*]	X 4003-HU-2*a*	28/28	0/28
nic 237	X [*mi-3*]	0/16	14/16[1]

1. Two of the sixteen ascospores picked were deficient in cytochromes aa_3 and *b*. This is characteristic of strains having lesions in the mitochondrial translation apparatus (*i.e.* [*poky*]). Since no [*mi-3*] ascospores were picked, it was concluded that the mutant characteristics of this strain were inherited maternally.

Figure 15. Differentially-labeled mitochondrial translation products from ^{14}C-labeled *nic* 240 (o—o) and ^{3}H-labeled [*mi-3*] (•—•) separated on a 10% Laemmli gel. Translation products were labeled at 9°C.


APPARENT
M_r ($\times 10^{-3}$)
41
600
300
0
120
60
0
^{3}H CPM
^{14}C CPM
30
44
GEL
SLICE

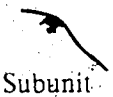


Figure 16. Subunit 1 immunoprecipitate of ^{3}H-labeled [*mi-3*] (•—•) and ^{14}C-labeled *nic* 240 (o—o) mitochondrial translation products. Translation products were labeled at 9°C and separated on a 10% Laemmli gel.

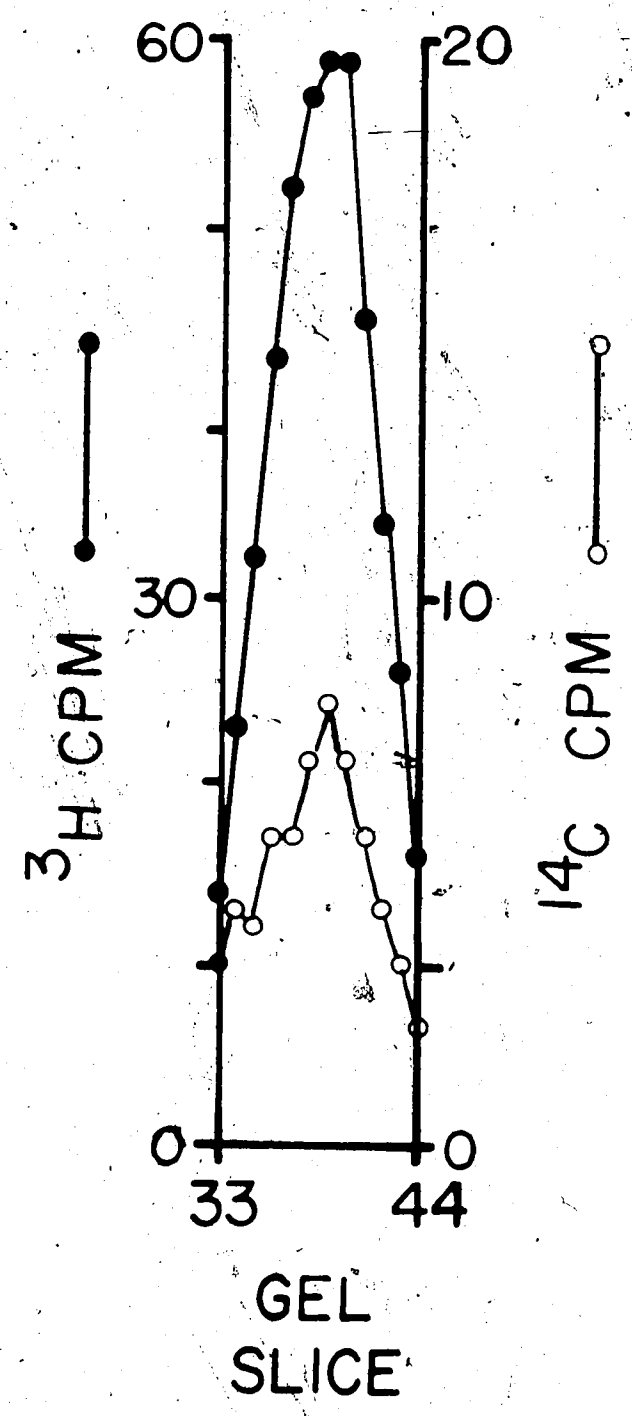
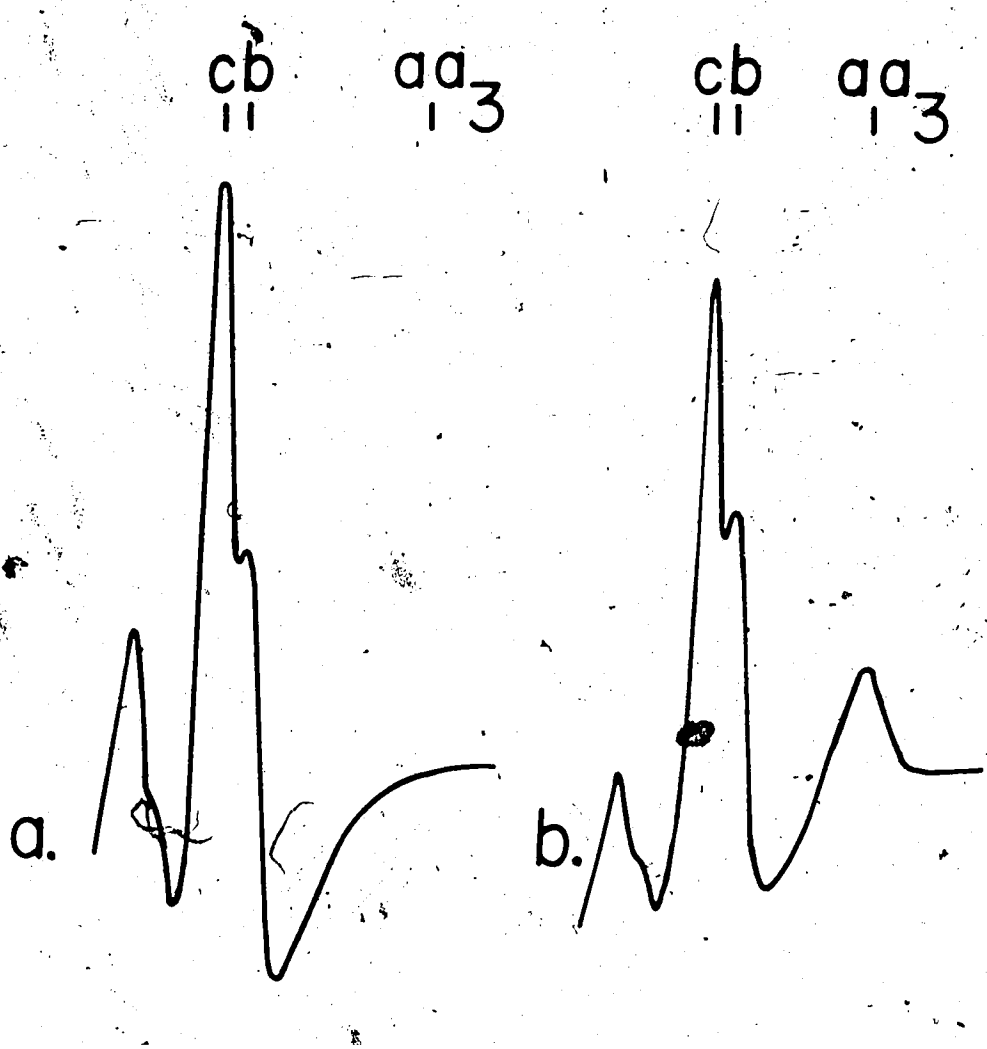

APPARENT
M_r ($\times 10^{-3}$)
41
60
30
0
20
10
0
^{3}H CPM
^{14}C CPM
33
44
GEL
SLICE

Figure 17. Cytochrome spectra of [*mi-3*] cultures grown in a) in the absence of antimycin A and b) in the presence of antimycin A (0.3 μg/mℓ).


cb
II
aa3
I
cb
II
aa3
I
a.
b.

Detection of an RFLP in the [mi-3] Mutant

Since the [*mi-3*] mutant of *N. crassa* accumulates the subunit 1 precursor polypeptide that bears an amino-terminal extension (Bertrand and Werner, 1979; Werner and Bertrand, 1979; Werner *et al.*, 1980), it was conceivable that examination of the subunit-1-encoding *oxi-3* gene might reveal an alteration relative to the wildtype. To facilitate identification of possible RFLPs affecting subunit 1 of cytochrome *c* oxidase in [*mi-3*], the mitochondrial EcoRI-3 DNA fragment from both [*mi-3*] and wildtype were cloned onto the bacterial plasmid pBR322 (Figures 5 and 18). The EcoRI-3 fragment carries the *oxi-3* gene which encodes cytochrome oxidase subunit 1 (Agsteribbe *et al.*, 1980; Macino, 1980). No size alterations were observed in the EcoRI-3 fragment of [*mi-3*] when compared with that of wildtype (Figure 18).

To facilitate the detection of possible smaller alterations, restriction digests of EcoRI-3 clones from [*mi-3*] and from the wildtype strains, 74*A* and Abbott 12a, were compared on polyacrylamide gradient gels. An electrophoretic variant of apparently higher molecular weight was detected in the [*mi-3*] clone upon analysis of RsaI digestion products of the cloned DNAs (Figure 19). Further restriction analysis, combined with the knowledge of the published wildtype DNA sequences (Burger *et al.*, 1982; de Jonge and de Vries, 1983) from this region, revealed that the alteration affected a *ca.* 270 bp HpaII/PstI fragment within the original *ca.* 630 bp RsaI fragment (Figure 19). The position of the RFLP relative to the *oxi-3* gene is shown in Figure 20.

To insure that the observed difference in the [*mi-3*] clone was not due to an artifactual mutation acquired during or after the cloning process, RsaI digests of total mitochondrial DNA were compared by gradient polyacrylamide gel electrophoresis. Figure 21 shows that it was also possible to detect the alteration in [*mi-3*] when comparing whole mitochondrial DNAs. No electrophoretic mobility differences other than in this region were observed to exist between [*mi-3*] and Abbott 12a (Figure 21). However, differences between the wildtype type I and type II mitochondrial DNAs and between [*mi-3*] and the type II wildtype mitochondrial

Figure 18. EcoR1 digests of EcoR1-3 clones and mitochondrial DNAs from the [*mi-3*] mutant and wildtype separated on an agarose gel. The plasmids pHBE3 and pLN2 are the wildtype and [*mi-3*] clones of EcoR1-3, respectively.

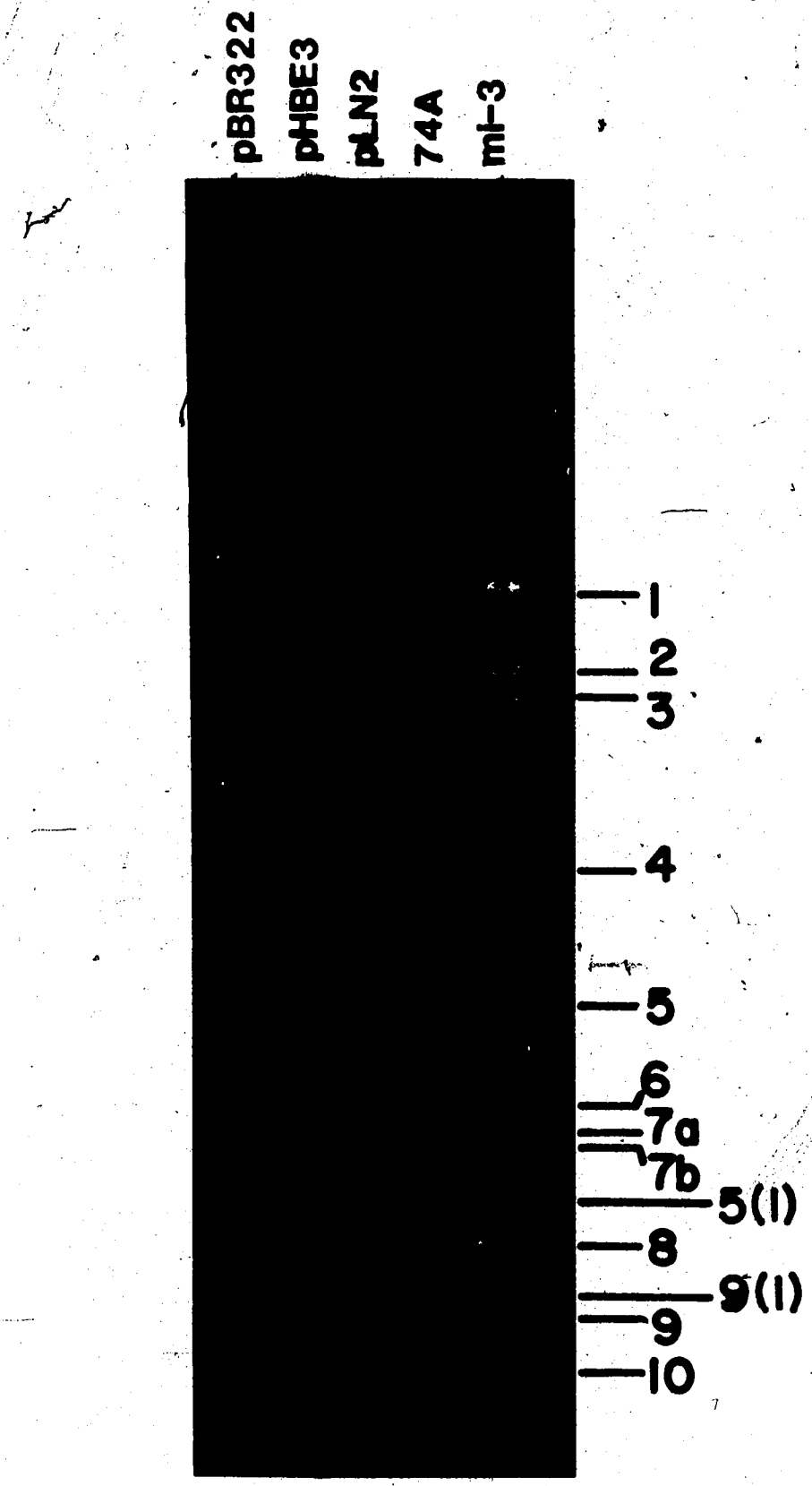

pBR322
pHBE3
pLN2
74A
ml-3
1
2
3
4
5
6
7a
7b
5(1)
8
9(1)
9
10

Figure 19. Polyacrylamide gradient gel showing electrophoretic differences observed in RsaI and PstI/HpaII digestion products between cloned [*mi-3*] (pLN2) and wildtype (pHBE3) EcoRI-3 mitochondrial DNA fragments. The top arrow indicates the RsaI products of differing molecular weight; the bottom arrow the differing PstI/HpaII products. The number of base pairs in two marker bands is indicated.

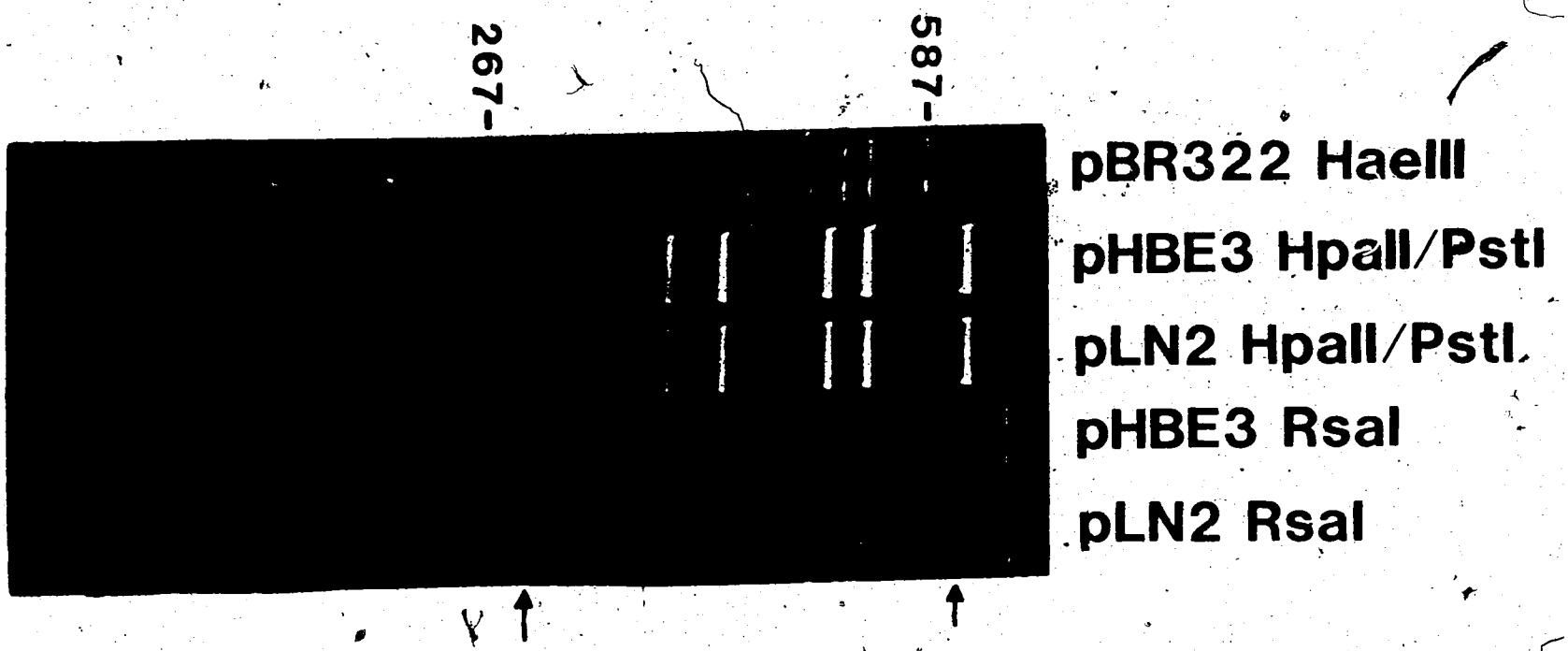

267-
587-
pBR322 HaeIII
pHBE3 HpaII/PstI
pLN2 HpaII/PstI
pHBE3 RsaI
pLN2 RsaI

Figure 20. Partial restriction map of the region of mitochondrial DNA encoding the cytochrome oxidase subunit 1 gene. The areas encompassed by the a) PstI/HpaII and b) RsaI fragments in [*mi-3*] are indicated. RsaI sites occur within all PstI palindromes (Pst Pal).

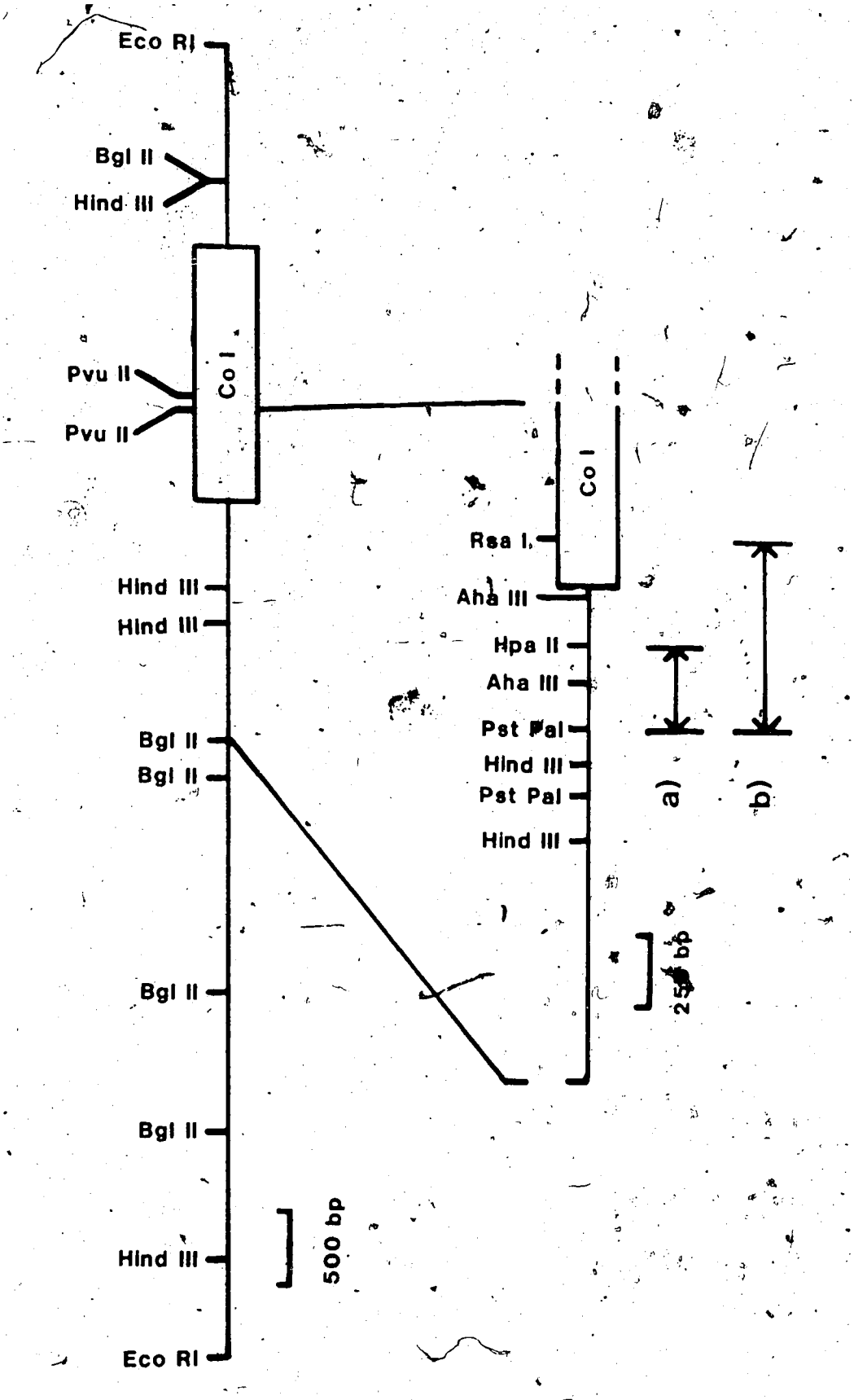
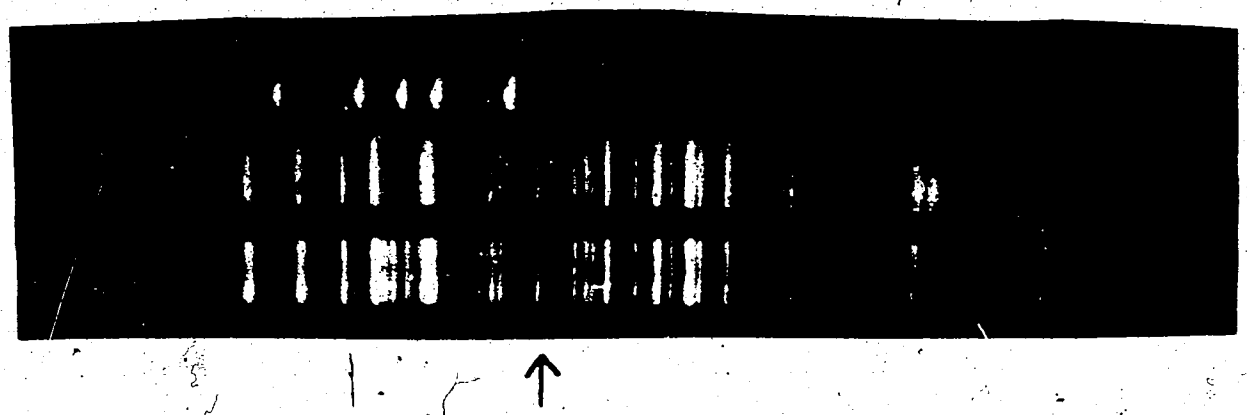

Eco RI
Bgl II
Hind III
Co I
Pvu II
Pvu II
Hind III
Hind III
Bgl II
Bgl II
Bgl II
Bgl II
Hind III
Eco RI
500 bp
Co I
Rsa I
Aha III
Hpa II
Aha III
Pst Pal
Hind III
Pst Pal
Hind III
a)
b)
25 bp

Figure 21. RsaI digestion products of [*mi-3*] and wildtype (Abbott 12*a*) mitochondrial DNAs separated on a polyacrylamide gradient gel. The arrow indicates the position of the restriction fragment length polymorphism.


587-
pBR322 HaeIII
Abbott 12
mi-3

DNA from 74*A* did exist. These differences were attributed to polymorphisms inherent in the type I and type II mitochondrial DNAs.

DNA Sequence of the RFLP

In order to determine the nature of the alteration giving rise to the RFLP, the DNA sequence of the 270 bp HpaII/PstI fragment from [*mi-3*] was determined and compared to the published wildtype sequences (Burger *et al.*, 1982; de Jonge and de Vries, 1983). The sole difference observed was a single A/T bp insertion in an A/T rich palindromic region (Figure 22). On the basis of the difference observed in electrophoretic mobility, an insertion of 5 to 10 bp was expected. Anomalies in migration rates of restriction fragments containing 1 bp changes have been reported previously (Ross *et al.*, 1982; Singh *et al.*, 1987).

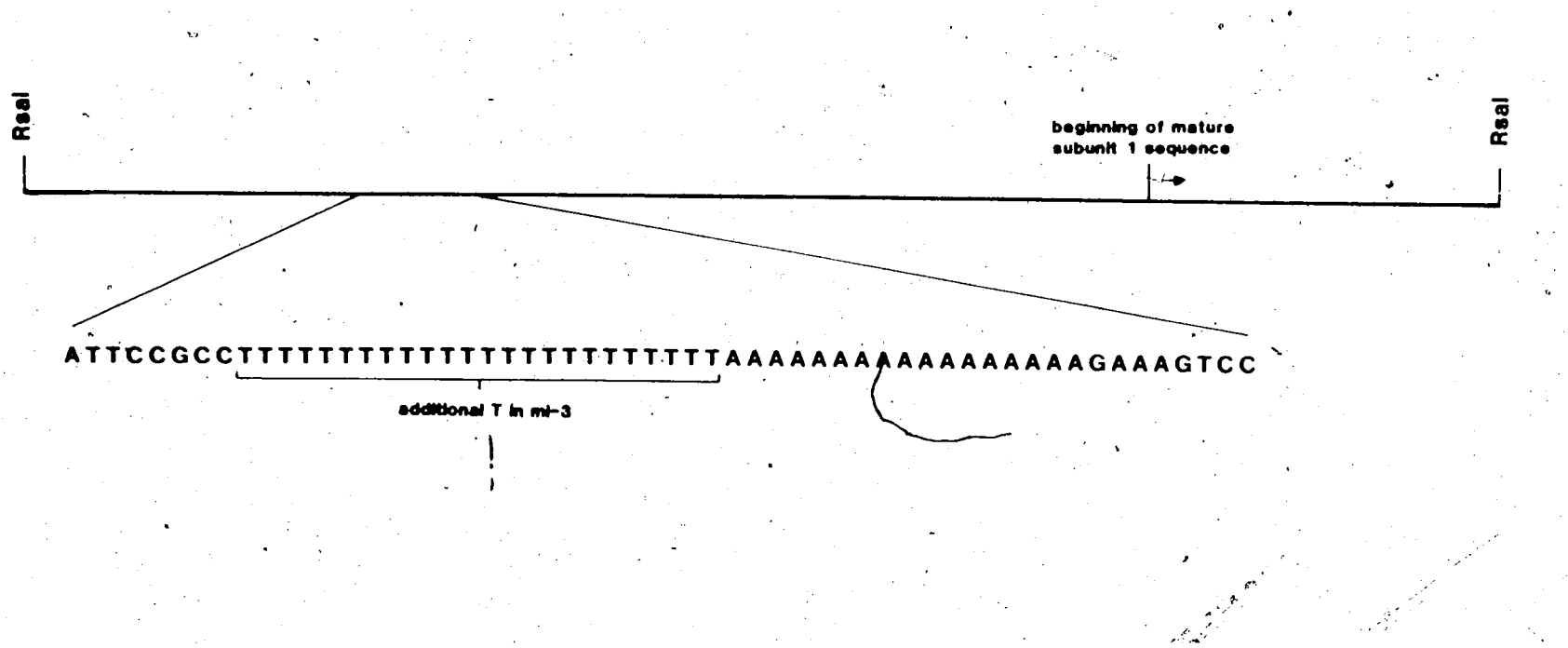
Interestingly, the unusual migration pattern of the fragment carrying the single base pair insertion was destroyed by digestion with the restriction endonuclease DraI (data not shown). The DraI site (5' TTT↓AAA 3') is located at the junction of the poly T and poly A stretches (Figure 22). Digestion with DraI would destroy any intramolecular secondary structure formed by this palindromic region. However, it is not immediately obvious how insertion of a single non-mismatched base pair might alter the secondary structure and lead to the anomalous electrophoretic mobility observed.

Genetic Relationship of the RFLP to the Mutant Phenotype

In order to test genetically the relationship between the [*mi-3*] phenotype and the alteration found in the HpaII/PstI fragment, heterokaryons were forced between a wildtype strain and an [*mi-3*] strain. It was reasoned that [*mi-3*] should predominate over the wildtype component in heterokaryons after repeated subculturing as had been reported for other mutants of *Neurospora* (Diacumakos *et al.*, 1965; Mannella and Lambowitz, 1978).

The heterokaryons were constructed using components that contained type I mitochondrial DNA ([*mi-3*]) and type II mitochondrial DNA (*nic* 240) so that recombination

Figure 22. DNA sequence of the region preceding the *oxi-3* gene that contains the alteration found in [*mi-3*].


RsaI
beginning of mature
subunit 1 sequence
RsaI
ATTCCGCCTTTTTTTTTTTTTTTTTTTTTTTTTTTAAAAAAAAAAAAAAAAAAGAAAGTCC
additional T in mi-3

of mitochondrial DNA can be monitored by following the unidirectional gene conversion to the type II EcoRI-5 and type I EcoRI-9 fragments as described previously (Mannella and Lambowitz, 1979). Thus, any heterokaryons that acquired the [*mi-3*] phenotype should have both the fragment that contains the [*mi-3*] lesion from the incoming type I mitochondrial DNA plus the type II EcoRI-5 fragment from the wildtype component of the heterokaryon. If the alteration originally identified in the RsaI fragment could be shown to be present in all heterokaryons that have acquired the [*mi-3*] phenotype, this could be construed as good evidence that the alteration is, in fact, responsible for the [*mi-3*] phenotype.

After repeated subculturing, 10 out of 30 heterokaryons were found to have become phenotypically [*mi-3*] as determined initially by the decline in their growth rates. This was confirmed by spectral analysis showing a deficiency of cytochrome *aa*$_3$ (Figure 23). This result demonstrates that the [*mi-3*] extranuclear mutant can be suppressive over wildtype mitochondria as are group I mutants. Since more subculturing passages of the heterokaryons were required than was reported for group I mutants (Mannella and Lambowitz, 1978), the [*mi-3*] mutant appears to be less efficient in this suppressive process.

Restriction analysis of the mitochondrial DNA isolated from these heterokaryons was carried out by digestion with EcoRI and the products were examined by agarose gel electrophoresis. All heterokaryons possessed the type II mitochondrial DNA version of EcoRI-5 and 3 of 10 had the type I EcoRI-9 fragment, thus demonstrating that mitochondrial DNA recombination had taken place. We did not observe the unidirectional conversion to the type I EcoRI-9 fragment as described by Mannella and Lambowitz (1979) (Figure 24).

Whole mitochondrial DNAs isolated from the heterokaryons were compared by gradient polyacrylamide gel electrophoresis following RsaI digestion. These data are shown in Figure 25 for 4 of the heterokaryons. The most important observation made from Figure 25 is that heterokaryons 10-9-1 and 10-45-1 carry the [*mi-3*] version of the 630 bp RsaI fragment, while heterokaryon 10-45-7 carries the wildtype version of the fragment. At least 4 of the remaining 6 heterokaryons that had acquired the [*mi-3*] phenotype also appear to carry the

Figure 23. Cytochrome spectra of [*mi-3*], wildtype (*nic* 240) and ten heterokaryons that were constructed and propagated as described in the text.

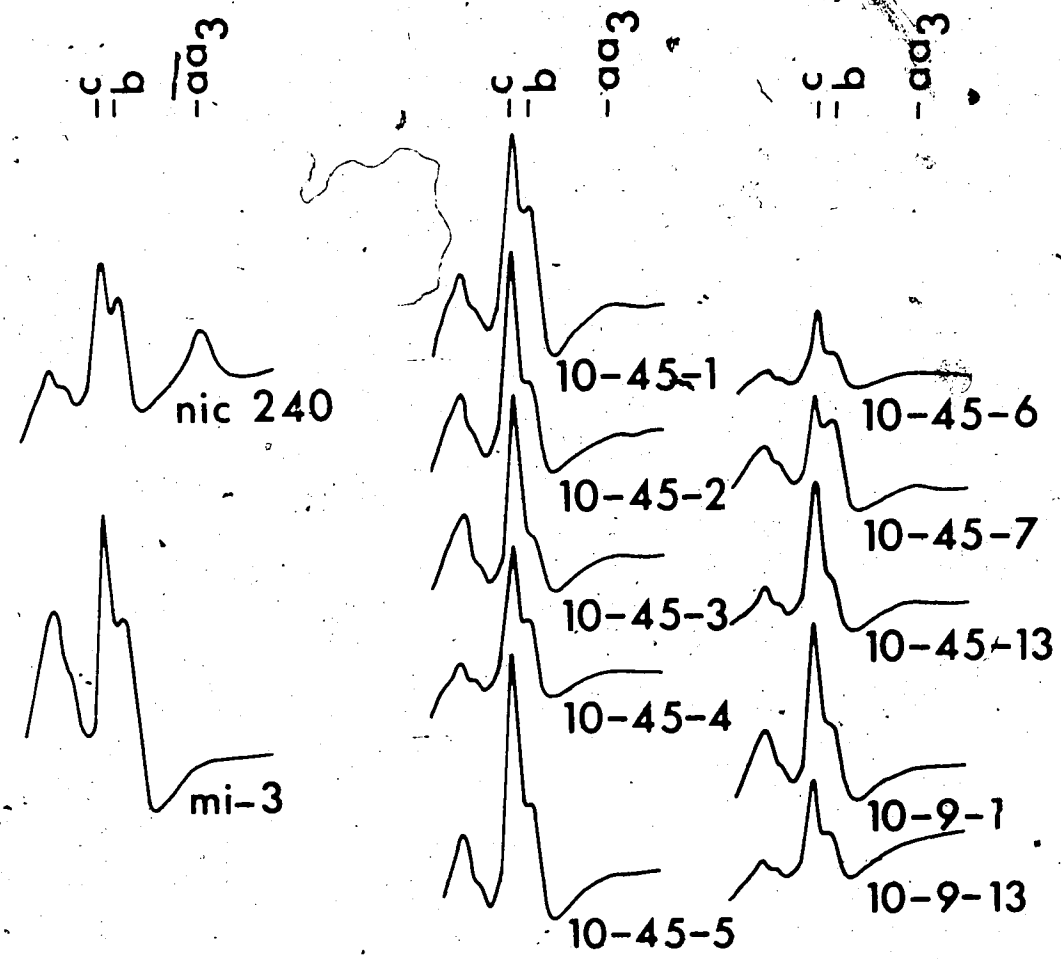

c
b
aa3
nic 240
mi-3
10-45-1
10-45-2
10-45-3
10-45-4
10-45-5
10-45-6
10-45-7
10-45-13
10-9-1
10-9-13

Figure 24. Agarose gel electrophoretic analysis of EcoRI-digested mitochondrial DNAs from [*mi-3*] (type I mitochondrial DNA), 74*A* (type II mitochondrial DNA) and the heterokaryons. The position of all type II EcoRI fragments and the type I EcoRI-5 and EcoRI-9 fragments is indicated.

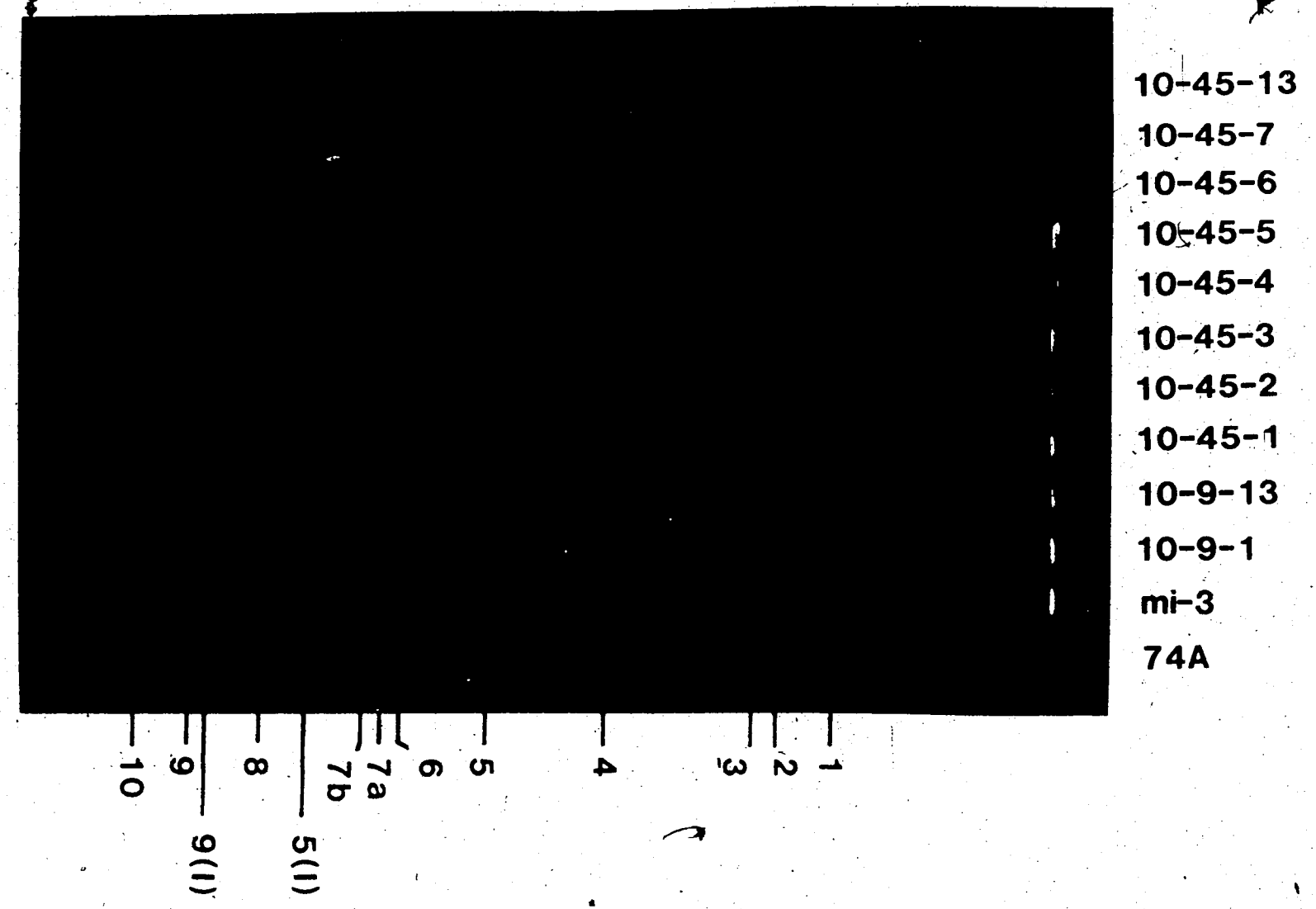
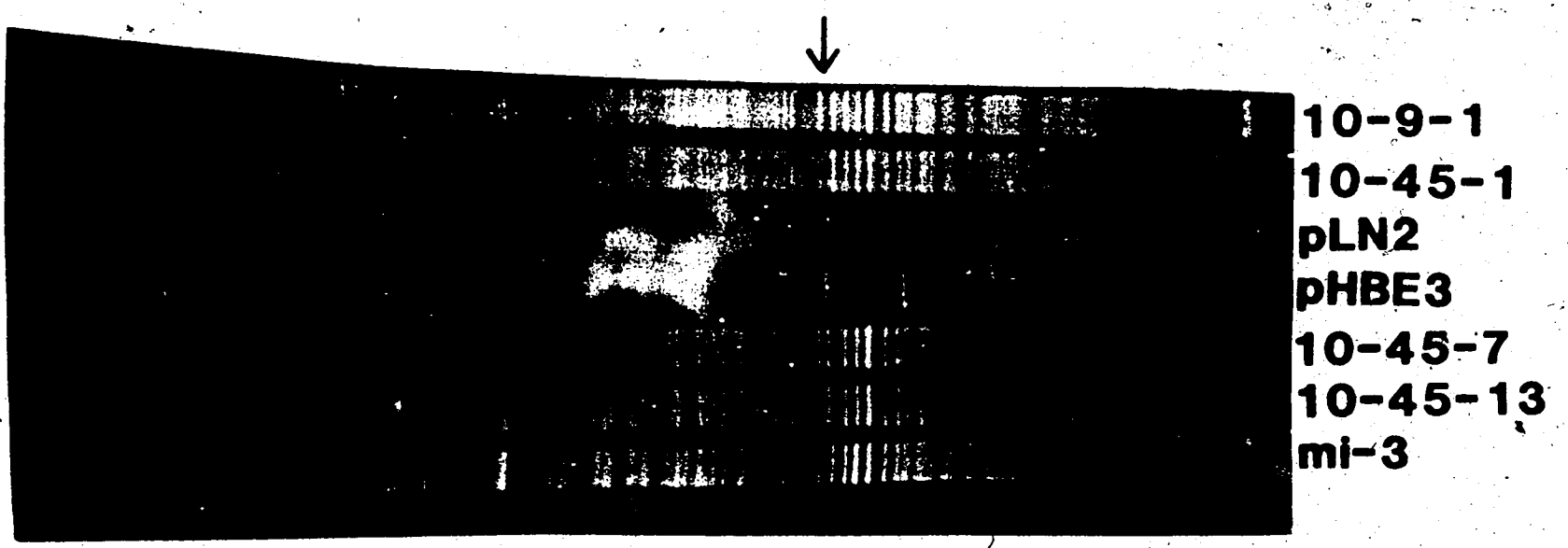

10-45-13
10-45-7
10-45-6
10-45-5
10-45-4
10-45-3
10-45-2
10-45-1
10-9-13
10-9-1
mi-3
74A
10
9
9(I)
8
5(I)
7b
7a
6
5
4
3
2
1

Figure 25. Polyacrylamide gradient gel electrophoresis of RsaI digestion products of the mitochondrial DNAs from four heterokaryons is shown. The arrow indicates the region containing the restriction fragments of interest. The differences described in the text are most easily observed by comparing the distance between the variable band and the band migrating immediately below it. The pLN2 and pHBE3 plasmid provide controls for the [*mi-3*] and wildtype fragments, respectively.


10-9-1
10-45-1
pLN2
pHBE3
10-45-7
10-45-13
mi-3

wildtype fragment (data not shown). The results are less clear for heterokaryon 10-45-13 which appears to possess a mixed population of the 630 bp RsaI fragment. Regardless, genetic analysis of the heterokaryons suggests that the observed difference is not responsible for the [*mi-3*] mutant phenotype.

DNA Sequence Analysis of the [mi-3] oxi-3 Gene

Since no alteration conferring the mutant phenotype was detected through restriction analysis, it was deemed necessary to sequence the [*mi-3*] subunit 1-encoding *oxi-3* gene including the 5' flanking region (Figure 26). The sequence from the [*mi-3*] mutant was largely determined on one strand only because the nucleotide sequence of the wildtype subunit 1 gene had previously been published (Burger *et al.*, 1982; de Jonge and de Vries, 1983). Whenever changes relative to the wildtype sequence were detected, the sequence of the complementary strand was determined. If a change in the sequence of the extranuclear mutant was confirmed, the Abbott 12*a* sequence from the corresponding region was checked to determine whether or not the change was a simple polymorphism that existed between type I and type II mitochondrial DNAs. The first nucleotide of the mature coding sequence was designated as +1 (Figure 26).

Five changes relative to the wildtype sequences were observed in the [*mi-3*] *oxi-3* gene, only two of which were contained in the coding region (Figure 26 and Table 9). The first change in the intergenic region between *cob* and *oxi-3* occurs at position -468. The [*mi-3*] mutant sequence contains an additional nucleotide and at least one more change. The Abbott 12*a* strain had a sequence identical to that of the mutant. The wildtype strain used in the de Jonge and de Vries (1983) study was not described. Consequently, at that time, we were unable to discern whether or not this change was due to different mitochondrial DNA types.* On the other hand, our sequence matched the 18 bp consensus sequence of Pst I palindromes characteristic of *Neurospora* mitochondrial DNA (Yin *et al.*, 1981).

*Subsequently, we learned (H. de Vries, pers. comm.) that 74*A* was the wildtype strain used by de Jonge and de Vries (1983).

Figure 26. Partial restriction map and the DNA sequencing strategy for the *oxi-3* region of *N. crassa* mitochondrial DNA. The first nucleotide of the subunit 1 mature coding sequence is designated +1. Horizontal arrows show the extent and direction of sequencing from M13 clones. Arrows beginning with short vertical lines indicate sequences obtained for [*mi-3*] and those beginning with filled circles designate Abbott 12*a* sequences. Horizontal arrows with broken lines indicate sequences beginning at sites outside the region. Vertical arrows and the corresponding numbers indicate the position of differences detected in the [*mi-3*] sequence. The enlarged darkened area shows the mature *oxi-3* coding region.

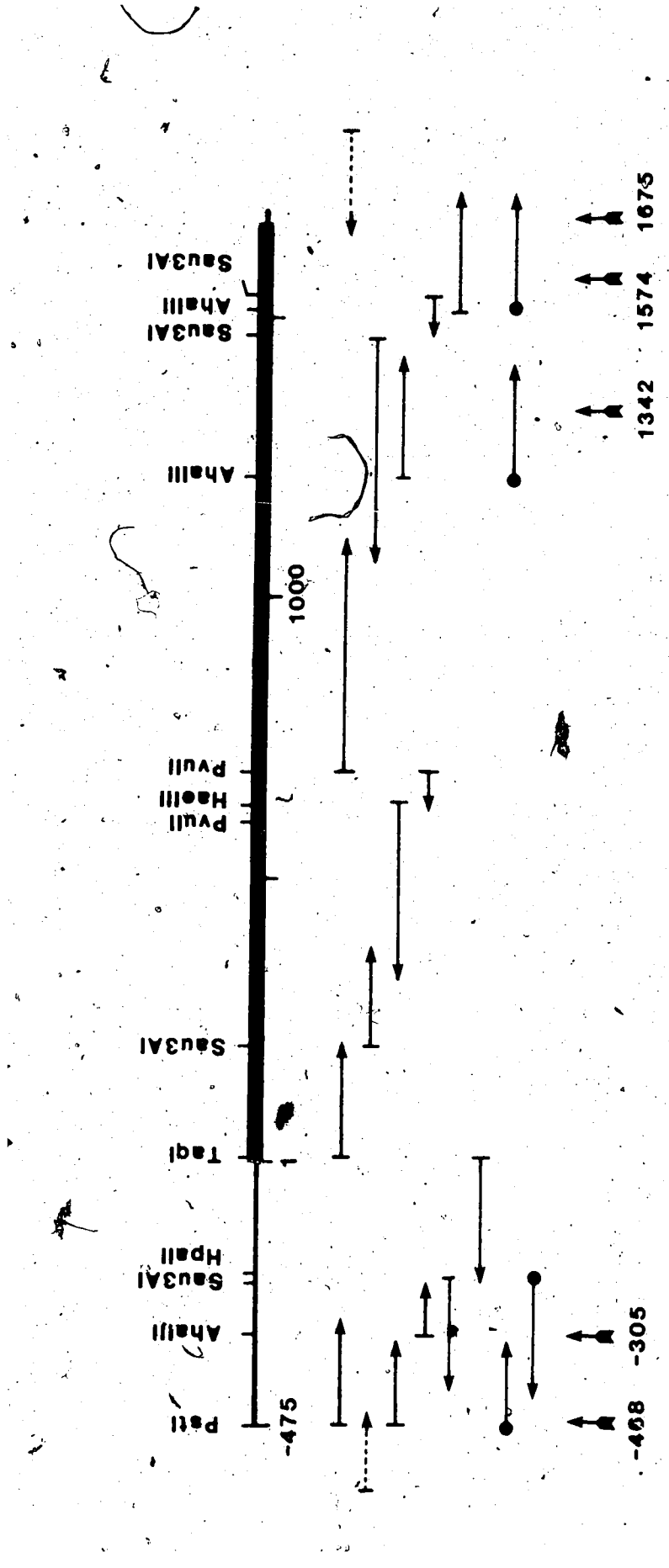

Sau3AI
AhaIII
Sau3AI
AhaIII
1000
PvuII
HaeIII
PvuII
Sau3AI
TaqI
Sau3AI
HpaII
AhaIII
PstI
-475
1675
1574
1342
-305
-468

Table 9. Summary of differences detected in the DNA sequence of the [*mi-3*] *oxi-3* gene and its flanking regions versus the corresponding region in wildtype.

Differences Observed	References
-468 CAGG—CAGG	de Jonge and de Vries, 1983; de Vries *et al.*, 1985
CAGGGCGGG	Abbott 12*a*, [*mi-3*] (this study)
-305 $(T)_{24}A(A)_{16}$	Burger *et al.*, 1982; de Jonge and de Vries, 1983
$(T)_{25}A(A)_{16}$	[*mi-3*] (this study)
1342 GAC (asp)	Burger *et al.*, 1982; de Jonge and de Vries, 1983; Abbott 12 (this study)
TAC (tyr) -mature subunit 1 codon 448	[*mi-3*] (this study)
1574 GCG (ala)	Burger *et al.*, 1982
GTG (val) -mature subunit 1 codon 525	de Jonge and de Vries, 1983; Abbott 12*a*, [*mi-3*] (this study)
1675 TTTTCTCGAA	Burger *et al.*, 1982; de Jonge and de Vries, 1983
TTTTCTTGAA	Burger and Werner, 1986; Abbott 12*a*, [*mi-3*] (this study)

The second alteration corresponds to the A/T bp insertion in the A/T rich palindrome at position -305 that was detected as an RFLP, as previously mentioned (Figure 26 and Table 9). The sequence of the Abbott 12*a* wildtype was not determined, therefore we cannot ascribe this change to a mitochondrial DNA type polymorphism, but genetic analysis with forced heterokaryons demonstrated that this insertion was not associated with the [*mi-3*] mutation as

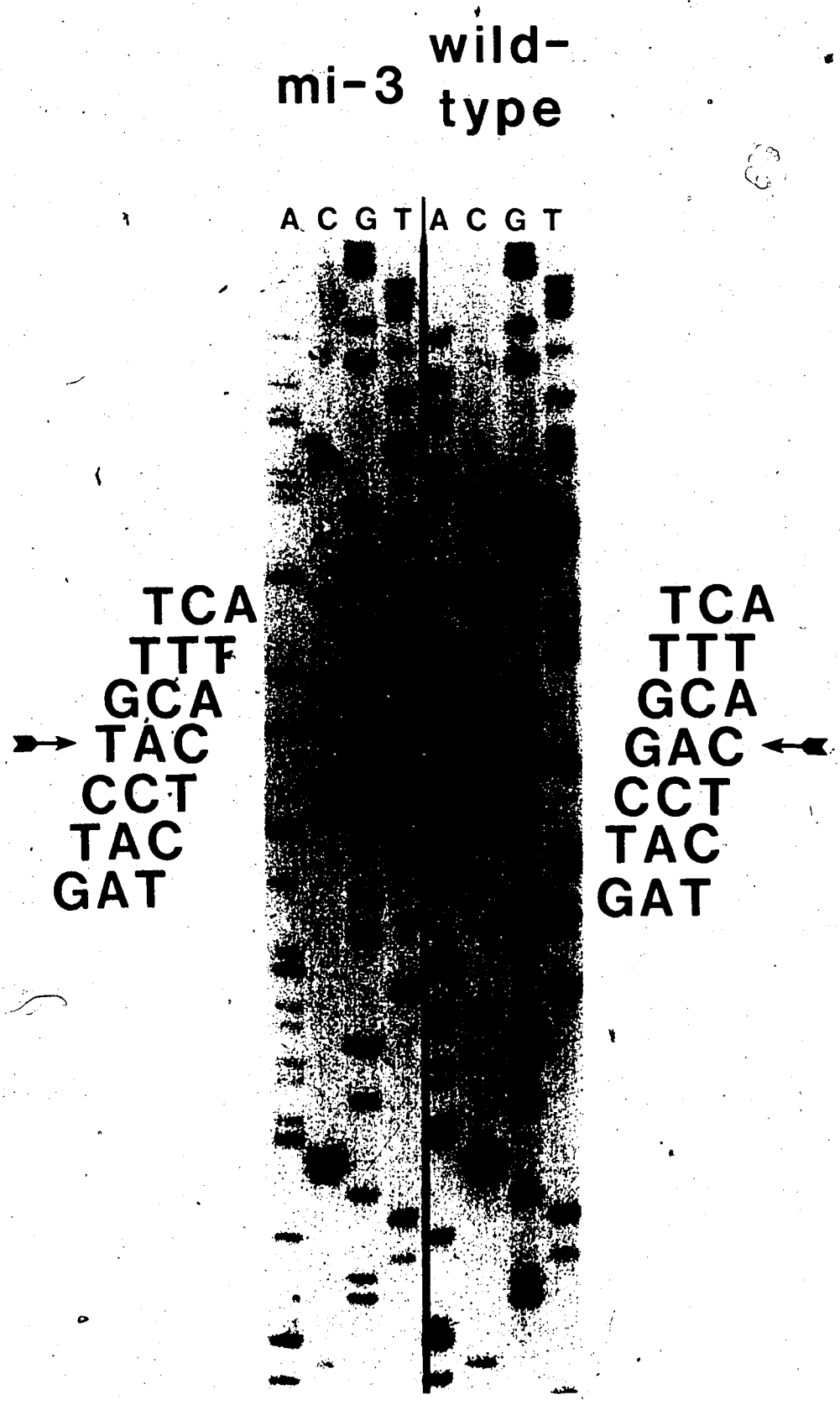
discussed previously on page 97.(Figure 25).

The final change located ouside the *oxi-3* coding region was located immediately downstream of the TAA termination codon (Figure 26 and Table 9). The published sequences (Burger *et al.*, 1982; de Jonge and de Vries, 1983) predict the presence of a TaqI site (5' T↓CGA 3'). The existence of this site could not be confirmed using cloned mitochondrial DNA from both the mutant and the wildtypes. Since the change is outside the *oxi-3* coding region, it is not likely that it would confer the cytochrome oxidase deficiency upon the [*mi-3*] mutant. Its presence was attributed to either a DNA sequencing error or a genetic polymorphism. In a subsequent publication by Burger and Werner (1986), their sequence completely matched the sequence published by Lemire and Nargang (1986).

Only two differences affected the [*mi-3*] *oxi-3* gene sequence (Figure 26 and Table 9). The first change affected amino acid 525 of the 555 amino acid subunit 1 polypeptide. The [*mi-3*] and Abbott 12*a* sequences matched that of de Jonge and de Vries (1983) in that a valine residue was specified at this position. Burger and Werner (1982) have an alanine at this position. Since the de Jonge and de Vries (1983) 74A strain (type II) specifies a valine at position 525 like the type I strains, [*mi-3*] and Abbott 12*a*, it was thought unlikely that a genetic polymorphism would be present in an essential and well conserved structural gene within the same species. It is likely that a sequencing error was made by Burger and Werner (1982) since their sequence was obtained from 74*A*, the same strain used by de Jonge and de Vries (1983). Whether or not this change is a genetic polymorphism or a sequencing error is irrelevant. We can exclude an association with the [*mi-3*] lesion since the codon specifying a valine residue at this position is present in at least two different wildtype strains.

The second alteration found within the [*mi-3*] subunit 1 gene is a G to T transversion that specifies a tyrosine at position 448 of the mature col polypeptide instead of the usual aspartic acid residue (Table 9 and Figures 26 and 27). The Abbott 12*a* strain contains the wildtype amino acid, aspartic acid, at this position (Table 9). Therefore, this missense mutation cannot be attributed to a mitochondrial DNA type I polymorphism. The severity of

Figure 27. Section of a DNA sequence gel autoradiogram that shows the nucleotide substitution affecting codon 448 of the mature subunit 1 gene in [*mi-3*].


mi-3
wild-type
A C G T A C G T
TCA
TTT
GCA
TAC
CCT
TAC
GAT
TCA
TTT
GCA
GAC
CCT
TAC
GAT

the amino acid substitution (Grantham, 1974) makes it a likely candidate for the mutation conferring the respiratory-deficient phenotype upon the [*mi-3*] mutant.

To determine whether or not this change segregated with the mutant phenotype, heterokaryons were forced between *nic* 240, a wildtype strain, and [*mi-3*]^{s} as previously described. The sequence of the subunit 1 coding region containing the change was determined in 6 of 10 slow-growing heterokaryons. All six contained the G to T transversion mutation (Figure 28). Additionally, the sequence of this region from a fast-growing heterokaryon was determined and it was identical to the sequence characteristic of wildtype strains in that an aspartic acid was specified at position 448 (Figure 28). The data suggest that the missense mutation segregates with the mutant phenotype.

At this point it should be mentioned that the heterokaryons were grown at least once along the length of a race tube after the 15th generation of subculturing to make them homoplasmic. It is clear from the heterokaryon mitochondrial DNA run on polyacrylamide gradient gels that at least some, if not all, consist of mixtures of mitochondrial DNA molecules (Figure 25). Therefore, it is possible that the "correct" allele was conveniently cloned in all seven cases. But this is unlikely since the mutant and wildtype phenotype conferred upon the heterokaryons is due to a predominance of mitochondrial DNA molecules harbouring the [*mi-3*] mutation and wildtype allele, respectively.

A second line of evidence suggests the functional importance of at least an acidic residue at position 448. When cytochrome *c* oxidase subunit 1 polypeptides from various organisms are aligned on the basis of amino acid homology,[10] it was noted that there was complete conservation of the aspartic acid residue at this position with the exception of *S. pombe* (Lang, 1984) which specified the closely related amino acid glutamic acid (Grantham, 1974; Table 10). Conservation of an acidic amino acid in one of the few hydrophilic regions of the cytochrome oxidase subunit 1 polypeptide can be construed as good circumstantial

[10]The protozoan subunit 1 sequences were not included in this table because of their limited homology to other eukaryotes (de la Cruz *et al.*, 1984; Hensgens *et al.*, 1984; Pritchard *et al.*, 1986; Ziaie and Suyama, 1987).

Figure 28. Portion of a DNA sequence gel autoradiogram that shows the sequence of the non-coding strand for the region of the amino acid substitution affecting codon 448 of the mature subunit 1 gene in heterokaryons formed between [*mi-3*] and wildtype. A) slow-growing heterokaryon B) fast-growing heterokaryon. The slow-growing heterokaryon carries the transversion mutation in codon 448, characteristic of [*mi-3*] strains, while the fast-growing heterokaryon has retained the wildtype sequence in this region.

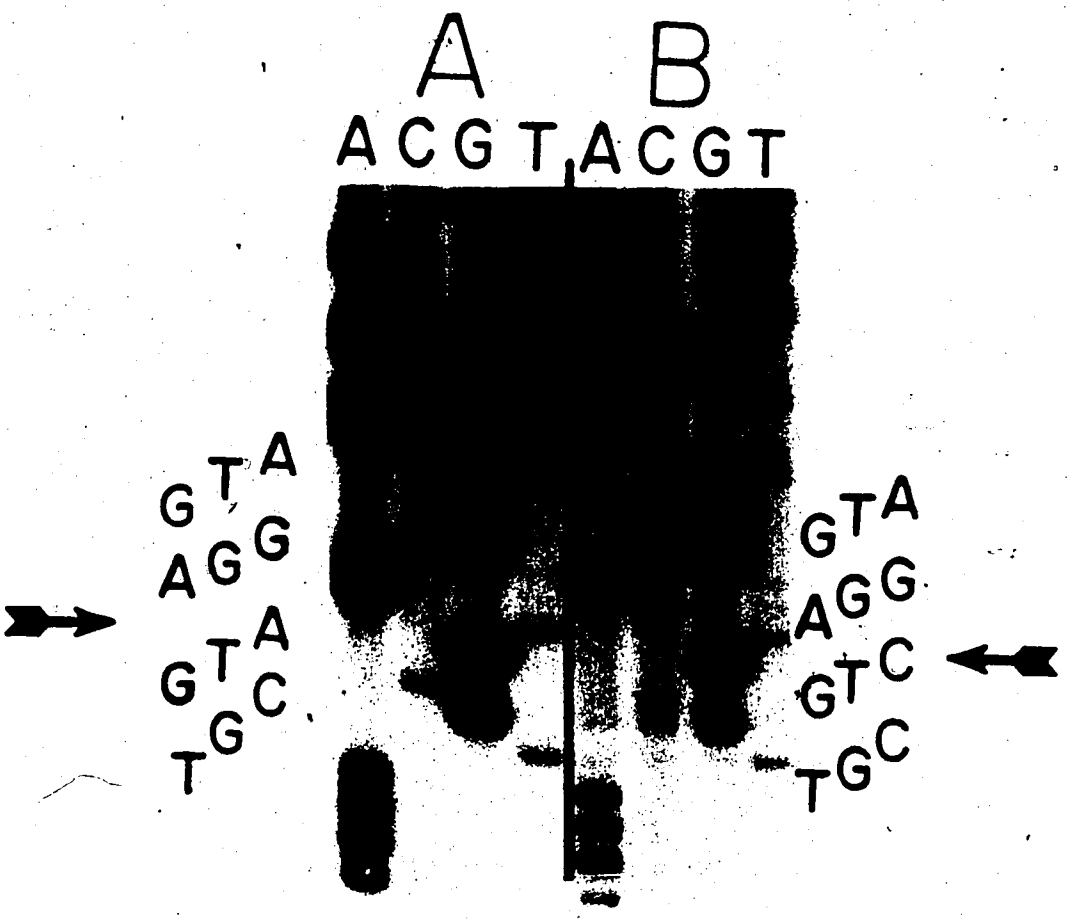

A B
ACGT ACGT
GTA
AGG
GTA
GTC
TGC
GTA
AGG
GTC
TGC

Table 10. Conservation of an acidic residue at position 448 of subunit 1. Only amino acids different from the *N. crassa* type II sequence are indicated in the sequences from the other organisms. Amino acids are given in the standard 1-letter code.

ORGANISM	AMINO ACID SEQUENCE
N. crassa type II mtDNA	PRR I SDYP D AFSGWNL ISSF
N. crassa type I mtDNA [mi-3]	Y
A. nidulans	Y L I
C. reinhardtii	MF A C A AV
D. melanogaster	Y YTT IV TI
D. yakuba	Y YTT VV TI
Bovine	Y YTM T M
Human	Y YTT IL V
Maize	P YA AL
Mouse	Y YTT TV M
Rat	Y YTT TV M
S. cerevisiae	P A YVA I
S. pombe	P E V FV I
Wheat	P YA AL
X. laevis	Y YTL TV I
P. anserina	A

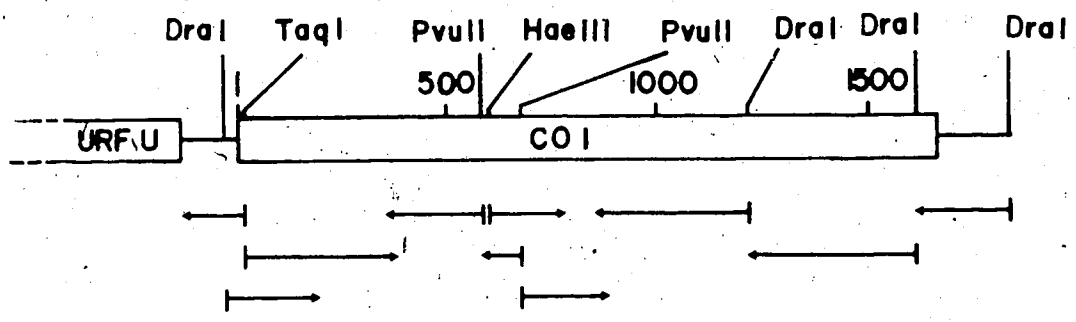
evidence of the importance of this residue.

In conclusion, all of the above evidence strongly indicates that the missense mutation in codon 448 of the [*mi-3*] *oxi-3* gene imparts the mutant phenotype. It is not immediately obvious how all the information on [*mi-3*] can be explained by this mutation. A missense mutation close to the subunit 1 C-terminus may prevent the proteolytic removal of the amino-terminal extension by preventing the polypeptide from adopting a conformation compatible with processing. This proposal does not easily explain how antimycin A suppresses the cytochrome *aa*$_3$ deficiency and allows conversion of the precursor subunit 1 polypeptide to the mature form. An explanation for the observed cytochrome oxidase subunit 2 deficiency is also not readily obvious. Thus, though it seems unlikely, one or more mutations elsewhere may act in whole or in part to manifest the mutant phenotype characteristic of [*mi-3*] strains.

Molecular Characterization of the [exn-5] Mutant

Previous studies on the [*exn-5*] mutant did not reveal the location or nature of the mutation leading to its abnormal phenotype. However, it was noted that there was a slight but consistent displacement of the [*exn-5*] subunit 1 polypeptide towards the cathode in SDS polyacrylamide gels. It was concluded that the degree of displacement was insufficient to establish whether or not the protein did in fact have a higher molecular weight (Bertrand and Werner, 1979). Since the [*mi-3*] mutant has a mutation in the *oxi-3* gene (Lemire and Nargang, 1986) and both extranuclear mutants are suppressed by a common suppressor, namely *su-1* (Bertrand *et al.*, 1976), and [*exn-5*] may have a subunit 1 polypeptide with a slightly different molecular weight (Bertrand and Werner, 1979), it was reasoned that examination of the [*exn-5*] *oxi-3* gene was the most logical starting point to search for the [*exn-5*] mutation. The DNA sequence of the [*exn-5*] subunit 1 gene was determined using the strategy outline in Figure 29. Comparison of the *oxi-3* coding sequence to the published wildtype sequences (Burger *et al.*, 1982; de Jonge and de Vries, 1983) failed to reveal any changes.

Figure 29. DNA sequencing strategy and a partial restriction map of the [*exn-5*] *oxi-3* gene. The arrows indicate the direction and extent of sequences. The first nucleotide of the mature coding sequence is designated as +1.

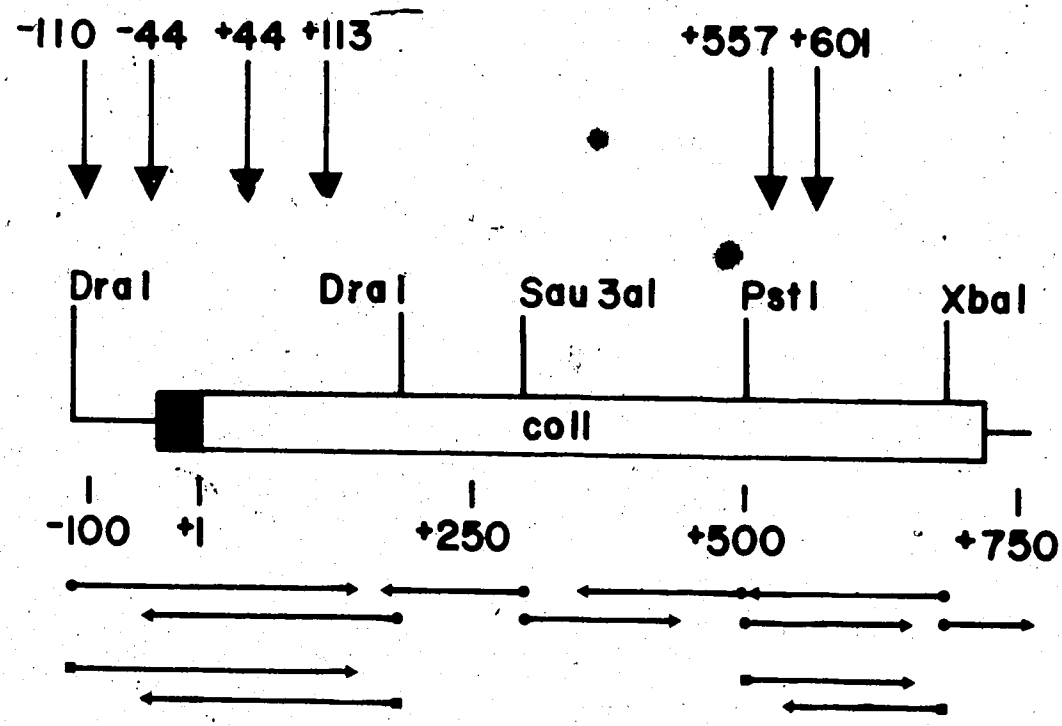

DraI
TaqI
PvuII
HaeIII
PvuII
DraI
DraI
DraI
500
1000
1500
URF\U
CO I

Both [*exn-5*] and [*mi-3*] are phenotypically similar and are suppressed by a common suppressor suggesting that they have acquired genetically similar lesions. It is now established that the *N. crassa* subunit 1 polypeptide is synthesized as a precursor (Van't Sant *et al.*, 1981; Van't Sant and Kroon, 1983) and that [*mi-3*] accumulates the precursor form of cytochrome oxidase subunit 1 (Bertand and Werner, 1979; Werner and Bertrand, 1979; Werner *et al.*, 1980). The only other mitochondrially-encoded subunit that is synthesized as a precursor is subunit 2 (van den Boogaart *et al.*, 1982b), therefore it was reasoned that the [*exn-5*] mutation may affect the subunit 2-encoding *oxi-1* gene (van den Boogaart *et al.*, 1982b; Macino and Morelli, 1983). The decision was made to sequence the mutant *oxi-1* gene. The sequencing strategy is oulined in Figure 30. Any sequence differences detected were compared to the nucleotide sequence of the corresponding region in the parental wildtype, 74A (van den Boogaart *et al.*, 1982; Macino and Morelli, 1983).

DNA sequencing revealed six alterations, two of which are not within the *oxi-1* coding region (Figure 30 and Table 11). The first change was the presence of an additional T about 110 bp upstream of the mature coding sequence (Figure 30 and Table 11). The Macino and Morelli (1983) sequence may be incorrect since the 74*A* sequence presented here and the previously published partial sequence of van den Boogaart *et al.* (1982b) are both in complete agreement with the [*exn-5*] sequence in this region. Alternatively, the alteration may be a polymorphism since it is located in an intergenic region. Nevertheless, since the mutation does not affect the *oxi-1* gene and at least two wildtype strains contain the insertion, it is unlikely that it confers the cytochrome *aa*$_3$ deficiency to [*exn-5*] strains.

The second change affects nucleotide -44, a C is replaced by a T in [*exn-5*] (Figure 30 and Table 11). The published wildtype sequences (van den Boogaart *et al.*, 1982b; Macino and Morelli, 1983) as well as the 74*A* sequence (this study) are all in complete agreement with each other, but they differ from [*exn-5*] at this position. It is peculiar that a polymorphism would exist in the [*exn-5*] strain, but be absent in its parental wildtype, 74*A*. It has been observed however, that mutational hotspots are often located in regions of base repeats

Figure 30. DNA sequencing strategy and a partial restriction map of the [*exn-5*] *oxi-1* gene. The horizontal arrows indicate the direction and extent of sequences. Horizontal arrows beginning with closed circles indicate sequence obtained from [*exn-5*] clones, while those beginning with filled squares indicate sequence obtained from wildtype clones. Vertical arrows and the corresponding numbers indicate the location of the alterations detected. The boxed area indicates the extent of the coII gene. The darkened box shows the presequence. The first nucleotide of the mature coding sequence is designated as +1.


-110
-44
+44
+113
+557
+601
Dra I
Dra I
Sau 3a I
Pst I
Xba I
coll
-100
+1
+250
+500
+750

(Okada *et al.*, 1972). The polymorphism is situated in a stretch of T residues (Table 11). Since the base substitution occurs outside the subunit 2 coding region, it is not likely that it confers the respiratory-deficient phenotype to [*exn-5*] strains.

A nucleotide change within the subunit 2 coding sequence affected codon 38 of the mature polypeptide replacing a valine with a glycine residue (Figure 30 and Table 11). The Macino and Morelli (1983) sequence may be incorrect since the 74*A* sequence presented here and the previously published sequence of van den Boogaart *et al.* (1982b) are both completely identical to the [*exn-5*] sequence in this region. It is unlikely that a well conserved protein would have an amino acid substitution within the same species.

Another alteration affected nucleotides 556 and 557 of the mature coding sequence (Figure 30 and Table 11). The 74*A* and [*exn-5*] sequence determined in this study predict the codon AGG (serine), while the published sequence (Macino and Morelli, 1983) has a GAC codon (aspartic acid) at this position. A serine residue appears to be conserved at this position upon examination of other subunit 2 sequences (Barrell *et al.*, 1979; Young and Anderson, 1980; Anderson *et al.*, 1981; Bibb *et al.*, 1981; Fox and Leaver, 1981; Grosskopf and Feldmann, 1981; Anderson *et al.*, 1982; Brown and Simpson, 1982; Clary and Wolstenholme, 1983a; de Bruijn, 1983; Hiesel and Brennicke, 1983; Bonen *et al.*, 1984; Kao *et al.*, 1984; Clary and Wolstenholme, 1985; Moon *et al.*, 1985; Roe *et al.*, 1985; Grabau, 1987; Ramhamack and Deeley, 1987), the sole exception being *S. cerevisiae* which also has an aspartic acid residue at this position (Coruzzi and Tzagoloff, 1979). We find it unlikely that this is a polymorphic site since severe amino acid substitutions (Grantham, 1974) are not likely to be tolerated in conserved proteins within a species. Nevertheless, since at least the sequence of the [*exn-5*] parental wildtype is completely identical to the mutant sequence in this region, this excludes the possibility that this change is responsible for the respiratory-deficient characteristics in [*exn-5*].

Another amino acid substitution resulted when a G was present at position 601 in 74*A* and [*exn-5*], instead of an A as determined by Macino and Morelli (1983) (Figure 30 and

Table 11. Summary of differences detected in the DNA sequence of the *oxi-1* gene in [*exn-5*] versus the corresponding region in wildtype. The first nucleotide of the mature coding sequence is designated as +1.

Differences Observed	References
-110 TATA-TTT	Macino and Morelli, 1983
TATATTTT	van den Boogaart *et al.*, 1982b; 74*A*, [*exn-5*] (this study)
-44 TTTCTTTTTTT	van den Boogart *et al.*, 1982b, Macino and Morelli, 1983; 74*A* (this study)
TTTTTTTTTTT	[*exn-5*] (this study)
44 ACT (thr)	van den Boogaart *et al.*, 1982b; Macino and Morelli, 1983; 74*A* (this study)
ATT (ile) -mature subunit 2 codon 15	[*exn-5*] (this study)
113 GTT (val)	Macino and Morelli, 1983
GGT (gly) -mature subunit 2 codon 38	van den Boogaart *et al.*, 1982b; 74*A*, [*exn-5*] (this study)
557 GAC (asp)	Macino and Morelli, 1983
AGC (ser) -mature subunit 2 codon 174	74*A*, [*exn-5*] (this study)
601 AGG (arg)	Macino and Morelli, 1983
GGG (gly) -mature subunit 2 codon 189	74*A*, [*exn-5*] (this study)

Table 11). This results in glycine being specified by codon 189 instead of an arginine. The Macino and Morelli (1983) sequence is probably incorrect since examination of other subunit

2 sequences at this position shows that a serine residue is always present at this position (Barrell *et al.*, 1979; Young and Anderson, 1980; Anderson *et al.*, 1981; Bibb *et al.*, 1981; Fox and Leaver, 1981; Grosskopf and Feldmann, 1981; Anderson *et al.*, 1982; Brown and Simpson, 1982; Clary and Wolstenholme, 1983a; de Bruijn, 1983; Hiesel and Brennicke, 1983; Bonen *et al.*, 1984; Kao *et al.*, 1984; Clary and Wolstenholme, 1985; Moon *et al.*, 1985; Roe *et al.*, 1985; Grabau, 1987; Ramharack and Deeley, 1987).

A C to T transition mutation in the 15th codon of the mature subunit 2 sequence was also detected. (Figures 30 and 31; Table 11). The mutation leads to the replacement of threonine by an isoleucine residue in [*exn-5*]. The published sequences (van den Boogaart *et al.*, 1982b; Macino and Morelli, 1983) as well as the parent 74*A*, all have the ACT codon (threonine) at this position; [*exn-5*] has the nucleotide sequence ATT (isoleucine). This change is relatively drastic because an amino acid with a polar side chain (threonine) is replaced by one with a hydrophobic side chain (isoleucine) (Grantham, 1974). Comparison of subunit 2 sequences from other organisms reveals that the amino acid at position 15 is conserved, without exception, as either a threonine or serine indicating that a hydroxylated amino acid may play a key functional or structural role (Table 12). [11] It is likely that this is the mutation in [*exn-5*] that leads to its characteristic maternally inherited cytochrome *aa*$_3$-deficient phenotype. No genetic evidence substantiating this claim has been obtained at this time. Incompatibilities between strains prevented the formation of heterokaryons, therefore, it is not possible to determine if there exists a correlation between the presence of this transition mutation and the mutant phenotype as demonstrated in the case of the [*mi-3*] *oxi-3* mutation (Lemire and Nargang, 1986). It is peculiar that [*exn-5*] appeared to synthesize a normal subunit 2 polypeptide, albeit at slightly lower levels (Bertrand and Werner, 1979). Thus, the possibility that one or more mutations elsewhere in the mitochondrial genome confer the slow-growth and cytochrome oxidase-deficient phenotype upon the [*exn-5*] mutant

[11] The protozoan subunit 2 sequences were not included in this table because of their distinct nature and limited homology to other eukaryotic coII sequences (de la Cruz *et al.*, 1984; Hensgens *et al.*, 1984.

Figure 31. Portion of a DNA sequence gel autoradiogram that shows the sequence of the coding strand of [*exn-5*] and wildtype in the region containing the transition mutation affecting the 15th codon of the mature subunit 2 gene. The sequence was obtained using dITP mixes (see Appendix).

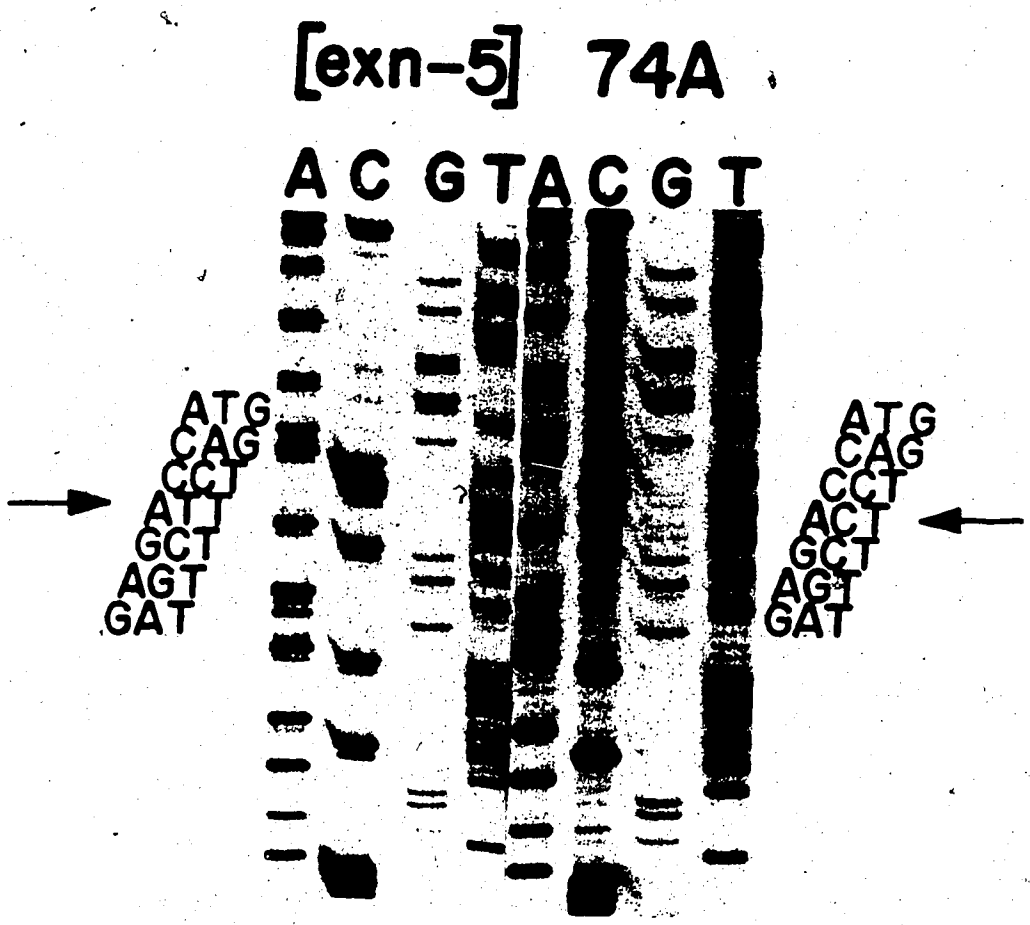

[exn-5] 74A
A C G T A C G T
ATG
CAG
CCT
ATT
GCT
AGT
GAT
ATG
CAG
CCT
ACT
GCT
AGT
GAT

Table 12. Conservation of a hydroxylated residue at position 15 of mature subunit 2. Only amino acids different from the 74*A* sequence are indicated in the sequences from other organisms. Amino acids are given in the standard 1-letter code.

ORGANISM	AMINO ACID SEQUENCE
N. crassa 74A [exn-5]	DAPSPWGIYFQDSATPQMEGLVELHD
Bovine	MAY MQLG ATS I E LHF
D. melanogaster	MSTWANLGL S L Q IFF
D. yakuba	MSTWANLGL S L Q IFF
Human	MAHAAQVGL ATS I E ITF
Maize	AE QLGS A M Q IID H
Mouse	MAY FQLGL ATS I E MNF
Oenothera	AE QLGS A M Q IID H
Pea	AE QLG A M Q IID H
Rat	MAY FQLGL ATS I E TNF
Rice	AE QLGS A M Q IID H
S. cerevisiae	V T YAC NQ IL
Soybean	AQ QLG A M Q IID H
X. laevis	MAH SQLG A S I E LHF
Wheat	AE QLGS A M Q IID H
Monkey	MAH VQLSL ATS I E ITF

cannot be excluded.

The su-1 gene

The cytochrome *aa*$_3$ deficiency of both group II extranuclear mutants is suppressed by the nuclear suppressor, *su-1*. Even though the mutations thought to be responsible for both the [*mi-3*] and [*exn-5*] mutant phenotypes have been localized to the *oxi-3* (Lemire and Nargang, 1986) and *oxi-1* (this study) genes, respectively, much more needs to be known to more fully understand the exact mechanism by which the mutations impart their characteristic respiratory-deficient phenotypes. Therefore, the cloning and characterization of the nuclear gene *su-1* was undertaken. It was hoped this would reveal clues about the nature of the suppressive activity that relieved the cytochrome *aa*$_3$ deficiency in the two cytoplasmic mutants as well as uncovering information concerning the coordinate expression of the nuclear and mitochondrial genomes.

A genomic cosmid DNA library from a *su-1* [*mi-3*] strain was constructed in the cosmid vector, pSV50 (Vollmer and Yanofsky, 1986). For a 99% probability that a given DNA sequence would be represented in the library (using 27,000 kbp as the genome size (Vollmer and Yanofsky, 1986) and 40 kbp as the average size of insert (Vollmer and Yanofsky, 1986)), 5000 individual clones (80% insert frequency) were picked (Clarke and Carbon, 1976) into 96 well microtitre dishes as described by Vollmer and Yanofsky (1986). This genomic library was used in all subsequent *N. crassa* transformation experiments.

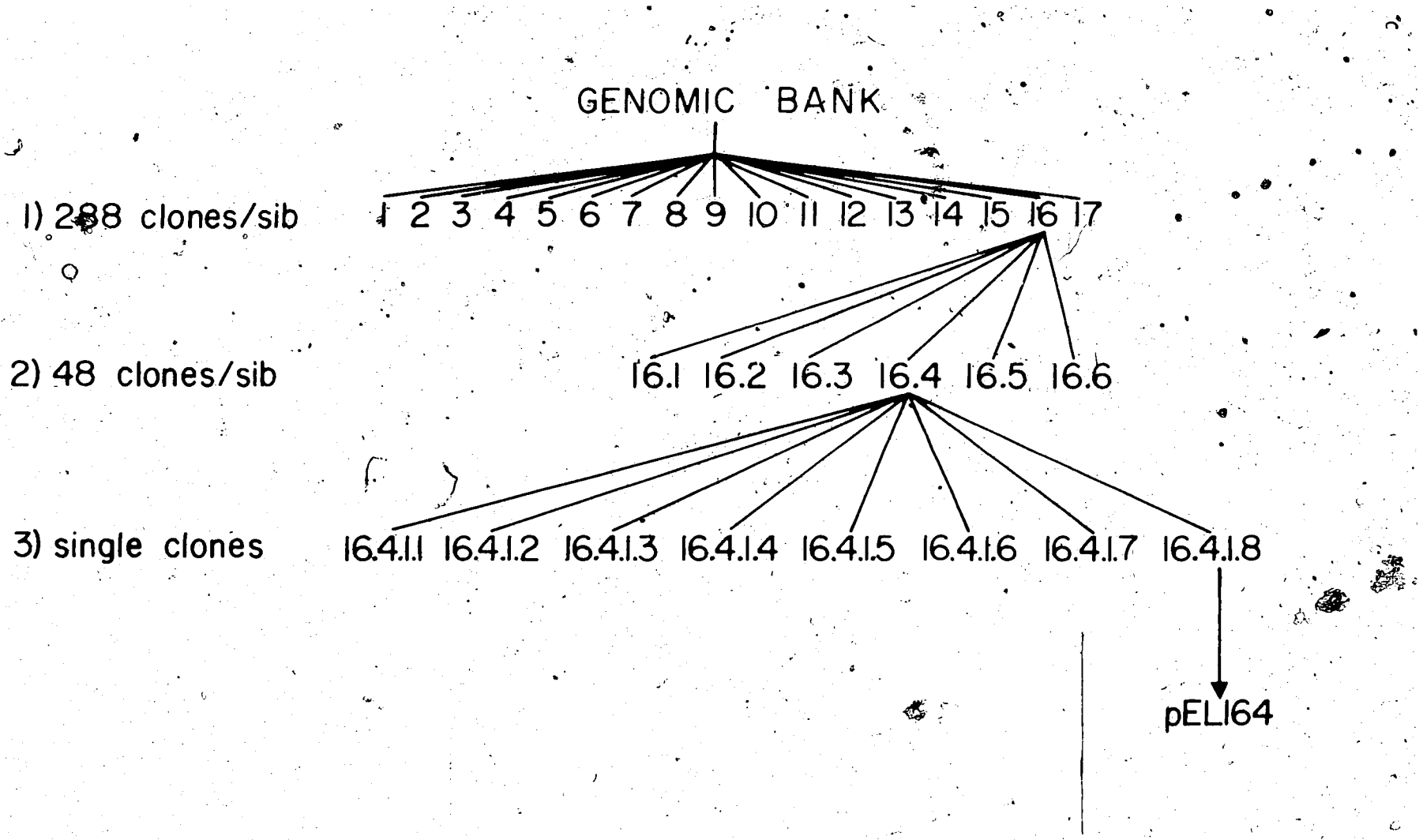
First round transformations indicated that DNA from sibs 12, 13, 14 and 16 rescued the [*mi-3*] mutant more efficiently than DNA preparations from the other sibs. Second round DNAs from each of these sibs were isolated and used in separate transformation experiments, however ambiguous results were repeatedly obtained. This led to the conclusion that the selection scheme, (*i.e.* looking for fast-growing transformants (*su-1*, [*mi-3*]) in a background of slow-growers ([*mi-3*])), was not adequate. An alternative approach, chromosome walking from an adjacent locus, was considered feasible.

In the original paper citing the discovery of *su-1*, the suppressor was mapped to linkage group I, about 4 centimorgans (9/201 recombinants) away from the *al-2* locus, apparently proximal to the centromere (Gillie, 1970; Perkins *et al.*, 1982). Vollmer and Yanofsky (1986) estimated that the average insert size of the library (40 kbp) was roughly equivalent to 1.5% recombination. This suggested that the *su-1* gene was >100 kbp away from *al-2* and could be reached by a minimum of three walks. The success of this approach (chromosome walking from *al-2*) was contingent upon being able to identify the clone carrying the suppressor gene by transformation of an [*mi-3*] strain. It was felt that the selection scheme previously described would permit identification of the correct clone under conditions where the transforming DNA consisted of a unique cosmid clone rather than a mixed population as was the case in the sib-selection procedure. At this point, it should be mentioned that ambiguous results are more often than not the norm for the first two rounds of transformation with cytochrome-deficient mutants (Akins and Lambowitz, 1985). Transforming with single clones virtually circumvents this problem.

The *AL-2* gene was isolated from the *su-1* [*mi-3*] cosmid library by transforming *nic 240* (*nic-1, al-2*) and rescuing its albino phenotype. To achieve this, transformants were not overlayed onto benomyl plates with top agar as was normally done (Akins and Lambowitz, 1985; Vollmer and Yanofsky, 1986). Rather, the sphaeroplasts were transferred to regeneration media (see appendix) and grown at 25°C until the cell walls regenerated (about 4 hours). These were subsequently spread onto benomyl plates using a glass spreader after washing. This modification was essential because it maximized the number of conidiating transformants. This in turn made the detection of non-albino transformants possible.

The first two rounds of sib-selection localized *AL-2* to sib 16.4, thus limiting the search to 48 clones (Figure 32). Instead of proceeding to the third and fourth rounds of transformation using CsCl-banded cosmid DNA, the DNA was isolated by a modified rapid plasmid procedure incorporating a LiCl step to remove the RNA (see Materials and Methods). DNAs from only 8 of the possible 48 clones were isolated in this manner to test

Figure 32. Cloning of the *N. crassa AL-2* gene by sib selection.


GENOMIC BANK
1) 288 clones/sib
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
2) 48 clones/sib
16.1 16.2 16.3 16.4 16.5 16.6
3) single clones
16.4.1.1 16.4.1.2 16.4.1.3 16.4.1.4 16.4.1.5 16.4.1.6 16.4.1.7 16.4.1.8
pEL164

the feasibility of this approach. Fortuitously, 1 of the 8 clones tested rescued the albino phenotype. Clone 16.4.1.8 of the *su-1* [*mi-3*] genomic library harbors the *AL-2* gene (Figure 32). Transformation of [*mi-3*] sphaeroplasts demonstrated that the cosmid clone, as was expected, did not carry the [*su-1*] gene.

Before chromosome walking is initiated, a linkage map of the region must be constructed to accurately map the *su-1* gene on chromosome I. The identification and cloning of markers on either side of the suppressor locus would help to limit the search. Crosses involving strains carrying the following linkage group I markers are being performed: *arg-6*, *al-1*, *hom*, *un-7*, and *cyh-1*.

IV. Concluding Remarks

Missense mutations in the *oxi-3* and *oxi-1* genes of [*mi-3*] and [*exn-5*], respectively, have been detected by DNA sequence analysis (Lemire and Nargang, 1986; this study). These changes are relatively severe based on the parameters of Grantham (1974) and both affect relatively conserved domains of the respective polypeptides (Tables 10 and 12). At least the transversion mutation in [*mi-3*] appears to segregate with the mutant phenotype (Lemire and Nargang, 1986) indicating a genetic relationship with the [*mi-3*] mutation. No genetic evidence supporting the hypothesis that the *oxi-1* transition mutation and the [*exn-5*] mutation are one and the same is available at this time.

A definitive test that would demonstrate whether or not the missense mutations in the *oxi-3* and *oxi-1* genes impart the mutant phenotypes to [*mi-3*] and [*exn-5*], respectively, would be by transformation rescue experiments, that is, by introduction of a wildtype version of the affected gene. The transformed mutants would be expected to be respiratory competent. The cytoplasmic transformation of *S. cerevisiae* using purified mitochondrial DNA has been reported (Maqueda *et al.*, 1984). Two cytoplasmic mutant strains were rescued using this procedure. An erythromycin-sensitive strain was transformed to an an erythromycin-resistant phenotype and a respiratory-deficient strain (ρ^-) became respiratory-competent after transforming with mitochondrial DNA from a ρ^+ donor strain (Maqueda *et al.*, 1984). Transformation of the mutants with wildtype mitochondrial DNA and DNA sequence analysis of the regions containing the missense mutations in the wildtype transformants should establish the importance of the changes in conferring the cytochrome oxidase-deficient phenotypes. But as of yet, there are no reports of successful cytoplasmic transformation experiments in *Neurospora crassa*.

A second approach being developed involves the chemical synthesis of mitochondrial genes (Gearing *et al.*, 1985). A synthetic gene was made using the universal rather than the mitochondrial genetic code and was fused to a suitable N-terminal targeting presequence (Gearing *et al.*, 1985; Gearing and Nagley, 1986). The *in vitro* import of a synthetic ATPase

subunit 8 polypeptide, normally-specified by the mitochondrial genome, into yeast mitochondria was reported (Gearing and Nagley, 1986). Transformation with synthetic *oxi-1* and *oxi-3* genes linked to suitable N-terminal presequences should rescue the [*exn-5*] and [*mi-3*] mutants, respectively, if the missense mutations detected conferred the respiratory-deficient phenotypes. Problems may arise in these transformants since the appropriate cytochrome oxidase subunit probably would not be produced in stoichiometric amounts, as is the case in wildtype strains. Furthermore, assembly of this subunit into the holoenzyme complex may be prevented if the artificially-attached presequence is not correctly removed. But it is encouraging to note that there is a precedent for a function normally specified function by the mitochondria being provided by a nuclear gene. The cytochrome oxidase subunit 1 polypeptide is found in the nucleus in the *Podospora mex1* mutant and appears to provide the normally specified mitochondrial function. (Vierny *et al.*, 1982; Wright and Cummings, 1983; Timmis and Scott, 1984).

An understanding of the primary structure of the col precursor and mature polypeptides is necessary to fully comprehend the respiratory-deficient phenotype of [*mi-3*] strains. The use of synthetic antigens, homologous to different portions of the col precursor, to produce antisera used for immunoblots proved unsuccessful. The use of longer peptides as immunogens may circumvent this problem. Alternative approaches to resolve the primary structure of the col forms also merit an attempt. Partial amino acid sequence analysis of the [*mi-3*] subunit 1 precursor polypeptide would determine the length and sequence of the N-terminal presequence. Since the [*mi-3*] mutant accumulates the precursor form (Bertrand and Werner, 1979; Werner and Bertrand, 1979; Werner *et al.*, 1980), isolating sufficient amounts for amino acid sequencing should not be difficult. This is contingent upon the precursor form being relatively stable and that its amino-terminal methionine residue residue can be deformylated to remove the block (Werner and Bertrand, 1979; Werner *et al.*, 1980). The C-terminal residue could be determined by conventional means such as hydrazinolysis or the use of carboxypeptidase (Lehninger, 1976). The presence of different C-terminal residues

would be good evidence in support of the C-terminal processing hypothesis.

Therefore, until the primary structure of the mature and precursor subunit 1 polypeptides is determined, the possibility remains that either N- or C-terminal processing or both may be involved in the maturation of cytochrome oxidase subunit 1. The use of alternative initiation codons has not been eliminated nor has the possibility that some other post translational modification event like phosphorylation has occurred.

Bertrand and Collins (1978) proposed a model to explain the genetic data on the regulation of cytochrome aa_3 biosynthesis in *Neurospora*. They suggested that at least two control circuits regulated the expression of cytochrome aa_3. The first circuit, which includes the *cyt-2-1*, [*mi-3*], and *cya-3-16* genes, controlled the constitutive expression of cytochrome oxidase, while the second circuit modulated the levels of cytochrome oxidase when electron flow was blocked through the cytochrome *bc*$_1$ segment of the respiratory chain by chemical (*i.e.* antimycin A, HNQO) or genetic means (*i.e. cyb-1, cyb-2*) (Bertrand and Collins, 1978; Bertrand, 1980).

The recent evidence presented herein and elsewhere (Lemire and Nargang, 1986) now indicates that several flaws exist in the model. First, it was proposed that *cyt-2-1* and the two extranuclear mutants, [*mi-3*] and [*exn-5*], had mutations in genes that regulate the expression of cytochrome oxidase rather than in ones that code for structural components (Bertrand *et al.*, 1976; Bertrand and Collins, 1978; Bertrand, 1980). Evidence gathered from this study has shown that both the [*mi-3*] and [*exn-5*] mutants have missense mutations in cytochrome oxidase structural genes.

The gene affected in the *cyt-2-1* strain has been determined (Drygas, M.E., R.A. Akins, A.M. Lambowitz and F.E. Nargang, in preparation). The *cyt-2-1* mutation does not affect a structural component of cytochrome oxidase as in the two extranuclear mutants, but rather the gene for cytochrome *c* heme lyase, an enzyme that post-translationally modifies apocytochrome *c* by attaching the heme prosthetic group (Dumont *et al.*, 1987; Nicholson *et al.*, 1987; Drygas, M.E., R.A. Akins, A.M. Lambowitz and F.E. Nargang, in preparation).

Thus, it appears that the cytochrome aa_3 deficiency in *cyt-2-1* is merely a secondary effect of the heme lyase mutation. A similar phenomenon regarding the cytochrome aa_3 deficiency is observed in the yeast heme lyase mutant, *cyc3*, particularly under repressing conditions (Sherman *et al.*, 1965; Dumont *et al.*, 1987). It is not yet known how a cytochrome *c* heme lyase mutation affects the expression of cytochrome aa_3.

The modulator circuit proposed by Bertrand and Collins (1978) appears to exist. Increased levels of cytochrome aa_3 are observed when electron transport is blocked genetically or chemically at complex III of the respiratory chain, but not exclusively as proposed. Antimycin A and HNQO (Figure 1), as well as the nuclear mutations, *cyb-1* and *cyb-2*, induce the production of cytochrome aa_3 in [*mi-3*], but so does oligomycin, an inhibitor of the mitochondrial ATPase (Bertrand and Collins, 1978; Bertrand, 1980; Tzagoloff, 1982). Oligomycin does block electron flow, but only indirectly because ATP production and the flow of electrons are coupled (Lehninger, 1976; Tzagoloff, 1982). Consequently, electron flow is not specifically impaired in the cytochrome bc_1 segment. Additionally, the *cyb-3* allele (West and Pittenger, 1977), a nuclear mutation resulting in a cytochrome *b*-deficient phenotype similar to *cyb-1*, does not induce the production of cytochrome aa_3 in [*mi-3*] (Bertrand, 1980). From these observations, it can be concluded that the impairment of electron flow can sometimes modulate levels of cytochrome aa_3 in some cytochrome aa_3-deficient mutants. Blockage of electron transport at cytochrome *b* does not always increase the levels of cytochrome aa_3 and inhibitors that do not specifically act at complex III may behave similarly to antimycin A with respect to its effects on cytochrome aa_3 levels.

It should also be noted that the chemical and genetic means used do not exclusively impair the flow of electrons. Pleiotropic effects are observed. For example, the *cyb-1* mutation, as well as antimycin A and oligomycin, are all known to induce the alternate oxidase in *Neurospora* (Bertrand *et al.*, 1976; Edwards *et al.*, 1974; Szakacs and Bertrand, 1976; Nargang, 1982). The induction of the alternate oxidase is probably not the critical component required for the modulation of cytochrome aa_3 levels since *cyb-2* mutants are

unable to induce the alternate oxidase, yet this allele is as effective as *cyb-1* in increasing cytochrome aa_3 levels in [*mi-3*] (Bertrand and Collins, 1978; Bertrand, 1980; Nargang, 1982). Chemicals such as cyanide and ethidium bromide induce the alternate oxidase in *Neurospora* (Lambowitz and Slayman, 1971; Edwards and Rosenberg, 1976), yet there are no reports that they lead to increased levels of cytochrome aa_3. Additionally, the [*mi-3*] mutant already respires predominantly by the alternate oxidase (von Jagow *et al.*, 1973).

Oligomycin and antimycin A also disrupt the membrane potential and, consequently block the import of proteins synthesized on cytoplasmic ribosomes (Hay *et al.*, 1984). The inhibition of protein import into mitochondria is probably not what induces cytochrome aa_3 production since cytoplasmically synthesized polypeptides are essential for the biogenesis of cytochrome aa_3. The concentration of antimycin A (0.3 μg/mℓ) used to achieve cytochrome aa_3 production in [*mi-3*] is probably insufficient to completely inhibit electron flow, and consequentially, protein translocation. The possibility exists that some proteins (*i.e.* a repressor of cytochrome aa_3 production) are less efficiently translocated into the mitochondria than others under these conditions, but as of yet there is no evidence to support the existence of such a regulatory protein.

The various aspects of mitochondrial activity affected by these chemicals makes it difficult to determine exactly what modulates cytochrome aa_3 expression in [*mi-3*]. Consequently, it may be worthwhile to determine if other inhibitors of the respiratory chain such as rotenone and azides (Figure 1) affect the levels of cytochrome aa_3 in [*mi-3*] and other cytochrome aa_3-deficient mutants. It would be particularly interesting to see if oligomycin induces cytochrome aa_3 expression in the presence of 2,4-dinitrophenol (an uncoupler), since electron transfer would be expected to resume, independent of ATP synthesis. Experiments of this nature may help to localize the critical factor(s) involved in the modulator circuit.

The model assumes that spectrally detectable cytochrome aa_3 is synonymous with cytochrome oxidase activity (Bertrand and Collins, 1978). This may be a major error on the part of the authors since it has been suggested that the presence of cytochrome aa_3 may not

always be associated with cytochrome *c* oxidase activity (Nargang *et al.*, 1979). In fact, it has been shown that choline starvation of *N. crassa* cultures results in a decrease in the amount of cyanide-sensitive respiration, despite the normal amounts of cytochrome *aa*$_3$ (Luck, 1965; Juretić, 1976; Nargang *et al.*, 1979). The cytochrome *c* oxidase activity was never determined in antimycin A-supplemented cultures of [*mi-3*], *cyt-2-1* or *cya-3-16* (Bertrand and Collins, 1978; Bertrand, 1980). It has been observed that only [*mi-3*], but not *cya-3-16* nor *cyt-2-1* has a markedly stimulated growth rate along with the induction of cytochrome *aa*$_3$ expression in the presence of antimycin A (Bertrand and Collins, 1978). This may possibly reflect an increase in cytochrome oxidase activity along with the elevated levels of cytochrome *aa*$_3$ in [*mi-3*], while in *cyt-2-1* and *cya-3-16* cultures there is no increase in enzymatic activity associated with the presence of cytochrome *aa*$_3$. Other factors may explain this particular observation, but this scenario is presented to stress the fact that the enzymatic and spectrally-detected components of complex IV may be distinct and should be quantitated separately rather than assuming that a change in one corresponds to a similar change in the other.

Since the [*mi-3*] and [*exn-5*] mutants are genetically similar (Bertrand *et al.*, 1976), they must have mutations affecting a similar step involved in the biogenesis of cytochrome *aa*$_3$. Based on the evidence gathered in this study, it is conceivable that the extranuclear mutations in [*mi-3*] and [*exn-5*] affect the processing of both subunit 1 and 2 precursor polypeptides, respectively. Both cytochrome oxidase subunits 1 and 2 are synthesized as precursors in *Neurospora* (Van't Sant *et al.*, 1981; van den Boogaart *et al.*, 1982b; Van't Sant and Kroon, 1983). Thus, the assumption would be that the missense mutations in the two cytoplasmic mutants lead to a conformational change in the polypeptide which alters a protease recognition and/or binding site in a manner that is not compatible with efficient processing.

The two cytoplasmic mutants are suppressed by a common suppressor, namely *su-1*. Thus, the *su-1* gene might encode a protease, similar to the *TS2858* gene product identified in

yeast (Pratje *et al.*, 1983; Pratje and Guiard, 1986). This gene appears to be required for the removal of the yeast cytochrome oxidase subunit 2 presequence (Pratje *et al.*, 1983; Pratje and Guiard, 1986). Since cytochrome oxidase subunit 1 in yeast is not synthesized as a precursor (Mannhaupt *et al.*, 1985), there is no analogous function required for proteolytic cleavage of this protein.

The *su-1* mutation has no detectable phenotype in wildtype strains (Bertrand and Collins, 1978), suggesting that the wildtype function is unaffected by this mutation. It appears that the *su-1* gene product may have been modified to recognize and process the mutant precursor forms more efficiently. Alternatively, the *su-1* mutation may be a promoter-up mutation which increases the levels of expression of this gene. Conceivably, the increased levels of processing activity would enable the poorly processed precursor substrates to be more efficiently post-translationally modified.

If the precursor and mature forms of subunit 1 had been resolved on SDS polyacrylamide gels, the development of an *in vitro* assay using wildtype, [*mi-3*] and suppressed [*mi-3*] (*su-1*, [*mi-3*]) mitochondria would have helped to test this hypothesis. At least the processing activity, encoded by the *MAS1* gene (Yaffe *et al.*, 1985), previously described (Böhni *et al.*, 1983), is sensitive to chelating agents. There is no report of any such experiment being tried with the *TS2858* gene product. Possibly, the addition of EDTA to the *su-1* extracts would affect the processing of the subunit 1 precursor and lend support to this hypothesis. In addition, such an assay would allow isolation of the *su-1* activity.

As for the cytochrome oxidase subunit 2 deficiency, characteristic of [*mi-3*] strains (Bertrand and Werner, 1977), this may merely reflect the instability of this particular subunit when not assembled into the holoenzyme complex. Suppression by antimycin A allows processing of the subunit 1 precursor to proceed, which subsequently permits assembly of the constituents into the multimeric complex (Werner and Bertrand, 1979; Werner *et al.*, 1980). Further experimentation is required before a model incorporating the observation that cytochrome aa_3 levels are induced in specific mutants by both chemical and genetic means.

It appears that mitochondrial biogenesis involves complex interactions. The levels of expression of the different cytochromes appear to be coordinately regulated. It is well documented that cytochrome *c* levels are elevated in cytochrome aa_3 mutants. The *cyt-2-1* mutant, which has a defective cytochrome *c* heme lyase gene (Nargang *et al.*, 1988), is also deficient in cytochrome aa_3. This suggests that the levels of expression of cytochrome aa_3 and *c* are affected by the levels of cytochrome *c* and aa_3, respectively. Cytochrome *b* and *c* levels likewise show a similar relationship. Excess cytochrome *c* is also present in *cyb* mutants and wildtype levels of cytochrome *b* are present in *cyb-1, cyt-2-1* double mutants (Bertrand and Collins, 1978). Thus, it appears that the levels of one cytochrome are dependent on the levels of the other cytochromes, rendering it more difficult to propose models and demonstrating the complexity of the system. This emphasizes the need for further experimentation to more fully understand the intricacies of mitochondrial biogenesis.

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Appendix

I. Bacterial Media

L-broth
(Lennox, 1955)

10 g	bacto-tryptone (Difco)
5 g	yeast extract (Difco)
5 g	NaCl
1 g	D-glucose

The pH was adjusted to 7.4 with 5 N NaOH. The solution was made up to 1 ℓ with water and sterilized by autoclaving.

D.M. Salts + Thiamine + Glucose
(Davis and Mingioli, 1950)

50 mℓ	20X D.M. stock
1 mℓ	10% $MgSO_4$
1 mℓ	10 mM thiamine

The solution was made up to 500 mℓ with water and sterilized by autoclaving. A 1% (w/v) solution of sterile D-glucose (500 mℓ) was added when the above solution had cooled.

20X D.M. Stock

20 g	$(NH_4)_2SO_4$
60 g	KH_2PO_4
140 g	K_2HPO_4

This was made up to 1ℓ with water and 5 mℓ of chloroform was added as a preservative.

II. Neurospora Media and Solutions:

Vogel's Medium
(Davis and de Serres, 1970)

20 mℓ	50X Vogel's stock
1 mℓ	1X trace elements
1 mℓ	biotin solution
15 g	table sugar

Supplements were added as necessary and the solution was made up to 1 ℓ with water before autoclaving.

0.5X Vogel's

10 mℓ	50X Vogel's stock
0.5 mℓ	biotin solution
0.5 mℓ	1X trace elements
7.5 g	table sugar

Supplements were added as required, but only 1/2 the amount added to Vogel's and crossing media. The solution was made to 1 ℓ with water and sterilized by autoclaving.

50X Vogel's

(Davis and de Serres, 1970)

125 g	$Na_3citrate{\cdot}2H_2O$
250 g	KH_2PO_4
100 g	NH_4NO_3
10 g	$MgSO_4{\cdot}7H_2O$
5 g	$CaCl_2{\cdot}2H_2O$

The first three ingredients were dissolved sequentially in 650 mℓ of water. The $MgSO_4$ and $CaCl_2$ were dissolved in 35 mℓ and 100 mℓ of water respectively. These two solutions were added slowly and sequentially to the first. When completely dissolved, it was made up to 1 ℓ with water. Chloroform (5 mℓ) was added as a preservative.

4X Trace Elements

(Davis and de Serres, 1970)

50 g	$Citric\ Acid{\cdot}1H_2O$
50 g	$ZnSO_4{\cdot}7H_2O$
10 g	$Fe(NH_4)_2(SO_4){\cdot}6H_2O$
2.5 g	$CuSO_4{\cdot}5H_2O$
0.5 g	$MnSO_4{\cdot}1H_2O$
0.5 g	H_3BO_3
0.5 g	$Na_2MoO_4{\cdot}2H_2O$
20 mg	$CoCl_2$

Water was added to 1 ℓ. A 1X stock was made to use as a working solution. Both were stored at 4°C.

Biotin Solution

(Davis and de Serres)

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

Supplements

(Davis and de Serres, 1970)

L-amino acids and vitamins were added to a final concentration of 200 μg/mℓ and 10 μg/mℓ, respectively, when required.

Acid-Washed Sand

A large glass container 3/4 full with sand (Ottawa sand or sea sand) was filled with concentrated HCl and left overnight in the fumehood. The next day, the acid was decanted and the sand was washed with water until the pH was neutral. The sand was dried in an oven, dispensed into jars and stored at 4°C.

Crossing Media
(Davis and de Serres, 1970)

50 mℓ	20X Westergaard's
1 mℓ	1X trace elements
1 mℓ	biotin solution
15 g	table sugar
15 g	agar

4 N KOH was added to pH 6.5 and the solution was made to 1 ℓ with water and sterilized by autoclaving. The carbohydrate concentration was reduced to 0.1% or eliminated completely and substituted by the presence of filter paper to help improve fertility. Supplements were added as required.

20X Westergaard's
(Davis and de Serres, 1970)

20 g	KNO_3
20 g	KH_2PO_4
2 g	NaCl
2.6 g	$CaCl_2 \cdot 2H_2O$
10 g	$MgSO_4 \cdot 7H_2$

The first three ingredients were dissolved in 650 mℓ of water. The $CaCl_2$ and the $MgSO_4$ were each dissolved in 50 mℓ of water and added slowly and sequentially to the rest of the solution. The solution was made up to 1 ℓ with water and 5 mℓ of chloroform was added as a preservative.

Viability Plates

20 mℓ	50X Vogel's
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 water. After autoclaving, 100 mℓ of 10X sugars solution was added prior to pouring the plates.

10X Sugars

2.0 g L-sorbose
g D-fructose
5 g D-glucose
2 g myo-inositol

This was made up to 1 ℓ with water and sterilized by autoclaving.

Top Agar

20 mℓ 50X Vogel's stock
182 g sorbitol
1 mℓ 1X trace elements
10 mℓ vitamins mix
15 g agar

Supplements were added as required before the solution was made up to 900 mℓ. After autoclaving, 100 mℓ of 10X sugars solution was added. For determining sphaeroplast viability, no benomyl was added. For transformations, 2 mℓ of benomyl Solution (0.25 mg/mℓ in 95% ethanol) was added at the same time as the 10X sugars.

Vitamins Mix

30 mg riboflavin
100 mg thiamine
75 mg pyridoxine
5 mℓ *p*-aminobenzoic acid (2 mg/mℓ)
200 mg choline chloride
1 mℓ folic acid
4 g myo-inositol
0.5 g adenine sulfate
0.5 g uracil
0.5 g L-methionine
0.5 g L-arginine
1 g L-lysine
0.1 g L-threonine
0.5 g L-serine
0.2 g L-homoserine
0.5 g L-histidine
0.1 g L-isoleucine
0.2 g L-valine
0.2 g L-leucine
0.5 g L-proline

The riboflavin was dissolved in 100 mℓ of water containing 4 drops of 5 N NaOH before adding to the rest of the solution. After adding water to 1 ℓ, the solution was filter sterilized and stored in dark bottles at 4°C.

Benomyl Plates

Benomyl plates were prepared exactly like viability plates except that 2 mℓ of benomyl solution (0.25 mg/mℓ in 95% ethanol) was added when the 10 X sugars were added.

Regeneration Media:

5 mℓ 50X Vogel's stock
10 g sucrose
2.5 g D-glucose
0.25 mℓ 1X trace elements
0.25 mℓ biotin solution
0.5 mℓ vitamins mix

Supplements (same amounts as in 0.5X Vogel's) were added, if required. The solution was made to 250 mℓ with water and autoclaved. After the solution had cooled, 250 mℓ of a sterile 1 M $MgSO_4$ solution was added as well as 0.5 mℓ of a benomyl solution (0.25 mg/mℓ in 95% ethanol).

III. DNA Sequencing Mixes and Solutions:

Regular Mixes

A mix:

40 µℓ 0.5 mM dCTP
40 µℓ 0.5 mM dGTP
40 µℓ 0.5 mM TTP
40 µℓ 10X pol buffer
140 µℓ dH_2O
1 µℓ 10 mM ddATP

C mix:

4 µℓ 0.5 mM dCTP
40 µℓ 0.5 mM dGTP
40 µℓ 0.5 mM TTP
40 µℓ 10X pol buffer
120 µℓ dH_2O
2 µℓ 10 mM ddCTP

G mix:

40 µℓ 0.5 mM dCTP
4 µℓ 0.5 mM dGTP
40 µℓ 0.5 mM TTP
40 µℓ 10X pol buffer
110 µℓ dH_2O
4 µℓ 10 mM ddGTP

T mix:

40 µℓ 0.5 mM dCTP
40 µℓ 0.5 mM dGTP
4 µℓ 0.5 mM TTP
40 µℓ 10X pol buffer
100 µℓ dH_2O
8 µℓ 10 mM dTTP

dITP Mixes
(Sanger *et al.*, 1982)

A mix:

20 µℓ 0.5 mM dCTP
100 µℓ 0.5 mM dITP
20 µℓ 0.5 mM TTP
20 µℓ 10X pol buffer
40 µℓ dH_2O
0.25 µℓ 10 mM ddATP

C mix:

2 µℓ 0.5 mM dCTP
100 µℓ 0.5 mM dITP
20 µℓ 0.5 mM TTP
20 µℓ 10X pol buffer
60 µℓ dH_2O
1.5 µℓ 10 mM ddCTP

G mix:

20 µℓ 0.5 mM dCTP
3 µℓ 0.5 mM dITP
20 µℓ 0.5 mM TTP
20 µℓ 10X pol buffer
140 µℓ dH_2O
1.5 µℓ 1 mM ddGTP

T mix:

20 µℓ 0.5 mM dCTP
100 µℓ 0.5 mM dITP
2 µℓ 0.5 mM TTP
20 µℓ 10X pol buffer
60 µℓ dH_2O
4 µℓ 10 mM dTTP

Deazaguanine Mixes
(Barr *et al.*, 1986)

A mix:

40 µℓ 0.5 mM dCTP
40 µℓ 0.5 mM c^{7}dGTP
40 µℓ 0.5 mM TTP
40 µℓ 10X pol buffer
140 µℓ dH_2O
1 µℓ 10 mM ddATP

C mix:

4 µℓ 0.5 mM dCTP
40 µℓ 0.5 mM c⁷dGTP
40 µℓ 0.5 mM TTP
40 µℓ 10X pol buffer
120 µℓ dH_2O
2 µℓ 10 mM ddCTP

G mix:

40 µℓ 0.5 mM dCTP
4 µℓ 0.5 mM c⁷dGTP
40 µℓ 0.5 mM TTP
40 µℓ 10X pol buffer
110 µℓ dH_2O
4 µℓ 10 mM ddGTP

T mix:

40 µℓ 0.5 mM dCTP
40 µℓ 0.5 mM c⁷dGTP
4 µℓ 0.5 mM TTP
40 µℓ 10X pol buffer
100 µℓ dH_2O
8 µℓ 10 mM dTTP

^{35}S Mixes
(New England BioLabs, Inc.)

A mix:

19 µℓ 0.5 mM dCTP
19 µℓ 0.5 mM dGTP
19 µℓ 0.5 mM TTP
25 µℓ 10X pol buffer
163 µℓ dH_2O
5 µℓ 1 mM ddATP

C mix:

3 µℓ 0.5 mM dCTP
27 µℓ 0.5 mM dGTP
27 µℓ 0.5 mM TTP
25 µℓ 10X pol buffer
160 µℓ dH_2O
7.5 µℓ 10 mM ddCTP

G mix:

27 µℓ 0.5 mM dCTP
3 µℓ 0.5 mM dGTP
27 µℓ 0.5 mM TTP
25 µℓ 10X pol buffer
160 µℓ dH_2O
7.5 µℓ 10 mM ddGTP

T mix:

27 µℓ 0.5 mM dCTP
27 µℓ 0.5 mM dGTP
3 µℓ 0.5 mM TTP
25 µℓ 10X pol buffer
153 µℓ dH_2O
15 µℓ 10 mM dTTP

Chase:

20 µℓ 10 mM dATP
20 µℓ 10 mM dCTP
20 µℓ 10 mM dGTP
20 µℓ 10 mM dTTP
120 µℓ dH_2O

10X Pol Buffer

0.7 mℓ 1 M Tris·HCl, pH 8.0
142 mg $MgCl_2 \cdot 6H_2O$
1 mℓ 5 M NaCl

The solution was made up to 10 mℓ with water.

Dye-Formamide Mix

10 mg xylene cyanole FF (Kodak)
10 mg bromphenol blue
400 µℓ 0.25 M EDTA, pH 8.0
9.5 mℓ deionized formamide[1]

1. Deionized formamide was prepared as follows: To 50 mℓ of formamide was added 5 g of mixed-bed resin (Bio-Rad AG 501-X8, 20-50 mesh). This was stirred for 30 minutes. The formamide was decanted into a clean beaker and more mixed-bed resin (5 g) was added. After mixing for 30 minutes, the formamide was decanted into a clean beaker and filtered to remove any resin. The deionized formamide was stored at -20°C.

IV. ELISA Buffers:

Coating Buffer (pH 9.6)
(Voller, 1980)

4.29 g $Na_2CO_3 \cdot 10H_2O$
2.93 g $NaHCO_3$
0.2 g NaN_3

The solution was made to 1 ℓ with water and was stored at 4°C.

PBST (pH 7.4)
(Voller, 1980)

8.0 g NaCl
0.2 g KH_2PO_4
0.46 g Na_2HPI_4
0.2 g KCl
0.2 g NaN_3
0.5 mℓ tween 20

The solution was made up to 1 ℓ with water and was stored at 4°C.

ELISA Substrate Solution
(Voller, 1980)

2 X 5 mg tablets of *p*-nitrophenylphosphate (Sigma 104 phosphatase substrate) were dissolved in 10 mℓ of 10% DB Buffer.

10% DB Buffer
(Voller, 1980)

97 mℓ diethanolamine
0.2 g NaN_3
700 mℓ dH_2O

The pH was adjusted to 9.8 with concentrated HCl before adding water to 1 ℓ. The buffer was stored at 4°C.

V. Hybridization Buffers:

50X Denhardt's

5 g ficoll
5 g polyvinylpyrrolidone
5 g BSA

The solution was stored at -20°C after adding water to 500 mℓ.

20X SSPE

21.04 g NaCl
2.4 g NaH_2PO_4
8 mℓ 0.25 M Na_2EDTA, pH 8.0

The pH was adjusted to 8.3 with 1 M NaOH before adding water to 100 mℓ.

VI. Miscellaneous Solutions:

Salt-Saturated Isopropanol

100 g NaCl
10 mℓ 1 M Tris·HCl, pH 7.3
250 mℓ distilled water
500 mℓ isopropanol

The mixture was mixed thoroughly and the phases were allowed to separate. The top phase was used to extract the ethidium bromide from aqueous solutions.

Protease K Solution

30 mg of protease K (Sigma) was dissolved in 5 mℓ of 100 mM Tris·HCl, pH 7.3, 150 mM NaCl, 1 mM EDTA and incubated in a 37°C water-bath. After 60 minutes, 5 mℓ of glycerol was added and the well-mixed solution was stored at -20°C.

OLB Buffer

OLB buffer was made by mixing solutions A:B:C in a ratio of 100:250:150 and stored at -20°C.

Solution O: 1.25 M Tris·HCl, pH 8.0, 0.125 $MgCl_2$ (stored at 4°C).

Solution A: 1 mℓ of solution O + 18 μℓ of β-mercaptoethanol + 5 μℓ each of 0.1 M solutions of dATP, dGTP and TTP in 3 mM Tris·HCl, pH 7.0, 0.2 mM EDTA (stored at -20°C).

Solution B: 2 mM Hepes, pH 6.6 (titrated with 4 M NaOH and stored at 4°C).

Solution C: Hexadeoxyribonucleotides (P-L Biochemicals) made to 90 OD Units/mℓ in 3 mM Tris·HCl, pH 7.0, 2 mM EDTA (stored at -20°C).