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THE UNIVERSITY OF ALBERTA

Nucleo-Mitochondrial Interactions: Protein Import and Assembly of Multi-Subunit Complexes in *N. crassa*

BY

© Troy Anthony Alan Harkness

A thesis
submitted to the faculty of graduate studies and research
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

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For my family, old and new, especially Terra.

Abstract

Mitochondrial biogenesis involves the co-ordinated action of both the nuclear and mitochondrial genomes. The study of how cytoplasmically synthesized precursor proteins are transported across mitochondrial membranes and subsequently assembled into higher-order protein complexes should yield information regarding the complexity of the processes involved. Two cytoplasmically synthesized precursors, MOM19, the mitochondrial protein import receptor, and NUO78, the largest subunit of the NADH dehydrogenase complex (complex I), were examined in this study.

Mom-19^{+} was mutagenized in the filamentous fungus, *Neurospora crassa*, using the phenomenom of RIP (repeat induced point-mutation). This procedure involves integrating a second copy of *mom-19*$^{+}$ at an ectopic site within the genome to produce a duplication. When this duplication strain is crossed to a strain harbouring only the resident copy of *mom-19*$^{+}$, the repeated sequences within the transformed nucleus are altered by methylation and GC to AT transitions, effectively inactivating the function of the protein. Since *mom-19*$^{+}$ was considered to provide an essential function to the cell, a variation of the RIP procedure, called "sheltered RIP", was used to isolate the *mom-19* mutants. This procedure allowed the mutagenized nucleus to be maintained within a heterokaryon containing a complementing, or sheltering, wild-type version of the *mom-19*$^{+}$ gene. Incorporation of a gene that specifies resistance to an inhibitor of growth in the nucleus harbouring the inactivated gene allows alteration of the ratio of the mutant nucleus over the wild-type nucleus so that the mutant phenotype can be studied. Strains selected from the sheltered RIP cross, that exhibited a growth defect in the presence of the inhibitor, were characterized further. Western analysis revealed that one of the slow-growing isolates lacked

the MOM19 protein. For cells lacking MOM19, the following results were observed: i) cells grew extremely slowly and eventually senesced, ii) MOM19 depleted mitochondria underwent gross morphological changes, iii) *in vitro* import into isolated mutant mitochondria was deficient for the majority of precursors tested, iv) the depletion of MOM19 appeared to affect the activity of another component of the import apparatus, MOM22, and v) a second receptor on the mitochondrial outer membrane, MOM72, did not compensate for the lack of MOM19 during *in vitro* import.

NUO78 deficient strains were generated and selected in the same manner as described for MOM19. The lack of NUO78 in *N. crassa* cells only slightly reduced the growth rate as compared to wild-type cells, but had a marked affect on the ability of the strains to produce conidia. This contrasts with previous findings in *N. crassa* where strains defective in other subunits of complex I have not been reported to be associated with a recognizable phenotype. These results suggest that NUO78 plays an important role in the assembly, maintenance and/or function of complex I.

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My parents never seemed to waver in their support no matter how stupid I seemed to be or how much trouble I was causing. I don't believe I would have made it though the my early years here without that support. I think my finest accomplishment during my years in Edmonton, however, was convincing Terra to marry me. It is truly amazing how life changes when one suddenly has a great future to share.

Lastly, I am grateful for the finanial support from the Alberta Heritage Foundation for Medical Research, the Department of Genetics and the University of Alberta.

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List of abbreviations:

A	adenine
AAC	adenosine triphosphate/adenosine diphosphate carrier
A. eutrophus	*Alcaligenes eutrophus*
A. niger	*Aspergillus niger*
arg	gene encoding arginine biosynthetic protein
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BSA	bovine serum albumin
bisacrylamide	*N,N'*-methylenebisacrylamide
bp	base pair
C	cytosine
CCHL	cytochrome *c* heme lyase
cDNA	complementary deoxyribonucleic acid
Ci	Curie
Complex I	NADH:ubiquinone oxidoreductase
Complex II	succinate:ubiquinone oxidoreductase
Complex III	ubiquinone:cytochrome *c* oxidoreductase
Complex IV	cytochrome *c* oxidase
Complex V	F_1F_O ATPase
CoxIV	cytochrome *c* oxidase subunit IV
CoxVa	cytochrome *c* oxidase subunit Va
CS	citrate synthase
C-terminus	carboxy terminus
Cyt *c*	cytochrome *c*
Cyt c_1c	cytochrome c_1 presequence fused to cytochrome *c*
dATP	2'-deoxyadenosine 5'-triphosphate
ddNTP	2',3'-dideoxynucleotide 5'-triphosphate
DHFR	dihydrofolate reductase
DNA	deoxyribonucleic acid
DnaJ	deoxyribonucleic acid protein J
DnaK	deoxyribonucleic acid protein K
dNTP	2'-deoxynucleotide 5'-triphosphate
DTT	dithiothreitol
E. coli	*Escherichia coli*

EDTA	ethylenediaminetetraacetic acid
ER	endoplamic reticulum
F_1b	F_1-ATPase subunit b
Fab	antigen binding subunit of IgG
FAD	flavin adenine dinucleotide
Fe/S	Rieske iron-sulfur protein
FMN	flavin mononucleotide
fpa	*p*-fluorphenyl alanine
G	guanine
GIP	general insertion pore
GTP	guanosine triphosphate
H II	Host strain II
H IV	Host strain IV
hph	gene encoding bacterial hygromycin B phosphotransferase
Hsp10	heat shock protein of 10 kd
Hsp60	heat shock protein of 60 kd
Hsp70	heat shock protein of 70 kd
IDH	isocitrate dehydrogenase
IgG	immunoglobulin G
IMP1	inner membrane peptidase
IMS	intermembrane space
inl	gene encoding inositol biosynthetic protein in *Neurospora crassa*
inv	gene encoding sucrose utilizing protein in *N. crassa*
Isp6	import site protein of 42 kd
Isp45	import site protein of 45 kd
ITP	inosine triphosphate
kb	kilo base
kd	kilo dalton
LG II	linkage group II
LG IV	linkage groug IV
LHON	Leber's hereditary optic neuropathy
M	Mauriceville *N. crassa* strain
M II	Mate strain II
M IV	Mate strain IV
Mas5	mitochondrial assembly protein #5

Mas6	mitochondrial assembly protein #6 of 23 kd
Mas20	mitochondrial assembly protein of 20 kd
mas-20	gene encoding Mas20
Mas70	mitochondrial assembly protein of 70 kd
mas-70	gene encoding Mas70
MDJ1	mitochondrial DnaJ
mei	gene encoding protein involved in meiosis
MGE	mitochondrial GrpE
mg	milligram
ml	millilitre
mM	millimolar
MOM7	mitochondrial outer membrane protein of 7 kd
MOM8	mitochondrial outer membrane protein of 8 kd
MOM19	mitochondrial outer membrane protein of 19 kd
mom-19	gene encoding MOM19
MOM22	mitochondrial outer membrane protein of 22 kd
MOM30	mitochondrial outer membrane protein of 30 kd
MOM38	mitochondrial outer membrane protein of 38 kd
MOM72	mitochondrial outer membrane protein of 72 kd
MOPS	3-(*N*-morpholino)propanesulfonic acid
MPI1	mitochondrial protein import protein #1 of 45 kd
MPI2	mitochondrial protein import protein #2
MPP	mitochondrial processing protease
MSF	mitochondrial import stimulation factor
mtDNA	mitochondrial deoxyribonucleic acid
mtHsp70	mitochondrial heat shock protein of 70 kd
mtr^R	4-methyltryptophan resistant
N-1	iron-sulfur cluster in peripheral arm of complex I
N-2	iron-sulfur cluster in membrane arm of complex I
N-3	iron-sulfur cluster in peripheral arm of complex I
N-4	iron-sulfur cluster in peripheral arm of complex I
NAD^+	nicotinamide adenine dinucleotide, oxidized form
NADH	nicotinamide adenine dinucleotide, reduced form
N. crassa	*Neurospora crassa*
ND1	NADH dehydrogenase mitochondrial encoded protein #1
ND2	NADH dehydrogenase mitochondrial encoded protein #2

ND4	NADH dehydrogenase mitochondrial encoded protein #4
ND5	NADH dehydrogenase mitochondrial encoded protein #5
NDH I	NADH dehydrogenase I
NDH II	NADH dehydrogenase II
NQO	NADH:coenzyme Q oxidoreductase
N-terminus	amino terminus
NUO78	NADH:ubiquinone oxidoreductase subunit of 78 kd
nuo-78	gene encoding NUO78
O	Oak-Ridge *N. crassa* strain
O_2	oxygen
p32	protein of 32 kd
PAGE	polyacrylamide gel electrophoresis
PBF	presequence binding factor
PCR	polymerase chain reaction
P. denitrificans	*Paracoccus denitrificans*
pet	*petite*
pF51-CCHL	N-terminal 51 amino acids from F_1b fused to CCHL
PK	proteinase K
PMSF	phenylmethylsulfonyl fluoride
pyr	gene encoding pyrimidine biosythetic protein
RFLP	restriction fragment length polymorphism
RIP	repeat induced point-mutation
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulfate
SEM	sucrose, EDTA and MOPS
S. typhimurium	*Salmonella typhimurium*
SRP	signal recognition particle
SSC1	yeast mtHsp70
Su9	subunit 9 of F_O-ATPase
T	thymine
TAE	tris; acetate; EDTA
thr	gene encoding threonine biosynthetic protein
Tris	Tris (hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid

trp	gene encoding tryptophan biosynthetic protein
ts	temperature sensitive
mg	microgram
ml	microlitre
mM	micromolar
URF	unidentified reading frame
UV	ultraviolet radiation

1 The role of nucleo-mitochondrial interactions for the biogenesis of mitochondria: protein import and assembly of multi-subunit complexes

1·1 Introduction

Mitochondria, the double-membraned organelles contained within virtually all eukaryotes, are responsible for fulfilling the energy requirements of the cell (reviewed in Tzagoloff, 1982; Attardi and Schatz, 1988; Pon and Schatz, 1991). The organelle can be divided into four subcompartments: the inner and outer membranes, the intermembrane space and the matrix (Hogeboom *et al.*, 1948; Palade, 1953). Hundreds of proteins, the vast majority of which are encoded in the nucleus, are required for proper mitochondrial function. These mitochondrial proteins must be specifically localized to a particular subcompartment in order to carry out their assigned function. The mitochondrial genome (Schatz *et al.*, 1964), located within the matrix, has a limited coding capacity and generally specifies only a few subunits of the electron transport chain, the tRNAs required for the translation of these proteins within the organelle, and mitochondrial rRNAs (McLean *et al.*, 1958; Tzagoloff, 1982; Hatefi, 1985).

The biogenesis of mitochondria requires the co-ordinated action of both the nuclear and mitochondrial genomes (Grivell, 1989). Nuclear encoded mitochondrial proteins are targeted to the outer membrane of mitochondria by signals located within the coding region of the protein (Hurt and van Loon, 1986; Schatz, 1987). Some of the major functions of proteins within the organelle include electron transport, oxidative phosphorylation, the citric acid cycle and fatty acid metabolism (Tzagoloff, 1982), as well as the recognition and import of the mitochondrially targeted precursors from the cytosol (Hartl and Neupert, 1990; Glick and Schatz, 1991; Kiebler *et al.*, 1993). A series of

Saccharomyces cerevisiae mutants, affected in discrete steps of mitochondrial biogenesis, have been utilized to identify nuclear genes encoding mitochondrial proteins (Tzagoloff and Dieckman, 1990; Baker and Schatz, 1991).

There is good evidence that the physiological state of mitochondria can influence nuclear gene expression. There are several examples of this so-called "retrograde regulation" (Vandana *et al.*, 1987; Butow *et al.*, 1988; Forsburg and Guarente, 1989). One of the best characterized involves the elevated levels of nuclear CIT2 transcription in response to mitochondrial dysfunction (Liao and Butow, 1993). Similar retrograde regulation was seen with the mutliple ADP/ATP translocator genes in human cells (Lunardi and Attardi, 1991), with the *N. crassa cyt-21* gene that encodes a mitochondrial ribosomal protein (Kuiper *et al.*, 1988) and with the induction of the alternate oxidase in respiratory deficient strains of *N. crassa* (Lambowitz and Slayman, 1971; Edwards *et al.*, 1974).

The work in this thesis focuses on two events that are essential for the proper activity of mitochondria: i) the import of protein precursors targeted to *Neurospora crassa* mitochondria, and ii) the association and assembly of multi-subunit complexes composed of proteins encoded by both the *N. crassa* nuclear and mitochondrial genomes. With regard to the former process, mutants affecting the import apparatus have been constructed and analyzed. As an example of the latter process, complex I (NADH:ubiquinone oxidoreductase) of the electron transport chain has been a focus in this study. This enzyme is composed of more than 30 subunits, 7 of which are derived from mtDNA. The remaining subunits must be imported from the cytosol.

1·2 Mitochondrial Protein Import

1·2·1 The role of cytosolic factors

The initial events in mitochondrial biogenesis involve the recognition of mitochondrial-targeted precursors and the subsequent translocation and sorting processes that follow. Import across the mitochondrial outer membrane requires the precursor to be in an extended, unfolded state (Schleyer and Neupert, 1985; Rassow *et al.*, 1989) which requires the action of cytosolic factors (Argan *et al.*, 1983; Pfanner and Neupert, 1987; Ono and Tuboi, 1988), such as members of the DnaK (Deshaies *et al.*, 1988; Murakami *et al*, 1988; Stuart *et al.*, 1994) and DnaJ (Caplan and Douglas, 1991; Caplan *et al*, 1992; Atencio and Yaffe, 1992) families of chaperones, as well as ATP hydrolysis (Pfanner *et al.*, 1987a; Verner and Schatz, 1987; Pelham, 1988; Flynn *et al.*, 1989). A protein complex anchored in the mitochondrial outer membrane initially recognizes and receives the precursor (Attardi and Schatz, 1988; Pfanner *et al.*,1988; Hartl *et al.*, 1989). The subsequent transfer of the precursor from the chaperones to the outer membrane complex requires ATP hydrolysis (Eilers *et al.*, 1987; Chen and Douglas, 1987), although the requirement for chaperones and ATP hydrolysis can be circumvented if the precursor is first denatured *in vitro* with urea (Becker *et al.*, 1992).

The interaction of the mitochondrial targeted precursor with cytosolic Hsp70, a DnaK homolog, is thought to stabilize the denatured structure of the precursor (Gething and Sambrook, 1992). This is most likely accomplished by preventing the precursors from folding into a conformation that cannot be unfolded, or by preventing aggregation (Gething and Sambrook, 1992; Skowyra *et al.*, 1990). Depletion of cytosolic Hsp70 in the yeast, *S. cerevisiae* (the term yeast will refer to *S. cerevisiae* for the remainder of this thesis), resulted in

accumulation of uncleaved mitochondrial precursor proteins and death (Deshaies *et al.*, 1988). Unlike the signal recognition particle (SRP) that directs precursor proteins into the endoplasmic reticulum (ER; Bernstein *et al.*, 1989), cytosolic Hsp70 does not interact specifically with receptors on the mitochondrial surface (Ellis, 1987).

In *E. coli.*, DnaK interacts with unfolded polypeptides to maintain their structure (Langer *et al.*, 1992). The ATP-dependent release of the precursors to a second chaperonin, GroEL (Hsp60 in eukaryotes; Hartl *et al.*, 1992), which catalyses the complete folding of precursors, is controlled by specific cofactors, DnaJ and GrpE, that co-operatively stimulate the ATPase activity of DnaK (Langer *et al.*, 1992; Schröder *et al.*, 1993). The DnaJ homologues in the cytosol of yeast (Sec63; Rothblatt *et al.*, 1989; Scj1; Blumberg and Silver, 1991; Ydj1, Caplan and Douglas, 1991; Caplan *et al*, 1992; Mas5, Atencio and Yaffe, 1992) have been found to be non-essential components implicated in efficient post-translational import into mitochondria. In the eukaryotic cytosol, a homologue to GrpE, if it exists, remains undetected.

The situation in mammalian cells for maintenance of an unfolded conformation for precursor proteins seems to be more complex. Import of precursors with cleavable presequences into rat liver mitochondria appears to require a presequence-binding factor (PBF) of 50 kd (Murakami *et al.*, 1990; Murakami and Mori, 1990). Rabbit PBF contains acidic domains at both the N- and C-termini that are proposed to interact with the positively charged amino acids of the presequence. A second cytosolic factor, mitochondrial import stimulation factor (MSF), a heterodimer of 30 and 32 kd subunits, is thought to stimulate the import of the precursor of the matrix-localized protein adrenodoxin possibly by depolymerizing and unfolding of the oligomeric precursor in an ATP-dependent fashion (Hachiya *et al.*, 1993).

1·2·2 Import Pathway into the Mitochondrial Matrix

Mitochondrial precursor proteins destined for different subcompartments of mitochondria carry different targeting signals (Hartl and Neupert, 1990; Glick and Schatz, 1991; Kiebler *et al.*, 1993a). Proteins destined for the matrix generally contain cleavable hydrophilic N-terminal presequences, whereas some proteins targeted for the inner or outer membranes contain signals within the mature parts of the precursor protein. These latter signals have not yet been well defined (see below). Submitochondrial sorting signals are often also found at the N-terminus, following the mitochondrial targeting signal, as a stretch of hydrophobic amino acids of variable length. Submitochondrial sorting signals show striking similarity to chloroplast thylakoid transfer signals, as well as ER and bacterial translocation signals (von Heijne *et al.*, 1989; Robinson *et al.*, 1994).

The most extensively studied signals are the mitochondrial matrix-targeting signals (Hartl and Neupert, 1990; Glick and Schatz, 1991). The signals are highly-degenerate sequences of 15-35 amino acids that can adopt an amphiphilic α-helix and are usually rich in positively charged and hydroxylated residues. Import into the matrix requires an electrochemical potential across the inner membrane and ATP hydrolysis in the matrix. Upon arrival into the matrix, the presequences are removed by specific proteases. The matrix-targeting signal is necessary and sufficient to direct chimeric protein sequences into the mitochondrial matrix. The matrix-targeting signals are structurally related to imported chloroplast stromal protein presequences (Ko and Cashmore, 1989; von Heijne *et al.*, 1989; Robinson *et al.*, 1994). These chloroplast protein presequences are required for translocation across the chloroplast envelope membranes and are cleaved by stromal proteases.

1·2·2·1 Outer membrane import machinery

Proteins destined for the matrix appear to follow a general pathway into mitochondria (reviewed in Hartl and Neupert, 1990; Glick and Schatz, 1991; Kiebler *et al.*, 1993). The unfolded precursors are first recognized by the import receptors on the surface of mitochondria (see above). Two receptors have been identified: MOM19 (Söllner *et al.*, 1989) and MOM72 (Söllner *et al.*, 1990) in *N. crassa* (mitochondrial outer membrane proteins of 19 kd and 72 kd, respectively) and the homologues Mas20 (Ramage *et al.*, 1993) and Mas70 (Hines *et al.*, 1990) in *S. cerevisiae* (mitochondrial assembly proteins of 20 kd and 70 kd, respectively). *In vitro* studies using isolated *N. crassa* mitochondria have shown that virtually all mitochondrial-targeted precursors utilize MOM19 as the major receptor (Söllner *et al.*, 1989; Schneider *et al.*, 1991). MOM72 seems to be limited to the import of a class of proteins structurally related to the inner membrane ATP/ADP carrier protein (AAC; Söllner *et al.*, 1990). *N. crassa* strains deficient in either MOM19 or MOM72 had not yet been described prior to the work described in this thesis. In yeast, disruption of both Mas20 and Mas70 have been described. Neither protein was found to be essential to the organism (Ramage *et al.*, 1993; Riezman *et al.*, 1983). However, Mas20 deficient cells exhibited a *pet* phenotype (Tzagoloff and Dieckman, 1990) in that they did not grow on non-fermentable carbon sources, but were able to grow at reduced rates on medium containing glucose (Ramage *et al.*, 1993). Respiratory deficiency caused by the Mas20 mutant could be suppressed if Mas70 was over-expressed, supporting the notion that the functions of the two receptors overlap (Ramage *et al.*, 1993). Mutants of Mas70 showed a long lag in forming colonies on glucose medium and barely grew on nonfermentable carbon sources at 37°C (Riezman *et al.*, 1983). The temperature sensitivity of

the *mas-70* disruptant suggests that Mas70 plays a role in stabilizing the receptor complex. Mitochondrial import studies using intact yeast cells carrying a *mas-70* null mutation have shown that the ability to import the F_1-ATPase α- and β-subunits as well as cytochrome c_1 is reduced (Hines *et al.*, 1990). Additional *in vivo* studies have shown that Mas70 is an import receptor for most, if not all authentic mitochondrial precursor proteins, suggesting a more general role in import (Hines and Schatz, 1993). However, import of F1-β into isolated mitochondria was only partially inhibited by antibodies to either Mas20 or Mas 70 (Ramage *et al.*, 1993). Yeast strains carrying mutations in both Mas20 and Mas70 are not viable, even on glucose, presumably due to a severe reduction of import rates. Taken together, these results suggest that Mas20 and Mas70 have overlapping specificities, though the relatively milder phenotype of *mas-70* mutants suggests that Mas20 may play a more important role. This interpretation disagrees somewhat with the results obtained in *N. crassa* which suggests a very specific role for MOM72 and a general role for MOM19. In fact, *in vitro* studies suggest that MOM19 may be able to substitute for MOM72, but not vice-versa (Steger *et al.*, 1990). Interestingly, a consensus motif called the tetratricopeptide repeat has been found in MOM19/Mas20 and MOM72/Mas70 (Boguski *et al.*, 1990; Sikorski *et al.*, 1990; Ramage *et al.*, 1993). Other members of this family participate in cell cycle control and in cytoskeletal functions.

Other putative receptors have been identified. in yeast, a mitochondrial integral membrane protein of 32 kd (p32) was identified using anti-idiotypic antibodies, which mimic a chemically synthesized signal peptide of the precursor of cytochrome *c* oxidase subunit IV (Pain *et al.*, 1990). p32 was localized to the outer membrane by immunoelectron microscopy and by Fab fragments against purified p32 that blocked import of precursor proteins into

isolated mitochondria. A p32 disruption mutant resulted in an inability to grow on non-fermentable carbon sources only, showing p32 was not essential for viability. Sequence analysis unexpectedly revealed p32 to be identical to the mitochondrial phosphate carrier protein, an inner membrane protein (Phelps *et al.*, 1991). The putative dual function of p32 remains to be elucidated. Other studies report the purification of proteins of 28 kd from mitochondria of rabbit heart, rat liver, bovine adrenal cortex and *Schizosaccharomyces pombe* (Font *et al.*, 1991), and of 29, and 52 kd from rat liver mitochondria (Ono and Tuboi, 1990; Ono and Tuboi, 1991) that may be putative receptor proteins.

Following interaction with the receptor(s), the precursor is then transferred to a complex of proteins that make up the general insertion pore (GIP), first identified in *N. crassa* (Pfanner and Neupert, 1987; Pfaller *et al.*, 1988). Unlike translocation across the inner membrane, import of proteins across the outer membrane does not require an electrochemical potential. The GIP was initially thought to be, or be part of, an import channel that spans the two membranes. These import channels were thought to be situated at contact sites where the two membranes are closely apposed (Schwaiger *et al.*, 1987). It is now clear, however, that each membrane contains unique translocation machinery that may act in concert during the translocation of matrix proteins (Hwang *et al.*, 1989; Mayer *et al.*, 1993). Studies using an outer membrane vesicle system established the identity of an independent import apparatus (Mayer *et al.*, 1993). These vesicles can translocate precursor proteins of the outer membrane and IMS in a receptor-dependent fashion in the absence of a membrane potential. Import of precursors requiring a membrane potential, which is not present in the vesicles, is not supported, indicating that some essential component is absent, perhaps components of the inner membrane.

Crosslinking or coimmunoprecipitation studies in both yeast (Moczko *et al.*, 1992) and *N. crassa* (Kiebler *et al.*, 1990; Söllner *et al.*, 1992) have shown that several proteins are associated in mitochondrial outer membranes including MOM72, MOM38, MOM30, MOM22, MOM19, MOM8 and MOM7, or the yeast correlates. MOM38 has been shown to be a component of GIP in *N. crassa* (Kiebler *et al.*, 1990). Isp42, the yeast equivalent of MOM38 (import site protein; Vestweber *et al.*, 1989; Baker *et al.*, 1990), and Isp6 (Kassenbrock *et al.*, 1993) are proposed to be part of the yeast GIP and have been isolated and cloned. The disruption mutant of Isp42 is lethal in yeast, emphasizing its importance in mitochondrial biogenesis and function (Baker *et al.*, 1990). Isp6 was isolated as an over-expression suppressor of an Isp42 temperature-sensitive (ts) mutant. The disruption of Isp6 did not produce a phenotype, however, it was lethal when combined with the ts allele of Isp42 at the permissive temperature (Kassenbrock *et al.*, 1993). Antibodies against Isp42 immunoprecipitated a complex of proteins that included Isp6, showing a direct interaction between the two proteins. Since the genes for MOM7 and MOM8 have not been cloned, a direct sequence comparison with Isp6 has not been possible. However, it is doubtful that either MOM7 or MOM8 are related to Isp6 since they both respond differently to externally added protease than Isp6 (Söllner *et al.*, 1992; Kassenbrock *et al.*, 1993). Furthermore, Isp6 showed mobility on an acrylamide gel inconsistent with identity with MOM8 and was readily labelled with ^{35}S-methionine, whereas MOM7 was not (Kassenbrock *et al.*, 1993). This, however, does not entirely rule out the possibility that they are related.

Passage of precursors from the receptors to the GIP is thought to be mediated by MOM22 in *N. crassa* (Kiebler *et al.*, 1990; 1993b). The protein spans the outer membrane with its N-terminus facing the cytosol. Antibodies against MOM22 block import of all precursors tested *in vitro,* but do not prevent

binding to outer membrane receptors (Kiebler *et al.*, 1993b). These results, coupled to the observation that the N-terminus of MOM22 is highly acidic, encouraged the idea that this domain interacts with precursor presequences on route from the receptors to the GIP. The yeast homologue has not been identified, however a protein of similar size in the import complex is a likely candidate (Moczko *et al.*, 1990). The known proteins of the outer membrane import apparatus are listed in Table 1·1.

1·2·2·2 Inner membrane import machinery

The existence of a separate inner membrane import machinery was first observed when import was restored to "protease-shaved" mitochondria by selectively rupturing the outer membrane, thereby exposing inner membrane import sites (Ohba and Schatz, 1987). Components of the inner membrane import machinery are not well characterized. A 45 kd peripheral component of the inner membrane was isolated from yeast using two independent approaches. The first utilized a genetic screen designed to isolate mitochondrial import mutants (Maarse *et al.*, 1992). Using this procedure 19 recessive nuclear mutations were isolated. In addition to the mutant of the 45 kd protein (MPI1), which was found to be essential, the SSC1 gene, coding for mitochondrial Hsp70 and a gene coding for a protein (MPI2) that interacted with mtHsp70 were identified. MPI2 may be the mitochondrial homologue of bacterial DnaJ or GrpE.

The second approach utilized mitochondria depleted of matrix ATP to arrest precursors translocating across the inner membrane (Scherer *et al.*, 1992). Proteins within the matrix, such as mtHsp70 and Hsp60, require ATP to function properly in translocation and folding of precursors. Precursors stuck in the inner membrane were then cross-linked to adjacent proteins. A protein of

Table 1·1 Components of the mitochondrial protein import machinery in the mitochondrial outer membrane.

Organism:		
N. crassa	***S. cerevisiae***	**Function:**
MOM72	Mas70	Receptor component of import complex (Söllner *et al.*, 1990; Hines *et al.*, 1990); contains seven repeats of an amino acid motif found in proteins involved in mitosis and cytoskeletal interactions (Schatz and Glick, 1991). -**MOM72**: specific recognition of a subset of precursors; no report of a MOM72 deficient strain. -**Mas70**: may act as a more general receptor; overlapping specificity with Mas20 (Ramage *et al.*, 1993). Null mutant exhibits a slow growth phenotype (Riezman *et al.*, 1983); *in vivo* studies suggest import of all precursors may be reduced (Hines and Schatz, 1993).
MOM38	Isp42	Integral component thought to form the general insertion pore (GIP). -**MOM38**: can be cross-linked to imported precursor intermediates; no report of a MOM38 deficient strain. -**Isp42**: can be cross-linked to imported precursor intermediates. Null mutant is inviable.
MOM30	?	In *N. crassa*, coimmunoprecipitated with antibodies against MOM19; function unknown (Söllner *et al.*, 1992).
MOM22	?	In *N.crassa*, coimmunoprecipitates with antibodies against MOM19 (Kiebler *et al.*, 1990); *in vitro* studies suggest a role in transferring precursors from the receptors to the GIP (Kiebler *et al.*, 1993b). Disruption mutant is lethal in *N. crassa* (F. Nargang, unpublished).

Organism:		
N. crassa	***S. cerevisiae***	**Function:**
MOM19	Mas20	Protein import receptor (Söllner *et al.*, 1989; Ramage *et al.*, 1993). *In vitro* studies show this protein to be responsible for import of majority of precursors in *N. crassa* (Söllner *et al.*, 1989; Schneider *et al.*, 1991; this study). Contains a single repeat of the amino acid motif already described for MOM72/Mas70 (Ramage *et al.*, 1993). -**MOM19**: antibodies against MOM19 block import of most tested precursors. Antibodies against MOM19 coimmunoprecipitate the import complex (Kiebler *et al.*, 1990; Söllner *et al.*, 1992). Null mutant is eventually inviable (this study). -**Mas20**: import of tested precursors is blocked with antibodies against Mas20 (Ramage *et al.*, 1993). Null mutant is inviable on nonfermentable carbon sources and exhibits a *pet* phenotype on glucose (Ramage *et al.*, 1993); can be suppressed when combined with overexpressed Mas70 (Ramage *et al.*, 1993).
MOM8	?	In *N. crassa*, coimmunoprecipitates with antibodies against MOM19 (Söllner *et al.*, 1992); thought to be part of the GIP. No reports of mutant strains.
MOM7	?	As MOM8.
?	Isp6	Isolated as overexpression suppressor of Isp42 temperature sensitive (ts) mutants; can be coimunoprecipitated with antibodies against Isp42 (Kassenbrock *et al.*, 1993). Null mutant does not have a phenotype, but when combined with the Isp42 ts allele, it is inviable on all media (Kassenbrock *et al.*, 1993). Biochemical analysis suggests that neither MOM8 nor MOM7 represent the *N. crassa* homologue of Isp6 (Kassenbrock *et al.*, 1993).

45 kd was identified, called Isp45, that was shown to be identical to MPI1 by tryptic peptide analysis (Horst *et al.*, 1993).

A second component of the inner membrane import apparatus has been identified in *S. cerevisiae* (Emtage and Jensen, 1993; Ryan and Jensen, 1993). Mutants of the 23 kd protein, Mas6, were found to be temperature sensitive for viability and accumulated mitochondrial precursor proteins at the restrictive temperature. Yeast cells disrupted for Mas6 were inviable at all temperatures and carbon sources. Mas6 was localized to the inner mitochondrial membrane and antibodies against Mas6 blocked import into isolated mitochondria only when the outer membrane was opened by osmotic shock (Emtage and Jensen, 1993). Further studies showed that Mas6 could be cross-linked to an imported protein arrested in transit through the inner membrane and that Mas6 could be coimmunoprecipitated with at least two other proteins that could also be cross-linked to arrested precursors (Ryan and Jensen, 1993). The two additional proteins, of 10 and 60 kd, were not considered to be MPI1/Isp45 and it was concluded that Mas6 and MPI1/Isp45 probably do not interact. These results suggest that Mas6 is a component of a mitochondrial inner membrane import channel complex that interacts with mitochondrial precursors during import.

1·2·2·3 Import components in the matrix

Once a precursor's N-terminus is through the inner membrane and exposed to the matrix, it binds to a 70 kd protein (mtHsp70; Craig *et al.*, 1989; Scherer *et al.*, 1990; Hartl *et al.*, 1992). MtHsp70 appears to pull precursors into the matrix by multiple rounds of binding and releasing in an ATP-dependent fashion (Kang *et al.*, 1990; Neupert *et al.*, 1990). By this mechanism, unidirectionality is conferred upon precursors translocating across the mitochondrial membranes

(Neupert *et al.*, 1990; Simon *et al.*, 1992). The completely translocated and unfolded precursor is then transferred to the Hsp60 folding machinery (Cheng *et al.*, 1989; Ostermann *et al.*, 1989). Hsp60, a bacterial GroEL homologue, is a large chaperone composed of 14 identical 60 kd subunits, which hydrolyzes ATP in order to function (Cheng *et al.*, 1989; Reading *et al.*, 1989). GroEL functions with a smaller component, GroES, that has been shown to have a homologue in mitochondria (Hsp10; Lubben *et al.*, 1990). Mitochondrial homologs to other *E. coli* proteins involved in folding have recently been identified. MDJ1, a DnaJ homologue found in the mitochondrial matrix, was found to be non-essential in yeast for import, folding and refolding of proteins, but seemed to increase the efficiency of these processes (Rowley *et al.*, 1994). Yeast cells harboring a disrupted *GRPE* gene, a GrpE homologue, were inviable (Bolliger *et al.*, 1994).

The N-terminal presequence of precursors that have reached the matrix is removed by the matrix processing peptidase (α- and β-MPP; Hawlitschek *et al.*, 1988; Schneider *et al.*, 1989; Arretz *et al.*, 1991). Interestingly, in *N. crassa*, β-MPP is identical to the large subunit of the inner membrane cytochrome bc_1 complex (complex III) while α-MPP is soluble within the matrix. In yeast, both α- and β-MPP are soluble whereas in potato mitochondria, MPP activity is exclusively associated with the bc_1 complex (Röhlen *et al.*, 1991; Stuart *et al.*, 1993).

1·2·3 Pathways to the Intermembrane Space (IMS)

1·2·3·1 Cytochrome c *Heme Lyase (CCHL)*

Proteins that eventually reside in the intermembrane space (IMS) take one of a number of routes to that subcompartment. At least three different pathways

have been defined (reviewed in Stuart *et al.*, 1993). The cytochrome *c* heme lyase (CCHL), a peripheral component of the inner membrane, uses the MOM19-GIP receptor complex to reach its final destination in the IMS without translocating across the inner membrane (Lill *et al.*, 1992). The ability of CCHL to be imported in the absence of ATP suggests that it does not require cytosolic factors, while import without a membrane potential suggests that the precursor does not come in contact with the inner membrane. The mitochondrial-targeting signal must also be within the mature protein since CCHL lacks a cleavable presequence.

The above results also show that the inner and outer membrane import machinery can act independently of one another. Further work using a fusion protein containing the matrix-targeting N-terminal presequence from the F1-ATPase β subunit (F1-β) fused to CCHL presented strong evidence supporting the dynamic interactions of the inner and outer membrane machineries (Segui-Real *et al.*, 1993). In the absence of a membrane potential the fusion protein followed the signals residing in the CCHL moiety and was imported to the IMS. In the presence of a membrane potential, the fusion protein would translocate across both membranes to the matrix. In this case, the presequence was processed. Precursors imported into the IMS could be chased into the matrix when a membrane potential was restored, confirming that the machineries of the inner and outer membrane could act independently.

1·2·3·2 Cytochrome C

The most unique pathway to the IMS is that taken by cytochrome *c*. The heme-free apoprotein does not contain an N-terminal cleavable presequence and apparently can insert spontaneously into and partly across the outer membrane (Stuart and Neupert, 1990). Protease-sensitive components on the

surface of mitochondria have not been found that mediate apocytochrome *c* binding and import (Stuart *et al.*, 1990). Mitochondrial-specific targeting of cytochrome *c* may be ensured by a high affinity interaction with CCHL, which resides in the IMS (Nicholson *et al.*, 1987; Nargang *et al.*, 1988), and by another cytochrome *c* specific factor, cyc2 (Dumont *et al.*, 1993). The driving force responsible for cytochrome *c* translocation into the IMS appears to be the refolding of the polypeptide as a result of the covalent attachment of heme, catalyzed by CCHL (Nicholson *et al.*, 1988; Dumont *et al.*, 1988).

1·2·3·3 Cytchromes b_2 *and* c_1

A third pathway, which has been the topic of much controversy, concerns the import of proteins with a bipartite presequence to the IMS (van Loon *et al.*, 1986). The signal consists of a positively-charged matrix-targeting sequence, followed by a hydrophobic sorting signal. The latter sequence resembles the bacterial and ER leader sequences (Randall and Hardy, 1989; Wickner *et al.*, 1991). Such proteins, for example, cytochromes b_2 and c_1, are processed in a two step fashion. Cleavage of the matrix-targeting signal occurs in the matrix by MPP (see above). The second hydrophobic signal is cleaved by the membrane-bound protease, called inner membrane peptidase 1 (IMP1), in the IMS (Schneider, A. *et al.*, 1991). IMP1 shows sequence similarity with the *E. coli* leader peptidase (Behrens *et al.*, 1991).

The sorting signal has been proposed to work in one of two ways. According to the "stop-transfer" model (van Loon *et al.*, 1987; Glick *et al.*, 1992), the first signal targets the precursor to the matrix, whereas the second signal, because of its hydrophobicity, prevents complete translocation across the inner membrane. Subsequent sorting to the IMS could then occur by lateral diffusion in the inner membrane. Conversely, the "conservative sorting" hypothesis

(Hartl *et al.*, 1987; Koll *et al.*, 1992; Schwarz *et al.*, 1993) states that the first signal targets the precursor completely into the matrix whereupon it embarks on an export pathway back across the inner membrane to the IMS as directed by the sorting signal. This model is believed to reflect the route of protein export in bacteria, which are proposed to be the evolutionary ancestors of mitochondria. Evidence favouring both models has been generated (Cheng *et al.*, 1989; Koll *et al.*, 1992; Schwarz *et al.*, 1993; Glick *et al.*, 1992), however the debate continues.

1·2·4 Sorting to the inner membrane

Import into the inner membrane appears to follow several pathways. The ATP/ADP carrier (AAC), a member of a family of structurally related homo-oligomeric proteins, is synthesized without a cleavable presequence (Smagula and Douglas, 1988). Targeting information seems to reside in triplicate internal segments (Pfanner *et al.*, 1987b) which are located between amino acids 72 and 111 (Smagula and Douglas, 1988). The majority (70%) of imported AAC is received by MOM72/Mas70 (Söllner *et al.*, 1989; Hines *et al.*, 1990) and transferred to the GIP via MOM22 (Kiebler *et al.*, 1993b) in an ATP dependent reaction (Pfanner and Neupert, 1986, 1987a). Yeast Mas70 mutants still import AAC at approximately 30% wild-type levels. Mitochondria isolated from *N. crassa* were treated with antibodies against either MOM19 or MOM72 in order to determine the role played by the two receptors in AAC import (Steger *et al.*, 1990). The antibodies blocked import with different efficiencies, suggesting that the two receptors act in parallel for AAC import. It was proposed that AAC is next transported into contact sites via MOM22 and the GIP (Kiebler *et al.*, 1993), whereupon the protein diffuses laterally into the inner membrane. This mechanism is implied by a requirement for an

electrochemical potential across the inner membrane, but the lack of an interaction with matrix Hsp60, which is required for folding of precursors newly imported into the matrix (Mahlke *et al.*, 1990). In the absence of an electrochemical potential, AAC accumulates at the GIP level where it is not exposed on the mitochondrial surface (Pfanner and Neupert, 1987b). AAC cannot be translocated across the outer membrane in experiments using outer membrane vesicles, showing that AAC must contact either the inner membrane, possibly at contact sites, or an IMS component (Mayer *et al.*, 1993) for translocation to occur.

Another inner membrane protein structurally related to AAC, the uncoupling protein from mammalian brown fat mitochondria, also lacks a cleavable presequence (Agnila *et al.*, 1987). The synthetic attachment of a matrix-targeting signal to the uncoupling protein results in the translocation of the hybrid to the matrix and it fails to enter the inner membrane even following cleavage of the presequence in the matrix (Liu *et al.*, 1990). This suggests that the uncoupling protein follows the same pathway as AAC by directly transferring from the import sites to the inner membrane. A third structurally related protein, the bovine mitochondrial phosphate carrier, is synthesized with a cleavable presequence, suggesting its mode of import differs from AAC and the uncoupling protein. This protein may be routed through the matrix (Runswick *et al.*, 1987).

Components of the respiratory chain generally follow the standard route of import in that they are synthesized with a cleavable presequence and translocated completely into the matrix before assuming their mature configuration in the inner membrane (reviewed in Glick and Schatz, 1991; Hartl and Neupert, 1990). However, CoxVa, a component of cytochrome *c* oxidase that is synthesized with a cleavable presequence (Glaser *et al.*, 1988) and

requires both a membrane potential and ATP hydrolysis for import deviates from the standard pathway in one important respect. Surprisingly, the CoxVa precursor is imported into trypsin-pretreated mitochondria with efficiencies equal to wild-type. CoxVa precursors that were arrested during import into trypsin-pretreated mitochondria were found at contact sites, presumably at the GIP, implying that the CoxVa precursor bypasses the receptors and interacts directly with the GIP, becoming translocated into the inner membrane (Miller and Cumsky, 1991). CoxVa also contains a hydrophobic sequence in the C-terminal third of the protein that is necessary for proper inner membrane localization; deletion of this sequence results in misrouting to the matrix (Glaser and Cumsky, 1990). Furthermore, overproduction of a form of CoxVa that lacks a presequence circumvents the leader sequence requirement for import into mitochondria (Dircks and Poyton, 1990), suggesting the hydrophobic sequence may be sufficient for localization.

In general, hydrophobic stretches of amino acids have been demonstrated to be important for import across biological membranes (von Heijne *et al.*, 1989) though the role of these stretches is a subject of debate (see above for import of cytochromes b_2 and c_1). For example, hydrophobic stretches in F_o-ATPase subunit 9 apparently fail to stop translocation across the inner membrane (Mahlke *et al.*, 1990), whereas the hydrophobic sequence in CoxVa appears to stop the transfer of the precursor in the inner membrane (Glaser *et al.*, 1990). Studies using a hydrophobic ER stop-transfer sequence from the vesicular stomatitis virus G protein, fused to the N-terminus of the normally matrix-localized precursor, pre-ornithine carbamyl transferase, resulted in import of the fusion protein to the inner membrane. Conversely, placement of the same hydrophobic sequence near the C-terminus resulted in outer membrane localization (Nguyen *et al.*, 1988). Therefore it is not clear if the

hydrophobic sequence acts as a "stop-transfer" signal that prevents the complete translocation of the precursor across the inner membrane or acts similar to a bacterial export sequence and is therefore consistent with the conservative sorting model.

1·2·5 Targeting to the outer membrane

The extensive study of insertion of proteins into the mitochondrial outer membrane has demonstrated several routes by which precursors destined for the outer membrane are targeted to and recognized by mitochondria. Outer membrane proteins typically are not synthesized with a cleavable presequence and an electrochemical potential across the inner membrane is not required for their placement in the outer membrane (reviewed in Glick and Schatz, 1991). Nonetheless, it is likely that the outer membrane proteins share the same import apparatus utilized by proteins previously discussed. This is implied by two observations. Firstly, the placement of a hydrophobic stop-transfer signal near the C-terminus of a matrix protein resulted in mistargeting to the outer membrane (see above; Nguyen *et al.*, 1988). Secondly, a water-soluble form of the mitochondrial outer membrane protein, porin (Pfaller *et al.*, 1985), which binds tightly to isolated *N. crassa* mitochondria, was shown to interact with the same outer membrane import components as many matrix-targeted precursors, and block their import (Pfaller *et al.*, 1988).

The outer membrane proteins porin (Schneider *et al.*, 1991) and MOM72 (Söllner *et al.*, 1990) require MOM19 as a receptor, since their import is inhibited if mitochondria are pre-incubated with antibodies against MOM19. A different dependence was found for MOM38 (Keil *et al.*, 1993) and for MOM22 (Keil and Pfanner, 1993). These two proteins appears to require both MOM19 and MOM72 for efficient import. This was shown using mitochondria

separately pre-incubated with antibodies against MOM19 and MOM72. Import was reduced by 80-90% for both MOM22 (Keil and Pfanner, 1993) and MOM38 (Keil *et al.*, 1993) when antibodies against either MOM19 or MOM72 were used. The effects of using both antibodies simultaneously are not known since this experiment was not done. The import of MOM19, on the other hand, does not appear to require any protease sensitive mitochondrial surface receptors (Schneider *et al.*, 1991). However, when yeast mitochondria were pre-incubated with antibodies against Isp42 (the yeast MOM38 correlate), subsequent assembly of *N. crassa* MOM19 in the outer membrane was blocked (Schneider *et al.*, 1991). The requirement of both receptors for MOM38 and MOM22 was proposed as a mechanism to increase specific targeting to mitochondria by a double-check system (Kiebler *et al.*, 1993a; Keil *et al.*, 1993). The requirement for precise control of MOM38 targeting becomes obvious with the finding that MOM38 may target MOM19 to the mitochondrial outer membrane. That is, if MOM38 was inserted into other cellular membranes, which in turn led to MOM19 being targeted to other membranes, this could presumably result in deleterious effects caused by mistargeting of all the precursors recognized by MOM19.

The targeting signals of outer membrane proteins have been analyzed in several proteins (Glick and Schatz, 1991). The N-terminal domain of Mas70 resembles a bipartite signal sequence; the first 12 amino acids can act as a matrix-targeting signal, whereas residues 10 to 37 act as a hydrophobic stop-transfer signal. It appears that Mas70 in yeast is initially directed to the matrix and the hydrophobic domain acts as a stop-transfer signal for the outer membrane (Hase *et al.*, 1984; Hurt *et al.*, 1986). This is opposed to what was seen when a stop-transfer signal was placed at the N-terminus of a matrix-localized protein; it was misrouted to the inner membrane (see above; Nguyen

et al., 1988). Unexpectedly, it was found that the N-terminal domain of MOM72, the *N. crassa* homologue of yeast Mas70, lacked a matrix-targeting signal (Steger *et al.*, 1990) but did contain the hydrophobic membrane-spanning domain. The targeting information for porin, which also lacks a matrix-targeting signal, seems to reside in both the N- and C-terminal domains (Hamajima *et al.*, 1988), whereas MOM38 and Isp42 do not contain typical mitochondrial targeting sequences (Kiebler *et al.*, 1990; Baker *et al.*, 1990).

1·3 Assembly of multi-subunit complexes within the inner membrane

Many enzymes within the mitochondria are composed of multiple subunits encoded by both mitochondrial and nuclear DNA. To gain insight into the complex process of assembling such enzymes, I have used complex I as a model system (reviewed in Weiss *et al.*, 1991; Walker, 1992). Complex I is the first of three proton pumping complexes involved in electron transport. Complex I, as well as complexes III and IV, link electron transport with proton translocation across the inner membrane, producing an electrochemical potential that subsequently drives ATP synthesis (Tzagoloff, 1982). Electrons are passed from NADH, generated by the citric acid cycle, to complex I, which then passes the electrons to ubiquinone. For this reason, complex I is also referred to as NADH:ubiquinone oxidoreductase.

This complex is by far the largest and most intricate of the inner membrane complexes. It is associated with one FMN, four known iron-sulfur clusters designated N-1, N-2, N-3 and N-4, perhaps two to four more iron-sulfur centers (Ragan, 1987; Walker, 1992), and probably one bound ubiquinone. A variety of inhibitors, such as rotenone and piericidin A, are proposed to block electron flow from the iron-sulfur cluster N-2 to ubiquinone by steric hindrance or by a

conformational change that prevents passage of electrons to ubiquinone (Weiss *et al.*, 1991; Singer and Ramsay, 1992; Walker, 1992).

The *N.crassa* enzyme contains approximately 30 protein subunits. In bovine mitochondria, approximately 40 subunits have been identified and sequenced (see Walker *et al.*, 1992 and references therein). In both cases, seven of the the subunits are encoded by mtDNA. The remaining subunits are encoded in the nucleus, translated in the cytoplasm, and translocated into mitochondria presumably by utilizing the import machinery of the mitochondrial outer membrane. Not all subunits are synthesized with a cleavable presequence (Walker *et al.*, 1992), therefore it is not known precisely how the subunits are imported.

Three of the remaining four complexes involved in oxidative phosphorylation contain subunits derived from both mitochondrial and nuclear genomes (reviewed in Hatefi, 1985; Tzagoloff, 1982). Complex II (succinate:ubiquinone oxidoreductase) transfers electrons from succinate to ubiquinone, but is not coupled to proton pumping. All 4 of the subunits from this enzyme are encoded in the nucleus. Complex III, also known as the bc_1 complex or ubiquinone:cytochrome *c* oxidoreductase has only one of its nine subunits encoded by the mitochondrial genome (the cytochrome *b* apoprotein). Complex IV (cytochrome *c* oxidase) catalyzes the flow of electrons from cytochrome *c* to the final electron acceptor, O_2. This complex consists of 3 mitochondrial and approximately 8 nuclear encoded proteins.

Complex I, along with complexes III and IV, pump electrons from the matrix to the IMS producing a proton gradient. The excess protons in the IMS flow through complex V, the F_1F_0 ATPase, back across the inner membrane to generate ATP (Tzagoloff, 1982; Hatefi, 1985). This complex consists of two subcomplexes: the hydrophilic F_1 subcomplex, which faces the matrix, is

responsible for catalyzing the synthesis of ATP while the hydrophobic F_o subcomplex acts as the proton channel. F_1 contains 5 proteins in different stoichiometries whereas F_o is made up of 4 proteins. Both mitochondrial and nuclear genomes contribute to this enzyme, however, the contributions vary in different organisms.

Complex I has recently been studied extensively in *N. crassa* and bovine (Weiss *et al.*, 1991; Walker, 1992). The impetus for studies on complex I, which has proven difficult due to the enormous complexity of the enzyme, has come from several areas. i) The development of targeted gene replacement (Nehls *et al.*, 1992) and gene RIP (repeat induced point-mutation; a method of generating mutations in specific genes in *N. crassa* and is discussed fully in section 2·1 and Selker, 1990) in *N. crassa* has made it possible to study the effects of mutant genes . ii) A broad spectrum of degenerative mitochondrial diseases in humans have been associated with defects in complex I (Schapira, 1993; Shoffner and Wallace, 1992). iii) Simpler related forms of the NADH dehydrogenase enzyme have been found in fungi and bacteria which allow comparative studies (Calhoun and Gennis, 1993; de Vries *et al.*, 1992). iv) Sequencing of subunits from complex I (Walker *et al.*, 1992) has allowed the functions of several subunits to be determined. v) Comparative electron microscopic and biochemical studies have revealed that complex I is composed of two distinct arms that are assembled independently of one another (Freidrich *et al.*, 1989; Tuschen *et al.*, 1990), suggesting that the two arms evolved separately.

1·3·1 Generation of mutants in complex I

As described above, the major role of complex I in the eukaryotic cell is to transfer electrons from matrix-generated NADH to ubiquinone in the electron

transport chain. Furthermore, electron transport is coupled to proton pumping through this enzyme. Humans with defects in complex I present a variety of debilitating diseases. On the other hand, the lack of complex I in *S. cerevisiae* suggests that the complex is not essential, though it should be noted that *S. cerevisiae* is not an obligate aerobe. To address the question of the importance of complex I in *N. crassa*, mutants have been generated in subunits of the complex using either gene disruption (Nehls *et al.*, 1992) or RIP (Alves and Videira, 1994).

Inactivation of the gene encoding the 21.3 kd subunit of the membrane arm by gene disruption resulted in a mutant strain that grew almost as fast as the wild-type parental strains (Nehls *et al.*, 1992). Assembly studies revealed that the peripheral arm accumulated in the absence of any assembled membrane arm. Studies using isolated mitochondrial membranes from the mutant and wild-type revealed that the remaining NADH:oxidoreductase activity resembled the activity seen in wild-type cells grown in the presence of rotenone, piericidin A, or chloramphenicol (Friedrich *et al.*, 1989). As discussed below, it is not clear if this activity is due to a completely different NADH dehydrogenase or if it arises from the altered complex I due to a second ubiquinone binding site in the peripheral arm (see below and Weiss *et al.*, 1991).

RIP was used to generate mutants in the gene coding for the 21.3 kd component of the peripheral arm (Alves and Videira, 1994). This mutant was also capable of growing at close to wild-type rates. Complex I was still assembled in this mutant, but at a much reduced level. Therefore, all potential activities are still present in this mutant, but at reduced levels.

Mutants in the *Salmonella typhimurium* complex I-like enzyme have been described that have little effect on growth rate (Archer *et al.*, 1993). Likewise, disruption of the homologous enzyme in *E. coli* had little effect (Calhoun and

Gennis, 1993). In both cases, there are simple enzymes that may be sufficient to cover for the absence of the complex enzyme. Disruption of the gene encoding the 51 kd subunit of the putative peripheral arm of *Aspergillus niger* complex I, which is proposed to contain the binding sites for NADH, FMN and 1 iron-sulfur cluster, resulted in the complete loss of assembled peripheral arm (Weidner *et al.*, 1992). In this case, the mutant grew significantly slower than the parental strain, perhaps emphasizing the importance of the potential functions carried out by the peripheral arm.

In vitro studies using a human cell line defective in the mtDNA encoded ND4 gene, revealed that all or most of the mtDNA encoded subunits were absent from the immunoprecipitated complex (Hofhaus and Attardi, 1993). Furthermore, there was a complete loss of NADH-dependent respiration. It is important to note that different human tissues utilize energy at different rates and that it is the tissues that demand the highest levels of energy that invariably show a phenotype in response to complex I deficiencies.

1·3·2 Human diseases associated with complex I

The organs of the human body utilize energy at various rates. Those with the highest demand for ATP, such as the central nervous system and skeletal muscle, are at possible risk of disease if any one of the 50 or so proteins involved in oxidative phosphorylation, encoded by the nuclear and mitochondrial genomes, becomes defective (reviewed in Weiss *et al.*, 1991; Walker, 1992). There have been a broad spectrum of diseases associated with defects in the respiratory chain components that show a remarkable heterogeneity of symptoms. The disorders associated with complex I range from debilitating muscle and central nervous system disorders to diseases of

less severity affecting the heart, kidney and liver (reviewed in Weiss *et al.*, 1991; Walker, 1992; Shoffner and Wallace, 1992; Schapira, 1993).

1·3·3 Simpler forms of the NADH dehydrogenase

Prokaryotes have been found to contain enzymes similar to complex I that have noncovalently bound FMN, multiple iron-sulfur clusters and a rotenone- or piericidin A-sensitive ubiquinone binding site (Anraku, 1988). The prokaryotic complex I is simpler than the mitochondrial counterpart. The isolated complex from *Paracoccus denitrificans* contains approximately 15 subunits (Yagi *et al.*, 1992) encoded by the the *NQO* gene cluster. This cluster has been cloned and sequenced, revealing 14 known structural genes and 6 URFs. The genes are homologous to 7 of the bovine mitochondrial encoded subunits and 6 nuclear encoded subunits, including the 51 kd and 75 kd subunits thought to contain the NADH, FMN and 3 iron-sulfur cluster binding sites (Walker *et al.*, 1992; Walker, 1992; Weiss *et al.*, 1991). The analogous complexes from *E. coli* (Weidner *et al.*, 1992) and *S. typhimurium* (Archer *et al.*, 1993) appear to be even simpler than the *P. denitrificans* enzyme.

It has been found that mitochondria of plants, fungi and yeast contain two additional NADH dehydrogenases that consist of a single polypeptide with a noncovalently bound FAD as the only prosthetic group. These rotenone- and piericidin A-insensitive enzymes are found on both sides of the inner membrane and oxidize either matrix or cytosolic NADH. The oxidation of NADH by these enzymes is not coupled to proton pumping (reviewed in Weiss *et al.*, 1991; Walker, 1992). *S. cerevisiae* lacks an enzyme analogous to complex I, containing only the internal and external simple enzymes, whereas mammalian mitochondria lack both the simple enzymes and only contain complex I (de Vries and Grivell, 1988). *E. coli* has also been shown to contain

a single polypeptide NADH dehydrogenase that is not coupled to proton pumping (Young and Wallace, 1976) as well as the more complex enzyme. Sequencing of the *S. cerevisiae* internal enzyme (de Vries *et al.*, 1992) and the simple *E. coli* enzyme (Young *et al.*, 1981) has shown these two enzymes to be homologous. Yeast cells containing a disruption in the internal enzyme were incapable of growth on nonfermentable carbon sources (de Vries *et al.*, 1992). Disruption of either the simple or complex enzyme in *E. coli* was not lethal, however, a cell lacking both enzymes was inviable (Calhoun and Gennis, 1993). It has been proposed that the simple enzymes play an important role in the regulation of the intracellular redox balance, whereas in mammalian mitochondria, this redox balance is maintained by shuttle systems within the inner membrane, thereby eliminating the need for the external and internal simple enzymes (de Vries and Grivell, 1988).

1·3·4 Sequences and functions of complex I subunits

Two different strategies have been taken to sequence complex I subunits from *N. crassa* and bovine. For *N. crassa*, cDNAs have been isolated by screening expression libraries with antibodies against isolated subunits and by sequencing mtDNA (Weiss *et al.*, 1991; Walker, 1992; Videira *et al.*, 1990a, 1990b). The genes for approximately 22 subunits have been sequenced, identifying the putative binding sites for iron-sulfur clusters N-3 and N-4 to conserved cysteine residues (75 kd subunit), the binding sites for NADH, FMN and iron-sulfur cluster N-1 (51 kd subunit), iron-sulfur cluster N-2 (ND5), the ubiquinone binding site (ND1) and another potential iron-sulfur protein (22 kd; Videira *et al.*, 1990b). The 75, 51 and 24 kd subunits are related to subunits of the *Alcaligenes eutrophus* NAD^+ hydrogenase and very likely constitute a structural and functional unit. The mitochondrial encoded subunits ND2, ND4

and ND5 show significant identity to each other, suggesting that these genes evolved from a single ancestral gene. Furthermore, the 9.6 kd subunit shows sequence similarity with a bacterial and chloroplast acyl-carrier protein, suggesting complex I may have a function in fatty acid metabolism (Sackmann *et al.*, 1991). Finally, a 40 kd subunit has been found to be related to a family of protein peptidases, including subunits of complex III and β-MPP of mitochondria (Röhlen *et al.*, 1991).

The approach used to isolate the bovine complex I subunits entailed the design of DNA primers from partial protein sequence for use in PCR reactions (Walker *et al.*, 1992; Walker, 1992). The PCR products were then used to screen phage M13 libraries of bovine genomic DNA. The bovine subunits shown to bind NADH, FMN, the iron-sulfur clusters and ubiquinone were found to be homologous to the equivalent *N. crassa* subunits. A bovine subunit was also found to be homologous to the *N. crassa* subunit that exhibited similarity to an acyl-carrier protein. Unfortunately, the functional identity of the remaining subunits could not be deduced from the primary sequence.

It has been suggested that some of the subunits of complex I may be involved in the binding of other matrix enzymes, especially metabolically linked dehydrogenases (Moore *et al.*, 1984; Sumegi and Srere, 1984; Fukushima *et al.*, 1989) since binding of these dehydrogenases to complex I would result in a functionally logical enzyme organization. Thus, NADH production and oxidation could be maintained in an efficient coupled manner. Analysis of the bovine sequences did not provide any obvious clues as to which subunits may be involved (Walker, 1992). However, studies using isolated mitochondria containing a mutation in the ND4 gene from patients with Leber's hereditary optic neuropathy (LHON) found that the rate of oxidation of NAD-linked substrates, but not succinate, is decreased (Majander *et al.*, 1991). This

suggests that the mutation in ND4 may affect binding of other NADH-dependent dehydrogenases to complex I, thereby promoting electron transfer through complex I.

1·3·5 Independent assembly of the two subcomplexes

Comparative electron microscopy and biochemical studies have shown that complex I in *N. crassa* is constructed of two distinct parts arranged at right-angles to give an overall L-shaped structure (Hofhaus *et al.*, 1991). One part protrudes from the inner membrane into the matrix space and contains approximately 15 nuclear encoded subunits. This arm, called the peripheral arm, contains the NADH, FMN and three iron-sulfur cluster binding sites (N-1, N-3, N-4). The membrane arm, which is composed of approximately 17 subunits, contains all 7 mitochondrial encoded subunits and the N-2 iron-sulfur cluster and ubiquinone binding sites. It has been observed that in *N. crassa*, if mitochondrial protein synthesis is inhibited by chloramphenicol, the peripheral arm does not associate with any subunit of the membrane arm (Friedrich *et al.*, 1989). Furthermore, pulse-labelling experiments in *N. crassa* have shown that the membrane arm subunits accumulate prior to final assembly with the peripheral arm (Tuschen *et al.*, 1990). One interpretation of these results is that the two components evolved independently of one another and later began to interact. This hypothesis is also supported by sequence analysis which has revealed identity between components of the membrane arm of complex I and an *E. coli* formate hydrogenylase (Böhm *et al.*, 1990) and between components of the peripheral arm and the *A. eutrophus* NAD^+ hydrogenase (Tran-Betcke *et al.*, 1990). The different arms of the complex may have conceivably evolved in different prokaryotic hosts, originally carrying out different functions.

While the peripheral arm contains the binding sites for NADH, FMN and at least 3 iron-sulfur clusters, it has not been demonstrated if it is capable of donating electrons to the electron transport pathway. If the cells are grown in the presence of chloramphenicol, in which only the peripheral arm assembles, the NADH dehydrogenase activity differs from complex I in that it shows a lower affinity for ubiquinone and is insensitive to rotenone and piericidin A (Friedrich *et al.*, 1989). While it is possible that this activity is indicative of an alternative NADH dehydrogenase, the authors suggest that the peripheral arm may be capable of donating electrons to ubiquinone through a second ubiquinone binding site located in the peripheral arm. This binding site may act as a reduction site for an internal ubiquinone in the larger complex I (Weiss *et al.*, 1991). It is pointed out however, that this hypothesis is highly speculative.

In what appears to be an adaptation to the lower affinity for ubiquinone when *N. crassa* cultures are grown in chloramphenicol, mitochondria increase the ubiquinone content of the inner membrane 5 to 8 times the levels of control cells (Friedrich *et al.*, 1989). This suggests that some signal is sent from the mitochondria to the nucleus to increase the ubiquinone level. This is reminiscent of retrograde regulation proposed to explain how nuclear encoded genes can be regulated by the functional state of mitochondria (Butow *et al.*, 1988).

1·4 Objectives of this study

The present study was designed to address two aspects of mitochondrial biogenesis using genetic and biochemical approaches. First, the specific roles of selected proteins in mitochondrial protein import, and second, the assembly of imported proteins into multi-subunit complexes. The technique of

"Sheltered" RIP was used to generate mutations in the genes *mom-19* and *nuo-78.*

In previous biochemical studies, MOM19, of *N. crassa,* was shown to be a receptor on the mitochondrial outer membrane that is responsible for the import of the majority of mitochondrial targeted precursors. To determine if MOM19 plays an essential role in protein import and cell viability, the gene encoding the protein was inactivated by RIP. During the course of the work in this thesis, the yeast gene *mas-20* was isolated in another laboratory (Ramage *et al.*, 1993). Sequence comparisons and functional studies in cells with *mas-20* null alleles suggested that Mas20 was a functional equivalent of MOM19. Because MOM19 is predicted to play an important role in the cell, the technique of "sheltered RIP" was used to generate the mutants so that the mutant nucleus would be sheltered, or complemented, in a heterokaryon by a second wild-type nucleus. This enabled the mutant allele to be maintained for further study if it indeed proved to be essential.

In an unrelated project, designed to address the mechanism of assembly of complex enzymes in mitochondria, a protein thought to be essential for electron flow through complex I was inactivated by RIP. NUO78, the largest subunit of complex I, is thought to contain binding sites for at least 2 iron-sulfur clusters. Since electrons are routed through these iron-sulfur clusters, the electron transport and presumably the proton pumping activity of complex I should be abolished. It has previously been suggested that in *N. crassa* an alternate NADH dehydrogenase, or the peripheral arm of complex I alone, may be sufficient to back-up the complex I activity (Friedrich *et al.*, 1989, Weiss *et al.*, 1991; Nehls *et al.*, 1992). On the other hand, results in *Aspergillus niger* suggested that the loss of the complex I peripheral arm may be deleterious to viability (Weidner *et al.*, 1992). In *N. crassa,* the effects of totally disrupting

peripheral arm assembly have not been demonstrated. Therefore, the *nuo-78* gene was also inactivated using sheltered RIP to protect against possible deleterious effects on the cell. The lack of NUO78 protein in one nucleus of the heterokaryon generated by sheltered RIP could result in the loss of the peripheral arm under selective conditions, thereby allowing the study of the phenotype and effects on complex I function.

1·5 References

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2 Inactivation of the *Neurospora crassa* gene encoding the mitochondrial protein import receptor MOM19 by the technique of "sheltered RIP"

2.1 Introduction

The filamentous fungus, *Neurospora crassa*, has proven to be an excellent organism for many biochemical investigations. The ease by which biochemical questions can be addressed has been contrasted, until very recently, by the relative difficulty of isolating mutants in specific target genes. In the yeast *Saccharomyces cerevisiae*, there are various methods for creating null alleles of genes that have been cloned. Perhaps the most common is by integrative replacement of the endogenous gene with a copy of the gene that has been interrupted by a large DNA sequence, usually a selectable marker (Rothstein, 1991). This approach has achieved limited success in *N. crassa* (Frederick *et al.*, 1989; Nehls *et al.* 1992) but the frequency of replacement by homologous integration may be very low for many *N. crassa* genes (Asch and Kinsey, 1990). Recently a method for increasing the efficiency of using gene disruption in *N. crassa* has been described (Aronson *et al.*, 1994). An efficient alternative to methods of disruption based on homologous replacement is to utilize the RIP (repeat induced point mutation) phenomenon that has been described in *N. crassa* (Selker, 1990). The process of RIP affects duplicated DNA sequences present in a nucleus that participates in a genetic cross and effectively results in the disruption of both copies of the duplication by the generation of numerous GC to AT transitions. Thus, a strain engineered by genetic transformation to contain a duplication of any *N. crassa* gene could be used to generate mutants specifically in that gene. This approach has already been

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used to create mutants in various genes in *N. crassa* (Fincham *et al.*, 1989; Glass and Lee, 1992). Thus, gene RIP in *N. crassa* and gene replacement procedures in yeast both result in the isolation of strains with non-functional target genes.

However, for genes providing important functions to the cell, the rather simple approach described above would not permit the isolation of mutants since they might be inviable and/or have severely reduced germination rates. If such a gene was essential for growth or germination of ascospores, a certain percentage of tetrads, related to the frequency of RIP of the target sequence, should produce only four viable spores instead of the usual eight. The latter situation is analogous to sporulation of a diploid yeast carrying a disruption in one copy of a gene, where failure to isolate the relevant haploid strain indicates that the target gene is essential for germination and/or growth (Rothstein, 1991). In both organisms, the genetic data is useful but haploid strains with the mutant phenotype, which might be utilized for further study, are not produced because they are inviable. In yeast, one way to overcome this problem is by supplying an extra copy of the target gene, under the control of a promoter that is induced by galactose and is repressed by glucose, to the disruptant-containing nucleus of the diploid prior to meiosis. Therefore, in the presence of galactose and the absence of glucose, the disruptants will germinate and grow since the spores are supplied with the essential gene product (Baker *et al.*, 1990; Schneider and Guarente, 1991). However, when such a culture is shifted to medium lacking galactose and containing glucose the gene product is gradually diluted out of the cells so that the effect of the deficiency can be monitored in the cells at various stages of depletion of the product. Unfortunately, there are no promoters with a suitable degree of control currently available for performing similar experiments in *N. crassa*. Therefore, an

extension of the RIP procedure, which is referred to as "sheltered RIP", has been developed to achieve a similar outcome (Metzenberg and Grotelueschen, 1992a; the method is described with the aid of figures for the specific inactivation of *mom-19*$^{+}$ in section 2·3). This technique allows the isolation of a mutant gene in one nucleus, even if that gene is essential for the survival of the organism, by sheltering the nucleus carrying the mutant gene in a heterokaryon with an unaffected nucleus. Furthermore, the inclusion of a selectable marker in the nucleus harbouring the RIPed gene makes it possible, by growing cultures under the appropriate conditions, to shift the nuclear ratios in the heterokaryons at will, to a state in which the nucleus containing the RIPed gene predominates. This gives rise to a condition in which the product of the gene that has been RIPed is present at very suboptimal levels and allows the study of the mutant phenotype. The latter situation should be analogous to the conditions created when the yeast disruptant is shifted to glucose medium lacking galactose. In both cases, alterations in the composition of the medium can be used to "control" the amount of the essential product present in the culture.

In this chapter, the successful application of sheltered RIP is described for generating mutations in the *mom-19*$^{+}$ gene. As discussed fully in section 1·2·2·1, the product of the gene, MOM19, is a protease-sensitive surface component of the protein import complex of the mitochondrial outer membrane (Söllner *et al.*, 1989). Biochemical studies, using MOM19 specific antibodies, have demonstrated that MOM19 serves as the initial receptor site for the import of the vast majority of nuclear-encoded mitochondrial preproteins (Söllner *et al.*, 1989). A second receptor, MOM72, was found to play a more specialized role in the import of the ATP/ADP carrier (Söllner *et al.*, 1990). Following interaction with MOM19 or MOM72, the preproteins are transferred to a general insertion

pore called GIP (Pfaller *et al.*, 1988). A major component of GIP is MOM38 (Kiebler *et al.*, 1990) or its yeast homologue, ISP42 (Baker *et al.*, 1990). Transfer of the preproteins from the receptor stage to the GIP stage is thought to be facilitated by a membrane-spanning component of the receptor complex called MOM22 (Kiebler *et al.*, 1993).

Since MOM19 plays such a pivotal role in the import of the majority of proteins into mitochondria, it was suspected that inactivation of the gene encoding the protein might have severe consequences on *N. crassa* cells. For this reason, sheltered RIP was used to isolate mutants in this gene. Here both the genetic details of the procedure as applied to *mom-19*$^{+}$ and the characteristics of a mutant strain which is grossly deficient in the MOM19 protein when grown under appropriate conditions are described.

2.2 Materials and Methods

2·2·1 Strains and media.

Growth and handling of *N. crassa* strains was as described in Davis and de Serres (1970). All strains used in this study are listed in Table 2·1. The Host IV and Mate IV strains carry complementary nutritional markers on linkage group IV (LG IV) that allow for specific selection of LG IV disomic ascospores following crosses of Mate IV and transformed derivatives of Host IV (see Results). LG IV disomics are the only spores generated from the cross that are capable of growth on basal media lacking tryptophan (*trp-4* marker) and uridine (*pyr-1* marker). Thus, we define basal medium as the medium capable of supporting growth of disomics or heterokaryons containing both the Host and Mate LGs IV. The medium contains Vogel's salts including trace elements and biotin (Davis and de Serres, 1970), 1.5% glucose, and inositol (50 µg/ml). When required, uridine (1 mM) and tryptophan (0.5 mM) were added to the

medium. *p*-fluorophenylalanine (fpa) was used at various concentrations as given in the text. Though not required by the strains used in this study, either leucine (1 mM) or arginine (1 mM) were also present in media because they are part of a standard medium used in the laboratory for similar experiments.

2·2·2 Plasmid construction

Both genomic and cDNA versions of the *mom-19*$^{+}$ gene were cloned previously (Schneider *et al.*, 1991). The genomic clone was utilized to construct a plasmid, pKSH6 (Fig. 2·1) that is a derivative of pBR322 containing the *mom-19*$^{+}$ sequence and a bacterial *hph* gene (hygromycin B phosphotransferase) expressed by a *trpC* promoter from *Aspergillus nidulans* (Cullen *et al.*, 1987). Resistance to hygromycin allows selection of transformants in *N. crassa* (Staben *et al.*, 1989). The *N. crassa* DNA in pKSH6 totals 1607 bp and extends only 39 bp upstream of the first base of the *mom-19*$^{+}$ cDNA and 108 bp downstream of the cDNA end point. In experiments designed to generate RIP mutations in specific target genes it is desirable to limit the amount of non-*mom-19*$^{+}$ DNA in the construct to prevent RIP of neighboring genes.

2·2·3 Isolation of mitochondria

Mycelia from liquid cultures was harvested by filtration. The mycelia was weighed and ground by hand using a mortar and pestle in the presence of 1.5 g of acid washed quartz sand and 1 ml of grinding buffer (0.44 M sucrose; 10 mM Tris-HCl, pH 7.5; 0.2 mM ethylenediaminetetraacetic acid; 0.2 mM phenylmethylsulfonyl fluoride) per gram of mycelia. When a smooth paste was formed, the slurry was centrifuged at 3000 rpm in a Sorvall SS-34 rotor for 10 min to remove nuclei, cell debris and sand. The supernatant was transferred to a clean tube and centrifuged for 20 min at 12,000 rpm in the same rotor. The

Table 2·1 *N. crassa* strains used in this study.

N. crassa strains

Strain	Genotype or origin
Host IV[a]	LG I, *a*; LG IV, *pyr-1 mom-19$^+$ mtrR trp-4$^+$*; LG V, *inl inv mei-2.*
Mate IV[b]	LG I, *A*; LG IV, *pyr-1$^+$ mom-19$^+$ mtrS trp-4*; LG V, *am inl inv mei-2.*
TI 26.3	Isolate from tranformation of Host IV with pKSH6 (see Fig. 2·1), contains single ectopic copy of *mom-19$^+$*, hygromycin resistant.
TI 28.3	As TI 26.3
TI 40.3	As TI 26.3
28.1	Ascospore isolate of Mate IV x TI 28.3, selected as a heterokaryon on basal medium, status of *mom-19* gene unknown.
28.16	As 28.1, except that one component of the heterokaryon, containing the LG IV derived from the Host IV strain, is known to contain a RIPed allele of *mom-19*. Both components of the heterokaryon contain an ectopic RIPed version of the *mom-19* sequence derived from integration of pKSH6.
28.17	As 28.16.
28.18	As 28.1.
28.23	As 28.1.
M17.60	Homokaryon isolated from 28.17 containing RIPed allele of *mom-19*
M17.63	As M17.60.
M17.65	As M17.60.
M17.67	As M17.60.
M17.69	As M17.60.
M17.70	As M17.60.
M17.71	As M17.60.
M17.72	As M17.60.
M17.73	As M17.60.
M17.74	As M17.60.
M17.75	As M17.60.
M17.76	As M17.60.

17.43.1	Transformant of strain 28.17 obtained by transformation with a cosmid containing the *mom-19*$^{+}$ gene and approximately 35 kb of additional *N. crassa* genomic DNA. Isolated on the basis of increased growth rate on basal medium plates containing sorbose and supplemented with 600 mM fpa plus uridine. Shown to be a homokaryon containing LG IV from Host IV. MOM19 protein present.
17.67.1	As 17.43.1 except that transformation was with a plasmid containing the *mom-19*$^{+}$ gene and an additional 4 kb of *N. crassa* genomic DNA.
17.94.2	As 17.43.1 except that transformation was with a plasmid containing *mom-19*$^{+}$ cDNA.

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[a] The Host IV strain used in these experiments is carried as an alternate Host IV at the Fungal Genetics Stock Center (strain #7270), the standard Host IV strain (Fungal Genetics Stock Center strain #7254) is *pan-1* instead of *pyr-1*.

[b] This strain is used as a heterokaryon with an inactive mating type strain (allele a^{m1}) carrying an *ad-3B* marker so that nutritional complementation is possible with the Mate IV nucleus. Thus, the heterokaryotic strain can be grown as a prototroph on minimal medium to increase fertility in genetic crosses (Griffiths and DeLange, 1978; Griffiths, 1982; Perkins, 1984).

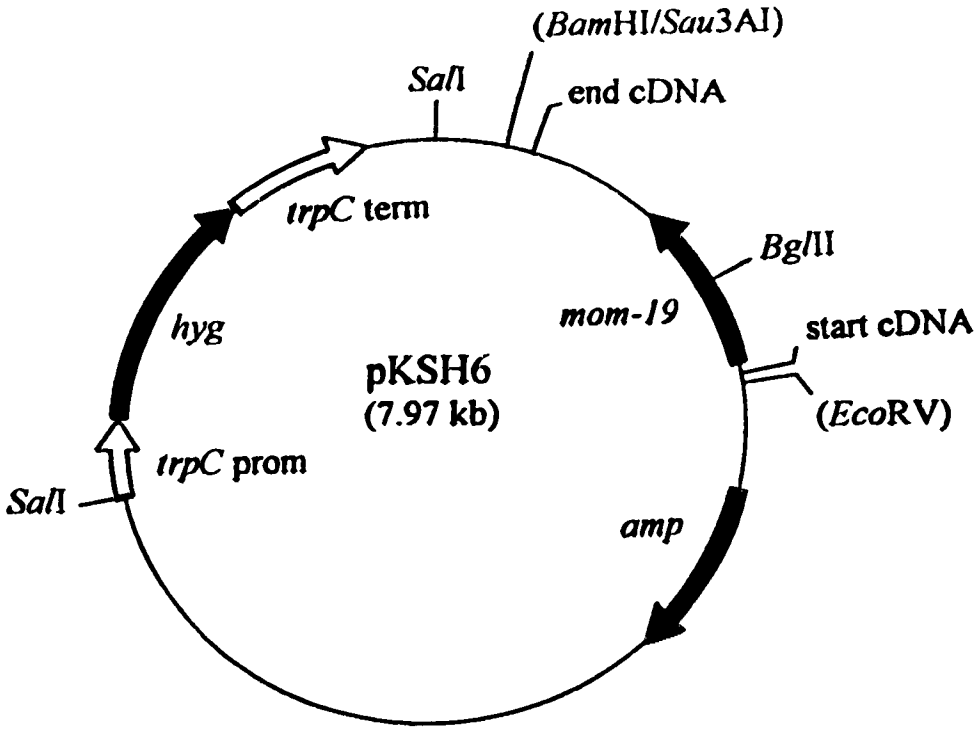


Figure 2·1. Plasmid pKSH6. Names of genes are indicated inside the circle. Filled arrows indicate the extent of coding sequences, open arrows indicate the promoter and terminator regions derived from *Aspergillus nidulans*. Restriction sites are indicated outside the circle. The sites in parentheses indicate the cloning sites and extent of the *mom-19*$^{+}$ genomic sequence in the plasmid. The positions that correspond to the start and endpoints of the *mom-19*$^{+}$ cDNA sequence are also indicated to demonstrate that the genomic DNA in the plasmid does not extend significantly past *mom-19*$^{+}$ sequence. *hyg*, hygromycin resistance gene; *amp*, ampicillin resistance gene.

supernatant was discarded and the mitochondrial pellet was washed once in grinding buffer. The final pellet was suspended in a small volume of grinding buffer and stored at -80°.

2·2·4 Analysis of genomic DNA for evidence of RIP

Restriction digests of genomic DNA with enzymes chosen to detect RIP were electrophoresed on 5% polyacrylamide gels (29:1, acrylamide:bisacrylamide) in 1X TAE buffer (40 mM Tris-acetate, pH 8.3; 1 mM EDTA) and electroblotted to nylon membranes using 0.5X TAE as the blotting buffer. The membrane was then placed on 3MM chromotography paper (Whatman) saturated with denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 10 min. Following denaturation, the membrane was neutralized by placing it on 3MM paper saturated with 3 M sodium acetate, pH 5.5 for 10 min. The membrane was then air dried and baked at 80° for one hour. Hybridization of the membrane to labelled probe was by standard techniques (Ausubel *et al.*, 1992) except that the hybridization and wash temperatures were reduced from 65° to 57°, since many of the restriction fragments were predicted to be small.

2·2·5 Other techniques

The standard techniques of agarose gel electrophoresis and Southern blotting of agarose gels, preparation of probes for DNA-DNA hybridizations, transformation of *E. coli*, isolation of bacterial plasmid DNA, and the polymerase chain reaction (PCR) using Vent polymerase (New England Biolabs, Beverly, MA) to minimize replication errors were performed as described in Ausubel *et al.* (1992). The following procedures were also performed using previously published procedures: separation of mitochondrial

proteins by polyacrylamide gel electrophoresis (Laemmli, 1970), western blotting (Good and Crosby, 1989), *N. crassa* DNA isolation (Schechtman, 1986), determination of mitochondrial protein concentration (Bradford, 1976), transformation of *N. crassa* spheroplasts (Schweizer *et al.*, 1981) with the modifications of Akins and Lambowitz (1985), and restriction fragment length polymorphism (RFLP) analysis of *N. crassa* genes (Metzenberg *et al.*, 1984; 1985). DNA sequences were obtained using Sequenase (United States Biochemical) according to the supplier's instructions. Database information with regard to the sequences obtained is given in the legend to Figure 2·5. Antibodies to various mitochondrial proteins were generously supplied by various individuals in the laboratory of W. Neupert. The *mom-19*$^{+}$ cosmid used in these studies was isolated from an *N. crassa* genomic library of strain NCN53 (su-1[*mi-3*]), described in Gessert *et al.* (1994), using previously isolated *mom-19*$^{+}$ cDNA as a probe. Cosmid 15.1.3.6 from this library was used in this study.

2.3 Results

2·3·1 Principles of "sheltered RIP"

It was suspected that the loss of MOM19 would have an extremely deleterious effect on the organism, and to overcome this problem, the procedure referred to as "sheltered RIP" was utilized to generate mutants of the *mom-19*$^{+}$ gene. The method utilizes the phenomenon of RIP (Selker, 1990), to destroy the target gene. When a DNA sequence is present in two copies in a single nucleus both copies are, with fairly high frequency, subjected to RIP in the ascogenous hyphae as the nucleus is prepared for karyogamy. Thus, any gene that has been cloned can be re-introduced into a strain by transformation to create a duplication in which both copies serve as targets for RIP. Most frequently the re-introduced gene integrates at an ectopic site (Asch and

Kinsey, 1990). The result of RIP is that both copies of the sequence in the transformed nucleus are effectively disrupted since they are altered by methylation and by GC to AT transitions. The single copy of the gene in the nucleus of the mating partner in the cross is unaffected by RIP. To allow the isolation of strains containing essential genes that have been RIPed during the cross, the technique employs a mutant allele of the *mei-2* gene in both partners of the cross. This mutation effectively eliminates meiotic recombination and also results in a high frequency of non-disjunction of chromosomes during meiosis (Smith, 1975). Thus, in crosses homozygous for *mei-2*, a variety of meiotic products are generated, many of them inviable because they are nullosomic for one or more chromosomes. However, a fraction of the meiotic products should be disomic for any specific chromosome. The desired products of a sheltered RIP cross will be disomic ascospores containing a RIP disruption of the target gene on one copy of a certain chromosome (derived from the transformation host) and a normal copy of the target gene on another copy of the same chromosome (derived from the mating partner of the cross). These disomic spores break down rapidly during vegetative growth into a heterokaryon composed of two different nuclei (Smith 1974). Even if the target gene is essential for viability, the normal copy of the gene in one component of the heterokaryon will complement, or shelter, the non-functional copy in the other component. Knowledge of the linkage group on which the target gene is located and appropriate genetic design of the strains used in the cross allows the direct selection of the desired dikaryons/heterokaryons from the progeny of the cross, as well as providing selectable markers that can be used to alter the ratio of the different nuclei in the ultimate heterokaryon (see below). Therefore, it is possible to produce a strain in which the nucleus containing the disrupted version of the gene should

be heavily favored numerically, though if the target gene provides an indispensible function it can never become the sole nuclear type. These skewed-ratio heterokaryons should allow any growth-limited phenotype to be studied. In principle, the technique should be useful for generating mutants of any cloned gene in *N. crassa* though it is particularly well-suited for genes that have an important or essential function (Metzenberg and Grotelueschen, 1992a). Below, the details of the technique specifically as applied to the *mom-19*$^{+}$ gene are described.

2·3·2 Transformants containing mom-19^{+} *duplications*

The principle of sheltered RIP, which employs various genetic markers on the chromosome carrying the target gene, requires that the chromosome or linkage group (LG) carrying the target gene locus be known. Therefore, *mom-19*$^{+}$ specific sequences were used as a probe to isolate *mom-19*$^{+}$ containing cosmids from a *N. crassa* library. One cosmid was then utilized as a probe to deduce the chromosomal location of the *mom-19*$^{+}$ locus in RFLP mapping studies (Fig. 2·2). A cosmid was used as a probe in this analysis in order to include a sufficient amount of genomic DNA to reveal RFLPs. The analysis revealed that *mom-19*$^{+}$ is located on LG IV near the *pyr-1* marker as shown in Metzenberg and Grotelueschen (1992b, 1993). Plasmid pKSH6, which carries the *mom-19*$^{+}$ gene (see Materials and Methods; Fig. 2·1), was used to transform the Host IV strain. This strain carries a number of relevant markers: the *pyr-1* and *trp-4*$^{+}$ alleles to be utilized in the selection of appropriate disomic ascospores following the eventual RIP cross, a recessive LG IV mutation imparting resistance to fpa or 5-methyltryptophan (*mtr*^{R}) which should allow shifts in nuclear proportions favoring the RIPed nucleus in the isolates ultimately obtained from the RIP cross, and a mutant allele of *mei-2* to generate

disomics in the RIP cross (Fig. 2·3, panel 1). The genetic make-up of appropriate transformants is shown in Fig. 2·3, panel 3.

Transformants were selected on media containing hygromycin. Sixty transformants were purified through two rounds of single colony isolation on hygromycin medium to insure that the transformants were homokaryotic. DNA was isolated from 35 of these transformants and examined for the presence of a single ectopically inserted copy of the *mom-19*$^+$ sequences derived from pKSH6, in addition to the resident copy of *mom-19*$^+$. This was tested by using a *mom-19*$^+$ specific sequence to probe Southern blots of genomic DNA isolated from the transformants and digested with either *Bgl*II or *Eco*RV plus *Sal*I. *Bgl*II has a recognition site within the coding region of *mom-19*$^+$ DNA and, in appropriate transformants containing a single ectopically integrated *mom-19*$^+$ sequence, should generate four bands when probed with *mom-19*$^+$ specific DNA: two bands from the ectopic copy and two from the resident copy. The size of the bands from the ectopic copy cannot be predicted and depends on the position of flanking *Bgl*II sites. Thus, appropriate strains should contain bands that correspond to those in the parental strain Host IV, and two additional bands. Both *Eco*RV and *Sal*I cut outside the *mom-19*$^+$ coding sequence and outside the *mom-19*$^+$ DNA present in pKSH6 (Fig. 2·1). Therefore, the double digest should generate two bands in appropriate transformants. One band should be 1.8 kb representing the ectopic sequence derived from integration of the *mom-19*$^+$ sequence in pKSH6. The other band represents the resident *mom-19*$^+$ sequence and should correspond to the band in the Host IV strain. Three strains that satisfied these criteria were identified from the analysis: TI26-3, TI28-3, and TI40-3 (Fig. 2·4). Each of these strains was used as the male parent in crosses to strain Mate IV, which carries the *mei-2* allele on LG V as well as nutritional markers on LG IV that are

	Ascospore Isolates:										matches to *mom-19*$^+$
CenI	MMOO	MMOO	MMOO	OOMM	OOMM	MMMM	MMOM	OOOO	OOMM	MM	29/34
pyr-1	MMMO	MMOO	MMOO	OOMM	OOMM	MMMM	MMMM	OOOO	MOMO	MM	32/34
mom-19$^+$	MMMO	MMO+	MM+O	OOMM	OOMO	MOMM	MMMM	OOOO	+O+O	MM	
Fsr-62	MOMO	MOOO	MMOO	OOMM	OMOO	MOMM	MMMM	MOOM	MOMO	MM	28/34

Figure **2.2.** RFLP mapping of *mom-19*$^+$ to linkage group (LG) IV. The *N. crassa* strains Mauriceville (M) and Oak-Ridge (O) were crossed to generate progeny. These two strains are known to contain many genetic polymorphisms which are manifested in restriction site differences in their genomes. Thirty-eight segregants were selected from ordered asci (Metzenberg *et al.*, 1984). DNA from these isolates was extracted and digested with *Sal*I. *Sal*I was previously found to be appropriate for detecting RFLPs with the *mom-19*$^+$ containing cosmid through a test gel of M and O DNA that was digested with various enzymes, blotted and hybridized to the *mom-19*$^+$ cosmid. The *Sal*I digested DNA from each test isolate was separated on 0.8% agarose and analyzed by Southern blotting using a radioactively labeled *mom-19*$^+$ containing cosmid as a probe. Strains were scored according to whether the RFLP was like the Mauriceville parent (M) or like the Oak-Ridge parent (O). The derived pattern was then compared to a list of compiled patterns from DNA fragments covering the entire genome. The greater the number of matches to a known gene, the closer the linkage. Four of the DNA samples for the *mom-19*$^+$ analysis were lost, therefore only 34 samples were available for comparison with the compiled RFLP patterns. By this criteria, genes, or fragments of DNA, can be localized with reasonable accuracy within the genome. The *CenIV*, *pyr-1* and *Fsr-62* markers are all known to be on LG IV (Metzenberg and Groteleuschen, 1993). +, indicates a DNA sample that was lost.

Figure **2·3**. Relevant genetic markers in appropriate initial transformants, the Mate IV strain, disomic progeny isolates, and subsequent heterokaryons. Only the genetic markers that are important for generation, selection, or manipulation of the strains are shown. These are indicated on specific linkage groups represented by horizontal lines. The position of *mom-19*$^{+}$ relative to the other markers on LGIV is shown as deduced from both the RFLP map (Metzenberg and Groteleuschen, 1992) and the genetic map of LGIV (Perkins, 1992). LGIV (H), linkage group IV derived from the original Host IV strain; LGIV (M), linkage group IV derived from the original Mate IV strain; LGV, linkage group V; any LG, any of the seven linkage groups found in *N. crassa*. Boxes with squared corners indicate nuclei, boxes with rounded corners indicate cells. **1**. The Host IV strain. **2**. The Mate IV strain. **3**. Desired initial transformants of the Host IV strain containing a single ectopic copy of *mom-19*$^{+}$. **4**. The two possible versions of disomic spores that would grow on media lacking both uridine and tryptophan. Each must contain one LG IV from the original host strain and one LG IV from the original mate strain to allow complementation of the auxotrophic *pyr-1* and *trp-4* markers. One type contains one unaltered copy and one RIPed copy (**4a**); the other contains two unaltered copies of *mom-19*$^{+}$ (**4b**). In both cases the ectopic copy of *mom-19*$^{+}$ may or may not be present depending on the meiotic segregation of the chromosome on which it was integrated. **5**. Each of the possible disomics breaks down into a heterokaryon in which one nucleus contains LG IV from the original host and the other LG IV from the original mate. The heterokaryon in **5a** contains the RIPed version of *mom-19*$^{+}$ in one nucleus and the wild-type version of the gene in the other, whereas the nuclei in the heterokaryon in **5b** both contain wild-type copies of *mom-19*$^{+}$.

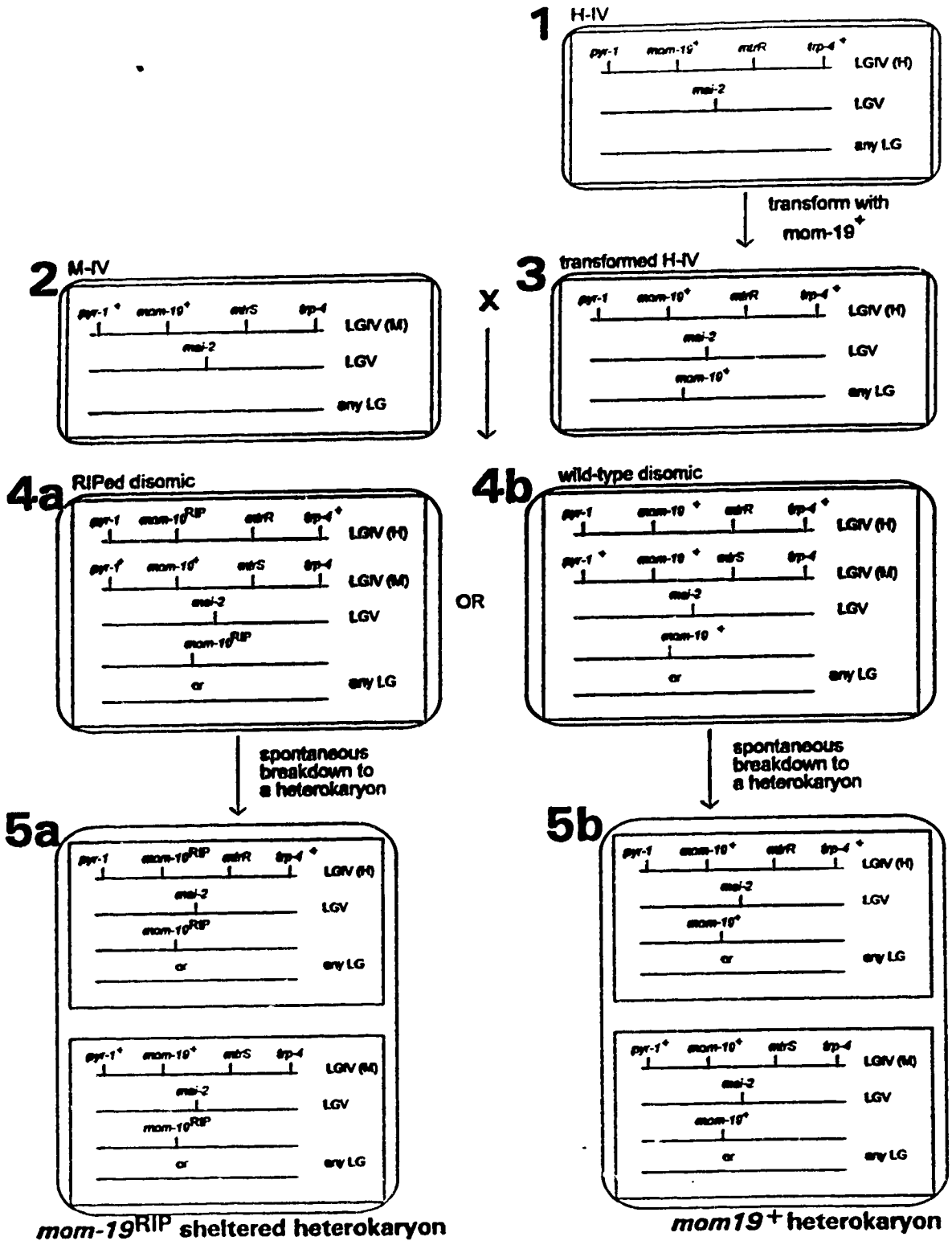
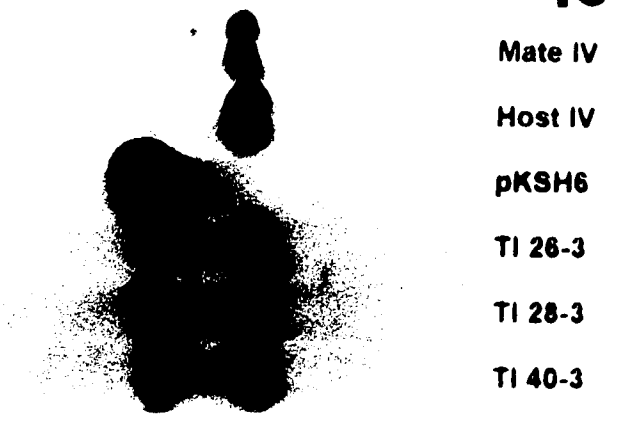

1
H-IV
pyr-1
mom-19+
mtrR
trp-4+
LGIV (H)
mei-2
LGV
any LG
transform with
mom-19+
2
M-IV
pyr-1+
mom-19+
mtrS
trp-4
LGIV (M)
mei-2
LGV
any LG
X
3
transformed H-IV
pyr-1
mom-19+
mtrR
trp-4+
LGIV (H)
mei-2
LGV
mom-19+
any LG
4a
RIPed disomic
pyr-1
mom-19RIP
mtrR
trp-4+
LGIV (H)
pyr-1+
mom-19+
mtrS
trp-4
LGIV (M)
mei-2
LGV
mom-19RIP
or
any LG
OR
4b
wild-type disomic
pyr-1
mom-19+
mtrR
trp-4+
LGIV (H)
pyr-1+
mom-19+
mtrS
trp-4
LGIV (M)
mei-2
LGV
mom-19+
or
any LG
spontaneous
breakdown to
a heterokaryon
spontaneous
breakdown to
a heterokaryon
5a
pyr-1
mom-19RIP
mtrR
trp-4+
LGIV (H)
mei-2
LGV
mom-19RIP
or
any LG
pyr-1+
mom-19+
mtrS
trp-4
LGIV (M)
mei-2
LGV
mom-19RIP
or
any LG
mom-19RIP sheltered heterokaryon
5b
pyr-1
mom-19+
mtrR
trp-4+
LGIV (H)
mei-2
LGV
mom-19+
or
any LG
pyr-1+
mom-19+
mtrS
trp-4
LGIV (M)
mei-2
LGV
mom-19+
or
any LG
mom19+ heterokaryon

Figure **2·4**. Southern analysis of restriction digests showing single inserted *mom-19*$^{+}$ sequence in strains TI26-3, TI28-3, TI40-3. Panel **1**: *Bgl*II digest of total cellular DNA from the indicated strains. Panel **2**: *Eco*RV plus *Sal*I digest of total cellular DNA from the indicated strains. Also shown is the 1.8 kb marker generated from a digest of plasmid pKSH6. The probe was a radioactively labeled *mom-19*$^{+}$ cDNA fragment that extends from nucleotides 245 to 1704 as shown in Fig. **2·6**, except that the intron sequences shown in the Figure are absent in the cDNA.


1
Mate IV
Host IV
TI 26-3
TI 28-3
TI 40-3


2
Mate IV
Host IV
pKSH6
TI 26-3
TI 28-3
TI 40-3

complementary to those on LG IV of the transformed strains (Fig. 2·3, panel 2). In the present study the analysis of the progeny from the cross involving TI28-3 is described.

2·3·3 Generation and characterization of mom-19$^+$ RIP mutants

Crosses between the Mate IV strain and transformant TI28-3 are homozygous for the *mei-2* allele so that during meiosis non-disjunction of chromosomes occurs and meiotic recombination is inhibited. Therefore, a fraction of the ascospores obtained from the cross should be disomics containing one LG IV derived from the Mate IV strain and one derived from the Host IV transformed strain (Fig. 2·3, panels 4a and 4b). Such ascospores can be directly selected because they are the only ones produced from the cross that are capable of growth on medium that lacks both uridine (*pyr-1* marker) and tryptophan (*trp-4* marker). When the cross is carried out a certain percentage of the nuclei carrying the duplication of *mom-19*$^+$ should undergo RIP of those sequences. Therefore, two types of disomics should emerge from the selection. The first type would contain one LG IV derived from the Host IV transformed strain with a RIPed version of the gene and one LG IV derived from the Mate IV strain carrying a wild-type *mom-19*$^+$ allele (Fig. 2·3, panel 4a). The second type would contain two wild-type alleles of *mom-19*$^+$. (Fig. 2·3, panel·4b). The spores of the first type are the desired isolates and their frequency will be related to the frequency of RIP of the *mom-19*$^+$ duplication in the original transformed host. Depending on the site of integration of the transformed ectopic *mom-19*$^+$ gene and the pattern of segregation of chromosomes during meiosis, the spores may or may not contain the ectopic integrated copy of *mom-19*$^+$ as well ("any LG", Fig. 2·3). If the ectopic copy is present in the spores that contain resident *mom-19* sequences that have bee-

subjected to RIP, the ectopic sequence should also have undergone RIP since both copies of a duplication are affected by the process (Selker, 1990).

Ascospores produced from the Mate IV x TI28-3 cross were plated on basal medium (see Materials and Methods), which lacks uridine and tryptophan. A total of 50 colonies that formed on this medium were picked to culture tubes containing basal medium and allowed to conidiate. Each strain was then taken through two rounds of purification by streaking to single colony isolates on basal medium to provide sufficient opportunity for breakdown of disomic nuclei (Smith, 1974) and to maintain the heterokaryons formed during the process of breakdown (Fig. 2·3, panels 5a and 5b). The isolates were then examined to determine which, if any, carried RIPs of *mom-19* in the *pyr-1 trp-4*$^{+}$ *mtrR* component of the heterokaryon. We tested for such strains by examining the growth properties of each isolate on medium that is both selective and permissive for the RIPed nucleus, that is, containing uridine and fpa but not tryptophan. The heterokaryotic strains isolated should all carry the recessive resistance marker *mtrR* and should therefore be capable of growth on this medium by spontaneous resolution to give *mtrR* homokaryons. However, if the nucleus carrying *mtrR* contains a RIPed copy of *mom-19*, and *mom-19*$^{+}$ is required to maintain a normal rate of growth, then such strains should not give rise to vigourously growing *mtrR* homokaryons. In such cases, the heterokaryons should exhibit a reduced growth rate at some critical concentration of the inhibitor, since the nucleus carrying the RIPed *mom-19* allele is forced to predominate in the heterokaryon. In Table 2·2 the results obtained with five candidate strains that exhibited altered growth patterns on the inhibitor relative to control strains are shown. The values shown in Table 2·2 that indicate slower growth rate, by rate of mycelial advance, may even

Table 2·2 Growth of heterokaryons on medium containing *p*-flurophenylalanine

Strain	Hours required to cover surface of slant[a] containing *p*-fluorophenylalanine at indicated concentration							
	0 µM	100 µM	200 µM	300 µM	400 µM	500 µM	600 µM	700 µM
TI28-3	24	24	24	24	24	24	24	48
Host IV	24	24	24	24	24	24	24	48
Mate IV	24-48	>96	no gr[b]	no gr	no gr	no gr	no gr	no gr
28.1	24-48	24-48	72	96	96	96	96	96
28.16	24-48	24-48	48	lost	48	48	48	48
28.17	24-48	24-48	72	72	72	72	72	72
28.18	24-48	24-48	>96	>96	>96	>96	>96	>96
28.23	24-48	24-48	48	48	48	48	48	48

[a] Slants were prepared in 16 x 150 mm tubes. Average length of the slant was about 80 mm. Conidia were inoculated at a single point in the center of the slant.

[b] no gr, no growth after 96 hr

underestimate the growth difference compared to wild-type since the density of mycelia for the slow growing strains is also reduced compared to wild-type.

As a test for RIP of *mom-19* that was not influenced by any prediction with regard to the functional importance of the gene, we examined the *mom-19* DNA in the strains discussed in Table 2·2 for evidence of restriction site alterations that should be produced by the RIP process. DNA was isolated from cultures of each of these strains, digested with restriction enzymes, and compared to digests of DNA from the parent strains used in the RIP cross by Southern analysis using a *mom-19*$^{+}$ specific sequence as the probe. It could be determined if alterations were simply due to methylation or to actual base pair changes by using the enzyme combinations described below. The enzymes *Sau*3AI and *Mbo*I both cut the sequence GATC. However, *Sau*3AI will not cut if the C is methylated and *Mbo*I will not cut if the A is methylated. Virtually all methylation in *N. crassa* is at cytosine residues (Selker, 1990) so that digestion with *Mbo*I should not be affected by methylation. *Hpa*II and *Msp*I both cut the sequence CCGG. *Hpa*II will not cut if either C is methylated. *Msp*I will not cut if the outer C is methylated. The enzymes were used in the combinations (*Sau*3AI plus *Hpa*II) and (*Mbo*I plus *Msp*I) to generate many restriction fragments thereby maximizing the chances of detecting alterations due to methylation or restriction site alterations. As shown in Fig. 2·5, panel 1, it is apparent that strains 28.16 and 28.17 have alterations with respect to control DNAs. For strain 28.16, the alterations may be explained by differences in methylation alone since there are no apparent differences from controls in the *Msp*I plus *Mbo*I digest. However, for 28.17, the alterations are likely the result of both methylation and mutation. For both strains we have shown that the alterations are specific for *mom-19*$^{+}$ DNA by stripping the blots and hybridizing with a different probe (Fig. 2·5, panel 2). In this case, no differences

Figure **2·5**. Southern analysis of restriction digests showing RIP in strains 28.16 and 28.17. DNA isolated from the indicated strains was digested with the restriction enzymes indicated. (H+S) = *Hpa*II+*Sau*3AI; (M+M) = *Msp*I+*Mbo*II. In panel **1**, the probe was a radioactively labeled *mom-19*$^{+}$ cDNA fragment as described in the legend to Fig. **2·4**. In panel **2**, the probe was radioactively labeled DNA derived from *nuo-78*$^{+}$ cDNA, used to show that the differences seen in panel 1 are specific to *mom-19*$^{+}$ sequence.

1
Mate IV (H+S)
28.16 (M+M)
28.16 (H+S)
28.17 (M+M)
28.17 (H+S)
TI 28-3 (H+S)
2
Mate IV (H+S)
28.16 (M+M)
28.16 (H+S)
28.17 (M+M)
28.17 (H+S)
TI 28-3 (H+S)

in banding patterns were seen in any strain. Although no alterations in *mom-19* were apparent in strains 28.1, 28.18, and 28.23 (data not shown), it is conceivable that changes have occurred that are not detected by the restriction enzymes used in the analysis.

2·3·4 DNA sequence analysis of mom-19^{RIP} alleles

To fully characterize the extent of RIP that had occurred in strains 28.16 and 28.17, *mom-19* DNA was amplified from these strains by PCR of DNA isolated from cultures grown under conditions that force the maintenance of the heterokaryon (basal medium). Since it had been demonstrated by Southern analysis that both strains carried the ectopic, or plasmid derived copy of *mom-19* in addition to the resident copies on the two LGs IV (Fig. 2·6, panel 1), two sets of PCR primers were used in these experiments. One set was designed to amplify the introduced copy of *mom-19* derived from plasmid pKSH6 and the other set was designed to amplify the resident genomic *mom-19* sequence (Fig. 2·6, panel 2). The characteristics of the primers used for PCR are listed in Table 2·3. The products of each reaction were cloned into bacterial vectors and their DNA sequences were determined. The strategy followed for sequencing the two copies of *mom-19* is shown in Fig. 2·6, panel 2. The sequence and location of the primers used are shown in Table 2·3. As expected, the products of the PCR reaction using the primers for the resident sequence gave rise to two types of clones. One type was of the wild-type *mom-19^{+}* sequence derived from LG IV of the Mate IV strain. The other type gave DNA sequence with alterations from the wild-type sequence. The latter type represents the RIPed version of the gene present on the LG IV originally derived from the transformed Host IV strain. For strain 28.17 a total of 44 changes from the wild-type sequence were observed in the 1134 base pairs of duplicated sequence

examined (Fig. 2·7, panel 1). All these alterations were of the type that characterize the RIP process, GC bp to AT bp transitions. The strand bias of mutation that is typical of RIP is also evident (Cambareri *et al.*, 1989). The mutations are predicted to result in a total of 18 amino acid substitutions in the MOM19 protein (Fig. 2·7, panel 1). Most of the amino acid changes are conservative but one, at nucleotide position 671, results in a Glu to Lys substitution. The ectopically integrated copy of *mom-19* in strain 28.17 has not been sequenced entirely but from 870 bp completed thus far, 32 transitions that would result in 12 amino acid substitutions have been found (Fig. 2·7, panel 2). It is extremely doubtful that any functional MOM19 is produced from this ectopic *mom-19* gene since the 5' splice junction of the third intron is also affected by one of the alterations. The sequence of the RIPed alleles from strain 28.16 has not been completed, but preliminary analysis indicates several transitions characteristic of RIP in this strain as well. The resultant amino acid changes determined thus far for strain 28.16 are shown in Fig. 2·7, panel 2.

2·3·5 MOM19 protein in mutant strains

To investigate further the status of the *mom-19* gene in the strains that showed evidence of RIP on Southern blots, strains 28.16 and 28.17 were examined for the presence of the MOM19 protein following growth as either heterokaryons without fpa or in the presence of uridine and 600 μM fpa. The latter conditions require that the nucleus containing the RIPed *mom-19* alleles be in a large majority in the cultures. Under such conditions the growth rate of both strains is reduced relative to controls as shown in Table 2·2. Mitochondria were isolated from these cultures, the proteins were extracted with detergent, and subjected to polyacrylamide gel electrophoresis. The separated proteins

Figure **2·6.** Southern analysis and PCR/sequencing strategy for the *mom-19* ectopic and endogenous copies. **1.** Southern analysis of restriction digests from putative RIP isolates showing the presence of the ectopic copy in strains 28.16, 28.17 and others. DNA isolated from the indicated strains was digested with the restriction enzymes *Eco*RV and *Sal*I, which cut immediately outside the *mom-19*$^+$ gene in the ectopic copy derived from pKSH6 (Fig. **2·1**) and at flanking sites for the resident copy. This results in one band for each copy of *mom-19*$^+$. The probe, as described in Figure **2·4**, was radioactively labeled *mom-19*$^+$ cDNA. The strains in panel **1a** represent the strains exhibiting a slow growth phenotype in the presence of fluorophenylalanine (fpa). The strains in panel **1b** represent a sample of the remaining strains that do not show a response to fpa. The latter were not examined further. **2.** Two sets of PCR primers were designed to amplify the two different copies of *mom-19* from the strains of interest. Primers specific for the 5' region of the endogenous copy (THA1) or the vector sequence 5' of the ectopic copy (THA7) were used in PCR reactions with a primer specific for the 3' region of both copies (THA8) to generate PCR products representing the resident and ectopic copies. *Eco*RI sites were added to the PCR primers specific for the 5' end of *mom-19*$^+$ (THA1 and THA7) and a *Bam*HI site was added to the primer specific for the 3' end (THA8) in order to facilitate cloning. The PCR products were digested with *Eco*RI and *Bam*HI and cloned into the bacterial vector pBlueScript. To reduce the number of primers for sequencing, these constructs were then digested with *Bgl*II and *Bam*HI to divide the gene into two parts and subcloned again into pBlueScript. The primers T3 and T7, specific for vector sequences in pBlueScript, were used to generate sequence from the internal *Bgl*II site. The orientation of the subcloned *Bam*HI/*Bgl*II fragment relative to the T3 and T7 primer sites on the vector cannot be predicted. The remaining sequence was generated as indicated using *mom-19*$^+$ specific primers. The sequences of the primers used are shown in Table **2.3.** ▪▪▪▪▪▪▪, *mom-19*$^+$ open reading frame; ——, 5' and 3' untranslated region; ▪▪▪▪▪, vector sequence surrounding ectopically integrated copy; ▬▬, cloning vector.

1a
TI28.3
MIV
28.1
28.11
28.16
28.17
28.18
28.23
28.26
1b
TI28.3
MIV
28.9
28.15
47.1
47.6
47.8
47.9
47.14
47.15
40.3
45.1
45.4
53.3
26.158

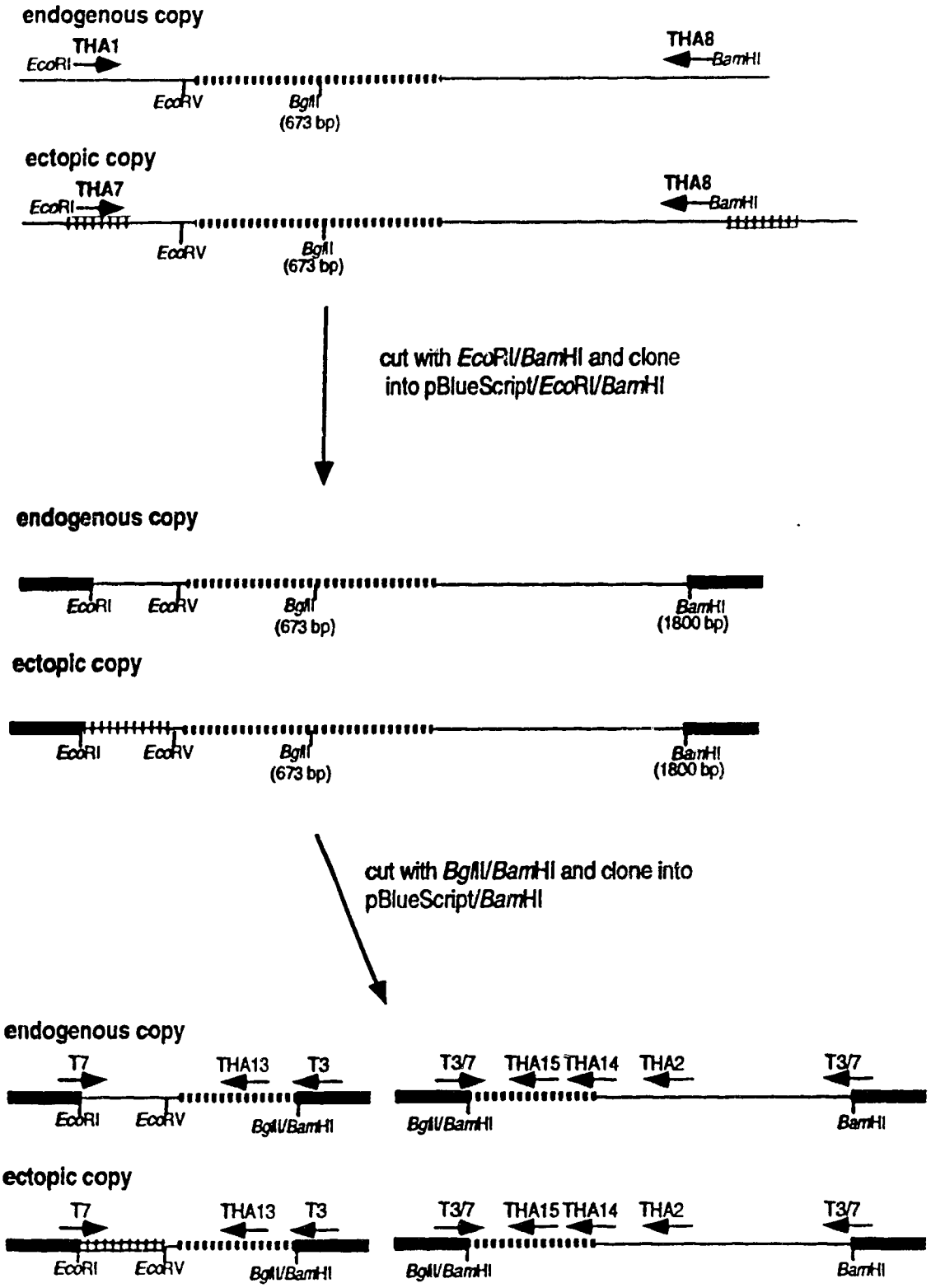

endogenous copy
THA1
EcoRI
THA8
BamHI
EcoRV
BglII
(673 bp)
ectopic copy
THA7
EcoRI
THA8
BamHI
EcoRV
BglII
(673 bp)
cut with EcoRI/BamHI and clone into pBlueScript/EcoRI/BamHI
endogenous copy
EcoRI
EcoRV
BglII
(673 bp)
BamHI
(1800 bp)
ectopic copy
EcoRI
EcoRV
BglII
(673 bp)
BamHI
(1800 bp)
cut with BglII/BamHI and clone into pBlueScript/BamHI
endogenous copy
T7
THA13
T3
T3/7
THA15
THA14
THA2
T3/7
EcoRI
EcoRV
BglII/BamHI
BglII/BamHI
BamHI
ectopic copy
T7
THA13
T3
T3/7
THA15
THA14
THA2
T3/7
EcoRI
EcoRV
BglII/BamHI
BglII/BamHI
BamHI

Table 2.3 Primers used for PCR and sequencing of mom-19 alleles

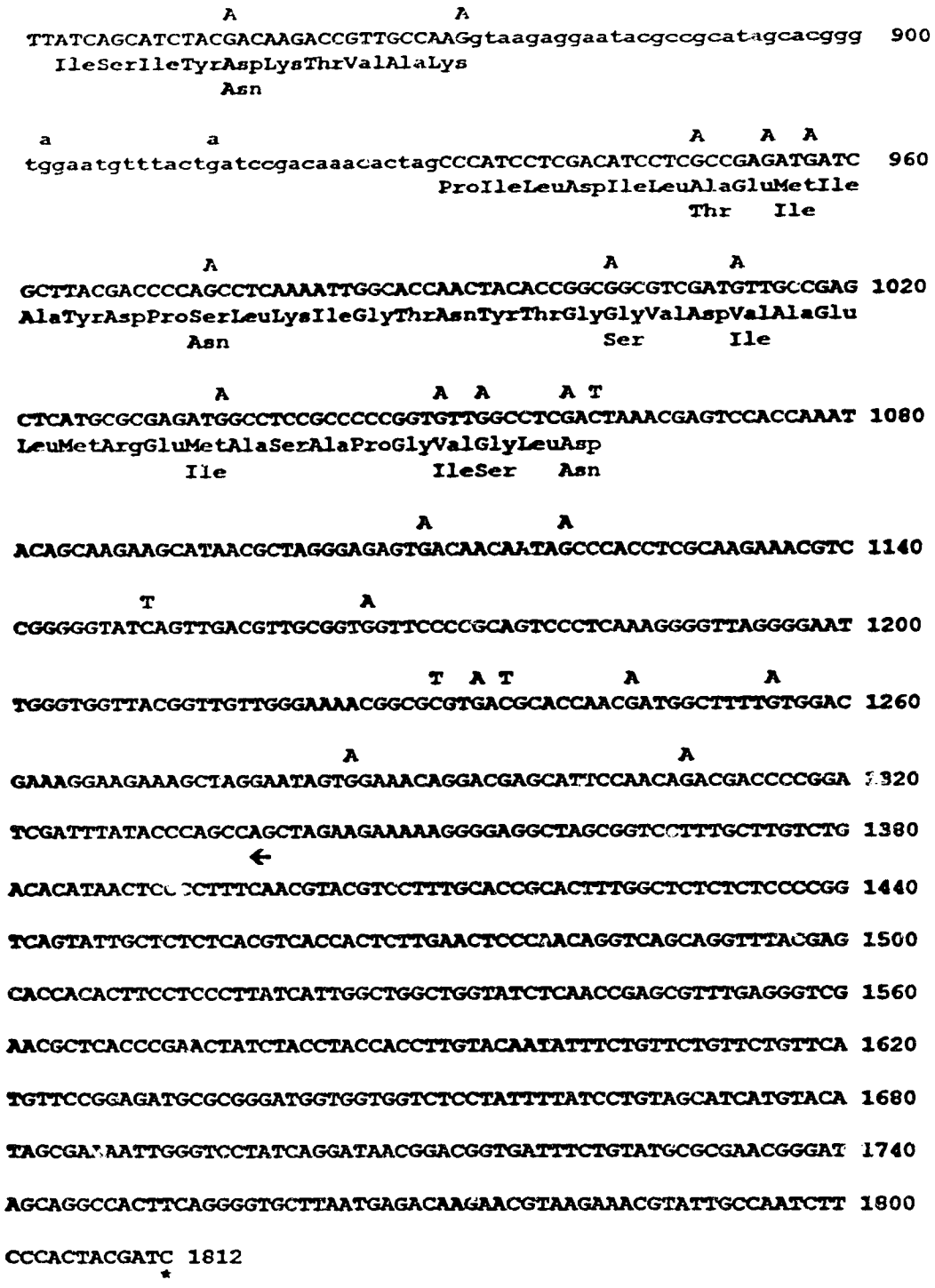
Primer	Use	Sequence (5' to 3')
THA 1	PCR	AAGAATTCTACGGGTACGATAATTACGG EcoRI 22 41
THA 2	PCR	AAGAATTCGCCTCCCCTTTTTCTTCTAG EcoRI 1330 1349
THA 7	PCR	AAGAATTCTAGGCATAGGCTTGGTTATG EcoRI vector sequence in ect. copy
THA 8	PCR	AAGGATCCGATCGTAGTGGGAAGATTGG BamHI flanking 3' end of gene
THA 13	sequencing	CTCGGCGTATTCTTTCT 551 535
THA 14	sequencing	AATTCCCCTAACCCCTT 1191 1175
THA 15	sequencing	GACTCGTTTAGTCGAGG 1072 1056

The numbers below each sequence correspond to the numbering system in Fig. 2-7, panel 1 and indicate the location and orientation of the primer with respect to the *mom-19* gene. The underlined sequence indicates the added restriction site. Two A residues were included prior to the restriction site in order to facilitate digestion with the desired enzymes.

Figure **2·7**. DNA sequence of the resident RIPed allele in strain 28.17 and summary of the resulting amino acid changes in the resident and ectopic copies from 28.16 and 28.17. **1**. Genomic DNA sequence of *mom-19*$^{+}$. Asterisks indicate the extent of the duplication (*ie.*, the *mom-19*$_{+}$ region cloned in pKSH6; Fig. **2·1**), which should serve as a target for RIP. The *Eco*RV and *Sau*3AI sites that define the extent of the cloned *mom-19*$^{+}$ region in pKSH6 (see Fig. **2·1**) are underlined. The two small arrows below the sequence delineate the region that has been sequenced. Lower case letters indicate intron sequences. The complete sequence of the wild-type gene is shown. Bases above the wild-type sequence indicate the positions where mutations were found in the RIPed version of the resident gene found on the original host LG IV in strain 28.17. The amino acid sequence of the protein encoded by the wild-type gene is indicated immediately below the sequence. Amino acid alterations resulting from RIP mutations in the endogenous RIPed copy of 28.17 are shown under the normal protein sequence. The sequence of the wild-type allele, as shown in this Figure, revealed minor errors in the sequence previously entered in Genbank (accession number M80528). These changes have been reported to Genbank and are entered under the previous accession number as is the sequence of the RIP allele shown in this Figure. **2**. Amino acid sequence of MOM19 protein deduced from the DNA sequence and changes identified in various RIP alleles. The extent of sequencing for each allele is indicated by dashed lines. The amino acid changes resulting from RIP in each allele are shown below the wild-type sequence.

1

```
AAAAACACAGTTTGTGTAGTGTACGGGTACGATAATTACGGTATGTGCAATGTTTTGACC    60
                                                  →
TGCAAGCTTACCTCTCCTCTAACCATCCAATCTTTGAGAGTGCTGTTGATGTCCATTGCT   120

TGTGAACCTTCCCGTGGAGCCAGTGTCATATACTGACCCCCACTTGCAGCAGTCTTTCCG   180

GTAGTACTAAGAAATCGAGAGGCTCGATATCCATCGCATCTTTCCCTACCATTATAATTT   240
                         *
                    A
CTCTCTCTCGACTCTCCCCCGCATCCACCATCACCACACAACGAGCCAACAAGATGCCGT   300
                                                   MetProSer

                                  A
CGCAAGCCGTCACCTACACCACCGCTGCCGTTGCGGCCGTCGCCACGGGTTTCCTCGgtt   360
  GlnAlaValThrTyrThrThrAlaAlaValAlaAlaValAlaThrGlyPheLeu
                                  Thr

                                                          a
agtttgcacttggatctcaacgtcaccactttctcgagatgtaaccattagtcgctgatg   420

    a                             A        A
ccttgcgctcccctaccttccagCCTACGCCGTCTACTTCGACTACAAGAGGCGCAATGA   480
                          AlaTyrAlaValTyrPheAspTyrLysArgArgAsnAsp
                                  Ile      Asn

                              A
CCCCGAGTTCAGGAGACAACTCAGGCGCAGCGCCAGGCGACAGGCTCGTCAGGAGAAAGA   540
 ProGluPheArgArgGlnLeuArgArgSerAlaArgArgGlnAlaArgGlnGluLysGlu
                           His

ATACGCCGAGTTGAGCCAGCAGGCCCAGAGACAACGAATCAGGCAAATGGTTGATGAGGC   600
 TyrAlaGluLeuSerGlnGlnAlaGlnArgGlnArgIleArgGlnMetValAspGluAla

     A                            A
CAAGGAGGAGGGCTTCCCTACCACTTCGGACGAGAAGGAGGCCTACTTCCTCGAGCAGGT   660
 LysGluGluGlyPheProThrThrSerAspGluLysGluAlaTyrPheLeuGluGlnVal

      A  A       A                a    a
CCAGGCTGGTGAGATCTTGGGCCAGGATCgtaagtgttttgccaaccaagcgagaagaag   720
 GlnAlaGlyGluIleLeuGlyGlnAspPro
       SerLys

                    a
gaacggaacttccgggacatgtgtcggctactaacactccattcgtaacagCCACCAAGG   780
                                                   ThrLysAla

       A        A                    A
CCATCGATGCCTCCCTCGCTTTCTACAAGGCCCTCAAGGTCTACCCTACGCCCGGCGACC   840
  IleAspAlaSerLeuAlaPheTyrLysAlaLeuLysValTyrProThrProGlyAspLeu
       Thr      Thr
```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IleSerIleTyrAspLysThrValAlaLys
Asn
tggaatgtttactgatccgacaaacactagCCCATCCTCGACATCCTCGCCGAGATGATC 960
ProIleLeuAspIleLeuAlaGluMetIle
Thr Ile
GCTTACGACCCCAGCCTCAAAATTGGCACCAACTACACCGGCGGCGTCGATGTTGCCGAG 1020
AlaTyrAspProSerLeuLysIleGlyThrAsnTyrThrGlyGlyValAspValAlaGlu
Asn Ser Ile
CTCATGCGCGAGATGGCCTCCGCCCCCGGTGTTGGCCTCGACTAAACGAGTCCACCAAAT 1080
LeuMetArgGluMetAlaSerAlaProGlyValGlyLeuAsp
Ile IleSer Asn
ACAGCAAGAAGCATAACGCTAGGGAGAGTGACAACAATAGCCCACCTCGCAAGAAACGTC 1140
CGGGGGTATCAGTTGACGTTGCGGTGGTTCCCCGCAGTCCCTCAAAGGGGTTAGGGGAAT 1200
TGGGTGGTTACGGTTGTTGGGAAAACGGCGCGTGACGCACCAACGATGGCTTTTGTGGAC 1260
GAAAGGAAGAAAGCTAGGAATAGTGGAAACAGGACGAGCATTCCAACAGACGACCCCGGA 1320
TCGATTTATACCCAGCCAGCTAGAAGAAAAAGGGGAGGCTAGCGGTCCTTTGCTTGTCTG 1380
ACACATAACTCCCTTTCAACGTACGTCCTTTGCACCGCACTTTGGCTCTCTCTCCCCGG 1440
TCAGTATTGCTCTCTCACGTCACCACTCTTGAACTCCCAACAGGTCAGCAGGTTTACGAG 1500
CACCACACTTCCTCCCTTATCATTGGCTGGCTGGTATCTCAACCGAGCGTTTGAGGGTCG 1560
AACGCTCACCCGAACTATCTACCTACCACCTTGTACAATATTTCTGTTCTGTTCTGTTCA 1620
TGTTCCGGAGATGCGCGGGATGGTGGTGGTCTCCTATTTTATCCTGTAGCATCATGTACA 1680
TAGCGAAAATTGGGTCCTATCAGGATAACGGACGGTGATTTCTGTATGCGCGAACGGGAT 1740
AGCAGGCCACTTCAGGGGTGCTTAATGAGACAAGAACGTAAGAAACGTATTGCCAATCTT 1800
CCCACTACGATC 1812

2

```
wt  MOM19            MPSQA VTYTT AAVAA VATGF LAYAV YFDYK RRNDP EFRRQ LRRSA RRQAR      50
end  MOM19-28.17     ----- ----- ---T- ----- ----I --N-- ----- ----- --H-- -----
ect  MOM19-28.17     ----- ----- -T--- ----- ---T- ----- K--N- ----- ----- --
end  MOM19-28.16     ----- ----- ----- ----- ----- ----- ----- ----- ----- ---
ect  MOM19-28.16     ----- ----- ----- ----- ----- ----- ----- ----- ----- -----

                     QEKEY AELSQ QAQRQ RIRQM VDEAK EEGFP TTSDE KEAYF LEQVQ AGEIL      100
                     ----- ----- ----- ----- ----- ----- ----- ----- ----- -SK--
                                                                          --
                                                                          --
                     ----- ----- ----- ----- ----- ----- ----- ----- ----- ---

                     GQDPT KAIDA SLAFY KALKV YPTPG DLISI YDKTV AKPIL DILAE MIAYD      150
                     ----- ----T --T-- ----- ----- ----- -N--- ----- ---T- I----
                     ---*- ----T --T-- ----- ----- ---N- -N--- ----- ----- I----
                     ----- ----- ----- ----- ----- ----- ----- ----- ----- I----


                     PSLKI GTNYT GGVDV AELMR EMASA PGVGL D                          181
                     -N--- ----- -S--I ----- -I--- --IS- N
                     ----- S---- ----I ----- ----- --I-- -
                     ----- S---- S---I ----- ----- ----- -
```

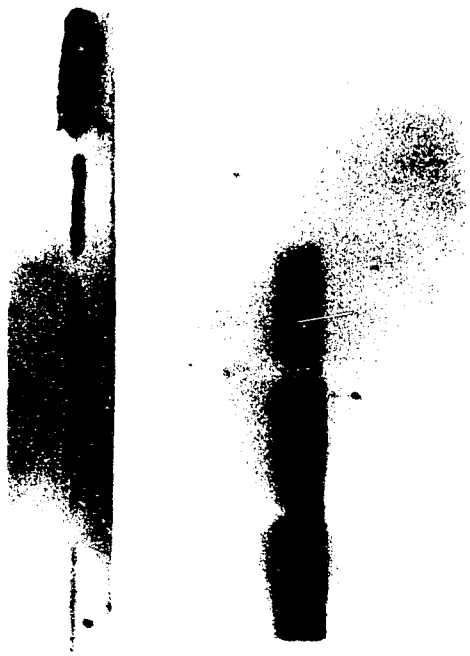
* altered splice junction between DP

were transferred to nitrocellulose membrane and probed with antibodies raised against the MOM19 protein. As shown in Fig. 2-8, panel 1, the MOM19 protein was undetectable in strain 28.17 when grown in the presence of fpa and uridine. When up to 200 µg of mitochondrial protein was loaded on the gels, MOM19 in mitochondria from these cultures remained undetectable (Fig. 2-8, panel 2). Inspection of the blots in Fig. 2-8 suggests that the amount of the protein is also reduced in 28.17 cultures grown as heterokaryons without fpa (Fig. 2-8, panels 1 and 2). The simplest explanation for this observation is that the heterokaryotic cells contain both RIPed and wild-type alleles of *mom-19* resulting in an overall reduction in MOM19 content.

2.3.6 Rescue of mom-19^{RIP} mutant

The ability to obtain evidence that nuclei containing the RIPed *mom-19* allele could be rescued by transformation with a wild-type copy of *mom-19*$^{+}$ was desired to verify that the effects observed were specific to *mom-19*$^{+}$. Both genomic and cDNA versions of *mom-19*$^{+}$ were used to transform mutant 28.17. Successful transformation was qualitatively assessed by the appearance of rapidly growing colonies on plates containing basal medium plus uridine and 600 mM fpa. By this criterion, both genomic and cDNA clones of *mom-19*$^{+}$ were capable of restoring rapid growth rate when transformed into spheroplasts of strain 28.17. In addition, three primary transformants were purified through one round of single colony isolation on basal medium containing uridine plus 600 µM fpa and examined for the presence of MOM19 protein in their mitochondria. Strain 17-43.1 was derived from transformation of strain 28.17 with a cosmid containing *mom-19*$^{+}$ and an additional 35 kb of *N. crassa* genomic sequence. Strain 17-67.1 was derived by transformation with a plasmid containing *mom-19*$^{+}$ and an additional 4 kb of genomic sequence.

Figure **2·8**. Western blot analysis of RIP strains. **1**. Forty μg of protein from isolated mitochondrial was loaded per lane. Cells were grown in the indicated medium: min, minimal medium; urid, basal medium plus uridine; het, basal medium; fpa+urid, basal medium containing 600 μM fpa plus uridine. Following electrophoresis, the proteins were electroblotted to nitrocellulose membrane and decorated with polyclonal antiserum to MOM19. **2**. Leftmost lane, 200 μg of protein; other lanes, 40 μg of protein. As a control, MOM38 was used to confirm the different amounts of protein loaded.



2

28.17 (inh+urid)

28.17 (inh+urid)

28.17 (het)

Mate IV (min)

TI28-3 (inh+urid)

1

Mate IV (min)

TI28-3 (urid)

28.16 (het)

28.17 (het)

TI28-3 (inh+urid)

28.16 (inh+urid)

28.17 (inh+urid)

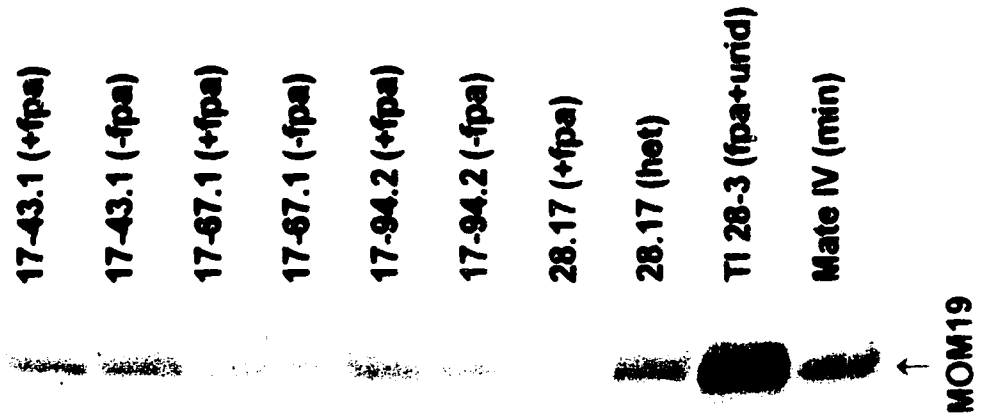
Strain 17-94.2 was obtained by transformation with a plasmid containing *mom-19*$^{+}$ cDNA. All three strains were found to be incapable of growth on basal medium or basal medium containing tryptophan. However, they were able to grow on basal medium containing uridine or uridine and fpa Thus, these strains are homokaryons containing the Host IV version of LG IV and do not contain the sheltering copy of *mom-19*$^{+}$ from the Mate LG IV. As shown in Fig. 2·9, panel 1, the mitochondria of all three transformed strains contain the MOM19 protein. The growth rate of these transformed strains was measured in "race tubes" (Davis and de Serres, 1970) and compared to control strains. There was little difference between the transformants and control strains when grown on medium that does or does not contain fpa (Fig. 2·9, panels 2 and 3, respectively). The ability of cDNA clones to rescue the mutant phenotype is significant since it proves that extension of RIP from duplicated *mom-19* sequence into neighbouring genes (Foss *et al.*, 1991) is not responsible for the phenotypes observed.

2.3.7 Homokaryons containing the mom-19^{RIP} allele

The finding that the MOM19 protein was absent from 28.17 cultures grown in the presence of uridine and fpa suggested that the protein might not be essential for growth. To test this hypothesis, it was reasoned that if *mom-19*$^{+}$ is an essential gene and strain 28.17 contains a null allele of the gene in the *pyr-1*, *trp-4*$^{+}$, *mtr*^{R} nucleus of the heterokaryon, then it should not be possible to isolate homokaryons of that nucleus. To determine if such homokaryons could be isolated, conidia from strain 28.17 was plated on basal medium containing uridine but lacking tryptophan and 78 single colony isolates were picked to tubes containing the same medium. These were allowed to conidiate and then tested for their ability to grow on basal medium. Isolates able to grow

Figure **2·9**. Analysis of transformants. Panel **1**: Western blot of mitochondria isolated from transformants grown on basal medium plus uridine. The presence or absence of 600 μM fpa in the growth medium is indicated on the Figure. The control strains were Mate IV grown in minimal medium, TI28-3 grown in basal medium plus uridine and fpa, 28.17 grown in basal medium as a heterokaryon (het), and 28.17 grown in basal medium plus uridine and fpa. Eighty μg of protein purified from mitochondria from each of the indicated strains was electrophoresed, transferred to nitrocellulose membrane, and decorated with polyclonal antiserum to MOM19. Panel **2**: Growth of transformants 17-43.1 (▲), 17-67.1 (**x**), and 17-94.2 (*) and control strains TI28-3 (◆) and 28.17 (■) in race tubes containing basal medium plus uridine and 400 μM fpa. Panel **3**: Growth of transformants on medium lacking fpa. Symbols for the transformant growth curves 17-43.1, 17-67.1, and 17-94.2 are as in Panel **2**. The transformants were grown on basal medium containing uridine. For a control to indicate the growth rate of the untransformed strain as a heterokaryon, strain 28.17 was grown on basal medium without fpa (◆). To indicate the slow growth rate when the RIPed nucleus is forced to predominate, strain 28.17 was grown on basal medium plus uridine and 400 mM fpa (■).

1


17-43.1 (+fpa)
17-43.1 (-fpa)
17-67.1 (+fpa)
17-67.1 (-fpa)
17-94.2 (+fpa)
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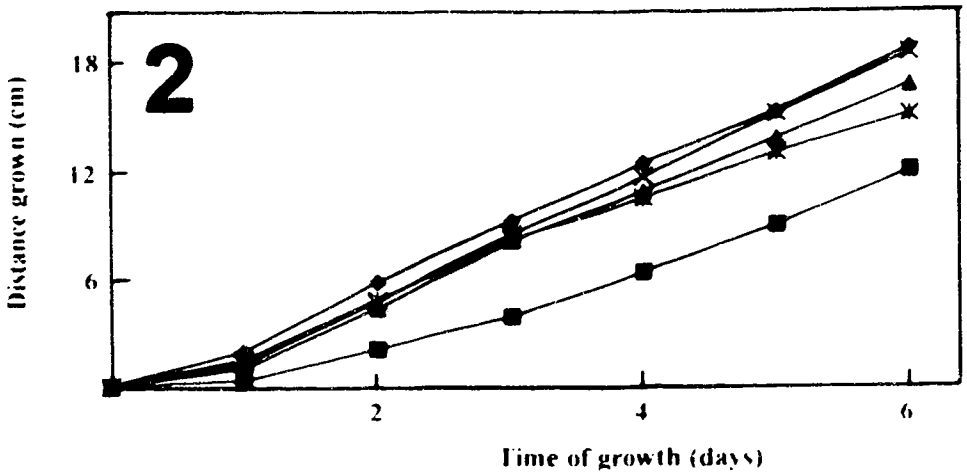
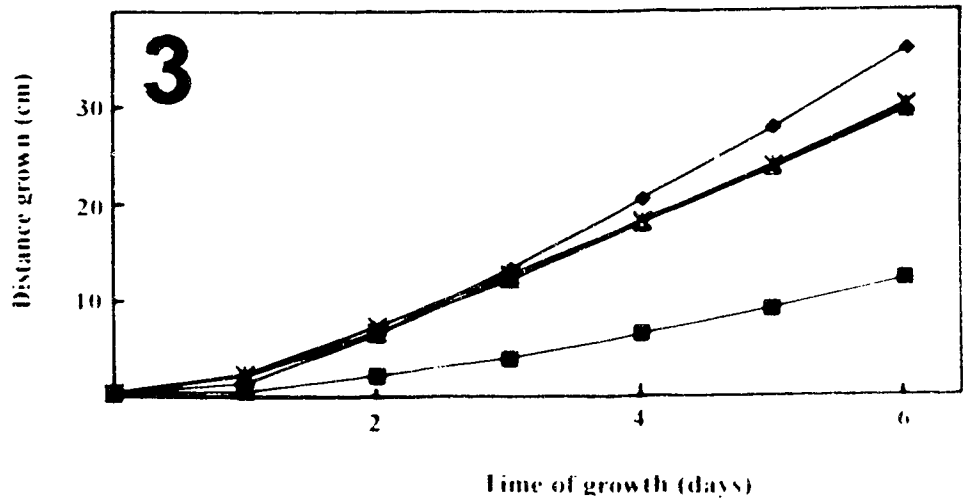
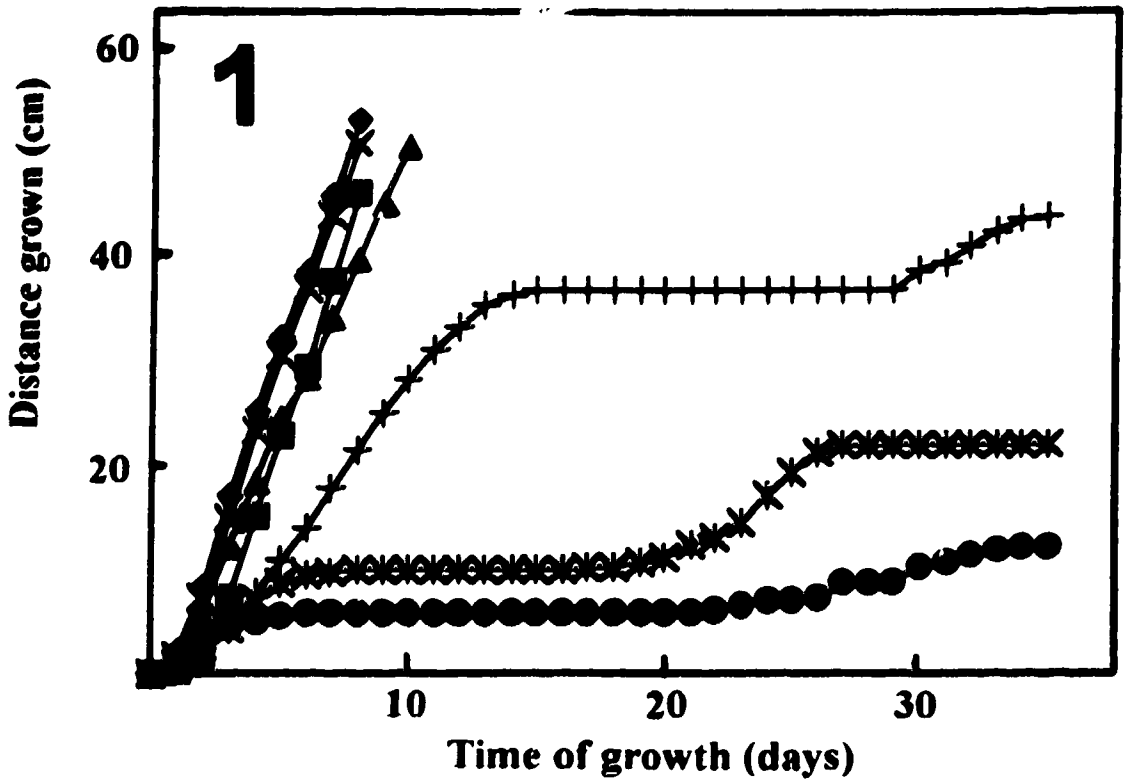
28.17 (+fpa)
28.17 (hot)
TI 28-3 (fpa+urid)
Mate IV (min)
MOM19

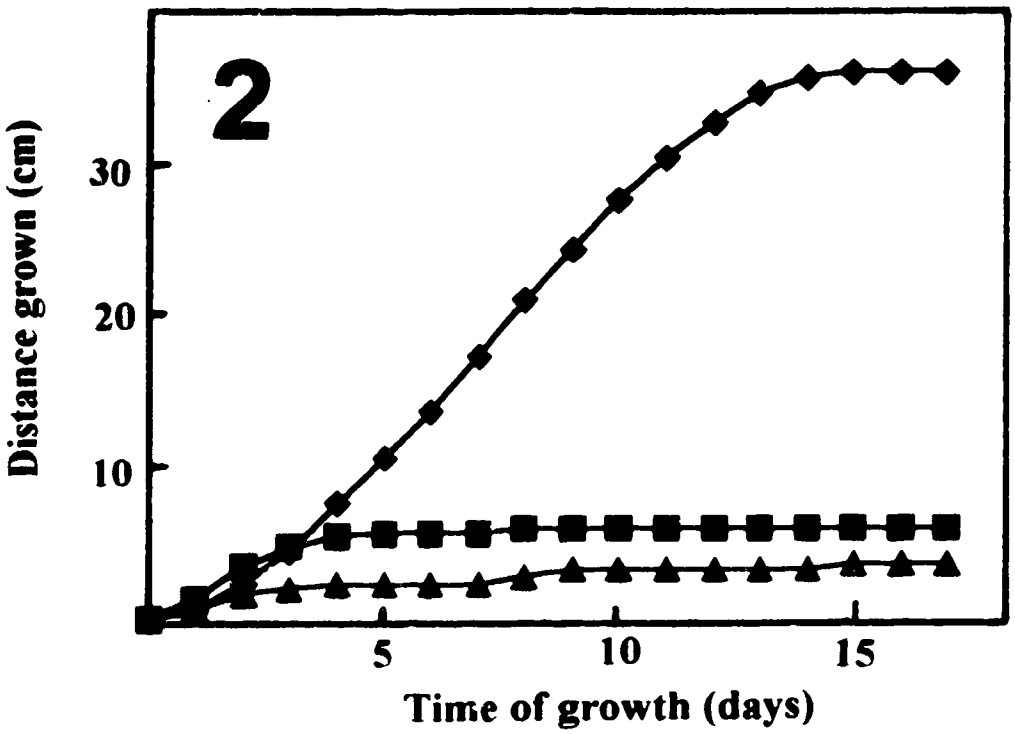

2
Distance grown (cm)
18
12
6
2
4
6
Time of growth (days)


3
Distance grown (cm)
30
20
10
2
4
6
Time of growth (days)

on basal medium must be heterokaryons, but isolates that cannot grow on this medium should be *pyr-1 trp-4*$^{+}$ homokaryons. We found that 64 of the colonies picked from the original plates were able to grow on basal medium, but 14 were unable to grow without added uridine. These 14 were presumably homokaryons containing only the RIPed version of *mom-19*. This interpretation was supported by showing that each of the 14 putative homokaryons was resistant to fpa. The *mom-19*^{RIP} homokaryons isolated in this fashion have a very slow growth rate and produce very few conidia. The density of mycelium formed in slants also appears to be thinner than with control strains. When the growth of 12 of the homokaryons in race tubes was examined, it was found that the strains exhibited a complex behaviour. In addition to their slow rate of growth, all the isolates stopped growing after various distances down the race tube while controls continued to grow until the end of the tube was reached. However, many of the homokaryons began growth again, usually from a small sector of the old growth front, after several days without growth. For at least some isolates, the stop-start growth pattern was repeated through several cycles. The growth patterns of representative strains are shown in Fig. 2·10, panel 1. The length of the period of growth in race tubes before the first cessation of growth is not a consistent characteristic associated with each of the individual homokaryotic strains since the amount of growth that precedes the stopped phase is progressively reduced when race tubes are inoculated with material from successive subcultures. This is shown in Fig. 2·10, panel 2 for strain M17-76, one of two homokaryons that grew over 35 cm when inoculated from the primary subculture of the strain. Hence the organism is apparently capable of short-term growth without a functional *mom-19* gene but not sustained long-term growth. Unfortunately, the further analysis these

Figure **2·10**. Growth patterns of *mom-19^{RIP}* homokaryons in race tubes. Panel **1**: Growth was measured for 50 cm or for 35 days. Control strains were Mate IV grown on minimal medium (■), Host IV grown on basal medium plus uridine (◆), TI28-3 grown on basal medium plus uridine (▲), and 28-17 grown as a heterokaryon on basal medium (x). Three strains that represent the range of growth capacity and the stop-start growth behaviour observed in *mom-19^{RIP}* homokaryons are also shown: M17-65 (•), M17-69 (*), M17-76 (+). These homokaryons were grown on basal medium containing uridine. As described for the crude measure of growth in slants (section 2·3·3) these curves underestimate the difference in growth of homokaryons versus wild-type because mycelial density is much lower for the homokaryons. Panel **2**: Effect of subculturing on extent of growth in race tube shown for homokaryon M17-76. Cultures were grown on basal medium plus uridine. First subculture (◆), second subculture (■), third subculture (▲).


1
60
40
20
Distance grown (cm)
10
20
30
Time of growth (days)


2
30
20
10
Distance grown (cm)
5
10
15
Time of growth (days)

homokaryotic strains has been hampered due to the inability to generate sufficient material from these strains.

2.4 Discussion

The technique of "sheltered RIP" was utilized to generate mutants of *mom-19*. In at least two of the heterokaryotic strains (28.16 and 28.17) isolated following the RIP cross, two different alleles of *mom-19*, one wild-type, the other containing many transition mutations characteristic of RIP, were demonstrated by DNA sequence analysis of PCR products. This suggests that the heterokaryotic strains have exactly the genetic make-up predicted. To obtain the desired mutants, LG IV complementing strains were examined for their ability to grow on basal medium supplemented with uridine and fpa and for evidence of RIP as seen on Southern blots following digestion of genomic DNAs with appropriate restriction enzymes. Evaluation of these two criteria alone were sufficient to identify the correct strains. Thus, the method of sheltered RIP should be useful as a general technique for the isolation of mutants in genes that are essential or have a severe effect on the growth of the organism. Appropriate strains for each of the seven *N. crassa* linkage groups are available (Metzenberg and Grotelueschen, 1992a). Perhaps the major problem with the method will be to obtain confirmation of RIP in those cases where target sequences are only mildly affected by RIP. Such a problem might occur when the target duplication is relatively small (Selker, 1990). Mild RIP could result in effective inactivation of a target gene, possibly by altering only one or two codons, but would be difficult to detect by examination for restriction site changes. In such cases the only way to demonstrate RIP may be by sequence analysis following PCR of target genes. In these instances it would

be useful to isolate DNA from cultures grown under conditions selective for the RIPed nucleus since this will increase the ratio of RIPed to non-RIPed alleles.

It is assumed that the generation of 18 amino acid substitutions in strain 28.17 has effectively disrupted the gene and results in a non-functional gene product. This is supported by the observation that the polyclonal antibody used to detect the protein on western blots reveals no protein in the mitochondria of 28.17 cultures grown in the presence of uridine and fpa. It seems likely that the altered protein is rapidly degraded, inefficiently sorted to mitochondria, or both (see section 3·3·1 as well). Although we cannot entirely eliminate the possibility that the protein retains a low level of activity despite lacking all antigenic sites found on the wild-type protein, this seems unlikely. The possibility that the phenotypic affects we observe in strain 28.17 are due to alterations in other genes, unintentionally altered during the RIP cross, is effectively eliminated by the observation that MOM19 and growth rates can be restored in cDNA transformants.

The inability to detect MOM19 in mitochondria from strain 28.17 grown in the presence of 600 mM fpa plus uridine suggests that MOM19 is not an absolute requirement for growth since the cells do grow, albeit at reduced growth rates, at this concentration of fpa in both slants (Table 2·2) and liquid cultures used for mitochondrial isolation. The isolation of *mom-19*^{RIP} homokaryons supports the notion that MOM19 is not essential. However, the slow and rather complex growth behaviour of the homokaryons makes it apparent that loss of MOM19 is deleterious for the organism. One possible explanation for the range of growth behaviours in the different homokaryons is that mitochondria containing functional MOM19 are initially present in the culture and are diluted out as growth occurs. Such mitochondria would be present in the conidia formed from the original heterokaryotic culture used for plating. The length of

time that each homokaryon continues to grow in the race tubes may be related to random factors that influence the number of mitochondria in different conidia and/or the amount of MOM19 present in the mitochondria of each initial isolate. It should also be noted that cessation of growth probably does not correspond immediately to the loss of MOM19, but rather to the depletion of essential components within the mitochondrion that are not replenished due to lack of MOM19. This might result in at least some delay in the appearance of phenotypic effects. It may also be that inefficient import via alternate routes, such as MOM72 or MOM22 can sustain slow growth for short periods. A possible explanation for the "stop-start" growth pattern of the homokaryons is that import via alternate routes may allow accumulation of essential proteins in mitochondria to occur very slowly. When a threshold level of these proteins is reached in mitochondria of a particular cell, growth begins again. These proteins might be nuclear encoded proteins involved in the maintenance or replication of mtDNA, since a similar pattern of stop-start growth has been observed previously in the so-called "stopper" extranuclear mutants of *N. crassa* (McDougall and Pittenger, 1966; Bertrand and Pittenger, 1969; 1972). In these cases, the stop-start growth pattern has been explained by changing ratios of different forms of mtDNA in the culture (Bertrand *et al.*, 1980; Gross *et al.*, 1984). However, it should be noted that yeast cells carrying a disrupted *mas20* gene, the homologue of *mom-19*$^{+}$, are inviable on nonfermentable carbon sources (Ramage *et al.*, 1993). This suggests that mutations in the equivalent gene in *N. crassa* would be lethal, since *N. crassa* is an obligate aerobe. The observation that continuous subculturing of MOM19 deficient homokaryons results in growth arrest (Fig. 2·10, panel 2) does indeed suggest that once the mutant *N. crassa* cells have fully depleted their limited store of functional MOM19, the cell is no longer capable of growth.

Mom-19 mutant strains should prove useful in understanding the functional role of the protein in the import process. To this end, a detailed biochemical investigation of MOM19 deficient cells was carried out as described in Chapter 3. The *mom-19* mutant should also be useful for investigating the assembly of the import apparatus. Furthermore, using sheltered RIP, as well as another newly developed technique, "sheltered disruption", attempts are being made to create mutants in other genes that encode proteins of the mitochondrial import machinery.

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3 A crucial role of the mitochondrial protein import receptor MOM19 for the biogenesis of mitochondria

3.1 Introduction

The biogenesis of mitochondria requires the coordinated action of both nuclear and mitochondrial genomes (Grivell, 1989). Proper function of mitochondrial processes depends on accurate import and suborganellar sorting of many preproteins synthesized on cytosolic ribosomes. Protein import into mitochondria is a complex process which requires two separate machineries in the outer and inner membrane each consisting of a large number of components (for reviews see Pfanner and Neupert, 1990; Pfanner *et al.*, 1992; Glick and Schatz, 1991; Segui-Real *et al.*, 1993b; Maarse *et al.*, 1992; Emtage and Jensen, 1993). The translocation machinery of the mitochondrial outer membrane is comprised of at least six components organized in a complex (Kiebler *et al.*, 1990; Moczko *et al.*, 1992). MOM19 and MOM72 of *Neurospora crassa* mitochondria have been reported to be involved in the initial step of recognition and binding of preproteins to the mitochondrial surface (Söllner *et al.*, 1989; Söllner *et al.*, 1990). MOM22 has recently been shown to function in the passage of preproteins from this receptor binding stage to a site where proteins are fully inserted into the outer membrane (Kiebler *et al.*, 1993). At least part of this site, the so-called "general insertion pore" (GIP; Pfaller *et al.*, 1988) consists of MOM38 and MOM7/MOM8 (Söllner *et al.*, 1992). So far, most of the functional analysis of preprotein passage across the receptor/GIP complex has been carried out *in vitro*. For instance, the relative specificities of MOM19 and MOM72 for various precursor proteins have been analysed in a

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biochemical approach by using MOM19- and MOM72-specific antibodies. Antibodies against MOM19 inhibited import of the majority of mitochondrial preproteins (Söllner *et al.*, 1989; Moczko *et al.*, 1993), while those against MOM72 appeared to be specific for a subset of proteins like the ADP/ATP carrier (AAC; Söllner *et al.*, 1990).

In order to understand the role of MOM19 in the initial reaction of protein import, it is important to investigate its function *in vivo*, e.g., by using mutants defective in MOM19. In yeast , Mas70p, the counterpart of *N. crassa* MOM72, has been found to be involved, at least *in vitro*, in the transport of a number of preproteins (Hines *et al.*, 1990; Hines and Schatz, 1993). The phenotype of a *mas70* disruption mutant, however, was rather inconspicuous showing slower growth only on non-fermentable carbon sources (Riezman *et al.*, 1983) and no drastic effects on protein import *in vivo* (Hines *et al.*, 1990). Despite the wealth of biochemical information obtained from *N. crassa*, mutants of the protein import machinery had not yet been described (prior to this work), thus precluding *in vivo* studies on the functional role of individual components.

In this chapter, the MOM19 deficient strain described in chapter 2 was utilized to study the phenotypic consequences of a lack of functional MOM19 on cell growth, cellular ultrastructure, mitochondrial protein composition, and protein import into mitochondria. The results establish a crucial role for MOM19 in the biogenesis of mitochondria. A detailed investigation of the small amount of protein import still detectable in isolated mutant mitochondria suggests a tight functional cooperation between MOM19 and MOM22. In addition, MOM72 does not serve as a general backup receptor for MOM19 implying that MOM19 is the major entry point for preproteins into mitochondria.

3.2 Materials and Methods

3·2·1 Neurospora strains and growth conditions

Strains of *N. crassa* used in this study were: (i) Host IV (H IV): LGI, *a*; LGIV, *pyr-1 mom-19*$^{+}$ *mtr*^{R} *trp-4*$^{+}$; LGV, *inl inv mei-2*; (ii) Mate IV (M IV): LGI, *A*; LGIV, *pyr-1*$^{+}$ *mom-19*$^{+}$ *mtr*^{S} *trp-4*; LGV, *am inl inv mei-2*; (iii) TI28.3, derived from H IV by integrating an ectopic copy of *mom-19*$^{+}$ into an unknown chromosome (linkage group, LG); (iv) 28.17, a heterokaryon containing one nucleus with chromosome IV derived from M IV and another nucleus containing chromosome IV derived from TI28.3 in which the two copies of *mom-19* have been inactivated by RIP (for genetic details see Chapter 2, section 2·3·3).

Cultures were grown at 25°C with vigorous aeration and bright illumination in basal medium containing Vogel's salts including trace elements and biotin (Davis and de Serres, 1970), 1.5% glucose and 50 mg/ml inositol. As required, media were supplemented with 1 mM uridine (for *pyr-1*). Liquid medium was inoculated with 1 to 5x10^{6} conidia/ml. *p*-Fluorophenylalanine (fpa, Sigma) was used at a concentration of 400 µM. In the absence of fpa all strains were grown for 16 to 20 hours, whereas in the presence of fpa, H IV and TI28.3 were grown for 24 hours, and the heterokaryon 28.17 was grown for 32 to 36 hours.

3·2·2 Biochemical procedures

The following published procedures were used: Preincubation of mitochondria with IgG (Söllner *et al.*, 1989); immunoblotting and detection by chemiluminescence utilizing the ECL system (Amersham Corp., Arlington Heights, IL) was according to Mayer *et al.* (1993); *in vitro* transcription and translation in reticulocyte lysate using [^{35}S]-methionine as radioactive label (Söllner *et al.*, 1991); immunoprecipitation by protein A-sepharose (Zimmermann and Neupert, 1980); protein determination using IgG as a

standard was performed with the dye binding assay (Biorad) or with the BCA reagent (Pierce); isolation of mitochondria (Stuart *et al.*, 1990); SDS-PAGE, fluorography (Nicholson *et al.*, 1987), and quantitation by laser scanning densitometry (Pfaller *et al.*, 1988); spectral analysis of cytochromes was according to Bertrand and Pittenger (1972).

3.2.3 Whole-cell protein extracts

Cellular protein extracts were prepared by grinding with 1 g quartz sand in 1 ml SEM buffer containing 1 mM PMSF and 1% SDS per gram of mycelia. The protein extract was clarified by centrifugation (4,000 xg, 5 min) and the protein concentration measured by the BCA method (Pierce). 125 µg protein was analysed for the content of MOM19 protein by SDS-PAGE and immunoblotting.

3·2·4 Mitochondrial protein synthesis

To 100 ml of cell culture, 100 µg/ml cycloheximide and, where indicated in Fig. 3·4, panel 1, 2 mg/ml chloramphenicol (CA) were added to block cytosolic and mitochondrial protein synthesis, respectively (Hallermayer *et al.*, 1977). After 3 min the cells were labelled with [^{35}S]-methionine (0.5 mCi; 1,100 Ci/mMol) for the indicated times and mitochondria were immediately isolated. Radioactively-labelled mitochondrial protein (70 µg/sample) was analysed by SDS-PAGE and fluorography. Quantitation was by laser densitometry.

3·2·5 Protein import into isolated mitochondria

A typical protein import reaction consisted of freshly isolated mitochondria (30 µg protein), 1 to 5 µl rabbit reticulocyte lysate containing radioactively labeled precursor proteins, 80% import buffer (250 mM sucrose, 3% (w/v) fatty acid free BSA, 80 mM KCl, 5 mM $MgCl_2$, and 10 mM MOPS/KOH, pH 7.2) in a

total volume of 100 µl. Samples were supplemented with an energy mix (2 mM ATP, 3 mM NADH, 10 mM creatine phosphate, and 100 µg/ml creatine kinase) in order to maintain a high ATP to ADP ratio. Import was performed at 10 to 25°C for 15 min. After chilling on ice, samples were immediately treated with 30 to 60 µg/ml proteinase K for 15 min on ice. Protease digestion was halted by the addition of 2mM phenylmethylsulphonyl fluoride (PMSF) from a freshly prepared 200 mM stock solution in ethanol, and samples were diluted with 1 ml SEM buffer (250 mM sucrose, 1 mM EDTA, and 10 mM MOPS/KOH, pH 7.2) containing 1mM PMSF. In the case of MOM38 import, 50 µg/ml trypsin was used which does not degrade imported MOM38, and protease was halted by addition of a 30-fold excess (w/w) of soy bean trypsin inhibitor. Mitochondria were reisolated by centrifugation for 12 min at 10,000 xg in a Beckman JA-18.1 rotor. Radioactive, imported proteins were subjected to SDS-PAGE and fluorography, and quantitated by laser densitometry.

3·2·6 Electron microscopy

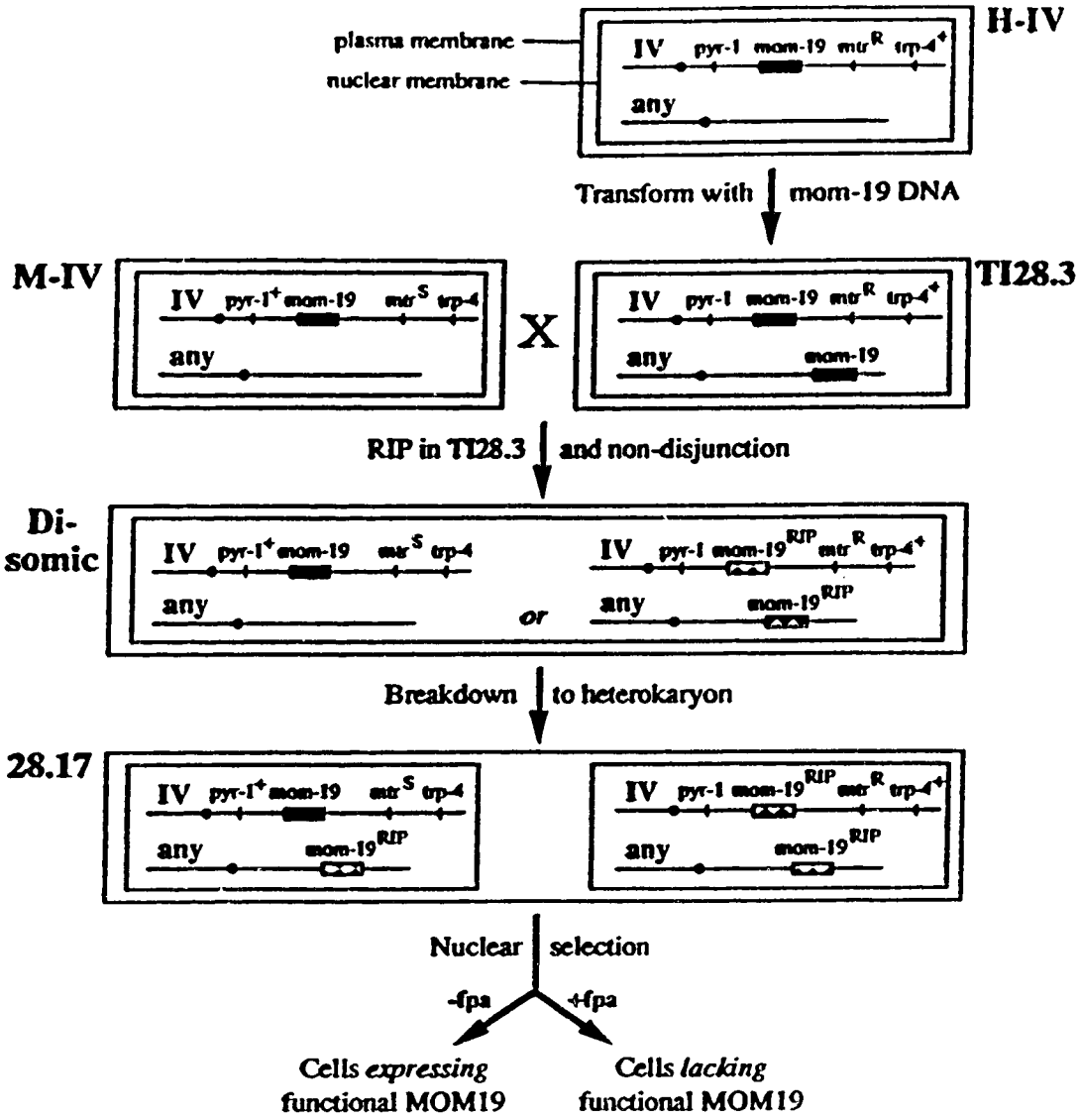
Hyphae were fixed in 1.5% $KMnO_4$ for 20 min at room temperature followed by intensive washing with H_2O till the suspension was colorless. Protoplasts and isolated mitochondria were fixed in 3% (v/v) glutaraldehyde in 0.1 M cacodylate buffer for 30 min on ice and subsequently postfixed in a mixture of 1% (w/v) OsO_4 and 1.5% (w/v) $K_2Cr_2O_7$. All samples were post-stained in 1% (w/v) uranyl acetate for 16 h at room temperature and sent to I. van der Klei (University of Groningen, The Netherlands) for analysis. Samples were prepared for electron microscopy by dehydration in a graded ethanol series, and embedded in Epon 812. Ultrathin sections were cut with a diamond knife and examined in a Philips EM 300.

3·3 Results

3·3·1 Depletion of MOM19 results in severely impaired growth of Neurospora cells.

The method of "sheltered RIP", as described in chapter 2, has been applied to inactivate the *mom-19*$^{+}$ gene in order to study the functional consequences of a deficiency of MOM19 protein in *Neurospora* cells and mitochondria. To examine further the effects of MOM19 depletion on the growth of *N. crassa* cells, the heterokaryotic strain 28.17 was grown in liquid media in the presence or absence of fpa to inhibit or allow synthesis of functional MOM19, respectively (Fig. 3·1). Cell mass was measured after harvesting the cells by filtration. This differs from measuring the rate in which hyphae extend in a race tube (Chap. 2, section 2·3·7) by measuring the rate in which whole cells increase their entire mass. As seen in Fig. 2·10, panel 1 (Chap. 2), the mycelial elongation rate of cells deficient in MOM19 in race tubes was reduced when compared to wild-type, but remained substantial. However, the observation that these strains exhibited diffuse mycelia in race tubes suggested that simple measurement of hyphal extension may not be an accurate measure of the rate in which the cells actually increase their mass. Therefore, growth of 28.17 in liquid culture was analyzed. For comparison, growth of TI28.3 cells was analysed in parallel. In the absence of fpa, both 28.17 and TI28.3 strains displayed exponential growth at comparable rates (Fig. 3·2, panel 1). After an initial lag phase, TI28.3 cells, in the presence of fpa , grew at a similar rate as cells in the absence of fpa. In contrast, 28.17 cells grown in the presence of fpa displayed a drastic reduction in growth rate after about 20 h. When the cells were diluted into fresh medium containing fpa, little further increase in mycelial mass was detectable (28.17 +fpa* in Fig. 3·2, panel 1). Upon inoculation of these cells into fresh medium lacking fpa, however, they were able to resume growth at wild type

Figure 3·1: Inactivation of the *mom-19*$^{+}$ gene by "sheltered RIP" (this figure is a modified version of Fig. 2·3). The host strain (H IV) carrying both the endogenous copy of *mom-19*$^{+}$ and the auxotrophic marker *pyr-1* (uridine requirement) on chromosome (linkage group) IV was transformed with genomic *mom-19*$^{+}$ DNA to produce the strain TI28.3. The transformant thus received a second functional copy of *mom-19*$^{+}$ (ectopic copy) on an unidentified chromosome. TI28.3 was crossed to the mate strain (M IV), which contained the *trp-4* mutant allele (tryptophan requirement) on chromosome IV. During the sexual cycle the duplicated *mom-19*$^{+}$ sequences in TI28.3 underwent mutational modifications according to the RIP mechanism (Selker, 1990). Normal chromosome pairing during meiosis was prevented by the inclusion of the *mei-2* mutant allele on chromosomes V in both H IV and M IV strains. This led to non-disjunction generating a series of aneuploid products including the desired disomic cell harboring both functional *mom-19*$^{+}$ and mutant *mom-19*^{RIP} alleles on the two chromosomes IV. Plating on media lacking uridine and tryptophan ensured that only the disomic colonies containing both chromosomes IV were present. The ectopic *mom-19*^{RIP} copy should segregate with the endogenous *mom-19*^{RIP} copy 50% of the time. Further culture of the disomic colonies on media lacking uridine and tryptophan allowed rapid and spontaneous breakdown of the disomic nucleus to a heterokaryon containing haploid nuclei with one of the two different chromosomes IV. The strain 28.17 which also contained the ectopic copy of *mom-19*^{RIP} was isolated. Synthesis of functional MOM19 was inhibited by growth in the presence of the inhibitor p-fluorophenylalanine (fpa; *mtr* as the genetic marker) which selected for cells containing *mtr* resistant (*mtr*^{R}) nuclei. Since cells harboring *mtr*^{S} do not grow in the presence of fpa, only defective MOM19 protein is produced, thus allowing the study of the effects caused by inactivation of *mom-19*$^{+}$.

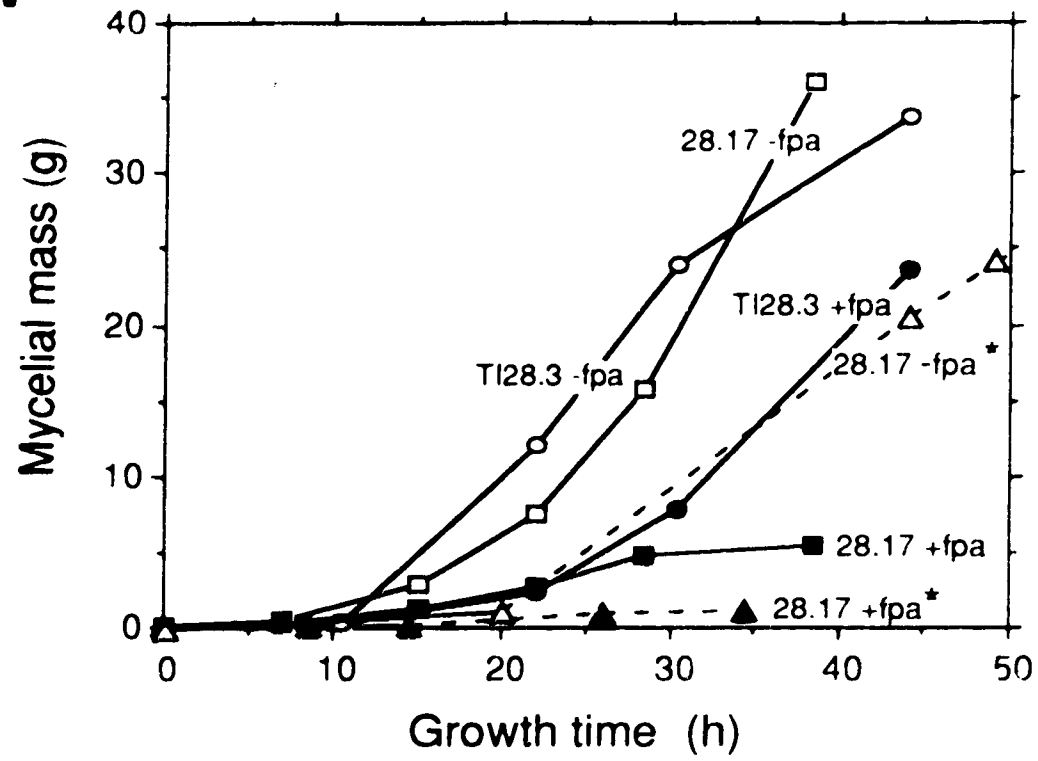

plasma membrane
nuclear membrane
H-IV
IV pyr-1 mom-19 mtr^R trp-4^+
any
Transform with mom-19 DNA
M-IV
IV pyr-1^+ mom-19 mtr^S trp-4
any
X
TI28.3
IV pyr-1 mom-19 mtr^R trp-4^+
any mom-19
RIP in TI28.3 and non-disjunction
Di-somic
IV pyr-1^+ mom-19 mtr^S trp-4
any
or
IV pyr-1 mom-19^RIP mtr^R trp-4^+
any mom-19^RIP
Breakdown to heterokaryon
28.17
IV pyr-1^+ mom-19 mtr^S trp-4
any mom-19^RIP
IV pyr-1 mom-19^RIP mtr^R trp-4^+
any mom-19^RIP
Nuclear selection
-fpa
+fpa
Cells expressing functional MOM19
Cells lacking functional MOM19

rates after a lag phase of 20 h (28.17 -fpa* in Fig. 3·2, panel 1). Comparable recovery of growth after exposure to fpa was observed for the fpa-sensitive strain M IV (data not shown). The results demonstrate that growth of 28.17 cells was severely impaired when fpa was used to inhibit the proliferation of cells expressing functional MOM19. The cells, however, remained viable even after prolonged exposure to fpa.

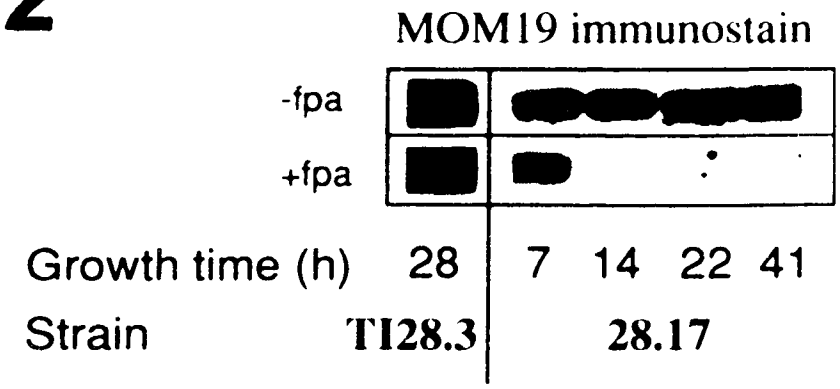
The amount of MOM19 protein in whole cell extracts was measured by immunoblotting. While fpa had no apparent influence on the amount of MOM19 during growth of TI28.3, MOM19 was undetectable by immunostaining in 28.17 cells after growth for 22 hours, the time when cells exhibited a marked reduction in growth rate (Fig. 3·2, panel 2). MOM19 was also not detectable by employing an antibody raised against a peptide representing a segment of MOM19 unaffected by RIP (data not shown). This excludes the possibility that the lack of detectable MOM19 was due to alteration of all antigenic sites recognized by the anti-MOM19 antibodies. TI28.3 cells contained threefold higher amounts of MOM19 as compared to wild type cells (Table 3·1), apparently because of the presence of the additional copy of *mom-19*$^{+}$. The content of MOM19 in 28.17 cells grown without fpa, on the other hand, was only about 20% of that found in H IV or M IV cells (Table 3·1; see also Chap. 2, section 2·3·5). The effects described above were not due to alterations introduced into 28.17 cells by RIP outside the coding region of *mom-19*, since transformation of 28.17 cells with *mom-19*$^{+}$ cDNA fully relieved the growth defect in homokaryotic isolates, and restored the MOM19 level to that observed in 28.17 cells grown as a heterokaryon without fpa (Chap. 2, section 2·3·6). Taken together, these results suggest an essential role for MOM19 during growth of *N. crassa* cells. This notion is corroborated by genetic evidence demonstrating that homokaryotic *mom-19*^{RIP} mutant cells derived from the

Figure 3·2: Depletion of functional MOM19 leads to a severe growth defect. **1)** Cells from the strains TI28.3 and 28.17 were grown at 25°C in the presence or absence of fpa in basal medium as indicated. At various times cells were harvested by filtration, and the wet cell mass was measured. After 38 h mycelia of strain 28.17 grown in the presence of fpa were backdiluted 40-fold into fresh basal medium with (28.17 +fpa*) or without (28.17 -fpa*) fpa, and growth was continued at 25°C for the indicated times before harvesting and weighing of the cells. The mycelial masses are given per liter of cell culture. **2)** Time course of MOM19 depletion. Cells were grown as described in **1)** and harvested at the indicated times by filtration. Whole cell protein extracts were analyzed by immunoblotting with antibody against MOM19.

1


Mycelial mass (g)
40
30
20
10
0
0
10
20
30
40
50
Growth time (h)
28.17 -fpa
TI28.3 +fpa
TI28.3 -fpa
28.17 -fpa*
28.17 +fpa
28.17 +fpa*

2


MOM19 immunostain
-fpa
+fpa
Growth time (h)
28
7
14
22
41
Strain
TI28.3
28.17

heterokaryotic 28.17 strain fail to generate conidia, grow very slowly, exhibit a "stop-start" phenotype and eventually senesce (Chap. 2, section 2·3·7). The availability of a MOM19 mutant thus allowed the role of MOM19 in growth of *Neurospora* cells to be investigated.

3·3·2 MOM19-deficient cells contain mitochondria with a grossly altered morphology and protein composition.

As MOM19 has been reported to represent an important component of the mitochondrial protein import machinery, MOM19-deficient cells were analyzed for the extent of morphological consequences resulting from the loss of MOM19. Cells of strains TI28.3 and 28.17 were grown for various times in the presence and absence of fpa, and examined by electron microscopy. In all cases mitochondrial profiles were clearly visible (Fig. 3·3). In the presence of fpa, 28.17 cells accumulated abnormal mitochondria with a distinct lack of cristae (panels 1 through 4), while in TI28.3, fpa caused no apparent changes (data not shown). The altered morphology of mitochondria in 28.17 cells grown with fpa appeared concomitantly with the depletion of MOM19 from the cell extracts suggesting that loss of MOM19 function is responsible for the altered morphology (compare Fig. 3·3, panels 2 through 4 with Fig. 3·2, panel 2). Interestingly, the lack of cristae in MOM19-deficient mitochondria resembles the morphology observed for mitochondria in r^0- and r^--yeast strains (Stevens, 1977; Stevens, 1981). Despite the substantial reduction in inner membrane content, outer and inner membranes were clearly distinguishable in these mitochondria. The number of mitochondrial profiles per cell and their size did not change significantly upon MOM19 depletion. Furthermore, in glutaraldehyde-fixed samples a lower number of mitochondrial ribosomes was visible in MOM19-deficient cells (dat not shown). Mitochondria in these cells are

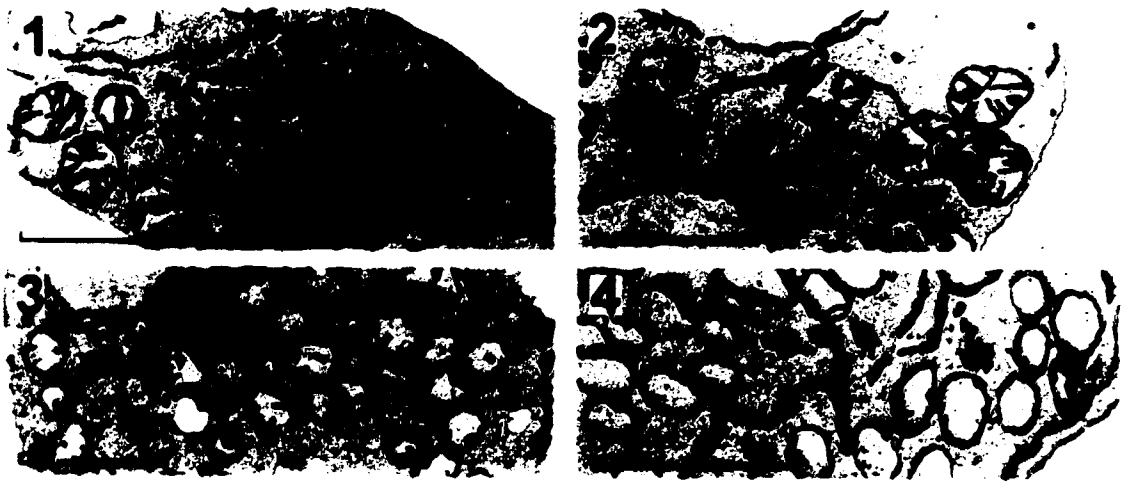


Figure 3·3: The mitochondrial ultrastructure is grossly changed in MOM19-deficient *N. crassa* cells. **1-4**) 28.17 cells were grown as described in Fig. 3·2, panel 1 in the absence of fpa for 15 h (**1**) or in the presence of fpa for 8 (**2**), 16 (**3**), and 32 h (**4**). Cells were examined by electron microscopy after fixation with $KMnO_4$.

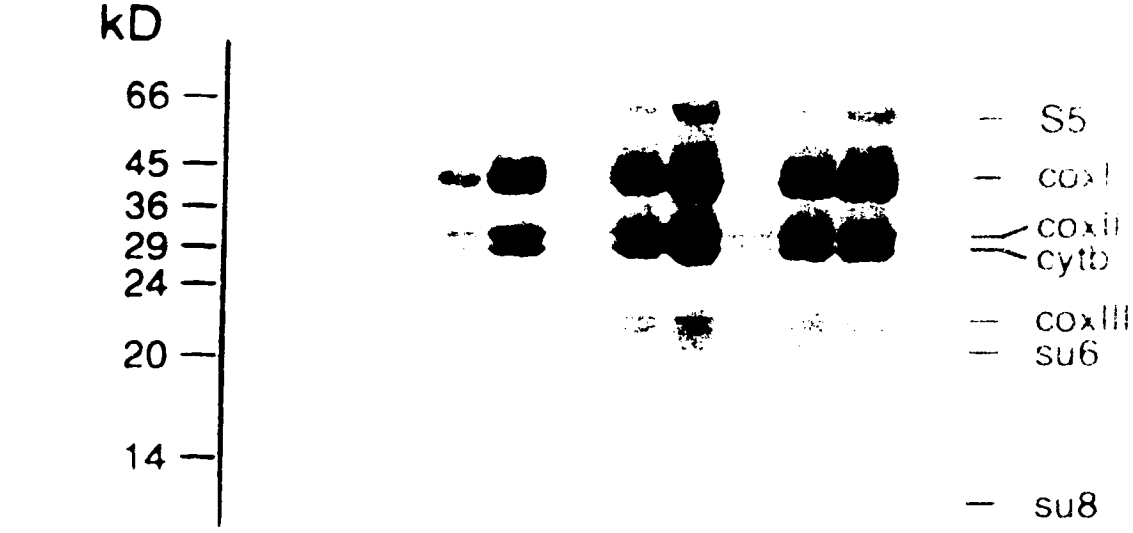
apparently not fragile or damaged since mitochondria isolated from MOM19-deficient cells appear as intact organelles despite the complete lack of cristae (data not shown). Taken together, these data demonstrate the importance of MOM19 for the biogenesis and morphology of mitochondria.

To confirm the observation of reduced numbers of mitochondrial ribosomes, organellar protein synthesis was measured *in vivo* in the presence of cycloheximide to inactivate cytosolic ribosomes. Under these conditions, protein synthesis was reduced more than 40-fold in MOM19-deficient cells compared to wild type cells or 28.17 cells without fpa (Fig. 3·4, panel 1). The results suggest that MOM19-deficient mitochondria would be defective in the expression of mitochondrial-encoded proteins, most of which are components of the oxidative phosphorylation pathway. This observation and the loss of cristae membranes (see above) in MOM19-deficient mitochondria made it likely that the content of cytochromes aa_3 and *b* would be reduced since both have components encoded by mtDNA. Absorption difference spectra was recorded for mitochondria (Bertrand and Pittenger, 1972) isolated from H IV and from 28.17 cells grown with and without fpa. Despite the marked reduction of MOM19 in 28.17 cells grown without fpa (Table 3·1; described further below in section 3·3·3), no apparent differences in the spectra from mitochondria of wild type cells were observed (Fig. 3·4, panel 2). On the other hand, mitochondria completely lacking MOM19 (from 28.17 cells grown with fpa) displayed a substantial decrease in cytochromes aa_3 and *b*, while cytochrome *c* appeared to be virtually unchanged.

The changes in the ultrastructure of mitochondria during MOM19 depletion in 28.17 cells were reflected by a changing mitochondrial protein pattern. While fpa had no apparent effect on the protein composition of wild type mitochondria, the steady state level of a number of proteins from mitochondria isolated from

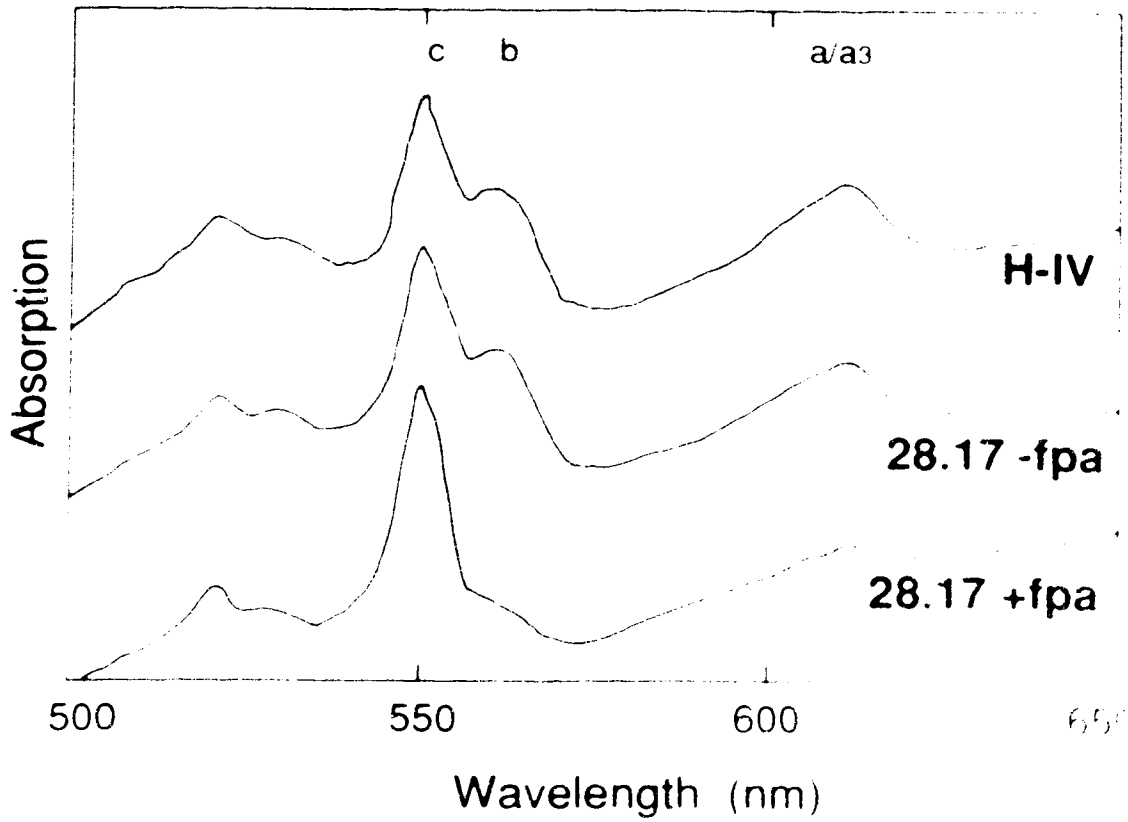
Figure 3·4: MOM19-deficient mitochondria have a strongly reduced protein synthesis activity and a deficiency in cytochromes. **1)** Strains H IV and 28.17 were grown at 25°C in the absence or presence of fpa as described in Fig. 3·2, panel 1. Chloramphenicol (CA) was added where indicated to inhibit mitochondrial protein synthesis. Cycloheximide was added to all cultures prior to the addition of [^{35}S]-methionine to block cytosolic protein synthesis. The cultures were incubated in the presence of radioactive label for the indicated times. Some of the mitochondrial protein synthesis products are indicated (S5, ribsosomal protein S5; coxI - III, subunits I - III of cytochrome *c* oxidase; cytb, cytochrome *b*; su6 and su8, subunits 6 and 8 of F_0-ATPase). Molecular weights of marker proteins are given on the left side of the figure. **2)** Mitochondria were isolated from strains H IV and 28.17 grown with and without fpa, and examined by differential absorption spectrophotometry (Bertrand and Pittenger, 1972). The spectra of mitochondria from H IV grown with or without fpa were identical. The absorption maxima of the cytochromes *aa3*, *b*, and *c* are indicated. Equivalent amounts of mitochondrial protein were used in each spectrum.

1



CA	-	-	+	-	-	+	-	-	+	-	-	+
time (min)	10	20	20	10	20	20	10	20	20	10	20	20
Mito.	28.17			H-IV			28.17			H-IV		
fpa	+			+			-			-		

2



28.17 cells strongly increased or decreased in concentration during growth in medium containing fpa, as assumed by examination of ponceau S and coomassie blue stained gels of mitochondrial proteins (data not shown). To investigate the specific changes of individual mitochondrial proteins, mitochondria were isolated from H IV, TI28.3, and 28.17 cells grown with or without fpa and analysed by immunostaining. The amounts of the other components of the protein import complex in the mitochondrial outer membrane was examined first. No significant changes in the steady-state levels were observed for the GIP protein MOM38 or the other surface receptor MOM72 upon complete depletion of MOM19, in 28.17 cells grown in the presence of fpa (Fig. 3·5, panel 1 and Table 3·1). In contrast, MOM22 was reduced by 80% in these cells as compared to wild-type cells of strain H IV. Even in 28.17 cells grown in the absence of fpa, where MOM19 is present at 24% of wild-type levels (Table 3·1), a twofold reduction of MOM22 was seen as a consequence of the decreased amount of MOM19. On the other hand, in TI28.3, which contains threefold higher amounts of MOM19 as a result of the second copy of *mom-19*$^{+}$, MOM22 appeared to be increased (Table 3·1). These results suggest that the steady-state levels of MOM19 and MOM22 are adjusted in a coordinated fashion. This might be a consequence of a direct interaction between these two proteins in the receptor complex which is also suggested by independent functional studies (see below, section 3·3·4).

Next, individual components of the outer membrane and the intermembrane space were analyzed for changes in their steady-state levels as a result of MOM19 inactivation (Fig. 3·5, panel 2). All proteins analysed from these sub-compartments including cytochrome *c* (see above and Fig. 3·4, panel 2) were not significantly altered in their levels irrespective of the amount of MOM19 present in the mitochondria. A similar result was observed for proteins from the

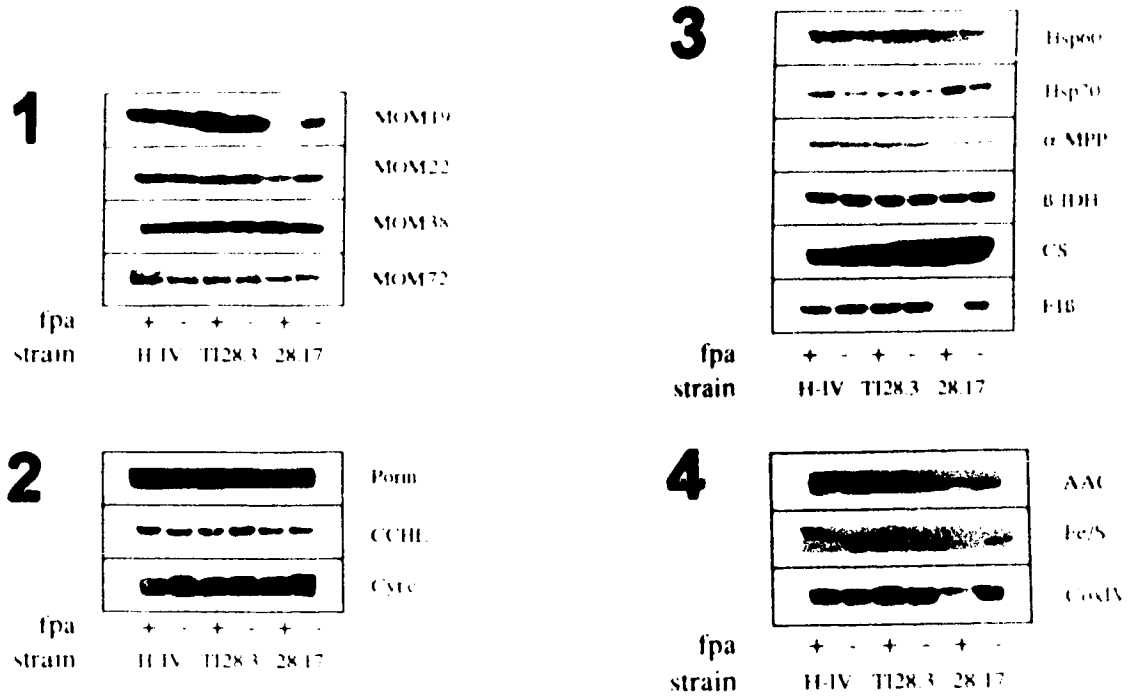


Figure 3·5: MOM19-deficient mitochondria display a largely altered protein composition. Mitochondria were isolated from strains H IV, TI28.3, and 28.17 grown in the presence or absence of fpa. 1 to 100 µg of mitochondrial protein was analysed by SDS-PAGE and immunoblotting for the relative amounts of the indicated components from **1**) the protein import complex of the outer membrane, **2**) the outer membrane and the intermembrane space, **3**) the matrix space, and **4**) the inner membrane. CCHL, cytochrome *c* heme lyase; Cyt c, cytochrome *c*; Hsp 60 and Hsp70, heat shock proteins of 60 and 70 kDa; α-MPP, α-subunit of matrix processing peptidase; ß-IDH, ß-subunit of isocitrate dehydrogenase; CS, citrate synthase; F1ß, ß-subunit of F_1-ATPase; AAC, ADP/ATP carrier; Fe/S, Rieske iron-sulfur protein; CoxIV, subunit IV of cytochrome *c* oxidase.

Table 3·1: Abundance of components of the protein import complex of the mitochondrial outer membrane in MOM19-deficient mitochondria.

N. crassa strain	MOM19	MOM22	MOM38	MOM72
H-IV or M-IV	=100	=100	=100	=100
TI28.3 -fpa	322	185	84	106
TI28.3 +fpa	280	128	92	87
28.17 -fpa	24	49	112	96
28.17 +fpa	n.d.	20	104	62

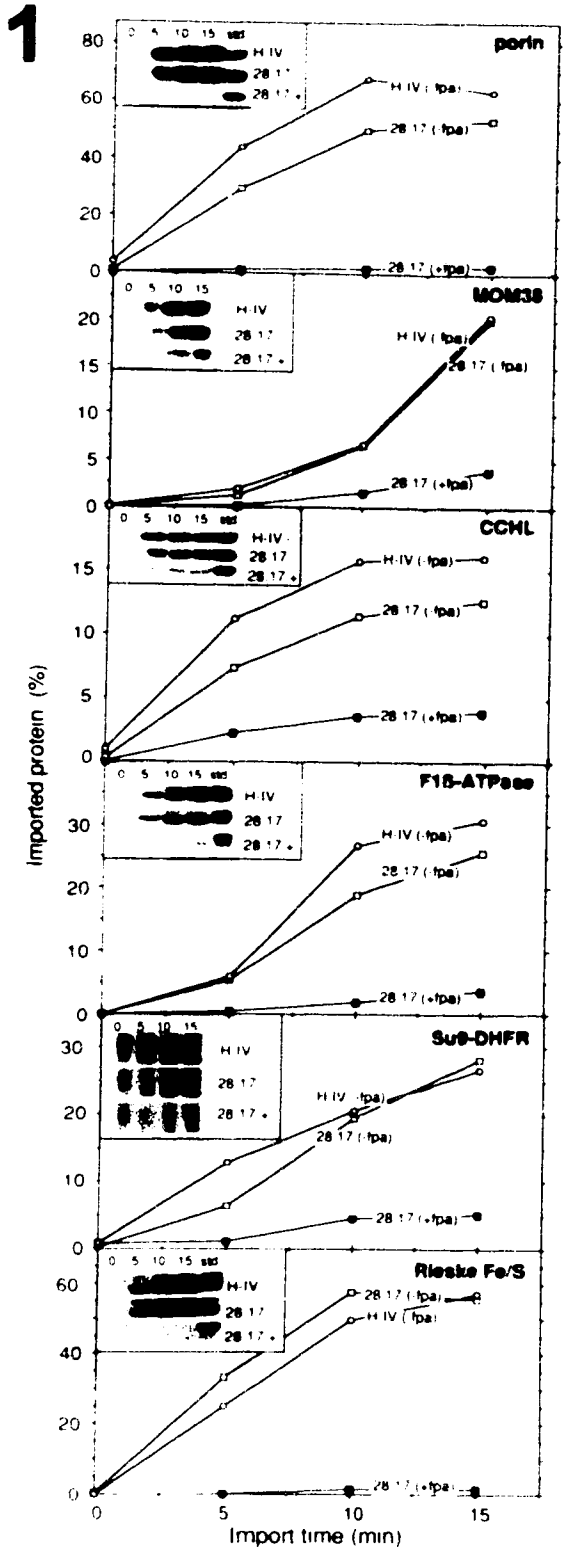
Mitochondria were isolated from the indicated strains grown in the presence or absence of fpa. Mitochondrial protein (10-100 μg) was analysed for the relative amounts of the various components of the protein import complex by SDS-PAGE and immunoblotting using the ECL luminescence detection kit (Amersham). Quantitation was performed by laser densitometry. Data are given relative to the values obtained for wild type strains H IV and M IV. n.d., not detectable. Values are the average of 5 to 10 different experiments.

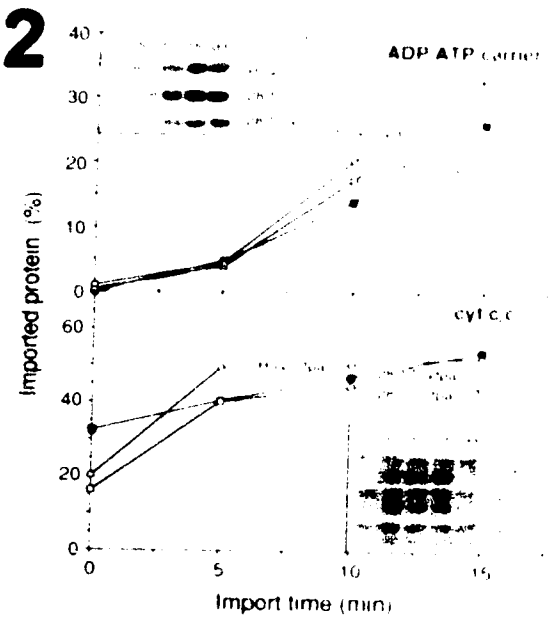
matrix space, with the exception of Hsp70 which showed a twofold increase, and the ß-subunit of F_1-ATPase, a protein tightly associated with the inner membrane (Fig. 3·5, panel 3). Components of the inner membrane, on the other hand, were reduced by factors of more than ten as a result of MOM19 depletion (Fig. 3·5, panel 4). This result is consistent with the substantial reduction in cristae membranes and with the decrease in cytochromes. From the data in Fig. 3·5 it becomes obvious that the steady-state levels of specific mitochondrial proteins were altered as a consequence of MOM19 inactivation. These effects are most likely due to a variety of secondary events, e.g., protein import and assembly, reduced expression of the mitochondrial gene products, altered regulation of expression, and different stability of the individual proteins.

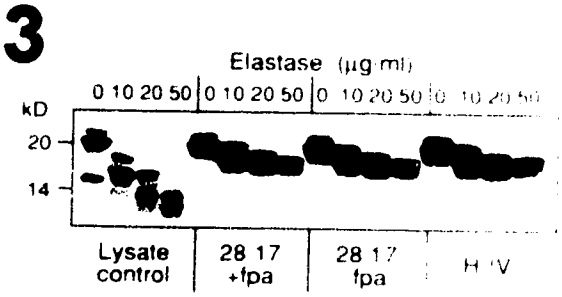
3·3·3 MOM19-deficient mitochondria are strongly impaired in the import of most, but not all precursor proteins.

To assess the consequences of a lack of functional MOM19 for the transport of individual mitochondrial preproteins, import into isolated mitochondria was measured. Mitochondria from strains H IV or 28.17 grown in the absence or presence of fpa were incubated with *in vitro* synthesized preproteins. Import into MOM19-deficient mitochondria (from 28.17 cells grown with fpa) was substantially reduced for a number of preproteins, in comparison to import into mitochondria derived from H IV wild type cells (Fig. 3·6, panel 1). Import into H IV mitochondria was not effected by fpa (not shown). These proteins included components of the outer membrane (porin; Kleene *et al.*, 1987 and MOM38; Keil *et al.*, 1993), the intermembrane space (cytochrome *c* heme lyase (CCHL); Lill *et al.*, 1992), the inner membrane (Rieske Fe/S protein; Hartl *et al.*, 1986 and Su9-DHFR, a fusion protein between subunit 9 of F_0-ATPase and dihydrofolate reductase; Pfanner *et al.*, 1987), and a protein from the matrix

Figure 3·6: Protein import into MOM19-deficient mitochondria is severely affected for most, but not all preproteins. Radioactively-labelled preproteins were incubated in import buffer (supplemented with an energy mix) with 30 µg freshly isolated mitochondria from strains H IV and 28.17 grown with and without fpa as indicated. To assure that small differences in the rates of import can be detected, import temperatures were kept at 10°C. After the indicated times import was terminated by transfer to 0°C and immediate protease treatment. Mitochondria were reisolated by centrifugation, and the samples were analysed for imported protein by SDS-PAGE and fluorography. Quantitation of the fluorographs was performed by laser densitometry. Ordinate values are given relative to the amount of radioactively-labelled protein associated with mitochondria after 15 min. The standard lane (std.) contains 30% of that material. Preproteins which were affected by MOM19 depletion are shown in **1**) and those unaffected by MOM19 deficiency are given in **2**) and **3**). Su9-DHFR, a fusion protein between subunit 9 of F_0-ATPase and dihydrofolate reductase; cyt c_1c, a fusion protein between the presequence of cytochrome c_1 and cytochrome *c*. Other abbreviations are as in Fig. 3·5. **3**) Import of MOM19 was for 10 min at 25°C (Mayer *et al.*, 1993). Samples were treated with the indicated amounts of elastase for 10 min at 25°C followed by addition of 1 ml of SEM buffer containing 1 mM PMSF and an incubation for 5 min at 25°C. Further analysis of the samples was as above. Import was estimated from the characteristic fragmentation pattern of MOM19 which is formed only after correct insertion of MOM19 into the outer membrane (Schneider *et al.*, 1991). In contrast, MOM19 precursor present in reticulocyte lysate becomes degraded at much lower concentrations of protease (Lysate control).


1
porin
MOM38
CCHL
F1β-ATPase
Su9-DHFR
Rieske Fe/S
H-IV
28 17
28 17 +
H-IV (-fpa)
28 17 (-fpa)
28 17 (+fpa)
Imported protein (%)
Import time (min)


2
ADP ATP carrier
cyt c
Imported protein (%)
Import time (min)


3
Elastase (μg/ml)
0 10 20 50
kD
20
14
Lysate control
28 17 +fpa
28 17 fpa
H-IV

space (ß-subunit of F_1-ATPase; Rassow *et al.*, 1990). Although the data is not shown in Fig 3·6, similar results were obtained for precursors of MOM22 (Keil and Pfanner, 1993), the α-subunit of matrix processing peptidase, α-MPP (Schneider *et al.*, 1989), and pF51-CCHL (Segui-Real *et al.*, 1993a), a fusion protein between the presequence of F_1ß-ATPase and CCHL. The effects varied for the individual preproteins from a threefold (for CCHL) to a more than tenfold reduction (porin). Even though strongly diminished, the residual import of matrix and inner membrane components into MOM19-deficient mitochondria was still fully dependent on the presence of a membrane potential (data not shown) required for the import of these proteins across the inner membrane (Schleyer and Neupert, 1985). This shows that the low amounts of protease-protected proteins observed in these experiments still represented authentic protein import. These findings demonstrate the important function of MOM19 in protein import into mitochondria. Surprisingly, import into mitochondria derived from 28.17 cells grown without fpa was only slightly reduced as compared to wild-type mitochondria (Fig. 3·6, panel 1) despite the substantial reduction of MOM19 by 80% in these mitochondria (see Table 3·1). This suggests that MOM19 is either not involved in the rate limiting step of the protein import reaction, or that under laboratory conditions, protein import into isolated mitochondria can occur at almost wild-type rates even when levels of MOM19 are greatly reduced. It is probable that a strain of *N. crassa* in the wild, with a level of MOM19 exhibited by the 28.17 heterokaryon, would be at a competitive disadvantage for utilizing available food sources.

Previous findings have shown that a number of mitochondrial proteins become imported into mitochondria independently of MOM19 function including the ADP/ATP carrier (AAC; Söllner *et al.*, 1990), cytochrome *c* (Stuart and Neupert, 1990), and MOM19 (Schneider *et al.*, 1991). Therefore, we expected

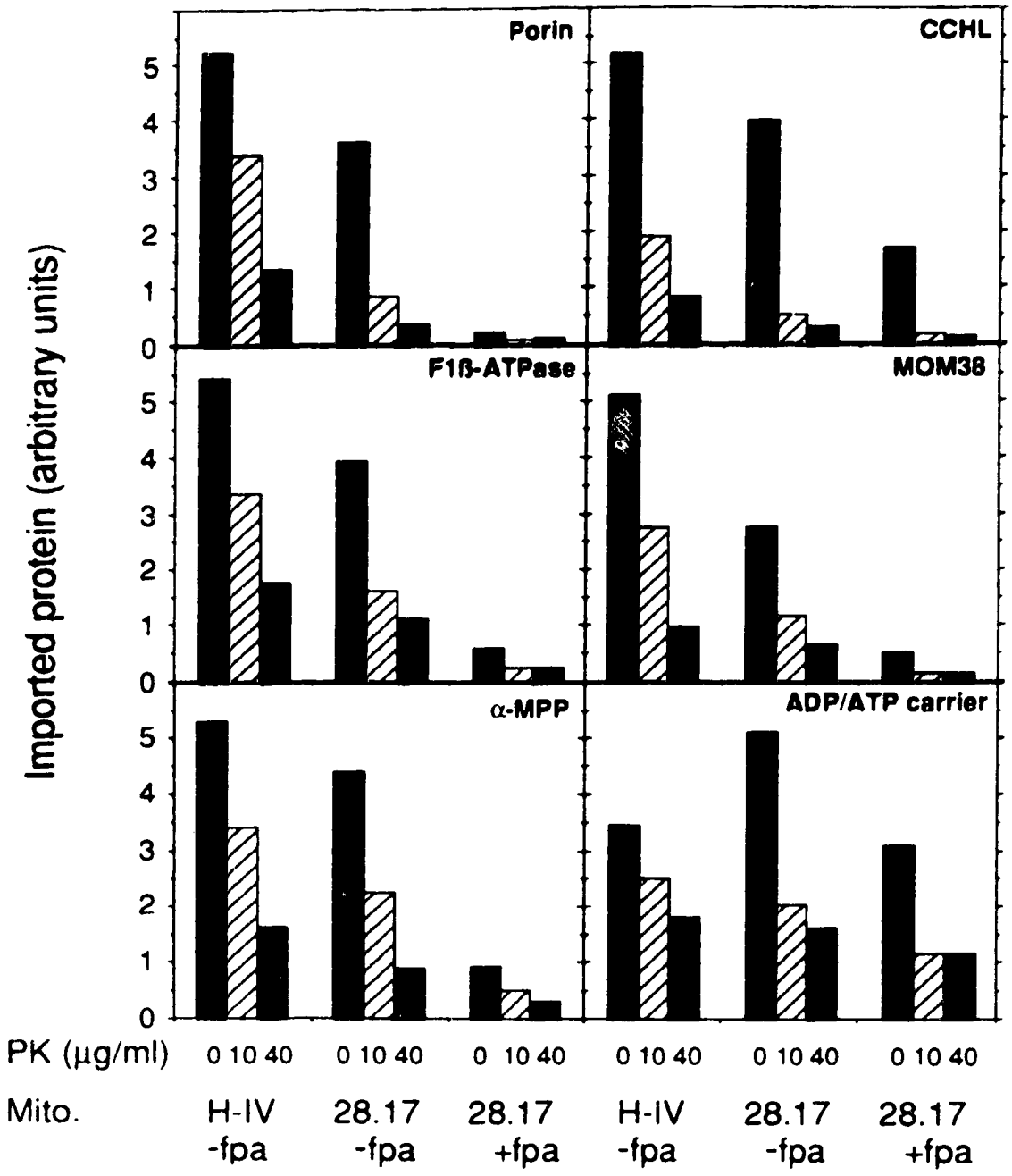
that import of these proteins should not be affected by the depletion of MOM19. Indeed, AAC was imported with equal efficiencies into mitochondria irrespective of their content in MOM19 protein (Fig. 3·6, panel 2). The fact that AAC needs a membrane potential for import into the inner membrane (Pfanner and Neupert, 1987) supports the conclusion drawn above that the largely diminished import of MOM19-dependent preproteins of the inner membrane and the matrix was not the result of a reduced membrane potential in MOM19-deficient mitochondria. As with the import of AAC, no significant import differences were observed between wild type and mutant mitochondria for a fusion protein between the presequence of cytochrome c_1 and cytochrome *c* (Fig. 3·6, panel 2). This protein is similar to cytochrome *c* in its import across the outer membrane which occurs independently of the receptor/GIP complex (Stuart *et al.*, 1990). Likewise, no effect of MOM19 depletion was observed on the insertion of MOM19 precursor into the outer membrane (Fig. 3·6, panel 3). The unchanged import of MOM19-independent precursor proteins convincingly demonstrates that MOM19-deficient mitochondria are still fully functional in performing protein import reactions which can occur without the participation of MOM19. For the majority of preproteins, however, MOM19 is needed for efficient transport.

3·3·4 MOM19 cooperates with MOM22 during protein import and is not substituted for by MOM72.

Previous studies have demonstrated that protein import can occur without the function of protease-sensitive components (Pfaller *et al.*, 1989). This so-called "bypass" import reaction occurs at a low efficiency, and preproteins are thought to enter the mitochondria at a later stage of the import reaction, possibly by directly engaging contact with constituents of GIP. However, it

should be noted that "bypass" import may also be due to a residual level of intact protein import components that may remain following protease treatment. MOM19-deficient mitochondria were examined in order to determine whether the small amount of protein import into these mitochondria occurred via a bypass or a protease-sensitive pathway. Import experiments were performed using mitochondria which had been pretreated with proteinase K (Fig. 3·7) or trypsin (data not shown). As previously observed, import of various precursor proteins into mitochondria derived from a wild type strain (H IV) was strongly dependent on the integrity of protease-sensitive components (Pfaller *et al.*, 1988). Similar results were obtained for mitochondria derived from strain 28.17 grown without fpa. Import into MOM19-deficient mitochondria (from strain 28.17 grown with fpa) unexpectedly appeared to be strongly susceptible to protease pretreatment suggesting that the import reaction in the absence of functional MOM19 cannot be considered to represent bypass import. This is supported by the observation that import into protease-pretreated wild type mitochondria (i.e. bypass import) was usually at least twice as high as import into mitochondria lacking MOM19 (Fig. 3·7, compare lanes with 40 µg/ml proteinase K in H IV with 0 µg/ml proteinase K in 28.17 +fpa). What might be the explanation for these findings? First, one has to take into consideration that wild type mitochondria used for bypass experiments still contain the membrane anchor of MOM19. This membrane-embedded protein fragment might still be attached to the protein import complex and thus fulfill an important function in the structural organization of the other subunits in the complex and/or provide some protection for the other subunits from protease degradation. The complete lack of MOM19, on the other hand, might result in a protein import complex of largely altered composition, stability, and access to protease. Therefore, the definition of "bypass" import used for wild-type

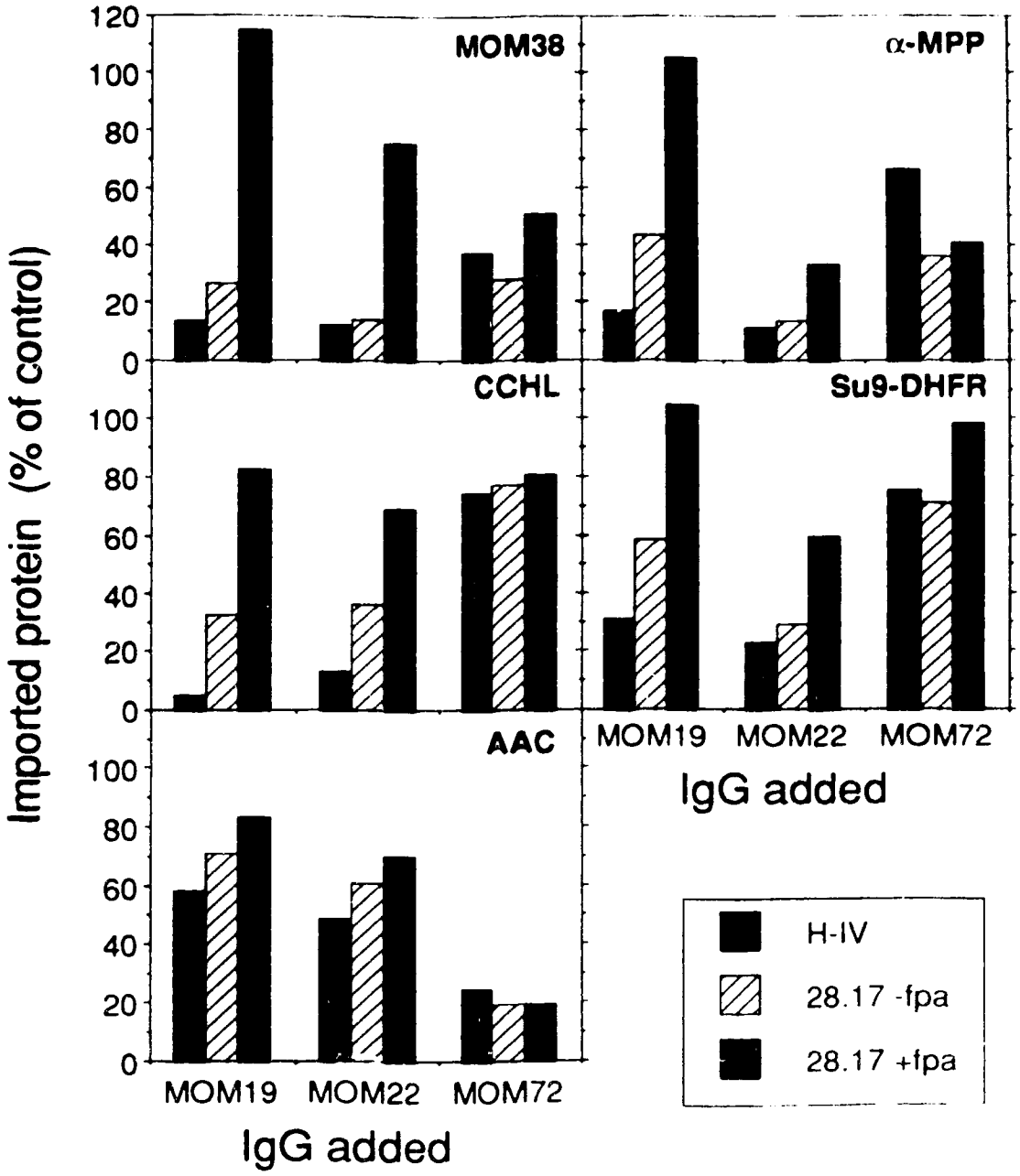
Figure 3·7: Protein import into MOM19-deficient mitochondria requires protease-sensitive components other than MOM19. Mitochondria (Mito.) freshly isolated from the indicated *Neurospora* strains were pretreated with the indicated amounts of proteinase K (PK) for 15 min at 0°C. After addition of 1 mM PMSF mitochondria were reisolated by centrifugation (7 min, 6,000 xg) and resuspended in import buffer. Before import of the indicated preproteins (15 min at 15°C) samples were supplemented with an energy mix. After protease treatment mitochondria were reisolated by centrifugation, and the samples analysed for imported protein by SDS-PAGE and fluorography. Quantitation of the fluorographs was performed by laser densitometry. Abbreviations are as in Fig. 3·5.


Porin
CCHL
F1ß-ATPase
MOM38
α-MPP
ADP/ATP carrier
Imported protein (arbitrary units)
0
1
2
3
4
5
PK (µg/ml) 0 10 40 0 10 40 0 10 40 0 10 40 0 10 40 0 10 40
Mito. H-IV -fpa 28.17 -fpa 28.17 +fpa H-IV -fpa 28.17 -fpa 28.17 +fpa

mitochondria may not be applicable for MOM19-deficient mitochondria since complete lack of the protein may render the remaining proteins of the complex (which probobly facilitate bypass import) more susceptible to protease action than in wild-type mitochondria. Second, protease sensitivity of import into MOM19-deficient mitochondria indicates the participation of protease-sensitive components other than MOM19. Possible candidates for such components might include i) MOM72 which may act as a backup system for MOM19 (see below), ii) MOM22 which has recently been characterized to have a functional role between receptor and GIP stage (Kiebler *et al.*, 1993), iii) MOM38, a putative component of GIP (Kiebler *et al.*, 1990), and iv) possibly other yet to be identified components of the import machinery.

To specifically address whether MOM22 function is still required for protein import after MOM19 depletion, and whether MOM72 can partially replace MOM19; mitochondria were pretreated before import with antibodies against MOM22 or MOM72 and, as a control, against MOM19. In wild type mitochondria (H IV) import of MOM38, α-MPP, CCHL, and Su9-DHFR was strongly decreased after blocking of MOM19 or MOM22 (Fig. 3·8). Comparable results were obtained for the ß-subunit of F_1-ATPase, Rieske Fe/S protein, and porin (data not shown). Import into mitochondria containing lower levels of MOM19 (28.17 -fpa) was inhibited to a lesser degree, showing further that import is not occurring at optimum efficiency in the heterokaryon. In mitochondria devoid of MOM19 (28.17 +fpa) pretreatment with anti-MOM19 antibodies had no inhibitory effect on the residual low levels of protein import (Fig. 3·8). These observations demonstrate that the antibodies specifically interferred with MOM19 function in wild-type cells. Similarly, only a minor inhibitory affect in MOM19 depleted mitochondria was seen using antibodies against MOM22 (Fig. 3·8). Apparently, the lack of MOM19 led to protein import which was not only strongly reduced

Figure 3·8: MOM19 cooperates with MOM22 during protein import, but cannot be functionally replaced by MOM72. 20 µg freshly isolated mitochondria were incubated in import buffer for 40 min at 0°C with 70 µg (MOM19 and MOM22) and 130 µg (MOM72) immunoglobulin G (IgG) specific for the indicated proteins. Mitochondria were reisolated by centrifugation and resuspended in import buffer. After addition of an energy mix import of the indicated preproteins was preformed for 15 min at 15°C. Following protease treatment mitochondria were reisolated by centrifugation, and the samples analysed for imported protein by SDS-PAGE and fluorography. Quantitation of the fluorographs was performed by laser densitometry. In order to account for the large differences of import into the various mitochondria (Figs. 3·6 and 3·7), data are given relative to the respective control import in the absence of IgGs. Preincubation of the mitochondria with 70 µg IgG isolated from preimmune serum yielded import efficiencies of 93 % (+/- 9 %). Standard deviation for import into H IV and 28.17 cells grown without fpa was 9%, whereas it was 18% for the low amounts of import into mitochondria from 28.17 cells grown with fpa.


Imported protein (% of control)
120
100
80
60
40
20
0
MOM38
α-MPP
CCHL
Su9-DHFR
AAC
MOM19
MOM22
MOM72
IgG added
H-IV
28.17 -fpa
28.17 +fpa

due to lack of MOM19, but the small amount of import activity remaining occurred largely independent of MOM22. Thus, import into MOM19-deficient mitochondria fairly efficiently bypasses MOM22 function. These data suggest a direct functional cooperation of these two proteins during the import reaction. The remaining weak dependence on MOM22 function in MOM19 depleted mitochondria shows that MOM22 still plays a role during import, though at reduced efficiency.

Using wild type mitochondria, antibodies against MOM72 displayed an inhibitory effect on the import of MOM38 and α-MPP, whereas the import of CCHL and Su9-DHFR was not affected (Fig. 3·8). Virtually identical inhibition by anti-MOM72 antibodies was seen using mitochondria with a low content of MOM19 (28.17 -fpa) or mitochondria deficient in MOM19 (28.17 +fpa) showing that MOM72 is used to the same extent in these mitochondria as in wild type controls. This observation is surprising for proteins like MOM38 and α-MPP whose import is partially inhibited by MOM72 antibodies in wild-type mitochondria even though the import of these proteins is drastically reduced in MOM19 deficient mitochondria. That is, since the import of these proteins is partially affected by MOM72 antibodies in wild-type mitochondria, it might be expected that the residual import in MOM19 depleted cells is due to MOM72 function. However, this does not appear to be the case because these proteins display no higher degree of dependence on MOM72 in MOM19 deficient mitochondria than in wild-type mitochondria (Fig. 3·8).

These observations suggest that MOM72 cannot functionally replace MOM19 during import of these proteins, at least not at the levels present in these mitochondria. The exact role of MOM72 during the import of MOM38 remains to be determined. Overlapping specificities of MOM19 and MOM72 have been reported, however, for the import of AAC in that this precursor can use MOM19,

when MOM72 is absent (Steger *et al.*, 1990). As expected from earlier observations, import of AAC into wild type mitochondria was only weakly affected by antibodies against MOM19, but strongly inhibited by anti-MOM72 antibodies (Fig. 3·8). In MOM19-deficient mitochondria, however, import of AAC was no longer inhibited by anti-MOM19 antibodies and occurred exclusively and at unchanged efficiency (see Fig. 3·6, panel 2 and Fig. 3·7) via MOM72. Thus, AAC can use either MOM72 or MOM19 for efficient import, whereas most other proteins appear to strictly depend on functional MOM19 for high levels of import.

3·4 Discussion

The novel technique of "sheltered RIP" for inactivating individual genes in *Neurospora crassa* provides a useful tool to analyze the roles of important or essential genes. Thus, sheltered RIP was utilized to enable investigation of the function of MOM19 *in vivo*. Experiments *in vitro* had suggested a participation of MOM19 in the initial steps of protein import into mitochondria of *N. crassa* (Söllner *et al.*, 1989) and yeast (Moczko *et al.*, 1993). In order to understand the role of MOM19 it was essential to study its action in the intact cell.

Deficiency in MOM19 as a result of inactivation of the *mom-19*$^{+}$ gene has drastic effects not only on the structure and function of mitochondria, but also on the behaviour of whole cells. Growth of cells slows down in conjunction with the falling levels of MOM19 and virtually stops, when MOM19 is fully depleted. This is undoubtedly related to the alterations occurring on both a morphological and a biochemical level. MOM19-deficient mitochondria display a nearly complete loss of cristae membranes and consist only of the outer membrane and the inner boundary membrane. Furthermore, the data suggest that mitochondria lose their capacity for oxidative phosphorylation and for protein synthesis. Clearly, MOM19 performs an essential function in the formation and

maintenance of mitochondria which are competent to provide energy for the cell.

The loss or drastic reduction of the ability to import proteins from the cytosol is a prominent characteristic of mitochondria deficient in MOM19. The impaired import competence holds for the majority of mitochondrial proteins (at least of those tested here) and includes proteins from all mitochondrial subcompartments. However, reduced import is not observed with all precursors, as some were found to be imported at the same rate into mutant and wild type mitochondria. This emphasizes that the changes caused by MOM19 depletion are not unspecific or general, but lead to defined and selective changes in the protein import pathway. Furthermore, this differential effect on various precursor proteins is in excellent agreement with the previously observed differential inhibitory effects of antibodies against the MOM19 and MOM72 components of the mitochondrial receptor complex. Finally, the results strongly argue that MOM19 is the major entry site for precursor proteins into mitochondria.

These findings provide detailed insight into the consequences of depletion of MOM19 on the structure and function of mitochondria. These consequences appear to be rather complex. On the one hand, the deficiency in the levels of most inner membrane components is consistent with their strongly reduced import. On the other hand, the steady-state levels of proteins from other compartments in mutant mitochondria and the import rates do not correspond to each other. An interesting example is the ADP/ATP carrier. Its precursor is imported into MOM19-deficient mitochondria with the same apparent efficiency as into wild type mitochondria. This is consistent with the previously observed preferential use of MOM72 as its import receptor. The observed level of the ADP/ATP carrier in MOM19-depleted mitochondria is, however, very low. It

seems therefore that some constituents of mitochondria cannot be assembled because other components limiting for assembly are missing, or because cristae membranes are lacking, and there is no space for integration. It will be interesting to determine whether the precursor of the ADP/ATP carrier becomes degraded, and if so, where and how degradation takes place.

The opposite behaviour was observed with porin. Import of this major component of the outer membrane was drastically reduced in MOM19-depleted mitochondria, but the steady-state level was nearly the same as in wild type mitochondria. Apparently, porin existing at the time when depletion of MOM19 starts is not degraded and/or the very low rates of import during the phase of depletion are linked to the slow rate of growth so that normal concentrations are maintained in the outer membrane. Degradation or limitations due to impaired assembly do not appear to have a diminishing effect on porin. A comparable interpretation may explain the unchanged levels of the highly protease-sensitive component MOM72 which has been shown to require MOM19 for its specific association with the outer membrane (Söllner *et al.*, 1990). The almost normal levels of matrix proteins in MOM19-deficient mitochondria may also reflect the absence of limitations regarding assembly of these proteins, since most of them are homooligomers or monomers. The availability of a mutant in which uptake of components is disturbed will be useful for investigating the regulation of assembly and may allow the identification of components critical for the assembly of the mitochondrial membranes.

Mitochondria of cells depleted in MOM19 contain virtually normal levels of MOM72. This and the import experiments using anti-MOM72 antibodies show that MOM72 does not act as a general backup receptor for MOM19. With the ADP/ATP carrier, for which MOM72 acts as major receptor, deletion of the

MOM72 correlate in yeast had only a limited effect on the targeting efficiency of AAC to mitochondria. This is because MOM19 can functionally replace MOM72 as a receptor (Steger *et al.*, 1990). It cannot be excluded, however, that MOM72 is capable of low efficiency recognition of a number of other components and that highly increased levels of MOM72, due to overexpression for example, could improve the efficiency of targeting precursors, even MOM19 dependent, to mitochondria, as is the case in yeast (Ramage *et al.*, 1993).

MOM38 and MOM22 represent a class of precursor proteins that apparently requires both MOM19 and MOM72 for targeting (Keil *et al.*, 1993; Keil and Pfanner, 1993). Antibodies against both MOM19 and MOM72 were found to inhibit import of these precursors. In agreement with these findings, import of both precursors was strongly reduced in MOM19 depleted mitochondria. The data in Fig. 3-8 also show that MOM72 alone cannot act as a backup system, even though MOM72 does interact with the preproteins. To date, it is unclear, how the entry of MOM38 is achieved by both receptors acting at the same time. However, a possible reason for this mode of import could be to increase the specificity of targeting by a double-check system. It seems logical that the targeting of MOM38 should be controlled with particular precision, since MOM38 is also believed to control the insertion of MOM19 into the outer membrane (Schneider *et al.*, 1991), and MOM38 may be able to form an essential part of the outer membrane translocation pore (Söllner *et al.*, 1992). Thus, incorrect insertion of MOM38 into other cellular membranes could lead to deleterious effects due to the subsequent mistargeting of mitochondrial proteins to other membranes.

The results presented in this chapter allow new insights into the functional interaction of components of the receptor complex. In particular, it appears that MOM19 and MOM22 may act in a cooperative fashion. This is suggested by

bypass of residual MOM22 during import of precursors into MOM19-deficient mitochondria. Antibodies against MOM22 have been shown to inhibit import of precursors into wild type mitochondria (Kiebler *et al.*, 1993) but, as seen in Fig. 3.8, MOM22 antibodies had only a small effect on the import of precursors into MOM19-deficient mitochondria. Obviously, proteins imported via MOM19 also require the function of MOM22, whereas in the absence of MOM19 preproteins enter the outer membrane mainly without the help of MOM22. Both proteins are constituents of the protein import complex of the outer membrane (Kiebler *et al.*, 1990). As shown in Table 3·1, the level of MOM22 is reduced in MOM19 depleted mitochondria. This may reflect a requirement for MOM19 to allow stable integration and maintenance of MOM22 in the outer membrane receptor complex. An analogous situation has been described for subunit IV of the cytochrome *c* oxidase complex (Dowhan *et al.*, 1985) in which a null mutant of Cox IV in yeast results in the inability of cytochrome *c* oxidase components to assemble. On the other hand, there might also be a regulatory mechanism that ensures a correlated synthesis of both components. The molecular basis of the cooperation between the two proteins is unknown. The availability of receptor-deficient mutants may now allow the dissection of the initial steps of protein translocation into mitochondria. Moreover, such investigations can take advantage of a recently developed system for the study of protein insertion and translocation into the isolated outer membrane (Mayer *et al.*, 1993), and thereby should allow novel insights into the molecular details of these complex processes.

3·6 References

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4 Inactivation of the *Neurospora crassa* gene encoding the 78 Kd subunit of Complex I by RIP is deleterious to the cell

4.1 Introduction

The rotenone-sensitive mitochondrial complex I of *Neurospora crassa*, (NADH:ubiquinone oxidoreductase), is composed of approximately 25 nuclear and 7 mitochondrial encoded proteins. The complex links electron transfer with proton translocation across the inner membrane, producing an electrochemical potential that subsequently drives ATP synthesis (reviewed in Weiss *et al.*, 1991; Walker, 1992). Recent studies have shown that the proteins composing complex I form structures referred to as the peripheral arm and the membrane arm. These arms assemble independently of one another and then come together to form an L-shaped structure (Friedrich *et al.*, 1989; Tuschen *et al.*, 1990). There is no obvious sequence similarity between components of the two arms, which are apparently responsible for different activities.

As reviewed in section 1·3·3, the transfer of electrons from NADH to ubiquinone can also be carried out in various organisms by enzymes with a much simpler structure than complex I. For example, the yeast *Saccharomyces cerevisiae* does not contain a complex I enzyme but does harbour single polypeptide enzymes on both sides of the inner mitochondrial membrane that are rotenone-insensitive and are not coupled to ATP synthesis (de Vries and Grivell, 1988; de Vries *et al.*, 1992). *N. crassa* and plants contain complex I as well as simple rotenone-insensitive enzymes. Mammals, on the other hand, seem to lack the the rotenone-insensitive enzymes (de Vries and Grivell, 1988; de Vries *et al.*, 1992). The lack of a complex I enzyme in *S. cerevisiae* suggests that complex I may not be essential for functional mitochondria. Indeed, two

recent papers report that in *N. crassa* subunits of the peripheral arm (Alves and Videira, 1994) and membrane arm (Nehls *et al.*, 1992) can be rendered non-functional, resulting in abberant assembly of the complex. In the former case, a small amount of the complex is still completely assembled, whereas in the latter case the fully assembled peripheral arm accumulates in the absence of the membrane arm. However, neither the reduction in, nor the lack of assembled complex I were reported to be associated with a significant effect on growth rate. On the other hand, in *Aspergillus niger,* disruption of the 51kd subunit of the peripheral arm resulted in a growth rate significantly slower than the parental strain (Weidner *et al.*, 1992). The 51 kd subunit contains binding sites for NADH, FMN and an iron-sulfur cluster and its disruption resulted in complete loss of peripheral arm assembly, whereas the membrane arm was fully assembled. Moreover, several human diseases are associated with complex I deficiencies (Weiss *et al.*, 1991; Walker, 1992). The effects of complex I abnormalities may be amplified in humans since they lack the simple forms of the enzyme.

The role of components of the peripheral arm in the assembly and function of complex I in *N. crassa* is further addressed in this chapter. The 78 kd subunit protein, NUO78, which is the largest in the peripheral arm, is thought to contain two iron-sulphur cluster binding sites. In this report, we describe the inactivation of the gene encoding this protein by repeat induced point-mutation (RIP). To control for any possible deleterious effects caused by the inactivation of this subunit, the mutant was generated by the sheltered RIP procedure described elsewhere (Chapter 2; Metzenberg and Groteleuschen, 1992a; Harkness *et al.*, 1994) and maintained as a heterokaryon that contained a wild-type *nuo-78*$^{+}$ allele in the sheltering nucleus. Homokaryotic isolates could subsequently be obtained that lacked NUO78 protein, demonstrating that the

gene is not essential for viability. These isolates had only a slightly reduced growth rate, but they failed to fully conidiate.

4.2 Materials and Methods

4·2·1 Strains and media.

Growth and handling of *N. crassa* strains was as described in Davis and de Serres (1970). All strains used in this study are listed in Table 4·1. The Host II and Mate II strains carry complementary nutritional markers on linkage group II (LG II) that allow for specific selection of LG II disomic ascospores following crosses of Mate II and transformed derivatives of Host II (see Results). LG II disomics are the only spores generated from the cross that are capable of growth on basal media lacking tryptophan (*trp-3* marker) and threonine (*thr-3* marker), but containing uridine (*pyr-3* marker). Thus, basal medium is defined as the medium capable of supporting growth of disomics or heterokaryons containing both the Host and Mate LGs II. The medium contains Vogel's salts including trace elements and biotin (Davis and de Serres 1970), 1.5% glucose, and inositol (50 µg/ml). When required, threonine (1 mM) and tryptophan (0.5 mM) were added to the medium. Uridine was used at various concentrations as given in the text for increasing the ratio of mutant to wild-type nuclei or at 1 mM to maintain heterokaryons. Though not required by the strains used in this study, leucine (1 mM) was also present in media because it is part of a standard medium used in my laboratory for similar experiments.

4·2·2 Plasmid construction

Nuo-78^{+} cDNA was cloned previously (Preis *et al.*, 1991). The cDNA was utilized to construct a plasmid, pGAH3 (Fig. 4·2) that is a derivative of pBR322 containing a portion of the *nuo-78*$^{+}$ cDNA sequence and a bacterial hph gene

Table 4.1 *N. crassa* strains used in this study

Strain	Genotype or origin
Host II	LG I, *A*; LG II, *thr-3 nuo-78*$^{+}$ *arg-12*^{S} *trp-3*$^{+}$; LG IV, *pyr-3*; LG V, *am*$^{+}$ *inl inv mei-2.*
Mate II	LG I, *a*; LG II, *thr-3*$^{+}$ *nuo-78*$^{+}$ *arg-12*$^{+}$ *trp-3*; LG IV, *pyr-3*; LG V, am_{132} *inl inv mei-2.*
TH 6.3	Isolate from tranformation of Host II with pGAH3, contains single ectopic copy of *nuo-78*$^{+}$, hygromycin resistant, two rounds of single colony isolation.
TH 49.3	As TH 6.3
TH 61.3	As TH 6.3
TH 62.3	As TH 6.3
49.6	Ascospore isolate of Mate II x TH 49.3, selected as a disomic/heterokaryon on basal medium, does not contain the ectopic copy of *nuo-78*$^{+}$. One component of heterokaryon contains RIPed, non-functional *nuo-78*$^{+}$ gene.
NCN 235	LG I, *a*; LG \ *n-2.*
NCN 251	LG I, *A.*
6.17	Homokaryotic ascospore isolate from NCN 235 x 49.6; LG I, *a*; LG II, *thr-3*$^{+}$ *nuo-78*^{RIP} may be *arg-12*^{S} or *arg-12*$^{+}$ *trp-3*$^{+}$; LG IV, *pyr-3*$^{+}$; LG V, *inl*$^{+}$ *inv*$^{+}$ may be *mei-2* or *mei-2*$^{+}$; LGVI, *pan-2.*
17.1	Ascospore isolate from NCN 251 x 6.17; may be *arg-12*^{S} or *arg-12*$^{+}$; may be *pan-2* or *pan-2*$^{+}$; contains *nuo-78*$^{+}$ allele.
17.2	As 17.1, except it contains *nuo-78*^{RIP} allele.
17.3	As 17.1.
17.4	As 17.2.

17.5	As 17.2.
17.6	As 17.1.
17.15	As 17.2.
17.20	As 17.1.
62.4	Ascospore isolate of Mate II x TH 62.3. As 49.6.
4.102	Homokaryotic isolate from 62.4 containing transformed H II component of heterokaryon and *nuo-78*$^{+RIP}$ allele.
62.5	As 62.4
5.103	Homokaryotic isolate from 62.5 containing transformed H II component of heterokaryon and *nuo-78*^{RIP} allele.

(hygromycin B phosphotransferase) expressed by a *trpC* promoter from *Aspergillus nidulans* (Cullen *et al.*, 1987). Resistance to hygromycin allows selection of transformants in *N. crassa* (Staben *et al.* 1989). The *N. crassa* DNA in pGAH3 includes the 2.3 kb carboxy terminal portion of the *nuo-78*$^{+}$ cDNA, including approximately 600 bp of the 3' untranslated region. In experiments designed to generate RIP mutations in specific target genes it is desirable to limit the amount of non-*nuo-78*$^{+}$ DNA in the construct to prevent RIP of neighboring genes.

4·2·3 Isolation of mitochondria

Mycelia from liquid cultures was harvested by filtration. The mycelia was weighed and ground by hand using a mortar and pestle in the presence of 1.5 g of acid washed quartz sand and 1 ml of grinding buffer (0.44 M sucrose; 10 mM Tris-HCl, pH 7.5; 0.2 mM ethylenediaminetetraacetic acid; 0.2 mM phenylmethylsulfonyl fluoride) per gram of mycelium. When a smooth paste was formed, the slurry was centrifuged at 4000 rpm in a Sorvall SS-34 rotor for 5 min to remove nuclei, cell debris and sand. The supernatant was transferred to a clean tube and centrifuged for 12 min at 12,500 rpm in the same rotor. The supernatant was discarded and the mitochondrial pellet was subjected to a second round of centrifugation to fully remove contaminating cellular membranes. The final pellet was suspended in a small volume of grinding buffer and stored at -80°.

Mitochondria for e.p.r. (electroparamagnetic resonance; currently being done by collaborators) analysis were isolated by differential centrifugation as described above. The pelleted mitochondria were then washed once in 20% sucrose; 10 mM Tris, pH 7.5; 0.2 mM EDTA and then resuspended in a minimal amount of 60% sucrose; 10 mM Tris, pH 7.5; 0.1 mM EDTA,

transferred to a SW41 centrifugation tube and layered successively with 55% sucrose; 10mM Tris, pH7.5 and 44% sucrose; 10mM Tris, pH7.5. The samples were spun for 90 min at 40,000 xg. The mitochondria were extracted from the 44%/55% interface. The mitochondria were washed once in grinding buffer and resuspended in a minimal amount of the same buffer to give a concentration of about 30 to 50 mg of mitochondrial protein per ml. The samples were then delivered to R. Rothary in the Department of Biochemistry at the University of Alberta to carry out the actual e.p.r. analysis.

4·2·4 Analysis of genomic DNA for evidence of RIP

Restriction digests of genomic DNA with enzymes chosen to detect RIP were electrophoresed on 5% polyacrylamide gels (29:1, acrylamide:bisacrylamide) in 1X TAE buffer (40 mM Tris-acetate, pH 8.3; 1 mM EDTA) and electroblotted to nylon membranes using 0.5X TAE as the blotting buffer. The membrane was then placed on 3MM chromotography paper (Whatman) saturated with denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 10 min. Following denaturation, the membrane was neutralized by placing it on 3MM paper saturated with 3 M sodium acetate, pH 5.5 for 10 min. The membrane was then air dried and baked at 80° for one hour. Hybridization of the membrane was by standard techniques (Ausubel *et al.*, 1992) except that the hybridization and wash temperatures were reduced from 65° to 57°, since the size of many of the restriction fragments was predicted to be small.

4·2·5 PCR sequencing

Fragments of *nuo-78*$^{+}$ DNA, RIPed and wild-type, were generated by PCR reactions and either cloned into bacterial vectors or directly sequenced. A typical PCR reaction contained primers flanking the gene plus genomic DNA

isolated from the appropriate *N. crassa* strain. Approximately 0.1 µg of genomic DNA was brought to a final volume of 10 µl, and to this, the following was added: 1 µl of each primer (0.25 mg/ml), 5.8 µl 10X Taq buffer, 5.8 µl 25 mM $MgCl_2$ (2.5 mM final concentration), 0.9 µl 10 mM dNTPs (0.15 mM final concentration), 0.4 µl Taq polymerase (Bio Can), H_2O to 58 µl, and overlaid with 30 µl paraffin oil. The reaction conditions were as follows: 1 cycle at 95°C for 1 min, followed by 30 cycles of 95°C for 30 sec, 49°C for 60 sec, and 73°C for 3 min on a RoboCycler (Stratagene). After a final cycle of 73°C for 2 min, the samples were shifted to 4°C. The reaction products were separated on 0.8% agarose gels, visualized by ethidium bromide staining and UV irradiation, excised and isolated using the protocol according to Geneclean (Bio 101). The DNA was extracted from the glassmilk with 13 µl of sterile ddH_2O; 12 µl was recovered. The DNA was prepared for sequencing by adding 1 µl of primer to the 12 µl of template and boiling for 8 min followed by flash freezing in a dry ice/ethanol bath for at least 2 min. The DNA samples were rapidly thawed and to each the following was added: 1 µl sterile ddH_2O, 1 µl 5X dGTP labelling mix, 1 µl 0.1 M DTT, 0.5 µl Mn buffer, 0.5 µl ^{35}S-dATP, and 0.25 µl sequenase (US Biochemicals). The reactions were incubated at room temperature for 2 to 5 min, after which 4.2 µl of the reaction was aliquotted to 2.5 µl of each of the 4 ddNTP termination mixes. These were incubated for a further 5 min at 37°C. To stop the reactions, 5 µl of the formamide stop mix was added. In regions of high GC content that were difficult to resolve using dGTP mixes, dITP mixes were used.

4·2·6 Whole cell PCR

Small samples of conidia from *N. crassa* cultures were suspended in 500 ml of sterile distilled H_2O. To each sample, 2.5 mg of lysing enzyme (Sigma)

was added, followed by a 10 to 15 min incubation at 37°C with shaking at 100 rpm. The samples were pelleted in a microcentrifuge with a 5 min spin and washed 2 times with 1X Taq polymerase buffer (10 mM Tris-HCl (pH 8.8); 0.5 M KCl). The pelleted samples were finally resuspended in 10 µl distilled H_2O. The PCR reaction mixes and conditions were as described in section 4·2·5. The products were separated in 0.8% agarose gels, stained in ethidium bromide and visualized under UV irradiation.

4·2·7 Other techniques

The standard techniques of agarose gel electrophoresis and Southern blotting of agarose gels, preparation of probes for DNA-DNA hybridizations, transformation of *E. coli*, isolation of bacterial plasmid DNA, and the polymerase chain reaction (PCR) using Vent polymerase (New England Biolabs, Beverly, MA) to minimize replication errors when individually cloned PCR products were to be sequenced were performed as described in Ausubel *et al.* (1992). Also see section 4·2·5 for specific PCR reaction conditions. The following procedures were also performed using previously published procedures: separation of mitochondrial proteins by polyacrylamide gel electrophoresis (Laemmli, 1970), western blotting (Good and Crosby, 1989), *N. crassa* DNA isolation (Schechtman, 1986), determination of mitochondrial protein concentration (Bradford, 1976), transformation of *N. crassa* spheroplasts (Schweizer *et al.*, 1981) with the modifications of Akins and Lambowitz (1985), and restriction fragment length polymorphism (RFLP) analysis of *N. crassa* genes (Metzenberg *et al.*, 1984; 1985). DNA sequences were obtained using Sequenase (United States Biochemical) according to the supplier's instructions. The gift of antibodies to NUO78 (J. Azevedo and A. Videira) is gratefully acknowledged.

4.3 Results

4·3·1 Rationale and Design of Sheltered RIP

Since *nuo-78*$^{+}$ may contain binding sites for two iron-sulfur clusters in Complex I (Preis *et al.*, 1991), it was reasoned that the effects of disrupting this gene might have a deleterious effect on the organism, therefore, the technique referred to as "sheltered RIP" was employed to mutagenize *nuo-78*$^{+}$. As previously described (Chapters 2; Metzenberg and Grotelueschen, 1992; Harkness *et al.*, 1994), this procedure allows the isolation of mutant alleles in one nucleus, while sheltering the mutant nucleus from any deleterious effects caused by the mutations, with a wild-type allele in a second nucleus in a heterokaryotic culture. The phenomenon of RIP (repeat induced point mutation) is utilized to generate GC to AT transition mutations (Selker, 1990). This is achieved by crossing a strain carrying two copies of *nuo-78*$^{+}$ DNA with an appropriate strain containing only the endogenous copy. A percentage of the nuclei containing the repeats undergo RIP, while the nuclei harboring only the single endogenous copy is unaffected. Inclusion of the *mei-2* mutant allele in partners of the cross causes disruption of chromosome pairing during meiosis, resulting in a high degree of nondisjuction (Smith, 1975). Disomic ascospores carrying a chromosome from the unaffected nucleus which houses the wild-type *nuo-78*$^{+}$ allele and a chromosome from the nucleus subject to RIP, carrying the *nuo-78*^{RIP} allele (or another wild-type allele if RIP did not occur), can then be selected if the chromosomes of interest carry complementing nutritional markers. The disomic ascospores will rapidly and spontaneously break down to heterokaryons (Smith, 1974); one nucleus containing the wild-type allele and the other containing the putative RIP allele. If the affected nucleus carries an appropriate marker on the chromosome

containing the RIPed gene, the ratio of RIPed to wild-type nuclei in a culture can be selectively enhanced so that the mutant phenotype can be studied. However, if the gene encodes an essential function, the culture can never become homokaryotic for the RIP nucleus. In these cases the concentration of inhibitor is adjusted so that a minimal amount of the essential gene product is supplied by the unaffected nucleus to maintain a basal level of growth but still allowing the mutant phenotype to be studied. The isolation and characterization of *nuo-78*^{RIP} alleles is described below.

4·3·2 Generation of duplication strains

In order to utilize the appropriate complementing markers when selecting for disomic ascospores, it is imperative to know the linkage group (LG) on which *nuo-78*$^{+}$ resides. Accordingly, *nuo-78*$^{+}$ specific sequences, derived from previously isolated cDNAs (Preis *et al.*, 1991), were used to isolate cosmids containing *nuo-78*$^{+}$ from an *N. crassa* library. RFLP mapping studies (Metzenberg *et al.*, 1984) were then carried out using a single cosmid as a probe to deduce the chromosomal location of the *nuo-78*$^{+}$ locus. The results demonstrated that the *nuo-78*$^{+}$ locus is on LG II (Fig. 4·1). A plasmid (pGAH3) carrying 2.3 kb of the carboxy terminal portion of the *nuo-78*$^{+}$ cDNA as well as the bacterial *hph* gene for hygromycin resistance (Fig. 4·2), was used to transform the strain designated Host II (H II). H II carries markers relevant to achieve the goals of sheltered RIP for genes on LG II (Fig. 4·3, panel 1; Metzenberg and Grotelueschen, 1992). The *thr-3* and *trp-3*$^{+}$ alleles on LG II allow selection for disomic ascospores following a cross with the Mate II (M II) strain, which is *thr-3*$^{+}$ and *trp-3* (Fig. 4·3, panels 2 and 3). The *arg-12*^{S} allele on the H II LG II suppresses the pyrimidine requirement caused by the *pyr-3* allele on LG IV by a fairly complex mechanism (see below, section 4·3·3) and

	Ascospore Isolates:										matches to *nuo-78*$^+$
CenII, arg-5	MMOO	MMOO	MMOO	OOMM	MMMM	MMMM	MMMM	OOOO	OOMM	OO	25/27
nuo-78$^+$	++++	MMO+	MM+O	OOMM	MMMM	MMMM	++MM	OMO+	O+MO	O+	
preg	OMOO	MMOO	MMOO	OOMM	MMMM	MMMM	MMMM	OMOO	OOMM	OO	26/27
Ncr-5	OMOO	++OO	M+OO	OOMM	MMMM	OMMO	MMMM	OMOM	+OMM	MO	19/23

Figure **4.1**. RFLP mapping of *nuo-78*$^+$ to linkage group (LG) II. The *N. crassa* strains Mauriceville (M) and Oak-Ridge (O) were crossed to generate progeny. These two strains are known to contain many genetic polymorphisms which are manifested in restriction site differences in their genomes. Thirty-eight segregants were selected from ordered asci (Metzenberg *et al.*, 1984). DNA from these isolates was extracted and digested with *Apa*I. *Apa*I was previously found to be appropriate for detecting RFLPs with the *nuo-78*$^+$ containing cosmid through a test gel of M and O DNA that was digested with various enzymes, blotted and hybridized to the *nuo-78*$^+$ cosmid. The *Apa*I digested DNA from each test isolate was separated on 0.8% agarose and analyzed by Southern blotting using a radioactively labeled *nuo-78*$^+$ containing cosmid as a probe. Strains were scored according to whether the RFLP was like the Mauriceville parent (M) or like the Oak-Ridge parent (O). The derived pattern was then compared to a list of compiled patterns from DNA fragments covering the entire genome. Since some DNA samples were lost (indicated by) not all 38 ascospores were available for comparison. The greater the number of matches to a known gene, the closer the linkage. By this criteria, genes, or fragments of DNA, can be localized with reasonable accuracy within the genome. The *CenII/arg-5*, *preg* and *Ncr-5* markers are all known to be on LG II (Metzenberg and Groteleuschen, 1993). +, indicates that DNA prepared from that isolate was lost.

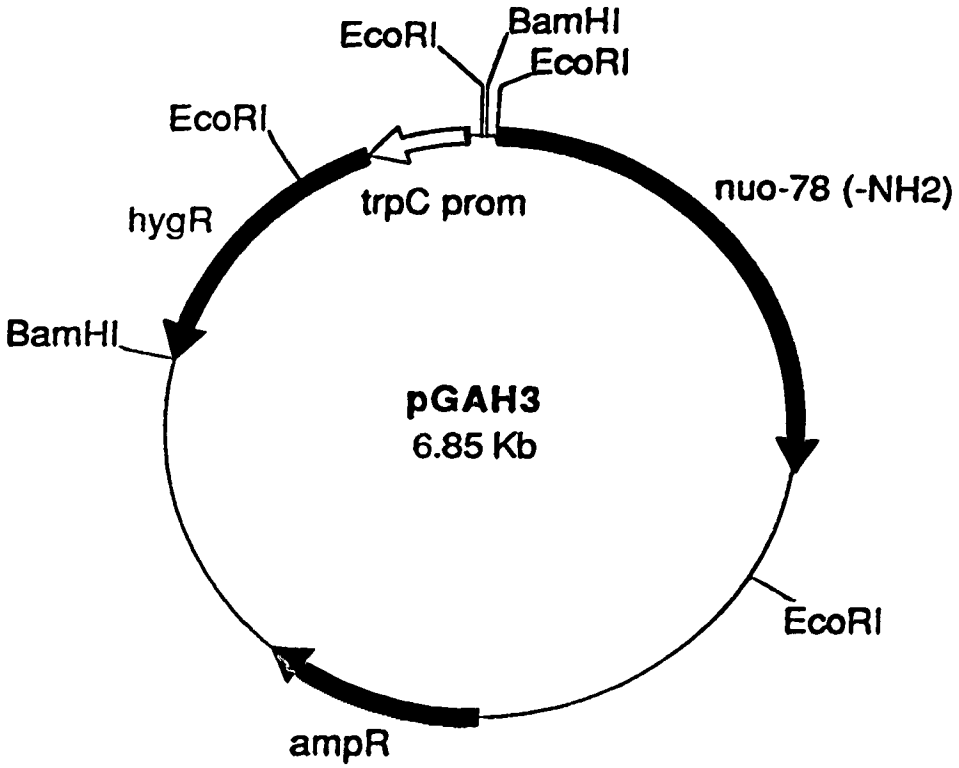


Figure **4·2**. Plasmid pGAH3. Names of genes are indicated. Filled arrows indicate the extent of coding sequences and the open arrow indicates the promoter region derived from *Aspergillus nidulans*. Restriction sites are also indicated. *hyg*, hygromycin resistance gene; *amp*, ampicillin resistance gene. The *nuo-78*$^{+}$ cDNA sequence on the plasmid lacks the N-terminal coding region.

will allow the nuclear proportions in the final sheltered RIP heterokaryon to be shifted in favor of the RIPed nucleus (Fig. 4·3, panel 4). A mutant allele of *mei-2* is utilized to generate disomic ascospores by disrupting chromosome pairing during meiosis.

Appropriate transformants of H II were selected by growth on hygromycin and were purified through two rounds of single colony isolation to insure that the transformants were homokaryotic. To identify isolates containing single, ectopic copies of *nuo-78*$^{+}$ sequences, in addition to the resident *nuo-78*$^{+}$ gene, thirty transformants were analyzed by restricting genomic DNA with enzymes that do, or do not cleave within the gene. Appropriate transformants, when restricted with *Eco*RI, which does not cut within the gene, should contain two bands when analyzed by Southern blotting using *nuo-78*$^{+}$ sequences as a probe; the 2.3 kb band from the ectopic integrant and a 3.4 kb band which was previously found to represent the endogenous copy (Fig. 4·4, panel 1). Digests using *Pvu*II, which has a single site within the gene, should produce four bands upon Southern analysis (Fig. 4·4, panel 2). Two of these bands will be from the ectopic integrant and will be an unpredictable size, and two will be from the endogenous copy, with sizes of 2.4 and 1.4 kb. Using these criteria, transformants TH6.3, TH49.3, TH61.3, and TH62.3 were identified for use in genetic crosses as the male parent to the LG II mate strain (M II).

4·3·3 Generation and characterization of nuo-78^{RIP} alleles

Strain M II was used as the female parent in genetic crosses with the appropriate transformants. This strain carries various markers essential for the goals of the sheltered RIP cross: the *thr-3*$^{+}$ *arg-12*$^{+}$ and *trp-3* alleles on LG II; and *pyr-3* (uridine requiring) on LG IV. The M II strain also carries the *mei-2* mutant allele, which must be homozygous in a cross to be effective. When the

Figure 4·3. Relevant genetic markers in appropriate initial transformants, the Mate II strain, disomic progeny isolates, and subsequent heterokaryons. Only the genetic markers that are important for generation, selection, or manipulation of the strains are shown. These are indicated on specific linkage groups represented by horizontal lines. The position of *nuo-78*$^{+}$ relative to the other markers on LG II is shown as deduced from both the RFLP map (Metzenberg and Grotelueschen, 1992b, 1993) and the genetic map of LG II (Perkins, 1992). LG II (H), linkage group II derived from the original Host II strain; LG II (M), linkage group II derived from the original Mate II strain; LG V, linkage group V; any LG, any of the seven linkage groups found in *N. crassa*. Boxes with squared corners indicate nuclei, boxes with rounded corners indicate cells. **1**. The H II strain containing only one copy of *nuo-78*$^{+}$. **2**. The cross of the M II strain with the appropriate transformed H II strain that carries two copies of *nuo-78*$^{+}$. **3**. The generation of disomic spores that would grow on media lacking both threonine and tryptophan, but containing uridine. Each must contain one LG II from the original host strain and one LG II from the original mate strain to allow complementation of the auxotrophic *trp-3* and *thr-3* markers. One type contains two unaltered copies of *nuo-78*$^{+}$; the other contains one unaltered copy and one RIPed copy. In both cases the ectopic copy of *nuo-78*$^{+}$ may or may not be present depending on the meiotic segregation of the chromosome on which it was integrated. **4**. Each of the disomics breaks down into a heterokaryon in which one nucleus contains LG II from the host and the other LG II from the mate. In one case, the LG II derived from H II in the heterokaryon contains the RIPed version of *nuo-78*$^{+}$, whereas in the other case, the LG II derived from H II contains the wild-type copy of *nuo-78*$^{+}$.

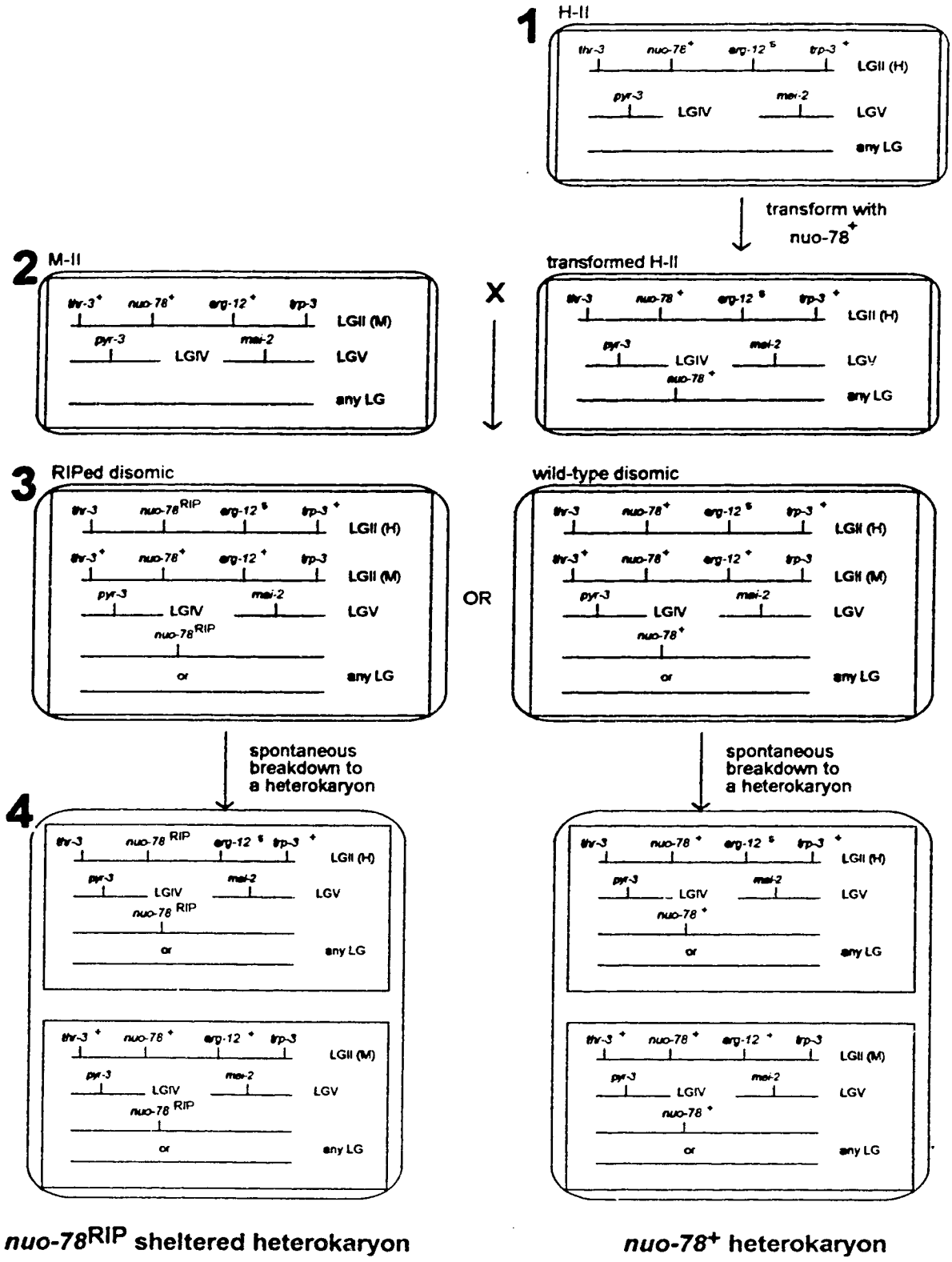

1
H-II
thr-3
nuo-78+
erg-12 S
trp-3 +
LGII (H)
pyr-3
LGIV
mei-2
LGV
any LG
transform with
nuo-78+
2
M-II
thr-3+
nuo-78+
erg-12+
trp-3
LGII (M)
pyr-3
LGIV
mei-2
LGV
any LG
X
transformed H-II
nuo-78+
3
RIPed disomic
nuo-78RIP
or
OR
wild-type disomic
spontaneous
breakdown to
a heterokaryon
4
nuo-78+ heterokaryon
nuo-78RIP sheltered heterokaryon

Figure **4·4**. Southern analysis of restriction digests showing single inserted *nuo-78*$^{+}$ sequence in strains TH49.3, TH61.3, and TH62.3. Panel **1**: *Pvu*II digest of total cellular DNA from the indicated strains. Panel **2**: *Eco*RI digest of total cellular DNA from the indicated strains. The probe was a radioactively labeled *nuo-78*$^{+}$ cDNA fragment that extends from nucleotides 784 to 3150 as shown in Fig. **4·9**, except that the intron sequences shown in the Figure are absent in the cDNA.

1

Mate II

Host II

TH 49.3

TH 61.3

TH 62.3

2

Mate II

TH 49.3

TH 61.3

TH 62.3

cross is carried out, a certain percentage of the H II nuclei harboring the duplicated *nuo-78*$^{+}$ sequences will undergo RIP of the repeat (Fig. 4·3, panel 2). The presence of the homozygous *mei-2* allele will result in the production of some ascospores disomic for LG II. The disomic ascospores of interest can be directly selected since they should be the only ones from the cross able to grow on medium lacking both threonine and tryptophan ,but containing uridine (Fig. 4·3, panel 3). Thus, the desired ascospores should contain one LG II from the mate, one LG II from the transformed host strain and be monosomic for the remaining linkage groups. The LG II from the transformed host will carry the RIP allele at a frequency equal to the frequency of RIP of that allele during the cross. The spores may contain the ectopic copy of *nuo-78*$^{+}$ as well, depending on the site of integration and the segregation pattern of the chromosomes during meiosis. If the endogenous copy of *nuo-78*$^{+}$ was subject to RIP, the ectopic copy will also exhibit a similar degree of alteration, since both copies of a duplication are affected by the RIP process.

Ascospores produced from the crosses were plated on basal medium containing uridine (see below) but lacking threonine and tryptophan. Although germination of spores was very poor, viable spores were isolated from the crosses involving TH6.3 (2 spores), TH49.3 (11 spores), TH61.3 (1 spore) and TH62.3 (10 spores). Following conidiation in culture tubes containing basal medium, each strain was then purified through two rounds of single colony isolation on the same medium to provide sufficient opportunity for breakdown of disomic nuclei and to maintain the strains as the heterokaryons formed during the process of breakdown (Fig. 4·3, panel 4).

To identify strains deficient in the mitochondrial NUO78 protein, cultures were grown in low concentrations of uridine, which should shift the nuclear ratios in favor of the nucleus carrying LG II from the potentialy RIPed H II strain.

The manipulation of uridine concentration in the medium allows the skewing of nuclear ratios by the following mechanism. The *arg-12^S* allele carried on the H II version of LG II, which will also carry the RIPed allele of *nuo-78$^+$* following the cross, affects the mitochondrial ornithine carbamyl transferase, reducing its activity over 98% without imposing an arginine requirement (Houlahan and Mitchell, 1947; Perkins *et al.*,1982). As a result, accumulated carbamyl phosphate overflows to the nucleus where it is used in the pyrimidine biosynthesis pathway, thus by-passing the carbamylphosphate synthase deficiency in that compartment caused by the *pyr-3* mutant. Thus, the *arg-12^S* allele suppresses the pyrimidine requirement of *pyr-3* strains, and growth of *pyr-3* heterokaryotic cultures in low levels of uridine forces the *arg-12^S* nucleus, containing the *nuo-78^{RIP}* allele, to predominate in the culture. Of course, the medium must also contain threonine to be permissive for that nucleus. The results in Table 4·2 show that there were several strains that grew very slowly in low concentrations of uridine, and not at all in medium entirely lacking uridine. The latter observation suggests that nuo-78 may be an essential gene (but see below, section 4·3·4).

In order to determine the state of NUO78 in the slow growing strains, mitochondria were isolated from cells grown in low concentrations of uridine in order to enhance the ratio of mutant to wild-type nuclei while still allowing growth to occur. The proteins were solubilized, separated by SDS-PAGE, transferred to nitrocellulose membrane, and then analyzed by immunoblotting with antibodies against NUO78. Four of the isolates, 49.6, 62.1, 62.4, and 62.5, were shown to be deficient in NUO78 (Fig. 4·5, panel 1). The result with 62.1 could not be repeated and was not analyzed further.

Table 4·2. Growth of heterokaryons on medium containing uridine at 30°C

	Hours required to cover surface of slant[a] containing uridine at indicated concentration						
Strain	0 μM	3 μM	6 μM	9 μM	10 μM	100 μM	1 mM
Host IV	30	30	30	30	30	30	30
Mate IV	28	28	28	28	28	28	28
49.3	no gr[b]	72	48-72	48	42	34	30
49.6	no gr	72	48-72	48	42	36	34
49.8	no gr	72	48-72	48	42	34	32
62.1	no gr	72	48-72	48	42	36	30
62.4	no gr	72	48-72	48	40	36	34
62.5	no gr	72	48-72	48	42	36	32

[a] Slants were prepared in 16 x 150 mm tubes. Average length of the slant was about 80 mm. Conidia were inoculated at a single point in the center of the slant.

[b] no gr, no growth after 96 hr

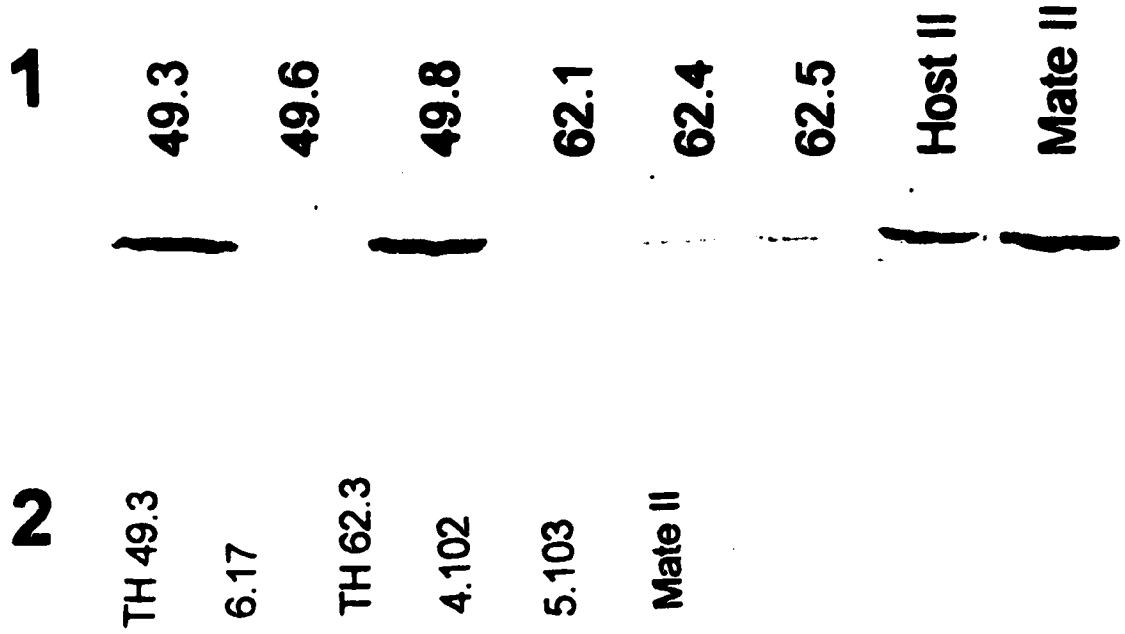


Figure **4·5**. Western blot analysis of potential RIP heterokaryotic and homokaryotic strains. **1**) A sample of the heterokaryotic strains generated from the sheltered RIP cross were analyzed for their NUO78 protein content. Each lane contained 150 µg of protein isolated from mitochondria. Cells were grown in medium containing threonine and low concentrations of uridine (3 µM) to enhance the proportion of RIPed nuclei to wild-type nuclei. Following electrophoresis, mitochondrial proteins were electroblotted to nitrocellulse membrane and decorated with polyclonal antiserum to NUO78. **2**) Homokaryotic isolates obtained from crossing (6.17) or vegatative isolation (4.102 and 5.103) from the heterokaryons 49.6, 62.4 and 62.5 were analyzed for their NUO78 protein content. Each lane contained 50 µg of mitochondrial protein. The gels and blots were treated in the same fashion as described in **1**.

4·3·4 Isolation of nuo-78^{RIP} homokaryotic strains

To determine if NUO78 was indeed essential for the cell, an attempt was made to isolate homokaryons from the strains that were shown to be deficient in the protein under growth conditions favoring the RIPed nucleus. Conidia from the heterokaryons were streaked onto plates containing threonine and lacking tryptophan and uridine. Such medium should allow growth of heterokaryotic strains or homokaryons of the *thr-3 trp-3*$^{+}$ nucleus, providing that the loss of NUO78 were not lethal to the cell (the desired homokaryons should not require uridine since they should contain the *arg-12*^{S} allele). Single colonies were isolated, allowed to conidiate and then tested for growth on minimal medium, ie., lacking threonine, tryptophan and uridine. Only heterokaryotic strains, containing both the *thr-3 trp-3*$^{+}$ and the *thr-3*$^{+}$ *trp-3* nuclei will grow on this minimal medium. Any isolates that do not grow on this medium must therefore be *thr-3 trp-3*$^{+}$ homokaryons. Such homokaryons were obtained from both 62.4 and 62.5 (Table 4·3). The results for 62.5 suggest that the nuclear ratios in this strain are strongly biased towards the RIPed nucleus in the heterokaryon. Strain 49.6, however, did not segregate *thr-3 trp-3*$^{+}$ homokaryons since all isolates grew on minimal media. In a reciprocal experiment, the three strains were tested for their ability to segregate *thr-3*$^{+}$ *trp-3* homokaryons, which would contain the sheltering nucleus only. All three strains were shown to segregate *thr-3*$^{+}$ *trp-3* homokaryons (Table 4·4), confirming that the strains were indeed heterokaryons.

Since homokaryons were obtained from the NUO78 deficient heterokaryons 62.4 and 62.5, it seemed likely that NUO78 was not essential for viability. However, the lack of homokaryons from 49.6 suggested two possibilities. Either the specific alteration in the 49.6 *nuo-78*^{RIP} allele was responsible for the lack of cell viability or a random alteration in the *thr-3 trp-3*$^{+}$ nucleus of the

Table 4·3 Generation of thr requiring homokaryons from the heterokaryotic cultures

	colonies examined	thr requiring homokaryons	heterokaryons
49.6	96	0	96
62.4	73	25	48
62.5	85	63	22

Table 4·4 Generation of trp requiring homokaryons from the heterokaryotic cultures

	colonies examined	trp requiring homokaryons	heterokaryons
49.6	49	44	5
62.4	36	11	25
62.5	21	2	19

strain affected an essential function. To distinguish these possibilities, an attempt was made to separate the *nuo-78*^{RIP} allele in 49.6 from any other unknown mutations by crossing with a wild-type strain, NCN235. Progeny were plated on complete media, and 117 germinated spores were cultured in tubes of the same medium. Since 49.6 is a heterokaryon composed of two different nuclei, each capable of undergoing nuclear fusion prior to meiosis with the NCN235 nucleus, only a fraction of the progeny spores will be the result of a fertilization with the H II nucleus. The isolates were tested for their tryptophan and threonine requirements by inoculating conidia on medium containing all required nutrients except tryptophan or threonine. Any isolates that were *thr-3*$^{+}$ *trp-3* would have arisen from fertilization by the M II sheltering nucleus, whereas the *thr-3 trp-3*$^{+}$ isolates would be the desired products from fertilization of NCN235 by the H II nucleus. Since the strain NCN235 is *thr-3*$^{+}$ *trp-3*$^{+}$, it is expected that half the progeny would be *thr-3*$^{+}$ *trp-3*$^{+}$ with their origin indeterminable. The results show that nearly half the isolates (55) were *thr-3*$^{+}$ *trp-3*, but none were *thr-3 trp-3*$^{+}$. The inability to isolate ascospores from the cross involving the RIPed nucleus may be due to underrepresentation of that nucleus in the heterokaryon, or an inability of the RIPed nucleus to progress through the cross and give rise to viable ascospores. Since some of the 62 *thr-3*$^{+}$ *trp-3*$^{+}$ spores could have contained the *nuo-78*^{RIP} allele as the result of crossing-over, primers specific for RIP sequence (see below for sequence data specific to the 49.6 RIP allele; Table 4·5 and Fig. 4·9, panel 2) were used in whole cell PCR reactions to identify the 49.6 RIP-specific *nuo-78* allele in any isolate. Only one of the three *thr-3*$^{+}$ *trp-3*$^{+}$ isolates that showed delayed conidiation in small slants tested in this fashion, 6.17, was found to contain the *nuo-78*^{RIP} allele. This result supports the notion that the nucleus containing the *nuo-78*^{RIP} allele is underrepresented in the cross.

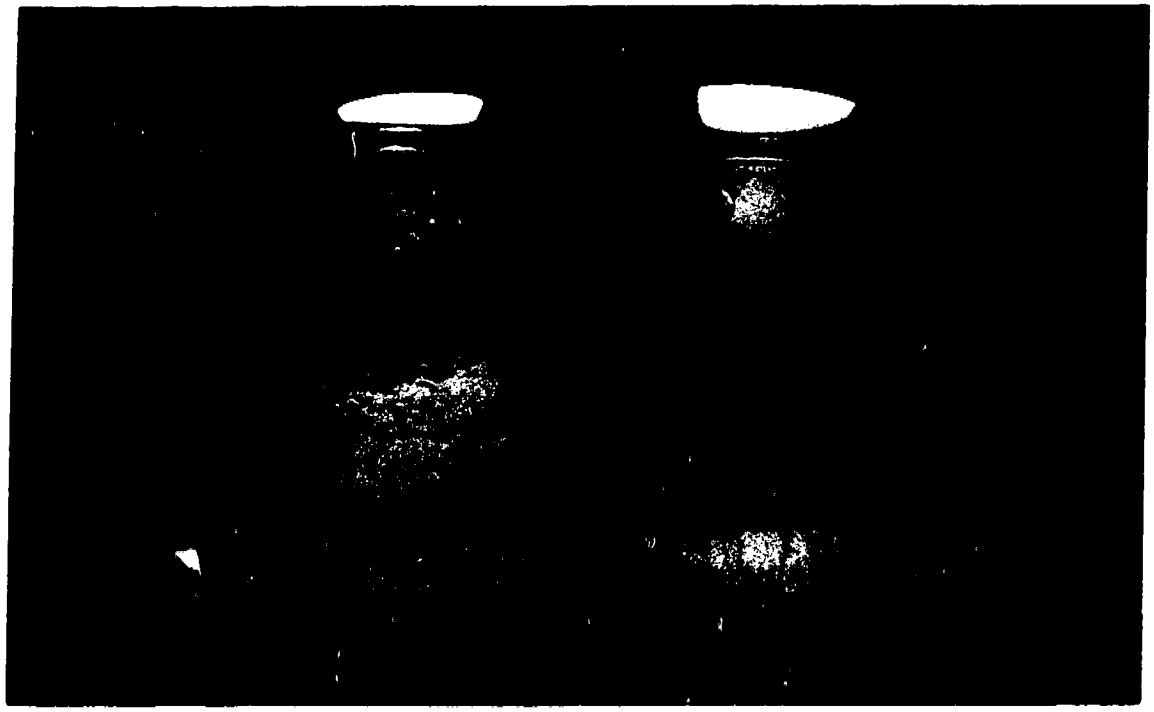
The 49.6 crossing isolate, 6.17, and the homokaryotic isolates from 62.4 and 62.5, 4.102 and 5.103 respectively, were analyzed for their NUO78 content. Mitochondrial proteins from these strains were separated by SDS-PAGE and analyzed by Western blotting with antibodies against NUO78. The results clearly show that homokaryotic isolates lacking NUO78 protein can be obtained (Fig 4·5, panel 2). These results are apparently in contradiction to the initial data (Table 4·2) that suggest NUO78 was essential. At least for 62.4 and 62.5, the difference can not be attributed to other mutations that have occurred in the RIPed nucleus, since homokaryons of that nucleus were isolated directly from the heterokaryons. This problem is as yet unresolved and is discussed in section 4·5.

To demonstrate that the poor recovery of the 49.6 RIP allele from the original (NCN235 x 49.6) cross was not due to a deleterious effect of the allele itself, but rather to unknown alterations in the 49.6 RIP nucleus that caused it to be underrepresented in the 49.6 heterokaryon, the 6.17 isolate was further crossed to the strain NCN251 and 36 ascospores isolates were obtained. These isolates exhibited a slight difference in the amount of conidiation when grown in the small slants used for picking ascospores. This difference was enhanced when the cultures were grown in conidia flasks (Fig. 4·6, panel 1). Growth of all 36 ascospore isolates in conidia flasks revealed that 16 isolates conidiated poorly while 20 conidiated normally. Western analysis on all 36 strains revealed that the isolates with poor conidiation lacked detectable NUO78 protein whereas the mitochondria from the fully conidiated strains contained NUO78. Fig. 4·6, panel 2, shows the results for 4 isolates with full conidiation and 4 with poor conidiation.

The growth rates of these 8 isolates were tested on medium containing either sucrose or glycerol as a carbon source in order to determine if growth of

Figure 4·6. Phenotype of strains deficient in NUO78. 1) Ascospore isolates from a cross between the wild-type strain NCN251 and the mutant strain 6.17, containing the *nuo-78*^{RIP} allele from the heterokaryon 49.6, were picked to small slants. Conidia from these isolates were used to inoculate flasks in order to enhance the conidiation difference seen with the isolates. The flask on the left is a fully conidiating strain (isolate 17.3) whereas the strain on the right is a poorly conidiating strain (isolate 17.2). 2) Mitochondrial proteins from four fully conidiating strains and four poorly conidiating strains were solubilized and separated on SDS-PAGE in order to determine the NUO78 content of these strains. The proteins were transferred to nitrocellulose membrane and decorated with antibody against NUO78. +, fully conidiating strain; -, poorly conidiating strain. TH 49.3 and 6.17 are control strains. Isolates 17.1 through 17.20 are different isolates of the NCN251 x 6.17 cross.

1



2

TH49.3	+
6.17	-
17.1	+
17.2	-
17.3	+
17.4	-
17.5	-
17.6	+
17.15	-
17.20	+

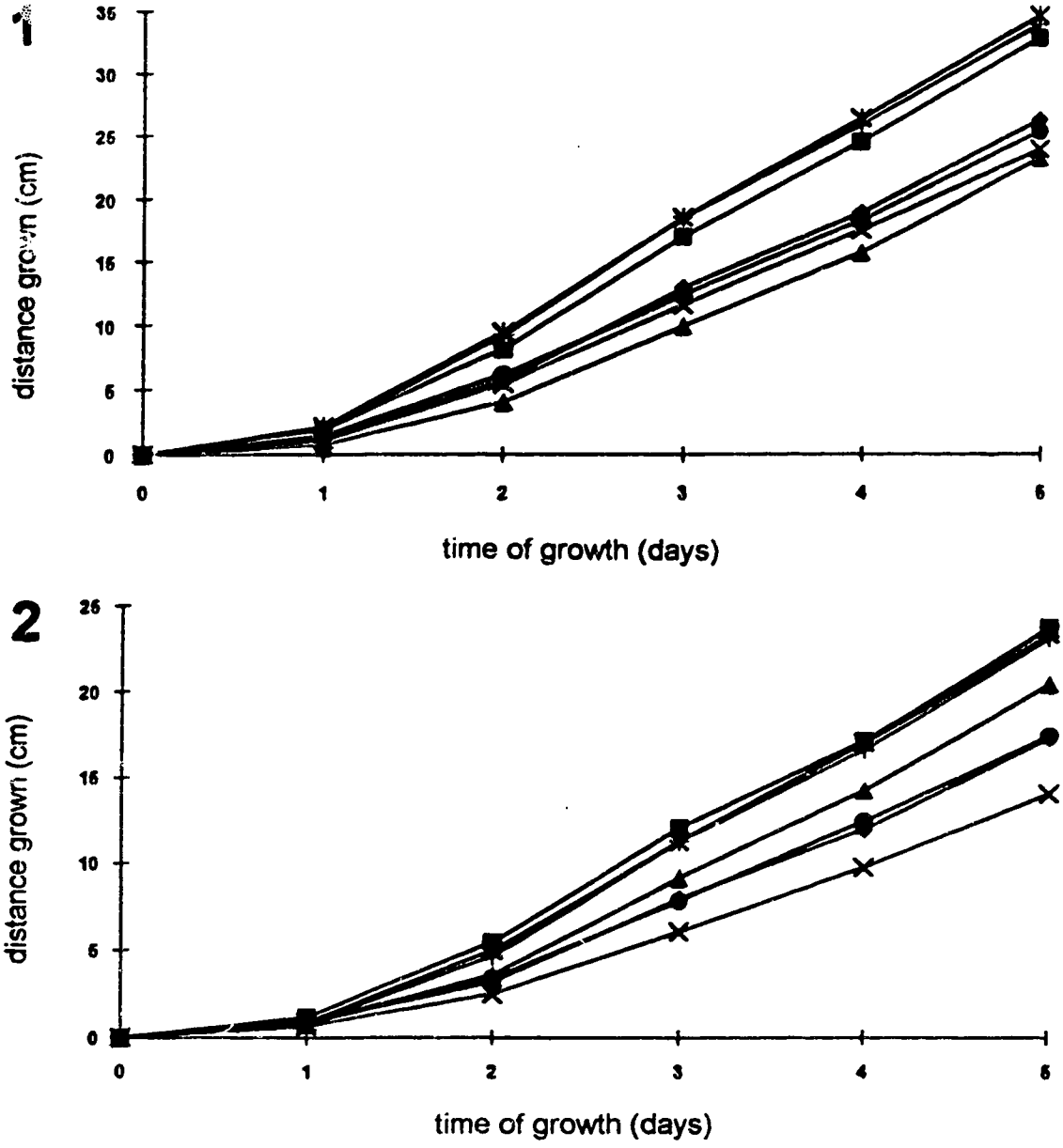


Figure 4-7. Growth rate analysis of ascospore isolates on medium containing either sucrose or glycerol as a carbon source. **1**) Growth of poorly conidiating isolates 17.2 (◆), 17.4 (▲), 17.5 (x), and 17.15 (●) compared to the fully conidiating strains 17.3 (+), 17.6 (*) and 17.20 (■) in race tubes containing sucrose. **2**) As in **1**), except glycerol was used as the carbon source.

the NUO78 deficient strains on a poor carbon source would enhance the severity of the phenotype. As shown in Fig. 4.7, panels 1 and 2, NUO78 deficient strains grow at a slightly slower rate than strains containing NUO78 regardless of the carbon source.

4·3·5 DNA analysis

To determine the number of copies of *nuo-78* in the heterokaryons, genomic DNA was isolated and digested with *Eco*RI, which does not cut within *nuo-78*$^+$ and should result in 1 band for each copy. Southern analysis, using *nuo-78*$^+$ specific sequences as a probe, demonstrated that the RIP isolates lacked the ectopic copy of *nuo-78*$^+$ (Fig. 4·8, panel 1). In order to demonstrate directly that RIP-type alterations were responsible for the loss of NUO78, genomic DNA from the homokaryotic and heterokaryotic versions of strains 49.6 and 62.5 was digested with *Hha*I, and compared to appropriate parental strains by Southern blot analysis. *Hha*I cuts the sequence CGCG with no apparent sensitivity to methylation. Therefore, this analysis will identify changes in sequence, but not in methylation patterns. The results revealed that the homokaryotic strains lacking NUO78 were altered with regard to their restriction fragment patterns, as expected for sequences that have undergone RIP (Fig. 4·8, panel 2). Cultures for DNA isolation from the 49.6 and 62.5 heterokaryons were grown under conditions that force the maintenance of the heterokaryon, ie., minimal media. A heterokaryon harbouring nuclei of equal proportion should show bands of equal intensity representing both the altered and unaltered nuclei. However, the restriction patterns seen in these isolates was consistent with the skewing of nuclear ratios mentioned previously (see Table 4·3 and section 4·3·3). The pattern for the heterokaryon, 49.6, is similar to the pattern of the

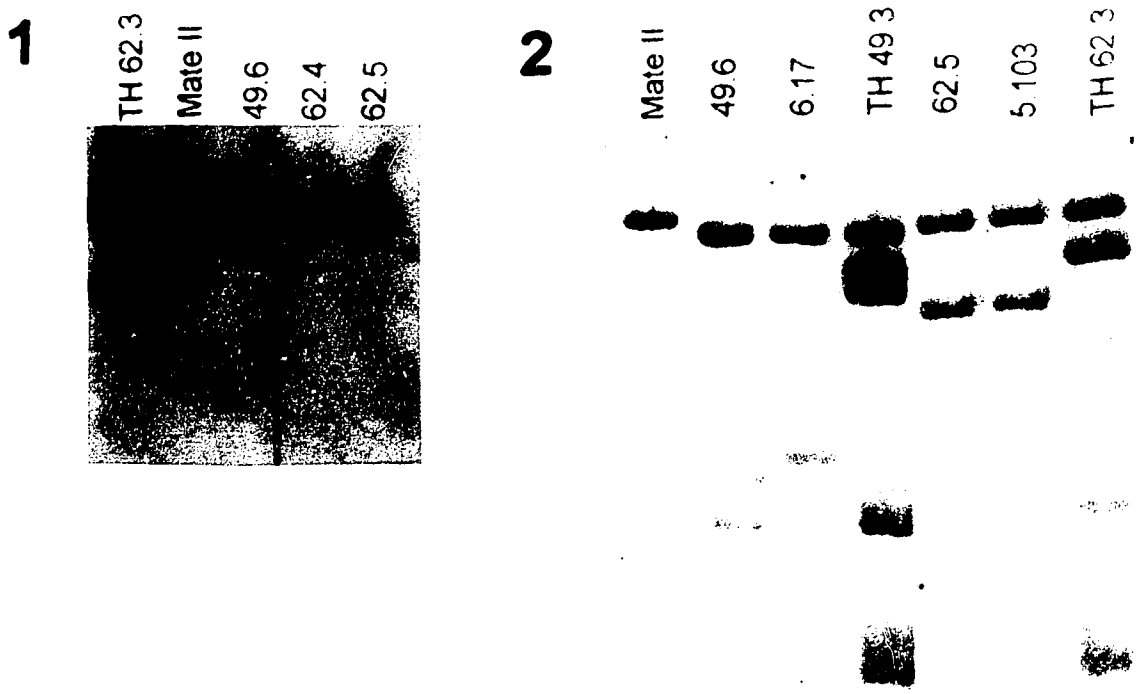


Figure **4·8**. Southern analysis of restriction digests showing the number of copies of *nuo-78* in the heterokaryotic strains and RIP in strains 49 the derived homokaryons and relevant parental strains. The pr was a radioactively labeled *nuo-78*$^{+}$ cDNA fragment as described in the legend to Fig. **4·4**. **1**) DNA isolated from the indicated strains was digested with *Eco*RI, which cuts outside of the gene. Therefore, one band should be present for each copy of *nuo-78*$^{+}$. **2**) DNA isolated from the indicated strains was digested with the restriction enzyme *Hha*I. *Hha*I is not sensitive to methylation, therefore any observed changes would be due to nucleotide substitutions only.

sheltering MII nucleus, whereas, the pattern for the heterokaryon, 62.5, is identical to the homokaryon 5.103, isolated directly from this strain.

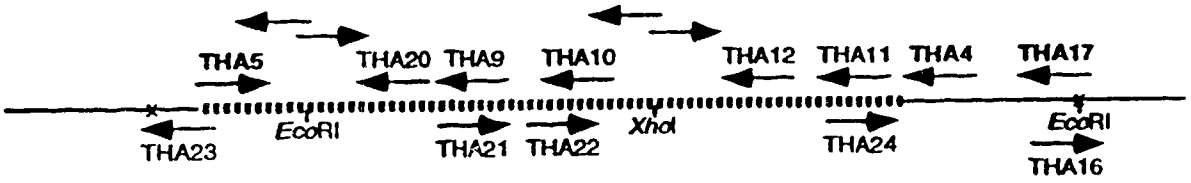
To determine the extent of the RIP alterations, the genomic sequence of the endogenous *nuo-78*^{RIP} gene, representing the RIPed allele originally obtained in heterokaryon 49.6, from ascospore isolate 6.17 was determined and compared to the previously published cDNA sequence and to our own wild-type genomic sequence (Fig. 4·9). The PCR, cloning and sequencing strategy is shown in Fig. 4.9, panel 1. The primers used are listed in Table 4·5. The sequence shown for this "49.6" allele is a composite of sequence determined from PCR amplified products of the original strain, 49.6, and the subsequent crossing isolate, 6.17, containing the same allele. Sixty-one transition mutations were revealed that were characteristic of RIP. In the case of the 49.6 allele, all were G to A. The changes would result in 39 amino acid changes, including the introduction of 4 stop codons. The sequence shown for "62.5" was obtained entirely from the RIPed allele from 5.103, which is the same allele as in the original heterokaryotic isolate, 62.5. This allele was also partially sequenced directly from PCR products. From approximately 1103 bp of sequence, 14 G to A and 27 C to T transitions were observed. These would result in 21 amino acid changes, including 3 stop codons.

The analysis of the genomic sequence in the RIPed alleles and the wild-type also revealed 5 introns, ranging in size from 66 to 106 bp, relative to the previously published cDNA sequence. Four were clustered within the first 260 bp of the cDNA sequence, upstream of the sequence used for the duplication (Fig. 4·9, panel 2). The fifth intron was at the extreme 3' end of the gene. Several discrepancies were observed between the sequence presented here and that of the published cDNA (Preis *et al.*, 1991). Three non-RIP-type nucleotide substitutions, relative to the previously published cDNA sequence,

Figure 4·9. PCR/sequencing strategy and sequence for the resident RIPed allele in the strains 6.17/49.6 and 5.103/62.5. 1) The primers designed for PCR (THA5 and THA17) flanked the region of *nuo-78*$^{+}$ used to generate the duplication in order to avoid mispairing due to any possible RIPed sequence. The PCR primer, THA4, was within the duplicated region. *Bam*HI sites were attached to THA4, THA5 and THA17 to facilitate cloning of the PCR products. When products were cloned, pBlueScript, cut with *Bam*HI, was used as the bacterial vector. When PCR products were used for direct sequencing, there was no need for digesting the products. The primers used for sequencing are also indicated and described in Table 4·4. The template used to obtain sequence 5' and 3' of the duplicated region (using primers THA23 and THA16) was created by cloning a 6.7 kb genomic fragment containing the wild-type version of *nuo-78*$^{+}$ from cosmid 3.6.2.1, isolated from one of the laboratories genomic libraries (su-1[*mi-3*]), into pUC19 to generate the plasmid pTH·H4. The arrows without names indicate sequence that was obtained by subcloning. The arrows with names indicate the location and orientation of the primers, but not the length of sequence obtained by each primer. All sequence was obtained with both short and long sequencing reactions. x, indicates the 5' and 3' ends of the *nuo-78*$^{+}$ cDNA; ▪▪▪▪▪▪▪▪, the *nuo-78*$^{+}$ gene; the duplication was generated by cloning the *Eco*RI fragment from the *nuo-78*$^{+}$ cDNA covering the C-terminal portion of the gene. 2) The *Eco*RI sites that define the extent of the cloned *nuo-78*$^{+}$ region in pGAH3 (see Fig. 4·2), which should serve as a target for RIP are underlined (at nucleotides 784 and 3150). The dashed lines above the sequence delineate the region that has been sequenced in each allele. Lower case letters indicate intron sequences. The complete sequence of the wild-type gene is shown. Bases above the wild-type sequence indicate the positions where mutations were found in the RIPed version of the resident gene, originally isolated in 49.6 or 62.5. However, the sequence shown as "49.6" is a composite of sequence obtained from 49.6 and 6.17. The sequence shown as "62.5" was derived entirely from the subsequent homokaryotic isolate, 5.103. Using DNA isolated from the homokaryotic strains as a template avoids the problem of isolating two different alleles of *nuo-78*$^{+}$, wild-type and RIPed, as was the case when dealing with the heterokaryons. The wild-type sequence obtained in this study differed from that of the published sequence (Preis *et al.*, 1991) at several locations . Bases in bold within the wild-type sequence represent bases in the published wild-type sequence that differed from the sequence obtained from this work. The changes observed in this study are indicated above the base shown in bold (on the line for "49.6") and were nonRIP-type. At four locations within the sequence, a single bp insertion/deletion was found as compared to the published sequence. Insertions are indicated by a + in the wild-type sequence with the base obtained in this study above it. Deletions are indicated by a + in sequence above the wild-type sequence. The corresponding base that is missing

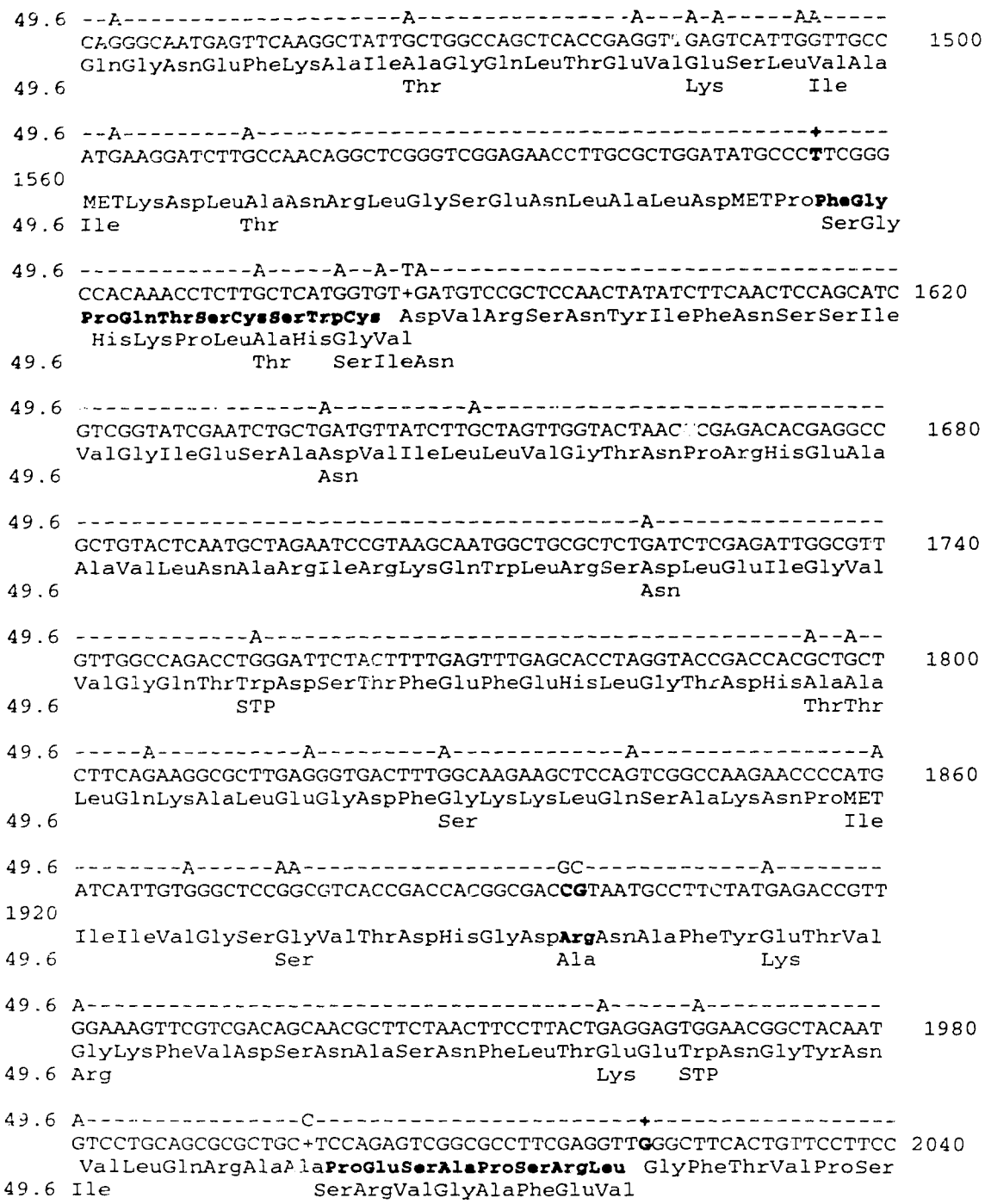
compared to the published sequence is indicated in bold below the +. The insertion/deletions were followed in all cases by a corresponding deletion/insertion that returned the reading frame to that of the published sequence. The amino acid sequence of the protein encoded by the wild-type gene is indicated immediately below the sequence. Amino acid alterations resulting from RIP mutations are shown under the normal protein sequence. Amino acids in the published wild-type sequence that are altered due to the nonRIP-type nucleotide substitutions are shown in bold with the changed amino acid below. An asterisk next to a changed amino acid indicates that the nonRIP-type alteration resulted in a closer match to the published bovine amino acid sequence (Preis *et al.*, 1991). The altered amino acids within the regions corresponding to balanced insertions/deletions do not increase the degree of homology of the wild-type sequence derived here with the published amino acid sequence.

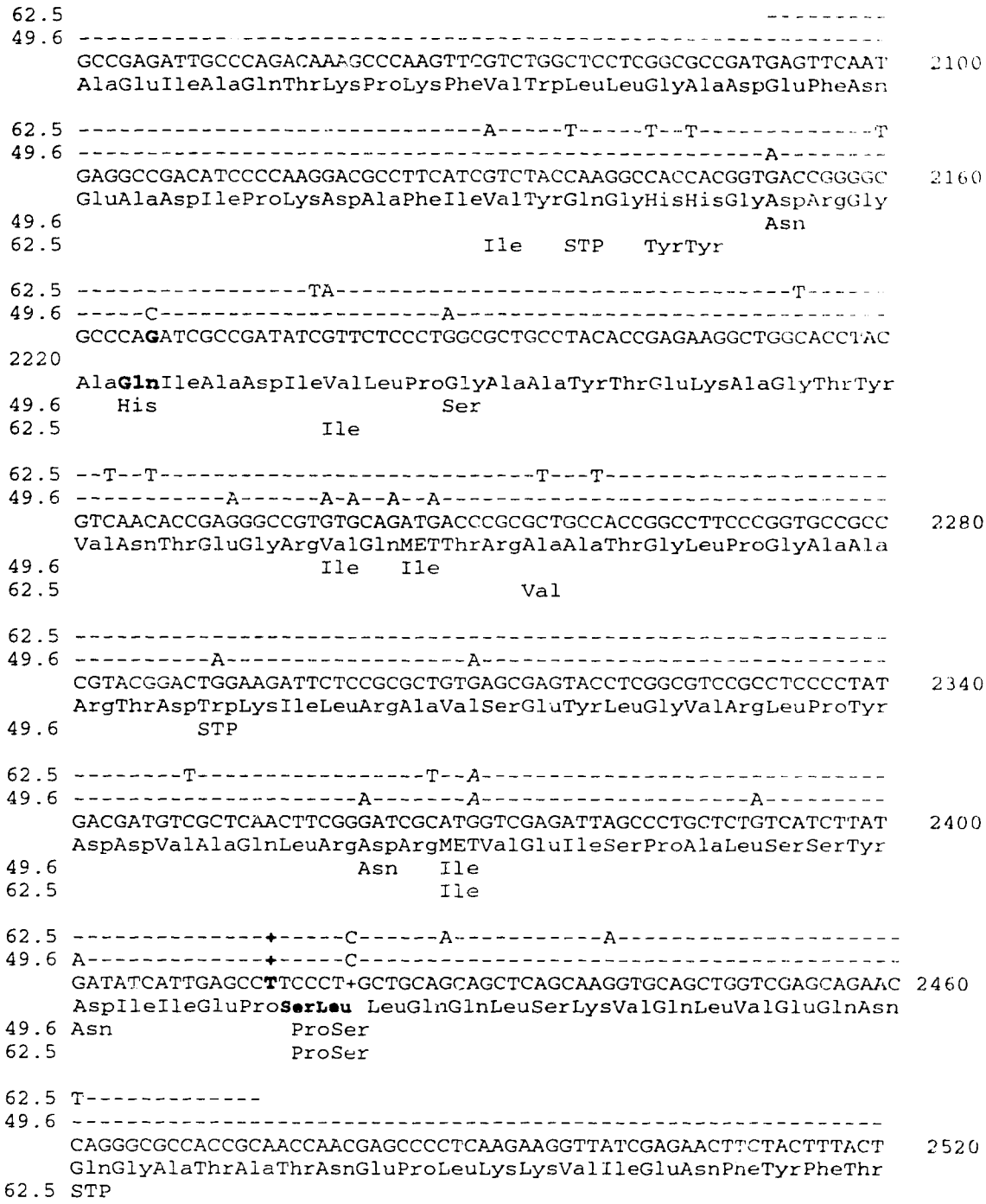
1



```
CCAAGTCCCCCTCCGACCGCCGTCGAGACGACGACACACGATGTTGAGGTCGACATTGTC      60
                                                  METLeuArgSerThrLeuSer

CCGTTCGGCTTGGCGGACTGGCAGGCATCAGGCGGCCAGGAACGCCAGCCGTGCCTTTTC     120
 ArgSerAlaTrpArgThrGlyArgHisGlnAlaAlaArgAsnAlaSerArgAlaPheSer

GGCCACAGCTCAGAGACCCGCAGACCTCGAGCTCACAATTGgtatgtctccatccagacc     180
 AlaThrAlaGlnArgProAlaAspLeuGluLeuThrIle

tccaccacatactagcatacaccgtgtatgatccccgtacccgtcggcgccgatactgac     240

tcgtgtctcccgcatctcacagATGGAAAGAAGGTCTCTATCGAGGgtgagcactcacaa     300
                       AspGlyLysLysValSerIleGlu

tgcgctgcgtcgaagtcctggctacggataatggggatgaggggggcccaaaaatacgga     360

catggtcgtcgctaatactcgaggaaaccaccagCTGGGTCGGCCTTGATCCAGGCTTGC     420
                                    AlaGlySerAlaLeuIleGlnAlaCys

GAAAAGGCCGGCGTTACCATTCCCAGgtatgcggaaaacggaccctgaatctcgggactg     480
GluLysAlaGlyValThrIleProArg

cccgtagaggttcttgctgactgttttgcagATACTGTTACCATGAgtacgtcacatgtg     540
                                    TyrCysTyrHisGlu

gctcgcccgctctcccaaattgatcgcagctccagtcactccagcaggcgtacctacgaa     600

gatcgactgacaaattgttggtaactttccagGAAGCTCATGATTGCGGGCAACTGCCGC     660
                                    LysLeuMETIleAlaGlyAsnCysArg

ATGTGCTTGGTCGAAGTCGAAAAGGTCCCGAAGCCCGTCGCGTCGTGCGCATGGCCCGTC     720
METCysLeuValGluValGluLysValProLysProValAlaSerCysAlaTrpProVal

CAACCTGGCATGGTCGTCAAGACCAACTCGCCCCTGACGCACAAGGCGCGCGAGGGTGTG     780
GlnProGlyMETValValLysThrAsnSerProLeuThrHisLysAlaArgGluGlyVal

49.6          ----T-----------------------------------------------
ATGGAATTCCTGCCCGCAAACCACCCCTTGGACTGCCCCATTTGCGACCAGGGTGGTGAG
840
METGluPheLeuProAlaAsnHisProLeuAspCysProIleCysAspGlnGlyGlyGlu
49.6             Leu*

62.5                          --------------------A-----------A-----
49.6 -GC---------A------A--A-----------------------------------------
TCGGATCTCCAGGACCAGTCGATGCGCTACGGCCGCGACCGTGGTCGGTTCCACGAAGTC
900
SerAspLeuGlnAspGlnSerMETArgTyrGlyArgAspArgGlyArgPheHisGluVal
49.6 Cys*
62.5                                                Ser          Lys

62.5 ----------G--------------------A-------------------------
49.6 ----------G-----------------------------------------------
GGCGGGAAGCAAGCGGTGGAGGACAAGAACATGGGTCCCCTCATCAAGACCTCCATGAAC
960
GlyGlyLysGlnAlaValGluAspLysAsnMETGlyProLeuIleLysThrSerMETAsn
49.6          Arg*
62.5          Arg*                  Ile
```

```
62.5 ----------------------------------------------A----------
49.6.----------A-A---A-A-----------------------------------------
     AGGTGTATTCAGTGCACGAGATGTGTGCGATTCGCGAACGATATTGCCGGCGCCCCGGAG   1020
     ArgCysIleGlnCysThrArgCysValArgPheAlaAsnAspIleAlaGlyAlaProGlu
49.6            Tyr   Lys
62.5                                                  Ser

62.5 ---------                                             ---------
49.6 ----------------------------A------A-----------------------
     CTGGGTTCGACCGGCCGTGGCAACGACCTGCAGATTGGTACCTACTTGGAGAAGAACCTC   1080
     LeuGlySerThrGlyArgGlyAsnAspLeuGlnIleGlyThrTyrLeuGluLysAsnLeu
49.6                                     Ser

62.5 ---------------A-----------------------------------------------
49.6 ---------------A----------------------------A-----------------
     GACTCTGAGCTTTCTGGTAACGTCATCGATCTCTGCCCTGTCGGTGCTCTGACCTCGAAG   1140
     AspSerGluLeuSerGlyAsnValIleAspLeuCysProValGlyAlaLeuThrSerLys
                  Ser                                 Thr
62.5              Ser

62.5 -------------------------------T---------T--------------------
49.6 ---------------------------------------------------------------
     CCATATGCTTTCCGTGCGCGCCCTTGGGAGCTGAAGAAGACGGAATCGATTGACGTTCTG   1200
     ProTyrAlaPheArgAlaArgProTrpGluLeuLysLysThrGluSerIleAspValLeu
62.5                                       Met

62.5 -----------------T------         ----A----------------T------
49.6 ----------------------------------------------A---------
     GACGGCCTGGGCTCCAACATTCGCGTCGACACTCGTGGCTTGGAGGTTATGCGCATTCTT   1260
     AspGlyLeuGlySerAsnIleArgValAspThrArgGlyLeuGluValMETArgIleLeu
49.6                                                   Ile
62.5                                    Ser

62.5 ---T----T--------------------------T--T--T--------------T---
49.6 -------------------------A----A-------------------------A-
     CCTCGCCTCAACGACGAGGTTAACGAGGAGTGGATCAACGACAAGACTCGCTTTGCCTGC   1320
     ProArgLeuAsnAspGluValAsnGluGluTrpIleAsnAspLysThrArgPheAlaCys
49.6                                 Stop                      Tyr
62.5    Cys

62.5 --------T-----------------T-----------------------------------
49.6 --------------------A-------------------------------------
     GACGGTCTCAAGACCCAGCGTCTTACCATCCCCCTAGTCCGAAGAGAAGGAAAGTTCGAG   1380
     AspGlyLeuLysThrGlnArgLeuThrIleProLeuValArgArgGluGlyLysPheGlu
49.6                    His

62.5 -T-----T--A-A----A----------------
49.6 ---------------------------------------------------------C--
     CCGGCGTCATGGGACCAGGCTTTGACCGAAATTGCACACGCTTACCAAACGCTGAACGCC
1440
     ProAlaSerTrpAspGlnAlaLeuThrGluIleAlaHisAlaTyrGlnThrLeuAsnAla
49.6                                                           Pro
62.5 Leu    LeuSTPAsn
```


49.6 --A----------A----------A---A-A-----AA-----
CAGGGCAATGAGTTCAAGGCTATTGCTGGCCAGCTCACCGAGGTTGAGTCATTGGTTGCC 1500
GlnGlyAsnGluPheLysAlaIleAlaGlyGlnLeuThrGluValGluSerLeuValAla
49.6 Thr Lys Ile
49.6 --A---------A----------+-----
ATGAAGGATCTTGCCAACAGGCTCGGGTCGGAGAACCTTGCGCTGGATATGCCCTTCGGG
1560
METLysAspLeuAlaAsnArgLeuGlySerGluAsnLeuAlaLeuAspMETProPheGly
49.6 Ile Thr SerGly
49.6 ----------A-----A--A-TA----------
CCACAAACCTCTTGCTCATGGTGT+GATGTCCGCTCCAACTATATCTTCAACTCCAGCATC 1620
ProGlnThrSerCysSerTrpCys AspValArgSerAsnTyrIlePheAsnSerSerIle
HisLysProLeuAlaHisGlyVal
49.6 Thr SerIleAsn
49.6 ----------A----------A----------
GTCGGTATCGAATCTGCTGATGTTATCTTGCTAGTTGGTACTAACTCGAGACACGAGGCC 1680
ValGlyIleGluSerAlaAspValIleLeuLeuValGlyThrAsnProArgHisGluAla
49.6 Asn
49.6 ----------A----------
GCTGTACTCAATGCTAGAATCCGTAAGCAATGGCTGCGCTCTGATCTCGAGATTGGCGTT 1740
AlaValLeuAsnAlaArgIleArgLysGlnTrpLeuArgSerAspLeuGluIleGlyVal
49.6 Asn
49.6 ----------A----------A--A--
GTTGGCCAGACCTGGGATTCTACTTTTGAGTTTGAGCACCTAGGTACCGACCACGCTGCT 1800
ValGlyGlnThrTrpAspSerThrPheGluPheGluHisLeuGlyThrAspHisAlaAla
49.6 STP ThrThr
49.6 -----A----------A---------A----------A----------A
CTTCAGAAGGCGCTTGAGGGTGACTTTGGCAAGAAGCTCCAGTCGGCCAAGAACCCCATG 1860
LeuGlnLysAlaLeuGluGlyAspPheGlyLysLysLeuGlnSerAlaLysAsnProMET
49.6 Ser Ile
49.6 --------A------AA----------GC----------A--------
ATCATTGTGGGCTCCGGCGTCACCGACCACGGCGACCGTAATGCCTTCTATGAGACCGTT
1920
IleIleValGlySerGlyValThrAspHisGlyAspArgAsnAlaPheTyrGluThrVal
49.6 Ser Ala Lys
49.6 A----------A-----A----------
GGAAAGTTCGTCGACAGCAACGCTTCTAACTTCCTTACTGAGGAGTGGAACGGCTACAAT 1980
GlyLysPheValAspSerAsnAlaSerAsnPheLeuThrGluGluTrpAsnGlyTyrAsn
49.6 Arg Lys STP
49.6 A----------C----------+----------
GTCCTGCAGCGCGCTGC+TCCAGAGTCGGCGCCTTCGAGGTTGGGCTTCACTGTTCCTTCC 2040
ValLeuGlnArgAlaAlaProGluSerAlaProSerArgLeu GlyPheThrValProSer
49.6 Ile SerArgValGlyAlaPheGluVal


62.5
49.6
GCCGAGATTGCCCAGACAAAGCCCAAGTTCGTCTGGCTCCTCGGCGCCGATGAGTTCAAT 2100
AlaGluIleAlaGlnThrLysProLysPheValTrpLeuLeuGlyAlaAspGluPheAsn
62.5
49.6
GAGGCCGACATCCCCAAGGACGCCTTCATCGTCTACCAAGGCCACCACGGTGACCGGGGC 2160
GluAlaAspIleProLysAspAlaPheIleValTyrGlnGlyHisHisGlyAspArgGly
49.6
62.5
Ile STP TyrTyr
62.5
49.6
GCCCAGATCGCCGATATCGTTCTCCCTGGCGCTGCCTACACCGAGAAGGCTGGCACCTAC
2220
AlaGlnIleAlaAspIleValLeuProGlyAlaAlaTyrThrGluLysAlaGlyThrTyr
49.6 His Ser
62.5 Ile
62.5
49.6
GTCAACACCGAGGGCCGTGTGCAGATGACCCGCGCTGCCACCGGCCTTCCCGGTGCCGCC 2280
ValAsnThrGluGlyArgValGlnMETThrArgAlaAlaThrGlyLeuProGlyAlaAla
49.6 Ile Ile
62.5 Val
62.5
49.6
CGTACGGACTGGAAGATTCTCCGCGCTGTGAGCGAGTACCTCGGCGTCCGCCTCCCCTAT 2340
ArgThrAspTrpLysIleLeuArgAlaValSerGluTyrLeuGlyValArgLeuProTyr
49.6 STP
62.5
49.6
GACGATGTCGCTCAACTTCGGGATCGCATGGTCGAGATTAGCCCTGCTCTGTCATCTTAT 2400
AspAspValAlaGlnLeuArgAspArgMETValGluIleSerProAlaLeuSerSerTyr
49.6 Asn Ile
62.5 Ile
62.5
49.6
GATATCATTGAGCCTTCCCT+GCTGCAGCAGCTCAGCAAGGTGCAGCTGGTCGAGCAGAAC 2460
AspIleIleGluProSerLeu LeuGlnGlnLeuSerLysValGlnLeuValGluGlnAsn
49.6 Asn ProSer
62.5 ProSer
62.5
49.6
CAGGGCGCCACCGCAACCAACGAGCCCCTCAAGAAGGTTATCGAGAACTTCTACTTTACT 2520
GlnGlyAlaThrAlaThrAsnGluProLeuLysLysValIleGluAsnPheTyrPheThr
62.5 STP

```
49.6 ------------------------------------------------------------
     GATGCCATTTCCAGAAGgttcgttctcttttgttacgctgacgacatctccacttccatc   2580
     AspAlaIleSerArg

49.6 ------------------------------------------------------------
     agatcataacgcagtatccactaaccgtccacaactcacagCTCCCCAACCATGGCCCGT   2640
                                              SerSerProThrMETAlaArg

49.6 ----------------G----------------------------+-----------T----
     TGCTCAGCCGCCAAGAAGACGGGTGATTCCCGAACCAACTTCATTGGCTCCGGCAT+GGAG 2700
     CysSerAlaAlaLysLysThrGlyAspSerArgThrAsnPheIleGlySerGlyMET Glu
49.6                 Glu                                AlaProAlaLeu

49.6 ------------------------------------------------------------
     GAGGATAGACCTATGGGTCAGTACGCCTATGGTGCATAGATAAGCATCAGGGATCAAGCG   2760
     GluAspArgProMETGlyGlnTyrAlaTyrGlyAlaStop

49.6 ------------------------------------------------------------
     TAGGCCAAAACAAGGCAAAGGTAAGGGGCGAATGAACAAGCAACAGACAGACAGATACAG   2820

49.6 --------------------T---------------------------------------
     CCAAACCAACAGACACTGGAACACAGTACAGTGCAGGACATATAAGACATGAAAAAAGAG   2880

49.6 ------------------------------------------------------------
     AGGAAGGAGGGTTTCCGTCTTTCCTTGCTTTCTCTTTTCAACTTGTTCTTACCGCATGCA   2940

49.6 --------------------------T---------------------------------
     TCCCAGTTGCTGCAAGTTGTTTTTTT+GAAGGACGATAAGAGACATACAGAGTTTTGTTCT   3000

49.6 ----------------------------------------------------ATCG--------
     TGCTCTTTGATTTATTTCTGTCCTAGCTTTGTGTTTATTGTGCATGTAT++++ATATTGAA   3060

49.6 ------------------------------------------------------------
     CGGATGATGTTGTGGAGGTTGTGGAGATAGGTGTTGGTTACAGTCAGGCCTAGAGTAGCA   3120

49.6 ------------------------------------
     GGGTTGTATGCAATCTCGTGCCGCTCGTGCCGAATTC   3150
```

Table 4-5 Primers used for PCR and sequencing of *nuo-78* alleles

Primer	Use	Sequence (5' to 3')
THA 4	PCR	AAGGATCCCAGTAAAGTAGAAGTTCTCG *Bam*HI 2520 2502
THA 5	PCR	AAGGATCCGAAAGAAGGGTCTCTATCGAG *Bam*HI 266 285
THA 9	sequencing	TCGTTGAGGCGAGGAAG 1274 1258
THA 10	sequencing	AGGTTTGTGGCCCGAAG 1570 1554
THA 11	sequencing	GCGATCCCGAAGTTGAG 2367 2351
THA 12	sequencing	CTCGAGTATTAGCGACG 2126 2110
THA 16	sequencing	AGTCAGGCCTAGAGTAG 3101 3117
THA 17	PCR	AAGGATCCCACGCCATGAGAGATGACTG *Bam*HI 3' flank → 5'
THA 18	RIP sequencing	CAATACACAAAATGTATGCG 970 589
THA 19	RIP sequencing	TTAATGATTTAACTTCGGTG 1495 1476
THA 20	sequencing	GGTTCTTCTCCAAGTAG 1078 1062
THA 21	sequencing	GATTGACGTTCTGGACG 1188 1204
THA 22	sequencing	ACGCTTACCAAACGCTG 1418 1434
THA 23	sequencing	CTCGAGTATTAGCGACG 383 368
THA 24	sequencing	GACGATGTCGCTCAACTTCG 2340 2359

were identified in both the 49.6 allele and the wild-type sequence that resulted in an increased similarity of the *N. crassa* amino acid sequence to that of bovine(Preis *et al.*, 1991). Three other nucleotide substitutions relative to the previously published sequence were identified that did not increase similarity. In addition, there were four single bp insertions balanced by four single bp deletions that resulted in four regions with a different amino acid sequence compared to the previously published sequence. All four regions were in areas of low identity to the bovine sequence and the changes do not increase or decrease overall similarity to the published protein sequence.

The 3' intron divided the 2.3 kb of the cDNA used to generate the duplication into a 1751 bp region containing the fifth and largest exon and a 528 bp fragment containing the last exon and the 3' untranslated region. All RIP-type transition mutations were found exclusively in the larger fragment and not in the smaller 528 bp fragment. This is in accordance with previous observations that duplications smaller than 1 kb tend to be missed by the RIP machinery (Selker, 1990) and suggests that breaks in a repeated region, such as that provided by the 84 bp fifth intron, disrupt the identification of repeated regions by the RIP machinery.

4.4 Discussion

The technique of sheltered RIP has again proved to be an efficient method to mutagenize specific genes *in vivo*. The finding from these experiments that inactivation of the *nuo-78*$^{+}$ gene has an affect in the mutant strain is the first reported demonstration in *N. crassa* that defects associated with complex I are indeed debilitating. Previous findings with *N. crassa* strains deficient in complex I subunits did not report any significant effects on growth (Nehls *et al.*, 1992; Alves and Videira, 1994). The heterokaryon generated following the

crosses involving the transformed strains and the mate strain grew at wild-type rates and fully conidiated, indicating that the deleterious effects caused by the lack of NUO78 protein is effectively complemented by products in the mate nucleus. The fact that the mutation is not lethal is not surprising when it is noted that other *N.crassa* strains defective in respiratory components, such as CoxI ([*mi-3*]; Lemire and Nargang, 1986) and cytochromes *b* (*cyb-1* and *cyb-2*; Bertrand *et al.*, 1977) and *c* (*cyt-2*, Drygas *et al.*, 1989), still grow, although at reduced rates. Furthermore, it appears that strains defective in respiratory components may be capable of up-regulating other components, such as the alternate oxidase in *N. crassa* (Lambowitz and Slayman, 1971; Edwards *et al.*, 1974), through a pathway that leads from the mitochondria to the nucleus (Butow *et al.*, 1988; Forsburg and Guarente, 1989) in an apparent attempt to compensate for deficiencies and to enhance viability. Similarly, *N. crassa* strains grown in the presence of chloramphenicol accumulate the peripheral arm of complex I and increase ubiquinone levels 5 to 8 fold (Friedrich *et al.*, 1989). An appealing hypothesis is that these cells are attempting to compensate for the reduced NADH dehydrogenase activity by increasing ubiquinone.

The initial results obtained by growing the heterokaryons on various concentrations of uridine is misleading. Growth on media lacking uridine should selectively enhance the proportion of RIPed nuclei compared to wild-type, resulting in the manifestation of a mutant phenotype. The complete lack of growth observed in these initial tests suggested that NUO78 was essential. This was a surprising result since previous complex I mutant strains of *N. crassa* did not manifest a recognizable phenotype (Nehls *et al.*, 1992; Alves and Videira, 1994) and an *A. niger* mutant strain lacking the peripheral arm simply showed a slow growth phenotype (Weidner *et al.*, 1992). In fact, the

subsequent generation of viable homokaryotic strains lacking NUO78 conclusively demonstrates that the gene is not essential.

It appears from the results described in this study that strain 49.6 contained a secondary mutation within the *thr-3 trp-3*$^{+}$ component of the heterokaryon that affected another essential gene. This mutation could have arisen spontaneously or by the integration of a fragment of DNA into an essential locus during the initial transformation. This secondary mutation resulted in a drastic under-representation of the RIPed nucleus in the heterokaryon as shown in Table 4·3. The second generation cross of the NUO78 deficient isolate from the first cross resulted in Mendelian segregation of the *nuo-78*^{RIP} allele among the progeny. This result demonstrates that the RIPed allele derived from 49.6 was not responsible for the severe phenotype observed in the 49.6 heterokaryon. These experiments also demonstrate the versatilty of the system in which nonessential RIPed alleles can be crossed into any desired genetic background for further study.

These data presented here demonstrate that *N. crassa* is an excellent organism to study the roles of various complex I subunits. Human cells lacking components of complex I suffer a number of side-effects. It is difficult to study the roles of nuclear encoded mitochondrial proteins in humans. Thus, the ability to manipulate *N. crassa* biochemically, and now genetically, allows the study of mutant nuclear genes that exhibit phenotypes which can be interpreted as being analogous to that observed in higher eukaryotes. Future work in this area might include the inactivation of the alternate dehydrogenases to elucidate their role in NADH oxidation. This would also be an ideal method to determine the functions of other subunits of complex I.

4·5 References

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5 General discussion

5·1 MOM19 deficient strains

The minimal size of the duplication required for recognition by the RIP machinery is considered to be approximately 1 Kb (Selker, 1990). With an ectopic copy of this size, approximately 10% of the progeny will be expected to undergo RIP. Furthermore, the proximity of the ectopic copy to the endogenous copy is thought to influence efficiency (Selker *et al.*, 1987). The genomic sequence used to generate the *mom-19*$^{+}$ duplication encompassed the region encoding the entire cDNA but very little flanking DNA, for a total of 1.6 Kb. It was predicted that the frequency of the RIP would be at about 10-20%. Between 20 and 100 spores were recovered from each of the crosses involving a strain with this *mom-19*$^{+}$ duplication and each was analyzed for its ability to grow on *p*-fluorophenylalanine (fpa), a toxic amino acid analog. Cells containing the RIPed version of *mom-19* carry the *mtr*^{R} allele, which blocks the transport of fpa into the cell, thereby allowing growth on fpa media unless the *mom-19*$^{+}$ gene is required for viability. It was found that only isolates obtained from the cross involving TI28.3 exhibited growth defects on fpa media. Southern analysis revealed that all tested isolates from the cross (9) involving TI28.3 contained the ectopic copy as well as the resident copy, whereas isolates from the other crosses showed that the ectopic copy did not always segregate with the endogenous copy. This suggests that the ectopic copy in TI28.3 had integrated into the chromosome harbouring the resident gene. This further implies that the proximity of the two copies in TI28.3 was sufficient to overcome the relatively small size of the repeat used for this study for recognition by the RIP machinery. It seems likely that the protein is missing in 28.17 because of rapid degradation of the altered product. However, one transition mutation was

found upstream of the translation start site. Although this alteration does not affect a known consensus sequence for transcription or translation, it is conceivable that it reduces the efficiency of either of these processes. In future experiments, use of repeats smaller than the one used here may prove to be too inefficient for practical use.

The initial finding that the cells deficient in MOM19 can still grow suggests that the protein is not essential for viability. The severe stop-start phenotype of the homokaryons does, however, indicate that cells without MOM19 suffer ill health. The demonstration that repeated sub-culturing of the homokaryons results in decreased growth can be explained by low levels of residual MOM19 that enables basal growth, while the gradual dilution of MOM19 during growth and sub-culturing leads to levels that do not support growth. The growth pattern observed for the homokaryons in race-tubes, ie. sectored growth from the old growth front, may be due to the generation of cells homoplasmic for the limited amount of MOM19-containing mitochondria. Heteroplasmic cells have been shown with time to segregate homoplasmic cultures (Hawse *et al*,. 1990), however this was observed in actively growing cultures. Furthermore, the finding that the yeast and *N.crassa* mitochondrial receptors contain repeated sequences previously shown to be characteristic of proteins involved in mitosis and interacting with the cytoskeleton (Sikorski *et al.*, 1990; Ramage *et al.*, 1993) might suggest that MOM19-containing mitochondria are selectively retained in growing cells by interacting with the cytoskeleton. This however, is purely speculative and remains to be investigated.

Growth of the heterokaryons in liquid culture containing fpa results in eventual cessation of growth that coincides with the depletion of MOM19. Furthermore, the mitochondria of these cells had undergone drastic morphological changes resulting in the loss of cristae membranes. Cristae

membranes normally contain the bulk of the respiratory complexes that produce ATP essential for *N.crassa* viability. Cytochrome spectra and western analysis revealed that the levels of inner membrane complexes are severely reduced. The inability of cytosolic proteins to be translocated across the mitochondrial membranes also renders the mitochondria incapable of protein synthesis, and presumably mtDNA replication and transcription, further reducing the mitochondrial synthesized proteins necessary for health. Moreover, the finding in yeast that the loss of the MOM19 homolog, Mas20, results in an inability to grow only on nonfermentable carbon sources, whereas the Isp42 null strains are incapable of growth on any carbon sources, suggests that enough proteins can translocate across the mitochondrial membranes in the absence of MOM19/Mas20 to maintain at least minimal levels of essential cellular operations. However, when the organism is totally dependent on mitochondrial function for energy production, the lack of MOM19/Mas20 cannot be overcome. Taken together, these findings suggest that MOM19/Mas20 is essential for mitochondrial function and cell viability in *N. crassa*.

In yeast, the *mas-20* disruptant strain could be suppressed if Mas70 was over-expressed (Ramage *et al.*, 1993). This implies that the two receptors have overlapping functions and co-operate during the translocation of precursor proteins. Furthermore, the disruption of both *mas-20* and *mas-70* in the same cell resulted in the inability to grow on any carbon sources tested, suggesting that the two receptors act synergistically in the same pathway. The *in vitro* findings with *N. crassa* do not suggest a similar conclusion. Antibodies against MOM72 did not influence the residual amount of import into isolated MOM19-deficient mitochondria, suggesting that MOM72 does not compensate for the loss of MOM19. It must be considered, however, that this experiment

was not conducted with a strain over-expressing MOM72, since such a strain does not presently exist.

The results presented in this thesis also demonstrate that MOM22, the protein that mediates the transfer of precursor proteins from the receptors to the proposed GIP (Kiebler *et al.*, 1993), is directly influenced by the presence of MOM19 and may directly interact with MOM19. The transformant strain, TI28.3, over-expresses MOM19 by about 3 times that of wild-type. MOM22 is also found to be present in the mitochondria of the transformant at levels higher than wild-type (Table 3·1). Furthermore, in MOM19-deficient strains, MOM22 is found to be reduced. Finally, import of precursors into MOM19-deficient mitochondria was found to be less dependent on MOM22 function than in wild-type. Several possibilities can be considered to explain these findings. First, the conformation of the complex may be influenced by the presence of MOM19, resulting in increased or decreased stability of some components. The N-terminus of MOM22 faces the cytosol, so conceivably, the accessibility of MOM22 to endogenous proteases may depend on the conformation of the complex. Secondly, MOM22 and MOM19 may work together to increase the efficiency of import. In the absence of MOM19, MOM22 may be incapable of efficiently fulfilling its responsibilities during import. Lastly, the GIP may be more exposed in MOM19-deficient mitochondria, allowing incoming precursors to interact directly with the translocation apparatus, thereby reducing dependency on the receptors.

5·2 NUO78 deficient strains

From approximately 20 heterokaryotic strains obtained following the sheltered RIP crosses involving TH49.3 and TH62.3 with M II, at least 3 were found to be deficient in NUO78 protein under the appropriate growth conditions.

The ability to isolate the RIPed alleles from all three heterokaryons as homokaryotic cultures indicates that cells deficient in NUO78 can survive. In view of the phenotypes displayed by other mutant cells of *N. crassa* that lack components of the respiratory chain, such as [*mi-3*] (COX I; Lemire and Nargang, 1986), [*exn-5*] (COX II; Lemire *et al.*, 1991), and *cyb-1* and *cyb-2* (complex III; Bertrand *et al.*, 1977), which display slow growth phenotypes, it is perhaps not surprising that cells deficient in NUO78 can still survive. The demonstration that the inability to fully conidiate segregates with the loss of NUO78 protein in a cross involving *nuo-78*$^{+}$ and *nuo-78*^{RIP} strains suggests that NUO78 function is important for proper conidial function.

5·3 Nature of the RIP machinery

The simplest explanation for the mechanistic action of RIP involves the deamination of cytosine and 5-methylcytosine to produce uracil and thymine. This suggests that one strand of the duplication is methylated followed by deamination of the 5-methylcytosine to produce the effects observed (Selker, 1990). Two results suggest that the RIP machinery is independent of the apparatus utilized for meiosis and general genome methylation Firstly, the observation that RIP still occurs in crosses homozygous for the *mei-2* mutation (Selker, 1990), demonstrates that pairing of the repeated sequences must occur by some other mechanism. Secondly, a methylation minus mutant in *N. crassa* is still capable of undergoing RIP (Foss *et al.*, 1993), indicating that methylation of repeated sequences is not carried out by general processes.

The observation that an 84 bp stretch of non-repetitive DNA, representing an intron of *nuo-78*$^{+}$ amidst repetitive sequences, is sufficient to break up the duplication into independent modules strengthens the idea that the RIP machinery pairs the two repeats together and initiates the mutational process on only those sequences that are homologous, as previously described

(Selker, 1990). Perhaps the "enzyme" requires contact with strands from the two repeats in order to function and any significant stretch of non-repetitive DNA may be sufficient to signal release. The observation that, 1) strains containing more than two copies of a repeat undergo RIP on two repeats at a time (Fincham *et al.*, 1989; Selker, 1990), 2) repeats as divergent as 10% still remain active targets for RIP (Selker, 1990), and 3) tandem duplications undergo RIP 100% of the time, all support a model for the RIP machinery. A RIP enzyme(s) may actively search the genome for repeated sequences and, upon identification of such a target, pair the repeats. Alternatively, physical pairing may be required for identification of repeats. In either case, the correct detection of the repeats activates the mutational activity. Tandemly aligned duplications may be the simplest to find, whereas smaller repeats dispersed throughout the genome are much more difficult to discern.

5.4 References

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