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The Isolation And Characterization Of The
Gene ro-4 That Is Responsible For A
Morphological Mutation In
Neurospora crassa

by

Malcolm John Robb, B.Sc. (Hons.)

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of
the requirements for the degree of
Master of Science

Ottawa-Carleton Institute of Biology
Carleton University
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Gene ro-4 That Is Responsible For A
Morphological Mutation In
Neurospora crassa"

submitted by Malcolm John Robb, B.Sc.
in partial fulfilment of the requirements
for the degree of Master of Science.

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Thesis Supervisor

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Chairman, Department of Biology

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ABSTRACT

The morphological *ro-4*⁺ gene, from *Neurospora crassa* has been isolated and partially characterized. The clone G21:5H possessing the gene for *pab-2*⁺ of the *N. crassa* auxotrophic mutant was isolated by complementation using the sib-selection. Clone G21:5H was used for chromosome walking to obtain the *ro-4* gene. The verification of the *ro-4* gene was carried out by isolating homokaryotic *ro-4*⁺ transformants with the *ro-4* gene, and by restriction fragment length polymorphism (RFLP) mapping. The characterization of the gene involved sequencing, RNA analysis (expression levels), observations of nuclear migration by staining with 4', 6-diamidino-2-phenylindole (DAPI) and functional disruption of the *ro-4* gene by means of repeat-induced point mutations (RIP).

The R04 protein appears to have a high homology to actin-related proteins, in particular actin-RPV, isolated from human brain tissue (Lees-Miller et al., 1992). *ro-4* transcript levels appear to be very low and through observations of RIP strains, the gene is not essential. The *ro-4* and RIP strains are similar in appearance and both display impairment of nuclear migration.

All evidence to date suggests the putative R04 protein is actin-related, forming one of the subunits belonging to a dynactin complex. The dynactin complex is an associated protein suspected of involvement with microtubule-based dynein motor proteins.

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List of Abbreviations

A_{nm}	absorbency (wavelength nm)
amp	ampere
Amp ^r	ampicillin resistance gene
ATP	adenosine triphosphate
cDNA	complementary DNA
C:I	96% chloroform, 4% isoamyl alcohol
Ci	Curie
cpm	counts per minute
DAPI	4',6-diamidino-2-phenylindole
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DEPC	diethyl pyrocarbonate
dGTP	deoxyguanosine triphosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dTTP	deoxythymidine triphosphate
DTT	dithiothreitol
EDTA	N,N-ethylenediaminetetraacetic acid
EtOH	ethanol
FGSC	Fungal Genetic Stock Centre
FITC	fluorescein isothiocyanate
Hyg ^r	hygromycin resistance gene
IPTG	isopropylthio β -D-galactoside
Kb	kilobase pair
KDa	kilodalton
MOPS	3-[N-morpholino] propanesulfonic acid
mRNA	messenger RNA
OLB	oligolabeling buffer
pI	isoelectric point
PBS	phosphate-buffered saline
P:C:I	50% phenol, 48% chloroform and 2% isoamyl alcohol
³² P	radioactive isotope of phosphorous
PEG	polyethylene glycol
pH	$-\log_{10}[H^+]$
RIP	Repeat-Induced Point
RFLP	Restriction Fragment Length Polymorphism
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
³⁵ S	radioactive isotope of sulphur
SDS	sodium dodecyl sulfate
SPB	spindle pole body
SSC	150 mM NaCl, 15 mM sodium citrate (pH 7.0)
SSPE	150 mM NaCl, 10 mM NaH ₂ PO ₄ , 1 mM EDTA (pH 7.4)

TBA-EDTA	89 mM Tris, 89 mM boric acid, 2 mM EDTA pH 8.0
TEA-EDTA	40 mM Tris-acetate pH 8.0, 2 mM EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
UV	ultraviolet
V	volts
X-Gal	5-Bromo-4-chloro-3-indolyl-beta-D-galactoside

I. INTRODUCTION

A) *Neurospora crassa* as a research organism

Neurospora crassa is a filamentous fungus belonging to the subdivision Ascomycotina. During the vegetative phase of its life cycle, *N. crassa* produces filamentous hyphae that grow away from the centre of inoculation, resulting in the formation of a mycelial mat. Asexual reproduction generates conidia and sexual reproduction generates ascospores. An inexpensive and defined medium has been established allowing both surface growth on solid agar or submerged growth.

Neurospora is an excellent organism for the study of eukaryotic molecular genetics since this organism is easily transformable. Transforming DNA usually integrates by non-homologous recombination since it can integrate at random throughout the genome often in multiple copies and in tandem repeats (Vollmer and Yanofsky, 1986; Fincham, 1989). The *Neurospora* genome has seven well mapped chromosomes with a total genome consisting of 27,000 Kb (Vollmer and Yanofsky, 1986). Any cloned DNA fragment from the *Neurospora crassa* genome can be assigned to a particular locus on the linkage map by a technique called restriction fragment length polymorphism (RFLP) mapping (Metzenberg et al., 1984). *N. crassa* also has the unique ability to disrupt duplicate sequences of DNA. The *Neurospora* genome does not consist of any known duplicate gene sequences except for rRNA. Duplicate DNA sequence disruption occurs

as a result of Repeat-Induced Point Mutations (RIP). Using this natural process one can disrupt any cloned gene to observe the resulting phenotype, if the gene was not essential (Selker, 1990).

B) The components of the fungal cytoskeleton

The cytoskeleton of fungi consist of microtubules and actin-filaments (F-actin) (Barnes et al., 1990). While intermediate filaments have been well characterized in higher eukaryotes, intermediate filament proteins have been found only recently in yeast (Madden et al., 1992; McConnell and Yaffe, 1992). Based on existing evidence, microtubules appear to play a role in nuclear division and migration, and in the stabilization of the orientation of the spindle pole body (SPB) (Barnes et al., 1990; Madden et al., 1992). F-actin appears to be located predominantly at growth sites (Drubin, 1991). In *Neurospora* this would be at the apex of elongating hyphae. F-actin is found at the bud site and developing neck in yeast, as in *Saccharomyces cerevisiae* (Drubin, 1991). The role of actin appears to be organizing surface growth elements such as chitin and membrane vesicles during cytokinesis (Barnes et al., 1990; Madden et al., 1992).

a) The role of microtubules in the fungal life cycle

A close relative of *N. crassa*, *Aspergillus nidulans*, has the most thoroughly characterized cytoskeleton among the filamentous

fungi. A total of five tubulin proteins have been isolated from *A. nidulans* to date. The five tubulin proteins consist of, two isotypes of α -tubulin, two isotypes of β -tubulin and a γ -tubulin (Morris and Enos, 1992). Although the tubulin isotypes differ from each other, they are interchangeable and do not seem to have different roles. Tubulin proteins are approximately 50-KDa. Both α and β -tubulin are assembled into microtubules (Darnell et al., 1986). γ -Tubulin has only been located at mitotic spindles. γ -Tubulin is believed to be involved in attaching microtubules to the mitotic spindle (Morris and Enos, 1992; Oakley et al., 1990).

Microtubules are involved in nuclear division, migration and bipolar SPB orientation. In yeast such as *S. cerevisiae*, it was previously suspected that microtubules were involved in selecting yeast bud sites. However, studies with conditional lethal mutations, or chemical inhibitors of microtubules such as benomyl or nocadazole have shown that bud site selection was independent of microtubules (Huffaker et al., 1988; Jacobs et al., 1988). Disruption of microtubules prevents both nuclear division and nuclear migration, leaving the budding cell without a nucleus and the mother cell uninuclear (Barnes et al., 1990). The establishment of a bud site leads to the accumulation of actin spots and the attachment of actin-filaments and microtubules that run parallel to the axis of growth (Barnes et al., 1993; Drubin, 1991).

According to Winey et al. (1993) the SPB for *S. cerevisiae* is the sole microtubule organizing centre. The SPB is constructed of microtubules approximately 4-5 nm long and is located in the

membrane of the nucleus (Darnell et al., 1986). At the initiation of mitosis the SPB duplicates. At this point they are side by side. The SPBs migrate in opposite directions until they are on opposite sides of the nucleus (Darnell et al., 1989), thus forming the bipolar SPB (Winey et al., 1993). Microtubule development begins with microtubules extending into the nucleus (spindle microtubules). Another set of microtubules also extend in the opposite direction away from the nucleus into the cytoplasm (astral microtubules) (Palmer et al., 1993). Astral microtubules are believed to secure the SPB to the bud site for proper nuclear migration into the daughter cell (Read et al., 1992). The spindle microtubules are believed to separate the duplicated set of chromosomes as they move away from each other towards the SPB on opposite sides (Barnes et al., 1990; Read et al., 1992; Winey et al., 1993; Kormanec et al., 1991).

Nuclear division and migration mediated by microtubules appears to be universal. The observations of syncytial embryos of insects has demonstrated that nuclear migration and placement are correlated with mitotic divisions (Foe and Alberts, 1983). According to Baker et al. (1993) nuclear division and migration are microtubule dependent at least during embryogenesis in *Drosophila*. Using immunofluorescence specific for microtubules, it was observed that during nuclear division and migration microtubules were very prominent (Baker et al., 1993).

b) Mutations affecting microtubule-dependent organelle migration

Evidence from initial research on *A. nidulans* and *S. cerevisiae* suggests that there is more than one mechanism involved in the migration of membrane-bound organelles (Oakley and Rinehart, 1985; Morris and Enos, 1992; Kormanec et al., 1991; McConnell et al., 1990; McConnell and Yaffe, 1992). When microtubule function is disrupted by benomyl or mutation, nuclear division and migration are prevented. However, mitochondrial migration is not affected (Oakley and Rinehart, 1985; Barnes et al., 1990).

Five temperature sensitive mutations affecting nuclear migration have been isolated from *A. nidulans*. These non-allelic mutations have been termed *nud*, for nuclear distribution. Under non-permissive temperatures, nuclear migration is prevented while mitochondrial migration is not significantly inhibited (Oakley and Rinehart, 1985; Meyer et al., 1988; Osmani et al., 1990). Filamentous fungi such as *N. crassa* and *A. nidulans* appear to have a regulated distribution of nuclei along the growing hyphae (Morris and Enos, 1992). Impairment in the cytoskeleton or motor proteins can lead to a disruption of proper nuclear migration. Strains of filamentous fungi displaying such mutations have germinating conidia containing several nuclei, while the hyphae display a few clumped nuclei or no nuclei at all (Oakley and Rinehart, 1992; Morris and Enos, 1992). If the cytoskeleton is the source of the mutation, and nuclear division is still intact, then the cytoplasmic microtubules of the SPB are probably the site of the

mutation. However, no disruption of the microtubule and actin cytoskeleton have been observed (Meyer et al., 1988; Osmani et al., 1990). If the cytoskeleton is intact, the defect in nuclear migration is likely to be in the motor proteins or their associated factors (Morris and Enos, 1992). Of the five mutations in *A. nidulans* that display the *nud* mutation, only *nudC* has been partially characterized. *nudC* produces a 22-KDa protein with a sequence that matches no existing protein (Osmani et al., 1990; Morris and Enos, 1992). In *N. crassa* the *ropy* mutant strains also have problems with nuclear migration.

Several similar mutations have been found in *S. cerevisiae*. Two examples of these temperature sensitive mutations are *mdm1* and *mdm2*. *mdm1* will retard both nuclear and mitochondrial migration, while *mdm2* only retards mitochondrial migration (McConnell et al., 1990; McConnell and Yaffe, 1992). These results suggest that mitochondrial and nuclear migration are the result of two different mechanisms (Oakley and Rinehart, 1985; Morris and Enos, 1992). Microtubules have been established as the highways for nuclear migration (Goldstein and Vale, 1992). What transports the mitochondria is not well understood. Cytoplasmic streaming was one idea, but this motive force should also be capable of transporting nuclei. Others have hypothesized that other variations of microtubules may exist that do not use β -tubulin and are resistant to benomyl, or that the actin cytoskeleton is involved with short fragments of microtubules (Oakley and Rinehart, 1985).

c) Actin

Actin can be grouped into two main divisions, major actins and minor actins. The major actins consist of two classes, class I and class II, which differ only at the amino terminus (Herman, 1993; Reisler, 1993). Class I actins are found in non-muscle cells (β and γ) and smooth muscle (γ). The non-muscle cells include lower eukaryotes such as fungi and protozoa. The class II actins (α) are found predominately in the skeletal muscle cells of vertebrates. Class III actins are actin isoforms, suspected of existing throughout the eukaryotic spectra (Herman, 1993). The recently discovered class III actins differ also at the amino terminus, but there are also greater deviations in some of the conserved regions described in the discussion (Reisler, 1993).

Major class I and II actins, come in two forms: G (globular) and F (filamentous). G-actin is an individual monomer protein. G-actin is a 45-KDa protein with dimensions of 3 X 5 X 6 nm, possessing a large and small domain separated by a cleft (Reisler, 1993; Steer; 1990). Actin is highly conserved across the eukaryotic spectrum. The comparison of the amino acid sequences of actin between all species shows approximately 85% amino acid similarity including comparisons between fungi and vertebrates. In the conserved regions, the similarities increase to 94%. Major actins are thought to be essential since disruption of the yeast Act1 gene is lethal (Herman, 1993).

A 43-KDa actin protein from *N. crassa* has been identified by

using anti-actin antibodies (Barja et al., 1991). 2-D gels revealed three actin isoforms, α , β and γ . The majority of actin detectable was the β isoform. Using FITC-phalloidin staining, actin cables were detected mainly in the hyphal apex. Actins spots were also detected randomly throughout the hyphae, similar to what is observed in the yeast. Cytochalasin A and B exposure prevented germination (Barja et al., 1991). The major actin gene from *A. nidulans* has been isolated by Fidel et al. (1988), only the sequence is complete. The exact role of the major actin in filamentous fungi is not known.

Filamentous actin (F-actin) exist as long thin filaments that participate in the formation of the cytoskeleton. The filaments are made of two parallel chains of G-actin wound around each other to form a helical structure (Darnell et al., 1980, Heath, 1990). Observed under an electron microscope, actin filaments bound with meromyosin head fragments gives the filament a chevron appearance leaving the ends with distinct features. The ends of the filaments are referred to as pointed (P) or barbed (B). The barbed end is able to bind G-actin, increasing the length of the filament. Filament formation is dependent on ATP and ion concentrations especially magnesium. Filament formation can also be stopped or reversed (filament dissociation) (Steer, 1990; Reisler, 1993).

d) The role of F-actin in hyphal extension

Actin filaments are one of the components involved in the

formation of the cytoskeleton. The other structures are microtubules and intermediate filaments. The cytoskeleton forms a 3-D network structure that is believed to support the membrane bound organelles in space. The two known non-structural roles of the actin cytoskeleton are cytoplasmic streaming and amoeboid locomotion (Darnell et al., 1986; Reisler, 1993).

Amoeboid pseudopod extension is believed to be the result of similar mechanisms as exist in hyphal extension. Amoebas have a high turnover rate for their plasma membrane (Steer, 1990). The membrane that is lost is being replaced by advancing golgi vesicles found at the tips of pseudopods and endocytosis at the rear. At the tips of the advancing pseudopods are found microfilaments attached to the plasma membrane running parallel to the pseudopod. Further back is found a network of actin and myosin thought to produce the motive force (Darnell et al., 1986; Steer, 1990).

Many physical and chemical processes are involved in hyphal extension. F-actin is believed to play a major role in this process. Evidence for this fact is the presence of abundant actin filaments found in the apical region of growing tips (Barja et al., 1991; Steer, 1990). Hyphal tip growth can be considered to involve two processes: first is the synthesis of the cell wall and plasma membrane which requires the exocytosis of the secretory vesicles at the point of hyphal extension (Barja et al, 1991). The other involves maintaining the cylindrical morphology characteristic of fungal hyphae as growth continues. The hyphae are also subject to directional change depending on the path of least resistance.

A logical question that arises, is what elements inside the cell are responsible for vesicle transport (Steer, 1990)? Microtubules are not found in the apical zone of the hyphae so they are not suspected of vesicle transport in the apex (Steer, 1990). However, microfilaments are quite concentrated at the apex. Interestingly, cytochalasins inhibit vesicle transport and exocytosis in fungi and tip growing cells of plants (Harold and Harold, 1986; Barja et al., 1991). Without vesicle transport, the tips may be starved for raw materials and their growth restricted.

One characteristic difference between the pseudopod extension of an amoeba and hyphal extension is the involvement of turgor pressure (Steer, 1990). The newly forming cell wall is a labile structure and the plasma membrane requires support or it could rupture, due to turgor pressure. The actin cytoskeleton is believed to provide this support by its attachment to the plasma membrane. The cytoskeleton is believed to be involved in determining cell shape and mediating hyphal extension in the same manner as extending pseudopods in amoeba (steer, 1990). These suggestions might have credibility due to the high concentrations of actin filaments and actin-associated proteins at the apex (Heath, 1990; Steer, 1990). As tip growth continues, new cytoskeletal links to the plasma membrane must be generated (Heath, 1990), as in the amoeba (Steer, 1990). It has been suggested that actin is recycled at the tip. As the actin cytoskeleton begins to leave the apical zone, it is disassembled to G-actin and sent back up to the apex to form new filaments (Steer, 1990).

Anderson and Soll (1986) state that in non-growing hyphae, actin is evenly distributed throughout the structure. Growing hyphae, have high concentrations of actin located at the apex. One apparent correlation is that the species with faster growing hyphae have higher concentrations of actin than their slower growing counterparts. *Candida albicans* in its stationary phase can be induced to bud when actin concentration is localized at the bud formation site. Hyphal extension can be induced by the presence of actin at the hyphal tip (Anderson and Soll, 1986).

The processes of tip growth can also be regulated by the distribution of ionic concentration. One ion of interest is Ca^{++} . High concentrations of Ca^{++} will slow or stop hyphal extension by competing with magnesium for ion binding sites on G-actin (Reisler, 1993). Actin's ability to hydrolyse ATP, to provide the energy used in filament formation is impaired in the presence of high Ca^{++} concentrations (Kraft et al., 1992). Ca^{++} can also activate dissociating proteins like gelsolin (Reisler, 1993).

C) The class III actin genes and proteins

Class III actin proteins have similar characteristics to class I and II actins (Herman, 1993). To date the best characterized class III actin protein is ACT2, an actin isoform isolated from *S. cerevisiae* (Schwob and Martin, 1992; Herman, 1993). The most prominent feature of ACT2 is the lack of looping domains necessary for binding other actin proteins, DNase I and myosin. The class III

actins appear to have 47% to 66% homology with the major actins. ACT2 is also slightly larger, possessing 391 amino acids as opposed to the 375 amino acids for the ACT1 (Herman, 1993; Schwob and Martin, 1992). The minor actins are more basic with pI's around 6.5 to 6.9. ACT1 is required for vegetative cell growth and has a role in sustaining cell shape and polarized cell growth. A disrupted *Act2* gene results in single cells that possess large buds. The cell cycle has been arrested at this stage. Cell growth has been arrested before completion of the first cell division. Disruption of the *Act1* gene results in single cells that possess no buds or very small ones (Schwob and Martin, 1992).

Other class III actin proteins have recently been discovered. A few examples are: ACT2 from *S. pombe* (Helfman et al., 1992), actin-RPV originally isolated from human brain tissue (Lees-Miller et al., 1992) and centractin (Clark and Meyer, 1992). The existence of actin-RPV is evidence that minor actins are also conserved in vertebrates. The exact role of actin-RPV is not well understood, but it is suspected to be involved with microtubule motor proteins (Lees-Miller et al., 1992). A virtually identical protein, centractin is associated with the centrosomes. Centractin and actin RPV can both theoretically hydrolyse ATP and bind divalent cations (Clark and Meyer, 1992; Lees-Miller et al., 1992; Goldstein and Vale, 1992). Some of the roles for class III actins could involve the securing of centrosomes to actin filaments and the mediation of microtubule and actin filament interaction (Herman, 1993; Goldstein and Vale, 1992).

D) Microtubule-associated motor proteins

The forces that propel membrane organelles along microtubules in extending hyphae or cells going through mitosis are thought to be generated by the motor protein complexes (Bloom, 1992; Vale, 1990).

a) The motor protein kinesin

The most thoroughly researched of these proteins are kinesin and dynein. Kinesin is distributed throughout eukaryotic cells and is comprised of four subunits. Two of the subunits are approximately 124-KDa and belong to a family of structurally related proteins involved in ATPase activity (Bloom, 1992; Vale, 1990). The site of ATP hydrolysis takes place in a highly conserved domain, 40% conserved in the family of similar proteins. The larger subunits are believed to be the source of the motor activity and binding sites that adhere to the microtubules, and the light chains are suspected to be the sites for binding to the membrane-bound vesicles (Bloom, 1992). The structure of the smaller subunit, which has an α -helical coiled-coil domain, supports this hypothesis. Kinesin is suspected of being one representative of a family of similar proteins (Bloom, 1992). Kinesin has been shown to be specific for plus end directed movement along the microtubules. The velocity of movement generated by kinesin is approximately $.5 \mu\text{m/s}$ (Kelly, 1990; Vale, 1990). However, evidence is surfacing that suggests kinesin or other proteins similar in structure can move in

both the plus and minus directions. At present, kinesin does not seem to require any associated proteins for motility (Bloom, 1992).

b) The motor protein dynein

Dynein is a much more complex motor protein. This protein is composed of two heavy subunits of approximately 300-KDa or 4460 residues and several smaller subunits such as three or four 70-KDa and four light 50-KDa subunits (Gill et al., 1991). The heavy subunits possess 4 ATP binding sites. The purpose of the smaller subunits has yet to be resolved and vary from species to species. Dynein has been shown to move membrane organelles in the minus direction along microtubules (Vale, 1990; Schroer and Sheetz, 1991). Dynein, isolated from detergent lysed amoeba *Reticulomyxa*, generates a velocity of approximately 10 $\mu\text{m/s}$ (Bloom, 1992). Hydrolysis of ATP is required to generate movement. The dynein isolated from *Reticulomyxa* appears to produce movement in both directions. All other dyneins which have been isolated have shown movement in only the minus direction. The dual directional movement for the dynein from *Reticulomyxa* could be due to different isoforms. If it is the same protein, then this could be evidence of dual directional movement. Dynein could also be a mitotic motor (Bloom, 1992). Plus and minus directional motors have been localized to the kinetochores. This minus directed dynein would move the chromosomes at the metaphase plate towards the SPB at anaphase (Bloom, 1992; Kelly, 1990).

c) Motor associated proteins

At present, the known roles for cytoplasmic dynein are: neurite extension, mitotic movements and vesicle movements via the cytoskeleton (Schroer et al., 1989). Experiments using in vitro assays have demonstrated that soluble factors associated with dynein are necessary to generate force (Schroer and Sheetz, 1991; Gill et al., 1991). Dynein supplies the force requirements and the soluble components (activators) appear to govern the magnitude and velocity of movement. Two main activators, 1 and 2, have been described as regulators of cytoplasmic dynein activity (Schroer and Sheetz, 1991). Purified dynein alone is immobile when bound to membrane material. The addition of activator 1 results in only basal motility and addition of activator 2 restored full activity to the dynein complex (Schroer and Sheetz, 1991). Recognizing that activator 2 is involved in velocity enhancement, and observing the correlation between velocity and ATPase requirement, suggests that activator 2 has a possible role in ATPase activity. Activator 1 is a 20S polypeptide complex called dynactin. This complex is composed of a doublet each with a mass of 150-KDa (Gill et al., 1991).

It has been demonstrated in vitro that actin-RPV is a major component of the dynactin complex (Lees-Miller et al., 1992). This association suggests its involvement in stimulating dynein movement for the translocation of membrane vesicles on microtubules. The dynactin complex is composed of a 160-KDa and 150-KDa dynactin subunit with the polypeptides of sizes, 62 ,50, 45, 37 and 32-KDa

(Gill et al., 1991; Lees-Miller et al., 1992). The 45-KDa subunit is the most abundant averaging 8 copies per complex. Immuno blot experiments with antibodies specific for actin, which do not cross react with actin-RPV demonstrate that actin is also present in the dynactin 45-KDa subunit. The 45-KDa subunit is believed to be composed of actin-RPV as well. Evidence for this comes from V8 protease digestion of peptides for amino acid sequencing. Also, antibodies directed against actin-RPV were detected in the 45-KDa subunit complex of the dynactin complex (Lees-Miller et al., 1992).

F) Objectives

The intent of this research was to isolate and characterize the gene *ro-4*, believed responsible for generating a morphological mutant in the fungus *Neurospora crassa*. The *ro-4* mutant displays a club- of broccoli-like appearance generated from hyphae that wind around each other. Hyphal pigmentation and conidial development are reduced with aerial hyphae that have a tendency to clump (Perkins et al., 1982). The morphology of this mutant appears to be caused by a defect in nuclear distribution as deduced by nuclear staining. The experimental approach was to first isolate the *pab-2* gene by sib-selection. The *pab-2* mutant is an auxotrophic mutant that is incapable of synthesising *p*-aminobenzoic acid (Perkins et al., 1982). Since the *ro-4* gene is in close proximity (<1 map unit) to the *pab-2* gene, chromosome walking was applied to isolate the *ro-4* gene.

II. METHODS AND MATERIALS

A) Strain types and growth conditions

a) Growth and maintenance of *Escherichia coli*

Strains DH5 α -MRC, DH5 α F' (supE44 Δ lacU169 (ϕ 80 lacZ Δ M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1) and BB4 (supF58 supE44 hsdR514 galK2 galT22 trpR55 metB1 tonA Δ LacU169 F'[proAB⁺ lacI^q lacZ Δ M15 Tn10(tet^r)] were grown and maintained on LB medium (1% tryptone, .5% yeast extract, 1% NaCl, .2% glucose and 1.5% agar) and when required ampicillin was added at a final concentration of 100 μ g/ml (LBA). For maximum growth rates, the bacterial cultures were incubated at 37°C and maintained at 4°C as they were or transferred to other LB plates as stab cultures.

b) Growth and maintenance of *N. crassa*

The main strains used were the wild-types (allele #: 74-OR23-1A, Fungal Genetic Stock Center (FSGC) #: 987 and allele #: 74-OR8-1a, FSGC #: 988), the auxotrophic mutant *pab-2* (allele #: H193, FSGC #: 4076) and the morphological mutant *ro-4* (allele #: B38, FSGC #: 2980). Other strains included the RFLP strains, two parental and eighteen progeny, FSGC numbers 4411-4430. The parental strain FSGC 4411 is an Oak Ridge laboratory strain possessing the markers: *al-2*, *nuc-2*, *arg-12*, *cot-1* and *inl-2*. The other parental strain, FSGC

4416, is a wild isolate Mauriceville-1c-A. All *Neurospora* strains were grown on Vogel's minimal medium (per liter: 15 ml 50 X Vogel's stock medium (per litre: 127 g Na_3 citrate $\cdot$ 2H $_2$ O, 250 g KH_2PO_4 , 100 g NH_4NO_3 , 10 g $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 5 g of $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ predissolved in 25 ml H $_2$ O added dropwise), 1 ml biotin stock, 1 ml trace elements, 15 g sucrose and 15 g agar) or modified Vogel's medium, the RFLP strains required the extra nutrients 1 mM L-arginine and 50 $\mu\text{g/ml}$ inositol. Crossing medium, which is similar to Vogel's has a reduced nitrogen content (per litre: 10 g NH_4NO_3). SFG medium, also similar to Vogel's except for the replacement of sucrose, with 1.5% sorbose, .2% fructose and .2% glucose. Selective conditions, when required used Hygromycin B at a final concentration of 150-200 $\mu\text{g/ml}$. Usually *N. crassa* was grown at room temperature and 37°C was used for transformations. *Neurospora* was stored on slant tubes at 4°C, cultures stored in this way were good for about 2 months.

B) DNA extraction and purification

a) Cosmid DNA purification using the alkaline method

The procedure for cosmid purification is a modified version of one outlined in Sambrooke et al. (1989). Cosmid DNA purification was identical for 96 and 8 clone pools and single clones. Bacteria grown up on LBA medium supplemented with chloramphenicol were collected by centrifugation and resuspended in 5 ml of solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, 1 mM EDTA). The following

solutions were added to the resuspended bacteria: .5 ml lysozyme solution (10 mg/ml lysozyme, 10 mM Tris-HCl pH 8.0) plus 10 ml of solution II (.2 N NaOH, 1% SDS). The solutions were mixed by inverting the tubes several times and then incubated at room temperature for 10 minutes. The basic solution was neutralized by adding 7.5 ml of solution III (5 M potassium acetate pH 4.5), the tubes again were mixed by inversion till the phases were homogeneous and placed on ice for 10 minutes. The solutions were centrifuged at 1935 RCF X g for 15 minutes at 4°C. The pellet, which contains cellular debris and SDS was discarded. The supernatant was transferred to a sterile polyalomal tube and a .6 volume of isopropanol was added. The solutions were incubated at room temperature for 10 minutes followed by centrifugation at 4340 RCF X g for 15 minutes at room temperature. The supernatant was discarded and the tubes were inverted, allowing most of the supernatant to drain off. The pellet was resuspended in 3 ml T₁₀E₁ (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and then 3 mls of 5 M LiCl was added. The samples were incubated on ice for 10 minutes followed by centrifugation for 10 minutes at 12,100 RCF X g at 4°C. The supernatant was transferred to a clean polyalomal tube and mixed with an equal volume of isopropanol. The solution was incubated for 10 minutes on ice and centrifuged at 12,100 RCF X g at 4°C for 10 minutes. The supernatant was discarded and the pellet briefly air dried and resuspended in 500 µl of T₁₀E₁. To the resuspended pellet was added 1 µl of 10 mg/ml of RNase and incubated at room temperature for 30 minutes. The cosmid DNA was transferred to a 1.5

ml microfuge tube and pelleted out by adding 500 μ l of 13% PEG (8,000), 1.6 M NaCl, mixing and centrifuging in an Eppendorff micro-centrifuge at 14,000 rpm for 5 minutes. The pellet was resuspended in 400 μ l of $T_{10}E_1$ and extracted once with an equal volume of phenol, phenol:chloroform and chloroform. The DNA was precipitated by adding 100 μ l of 10 M ammonium acetate and 2 volumes of 100% EtOH and collected by centrifugation at 14,000 rpm for 5 minutes at room temperature. The pellet was washed in 70% EtOH, dried under vacuum and resuspended in 100-200 μ l of T_1E_1 (1 mM Tris-HCL pH 8.0, .1 mM EDTA). Concentration was measured at A_{260} , A_{280} using the Hewlett Packard, 8452A diode array spectrophotometer.

b) Extraction and purification of *N. crassa* genomic DNA

Genomic DNA was isolated and purified using the procedure outlined by Metzenberg and Baisch (1981). *Neurospora* was grown in overnight shake cultures in 50 ml liquid Vogel's minimal medium at room temperature. The mycelia were harvested by vacuum filtration, washed with dH_2O and pressed dry using paper towels. The mycelia were placed at $-70^\circ C$ for six hours and lyophilized under vacuum for 8 hours using a speed vac (model, Savant SC100). The lyophilized mycelia were pulverized using a pestle and mortar with approximately 1 g of acid washed sand. To the pulverized mycelia was added 3.6 ml of TEA-EDTA extraction buffer (50% TEA-EDTA, .5% triton X-100, .25 mg/ml pre-digested pronase, 50 mM NaCl). The crude DNA solution was transferred to a 50 ml polyalomar tube and

incubated at room temperature for 2 to 3 days with occasional agitation. Following the incubation, the crude DNA solution was centrifuged at 5,000 RCF X g for 15 minutes at room temperature and the supernatant was transferred to a fresh 50 ml polyallomer tube. The DNA was precipitated by adding 10 ml of 100% EtOH. The supernatant was discarded, the pellet was briefly dried and 750 μ l of low salt buffer (1 mM Tris-HCl pH 7.4, 1 mM NaCl, .1 mM EDTA) was added to each pellet. Once the pellet was resuspended, 4.5 ml of ethanolic perchlorate reagent was added and incubated at room temperature for 30 minutes. The DNA was pelleted by centrifugation at 5,000 RCF X g for 10 minutes at room temperature. The pellet was once again dissolved in low salt buffer, followed by the addition of 375 μ l of high salt buffer (25 mM Tris-HCl pH 7.4, 100 mM NaCl, 2 mM EDTA) containing 300 μ g/ml of pre-boiled RNase A. The DNA solutions were incubated at 37°C for 2-3 hours. The DNA solutions were extracted once with an equal volume of C:I. The aqueous phase was transferred to a fresh 15 ml corex tube to which was added .54 volume of isopropanol. The DNA was collected by centrifugation at 12,100 RCF X g for 5 minutes at room temperature. The pellet was washed with 70% EtOH, dried under vacuum and resuspended in 1 ml $T_{10}E_1$.

c) Rapid preparations of *Neurospora* genomic DNA

This procedure of preparing *Neurospora* genomic DNA followed the techniques outlined by Raeder and Broda (1985). Fifty mg of freeze

dried mycelium, prepared in the same manner as the standard genomic DNA prep above, was ground using a spatula and a small pestle in a 1.5 ml microfuge tube. The ground mycelia was suspended in 500 μ l of extraction buffer (.2 M Tris-HCl pH 8.5, .25 M NaCl, 25 mM EDTA and .5% SDS). To the resuspended mycelia was added pre-digested pronase to achieve a final concentration of .5 mg/ml, followed by 350 μ l of phenol and 150 μ l of chloroform. After mixing by inversion, the crude DNA solution were centrifuged at high speed for 1 hour. The aqueous phase was transferred to a fresh microfuge tube containing 25 μ l of RNase (20 mg/ml) and incubated for 30 minutes at 37°C. After the incubation, an equal volume of C:I was added, mixed and centrifuged at high speed for 10 minutes. Following centrifugation, the aqueous phase was transferred to a fresh microfuge tube and .54 volume of isopropanol was added. The precipitated DNA was allowed to settle out to the bottom of the tube for 1 hour at 4°C. Most of the supernatant was removed, and the DNA was recovered by centrifugation. The pellet was washed with 70% ethanol, dried and resuspended in 100 μ l T₁₀E₁.

d) Rapid plasmid preparations

Rapid plasmid preparations were carried out by a modified version of the method outlined by Sambrooke et al. (1989). Single colonies were used to inoculate 3 ml of LB medium containing 100 μ g/ml of ampicillin. The cultures were grown overnight at 37°C at 200 rpm. Half of the overnight culture (1.5 ml) was transferred to

a fresh 1.5 ml microfuge tube and centrifuged at 1935 RCF X g for 5 minutes at room temperature. The recovered cells were resuspended in 100 μ l $T_{10}E_1$ and incubated at room temperature for 5 minutes. Two hundred μ l of .2 N NaOH, 1% SDS was added to the solution of resuspended cells. The cells were mixed thoroughly by inverting the tubes several times and placed on ice for 5 minutes. To the lysed cells was added 150 μ l of 3 M potassium acetate pH 4.8, mixed by a 2 second vortex at high speed and placed on ice for 5 minutes. The samples were centrifuged at high speed for 2 minutes at room temperature. The supernatant containing the plasmid DNA was transferred to a new 1.5 ml microfuge tube containing 900 μ l of 100% cold EtOH. The samples were placed on ice for 10 minutes and centrifuged at high speed for 5 minutes at room temperature. The plasmid pellet was washed once with 70% EtOH. The pellet was dried under vacuum and resuspended in 20 μ l of $T_{10}E_1$. The resuspended DNA could be treated with 1 μ l of 1 mg/ml RNase. This procedure allows rapid plasmid isolation that can be used for restriction enzyme analysis and DNA sequencing.

e) MagicTM plasmid preparations

The procedure was carried out as recommended by the manufacturers instructions, Promega. Overnight cultures grown in LBA medium were prepared in the same manner as described for rapid plasmid preps. The pelleted cells from the overnight culture were resuspended in 200 μ l of resuspension solution, $T_{10}E_1$. When the

cells were thoroughly resuspended 200 μ l of cell lysis solution .2 N NaOH, 1% SDS was added and mixed thoroughly by inversion several times. Neutralization solution was added, mixed well by inversion and centrifuged at high speed for 2 minutes. The supernatant was transferred to a fresh 1.5 ml microfuge tube. One ml of MagicTM DNA purification resin was added to each sample and mixed thoroughly. DNA purification was achieved by passing the DNA solution through a MagicTM prep mini-column. The DNA was eluted from the column with 50 μ l of water or T₁₀E₁.

C) Techniques used to isolate the *ro-4* and *pab-2* gene

a) Spheroplast preparation

Spheroplast preparation followed the procedure outlined by Vollmer and Yanofsky, (1986). One-week cultures of *N. crassa*, grown in 250 ml flasks containing Vogel's minimal medium, were harvested for their conidia by adding 25 ml of sterile water to each flask swirling vigorously, and then transferring the water to the next flask of the identical strain. Enough flasks were prepared to ensure the collection of enough conidia, $\approx 10^9$. The *ro-4* mutant has a reduced level of conidial development, therefore 4-5 flasks of conidiating mycelium were required. The flasks were usually rinsed with another 25 ml of water and transferred in series to the rest of the flasks. The conidia were filtered through nylon stockings into 250 ml centrifuge bottles and centrifuged at 6,500 rpm using

a GS3 rotor at 4°C for 5 minutes, resuspended in liquid Vogel's minimal medium and transferred to 500 ml flasks. The conidia were germinated for 4-6 hours with shaking using a gyrotory water bath shaker (New Brunswick Scientific, model G76) at 200 rpm and 30°C. The germinated conidia were transferred to 250 ml centrifuge bottles and collected by centrifugation at 6,500 rpm using a GS3 rotor at 4°C for 5 minutes. They were washed once in 60 ml sterile water and once in 40 ml 1 M sorbitol. The cell walls of the germinated conidia were removed by adding 14 ml of filter-sterilized 1 M sorbitol containing 20 mg of Novozyme 234 (Novo Biolabs). Novozyme 234 is a mixture of enzymes such as glucanases, chitinases and proteases capable of removing the cell walls of most fungi. The Novozyme solution was added directly to the centrifuge bottles, and the conidia were resuspended and incubated at 30°C with gentle shaking for 1 hour. At this stage the germlings were converted to spheroplasts. The spheroplasts were collected by centrifugation at 480 RCF X g, 4°C for 5 minutes. The spheroplasts were then washed once in 30 ml of 1 M sorbitol followed by two more washes in 30 ml of 1 M sorbitol, 50 mM Tris-HCl pH 8.0, 50 mM CaCl_2 . After completing the washes, the spheroplasts were resuspended in 16 ml of 1 M sorbitol, 50 mM Tris-HCl pH 8.0, 50 mM CaCl_2 and 4 ml PEG (4000), 50 mM Tris-HCl pH 8.0, 50 mM CaCl_2 and 200 μl DMSO. The spheroplasts were dispensed into 1 ml aliquots and stored at -70°C.

b) Sib-selection (*pab-2* gene isolation)

Sib-selection was carried out as described by Vollmer and Yanofsky, (1986). Sib-selection employed an *N. crassa* genomic library prepared by Orbach and Sachs, (1991). The genomic library was constructed using the cosmid vector pMOcosX (figure 1), a modified version of pDVCos constructed by Evans et al. (1989). The insert, genomic DNA fragments were isolated from *N. crassa* strain 74-OR23-1A and partially digested with *Mbo*I to generate fragments of genomic DNA ranging in size from 40-50 Kb. The packaging of the ligated vector with genomic DNA was carried out with Stratagene gold II and Stratagene XL extracts. The Stratagene gold II and XL extracts are high efficiency packaging extracts for the construction of genomic libraries. The gold II extracts have a packaging efficiency of 2×10^9 pfu/ μ g, packaging genomic DNA fragments in the size range of 38-51 Kb. The XL extracts has a lower packaging efficiency of 1×10^9 pfu/ μ g, packaging genomic DNA fragments in the size range of 47-51 Kb. The host bacteria used to propagate the clones was *E. coli* strain DH5 α -MCR.

The library consists of 50 microtiter plates each with 96 wells. The library consists of two sub-libraries, the G and X-library, each represented by 25 microtiter plates. The G-library was prepared using the gold II extracts and the X-library was prepared using the XL extracts. A single clone can be identified by the plate number (1-25) of either library, followed by the column number of the selected plate (1-12) and finally the horizontal row

Figure 1: The cosmid vector pMOcosX.

Restriction map of cosmid pMOcosX constructed by Orbach and Sachs, (1991). Displayed are the locations of the hygromycin resistance gene (Hyg^r), the double cos sites (cos) and the site of the genomic DNA insert. The genomic DNA insert is represented by the solid box. Note, that the insert DNA can be completely removed using the restriction enzymes *NotI* or *EcoRI*.

(A-H).

Sib-selection using the described library required the preparation of DNA from three types of cosmid-clone pools. Initial transformations were carried out with 96-clone pools of DNA from all the clones in an entire microtiter plate. Positive transformations with one of the 96-clone pools required the construction of twelve 8-clone pools from the suspect microtiter plate. Transformations were repeated with these 8-clone pools. The 8-clone pools generating positive transformation were identified and DNA from each of the eight single-clones was prepared. Transformations were again repeated and the single clones giving a positive transformation were identified and isolated for further study.

To prepare the pools of 96 cosmid clones, a 96-pin replicator was used to inoculate a 150 mm petri dish containing LBA medium. After overnight growth at 37°C, 6 ml of liquid LBA medium containing 2 mg/ml of chloramphenicol was added to the surface. Overnight incubation was repeated at 37°C. The cells were harvested using 6 ml of STF (100 mM sucrose, 10 mM tris-HCl pH 8 and 1 mM EDTA). The cells were pelleted at 3020 RCF X g for 5 minutes at room temperature followed by a wash using 10 ml of STE and centrifuged under the same conditions and resuspended in solution I. At this point cosmid DNA purification could proceed or the cells could be stored at -20°C until required.

The 8-clone pools were prepared similarly. A 96-pin replicator was used to inoculate a 150 mm petri dish containing LBA medium.

After overnight growth at 37°C, each column of bacteria was transferred to a separate 100 ml petri plate containing LBA. Each single colony from the column of bacteria was streaked onto an eighth of the plate. The rest of the procedure follows what has been outlined for the 96-clone pool DNA preparation. The exception was that 4 ml of LBA containing 4 mg/ml of chloramphenicol was added per plate.

The preparation of DNA from single clones was identical to the 8-clone pool method except that individual colonies were each streaked onto a separate 90 mm LBA plate.

c) Transformation of the spheroplasts

The transformation procedure follows the method outlined by Vollmer and Yanofsky, (1986). Five μ l of 1 to 2 μ g of cosmid DNA, 2 μ l of 50 mM spermidine-3HCl and 5 μ l of 50 mM heparin, 1 M sorbitol, 50 mM Tris-HCl pH 8.0, 50 mM CaCl₂ were added to a 1.5 ml microfuge tube. One hundred μ l of spheroplasts were added to the cosmid mixture, the solutions were gently mixed by up and down pipetting and incubated on ice for 30 minutes. Following the incubation, 1 ml of 40% PEG (4000), 50 mM Tris-HCl pH 8.0, 50 mM CaCl₂ was added and incubated at room temperature for 20 minutes. One hundred to 500 μ l of transformation mix was added to $\approx$ 10 ml of top agar (regular Vogel's medium containing 1 M sorbitol) at 50°C, mixed and poured onto 25 ml of vogel's minimal medium, containing 150 μ g/ml of hygromycin B. The plates were incubated at room

temperature for two days. Two days was sufficient to observe the distinct growth of positive transformed mutants such as *pab-2*.

D) Chromosome walking from *pab-2* to *ro-4*.

a) Construction of dot blot hybridization filters.

Dot blots were prepared using a modified protocol described by Sambrooke et al. (1989). Twenty μ l samples of DNA from each of the 96-clone pools, at a concentration range of .25 mg/ml to .50 mg/ml, were placed separately into the wells of a 96 well microtiter plate. This DNA was denatured by adding 20 μ l of 1M NaOH, 3M NaCl. The cosmid DNA solutions were incubated at room temperature for 30 minutes. After the incubation period, 40 μ l of 1.5 M NaCl was added giving each well a final volume of 80 μ ls.

Two pieces of N-Hybond nylon filter papers were cut to the size of the dot blot apparatus (12 X 9 cm Bio Rad). The 12 X 9 cm filter papers were placed on the dot blotter one at a time. To each well was added 40 μ l of denatured DNA solution. A slight vacuum was applied forcing the DNA in solution to pass through the filter paper. Upon completion, the filter papers were removed, washed in 2 X SSC and air dried. Each blot contained DNA from the entire stratagene genomic library.

The second filter type used to complete the chromosome walking permitted individual cosmid clones to be identified. A 96-pin replicator was used to stamp a microtiter plate and transfer the

inoculum to an N-Hybound filter cut to the size of a 150 mm petri plate placed on top of LBA medium. Once the bacterial colonies had grown to the desired size, the colonies were lysed and the DNA was denatured on the filter paper.

b) Probe preparation for chromosome walking.

The cosmid clone G21:5H, one of the four clones capable of a positive transformation for *pab-2* was used as a source for the probes. Since *EcoRV* and *BglIII* do not digest the cosmid vector, they were used to remove most of the insert DNA of G21:5H. *EcoRV* was selected because it removed most of the genomic insert DNA leaving end fragments of the sizes 5 Kb and 2 Kb. The clone G21:5H digested with *EcoRV* was self-ligated and amplified in *E. coli* strain DH5 α F'. After purification of the self-ligated G21:5H, *EcoRV* and *NotI* were used to remove the 5 Kb and 2 Kb fragments from the cosmid vector. The genomic DNA fragments were isolated by the freeze squeeze technique from a .8% agarose gel. The isolated DNA fragments were used to construct probes as described on page 36.

E) Preparation of competent cells

Three protocols were used: CaCl₂ treated cells, the Hanahan procedure and the one step preparation method.

a) CaCl_2 cell treatment method

The CaCl_2 method of preparing competent cells follows the method outlined by Sambrooke et al. (1989). Overnight colonies of *E. coli* strain DH5 α F', grown at 37°C on LB medium, were used to inoculate liquid 50-100 ml LB medium. The cultures were grown at 37°C at 200 rpm to an $A_{600} \approx .5$. The cells were placed into ice-cold 50 ml polyalomar tubes and centrifuged at 1935 RCF X g for 10 minutes at 4°C. The supernatant was discarded and the tubes inverted for one minute to drain the last traces of LB. The cells were resuspended by briefly vortexing in 10 ml of ice cold .1 M CaCl_2 and placed on ice for 15 to 30 minutes. The cells were recovered by centrifugation at 1935 RCF X g at 4°C for 10 minutes. The supernatant was discarded and the cells were resuspended in 2 ml of ice cold .1 M CaCl_2 , 15% glycerol for every 50 ml culture. The resuspended cells were dispensed in 200 μ l aliquots using a cold pipette tip, placed into cold 1.5 ml centrifuge tubes and incubated on ice for 1 to 4 hours. After incubation, the cells could be used immediately for transformation or stored at -70°C.

b) The procedure of Hanahan

This procedure, developed by Hanahan et al. (1983), is used to generate highly competent cells.

Using a flamed wire loop, *E. coli* strain DH5 α F' was streaked onto SOB (2% tryptone, .5% yeast extract, .05% NaCl, 2.5 mM KCl and

10 mM MgCl_2) plates from a frozen stock (-70°C). The cells were incubated at 37°C for 16 hours. Isolated colonies were placed into 50 ml of liquid SOB medium containing 20 mM MgSO_4 in a 500 ml flask. The cells were dispersed by vortexing. The cultures were grown to a density not exceeding 10^8 cells/ml at 37°C with shaking at 200 rpm. A_{600} readings after the first hour of incubation were taken every 20 to 30 minutes to ensure that the cell concentration does not exceed the limit. The cells were transferred to ice-cold 50 ml polyalomar tubes, placed on ice for 10 minutes and then recovered by centrifugation in an SS34 rotor at 4000 rpm at 4°C for 10 minutes. The medium was discarded and the tubes inverted for 1 minute to remove any remaining media. The cells were resuspended in 20 ml of ice cold transformation buffer (FSB) by gentle vortexing. The resuspended cells were incubated on ice for 10 minutes and recovered by centrifugation at 1935 RCF X g at 4°C for 10 minutes. The FSB was decanted and the cells resuspended by gentle vortexing in 4 ml of ice-cold FSB per 50 ml culture. Once the cells were resuspended, 140 μl of DMSO was added, swirled and placed on ice for 15 minutes. An additional 140 μl of DMSO was added, swirled and placed back on ice. The cells were loaded into 1.5 ml ice-cold microfuge tubes in 100 μl aliquots. The competent cells were stored at -70°C until required.

c) The one step preparation of competent cells

The one-step preparation of competent cells was done according

to the methodology of Chung et al. (1989). Overnight colonies of strain DH5 α F', grown on LB medium, were placed into 50 ml of liquid LB medium and grown to an A₆₀₀ of .3 to .4 (early exponential phase). Using this procedure, cells rapidly lose their competency past an A₆₀₀ of .4. The cells were collected and pelleted by centrifugation at 1935 RCF X g at 4°C for 10 minutes. The cells were resuspended by gentle swirling in 1/10th the original volume in ice-cold transformation buffer 10% w/v PEG (3350 MW), 5% DMSO and 30 mM MgSO₄ in LB media pH 6.5 (TSS). The cells were then dispersed into 1.5 ml ice-cold microfuge tubes in 100 μ l aliquots. The cells were ready for use or storage at -70°C. Cells prepared using this technique had a reasonable transformation frequency of 10⁵-10⁶ transformants per μ g of plasmid DNA. This technique of producing competent cells replaced the CaCl₂ treatment method based on its simplicity.

F) Techniques for DNA analysis and manipulation

a) Freeze-squeeze isolation of DNA from agarose gels

DNA fragments were separated and purified from agarose gels as outlined by Tautz and Renz, (1982) and Vogelstein (1986). DNA bands were cut from .8% agarose gels, placed in a 1.5 ml centrifuge tube and equilibrated with 10 volumes of .3 M sodium acetate, 1 mM EDTA at room temperature in the dark for 15-45 minutes. Each gel slice was transferred to a 500 μ l centrifuge tube packed with glass wool

with a hole punctured in the base. The 500 μ l centrifuge tube was inserted into a 1.5 ml centrifuge tube and stored at -20°C for 15 minutes. The gel slices were then centrifuged for 10 minutes starting at a slow rate increasing to the maximum centrifugation rate. The eluted DNA solution was transferred to a sterile microfuge tube. The DNA restriction fragments were precipitated by the addition of 2.5 volumes of 100% cold EtOH and 1/100 volume of 1M MgCl_2 , 10% acetic acid solution, placed on ice for 15 minutes and then centrifuged at high speed. The DNA pellet was washed with 70% EtOH, .1M sodium acetate pH 6.0, then in 100% EtOH and dried under vacuum. The DNA was resuspended in a small volume of T_1E_1 and spun again to pellet out residual agarose. The DNA prepared by this manner was used mainly for subcloning and DNA probes.

b) DNA ligation to cloning vectors

This procedure was performed as outlined by Sambrooke et al. (1989). For cohesive ends, approximately 100 ng of insert DNA (isolated with freeze squeeze technique) (3 μ l) was mixed with 100 ng of vector DNA (1 μ l), however these volumes can vary depending on DNA concentration. To the linearized vector and insert DNA was added four μ l of 5 X ligation buffer (50 mM Tris-HCl pH 7.6, 10 mM MgCl_2 , 5% PEG 8000, 1 mM ATP, 1 mM DTT), 13.5 μ l of water and .5 μ l of T4 DNA ligase. The ligation reactions were allowed to proceed at room temperature for 3-4 hours. Blunt end ligations were identical except .2 μ l of T4 DNA ligase was used and larger amounts of insert

DNA were added.

c) Transformation of Bacteria

This procedure was performed as outlined by Sambrooke et al. (1989). One or two hundred μ l of competent cells, thawed and kept on ice was added to 2.5-5 μ l (40 ng) of ligated DNA. The cells were gently shaken and placed on ice for 30-40 minutes. The transformed cells were heat shocked at 42°C for 2 minutes, 90 seconds for the Hanahan procedure and not at all for the one step procedure, and placed back on ice immediately. To permit expression of the ampicillin resistance gene, 1 ml of LBA was added to the transformed cells followed by incubation at 37°C for 1 hour. The cells at this stage were ready to be plated or stored at 4°C.

The identification of subcloned DNA using the cloning vectors pBluescript or pUC, was made using 50 μ l of X-Gal and 10 μ l of IPTG spread onto LBA plates with the transformed bacteria. Bacterial colonies containing self-ligated cloning vectors appeared blue, and colonies with subcloned DNA fragments appeared white.

d) The preparation of ^{32}P labelled probes

The preparation and purification of ^{32}P labelled DNA probes follows the outline prepared according to Feinberg and Vogelstein (1984).

One hundred to 200 ng of linearized template (2 μ l) was added to

80 ng of random hexamer primers pd(N)_6 (2 μl) although volumes can vary depending on concentration. The mixed template solution was boiled for two minutes and quick chilled on ice. Two μl of 10 X OLB (.5 M Tris-HCl pH 6.9, 100 mM MgSO_4 , 1 mM DTT), 2 μl of .5 mM dTTP, dCTP, dATP, 9 μl of H_2O , 1 μl of Klenow enzyme and 2 μl of (α - ^{32}P) dGTP (3,000 Ci/mmol from Dupont) was added to the template solution in the order described. The labelling reaction was incubated at room temperature in a protective environment for 2 hours. After stopping the reaction with 2 μl of .25 M EDTA, 3 μl of tRNA (10 mg/ml) and 76 μl of STE was added. The crude probe solution was run through a G-50 spun column removing free nucleotides. The purified probe solution was counted on a scintillation counter and before use was denatured by boiling for two minutes followed by a quick chill on ice.

e) Southern blotting and analysis by hybridization

i) Southern blotting

Southern blotting and hybridization was performed essentially as outlined in Sambrooke et al. (1989). After electrophoresis, the agarose gel was placed in denaturing solution (.4 M NaOH, 1 M NaCl) and slowly swirled on a shaker for 30 minutes. The denaturing solution was replaced with neutralizing solution (1.5 M NaCl, .5 M Tris-HCl pH 7.2) and the gel was slowly swirled for 30 minutes.

During the 30 minute incubation, a Hybond-N (Amersham) nylon membrane, two Whatman 3 MM filter papers and a stack of paper

towels were cut to size of the gel. A Whatman 3 MM filter paper had to be cut that would form a wick between a reservoir (containing 10-20 X SSC) and the gel. The wick was placed on an acrylic block to elevate the gel above the reservoir. After treating the gel with neutralization solution, the gel was transferred to the filter paper to be used as the wick. The nylon filter was placed in dH_2O , then 2 X SSC briefly. The nylon membrane was placed in direct contact with the gel. The two Whatman filter papers cut to size of the gel were placed on top of the nylon filter, followed by the stack of paper towels not exceeding 6 cm in height. The stack of paper towels was compressed with a glass plate and 1-1.5 Kg weight. The reservoir was filled with 10 to 20 X SSC. After the overnight capillary transfer, the nylon membrane was removed and rinsed in 2 X SSC and air dried. After the membrane was thoroughly dry, it was wrapped in saran wrap and exposed to short wave UV for 2-5 minutes.

ii) Pre-hybridization

The DNA-bound, Hybond-N nylon membrane was placed in a hybridization tube with pre-hybridization solution (6 X SSC, 5 X Denhardt's solution, .5% SDS and denatured salmon sperm DNA). The volume of pre-hyb solution added was calculated out to be .2 ml/cm² of the size of the membrane to be probed. Pre-hybridization was carried out in a hybridization chamber (Robbins Scientific model 310) for 1 to 4 hours at 68°C.

iii) Hybridization

The hybridization solution was identical to the pre-hybridization solution except the volume added corresponds to .05 ml/cm₂ for the size of the membrane. Approximately 150,000 cpm of probe was added per ml of hybridization solution. Hybridization was carried out for at least 12 hours using the same apparatus as for pre-hybridization. After hybridization the membrane was washed four times at 68°C: twice with 50 ml per membrane of 2 X SSC for 15 minutes, once with 50 ml of 2 X SSC, .1% SDS for 30 minutes and finally once for 10 minutes in 50 ml of .1 X SSC at 68°C.

After the high stringency wash, the membrane was wrapped in saran wrap and exposed to X-ray film.

G) Techniques used to demonstrate that the correct gene has been isolated

a) Preparation of homokaryon transformed strains

The procedure for isolating homokaryons was carried out as described by Fincham (1989). Conidia are usually multinuclear, possessing 2 to 3 nuclei each. The initial transformants of *N. crassa* usually have only one nucleus per conidium being transformed.

Transformed strains were identified under selective conditions. Second generation conidia were isolated from transformed strains grown from individual conidia. The process was repeated by

isolating individual conidia from the second generation under selective conditions. This repeated plating and selection resulted in the isolation of conidia that should have contained only the original transformed nuclei.

b) Construction of a Restriction Fragment Length Polymorphism (RFLP) map

The RFLP mapping procedure was carried out as described by Metzenberg et al. (1984). RFLP strains, consisting of two parentals and 18 progeny, were from the FGSC. The parental strains have different genetic backgrounds. Parental strain 4411 is an Oak Ridge laboratory strain possessing the markers: *al-2*, *nuc-2*, *arg-12*, *cot-1* and *inl-2*. The other parental strain, 4416, is a wild isolate Mauriceville-1c-A. The restriction enzymes *EcoRI*, *HindIII*, *BglII*, *SmaI* and *PstI* were tested to search for a useful RFLP near the *ro-4* gene. The DNA from the parental strains was digested with the above mentioned enzymes and loaded onto a .8% agarose gel. Each well was loaded with 4 μ g of digested DNA. The DNA was Southern blotted onto N-Hybound membranes and probed with linearized pMRB1 or pMRSB1 labelled with 32 P. *PstI* was the most suitable enzyme making a clear distinction between the two parental strains. All 20 strains were digested with *PstI*, Southern blotted and probed as outlined for the parental strains.

H) Techniques applied to characterize the *ro-4* gene

a) Subcloning restriction fragments from the *ro-4* gene

The 4.3 Kb fragment containing the *ro-4* gene was isolated from X9:2B using the restriction enzymes *NotI* and *BglII* and cloned into pBSSK+ (designated pMRNB1). The smallest fragment generated by restriction enzymes capable of positively transforming *ro-4* was a 3.9 Kb fragment (clone pMRSB1) using the restriction enzymes *SmaI* and *BglII*. A restriction map of pMRSB1 is displayed in figure 2, which was prepared by the techniques outlined by Kovacic and Wang (1979).

b) The generation of deletion clones using exonuclease III

Exonuclease III digestions were carried out following the procedure outlined by Sambrooke et al. (1989). The restriction enzymes *KpnI* and *HindIII* were chosen for the double digest of pMRNB1 since they do not cut into the target DNA. *HindIII* cuts closer to the target sequence leaving a recessed 3' terminus, and *KpnI* digestion leaves a four base, 3' overhang blocking exonuclease III activity and thereby preventing digestion into the cloning vector.

Upon completion of the restriction enzyme digestion, the linearized DNA was purified by extracting with an equal volume of phenol:chloroform. The aqueous phase was transferred to a fresh 1.5

Figure 2: Restriction map of the cloned 3.9 Kb DNA fragment containing the ro-4 gene, pMRSB1.

The restriction sites are: *Sma*I (S); *Eco*RV (E); *Pst*I (P); *Xho*I (X) and *Bgl*II (B). The open boxes represent the approximate location of the open reading frames. The open reading frames represent 1,140 nucleotides. The direction of transcription is left to right. The depressions between the open boxes are suspected introns. The arrows numbered 1-11 represent the most useful deletion clones for sequencing.

.5Kb

ml microfuge tube and the DNA was precipitated by adding 1/10th the volume of 3 M sodium-acetate pH 5.2, and 2 volumes of 100% EtOH. The solution was mixed by inversion several times and placed on ice for 10 minutes. The DNA was collected by centrifugation at high speed for 5 minutes at room temperature. Following the first spin, the supernatant was discarded and the pellet was washed with .5 ml of 70% EtOH. The pellet was dried under vacuum and resuspended in 30 μ l of 1 X exonuclease buffer (10 X exonuclease III buffer: .66 M Tris-Cl pH 8.0 and 66 mM $MgCl_2$). The solution was stored on ice until required. In order to obtain digestions throughout the desired fragment, twelve 1.5 ml microfuge tubes were placed on ice each containing 7.5 μ l of S1 reaction mixture, S1 nuclease (60 units), 10 X S1 buffer and 172 μ l of water. To the DNA solution in exonuclease buffer was added 2 μ l of 180 units/ μ l of exonuclease III enzyme. Approximately 150 units of exonuclease III was added for every picomole of recessed 3' termini. The tube containing the target DNA and exonuclease III was briefly vortexed and placed at 37°C initiating the digestion reaction. At 30 second intervals, 2.5 μ l samples were removed and placed into the appropriate labelled ice-cold microfuge tube containing S1 reaction mixture. The S1 tubes were transferred to a water bath set at 30°C and incubated for 30 minutes. Upon completion of the 30 minute incubation, 1 μ l of S1 stop mixture (.3 M Tris base and 50 mM EDTA pH 8.0) was added. Then each tube was gently vortexed and placed at 70°C for 10 minutes to destroy any exonuclease and S1 activity. The DNA samples were analyzed by gel electrophoresis to demonstrate success or

failure of generating deletion clones. The samples of choice were pooled. For each pooled sample, 1 μ l of Klenow mixture was added for every 10 μ l of pooled sample. The pooled samples were then incubated at 37°C for 5 minutes. Klenow enzyme without nucleotides removes 3' overhangs. To each 10 μ l of pooled DNA was added 1 μ l of a solution containing all the DNA nucleotides each at a concentration of .5 mM. The solution was gently mixed by pipetting up and down and incubated at room temperature for 15 minutes. The deleted clones were collected by adding 1/10th the volume of 3 M sodium acetate pH 5.2, and 2.5 volumes of 100% EtOH. The DNA samples were incubated on ice for 5-15 minutes and centrifuged at high speed for 5 minutes. The pooled DNA samples were resuspended in 10 μ l of distilled water and ligated. Transformations were carried out as described by the Hanahan procedure.

c) Sequencing of the ro-4 gene

i) Preparation of the template DNA

Deletion clones were screened by *EcoRV* digestion and comparison to pMRNB1. Approximately 10-15% of all screened deletion clones were suitable for sequencing. pBluescript was used as the cloning vector for the template involved in sequencing. The templates included were pMRSB1, subcloned restriction fragments (Table 1) and deletion clones of pMRNB1. To carry out the sequencing reactions approximately 1.5 to 2 μ g of DNA was required. Template DNA was obtained from either rapid mini-preps or magic preps. The rapid

Table 1. Subclone Constructs

Subclones	Size of insert	Restriction Enzymes used to Generate Fragments
	DNA (Kb)	
pMRE1	0.9	<i>EcoRV</i>
pMRE2	1.1	<i>EcoRV</i>
pMRE3	1.5	<i>EcoRV</i>
pMRP1	1.4	<i>PstI</i>
pMRP2	2.0	<i>PstI</i>
pMRX1	0.3	<i>XhoI</i>
pMREX2	0.7	<i>EcoRV</i> , <i>XhoI</i>
pMREP1	0.18	<i>EcoRV</i> , <i>PstI</i>
pMREP2	0.45	<i>EcoRV</i> , <i>PstI</i>
pMRNE1	1.0	<i>NotI</i> , <i>EcoRV</i>
pMRNP1	1.2	<i>NotI</i> , <i>PstI</i>
pMRSB1	3.9	<i>SmaI</i> , <i>BglII</i>
pMRNB1	4.5	<i>NotI</i> , <i>BglII</i>
pMRB1	5.5	<i>BglII</i>
pMRBX1	3.7	<i>BglII</i> , <i>XhoI</i>
pMRBS2	1.6	<i>BglII</i> , <i>SmaI</i>
pMRH1*	2.4	<i>HindIII</i> , <i>KpnI</i>

mini-preps were treated with RNase A and extracted with an equal volume of Phenol:Chloroform, precipitated with ethanol and resuspended in 12 μ l of sterile water. Eight μ l of the mini-prep DNA or 8 μ l of magic prep DNA containing 1.5-2 μ g of DNA was added to 2 μ l of 2 M NaOH for denaturation. The solution was vortexed gently, followed by quick centrifugation and incubated at room temperature for 10 minutes. Denatured template DNA was collected by adding 3 μ l of 3 M sodium acetate pH 4.8, 7 μ l of sterile water and 60 μ l of 100% EtOH. The samples were incubated on ice for 5-10 minutes and centrifuged at 14,000 rpm for 5 minutes. The denatured DNA pellet was washed with 70% EtOH. The pellet was dried under vacuum and resuspended in 10 μ l of sterile water. The resuspended template DNA is ready for primer annealing.

The sequencing reactions were carried out using the T⁷Sequencing™ Kit and techniques outlined by Pharmacia. To the 10 μ l resuspended template was added 2 μ l of annealing buffer and 2 μ l of primer solution (2-4 pmoles of primer). The samples were mixed and briefly centrifuged followed by incubation at 37°C for 20 minutes and at room temperature for 10 minutes.

The radioactive nucleotides used were [α -³⁵S]dATP (>600 Ci/mmol) from Dupont.

ii) The preparation and running of the DNA sequencing gel

The preparation of the glass sandwich used to house the acrylamide sequencing gel had the dimensions (42 X 33 X .4) cm. The larger plate was washed with .1% SDS and rinsed under cold tap

water. The plate was wiped with .5 N NaOH, left sitting for 5-15 minutes and rinsed with tap water. Following the rinse, the plate was wiped with 5-10 ml of 95% EtOH and polished dry. The smaller plate was washed with .1% SDS, rinsed with tap water and wiped dry. The plate was placed in the fume hood and 5 ml of 5% dichloromethylsilane in chloroform was spread over the plate evenly on one side. The plate stood for five minutes to allow the silicon layer to dry. After 5 minutes, 5-10 ml of 95% EtOH was spread over the plate and allowed to stand for 5 minutes and polished dry using kimwipes. The glass plates were clamped and taped together using 3M yellow electrical tape. At this stage the acrylamide gel was ready to be poured.

The gel solution was composed of 42% ultra pure urea, 6% acrylamide (addition of 14.5 ml of 40% acrylamide stock solution), 1 X TBE buffer (10 ml 10 X TBE buffer) and 40.5 ml of water to make the volume up to 99.2 ml. The acrylamide gel solution was filtered through a whatman #1 filter paper and degassed for 5 minutes. After degassing .8 ml of 10% ammonium persulfate and 30 μ l of TEMED was added. The solution was swirled and the gel cast immediately.

The gel was pre-run for 15-30 minutes at 1600-1900 V, 35-45 amp and 45-60 W using the model S2 sequencing apparatus from BRL. Before the samples were loaded, they were heated at 75-80°C for 2 minutes. The gel was run at the same settings for the pre-run. Upon completion of the run (about 4-6 hours) the glass sandwich was removed from the gel apparatus and the plates were separated. The gel still attached to the large glass plate was placed in fixing

solution (10% glacial acetic acid and 10% methanol) for 20 minutes. The acrylamide gel was removed from the fixing solution and as much fixing solution was removed from the gel as possible. The gel was peeled away from the glass plate by gently applying a piece of 3 mm whatman filter paper (cut slightly larger than the gel) to the surface of the gel. The gel was wrapped in saran wrap and dried for 20 minutes using a gel dryer (Bio-Rad model 583) and exposed to X-ray film for 2 days.

d) Repeat-Induced Point Mutations (RIP)

RIP was carried out as described by Selker (1990). This procedure is designed to inactivate the functional resident copy of the cloned gene. The Hygromycin B resistance gene (HygB^r) was isolated from the cloning vector pCSN43 using the restriction enzymes *HindIII* and *KpnI*. The HygB^r gene was then subcloned into pMRSB1 creating pMRH1 (see figure 3). Verification of a successful subcloning was demonstrated by restriction enzyme analysis, and the results of a positive transformation of *ro-4* and its ability to grow on medium containing HygB. The clone pMRH1 was used to transform the wild-type strain 74-OR8-1a. Transformed strains were observed for their ability to resist hygB. Homokaryons were selected for, and used to carry out sexual crosses. The ascospores were collected and spread out on SFG medium. The plates were incubated at 60°C for 45 minutes to destroy existing hyphae or conidia. When fungal colonies began to appear, slow growing

Figure 3: Construction of plasmid pMRH1.

Using the restriction enzymes *HindIII* and *KpnI* the *Hyg^r* gene was removed from pCSN43. pCSN43 is a modified pBluescript cloning vector possessing the *Hyg^r* gene. pMRSB1 was double digested using the same restriction enzymes, followed by the insertion and ligation of the *Hyg^r* gene, producing the plasmid pMRH1. The plasmid pMRH1 was used to transform the wild-type strain 74-OR8-1a in preparation for the generation of RIP strains.

Hyg^r / Amp^r
Hyg^r

Hyg^r / Amp^r
Hyg^r

Hyg^r / Amp^r
Hyg^r

colonies were cut out of the medium and placed onto Vogel's minimal medium in slants. The morphology of the growing RIP strains was observed. The strains that looked ropy were subject to further analysis.

e) Nuclear staining using DAPI

Germinated conidia were washed once in a 1.5 ml microfuge tube with 1 X PBS and 1 X PBS containing 10% triton X-100. The conidia were resuspended in 1 X PBS. A small sample of germinated conidia were applied to the surface of a cover slip and allowed to dry. The coverslip was dropped onto a slide with 2-5 μ l of 1 μ g/ml DAPI in 50% glycerol and 1 mg/ml p-phenylene diamine in 50% glycerol. The edges of the slide were sealed with nail polish. The stain was visualized under a fluorescence light microscope equipped with a mercury lamp.

I) RNA Preparation and Analysis

a) RNA extraction

RNA extraction was performed by the technique of Reinhart et al. (1981). Growth and treatment of the hyphae was similar to DNA Extraction. To each sample of pulverized mycelia was added 10 ml of extraction buffer (100 mM Na-acetate, 1 mM EDTA, 5% SDS, pH 5.0) and 10 ml of P:C:I. The mycelia were further ground in the

extraction mixture. This slurry was transferred to a 50 ml polyalomar centrifuge tube and centrifuged at 12,100 RCF X g for 10 minutes at 4°C. The aqueous phase was transferred to a new tube containing 120 µl of pre-digested pronase (10 mg/ml) and incubated for one hour at room temperature. The aqueous phase was extracted with P:C:I two more times. The crude RNA mixture was precipitated by adding two volumes of 100% EtOH. The pellet was resuspended in 1.8 ml of DEPC-treated water at 4°C. Three volumes of 4M Na-acetate pH 6.0, 10 mM EDTA was added to the RNA solution. The solution was incubated for 10 minutes at 4°C, then centrifuged at 12,100 RCF X g at 4°C. The pellet was washed with 70% EtOH. Each pellet was then resuspended in 2 ml of DEPC-treated water. Absorbance was measured at $A_{260,280}$. The crude RNA samples were precipitated with 1/13th volume of 4 M Na-acetate, pH 6.0, 10 mM EDTA and 2 volumes of EtOH. The crude RNA samples were resuspended at a concentration of 2 mg/ml.

b) Purification of the Poly(A⁺) RNA.

Poly(A⁺) RNA was prepared by the method of Aviv and Leder (1972). Oligo(dT)-cellulose was suspended in .1 N NaOH. The slurry of Oligo(dT) was added to rate controlled columns. The Oligo(dT) packed columns were washed with 3 column volumes of DEPC-treated water. The columns were further washed with 1 X column loading buffer (20 mM Tris.Cl pH 7.6, .5 M NaCl, 1 mM EDTA pH 8.0, .1% sodium lauryl sarcosinate). Lauryl sarcosinate was added after

autoclaving when the solution had cooled to 65°C and the lauryl sarcosinate has been heated to 65°C.

The crude RNA sample was heated to 65°C for 5 minutes and cooled quickly on ice. An equal volume of 2 X column loading buffer was added to the RNA solution. The RNA solution was applied to the columns. The eluate was collected in sterile tubes. When all the RNA solution had entered the column, one volume of 1 x loading buffer was added. When all of the eluate had been collected the solution was again heated to 65°C, quickly cooled and reapplied to the top of the column. The columns were washed with 5 to 10 volumes of 1 X loading buffer. The eluate was collected in fractions and absorbency was measured to determine when the tRNA and rRNA had been removed.

The mRNA was eluted with 2 to 3 column volumes of DEPC-treated water and the absorbance measured. The RNA samples were precipitated with 3 M sodium acetate (pH 5.2) at a final concentration of .3 M and 2.5 volumes of EtOH. The samples were mixed, incubated on ice and centrifuged at 10,000 rpm for 5 minutes. The pellet was washed with 70% EtOH and air dried. The pellet was resuspended in 500 μ l of DEPC-treated water, the A_{260} was measured, and stored at -20°C.

c) Northern blot analysis

i) Preparation of a formaldehyde agarose gel

The formaldehyde gel was prepared by the method outlined by

Sambrooke et al. (1989). Five to 20 μ g from each sample of purified mRNA was transferred to a sterile 1.5 ml microfuge tube. The mRNA samples were dehydrated in the speed vac and resuspended in 20 μ l of gel running mixture.

The gel was first prepared by adding .88 g of agarose with 46 ml of water and placed in a microwave until the agarose was in solution. The agarose solution was allowed to cool to 60°C at which point 13.2 ml of 37% formaldehyde (2.2 M final solution) and 14.5 ml of 5 X gel running buffer (.1 M MOPS pH 7.0, 40 mM sodium acetate, 5 mM EDTA pH 8.0) was added.

The RNA samples were heated at 65°C for 15 minutes and cooled on ice, followed by a brief centrifugation to deposit all the liquid. To each sample tube was added 2 μ l of formaldehyde gel-loading buffer (50% glycerol, 1 mM EDTA pH 8.0, .25% bromophenol blue and .25% xylene cyanol FF). The gel before sample loading was pre-run for 5 minutes at 5 V/cm of gel (70 volts). After the warm up run the samples were immediately loaded. The gel was run at 3-5 V/cm. The buffer was recirculated every 1.5 to 2 hours. After the completion of the run ($\approx$ 5 hours) the gel was photographed and prepared for northern blotting.

ii) Northern blotting

The gel was treated in several changes of distilled water to remove the formaldehyde. The probe used was pMRE2 as this subclone contains most of the ro-4 gene. The probe was constructed as described earlier. The blotting and hybridization protocol is

similar to that outlined for the Southern blotting except for changes in the prehybridization and hybridization solutions and temperatures. The prehybridization solution is composed of 7.5 X SSPE, 50% formamide, 1.25 ml of 100 X Denhardt's solution and .02 mg/ml of denatured salmon sperm DNA. Pre-hybridization was carried out for a period of 8-20 hours at 48°C using .2 ml/cm². The hybridization solution was identical to the pre-hyb except the 5.0 ml of water was replaced by 5 ml of 50% dextran sulphate. Ten to 20 ng of probe was added per ml of hybridization solution. The hybridization was carried out for at least 12 hours at 48°C.

After the hybridization, the blots were washed twice for 15 minutes at 48°C in 5 X SSPE and then for 30 minutes at 48°C in 1 X SSPE, .1% SDS. The last wash was carried out at room temperature in a glass dish in .1 X SSPE, .1% SDS for 30 minutes.

The hybridized filters were exposed to X-ray film for a duration of time dependent on the strength of the signal. Seven day exposure time is not uncommon with northern blots.

J) Attempts at isolation of a cDNA clone of ro-4

a) Screening the λ Zap library

Screening the λ Zap library was carried out according to the procedure of Sambrooke et al. (1989). A single colony of *E. coli* BB4 was used to inoculate 50 ml of LB medium supplemented with .2% maltose in a 250 ml flask. The culture was grown overnight at 37°C

with shaking at 200 rpm. Bacterial growth in the presence of maltose permits more efficient adsorption of the phage lambda. The overnight culture of cells was centrifuged at 1,935 RCF X g for 10 minutes at room temperature. The supernatant was discarded and the cells were resuspended in 20 ml of .1 M MgSO_4 resulting in approximately 1.6×10^9 cells/ml

Several tenfold serial dilutions of the bacteriophage were prepared in SM medium. The lambda Zap cDNA library used was constructed from mRNA isolated from mycelia. One hundred μl of diluted bacteriophage was added to 100 μl of BB4 cells in a sterile 13 mm X 100 ml tube. This was carried out for each phage dilution in duplicate. The bacterial/phage mixtures were gently mixed and incubated for 20 minutes at 37°C. Upon completion of the incubation, 3 ml of molten agarose (47°C) was added to each tube and gently vortexed. The molten agar mixture was poured onto petri plates containing 25-35 ml of LB medium. The plates were incubated overnight at 37°C. The plates showing the best distribution of phage plaques were selected for blotting to Hybond-N filters cut to the sizes of the petri plates. The blotting of the phage plaques was carried out as recommended by Amersham. The phage blots were probed with pMRE2 as outlined for southern blotting.

III. RESULTS

A) Isolation of the *ro-4* gene

a) Cosmid clones isolated by sib-selection of *pab-2* and chromosome walking

Sib-selection was used to identify clones in the genomic cosmid library that resulted in a positive transformation of *pab-2*. Four clones were isolated by this technique: G21:5H, G9:2C, G1:11E and G9:10F. Classical crossing experiments have demonstrated that the *ro-4* locus is less than 1 map unit from *pab-2* (Perkins et al., 1982). In *Neurospora*, 1 map unit is approximately 40 Kb (Fincham, 1989), the size of the genomic DNA inserts in the cosmid cloning vectors. Due to difficulties in selecting for transformants of *ro-4* and the close proximity of the *pab-2* locus, it was decided that chromosome walking outlined by Sambrooke et al. (1989) and examination of the phenotypes of single clone transformations would be the best approach to obtain the *ro-4* gene.

EcoRV is one of several enzymes that will not cut into the pMOcosX vector (figure 1). Removing most of the insert DNA from clone G21:5H using *EcoRV*, and self-ligation, allowed the remaining insert DNA to be used as ³²P probes for chromosome walking in both directions. The insert DNAs remaining after *EcoRV* digestion were, a 2 and 5 Kb fragment from the extreme ends of the cosmid clone.

Table 2 displays three columns of cosmid clones: column A

**Table 2. Cosmid Clones Isolated by Sib-selection and
Chromosome Walking**

A	B	C
G21:5H	X9:2B	G5:12C
G9:2C		G4:7B
G1:11E		X4:6A
G9:10F		G5:3A
G20:9E		
X2:12C		
X2:12D		
X4:6A		

represents clones capable of positive transformation for both *pab-2* and *ro-4*. Column B represents a clone only capable of a positive transformation of *ro-4*. In column C are clones that cannot positively transform either of the two mutants. No cosmid clones capable of only *pab-2* positive transformation have been isolated. Restriction digest comparisons of the cosmid clones isolated by sib-selection and X9:2B have been made using *EcoRV* and *PstI*. All five cosmid clones have some restriction bands in common (not including those from pM0cosX), however, each one is unique. Probing of the cosmid clones with the *ro-4* gene was not performed, since fragments used for chromosome walking from clone G21:5H detected all the clones, including those isolated by sib-selection. This suggests all cosmids presented in table 1 have overlapping regions.

b) The cloning and construction of a restriction map of the *ro-4* gene

Clone X9:2B was chosen for the isolation of the *ro-4* gene, because it was suspected that the *ro-4* gene was located at one of the extreme ends of the insert genomic DNA. This was strongly suspected because of the inability of X9:2B to transform *pab-2*. Enzymes that do not cut into the cosmid pM0cosX were chosen to remove most of the insert DNA for the self-ligation of X9:2B, as outlined for the self-ligation of G21:5H. The *BglIII* digested, self-ligated clone X9:2B was still capable of *ro-4* positive transformation while this was not true for *SmaI*, *EcoRV* and *XhoI*

self ligated X9:2B. Removing most of the insert DNA makes it easier to pick out DNA bands for subcloning.

A 4.3 Kb *NotI/BglIII* fragment was found to be capable of positively transforming *ro-4* isolated from the *BglIII* self ligated X9:2B. The fragment was subcloned into pBluescript SK+, clone pMRNB1. The smallest fragment generated by restriction enzymes capable of positively transforming the *ro-4* mutant is a 3.9 Kb *SmaI/BglIII* fragment, clone pMRSB1. A restriction map of the subclone pMRSB1 is displayed in figure 2. Table 1 lists all the subclones created from the clone pMRNB1. The construction of clone pMRH1 indicated by *, is displayed in figure 3.

B) Characterization of the *ro-4* mutant

a) Observations of the mycelial mats and hyphae of the wild-type strain 74-OR8-1a and *ro-4*

The mutant *ro-4*, grown on Vogel's medium in slants or plates, has a phenotype that deviates considerably from wild type strains. The *ro-4* mutant possesses a reduced amount of conidia having a tendency to clump. The hyphae also appear discoloured, indicating reduced pigmentation, and they are more curled and branched than wild-type. As seen by the naked eye, the phenotype of *ro-4* and that of most *ropy* strains as described by Garnjobst and Tatum (1967) display a broccoli- or club-like appearance. These features are better understood by observing figure 4. Plate A, displayed in

Figure 4: Comparison of four strains of *Neurospora*.

The *N. crassa* strains were grown on Vogel's minimal medium at room temperature for 2 days. A) The wild-type strain 74-OR8-1a. B) The *ro-4* mutant, (C) and (D) are RIP strains 3 and 4. The RIP strains are the result of incapacitating the *ro-4* gene by means of repeat-induced point (RIP) mutations. Note the similar appearance between the RIP strains and the *ro-4* mutant.

A

B

C

D

figure 4 is the wild-type strain 74-OR8-1a which would appear bright orange and hyphal growth covered the entire 100 mm petri dish within 30 hours. Plate B, the *ro-4* mutant would appear as a dull yellow which would require 3 to 4 days of growth to cover the entire surface of a standard 100 mm petri dish. The *ro-4* mutants display what appears to be slower growth especially at higher temperatures. Since the hyphae of *ro-4* are curved, this could account for what appears to be slower growth, since colony spreading would be reduced. However, if cell wall components are not being incorporated into the apical cell wall at wild-type rates, its growth rate may also be impaired. The transformed *ro-4* mutants take on an almost wild-type appearance. Pigmentation and growth rate are restored and no observed clumping of the conidia takes place.

However, the hyphae do not display complete wild type morphology as slight curving is still present, partial *ro-4* morphology still exists, although this is reduced by repeated isolation for homokaryons. The isolation of homokaryons implies that all the nuclei in germinating conidia possess a copy of the transforming DNA. Comparisons between the hyphae are presented in figure 5. The hyphae of the wild-type are quite straight compared to the *ro-4* and the *ro-4* transformed with pMRSB1. Despite the curvature of the transformed *ro-4*, the hyphae are more wild-type in appearance than *ro-4*.

Figure 5: Comparisons of hyphae observed by SEM.

All three strains were prepared under identical conditions. The *Neurospora* strains were grown on Vogel's minimal medium for 30 hours at 37°C. A) The wild-type strain 74-OR8-1a. B) The *ro-4* mutant. C) Homokaryon transformed *ro-4* mutant using clone pMRSB1.

A

B

C

b) Observations of nuclei stained with DAPI

Staining the nuclei in germinating conidia grown in Vogel's minimal medium with DAPI reveals the impaired nuclear migration in developing *ro-4* hyphae. Conidia were germinated for 6, 8 and 12 hours. The 8 hour germination period produced the most easily photographed results. Photographs of the DAPI-stained nuclei are presented in figure 6. Nuclei in the wild-type strain are relatively evenly spaced throughout the hyphae. The nuclei in the germinating *ro-4* are quite restricted in their ability to migrate into the germ tube. The photograph in figure 6 displays one representative example of a germinating *ro-4* conidia. Although, 12 hours after germination the nuclei begin their migration along the growing *ro-4* hyphae (data not shown). Nuclear migration in the RIPPed strains appears to be more inhibited than *ro-4*.

c) Experimental evidence suggesting the correct gene has been isolated

Two experimental techniques were employed to demonstrate the clone pMRSB1 contains the *ro-4* gene. The first experiment involved isolating homokaryons transformed with the *ro-4* gene. The second experiment uses the construction of an RFLP map, using pMRB1 or pMRNB1 as a probe.

Figure 6: Stained nuclei in germinated conidia using DAPI.

The conidia were geminated for 8 hours at room temperature and stained with the DNA specific dye DAPI. (A) Stained nuclei in the wild-type strain, note the even distribution throughout the hyphae. (B) The bright field image of (A). (C) Stained nuclei in the *ro-4* mutant. The transport of nuclei in the growing hyphae of *ro-4* is impaired. Note how the nuclei in *ro-4* are clumped at the point of germination. (D) A bright field image of (C). (E) Stained nuclei in RIP strain 3, note how the nuclei, similar to *ro-4* are located in the conidia and no nuclei are in the germ tubes. (F) A bright field image of (E).

A

B

C

D

E

F

a) Homokaryon observations

ro-4 is a morphological mutant. Upon receiving a complementary wild-type gene, the mutant should display wild type morphology. Transformed *ro-4* mutants initially do not display a complete reversion to wild-type. Given 3 days of growth however, the transformed *ro-4* begins to display an increasingly more wild-type morphology. The isolation of homokaryons by repeated selection of individual conidia from a transformed strain results in the recovery of strains with a nearly wild-type morphology. Upon germination the transformed homokaryons display an immediate wild-type like appearance (figure 5).

b) RFLP mapping of the *ro-4* gene

Linkage analysis using restriction fragment length polymorphism (RFLP) mapping is designed to map a particular cloned gene or fragment to an approximate location on the linkage map. If a mutation has been previously mapped by classical crossing experiments, the RFLP serves to demonstrate that the cloned DNA maps to the correct region of the genome as the genetic marker. If a suitable RFLP can be detected linked to cloned DNA, the segregation pattern in random progeny from a cross between two different strains can be used to map that fragment (Metzenberg et al., 1984). The desired results should be a close or exact match to link the markers.

The restriction banding pattern for parental strain 4411 and all similar progeny is represented by (O) that have the same banding pattern. The strain 4416 is labelled (M) and likewise for all similar progeny. The pattern of (O) and (M) is compared to other RFLP's and the location of the *ro-4* gene can be determined. The restriction enzyme used was *Pst*I, and the southern blot was probed with pMRB1 and pMRNB1. The data using probe pMRNB1 is displayed in figure 7.

The genes *inl* and *chs-1* are in close proximity to the *ro-4* gene and the comparisons between RFLP maps is very close. The RFLP for *ro-4* matches 95% of the banding pattern for both genes. No other RFLP patterns come as close, suggesting the correct gene has been isolated.

D) Characterization of the *ro-4* gene

a) Sequence analysis of the *ro-4* gene

Gene characterization begins with the gene sequence. The *ro-4* gene was sequenced in both directions using the subclones outlined in table 1, exonuclease deletion clones (figure 2) and custom-designed primers (figure 8). Deletion clones 1 to 3 were capable of *ro-4* positive transformations. Deletion clones 4 and up resulted in negative *ro-4* transformations. These transformation results indicate that deletion clones 1 to 3 contained the entire *ro-4* gene and only the sequence contained within deletion clone 1 was

Figure 7: RFLP analysis demonstrating the linkage map location of the clone pMRNB1.

RFLP analysis denotes (O) as parental strain 4411 and (M) as parental strain 4416. All progeny strains with similar banding patterns to strain 4411 are indicated by (O), and (M) denotes progeny strains similar to 4416. The RFLP stains 4411 to 4430 were digested with PstI and probed with pMRNB1. The RFLP map of pMRNB1 compared to nearby genes (*inl* and *chs-1*) on chromosome V suggests the clone pMRNB1 contains the *ro-4* gene (Metzenberg et al., 1984).

Banding Pattern Comparisons for Strains

4411 to 4430

	11		16		30																
<i>ro-4</i>	(O)	O	O	M	O	(M)	M	O	M	M	M	M	M	M	M	O	M	O	M	M	M
<i>inl</i>	(O)	O	O	M	O	(M)	M	M	M	M	M	M	M	M	M	O	M	O	M	M	M
<i>chs-1</i>	(O)	O	O	M	O	(M)	M	O	M	M	M	M	M	M	M	M	O	M	M	M	M

(O)

(M)

4411

4430

Figure 8: Nucleotide sequence and hypothesized amino acid sequence of the ro-4 gene.

The sequence presents the putative introns written in lower case. The putative intron splice sites and their consensus sequences are underlined. The putative CAT and TATA box are represented in bold. The italicized/bold regions represent the primers used for sequencing. The 5' and 3' terminus sites are based on evidence supplied by the sequence.

GGCTGTTTCCCATCGTCAGACCTTCGGGGGATTTTGCGGACAGTGTATCACGGTGCGCAA
 CGCGATCCAAGCTAACAACTCATAATCACCATCTTCAGAAGGCCCAACACAGACAAACTG
 ACGAGCCGAGGTTCAACATCATTAAATTCGTCCTTTTGGAGGATGAAGGACCTGCTGAAG
 TCTCGGAAATGGAAGTTGTAAGCGGGAGCACCCCGATGAAGCACAAGTGAAAAGTCAACC
 GACGGACTAAAGAGAGATAAGATACACCATATCTCAATGCCATAGACATGAGCATTACAC
 TACAACTATCATACTTCTTTTTGCAAAGTGTCCCTTCAACATGTGCTCTTGTAAGTGAA
 GCTAGCCCCGCGATATCGCTGAAGTGATCTGCATGGAAGTTGGGCAAGACAAGGGGGAGG 60
 GGGAGCTTCAGATCCACTGCTGAGGATGGCTCGTGCACCTCCACCCTCACCGGCGCTGAT
 TGGCTGGATCTAATCAAGCTCTCTGACCGACAGCCCAATTCGAACCGGCACGTCCCTGCA 180
 GCAGAAACGCCCTTTTCGAACGAATCGAGGCCAGTCCGTCTGGATGTCGCGTCTGTGCT
 ACCAGAAAACATCCATCTCTGCGACCGGACGACTGACTGACTTTCTGCGGCTATCCGATC 300
 TTGAAGTCTTTCGCAGAGACTCTCAACCTGTAAACCGACAACCGTCAACCGAACCTGGA
 CCTTCCCACGTCCCCACCAAACAACCCAGCTGCTGGCAGACAACCTCACACCCACTCGA 420
 TTTCCGCCTGGCAAATGCCTTCATGACATCCTGATTGTTGACGATACACGATTGCAAACG
 ACCACTACATAACAACCTTTCTGTTTACTTTAGGTCTTTGACCGCACAAACGGCCTACCAC 540
 CGAAGCCCACTCCCGCAGAGACACGAAGACCCCTTGTCGAAGCCTAGCCTTACCCCGCCT
 CCGCGATTCTACGATAGCATCACCACCCACCGCTGGGACGGTTAGGTGGCAGCGACGGAC 660
 AGCGGATAGCCTTCAACAACCTTACATGTCCACAATACGACCTACCCTTTACATAACA
 ACAATGACAGACTCTTTCGACAACGCGCCCATTGTGCTCGACAATGGCTCGGGCACCATT 780
 MetThrAspSerLeuHisAsnAlaProIleValLeuAspAsnGlySerGlyThrIle 19
 CGCGCCGGCTTCGCGGGAGACGACGTACCAAAATGCCACTTCCCCTCCTTCGTAGGGAGG
 ArgAlaGlyPheAlaGlyAspAspValProLysCysHisPheProSerPheValGlyArg 39
 CCAAAGCACCTACGAGTGCTGGCCGGCGCATTAGAAGGCGAGGTGTTTATCGGCCAAAAG 900
 ProLysHisLeuArgValLeuAlaGlyAlaLeuGluGlyGluValPheIleGlyGlnLys 59
 GCGGCCTCAGAGTTGCGCGGACTGCTCAAGATCCGATATCCGCTGGAGCACGGCATCGTG
 AlaAlaSerGluLeuArgGlyLeuLeuLysIleArgTyrProLeuGluHisGlyIleVal 79
 ACGGATTGGGACGATATGGAGAAGATTTGGGCATATGTATACGATGAGGGGTTGAAGACG 1020
 ThrAspTrpAspAspMetGluLysIleTrpAlaTyrValTyrAspGluGlyLeuLysThr 99
 CTCAGTGAAGAGgttcgttctcccatattcttttggtcagaacaagacaaaaaaaaaaaaa
 LeuSerGluGlu 103
 aaaaaagcggtagctaacaacgggtccaactgctagCATCCAGTCCTCCTAACCGAACCA 1140
 HisProValLeuLeuThrGluPro 111
 CCCCTCAACCCGCGCGCAAACCGCGACACAGCCGCCAGATCCTCTTCGAGACCTTCAAC
 ProLeyAsnProArgAlaAsnArgAspThrAlaAlaGlnIleLeyPheGluThrPheAsn 131
 GTACCTGCTCTCTACACCTCCATCCAAGCCGTCCTGTCTGCTCTACGCCTCGGGGCGAACG 1260
 ValProAlaLeuTyrThrSerIleGlnAlaValLeySerLeyTyrAlaSerGlyArgThr 151
 ACAGGCGTGGTTCTCGACTCGGGCGACGGTGTTTCGCATGCCGTGCCGTCTACCAGGGC
 ThrGlyValValLeuAspSerGlyAspGlyValSerHisAlaValProValTyrGlnGly 171
 TTCACCGTGCCCAACAGCATCCGGCGCATCGACGTCGAGGGCGAGACGTGACCGAGTAC 1380
 PheThrValProAsnSerIleArgArgIleAspValAlaGlyArgAspValThrGluTyr 191

CTGCAGACGCTGCTGCGCAAGAGCGGCTATGTGTTCCATACGAGCGCTGAGAAGGAGGTG
 LeuGlnThrLeuLeuArgLysSerGlyTyrValPheHisThrSerAlaGluLysGluVal 211

GTCCGGCTGATCAAAGAGAGCGTCACGTACGTGGCGCACGATCCGCGCAAAGAGGAGAAG 1500
 ValArgLeuIleLysGluSerValThrTyrValAlaHisAspProArgLysGluGluLys 231

GAGTGGGCGGCGGCCAAGATGGATCCGGCCAAGATTGCCGAGTATGTCTTGCCGGATGGA
 GluTrpAlaAlaAlaLysMetAspProAlaLysIleAlaGluTyrValLeuProAspGly 251

AACAAAGTTGAAGgtatgttaagttttattcctctcctgccattaccaatggcgtgaggtt 1620
 AsnLysLeuLys 255

gggaaagaaaaagagacatcgatgcagatactgacacccaccaagATAGGAGCAGAGCG
 IleGlyAlaGluArg 260

TTTCCGCGCCCCGGAAATCCTCTTCGACCCCGAGATTATCGGTCTCGAGTATCCCGGCGT 1740
 PheArgAlaProGluIleLeuPheAspProGluIleIleGlyLeuGluTyrProGlyVal 280

GCACCAGATAGTGGTTGACTCGATCAACCGGA AGACTTGGACCTGCGCAGGGACTTGTA
 HisGlnIleValValAspSerIleAsnArgThrAspLeuAspLeuArgArgAspLeuTyr 300

CTCCAACATTGTGTTATCCGGAGGCAGCACGCTCACAAAGGGTTTCGGCGATCGCTTGCT 1860
 SerAsnIleValLeuSerGlyGlySerThrLeuThrLysGlyPheGlyAspArgLeuLeu 320

CACGGAAGTGCAGAAGCTCGCGGTTAAAGACATGCGGATAAAGATTTTTGCGCCCGCGGA
 ThrGluValGlnLysLeuAlaValLysAspMetArgIleLysIlePheAlaProProGlu 340

GAGGAAGTACTCGACCTGGATCGGTGGTAGTATTTTGGCGGGTTTGAGCACATTTAGAAA 1980
 ArgLysTyrSerThrTrpIleGlyGlySerIleLeuAlaGlyLeuSerThrPheArgLys 360

Ggtatgtcctcacatttgtctctgtgtttgcgcgtggacgagaaggaaacttcacacaaat

ggggccatcatgtgtgctaacaaatgtgtgacaatgaacagATGTGGGTTAGCATTGACG 2000
 MetTrpValSerIleAsp 366

ACTGGCACGAGAATCCGGATATCAATCCATACGAAGTTTACATGATGATGATAACGAC
 AspTrpHisGluAsnProAspIleIleHisThrLysPheThr 380

AAGACTTGGGGGAGCGAGCGAGCCGGAGAGCAAGGACACCGGTTGTCTAATAAACAAAAA 2120
 TCCAATTTTCACCGTACATCATCTCTATCCAGACTTACCAGCCACATACCACCTTCTTC
 TGTGCTACCTGTCTGGGCTAGAATCTAGCGCCTCAGAATCTTGGCTTTTGGCAAGTGGAA
 AAAGGGTGGTTTTAGCCTTGACAAGTATACCTTTACGGAAATCATATGTTCCGATCGATT
 CTGGGGGGGACGAAGTATTATATGCCATTTCACTCTGAGGCTGGGGCGAAAAAGGGGGGAG
 GCTCCTAGTACATAAAGAGGCTGG

sequenced in both directions. The deletion clones combined with the subclones allowed the complete sequencing of the 3'→ 5' strand containing the putative *ro-4* gene. Using the custom-designed primers in combination with the subclones completed the sequence for the 5'→ 3' DNA strand. The deletion clones were screened using *EcoRV* on a .8% agarose gels compared to *EcoRV* digests of pMRNB1. Rapid plasmid preps were used for the initial sequencing of deletion clones. The deletion clones that supplied the most data (figure 2), were prepared using magicTM column preps. The amino acid sequence deduced from the nucleotide sequence was submitted to Genbank for a similarity search (Altschul et al., 1990; Gish and states, 1993). The results demonstrated high sequence similarity to actin and actin-related proteins. No other type of protein had sequence homology in the data sent back from genbank. The protein with the highest level of identity and similarity is the human actin-related protein, actin-RPV, with an identity of 66% and similarity of 74%. The amino acid sequence of R04 (*ro*) is compared to three other actin proteins in figure 9. The three proteins are a major γ -actin (γ) belonging to *A. nidulans*, an α -skeletal mouse actin (α) and the human actin-RPV (*rp*). The sequence data is displayed in figure 8. The R04 and RPV proteins have a 66% level of similarity compared to both the α and γ -actin. The level of similarity between the α and γ -actin is 92%.

From the data presented in figure 9, four functional domains can be described. These domains include suspected amino acids that interact with actin, myosin, ATP and Ca^{++} . The amino acids that are

Figure 9: A comparison of amino acid sequences between the hypothesized R04 protein (ro), the human actin-RPV (rp), the rabbit skeletal α -actin (α) and the major γ -actin from *A. nidulans* (γ).

The amino acid sequence comparisons were constructed based on comparative analysis from Genbank. (m) Residues that interact with myosin, (a) residues that interact with actin, (n) residues that interact with ATP and (c) residues that interact with Ca^{++} . The N terminus and C terminus regions of the R04 protein have not yet been defined, however the most likely sequence defining these regions has been included.

2 OF 2

PM-1 3½" x 4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT

60

ro M--TDS-LHNAPIVLDNGSGTIRAGFAGDDVPKCHFSPFVGRPKHLRVLAGALEGEVFIG
 rp M-ESYDVIANQPVIDNGSGVIKAGFAGDQIPKYCFPNYVGRPKHVRVMAGALEGDIFIG
 α MCDEDE---TTALVCDNGSGLVKAGFAGDDAPRAVFPSIVGRPRHQGVVMGMGQ-DSYVG
 γ M--EEEV----ALVIDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHHGIMIGMGQKDSYVG
 mmm c nnn n mmm aaaaaa

120

ro QKAASELRGLLKIRYPLEHGIVTDWDDMEKIWAYVYDE-GLKTLSEEHVLLTEPPLNPR
 rp PK-AEEHRLLSIRYPMEHGIVKDWNMERIWQYVYSKDQLQTFSEEHVLLTEAPLNPR
 α DE-AQSKRGILTLYPIEHGIITNWDDMEKIWHHTF-YNELRVAPEEHPTLLTEAPLNPK
 γ DE-AQSKRGILTLYPIEHGVVTNWDDMEKIWHHTF-YNELRVAPEEHVLLTEAPINPK
 maaa mm aaa

180

ro ANRDtaaQILFETFNVPALYTSIQAVLSLYASGRTTGVVLDSGDGVSHAVPVYQGFTVPN
 rp KNRERAAEVFFETFNVPALFISMQAVLSLYATGRTTGVVLDSGDGVTHAVPIYEGFAMPH
 α ANREKMTQIMFETFNVPAMYVAIQAVLSLYASGRTTGVVLDSGDGVTHNVPIYEGYALPH
 γ SNREKMTQIVFETFNVPAFYVSIQAVLSLYASGRTTGVVLDSGDGVTHVVPYIEGFALPH
 c c nnn aaaaaa

240

ro SIRRIDVAGRDVTEYLQTLRLKSGYVFHTSAEKEVVRLIKESVTYVAHDPKKEKEWAAA
 rp SIMRIDVAGRDVSRFLRLYLKKEGYDFHSSSEFEIVKAIKERACYLSINPQKDE-----
 α AIMRLDLAGRDLDYLMKILTERGYSFVTTAEREIVRDIKEKLCYVALDFENEM--ATAA
 γ AISRVDMAGRDLDYLMKILAERGYTFSTTAEREIVRDIKEKLCYVALDYEQEIQTASQ-
 aaa aaaaa nn

300

ro KMDPALIAEYVLPDGNKLKIGAERFRAPEILFDPEIIGLEYPGVHQIVVDSINRTDLDLR
 rp -TLETEKAQYYLPDGSTIEIGPSRFRAPELLFRPDLIGEESGIEHVLVFAIQKSDMDLR
 α SSSSLEKS-YELPDGQVITIGNERFRCPETLFQPSFIGMESAGIHETTYSIMKCDIDIR
 γ -SSSLDKS-YELPDGQVITIGNERFRAPKALFQPSVLGLESGGIHVTTFNSIIKCDVDVR
 aaa aaaa aaaaa

360

ro RDLYSNIVLSGGSTLTGFGDRLLTEVQKLAVKDMRIKIFAPPERKYSTWIGGSILAGLS
 rp RTLFSNIVLSGGSTLFGFGDRLLSEVKKLAPKDVKIRISAPQERLYSTWIGGSILASLD
 α KDLYANNVMSGGTTMYPGIADRMQKEITALAPSTMKIKIIAPPERKYSVWIGGSILASLS
 γ KDLYGNIVMSGGTTMYPGISDRMQKEITALAPSSMKVKIIAPPERKYSVWIGGSILASLS
 nnnnn aaa n mm

385

ro TFRKMWVSIDDWHENPDI-IHTKFT
 rp TFKKMWVSKKEYEEDGARSIRKTF
 α TFQQMWITKQYDEAGPSIVHRKCF
 γ TFQQMWISKQEFDESGPSIVHRKCF
 mmm

suspected of being involved with actin-actin interactions such as filament formation are highly conserved in the major actins, such as the α -skeletal mouse actin and the γ -actin displayed in figure 9. The amino acids Q⁴⁶, G⁴⁷, E¹⁷⁴, A¹⁷⁷, E²⁰², R²⁰³, F²⁷⁶, C²⁹⁵, P³³², S³³³ and T³³⁴ are conserved amino acids that exist in the typical major actin involved with actin-actin interactions. These amino acids have been substituted with L⁴⁶, R⁴⁷, Q¹⁷⁴, T¹⁷⁷, K²⁰², S²⁰³, I²⁷⁶, T²⁹⁵, V³³², K³³³ and D³³⁴ in the R04 protein. total of 44 amino acids are thought to be involved in actin-actin interactions. These substitutions represent 25% of the 44 amino acids. The actin-RPV also has a 25% amino acid change, mostly in the same areas as R04. The NH₂-terminus is believed to be the part of the actin protein that is most active in its interactions with myosin. The mouse α -skeletal actin has four acid residues and the γ -actin has 3 acid residue in their NH₂-terminus, while the actin-RPV has two and the R04 has one. The acid residues are believed to give the NH₂-terminus the myosin interacting capabilities. The amino acids E⁶, D⁸, E⁹, K⁶⁷, E¹⁰⁰, Y³⁷² and D³⁷³ are conserved amino acids that are found in the typical major actins that interact with myosin. R04 has these amino acids substituted with S⁶, L⁸, H⁹, L⁶⁷, G¹⁰⁰, W³⁷² and H³⁷³. There are a total of 19 suspect amino acids involved with myosin interactions present in the major actins, R04 displays a 37% difference in this conserved area. The actin-RPV has 8 amino acid substitution differences, but are not all the same amino acids as R04. The amino acids involved in Ca⁺⁺ interactions are conserved for both the R04 and actin-RPV compared to the major actins. The

amino acids involved in ATP interactions are highly conserved for both the R04 and RPV. Thirteen out of the fifteen amino acids involved in ATP interactions are conserved for R04 and fourteen of the fifteen are conserved for actin-RPV. The amino acids L²¹ and Y³¹⁶ representing the major actins have been substituted with T²¹ and T³¹⁶ for the R04 protein. With this degree of conservation for amino acids involved with ATP and Ca⁺⁺, the R04 protein should interact with these molecules to the same degree as the major actins and assume the same tertiary structure. The other amino acid differences between the R04 and the major actins would probably be involved with the protein's final tertiary structure. Assuming this is correct, one would assume the R04 protein has a similar tertiary structure as the major actins.

b) Measurements of the *ro-4* transcript levels

The failure to detect *ro-4* transcripts, or to isolate a cDNA clone from a λ Zap library, seems to imply that the level of expression of the *ro-4* gene is quite low during the vegetative life cycle. The purpose of attempting to isolate a cDNA clone was to map the 3' terminus and confirm putative intron/exon boundaries. Primer extension was also attempted to map the 5' terminus, however due to suspected low levels of the transcript my attempts at primer extension were not successful.

Two northern blots were prepared (figure 10). Both blots were identical, the blots had two lanes, one for the WT and the other

Figure 10: Transcript expression level comparisons of ro-4 to wild-type 74-OR8-1a.

Blot (A), represents the expression levels of a ribosomal proteins transcript. The probe used was a subclone of a ribosomal protein gene, only recognized as a ribosomal protein, clone rps8. Blot (B) was probed with clone pMRE2. Lanes (a) and (b) of both blots are identical, (a) is mRNA from the wild-type strain 74-OR23-1A and lane (b) is ro-4 mRNA.

A a b

B a b

for *ro-4*. The amount of mRNA added to each lane was 20 μ g. Blot A was probed with a ribosomal protein gene. This blot was exposed for 12 hours without a reflective screen. From the large bands visible it can be seen that this particular transcript is quite abundant. Blot B was probed with pMRE2, which possess about 90% of the *ro-4* gene and was exposed for 10 days with a reflective screen. This blot, like many others attempted even after lengthy exposure times, dose not display any mRNA hybridizing to the *ro-4* probe.

E) Repeat-Induced Point Mutations of the *ro-4* gene

The last experiment was designed to neutralize any functional capacity of the *ro-4* gene. This is carried out by a technique called repeat-induced point mutations (RIP). This technique involves transforming wild type strains with the desired gene of interest, along with a selectable marker. The marker is used to determine if the wild-type has been transformed, because there should be no morphological differences. Having transformed the wild type with clone pMRH1 (shown in figure 3), homokaryons had to be isolated. Conidia from the isolated homokaryons served as the male strains in crosses with the opposite mating type (female), wild-type strain 74-OR23-1A. The modified Vogel's medium has a reduced level of nitrogen stimulating the production of perithecia. It is during the sexual cross that RIPPING occurs, between fertilization and nuclear fusion (Selker, 1990).

RIPPed strains 3 and 4 are displayed in figure 4. The

morphologies of the RIP strains observed in figure 4 are ropy-like. Observations of the RIP strains suggest that they are more feeble than the *ro-4* mutant. The growth of the RIPPed strains in figure 4 are a bit more advanced due to earlier germination. The pigmentation of the *ro-4* mutant is reduced upon comparison the wild-type strains as explained earlier. The pigmentation of the RIPPed strains has been reduced further which appears as a pale yellow. The DAPI staining of RIPPed strain 3 in figure 6 demonstrates the defect in nuclear migration at that stage of development. The hyphae also appear less developed than that of the wild-type and *ro-4*.

A total of six RIP strains have been isolated, all possessing the same phenotype. Obtaining six RIP strains out of a possible 100 colonies is a relatively high frequency. All the colonies isolated in the search for RIP strains were either of wild-type or ropy morphology. To demonstrate that RIP was sufficient to disrupt the function of the *ro-4* gene, the DNA from *ro-4*-like (RIP) strains were digested with *EcoRV*, *PstI* and *PvuII*. Figure 11 presents a southern blot demonstrating differences in restriction digests between RIP strain 3 and 4, a wild-type and the homokaryon transformed wild-type with the *ro-4* gene. RIP strain 3 has the restriction sites *PstI* and *EcoRV* eliminated with a slight variation in the restriction banding pattern of *PvuII*. Final proof to demonstrate loss of gene function would be to demonstrate the elimination of the transcript or obtain the sequence of the target genes. The final conclusion, the *ro-4* gene, especially in RIP

strain 3 has probably been destroyed. This suggests that the ro-4 gene under laboratory conditions is not essential.

Figure 11: Southern blots comparing restriction digest patterns of non-RIP to RIP strains.

The upper case letters represent the type of restriction enzyme, (A) *PvuII*, (B) *EcoRV* and (C) *PstI*. The lower case letters represent the *Neurospora* strain. (a) The transformed homokaryon wild-type strain 74-OR8-1a, (b) RIP strain 3, (c) RIP strain 4 and (d) is the wild-type strain 74-OR8-1a. Note the loss of restriction sites for the RIP strains, especially RIP strain 3. Clone pMRNB1 was used as the probe.

A a b c d

B a b c d

C a b c d

IV. DISCUSSION

Isolating genes from morphological mutants of any organism will certainly further the understanding of the biochemistry underlying development and growth. The development of molecular techniques applied to eukaryotic microorganisms makes *Neurospora* and many types of fungus excellent organisms to gain such knowledge. The type of morphological mutant examined in this research project belongs to a class of *N. crassa* mutants classified as *ropy*. During germination the nuclei in many *ropy* strains have difficulty migrating as compared to wild-type strains (Vierula and Robb unpublished observations). At present, the molecular basis for this phenotype is not known. Choosing to work with the *ropy* mutants was based on curiosity and possible gains in obtaining genes not presently characterized, which would likely be linked to morphology.

A) Interpretation of evidence suggesting the isolation of the *ro-4* gene

The auxotrophic mutant *pab-2* of *N. crassa* was an ideal strain for the application of sib-selection. Without *p*-aminobenzoic acid the *pab-2* mutant displays very sparse growth. This provided excellent selection conditions with which to identify transformations, with the complementary gene from the cosmid library. Having isolated four cosmid clones capable of positive

transformation of the *pab-2* mutant, clone G21:5H was used for chromosome walking to obtain the *ro-4* gene. Single clone transformations had to be applied to the *ro-4* mutant because of the more difficult selection conditions.

After successfully isolating cosmid clones from the *N. crassa* genomic library suspected of positively transforming the *ro-4* mutant, it had to be demonstrated that these clones actually contained the *ro-4* gene.

As outlined in the results, the positive transformation of the *ro-4* mutant did not result in a completely wild-type morphology. This observation suggested that the correct gene had not been isolated. However, there are several possible explanations for this. One is a recently identified phenomenon known as quelling, which involves methylation of repeated DNAs during the vegetative life cycle and prevents normal gene function (Romano and Macino, 1992). Alternatively, since the original mutant allele is still present, the protein from the mutant *ro-4* gene could still interact deleteriously with other proteins in the cell. Finally, it had to be demonstrated that the transformation was not the result of suppression of a point mutation by the introduction of a possible suppressor gene.

Positive transformations of the *ro-4* mutant had an initial appearance of a ropy morphology until the third day. At this point, full colour was restored, growth was rapid and aerial hyphae were no longer clumped. However, the hyphae did not display complete wild type morphology as they grew with slight curving as shown in

figure 5. After selectively isolating homokaryotic *ro-4* transformants, a much more wild-type morphology was evident. Most conidia are multinuclear, each conidia usually possess two to three nuclei. However transformations usually transform only one nucleus. The repeated plating of conidia from transformed strains and isolation of these conidia individually under selective conditions, results in the isolation of a homokaryon. The selection of homokaryons ensures a ratio of one, two or maybe more transformed complementary alleles opposed to the single mutant allele (Fincham, 1989).

The RFLP results demonstrate that the clones resulting in positive transformations of the *ro-4* mutant are from the area mapped on linkage map V (Perkins et al., 1982). Using the enzyme *Pst*I and the subclone pMRWB1 as a probe, an RFLP difference was detected between the two parental RFLP strains 4411 and 4416. The data presented in figure 7 demonstrates that the closest matched RFLP's are from genes located nearest the *ro-4* gene. RFLP maps of genes on other linkage groups, or further from the *ro-4* locus on the same linkage group, have less in common or no similarity at all. Using the theory of RFLP mapping outlined in the results, making a data comparison, one can easily see that the cloned DNA fragment carrying the *ro-4* gene is from the area predicted by the linkage map.

The RIP experiments, used to disrupt the function of a cloned gene demonstrates if it is essential or not. If the gene is not essential, one can observe the null phenotype (Selker, 1990; Jarai

and Marzluf, 1991). Since the RIP experiments using the *ro-4* gene resulted in a ropy morphology, and transforming the RIP strains with the *ro-4* gene results in restoration to wild-type morphology, this is fairly conclusive evidence that the *ro-4* gene has been isolated. Since the 5.5 Kb clone pMRB1 can complement both the *ro-4* and *pab-2* mutants demonstrates a close linkage between them, which is consistent with classical crossing experiments (Perkins et al., 1982). All four lines of evidence support the premise that the *ro-4* gene has been isolated.

B) Implications of gene characterization.

To understand the genetic basis for the *ro-4* phenotype, I set out to characterize the *ro-4* gene by sequencing, RNA analysis and mutagenesis by RIP.

a) Sequence analysis

The DNA sequence comparisons revealed strong similarities to actin, with the highest homologies to minor actins or actin-related proteins. The protein with the most in common is the human actin-related protein actin-RPV (Altschul et al., 1990; Gish and States, 1992).

Assuming the putative 5' transcription start site, 3' termination site and intron boundaries are accurate, the *ro-4* gene has 380 ± 10 residues. In comparison, the human actin-RPV has 376

amino acids and the typical major actin has 375 (Lees-Miller et al., 1992). The putative 5' and 3' sites are based upon comparisons to actin and actin-related genes. At the 3' termination site there is a string of termination codons and sequence similarity. At the 5' start site there is an appropriate Met residue followed by highly conserved sequences found in minor and major actins alike (figure 8, 9). The intronic sequences had no similarities to anything in Genbank (Altschul et al., 1990; Gish and States, 1993). The ro-4 gene has a 64% identity and 74% amino acid sequence similarity compared to the human actin-RPV (Lees-Miller et al., 1992). The isoelectric point of major actins is about 5.0 and for minor actins and actin-related proteins the pI is about 6.5 as is for the human RPV. The pI for the deduced R04 protein is around 6.45. A pI over six is a characteristic displayed by actin-related proteins. However, Barja et al. (1991) have shown by 2 dimensional gel electrophoresis that the actin proteins isolated from *N. crassa* have a pI of 6.4. Therefore, it is possible that the R04 protein is a major actin isoform.

Major actins from all studied eukaryotes can bind other actins forming F-actin, and these F-actins can bind the motor protein myosin. Based on sequence analysis, the areas suspected of actin-actin interactions and myosin-actin interactions have deviated in the actin-related proteins. In the major actin sequence there are a suspected 44 amino acids involved in actin-actin binding. These sequences are highly conserved. Based on the R04 genomic DNA sequence, ten of the amino acids out of the forty four involved

with actin-actin binding have been substituted with different amino acids. The amino acids that have deviated that are involved with actin-actin interaction are suspected of being the most critical (Holmes et al., 1990). There are nineteen amino acids that are highly conserved that are suspected to be involved in actin-myosin interactions present in the major actins. Out of these highly conserved nineteen amino acids seven have been substituted in R04. This suggests the actin RPV's are incapable of interacting with myosin and cannot or have difficulty forming actin filaments. The residues which dictate actin secondary structure are mostly conserved, which implies the R04 protein should have the same globular structure as the major actins. Ca^{++} binding sites are completely conserved and out of the 15 suspect amino acids involved in ATP interactions only two amino acids have been substituted, implying that the R04 protein should be able to carry out functions with these molecules in the same manner as the major actins.

Site specific mutagenesis at the NH_2 -terminus has led to some insight into the behaviour of the major actins. All of the actin genes sequenced to date have 2 to 4 acidic residues confined to the NH_2 -terminus (Cook et al., 1992; Herman, 1993). The R04 protein has 1 acid residue Asp^3 and actin-RPV has two. The acidic residues are believed to be involved in binding several actin-binding proteins such as myosin. Actin and myosin binding in the presence of ATP can be prevented by applying antibodies specific for the amino terminus of actin (Cook et al., 1992). Yeast Act1 is not post-translationally modified as in many other actins found in other species. Some new

Act1 alleles with slight amino terminal modifications have interesting effects. Adding an extra Cys to the amino terminus had no effect at all on the actin in carrying out its role despite the fact that 1/3 of all the actins had unacylated amino termini. Two mutant genes for actin were constructed. One had the acid residues Asp² and Glu⁴ replaced with Asn and Gln (Cook et al., 1992). The other gene had the first three residues deleted from the amino terminus. The actin was assayed by examining effects on invertase secretion, *in vitro* and *in vivo*. The evidence gathered suggests that removing the acidic charges results in a bundling of the actin filaments. There was an observable decrease in the ability of the actin to activate the ATPase activity of rabbit myosin S1. However, the mutant actins were capable of sustaining their normal functions.

The disruption of the single tropomyosin gene *Tpm1* belonging to *S. cerevisiae* did not result in death of the cell, but only the disappearance of the F-actin that form during budding. These examples demonstrate that actin has many roles, some of which are non-essential (Liu and Bretscher, 1989).

b) Transcript analysis

Attempts have been made to isolate the *ro-4* cDNA clone from the *N. crassa* cDNA library in the λ Zap vector (stratagene). No clone was detectable. From the Northern blot experiments, no detectable *ro-4* mRNA transcript has been found. The Northern blots in figure

10 used 20 ug of purified mRNA with no detectable levels of a ro-4 transcript. Blot A was probed with a ribosomal protein gene and exposed overnight. As can be seen from panel A (figure 10) the level of gene expression is quite significant. Blot B was probed with clone pMRE2 and exposed for ten days without detection of a signal, as presented by panel B (figure 10). In addition, a primer extension experiment was carried out, but this was also unsuccessful. All the difficulty with the mRNA experiments can probably be attributed to low levels of gene expression.

c) Analysis by Repeat-Induced Point (RIP) mutations

Gene inactivation by RIP is conducted by transforming wild type strains with the gene of interest in association with a selectable marker gene. The homokaryotic transformed strain, still displaying a wild type morphology, is crossed with the standard wild-type 74-OR23-1A. Gene disruption (RIP) actually occurs during nuclear fusion and fertilization (Selker, 1990). With the exception of rRNA's, the genome of *N. crassa* has only single copy genes. Multiple sequences are disrupted by methylation of the cytosine nucleotides and subsequent high frequency replacement of GC with AT effectively destroying the gene's function. Approximately 10-15% of the progeny experience the RIPPING process (Selker, 1990; Fincham, 1990; Jarai and Marzluf, 1991). If the gene is essential, strains with an inactive gene are never retrieved. Only wild-type progeny are recovered. The non-essential gene can result in producing a

similar or more extreme form of the original mutant phenotype. The reintroduction of the wild type gene by transformation should restore the wild type morphology.

The RIP experiment using the *ro-4* gene produced strains with a more extreme ropy phenotype. All the recovered ropy strains were identical in appearance. When transformed with the *ro-4* wild-type gene, the wild-type morphology was restored. This result suggests that no other gene that could possibly be present in the clone pMRSB2 was involved in the RIP process. The disruption of the *ro-4* gene alone was responsible for creating the ropy phenotype.

Analysis of the RIPPed strains by Southern blotting revealed that they have lost some of the restriction sites present in the wild-type and transformed wild-type *ro-4* gene as can be seen in figure 11. The loss of restriction sites suggests that RIP has extensively mutated the functional gene (Fincham, 1990; Jarai and Marzluf, 1991). The conclusion drawn from the RIP experiment demonstrates that the *ro-4* gene is not essential under laboratory conditions, but is probably important for vigorous growth and reproduction. Nuclear migration is impaired, conidial development is reduced and temperature sensitivity is increased.

Two RIPPed strains were also examined by DAPI staining of germlings. From the germinating conidia, it can be clearly seen that nuclear migration is impaired. The evidence suggests that the impairment of nuclear migration is even more extreme than in the original *ro-4* mutant. The germ tubes of the RIP strains also appear to be more feeble upon comparison to the *ro-4* mutant germ tubes

(figure 6).

C; Hypothesis based on morphological and genetic evidence.

An important observation of the *ro-4* phenotype is that nuclear migration is impaired in the growing hyphae (figure 6). Most *ropy* strains tested for nuclear migration demonstrate this characteristic including the RIP strains. If nuclear migration is impaired, this implies other cellular organelles may also have problems migrating (secretory vesicles).

In *A. nidulans*, treatment with tubulin inhibitors such as Benomyl or mutations in β -tubulin proteins result in impaired nuclear migration and division. However, mitochondrial migration was unaffected. Similar observations have also been made in *S. cerevisiae*: microtubule inhibitors or mutations affecting microtubules result in blocked passage of nuclei. The role of actin is less clearly understood, because mutations in actin usually kills the cell. Palmer et al. (1993) used a conditional lethal mutant *Act1* in *S. cerevisiae* to examine the role of cytoplasmic actin. When synchronized cells were shifted to non-permissive conditions between anaphase and cytokinesis, it was observed that the mother cells had predominately two nuclei and the daughter cells had no nuclei. When actin is disrupted at this stage of the life cycle, the bipolar SPB appears to be unable to orient itself.

Therefore the *ro-4* morphology could be the result of an impaired cytoskeleton as the *ro-4* gene is very similar to actin and

organelle transport is known to be cytoskeleton dependent. The examples presented by Cook et al. (1992) and Liu and Bretscher (1989) have demonstrated certain mutations and roles involving the major actin are not always essential. Actin related proteins have also been suspected of linking the actin cytoskeleton to the microtubules, or securing microtubules to the centrosomes (Herman, 1993). If this were the case, cytoskeleton interactions would be weakened by a mutation in such a protein. If the cytoskeleton is impaired, as will have to be demonstrated for continuing research, the ro-4 morphological defect could be a direct result.

If the cytoskeleton is not impaired, motor protein defects that use the cytoskeleton 'highways' to transport membrane organelles are likely to be at fault (Goldstein and Vale, 1992). This suggests that the migration or positioning of nuclei or other membrane-bound organelles could be contributing to the morphological deviation. Another possible cause of the morphological defect, assuming the secretory vesicles also have problems migrating, is that enzyme or precursor delivery to the apex is aberrant resulting in a decrease in tip growth. Since the nuclei are the only known source of a base for microtubule extension, it is possible that microtubules from nuclei near the tips extend towards the growing tip supplying a structural support for straight hyphae growth. However, staining for cytoskeletal components detects only actin in the apex (Heath, 1990). As explained earlier by Palmer et al. (1993) the disruption of actin during nuclear division prevents proper bipolar spindle orientation. This suggests that the actin cytoskeleton binds the

astral microtubules emanating from the nucleus.

All the experimental evidence suggests that the R04 protein is an actin-like protein. From the DAPI stains of germinating conidia it is clear that nuclear migration is impaired for both the *ro-4* and RIP strains. Nuclear division is not impaired, suggesting that microtubule assembly proceeds normally. The sequencing data also suggests that R04 is an actin-like protein based on its sequence homology to the human actin-RPV and the amino acid differences in the assumed areas that involve actin-actin and actin-myosin interactions. These are the distinguishing features of some actin-related proteins. The studies of the gene for an actin-related protein associated with the centrosomes has demonstrated that its transcript expression level is very low (Clark and Meyer, 1992). The *ro-4* transcript level has also been demonstrated to be very low. However, the possibility that the actin cytoskeleton could be impaired cannot be ruled out. The cytoskeleton will have to be examined to determine if any impairment exists. If no impairment is detectable, analysis of the dynactin complex is the next logical approach. As described earlier the 45-KDa subunit belonging to the human dynactin complex has been determined to consist of both the major actin and actin-RPV, as determined by antibodies that do not cross react with each other (Lees-Miller et al, 1992). The dynactin complex of *N. crassa* can be tested using the same techniques. Isolating and immunoblotting subunits of dynactin should reveal if the R04 protein is present. A low level of *ro-4* mRNA transcript implies that the R04 protein is probably not involved with multiple

cellular roles.

The *ro-4* allele is currently being isolated. Having isolated the *ro-4* mutant gene, the mutation should be localized and a possible explanation as to how the mutation is affecting the mechanics of the system may be derived and how the molecule itself behaves. The isolation of the cDNA is also being attempted. Having the cDNA will answer all the questions pertaining to the location of the 5', 3' sites and splice sites for introns.

At present, cellular processes such as the ability of chromatin to condense, SPB formation, the migration of chromosomes to their prospective poles, and even nuclear division and nuclear migration are not understood. This research, should provide further insights into these areas of fundamental interest.

V. CONCLUSION

(1) The *ro-4* gene has been isolated, which is responsible for the *N. crassa ro-4* morphological mutation. The gene was isolated by chromosome walking using the clone G21:5H isolated by sib-selection of the *N. crassa pab-2* mutant.

(2) The *ro-4* clone was verified by isolating homokaryons transformed with the *ro-4* gene. The transformed homokaryons had a near wild-type appearance. RFLP mapping was used successfully to demonstrate that the *ro-4* clone was from the area indicated on chromosome V.

(3) The complete sequence data of the *ro-4* gene has demonstrated high homologies to the human actin-related protein RPV. The amino acid identity is 66% and the amino acid similarity is 74%. The transcript level of the *ro-4* gene is assumed to be very low as it was never detected. Disrupting the functional *ro-4* gene by RIPPING has demonstrated the gene is not essential under laboratory conditions and produces a mutant with a more severe, but similar phenotype to the *ro-4*. Observations of nuclear migration by means of DAPI staining has demonstrated that the nuclei belonging to the *ro-4* and RIP strains is impaired upon comparison to the wild type (figure 6).

(4) All the evidence, especially the gene sequence suggests the R04

protein is an actin-related protein that is part of the dynactin complex associated with the motor protein dynein.

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