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An Analysis of the *ro-4* gene
and its product
in *Neurospora crassa*.

by

Margaret Ann Wilson, B.Sc(Hons), B.Ed.

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

Ottawa-Carleton Institute of Biology
Carleton University
Ottawa, Ontario.

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"An Analysis of the *ro-4* gene and its product
in *Neurospora crassa*."

submitted by Margaret Ann Wilson, B.Sc., B.Ed.
in partial fulfillment of the requirements
for the degree of Master of Science.

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Abstract

The *ropy* mutations of *Neurospora crassa* are morphological mutations generally associated with a highly branched growth habit and aberrant nuclear distribution. The locus *ro-4* has previously been found to encode an actin-related protein. The *ro-4* gene of a mutant strain has been sequenced and found to contain a premature termination signal resulting from a frameshift mutation caused by a single base-pair deletion.

Antibodies to a synthetic peptide from the *RO4* protein raised in rabbits and used to probe proteins from the wild-type as well as the *ro-4* mutant strains, uniquely recognized a protein with an electrophoretic mobility slightly greater than the major actin, which was present in all strains tested.

One strain designated as a *ro-4* mutant shows typically *ropy* colony morphology when young but is similar to wild-type in growth rate and nuclear distribution and lacks the major actin epitope recognized by a generally reactive monoclonal antibody.

This thesis is dedicated
to my mother and father.

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List of Abbreviations

Arp	actin-related protein
ATP	adenosine triphosphate
cDNA	complementary DNA
cpm	counts per minute
DAPI	4',6-diamidino-2-phenylindole
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DEPC	diethyl pyrocarbamate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dTTP	deoxythymidine triphosphate
DTT	dithiothreitol
EDTA	N,N-ethylenediaminetetraacetic acid
EtOH	ethanol
FGSC	Fungal Genetic Stock Centre
IPTG	isopropyl thio β -D-galactoside
Kb	kilobase-pair
kD	kilodalton
mAb	monoclonal antibody
MOPS	3-[N-morpholino] propane sulphonic acid
mRNA	messenger RNA
OLB	oligo-labelling buffer
PAGE	polyacrylamide gel electrophoresis
pI	isoelectric point
PBS	phosphate buffered saline
^{32}P	radioactive phosphorus isotope
pH	$-\log_{10}[\text{H}^+]$
RIP	repeat induced point
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
kNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
S	Svedberg units
^{35}S	radioactive sulphur isotope
SDS	sodium dodecyl sulphate
SPB	spindle pole body
SSC	150mM NaCl, 15mM sodium citrate (pH 7.0)
SSPE	150mM NaCl, 10mM NaH_2PO_4 , 1mM EDTA (ph 7.4)
TBE	10mM Tris, 1mM EDTA (ph 7.6)
Temed	N,N,N',N'-tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
UTR	untranslated region
uv	ultraviolet
V	volts
W	watts
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactoside

I. Introduction

The mold *Neurospora crassa*

N. crassa is a classic model system in the study of eukaryotic genetics. It was with this organism that Beadle and Tatum elaborated their one gene - one enzyme theory of inheritance in 1942. *Neurospora* is a filamentous ascomycete (order Sphaeriales, family Sordariaceae) which occurs naturally on burned soil or vegetation in warm countries, but otherwise is better-known as a troublesome contaminant in bakeries and on stored bread.

Certain characteristics of this fungus recommend it for genetic studies. Its nutritional requirements are confined to simple mineral salts, a carbon source and biotin, which means that auxotrophic mutants are easily identified. It is haploid, meaning that mutations are not masked, and mutants are readily obtained by irradiation of conidia. Normally growth is rapid, with asexual reproduction possible within 24 hours, and this facilitates the study of segregation of genetic defects. The production of ordered tetrads during the sexual cycle illustrates the order of the 4 chromatids during the first meiotic division of ascus development.

Germlings arise from micro- or macroconidia, or from ascospores. Growth is by addition of material at the hyphal tips and by branching (Springer, 1993). At intervals of about 50 μ m, chitinous septa are laid down, although these contain a

central pore that allows movement of both cytoplasm and nuclei (Springer and Yanofsky, 1989). As the hyphae extend their tips into fresh areas of substrate, the nuclei must divide and distribute themselves along the length of the growing hypha, in order to continue to supply the tip with needed materials.

The development of the asexual macroconidia can be induced by dessication or depletion of carbon from the medium, and begins with the growth of aerial hyphae. Conidiation begins when an aerial hypha ceases to grow by apical extension but instead undergoes repeated apical budding. In the early stages of conidiation the constrictions between these buds are slight; at this stage of "minor constriction" the hypha may still revert to strictly vegetative growth. The hypha is committed when it exhibits "major constriction", in which the width of the joints between buds narrows considerably. Roughly 10 hours after induction, septal formation in these joints follows, completing the process (Springer and Yanofsky, 1989). The movement of nuclei during conidiation is finely regulated. Nuclei apparently enter the conidiophore while it is being formed, but enter the bud only after it has finished growing (Springer and Yanofsky, 1989) and commonly undergo mitotic divisions to produce a multinucleate spore.

Mitosis in *Neurospora* is not accompanied by the breakdown of the nuclear membrane. The nucleolus breaks down first, and this event is followed by the division of the Spindle Pole Body (SPB) residing outside the nucleus, and migration of its

daughters to opposite poles (Webster, 1986). On the inner side of the nuclear membrane opposite these spindle bodies, microtubules appear which span the nucleus in a parallel bundle. The chromosomes are attached to these microtubules by kinetochores. The nucleus elongates and its membrane narrows in the middle to form two daughter nuclei, in each of which a nucleolus then appears (Webster, 1986).

A group of *N.crassa* mutants known to be nuclear distribution mutants are known by the older designation of *ropy*, named for the appearance of their hyphae. At least 7 genes are thought to be involved in the *ropy* phenotype: *ro-1*, *-2*, *-3*, *-4*, *-6*, *-7*, and *-10*. The *ropy* strains are some of a large array of morphological mutants generated in *N.crassa*, mostly as a result of uv irradiation. Their curled, twisted hyphae are associated with slow growth and a striking "starburst" appearance of growth on solid culture media (Figure 1).

Nuclear movement in filamentous fungi

Much of the knowledge in this area has come to us from studies of mutant strains in two organisms: *N. crassa* and *Aspergillus nidulans*. In *Aspergillus*, mutant strains which show an inability to distribute nuclei properly are called *nud* (nuclear distribution) mutants. Five *nud* genes have been identified so far, *nud A*, *C*, *F*, *G*, and *H*; all are temperature sensitive. The elucidation of the mechanism of nuclear

Figure 1. Colony morphology of the wild-type and *ropy* mutants:
The cultures were grown on VMM at RT. They are:

<i>ro-1</i> (146)	<i>ro-2</i> (52)	<i>ro-3</i> (43)
<i>ro-6</i> (3627)	<i>ro-7</i> (4025)	<i>ro-10</i> (3618)
<i>ro-4</i> (1511)	<i>ro-4</i> (1529)	<i>ro-4</i> (2980)
wild-type	<i>ro-4</i> (1529) (2 ³ / ₄ days old)	wild-type (2 days old)

All cultures are four days old except where noted.

movement in fungi has been sought by looking for the products of these and of the *N.crassa* *ropy* genes. Both *nudA* (Xiang et al, 1994) and *ro-1* (Plamann et al.,1994) have been found to code for the cytoplasmic dynein heavy chain of their respective organisms, implying that the microtubule system is the basis of nuclear migration.

The product of the *nudC* gene is a 22 or 23 kD protein which exhibits similarity to an expressed sequence tag of *Caenorhabditis elegans* and is also conserved in rats and in *Drosophila* (Morris et al.,1995). In *nudC* mutants, microtubules form normally but nuclei are not distributed in the hyphae subsequent to mitosis (Osmani et al.,1990). Interestingly, the *nudC* mutation can be suppressed by the presence of *nudF* as an extra-copy on a plasmid (Morris et al.,1995). At the same time, the levels of NUDF protein in the *nudC* mutant are decreased, even when an exogenous promoter controls the transcription of *nudF*. The two protein products do not co-sediment (Xiang et al.,1995). The *nudF* gene, which codes for a 49 kD protein, shows similarity to other genes involved in cell signalling, such as those for human LIS1 and the β -subunits of heterotrimeric G proteins (Morris et al.,1995). G protein α -subunits recently described in *Neurospora crassa* may regulate Ca^{2+} channels, by analogy with similar proteins in mammals. At least two colonial mutants in *Neurospora* are known to be deficient in Ca^{2+} in their growing tips, which seems to dictate their phenotype (Turner

and Borkovich, 1993).

There are other *A. nidulans* genes (*apsA* and *apsB*) in which mutations affect nuclear migration during conidiation only and not during mycelial growth (Morris et al., 1995). In *apsA* mutants, nuclei usually fail to enter the primary sterigmata, thus preventing conidiation; however if one does reach this structure, the event is succeeded by normal conidiation, including migration of nuclei into the growing chain of spores (Fischer and Timberlake, 1995).

Further evidence for the involvement of the microtubule system came from studies of the "wall-less" mutant of *N.crassa*. In this as well as in hyphae and protoplasts, organelle movement was blocked by nocadazole but not affected by cytochalasin D up to 20 μ M (Steinberg and Schliwa, 1993). The rate of nuclear movement was measured as roughly 2 μ m per second.

Microtubules

Microtubules are hollow 24 nm polymers of tubulin, generally composed of 13 "protofilaments" of α/β -tubulin dimers in a left-handed helix. The orientation of the heterodimers in the molecule confers polarity, the "plus" end being more dynamic, where subunits are both added and removed faster than at the "minus" end.

A third form of tubulin, gamma-tubulin, is found exclusively in microtubule organizing centres (MTOCs). In

fungi it is responsible for nucleating tubulin dimers on the spindle pole body by binding β -tubulin and possibly ensuring the polarity of the microtubule (Morris and Enos, 1992). However two different gamma-tubulin antibodies failed to label two sites of microtubule nucleation in *Allomyces macrogynus* (Roberson and Vargas, 1994).

There is some evidence that in fungal germlings, microtubules are nucleated at the apex of the hyphal tip rather than in the perinuclear region. In studies in which microtubules of *Uromyces phaseoli* germlings were destabilized and then allowed to re-polymerize, FITC-labelled anti-tubulin first appeared at the hyphal tip and extended rearward (Hoch and Staples, 1985). Similar studies done with *Allomyces macrogynus* suggest that microtubules are nucleated both from the Spitzenkörper (an electron-dense apical body of growing hyphae) and from the centrosome (Roberson and Vargas, 1994). These studies indicated that the centrosome was responsible only for the nucleation of spindle microtubules, whereas cytoplasmic microtubules originated primarily from the Spitzenkörper. However the centrosome may be the site of nucleation for a network of microtubules reported surrounding the nuclear envelope.

More recently the role of microtubules in nuclear movement has been complicated by the discovery of a cold-sensitive α -tubulin mutation that results in fewer and more labile microtubules at both permissive and restrictive

temperatures. In addition, at restrictive temperature, it apparently disrupts nuclear distribution, though in combination with mutations in *nud* genes A,C,G and F of *A. nidulans* it suppresses their phenotype. Low concentrations of the microtubule-inhibiting drug benomyl mimic this effect. (Willins et al.,1995).

Oakley and Morris (1981) discovered a single heat-sensitive mutation (benA33) in a β -tubulin which prevented both nuclear division and nuclear migration, though the microtubules are assembled normally. The authors hypothesize that it is the disassembly which is required for full function and which is prevented in this mutant.

Desai and Mitchison (1995) have proposed a mechanism for microtubule-based chromosome movement at Anaphase B whereby the kinetochore microtubule depolymerizes at the site of the kinetochore and motor proteins situated there couple this depolymerization to chromosome movement.

Motor proteins associated with microtubules

In addition to dynein one other motor protein associates with microtubules; kinesin. Kinesin generates force in the anterograde direction (from "+" end to "-" end) while dynein generates force in the opposite direction (Preston et al., 1990). Thus any organelle moved by kinesin will usually translocate from "-" to "+" end, while conversely dynein-associated movement is normally "minus-end directed".

Cytoplasmic dynein is known to be associated with kinetochores and the microtubules of the mitotic spindle. It consists of two large subunits ("heavy chains") of about 400kD, three intermediate chains of about 74kD and four light chains of about 55kD. The two large subunits are positioned like antennae arising from the rest of the complex and are the "heads" which interact with the microtubules. Although much cytoplasmic dynein appears to be present in soluble form, able to be extracted by co-assembly with microtubules (Paschal et al., 1987), immunolocalization studies show also an association with membrane-bound structures (Collins, 1994). Dynein is actually capable of binding to pure phospholipid vesicles (Lacey and Haimo, 1992 in Collins, 1994).

The dynactin complex

Two polypeptides, 150kD and 45kD, frequently co-purify with cytoplasmic dynein (Paschal and Vallee, 1987). These are two components of the "dynactin complex" (for dynein activating, Gill et al, 1991), also known as the "Glued" complex as it was first named in *Drosophila*. The 150kD polypeptide ("dynactin") exists as a doublet comprising the "heads" of the complex, and in *N.crassa* is coded for by the *ro-3* gene (Plamann et al., 1994). The remainder of the complex consists chiefly of a 10x37 nm filament, a helix with a pitch very similar to that of the conventional f-actin (51.3 to 51). This filament is actually composed of the 45kD polypeptide

(Schafer et al., 1994), which is an actin-related protein (Arp) whose sequence is similar to the *ro-4* gene of *N. crassa* (Plamann et al., 1994). Both an α - and β -capping protein (37 and 32kD) are found on the barbed end of the filament, and a 62kD component on its pointed end. Near the capping protein a thinner 24nm long filament forms a sidearm to the main filament, ending in the two globular heads (Schafer et al., 1994). Recently p50 has been overexpressed with the result that mitosis was disrupted and spindles were distorted (Echeverri et al., 1996). The overexpression also reduced the association of dynactin and cytoplasmic dynein with kinetochores during prometaphase. The subunits p27 and p24 have not yet been localized. All cosediment at 20S (Lees-Miller et al., 1992).

Arp1 (pI 6.8) and actin are present in the dynein activating complex in a ratio of about 10:1. In addition to these two 45kD proteins there is a third 45kD protein with pI 6.7. The rest of the components of the complex are present at about .7 of the amount of the combined actins, though on the whole they are present in the cell in much lower quantities (Paschal, 1993). While *in vitro* studies show purified dynein to be capable of moving along microtubules, the dynactin complex is required in order for dynein to power microtubule-based vesicle movements (Gill et al, 1991).

Insights into nuclear movement from yeast studies

Saccharomyces cerevisiae has a single actin gene, ACT1, actin-related protein genes, ACT2, ACT3 (Harata et al., 1994), ACT3 (Clark and Meyer, 1994) and ACT5 (Muhua et al., 1994), two α -tubulin genes (TUB1 and TUB3) and a β -tubulin gene (TUB2) (Barnes et al., 1990). The Clark and Meyer ACT3 is classed as an Arp1 (Actin-related protein - see below) whereas the sequence of the Harata et al. ACT3 diverges greatly from known actins.

The cytoplasmic dynein heavy chain in this yeast is encoded by the gene DYN1. Disruption of this gene results in misalignment of the spindle during budding with the subsequent production of binucleate mother cells and anucleate daughter cells (Eschel et al., 1993). The spindle elongates normally, and as anaphase B progresses can be seen bent within the confines of the mother cell, unless it spontaneously becomes properly oriented. This phenotype is shared by a temperature sensitive tub2 mutant (tub2-401) in which spindle microtubules but not astral microtubules are assembled at the restrictive temperature (Sullivan and Huffaker, 1992). There was no evidence of exceptional chromosome loss in homozygous dyn1 diploids, implying either that cytoplasmic dynein is not involved in chromosomal migration to the poles, or that its role in this process is redundant (Eschel et al., 1993). Conversely, reduced chromosome movement was evident in *S. cerevisiae* suffering from disrupted astral microtubules,

whereas the spindle microtubules were not involved (Palmer et al.,1992). In the same study it was demonstrated that disruption of actin function also led to an increase in binucleate mother cells.

Similarly, migration of nuclei into mating projections of *S. cerevisiae* was defective in actin mutants, and tubulin mutants were both unable to move their nuclei into projections and to orient the SPB of the nucleus toward the projection tip (Read et al.,1992).

Capping proteins have been studied in *S. cerevisiae* without as yet revealing an effect on nuclear migration (Amatruda et al.,1992, Amatruda and Cooper, 1992).

Actin-related proteins

Actin-related proteins are between 35 and 55% identical to conventional actin (Schroer et al.,1994), generally having conserved the nucleotide and divalent cation binding sites (Schafer et al, 1994). There are at least 5 classes of Arps, with similarity among the classes no greater than similarity to actin; probably each has a distinct function (Schafer et al., 1994). The first class of Arps, "Arp1", of which R04 is a member, is defined as being the most similar to conventional actin (47-56% amino acid identity, Schroer et al.,1994). This class also includes centractin, and both Act3p and Act5p of *Saccharomyces* (Schroer et al.,1994).

Centractin (obtained from an MDCK (canine) cDNA library)

is expressed ubiquitously (across cells) at low levels. Indirect immunofluorescence indicated brightest staining in the perinuclear region of each cell against a faint cytoplasmic background stain, and occasionally two bright perinuclear spots, a pattern similar to that of centrosomes (Clark and Meyer, 1992).

Act3 of *Saccharomyces cerevisiae* encodes a nonessential polypeptide which in mutant strains results in a misalignment of the spindle in budding cells, yet not in an increase of anucleate or multinucleate cells (Clark and Meyer, 1994). Conversely, the Act5 gene is responsible for both of these phenomena when mutated. When in addition the dynein heavy chain gene of these cells is mutant their phenotype is unchanged, pointing to a shared function (Muhua et al., 1994).

Whereas conventional actins have a pI of 5.4-5.9, that of actin-related proteins is higher, around 6.2 in the case of an Arp from a human carcinoma cDNA (Lees-Miller et al., 1992a). Most of the non-conserved amino acids (compared to actin) are in surface loops of the actin structure. Conservation occurs in the nucleotide- and cation-binding regions (Clark and Meyer, 1992), and in sites involved in actin polymerization and in interaction with myosin alkaline light chain, but not the myosin head (Lees-Miller et al., 1992a).

In *N. crassa*, the characteristics of the ro-4 mutation can also be shown by "ripped" strains. Ripping (for repeat-induced point mutation) is a function of the organism

Neurospora crassa which occurs between fertilization and nuclear fusion. At that point, any duplications of sequence (with the exception of rRNAs) will be subject to numerous, scattered point mutations, predominantly G-C to A-T transitions (Selker et al., 1988) as well as to extensive cytosine methylation (Selker et al., 1993). Thus by transforming the wild-type *N. crassa* strain with the *ro-4* gene and then performing sexual crosses, four "ripped" strains were obtained (Robb, 1993) which share the *ropy* phenotype.

Two strains originally described as *ro-5* and *ro-8* and mapped to distal regions of the fifth linkage group (see Appendix 1) were subsequently identified as alleles of *ro-4* (Perkins, 1969). The gene encoded by *ro-4* has strong similarity to other actin-related proteins (Robb, 1993; Plamann et al., 1994)

Regulation of nuclear movement

In cultured fibroblasts binding of dynein to lysosomes has been shown to be inhibited by either serum starvation, low extracellular calcium, or okadaic acid, a phosphoprotein phosphatase inhibitor (Lin and Collins, 1993, Lin et al., 1994). This dissociation was associated with an increase in phosphorylation of the dynein heavy chain, leading Lin et al. (1994) to venture the theory that regulation of retrograde organelle movement may be dependent on alterations in membrane binding. However Dillman and Pfister (1994) found that the

dynein heavy chain (of bovine brain tissue) was phosphorylated to a lesser extent in an anterograde (presumably inactive) pool than in the entire cellular pool, suggesting that phosphorylation is a feature of active dynein. In transport of pigment granules, protein dephosphorylation is required for microtubule-based minus-end directed motility (cited in Dillman and Pfister, 1994).

An aberrant distribution of nuclei is associated with a highly-branched growth habit in the *ropy* mutants of *N. crassa*. Dicker and Turian (1990) were able to achieve excessive branching in the wild-type by application of the calcium channel blocker Verapamil, and refined these results further by restoring a wild-type morphology to hyper-branching *N. crassa* mutants ("frost" and "spray") by addition of calcium to the medium. The loss of a calcium ion gradient in hyphal tips by application of an ionophore is associated with loss of apical dominance of the tip, manifested by emergence of multiple branches from just behind the tip, whereas when external Ca^{2+} was decreased and surface-bound Ca^{2+} lost, the hyphae became stubby (Schmid and Harold, 1988).

Both of these studies call into question an earlier conclusion that Ca^{2+} induces branching in *N. crassa* (Reissig and Kinney, 1983) based upon the increase in branching with application of the same ionophore, but also upon the observation that prior calcium starvation of the medium reduced this effect. The authors also note that cAMP reduces

the branching response as well.

Calcium is the predominant second messenger in cells. It is associated with IP_3 and diacylglycerol in a cascade distinct from the one involving cAMP. Both paths share a dependence on G proteins for their initial transduction of the signal from the receptor. The mode of action of calcium may be from binding directly to a regulatory site of the protein involved in the response, or by activating a protein kinase which then phosphorylates (activates) the protein in question. A common intermediate (at least in plant and animal cells) is calmodulin, which when binding Ca^{2+} ions can alter the activity of many enzymes in the cell; principally the Ca-kinases (for calcium/calmodulin-dependent). These kinases phosphorylate serine and threonine residues of proteins.

Objectives

The wild-type *ro-4* cDNA was sequenced in order to confirm that the positions of the introns were as postulated by Robb (1993). A comparison between the *ro-4* sequence and other Arp sequences was pursued with the intent of finding shared functional domains, or conversely divergent regions which might be indicative of unique features.

The phenotype of the *ro-4* mutant strains differs markedly from wild-type (Figure 1). The bulk of the research herein was pursued to examine the genetic basis of this phenotype. It was of interest then, to obtain the sequence of the mutant

allele of strain (Fungal Genetics Stock Centre number) 2980 to be able to relate the altered sequence to its particular phenotype, which is the most extreme of the three mutant strains. These three *ro-4* mutants (Table 1) are all products of uv-irradiation, so it was presumed that their mutations were probably point mutations produced as a result of faulty repair of thymine-dimers. Since the mutant strain 2980 had been shown to have reduced *ro-4* mRNA levels (Robb et al., 1995) it was possible that its mutation occurred in the promoter region. The other mutants 1511 and 1529 were expected to also suffer point mutations and thus the restriction fragments produced by digestion of their DNA were not expected to vary from wild-type. This was tested by Southern analysis.

The sequence data indicated that the product of the *ro-4* gene would be missing in strain 2980. This was tested by Western analysis first using an antibody against the major actin, and secondly using purified polyclonal antisera raised against a synthetic peptide from the *RO4* protein.

Four "RIP" strains in which the *ro-4* gene was effectively knocked out (Robb, 1993) were expected to have reduced mRNA levels (comparable to that of 2980) as a result of point mutations in the promoter (among other regions) or which resulted in premature stop codons. The latter are associated with early degradation of mRNA (Muhua et al., 1994). Conversely, the mutants 1511 and 1529 would be expected to have levels similar to those of wild-type. These theories

were tested by a Northern analysis in which *ro-4* antisense RNA was used as a probe. A theoretical estimate of expression was obtained based on codon usage.

The results of the Western analysis, together with the early mapping data of the mutant strain 1529 led us to question its designation as a *ro-4* mutant. Phenotypic studies were carried out to allow comparisons of this strain to wild-type and to other *ropy* mutants. These included nuclear distribution in germlings and in ungerminated conidia, growth at normal and high temperatures, and culture morphology.

II. Methods

Culturing of *Neurospora crassa* strains in liquid and on solid media

N. crassa strains (Table 1), were grown on VMM (Vogel's Minimal Medium) slants from silica stocks by shaking a few grains of silica onto the surface of the slant, shaking the tube to expose the maximum surface to the medium, and incubating at room temperature. These were subcultured each month onto fresh slants. To obtain mycelium for the purposes of extracting DNA or protein, conidia obtained from the slant cultures, or from 50 ml solid VMM cultures were used to inoculate 50 mls of liquid VMM. These liquid cultures were incubated with shaking (250 rpm) for 1 to 2½ days, after which the mycelium was strained out, pressed to expel excess liquid, frozen and dessicated in a Speed-Vac (Savant SC100).

For studies on growth rate and colony morphologies, 8 cm petri dishes containing about 20 ml solid VMM were inoculated in the centre and incubated at room temperature (ca. 25°C) for the duration of the study, or up to 4 days for photographs.

Strains will hereafter be referred to by their FGSC stock number, except wild-type, which will be designated WT. In addition to those listed in Table 1, four strains generated by "ripping" (Robb, 1993) were used and designated R1, R2, R4 and R5.

Table 1: Strains of *Neurospora crassa* used in this research

Description	Strain Designation	Fungal Genetics Stock Centre #	Genetic Background
Wild-type	74-OR23-1A	987	St. Lawrence
ro-1	B-15	146	*
ro-2	B20	52	St. Lawrence 3
ro-3	R2354	43	Lindegren
ro-4 (=ro-8)	R2520	1511	Mauriceville
ro-4 (=ro-5)	R2428	1529	Mauriceville
ro-4	B38	2980	St. Lawrence
ro-6	R2431	3627	St. Lawrence 3
ro-7	R2470	4025	St. Lawrence 5
ro-10	AR7	3618	Mauriceville

* Background not given in Fungal Genetics Stock Centre Catalogue of strains, 1990

All of the mutants were generated by ultra-violet irradiation.

Culturing of bacterial strains

Escherichia coli strains BB4 and DH5 α F' were streaked on LB plates, or grown in liquid LB medium in shaker baths (250 rpm), all at 37°C. Stocks of these cultures were transferred to 10% glycerol and stored at -70°C.

Methods for DNA Analysis

Rapid preparation of DNA from filamentous fungi:

About 50 mg lyophilized mycelium was ground in a 1.5 ml microfuge tube and suspended in extraction buffer (0.2M Tris, pH 8.5, 0.25M NaCl, 25mM EDTA, 0.5% SDS) with pronase added at 0.5 mg/ml. After 15 minutes the mixture was extracted with phenol-chloroform and spun for 60 minutes at 13000 rpm in an eppendorf Centrifuge 5415C. The aqueous phase was then transferred to a new tube and treated with RNase for 30 minutes at 37°C. One volume of phenol-chloroform was used to extract the solution which was spun for 10 minutes at 13000 rpm. A 0.54 volume of isopropanol was added to the aqueous phase to precipitate the DNA, which was pelleted by a 5 second spin. The pellet was then washed with 70% ethanol, dried and resuspended in T₁E_{0.1} (Raeder and Broda, 1985).

Restriction enzyme digestion of DNA

Digestion of DNA with restriction enzymes was carried out according to the specifications provided by New England Biolabs using the appropriate NEB buffer provided and approximately 1 unit of enzyme for every 5 μ g of plasmid DNA, or 2.5 μ g of genomic DNA.

Amplification of DNA by Polymerase Chain Reaction

In each reaction, 30 ng of template DNA (in a volume of 3 μ l) and 25 pmoles of each specific primer were used. The balance of the reaction mix consisted of 0.6 μ l of a 15mM stock of dNTPs, 5 μ l of the 10X PCR buffer provided with the enzyme, all in a thin-walled 0.5 ml tube. $MgCl_2$ at 1.5mM was added when the polymerase used was TaqTM rather than VentTM. Sterile distilled water was added to a final volume of 50 μ l, and this was overlaid with 30 μ l sterile mineral oil. The tubes were placed into the wells of a Perkin-Elmer Cetus Thermocycler, sealed with a dab of mineral oil, and subjected to 36 cycles of : 1 minute at 95°C (in the first cycle this step was extended to 5 minutes), 1 minute at 55°C, and 1.5 minutes at 72° followed by a cold soak at 4° until they were collected. (2.5 μ l enzyme was added immediately after the initial 5 minutes at 95°C.) The reactions were then extracted with 50 μ l of chloroform and the aqueous phase retained following a quick spin. Eight microlitres of reaction product was run on a 0.8% agarose mini-gel to determine the presence or absence of product and its size.

The template DNA was genomic, or cDNA obtained by priming a pool of polyA-enriched 74A mRNA with polyT Race Primer (Frohman, 1990) in the case of RT (Reverse Transcriptase PCR).

Separation of DNA by an agarose gel system

A 0.8% agarose gel containing 0.05% ethidium bromide was prepared in 0.5X TBE buffer. The DNA sample was mixed with a

Table 2: Primers used in sequencing and amplifying ro-4

ro4c: -866 → -847	AAGCACAAGTGAAAAGTCAA
MRobb4: -364 → -348	ACCCTTCCCACGTCCCC
MRobb2: -57 → -41	TAGCCTTCAACAACCTT
MRobb3: 281 → 297	TGAGGGGTGAAGACGC
MRobb1: 809 → 825	GATTGCCGAGTATGTCT
ro4e: 942 ← 961	GGAAACGCTCTGCTCCTATC
ro4j: 1430 ← 1449	TCCCCCAAGTCTTGTCGTTA
ro4a: -753 → -735	GTCCCTTCAACATGTGCTCT
ro4h: -44 → -25	CCTTACATGTCCACAATACG
Rc1b: 457 → 475	TCCTCTTCGAGACCTTCAA
Rc3a: 992 → 1011	TTATCGGTCTCGAGTATCCC
ro4d: 1377 ← 1396	ATCCGGATTCTCGTGCCAGT
Rc1N: -151 → -135	TTGTCGAAGCCTAGCCT
ro4g: 451 ← 471	AGGTCTCGAAGAGGATCTGG
Rc2N: 1019 → 1036	CCAGATAGTGGTTGACT
ro4i: -2 → 17	CAATGACAGACTCTTTGCAC
Rc5a: 288 ← 307	TCTTCACTGAGCGTCTTCAA
Rc2 : 712 ← 731	TTTGATCAGCCGACCACCT

Positions are given relative to the first nucleotide of the first exon.

third of its volume of 6X loading buffer and 10 μ l loaded into the well. A marker consisting of 10 μ l of Lambda phage DNA cut with *Hind*III (2 μ l Lambda/*Hind*III stock, 2 μ l NEB buffer 3, 16 μ l dH₂O, 6 μ l 6X loading buffer) was also loaded.

Recovery of DNA from agarose gel fragment

The desired band was sliced from the gel using a clean razor, placed into a 1.5 ml microfuge tube with 2.5-3X its volume of 6M NaI solution, and heated at 50°C with occasional tapping until dissolved about 5 minutes later. Five microlitres of Glass MilkTM was then added, mixed by tapping and the tube left on ice for 5 minutes. A 5 second spin produced a pellet which was washed three times (by drawing the suspension up and down a 1000 μ l pipette tip) with 400 μ l New WashTM after discarding the supernatant. After each wash the mixture was spun briefly (1-2 seconds) to collect the pellet, and finally the pellet was resuspended in 10 μ l dH₂O and incubated at 50°C for 3-5 minutes to release the DNA into solution. After a 30 second spin the supernatant was carefully removed with a pipette and transferred to a fresh microfuge tube, where it was stored at 4°C.

Recovery of DNA from agarose gel by the Freeze-Squeeze Method

The desired band was cut out of the gel illuminated by longwave uv light, and equilibrated in 0.3M NaOAc pH 7, 1mM EDTA for 15-45 minutes. A 0.5 ml microfuge tube was partly stuffed with silanized glass wool and a hole punched in the bottom. The gel fragment was added and this tube then placed

inside a 1.5 ml eppendorf and frozen at -70 or -20°C . Once frozen the tube was centrifuged for 10 minutes at $12000\times g$ to allow the DNA to collect in the bottom of the larger tube. To this eluted DNA 0.01 volume of 1M MgCl_2 in 10% acetic acid was added and mixed, followed by 2.5 volume of EtOH. The mixture was allowed to sit on ice for 1 hour after which it was centrifuged 20 minutes at $12000\times g$ and the supernatant replaced with 70% ethanol, 0.1M NaOAc pH 6. After another spin the supernatant was replaced with 100% EtOH and the tube subjected to a final spin before the supernatant was poured off and the pellet dried in a dessicator. The pellet was cutomarily resuspended in 5 to 10 $\mu\text{l T}_1\text{E}_{0.1}$.

Phenol/Chloroform extraction of DNA

A preparation of DNA was brought up to 100 μl by the addition of TE. To this was added an equal volume of phenol/chloroform (1:1) which was mixed to an emulsion by vortexing. This was centrifuged at $12000\times g$ for 15 seconds and the aqueous phase withdrawn by pipetting. The aqueous phase was then subjected to the same procedure as above, once or twice more. In some cases, another 100 μl TE was added to the phenol/chloroform remaining after the first aqueous phase has been removed to further extract any remaining DNA. The resulting aqueous phases were pooled and an equal volume of chloroform added, mixed in, and then separated by centrifugation as above. The final aqueous phase was again withdrawn by pipette and the DNA recovered by Ethanol

precipitation.

Ethanol precipitation of DNA

The volume of a solution of DNA was measured and 0.1 volume of 3M NaOAc pH 5.2 was added. This was mixed together and then twice the total volume of EtOH was added and also mixed. This was left on ice or in the refrigerator for at least one hour, and then centrifuged for 10 minutes at 12000xg. The supernatant was poured off and the pellet washed with 70% ethanol, and then dried under vacuum. The dry pellet was resuspended in 10-20 μ l $T_1E_{0.1}$.

Preparation of vector DNA for ligation

The BluescriptTM SK plasmid was digested with a restriction enzyme(s) appropriate for its ligation with the DNA to be introduced. In the case in which sticky ends were produced by the *Pst*I digestion, re-ligation was prevented by treating the digested plasmid with calf intestinal alkaline phosphatase (CIP) which removes 5' phosphate groups. The plasmid DNA was extracted from the digestion mix with phenol-chloroform and precipitated with ethanol. It was resuspended in 90 μ l of 10mM Tris (pH 8.3) and 10 μ l of 10X CIP dephosphorylation buffer (10mM ZnCl₂, 10mM MgCl₂ and 100mM Tris pH 8.3) and 0.023 units of CIP were added and the reaction incubated for 30 minutes. Following this SDS to 0.5% and EDTA (pH 8) to 5mM were added and mixed. Proteinase K was then added to a final concentration of 100 μ g/ml and incubation at 56°C proceeded for 30 minutes, after which the reaction was

cooled to RT and extracted with phenol followed by phenol-chloroform. The DNA was precipitated with ethanol and 3M NaAc pH 7.

Ligation of DNA fragment into plasmid vector

To clone the 3' end of the *ro4* gene 26 ng of *Pst*I/*Bam*HI digested Bluescript™ was ligated with 22 ng of 2kb *Pst*I/*Bgl*II fragments from the mutant 2980. The reaction was carried out overnight at 15°C in a 10 µl volume including 1 µl of 10X Ligase Reaction buffer and 0.2 µl T₄ DNA Ligase.

In cloning the 5' end of the gene it was necessary to combine 7.5 ng of *Pst*I/CiP-treated Bluescript™ and 36 ng of 1.2kb *Pst*I digested 2980 DNA (roughly a 2:1 insert:vector ratio) in a 20 µl reaction volume containing 4 µl of 5X Ligase reaction buffer and 0.5 µl T₄ DNA Ligase. Ligation proceeded for 5 hours at room temperature, after which the DNA was precipitated with 3M NaOAc (pH 5.2) to 10% and absolute ethanol to three times the volume. The dried pellet was resuspended in 5 µl T₁E_{0.1} and used directly in the electroporation procedure.

Transformation of *E. coli* cells with foreign DNA and detection of transformed colonies

i) Preparation of competent cells

Competent cells were obtained by inoculating 500 ml of LB broth with 5 ml of a fresh overnight culture of DH5αF' cells derived from a single colony. The culture was incubated on a shaker bath at 37°C until an Abs₆₀₀ of 0.2 to 0.5 was reached.

The culture was then chilled on ice for 15 to 30 minutes, then decanted into two centrifuge tubes and spun at 4000xg for 15 minutes in a cold rotor. The supernatant was allowed to drain off, and the cells resuspended in the same volume of ice-cold, sterile 10% glycerol. These suspensions were spun at 4000xg for 15 minutes two more times, resuspending in 100 ml and 10 ml cold sterile glycerol respectively. The suspension was then aliquoted into microfuge tubes and stored at -70°C.

ii) Transformation of competent cells

To transform these cells, 40 μ l of cell suspension was mixed with 2 μ l of transforming DNA and the mixture transferred to a 0.1 cm electroporation cuvette and kept on ice. The cuvette was then placed in the electroporation chamber and subjected to a voltage of 180 kV. Immediately, the mixture was removed and 1 ml of SOC medium added. The SOC medium consisted of 2% Bacto-tryptone, 0.5% Bacto yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl₂ and 20mM glucose.

After a 60 minute incubation at 37°C, the cell mixtures were plated onto LBA (LB + ampicillin) supplemented with X-gal and IPTG in volumes of 10 to 50 μ l respectively, and incubated at 37°C until blue or white colonies formed. The white colonies were replica-plated onto LBA and then transferred to nylon membranes to be probed.

Rapid preparation of DNA from plasmids

A single colony from a fresh plate of DH5 α F' *E. coli* was used to inoculate 2 mls of LB or LBA liquid medium and

cultured overnight at 37°C with shaking. The next day, 1.5 mls of the culture was centrifuged in a 1.5 ml microfuge tube for 60 seconds and the supernatant poured off. The cells were resuspended in 100 μ l TE buffer and left at RT 5 minutes, then 200 μ l of 0.2N NaOH, 1%SDS was added and the tubes placed on ice for 5 minutes. Next 150 μ l of 3M KAc (pH 4.8) was added and the mixture vortexed at high speed for two minutes and left on ice for a further 5 minutes. After a 4 minute spin the supernatant was mixed with 900 μ l EtOH and allowed to sit 10 minutes to an hour at RT. The nucleic acids were then pelleted by a short (1-2 minute) spin and washed with 70% ethanol, then dried and resuspended in 20 μ l $T_1E_{0.1}$. For sequencing reactions, these were digested with 10 μ g of RNase A for 30 minutes to 2 hours (at 37°C), then collected by phenol-chloroform extraction and ethanol-precipitation.

Southern blotting

The agarose slab gel was immersed in denaturing solution (1M NaCl, 0.4M NaOH) with shaking for about 40 minutes, then in neutralizing solution (1M Tris-Cl pH 7.4-7.5, 1.5M NaCl) for about 20-30 minutes. The gel was then placed onto a 0.5 cm thick plexiglass slab which was wrapped in 1 thickness of Whatman 3MM filter paper and partially immersed in 2X SSC. A similar-sized piece of Hybond-N nylon membrane (Amersham) which had been equilibrated in 2X SSC was laid on top of the gel, followed by two pieces of filter paper and a stack of paper towels about 10 cm thick. The dish and its contents was

wrapped in plastic wrapping and a weight (glass baking dish) placed on the top. Transfer of the DNA by capillary action was allowed to proceed overnight. The next morning the pile was taken apart, the gel discarded and the nylon membrane allowed to air-dry, then baked at 80°C for about 2 hours.

Transfer of bacterial DNA from replica plates to nylon membrane

One Hybond-N nylon membrane (Amersham) was laid directly onto the LB plate containing the bacterial colonies, and allowed to rest there 1-2 minutes, until the colonies adsorbed to it. The membrane was then lifted off and laid (colony side up) onto filter paper saturated with 10% SDS for 3 minutes, then onto filter paper saturated with denaturing solution (0.5N NaOH, 1.5M NaCl) for 5 minutes, then onto a filter paper saturated with neutralizing solution (1.5M NaCl, 0.5M Tris-Cl pH 7.4) for 5 minutes, and finally onto a filter paper saturated with 2X SSC for 5 minutes. The membrane was allowed to air dry and was then baked at 80°C for 15 minutes.

Preparation of a digoxigenin-labelled probe for southern hybridizations

Eighty nanograms of the desired DNA fragment in 8 μ L was boiled for 10 minutes with 5 μ l dH₂O and 2 μ l of dp(N)₆ at 40 ng/ μ l. The mixture was then chilled quickly on ice, spun down, and to it was added 2 μ l of 10X oligolabelling buffer, 1 μ l Klenow enzyme (DNA Polymerase I, large fragment, @ 5000 units/ml, NEB) and 2 μ l digoxigenin labelling mix (Boehringer

Mannheim). This was incubated at 37°C for 1 hour and placed on ice. Half a microlitre of 0.5M EDTA, 3 μ l of tRNA at 10 mg/ml, and 76 μ l of STE (100mM NaCl, 10mM Tris pH8, 1mM EDTA) was added and the probe collected by running this mixture through a Sephadex column packed in a sterile pasteur pipette plugged with silanized glass wool.

Probing of membrane-bound DNA with specific nucleic acid probes

The nylon membranes from either colony lifts or gel transfers were pre-hybridized by dipping them briefly (about 5 seconds) in a solution of 0.1% gelatin (melted and cooled to RT). They were then inserted into hybridization chamber tubes and hybridization solution (5X SSC, 0.1% N-laurylsarcosine, 0.02% SDS, 2% dried skimmed milk powder, with 0.2% salmon sperm DNA and about 0.3% probe, boiled together for 5 minutes, in volume equal to 0.05 ml per cm of membrane) was added. Hybridization proceeded overnight, and the next morning the membranes were washed in 2X SSC with 0.1% SDS (2x15 minutes), 0.1X SSC with 0.1% SDS (2x15 minutes), and washing buffer (0.3% Tween-20 in 100mM maleic acid and 150mM NaCl, pH 7.5). They were then incubated for 30 minutes first in buffer 2 (1% milk powder in buffer 1 (100mM maleic acid and 150mM NaCl)), then for 30 minutes in buffer 2 with anti-dig-AP added at a rate of 1:10,000. Following this incubation they were again washed in washing buffer (2x15 minutes), and finally equilibrated briefly (5 minutes) in buffer 3 (100mM Tris-Cl pH

9.5, 100mM NaCl, 50mM MgCl₂). They were then placed, DNA side up, in clear plastic sheet protectors and 1 to 2 mls of Lumigen PPD (Boehringer Mannheim) diluted in buffer 3 (1 part of 10 mg/ml stock in 100 parts buffer 3) was dropped onto them and distributed evenly as the sheet protector was closed and smoothed flat. The sheet protector was placed in a photographic cassette and incubated for 10 minutes at 37°C after which a piece of Kodak film was placed on top and the whole left in the dark for exposure to take place overnight.

Preparation of sequencing reactions

For each template/primer combination the following steps were followed. To denature the template, 1.5 to 2 µg DNA in 8 µl was added to 2 µl 2M NaOH and incubated 10 minutes at room temperature. The denatured DNA was precipitated by mixing in 3 µl of 3M NaOAc (pH 4.8) and 7 µl dH₂O, followed by 60 µl EtOH and leaving on ice for 15 minutes. The mixture was then centrifuged 10 minutes, the supernatant poured off and the pellet resuspended in 7 µl dH₂O.

To this was added 1 µl primer (40ng) (Table 2) and 2 µl of reaction buffer provided in the USB "Sequenase 2" sequencing kit. Annealing occurred after this mixture was heated to 65°C for two minutes and then allowed to slowly cool to below 35°C. This preparation was then spun down, cooled on ice and used within 4 hours.

For each template/primer combination four tubes were prepared by adding 2.5 µl of one of A,C,G or T labelling mix

(provided in kit) to each, and allowing them to reach 37° in a heat block.

To each annealed template/primer combination was added 1 μ l of 0.1M DTT (dithiothreitol), 2 μ l of labelling mix (provided in kit and diluted 5X in water), 0.5 μ l of 35 SdATP and 2 μ l SequenaseTM Version 2 T7 polymerase (provided and diluted 8X in dilution buffer). This mixture was incubated 5 minutes at room temperature and then 3.5 μ l of it was added to each tube of A,C,G,T and the polymerizing reaction allowed to proceed at 37°C for 5 minutes, after which it was ended by adding 4 μ l of Stop solution (provided).

These reactions were stored in the freezer, and heated at 80-95°C for 2-5 minutes prior to loading.

Sequencing using the CircumVent Cycle sequencing method

Template DNA obtained from PCR was first purified in a Centricon spin filter (molecular weight cut-off 125 nucleotides double-stranded) according to the instructions provided.

Three microlitres of A,C,G and T sequencing mixes (provided) were placed into four 0.5 ml thin-walled microcentrifuge tubes.

In a 0.5 ml microfuge tube 100 ng of template DNA was mixed with 1.2 pmole primer (Table 2), 1.5 μ l 10X CircumventTM Sequencing Buffer, 1 μ l 30X Triton X-100 and distilled water to 12 μ l. Two microlitres of [α - 35 S]dATP and 2 units of VentTM(exo-)DNA polymerase was added and 3.2 μ l of the

resulting solution added to each of the four A,C,G and T tubes. Each reaction mixture was then overlaid with sterile mineral oil and the tubes then put in the thermocycler and run through 20 cycles of : 20 seconds at 95°C, 20 seconds of 55°C, 20 seconds of 72°C. The reactions were terminated by adding 4 μ l of Stop/Loading dye (provided) to each and stored in the freezer. They were run as above, after a 2 to 5 minute heating at 80-95°C.

Preparing and running the sequencing gel

A 6% acrylamide gel was prepared by dissolving 33.6 g urea in 8 mls 10X TBE (9M Tris,9M Boric acid,20mM EDTA) and 32.4 mls dH₂O, then adding 11.5 mls of 40% acrylamide (including 2% bis-acrylamide) and degassing for 5 minutes. The solution was filtered through Whatman #1 in a buchner funnel and 24.5 μ l TEMED and 600 μ l of 10% ammonium persulphate was added. Using a 50 ml pipette the solution was dropped between two gel plates (the shorter siliconized and the taller etched with NaOH) spaced 0.5 mm apart with plastic spacers, and taped sides and bottom. Well combs were placed flat against the top and the gel was allowed to set for at least one hour. Prior to running the sequencing reactions the well combs were removed and the top of the gel flushed with 0.5X TBE. The tape was removed and the gel was placed in the sequencing apparatus, the upper and lower buffer chambers filled with TBE and the well combs inserted between the plates to provide 100 wells. The gel was pre-run at 60W constant

power for about half an hour. Then 1.5-2.5 μ l of each sequencing reaction was pipetted into each well, and the gel run again at 60W for between 1 and 5 hours.

After the run the shorter gel plate was removed and the gel was fixed in methanol and acetic acid for 15 minutes while resting on the taller plate. It was then transferred to 3MM filter paper, covered in plastic wrap and dried in a gel dryer under vacuum for an hour. The plastic wrap was removed and the gel inserted into a film cassette with a piece of Kodak film and left in the dark to expose for between 4 days and 2 weeks.

Methods for RNA Analysis

Extraction of total RNA from fungal cultures

Mycelia of isolates grown in liquid culture (500 mls LB) for 34 to 58 h was harvested by straining through "MiraclothTM" and expressing the excess liquid between paper towelling. The mycelium was then cut into squares and frozen at -20°C, after which it was dried in a Speed-Vac. The dried mycelium was ground with roughly one gram of acid-washed sea sand and quickly mixed with 20 mls of a 1:1 mixture of extraction buffer (100mM sodium-acetate, pH 5.0, 1mM EDTA, 5%SDS) and phenol/chloroform /isopentyl alcohol (P/C/I at 26:25:1).

The mixture was then centrifuged at 10,000 rpm for 10 minutes at 4°C, after which the aqueous phase was mixed with 1.2 mg pre-digested pronase and left to incubate 1 h at room

temperature. This P/C/I extraction was repeated twice more and the RNA was precipitated with 2 volumes of ethanol. The resulting pellet was resuspended in 1.8 mls DEPC-treated water at 4°C, then re-precipitated with 3 volumes of 4M sodium acetate, pH 6.0, 10mM EDTA. After sitting at 4°C for 10 minutes the suspension was centrifuged at 10,000 rpm for 20 minutes at 4°C. The pellet was then rinsed with 70% EtOH, and resuspended in 2 ml DEPC-treated water containing 0.5% SDS. A final precipitation was performed with 1/13th volume of the above sodium acetate solution and 2 volumes EtOH, and finally resuspended in DEPC-treated water to a concentration of 2 mg/ml and frozen at -70°C.

One total RNA preparation was prepared for each of isolates WT, 1511, 1529, 2980, R1, R2, R4, and R5.

Enrichment of total RNA for poly-A mRNA

For each RNA preparation referred to above, one Dispacolumn (BioRad) was loaded with 3 mls of oligo(dT)-cellulose (packed volume) and washed with 10 mls sterile water. Each column was then washed with sterile 1X column-loading buffer (20mM Tris-Cl (pH 7.6), 0.5M NaCl, 1mM EDTA (pH 8), 0.1% sodium lauryl sarcosinate) until the pH of the effluent was reduced to below 8. The total RNA extract was heated to 65°C for 5 minutes, then cooled to RT and diluted 1:1 with 2X column-loading buffer. This solution was poured into the column, followed by about 3 mls of 1X column-loading buffer, and the eluate collected, heated to 65°C and again

passed through the column. The column was then washed with 40 to 50 mls of 1X column-loading buffer until the flow-through demonstrated an ABS_{260} of +0.0005 or less. The poly-A enriched mRNA was then eluted by adding roughly 9 mls of sterile elution buffer (10mM Tris-Cl pH 7.6), 1mM EDTA (pH 8, 0.5% SDS). The eluate was collected and precipitated with 1/13th volume of 4M sodium acetate (pH 5.2) and 2.5 volumes of ethanol. After 30 minutes the mixture was centrifuged at 10,000xg for 15 minutes at 4°C and the pellet rinsed with 75% ethanol. The pellet was then air-dried and resuspended in 400 μ l of DEPC-treated water. The absorbance at 260 nm of a 200X dilution was taken to estimate yield.

Separation of poly-A enriched mRNA

An agarose/formaldehyde gel was prepared in formaldehyde gel-running buffer (0.02M MOPS (pH 7), 8mM sodium acetate, 1mM EDTA (pH 8)) to provide a 1.2% agarose gel with 2.2M formaldehyde. A volume of poly-A enriched mRNA solution sufficient to provide 20 μ g of mRNA was dried in a speed vac and resuspended in 20 μ l of sample buffer (2 μ l 5X formaldehyde gel-running buffer, 3.5 μ l formaldehyde, 10 μ l formamide, 4.4 μ l water, 10 μ g EtBr). Before loading the samples were incubated for 15 minutes at 65°C and then chilled on ice, and the gel was pre-run at 5 V/cm for 5 minutes. Finally 2 μ l of formaldehyde gel-loading buffer (50% glycerol, 1mM EDTA (pH 8), 0.25% bromophenol blue, 0.25% xylene cyanol FF) was added to each sample and they were loaded into the

wells. A marker provided by Boehringer Mannheim was treated in the same way as the samples (but with only 10 μ g used per lane) and also loaded. The gel was run at 70 V for 3 h, with the buffer in the two chambers mixed at the half-way point. At the end of the run the RNA was transferred to nylon membrane.

Northern blotting

Following the gel run, the mRNA gel was soaked for 15 minutes in DEPC-treated water to remove formaldehyde, then for 45 minutes in 20X SSPE (3M NaCl, 0.2M $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 20mM EDTA). The gel was then placed on a plexiglass support wrapped in 3MM filter paper and a piece of Amersham HybondN nylon membrane (cut to fit) laid on top. A test tube was used to roll out any bubbles that may have come between the gel and the membrane. Over this was placed two squares of filter paper the same size as the gel and over these a stack (roughly 5-6 cm) of paper towels. The entire package was placed in a glass baking dish with 20X SSPE added to the level of the gel. Plastic wrap was used to prevent evaporation, and another glass baking dish was placed on top to ensure good contact. Transfer of RNA was allowed to occur overnight, after which the stack was disassembled and the membrane soaked briefly in 6X SSPE to remove agarose, air-dried and baked 1-2 hours at 75°C.

Preparation of radioactive RNA probe

The template consisted of the E2 fragment of the *ro-4* gene (Figure 3) cloned into the Bluescript vector. Two

micrograms of template DNA was linearized by digestion with *Sma*I, which is distal to the T7 promoter site, and treated with Proteinase K (@100 μ g/ml overnight) to remove RNases. It was then extracted with phenol/chloroform and precipitated with ethanol. The pellet was resuspended in RNase-free water. This template was combined with NTPs, [α -³²P]UTP and T7 RNA polymerase as per the instructions given with the Maxiscript kit (Ambion), and the reaction allowed to proceed for one hour. Residual DNA was digested with DNaseI and the probe was purified by passing through a spin column (Sephadex G-50) with 5 μ g yeast tRNA to improve recovery.

Hybridization

The nylon membrane to which the mRNA had been bound was pre-hybridized at 68°C overnight in 0.2 ml/cm² of 6X SSPE, 5X Denhardt's reagent, 0.5% SDS and 100 μ g/ml salmon sperm DNA in a Hybridization Incubator (Model 310, Robbins Scientific).

Probe having at least $1-2 \times 10^7$ cpm was added directly to the pre-hybridization solution and allowed to hybridize overnight at 68°C. The blot was then washed in four changes of 5X SSPE, two of which were left on for 15 minutes, then washed in 1X SSPE, 0.1% SDS for half an hour, then in 0.1X SSPE, 0.1% SDS for 15 minutes. The blot was then air-dried briefly and placed in a sheet protector between two intensifying screens in a cassette with a piece of film. Exposure took place overnight.

Methods for Protein Analysis

Preparation of fungal protein extracts for one- and two-dimensional polyacrylamide gel electrophoresis

Fungal isolates were grown in liquid VMM (Vogel's Minimal Medium) on shaker baths for 1 to 3 days. The mycelium was then filtered through MiraclothTM, rinsed with distilled water and the excess moisture pressed from it. The resulting cake was cut into small squares and frozen. Before use, one or two squares were placed in a microfuge tube and dried fully in the Speed-Vac. The resulting material was ground in protein extraction buffer containing 10mM Tris pH7.6, 1mM EDTA, 2mM DTT, 0.5% SDS, 1 μ M leupeptin, 1 μ L pepstatin and 1mM PMSF (phenylmethylsulfonylfluoride).

Eventually, a more complete protease inhibiting mixture was used, containing 1mM PMSF, 1mM Benzamidine, 1mM ϵ amino-n-caproic acid, 2mM DTT, and 1 μ g/ml each of pepstatin, leupeptin, TPCK (n-tosyl-L-phenylalanine chloromethyl ketone) and aprotinin.

One-dimensional polyacrylamide gel electrophoresis

Acrylamide slab gels (0.75 mm) were prepared according to the instructions (BioRad Minigel) to provide a 4% stacking gel and 10% resolving gel. The resolving gel was made with 3.64 ml dH₂O, 2.5 ml of 1.5M Tris (pH 8.8), 100 μ l of 10% SDS and 3.33 ml of 30% acrylamide (2.67% bis-acrylamide) added just prior to de-gassing for 15 minutes. To induce polymerization 50 μ l of 10% ammonium persulphate and 5 μ l of TEMED were added

before pipetting the solution between the gel plates. The resolving gel was overlaid with water as it polymerized. The stacking gel consisted of 6.1 ml dH_2O , 2.5 ml 0.5M Tris (pH 6.8), 100 μl and 1.3 ml of 30% acrylamide (2.67% bis-acrylamide), mixed with 50 μl of 10% ammonium persulphate and 10 μl TEMED after de-gassing.

Extracts of total protein were prepared at 2 $\mu\text{g}/\mu\text{l}$, and mixed 1:1 with 2X gel-loading buffer. These mixtures were boiled 5 minutes and then 10 to 15 μl s loaded in each lane. The gel was run in buffer consisting of 3 g Tris, 14.4 g Glycine and 1 g SDS per litre at 180 V for 45 minutes to 1 hour, then stained with Coomassie Blue, or soaked in transfer buffer if a Western analysis was to follow. The transfer buffer consisted of 3 g Tris, 14.4 g Glycine and 200 ml methanol per litre.

Two-dimensional polyacrylamide gel electrophoresis

Protein was extracted from lyophilized cubes of mycelium in a buffer containing 10mM Tris-Cl (pH 7.6), 5mM MgCl_2 , 0.25 mg/ml RNase A, 0.5 mg/ml DNase I, 0.1% SDS and 1mM PMSF. Tube gels were prepared according to instructions provided with BioRad's Mini-Protean II 2-D gel apparatus. The gel solution contained 5.5 g urea, 1.33 ml acrylamide (28.38 g acrylamide with 1.62 g bis-acrylamide), 2 ml of 10% deionized Triton X-100, 400 μl Bio-Lyte 5/7, 100 μl Bio-Lyte 3/10 and 1.97 ml dH_2O . It was warmed at 45°C to dissolve the urea and de-gassed 15 minutes, after which 10 μl each of 10% ammonium

persulphate and TEMED was added. The upper buffer was 100mM NaOH in 250 ml dH₂O de-gassed thoroughly, and the lower was 10mM H₃PO₄ de-gassed thoroughly. The tubes were pre-electrophoresed for 200V for 10 minutes, 300V for 15 minutes and 400V for 15 minutes before having the upper buffer withdrawn and replaced with the protein samples. These were suspended 1:1 in 2X sample buffer of 9.5M urea, 2% Triton X-100, 5% β-mercaptoethanol, 1.6% of Bio-Lyte 5/7 and 0.4% of Bio-Lyte 3/10, and incubated at RT 15 to 20 minutes. Twenty-five micrograms of total protein extract was commonly loaded into each tube and overlaid with Overlay buffer. This was comprised of 9M urea, 0.8% Bio-Lyte 5/7, 0.2% Bio-Lyte 3/10 and 500 μl of a 0.05% Bromophenol blue stock solution in 10 ml. In most cases it was then diluted 1:1 with dH₂O to achieve the proper density. Tubes were then pre-electrophoresed at 500V for 10 minutes and electrophoresed at 750 V for 3.5 h. Following separation the gels were ejected carefully from the glass tubes and stored individually at -20°C. To obtain the second dimension a tube gel was laid across the surface of a 10% polyacrylamide mini-gel (1 mm thickness, prepared by doubling the recipe given above) and the usual current applied.

Fractionation of total proteins

About 1 g fresh, pressed fungal mycelium was ground with 1 g washed sand and 10 mL protein extraction buffer (with full range of protease inhibitors as above). The slurry was

centrifuged 10 minutes at 2,500 rpm (700xg) in a Sorval SS-34 rotor at 4°C. The supernatant was poured off (to obtain three other fractions from it) and the pellet washed in 3 mls of buffer and retained for later use.

The supernatant was centrifuged 20 minutes at 9500 rpm (10,000xg) in the same conditions as above. The new supernatant was poured off and retained while the pellet was washed in 2 mls buffer and spun again to collect, then resuspended in 500 μ L sucrose buffer (0.25M sucrose, 50mM Tris pH 7.6, 50mM KCl) to provide the 10K fraction.

The most recent supernatant was then centrifuged 60 minutes at 27,000 rpm (100,000xg) in a Beckman SW40 rotor, at 4°C. The resulting supernatant was poured off and became the cytosolic fraction. The pellet was resuspended in 500 μ l sucrose buffer (100K fraction).

To obtain soluble proteins representing the nuclear fraction, 0.6 g of the first pellet was mixed with 1 ml protein extraction buffer, incubated 15 minutes on ice and then centrifuged 4 minutes at 7,000 rpm and 15 minutes at 14,000 rpm. The resulting supernatant was referred to as the nuclear fraction.

Each fraction was analysed by spectrophotometry to determine the concentration ($183A_{230} - 75.8A_{260} = \mu\text{g/ml protein}$) and dilutions were made to 2 mg/ml protein. PAGE gels using fractionated proteins were loaded with 10 μ g per lane.

Western blotting

The gel was equilibrated in transfer buffer (25mM Tris, 192mM glycine, 20% methanol) for 15-30 minutes. One piece of nitrocellulose membrane (Hybond-ECL, Amersham) and 6 pieces of filter paper, all cut to the same size as the gel, were also wetted in transfer buffer. Using the Trans-Blot Semi-dry electrophoretic transfer cell (BioRad), the membrane was placed atop 3 pieces of filter paper, and overlaid with the gel and the other three filters. Air bubbles were rolled out after each layer. Transfer was accomplished at 10-15 V for about 20 minutes (for mini-gels), or 20 V for 50 minutes (for larger gels). Following transfer the membrane was stained with PonceauS (0.2% in 0.3% TCA) to assess completeness of transfer, and the gel was stained in Coomassie (Coomassie Brilliant Blue R250 in 10% Acetic acid, 40% methanol) and in some cases silver-stained according to the instructions (BioRad).

Western analysis

The nitrocellulose membrane was blocked in TBS-T (Tris-buffered saline with 0.1% Tween 20) containing 2% skimmed milk powder for 2 h. This liquid was then replaced with 1% skimmed milk in TBS-T containing the primary antibody at 1:500 dilution, and the blot incubated for 1 hour. The blot was then washed 4x15 minutes with TBS-T before being incubated in the appropriate (anti-rabbit or anti-mouse) secondary antibody at a 1:2000 dilution in TBS-T and 1% skimmed milk. Four more

15 minute washes in TBS-T followed this step, after which the antibody-labelled proteins were detected using the Enhanced Chemi-Luminescence method of Amersham (according to the instructions accompanying the kit). All incubation steps were carried out with gentle shaking at room temperature.

The monoclonal anti-actin antibody was catalogue number 1 378 996 from Boehringer Mannheim.

Preparation of polyclonal antibodies from rabbits

Two peptides were chosen from the ro-4 sequence (Figure 2) and synthesized by the Peptide Synthesis Lab (Ottawa Centre for Cancer Research, University of Ottawa, 451 Smythe Road, Ottawa) on an 8-branch lysine tree. The choice of peptides was made on the basis of hydrophilicity of the region to ensure that it would represent an exposed epitope, and relative uniqueness. The latter consideration was examined by sending the sequences (11 and 13 amino acids, respectively) to GenBank (Bethesda, Md) to search for similar sequences. None were found. The peptide preparations were resuspended to a concentration of 10 mg/ml and stored at 4°C. Two New Zealand White rabbits per antigen were each injected subcutaneously with 0.25 mg of antigen suspended in 1 ml of RIBI Adjuvant (MPL+TDM+CWS Emulsion, R-730, from RIBI ImmunoChem Research, Inc., 533 Old Corvallis Road, Hamilton, MT). Four weeks later each received a booster injection of the same composition. Pre-bleeds of 2 ml per rabbit were conducted prior to the first injection, and post-bleeds began two weeks after the

first boost.

Affinity purification of polyclonal antibodies

As a preliminary purification step, antiserum was either heated to 56°C for 30 minutes, or run through a DEAE (diethylaminoethyl) Affi-gel Blue GelTM (Bio-Rad) to remove albumin and other proteins with dinucleotide folds. Affinity chromatography using the peptides as ligand was carried out on Affi-gel 10 according to instructions from BioRad.

First 30 mg of ligand was dissolved in 2 mls of 0.1M MOPS pH 7.5. Coupling to 1.3 ml of Affi-gel 10 as allowed to proceed for 4h at 4°C with gentle agitation. Then 300 μ l of 1M ethanolamine (pH 8) was added to block unreacted sites; this proceeded under the same conditions for 1½ hours. The gel bed was then added to a column and washed with 15 to 25 mls of the elution solution: 0.1M glycine-HCl pH 2.5. Five mls of previously-heated serum was then poured through the column which was then washed with about 50 mls PBS. The now-specific antiserum was eluted with 2.5 ml 0.1M Glycine-HCl pH 2.5 into a falcon tube containing 67 μ l 1.5M Tris-Cl pH 8.8 to neutralize the pH, and dialysed against 1 litre PBS for 2 days.

Ten mls of rabbit serum was dialysed against application buffer (20mM Tris-HCl pH 8.0, 25mM NaCl, 0.02% NaN₃) for 2 days.

The DEAE Affi-Gel Blue Gel bed was prepared by washing the gel with 5 volumes of pre-wash buffer (0.1M acetic acid,

1.4M NaCl, 40% isopropanol) followed by 10 volumes of application buffer (0.02M Tris-Cl pH 8.0, 0.025M NaCl, 0.02% NaN_3), then transferring it to the column. Once in the column, it was eluted with 3 volumes (105 ml) of application buffer, after which 13 mls of serum was added. The gel was then eluted with 3 more volumes of application buffer which were collected in 10 ml fractions and tested for light absorbance at 260 and 280 nm to monitor the progress of the elution. The first 50 mls was then passed through the second-epitope coupled column. This column was washed with PBS and eluted with 2.5 mls 0.1M Glycine-HCl pH 2.5 as above. The eluate was neutralized and dialysed against PBS.

Unfortunately, the preliminary Glycine-HCl wash of the RANRDTAAQIL column was mistakenly omitted so the final eluate may have contained uncoupled ligand as well as antibodies. However the early results (including dot blots) of this preparation were more promising than those using the antisera derived from the KEEKEWAAAKMDP peptide, so the former was used in the Western analyses presented here.

The DEAE Affi-Gel Blue GelTM column was regenerated with 2 volumes of regeneration buffer (2M guanidine HCl in application buffer) followed by 3 volumes of application buffer.

Methods for Phenotypic Analysis

Nuclear and germling staining

Conidia were obtained from slant cultures grown 10 days to 3 weeks by adding 1 to 2 ml dH₂O to the slant and vortexing to loosen conidia from the culture. The resulting suspensions were kept refrigerated in eppendorfs for up to 2 weeks. For nuclear staining, 60μL of settled conidia were mixed with 500mL PBS then spun very briefly (about a second) in a microcentrifuge. The supernatant was poured off and 20 μl of 10 μg/ml DAPI was added to the roughly 20 μl remaining. The mixture was allowed to incubate at room temperature for 10-15 minutes, then spun again as briefly, tipped to pour off the supernatant which was replaced with 500mL dH₂O to wash the conidia. After a final brief spin the supernatant was poured off and about 6 μl was smeared onto a slide in two smears. To each smear about 10 μl of 90% glycerol was added and a coverslip dropped on top and sealed with nail polish. For septal staining 10 μl of a 10 μg/ml stock solution of calcofluor was added prior to applying the coverslip.

Conidia were inoculated onto slides smeared with two drops of liquid VMM (about 20 μl per drop) and left at room temperature to incubate for 4 to 5 hours (3h for wild-type). Dessication was prevented by keeping the slides on moist filter paper in petri dishes. After incubation the smears were carefully washed by pipetting about 100-500 μl of PBS onto the slide and then pipetting or wicking it off with torn

filter paper. Ten microlitres of 10 $\mu\text{g/ml}$ DAPI was then added to each smear, left to incubate 10-20 minutes, then rinsed off with dH_2O in the same manner as the PBS rinse. Cover slips were affixed with 10 μl of 90% glycerol and sealed with nail polish.

The slides were viewed on a Zeiss microscope equipped with a mercury burner.

III. Results

The *ro-4* wild-type sequence

a) Sequencing of *ro-4* cDNA for determination of intron positions:

It was necessary to amplify *ro-4* cDNA directly from a *N.crassa* cDNA pool in order to perform sequencing reactions, as previous attempts to obtain a *ro-4* cDNA clone by screening three different cDNA libraries had failed (Robb, and Vierula, personal communications). Amplification of *ro-4* cDNA was achieved using the Polymerase Chain Reaction and a total cDNA pool. The primer pair *ro4h* and *ro4d* yielded a product just over 1kb (the expected size for that region) which hybridized strongly with the E2 probe (see Figure 3 and Table 2 for location of primers and probes). The primer pair *ro4h* and *ro4g* yielded a slightly larger product when WT genomic DNA was used as template, than when WT cDNA was used. Both products bound the E2 probe in Southern blots.

The product was sequenced using Circumvent Cycle Sequencing to establish the positions of the three introns in the *ro-4* gene by comparison with the genomic sequence. The successful primers were situated between 300 and 500 bp upstream of each intron. The comparison confirmed that the introns began and ended where Robb (1993) had postulated (Figures 2 and 3). The introns do not interrupt any major secondary structures (Figure 2).

Figure 2. Comparison of amino acid sequence between Arp1s: Approximate binding sites for calcium ion (c), nucleotide (n), actin (a) and myosin (m) are noted below each comparison block, as are secondary structure features of α -helices and β -strands (with reference to α -actin, Weber and Kabsch, 1994).

The sequences are: ro-4 (ro4, Robb, 1993), actin-related protein vertebrate (rpv, Lees-Miller et al., 1992b), the yeast actin-related protein 5 (ac5, Muhua et al., 1994), *Drosophila* Actr87c (D87, Fyrberg et al., 1994) and *Aspergillus* gamma actin (Asg, Fidel et al., 1988). Similar residues conserved across the four Arp1s are given in bold. The five residues at which ro-4 differs from the other three Arp1s are underlined. The 11 and 13 amino acid regions printed in italics are the two peptides against which antibodies were raised.

The last residue of each exon of ro-4 is marked with ",".

ro4 M.T..DS..LHNAPIVLDNGSGTIRAGFAGDDVPKCHFSPFVGRPKHLRVLAGALEGE
 rpv MESY.DV..IANQPVVIDNGSGVIKAGFAGDQIPKYCFPNYVGRPKHVRVMAGALEGD
 Ac5 MDQLSDSYALYNQPVVIDNGSGI IKAGFSGEERP KALEYCLVGNTKYDKVMLEGLOGD
 D87 MEPY.DV.VV.NQPVVIVNGSGVIKAGFAGEHIPKCRFPNYRGRPKHVRVMAGALEGD
 Asg ME...EF...VAALVIDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHH....GIMIG.
 m mm c nnn n mmmmm aaa aaaa
 BBBB BBBB BBBB BBB

ro4 VFIGQK....A..ASELRGLLKIRYPLEHGIIVTDWDDMEKIWAYVY..D.EGLKTLSE
 rpv IFIGPK.....AEEHRGLLSIRYPMEHGIIVKDWNDMERIWQYVYSKD..QLQTFSE
 Ac5 TFIGN!.....AQKLRLGLLKIRYPIKHGVVEDWDSMELIWSYVL.NEVLQQLQONIG.
 D87 IFVGPKE.....AEEHRGILSIRYPMEHGIIVTDWNDMERIWSYIYSKA..QLATFTE
 Asg ..MGQKDSYVGDEAQSKRGILTLRYPIEHGVVTNWDDMEKIWHHTFYNELRVAPE...
 BB maa aaaaaaaaaaaaaa mmm

ro4 EHPVLL.TEPPLNPRANRDTAAQILFETFNVPALYTSIQAVLSLYASGRTTGCVVLD SG
 rpv EHPVLL.TEAPLNPRKNRERAAEVFFETFNVPALFISMQAVLSLYATGRTTGCVVLD SG
 ac5 EHP.LLITEAPMNPLKNREQMAQVLFETFDVSALYVSNPAVLSLYASGRTTGCVVLD SG
 d87 DHPVLL.TEAPLNPRNRREKAAEFFFEGINAPALFVSMQAVLSLYATGRVT.VVLD SG
 asp EHPVLL.TEAPINPKSNREKMTQIVFETFNVPAFYVSIQAVLSLYASGRTTGIVLD SG
 aaa c c
 BBB BB aaaaaaaaaaaaaa BBBB BB aaaaaaaaaa BBBB BB

ro4 DGVSHAVPVYQGF TVPNSIRRIDVAGR DVTEYLQ TLL.RKS.GYVFHTSAEKEIVRLI
 rpv DGVTHAVPIYEGFAMPHSIMRIDVAGR DVSRLRLYL.RKE.GYDFHSSSEFEIVKAI
 ac5 EGYCSTVPIYDGFALPASMMRMDIGGADITEQLQFQL.RKSAGVSLFSSSEREIVRTM
 D87 DGVTHAVPIYEGFAMPHSIMRVDIAGR DVTRYLKT.LIFRE.GFNFRSTA EFEIVRSI
 asp DGVTHVVPYIYEGFALPHAISRVDMAGRDLTDYLMKILAFR..GYTFSTTAEREIVRDI
 nnn aaaaaaa aa a aaaaa
 BBBB BB aaaaaaaaaaaaaa aaaaaaaaaaaaaa

ro4 KESVTYVAHDPRKEEKEWAAAKMDPAKIAEYVLPDGNKLGIGAERFRAPEILFDPEII
 rpv KERACYLSINPQKDE..TL....ETEK.AQYVLPD GSTIEIGPSRFRAPPELLFRPDLI
 ac5 KE.VCYLAKNIKKEEEKYLQGTQDL..ISTFKLPDGRGCI EVGNDRYRAPEILFSPQII
 D87 KEKVCYLATNPQKEE..TV....ETEKFA.YKLPDGKIFEIGPARFRAPEAVFRPDLL
 asp KEKLCYVALDFEQ EIQTASQSSSLEKS..YELPDGQVITIGNERFRAPKALFQPSVL
 nn aaa aa
 aaa BBBB aaaaaaaaaa

ro4 GLEYPGVHQIVVDSINRTDLDDLRLDLYSNIVLSGGSTLTGFGDRLLTEVQKLAVKDM
 rpv GEESEGIHEVLVFAIQSDMDLRLTLFSNIVLSGGSTLFGFGDRLLSEVKKLAPKDV
 ac5 GLGYDGLSDMCMQSIWKVDLRLKPLLSII LSGGTTTLKGFGDRMLWDLEALTKGTS
 D87 GEECEGIHDVLMYSIEKSDMDLRKMLYQNIIVLSGGSTLFGFGDRLLSELKKHS AKDL
 asp GLESGGIHVTTFNSIIKCDVDVRKDLYGNIVMSGGTTMYPGISDRMQKEITALAPSSM
 aa aaaaa nnnnn aaa
 BBBB aaaaaaaaaa

ro4 RINIFAPPERKYSTWIGGSILAGLSTFRKMVVSIDDWHENPDI.I.HTK.FT
 rpv KIRISAPQERLYSTWIGGSILASLDTFKKMVSKKEYEEDGARS I.HRKT F
 ac5 KIKIIAPSERKYTTWIGGSILTGLSTFORLWTKKSDWLEDSTKVYSNL M
 D87 KIRIAAPQERLYSTMGGGSILASLDTFKKMWISKREYEEEGQK.AVHRKTF
 Asg KVKIIAPPERKYSVWIGGSILASLSTFQQMWISKQEYDE.SGPSIVHRKCF
 n mm mmmmm
 BB aaaaaa

Figure 3. Map of the *ro-4* gene: There are four exons at positions 1→308, 393→848, 943→1257 and 1358→1417. The cutting sites of the restriction enzymes *EcoRV* (E), *PstI* (P), *SphI* (S), *BamHI* (B), *XhoI* (X) and *ScaI* (Sc) are as marked. The segments E1 and E2 are restriction fragments produced by *EcoRV*. In addition the region is bounded by an upstream *SmaI* site and a *BglII* site downstream, roughly 1.6 kb from the end of the coding region.

The translation initiation start site diverges from consensus for *N.crassa* at only 3 of 10 bases. The 5'-intron donor sites and 3' acceptor sites agree with consensus at 76% and 67% of nucleotides respectively, across the three introns (Figure 4). In contrast the internal branchpoints of the introns agree closely (90%) with the consensus.

b) Comparison of ro-4 amino acid sequence with other Arp1s

Using the similarity criteria of Lees-Miller et al. (1992a), the five residues at which *ro-4* differs from the other Arp1s (Figure 2) are also different from analagous residues in α - and β -centractin (Clark et al., 1994), *Schizosaccharomyces pombe* act2 (=Arp3, Lees-Miller et al, 1992a), *Saccharomyces cerevisiae* ACT2 (=Arp2, Schwob and Martin, 1992), both Arp2* and Arp3 from *Acanthamoeba castellanii* (Kelleher et al., 1995), *Saccharomyces cerevisiae* ACT1* (in Harata et al., 1994), *Drosophila melanogaster* actin-related proteins Act5c, Act53D, Actr14D and Actr66B (in addition to Actr87C shown in the comparison, Fyrberg et al., 1994) and major actins from *Oxytricha nova*, *Tetrahymena thermophila*, *Trypanasoma brucei**, *Schizosaccharomyces pombe* and human cytoplasmic gamma actin (Lees-Miller et al., 1992a). Interestingly, maize major actin shares the T₁₇₃ with *ro-4*, and three others (marked with asterisks above) have a serine at that site. One actin-related protein which shares two of the five residues with *ro-4* (T₁₈ and T₁₇₃) is *Saccharomyces cerevisiae* ACT3, which is the most distantly related of actin-

Figure 4: Sequence features of the *ro-4* gene as compared with consensus for *Neurospora crassa* genes (Edelmann and Staben 1994). Bold lettering designates *ro-4* sequence while consensus is given in normal type.

Consensus translation initiation site compared with that of ro4:

CAM¹ATGGCT
AACAAATGACA (-7 to +6)

Consensus 5'-intron donor site compared to that of ro4:

first intron: G[^]GTTTCGTTCTCCCA
second intron: G[^]GTATGTTAAGTTT
third intron: G[^]GTATGTCCTCACA
ro4 consensus: G[^]GTWYGTYMWSHYW
G[^]GTAAGTnnYCnYY

Consensus internal branchpoint:

first intron: AGCTAACAACGGGTCC
second intron: TACTGACACCCACCAA
third intron: TGCTAACAAATGTGTG
ro4 consensus: WRCTRACAMMBRBBHV
WRCTRACMnnnnnnnYY

Consensus 3'acceptor site:

first intron: GCTAG[^]
second intron: CAAAG[^]
third intron: AACAG[^]
ro4 consensus: VMHAG[^]
WACAG[^]

where Y = C,T
R = A,G
M = A,C
K = G,T
W = A,T
S = G,C
D = A,G,T
H = A,C,T
V = A,C,G
B = C,G,T
N = any base

related proteins (Kelleher et al., 1995), yet the Arp1 most closely related to *ro-4*, *Pneumocystis carinii* actinII, shares only the T₁₃₇ with it (Genbank Accession data).

The lysine at position 365 (which is replaced in *ro-4* by isoleucine) is conserved identically in all other proteins listed here, except *D melanogaster* Actr53D which is only expressed in testes (Fyrberg et al., 1993), and *Pneumocystis carinii* actinII. The first four residues (18, 137, 170 and 173) are similar to actin-rpv in 61%, 100%, 96% and 56% of proteins listed above. All five of these sites reside in the same quadrant of the three-dimensional structure at which the carboxy and amino termini are located, both of which are also relatively unconserved in *ro-4* (Figure 2). The proposed binding sites of many actin-binding proteins, including myosin, are located in this quadrant (Hennessy et al., 1993).

Another feature of interest in the sequence comparison is the absence in *ro-4* of the conserved peptide RK(A,C,T)F at the carboxy terminus which has been proposed to determine the folding pathway by binding to a peptide in the chaperonin complex (Burns and Surridge, 1994). Chaperonin-folded actin-rpv has been shown to be capable of co-polymerizing with actin filaments (Melki et al., 1993), although *Acanthamoeba castellanii* Arps 2 and 3 display the same peptide yet are probably unable to interact with actin as more than a barbed end nucleator (Kelleher et al., 1995). A profilin binding site that is conserved in Arp2s (Kelleher et al., 1995) is

absent from *ro-4* and the other *Arp1s* presented in Figure 2.

Sequencing of the *ro-4* mutant allele:

The strain 2980 is a uv-induced mutant whose mutation was mapped to a locus designated *ro-4* (Garnjobst and Tatum, 1967, Appendix I). It was desirable to obtain the sequence for this allele in order to gain insight into how the mutated protein gives rise to the mutant phenotype.

Genomic DNA from this strain was cut into two *ro-4* fragments which were cloned into the BluescriptTM plasmid and sequenced.

The strain 2980 was found to contain a single deletion (Figure 5) within the first exon of the gene. The remainder of the coding region of the gene was found to be identical to the wild-type sequence. To confirm this result, a PCR was carried out using freshly prepared 2980 DNA as template and the region of the deletion was then sequenced using CircumventTM Cycle Sequencing.

The result of this deletion is a frameshift mutation which codes for several mis-sense amino acids and then a premature termination codon (Figure 6). If translated, the mutant protein would be only 8.2 kD in size.

Restriction fragment length polymorphism analysis of *ro-4* mutant strains:

The three *ro-4* mutant strains were expected to have single point mutations as a result of uv-irradiation. This

Figure 5. The *ro-4* mutant has a single base-pair deletion: The sequence on the left is that of the *N. crassa ro-4* mutant 2980, the corresponding region of wild-type sequence is on the right. The arrow marks the position of the adenine deletion in the *ro-4* mutant. Above (downstream of) the deletion the mutant sequence is frame-shifted relative to wild-type.

Figure 6. Putative amino acid sequence of the *ro-4* mutant: The nucleotide sequence of the first exon and part of the first intron of *ro-4* is presented, followed by the amino acid sequence of the wild-type (in bold) and mutant (in normal type) The prematurely terminated *ro-4* sequence codes for a mutant protein of 78 amino acids and 8.2 kD molecular weight, compared to the wild type protein of 380 amino acids and roughly 42.8 kD weight.

* site of mutation

ATG ACA GAC TCT TTG CAC AAC GCG CCC ATT GTG CTC GAC AAT
 +41
 GGC TCG GGC ACC ATT CGC GCC GGC TTC GCG GGA GAC GAC GTA CCA
 86
 AAA TGC CAC TTC CCC TCC TTC GTA GGG AGG CCA AAG CAC CTA CGA
 131
 GTG CTG GCC GGC GCA TTA GAA GGC GAG GTG TTT ATC GGC CAA AAG
 176
 GCG GCC TCA GAG TTG CGC GGA CTG CTC AAG ATC CGA TAT CCG CTG
 221
 GAG CAC GGC ATC GTG ACG GAT TGG GAC GAT ATG GAG AAG ATT TGG
 266
 GCA TAT GTA TAC GAT GAG GGG TTG AAG ACG CTC AGT GAA GAG
 308
 GTTCGTTCTCCCATATTCTTTTGGTCAGAACAAAGACAAAAAAAAAAAAAAAAAAGCGGTA
 368

*
 MTDSLHNAPIVLDNGSGTIRAGFAGDDVPKCHFSPFVGRPKHLRVLAGALEGEVFSANRRPQ
 MTDSLHNAPIVLDNGSGTIRAGFAGDDVPKCHFSPFVGRPKHLRVLAGALEGEVFIGQKAAS
 SCADCSRSDIRWSTAS
 ELRGLLKIRYPLEHGIVTDWDDMEKIWAYVYDEGLKTLSEHPVLLTEPPLNPRANRDTAAQ
 ILFETFNVPALYTSIQAVLSLYASGRTTGVVLDSGDGVSHAVPVYQGFTVPNSIRRIDVAGR
 DVTEYLQTLRLKSGYVFHTSAEKEIVRLIKESVTYVAHDPRKEEKWAAAKMDPAKIAEYVL
 PDGNKLKIGAERFRAPEILFDPEIIGLEYPGVHQIVVDSINRTDLRLRDLYSNIVLSGGST
 LTKGFGDRLLTEVQKLAVKDMRIKIFAPPERKYSTWIGGSILAGLSTFRKMWVSIDDWHENP
 DIIHTRFT

was tested by Southern analysis of restriction fragments generated by four restriction enzymes, in the wild-type and three mutants. Although this will not prove the existence of a point mutation it can demonstrate that no major deletions or insertions are present.

The fragments produced by four restriction enzymes known to have sites within the gene were probed first with the E2 probe (see Figure 3) to reveal changes within the coding region, and secondly with a 4.3 kb probe (from the cosmid originally used to transform ro-4) which recognizes the surrounding sequence.

The fragment produced by the enzyme *EcoRV* spans 90% of the coding region of the gene and migrates the same distance in all four strains (Figure 7, upper panel, Table 3) indicating the absence of major deletions or insertions within this region. Within this 90%, the strains 1529 and 2980 are similar to wild-type in their pattern of restriction fragments (Figure 7, Table 3). The strain 1511 differs in the fragments produced by *PstI*, *ScaI* and *XhoI*, however in each case one fragment is shared with the other strains and thus at least two recognition sites are probably undisturbed. (The pattern of fragments produced by digestion of wild-type DNA with *ScaI* can be deduced from the results shown in the lower panel.)

When a larger probe was used, again it was seen that at least one fragment was shared across all strains, however differences (from wild-type) existed in fragments from 1529

Figure 7. Restriction fragment length polymorphism analysis: Genomic DNA of the strains WT (a), 1511 (b), 1529 (c) and 2980 (d) was digested with each of the following enzymes: *EcoRV* (E), *PstI* (P), *ScaI* (S) and *XhoI* (X). The probe used in the upper panel was an E2 fragment which includes 90% of the coding region of the *ro-4* gene. The lower panel was probed with a 4.3 kb fragment originating from a cosmid clone which contains the entire gene. The size markers are given in kb. The sizes of the restriction fragments are given in Tables 3 and 4.

Table 3: Restriction fragments recognized by the E2 probe. Both strain 1511 and 1529 are from a Mauriceville genetic background, while WT and 2980 are derived from St. Lawrence. The estimated sizes of fragments generated by the enzymes *EcoRV* (E), *PstI* (P), *ScaI* (S) and *XhoI* (X) and detected using the E2 probe, were calculated using the DNAfrag program. Fragments given in brackets are assumed to be undigested genomic DNA.

expected fragment size(kb):		actual size (kb):			
		<u>74 (WT)</u>	<u>1511</u>	<u>1529</u>	<u>2980</u>
<i>EcoRV</i>	1.183	1.1	1.1	1.1	1.1
<i>PstI</i>	1.205	1.18 7.0	1.18 (24.58)	1.18 7.0	1.18 7.83
<i>ScaI</i>			2.73 (46.84)	10.14 (46.84)	10.14 (46.84)
<i>XhoI</i>		2.48 7.83	7.34 (20.62)	2.48 7.83	2.42 7.34

Table 4: Restriction fragments recognized by the 4.3kb probe. Both strain 1511 and 1529 are from a Mauriceville genetic background, while WT and 2980 are derived from St. Lawrence. The estimated sizes of fragments generated by the enzymes *EcoRV* (E), *PstI* (P), *ScaI* (S) and *XhoI* (X) and detected using the 4.3 kb probe, were calculated using the DNAfrag program. Fragments given in brackets are assumed to be undigested genomic DNA.

expected fragment size(kb):		actual size (kb):			
		<u>74 (WT)</u>	<u>1511</u>	<u>1529</u>	<u>2980</u>
<i>EcoRV</i>	0.923	1.08	1.1	1.08	1.08
			1.2		
	1.183	1.31	1.3	1.31	1.3
		1.64		1.64	1.7
			2.1		
			4.35		
		4.98		4.98	
					5.41
<i>PstI</i>	1.205	1.38	1.38	1.38	1.42
		2.14			2.22
			4.98	5.19	
		5.86			
			6.15	6.15	
					6.72
<i>ScaI</i>			2.93		
			4.06		
		8.39	8.39	9.46	10.21
		(23.25)	(28.11)	(35.5)	(35.5)
<i>XhoI</i>		2.74		2.74	2.74
		7.52	8.39	8.39	8.39
			(23.25)		

and especially 1511 (Figure 7, lower panel). Some of these can probably be attributed to the genetic background of these two strains, which is Mauriceville as opposed to the St. Lawrence background of wild-type.

The largest "fragments" (above 20 kb, see Tables 3 and 4) are probably undigested DNA. All four enzymes are palindromic six base-pair cutters, so that we can predict a mean fragment size of about 5000 bp in a random sequence of nucleotides (calculated as the reciprocal of the probability of finding a restriction site: $(\frac{1}{4})^6$). In fact the mean size of fragments binding the E2 probe is 4.36 kb (± 3.4) and that of fragments binding the larger 4.3 kb probe (a *Bgl*III to *Not*I cosmid fragment) is 3.97 (± 2.85).

The E2 probe did not label the 2 kb *Pst*I fragment seen in the wild-type lane labelled with the 4.3kb probe, suggesting that an additional *Pst*I site near the gene is upstream of the 5' end *Pst*I site.

The expression of *ro-4*

The abundance of the *ro-4* mRNA was expected to be lower than wild-type in the RIP strains, yet comparable with the wild-type in strains 1511 and 1529.

a) *ro-4* mRNA levels in the wild-type and three mutant strains:

A Northern analysis was performed on polyA-enriched RNA extracted from WT, 1511, 1529, 2980, and the RIP strains R1, R2, R4 and R5. The probe used was a *ro-4* antisense RNA.

Figure 8. Northern analysis of *ro-4* expression: The left panel illustrates the position of the 26S and 17S ribosomal RNAs remaining in the ethidium bromide-stained preparations of polyA-enriched RNA, as visualized on a nylon membrane by uv light. The right panel is an autoradiograph taken after the nylon membrane was probed with a ^{32}P -labelled anti-sense RNA probe representing the E2 fragment of *ro-4*.

Lanes a, b, c and d contain mRNA extracted from the RIP strains R5, R4, R2 and R1; columns e, f and g represent the *ro-4* mutant strains 2980, 1529 and 1511, and column h is wild-type.

a b c d e f g h

a b c d e f g h

Three bands appeared in the wild-type lane on the Northern blot (Figure 8) with approximate sizes 2.9, 2 and 1.65 kb. The two largest bands appeared in all other strains, with the possible exception of R1 whose lane is smudged. However the intensity of the lowest band varied across the strains; it is extremely faint in 2980 and R5, and is absent in R2. The strain R4 has additionally a band smaller than even the smallest of wild-type. The largest of the three wild-type bands migrated at the same rate as one of two bright bands, presumed to be ribosomal RNA (26S and 17S species), which were visible on the nylon transfer (viewed under uv, Figure 8). However the intensity of the labelling did not correspond to the intensity of the ethidium bromide staining in these two bands, suggesting that the rRNA was not recognized by the probe. This is consistent with the fact that the lower of the two bright bands seen on the nylon transfer did not appear to label at all with the probe, and is situated between the two smaller wild-type bands seen on the Northern.

b) Predicted expression level of wild-type *ro-4*:

The codon adaptation index (C.A.I.) was calculated for *ro-4* based on a reference set of three highly expressed *N. crassa* genes (see Appendices II and III). The result was a value of 0.489. This is only slightly lower than the value for the most lowly-expressed histone proteins in yeast (0.532), whereas the lowest value for C.A.I. among the yeast

genes was 0.098 and the highest was 0.929 (Sharp and Li, 1987). This suggests that *ro-4* may be expected to have a moderate level of expression.

Protein analysis of the wild-type and *ro-4* mutant strains

Initially an antibody to conventional actin was used in Western analyses on proteins of the WT and *ro-4* mutant strains in the hope that it would recognize the *RO4* protein. Although this approach yielded some interesting findings, eventually we determined to develop a more specific antibody with which to seek the *RO4* protein. Ideally such an antibody would enable us to compare the size, pI and cellular location of a mutant allele with the wild-type version, and in particular confirm that the *RO4* protein is absent in the strain 2980, as the sequence data suggests.

a) Western analysis of total proteins from wild-type and *ro-4* mutant strains with a monoclonal anti-actin antibody:

PAGE-separated total proteins of WT, 1511, 1529 and 2980 were transferred to nitrocellulose and probed with Boehringer-Mannheim monoclonal anti-actin (1 378 996) in a Western analysis. Only one band was clearly labelled, of a mass consistent with actin. This band appeared in all strains except 1529 (Figure 9), though polyclonal anti-actin as well as polyclonal anti-tubulin labelled all four strains.

When the same antibody was used to probe 2-dimensional separations of total proteins, a single, elongated spot

Figure 9. One and two-dimensional Western analysis of major actin: Western analysis of the major actin in the wild-type and three *ro-4* strains. A monoclonal anti-actin was used to probe one- and two-dimensional blots of total proteins (upper panel). In the lower panel the blots were probed with both the monoclonal anti-actin and a polyclonal anti-tubulin. In the 2-D blots the proteins were separated by iso-electric focusing followed by denaturing SDS PAGE. The position of the tubulin is marked with an arrow. Size markers are given in kD.

suggestive of two equal sized isoforms was labelled in WT, and could also be seen in 1511 and 2980, but not in 1529. This spot appeared in the centre of the blot, implying a near-neutral pI. When this antibody was combined with polyclonal anti-tubulin, another spot appeared above the major actin and offset to the acidic side (the basic side of the gel can be identified by the dark vertical band representing the top of the tube gel).

b) Western analysis of wild-type and *ro-4* mutant strains with a RO4 antibody:

A synthetic peptide representing sequence from a region of the RO4 protein (Figure 2) was injected into rabbits to raise antibodies against RO4. The peptide occurred after the point of premature termination later discovered in the strain 2980 allele of *ro-4*. When bleeds indicated the serum could recognize dot blots containing 20-200 ng of the antigen the rabbits were bled and the serum collected. The serum was purified against DEAE-Affigel Blue Gel and against the antigen coupled to Affi-gel in an affinity column.

Since serum commonly contains antibodies against actin (unpublished results, Pollard, pers.comm.) it was necessary to probe blots of separated proteins with both pre-immune serum and with immune serum, and compare the results. Proteins that are only labelled in the immune serum are more likely to exhibit the epitope against which the antibody was raised.

Using immune serum to probe a blot of wild-type and *ro-4*

mutants (10 μ g total protein per lane), two bands unique to the immune vs the pre-immune and having a mass estimated by the program BANDSIZE at 41.6 and 40.3 kD were seen (Figure 10a). The lower of these two bands occurred only in the wild-type and strains 1511 and 1529 (the pattern expected of RO4), whereas the upper appeared across all lanes. When I attempted to repeat this result (Figure 10b), the two bands were estimated at 42.3 and 40.4 kD and both appeared across all strains, though the lower was faint in 2980 and the R1P strains. Although the latter migrated at the same distance as a protein labelled faintly by the pre-immune serum, this could merely be due to the similarity between the major actin (to which many animals harbour antibodies) and RO4.

c) SDS PAGE and Western analysis of degraded wild-type proteins:

To determine if degradation of protein extracts was playing a role in the lower molecular weight bands seen in the blots probed with immune serum, wild-type protein extracts were subjected to varying degrees of degradative conditions, then separated by PAGE and probed with pre-immune and immune sera. Although the silver-stained gel revealed the disappearance with time (more pronounced in the absence of protease inhibitors) of a protein of the same MW as actin (Figure 11), no difference was seen between the degradation conditions in the resulting Western analysis probed with either pre-immune serum or anti-RO4 antibody (Figure 12).

Figure 10a. Western analysis using R04 antibodies, trial 1: Both panels are western analyses detected by the chemiluminescent method, and contain separated total proteins (10 μ g per lane) from the wild-type, the three ro-4 strains and four ro-4 RIP strains. The left panel was probed with pre-immune serum while the right was probed with serum from the same rabbit after it had been immunized with a ro-4 peptide. This serum was purified against DEAE Affi-gel and by affinity chromatography with the peptide itself.

The positions of molecular weight markers are given in kD. The two bands marked with arrows are estimated to be 41.6 and 40.3 kD by the BANDSIZE program.

2 of/de 2

PM-1 3½"x4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT

PRECISIONSM RESOLUTION TARGETS

Figure 10b. Western analysis using R04 antibodies, trial 2: Both panels are western analyses detected by the chemiluminescent method, and contain separated total proteins (10 μ g per lane) from the wild-type, the three ro-4 strains and four ro-4 RIP strains. The left panel was probed with pre-immune serum while the right was probed with serum from the same rabbit after it had been immunized with a ro-4 peptide. This serum was purified against DEAE Affi-gel and by affinity chromatography with the peptide itself.

The positions of molecular weight markers are given in kD. The two bands marked with arrows are estimated to be 42.4 and 40.4 kD.

Figure 11. Silver-stained gel of degraded proteins: Proteins of the WT *N.crassa* were extracted in either TE with protease inhibitors, or TE with no inhibitors, and allowed to sit at room temperature (or 37°C in one of the 2' hour treatments) for between 0 and 24 hours before being loaded into an SDS polyacrylamide gel. The size markers are given in kD.

Figure 12. Western analysis of degraded proteins. Proteins from the gel shown in Figure 11 were transferred to nitrocellulose and probed with pre-immune (left panel) or immune (right panel) serum raised against a RO4 peptide. The size markers are given in kD.

inhib				none				inhib							
0	3	8	24	0	3	8	24	0	3	8	24				
				heat								heat

d) SDS PAGE and Western analysis of fractionated proteins from the wild-type and ro-4 mutant strains:

A comparison of PAGE-separated, silver-stained proteins from the cytosolic (100K supernatant) fraction of wild-type and the ro-4 mutant strains very clearly indicates the absence of a protein or proteins roughly the molecular weight of actin in the lanes of 2980 and the RIP strains R2 and R4, which is present in the wild-type and the other ro-4 mutant strains (Figure 13).

When four fractions were probed with the anti-RO4 antibody in a Western analysis a faint band unique to the immune serum was visible in both the nuclear and cytosolic fractions (Figure 14), of all strains tested, but not in the 10K nor 100K pellet fractions.. The molecular weight of this band was estimated at 40.75 to 42.2 kD by the BANDSIZE program.

Two-dimensional blots were loaded with 25 μ g of cytosolic fraction proteins and probed with pre-immune and immune sera, however the results were too faint to be interpreted.

Phenotypic characteristics of the wild-type and ropy strains

The strain 1529, classified as a ro-4 mutant (Perkins, 1969) in contravention of mapping data (Appendix I) was shown by Western analysis to have a mutation in the major actin gene. Furthermore, its colony morphology appeared typically

Figure 13. Comparison of silver-stained proteins of cytosols. The silver-stained SDS PAGE gel was loaded with 15 μ g of cytosolic proteins. The sizes of marker bands in lane a are given in kD. Lane b is WT, c is 1511, d is 1529, e is 2980, f and g are the RIP strains R2 and R4.

Figure 14. Western analysis of fractionated proteins: Panel A portrays pre-immune (left side) and immune-probed (right side) lanes of cytosolic proteins from 1511 (a) and 1529 (b). Panel B shows pre-immune (left side) and immune-probed (right side) nuclear fraction proteins from the same two strains. Panels C and D are analagous results in which WT (c) and 2980 (d) proteins are probed. The arrow heads mark the position of the 43 kD marker.

C c d c d D c d c d

[REDACTED]

ropy only briefly, as it soon colonized the entire petri dish and became indistinguishable from wild-type. It occurred to us that its mutation might actually be in the major actin gene which must therefore be very close to the *ro-4* locus.

Four phenotypic characteristics were examined in order to more accurately describe the strain 1529 and either support or oppose its present classification.

a) Nuclear staining of wild-type and *ropy* germlings:

Four to five-hour germlings of *ropy* mutants were stained with DAPI or DAPI and calcofluor in order to assess their capacity for proper nuclear distribution, in comparison with wild-type germlings grown for 3 hours. All exhibited poor distribution with the exception of the strain 1529, which appeared wild-type (Figure 15).

The position of the nuclei in mutant germlings was almost exclusively along the perimeter of the wall furthest from the point of emergence of the germ tube.

b) Distribution of nuclear number in conidia:

For each *ropy* mutant strain, including 3 *ro-4* mutants and one RIP strain, the number of nuclei were counted in 200 conidia where any could be seen (proving that they were stained successfully), and means and standard deviations calculated (Appendix IV). The wild-type was distinguished by few nuclei per conidium and very low deviation from the mean (1.87 ± 0.67). In comparison, all the *ropy* mutants including strain 1529 showed large standard deviations from the mean,

Figure 15. Nuclear distribution in *N.crassa* germlings: Germlings of wild-type, 2980 and 1511 were stained with DAPI and calcofluor; all others were stained with DAPI only. The small dash in the *ro-7* panel indicates the tip of the germ tube. The scale bar given in the third panel represents 10 μm and applies to all.

Wild-type	Wild-type	<i>ro-4</i> (1529)
<i>ro-4</i> (2980)	<i>ro-4</i> (2980)	<i>ro-4</i> (1511)
<i>ro-1</i> (146)	<i>ro-2</i> (52)	<i>ro-3</i> (43)
<i>ro-6</i> (3627)	<i>ro-7</i> (4025)	<i>ro-10</i> (3618)

which were however not statistically significant except in the case of strain 52 (*ro-2*) ($F=35.29$, $p<0.05$). This result implies that the mechanism for moving discrete numbers of nuclei into the immature conidia is deficient in the strain 52 compared to wild-type.

c) Growth of the wild-type and the *ropy* mutants at normal and high temperatures:

As seen in Figure 16a, the wild-type strain grew very quickly, around 0.17 cm/hr. For the most part, the *ropy* mutants exhibited a considerably slower rate of growth, with the exception of the *ro-4* mutant strain 1529 (Figure 16b), which grew as quickly as the wild-type. Interestingly, the *ro-4* RIP strains were intermediate in their rate of growth between the wild-type and the two *ro-4* mutant strains 1511 and 2980 (Figure 16b).

At high temperatures (40°C) the RIP strains showed distinct responses (Figure 17b). R4 was affected severely and did not exhibit appreciable growth until about three days had passed, at which point it was distinguished by an unusual degree of mycelium within the agar. Conversely, R2 and R5 showed no inhibition of growth and the effect on R1 was intermediate.

Among the *ropy* mutants the difference in growth rate at the two temperatures was less pronounced (Figure 17a), although 3627 nearly approached the wild-type growth rate at higher temperatures.

Figure 16a: Growth of wild-type and ropy mutant strains at room temperature. Cultures were grown on Vogel's Minimal medium at between 23° and 25°C. Each point is a mean of 5 replicates, with no standard deviation greater than 0.3 cm.

colony diameter (cm)

Figure 16b: Growth of wild-type and *ro-4* mutant strains, including the ripped strains at room temperature. Cultures were grown on Vogel's Minimal Medium at between 23° and 25°C. Each point is a mean of 5 replicates, with no standard deviation greater than 0.3 cm.

Figure 17a: Growth of wild-type and *ropy* mutant strains at high temperature. Cultures were grown on Vogel's Minimal medium at between 40° and 43°C. Each point is a mean of 3 replicates with standard deviations no greater than 0.6 cm except where indicated.

Figure 17b: Growth of wild-type and *ro-4* mutant strains, including the RIP strains at high temperature. Cultures were grown on Vogel's Minimal medium at between 40° and 43°C. Each point is a mean of 3 replicates with standard deviations no greater than 0.6 cm except where indicated.

colony diameter (cm)

d) Colony morphology of wild-type and ropy mutant strains:

Figure 1 clearly emphasizes the advantage of the wild-type strain over the ropy mutants in terms of growth rate, since it completely covers the petri dish within 4 days. The wild-type was also photographed at an earlier stage so a more useful comparison can be drawn between the growth habits of it and of the ropy strains. At 2 days old the wild-type has grown across most of the plate, displaying fine, sparsely-branched hyphae and little if any conidiation. In contrast mutant strains of the same size have formed relatively compact colonies with profuse aerial hyphae and some conidiation. The *ro-4* mutant strain 1529 has been photographed at 4 days, by which time it also has filled the plate, and $2\frac{3}{4}$ days at which point it appears ropy.

IV. Discussion

Sequence features of the wild-type *ro-4* gene in *Neurospora crassa*:

The positions of the three introns in the *ro-4* gene had tentatively been identified (Robb, 1993) based on sequence similarity with the mammalian actin-related protein and conserved exon/intron junction sequences. These were confirmed by sequencing wild-type *ro-4* cDNA from the strain 74A, and comparing this sequence to the genomic sequence. All begin after the third position of the preceding codon (phase 0). With lengths of 84, 94 and 100 nucleotides, they are longer than is typical for *N. crassa* (60) and are less G/C rich; 38%, 42% and 45%, rather than 49% (Edelmann and Staben, 1994). The first two introns (positions 100 and 248) align with introns in the actin gene of *Volvox*, but not found in any other major actin genes known as of March, 1993 (Weber and Kabsch, 1994). Among the other members of the Arp1 family, *Drosophila* Actr87c has five introns none of which correspond to those in *ro-4*. The introns of *ro-4* do not interrupt any major structural domains (Figure 2).

All three introns contain 5' and 3' splice sites and internal branch points as delineated by Edelmann and Staben, (1994), with some differences from the consensus sequence as shown in Figure 4. The 5' end of the translation initiation site, the 5' intron donor site and the internal branchpoint

adhere more closely to the consensus sequences for the species than the 3' acceptor sites.

Sequence of the *ro-4* mutant allele in strain 2980:

The sequence of the strain 2980 was obtained by sequencing clones containing the 3' or 5' restriction fragments of the *ro-4* gene ligated into the BluescriptTM plasmid. A single bp deletion was found at position +165 (relative to the translation start site). The resulting frame-shift mutation results in an altered sequence of amino acids from number 56 to number 79 at which point there is a premature stop signal (Figure 6). The resulting peptide has a putative MW of only 8.2 kD compared to 42.8 kD (Plamann et al., 1994) for the wild-type protein.

A mutant protein of this nature would be missing most of the interactive sites of the functional protein. The wild-type *ro-4* sequence is conserved at most of the sites responsible for nucleotide and calcium binding (Robb, 1993). In addition the wild-type *ro-4* may be capable of forming heterofilaments with conventional actin, based on its similarity to cetractin which has these capabilities (Melki et al., 1993).

A foreshortened coding sequence may result in a shortened lifespan for the transcribed mRNA. Muhlrad and Parker (1994) showed that in yeast, premature translational termination led to decapping of the 5' end of the transcript, after which

the transcript was degraded both in the 5'→3' and 3'→5' directions. This may mean that the putative 8.2 kD product of the mutant allele is not translated.

Expression of *ro-4* in the wild-type and *ro-4* mutant strains:

Robb *et al.*, (1995) reported that the *ro-4* gene hybridizes to two bands of 1.7 and 2.1 kb. The two lower bands labelled in the wild-type lane (Figure 8) of estimated sizes 1.65 and 2 kb probably correspond to the 1.7 and 2.1 kb species reported above. It is the 1.7 kb band which is affected in the mutants 2980, R5 and R2. Plamann *et al.* (1994) found that the cDNA for *ro-4* began roughly 370 bases upstream of the initiation codon. This would yield an mRNA molecule of roughly 1.5 kb, not including any 3' untranslated region. Harata *et al.* describe mRNA for *act3* in yeast as 1.7-1.8 kb (1994), with the encoded essential protein being in excess of 54 kD. From these results it is most plausible therefore that the 1.7 kb species represents the *ro-4* transcript. The weak labelling of this species in 2980 is consistent with the possibility that its mRNA is quickly degraded, as proposed above.

Clark and Meyer (1992) used a centractin cDNA (later identified as α -centractin) to probe poly(A)⁺mRNA of human tissues and detected a single species of mRNA of about 3.1 kb (due to a large 3' untranslated region). In 1994 Clark *et al.*

found that the mRNA recognized by a β -centractin probe was about 2.4 kb in multiple human tissues, and that the α -centractin probe also detected a gamma-centractin with an mRNA of about 1.4 kb (Clark et al., 1994). Possibly the 2.9 kb uppermost band in the wild-type lane represents an analogous species of mRNA to that reported by Clark and Meyer (1992), and is indicative of multiple isoforms of RO4 encoded in the *N.crassa* genome. This theory has in its favour the Western blot results in which the anti-RO4 serum recognizes a protein in the 2980 mutant strain whose sequence terminates upstream of the epitope site. However if multiple isoforms existed they could be expected to interfere with Southern analyses, for example restriction fragment length analyses. The Southern analyses presented above are quite clear, even though the stringency conditions (hybridization carried out at 68°C) were comparable to those used in the Northern protocol. The most likely explanation is that the stringency used in the Northern analysis was inadequate for RNA-RNA hybrids and that the 2.9 kb band does not represent a species of *ro-4* mRNA.

Clark and Meyer (1992) estimated that centractin mRNA levels must be far less abundant than those of the major actin, based on the length of exposure time required in a Northern (10X longer for centractin). Fyrberg et al. (1994) also report that mRNAs for actin-related proteins comprise far less of the mRNA pool than do mRNAs for the major actins. The calculated value for Codon Adaptation Index for *ro-4* (0.489)

is indicative of a moderately-expressed protein however. There is a possibility that the reference set did not contain enough highly-expressed genes to be a reliable standard.

Protein analysis of the wild-type and *ro-4* mutant strains:

a) Western analyses using a major actin antibody:

A monoclonal anti-actin obtained from Boehringer Mannheim labelled a protein of roughly the same molecular weight as actin in the wild-type and two of the *ro-4* mutant strains. However it failed to recognize any protein extracted from the strain 1529. The *ro-4* mutant strain 1529 must have a mutation in its major actin gene which disrupts the mAb epitope. It is conceivable, given its dissimilarities to the other *ro-4* mutant strains in terms of growth rate and nuclear distribution that the mutation in 1529 is actually in the major actin, and not in *ro-4*, in other words the major actin may be located close to *ro-4* in *N.crassa*.

Barja et al.(1991) reported that the major actin in *N.crassa* has a molecular weight of 43 kD and pI of 6.4. It consisted of α -, β - and gamma-isoforms with a preponderance of β -actin. Only two isoforms were distinctly visible in the anti-actin Western analysis (Figure 9) which are tentatively identified as β -and gamma-actin, based on relative size and pI compared with Barja's results. Neither of these appeared in strain 1529, implying that they are both encoded by the same gene, and therefore that differences in pI are due to post-

translational modification.

The epitope of this Boehringer Mannheim anti-actin antibody is presumably well-conserved among actins, since the antibody reacted with all six known vertebrate isoactins as well as *Dictyostelium discoideum* and *Physarum polycephalum* actins (Lessard, 1988). It was developed using chicken gizzard (rich in gamma actin) as the immunogen. The binding site on skeletal actin overlaps that of DNase I. In addition, the antibody binds an SV8 (V8 protease) proteolytic subunit comprising the amino-terminal 2/3 of the molecule and overlaps the epitope of the skeletal actin antibody, but does not interfere with actin polymerization.

A polyclonal antibody against actin exists which labels all three isoforms of actin on Western blots, yet *in situ* recognises only specific manifestations of actin; namely myofibrils, microvilli of brush border epithelium, and "comet tails" of *Listeria monocytogenes* (Schrader et al., 1994). The authors hypothesize that the epitope may only be accessible in certain structures, or certain conformations. This could only explain the absence of binding in 1529 if the proteins were not already denatured as they are in SDS PAGE.

The mutations *ro-4* (2980), *ro-8* (1511) and *ro-5* (1529) were originally mapped to separate loci at 16 (*ro-4*), 46 (*ro-5*) and 70 (*ro-8*) mu from the centromere (Appendix I), even though tests for heterokaryon complementation were negative (Morgan et al., 1967). They were since assigned to the same

locus (Perkins et al., 1969) on the basis of non-recombination with the *paba2* locus, and inability to produce complementation in heterokaryons, a result that may indicate a dominant mutation in one of the strains. The possibility exists that the loci for the *ro-4* and 1529 mutations are on opposite sides of the *paba2* gene at different loci.

b) Western analyses using a *RO4* antibody:

The epitope against which the *RO4*-specific antibody was raised was chosen prior to the discovery of the mutation in strain 2980. The result of this is that the purified anti-*RO4* serum cannot recognize any truncated polypeptide in that *ro-4* strain, since the epitope is after the point of premature termination. In one-dimensional Western analyses probed with purified anti-*RO4* serum two bands with roughly the same electrophoretic mobility as actin were recognized by the anti-*RO4* antibody (Figure 10a and b) migrating at between 40.3 and 43.3 kD. The lower of these two bands is the better candidate for the *RO4* protein as it was absent in strain 2980 and the RIP strains in one trial, and faint in these lanes in the second. Although the predicted size of *RO4* is 43.38 kD, the discrepancy is not unprecedented. Lees-Miller et al. (1992b) found that actin-rpv migrated faster than conventional actin and faster than predicted by its sequence 'whereas centractin migrated more slowly than regular actin (Clark and Meyer, 1992)). The upper band may then be either another actin-related protein, or an erroneous result. The yeast

Saccharomyces cerevisiae has at least three Arps (Schroer et al.,1994), the slime mold *Acanthamoeba castellani* has 2 (Kelleher et al.,1995) and *Drosophila* has four (Fyrberg et al.,1994). At least two isoforms of centractin are known to exist in humans; α - and β -centractin are 91% identical (96% similar) in their amino acid sequences, whereas a third isoform referred to as gamma-centractin was identified as a small mRNA species that was labelled with cDNA of α -centractin (Clark et al.,1994).

Since actin is a common allergen it would be understandable if actin-related proteins which are 35-55% identical to it (Schroer et al.,1994) might also be recognized by pre-immune sera.

Three other protein bands, of estimated sizes 95, 50 and 37 kD were labelled more strongly by immune than by pre-immune sera. Muhua et al.(1994) detected a 50 kD protein in western blots probed with anti-Act5p which was due to non-specific binding and not eliminated by pre-absorption against a null strain. None of the three could be identified, however it is presumed that each contains an epitope similar to that against which the antibody was raised.

Although Kelleher et al.(1995) reported that Arp2 of *Acanthamoeba castellani* is degraded by repeated boiling or over time, yielding lower MW breakdown products, this was probably not a factor in the present study, as there was no obvious difference in the lanes representing separated protein

extracts which had been allowed to degrade for various spans of time. The bands which were unique to the immune anti-serum were labelled equally across lanes (Figure 11).

In other studies of actin-related proteins the problem of scarcity interfered with recognition. Muhua et al., (1994) estimated that the Arp1 Act5p in *Saccharomyces cerevisiae* was present at less than 0.00022%, based on their inability to detect it in a Western blot. The antibody used in the present study was applied to Western analyses of total and fractionated proteins when it was able to detect between 20 and 200 ng of pure antigen applied in a dot blot. This represents 0.2% of a 10 μ g protein sample. Clark et al. (1994) estimated the abundance of α -centractin in HeLa cells at 0.006% of total cell protein, and β -centractin was estimated to be 10- to 20-fold less abundant. These amounts were obtained from protein immunoprecipitated with an antibody to p50. This was justified due to sucrose gradient fractionation studies that indicated that centractin co-sedimented with p150^{Glued} and p50 but was not present in a free cytosolic pool.

Kelleher et al. (1995) report a cellular concentration in *Acanthamoeba* of 1.9 μ M for Arp2 and 5.1 μ M for Arp3, compared to the major actin which is present at 200 μ M. These small concentrations are in the range of several actin-binding proteins such as α -actinin at 4 μ M and capping protein at 1.3 μ M. The results of the Northern blotting to survey expression

levels as well as the calculated C.A.I. to predict expression although not definitive, hint at a detectable level of RO4.

c) Analyses of fractionated proteins:

The one-dimensional gels of fractionated proteins indicate that a protein exists in the wild-type and strains 1511 and 1529 which does not exist (or does not silver-stain) in 2980. This could represent the RO4 protein. The similarity between cytosolic fraction proteins of 2980 and R2 is striking, and suggests that the result of the ripping in the strain R2 may have been a premature termination such as that present in 2980. However in growth studies there is no similarity between the two strains, especially at high temperature (Figure 16b). The generally unique profile of silver-stained proteins in 2980 relative to that of wild-type belies the fact that only one locus is mutated in the strain. It is possible that the *ro-4* product is designed to have a regulatory role as well. Lloyd et al., (1993) report that expression of non-muscle actin genes can affect the expression of both other actins and tropomyosin.

The faintness of the band bound by the anti-RO4 antibody in fractionated proteins casts doubt on either the continued activity of the antibody preparation or the efficiency of the fractionation procedure. Although between 2½ and 5 times as much protein was loaded per two-dimensional gel as per lane of a one-dimensional gel, the band is not as strong in the former as in the latter. A protein recognized by the RO4 antibody

was enriched in both the cytosolic fraction and the nuclear fraction compared to the 10K and 100K fractions. That this protein also appeared in strain 2980 implies that it is not the putative *RO4* protein identified on the basis of Western analyses of total proteins. Kelleher et al. (1995) found that anti-Arp2 from *Acanthamoeba* remained diffuse in the cytoplasm and failed to label any organelle including the nucleus, whereas Arp3 was localized to discrete spots within unidentified cytoplasmic lamellae. Both Arps remained in the cytoplasmic fraction even after centrifugation at 100,000 g for 90 minutes.

Clark and Meyer (1992) found that fluorescently-labelled anti-centractin localised predominantly to the microtubule organizing centre with faint cytoplasmic staining. When colcemid was used to depolymerize the bulk of the cytoplasmic microtubules the cytoplasmic staining was reduced. Later, Clark et al. (1994), determined the sub-cellular localization of both α - and β -centractin to be the cytosol, as part of the 20S dynactin complex.

Phenotypic analysis of wild-type and ropy strains:

One striking conclusion arising from the fact that the strain 1529 does not have faulty nuclear distribution is that the ropy phenotype at the level of colony morphology is not obligately associated with poor nuclear distribution. The strain 1529 grows nearly as quickly as WT but exhibits a far

greater degree of branching (which can be seen with the naked eye). This means that it actually has a greater rate of hyphal extension than WT.

In contrast the other *ro-4* strains 1511 and 2980 have a much slower growth rate, distinguishing them from strain 1529, although they too show extensive branching. This hints that the mutation in 1529 is not at the same locus as the mutations in 1511 or in 2980, and may in fact be in a closely linked major actin gene. The *ro-4* RIP strains all grow more quickly than 1511 and 2980, but not as quickly as either the wild-type or 1529, although their colony morphology is distinctly 'ropy'.

Huebschman (1952) reported that the number of nuclei in conidia of *N.crassa* was never zero, though it could be increased from 2.64 to 6.15 by growing the colonies on complete as opposed to minimal medium. In addition, he found that the number was independent of the age of the culture, suggesting that post-absission mitosis was not an important factor in determining the number.

The strain 52 which is least able to distribute nuclei among its conidia is also the slowest grower of all the *ropy* strains, a deficiency which is exacerbated at high temperatures. The strains 146, 43 and 4025 representing the loci *ro-1*, *ro-3* and *ro-7* show similar traits in terms of growth at normal and high temperatures, and so these may all (like *ro-1*) be involved in the dynein heavy chain aspect of

nuclear movement.

The high temperature effect shown by 52 and 2980 may be a result of the tendency of microtubules to polymerize at 37°C (in Preston et al., 1990). If nuclear movement is driven by microtubule depolymerization as suggested by Desai and Mitchison (1995), then conditions which promote this polymerization will put mutant strains at even more of a disadvantage. A Ca^{2+} deficiency such as that seen in frost and spray mutants would have the same effect, as Ca^{2+} can cause depolymerization of microtubules (in Preston et al., 1990).

From what is known of analagous genes in *Aspergillus* (in particular *NudF*, Xiang et al., 1995) the process of nuclear migration may involve a signalling pathway incorporating G proteins. In *N.crassa*, the interaction of G_i and G_o proteins with Ca^{2+} channels has been implicated in the branching morphology of frost and spray mutants (Turner and Borkovitch, 1993). Thus receptor proteins, other G protein subunits, and protein kinases are all candidates for elements of the pathway which results in nuclear distribution. In addition, the other proteins involved in microtubule-based movement (for example the numerous proteins of the dynactin complex, in addition to *ro-4*) may eventually be identified as the faulty link in the other *ropy* strains.

V. Conclusions

1. The introns in the wild-type *ro-4* gene of *Neurospora crassa* were confirmed to be at the positions proposed by Robb (1993).
2. The mutant *ro-4* allele in strain 2980 contains a premature termination signal and encodes a putative product of only 8.2kD which may not be translated.
3. There are three species of mRNA which hybridize to a *ro-4* antisense RNA in Northern analysis. Two of these may correspond to the previously reported 1.7 and 2.1 kb (Robb et al., 1995) mRNAs while the third is 2.9 kb. The 1.7 kb species is close to the predicted size.
4. The *ro-4* mutant strain 1529 was shown by Western analysis to lack the epitope recognized by a monoclonal antibody to the major actin, though polyclonal antibodies to both the major actin and to tubulin recognized protein of this strain.
5. Anti-serum raised against a synthetic peptide of *RO4* recognized two bands in the range of the 43 kD marker which were not labelled by pre-immune serum. The lower of the two was faint or absent in strain 2980 as

predicted by the sequence of the mutant allele.

6. A single protein was recognized by the anti-RO4 antibody in cytosolic and nuclear fractions of the wild-type and of the mutant strains 1511, 1529 and 2980. This protein was enriched in the nuclear and cytosolic fractions compared to the 10K and 100K fractions, and migrates below the major actin.
7. The growth rate and nuclear distribution of the strain 1529 is similar to that of wild-type, although its colony morphology is *ropy*. In contrast, the strains 1511 and 2980 show the *ropy* characteristics in their colony morphology, slow growth rate, and aberrant nuclear distribution.

Appendix I: Partial Map of Linkage Group V in *N. crassa*

from Morgan et al., (1967).
 Distances are in map units.

Appendix II: Calculation of C.A.I. (Codon Adaptation Index) for ro-4:

The Codon Adaptation Index was developed by Sharp and Li (1987) to predict the level of expression of a gene based on the similarity of its codon usage with that of a reference set of highly-expressed genes in the same species.

The formula for the C.A.I. is

$$\text{C.A.I.} = \left(\prod_{k=1}^L w_k \right)^{1/L}$$

where L is the number of amino acids in the protein which can be coded for by more than one codon, and w is measure of bias for each codon.

The value for w is obtained by first assigning weights to each codon in the reference set, such that each weight represents the proportion of times that codon is used in the genes of the set, and such that the sum of the weights of the codons for each amino acid is equal to the number of codons which code for that amino acid. For each codon in the protein then w is the fraction of the weight for the codon divided by the maximum possible weight for any codon coding for the same amino acid.

For example, the genes used to compile the reference set in this thesis were Neucrp3, NeuTyrA and His-3 (see Appendix II). In the case of aspartic acid, on average these genes used the codon

GAU 30% of the time, for a weight of 0.6
GAC 70% of the time, for a weight of 1.4

Thus the value of w for each occurrence of the codon GAU in the gene ro-4, is (0.6/1.4), whereas if the preferred codon GAC is used the value becomes 1 (that is, 1.4/1.4). The C.A.I is the product of all the w values for the amino acids in the coding region (with the exception of methionine and tryptophane), raised to the reciprocal of the number of amino acids used.

In the case of ro-4, which begins

5' ATG ACA GAC TCT TTG CAC 3'
(i.e. AUG ACA GAC UCU UUG CAC)

the C.A.I calculation begins

$$[(0.4/2.3) \times (1.4/1.4) \times (0.8/2.3) \times (1/2.8) \times (1.5/1.5) \dots]^{1/368}$$

and yields the value 0.489.

**Appendix III: Average Weights of Alternate Codons Compiled
from Three Highly-expressed Genes of *Neurospora crassa***

Amino Acid	Codon	Average Weight	Amino Acid	Codon	Average Weight
PHE	UUU	0.4	SER	UCU	0.8
	UUC	1.6		UCC	2.3
LEU	UUA	0		UCA	0.3
	UUG	1		UCG	0.7
	CUU	0.8	PRO	CCU	0.8
	CUC	2.8		CCC	2.3
	CUA	0.1		CCA	0.3
	CUG	1.3		CCG	0.6
ILE	AUU	0.8	THR	ACU	0.9
	AUC	2		ACC	2.3
	AUA	0.1		ACA	0.4
MET	AUG	1		ACG	0.5
VAL	GUU	0.7	ALA	GCU	1
	GUC	2.6		GCC	2.4
	GUA	0.2		GCA	0.2
	GUG	0.5		GCG	0.5
TYR	UAU	0.3	CYS	UGU	0.2
	UAC	1.7		UGC	1.8
ter	UAA	0.7	ter	UGA	0
	UAG	0	TRP	UGG	1
HIS	CAU	0.4	ARG	CGU	1.5
	CAC	1.5		CGC	2.1
GLN	CAA	0.4		CGA	0.5
	CAG	1.6		CGG	0.8
ASN	AAU	0.4	SER	AGU	0.3
	AAC	1.6		AGC	1.6
LYS	AAA	0.2	ARG	AGA	0.3
	AAG	1.8		AGG	0.8
ASP	GAU	0.6	GLY	GGU	1.2
	GAC	1.4		GGC	2
GLU	GAA	0.2		GGA	0.4
	GAG	1.8		GGG	0.2

Appendix IV: Distribution of nuclear number in nucleated conidia of wild-type and *ropy* mutant strains.

Two hundred nucleated conidia were counted from each of the strains. The means and standard deviations follow:

WT	1.87 ± 0.67
146 (ro-1)	3.68 ± 2.39
52 (ro-2)	4.68 ± 3.98
43 (ro-3)	2.71 ± 1.68
1511 (ro-4)	1.94 ± 1.47
1529 (ro-4)	3.46 ± 2.52
2980 (ro-4)	2.50 ± 1.71
R4	2.78 ± 1.8
3627 (ro-6)	2.74 ± 1.52
4025 (ro-7)	2.92 ± 1.58
3618 (ro-10)	2.95 ± 1.64

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