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**THE PHYSICAL AND MOLECULAR CHARACTERIZATION OF *un-24*,
A TEMPERATURE-SENSITIVE CONDITIONAL MUTATION IN *NEUROSPORA*
CRASSA DEFECTIVE IN RIBONUCLEOTIDE REDUCTASE.**

A Thesis

Presented to

The Faculty of Graduate Studies

of

Carleton University

by

Sheryl P.G. Hubbard

In partial fulfilment of requirements

for the degree of

Master of Science

July, 1997

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"The physical and molecular characterization of
un-24, a temperature-sensitive conditional
mutation in *Neurospora crassa* defective in
ribonucleotide reductase."

submitted by

SHERYL PATRICIA GODKIN HUBBARD

In partial fulfilment of the requirements
for the degree of Master of Science

Chair, Department of Biology

Thesis Supervisor

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Carleton University

Abstract

THE PHYSICAL AND MOLECULAR CHARACTERIZATION OF *un-24*, A TEMPERATURE-SENSITIVE CONDITIONAL MUTANT OF *NEUROSPORA* *CRASSA* DEFECTIVE IN RIBONUCLEOTIDE REDUCTASE.

Neurospora crassa strains carrying *un-24* display wild-type growth rates at 22°C, but little or no growth at temperatures above 35°C. This thesis describes the origin and characterization of *un-24*, and compares the DNA sequence of the mutant allele to its wild-type, OakRidge (OR), counterpart. Genetic mapping data indicate *un-24* is 2.6 to 4.5 map units centromere distal to the incompatibility gene, *het-6*. *un-24* and OR alleles cause an incompatibility reaction in Panama (PA) spheroplasts at both 30 and 37°C. The PA allele hybridizes to a DIG-labelled *un-24** probe, but does not have *un-24* complementation ability nor incompatibility function in OR spheroplasts.

RFLP analysis and temperature-sensitivity complementation were used to locate mutant and wild-type alleles at *un-24*. DNA sequencing revealed that *un-24* possesses three characteristic RIP mutations (G:C-A:T transition) in the large subunit of ribonucleotide reductase. The *N. crassa* OR ribonucleotide reductase gene potentially encodes a 824 amino acid polypeptide; the gene has 4 putative introns and *Mlu* elements.

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List of Abbreviations

<i>a</i> or <i>A</i>	mating type	LB	Luria-Bertani
<i>am</i>	amination deficient		medium
	mutation	MOPS	Modified Citrus
bp	Base Pair		Pectin
<i>cyh-1</i>	cycloheximide	RIP	Rearrangements
	resistant - 1		Induced
DAPI	4',6-diamidino		Pre-meiotically
	2-phenylindole	<i>ro-7</i>	ropy morphology 7
DIG	Digoxigenin	OR or O	OakRidge
DMF	Dimethyl	PA or P	Panama
	Formamide	PCR	Polymerase Chain
DNA	Deoxyribonucleic		Reaction
	acid	<i>pyr-4</i>	pyrimidine-4 deficient
dNTP	Deoxy-nucleotide		mutation
	triphosphate	RFLP	Restriction Fragment
Dp	partial diploid		Length Polymorphism
<i>Dp(AR18)</i>	<i>Dp(II-III)AR18</i>	TAE	Tris-acetate - EDTA
DTT	Dithiothreitol	<i>T(AR18)</i>	<i>T(II-III)AR18</i>
ECL	Enhanced	TE	Tris Cl-EDTA
	Chemiluminescence	<i>thr-2</i>	Threonine-requiring
EDTA	Ethylenediamine-		mutant
	tetraacetic	Tm	Melting Temperature
	Acid	<i>trp-1</i>	Tryptophan-requiring
EtBr	Ethidium Bromide		mutant
FGSC	Fungal Genetics	<i>T(P2869)</i>	<i>T(II-IV)P2689</i>
	Stock	<i>un</i>	unknown temperature-
	Center		sensitive mutation
<i>het</i>	heterokaryon	UV	Ultra Violet
	incompatibility	WT	wild-type
	locus	X-gal	5'-bromo-4-chloro-3-
<i>inl</i>	inositol-requiring		indolyl- β -D-
	mutant		galactoside
IPTG	isopropyl-thio- β -D-		
	galactoside		
kbp	Kilobase Pairs		

General Introduction and Literature Review

Introduction

General Introduction.

The fungi have had both a beneficial and detrimental impact on human affairs. They have proved useful as a source of antibiotics (penicillin) and food (mushrooms, quorn), and in the preparation of various foods (bread, soya sauce) and alcoholic beverages (beer and wine). Unfortunately, the fungi can also have very detrimental effects as plant and animal pathogens, and by the production of mycotoxins, such as aflatoxin in peanuts and deoxynivalenol in wheat and corn products. Because of their diverse role in nature, many scientists have sought to understand and control the fungi.

Due to its rapid growth, its ability to grow readily on defined media, and its non-pathogenic nature, the Ascomycete fungus, *Neurospora crassa*, has long been used as an experimental organism in laboratories. Over the years, it has been well characterized in terms of morphology, genetics and biochemistry. The relative simplicity of the fungus has allowed for the continuing implementation of *N. crassa* as a model for eukaryotic biological systems (Timberlake & Marshall, 1989). Indeed, the study of non-self recognition, termed heterokaryon incompatibility in *N. crassa* and related organisms, has provided insight into the biological control of pathogenic fungi (Leach and Yoder, 1974; Anagnostakis, 1982; Anagnostakis, 1983; Brasier, 1983).

Many nutritional, morphological and temperature sensitive mutants have

been found and characterized in *N. crassa*. Temperature-sensitive mutations have played a key role in the discovery and cloning of many essential genes (Davis & DeSerres, 1962). Temperature-sensitive mutants in *N. crassa* that have no known function have been used repeatedly as genetic markers during the isolation of many important genes, including those involved in the *Neurospora* mating system (Newmeyer *et al.*, 1973; Glass *et al.*, 1988; Perkins *et al.*, 1982). The mutation of interest in this study, *un-24*, is one of over thirty of these unknown function temperature-sensitive conditional mutations. It is characterized by near wild-type growth at 20°C, reduced growth at 30°C, and no growth at temperatures greater than 35°C. We have found that *un-24* is caused by a mutation in the large subunit of ribonucleotide reductase. This enzyme is required for the conversion of ribonucleotides to deoxyribonucleotides during the synthesis phase of mitosis (reviewed by Elledge *et al.*, 1992).

The following literature review provides a thorough overview of *N. crassa*, its life cycle, and the role of ribonucleotide reductase in the viability of cells. Temperature-sensitive mutations will also be reviewed, especially with respect to the role they play in the cloning and characterization of essential genes.

Biology of N. crassa.

Neurospora was first described by Shear and Dodge in 1927 (Perkins and Turner, 1988). It is a haploid, coenocytic, filamentous fungus that grows by the apical extension of tubular filaments called hyphae. The hyphae are made up of

multinucleated cells separated by perforated septae that allow the free transport of solutes and other macromolecules but which inhibit the movement of organelles, excluding nuclei and mitochondria (Perkins & Turner, 1988; Davis & DeSerres, 1962). Hyphal cells are bound by a plasma membrane and a distinct cell wall that is fortified with chitin (Davis & DeSerres, 1962). The formation of carotenoids in the mycelium (a connected network of hyphae) and conidia (asexual propagules) of many species of *Neurospora* give the fungus the distinguishing character of orange, or yellow-orange, billowy growth.

Neurospora has been isolated world-wide, but grows ideally in moist tropical, or subtropical, environments (Perkins & Turner, 1988). *N. crassa*, specifically, has been isolated most often in areas surrounding the Gulf of Mexico, the Caribbean, Africa and India (Perkins & Turner, 1988). The fluffy, orange mycelium can be found growing on burned substrates, especially those with pithy stems and sugary sap, or on unburned substrates, including corncobs, sugarcane, bagasse, or filter mud from sugarcane refineries (Perkins & Turner, 1988). Few samples have been isolated from soil (Perkins & Turner, 1988).

The life cycle of *N. crassa* embodies three stages, including vegetative growth (branching and apical extension), asexual reproduction and sexual reproduction (see Figure I-1). During vegetative growth, identical hyphae have the ability to fuse and grow as heterokaryons. If, however, genetic differences between strains exist at the mating-type locus or at one of a number of heterokaryon incompatibility (*het*) loci, an incompatibility reaction ensues that

Figure I-1: Life cycle of *Neurospora crassa*.

causes death within, and around, the fused cells (Garnjobst and Wilson, 1956).

During the incompatibility reaction, the cell's cytoplasm gradually becomes more granular, vacuolated and disorganized, until the cell is no longer viable (Garnjobst and Wilson, 1956). Subsequently, new growing tips are initiated in normal cells located back from the destroyed cells (Garnjobst and Wilson, 1956). Heterokaryon incompatibility is believed to prevent the spread of deleterious genetic elements throughout a population, and has been used to identify population structure as defined by "vegetative compatibility groups" (VCGs) (reviewed by Leslie, 1993). At its most basic, heterokaryon incompatibility appears to be a form of self/non-self recognition that occurs during the vegetative growth of *Neurospora* and other ascomycete fungi, including *Aspergillus* (Caten, 1972; Coenen *et al.*, 1994), *Podospora anserina* (reviewed by Bégueret *et al.*, 1994), *Ceratascystis ulmi* (responsible for Dutch Elm disease - Brasier, 1983), *Cochliobolus heterostrophus* (responsible for Southern Leaf Blight in corn - Leach and Yoder, 1974), and *Endothia parasitica* (causes Chestnut blight in Oaks - Anagnostakis, 1982; Anagnostakis, 1983).

Asexual reproduction in *N. crassa* involves the formation of chains of uninucleate microconidia and multinucleate macroconidia at the tips of aerial hyphae. The role of macroconidia in the life cycle of *N. crassa* lies more in the fertilization, rather than in dispersal, of the organism (Davis & DeSerres, 1962; Perkins & Turner, 1988). Macroconidia are produced in high numbers in actively growing cultures by the successive acropetal pinching off of lateral or terminal

branches (Lowry *et al.*, 1967). The spores are sized between 5-9 μm , appear spherical to barrel-shaped (Lowry *et al.*, 1957), and house up to thirty nuclei (Huebschman, 1952).

Microconidia are less viable cells that do not become airborne and which are produced by a manner distinctive from macroconidia (Perkins & Turner, 1988). They are usually produced later in the asexual life cycle of the fungus (Davis & DeSerres, 1962). Microconidia are borne at the tips of short, blunt, specialized hyphae called microconidiophores formed by the vegetative mycelium (Lowry, *et al.*, 1967). Partially formed microconidia appear to be pushed out from the conidiophore through a "collar-like" projection (Lowry *et al.*, 1967). Mature microconidia are ovoid to pear-shaped cells that average 2.5 by 3.5 μm in length (Lowry *et al.*, 1967).

N. crassa is a self-sterile organism, and sexual mating requires the presence of two distinct mating types (Raju, 1980). Strains of either A or a mating type can act as male or female partners (Raju, 1980). Initiation of the sexual cycle is stimulated by nitrogen starvation (Glass & Lorimer, 1991). Female reproductive structures, called protoperithecia, are formed by the spherical aggregations of hyphae of either mating type (Glass & Lorimer, 1991). A specialized hypha called a trichogyne extends from the protoperithecium and is attracted by mating-type-specific pheromones to a male element of the opposite mating type (Glass & Lorimer, 1991). The male element can either be an asexual conidium or a segment of a vegetative hypha (Glass & Lorimer, 1991).

Once fusion occurs between the male and female elements, the nucleus of the male is delivered through the trichogyne to the protoperithecium where the *A* and *a* nuclei form an ascogenous hypha (Glass & Lorimer, 1991; Raju, 1980). Unitunicate asci (singular - ascus), which construct and house the spores created during the sexual cycle of *N. crassa*, are formed from the penultimate cell of croziers formed in the ascogenous hypha (Raju, 1980). A crozier is created by the construction of two septae within the ascogenous hypha, such that two uninucleate cells, the stock cell and the apical cell (one of each mating type), and a binucleate penultimate cell (both mating types), are created (Raju, 1980). Ascus and ascospore development is initiated by the elongation of the penultimate cell of the crozier and the fusion of the *A* and *a* nuclei to form a diploid nucleus (Raju, 1980). Once karyogamy occurs, the nucleus immediately undergoes meiosis, followed by a round of mitosis, to create eight haploid nuclei (Raju, 1980).

Additional asci are formed by the fusion of the stalk and apical cells to establish new binucleate cells in which the nuclei divide and successive croziers are constructed (Raju, 1980). Invaginations of the wall membranes of the ascus result in the individual compartmentalization of the eight nuclei and some of the ascus cytoplasm (Raju, 1980). The effect is the formation of eight haploid spores within each ascus. Maturation of the ascospores involves the growth of the spores and the formation of spore ornamentation and pigmentation (Raju, 1980). The nuclei begin to divide once again, with the mature ascospores housing thirty or more haploid nuclei (Raju, 1980). More than 200 asci are formed within each

perithecium (Raju, 1980).

The time course of the sexual cycle takes approximately five to six days at 25°C (Raju, 1980). The development of perithecia from protoperithecia is initiated immediately after the arrival of the male nucleus into the system, and fertilization of other mature protoperithecia within the culture is inhibited by perithecial development (Raju, 1996; Raju, 1980). Mature perithecia in *N. crassa* and related Pyrenomycetes are characterized as being superficial, flask-shaped bodies with a black pigmentation (Glass and Lorimer, 1991). The first crozier appears within the perithecium two to three days post-fertilization and, five to six days after the sexual cycle is stimulated, perithecia containing asci at all developmental stages can be isolated (Raju, 1980). Spores are released from the unitunicate asci through an operculum (pore) at the tip of the ascus (Kendrick, 1992).

Genetics of N. crassa.

The haploid genome of *Neurospora* consists of about 38 900 kbp of DNA (Orbach, 1993) organized into 7 linkage groups (Perkins *et al.*, 1982; Vollmer & Yanofsky, 1986; Perkins, 1992). The genome is comprised of 2% foldback, 8% repetitive (90% of which is ribosomal DNA), and 90% single copy sequences (Krumlauf and Marzluf, 1980).

Extensive genetic maps of *N. crassa*'s linkage groups have been compiled and, in 1992, over 700 gene loci were known (Perkins, 1992). Many of these loci were determined by generating mutations in the laboratory through ultraviolet

treatment, x-radiation or neutron bombardment, with subsequent isolation of single strains on complete medium (Beadle and Tatum, 1945). To identify nutritional mutants, pure strains were plated on a minimal medium on which growth of the fungus depended upon the presence of all the biosynthetic pathways present in the original strain. Nutritional markers were then determined by supplementing the media on which the mutants were to be cultured with various single nutrients until the required chemical was determined (Beadle and Tatum, 1945). Temperature-sensitive strains and morphological mutants were also identified and catalogued after exposure to this type of mutagenesis (Beadle and Tatum, 1945; Morgan *et al.*, 1967; Garajobst and Tatum, 1967).

An interesting feature of *Neurospora* genetics is the selective pressure on the genome to maintain the haploid state. Two events have been observed in the laboratory that act to conserve copy number, namely RIP and escape, and which may be responsible for the production of the mutation at *un-24*. Selker *et al* (1987a; 1987b) first documented the molecular basis of RIPping (*Rearrangements Induced Pre-meiotically*). These authors noted that DNA introduced into the *N. crassa* genome was unable to conserve itself through sexual crosses. To understand RIP, they transformed *N. crassa* strains with plasmids possessing both unique DNA and DNA that was already represented within the natural genome (Selker *et al*, 1987a). Their results showed that unique DNA was left unaltered when entering into a cross, but that duplicated segments were altered at a high frequency. The alteration involved the methylation of cytosine nucleotides and the subsequent

transition of G:C base pairs to A:T base pairs. Further investigation revealed that RIPping occurred pre-meiotically in ascogenous hyphae, and few duplicated sequences recovered from crosses without alteration (Selker *et al.*, 1987a; Selker *et al.*, 1990). Glass and Lee (1992) successfully utilized RIP to identify functional regions of the A mating-type idiomorph.

Escape is a vegetative process that has been observed in laboratory strains that are diploid for specific segments of the genome (partial diploidy). Partial diploid strains are created by crossing normal sequence strains to translocation strains. Many partial diploid strains have the ability to grow as wild-type, however, duplications at certain locations display an inhibited, colonial morphology (Mylyk, 1975). This effect is particularly strong when duplications are heteroallelic at heterokaryon incompatibility loci. In fact, a number of heterokaryon incompatibility loci were originally identified using this "nuclear incompatibility" approach with various translocation strains (Mylyk, 1975).

The escape mechanism consists of a process by which partial diploids with inhibited morphology resume wild-type growth after an incubation of 8 to 10 days and has been observed in both heterokaryons and self-incompatible partial diploids (Pittenger, 1964; Delange and Griffiths, 1975; Newmeyer and Galeazzi, 1977; Vellani *et al.*, 1994; Smith *et al.*, 1996). Escape from mating type and *het-6* incompatibility is caused by large-scale deletions within the diploid region that effectively silence one or the other copy of the duplicated gene (Delange and Griffiths, 1975; Smith *et al.*, 1996). Mutations caused by imperfect excision can be

isolated when escaped partial diploids are brought through crosses with wild-type strains.

Temperature-sensitive, conditional mutations and un-24.

Temperature-sensitive, conditional mutants are defined as having wild-type growth rates and morphology when grown within a specific temperature range (termed permissive), but display a characteristic mutant phenotype at another temperature range (termed restrictive) (reviewed by Pringle, 1975). Temperature-sensitive mutations have provided a means by which essential genes can be identified and cloned by complementation.

The mutant phenotype induced by a shift to high temperature is presumed in most cases to be caused by missense mutations within the coding regions for certain essential proteins (Pringle, 1975). These mutations often involve small deletions or nonsense mutations near the ends of genes. Such mutated products are able to maintain their proper structure and function only at lower, less stringent, temperatures, although often normal function is present in small quantities even at restrictive temperatures. The mutant phenotype of temperature-sensitive strains is often reversible, which might aid in the characterization of certain mutations (Pringle, 1975).

Many temperature-sensitive mutations involve factors affecting cell growth and nuclear division. One interesting mutant, *bimGII*, in *Aspergillus nidulans* has a splicing defect in a protein phosphatase involved in cell cycle transitions (Hughes

et al., 1996). At permissive temperature, the splicing machinery is able to read through a mutation in the 5' splice site of one intron thus forming a normal product. At more stringent restrictive temperatures however, the intron is maintained in the final transcript resulting in a truncated protein phosphatase that interferes with the normal functioning of the enzyme (Hughes *et al.*, 1996). A group of temperature-sensitive cell division cycle (CDC) mutants in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* are mutant in ribonucleotide reductase, an enzyme required for the conversion of ribonucleotides to deoxyribonucleotides during the synthesis phase of the cell cycle (reviewed by Elledge *et al.*, 1992).

In Chapter 1, the derivation of the temperature-sensitive, conditional mutant, *un-24*, will be described. *un-24* was derived in a series of crosses undertaken to generate RIP mutations within the translocated region present in the *N. crassa* strain, *T(II-III)AR18*. One resultant mutant, *un-24*, displays wild-type growth at 20°C, reduced growth at 30°C, and no growth at 37°C. There are over thirty "unknown function" temperature-sensitive, conditional mutations (denoted *un*) in *N. crassa* that display growth patterns similar to those shown by *un-24* (reviewed by Perkins *et al.*, 1982). While the function of many *un* mutants is unknown, the mutation at *un-24* appears to affect the large subunit of ribonucleotide reductase (Chapter 2).

Ribonucleotide reductase.

Ribonucleotide reductase is a regulated enzyme responsible for the

reduction of ribonucleotides to deoxynucleotides during the synthesis phase of mitosis (reviewed by Elledge *et al.*, 1992). The enzyme is made up of two conserved subunits [60% identity between the mammalian and yeast homologs (Elledge and Davis, 1990)] with the structure, $\alpha_2\beta_2$. The large subunit, R1, is a dimer responsible for allosteric regulation of the ribonucleotide conversion. Each 90 kda monomer has unique binding sites for deoxynucleoside triphosphates and is involved in the regulation of substrate specificity, the maintenance of equal numbers of nucleotides in the synthesis pools, and the conservation of the ATP/dATP ratio. The small subunit, R2, is also a dimer, with each 45 kda monomer housing a binuclear ferric centre and a tyrosyl free radical that make up the active site of the enzyme (Norland *et al.*, 1990).

The levels of ribonucleotide reductase vary during the cell cycle and have been found at their highest during the synthesis (S) phase of the cell cycle (Elledge and Davis, 1990). mRNA levels of R1 in *Saccharomyces cerevisiae* (called RNR1) undergo a 15 to 30 - fold increase at late G1 and into S phase (Elledge and Davis, 1990). *MluI* elements (CGCG) have been implicated in upstream regulation of ribonucleotide reductase transcription in *S. cerevisiae* (Lowndes *et al.*, 1991), *Schizosaccharomyces pombe* (Lowndes *et al.*, 1992), and of other genes, including *POL1* (DNA polymerase 1), that are up-regulated during S-phase (Andrews and Herskowitz, 1990). *S. cerevisiae* is unusual in having RNR3, a homologue of RNR1, which is induced during DNA repair and activated by DNA damaging agents and compounds that block DNA synthesis through nucleotide depletion (Elledge

and Davis, 1989). RNR3 also forms a tetramer with RNR2. While RNR1 and RNR2 are essential to the activity of the enzyme, RNR3 can be disrupted without phenotypic effects (Elledge and Davis, 1990). Temperature-sensitive conditional mutants in RNR1 and RNR3 have been found in *S. cerevisiae* (*CRT* mutants - Zhou and Elledge, 1992) and *S. pombe* (*cdc22* - Fernandez Sarabia *et al.*, 1993). Serna and Stadler (1978) describe a temperature-sensitive mutant, *ndc-1*, in *N. crassa* that is inhibited at the beginning of the S-phase, and that responds similarly to RNR2 and RNR3 in the presence of compounds that block DNA synthesis through nucleotide depletion.

Hypothesis and objectives.

The experiments presented herein have been prepared as a series of manuscripts written by myself (S.P.G. Hubbard) and performed by myself, David J. Jacobson (Stanford University, California), N. Louise Glass (University of British Columbia, Vancouver, B.C.) and Myron L. Smith [credit is given to the experimenter (apart from myself) in the results sections]. The additional experimental work is unpublished and is included as background to my thesis work.

We hypothesized that the mutation at *un-24* was the result of a RIP- or an escape event in an essential gene caused by diploidy at, and around, the heterokaryon incompatibility gene, *het-6*. The objectives of each chapter are as follows:

Chapter 1 - (a) to recover *T(AR18)* diploidy-induced mutants at, or close to,

the heterokaryon incompatibility gene, *het-6*; (b) to characterize the resultant mutant, *un-24*, in terms of growth rate, nuclear distribution, cell wall characteristics and the viability of heat-shocked germlings.

Chapter 2 - (c) to localize *un-24*⁺ within a series of overlapping cosmids from the *T(AR18)* region by RFLP and complementation assays; (d) to clone wild-type and mutant copies of *un-24*; (e) to sequence wild-type and mutant copies of *un-24*; (f) to determine if *un-24* is caused by RIP or escape; (g) to search data banks for identity to the predicted protein.

Chapter 3 - (h) to determine if there is a temperature effect on the uptake of the nucleic acid stains, DAPI and Ethidium Bromide, in wild-type and mutant germlings.

In summary, we have found that the temperature-sensitive, conditional phenotype of *un-24* is caused by 3 transition mutations, characteristic of RIP, within the essential ribonucleotide reductase gene. Nuclear distribution and cell wall positioning appears to be similar for wild-type and *un-24* strains, although there may be a temperature-related increase in the uptake of DAPI and Ethidium Bromide in *un-24* strains. *un-24* is closely linked to the heterokaryon incompatibility gene *het-6*. An internal region of the ribonucleotide reductase coding region has an incompatibility-like effect on spheroplasts of a different genetic background. It is unknown whether this incompatibility is due to a portion of the ribonucleotide transcript or another gene within the same region of DNA.

Chapter 1:

Derivation and physical characterization of the temperature-sensitive mutation, *un-24*, in *Neurospora crassa*.

Sheryl P. G. Hubbard¹, David J. Jacobson², N. Louise Glass³
and Myron L. Smith¹

¹Biology Department, Carleton University, Ottawa, Ontario, Canada.

²Department of Biology, Stanford University, Stanford, CA ³ Biotechnology Laboratory and Department of Botany, University of British Columbia, Vancouver, B.C. Canada.

Introduction

There are over thirty "unknown function" temperature-sensitive conditional mutations (denoted "*un*") in *Neurospora crassa* (Perkins *et al.*, 1982). Strains carrying *un* mutations generally grow at wild-type rates at room temperature but display little to no growth at temperatures greater than 34 to 37°C (reviewed in Perkins *et al.*, 1982). While the function of many *un* loci in *N. crassa* is unknown, some have been found to play roles in phosphate accumulation (Houlahan and Mitchell, 1948), amino acid transport and membrane stability (Thwaites, *et al.*, 1973; Kappy and Metzenberg, 1967), or in the synthesis of critical cell components (Inoue and Ishikawa, 1975; Loo, 1975). Other *un* mutants affect the circadian rhythm of conidiation (Goto, *et al.*, 1994). In general, temperature-sensitive mutants in *Neurospora* and other fungi have provided insight into several indispensable cell functions, including respiration (Pittenger and West, 1979), amino acid production (Chow, *et al.*, 1995), macromolecular synthesis (Hartwell, 1967), cell cycling (Hughes *et al.*, 1996; Osmani *et al.*, 1990; Doonan and Morris, 1989; Osmani *et al.*, 1988; Serna and Stadler, 1978) and in cell division and septation (Harris *et al.*, 1994; Trinci and Morris, 1979). Additionally, temperature sensitivity has provided a means by which essential genes can be identified, isolated, and cloned via complementation.

un-24 was generated from a series of crosses performed to examine the possibility that RIP (*Rearrangements Induced Premeiotically*) (Selker *et al.*, 1987a)

occurs in large duplicated segments of DNA. Crosses were performed between translocation-bearing *T(II-III)AR18* [hereafter referred as *T(AR18)*] and normal sequence strains to generate partial diploid (Dp) progeny. Partial diploid strains are generally barren in crosses, although occasionally, a few progeny can be recovered. We hypothesized that some of these progeny would carry RIP mutations in genes found in the duplicated region. In particular, we were interested in recovering mutations at *het-6*, which is known to be within the *T(AR18)* translocation (Mylyk, 1975).

In this paper we, first, describe the derivation of *un-24* and, second, characterize *un-24* in terms of growth rate, nuclear distribution, cell wall characteristics, and the viability of heat-shocked germlings. Viability of heat-shocked and non-heat-shocked germlings was compared by regeneration experiments and indicate that *un-24* could be cloned by standard *Neurospora* transformation protocols.

Methods and Materials

Strains and culture conditions.

N. crassa strains used in this study are listed in Table 1-1. Except during experimental procedures as indicated, cultures were grown and maintained on 1X Vogel's salts (Vogel, 1964), 1.5% sucrose, 1.5% agar, and 1X growth supplements where necessary (Davis and DeSerres, 1970). Crosses were performed on synthetic medium (Westergaard and Mitchell, 1947) by standard methods (Davis and DeSerres, 1970). Growth rate measurements were carried out as described by Jacobson *et al.* (1995).

Microscopy.

Fluorescence microscopy performed using a Reichart Zetopan fluorescence microscope, equipped with a 2UGI UV exciter filter, a GG9 barrier filter and an HBO 200 W/4-L1 mercury lamp. Photography was performed with a Nikon UFX 35 mm camera using Tri-Xpan 400 Kodak film. Brightfield microscopy was performed with a Wilde Heerbrugg microscope.

Nuclear distribution and cell wall characteristics as affected by temperature.

Conidia of C8c-164 *un-24; trp-1; inl a* and C9-2 *thr-2 a* were germinated at 30°C to the 2-10 cell stage (3 hours to overnight) in 1 ml of liquid medium with appropriate supplements. Cells were used directly (time = 0) or exposed to 37°C

Table 1-1: *N. crassa* strains used in this study.

Strain	Source
<i>T(II-III)AR18a</i>	FGSC ¹ 2644
OR23-1V A	FGSC 2489
OR6 a	FGSC 4200
<i>Dp(II-III)AR18 A</i> DJ258-4	<i>T(II-III)AR18 X OR23-1VA</i>
<i>T(II-VI)P2869 A</i>	FGSC 1828
FGSC 1847 <i>trp-1; am a</i> (OR) ²	FGSC 1847
986-1 <i>ro-7 un-24 A</i> (OR)	
C5-44 <i>ro-7 un-24; trp-1A</i> (OR)	FGSC 1847 a X 986-1 A
RLM58-18 <i>het-6^{PA} het-C^{PA} pyr-4; inl a</i> (OR)	R.L. Metzenberg
C8c-164 <i>un-24; trp-1; inl a</i> (OR)	RLM58-18 a X C5-44
C8c-74 <i>trp-1; inl A</i> (OR)	RLM58-18 a X C5-44
C9-2 <i>het-6^{OR} het-C^{PA} thr-2 a</i>	57-30 <i>cyh-1; het-6^{OR} het-C^{OR} pyr-4 A</i> X C2(2)-5 <i>het-6^{PA} het-C^{PA} thr-2 a</i>

¹FGSC = Fungal Genetics Stock Center Department of Microbiology, University of Kansas Medical Centre, Kansas City.

²OR = heterokaryon compatible with Oak Ridge tester strains.

for 3 or 6 hours, at which point germling suspensions were removed to a 1.5 ml microcentrifuge tube and centrifuged at top speed (Eppendorf Centrifuge 5415C) for five minutes. The pellet was resuspended at an appropriate density in a solution of 1 µg/ml DAPI (Aldrich Chemical Company) and a 0.9% NaCl solution containing 0.1 µg/ml Calcafluor White M2R New (gift from Dr. Byron Johnson, Carleton University, Ottawa, Ontario). Nuclear distribution and cell wall characteristics were compared in mutant and wild-type germlings grown at 30°C and 37°C using fluorescence microscopy.

Viability of heat-shocked germlings.

Conidia of C8c-164 *un-24; trp-1; inl a* and C9-2 *thr-2 a* or C8c-74 *trp-1; inl A* were germinated and allowed to grow to the 2-10 cell stage. Cells from each strain were heat-shocked at 37°C for 0, 0.5, 1 or 3 hours and approximately 800 to 2000 *un-24* or wild-type germlings were plated onto Vogel's minimal medium with appropriate nutritional supplements and 1X FIGS (0.05% fructose, 0.02% inositol, 0.05% glucose, 2% L-sorbose). Cultures were incubated for 3 days at room temperature and the number of colonies was counted.

Results

Derivation of un-24 (performed by D.J. Jacobson).

Figure 1-1 shows the derivation of *un-24* and Table 1-2 gives the crosses used and progeny selected in the derivation of *un-24*. The cross of *T(AR18)a* (FGSC 2644) with the wild-type strain, OR23-1VA, produced 25% white, non-viable ascospores. The remaining mature, black ascospores were cultured on complete medium at 34°C for 2 days. Progeny with duplications were recognized by a colonial growth form. Colonial growth is characteristic of *T(AR18)* duplications, even when the same alleles occur at *het-6* (Perkins and Barry, 1977). The cause of the growth inhibition in *Dp(AR18)* strains is unknown, but it is less severe than *Dp(AR18)* strains that are heteroallelic at *het-6*.

Although wild-type growth rates were never achieved, 15 of the 17 progeny displayed an increased growth rate with elevated levels of conidiation as compared to the original *Dp(AR18)* offspring after 8 to 10 days at 25°C with high humidity. The "escaped" morphology was stable through repeated subculture. Only two of the 15 crosses [escaped *Dp(AR18)* progeny X wild-type OR6 strain] produced spores after 6 weeks, with the remaining crosses being completely barren. Only the cross yielding *un-24* will be discussed hereafter as no mutants of interest were recovered from the other cross.

One perithecius from the cross of interest contained 25% aborted white ascospores. 33 black ascospores were recovered and transferred to

Figure 1-1: Derivation of the temperature-sensitive mutation *un-24*.

N.B. Two points at which the *un-24* mutation could have occurred in derivation are indicated as "Deletion by escape" and "mutation by RIP".

complete medium to be grown at 25°C. After five days, 9 spores did not germinate, 4 grew at wild-type rate, 14 had partial diploid morphology, 2 displayed inhibited growth with dark agar morphology and 4 grew with a fine, sparse mycelium. After continued growth, only the 14 Dp progeny escaped to wild-type morphology. The 4 fast-growing progeny that had wild-type growth rates and the 14 "escaped" Dp strains were screened for auxotrophic and temperature sensitive mutations. One of the 4 progeny was temperature sensitive, displaying near wild-type growth at 25°C and no growth at 38°C. This mutant was prototrophic, and was therefore characterized as *un-24*, an unknown, temperature-sensitive mutation. The morphology of this strain, named DJ517-1a, was also distinctive, growing in a manner similar to the peach (*pe*) mutant (Perkins *et al.* 1982).

Linkage Mapping of un-24 (performed by D.J. Jacobson and M.L. Smith).

Further crosses demonstrated that the mutant morphology and the temperature sensitivity segregated independently, such that four distinctive progeny categories were produced: 20 parental wild-type; 23 parental peach-like morphology, temperature-sensitive; 21 peach-like morphology; and 18 wild-type morphology, temperature sensitive. The morphological peach-like mutation was not linked to *T(AR18)*, however it was found to be linked to the mating-type locus (25% recombination). One of the 18 temperature -sensitive wild-type morphology, strains, DJ826-1, was utilized for further experimentation. Crosses with both *T(AR18) A* and *T(II-VI)P2869 A* revealed that the temperature sensitive mutation

Table 1-2: Pedigree of the *un-24* mutant.

Parents	Progeny selected
<i>T(II-III)AR18 a</i> X <i>OR23-1VA</i>	<i>Dp(II-III)AR18 A</i>
<i>Dp(II-III)AR18 A</i> X <i>OR6a</i>	DJ517-1a <i>un-24 morph a</i>
DJ517-1a <i>un-24 morph a</i> X WT	DJ826-1 <i>un-24 a</i>
<i>un-24 A</i> X <i>pyr-4 ro-7 het-6^{OR}</i>	DJ960-2a <i>ro-7 un-24 het-6^{OR}</i>
1847 <i>trp-1 am a</i> (OR) X DJ986-1 <i>ro-7 un-24 het-6^{OR} A</i>	C5-44 <i>trp-1 ro-7 un-24 A</i>
C5-44 <i>trp-1 ro-7 un-24 A</i> X RLM58-18 <i>het-6^{OR} het-C^{OR} pyr-4 inl a</i>	C8c-164 <i>un-24 trp-1 inl a</i> (OR)

in strain *DJ826-1 a* segregates in a 1:2 ratio (mutant to wild-type), indicating that *un-24* is covered (heteroallelic) and recessive in the duplicated progeny. *T(II-VI)P2869* has a right breakpoint 5.6 kbp from the right breakpoint of *T(AR18)* (Smith and Glass, 1996), but extends distally beyond the left breakpoint of *T(AR18)* to the left telomere region of LGII (Perkins *et al.*, 1982).

Linkage of *un-24* to *het-6* was examined in two series of crosses. In the first series, one of the 18 *un-24*, wild-type morphology (Figure 1-1) was crossed to a marked strain of Oak Ridge *het-6* genotype to yield the strain *ro-7 un-24 het-6^{OR}* (DJ960-2a), which was then crossed to a marked Spurger *het-6* genotype, *het-6^{SP} thr-2* (DJ959-1A). Genetic map data from this cross is given in Table 1-3a. In a second series of crosses, C5-44 *trp-1 ro-7 un-24 A* was selected from the cross, 1847 *trp-1 am a* (OR) X DJ986-1 *ro-7 un-24 het-6^{OR} het-C^{OR}A*. C5-44 was then crossed with RLM58-18 *het-6^{PA}het-C^{PA} pyr-4 inl a*. The phenotypes of 220 progeny from this cross are given in Table 1-3b. These crosses revealed that *het-6* and *un-24* are separated by about 2.6 - 4.5 map units. Strain C8c-164 *un-24 trp-1 inl a* (OR) was selected from the second cross for use in subsequent experiments.

Growth rate of un-24 (performed by D.J. Jacobson, Stanford University).

A comparison of wild-type and *un-24* growth rates at temperatures between 15 and 37°C is depicted in Figure 1-2. *un-24* strains have near wild-type growth rates at temperatures between 15 and 25°C. At temperatures of about 30°C,

Table 1-3a: Location of *un-24* in relation to *het-6* based on the first series of crosses.

	Markers				Number of Progeny
	<i>ro-7</i>	<i>un-24</i>	<i>het-6</i>	<i>thr-2</i>	
P	+	+	SP	-	58
P	-	-	OR	+	57
	-	-	OR	-	16
	-	-	SP	-	2
	+	+	NT ^a	+	19
	+	-	OR	+	12
	+	-	SP	+	0
	-	+	OR	-	0
	-	+	SP	-	4
	-	+	OR	+	1
	-	+	SP	+	0
	+	-	OR	-	0
	+	-	SP	-	1
P		-	OR		85
P		+	SP		62
R		-	SP		3
R		+	OR		1

R/Total = 4/151 = 2.6%

^aNT = not tested. This class of progeny could not be tested for *het-6* because of lack of heterokaryon forcing markers.

Table 1-3b: Location of *un-24* in relation to *het-6* based on the second series of crosses.

	Markers				Number of progeny	
	<i>ro-7</i>	<i>un-24</i>	<i>het-6</i>	<i>het-C</i>	<i>pyr-4</i>	
P	-	-	OR	OR	+	82
P	+	+	PA	PA	-	86
	-	+	PA	PA	-	6
	+	-	OR	OR	+	4
	-	-	PA	PA	-	2
	+	+	OR	OR	+	6 ^a
	-	-	OR	PA	-	16
	+	+	PA	OR	+	10 ^a
	-	-	OR	OR	-	4
	+	+	PA	PA	+	2 ^a
	-	+	OR	PA	-	1
	+	-	PA	OR	+	0
	-	+	OR	OR	-	1
	+	-	PA	PA	+	0
P		-	OR			106
P		+	PA			104
R		-	PA			2
R		+	OR			8

R/Total = 10/220 = 4.5%

^a18/22 *ro-7*⁺, *un-24*⁺, *pyr-4*⁺ progeny carried other auxotrophic markers and could be tested for heterokaryon incompatibility genotype.

Figure 1-2: Growth rate of wild-type and *un-24 N. crassa* strains at 20, 25, 30, 34 and 37°C.

strains carrying *un-24* exhibit a marked reduction in growth rate when compared to wild-type strains.

Nuclear distribution and cell wall characteristics as affected by temperature.

Germings of C9-2 (wild-type) and C8c-164 (*un-24*) kept at 30°C were examined for the number of nuclei per μm of germling length, the nuclear distribution, the average cell width and the distance between septae. Cell wall staining was obvious almost immediately following Calcafluor application. Staining of the cell walls was bright, and septae were evident. For both strains, nuclei took at least 30 minutes to stain with DAPI, and only a few germings took up the stain. Nuclei were situated at regular intervals along the length of extending hyphae in both C9-2 and C8c-164.

When germings were incubated for 3 hours at 37°C, C9-2 (wild-type) and C8c-164 (*un-24*) were remarkably different. Nuclei were immediately visible upon application of the DAPI stain to heat-shocked C8c-164, but not in C9-2 cells. More than 30 minutes was required before any of the heat-shocked C9-2 cells displayed nuclear staining, at which time, cells with stained nuclei were rare. Similarly, after 6 hours at 37°C, the majority of *un-24* germings exhibited nuclear staining, whereas wild-type cells rarely had stained nuclei. Aside from the apparent differences in these nuclear staining characteristics, neither the distribution of the nuclei, nor the cell wall morphology appeared different in the two strains (Figure 1-3, plates A and B).

Viability of heat-shocked germlings.

We examined whether *un-24* germlings exposed to 37°C were viable when returned to permissive temperature. Approximately 800 to 2000 C8c-164 (*un-24*) and C9-2 (wild-type) or C8c-74 (wild-type) germlings were independently exposed to 37°C for 0, 0.5, 1 or 3 hours and subsequently transferred to room temperature for 3 days. Germlings exposed to 37°C grew regardless of length of exposure to restrictive temperature (see Table 1-4), indicating that the temperature-sensitive effects on *un-24* are reversible. The number of *un-24* colonies regenerating after exposure to 37°C for 3 hours was lower than in any other treatment, although at least half of the germlings survived. This indicates that prolonged exposure to restrictive temperatures stops growth of *un-24* strains but does not generally kill them.

Figure 1-3: Cell morphology and nuclear distribution in *un-24* and wild-type germlings stained with DAPI and Calcafluor.

Plate A - C9-2 (wild-type), bar = 20 μm ,
Plate B - C8c-164 (*un-24*), bar = 10 μm .

N = nucleus; S = septum.

Table 1-4: Number of C9-2 or C8c-74 (wild-type) and C8c-164 (*un-24*) germlings regenerating after exposure to 37°C for 0, 0.5, 1 and 3 hours. Cells were allowed to recover at room temperature for 3 days. The number of regenerating colonies is taken as an average of 4 experiments.

Time at 37°C (hours)	Number of regenerating colonies	
	wild-type	<i>un-24</i>
0	1501	1305
0.5	1531	1379
1	1417	1477
3	1414	751

Discussion

The objectives of this study were to test whether mutations within a specific region could be generated with large duplicated segments of DNA by a process presumably similar to RIP. *Dp(II-III)AR18* progeny were recovered from a cross between *T(AR18)* and the wild-type strain, *OR23-1VA*. *T(AR18)* possesses a 270 kbp region, including the *het-6* locus, from linkage group II that is inserted into linkage group III (Mylyk, 1975; Smith and Glass, 1996). Escaped *Dp(AR18)* offspring were taken through a second cross and the resultant progeny were screened for mutations. A temperature-sensitive mutant with peach-like morphology (DJ517-1a) was isolated and tested for linkage to *T(AR18)* and *het-6*.

The peach-like morphology of DJ517-1a segregated independently from the temperature-sensitive phenotype. Crossing a peach-like offspring that was not temperature sensitive with *T(AR18)* demonstrated that the mutant morphology was not within the duplicated *T(AR18)* region, but that it was linked to mating type. It is interesting that a mutation arose that was not within *T(AR18)*, but which was linked to the mating-type locus on LGI since this position should not be subjected to duplication rearrangement events. It is possible that some sequence identity exists between a region within *T(AR18)* and the chromosomal locus responsible for the peach-like morphology on LGI which might subject it to a mutational event.

Analysis of progeny from crosses between the original temperature-sensitive strain, DJ826-1, and either *T(AR18)* and *T(II-VI)P2869*, revealed that the

temperature-sensitive locus, called *un-24*, is covered by both translocated segments. This suggests that *un-24* may have resulted from molecular events that act to silence duplications. Linkage mapping indicated that *un-24* is 2.6 to 4.5 map units centromere distal to the *het-6* locus. The close proximity of *un-24* to *het-6* makes it a valuable marker for localizing this heterokaryon incompatibility locus.

There are two processes that may have occurred to produce the *un-24* mutation, namely a RIP event or an escape event, both of which are associated with duplicated sequences. RIP mutations occur in duplicated loci prior to meiotic DNA replication in ascogenous hyphae (Selker *et al.*, 1987a; Selker *et al.*, 1990). Cytosine nucleotides become methylated and subsequently undergo C to T transitions (Selker *et al.*, 1987a). After karyogamy, meiosis and a single round of mitosis, eight ascospores are produced within an ascus, 4 of which have been RIPped, and 4 of which are wild-type in nature. RIPping is a high frequency event and few duplicated sequences come through a cross without alteration.

It is also possible that the *un-24* mutation was caused by the "escape" event observed during vegetative growth of the slow-growing colonial partial diploids. Escape has been observed in both incompatible heterokaryons and self-incompatible partial diploids (Pittenger, 1964; Delange and Griffiths, 1975; Newmeyer and Galeazzi, 1977; Vellani *et al.*, 1994; Smith *et al.*, 1996), and is characterized by a sudden increase in the growth rate of colonies to near-wild-type levels. Smith *et al.* (1996) found that the molecular basis of escape from self-incompatible *het-6* partial diploids generated by crosses between *T(AR18)* (OR) and a *het-6*^{PA} strain was caused by deletions in either, but predominantly the

ectopic, copy of the duplication.

In our study, several slow-growing, colonial, partial diploids were observed to shift to a near wild-type growth rate after about 8 to 10 days. The unique inhibited growth of *Dp(AR18)* partial diploids is less severe than *Dp(AR18)* strains that are heteroallelic at *het-6*. The slow growth and colonial phenotype observed in what are presumably homoallelic *Dp(AR18)* strains may be caused by some feature of the translocated region that disrupts normal cellular function. Notably, the translocation in *T(AR18)* involves the insertion of an interstitial portion of LGII into LGIII (Perkins and Barry, 1977). The nature of *un-24* should be evident from the different observed specificity of RIP and escape: RIP causes G:C to A:T transitions whereas escape results in deletions of one or the other of the duplicated copies.

Similar to previously described *un* mutations, *un-24* grows poorly, or not at all, at temperatures above 34°C. Temperature-sensitive conditional mutations are useful for the study of essential genes (the use of temperature-sensitive mutants is reviewed by Pringle, 1975). The physical basis of slow growth at restrictive temperatures by mutants of several fungi, including *A. nidulans* (*sepA2* - Trinci and Morris, 1979; *nim*, *bim*, *sep* and *nud* - Bergen *et al.*, 1984; *bimGII* - Doonan and Morris, 1989; *sep E*, *G*, *H*, *I*, and *J* - Harris *et al.*, 1994), *Saccharomyces cerevisiae* (*ts* mutants - Hartwell, 1967), *Schizosaccharomyces pombe* (*mts3* - Gordon *et al.*, 1995), and *Beauveria bassiana* (HS strains - Hegedus and Khachatourians, 1994), to name a few, has been partially elucidated by microscopic examination of morphological features. Nuclear staining with DAPI has revealed problems in cell

cycling, and in cell and nuclear division (reviewed by Doonan, 1992). To determine what vital function of the cellular growth is affected by a mutation at *un-24*, we wanted to further characterize this mutant in terms of growth characteristics, cell morphology and nuclear distribution.

At 30°C, the growth rate of *un-24* strains is slightly lower, the production of conidia occurs at roughly the same time after inoculation, and conidial germination is slower in comparison to *un-24*⁺ strains. The growth rate of the mutant strain begins to decrease significantly from that of wild-type at temperatures above 30°C, until it is not appreciable at temperatures of 35°C and greater. Other *N. crassa un* mutants display a similar growth response to temperature, although some do grow slightly at 37°C (Perkins *et al.*, 1982).

bimGII and *nud* mutants in the closely related fungus, *Aspergillus nidulans*, involve factors affecting cell growth and nuclear division which can be observed by microscopic examination (reviewed in Doonan, 1992). To determine if *un-24* caused any gross morphological alterations, germplings of heat-shocked and non-shocked wild-type (C9-2) and *un-24* (C8c-164) were examined for nuclear distribution and cell morphology. DAPI (4',6-diamidino-2-phenylindole) (reviewed in Kapuscinski, 1995) is a semi-permeant, blue fluorescent dye that binds to A-T rich DNA sequences (Lin *et al.*, 1977), thus rendering nuclei visible in stained cells examined with fluorescence microscopy. Calcafluor does not enter the cell, but fluoresces when bound to cell wall proteins. No striking differences were observed in the number or distribution of nuclei in the heat-shocked and non-shocked wild-type and *un-24* germplings. Cross wall positioning and cell morphology were similar

among strains under each experimental condition.

un-24 germlings exposed to 37°C for 3 hours are able to regenerate when placed at permissive temperatures, indicating that the cells are still viable. This indicates that *un-24* could, therefore, be cloned by standard *Neurospora* complementation protocols which involve a brief exposure to molten top agar (Akins and Lambowitz, 1985). As *un-24* is closely linked to *het-6*, it will serve as a useful marker for the study of heterokaryon incompatibility at this locus.

Chapter 2:

The *Neurospora crassa* temperature-sensitive mutant, *un-24*, is defective in ribonucleotide reductase.

Sheryl P. G. Hubbard¹, David J. Jacobson², N. Louise Glass³
and Myron L. Smith¹

¹Biology Department, Carleton University, Ottawa, Ontario, Canada. ²Stanford University, Stanford, CA ³Biotechnology Laboratory and Department of Botany, University of British Columbia, Vancouver, B.C. Canada.

Introduction

un-24 is a temperature-sensitive conditional mutation in *Neurospora crassa* that is closely linked (2.6-4.5 map units) to the heterokaryon incompatibility gene *het-6* (Chapter 1). The mutation was isolated in a series of crosses undertaken to generate RIP (*Rearrangements Induced Premeiotically* - Selker *et al.*, 1987a) mutants in strains with a duplication of the translocated segment of *T(AR18)*. This translocation possesses a 270 kbp region of LGII covering *het-6* that has been inserted into the right arm of LGIII (Perkins and Barry, 1977). *un-24* was recovered from a cross between *T(AR18)* partial diploids and wild-type, and is characterized by wild-type growth at 25°C, reduced growth at 30°C, and no growth at temperatures greater than 35°C (Chapter 1). Based on previous work that showed a three hour exposure to 37°C is not lethal, *un-24* may be cloned by complementation using standard DNA transformation protocols.

We hypothesized that temperature sensitivity in *un-24* strains was caused by a RIP or an "escape" mutation at an essential gene. In this paper, we describe the localization of *un-24* to a 3.75 kbp fragment of DNA. DNA sequence analysis revealed an open-reading frame in this region with 41-45% amino acid identity to ribonucleotide reductase. Ribonucleotide reductase is an essential enzyme, which is responsible for the conversion of ribonucleotides to deoxyribonucleotides during the synthesis (S) phase of mitosis (reviewed by Elledge, *et al.*, 1992) and is conserved among a diverse group of organisms. A comparison of *un-24* and wild-

type DNA sequence revealed 3 GC→AT transitions associated with the temperature-sensitive mutation. The 3.75 kbp fragment of OakRidge (OR) DNA that encodes the wild-type ribonucleotide reductase complements the *un-24* mutation but, interestingly, causes an incompatibility reaction when transformed into cells of PANAMA (PA) genetic background.

Methods and Materials:

Strains and culture conditions.

N. crassa strains used in this study are listed in Table 2-1. Except during experimental procedures where indicated, cultures were grown and maintained on 1X Vogel's salts (Vogel, 1964), 1.5% sucrose, 1.5% agar, and 1X growth supplements, where necessary (Davis and DeSerres, 1970). *Escherichia coli* DH5 α cells (Gibco/BRL, Burlington, ON) and Inv α F' cells (Invitrogen, Carlsbad, CA) were used as recipients for bacterial transformations.

DNA isolation.

Neurospora DNAs were isolated by the method of Oakley *et al.* (1987). Plasmid and cosmid DNAs were either isolated by the alkaline lysis procedure with or without further purification by CsCl-Ethidium Bromide gradients (Sambrook *et al.*, 1989) or by passage through Qiagen columns (Qiagen, Chatsworth, CA), or with the Circle Prep Spin-Midi Kit (Bio 101, CA) as per manufacturer's recommendations.

DNA hybridizations.

Genomic DNAs were digested with restriction endonucleases (Boehringer Mannheim, Laval, P.Q.; New England Biolabs, Beverly, MA; Gibco/BRL, Burlington, ON), subjected to electrophoresis through 0.8% agarose (SeaKem,

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

Strain	Source
RLM58-18 <i>het-6^{PA} het-C^{PA} pyr-4 inl a</i> (OR) ¹	R.L. Metzenberg
C5-44 <i>ro-7 un-24; trp-1A</i>	FGSC ² 1847 <i>trp-1; am a</i> X DJJ986-1 <i>ro-7 un-24 A</i>
C8c-164 <i>un-24 trp-1; inl a</i> (OR)	RLM58-18 <i>a</i> X C5-44 <i>ro-7 un-24; trp-1A</i>
C8c-165 <i>un-24; trp-1; inl a</i> (OR)	RLM58-18 <i>a</i> X C5-44 <i>A</i>
C8c progeny	RLM58-18 <i>a</i> X C5-44 <i>A</i>
C2(2)-1 <i>het-6^{PA} het-C^{PA} thr-2 a</i>	RLM58-18 <i>a</i> X DJJ987-51 <i>ro-7 un-24 het-6^{PA}</i> (OR)
C9-2 <i>het-6^{OR} het-C^{PA} thr-2 a</i>	57-30 <i>cyh-1; het-6^{OR} het-C^{OR} pyr-4 A</i> X C2(2)-5 <i>het-6^{PA} het-C^{PA} thr-2 a</i>

¹OR = heterokaryon compatible with Oak Ridge tester strains.

²FGSC = Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical Centre, Kansas City.

FMC BioProducts, Rockland, Me; Agarose LE, Boehringer Mannheim), in 1X TAE (0.04 M Tris-acetate, 0.001 M EDTA), and transferred to Hybond N membrane according to the membrane manufacturers recommendations (Amersham, Oakville, ON).

The Orbach/Sachs *Neurospora* genomic library in the cosmid pMOcosX (Orbach, 1994) was obtained from the Fungal Genetics Stock Center (FGSC, Department of Microbiology, University of Kansas Medical School, Kansas City, Kansas), and handled by standard protocols (Vollmer and Yanofsky, 1986). Bacterial colony DNA transfer onto GeneScreen Plus (Du Pont GeneScreen Plus, Du Pont Canada, Inc., Lachine P.Q.) or Hybond N (Amersham) membranes was by methods outlined in Sambrook *et al.*, (1989).

Probe DNAs were labelled using either T7 QuickPrime (Pharmacia, Baie d'Urfe, P.Q.) with [α -³²P]-dCTP (Amersham) or with fluorescein-dUTP (ECL, Amersham), or using Klenow with digoxigenin (DIG)-dUTP (Boehringer Mannheim). Hybridization conditions (60°C for T7 probes or 68°C for DIG probes) and washes were done according to the membrane manufacturers' protocols. Hybridization signals were visualized following exposure to Kodak X-OMAT or Kodak Biomax MR film.

PCR Reactions.

PCR reactions were performed using the Expand High Fidelity kit (Boehringer Mannheim). The total reaction volume was 50 μ l, and the conditions

were 200 μ M of each dNTP, 300 nM of each primer, 1X Expand High Fidelity Reaction Buffer (Boehringer Mannheim) and 2.6 U Expand High Fidelity PCR System enzyme (Boehringer Mannheim). 2- to 10 ng of *Neurospora* genomic or plasmid (p31ECO16) DNA was used for reactions. Table 2-2 lists primers utilized in PCR reactions. Amplification was performed using an Amplitron II Thermolyn Thermocycler (Barnstead/Thermolyn, Ducusque, IA). DNA was denatured for 2 min at 94°C, and reactions were cycled 25 times as follows: 15s at 94°C, 30s at 55°C, 1 min/1.5 kb DNA at 68°C. This was followed by 10 minutes at 72°C and a 4°C chill. Reaction products were visualized by electrophoresis under conditions described above. PCR products were purified using Boehringer Mannheim's High Pure PCR product purification kit as per recommendations. Concentrations of purified PCR products were approximately 200 ng/ μ l.

Ligations and Cloning.

Ligation of p31ECO16 restriction enzyme fragments into the pIBI76 vector (The Clonar Biosystem, IBI, Connecticut) was accomplished by a 2.5 hour incubation at room temperature using 1U T4 DNA ligase in 1x Ligase Reaction Buffer (Gibco/BRL). Ligation of PCR products into the pCRII vector (Original TA Cloning Kit, Invitrogen) was performed overnight at 14°C by mixing approximately 150 ng of PCR product, 50 ng pCRII, and 4.0 units of T4 DNA ligase (provided with Original TA cloning kit) in 1x ligation buffer.

p31ECO16 was cloned into the pOKE103 vector (gift from R.L.

Table 2-2: Name and sequence of primers used for PCR amplification and sequencing of WT and mutant copies of *un-24*.

Primer and Direction	Sequence (5' to 3')	~T _m (°C)
6JP1	GGTTGCCCGTCTCAGTC	56
6JP2	CCTGAGGTGTATGAGGG	54
6JP3	GGTGACACGGCGCTGTG	58
6JP4	GTGTCAGACTGTCAGGCAGC	64
6JP5	GCATTCGTGCCACTGGCTCC	66
6JP6	GTGCGGGCTTAACCGCTG	60
6JP7	CGAGGGTCTGAAGCCG	54
6JP8	CCTTGCCTCTCTCGCCC	58
6JP9	TTGCCCATGGTGGGTTCG	58
6JP10	GGACGAGTTCGACTCGGC	60
6JP11	CTCCGGATGAGGTTGCCG	60
6JP12	CGGCAACCTCATCCGGAG	60
6JP13	CACTAACACCAGTCTGGGC	58
6JP14	TTCTCTCCCAGCAGCGGG	60
6JP15	GTTGCTCAGAAAGTGGAG	60
6JP16	ACGTCTATCGTGTTGAGG	54
6JP17	GCAGCTAGGAAAGATTATTG	58
6JP18	TGGCTACAGGATGAACATGG	56
6JP19	GTCCCTCTCCTTGTACCG	58
6JP20	CAAATGGAGGGACAAAC	52
6JP21	TTCTGGACGTGTAAAGTG	52
6JP22	CACATCTGCATCTAGCGTGC	62

~T_m = approximate annealing temperature of primer.

Metzenberg). pECO16M plasmid was subcloned by digesting 300 ng p31ECO16 DNA with *Mlu*I and isolating a 12 kbp fragment from a 0.7% agarose gel by freezing, centrifugation, and ethanol precipitation as follows. 100 µl TE buffer (1 mM tris-Cl, pH 8; 0.1 mM EDTA) was added to the excised gel slice in a microcentrifuge tube. The gel slice in TE was fragmented with a blunted P1000 tip and the mix was frozen at -70°C for 10 minutes. The mixture was thawed and centrifuged for 1 min at 13 000 rpm in an Eppendorf microcentrifuge (Eppendorf Centrifuge 5415C). The supernatant was removed to a column prepared by piercing the bottom of a 500 µl microcentrifuge tube, plugging the tube with sterile glass wool and placing it into a 1.5 ml microcentrifuge tube. The pellet was placed on the top of the glass wool. The column was centrifuged for 1 min at 3000 rpm. The eluate was saved, and the column was placed in a fresh 1.5 ml catch tube to be washed with 100 µl of fresh TE. The eluates were pooled and precipitated overnight at -20°C in 0.8 volume of isopropanol and ¼ volume of NH₄OAC. The DNA was pelleted by high-speed centrifugation at 4°C for 30 minutes, dried under vacuum and resuspended in 10 µl dH₂O. Approximately 150 ng DNA was recovered using this procedure. The 12 kbp band was recircularized by ligation using T4 DNA ligase as described above.

Bacterial transformation.

1 µl of ligation reaction was used to transform 50 µl DH5 α *E. coli* electrocompetent cells (prepared as recommended by BioRad) using a BioRad *E. coli* Pulser electroporator (BioRad, Burlington, ON). Cells were pulsed at 2.5 kv,

25 μ F and 200 ohms in 2 mm path width electroporation cuvettes (Invitrogen) and resuspended in SOC medium (2% bactotryptone; 0.5% yeast extract; 10 mM NaCl; 2.5 mM KCl; 10 mM MgCl₂; 20 mM glucose). 2 μ l of pCRII ligation reactions were transformed into Inv α F' cells by a 30s heat-shock at 42°C in the presence of β -mercaptoethanol with subsequent resuspension in SOC medium (described in the TA Cloning Kit manual, Invitrogen). Transformed cells were incubated for 1 hour with shaking at 37°C, and plated onto LB agar (1% bactotryptone; 0.5% yeast extract; 1% NaCl; 1.5% agar) with 100 μ g/ml Ampicillin (Sigma Chemical Company, St. Louis, MO), and Isopropylthio- β -D-galactoside (IPTG - 200 mg/ml) and 5'-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal -40 mg/ml in dimethyl formamide) where required. Plates were incubated overnight at 37°C. Single transformed bacterial colonies were grown overnight at 37°C with shaking in LB broth with 100 μ g/ml Ampicillin for plasmid DNA isolation.

Spheroplasts and DNA transformations of N. crassa strains.

Spheroplast preparation of *N. crassa* strains was similar to that of Schweizer *et al.* (1981), as modified by Royer and Yamashiro (1992). Ten 500 ml flasks, each containing 75 ml of Vogel's solid medium with appropriate supplements, were inoculated with *N. crassa* strains C8c-164, C8c-165, C9-2 or C2(2)-1, and incubated for 12-14 days at room temperature. Conidia were harvested in 1 L of Vogel's liquid medium with appropriate supplements and allowed to stand at 5°C for 12 hours with gentle shaking (100 rpm). The conidial suspension was then

transferred to 30°C with shaking (100 rpm) for about 8 hours, or until 80% of the conidia had germinated. Germlings were collected by vacuum filtration through miracloth (Calbiochem, La Jolla Ca.) and resuspended in 10 ml of 1 M Sorbitol with 5 mg/ml Novozym 234 (lot 4859, Interspex Products Ltd., Foster City, CA.) Cell wall digestion were from 45 minutes to 1.5 hours at 30°C with shaking (75 rpm), until approximately 80% of germlings were converted to spheroplasts. Two volumes of ice-cold 1 M Sorbitol were added to spheroplasts prior to centrifugation at 700 rpm, 4°C for 15 minutes. Spheroplasts were subsequently treated for transformation as described by Akins and Lambowitz (1985). 100 µl of spheroplast suspension (8.0×10^7 spheroplasts/ml) was mixed with 0.6 to 2 µg of transforming DNA. Co-transformations were with 300 ng pCSN44 (*hyg^R*) and 0.6 to 2 µg of test DNA. Transformed C8c-164 and C8c-165 spheroplasts were selected at 37°C on regeneration plates without hygromycin. Transformed C9-2 and C2(2)-1 spheroplasts were selected on regeneration plates with hygromycin B (235 U/ml, Boehringer Mannheim) at 30 or 37°C.

DNA sequencing.

Figure 2-1 shows the subclones that were used in the sequencing of *un-24*. Approximately 1- to 2.5 µg of plasmid DNAs, or 1.4 µg of PCR products, was used for sequencing. Table 2-2 indicates the primers that were used for direct sequencing of wild-type and mutant PCR product. Additional primers utilized for sequencing subclones were the universal T7 promotor region and -20 region. All

primers were used at concentrations of 3.75 pM. Sequencing was by the dideoxy DNA chain termination method (Sanger *et al.*, 1977) with the T7 Sequenase Kit (Amersham Life Sciences) used as recommended. Labelling was with [α - ^{35}S]-dATP. GC-rich sequence was corrected using dITPs (Amersham Life Sciences). Sequencing close to the primer was achieved by the addition of Mn buffer (0.1 M MnCl_2 ; 0.15M sodium isocitrate - Amersham Life Sciences).

Figure 2-1: Plasmid subclones used for DNA sequencing of *un-24*.

+ 6JP3/6JP6 C8c-164 PCR product was cloned into pCRII vector as a source of additional subclones.

Results

Locating un-24 by RFLP analysis (completed by M.L. Smith).

The position of *un-24* was determined with respect to a set of overlapping cosmids that spanned the translocated region in strain *T(AR18)* (Smith *et al.* 1996). Overlapping cosmids in the *un-24* region are shown in Figure 2-2a. The strategy used to analyze crossovers in the vicinity of *un-24* was as follows. It was determined that strains RLM58-18 and C5-44 differed in RFLP patterns within the *T(AR18)* translocated region. The RFLP pattern in strain RLM58-18 is designated as the Panama (PA) type, that of C5-44 is the Oak Ridge (OR) type. Unselected progeny from the cross of these two strains are numbered C8c-1, C8c-2, etc. The genotype of the C8c progeny was determined for markers *ro-7*, *un-24*, *het-6*, *het-C*, and *pyr-4* (Figure 2-2b), all on LGII, in addition to the unlinked markers, *inl* and *trp-1*. RFLP patterns were determined for progeny with crossovers between *ro-7* and *pyr-4* with cosmid probes X10:D:7, G8:F:7, G12:E:3, X1:E:7b, G8:G:1 and X14:C:1 (Table 2-3). Note that three distinct cosmids were at X1:E:7. In our library, X1:E:7b was the second most prevalent in the E:7 position of plate X1. In examining the RFLP data from strains C8c-106 and C8c-353 (Table 2-3), *un-24* is apparently located within, or to the right of, cosmid G8:G:1. However, from strain C8c-88, *un-24* is evidently within, or to the left of, cosmid G8:F:7.

Figure 2-2: Overlapping cosmids spanning the $T(II \rightarrow III)AR18$ translocation region on the left arm of LGII.

- (A) shows a contiguous set of cosmids in the vicinity of *un-24*. Values in parenthesis indicate insert size.
- (B) genetic markers used in this study that are on the left arm of LGII.

A)

B)

Table 2-3: Genetic markers and RFLP patterns of selected progeny from C5-44 X RLM58-18 identifying the approximate position of *un-24*. "O" and "P" indicate RFLP patterns are "OakRidge" or "Panama" - like, respectively. O/P and P/O are hybrid patterns that are mostly OakRidge, or mostly Panama, respectively.

Strain	Genetic Marker					Cosmid					
	<i>ro-7</i>	<i>un-24</i>	<i>het-6</i>	<i>het-C</i>	<i>pyr-4</i>	X10:D:7	G8:F:7	X1:E:7b	G12:E:3	G8:G:1	X14:C:1
C8c-106 <i>inl, trp-1 A</i>	+	-	O	O	+	P	P	P	P	P/O	O
C8c-164 <i>inl, trp-1 a</i>	+	-	O	O	+	O	O	O	-	O	O
C8c-165 <i>inl, trp-1 a</i>	+	-	O	O	+	-	O	O	-	O	O
C8c-353 <i>inl, trp-1 A</i>	+	-	O	O	+	P	P	P	P	P/O	O
C8c-19 <i>inl, pyr-4, trp-1 A</i>	-	+	P	P	-	O	O	O	-	P	P
C8c-36 <i>inl, pyr-4, trp-1 a</i>	-	+	P	P	-	O	O	O	O/P	P	P
C8c-168 <i>pyr-4 a</i>	-	+	P	P	-	O	O	O	P/O	P	P
c8c-196 <i>pyr-4, trp-1 a</i>	-	+	P	P	-	O	O	O	P/O	P	P
C8c-74 <i>inl, trp-1 A</i>	+	+	O	O	+	P	P	P	P/O	O	O
C8c-88 <i>inl A</i>	+	+	O	O	+	P	P/O	O	O	O	O
C8c-350 <i>inl A</i>	+	+	O	O	+	P	P	P	O	O	O
C8c-317 <i>pyr-4, trp-1 a</i>	-	-	P	P	-	O	O	O	O/P	P	P
C8c-199 <i>inl, pyr-4 A</i>	-	-	P	P	-	O	P/O	P	-	P	P
RLM58-18 <i>inl, pyr-4 a</i>	+	+	P	P	-	P	P	P	P	P	P
C5-44 <i>trp-1 A</i>	-	-	O	O	+	O	O	O	O	O	O

un-24 complementation (completed by M.L. Smith).

From the RFLP analysis, *un-24* was taken to be somewhere within the overlapping region between, and covered by, G8:F:7 and G8:G:1. Complementation of the temperature sensitivity of *un-24* was tested by transforming C8c-165 with cosmids G8:F:7, X1:E:7b, G12:E:3 and G8:G:1. Initially, transformants were regenerated at 30°C on plates containing hygromycin. Regenerated colonies were then subcultured to Vogel's solid medium and incubated at 37°C to test for growth at restrictive temperature. Table 2-4 gives the numbers of regenerating transformants recovered at 30°C in comparison to pMP6 (hygromycin resistant) transformants and untransformed colonies as controls. A larger number of transformants was observed when G12:E:3 and G8:G:1 DNAs were used as the transforming DNA. Complementation of *un-24* by cosmids G12:E:3 and G8:G:1 was also evident in these initial experiments by near wild-type regeneration times of from two to three days at 30°C, compared to regeneration times of from four to ten days for the controls and non-complementing cosmids.

We examined the possibility of using a restrictive temperature to select for *un-24* complementation with cosmid G12:E:3. It was found that equivalent numbers of transformants were recovered (about 44 colonies/μg DNA) whether the regeneration plates were left at 22°C or 30°C for three hours and then transferred to 37°C, or were immediately placed at 37°C following transformation (data not shown). Subsequently, selection for *un-24* complementation was done without hygromycin, by placing regenerating plates directly at 37°C.

Table 2-4: Number of *N. crassa un-24* transformants regenerating at 37°C following transformation with the *T(AR18)* cosmid library.

Strain	Cosmid					
	<u>pMP6</u>	<u>G8:F:7</u>	<u>X1:E:7b</u>	<u>G12:E:3</u>	<u>G8:G:1</u>	<u>H₂O</u>
C8c-164	60	30	10	>600	>600	0

Table 2-5: Number of *N. crassa un-24* transformants regenerating at 37°C following restriction enzyme digests of a) cosmid and b) subclone DNAs.

a) Restriction enzyme digests of cosmid G12:E:3

Strain	Restriction enzyme						
	<i>EcoRI</i>	<i>XhoI</i>	<i>PvuI</i>	<i>BamI</i>	<i>KpnI</i>	<i>HindIII</i>	uncut
C8c-165	80	0	7	3	4	1	313

b) Restriction enzyme digests of subclone TLP-1-31 of cosmid G8:G:1

Strain	Restriction enzyme								
	<i>Apal</i>	<i>NaeI</i>	<i>SacI</i>	<i>SphI</i>	<i>BamHI</i>	<i>KpnI</i>	<i>XbaI</i>	uncut	pMP6
C8c-165	5	1	57	0	2	2	180	429	0

Location of un-24 within G8:G:1 and G12:E:3 (completed by M.L. Smith).

Approximately 11 kbp of overlapping DNA is shared by cosmids G12:E:3 and G8:G:1 (Figure 2-2a). To locate *un-24* within this region, 1 µg of DNA from G12:E:3 was digested with the restriction enzymes indicated in Table 2-5. The digested DNAs were then concentrated under vacuum, and used to transform spheroplasts of C8c-165. The data presented in Table 2-5a indicate that digesting G12:E:3 with *EcoRI* does not eliminate complementation of *un-24*. Digesting cosmid G12:E:3 DNA with any one of the enzymes *BamHI*, *HindIII*, *KpnI* or *XhoI* reduced the number of regenerating colonies to near control levels. Digestion with the enzyme *PvuI* reduced the number of recovered transformants to an intermediate level. The left portion of cosmid G8:G:1, including the overlapping region of cosmid G12:E:3, was subcloned into pMOcosX vector using an internal *NotI* site and the *NotI* sites in the vector (Orbach, 1995) to give the subclone TLP-1-31. Digests of TLP-1-31 were carried out with the restriction enzymes given in Table 2-5b, before transforming into spheroplasts of C8c-165, as described above. From Table 2.5b, it is apparent that digesting TLP-1-31 with *XbaI* and *SacI* does not reduce *un-24* complementation, whereas digestion with the enzymes *ApaI*, *NaeI*, *SphI*, *BamHI* and *KpnI* significantly reduces the number of regenerating transformants. Presumably, the controlling or coding regions of *un-24* contain recognition sequences for those enzymes that significantly reduce the number of transformants regenerating at 37°C.

Next, three *EcoRI* fragments were subcloned from TLP-1-31 DNA into the

pOKE103 plasmid vector. After three days at 37°C, a total of 0, 275, 0 and 526 transformants had regenerated from spheroplasts transformed with Eco-7, Eco-16, Eco-18 and TLP-1-31 DNA, respectively. This identified the location of *un-24* within Eco-16. This fragment was cloned into pOKE103 and was designated as p31ECO16.

Location of un-24 within p31ECO16.

Based on a restriction enzyme map of p31ECO16 (Figure 2-3), the most likely location for *un-24* was in the vicinity of the *Bam*HI, *Hind*III and *Sph*I sites. Complementation experiments were undertaken using restriction fragments of p31ECO16 (Table 2-6) which localized *un-24* between *Mlu*I and *Eco*RI and this fragment was called pECO16M. pECO16M complemented *un-24*, giving an average of 172.4 transformants/1 µg DNA at a regeneration temperature of 37°C.

DNA sequencing was initiated in p31ECO16 and pECO16M and primers were designed based on these sequences. Figure 2-4 shows a restriction enzyme site map of the 3.75 kbp region of p31ECO16 flanked by *Bam*HI and *Hind*III sites and gives primer locations. Primer pairs in the region were used to generate PCR products for direct transformation into *un-24* (OR) spheroplasts to test temperature-sensitivity complementation. Transformation of PCR products into C8c-164 placed *un-24*⁺ within a 3.75 kbp region bound by the primers 6JP3 and 6JP6. The 6JP3/6JP6 PCR product was cloned into pCRII and is referred to as p3/6OR. p3/6OR gave an average 272 colonies per 0.6 µg transforming DNA (Figure 2-5,

Figure 2-3: Location of *un-24* within the cosmid G8:G:1.

Restriction enzyme sites are indicated by N (*Not*I), E (*Eco*RI), B (*Bam*HI), H (*Hind*III) and S (*Sac*I). Values in parentheses show insert size.

Cosmid G8:G:1 (~35 kb)

TLP-1-31 (~24 kb)

Eco-16 (~11.5 kb)

Figure 2-4: Restriction map of the 3.75 kbp fragment of p31ECO16 flanked by the restriction enzymes, *Bam*HI and *Hind*III, and the complementation/incompatibility function of restriction enzyme and PCR-generated fragments of p31ECO16.

✓ positive function; X does not function; () putative intron; * stop site; — primer (arrow points in 3' direction).

plate G). Complementation of *un-24* with subclones or fragments of PCR products smaller than 6JP3 and 6JP6 was unsuccessful (see Figure 2-4, 2.5 plate E, and Table 2-7).

Subclones of 6JP3/6JP6 fragments generated from C8c-164 and the PA strain, RLM58-18, do not complement *un-24* activity (0.7 and 1.3 *un-24* colonies regenerated/ 0.6 µg DNA, respectively, in 3 experiments). That the 6JP3/6JP6 *un-24* (data not shown) and PA (Figure 2.6b) PCR fragments contained DNA homologous to the same OR fragment was confirmed by Southern Blotting using the OR 6JP3/6JP6 fragment as a probe. Interestingly, the PCR profile of the PA strain was different from that of C8c-164 and p31ECO16 (wild-type), with three different PCR products in the former and only one each in the latter two samples (Figure 2.6a). Only the 3.75 kbp band produced by the PA strain hybridized to the OR probe.

Incompatibility function of un-24.

PCR products shown in Figure 2-4 were also used to transform spheroplasts that were OR or PA in the *un-24/het-6* region to evaluate whether these fragments carried incompatibility function. Incompatibility in this study is defined by a marked reduction in the number of PA colonies, in comparison to the number of OR colonies, that regenerate following DNA transformation. Co-transformations of C9-2 (OR at *het-6*) spheroplasts with the plasmid, pCSN44, conferring hygromycin-resistance,

Figure 2-5: C8c-164 (*un-24*) colonies after transformation with complementary, or non-complementary, WT and *un-24* DNA fragments.

Regeneration was at 37°C for 3-5 days.

Colonies shown in plates were transformed with (A) H₂O; (B) p31ECO16; (C) 6JP3/6JP2 p31ECO16 PCR fragment; (D) 6JP3/6JP6 p31ECO16 PCR fragment; (E) 6JP3/6JP1 p31ECO16 PCR fragment; (F) p3/6UN; and (G) p3/6OR.

Figure 2-6: 6JP3/6JP6 PCR products of p31ECO16 (WT) and RLM58-18 (PA) visualized by EtBr staining (A) and by DNA hybridization to a 6JP3/6JP6 OR probe (B).

(-) no DNA control

Standard is a lambda DNA digest with *Hind*III. Visible bands from bottom to top are 2.0, 2.2, 4.4 (faint), 6.6, 9.4 (faint) and 21 (faint) kbp.

and the control DNA, pUC128, gave an average of 79.7 regenerated colonies, while C2(2)-1 (PA at *het-6*) spheroplasts transformed with the same DNAs regenerated an average of 45.8 colonies, indicating that the OR spheroplasts were almost 2X more competent than the PA spheroplasts. Figure 2-7 shows that significantly fewer PA colonies, as compared to OR colonies, regenerate at 30 and 37°C following co-transformation with p3/6OR and pCSN44 (Figure 2-7), indicating that this fragment has incompatibility function. Furthermore, transforming *het-6^{OR}* and *het-6^{PA}* spheroplasts with p3/9OR, p10/9OR and p10/6 indicated that incompatibility is induced by a 1.75 kbp region, bound by primers 6JP9 and 6JP10, which is smaller than the region required for *un-24* complementation (Figure 2-4). The 6JP10/6JP9 DNA fragment that causes incompatibility in the *het-6^{PA}* spheroplasts is well within the boundaries of the region that complements *un-24*. The number of OR and PA colonies that regenerate following transformation with p3/6PA are not significantly different (Figure 2-7), indicating that the p3/6PA clone does not possess a functional incompatibility gene or that the incompatibility reaction at this locus is non-allelic.

To test whether the temperature-sensitive mutation at *un-24* affects the incompatibility function in the 6JP3/6JP6 region, p3/6UN was transformed into OR and PA spheroplasts at 30 and 37°C. The results obtained when OR and PA spheroplasts were transformed at either temperature with p3/6UN were similar to those obtained when transformations were with p3/6OR (Figure 2-7), indicating that *un-24* has incompatibility function and that the temperature-sensitive mutation at

Figure 2-7: Histogram depiction of the incompatibility function of p3/6UN, p3/6OR and p3/6PA at 30 and 37°C.

Colonies were co-transformed with pCSN44 (*hyg*^R). *het-6* incompatibility is taken as a lower number of PA colonies as compared to OR colonies following DNA transformation. Values represent the mean number of regenerated colonies in three experiments. Control regeneration temperature was 37°C. Differences in the numbers of PA and OR regenerated colonies are statistically significant to 5% (*) or 10% (**), or insignificant (p3/6PA), according to the Mann Whitney non-parametric test with tied ranks.

un-24 does not interrupt *het-6*.

DNA sequencing.

The DNA sequence from one strand of the entire 6JP3/6JP6 fragment that complements *un-24* was determined for both p31ECO16 and C8c-164. Sequence analysis revealed an open-reading frame of 2988 bp with 4 putative introns (Figure 2-8). While sequencing was not completed for the entire promotor region, a series of regulatory *MluI* elements (CGCG - Fernandez Sarabia *et al.*, 1993) were found upstream from *un-24*. Table 2-8 describes 4 putative introns found in *un-24** (*rir-1*) as based on *N. crassa* consensus intron acceptor, branch and donor sequences (Bruchez *et al.*, 1993; Edelman and Staben, 1994). Intron positions are demonstrated in Figure 2-4. The distances from the C of the branch point to the G of the 3' acceptor site were 17, 21, 16 and 19, for introns 1, 2, 3 and 4, respectively, which are within the consensus range of 14 to 30 nucleotides (Edelman and Staben, 1994). Splicing the putative introns gives the predicted amino acid a sequence identity of about 41 to 45% to the ribonucleotide reductase of humans, mice, *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Vaccinia* virus, and *Schizosaccharomyces pombe* (Figure 2-9). Intron 1 is in an identical position to an intron in *S. pombe* (Fernandez Sarabia *et al.*, 1993). The remaining introns are necessary to avoid stop codons that would truncate the ribonucleotide reductase. The putative introns result in a strikingly high level of overall identity to the ribonucleotide consensus sequences.

Figure 2-8: DNA sequence and predicted amino acid sequence of WT *N. crassa* ribonucleotide reductase.

Mutations in *un-24* are indicated above sequence. Amino acids are indicated below sequence.

CAAGTTTCGTGTTCTTCTGTTGTCCCTCCATTTTGGTTGTCAATAACTGTACTTGTTCCTTGTCCCTCTTCTTCACCTT
CTCCCCACACATCAGACATTATGTATGTCAAGAAGCGCGgtatgtttcacgactctcccttccctaccccaataaccatcat
M Y V K K R D
80
160
caggcccaatgctaactctctcctcatagATGGCCGCCAGGAGCGGTCCAGTTCGACAAGATCACAGCTCGTGTTCGA
G R Q E R V Q F D K I T A R V S R
240
T
GGCTCTCTTATGGCCTCGACATGAACCATGTTGATCCCTGTAGCCATTACACAAAAGGTCATCTCTGGCGTCTACGGTGGC
L C Y G L D M N H V D P V A I T Q K V I S G V Y G G
320
GTCACCACAGCACAACTAGACGATCTGgtaagcaccttctccccagtcctctccttgtagcgattataaaatcacgagct
T V T A Q L D D L
400
aacacataatcttccctagGCTGCCGAGACTGCCGCCCTACATGACCGTCACCCACCCGACTATGCCATCCTCGCCGCC
A A E T A A Y M T V T H P D Y A I L A A
480
CGTATCGCCGTCCAACTCCACAAGCAGACCAAGAAGCAATGGTCGCTCGTTATCAGCGAGCTGTACAACATATGTCAA
R I A V S N L H K Q T K K Q W S L V I S E L Y N Y V N
560
CCCCAGGACCGGAAAGCACTCCCCCATGATCGCCCAAGGACGTATACGAGTGTGTCTCATGCGCCACAAGGACGAGTTCGACT
P R T G K H S P M I A K D V Y E C V M R H K D E F D S
A A
CGGCAATTGTCTACGACCGTGACTTCAACTACCAATACTTCGGCTTCAAGACCCCTCGAACGATCATACCTTCTTAAAGTC
A I V Y D R D F N Y Q Y F G F K T L E R S Y L L K L
720
GATGGCCAGATTGCTGAGCGTCCCCAGCACATGATCATGCGTGTGCTCGGTATCTGGGAGACGACGTTGACGGGT
D G Q I A E R P Q H M I M R V A V G I W G D D V E R V
800

CATTGAGACTTACAACCTCATGTCCCTCAAGACCTTACCCACGCTTCTCCCACCCCTCTTCAACGCCGGTACTCCCCAGC 880
I E T Y N L M S L K T F T H A S P T L F N A G T P Q P
CCCAGCTCTCTTCGTGCTTCCCTGGTCGACATGAAGGAAGACAGTATCGAGGGCATCTACGATAACCCCTCAAGACCTGCGCC 960
Q L S S C F L V D M K E D S I E G I Y D T L K T C A
ATGATCTCTAAGATGGCTGGCGGTATCGGTCTCAACATCCACCGCATTCGTGCCACTGGCTCCTACATCGCTGGTACCAA 1040
M I S K M A G G I G L N I H R I R A T G S Y I A G T N
CGCACATCCAACGGTATCGTTCCCATGCTCCGTGTCTTCAACAACACCCGGCTACGTTGACCAGGGTGGCAACAAGC 1120
G T S N G I V P M L R V F N N T A G Y V D Q G G N K R
GTCCTGGTGCTTTCGCCCATCTACCTCGAGCCTTGGCACGCCGATGTCTTCGAGTTCCTTGACCTCCGCAAGAACCACGGT 1200
P G A F A I Y L E P W H A D V F E F L D L R K N H G
AAGGAAGAGGTGCGCGCTCGCGATCTCTTCCTTGCCCTCTGGATCCCCGATCTTTTCATGAAGCGTGTGTGAGCAGAACGG 1280
K E E V R A R D L F L A L W I P D L F M K R V E Q N G
CCAGTGGACTCTCATGTGCCCCTCACGAGTGCCCCCTGGCCCTTGCCCGACGTTTACGGTGACGAGTTCGAAGCTCTGTACGAAA 1360
Q W T L M C P H E C P G L A D V Y G D E F E A L Y E K
AGTACGAAAAGGAAGGTCGCAAGACCGTCAAGGCCCAAGAGCTTTTGGTATGCCATCTCTCGAGGCCCAGACTGAG 1440
Y E K E G K G R K T V K A Q K L W Y A I L E A Q T E
ACGGGCAACCCCTTCATGCTCTACAAGGATGCCCTGCAACCGCAAGAGTAACCAGAAGAACCCTGGGTACCATCCGCAGCTC 1520
T G N P F M L Y K D A C N R K S N Q K N L G T I R S S
CAACCTGTGCACGTAGATTATCGAGTACTCTGTCTCCGGATGAGGTTGCCGTTTGCAACCTTGCCCTCTCTCGCCCCCTTCCG 1600
N L C T E I I E Y S A P D E V A V C N L A S L A L S A
CCTTTATCGACTACGAGAAATGCCCTCTTACGACTTCAAGAAGCTGCACGAGGTCAAGTCGTCTCGCAACCTCAAC 1680
F I D Y E N A S Y D F K K L H E V T Q V V V R N L N

AAGATCATTGACATCAACCACTACCCCGTTAAGGAGGCCCAACAAGCAACATGCGTCACCGTCCCATTTGGTGTGCGGTGT
 K I I D I N H Y P V K E A H N S N M R H R P I G V G V 1760

TCAGGGTCTTGCTGATGCTTCTTGGCTCTCCGCATGCCCTTCGATTCTGACGGGCTAGCAAGCTCAACATCCAGATTT
 Q G L A D A F L A L R M P F D S D A A S K L N I Q I F 1840

TCGAGACCATCTACCACGCCCGCTCTTACTGCCCTCTTGCCAGCTCGCCAAGGAGCAGGGCCCGTACGCCACCTACGAAAGGC
 E T I Y H A A L T A S C Q L A K E Q G P Y A T Y E G 1920

TCACCCGCTCTCCCAAGGAATCCTCCAATACGACATGTGGAACGTACCCCTACCAACCTCTGGGACTGGACTGCTCTCAA
 S P V S Q G I L Q Y D M W N V T P T N L W D W T A L K 2000

GCGGGACATCAAGAAAGTACGGTGTTCGCAACAGTCTGCTGCTTGTCTCCCTATGCCCACTGCTTCCACTTCTCAGATTCCTTG
 A D I K K Y G V R N S L L L A P M P T A S T S Q I L G 2080

GCAACAACGAGTGCTTCGAGCCTTACACCTCTAACATCTAACACGACGTGTGCTCGCCGGCGAGTTCCAGGTCGTCAAC
 N N E C F E P Y T S N I Y Q R R V L A G E F Q V V N 2160

CCTTGGCTTCTCAGGGATCTTGTGGAGATGGGGCCTTTGGTCTTGATGCCCATGAAGAACCGCATCATTTGCTGAGGGCGGTTC
 P W L L R D L V E M G L W S D A M K N R I I A E G G S 2240

TATCCAGAACAATCCCAATGACATCAAGGCTCTCTATAAGACCGTCTGGGAGATTTCTCAGCGTACCATTG
 I Q N I Q S I P N D I K A L Y K T V W E I S Q R T I V 2320

TCAAGATGGCTGCCGATCGTGGTGCCTTCATTGATCAGTCCCAGTCCCCTCAACATTCACATGCGCGAACCACCATGGGC
 K M A A D R G A F I D Q S Q S L N I H M R E P T M G 2400

AAGATTACCAGCATGCAATTCGCCCGGCTGGAAGATGGGTCTCAAGACCGGCATGTACTCCGTACGCAGGCTGCTGC
 K I T S M H F A G W K M G L K T G M Y Y L R T Q A A A 2480

TCAGCCCATCCAGTTCACTGTAGACCAGGAGGCTCTCAGGGCCACGGATGACGAGTgccccagccccactcgggtcttaag
 Q P I Q F T V D Q E A L R A T D D E 2560

aagcgctcccctcccgctggcacttatacgtctatcgtgttgagggagaaacacttcgggccccccgcccgtatgcccagac 2640
tgggtgttagtggcactagcacaccccatcggcacccagggatgttcccacacccgccagcacacctcctcccactgagggttc 2720
ctgaaactctgtacagagtgaacaacaggcccccggtccacttgtctcgcgcgccaagagtgccggtttcaaggccgattgc 2800
gcccagaccctgagagcccccaagctctggccactgaccccgattgttaagACCGAGGACATTTGGCTCACCGCTGCTGGAGAG 2880
T E D I G S P L L E R
AAAGGAGGGCCAGAAATGAGGACGTTGGACGGAAGATAGCCAGGAGAGGGACGAGAAATATCTACTTAATGCTCCACTTTCTG 2960
K E G Q N E D V D E D S Q E R D E N I Y S N A P L S E
AGCAACAAGTTTGCTTGCTTgttaagtgacaagacttagccccctaccagacaccttgagacacacactaaccacatcaaaataaca 3040
Q Q V A A C
GGCGCCTGGAATCCCGGTGCCGACCCCTCCTCCTGCGAGATGTGCAGCGGTTAAGCCCCGCAC 3102
A W N P G A D P S S C E M C S G *

Table 2-8: Putative introns found in *rir-1*. Consensus sequences were determined by Edelman and Staben (1994) or Bruschez *et al.* (1993b).

Feature	Consensus	Intron #, length (bp), feature sequence and position (kbp)			
		1 (68)	2 (71)	3 (311)	4 (62)
5' donor	G [^] GTAAGTnnYCnYY	G [^] GTATGTTTCACGAC 120 to 133	G [^] GTAAGCACCTTCTC 347 to 361	A [^] GTGCCCCCAGCCCCA 2534 to 2547	T [^] GTAAGTGACAAGA 2976 to 2989
branch	WRC [^] TRACMnnnnnnYY	TGCTAACTCTCTCCTC 170 to 185	AGCTAACACATAATCT 397 to 412	GCCTGAGAGCCCCAAG 2826 to 2841	CACTAACACATCAAAAT 3016 to 3031
3'	WACAG [^]	CATAG [^] 185 to 189	CCTAG [^] 416 to 420	TTAAG [^] 2843 to 1847	AACAG [^] 3038 to 3042
acceptor					

Comparing the *un-24* sequence to wild-type reveals three point mutations characteristic of RIP (GC→AT transitions) at positions 247, 613 and 618 (Figure 2-10). The first mutation, at position 247, is a C→T transition which results in a cysteine residue present in wild-type to be exchanged for a tyrosine residue in *un-24* (see Figure 2-8 and Figure 2-11). Amino acid sequence comparison with other ribonucleotide reductases shown in Figure 2-9 reveals that the amino acid residue at the affected position (26) is either a serine or a cysteine residue. The mutation at position 613 is the result of a G→A transition that causes a wild-type valine at position 124 to be exchanged for an isoleucine. In the other ribonucleotide reductases analyzed, the amino acid at this position is either a valine or an isoleucine. The third mutation, at position 618, is also caused by a G→A transition, and results in the exchange of a wild-type methionine at position 125 for an *un-24* isoleucine. This position in other organisms is occupied by methionine, isoleucine, leucine or glutamate.

Figure 2-9: Sequence alignment of the ribonucleotide reductase from *N. crassa* (this study), human (AC P23921), mouse (AC P07742), *S. cerevisiae* (AC P21524), *S. pombe* (AC P36602), *C. elegans* (AC Q03604) and the *Vaccinia* virus (AC P20503).

* = amino acids are identical; . = changes in amino acids are conservative.

Accession numbers in parenthesis are from Swiss-Prot accessed through PROSITE (Bairoch *et al.*, 1995).

<i>N. crassa</i>	M-----YVKRDRGRQERVQFDKITARVSRLCYGLDMNHVDPVAITQKVI
human	M-----HVIKRDGRQERVMPFDKITSRIOKLCYGLNMFVDPQAQITMKVI
mouse	M-----HVIKRDGRQERVMPFDKITSRIOKLCYGLNMFVDPQAQITMKVI
<i>S. cerevisiae</i>	M-----YVIKRDGRKEPVQFDKITSRITRLSYGLDPNRIIDAVKVTQRII
<i>C. elegans</i>	MQRYNSTYVVKRDRGRKEDVHFQDKITSRIOKLCYGLNMFVDPVAVAIKVI
<i>Vaccinia</i>	M-----FVIKRNQYKENVMFQDKITSRIRKLCYGLNTDHDIPIKIAMKVI
<i>S. pombe</i>	M-----FVYKRDGRQEKVAFQDKITARVSRLCYGLDSDHVDPVEITQKVI
	* * * * * * * * * * *
<i>N. crassa</i>	SGVYGGVTTAQLDDLAAETAAYMTVTHPDYAILAARIAVSNLHKQTKKQW
human	QGLYSGVTTVELDTLAAETAATLTTKHPDYAILAARIAVSNLHKETKKVF
mouse	QGLYSGVTTVELDTLAAETAATLTTKHPDYAILAARIAVSNLHKETKKVF
<i>S. cerevisiae</i>	SGVYSGVTTVELDNLAAETCAYMTTVHPDYATLAARIAISNLHKQTTKQF
<i>C. elegans</i>	SGLYKGVTTVELDNLAAETAASMTTQHPEYALLAARIAVSNLHKKTNKF
<i>Vaccinia</i>	QGIYNGVTTVELDTLAAEIAATCTTQHPDYAILAARIAVSNLHKETKKLF
<i>S. pombe</i>	SGVYPGVTTIELDNLAAETAATMTTKHPDYAILAARIAVSNLHKQTEKVF
	* * * * * * * * * * *
<i>N. crassa</i>	SLVISELYNVNPRTGKHSPMIAKDVYECVMRHKDEFDSAIVYDRDFNYQ
human	SDVMEDLYNYINPHNGKHSPMIAKSTLDIVLANKDRLNSAIYDRDFSYN
mouse	SDVMEDLYNYINPHNGRHSPMVASSTLDIVMANKDRLNSAIYDRDFSYN
<i>S. cerevisiae</i>	SKVIEDLHDWINPATGKHAPMISDEIYNIVMENKDTLNSAIYDRDFQYT
<i>C. elegans</i>	SEVMKTLHEFHHPHTGKHAPMISDETWAIIEKNADKLSAIYDRDYSYT
<i>Vaccinia</i>	SEVMEDLFNYVNPKNKGKHSPIISSITMDIVNKYKDKLSVITYERDFSYN
<i>S. pombe</i>	STVVQQLHDYVNPKTDKPAPMISDKIYDIVMKHKDELDSAIYDRDFTYN
	* * * * * * * * * * *
<i>N. crassa</i>	YFGFKTLERSYLLKLDGQIAERPQHMIMRVAVGIWGDDVERVIETYNLMS
human	YFGFKTLERSYLLKINGKVAERPQHMLMRVSVGIHKEDIDAAIETYNLLS
mouse	YFGFKTLERSYLLKINGKVAERPQHMLMRVSVGIHKEDIDAAIETYNLLS
<i>S. cerevisiae</i>	YFGFKTLERSYLLRLNGEVAERPQHLVMRVALGIHGSIDIESVLKTYNLMS
<i>C. elegans</i>	YFGFKTLERSYLLKINKEIVERPQQMLMRVSGIHGDDITSAIETYNLMS
<i>Vaccinia</i>	YFGFKTLEKSYLLKINNKIVERPQHMLMRVAVGIHQWDIDSAIETYNLLS
<i>S. pombe</i>	FFGFKTLERSYLLRIDGKVAERPQHMIMRVAVGIHGEDI EAAIETYNLMS
	. * * * * * * * * * * *

<i>N. crassa</i>	KYGVNSLLAPMPTASTSQILGNNECFEPTYTSNIYQRRVLAGEFQVWNP
human	KYGIRNSLLIAPMPTASTAQILGNNESIEPTYTSNIYTRRVLSGEFQIVNP
mouse	KYGIRNSLLIAPMPTASTAQILGNNESIEPTYTSNIYTRRVLSGEFQIVNP
<i>S. cerevisiae</i>	KHGLRNSLTMAPMPTASTSQILGYNECFEPVTSNMYSRRLSGEFQVWNP
<i>C. elegans</i>	KHGIRNSLLMAPMPTASTAQILGNNESIEPTYTSNIYSRRVLSGDFQIVNP
<i>Vaccinia</i>	TYGLRNSLLVAPMPTASTAQILGNNESEVEPTYTSNIYTRRVLSGEFQVWNP
<i>S. pombe</i>	KHGIRNSLLVAPMPTASTSQILGFNECFEPTYTSNMYQRRVLSGEFQIVNP
	*.****.*****.****.*** ** ****. * ****. * **.
<i>N. crassa</i>	WLLRDLVEMGLWSDAMKNRIIAEGGSIQNIQSI PN DIKALYKTVWEISQR
human	HLLKDLTERGLWHEEMKNQIIACNGSIQSIPEI PDDLKQLYKTVWEISQK
mouse	HLLKDLTERGLWNKEMKNQIIACNGSIQSIPEI PDDLKQLYKTVWEISQK
<i>S. cerevisiae</i>	YLLRDLVDLGIWDDSMKQYLITQNGSIQGLPNVPQELKELYKTVWEISQK
<i>C. elegans</i>	HMLKDLVERGLWTDENKNRLIANNGSIQNIQSI PN DIKELYRTVWEISQK
<i>Vaccinia</i>	HLLRVLTERKLWNDEIKNRIMADGGSIQNT-NLPEDIKRVYKTVWEIPQK
<i>S. pombe</i>	WLLKDLVERDLWNEDMKNKLVMLDGSIQAIPEI PDDLKDLKTVWEISQK
	*. * . . * * * * *
<i>N. crassa</i>	TIVKMAADRGA FIDQSQSLNIHMRPTMGKITSMHFAGWKMGKLTGMYYL
human	TVLKMAAERGAFIDQSQSLNIHIAEPNYGKLTSMHFGWKQGLKLTGMYYL
mouse	TVLKMAAERGAFIDQSQSLNIHIAEPNYGKLTSMHFGWKQGLKLTGMYYL
<i>S. cerevisiae</i>	TIINMAADRAIYIDQSHSLNLFQAPSMGKITSMHFGWKKKGLKLTGMYYL
<i>C. elegans</i>	DIIEMAADRGA FIDQSQSLNIHMAKPSYAGITSMHFGWKKKGLKLTGMYYL
<i>Vaccinia</i>	TIIEKMAADRGA FIDQSQSMNIHIAADPSYKLTSMHFGWSLGLKLTGMYYL
<i>S. pombe</i>	TVIDYAADRGP FIDQSQSLNIHLKDPYSGKITSMHFGWKKKGLKLTGMYYL
	.. **.*. . *****.*.*. . * . . ***** ** *****
<i>N. crassa</i>	RTQAAAQPIQFTVDQE-----
human	RTRPAANPIQFTLNKE-----
mouse	RTRPAANPIQFTLNKE-----
<i>S. cerevisiae</i>	RTQAASAAIQFTIDQEVADQAATHIASVSELD RPVVPKGTKFSEQKAAS
<i>C. elegans</i>	RTKPAVNAVQFTVDKN-----
<i>Vaccinia</i>	RTKPA SAPIQFTL DKD-----
<i>S. pombe</i>	RTMAASAAIKFTVDPV-----
	** . * . . . *

<i>N. crassa</i>	ALRATDDEDEDIGSPLLERKEGQN-----EDVDEDSQE
human	KLKDKKEKVSKE-----EEEKE
mouse	KLKDKKEKALKE-----EEEKE
<i>S. cerevisiae</i>	ALTESSDNEKDASPVPSEQSSVSSAMSNVKLEDSVAPVPTETIKEDSDE
<i>C. elegans</i>	ALKTNQQA-----
<i>Vaccinia</i>	KIK-----
<i>S. pombe</i>	ALRARNEESNEENKKPVIKNG--K-----AEISAEPTK
	.
<i>N. crassa</i>	RDENIYSNAPLSEQQVAACAWNPGADPSSCEMCSG
human	RNTA-----AMVCSLE---NRDECLMCGS
mouse	RNTA-----AMVCSLE---NRECLMCGS
<i>S. cerevisiae</i>	KKCDIYNEK-----VIACT---APTPEACESCSG
<i>C. elegans</i>	-ETP-----ATVAESQ---D-EGCLMCSG
<i>Vaccinia</i>	---P-----LVVC-----DSEICTSCSG
<i>S. pombe</i>	EEIDIYNEKVL-----ACSIK---NPEACEMCSA
	* * *

Figure 2-10: DNA sequencing gels showing differences between wild-type and *un-24 rir-1*.

(A) mutation 1 - wild-type; (B) mutation 1 - *un-24*; (C) mutations 2 and 3 - wild-type; (D) mutations 2 and 3 - *un-24*.

Figure 2-11: Mutation map of the *un-24* hypothetical protein.

Position of mutations and changes in amino acid sequence are indicated in boxes.

(C) denotes conserved cysteine residues.

* represents the end of the protein.

Discussion

Mapping of un-24

The objectives of this study were to localize *un-24* by RFLP analysis and complementation assays, to clone and sequence wild-type and mutant *un-24* alleles to determine whether *un-24* is caused by a RIP or an escape event, and to determine if *un-24* has heterokaryon incompatibility activity. In Chapter 1, we report the results of a mutation hunt in the *T(AR18)* translocated region. A temperature-sensitive mutant, *un-24*, was found that grew at close to wild-type rates at room temperature (25°C), but ceased to grow at temperatures greater than 35°C. Possible causes of this mutation are RIP and deletion by escape of an essential gene in *Dp(AR18)* partial diploids (RIP and escape are reviewed in Chapter 1). RIP has previously been examined only in small segments of DNA which are integrated after transformation. A study was initiated in David Perkins' laboratory, Stanford University, to determine if duplications of larger chromosomal segments, such as those generated during the cross *T(AR18)* X Normal, are also subjected to RIPping. Escape would presumably be evident as deletions associated with *un-24* (Smith *et al.*, 1996). To determine if *un-24* was caused by RIP (GC to AT transitions) or escape (large-scale deletions of duplicated regions in *Dp* strains), the wild-type and *un-24* alleles were mapped, cloned, sequenced, and examined for deletions or transitions.

RFLP analysis of strains with crossovers around *un-24* mapped *un-24* to two

different locations on the left arm of LGII. While *un-24* maps to the right of cosmid G12:E:3 in most cases, RFLP patterns in 2 strains, C8c-88 and C8c-199, indicate *un-24* is to the left of G8:F:7 (see Table 2-3). The equivocal map position of *un-24* may indicate that crossover events in this region are accompanied by branch migration, heteroduplex formation and non-contiguous, converted tracts within the heteroduplex regions. Offspring recovered following this type of event could have discontinuous tracts of intermixed PA and OR DNA. Gene conversion may only occur in small DNA sections (reviewed by Alberts *et al.*, 1989) and in these strains the entire *un-24* locus may not have been converted.

The position of *un-24* was resolved by complementation assays using cosmids in the region as transforming DNA. The only cosmids that complemented the temperature-sensitive phenotype were G12:E:3 and G8:G:1 (over 600 colonies regenerated/1 µg G8:G:1 DNA compared to only 30 colonies for cosmid G8:F:7), suggesting that *un-24* was within the 11 kbp of DNA common to these two cosmids. Complementation studies were originally performed using the Hygromycin resistance gene, *hyg^R*, present within the cosmids, as a selectable marker. It was subsequently found that immediately transferring the transformed colonies to 37°C on medium without Hygromycin B was equally efficient for complementation screening. This indicated that temperature alone could be used as a selectable marker with *un-24* strains, thus effectively increasing the flexibility of restriction mapping and of vectors that could be used for subcloning since disrupting *hyg^R* was no longer an issue.

It was found that cutting the p31ECO16 subclone of G8:G:1 with *Mlu*I did not abolish *un-24* complementation and this was verified with the subclone, pECO16M. Based on partial DNA sequence of the wild-type region, primers 6JP2, 6JP3 and 6JP6 were designed. Transformation experiments revealed that 6JP3/6JP2 and 6JP3/6JP6 fragments did, indeed, complement *un-24*, although the number of regenerating transformants with P3/P6 complementation was low. It was hypothesized that these primers immediately flanked the *un-24* coding region. As recombination of transforming DNA into the *N. crassa* genome occurs at non-homologous sites, the coding region of small PCR fragments may be disrupted upon insertion. Transforming spheroplasts with DNA much larger than the locus in question would increase the probability of recombinational insertion of the fragment in an area outside the coding region. This was tested by cloning the P3/P6 fragment into pCRII (3.9 kbp in size), thus effectively increasing the area in which recombination could occur outside of the *un-24*⁺ coding region. The cloned fragment, p3/6OR, increased transformation efficiency 9-fold over the PCR fragment alone (272 transformants with clone versus 32 with PCR fragment). Cloning PCR fragments internal to 6JP3/6JP6 into pCRII indicated that the minimum region able to complement *un-24* was encoded by the 3.75 kbp fragment bound by the primers 6JP3 and 6JP6. Based on this functional analysis, the mutant *un-24* allele, bound by the primers 6JP3 and 6JP6, was cloned from genomic C8c-164 DNA into pCRII as p3/6UN.

un-24 is caused by RIP mutations in *rir-1*.

A comparison of wild-type and *un-24* DNA sequence in the 6JP3/6JP6 region revealed three characteristic RIP mutations (GC to AT) in the large subunit of ribonucleotide reductase, *rir-1*. The first mutation in *un-24* causes a change from a cysteine residue (amino acid position 26) in the wild-type to a tyrosine residue in the *un-24* mutant. This cysteine is conserved in an evolutionarily diverse group of organisms, and may therefore be important to the function of ribonucleotide reductase. Tyrosine has a large side group compared to that of cysteine which may impede the proper folding of the polypeptide at elevated temperatures. The second mutation (G to A), replaces a valine at position 124 in wild-type with an isoleucine in *un-24*. Other organisms examined for alignment possess either a valine or an isoleucine at this position, suggesting that this change would not affect the function of the predicted *un-24* ribonucleotide reductase. The third mutation results in a change from a methionine at position 125 in wild-type to an isoleucine in *un-24*. Either of these residues are present in this position in other organisms.

Many studies have been performed on temperature-sensitive ribonucleotide reductase mutants in bacteria and yeasts. Temperature-sensitive forms of ribonucleotide reductase in *S.cerevisiae* and *S. pombe*, have C-terminal mutations (Elledge and Davis, 1990; Fernandez Sarabia *et al.*, 1993). These mutants and the mutations at *un-24* may be useful for the molecular dissection of domains in ribonucleotide reductase. *un-24* complementation assays using cloned sequences that are altered at amino acid positions 26, 124 or 125, and wild-type throughout the

rest of the protein, would identify an essential region of wild-type ribonucleotide reductase. Similar experiments using the temperature-sensitive mutants in *S. cerevisiae* and *S. pombe* would also be informative.

The *N. crassa* ribonucleotide reductase possesses conserved cysteine residues at positions 218, 429, 444, 819 and 822 that appear to be involved in the active site of the enzyme (Stubbe, 1990; Fernandez Sarabia *et al.*, 1993). Site-specific mutagenesis of these conserved residues alters the oxidation/reduction chemistry and substrate binding of the enzyme (Stubbe, 1990). Fernandez Sarabia *et al.* (1993) report the presence of "Mlul elements" upstream of the coding region in *S. pombe* that are involved in the regulation of ribonucleotide reductase. Such Mlul elements were also found upstream from our ribonucleotide reductase sequence, and are present in the upstream region of other genes that are expressed at high levels during DNA synthesis (reviewed by Andrews and Herskowitz, 1990).

Neurospora's OR ribonucleotide reductase gene has 4 introns, located at positions 120, 347, 2534, and 2976. The introns we have identified have only been confirmed by the presence of *N. crassa* intron consensus sequences. mRNA transcript analysis will have to be performed in order to determine whether these introns are truly present within the DNA encoding for *nir-1*. However, the first putative intron found in *nir-1* is at an identical amino acid position to one found in *S. pombe* (Fernandez Sarabia *et al.*, 1993). While *S. pombe* does not possess our second putative intron, if this intron was not removed, the open-reading frame

would terminate at an in-frame stop site located in this region. Without the removal of introns 1 and 2, sequence identity in the highly conserved N-terminus of the ribonucleotide reductase would be lost. Without the removal of the third and fourth introns, the protein would terminate at stop sites located within these introns, resulting in the formation of a truncated product, and the loss of c-terminal cysteine residues involved in the interaction of ribonucleotide reductase with essential co-factors (Stubbe, 1990).

*Nuclear incompatibility of the *nir-1* region*

It is interesting that an evolutionarily conserved ribonucleotide reductase has some feature that causes an intra-specific incompatibility reaction between OR and PA *N. crassa* strains. In our experiments, nuclear incompatibility is recognized by a significantly reduced number of transformants regenerating after the addition of OR or *un-24 nir-1* into the PA genome (summarized in Figure 2-12). Because there exists an amino acid identity of 41-45% among the ribonucleotide reductase large subunits of wild-type *N. crassa*, *Vaccinia virus*, *C. elegans*, *S. cerevisiae*, *S. pombe*, mouse and human, it is hard to imagine a large-scale divergence at this locus between OR and PA strains of *N. crassa*.

DNA hybridization of PA, OR and *un-24* 6JP3/6JP6 PCR products showed that sequence homology exists between strains at *nir-1* (see Figure 2-6; results not shown for *un-24*). However, the experimentally-induced nuclear incompatibility reactions as caused by *nir-1* were not reciprocal; transforming the OR or *un-24*

allele into PA spheroplasts caused nuclear incompatibility, while transforming the PA allele into OR and *un-24* spheroplasts did not appear to give an incompatibility reaction. Transforming the *un-24* allele into PA strains at 30 and 37°C also caused an incompatibility reaction, indicating that the *un-24* mutation did not alter the function in this region. It was not tested whether transforming the PA allele into an *un-24* background at permissive temperature caused an incompatibility reaction, however, at 37°C the PA allele did not complement the temperature sensitivity of C8c-164 (*un-24*). The lack of transformants could be caused by an incompatibility reaction between the PA and *un-24* alleles, or the protein product of the PA allele simply may not complement *un-24*. It is also possible that the cloned PA allele is inactive, in which case it would not cause an incompatibility reaction in OR or *un-24* strains, nor would it be able to complement the *un-24* mutation. This, however, seems unlikely since a region of DNA that was functional for incompatibility was PCR amplified from *un-24* genomic DNA and *un-24* complementation was achieved with a PCR product from the OR genome.

It is also noteworthy that incompatibility function was achieved with a region of DNA internal to the smallest region that complemented the temperature-sensitive *un-24* mutation. Preliminary data obtained in our laboratory by Nadereh Mir-Rashed shows that a difference does exist between the OR and PA alleles within the region defined by the PCR primers 6JP3 and 6JP1 (see Figure 2-4 for a map of this region). The occurrence of heterokaryon incompatibility when PA spheroplasts are transformed with the cloned OR PCR fragment flanked by the

6JP10 and 6JP9 primers suggests that the difference exists in the region defined by 6JP10 and 6JP1. The *nir-1* protein region that corresponds to the sequence amplified by 6JP10 and 6JP9 contains 3 conserved cysteine residues involved in the active site of the large subunit, with one residue found in the internal sequence flanked by 6JP10 and 6JP1. Sequencing the PA allele within the region defined by 6JP10 and 6JP1 may localize differences between the 2 genes which may affect the active site of the large subunit of ribonucleotide reductase and which may be responsible for the incompatibility function.

It is also possible that the 1.75 kbp fragment flanked by 6JP10 and 6JP9 corresponds to a site in ribonucleotide reductase that is involved in protein-protein interactions. Active ribonucleotide reductase is made up of 2 large subunit proteins and 2 small subunit proteins that interact to form an $\alpha_2\beta_2$ tetramer (reviewed by Elledge *et al.*, 1992). Dissimilarity between OR and PA *RIR-1* might affect α_2 interactions that, in turn, effect binding with β_2 domains. In the scenario outlined in Figure 2-12b, an interaction between slightly different OR and PA *RIR-1* subunits in a PA background would form a "poison complex" that blocks ribonucleotide reductase activity and kills cells. Bégueret *et al.* (1994) suggest such an interaction might occur between *het* genes in *P. anserina* as some genes involved in heterokaryon incompatibility in this fungus seem to have primary cellular functions and contain regions involved in protein/protein interactions. They submit that irregular protein complexes, or "poison complexes", that are lethal to

Figure 2-12: (A) Summary of the ability of p3/6OR, p3/6PA, and p3/6UN to transform various recipient spheroplast strains at 22°C and 37°C, and (B) a possible mechanism causing incompatibility between OR and PA strains.

The hypothesis presented in Figure 2-12b assumes that *nir* is an incompatibility gene. Alternatively, another transcript may be present in the reverse direction.

RIR-1 = large subunit
RIR-2 = small subunit

A:

Transforming P3/P6DNA	Recipient Spheroplast Strain	Result
OR	PA - 22°C	X
PA	OR - 22°C	√
<i>un-24</i>	PA - 22°C	X
<i>un-24</i>	PA - 37°C	X
PA	<i>un-24</i> - 37°C	X

X = combination causes a nuclear incompatibility reaction;
 √ = combination is compatible.

B:

	ACTIVE
RIR-1 ^{OR} = ○		ACTIVE
RIR-1 ^{PA} = D		ACTIVE
RIR-2 ^{OR} = ■		ACTIVE
RIR-2 ^{PA} = □		INACTIVE Poison complex?
	ACTIVE

the cell's metabolism are produced upon co-expression of genes involved in heterokaryon incompatibility (Bégueret *et al.*, 1994).

One difficulty with suggesting that ribonucleotide reductase has incompatibility function within p10/9OR is that this region does not contain an active promotor, and therefore would not conceivably be transcribed. As transformation in *N. crassa* generally occurs by non-homologous recombination, p10/9OR would not likely recombine into the homologous region of *rir-1*. It is possible, though unlikely, that *rir-1* is a unique region within the *N. crassa* genome that tends towards site-specific recombination. It is also possible that the incompatibility function is caused by a second gene encoded in an opposite direction to the large subunit of ribonucleotide reductase on the complementary strand. The complementary strand to the ribonucleotide reductase sequence also contains a large open-reading frame that is within the 6JP10/6JP9 region. Sequence analysis within this region reveals an open-reading frame encoding a 564 amino acid protein with a *N. crassa* consensus start sequence (as defined by Bruchez *et al.*, 1993a) and 2 putative introns (data not shown). An amino acid identity of 21% exists between a 290 amino acid portion of this sequence and the heterokaryon incompatibility S and s alleles of *Podospora anserina*. However, DNA clones of these alleles do not complement *un-24* (data not shown).

The discovery of *un-24* may shed an interesting light on a number of different features of the *N. crassa* genome. While conservation of the large subunit of ribonucleotide reductase does exist in *N. crassa*, it is remarkable that an

intra-specific variation is present between the OR and PA strains. If nuclear incompatibility is due to intraspecific differences in *nir-1*, the notion proposed by Bégueret *et al.* (1994) that incompatibility may be caused by aberrant interactions between protein domains produced by the co-expression of variable alleles is validated. However, it is also possible that incompatibility is caused by a second gene encoded in the reverse direction to *nir-1*. The possibility that this second open-reading frame, transcribed from the complementary strand of *nir-1*, causes incompatibility requires further study. The three mutations at *un-24*, and the incompatibility function of *nir-1*, may provide a molecular means to dissect the active domains of ribonucleotide reductase. Additionally, *un-24* may be used to clarify aspects of nuclear incompatibility, and serve as a closely linked marker to *het-6*.

Chapter 3:

Other Experiments

N.B. The material presented in this chapter was originally prepared as part of Chapter 1. The results presented herein concerning nuclear staining with DAPI and/or EtBr were consistent for the first 5 experiments, but could not be repeated in 5 subsequent experiments. The reason for the change in DAPI/EtBr nuclear staining has not as yet been determined.

Introduction

un-24 is a temperature-sensitive mutant in *Neurospora crassa* that was caused by a RIP event (GC-AT transition) in duplicated offspring derived from a cross between the translocation strain, *T(AR18)* and a wild-type strain (Chapter 1). It was shown (Chapter 2) that *un-24* is the result of 3 RIP-like mutations in the large subunit of ribonucleotide reductase, an enzyme responsible for the conversion of ribonucleotides to deoxyribonucleotides during DNA synthesis. *un-24* is characterized by near wild-type growth rates at temperatures below 30°C, with negligible growth at 35°C and above. Nuclear distribution and cell morphology of C8c-164 (*un-24*) appear to be the same as the wild-type strain, C9-2 (Chapter 1).

During initial experiments, we found that exposing *un-24* germlings to restrictive temperatures caused an increase in the number of cells with nuclei stained by DAPI. Wild-type cells took at least 30 minutes before stained nuclei were evident. In this chapter, I describe results obtained when wild-type (C9-2) and mutant (C8c-164) germlings were stained with the nucleic acid stains, DAPI and EtBr, after exposure to restrictive temperatures for 0 to 5 minutes. In the first 5 of 10 experiments, it was found that nuclei in heat-shocked *un-24* germlings had a tendency to stain more readily with DAPI and EtBr than did heat-shocked wild-type cells. These nuclear staining characteristics suggest an interaction between ribonucleotide reductase and the uptake of DAPI and EtBr through cell membranes.

Methods and Materials

Strains and culture conditions.

N. crassa strains used in this study are listed in Table 3-1. Except during experimental procedures where indicated, cultures were grown and maintained on 1X Vogel's salts (Vogel, 1964), 1.5% sucrose, 1.5% agar, and 1X growth supplements where necessary (Davis and DeSerres, 1970),

Microscopy

Fluorescence microscopy was performed using a Reichart Zetopan fluorescence microscope equipped with a 2UGI UV exciter filter, a GG9 barrier filter and an HBO 200 W/4-L1 mercury lamp. Photography was performed using a Nikon UFX 35 mm camera using Tri-Xpan 400 Kodak film. Brightfield microscopy was with a Wilde Heerbrugg microscope.

Nuclear staining of heat shocked cultures.

C9-2 *thr-2 a* and C8c-164 *un-24; trp-1; inl a* were germinated and allowed to grow to the 2-10 cell stage in 1 ml of medium at 30°C. Cultures were transferred to microcentrifuge tubes and germlings were concentrated by centrifugation and the removal of 950 µl of the supernatant. Germling suspensions were maintained at 30°C using an adjustable temperature heating block (Lab-line Instruments, Melrose Park, IL). Microscope slides were placed on a second

Table 3-1: *N. crassa* strains used in this study.

Strain	Source
C8c-164 <i>un-24; trp-1; inl a</i> (OR ¹)	RLM58-18 <i>het-6^{PA} het-C^{PA} pyr-4; inl a</i> X C5-44 <i>ro-7 un-24; trp-1A</i> .
C9-2 <i>het-6^{OR} het-C^{PA} thr-2 a</i>	57-30 <i>cyh-1; het-6^{OR} het-C^{OR} pyr-4 A</i> X C2(2)-5 <i>het-6^{PA} het-C^{PA} thr-2 a</i>

¹OR = heterokaryon compatible with Oak Ridge tester strains.

heating block and adjusted to 35°C by monitoring the surface temperature with a copper/constantan Barnant 115 thermocouple thermometer (Barnant, Barrington, IL). 5 µl of C8c-164 or C9-2 germling suspension was transferred to microscope slides pre-warmed to 35°C. 5 µl of 1 µg/ml DAPI (Aldrich Chemical Company) and 5 µl of 0.1 µg/ml Calcafluor (gift of Dr. Byron Johnson) were placed onto the cell suspension. After 0, 0.5, 1.5, 3 or 5 minutes, the slide was removed, covered with a glass coverslip, and examined by fluorescence microscopy. About twenty germlings were examined for the presence or absence of nuclear stain. Similar experiments were done where DAPI stain was replaced with 1 µl of 84.5 µM EtBr.

To further characterize the nuclear staining of strains carrying *un-24*, 5 µl of a germling suspension was placed onto glass microscope slides pre-warmed to 37°C. After 5 minutes, the slides were incubated at 28°C for 5 minutes, followed by the addition of 5 µl of DAPI and 5 µl Calcafluor. Coverslips were placed on the samples, and germlings were examined by fluorescence microscopy.

Results

Nuclear staining as a function of time at 35°C.

Germlings kept at 28°C were transferred to elevated (35°C) temperatures for 0, 0.5, 1.5, 3.0 and 5.0 minutes prior to application of DAPI and calcafluor (Table 3-2). Figure 3-1 shows that the number of C8c-164 germlings with DAPI-stained nuclei increases with increasing time of exposure to 35°C until, at 4 to 5 minutes, approximately 70% of the germlings have stained nuclei. C9-2 germlings did not show an increase in DAPI nuclear staining with increased exposure to 35°C temperatures. Results were consistent during the first 5 of 10 experiments. Heat-shocked C8c-164 germlings did not exhibit nuclear staining with DAPI in subsequent experiments.

To determine whether the effect of heat-shocking C8c-164 cells was reversible, cells of both strains were exposed to 37°C for 5 minutes, and then transferred to 28°C for a further 5 minutes prior to the addition of DAPI. Nuclear staining still occurred in about 76% of the C8c-164 germlings as compared to approximately 5% of the C9-2 germlings. This indicated that the effect of increased temperature on C8c-164 was not reversible over the short period of time in these experiments.

Nuclear staining of heat-shocked germlings by Ethidium Bromide.

Upon the addition of EtBr, germlings appeared reddish in colour and

exhibited a low amount of background fluorescence. The fluorescence of nuclei stained with EtBr, however, was exceptional (Figure 3-2, plates A and B). The fluorescent signal produced by chelated ethidium bromide was much stronger than the background staining in the unstained germlings and in the DAPI-stained nuclei. Similar to DAPI experiments, EtBr nuclear stain was evident in 76% of heat-shocked C8c-164 germlings, significantly higher than non-shocked cells and than heat-shocked and non-shocked C9-2 germlings (Table 3-3). Again, the increase in the number of nuclei stained with EtBr was only observed during the first series of experiments.

Figure 3-1: Relationship between time at 37°C and the number of *un-24* and wild-type germlings stained with DAPI.

Figure 3-2: Nuclear staining of *un-24* germlings with Ethdium Bromide.

N = nucleus; S = septa; bar = 10 μm .

Table 3-2 Percentage of C9-2 (wild-type) and C8c-164 (*un-24*) germlings with nuclei stained by DAPI. Hyphae were placed at 35°C from 0-5 minutes prior to adding DAPI. Percentage is based on counts of at least 20 germlings for each treatment.

Time at 35°C (min)	% staining	
	C9-2	C8c-164
0.0	4.76	4.55
0.5	0.00	9.09
1.5	9.09	38.10
3.0	4.76	66.67
5.0	0.00	70.83

Table 3-3: Percentage of C9-2 and C8c-164 germlings with Ethidium Bromide-stained nuclei. Germlings have been placed at 35°C for 3 minutes (heat-shocked) or have been left at room temperature (non-shocked).

Time at 37°C	% staining	
	C9-2	C8c-164
0	9.52	5.26
3	7.14	76.00

Discussion

The objective of this study was to identify differences in the number of nuclei stained with the nucleic acid stains, DAPI (4'6-diamidino-2-phenylindole) and EtBr (Ethidium Bromide) between heat-shocked wild-type and *un-24* phenotypes. As shown in Chapter 1, nuclear distribution and cell morphology appear to be similar between C9-2 (wild-type) and C8c-164 (*un-24*) strains. During nuclear staining experiments with DAPI, it was observed that a large number of C8c-164 germlings had DAPI-stained nuclei (see Chapter 1). In the experiments presented herein, heat-shocked and non-shocked C9-2 and C8c-164 germlings were exposed to 35°C for different times to observe the effects of temperature on nuclear staining.

In the first 5 of 10 experiments, it was found that DAPI and EtBr were taken up more readily in heat-shocked, temperature-sensitive *un-24* cells than in wild-type cells. DAPI is a semi-permeant, blue fluorescent dye that binds to A-T rich DNA sequences (Lin *et al.*, 1977). Because of the relationship of DAPI to the anti-trypanoside, berenil, there is much interest in how DAPI and related compounds are taken into the cell (Kapuscinski, 1995). As EtBr is actively taken up into live fungal cells through monovalent cation channels in the presence of substrate (Peña and Ramirez, 1975), a mutant that takes it up in the absence of substrate might prove to be useful in the study of cation transport. *un-24* may offer a tool to study the uptake of DAPI, EtBr, and related compounds if the observations recorded in the first 5 experiments are accurate.

We suggest at least 4 hypotheses with respect to *un-24* and the temperature-related uptake of DAPI and EtBr. Increased incidence of nuclear staining at higher temperatures in the *un-24* compared to wild-type germlings may be caused by: 1) a disruption in the cell wall or cell membrane; 2) damage to cell membrane channels; 3) a breakdown in membrane pumps; or 4) a conformational change in DNA. Background staining of C9-2 germlings and non-shocked C8c-164 germlings was attributed to cell damage during handling of the cells.

Haugland (1996) and Kulik and Derif (1995) report that, without fixation, DAPI only stains the nuclei of living cells. In order for DAPI to penetrate the cell wall of higher plants and fungi, cells must be fixed (Kamo and Grieslach, 1993), suggesting that the cell wall acts as a barrier to the stain. Indeed, Kamo and Grieslach (1993) found that unfixed plant protoplasts had an increased sensitivity to DAPI staining. This supports the hypothesis that *un-24* has an effect on cell wall and/or membrane stability.

EtBr (1,7-diamino-10-ethyl-9-phenylphenanthridinium bromide) is a cationic dye that binds DNA with little or no sequence preference at a stoichiometry of one dye molecule per 4 to 5 bp of DNA (Haugland, 1996). Once bound to DNA, the UV fluorescence of EtBr increases 20 to 30 fold over unbound EtBr (Haugland, 1996). In viable cell systems in the absence of glucose, the cell membrane is relatively impermeant to low concentrations of EtBr, while concentrations greater than 5 to 20 μM (depending on the organism and strain) are actively pumped into yeast cells (*Kluyveromyces lactis* and *Saccharomyces cerevisiae*) through monovalent cation

channels using a K^+ -ATPase in a process requiring energy (Midoux *et al.*, 1995; Miranda *et al.*, 1995; Brunner *et al.*, 1982; Peña and Ramirez, 1975). Puchkov and Melkozernov (1994) found the same activity occurring in the gram-positive bacteria *Micrococcus luteus*, however, *Escherichia coli* K-12 cells will not stain unless their permeability barriers are damaged. Concentrations of dye greater than 500 μ m penetrated the cells by means other than monovalent cation channels and affected many cell functions besides K^+ transport (Peña and Ramirez, 1975).

EtBr penetration into heat-shocked *un-24* mutants implies that native resistance to the dye is reduced. Degradation of cell membranes of HL60 promyelotic cells and yeast cells, and of permeability barriers of *E. coli*, by chemical means causes an increase in EtBr uptake and fluorescence (Midoux *et al.*, 1995; Miranda *et al.*, 1995; Brunner *et al.*, 1982; Peña and Ramirez, 1975), indicating that *un-24* may have damaged cell membrane components (channels or otherwise) brought on by heat-shock.

Interestingly, Kappy and Metzenberg (1967) report that *un-3* mutants of *N. crassa* (strain 55701) are defective in K^+ transport, as well as in the uptake of amino acid analogues. These authors suggest the temperature-sensitive mutation produced at *un-3* might affect the basic structure of the cell membrane that, in itself, may not be lethal, but which could affect any number of unrelated permeases and other cell proteins. EtBr results suggest that *un-24* might also have abnormal K^+ transport properties.

Working in a cell free system, Akao *et al.* (1996) found that EtBr/DNA

fluorescence was affected by changes in DNA conformation in a DNA/bilayer-forming lipid complex. Fluorescence was quenched in the presence of the lipid, and was regained when the DNA/lipid complex was disrupted by higher temperatures or by chemical means (Akao *et al.*, 1996). Loss of fluorescence was thought to be caused by a tight interaction between the DNA and the lipid, such that the ability of EtBr to intercalate between bases was reduced (Akao *et al.*, 1996). A change in DNA conformation caused by increased temperature might increase the EtBr-induced fluorescence in *un-24*. This explanation, however, would only be valid if the nuclei were already in contact with EtBr. As reported by Peña and Ramirez (1975), glucose is required for active EtBr uptake in yeast. It is presently unknown if glucose has the same effect on EtBr uptake in wild-type *Neurospora*, however, concentrations of EtBr were sufficiently high (84.5 μM) to support the notion that the cells are resistant to passive EtBr uptake in the absence of substrate. Although DNA conformation in heat-shocked *un-24* might occur, the increased EtBr fluorescence is unlikely due to conformational change alone.

un-24 involves a mutation in a ribonuclease reductase responsible for the conversion of ribonucleotides to deoxyribonucleotides during DNA synthesis (for a review, see Elledge, *et al.*, 1992). Expression of ribonucleotide reductase fluctuates during the cell cycle with maximum levels occurring during S-phase (Lowden and Vitols, 1973). It is possible that the inability to repeat the observations achieved during the first 5 experiments was due to the cells not being at a stage in the cell cycle during which ribonucleotide reductase levels were sufficiently high for

a temperature effect to be detected. It is unknown how ribonucleotide reductase could affect the cell membrane structure.

The results presented herein suggest that *un-24* may cause a defect in cell membrane or cell wall structure that affects the ability of the nuclear stains, DAPI and EtBr, to penetrate into the nucleus. If a membrane defect does occur in response to temperature, *un-24* may prove useful in the study of numerous functions, including DAPI and EtBr uptake, cation transport, membrane structure, cell cycling and the interaction of ribonucleotide reductase with cell membrane structures. Further investigation is required to verify whether the results herein were an artifact or whether *un-24* does, indeed, affect membrane structure.

Summary

Summary

The temperature-sensitive conditional mutant, *un-24*, was derived from a cross of a wild-type strain with a partial diploid, *Dp(AR18)*, that possessed a translocated duplication of a 270 kbp region of LGII that spans the heterokaryon incompatibility gene *het-6*. The original *un-24* strain grew with a peach-like morphology and displayed a temperature-sensitive conditional phenotype expressed at temperatures of 35°C and greater, that was later found to be reversible. While the temperature-sensitive *un-24* mutation was mapped to the *T(AR18)* translocated region within 2.6–4.5 map units from *het-6*, the peach-like morphology was linked to the mating-type locus on LGI. Nuclear distribution and cell morphology appeared to be similar between wild-type and *un-24* strains, although heat-shocked *un-24* cells appear to take up the nucleic acid stains DAPI and Ethidium Bromide at a greater rate than non-shocked *un-24* germlings and wild-type cells.

The location of *un-24* was determined by RFLP analysis of crossover progeny using a set of overlapping cosmids that span the translocated region. *un-24* was localized to 11 kbp of overlapping DNA found within the OR cosmids G8:G:1 and G12:E:3. Complementation assays were used to further position *un-24* to within a 3.75 kbp fragment bound by the PCR primers, 6JP3 and 6JP6. While a 6JP3/6JP6 OR subclone (p3/6OR) successfully complemented the mutation at *un-24*, the same fragment from a PA background did not.

Cloning and sequencing wild-type and *un-24* alleles revealed that *un-24* possesses 3 characteristic RIP (G:C to A:T transition) mutations at positions 247, 614 and 618 of the large subunit of ribonucleotide reductase, *rir-1*. 41-45% amino acid identity exists among the large subunits of wild-type *N. crassa*, vaccinia virus, *Caenorhabditis elegans*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, mice and humans. 4 putative introns were found in *rir-1* and intron 1 is conserved in *S. pombe*. Cysteine residues involved in the active site of the enzyme are present within all organisms, including *N. crassa*. Additionally, the promotor region of *rir-1* has *Mlu* elements that are also found in *S. pombe*.

Both p3/6OR and p3/6UN subclones caused an incompatibility reaction in the PA background, but nuclear incompatibility was not observed when p3/6PA was transformed into OR. Nuclear incompatibility in transformed spheroplasts was recognized by reduced growth as compared to controls. OR-induced incompatibility is defined by a 1.75 kbp fragment that is internal to the area that complements *un-24*. It is unclear at this time whether incompatibility is caused by an internal portion of *rir-1* or by another open-reading frame encoded on the opposite DNA strand.

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