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***su(un-24)-1*, a suppressor of a temperature sensitive ribonucleotide reductase mutation in
Neurospora crassa: Characterization and PCR-based mapping**

A thesis presented to the Faculty of Graduate Studies of Carleton University

by

Moshi Kotierk

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

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Abstract

un-24 encodes a temperature sensitive form of the *Neurospora crassa* large subunit of ribonucleotide reductase, an enzyme essential for DNA precursor synthesis. We have identified a spontaneous mutation, *su(un-24)-1*, that suppresses *un-24* temperature-sensitivity over a broad range of temperatures. This suppressor may act by increasing intracellular osmotic pressure or by directly stabilizing the UN-24 protein. Characterization of *rnr-2*, the small subunit of ribonucleotide reductase, allowed us to conclude that the suppressor is not due to a mutation in *rnr-2*. The phenotypic ratios of progeny from a cross between *eth-1* and *su(un-24)-1* strains suggest that *su(un-24)-1* is not operating by an osmotic mechanism. To identify *su(un-24)-1* using a map-based approach, we utilized the complete genome sequence of *N. crassa* to develop polymorphic PCR-based markers located throughout the nuclear genome and on the mitochondrial DNA. None of the markers significantly cosegregated with *su(un-24)-1*. Several gaps were identified that may contain *su(un-24)-1*.

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List of Abbreviations

~	about
A	adenine
<i>aap-2</i>	<i>amino acid permease-2</i>
<i>acr-2</i>	<i>acriflavine resistant-2</i>
AFLP	amplified fragment length polymorphism
APLP	amplified product length polymorphism
<i>arg-10</i>	<i>arginine-10</i>
<i>arg-13</i>	<i>arginine-13</i>
<i>ars-1</i>	<i>aryl sulfatase-1</i>
BLAST	Basic local alignment search tool
bp	base pair
C	cytosine
<i>cax</i>	<i>calcium exchanger</i>
cm	centimeter
<i>Cen-III</i>	<i>Centromere-III</i>
<i>Cen-V</i>	<i>Centromere-IV</i>
<i>crp-4</i>	<i>cytoplasmic ribosomal protein-4</i>
<i>cya-4</i>	<i>cytochrome a-4</i>
°C	degree Celsius
dCTP	deoxycytidine 5' triphosphate
ddH ₂ O	distilled, deionized water
dH ₂ O	distilled water
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
<i>eas</i>	<i>easily wettable</i>
EDTA	ethylene diamine tetracetic acid
<i>eth-1</i>	<i>ethionine resistant-1</i>
FGSC	Fungal Genetic Stock Centre
<i>fl</i>	<i>fluffy</i>
<i>fr</i>	<i>frost</i>
<i>Fsr-17</i>	<i>5S rRNA-17</i>
G	guanine
g	gravity
<i>inl</i>	<i>inositol</i>
kV	kilovolt
L	left
λ	lambda

LB	Luria-Bertani medium
<i>leu-6</i>	<i>leucine-6</i>
LG	linkage group
M	molar
MA	Mauriceville
<i>mat</i>	<i>mating type</i>
<i>mat-A</i>	<i>mating type A</i>
<i>mat-a</i>	<i>mating type a</i>
<i>met-6</i>	<i>methionine-6</i>
μl	microlitre
μM	micro molar
mg/ml	milligram per milliliter
ml	milliliter
mm	millimeter
mM	millimolar
mt	mitochondrial
mV/cm	millivolt per centimeter
<i>nit-6</i>	<i>nitrate nomutilizer-6</i>
<i>nmr</i>	<i>nitrogen metabolite regulation</i>
<i>nmt-1</i>	<i>no message in thiamine-1</i>
<i>nuo19.3</i>	<i>NADH:ubiquinone oxidoreductase 19.3</i>
<i>nuo21.3c</i>	<i>NADH:ubiquinone oxidoreductase 21.3c</i>
<i>nuo29.9</i>	<i>NADH:ubiquinone oxidoreductase 29.9</i>
OD ₆₀₀	Optical density at 600 nanometers
OR	Oak Ridge
%	percent
%GC	percent GC
PCR	polymerase chain reaction
pH	the negative logarithm to the base 10 of the hydrogen ion concentration
³² P	phosphorus radioisotope 32
<i>poi-2</i>	<i>plenty of it-2</i>
<i>pyr-1</i>	<i>pyrimidine-1</i>
<i>pyr-2</i>	<i>pyrimidine-2</i>
<i>pyr-6</i>	<i>pyrimidine-6</i>
R	right
RAPD	randomly amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid

RNR	ribonucleotide reductase
<i>rnr-2</i>	<i>ribonucleotide reductase small subunit</i>
RNR-2	ribonucleotide reductase small subunit protein
<i>rnr-2^{MA}</i>	ribonucleotide reductase small subunit, Mauriceville allele
<i>rnr-2^{OR}</i>	ribonucleotide reductase small subunit, Oak Ridge allele
<i>ro-11</i>	<i>ropy-11</i>
<i>ro-7</i>	<i>ropy-7</i>
<i>ro-7⁺</i>	<i>ropy-7</i> , wild type allele
rpm	rotations per minute
SDS	sodium dodecyl sulfate
<i>su(un-24)-1</i>	<i>suppressor -1 of un-24</i>
<i>su(un-24)-1⁺</i>	wild type allele of <i>suppressor-1 of un-24</i>
TAE	Tris-acetate, EDTA
<i>Taq</i>	<i>Thermus aquaticus</i>
<i>thr-2</i>	<i>threonine-2</i>
<i>thr-2⁺</i>	wild type allele of <i>threonine-2</i>
T	thymine
T _m	melting temperature
<i>tom70</i>	<i>translocation of outer mitochondrial membrane 70</i>
<i>trp-1</i>	<i>tryptophan-1</i>
<i>un-24</i>	<i>ribonucleotide reductase large subunit</i>
UN-24	ribonucleotide reductase large subunit protein
UV	ultraviolet light
v:v	volume to volume
v:v:v	volume to volume to volume
w:v	weight to volume
Xgal	5-Bromo-4-Chloro-3-indolyl-β-D-galactoside

General Introduction

Introduction

This introduction provides basic information regarding fungi, followed by a focus on *Neurospora crassa* and its advantages as a research organism. The goals of this thesis are then introduced and outlined.

Fungi are a major group of eukaryotic organisms that derive their energy by catabolizing presynthesized organic matter. Within the fungal kingdom, there are three divisions, Zygomycetes, Ascomycetes and Basidiomycetes, based on sexual reproductive structures (Kendrick, 1992). There is a remarkable diversity of fungi within each of these three groups, and, as such, their roles in our ecosystems are diverse. As opportunists, fungi decay fruit and buildings, and cause disease in plants and animals. Examination of bedding plants in a garden store, for example, may identify several plants with fungal infections. A survey through a hardware or grocery store would reveal a number of antimicrobial and antifungal products manufactured to limit fungal encroachments on our lives.

In contrast to their negative roles, fungi also have beneficial roles in our lives. Fungal decay is a critical step for returning nutrients to primary producers in a utilizable form. Fungi also form important associations with photosynthetic organisms. In mycorrhiza, fungi help plants acquire nutrients, and in lichens, provide protection to algae and cyanobacteria. Humans have also directly utilized fungi for benefit. Many fungi are consumed, or are involved in the processing of many of our foods, including bread, cheese and alcohol and in the industrial synthesis of many products. Fungi have also played an important role in biology as model eukaryotic organisms. Examples of fungi commonly used in research include *Saccharomyces cerevisiae*,

Schizosaccharomyces pombe, *Aspergillus nidulans*, and *Neurospora crassa*.

Advantages to use of these organisms in research include short life cycles, small genomes, and ease of manipulation.

N. crassa in particular has been heavily utilized as a model organism. Prior to the description of its sexual stage in the late 1920s (Shear and Dodge, 1927), *Neurospora* was known in the 1800s as a common contaminant in bakeries. Following the description of *Neurospora*'s sexual cycle, the ease of manipulation *Neurospora* and its simple genetic analysis has aided research.

The *Neurospora* lifecycle

Neurospora's life cycle is composed of two stages (Figure 0-1). The vegetative stage consists of a macroconidial and a microconidial cycle. During these cycles, hyphae emerge from conidia deposited onto a suitable substrate. Hyphae grow into new areas by tip elongation and eventually form aerial hyphae. The aerial hyphae differentiate to form many orange-colored macroconidia. The conidia are dispersed to new environments, germinate and re-initiate the vegetative cycle.

Most of the sexual cycle occurs within a mass of hyphae, a protoperithecium, that develops into a protective perithecium surrounding reproductive ascogenous cells. Mating occurs between two *N. crassa* strains of different mating types. Special hyphae (trichogynes) emanating from the protoperithecium fuse with cells of the other mating type allowing the translocation of a fertilizing nucleus through the trichogyne to the protoperithecium. Following several mitotic divisions of the nuclei, nuclear fusion occurs in a cell of hook-shaped croziers. Immediately following nuclear fusion, a meiotic division produces four haploid nuclei. The nuclei undergo two mitotic divisions resulting

Figure 0-1: The life cycle of *Neurospora crassa*. The image is redrawn from Fincham *et al.* (1979).

in eight ascospores, each with two nuclei. The eight ascospores are packaged as a linear “tetrad” in a sac-like ascus. Mature asci protrude through an apical pore in the perithecium and disperse ascospores by forcefully ejecting them from the perithecium. The ascospores lay dormant until activation by heat or chemical cues. The emergence of a germ tube from the ascospore renews the vegetative lifecycle.

Benefits of using *Neurospora crassa*

Benefits of using *Neurospora crassa* as a research system are attributable either to the biology of *N. crassa*, or to its history as a research organism. At about 43 Mb, the genome is relatively small, and genetic analysis is further simplified by the haploid lifestyle of the fungus. The nuclear genome is distributed over seven chromosomes that range in size from 4.0 Mb for linkage group VI and VII to 10.3 Mb in linkage group I (Orbach *et al.*, 1988; Orbach, 1992).

In addition to the benefits inherent in working with *Neurospora crassa*, its long use as a research organism has added many resources. Commonly used techniques and laboratory strains are available (Davis and De Serres, 1970; Newmeyer *et al.*, 1987) including defined media that promote the sexual or the vegetative cycle. Detailed linkage maps, with more than 1000 loci, have been developed for all seven chromosomes (Perkins *et al.*, 2001). The linkage groups have been generated by identifying co-segregation of markers with other, previously known markers.

Mapping based approaches have been used extensively to locate genes of interest in *N. crassa*. Several strains with chromosome rearrangements and marked centromeres or telomeres are available for this purpose (Perkins, 1972). These can be useful in mapping a gene to linkage group, but it may require subsequent crosses to get a high

resolution of the gene position. The use of molecular markers can expedite identifying map position of genes. Several types of molecular markers have been used in *N. crassa* including DNA restriction fragment length polymorphisms (RFLPs, Metzenberg *et al.*, 1985) and randomly amplified polymorphic DNAs (RAPDs, Williams *et al.*, 1991). An RFLP mapping kit has been generated that takes advantage of DNA sequence differences that are present between standard laboratory strains and strains from the wild (Metzenberg *et al.*, 1984). Two progeny sets were created from two crosses between standard “Oak Ridge” laboratory strains and a wild type “Mauriceville” background strain. Initially, there were as few as no markers on a linkage group, to as many as seven markers on a linkage group for the mapping kit. These markers have been continually expanded upon, and now, 17 to 30 markers with different segregation patterns are on each linkage group (Nelson and Perkins, 2000). The mapping data sets illustrate the many DNA sequence polymorphisms that are present between these two strains. Many polymorphisms have been identified by using clones or clone fragments as probes for size separated restriction enzyme digestions of genomic DNA from the RFLP mapping kit progeny (e.g. *nit-6* [Exley, *et al.*, 1993]; *arg-13* [Liu and Dunlap, 1996]; *nmt-1* [McColl *et al.*, 2003]).

Several resources are also available for identifying mutated genes in *N. crassa*. Genomic libraries are available for screening the *N. crassa* genome (e.g.s Vollmer and Yanofsky, 1986; Orbach, 1994; and Kelkar *et al.*, 2001), making it easier to characterize mutations by complementation of the mutation (eg. the mating type locus [Glass *et al.*, 1988]; *cot-3* [Propheta *et al.*, 2001]).

In this thesis, a mutation, *su(un-24)-1*, that suppresses an osmotically remediated, temperature-sensitive mutation in the large subunit of ribonucleotide reductase (*un-24*) is examined. Ribonucleotide reductases are a conserved class of essential enzymes that catalyze the synthesis of deoxyribonucleotides from ribonucleotides. In *N. crassa* a mutation (*un-24*) generated in the large subunit of ribonucleotide reductase results in an osmotically-remediated, temperature-sensitive growth phenotype (Smith *et al.*, 2000a). The mutation was generated by an endogenous mechanism of mutating duplicated DNA segments, producing GC→AT transitions, known as Repeat Induced Point mutations (RIP; Selker *et al.*, 1989; Selker, 1990). Three point mutations were identified. One mutation led to a non-conservative amino acid substitution that is believed to lead to improper protein folding above 34 ° C, leading to growth arrest of *un-24* strains. Increasing the solute concentration appears to permit proper protein folding during synthesis, ameliorating the temperature-sensitivity.

su(un-24)-1 may suppress *un-24* temperature sensitivity by two mechanisms. First, *su(un-24)-1* may act by altering the solute concentration in the cell allowing protein folding to occur. Secondly, *su(un-24)-1* may directly interact with *un-24* to allow proper protein folding. For the second case, a suppressor of a temperature sensitive mutation in the *Schizosaccharomyces pombe* large subunit of ribonucleotide reductase was identified as a mutated small subunit of ribonucleotide reductase (Fernandez Sarabia *et al.*, 1993). The first chapter of this thesis characterizes the effect *su(un-24)-1* has on the growth rate of *un-24* strains. Several predictions are then tested to determine if the suppressor is operating by an osmotic mechanism, or by directly interacting with *un-24*. The second chapter of this thesis describes a mapping-based approach to identifying *su(un-24)-1*. For

this, PCR-based markers across all linkage groups were developed that are polymorphic between Oak Ridge laboratory and Mauriceville wild-type strains.

Chapter 1:
Characterization of $su(un-24)-1$

Introduction:

Ribonucleotide reductase is an evolutionarily conserved enzyme that catalyzes the conversion of RNA precursors into DNA precursors (Jordan and Reichard, 1998). The type I enzyme, found in eukaryotes and some prokaryotes, is composed of four subunits – two identical large subunits and two identical small subunits. In *N. crassa*, *un-24* codes for the large subunit of ribonucleotide reductase (Smith *et al.*, 2000a). In addition to DNA precursor synthesis, *un-24* is involved in heterokaryon incompatibility (Smith *et al.*, 2000b), a form of non-self recognition that results in the death of cells containing nuclei that are dissimilar at heterokaryon incompatibility loci (Glass *et al.*, 2000). The *un-24^{ts}* mutation in *N. crassa* leads to an osmotically-remediated, temperature-sensitive growth defect, due to a mutation in the allosteric activity site of the large subunit of ribonucleotide reductase. It was proposed that, at temperatures above 34°C, UN-24 is improperly folded during protein synthesis, resulting in non-functional ribonucleotide reductase, a decrease in available dNTPs and growth arrest (Smith *et al.*, 2000a). Temperature-sensitivity of strains carrying *un-24^{ts}* is osmotically remediated by, for example, the addition of 1M sorbitol to the medium. The high solute concentrations are hypothesized to allow proper protein folding by modifying the interaction between UN-24 amino acid residues and their environment.

During growth studies of the *un-24^{ts}* strain C8c-164, a revertant, 164-4, was isolated that appears to be associated with a spontaneous suppressor mutation, *su(un-24)-1*. There are two mechanisms by which *su(un-24)-1* may suppress temperature sensitivity of *un-24^{ts}* strains. First, given that *un-24^{ts}* can be osmotically remediated, *su(un-24)-1* may alleviate temperature sensitivity by increasing the intracellular solute concentration.

Alternately, *su(un-24)-1* may code for a protein that interacts directly with the large subunit of ribonucleotide reductase to allow proper protein folding at restrictive temperatures.

In this chapter the effect of *su(un-24)-1* on the growth rate of *un-24^{ts}* strains is examined and two hypotheses regarding the possible mechanism of *su(un-24)-1* are tested. First, a mutation in the small subunit of ribonucleotide reductase was shown to suppress a temperature-sensitive mutation in the large subunit of ribonucleotide reductase of *Schizosaccharomyces pombe* (Fernandez Sarabia *et al.*, 1993), raising the possibility that *su(un-24)-1* in *N. crassa* may be due to a mutation in the gene for the small subunit of ribonucleotide reductase (*rnr-2*). To determine if *su(un-24)-1* was due to a *rnr-2* mutation, the *N. crassa rnr-2* gene was characterized by designing PCR primers to amplify a fragment of the gene and used as a probe to screen *N. crassa* genomic DNA library clones. Analysis of naturally occurring polymorphisms at *rnr-2* was used to test whether or not *su(un-24)-1* and *rnr-2* co-segregate in progeny sets. Our second hypothesis was that *su(un-24)-1* suppresses *un-24* temperature sensitivity by increasing intracellular osmotic pressure. If this is the case, it would be expected that *su(un-24)-1* would suppress other osmotically remediated mutations. To test this prediction we examined whether or not *su(un-24)-1* also suppresses *eth-1*, an osmotically-remediated mutation that confers temperature sensitivity, ethionine resistance and an inability to repress transcription of genes normally repressed by methionine (Metzenberg *et al.*, 1964; Metzenberg, 1968).

Materials and Methods:

Strains and Growth Conditions

The *Neurospora crassa* strains used in these studies are listed in Table 1-1. The strains were grown on Vogel's medium N (Vogel, 1964) with 1.5 % sucrose, 1.5 % agar and supplements as required (Davis and de Serres, 1970). Crosses were performed on synthetic crossing medium (Westergaard and Mitchell, 1947). Ascospores were collected by adding sterile dH₂O, vortexing, and then recovering suspended ascospores in the water. Ascospores were plated onto Vogel's medium and subjected to 60°C for 50 - 60 minutes to induce germination. Single ascospore germlings were isolated under a dissecting microscope and transferred to appropriate growth medium in test tubes. Progeny were scored for their phenotype by inoculating conidial suspensions onto spot testing medium (1x Vogel's salts, 0.8 % sorbose, 0.4 % sucrose, 1.5 % agar, and supplements as required, Davis and de Serres, 1970).

Growth Rate Determinations

Growth rates for 164-4, C8c-164 and 74ORS were determined by inoculating each strain onto 16 mm diameter X 150 mm long test tube slants containing Vogel's medium, tryptophan and inositol, with or without sorbitol (0.5 M or 1 M; Sigma Chemical Company, Oakville, ON) as an osmoticum. The test tubes were placed inside a temperature gradient incubator TN-3 (Scientific Industries Inc., Bohemia, NY) with a temperature gradient between 21 to 46 °C. Growth rates (mm/hr) were recorded twice daily.

Neurospora DNA isolation

DNA was isolated from *Neurospora crassa* by the method of Oakley *et al.*

Table 1-1: *Neurospora crassa* strains used in this study

Strain	Genotype	Source
74ORS-6	Oak Ridge Wild-type <i>a</i>	FGSC# 4200
C8c-164	<i>un-24; trp-1; inl a</i>	Smith <i>et al.</i> , 2000a
164-4	<i>un-24; trp-1; inl; su(un-24)-1 a</i>	This study
2225	Mauriceville-1c-A Wild-type	FGSC #2225
1212	<i>eth-1 A</i>	FGSC #1212
1220	<i>eth-1 a</i>	FGSC #1220
DJ987-51	<i>ro-7 thr-2 A</i>	D. J. Jacobson
FGSC# 4450-4487	RFLP mapping strains	FGSC # 4450-4487

(1987). Four day old *N. crassa* mycelium was harvested from liquid Vogel's medium by vacuum filtration through 9 cm No. 2 Whatman filter paper and lyophilized overnight at -20 °C. After pulverizing the dried mycelium with a glass rod, approximately 100-200 µl of the powder was transferred to a 1.5 ml microcentrifuge tube, resuspended in 400 µl of salt detergent solution (2 M NaCl, 4 mg/ml sodium deoxycholate and 10 mg/ml Brij 58 [Sigma Chemical Company]) and incubated at room temperature for 20 minutes. 300 µl of supernatant was then recovered after centrifugation for 10 minutes at 14,000 rpm in a microcentrifuge (Eppendorf Centrifuge 5415C, Brinkmann Instruments, Westbury, NY) and transferred to a 1.5 ml microcentrifuge tube. About 1.2 mls of trichloroacetic acid-ethanol solution was mixed with the supernatant by inversion and then incubated for at least 20 minutes at -20 °C. Following centrifugation for 1 to 2 minutes at 14,000 rpm, the nucleic acid pellet was washed with 400 µl of ice-cold 70 % ethanol. After brief drying in a speed vac (Savant, Hicksville, NY), the pellet was resuspended in 300 µl of freshly prepared RNaseA solution and incubated for 1 hour in a 50 °C water bath. After RNaseA treatment, carbohydrates were separated by vigorous mixing of the solution with 300 µl of chloroform: isoamyl alcohol (24:1 [v:v]) and a 5 minute centrifugation at 14,000 rpm. 200 µl of the aqueous phase was then transferred to a new 1.5 ml microcentrifuge tube, mixed gently with 80 µl of 5.0 M ammonium acetate and 280 µl of isopropanol. Following an incubation at room temperature for 10 minutes, and a centrifugation for 2 minutes at 14,000 rpm, the DNA pellet was washed with 400 µl of ice cold 70 % ethanol and thoroughly dried in a speed vac. DNA pellets were resuspended in 20 to 40 µl of water.

Polymerase Chain Reaction

The primers and reaction conditions used in polymerase chain reactions (PCRs) are listed in Table 1-2. The PR2-3 and PR2-4 primers were manually designed based on conserved regions of the *rnr-2* genes from *Spisula solidissima*, *Homo sapiens*, *Glycine max*, *Arabidopsis thaliana*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Trypanosoma brucei*. All primers were synthesized at the University of Ottawa Biotechnology Research Institute (Ottawa, Ontario). 50 µl PCRs, consisting of template DNA (~20ng), 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM of MgCl₂, 0.2 mM dNTPs (Roche, Laval, QC), 0.5 µM of each primer, and 1.25 Units of *Taq* polymerase (GibcoBRL, Burlington, ON), were performed in sterile, thin-walled PCR tubes with an Amplifon II thermocycler (Barnstead, Dubuque, Iowa). PCRs were performed by first putting each template into their PCR tubes on ice. A master mix would then be prepared on ice for all the samples by addition of the buffer, MgCl₂, primers, ddH₂O and *Taq* Polymerase. A control with no template was used in all experiments. All PCRs consisted of an initial three minute denaturation at 95 °C followed by 30 cycles of denaturing for 30 seconds at 95 °C; annealing for 30 seconds and extending at 72 °C as given in Table 1-2. PCRs were concluded with a 3 minute, 72 °C final extension period and a 4 °C soak temperature for an indefinite time.

DNA Digestion and Ligation

DNA was digested with restriction enzymes as recommended by their manufacturer (New England Biolabs, Mississauga, ON; GibcoBRL) in 10 to 20 µl volumes. Ligations were performed using T4 DNA ligase (New England Biolabs), overnight in a 16°C waterbath.

Table 1-2: The primer sequences and reaction conditions used in this study.

Name:	Sequence (5' >3'):	Annealing Temperature	Extension Time
PR2-3	AATTTGGCAATTCTACAAGAAGG	46°C	0:30
PR2-4	TTGGAAAAAGTAAGACCAGGCAT		
PR2-7	TGTGGGCACCCACGTGCG	54°C	1:40
PR2-8	CAGCCATGATCTTGAGTCC		

Agarose Gel Electrophoresis

DNA was size separated in 0.7 % (w/v) agarose gels (Roche) containing ethidium bromide (Sigma Chemical Company) in 1x TAE (0.04M Tris-Acetate, 0.001M EDTA). A voltage of 14 mV/cm was used for 30 to 60 minutes in a Wide Mini-Sub Cell (BioRad, Mississauga, ON) with an electrophoresis power supply (Pharmacia) prior to visualization by UV illumination. Gels were photographed with a Polaroid gel documentation system using Polaroid 667 film.

Southern Transfer

Following electrophoresis, DNA was transferred to nylon membranes (Hybond, Amersham Pharmacia Biotech, Baie d'Urfé, QC). DNA was first depurinated by a 6 to 10 minute incubation in 0.25M HCl with gentle shaking. After rinsing in distilled water, the gel was incubated twice in 1.5M NaCl, 0.5M NaOH for 15 minutes each time. The gel was then neutralized in 1M TrisHCl (pH 7.4), 1.5M NaCl for 15 minutes and washed in 2x SSC for 15 minutes. The gel was then placed on a flat surface and 2x SSC poured over it. A pre-wetted nylon membrane, cut to the same size as the agarose gel was placed over the gel and air bubbles, located between the membrane and the gel, were removed by rolling a glass pipette over the membrane. The gel and membrane were then covered with plastic wrap that extended into the underlying 2x SSC pool. A window was cut in the plastic wrap and three layers of Whatman filter paper was placed over the window. A paper towel stack was placed over the filter paper to absorb the 2x SSC. After twenty minutes, the bottom layers of paper towels were replaced with fresh paper towels. The next morning, the transfer was stopped and the agarose gel wells were marked on the

membrane with a pencil. The DNA was fixed to the membrane with UV illumination for 15 minutes.

λ library screening

XLI Blue MRA cells with P2 lysogen, grown to an OD₆₀₀ of about 0.5 were infected with a λ FIX II (Stratagene, La Jolla, CA) genomic library. The cells were centrifuged at 1000rpm for 10 minutes (IEC Centra MP4R, International Equipment Company, Needham Heights, MA) and resuspended in 10 mM MgSO₄ to an OD₆₀₀ of 0.5. Lambda viruses, forming about 300 plaques per Petri plate, were then incubated with the cells at 37°C with slow shaking. After the addition of 3 mLs of warm top agar (LB broth base (GibcoBRL) with 0.7 % agarose) the cells were poured onto solid LB plates, cooled and incubated overnight at 37°C. After Petri plates were chilled for 30 minutes at 4°C, plaques were transferred to a circular nylon membrane (Hybond-N, Amersham pharmacia biotech). The membrane was pierced three times with a needle as it overlay the agar surface, providing landmarks for the alignment of the membrane and the Petri plate. Membranes were incubated in denaturing solution (1.5M NaCl, 0.5M NaOH) for 5 minutes, dried briefly, incubated in neutralization solution (1M Tris HCl, 1.5M NaCl) for 15 minutes and then incubated for 10 minutes in 2x SSC. After briefly air-drying the membrane, PCR products or plasmids bearing the PCR product were placed on the membrane at each pin puncture as positive controls for subsequent hybridization and autoradiographic steps. The DNA was then UV-fixed to the membrane for 15 minutes and probed with labeled PCR product.

λ DNA Isolation

λ DNA was isolated using the liquid culture isolation method in Sambrook *et al.*, (1989). XLI Blue MRA cells with P2 lysogen were grown in LB broth, infected with λ particles and incubated at 37°C with shaking in 4 ml of NZCYM medium for nine to fifteen hours. After the addition of 100 µl of chloroform, the solution was incubated again at 37°C for 15 minutes with shaking. The medium was then centrifuged at 8000g (Sorvall RC-5B Refrigerated Superspeed Centrifuge, Mandel Scientific, Guelph, ON) for 10 minutes at 4°C. The supernatant was recovered to a centrifuge tube and RNase A and DNase I treated for 30 minutes at 37°C. An equal volume of λ diluent with polyethylene glycol 8000 and 2M NaCl was added after RNase and DNase treatment and incubated for 1 hour at 0°C. λ particles were then isolated by centrifuging at 10,000g for 10 minutes at 4°C. After the supernatant was recovered, 5 µl of 10 % SDS and 5 µl of 0.5M EDTA was added and the tube incubated at 68°C for 15 minutes. This was followed by a phenol: chloroform: isoamyl alcohol (25:24:1 [v:v:v]) extraction with an equal volume, centrifugation at 14,000 rpm for 2 minutes and an additional chloroform: isoamyl alcohol (24:1 [v:v]) extraction with 200 µl. After a centrifugation at 14,000 rpm for 2 minutes, λ DNA was precipitated with an equal volume of isopropanol and incubated at -20°C for 20 minutes. This was followed by a 14,000 rpm centrifugation at 4°C for 15 minutes. After washing the pellet with ice-cold 70 % ethanol, the pellet was resuspended in 50 µl of TE (pH 8.0).

DNA Labelling and Hybridization

1 µl of PCR product was labelled with [α -³²P] dCTP (Amersham Biosciences) by using the Random Prime DNA Labelling Kit (Boehringer Mannheim). PCR product and

11.5 μ l of dH₂O were boiled for 5 minutes. After quenching the solution on ice, 4 μ l of reaction mix (dNTPs, Mg, hexanucleotide primers), 2.5 μ l [α -³²P] dCTP and 1 μ l of Klenow polymerase were mixed and incubated at 37°C for 1 hour. To separate incorporated and unincorporated dNTPs the volume was increased to 200 μ l with dH₂O and passed through a Sephadex G50 column. The labeled probe was then mixed with hybridization solution (5x Denhardt's, 5x SSPE, 0.5 % SDS, 10 μ g/ml sheared single stranded salmon sperm DNA) and incubated overnight with the membrane in a hybridization oven (VWR Scientific, Mississauga, ON). After hybridization, the membranes were washed twice, for 10 minutes each time, with Wash 1 (2x SSC, 0.5 % SDS), twice, for 10 minutes each time, with Wash 2 (0.1x SSC, 0.5 % SDS) and then wrapped in plastic wrap. Film (Kodak BioMax MR, Mandel Scientific) was exposed to the membrane at -80°C with an intensifying screen.

Bacterial Transformation

Forty microliters of *Escherichia coli* DH5 α cells (GibcoBRL, Burlington, ON) were transformed with 1 μ l of DNA. Electroporation cuvettes with a 2 mm path length (InVitrogen, Burlington, ON or EquiBio, Needham Heights, MA) were used for 2 kV electroporations with an E. coli Pulser (BioRad). Immediately after electroporation, 1 ml of SOC solution was added to the cells. The cells were then transferred to a sterile test tube and incubated at 37°C for one hour with or without shaking. After incubation, the solution was plated onto LB plates containing Xgal and an appropriate antibiotic selection.

Plasmid DNA Isolation and DNA sequencing

Plasmid DNA was isolated from *Escherichia coli* DH5a cells using either the Wizard Plus minipreps DNA purification system (Promega, Madison WI), or an alkaline lysis miniprep modified from Birnboim and Doly (1979). Plasmid DNAs were resuspended in 50 µl of ddH₂O.

All DNA samples were sequenced by Canadian Molecular Research Services (Ottawa, ON).

Computer Analysis

All DNA sequences were edited with MacDNASIS Pro v3.0 (Hitachi Software Engineering Co., Ltd) on a Power Macintosh 6500/225 to remove plasmid sequences, primer sequences, generate contiguous sequences, and translate the DNA. Clustal V (Higgins, *et al.*, 1992) was used in a Power Macintosh G3 to align the amino acid sequences. BLAST tools were used in all searches (Altschul, *et al.*, 1997).

RFLP Mapping

The RFLP mapping progeny were scored for their genotype at *rnr-2* by digestion of the PR2-7/PR2-8 PCR product with *Ava*I, an enzyme that revealed an RFLP between the two parents of these progeny. Digested PCR products were separated on 2 % agarose gels as described above.

Results:

su(un-24)-1 suppression of *un-24* temperature sensitivity

To measure the influence of *su(un-24)-1* on the growth rate of *un-24^{ts}* strains, the growth rate for 74ORS (wild-type), C8c-164 (*un-24^{ts}*) and 164-4 (*un-24^{ts} su(un-24)-1*) was determined between 21°C to 46°C on media supplemented with either 0M, 0.5M or

1M sorbitol. On Vogel's medium with 0 M sorbitol (Figure 1-1a), 74ORS had a peak growth rate of ~4 mm/h at ~32.5 °C and did not grow at temperatures above ~46 °C. Strain C8c-164 had a maximum growth rate of ~1.9 mm/h at ~30 °C and did not grow above ~32.5 °C. Strain 164-4 had a peak growth rate of ~2.2 mm/h at ~35 °C and did not grow above ~46 °C.

On Vogel's medium supplemented with 0.5 M sorbitol (Figure 1-1b), 74ORS had a peak growth rate of ~3.5 mm/h at 32 °C and did not grow beyond ~46.5 °C. The C8c-164 peak growth rate of ~1.4 mm/h was at ~29 °C. C8c-164 stopped growing by 34 °C. 164-4 had optimal growth rates of ~2.7 mm/h at 36.6 °C and stopped growing by about 42.5 °C.

On Vogel's medium supplemented with 1 M sorbitol (Figure 1-1c), 74ORS had maximum growth at ~35 °C with a rate of ~2.7 mm/h and stopped growing by ~44.5 °C. The C8c-164 growth rate peak of about 1.4 mm/h at ~30 °C. At 39 °C, C8c-164 does not grow. The 164-4 growth rate peaked at ~3 mm/h at ~39 °C. 164-4 stopped growing by 44.5 °C.

Characterization of *rnr-2*

Since the DNA sequence of a small subunit of ribonucleotide reductase was not available before 2001 (*Neurospora crassa* genome project) we designed primers for areas of the gene conserved amongst a variety of eukaryotic organisms. The PCR primers PR2-3 and PR2-4 amplified a 573 bp product from *N. crassa* genomic DNA. An open reading frame within this sequence had 47 % similarity and 28 % identity to the *Escherichia coli* small subunit of ribonucleotide reductase 1. Using this PCR product as a probe, the entire *rnr-2* gene was isolated from a λ library containing genomic DNA

Figure 1-1: The growth rates of 74ORS-6 (Wild-type), C8c-164 (temperature sensitive) and 164-4 (temperature sensitive suppressed) on Vogel's medium supplemented with, a) 0 M sorbitol, b) 0.5 M sorbitol, or c) 1 M sorbitol, at temperatures ranging from 21-46 °C. Four to twelve replicates were used to calculate means ($\pm$ standard deviation) with the exceptions of 74 ORS-6 on 1.0 M sorbitol at 21 °C and 25 °C; 74 ORS without sorbitol at 32 and 33 °C and 164-4 without sorbitol at 32 °C which had two to three replicates.

from *N. crassa* strain FGSC# 1131. After screening approximately 10,000 plaques, three clones were identified that hybridized to the PCR product. Two clones, λ 3-3.3 and λ 5-3.3, were identified as containing overlapping regions that hybridized to the PCR product. Sequencing λ 5-3.3 pUC118 subclones, 5-5 and 5-4 and the λ 3-3.3, pUC118 subclone, pR2Sac6, gave us the DNA sequence for the entire gene. The 1443 nucleotide *N. crassa rnr-2* gene encodes a 410 amino acid protein (Figure 1-2). Two putative introns, 136 and 74 nucleotides in length, are located in the 5' end of the gene. Comparison of the inferred *N. crassa rnr-2* protein sequence to the *rnr-2* protein from other organisms revealed ~60 % similarity over the entire gene including conserved residues that are important in free radical generation and iron binding (Figure 1-3). *rnr-2* was mapped to the right arm of LG III, near *thi-4* (Figure 1-4), using an *AvaI* RFLP identified within the PR2-7/PR2-8 PCR product.

rnr-2 and *su(un-24)-1* do not co-segregate

If *su(un-24)-1* is a result of a mutation in *rnr-2*, *su(un-24)-1* and *rnr-2* should co-segregate. To determine if this was the case, we analyzed progeny from a cross between, DJ987-51 *ro-7 thr-2; rnr-2^{MA} A* and 164-4 *un-24; rnr-2^{OR} trp-1; inl; su(un-24)-1 a*. The inheritance of *rnr-2* from either the MA (Mauriceville) or OR (Oak Ridge) parent was distinguishable by an *AvaI* RFLP in PR2-7/PR2-8 PCR products. *un-24^{ts}* progeny were selected by isolating *ro-7⁺* and *thr-2⁺* progeny (see Figure 1-5 for markers near *un-24* on linkage group II). *ro-7⁺ thr-2⁺* progeny that grew at 37°C were likely *su(un-24)-1*, while those incapable of growth at 37°C are *su(un-24)-1⁺*. Eleven of these putatively *un-24^{ts}* suppressed progeny were scored for their genotype at *rnr-2*. Six of the eleven progeny were recombinant for *su(un-24)-1* and *rnr-2*; 2 were *su(un-24)-1, rnr-2^{MA}* and 4 were

Figure 1-2: DNA Sequence and the predicted amino acid sequence of the Panama allele of the small subunit of *Neurospora crassa* ribonucleotide reductase.

1201 GATCCAGGAAATCATTGTCGATGCCGTCAGAATTGAGCAGGAGTTCCTCACTGAGGCCCT 1260
I Q E I I V D A V R I E Q E F L T E A L

1261 CCCTTGCGCCCTTCTTGGCATGAACGCCGACCTCATGAAGCAGTACATCGAGTTCGTTCGC 1320
P C A L L G M N A D L M K Q Y I E F V A

1321 CGATCGTCTCCTTGTCGCCCTCGGCAACGAGAAGATCTACAGGTCGACTAACCCCTTCGA 1380
D R L L V A L G N E K I Y R S T N P F D

1381 CTTTCATGGAGAACATTTCTCTCGGTGGCAAGACCAACTTCTTCGAGAAGCGTGTGGCGA 1440
F M E N I S L G G K T N F F E K R V G D

1441 CTACCAGAAGGCTGGTGTTCATGAACAGCACCAAGAAGGCTGACGCTGATGCGGAGGTGGC 1500
Y Q K A G V M N S T K K A D A D A E V A

1501 CAAGAACGAGAACGGCGGTGACTTCACCTTCGACGAGGACTTTTAAATGCCGTTGTCCTT 1560
K N E N G G D F T F D E D F *

1561 GCCGTTTCTCGTTTCTCTTTGCGACGCCTCTTATGACTTTGTTTCGTCAATACCCCTTC 1620
1621 ACCACACTGTACCTTTCCGTTCTGATCTCACACCGAACATCCCA 1664

Figure 1-3: Amino acid sequence alignment between the small subunit of ribonucleotide reductase from *Neurospora crassa*, *Aspergillus nidulans* (AAG40862), *Saccharomyces cerevisiae* (P09938), *Schizosaccharomyces pombe* (P36603), *Homo sapiens* (NP_001025) and *Mus musculus* (P11157). Amino acid residues of the iron binding domain are underlined. The tyrosine free radical is in bold.

N. crassa MSVQTSPSKQVTSGIQNLNM-DSPAKKLDGFGATDKENKPFDEDLAKLEAE
A. nidulans MSAQLTPSKQAASSLENLKMNDSPVKKLNFEEAGKENAPVSNPMV----D
S. cerevisiae MP-KETPSKAAADALSdleIKDSKSNLNKELETLREENRVKSDM--LKEK
S. pombe MGLEH-----LEEFsyPKehGEEVEYDSEQGVRKIYVKSI---KET
H. sapiens MSLSRVPLAPITDF-QQLQL--SPLKGLSLVDKENTPPALSGTRV-LASK
M. musculus MLSVRTPLATIADQ-QQLQL--SPLKRLTLADKENTPPTLSSTRV-LASK

N. crassa	IDAEHNANKKAAEAKMA-PTLKPEEANEPLLTENPQRFVLFP	IKYHEIW
A. nidulans	VPATKPATEKPEPSKAA-LDVKAIEANEPLLQENPHRFVLFP	IKYHEIW
S. cerevisiae	LSKDAENHKAYLKSHQVHRHKLKEMEKEEPLLNEDKERTVLFP	IKYHEIW
S. pombe	FNFDNVSEEEKQEGGDYY-LGKKEDELDEVVLRPNPHRFVLFP	IKYHEIW
H. sapiens	TARRIFQEPTEPKTKAAA-PGVE----	DEPLLRENPRRFVIFPIEYHDIW
M. musculus	AARRIFQDSAELESKAPTNPVSVE----	DEPLLRENPRRFVVFPIEYHDIW

* * * * *

N. crassa QMYKKAEEASF⁻TAAEIDLSKDLDHWNRLNDEKFFISHILAFFAASDGI
A. nidulans QMYKKAEEASF⁻TAAEIDLSKDLDHWNRLNEDERYFISRVLAFFAASDGI
S. cerevisiae QAYKRAEEASF⁻TAAEIDLSKDIHDWNNRMNERFFFISRVLFAFFAASDGI
S. pombe QFYKKAEEASF⁻TAAEIDLSKDLVDWDNKLNADERYFISTVLAYFAFFAASDGI
H. sapiens QMYKKAEEASF⁻TAAEEVDLSKDIQHWE-SLKPEERYFISHVLAFFAASDGI
M. musculus QMYKKAEEASF⁻TAAEEVDLSKDIQHWEA-LKPDERHFISHVLAFFAASDGI
* *.*****.*.....*.***.*****.

N. crassa VNENLVERFSGEVQIPEARCFYGFQIMMENIHSET**Y**SLLDITYIKEPSQR
A. nidulans VNENLLERFSGEVQIPEARCFYGFQIMIENIHAET**Y**SLLDITYIKEPKQR
S. cerevisiae VNENLVENFSTEVQIPEAKSFYGFQIMIENIHSET**Y**SLLDITYIKDPKES
S. pombe VNENLLERFSSEVQIPEARCVYGFQIMIENIHSET**Y**SLLLDITYIREPKKE
H. sapiens VNENLVERFSQEVQITEARCFYGFQIAMENIHSE**M**YSLLDITYIKDPKER
M. musculus VNENLVERFSQEVQVTEARCFYGFQIAMENIHSE**M**YSLLDITYIKDPKER
*****.* ** ***. **. *****.*****.* *****.*****.*

N. crassa TYLFNAIDTIPCIRKKADWALRWITDKSSTFAQRLVAFAAVEGIFFGSGAF
A. nidulans TYLFDAIDTIPCIRKKADWAIKWIQDKESTFAQRLVAFAAVEGIFFGSGF
S. cerevisiae EFLFNAIHTIPEIGKEAEWALRWIQDADALFGERLVAFASIEGVFFSGSF
S. pombe QRHFDAILTMGSIKAKAKWALRWINDEDSYAIRLVAFAAVEGIFFGSGF
H. sapiens EFLFNAIETMPCVKKKADWALRWIGDKEATYGERVVAFAAVEGIFFGSGF
M. musculus EYLFNAIETMPCVKKKADWALRWIGDKEATYGERVVAFAAVEGIFFGSGF
* . ** * . . ** * . * . . . * . * . * . * . * . * . *

N. crassa ASIFWLKKRGLMPGLTFSNELISRDEGLHTDFACLLFSHLNNRPSKQLIQ
A. nidulans ASIFWLKKRGLMPGLTFSNELISRDEGLHTDFACLLFSHLNWRPSKKVVE
S. cerevisiae ASIFWLKKRGMPGLTFSNELICRDEGLHTDFACLLFAHLKNKPDPAIVE
S. pombe ASIFWLKKRGLMPGLTFSNELICRDEGLHTDFACLMFSHLKHRPGRKVVE
H. sapiens ASIFWLKKRGLMPGLTFSNELISRDEGLHCDFACLMFKHLVHKPSEERV
M. musculus ASIFWLKKRGLMPGLTFSNELISRDEGLHCDFACLMFKHLVHKPAEQVR
*****.***** ***** ***** *

N. crassa	EIIIVDAVRIEQEFLTEALPCALLGMNADLMKQYIEFVADRLLVALGNEKI
A. nidulans	DIIVEAVAIEKEFLTDALPCALLGMNAKLMCQYIEFVADRLLVALGNKEY
S. cerevisiae	KIVTEAVEIEQRYFLDALPVALLGMNADLMNQYVEFVADRLLVAFGNKKY
S. pombe	AIIVEAVDIEKEYFTDALPVSLGGMNKDLMCQYIEFVADRLLVALGNDKY
H. sapiens	EIIINAVRIEQEFLTEALPVKLIGMNCTLMKQYIEFVADRLLMELGFSKV
M. musculus	EIITNAVRIEQEFLTEALPVKLIGMNCTLMKQYIEFVADRLLMELGFNKI

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N. crassa	YRSTNPFDPMENISLGGKTNFFEKRVDYQKAGVMNSTKKADADA ⁻ EVAKN
A. nidulans	FNATNPFDPMESISLAGKTNFFEKRVDYQKAGVMAS ⁻ TKK-DPKQDET ⁻ KT
S. cerevisiae	YKVENPFDPMENISLAGKTNFFEKRVS ⁻ DYQKAGVMSKSTKQEA-----
S. pombe	YNVTNPFDPMENISLAGKTNFFEKKVS ⁻ DYQIAGVMSGTKRAE-----
H. sapiens	FRVENPFDPMENISLEGKTNFFEKRVG ⁻ EYQRMGMSSPT-----
M. musculus	FRVENPFDPMENISLEGKTNFFEKRVG ⁻ EYQRMGMSSN ⁻ ST-----

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N. crassa	ENGGDFTFDEDF
A. nidulans	SDGNGLSFDEDF
S. cerevisiae	---GAFTFNEDF
S. pombe	KDDHTFTIDEDF
H. sapiens	--ENSFTLDADF
M. musculus	--ENSFTLDADF

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Figure 1-4: The RFLP segregation pattern of *rnr-2* and *thi-4*. *thi-4* is located on the right arm of linkage group III, near the centromere. "O" indicates that the marker was inherited from FGSC # 4488, while "M" indicates that the marker was inherited from FGSC# 2225. "-" indicates that the progeny was not scored at that marker.

Figure 1-5: Schematic of linkage group II, showing the location of markers near *un-24* in 164-4 and 987-51.

su(un-24)-1⁺, *rnr-2*^{OR}. Of the remaining five progeny, 2 were *su(un-24)-1*, *rnr-2*^{OR} and 3 were *su(un-24)-1*⁺, *rnr-2*^{MA}, indicating that *su(un-24)-1* and *rnr-2* are not linked (p=1, two tailed Fisher's exact test).

su(un-24)-1 does not suppress osmotically-remediated, *eth-1*, ethionine resistance

If *su(un-24)-1* suppresses *un-24*^{ts} by an osmotic mechanism, it is expected to also suppress other osmotically remediated mutations. In *N. crassa*, the osmotically-remediated mutation *eth-1* confers several phenotypes, including ethionine resistance. To determine if *su(un-24)-1* can suppress ethionine resistance, we crossed a *un-24*⁺ *su(un-24)-1* strain, 25-7, to *eth-1* strain FGSC# 1212. If *su(un-24)-1* suppresses *eth-1*, one quarter of the progeny would be ethionine resistant. If *su(un-24)-1* does not suppress *eth-1*, one half of the progeny would be ethionine resistant. In 156 progeny, 81 progeny grew as well on spot testing medium containing ethionine (0.5 mM) as on spot testing medium without ethionine. 75 progeny had reduced growth on spot testing medium containing ethionine (0.5mM) compared to their growth on spot testing medium without ethionine. Based on a ratio of 81:75, we cannot reject that one half of the progeny from this cross are ethionine resistant (Chi squared p=0.6). We can reject that one quarter of the progeny are ethionine resistant (Chi squared p=8 x 10⁻¹⁵). This indicates that *su(un-24)-1* does not suppress ethionine resistance due to *eth-1*.

Discussion:

C8c-164 was previously identified as possessing an osmotically-remediated temperature-sensitive mutation in *un-24* (Smith *et al.*, 2000a). The osmotic-remedial, temperature-sensitivity was confirmed in our experiments. In addition, a suppressor of *un-24* was identified, *su(un-24)-1*, that had not been characterized.

The growth rates of 164-4, a *un-24*, *su(un-24)-1* strain, indicate that *su(un-24)-1* suppresses *un-24* temperature sensitivity over a wide range of temperatures at 0, 0.5 and 1 M sorbitol concentrations. For example, 164-4 grows as well as 74ORS above ~37 °C on medium that does not contain sorbitol. The growth rate of 164-4 is improved by increasing the osmoticum concentration in the medium. The 164-4 growth rates are higher on media supplemented with 0.5 M or 1 M sorbitol than on medium without sorbitol. Interestingly, on medium supplemented with 1 M sorbitol, *su(un-24)-1* appears to enable 164-4 to grow better than wild type at high temperatures. It is not possible to say if these enhanced growth rates are a result of an interaction between *su(un-24)-1* and sorbitol, or if it is due to an interaction between *un-24* and sorbitol. This could be determined by determining growth rates of *su(un-24)-1* strains without *un-24^{ts}* on media of different sorbitol concentrations. The growth rate data also suggests that *su(un-24)-1* is not operating by an osmotic mechanism. Osmotic remediation of C8c-164 is evident up to ~39 °C, however, *un-24 su(un-24)-1* strains are capable of growing at temperatures higher than 39 °C. The observation that *un-24* osmotic remediation ceases, whereas *un-24 su(un-24)-1* strains continue to grow above ~39 °C, suggests that *su(un-24)-1* is not operating by an osmotic mechanism.

Characterization of the *N. crassa rnr-2* allowed us to determine whether *su(un-24)-1* is a mutation in *rnr-2*. The *rnr-2* gene was 1,443 nucleotides in length and encoded 410 amino acid residues. It has similarity to other small subunit ribonucleotide reductases including the *S. pombe rnr-2*, a mutant of which is reported to suppress a temperature sensitive mutation in the large subunit of ribonucleotide reductase (Fernandez Sarabia *et al.*, 1993). Segregation analysis allowed us to determine that

su(un-24)-1 and *rnr-2* do not co-segregate, indicating that *su(un-24)-1* is not an altered form of *rnr-2*. Some organisms, (e.g. *Saccharomyces cerevisiae*, *Homo sapiens*, *Mus musculus*), possess more than one copy of the RNR genes. Probing genomic DNA digests with the PR2-3/PR2-4 PCR product, however, revealed only one fragment that hybridized to the probe. In addition, BLAST searches of the *N. crassa* genome project, which had recently become available (Galagan *et al.*, 2003), with the *rnr-2* DNA and protein sequences did not identify other sequences that resemble *rnr-2* with an E value of less than e^{-3} . These observations indicate that only one *rnr-2* is present in the *N. crassa* genome.

Characterization of *rnr-2* also provided insight regarding the possible heterokaryon incompatibility function of *rnr-2*. Unlike *un-24*, where there are significant differences in protein sequences between incompatibility alleles (Smith *et al.*, 2000b), comparisons of RNR-2 revealed no amino acid sequence differences between the Panama and Oak Ridge genetic backgrounds. This, and the lack of an identified heterokaryon incompatibility locus near *rnr-2*, near the centromere of linkage group III (Perkins, *et al.*, 2001), suggests that *rnr-2* is not involved in heterokaryon incompatibility.

To test the hypothesis that *su(un-24)-1* is acting by increasing intracellular osmotic pressure, we crossed a *su(un-24)-1* strain to an *eth-1* strain. *eth-1* is an osmotically-remediated, temperature-sensitive mutation that confers resistance to ethionine. Our observation of 81 ethionine resistant progeny of 156 progeny indicates that *su(un-24)-1* is not acting by an osmotic mechanism.

In this chapter, the suppressor, *su(un-24)-1*, of *un-24* temperature-sensitivity was characterized. It is not due to a mutation in the small subunit of ribonucleotide reductase,

unlike the situation in *S. pombe*. Crosses between *eth-1* and *su(un-24)-1* strains suggest that *su(un-24)-1* is not suppressing *un-24* temperature-sensitivity by an osmotic mechanism. In the following chapter, we describe the development of PCR-based markers that are polymorphic between Oak Ridge and Mauriceville strains and apply them to the mapping of *su(un-24)-1*.

Chapter 2

The mapping of *su(un-24)-1* with PCR-based markers

Introduction

A mutation in *N. crassa* strain 164-4 that suppresses a temperature-sensitive mutation in the large subunit of ribonucleotide reductase (*un-24*) was characterized in Chapter 1. Strains carrying the temperature sensitive version of the large subunit of ribonucleotide reductase (*un-24^{ts}*) do not grow at temperatures above ~34°C (Smith *et al.*, 2000a), unless they also carry the suppressor mutation, *su(un-24)-1*. *su(un-24)-1* may suppress *un-24* temperature sensitivity by two mechanisms. In Chapter 1, we provide evidence that *su(un-24)-1* does not suppress *un-24^{ts}* by altering the osmotic conditions within the cell. To examine the second possible mechanism, that *su(un-24)-1* ameliorates protein folding deficiencies of UN-24 at restrictive temperatures, would require molecular characterization of the suppressor gene. Knowledge of the gene's DNA sequence may allow us to determine what the suppressor protein is, and how it interacts with UN-24 at a molecular level.

Two strategies are available for identifying and characterizing *N. crassa* gene mutations. Transformation-based identification of complementing clones can lead to the identification of mutated genes. Several genomic DNA libraries are available to identify genes by complementation (e.g. the Orbach/Sachs pMOcosX libraries [Orbach, 1994]; pSV50 libraries [Vollmer and Yanofsky, 1986]). However, these pre-made libraries can only be used to characterize recessive mutations by complementation. To identify and characterize dominant mutations, new genome libraries would need to be created from the mutated strain.

Another approach that can lead to the characterization of mutations is the identification of genetic markers that co-segregate with the mutation of interest. Co-

segregation is detected as a recombination frequency between the marker and the mutation significantly below 50%. Phenotypic (e.g. auxotrophic, morphological) markers have been primarily used to map genes of interest. Several resources, including special marked strains are available for this purpose through the Fungal Genetic Stock Center (e.g. Perkins, 1972; Metzenberg *et al.*, 1984, McClusky, 2003). Refining the map position of a gene, however, often requires subsequent crosses between strains bearing additional markers in selected areas.

Molecular markers can also be used for co-segregation analysis. RAPD and AFLP markers are most useful for these studies since neither method requires prior knowledge about DNA sequences (Williams *et al.*, 1991; Vos *et al.*, 1995). One difficulty of both of the above methods is that upon finding a marker that co-segregates with the mutation of interest, the co-segregating RAPD or AFLP marker must be isolated and characterized. This can be difficult since AFLP and RAPD PCR profiles are usually complex, with many co-migrating fragments.

A more direct approach is to use previously characterized genetic markers for co-segregation analysis. With the recent sequencing of the *N. crassa* genome (Galagan *et al.*, 2003), it is possible to develop molecular markers for particular regions of the genome. In this chapter, DNA sequences from the *N. crassa* genome project are utilized to develop a set of PCR-based markers for 164-4, an Oak Ridge background *un-24^{ts}*; *su(un-24)-1* strain, and FGSC# 2225, a Mauriceville background wild-type strain. Mapping with this approach could make use of a nearly unlimited number of genetic markers, and additional PCR markers can be developed within specific regions of the genome to refine the position of a gene of interest. Seventeen polymorphic PCR-based

markers were developed in this study that, together with the phenotypic markers available, significantly co-segregated with ~70% of the *N. crassa* nuclear genome and the mitochondrial DNA. Analysis of co-segregation between *su(un-24)-1* and each marker was performed to identify the location of the mutation.

Materials and Methods:

Fungal Strains, Growth Conditions and DNA isolation

The *N. crassa* strains that were used are listed in Table 2-1. All strains were grown on Vogel's minimal medium with supplements as required (Davis and de Serres, 1970). *N. crassa* DNA was isolated using the method of Oakley *et al.*, (1987) as described in Chapter 1. Crosses were performed in test tubes containing synthetic crossing medium (Westergaard and Mitchell, 1947). 164-4 was used as the protoperithecial parent and conidia of FGSC# 2225 were used to fertilize 164-4. Ascospores were collected by adding sterile dH₂O, vortexing the cross tube, and then recovering suspended ascospores in the water. Ascospores were plated onto Vogel's medium and subjected to 60°C for 50 - 60 minutes to induce germination. Single ascospore germlings were isolated under a dissecting microscope and transferred to appropriate growth medium in test tubes. *un-24^{ts}* progeny were identified by growing the progeny at 37 °C on spot testing medium. *un-24^{ts} su(un-24)-1* progeny were identified by preselecting progeny that grow slower than wild type at 37 °C on Vogel's medium and then PCR amplifying the *un-24* gene from each progeny strain with the 6JP11/6JP6 primer pair. Digesting the 6JP11/6JP6 PCR product with the *PvuI* restriction enzyme allowed us to determine if the progeny had an Oak Ridge *un-24^{ts}* or a Mauriceville *un-24⁺* according to the method outlined in Mir-Rashed *et al.* (2000).

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

Strain	Genotype	Source
164-4	<i>un-24; trp-1; inl su(un-24)-1 a</i>	Chapter 1
FGSC# 2225	Mauriceville-1c A Wild-type	FGSC
FGSC# 4317	<i>fl A</i>	FGSC
FGSC# 4347	<i>fl a</i>	FGSC
FGSC# 4450 to # 4487	RFLP mapping strains	FGSC
25-1 to 25-114	111 progeny of the 164-4 X 2225 cross	This study

Primer Design and PCR amplification

The *un-24* genotype was identified using primer pairs as described above. The *nmt-1* primers were provided by Dr. John Vierula at Carleton University, Ottawa, Canada. Primers for *arg-13* and *nit-6* were manually designed to amplify parts of the open reading frame (ORF) from each gene. PNCU04622 primers were manually designed to amplify a region with similarity to *het-6*. The *Pmt* primers were manually designed to amplify a size difference present in Oak Ridge and Mauriceville mitochondrial DNAs (Collins *et al.*, 1981; Taylor *et al.*, 1986). PrimerQuest was used to design all other primers to amplify non-coding regions between adjacent ORFs, as identified by the Whitehead Institute. PrimerQuest, provided over the world-wide-web by Integrated DNA Technologies (www.idtdna.com), was set to design primers of length, T_m , and %GC of 24 bases, 60 °C and 50 % respectively. Primers were synthesized by Sigma Genosys (Oakville, ON) or Qiagen Operon (Alameda, CA). PCRs were performed in a final volume of 25 μ l, and consisted of template DNA (~20ng), 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM of $MgCl_2$, 0.2 mM dNTPs (Roche, Laval, QC), 0.5 μ M of each primer, and about 1.25 Units of *Taq* polymerase (Invitrogen, Burlington ON or prepared at Carleton University, Ottawa ON), were performed in sterile, thin-walled PCR tubes with a TGradient thermocycler (Biometra, Montreal, PQ). PCRs, using DNA from 164-4 and FGSC 2225, consisted of an initial 3 minute denaturation at 95°C followed by 30 cycles of: denaturation for 30 seconds at 95°C, annealing for 30 seconds and extended at 72 °C. Annealing temperatures and extension times for each marker is provided in Table 2-2 and Table 2-3. For each primer pair, PCRs were optimized by calculating the T_m for each primer using the Wallace rule (G or C bases add

Table 2-2: Details of *N. crassa* PCR-based polymorphisms, including marker location, primer sequences, PCR conditions and polymorphism type.

Linkage Group, arm and region ¹	Primer sequence (5' > 3')	Annealing Temp. Extension time (min:secs)	Polymorphism ²		
			Type	OR	M
LG IIL <i>fr</i>	FrP1: CGAGGCAGAGTAGGAAGAACAA FrP2: ATGAGTGTTGGAGGGAAGGTATG	60 °C 1:00	+/-	1012	-
LG IIR <i>met-6</i>	Met6P1: TAAGGACGAAGGAGAGGTTCTGGA Met6P2: AAGAGGTGATGATGGGTGATGGTG	60 °C 2:10	+/-	2106	-
LG IIR <i>arg-13</i>	Arg13P2: GCTCGCACCGCGCTTCAGC Arg13P3: GCTGTAGTGTAACTACTCAAGG	57 °C 2:30	RFLP <i>XmaI</i>	1760, 115	1875
LG IIL <i>un-24</i>	6JP6: GTGCGGGCTTAACCGCTG 6JP11: CTCGGATGAGGTTGCCG	60 °C 1:40	RFLP <i>PvuI</i>	785, 767	1552
LG IIR <i>cya-4</i>	Cya4P1: TCGCCCAATTCCCTCCCAATTCTTC Cya4P2: GCATCTGCTCCCATCTCTTCTCAA	68 °C 2:00	RFLP <i>PstI</i>	1273, 681	1273, ~600
LG IIR <i>nmt-1</i>	NMT1-F: GCGCAACATGTCTACCGA NMT1-2R: ACCGCAGCAAGCCACATT	57 °C 1:30	RFLP <i>SaII</i>	883, 290	~600, ~280, ~290
LG IIIL <i>act-2</i>	Act2P1: ATGGAGAAAGTCTTCGCAAACTGG Act2P2: ATGGTGAGATGGAGAACTGGTTGG	68 °C 1:45	APLP	1706	1200
LG IIIR <i>ro-11</i>	Ro11P1: TGCCGCAGAAAGTTGTTCAATCTGG Ro11P2: GACAACAGTGAGAAAGGTGGTGA	64 °C 1:45	APLP	1669	3000
LG IIVR <i>pyr-1</i>	Pyr1P1: ACCATCACCAGAAATCAAGTCCGAG Pyr1P2: TACCGGCAACTAGGATAACCTTC	64 °C 2:30	RFLP <i>MseI</i>	1300, 1000	1900, 400
LG IIVR <i>pyr-2</i>	Pyr2P1: TGTGTGAACCTTACCTTACGGGTG Pyr2P2: GCCTTGAGGAAGTCAGCCTTGTA	52 °C 1:30	RFLP <i>BglII</i>	627, 482	1109

LG VR NCU04622	NCU04622P3: AAGCAGCGTTCTCCGTTTCG NCU04622P4: GGGGCAGTTGGTGGTAGGG	64 °C 4:00	+/-	3805	-
LG VR <i>pyr-6</i>	Pyr6P1: TGTAGTTAGCTCGGCTTGGTGTC Pyr6P2: GATAGACAGCATGAGCCATGGAA	64 °C 2:30	RFLP <i>FokI</i>	926, 747, 242, 156	600, 747, 300, 242, 156
LG VIL <i>nit-6</i>	Nit6P2: GTAGAGGTGGGCAGGAGGG Nit6P3: CTAGCGACATCAATCCCCGG	60 °C 2:00	RFLP <i>XhoI</i>	1356, 326	1682
LG VIR <i>nuo21.3c</i>	Nuo213cP1: GGAGAACCAAGCGTAACGGTAG Nuo213cP2: TAACAAACCTACCCGCAACAGGAG	64 °C 2:20	RFLP <i>KpnI</i>	1069, 1013	2082
LG VIIL NCU06045	NCU06045P1: GAACAGCTAGCATCCGCTCCTTA NCU06045P2: AAACTCAGTTCTCCCTCACAAGG	60 °C 1:50	RFLP <i>HhaI</i>	699, 587, 332, 145	600, 587, 332, 145, 100
LG VIIR <i>arg-10</i>	Arg10P1: TCGAGGAGGATATCTCCAAGGTGT Arg10P2: CTTGGTTGTCGGTAGGTAGCTGTT	60 °C 1:30	RFLP <i>HindIII</i>	1106, 331	1437
mitoch Pmt	mtP1: CGTATTCTAGGGAAGATGCTCTCCC mtP2: CGCAGTAATACCTTATGGACCGTCA	66 °C 2:00	APLP	1831	~700

¹Marker region, primer names and sequences are based on nearby mapped loci or the Whitehead Institute's NC locus designation

(Version 1) for assembly version 3.

²+/- = Presence / absence of a PCR product; RFLP = restriction fragment length polymorphism with given restriction enzyme; APLP = amplified product length polymorphism. Approximate fragment sizes (bp) are given for each under OR (Oak Ridge) and M (Mauriceville).

Table 2-3: Details of additional *N. crassa* PCR-based polymorphisms, including marker location, primer sequences, PCR conditions and polymorphism type.

Linkage Group, arm and name	Primer sequence (5' > 3')	Annealing Temp. Extension time (min:secs)	Polymorphism ²		
			Type	OR	M
LG IR <i>nuo19.3</i>	Nuo193P1: TTATTAAACCGGAAGGGTGAGACGG	56 °C	RFLP	517, 436, 420,	~430, ~430,
	Nuo193P2: CTAGTGAAGCGCTCATGTATGGCA	2:10	<i>HpaII</i>	265, 209, 8	~430, 265, 209, 8
LG IIR <i>eas</i>	EasP1: CGGCAACTCCTTCCTCATCATCAA	64 °C	RFLP	859, 816, 266,	~840, 266, 11
	EasP2: TAACGGAGCCGGTACATTTCTTG	2:30	<i>HpaII</i>	11	
LG II <i>leu-6</i>	Leu6P1: TGTTCCTGTTAACTCGTGTCTCC	64 °C	+/-	2331	-
	Leu6P2: ATGACCTGACCATCAAGCTACAG	2:10			
LG VR PNCU04662	NCU04662P1: TTCCCTCACCATCACCCACCTTCATC	56 °C	RFLP	720, 680, 397,	720, ~650, 397,
	NCU04662P2: CATCAACACCAACACACATATCCC	2:10	<i>MseI</i>	205, 122	205, 122
LG VR <i>nmr</i>	NMR1: TGAAGAGGAGAATCCGCAAGGTTT	50 °C	RFLP	885, 866	~950, ~800
	NMR2: AATGGTGGAGTTGATTTCCAGAGC	2:30	<i>NruI</i>		
LG VR PNCU04193	NCU04193P1: GCGGGTTGTTGTTGTTAGGAG	68 °C	RFLP	794, 672	~700, 672
	NCU04193P2: ACTGGTGATAGCCCCCAATAGAAG	1:30	<i>PstI</i>		
LG VR PNCU04221	NCU04221P1: TGGGTCAACGCCAGTTATGTCTAC	68 °C	RFLP	2687, 1099	~2000, 1099, ~800
	NCU04221P2: AGGTTGATGAGGAAATCTGGTCCG	4:30	<i>KpnI</i>		
LG VR PNCU04233	NCU04233P1: CGAGTGATGAAGAGGTGCAGATGA	68 °C	APLP	1537	~1400
	NCU04233P2: ATCTCAGTGTGCGACTGACTCTG	1:36			
LG VR <i>Tom70</i>	Tom701: AGGAGATTGAGGATGTCTGTTGGC	60 °C	RFLP	2173, 253	2426
	Tom702: CAAATGGTACTGCTTGTGTTGTG	2:40	<i>KpnI</i>		
LG VR	NCU04275P1: GTAATGCAGCTCCTTCGTGGTA	68 °C	RFLP	1109, 603,	1109, ~350,

PNCU04275	NCU04275P2:	AAGACGGTGCTTGATGGTCGTATC	2:00	MseI	109, 19	~250, 109, 19
LG VR	NCU07247P1:	GATGGCGTTTGGACTGTTGAGTTG	56 °C	+/-	1869	-
PNCU07247	NCU07247P1:	TCGATCTGATAGTTGCCGTTGGTCG	2:40			
LG VR	NCU07154P1:	CGGGTAGTTGGAACAAGGTAGCA	56 °C	RFLP	1035, 409,	~900, 409, 292
PNCU07154	NCU07154P2:	AGGAGGTAGATGGGTTTGATTGGG	1:45	MseI	292	

¹Marker region, primer names and sequences are based on nearby mapped loci or the Whitehead Institute's NC locus designation

(Version 1) for assembly version 3.

²+/- = Presence / absence of a PCR product; RFLP = restriction fragment length polymorphism with given restriction enzyme; APLP = amplified product length polymorphism. Approximate fragment sizes (bp) are given for each under OR (Oak Ridge) and M (Mauriceville).

4 °C and T or A bases add 2 °C to the T_m [Old and Primrose, 1994]) and then performing a PCR with an annealing temperature gradient of 4, 8, 12 and 16 °C below the calculated T_m . The extension time was set to 1 minute for every 1000 base pairs of expected PCR product.

Polymorphism identification and scoring

PCR products were electrophoresed in 0.7% (w:v) agarose gels, as described in Chapter 1, to confirm that amplifications were successful. APLPs and presence/absence polymorphisms were identified using agarose gel electrophoresis. When polymorphisms were not apparent, PCR products were digested with selected restriction enzymes for 2 hours as recommended by the manufacturer (New England Biolabs, Mississauga, ON; GibcoBRL, Burlington, ON; Invitrogen, Burlington, ON). Digested DNAs were separated on 6% acrylamide (27.5:1 acrylamide: bis acrylamide) gels with the Universal Mutation Detection System (BioRad, Mississauga, ON) with a 130 volt power supply for 2 hours, or on 0.7-2% agarose gels. After electrophoresis, acrylamide gels were incubated in 1x TAE (0.04M Tris-Acetate, 1 mM EDTA [pH 8.0]) containing ethidium bromide and visualized using an AlphaImager gel documentation system (Alpha Innotech Corporation, San Leandro, CA).

Once identified in the parental strains, polymorphisms were examined in progeny from a cross between 164-4 and 2225. The *trp-1* and *inl* marker were scored on spot testing medium without tryptophan or inositol. Mating type was scored by inoculating *mat-a* and *mat-A* fluffy strains separately onto Petri plates of synthetic crossing medium. After the fluffy strains grew on the Petri plate, conidial suspensions of each progeny were overlain and after about four days, mating reactions were scored based on the presence of

perithecia. Multiplex PCRs were performed for +/- markers to include positive controls for the addition of DNA. Significant co-segregation of the PCR-based polymorphism and the suppressor was identified by analysis of 2 X 2 contingency tables with Fisher's exact test, available online at www.matforsk.no/ola/fisher.htm.

Analysis of Markers

In order to identify gaps requiring additional markers, the coverage that each of our markers provided was estimated. This consisted of two steps. First, the map distances between adjacent markers in the year 2000 *Neurospora* RFLP mapping kit were determined using the segregation patterns for 34 of the 38 *N. crassa* RFLP mapping kit progeny (Nelson and Perkins, 2000). This data set consists of 38 progeny scored for M (Mauriceville) or O (Oak Ridge) alleles at markers on each of seven linkage groups (LG). Four progeny (FGSC# 4458-4461) were excluded in these calculations since they were all the products of the same meiosis (i.e. a tetrad) and their inclusion would represent a single recombination event twice. In addition to the RFLP mapping data, the *un-24* segregation pattern for the RFLP mapping strains was provided by M. L. Smith. The segregation patterns given in the RFLP mapping kit were converted from "O"s, "M"s and "-"s into "1"s, "0"s and "2"s, respectively. Microsoft Excel was used to score the frequency of recombination events between adjacent markers among the 34 progeny in the RFLP mapping strain data set.

The second part of this analysis involved, i) identifying proxies for our PCR-based and phenotypic markers from amongst the RFLP mapping kit markers and ii) estimating the region that co-segregated significantly with the proxy. The best possible proxies were selected according to the following. First, the locus could be used directly

in cases where the same locus was used in both our dataset and the RFLP mapping data set (e.g.s *fr*, *mat*, *arg-13*, *un-24*, *cya-4*, *trp-1*, *pyr-1*, *inl* and *nuo21.3c*). Second, a RFLP marker was selected as a proxy of our marker if the two markers were located on the same *Neurospora* genome project contig or supercontig (e.g. *met-6* and *aap-2*, *pyr-2* and *nuo29.9*, *pyr-6* and *crp-4*, NCU06045 and *ars-1*). Third, proxies were identified based on co-segregation of the PCR markers to RFLP markers (*Cen-III* and *acr-2*, 00032 and *ro-11*, *Cen-V* and NCU04622). This co-segregation analysis was done with the RFLP mapping strains. Finally, proxies were selected that were from the same chromosomal area (*nmt-1* maps close to *Fsr-17* [McColl *et al.*, 2003]; *nit-6* and *cax* are both on the left arm of LG VI [Perkins *et al.*, 2001; Nelson and Perkins, 2000]; *poi-2* was selected as a proxy for *arg-10* since *arg-10* is approximately 18 map units from *cpc-2* [Perkins *et al.*, 2001] and *cpc-2* is on the same supercontig as *poi-2*). The range of markers that appeared to co-segregate with the proxy was then estimated. In order for this analysis to be useful the genetic and physical distances between the *Neurospora* RFLP mapping cross and our *su(un-24)-1* mapping cross have to be similar.

Results:

Polymorphism development

Seventeen polymorphic PCR-based markers were developed throughout the *N. crassa* genome and the mitochondrial DNA (Figure 2-1). The primer sequences, location and PCR conditions are given for each marker in Table 2-2. *Amplified Product Length Polymorphisms* (APLPs) are evident when PCR products are of different sizes. Three markers, *acr-2*, *ro-11*, and *Pmt*, were APLPs. The presence (+) or absence (-) of PCR

Figure 2-1: The position of PCR-based markers on each of the seven linkage groups and the mitochondrial DNA are indicated by arrows. The nuclear maps are based on Perkins *et al.* (2001). The numbers beneath the mitochondrial map indicate Oak Ridge *EcoRI* fragments as designated in Taylor and Smolich (1985). The mitochondrial map is not on the same scale as the nuclear maps. The mitochondrial genes indicated are as in Perkins *et al.* (2001).

product in the Oak Ridge and Mauriceville strains, respectively was also used as a polymorphism. Three markers, *fr*, *met-6* and PNCU04622, were +/- polymorphisms. Finally, Restriction Fragment Length Polymorphisms (RFLPs) are used to identify internal sequence differences in PCR products that are the same size. Eleven markers, *arg-13*, *un-24*, *cya-4*, *nmt-1*, *pyr-1*, *pyr-2*, *pyr-6*, *nit-6*, *nuo21.3c*, NCU06045 and *arg-10*, were RFLPs.

Co-segregation of the markers and *su(un-24)-1*

From the cross, 164-4 X 2225, one hundred eleven progeny were grown at 37 °C. Thirty one of these progeny were temperature-sensitive. Analysis of *un-24* in 32 progeny capable of growth at 37 °C allowed us to identify 9 *un-24^{ts} su(un-24)-1* progeny and 23 *un-24⁺ su(un-24)-1⁺* or *su(un-24)-1* progeny. Initial analysis of eleven *un-24^{ts}* and seven *un-24^{ts} su(un-24)-1* progeny indicated that *pyr-6* on LG V significantly co-segregated with *su(un-24)-1* (4 mismatches out of 18, two-tailed Fisher's exact test, $p = 0.05$). To refine the position of *su(un-24)-1*, additional primers were designed to amplify regions on either side of *pyr-6* (Table 2-3). In an effort to fill a gap between supercontigs 13 (containing *pyr-6*) and 35 (containing markers PNCU07154 and PNCU07247) we developed *nuo19.3*, a supercontig 12 marker, and PNCU04662, a supercontig 15 marker. Supercontigs 12 and 15 were identified as candidate supercontigs using microsynteny between *N. crassa* and *Magnaporthe grisea* (Magnaporthe Sequencing Project. Ralph Dean, Fungal Genomics Laboratory at North Carolina State University [<http://www.fungalgenomics.ncsu.edu>], and The Center for Genome Research [<http://www.broad.mit.edu>]). *N. crassa* and *M. grisea* have previously been shown to have regions of microsynteny (Hamer *et al.*, 2001; Dawl *et al.*, 2003; Kutil *et al.*, 2004).

The relative position of LG V markers is provided in Figure 2-2. The co-segregation frequencies of these additional linkage group V markers and *su(un-24)-1* did not increase (Table 2-4), leading us to conclude that *su(un-24)-1* is not located in this region.

To avoid false significant co-segregation and to increase the power of developed markers, 15 additional *un-24^{ts}* progeny and 2 additional *un-24^{ts} su(un-24)-1* progeny were examined. None of the 17 PCR-based markers significantly co-segregated with *su(un-24)-1* (Table 2-5). Three markers, *cya-4*, *nmt-1* and Pmt, had p-values close to, or below $p=0.05$. Examination of the *cya-4* and Pmt segregation patterns indicate that the low p-value is due to an over representation of non-parental combinations (i.e. “repulsion”). The similarity of the *nmt-1* segregation pattern to the *su(un-24)-1* segregation pattern prompted the development of additional markers on either side of *nmt-1* (Table 2-3). The position of *eas* and *leu-6* with respect to *nmt-1* is provided in Figure 2-3. The segregation pattern for these additional markers departed from that expected for markers that co-segregate with *su(un-24)-1* (Table 2-5). So even though, with 11 mismatches out of 34 progeny, *nmt-1* co-segregates with *su(un-24)-1*, the co-segregation does not improve with additional markers that flank *nmt-1*. This indicates that *su(un-24)-1* is not likely to be close to *nmt-1*.

Coverage estimates of PCR-based markers

To determine where additional markers need to be developed to fill gaps, areas that co-segregate with our PCR-based markers were estimated using the segregation patterns of the RFLP mapping kit (Figure 2-4). Since, with 34 progeny, 32 map units

Figure 2-2: The position of additional markers near *pyr-6* on LG V.

Table 2-4: The genotype at a) seven loci near *pyr-6*, on linkage group V, in 7 suppressed temperature-sensitive progeny and 11 temperature-sensitive progeny and b) of two loci near *pyr-6* in 2 suppressed temperature-sensitive and 14 temperature-sensitive progeny. “O” indicates that the marker was inherited from 164-4, while “M” indicates that the marker was inherited from FGSC# 2225. “-” indicates that the progeny was not scored at that marker. The two-tailed p-value, at right, was determined using Fisher’s exact test to analyze 2X2 contingency tables between the markers and *su(un-24)-1*.

a)

Progeny #	6	18	19	20	21	23	50	51	54	61	62	suppressed, temperature sensitive						p-value
<i>su(un-24)-1</i>	M	M	M	M	M	M	M	M	M	M	M	37	57	67	79	80	90	106
<i>nmr</i>	O	O	M	O	M	O	M	M	M	M	O	O	O	M	O	O	O	O
PNCU04193	O	O	M	O	M	O	M	-	-	-	-	O	O	M	O	O	O	O
PNCU04221	O	O	M	O	M	O	M	M	M	M	M	O	O	M	O	O	O	O
PNCU04233	M	O	O	O	M	-	M	M	M	M	M	O	M	M	O	O	O	O
<i>Tom70</i>	M	-	O	O	M	M	M	M	M	M	M	O	M	M	O	O	O	O
<i>pyr-6</i>	M	M	O	M	M	O	M	M	M	M	M	O	M	M	O	O	O	O
PNCU07247	M	M	O	M	M	O	M	M	O	M	M	O	M	M	M	M	O	-

b)

Progeny #	2	22	35	38	39	45	48	70	77	92	94	96	103	104	suppressed temperature sensitive			p-value
<i>su(un-24)-1</i>	M	M	M	M	M	M	M	M	M	M	M	M	M	M	46	102		
<i>Tom70</i>	O	M	M	M	M	M	O	O	M	M	O	O	O	O	O	O	O	0.47
PNCU04275	O	M	M	M	O	M	O	O	M	M	O	O	O	O	O	O	O	0.5
<i>pyr-6</i>	O	M	M	M	O	M	O	O	M	M	O	O	O	O	O	O	O	0.5

Table 2-5: The genotype at seventeen PCR-based markers and three phenotypic markers, *mat*, *inl* and *trp-1* of thirty-four temperature-sensitive progeny. In addition, progeny were scored at *eas* and *leu-6*, subsequently developed near *nmt-1* and at *nuo19.3* and PNCU04622. “O” indicates that the marker was inherited from 164-4, while “M” indicates that the marker was inherited from FGSC# 2225. “-” indicates that the progeny was not scored at that marker. The two-tailed p-value was determined using Fisher’s exact test to analyze 2X2 contingency tables between the markers and *su(un-24)-1*.

Figure 2-3: The position of additional markers near *nmt-1* on LG II.

Figure 2-4: The estimated cosegregating regions of linkage group I (a), linkage group II (b), linkage group III (c), linkage group IV (d), linkage group V (e), linkage group VI (f), and linkage group VII (g). *aap-2* was used as a proxy for *met-6*. *Fsr-17* was used as a proxy for *nmt-1*. *Cen-III* and 00032 were used as proxies for *acr-2* and *ro-11*, respectively. *nuo29.9* was used as a proxy for *pyr-2*. *Cen-V* and *crp-4* were used as proxies for NCU04622 and *pyr-6* respectively. *cax* was used as a proxy for *nit-6*. *ars-1* and *poi-2* were used as proxies for NCU06045 and *arg-10* respectively. The positions of the proxies are indicated by arrows.

a) LGI

b) LG II

c) LG III

d) LG IV

e) LGV

f) LGVI

g) LGVII

was found to correspond to the limit of significant co-segregation (Fisher's exact test, two-tailed $p < 0.05$), markers that are within 32 map units of each other were classified as significantly co-segregating. Overall, 29.7 % of the estimated 1399.1 map unit genome is expected not to significantly co-segregate with any of the PCR-based markers used with the 34 progeny utilized in this study.

Based on analysis of Figure 2-4, gaps were found on each of seven linkage groups as listed in Table 2-6. Linkage group VI has the largest uncovered region with approximately 69.8 % of it not covered by a marker. This is followed by LG VII at 35.9 %. Linkage groups III, I, IV, V and II are estimated to have 29.2, 20.5, 15.5, 6 and 3.7 % of each linkage group not covered by a marker.

Discussion:

In this chapter, PCR-based markers that are polymorphic in the Oak Ridge background strain, 164-4, and the Mauriceville background strain, FGSC #2225, were developed. The PCR-based markers are distributed throughout the seven *N. crassa* linkage groups and the mitochondrial DNA (Figure 2-1). Development of these markers was based on the recently completed genome sequences for the Oak Ridge standard strain 74-OR23-1VA (Galagan *et al.*, 2003). The expected restriction fragment patterns, based on the genome sequence, were obtained in our Oak Ridge background strain for all but the *pyr-I* marker. For the *pyr-I* marker, the Mauriceville background strain had the expected Oak Ridge pattern and our Oak Ridge background strain did not. We found the most efficient way to find PCR-based polymorphisms was to use primers placed within adjacent ORFs to obtain amplification products of non-coding, intergenic sequences. Polymorphisms in these non-coding regions are present in most cases because of the

Table 2-6: A list of estimated unchecked intervals and their sizes.

LG	LG Size (map units)	Uncovered intervals	Interval sizes (map units)
I	250.7	AP11h.4 - <i>vma-4</i> 00036 - 12:8B	29 22.4
II	189.8	<i>ssr-5</i> - <i>hH3</i>	7.1
III	262.4	LZE4 - X14:1F AP32a.2 - 00032 <i>cat-3</i> - Tel-IIIIR	3.5 32.9 40.5
IV	149.5	Tel-IVL - <i>sdv-1</i> <i>ndp64</i> - Tel-IVR	18.8 4.4
V	153.3	AP8w.4 - AP5c.3	9.2
VI	251.5	<i>tf2d</i> - <i>con-11</i>	175.6
VII	141.9	Tel-VIIIL - AP11c.3 NP4A9 - AP5i.2	45.6 5.4

many sequence differences between the Oak Ridge and Mauriceville strains (Metzenberg and Grotelueschen, 1993).

The polymorphic PCR-based markers were applied to the mapping of *su(un-24)-1* by co-segregation analysis. Several markers had p-values at ~0.05. One marker, *cya-4* on LG II, had a p-value less than 0.05. Examination of the segregation pattern, however, shows that an over representation of recombinants occurs between *cya-4* and *su(un-24)-1*. This is also the case for the *Pmt* marker, in which the mitochondrial DNA was largely inherited from 164-4. This is consistent with past observations of maternal mitochondrial inheritance in the genus *Neurospora* (Lee and Taylor, 1993).

Although *nmt-1* on LG II and *pyr-6* on LG V have segregation patterns most like *su(un-24)-1*, *su(un-24)-1* is likely not at one of these locations. The segregation pattern of additional markers near both markers depart further from the expected *su(un-24)-1* pattern, suggesting that *su(un-24)-1* is not in either area and that observed co-segregation is by chance.

Regions with multiple markers that significantly co-segregated with *su(un-24)-1* were not identified. Coverage estimates of the linkage map by my PCR-based markers revealed 12 regions where co-segregation would likely not be detected. These non-covered regions represent 20.5, 3.7, 29.2, 15.5, 6.0, 69.8 and 35.9 % of LGs I, II, III, IV, V, VI and VII, respectively. Linkage groups VI and VII have the lowest level of coverage. The development of additional markers in these locations is a priority and may identify the location of *su(un-24)-1*.

The PCR-based markers can be used to identify any type of mutation in four steps. 1) Crossing an Oak Ridge-background *mat-a* strain bearing the mutation of

interest with the Mauriceville-background strain, FGSC# 2225. 2) Locating the mutation to linkage group by identifying PCR-based polymorphism(s) that co-segregate with the mutation. 3) Generation of additional markers in the region of interest to carry out high-resolution mapping of the mutation and to verify co-segregation patterns. 4) Identification of the mutated gene by, for example, complementation with co-segregating open reading frames (ORFs) based on the *N. crassa* genome sequence.

The markers developed in this chapter can also be used to improve upon the *Neurospora crassa* genome project. In assembly version 3, 106 of 173 supercontigs larger than 2 kb were not anchored to a linkage group and nine were anchored to two linkage groups (Galagan *et al.*, 2003). For example, supercontig 12 contained two markers from linkage group I and three markers from linkage group VI and was assigned to both linkage groups. The segregation pattern for the supercontig 12 marker, *nuo19.3*, placed it on linkage group I, between *met-6* and *arg-13*. Similarly supercontig 35 was also assigned to linkage group III and V in assembly version 3 (Galagan *et al.*, 2003). The segregation patterns of supercontig 35 markers, PNCU07154 and PNCU07247, allowed us to assign supercontig 35 to the right of *pyr-6* on linkage group V. We were also able to orient the supercontig; PNCU07154 was telomere proximal, while PNCU07247 was centromere proximal.

In this chapter, polymorphic PCR-based markers were developed between Oak Ridge laboratory and Mauriceville wild-type strains. The PCR-based markers were used in conjunction with three phenotypic markers to map *su(un-24)-1*. Although a few regions may contain *su(un-24)-1*, the development of additional markers in these regions suggests that *su(un-24)-1* is not at these positions. No region was identified that likely

contains *su(un-24)-1*, leading us to assess the coverage of the genome that the markers provide. This assessment revealed that the PCR-based and phenotypic markers covered over 70% of the genome and highlighted several gaps that may contain *su(un-24)-1*.

Summary and Conclusions

Ribonucleotide reductase (RNR) is an enzyme that converts RNA precursors into DNA precursors, and is thus essential to life. A suppressor, *su(un-24)-1*, of a temperature-sensitive mutation in the *Neurospora crassa* large subunit of ribonucleotide reductase (*un-24*) has been identified. Characterization of the growth rates of an *un-24 su(un-24)-1* strain at various temperatures on medium without or with sorbitol (0.5 or 1 M) indicates that *su(un-24)-1* is an effective suppressor of *un-24* temperature-sensitivity under all the conditions examined.

The mechanism that *su(un-24)-1* utilizes to suppress *un-24* temperature sensitivity was examined. *un-24* is believed to cause an osmotically-remediated, temperature-sensitivity due to improper protein folding at restrictive temperatures. As a result, *su(un-24)-1* may then suppress *un-24* temperature sensitivity by altering the osmotic condition within the cell, or by interacting directly with the protein to allow proper protein folding. Since the small subunit of ribonucleotide reductase (*rnr-2*) of *Schizosaccharomyces pombe* had previously been shown to suppress a temperature-sensitive mutation in the large subunit of ribonucleotide reductase (Fernandez Sarabia *et al.*, 1993), we characterized the *N. crassa rnr-2*. Segregation analysis of *rnr-2* and *su(un-24)-1* indicates that they are not the same gene.

A prediction based on the osmotic mechanism hypothesis was tested by examining the progeny of a cross between a *su(un-24)-1* strain and a *eth-1* strain. Since *eth-1* is an osmotically-remediated mutation, if *su(un-24)-1* is operating by an osmotic mechanism, it should suppress the *eth-1* mutation. *su(un-24)-1* suppression of *eth-1* would result in one quarter of the progeny with the *eth-1* phenotype. If *su(un-24)-1* does

not suppress *eth-1*, one half of the progeny with the *eth-1* phenotype. The observation of 81 *eth-1* progeny of 157 progeny examined indicates that *eth-1* is not suppressed by *su(un-24)-1*. Since *eth-1* is not suppressed by *su(un-24)-1*, *su(un-24)-1* appears not to suppress *un-24* temperature-sensitivity by an osmotic mechanism. This conclusion is supported by the growth rates of *un-24* and *un-24 su(un-24)-1* strains at restrictive temperatures with or without osmotic remediation. *un-24* osmotic remediation ceases at high temperatures but *un-24 su(un-24)-1* strains are capable of growth at all restrictive temperatures. This further suggests that *su(un-24)-1* is not operating by an osmotic mechanism.

In Chapter 2 of this thesis, a mapping approach using PCR-based markers was employed in order to identify *su(un-24)-1*. Seventeen polymorphic markers were developed between 164-4, an *un-24 su(un-24)-1* Oak Ridge laboratory strain and FGSC #2225, a Mauriceville wild type strain. Analysis of the PCR markers in conjunction with several available phenotypic markers revealed two locations that may contain *su(un-24)-1*. Further examination of these regions, however, suggest that *su(un-24)-1* is not at these locations. Estimation of regions of the genome that significantly co-segregate with a marker suggest that the markers provide coverage of over 70 % of the genome. Several unmarked regions were highlighted that may contain *su(un-24)-1*. Additional markers in these regions are currently being developed and may help to locate and identify *su(un-24)-1*. These markers can be used to map any mutation in an Oak Ridge background strain.

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