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The Isolation and Characterization of a Putative
Thiamine Biosynthetic Gene in *Neurospora crassa*

by

Dorothy McColl, B. Sc. (Hons.)

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

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

August, 1997

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"The Isolation and Characterization of a Putative Thiamine
Biosynthetic Gene in *Neurospora crassa*"

submitted by Dorothy McColl, B.Sc.
in partial fulfilment of the requirements
for the degree of Master of Science.

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Abstract

A polyclonal antiserum, raised against a crude cell wall extract of *Neurospora crassa*, recognized ten to fifteen prominent antigens in whole cell extracts as well as three that were enriched in the cell wall fraction. Immunogold labeling confirmed that the polyclonal antibodies recognized antigenic sites predominantly associated with the cell wall but there was also some labeling in the cytoplasm. Immunoscreening the Lambda Zap I⁺ cDNA expression library with this antiserum resulted in the isolation of six positive clones, one of which was selected for further study. Sequence analysis revealed an ORF of 1029 bp that encodes a putative 342 aa polypeptide with one 59 bp intron, a TGA termination codon, a predicted TATA box, and an inverted binding site for the *CPC1* regulatory protein. The DM1 clone has strong amino acid identity with the tightly regulated and completely thiamine-repressible *nmt1* of *Schizosaccharomyces pombe*, *Aspergillus parasiticus*, and *Saccharomyces cerevisiae*. In contrast to these three organisms, the *nmt1* gene of *N. crassa* is only moderately repressed by thiamine. Single amino acid limitation using 3-amino-1,2,4-triazole to inhibit histidine biosynthesis resulted in upregulation of *nmt1* expression. However, this upregulation was blocked by exogenous thiamine. Western blot analysis using cDM1 affinity purified antibodies demonstrated that the fusion protein expressed by the cDM1 phage is approximately 37 kD, is not *N*-glycosylated, and is

not likely associated with the *N. crassa* cell wall. Functional cDM1/pMA1-c2 fusion clones were prepared for the purification and further characterization of the *NMT1* protein.

Dedication

This thesis is dedicated to the spirit and memory of:

Donald Wightman (22 June, 1963 - 08 January, 1997)

and Missy (07 June, 1983 to 09 April, 1997).

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"I get by with a little help from my friends".

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Appendix 5. Mid-range molecular weight standard fragment sizes.

Appendix 6. Recombination event between Mauriceville and Oak Ridge parental strains.

Appendix 7. Formation of thiamine and thiamine pyrophosphate (Young, 1986).

Appendix 8. Linkage group II (Metzenberg et al., 1984).

Abbreviations

A_{nm}	absorbance (wavelength nm)
amp	ampere
Amp ^r	ampicillin resistance gene
APS	ammonium persulfate
AT	3-amino 1,2,4-triazole
ATP	adenosine triphosphate
BSA	bovine serum albumin
ccg-2	clock controlled gene
cDM1	complementary DM1 DNA
cDNA	complementary DNA
C:I	96% chloroform: 4% isoamyl alcohol
Ci	Curie
CPC1	cross pathway control regulatory protein
cpm	counts per minute
crps-7	cytoplasmic ribosomal protein small subunit
DAPI	4', 6' diamidino-2-phenylindole
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
DEPC	diethyl pyrocarbonate
dGTP	deoxyguanosine triphosphate
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
ECL	enhanced chemiluminescence
EDTA	N, N-ethylenediaminetetraacetic acid
EtOH	ethanol
FGSC	Fungal Genetics Stock Centre
FITC	fluorescein isothiocyanate
Fsr-17	five S ribosomal gene -17
gDM1	genomic DM1 DNA
gDNA	genomic DNA
IPTG	isopropyl- β -D-thiogalactoside
K-Ac	potassium acetate
kilobase	kb
kbp	kilobase pair
kDa	kilodalton
LB(A)	Luria-Bertani (Ampicillin)
MeOH	methanol
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger RNA
NaAc	sodium acetate
nmtI	no message in thiamine
O/N	overnight
OsO ₄	osmium tetroxide
³² P	radioactive isotope of phosphorous
PA	50 mL polyallomer centrifuge tube
PBS(-T)	phosphate buffered saline (0.05% Tween-20)
P:C:I	50% phenol: 48% chloroform: 2% isoamyl

	alcohol
PEM	proteinaceous extracellular coat
PMSF	phenylmethylsulfonylfluoride
PP	50 mL polypropylene centrifuge tube
psi	pounds per square inch
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
³⁵ S	radioactive isotope of sulphur
SDS	sodium dodecyl sulphate
SSC	150 mM NaCl, 15 mM sodium citrate (pH 7.0)
SSPE	150 mM NaCl, 10 mM NaH ₂ PO ₄ , 1 mM EDTA (pH 7.4)
STE	1 0.1 M NaCl, 10 mM Tris.Cl (pH 8.0), 1 mM
EDTA	(pH 8.0)
TCA	trichloroacetic acid
TDP	thiamine diphosphate
TE	10 mM Tris, pH 7.6; 1 mM Na ₂ EDTA
TEMED	N, N, N', N'-tetramethylethylenediamine
TPCK	n-tosyl-L-phenylalanine chloromethyl ketone
Tris-HCl	Tris(hydroxymethyl)aminomethane
TBS(-T)	Tris Buffered Saline: 20mM Tris, pH 7.5; 150mM NaCl, (0.05% Tween-20)
UV	ultraviolet
V	volts
VMM	Vogel's Minimal Medium
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactoside

I. INTRODUCTION

A. The importance of fungi

In recent years, increased attention has been given to fungal research as more groups recognize the commercial value of these organisms. The ability of some fungi to produce and excrete large quantities of hydrolytic enzymes has been very beneficial for biotechnology companies (Wessels, 1993). Most agricultural interest has traditionally been limited to pathogenic plant/fungal interactions, however, the focus has begun to shift in the direction of potentially symbiotic relationships between plants and fungi (Wessels, 1993; Read, 1991). The rapidly increasing population of immunocompromised patients has demonstrated the medical need for safe and effective antifungal agents. In this instance, the fungal cell wall and its development may serve as the target for such therapeutic agents (DeBono and Gordee, 1994).

B. *Neurospora crassa*

Neurospora crassa is a filamentous fungal saprophyte that has been isolated from charred vegetation and sugar cane cuttings, and can also be found as a contaminant in bakeries (Webster, 1980). The genus belongs to the class Ascomycetes whose characteristic feature is the sexually-produced spores which develop within a sac or ascus, typically containing eight ascospores (Webster, 1980). Their vegetative structure consists of septate filaments each containing several nuclei. Electron micrographs have shown that the septum is perforated

by a pore that is wide enough to allow the passage of mitochondria and nuclei, enabling cytoplasmic continuity between adjacent segments (Shatkin and Tatum, 1959).

Neurospora crassa has been a useful organism for studying basic genetic mechanisms, biochemical pathways, and cellular physiology (Perkins, 1992). This mold possesses a number of features which make it an excellent choice for molecular studies of fundamental eukaryotic processes. These features include: a small haploid genome (4.7×10^7 bp), extensive knowledge of its metabolism and nutrition, and a considerable amount of genetic and cytogenetic information (Perkins, 1992).

C. Fungal morphology

It was originally suggested by deTerra and Tatum (1961, 1963) that the rigid cell wall of *Neurospora* and other fungi must play an important role in determining the morphology of the organism. This hypothesis was based on the observation that treatment of the elongate hyphal filaments with carbohydrases gives rise to spherical, osmotically sensitive protoplasts.

The tubular morphology of fungal hyphae derives from the fact that cell wall expansion is primarily confined to the hyphal apex (Sietsma et al., 1995). Vesicles fill the apical zone of growing hyphae and the distribution of apical vesicles was found to correspond to the gradient of wall synthesis (Gooday and Trinci, 1980). This would suggest that vesicles supply the necessary components for extension of the hyphal

wall. An attractive model for fungal growth developed by Bartnicki-Garcia et al. (1989) proposes that the continuous, unidirectional movement of a vesicle-generating or vesicle-releasing apparatus, responsible for delivering these enzymes membrane-bound and precursors, will generate the hyphal morphology.

D. Composition of the *Neurospora* cell wall

Early studies of the chemical composition of the *Neurospora* cell wall revealed that it consists predominantly of polysaccharides (deTerra and Tatum, 1961; Mahadevan and Tatum, 1965). As in other ascomycete fungi, these carbohydrates are now known to be primarily chitin and glucans (Hunsley and Burnett, 1970). Chitin is the most characteristic polysaccharide of fungal cell walls. It is an unbranched polysaccharide made of *N*-acetylglucosamine (GlcNAc) joined through β -1,4 bonds whose chains are able to crystallize and form long microfibrils (Ruiz-Herrera, 1992).

Glucans are more abundant than chitin and include the β -glucans containing variable proportions of β -1,3 and β -1,6 linkages. Unlike chitin, the glucans form a much more amorphous matrix. Other minor components include proteins and substances such as lipids, pigments, and inorganic ions (Gander, 1974).

Hunsley and Burnett (1970) used electron microscopy, coupled with enzymatic digestion of chitin, glucans or proteins, to examine the composition of the cell wall of *N.*

crassa. In their model derived from these studies, the hyphal wall is a multi-layered composite structure consisting of a primary wall next to the plasmalemma and an outer secondary wall. In the tapered region at the tips of growing hyphae known as the extension zone (appendix 1), the cell is bounded by a 50 nm primary wall consisting of a 20 nm layer of chitin microfibrils covered by a thin layer of what is thought to be proteins and glycoproteins (Gooday and Trinci, 1980). In *N. crassa*, the primary wall formed at the hyphal tip eventually develops into the inner layer of the mature wall (Gooday and Trinci, 1980).

Behind this apical extension zone, the maturing wall gradually expands to a thickness of about 125 nm, within 250 μ m of the tip; the result of secondary wall growth (Trinci, 1978; Gooday and Trinci, 1980). This secondary wall consists of an apparent glycoprotein reticulum which is embedded in an amorphous layer of glucans and a laminarin-like glucan with predominantly β -1,3 linked with some β -1,6 linkages (Gooday and Trinci, 1980; Garraway and Evans, 1984). These regions of mixed mature wall are not found as very discrete layers, but rather they tend to grade into each other (Burnett, 1979).

Based on studies of several different fungi, it appears that the mechanically nonrigid and plastic hyphal apex is eventually transformed into a firm and elastic mature fungal cell wall (Sietsma et al., 1995). Trinci and Collinge (1975) suggested that rigidification might be the result of the

formation of cross-linkages between existing wall polymers. Studies have shown that stretches of these chitin polymers are deacylated immediately after synthesis and before crystallization has occurred (Ariko and Ito, 1975; Davis and Bartnicki-Garcia, 1984). This is followed by a delay of several minutes between enzymic polymerization and spontaneous crystallization of the chitin polymer chains. During this interval, the insoluble and partly deacylated cationic chitin chains are able to bind to the anionic β -glucans and form an insoluble complex in the fungal wall (Datema et al., 1977b). Further evidence of cross-linking was provided by Sietsma and Wessels (1981) when they described the occurrence of covalent linkages between the branched β -1,3 glucans and chitin chain.

In all Ascomycetes examined, depolymerization of chitin with chitinase releases a soluble form of β -glucans. According to Wessels (1990), Ascomycetes always appear to contain a chitin/ β -glucan complex in which the two distinct polymers are covalently linked to each other, while each polymer can form hydrogen bonds with homologous chains. Structural differences are apparent when one compares a newly-constructed elastic apex to the subapical wall that is more rigidified and plastic in its form. The microfibrils and the outer cementing matrix of glucans function to resist stretching and compressing of the cell wall, respectively (Sietsma et al., 1995).

E. Fungal cell wall proteins

Proteins typically constitute 3 to 20% of the fungal cell wall's dry weight although this value depends on the organism's form or stage of development (Ruiz-Herrera, 1992). For example, the thermal dimorph *Paracoccidioides brasiliensis* has a larger amount of protein in the mycelial form (24 to 41%) than in the yeast form (Kanetsuna et al., 1969). The presence of a peptide component of the hyphal wall of *N. crassa* was first suggested by the early studies of deTerra and Tatum (1963) and Mahadevan and Tatum (1965). Wrathall and Tatum (1973, 1974) reported that 10% of the dry weight of the hyphal wall consists of protein and that this protein could be released from the wall by alkali extraction. As described earlier, Hunsley and Burnett (1970) were then the first to suggest that proteins may constitute a discrete wall layer. In addition, these studies revealed the presence of loosely bound proteins associated with the fungal wall. Although considerable research has been focused on the fungal cell wall since these early studies, characterization of the protein component remains incomplete.

More recently, Martinez et al. (1991) have described a proteinaceous extracellular coat (PEM) which is excreted into the liquid growth medium by the slime variant of *N. crassa*. This triple mutant strain is unable to synthesize glucans and chitin *in vivo*, and it is propagated as protoplast-like cells in an osmotically stabilized medium. This PEM is thought to

consist of cell wall proteins since the electrophoretic pattern of these proteins is similar to proteins which co-purify with the cell wall of wild type strains. In addition, there is some immunological cross-reactivity between the PEM proteins and wall-associated proteins. It appears that the proteinaceous component that is typically attached to the normal cell wall is being released uninhibited into the medium in the absence of the structural polysaccharides by the slime strain (Martinez et al. 1991).

Most proteins present in the cell walls of yeasts are glycoproteins that serve in a structural capacity and various other roles (Gander, 1974). For example, following digestion *C. albicans* cell wall preparations with Zymolyase, two principle proteinaceous components have been released, a high molecular mannoprotein material (>120 kDa) and 31 - 34 kDa mannoprotein.

Glycoproteins are made up of a protein moiety and covalently-bound carbohydrate chains which are joined through two different types of linkages. The O-linked glycans are attached by a glycosidic bond between the oligosaccharide and the hydroxyl group of a threonine or serine. The sugar moiety of O-linked glycans can be quite variable depending on the nature of the glycoprotein. In *N. crassa*, mannose and galactose-containing oligosaccharides have been released from O-linked cell wall glycoproteins (Nakajima et al., 1984). In contrast, the N-linked glycans are universally joined through

N-acetylglucosamine (GlcNAc) to an asparagine residue. The next four sugars are also the same in all glycoproteins studied to date; a GlcNAc followed by 3 mannose units. Both GlcNAc residues are joined through β -1,4 linkages; the first mannose is bound by a β -1,4 linkage, and the other two mannosyl units are bound to this mannose by an α -1,6 bond and an α -1,3 bond. A common sequence surrounding the asparagine is -X-Asn-X-Thr/Ser- where X may be any amino acid residue (Mathews and van Holde, 1990; Ruiz-Herrera, 1992). Nakajima et al. (1984) suggested that both kinds of linkages could be found associated with *N. crassa* glycoproteins.

Another class of cell wall proteins has been identified in several fungi including *N. crassa* (Dempsey and Beever, 1979). Hydrophobins are low molecular weight, hydrophobic, cysteine-rich, proteins that are responsible for the hydrophobic characteristic of aerial fungal structures (Wessels, 1996). Two hydrophobins of *Schizophyllum commune* are known to coat the outer surface of aerial hyphae and fruiting bodies with an insoluble protein film. The hydrophobins are excreted as monomers which self-assemble into a network of parallel rodlets that are typically 10 nm thick (Wessels, 1996). Regular arrays of small fibers or rodlets are a common surface feature of many dry-spored fungi including *N. crassa* (Dempsey and Beever, 1979). It was demonstrated that the eas (easily wettable) mutant of *N. crassa* did not produce the small fibers which suggested that

these structures were responsible for making the spores water repellent; a conclusion confirmed by transferring the hydrophobin-encoding *eas* (*ccg-2*) gene to an *eas* mutant of *N. crassa* (Bell-Pedersen et al., 1992).

Some cell wall proteins are involved in specific recognition of fungi (Ruiz-Herrera, 1992). For example, the yeast *S. cerevisiae* has cell surface receptors characterized by seven transmembrane segments (7-TMS) that are involved in the agglutination of cells prior to mating (Dohlman et al., 1991). The two haploid mating types of the yeast, MAT α and MATa, each secrete peptide pheromones, α -factor and a-factor respectively, that bind to the specific 7-TMS receptor on the surface of cells of the opposite mating types (Cross et al., 1988; Marsh et al., 1991). Pheromone binding initiates a set of physiological changes required for conjugation.

Filamentous fungi can discern between self and non-self, a feature that is important because they undergo hyphal fusion either within an individual colony or between hypha of genetically different colonies (Glass and Kulda, 1992). To prevent heterokaryon formation between isolates of different backgrounds, the filamentous fungus *N. crassa* has at least 11 loci involved in the regulation of this process (Perkins, 1988). The product of one such locus, *het-C^{OR}* (where *het* refers to heterokaryon incompatibility), features a glycine-rich C-terminal domain that is typical of various extracellular and cell envelope proteins which suggests that

HET-C^{OR} might be associated with the fungal cell wall (Saupe et al., 1996)

F. Fungal cell wall-associated proteins

At least some of the enzymes involved in the biosynthesis or cross linking of chitin and glucan chains will be part of this wall associated group of proteins. Active forms of the enzymes for chitin and β -1,3 glucan synthesis are thought to be present in the apical plasma membrane (Sietsma and Wessels, 1990). These proteins are manufactured in the endoplasmic reticulum of the cytoplasm and are transported to the apex in vesicles where they are inserted into the wall when the vesicles fuse with the apical plasma membrane (exocytosis). It has been demonstrated that the contents of some of the vesicles have chitin synthase activity and are called chitosomes (Bartnicki-Garcia et al., 1979). Chitin synthases have been localized to chitosomes that are most plentiful at the hyphal tip (Sietsma et al., 1996). Crude membranous fractions from several fungi have also been used as a source of glucan synthetase activity to demonstrate the *in vitro* synthesis of β -glucans from uridine-5'-diphosphate-*D*-glucose (UDPGlc) (Ruiz-Herrera, 1992).

In *N. crassa*, the chitin synthase 1 structural gene (*chs-1*) is thought to play a major role in cell wall biogenesis since *chs-1* mutants are slow-growing and form hyphae with morphological abnormalities (Yarden and Yanofsky, 1991). In contrast, *chs-2* encodes a non-essential product since mutant

progeny were indistinguishable from the wild type. However, these strains do have reduced chitin synthase activity (as measured *in vitro*) (Beth Din and Yarden, 1994). CHS-4 may serve as an auxillary enzyme for chitin synthesis since *chs-4*(RIP) (Repeat-Induced Point) mutants show wild type growth under standard conditions (Beth Din and Yarden, 1996). However, when grown in the presence of sorbose (a carbon source that induces morphological changes accompanied by elevated chitin content), chitin levels in the *chs-4* strain were markedly lower than those observed in the wild type.

Cell walls also maintain some plasticity and mature walls are broken down to permit hyphal branching and hyphal fusions. As early as 1970, Mahadevan and Mahadkar had described a cell-wall-bound enzyme in *N. crassa* that was capable of hydrolyzing the β -1,3 glucan wall polymer and functioned as part of the branching process. Chitinases hydrolyze the β -1,4 glucosidic bonds of chitin which yields chitin oligomers by endo-activity and diacetyl chitobiose by exo-activity (Ruiz-Herrera, 1992). It has long been suspected that endochitinase activity is involved in apical extension of fungal hyphae (Gooday and Gow, 1990). Intracellular locations of chitinases involved in the regulation of chitin degradation in fungal cells have included the cell wall of *Aspergillus* (Polacheck and Rosenberger, 1978) and the periplasmic space of *S. cerevisiae* (Elango et al., 1982). The yeast chitinase contains about 18% carbohydrate, more specifically it is a non-asparagine linked mannoprotein

(Correa et al., 1982). Chitinase activity has also been demonstrated in *C. albicans*, both in soluble cytoplasmic extracts and microsomal preparations (Dickinson et al., 1991). Three chitinase-encoding genes (*cht1*, *cht2*, and *cht3*) have been isolated from a *C. albicans* expression library and it has been shown that transcription of *cht2* and *cht3* is greater in the yeast form than in the mycelial form (McCreath et al., 1995). A transcript of *cht1* could not be detected in either growth form.

In addition to its basic exo-skeletal function, the cell wall serves as a protective barrier between the fungus and the environment. However, the wall's physical attributes also render it impermeable to many of the larger nutrients in culture medium that the fungus requires for growth. Uptake of nutrients is accomplished by the accumulation and secretion of hydrolytic enzymes that digest the impermeable compounds into smaller products. The smaller products can then be transported through the cell wall where they are further metabolized. Pho4, an acid phosphatase of the fission yeast, *Schizosaccharomyces pombe*, is an example of such an enzyme (Schweingruber et al., 1986). It is a cell-surface glycoprotein that is mainly localized in the cell wall and partly secreted into the growth medium. Pho4 catalyses the removal of phosphate from its organic substrate before the phosphate can be transported into the cell (Schweingruber et al., 1986).

The fungal cell wall is a complex, multilayered, dynamic, structure that is modified for a variety of different functions such as mating, dispersal, invasion, and adaptation to hosts. The ability of *Candida albicans* to change from a yeast to a mycelial morphology by altering the composition of the cell wall is thought to give additional advantages to this pathogenic fungus in its ability to simultaneously colonize different tissues and avoid host defence mechanisms (Eroles et al., 1997). In addition, many fungi such as the smut fungus *Microbotryum violaceum* produce long, flexible, unbranched, appendages termed fimbriae which are required for cell-to-cell adhesions during mating and pathogenesis (Celerin et al., 1995). Since it is the outermost fungal wall surface that functions as an interface during all of these interactions, it must contain key factors involved in cell-to-cell communication. This communication allows the cell to regulate not only its rate of growth but its entire life cycle as it responds to positive or negative tropic stimuli (Ruiz-Herrera, 1992). The cell surface proteins such as fimbrial subunits and acid phosphatase are also known to be glycosylated (Castle et al., 1992).

The fungal cell wall also undergoes changes in response to environmental cues. Ambient light is a major factor that influences fungal development and morphogenesis. For example, expression of the *N. crassa* hydrophobin-encoding easily wettable eas gene (also known as clock controlled gene 2 [*ccg-*

2)), is positively regulated by light (Dunlap, 1993). Under favourable conditions fission yeast reproduce by means of the mitotic cell cycle. When glucose and nitrogen are in limiting amounts, the cells become sexually agglutinative as a result of changes to the protein component of the outer cell wall and diploid zygotes are formed by conjugation (Egel, 1989). Furthermore, low levels of the co-factor thiamine have been shown to inhibit sexual agglutination and zygote formation in *S. pombe* (Schweingruber and Edenharter, 1990). Little is known about how nutritional signals are sensed by the cell and translated into such dramatic results.

Recently, another class of fungal cell wall-associated proteins, the galectins, was described in the inky cap mushroom, *Coprinus cinereus* (Cooper et al., 1997). Previously known for their animal origin, the galectins belong to a genetically related family of β -galactose-binding lectins. Their affinity for oligosaccharides found on glycoconjugates on cell surfaces or in extracellular matrix has suggested that galectins function extracellularly by binding to such ligands.

Surprisingly, several enzymes involved in primary metabolism have also recently been shown to be cell wall-associated. A glycolytic enzyme has been identified in *C. albicans* that has extensive sequence homology with genes specifying 3-phosphoglycerate kinase (3-PGK) which catalyzes the substrate-level phosphorylation of ADP from 1,3-bisphosphoglycerate (Alloush et al., 1997). 3-PGK has been

detected at the outer surface of the cell membrane and throughout the cell wall, as well as in the cytoplasm; even though PGK activity was observed only in the cytosolic fraction. While screening for genes in cDNA expression libraries of yeast cells and mycelia whose products are components of the *C. albicans* cell wall, Eroles et al. (1997) identified two known cytoplasmic proteins, enolase and Hsp70p (Sundstrom and Aliaga, 1992; Pelham, 1986) that were also associated with the cell wall. Enolase catalyzes the reaction that generates the super-high-energy compound, phosphoenolpyruvate, which participates in the second substrate level phosphorylation of glycolysis (Mathews and van Holde, 1990).

To further the understanding of the protein component of the *N. crassa* cell wall, I have used polyclonal antibodies raised against a crude cell wall extract to demonstrate that unique proteins exist in this fraction. These same antibodies were used to screen an *N. crassa* mycelial cDNA expression library in Lambda Zap I⁺ from which 6 positive clones were isolated; one of these clones, cDM1, is characterized in this thesis.

G. Hypothesis, rationale, and objectives

(1) HYPOTHESIS

The hypothesis for my research project is that a discrete

group of proteins exists as an integral part of the *N. crassa* cell wall. Since many of these proteins are likely to co-purify with the cell wall fraction, it should be possible to clone and characterize their corresponding coding sequences following selection by immunological screening techniques.

(ii) RATIONALE:

The protein composition of the cell wall from *N. crassa* is not well defined (Martinez et al., 1991). Even the fundamental question of whether proteins are real components of fungal walls has persisted as a controversial issue for a long time (Ruiz-Herrera, 1992). Some researchers are still of the opinion that cytoplasmic proteins may become tightly adsorbed to the walls during cell breakage and contaminate the preparations (Ruiz-Herrera, 1992). Therefore, when cell walls are purified and a significant fraction of the material is identified as being proteinaceous, there is an element of uncertainty as to the source of that protein (Garraway and Evans, 1984).

(iii) OBJECTIVES

One of the objectives was to demonstrate that there is a distinct set of proteins that are primarily associated with the cell wall. To achieve this, polyclonal antiserum was raised against a crude cell wall extract of *N. crassa* mycelia. Western blotting and electron microscopy with immunogold

labeling were used to demonstrate that the antiserum primarily recognizes antigenic sites at the cell wall and at least some of these antigens are proteins that are enriched in the cell wall fraction. The other objective was to demonstrate the feasibility of using this complex polyclonal antiserum to identify clones whose cDNA encode proteins which co-purify with the fungal cell wall.

This thesis also reports on the initial characterization of one of the clones recovered in this immunological screen.

II. MATERIALS AND METHODS

A. Maintenance and storage of cultures

(i) **Fungal cells:** *Neurospora crassa* wild type strain (74-OR23-1A) was grown on VMM [2% (v/v) 50X VMM stock solution (per litre contains: 127 g Na_3 citrate $\cdot$ 2H $_2$ O, 250 g KH_2PO_4 , 100 g NH_4NO_3 , 10 g $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, and 5 g $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ pre-dissolved in 25 mL dH $_2$ O); 1.5% (w/v) sucrose; 0.1% (v/v) VMM trace elements; 0.1% (v/v) biotin stock] (Vogel, 1964). A few crystals from the silica stock culture were applied to the surface of the slant which was then shaken to maximize the exposure of the organism to the medium. The slants were incubated at room temperature. Fresh stock cultures were maintained by subculturing once a month onto VMM slants.

(a) **thiamine experimental cultures:** To study the effect of thiamine on the *nmt1* gene expression, liquid medium was prepared by adding 25 mL of the appropriate thiamine-HCl (Sigma) stock solutions to a 250 mL erlenmeyer flask containing 20 mL of 2X liquid VMM. The thiamine stock solutions of 0, 0.05, 0.10, 0.20 0.5, 1.0, 2.0 and 5.0 μM of thiamine-HCl (337.3 FW) were prepared in autoclaved dH $_2$ O. These thiamine solutions were filter sterilized with a 0.2 μm syringe filter. *N. crassa* conidia (approximately 5×10^8 spores) were harvested from VMM slant cultures by resuspension in 5 mL of sterile dH $_2$ O and used to inoculate 45 mL of VMM/thiamine liquid medium. Fungal cultures were incubated O/N at 25°C with mechanical agitation of 250 rpm and the cells

were harvested on Mira Cloth™ (CalBiochem) using vacuum suction to remove the culture medium. The mycelia were rinsed with dH₂O and pressed with paper towels to remove the excess liquid. The tightly packed mycelia were cut into 0.5 cm² cubes and stored at -70°C.

(b) CPC experimental cultures: To test for cross pathway control (Orbach et al., 1990) of the pDM1 cloned gene, the following 5 mL supplements were added to VMM (50 mL final volume): 0.05 M thiamine-HCl (Sigma), 1X MEM amino acid supplement (Sigma), or 5 mM 3-amino-1,2,4-triazole (Sigma) as indicated in the figure legends. The 1X amino acid supplement contained: arginine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, and valine. The mycelial cultures were grown, harvested and stored as previously described.

(ii) Bacteria: *Escherichia coli* strains BB4, DH5αF' and BNN132 were streaked out on LB plates (1% tryptone, 0.5% yeast extract, 1% NaCl, and 1 N NaOH was used to adjust the pH to 7.5; 1.5% agar) plates or grown in liquid LB medium in an agitating water bath (250 rpm). All were incubated at 37°C unless otherwise indicated. Liquid stocks of these cultures were prepared in 15% glycerol and stored at -70°C.

B. Preparation and transformation of competent bacteria

Competent *E. coli* cells were prepared essentially as described by Sambrook et al. (1989). A single colony of *E.*

coli DH5 α F' was used to inoculate 50 mL of LB which was then incubated O/N at 37°C with agitation (250 rpm). One mL of the fresh O/N culture was used to inoculate 100 mL of LB. The cells were grown at 37°C with agitation to an A₆₀₀ of 0.5 to 0.7 (early to mid log growth phase). To harvest the cells, the contents of the flask were poured into 2 sterile, ice cold 50 mL polypropylene tubes and cooled on ice for 30 minutes. The tubes were centrifuged for 10 minutes at 4000 rpm in a SorvallTM RC-58 Refrigerated Superspeed Centrifuge (Dupont) at 4°C. The supernatant was carefully discarded and the pellets were gently resuspended in 10 mL of ice-cold 0.1M CaCl₂ and stored on ice. The cells were recentrifuged for 10 minutes at 4000 rpm and the supernatant was carefully removed and discarded. The tubes were inverted and allowed to drain for approximately one minute. The pellets were gently resuspended in 2 mL of ice-cold 0.1M CaCl₂, 15% glycerol and 3.5% DMSO (X2). Cells were aliquoted into 200 μ L volumes and stored at -70°C.

For the transformation of competent bacterial cells, fifty ng of transformant DNA (10 μ L volume) were carefully added to a 200 μ L aliquot of competent DH5 α F' cells and stored on ice for 30 minutes. The cells were transferred to a 42°C water bath for 90 seconds. The cells were then transferred to an ice bath for 2 minutes. Eight hundred μ L liquid LB were added to the cells and the cells were incubated at 37°C for 45 minutes. During this time, LBA plates were prepared ie. 10 μ L

of 100 mM IPTG and 50 μ L of 2% X-gal were applied to the plates using a bent glass rod. Volumes of 50, 100 and 200 μ L of the transformed cells were spread onto LBA plates and incubated O/N at 37°C.

C. Isolation of the *nmt1* cDNA

A cDNA expression library in Lambda Zap I⁺ (Short et al., 1988), derived from mycelial mRNA from the *Neurospora crassa* wild type strain, 74-OR23-1VA (Orbach et al. (1990) was obtained from the FGSC. To prepare host cells for this library, fifty mL of LB/0.2% maltose broth were inoculated with *E. coli* BB4 host cells and incubated O/N at 37°C with moderate agitation (250 cycles/minute). The culture was centrifuged at 6000 rpm for 10 minutes in a SorvalTM RC-5B centrifuge (Dupont). The pellet was resuspended in 20 mL of sterile 0.01M MgSO₄·7H₂O and the cells were stored at 4°C until required.

Ten-fold serial dilutions (10^{-1} to 10^{-7}) of the *N. crassa* cDNA library (titre: 7×10^9 pfu/mL) were prepared in 100 μ L aliquots of SM medium (100 mM NaCl, 10 mM MgSO₄·7H₂O, 20 mM Tris-HCl (pH 7.5), 0.01% gelatin. One hundred μ L of the BB4/MgSO₄·7H₂O cells were added to the 10^{-5} , 10^{-6} , and 10^{-7} phage dilutions. The desired range of platings was 10^{-2} to 10^{-5} plaques. The Lambda phage/BB4 preparations were incubated at 37°C for 25 minutes with intermittent agitation. The preparations were mixed gently in 3.5 mL of molten (47°C) LB agarose (0.7%), poured over the surface of the LB plates

(1.5% agar), and incubated for 3 hours at 42°C. Circular nitrocellulose membranes (HybondTM-ECL; Amersham) were soaked in 10 mM IPTG for 10 minutes and then dried at 37°C for approximately 20 minutes. Each LB plate was overlaid with a nitrocellulose membrane and incubated O/N at 37°C. Before each membrane was removed, puncture marks were introduced into the membrane and agar for orientation and India ink was used to demark the orientation points in the agar.

The membranes were washed in TBS for 10 minutes at room temperature with gentle agitation. They were stained in 0.2% Ponceau S in 0.3% trichloroacetic acid for 10 minutes, destained in dH₂O with a final wash in TBS-T. Using the method for immunoblotting of Young and Davis (1985), membranes were blocked in a solution of 5% nonfat milk prepared in TBS [20 mM Tris-HCl (pH 7.5), 150 mM NaCl] for 1 hour. (All subsequent incubations and washes were performed at room temperature and on a gently rotating platform). The membranes were washed in TBS-T [TBS; 0.05% (v/v) Tween-20] two times for 5 minutes/wash.

The membranes were then incubated for 1.5 hours in a solution of 2.5% nonfat milk in TBS which contained a 1/500 dilution of polyclonal anti-cell wall antiserum. *N.crassa* cell walls had previously been isolated by J. Vierula using the procedure by Martinez et al. (1991). Polyclonal antibodies were prepared by injecting rabbits with this crude cell wall fraction from *Neurospora* (Huet, 1994). The

membranes were washed 3 times (5 minutes/wash) with TBS-T.

The membranes were incubated for 1 hour in a solution of 2.5% nonfat milk in TBS which contained a 1/3000 dilution of goat anti-rabbit HRP conjugated IgG secondary antibody. The membranes were then washed 4 times (5 minutes/wash) in TBS-T.

To complete the visualization step, an ECL system (Boehringer Mannheim) was used, in combination with the antibodies, to screen for positive clones. The membranes were incubated in a 100:1 preparation of the substrate A solution and starting solution B ($125 \mu\text{L}/\text{cm}^2$) for 1 minute. The membranes were then exposed to KodakTM XAR Scientific Imaging Film for 1 minute. The autoradiographic film was developed in GBX Developer for 5 minutes and GBX Fixer for 15 minutes. Five plaques on the 10^{-5} dilution membrane were recognized by the *N. crassa* cell wall antibodies. A sterile pasteur pipet was used to transfer each of the 5 positive clones (agar plugs) to a sterile 1.5 mL microcentrifuge tube containing 1 mL of SM medium and 1 drop of chloroform. The clones were labeled cDM1 through cDM5. [This thesis is based on the characterization of cDM1].

Screening of the cDM1 phage stock was continued until re-infection of BB4 cells and plating on LB medium resulted in a pure culture of positive, isolated plaques. For the recovery of plasmid DNA, 100 μL of BB4 cells in 10 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ were infected with 10 μL of cDM1 particles (an average plaque yields 10^6 to 10^7 infectious bacteriophage particles (Sambrook

et al., 1989) and incubated at 37°C for 20 minutes. Five mL of LB/0.05% maltose/10 mM $\text{MgSO}_4 \cdot \text{H}_2\text{O}$ were added to the culture which was subsequently incubated at 42°C for 2 hours. The culture was superinfected with 100 μL of R408 helper phage (7.5×10^{10} pfu/mL) and incubated at 37°C for 3 hours. During this time, LBA (100 μM ampicillin) plates were prepared and 10 μL of 100 mM IPTG and 50 μL of 2% X-gal were applied to the plates. Volumes of 50, 100 and 200 μL of the infected BB4 cells were similarly applied to the plates. Positive cDNA subclones were chosen on the basis of blue/white selection.

D. DNA Isolation

(i) Extraction of plasmid DNA from bacteria

The alkaline lysis method (Birnboim and Doly, 1979) was used to prepare plasmid DNA. Two mL of LBA medium were inoculated with a single white colony of a positive cDNA subclone stock. The cultures were incubated O/N at 37°C with agitation. Each culture was centrifuged for 2 minutes at 14,000 rpm. The supernatants were discarded and the cells were resuspended in 100 μL of TE [0.3 M Tris-HCl (pH 8.0), 50 mM EDTA] and incubated for 5 minutes at room temperature. Two hundred μL of 0.2 N NaOH/1% SDS solution were added and the cells were incubated on ice for 5 minutes. Following the addition of 150 μL of 3M K-Ac, pH 4.8, the suspensions were vortexed for 2 seconds, incubated on ice for 5 minutes, and then centrifuged at 14,000 rpm for 5 minutes. The supernatants were transferred to sterile microcentrifuge

tubes, mixed with 900 μL of absolute EtOH, incubated for at least 5 minutes at room temperature, and the precipitated DNA was recovered by centrifugation at 14,000 rpm for 5 minutes. The supernatants were discarded and the DNA pellets were washed with 1 mL of 70% EtOH. The pellets were centrifuged at 4,000 rpm for 5 minutes and the supernatant were discarded. The plasmid DNA was vacuum dried for 15 minutes and then resuspended in 20 μL of TE. The concentration of extracted plasmid DNA (and genomic DNA) was based on 50 μg of double-stranded DNA and 40 μg of single-stranded DNA (or RNA) having an absorbance of 1 at A_{260} (Sambrook et al., 1989).

If the rapid plasmid preparation was to be used for sequencing, one μL of RNase A (10 mg/mL) was added to 20 μL plasmid DNA and incubated at 37°C for 30 minutes. Eighty μL of TE buffer were added, followed by the addition of 100 μL of P:C:I (50% phenol: 48% chloroform: 2% isoamyl alcohol). The preparation was vortexed briefly and then centrifuged at 14,000 rpm for 5 minutes. The aqueous (upper) layer (approximately 80 to 85 μL) was transferred to a fresh microcentrifuge tube. One tenth volume (approximately 8.5 μL) of 3M Na acetate (pH 5.2) and 200 μL of absolute EtOH were added. The preparation was centrifuged at 14000 rpm for 5 minutes. The supernatant was discarded. The pellet was rinsed in 70% EtOH and centrifuged at 14000 rpm for 5 minutes. The supernatant was discarded and the pellet was dried under vacuum for 15 minutes. The pellet was resuspended in 8 μL of

TE.

Alternatively, DNA was purified using the Wizard™ Minipreps kit (Promega) according to manufacturer's instructions. Briefly, O/N cultures of positive cDNA subclones were grown, lysed, and neutralized as was described previously for the alkaline lysis method. Once centrifuged, the cleared supernatant was combined with one mL of Wizard™ Minipreps DNA Purification Resin and transferred into a syringe that was attached to a Wizard™ Minicolumn. The plunger pushed the slurry into the Minicolumn that was further flushed with two mL of Column Wash Solution (Promega). The Minicolumn was centrifuged at 14,000 rpm for 1 minute to dry the resin and transferred to a new microcentrifuge tube. The DNA was eluted with 50 μ L of 60°C TE and stored at 4°C until required.

(ii) Extraction of *N. crassa* genomic DNA

Genomic DNA was extracted following the method of Metzenberg and Baisch (1981). Briefly, *N. crassa* mycelia from VMM slants were resuspended in 5 mL of sterile dH₂O and used to inoculate 50 mL of VMM. The cultures were grown O/N at 25°C with agitation (250 rpm). The mycelia were harvested by vacuum filtration, washed with dH₂O, and pressed dry between paper towels. The fungal mass was cut into pieces of approximately 5 mm², frozen at -70°C and lyophilized for about 8 hours. The mycelial squares were crumbled in a 1.5 mL microcentrifuge tube using a pestle and about 25 mg of sea

sand. The mycelia were further pulverized for 20 seconds by holding the tube against a Vortex mixer running at maximum speed.

The crushed preparation was transferred to a 15 mL polypropylene centrifuge tube to which 3.6 mL of TEA-EDTA extraction buffer [(100 mL): 50 mL TEA-EDTA stock (in 500 mL: 73 g EDTA, 350 mL dH₂O, 110 mL triethylamine {pH 8.0-8.2}); 45.3 mL dH₂O, 2.5% Triton X-100, 1.25 mL predigested Pronase, 1.00 mL 5 M NaCl were added. The preparation was resuspended in the buffer with some manual agitation. Incubation was carried out for about 36 hours at 37°C with occasional manual agitation. The preparation was centrifuged at 6500 rpm for 20 minutes at 4°C. The supernatant was transferred to a fresh 15 mL centrifuge tube to which 10 mL of cold absolute EtOH were added. The preparation was stored O/N at 4°C to allow for the precipitation of the DNA.

To recover the precipitate, the preparation was centrifuged at 6500 rpm for 20 minutes at 4°C. The pellet was vacuum dried and resuspended in 1.5 mL of low salt buffer [1 mM NaCl, 1 mM Tris-HCl (pH 7.4), 0.1 mM Na₂EDTA] followed by the addition of 9 mL of ethanolic perchlorate reagent prepared by adding 140 g NaClO₄·H₂O and 36 mL dH₂O, warming carefully to 60-70°C, filtering a glass fiber filter, and adding 381 mL Absolute EtOH immediately. The preparation was incubated for 30 minutes at room temperature. The precipitate was recovered by centrifugation at 6500 rpm for 20 minutes at 4°C and vacuum

drying of the pellet. The pellet was resuspended in 750 μ L of low salt buffer, followed by the addition of 375 μ L of high salt buffer [100 mM NaCl, 25 mM Tris-HCl (pH 7.4), 2 mM Na₂-EDTA] containing 300 μ g/mL of preboiled (15 minutes) RNase A. The preparation was incubated for 3 hours at 37°C.

DNA extraction was carried out with equal volume of C:I, followed by centrifugation at 10,000 rpm for 10 minutes. The upper aqueous phase was transferred to a clean 15 mL centrifuge tube and a 0.54 volume of isopropanol was added. The precipitated DNA was collected by centrifugation at 10,000 rpm for 10 minutes. The pellet was rinsed with 70% EtOH, followed by centrifugation at 10,000 rpm for 10 minutes. The EtOH was drained from the pellet by carefully inverting the tube and finally by vacuum drying the pellet. The pellet was dissolved in 200 μ L of low salt buffer and 50 μ L of 5X high salt buffer. Concentration of the gDNA was determined as was previously described for plasmid DNA (Sambrook et al., 1989).

E. DNA manipulations

(i) Isolation of DNA fragments

Restriction endonuclease digestions were carried out as recommended by the supplier (New England BioLabs) using the appropriate NEB buffer provided and approximately 1 unit of enzyme for every 5 μ g of plasmid DNA or 2.5 μ g of genomic DNA. DNA digests, including a Lambda/*Hind*III standard (appendix 2), were analyzed on 1% agarose gels and electrophoresed at 60 V for 2 hours. The DNAFRAG program (Schaffer and Sederoff,

1981) was used to determine the size of DNA fragments on these gels. DNA ligations and other manipulations were essentially as described by Sambrook et al. (1989). DNA fragments were all subcloned into pBSKS⁺ (Stratagene).

DNA fragments were isolated from agarose gels by carefully cutting out the desired band using a fine-ended spatula and transferring to a preweighed microcentrifuge tube. The tube/fragment was then reweighed to determine the weight of the DNA/agarose slice. Approximately 3 volumes of 6N NaI solution were added to the slice. The sample was incubated at 50°C with occasional tapping until the agarose slice was completely dissolved. Eight μ L of Glass MilkTM were added to the mix which was then stored O/N at 4°C. The tube was centrifuged at 14,000 rpm for 5 minutes. The supernatant was carefully removed and discarded. The pellet was resuspended and washed in 400 μ L of New Wash solution [50% EtOH, 50 mM NaCl, 10 mM Tris-HCl (pH 7.6), 2.5 mM EDTA. The tube was again centrifuged at 14,000 rpm for 5 minutes. The wash step was repeated two more times. After the third wash, the tube was centrifuged for 5 minutes and the supernatant was removed. To ensure that all the wash solution was removed, the tube was again centrifuged for 1 minute and the remaining few μ L were drawn off with a P20. The pellet was resuspended in 15 μ L of dH₂O and incubated at 50°C for 5 minutes. The tube was centrifuged for 2 minutes and the supernatant was transferred to a fresh microcentrifuge tube. The approximate

concentration of the extracted DNA was determined by electrophoresing a 1 μ L aliquot on a 1% agarose gel and comparing the intensity of the extracted band with the Lambda/HindIII standard bands.

(ii) Generation of nested deletions

Generation of a nested set of deletion mutants was performed according to Sambrook et al. (1989). Briefly, 10 μ g of cDM1 were digested with HindIII and KpnI which created a 5' overhang and 3' overhang, respectively. The DNA was purified by phenol:chloroform extraction. The aqueous layer (approximately 43 μ L) was transferred to a new microcentrifuge tube to which was added 4.3 μ L (1/10th volume) of 3 M Na-acetate, pH 5.2. Two volumes of absolute EtOH were added to the tube. The DNA was stored on ice for 10 minutes, followed by centrifugation at 14,000 rpm for 5 minutes. The supernatant was carefully removed and discarded. One mL of 70% EtOH was used to wash the pellet which was again centrifuged at 14,000 rpm for 2 minutes. The supernatant was removed and the pellet was briefly dried under vacuum. The DNA pellet was resuspended in 60 μ L of 1X exonuclease III buffer [10X exonuclease buffer: 0.66 M Tris-HCl (pH 8.0), 66 mM MgCl₂].

7.5 μ L of the S1 reaction mixture [60 units S1 nuclease (400 u/ μ L), 27 μ L 10X S1 buffer {5.0 mL 5 M NaCl, 1.1 mL 3 M K-Ac (pH 4.5), 5.0 mL glycerol, 20.0 μ L 1M ZnSO₄}, 172 μ L dH₂O] dispensed into 20 numerically labeled microcentrifuge

tubes stored on ice. The resuspended DNA was incubated at 37°C for 5 minutes. A 2.5 mL aliquot was transferred to the first microcentrifuge tube and stored on ice. 5.1 μ L of the exonuclease III (118 units/ μ L) were added to the DNA stock. The tube was briefly vortexed and immediately returned to the 37°C water bath. At 30 second intervals, 2.5 μ L aliquots were removed from the DNA stock tube and transferred to successively labeled microcentrifuge tubes containing the S1 reaction mixture and stored on ice. The 20 aliquots were incubated together at 30°C for 30 minutes. One μ L of S1 stop mixture (0.3 M Tris-HCl, 50 mM EDTA (pH 8.0) was added to each tube, followed by incubation at 70°C for 10 minutes. The samples were stored at 4°C until required.

Four μ L of each sample were run on a 1% agarose gel to determine the extent of the exonuclease III digestion. The samples were subsequently pooled into 5 microcentrifuge tubes ie. samples 2 to 4, 5 to 7, 8 to 10, 11 to 13, and 14 to 16. Samples 17 to 20 showed complete digestion of the cDNA insert and were discarded. The volume in each of the 5 pools was brought up to 30 μ L with TE.

One μ L of Klenow mixture [(for 30 samples): 20 μ L dH₂O, 6 μ L 1 M MgCl₂, 3 μ L 0.1 M Tris-HCl (pH 7.6), 3 units Klenow fragment of *E. coli* DNA polymerase I (5u/ μ L)] was added for each 10 μ L of pooled sample. The samples were incubated at 37°C for 5 minutes. One μ L of a solution containing the four dNTP's (0.5 mM) was added for each 10 μ L of pooled sample and

the pooled samples were incubated for 15 minutes at room temperature. Forty μL of ligase mixture [(for 24 samples): 550 μL dH_2O , 100 μL 10X ligation buffer {500 mM Tris-HCl (pH 7.6), 100 mM MgCl_2 , 100 mM dithiothreitol, 500 $\mu\text{g}/\text{mL}$ BSA}, 100 μL 5 mM rATP, 250 μL 30% (w/v) polyethylene glycol (PEG 8000), 5 Weiss units bacteriophage T4 DNA ligase (1u/ μL) were added for each 10 μL of pooled sample. The sample components were mixed and were continued to be incubated at 4°C O/N. The ligation reactions were used to transform competent cells as was previously described.

F. Analysis of DNA

(i) Electrophoresis of DNA

DNA resolved by agarose gel electrophoresis was transferred to membranes essentially according to the method of Southern (1975). Four μg of genomic DNA were loaded per lane on a 1% agarose gel. The gel was run for 4 hours at 60 volts, followed by a brief wash in dH_2O . The gel was then immersed in a denaturing solution (0.5 N NaOH, 1.5 M NaCl) for 45 minutes. It was washed in dH_2O for 15 minutes and then neutralized [1.5 M CaCl_2 , 0.5 M Tris-HCl (pH 7.4)] for 30 minutes and then for 15 minutes. The gel was rinsed briefly in dH_2O . Two plexiglass supports were covered with Whatman paper and placed in a glass baking dish. 20X SSC was then added to the dish, just below the height of the two supports. The gel was laid well-side down on the supports and edged with parafilm. A gel-sized sheet of hybridization transfer

membrane (HybondTM-N, Amersham), previously wetted in dH₂O and soaked for 5 minutes in 20X SSC, was placed on top of the gel. Two gel-sized sheets of Whatman paper, previously soaked in 2X SSC, were placed on top of the nylon. Any air bubbles were removed from the gel, nylon and Whatman paper by rolling a pasteur pipet over each layer. A stack of approximately 8 cm of slightly smaller than gel-sized paper towels were placed on top of the two sheets of Whatman paper. SaranTM was used to completely cover the baking dish and its contents. The transfer of DNA from the gel to nylon was carried out for at least 12 hours. The position of each well was marked on the filter with a soft pencil. The nylon filter was soaked in 2X SSC for 5 minutes, air dried for 30 minutes and finally baked at 80°C for 30 minutes.

(ii) Preparation of labeled DNA probes

The [α -³²P]dCTP probe was prepared using the Ready To GoTM kit (Pharmacia Biotech). Fifty ng of the 1035 nt PstI fragment from cDMI was denatured by heating for 3 minutes at 95-100°C. The fragment was immediately placed on ice/5N NaCl for 2 minutes, and then centrifuged briefly at 14,000 rpm. The denatured DNA, 50 μ Ci of [α -³²P]dCTP (3000Ci/mmol) and dH₂O (to 50 μ L) were added to the Ready To GoTM bead and mixed by gently vortexing. The preparation was incubated at 37°C for 45 minutes. Following the incubation step, 0.1 μ L of 0.5 M EDTA and 3 μ L of yeast tRNA (10 mg/mL) were added to the probe preparation. Total volume of the probe was brought to

100 μ L with 1X STE (pH 8.0). Separation of the non-incorporated and incorporated radio-labelled DNA was accomplished by spun-column chromatography.

Spun columns were prepared according to Sambrook et al. (1989). Briefly, a 1-ml disposable syringe was plugged with a small amount of glass wool. The syringe was filled with Sephadex G-50, equilibrated in 1X STE buffer. The syringe was inserted into a 15-mL disposable plastic tube and centrifuged at 1600g for 4 minutes at room temperature in a swinging-bucket rotor in an IEC HN-SII bench centrifuge. This step was repeated until the volume of packed resin was approximately 0.9 mL. The column was flushed with 0.1 mL of 1X STE buffer and recentrifuged. This step was repeated two more times. Columns, which were not immediately used, were filled with 1X STE buffer and stored at 4°C.

The probe preparation was loaded into the spun column, centrifuged at 1600g for 4 minutes, and collected into an uncapped microcentrifuge at the bottom of the 15-mL disposable plastic tube. The probe was transferred to a capped microcentrifuge tube and stored at 4°C. The strength (cpm/ μ L) of the probe was obtained by analyzing a 2 μ L aliquot in an LKB Liquid Scintillation Counter model Rackbeta 1217. The [α -³²P]dCTP crps-7 probe that was used as an internal control was also prepared using this protocol.

(iii) Hybridisation of Southern blots

The blot was prehybridized for at least one hour at 68°C

in 0.2 mL/cm² hybridization solution [6X SSC, 5X Denhardt's reagent, 0.5% SDS, boiled salmon sperm DNA (1/100 of the total volume of pre/hybridization solution). 50X Denhardt's reagent: 1% (w/v) Ficoll, 1% (w/v) polyvinylpyrrolidone, 1% (w/v) BSA] was calculated as 0.2 mL/cm² of nylon filter. The Southern blot was hybridized with the [α -³²P]dCTP probe (500,000 cpm/mL hybridization solution) for at least 12 hours at 68°C. Volume of the hybridization solution was calculated as 0.05 mL/cm² of the nylon filter. Following hybridization, the blot was first washed in 2X SSC for 15 minutes at 68°C. The blot was washed a second time in a solution of 2X SSC, 0.1% SDS for one hour at 68°C. The final wash contained 0.1X SSC for 10 minutes at 68°C. The blot was slightly air dried and enclosed in two sheets of transparent plastic. The blot was overlayed with a sheet of autoradiographic film, stored O/N at -20°C, and then processed as was previously described.

(iv) RFLP analysis

The RFLP mapping procedure was carried out as described by Metzenberg et al. (1984). Briefly, two FGSC parental strains, 4411 and 4416, were selected for their different genetic backgrounds where 4411 has an Oak Ridge ancestry and carries the following markers: *al-2*, *nuc-2*, *arg-12*, *cot-1*, *inl-a*; 4416 originates from a wild Mauriceville-1c-A lineage. A Southern blot (prepared by J. Vierula) that included DNA from the two parentals and restricted with *Pst*I, *Hind*III, *Eco*RI and *Bam*HI was used to find a useful RFLP. Following

hybridization of the Southern with the [α - 32 P]dCTP probe, *EcoRI* was observed to be the appropriate enzyme. Four μ g of DNA from strains 4411, 4416 and their 18 progeny (4412 to 4415 and 4417 to 4430) were then digested with *EcoRI*. The digests underwent Southern analysis and [α - 32]dCTP probing as was previously described.

(v) Preliminary characterization of cDM2 to cDM6

E. coli BB4 host cells were harvested and stored in 10 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ as was previously described. Two hundred μ L of the BB4 cells were suspended in 3.5 mL of LB/0.7% agarose and poured onto the surface of an LB plate. The top agarose was allowed to set for 15 minutes. The bottom of the petri dish was divided into 6 equal sections and labelled cDM1 through cDM6. A 20 μ L aliquot from each of the original cDNA phage stocks (10^{-2} dilution in SM medium) (including cDM1) was carefully pipetted into its appropriately-labelled section. The aliquot was absorbed by the LB agar within approximately 15 minutes. The plate was incubated O/N at 37°C.

The plate was chilled at 4°C for 30 minutes before being overlayed with a circular nylon filter for approximately 2 minutes. During that time, three orientation points were marked through the filter and agar. The DNA was denatured, neutralized and washed (5 minutes/step) using the same solutions as for Southern blots. It was dried at room temperature for 30 minutes and baked at 80°C for 30 minutes. The filter was prehybridized, hybridized with the [α - 32 P] dCTP

*Pst*I probe and visualized as described for Southern analysis.

G. Isolation of genomic *nmt1* clones

A genomic DNA expression library in Lambda BARGEM7 (Pall and Brunelli, 1993) and constructed using gDNA from *N. crassa* wild type strain 74-OR-23-1A (Pall and Brunelli, 1994) was obtained from the FGSC. *E. coli* BB4 host cells were harvested and stored in 10 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ as was previously described for screening the *N. crassa* cDNA library. Ten-fold serial dilutions of the *N. crassa* genomic library (titre: 7.8×10^8 pfu/mL) were prepared in 100 μL of SM medium. An equal volume of the phage library stock was used to infect a 100 μL aliquot of BB4 cells, resulting in platings of 10^{-2} to 10^{-5} . The phage/BB4 preparations were incubated at 37°C for 15 minutes. The preparations were then mixed gently in 3.5 mL of molten (47°C) LB agar (0.7%), poured over the surface of the LB plate and incubated O/N at 37°C.

The plates were chilled at 4°C for 30 minutes before being overlayed with circular nylon hybridization transfer membranes (Hybond-NTM, Amersham) for approximately 2 minutes. Three orientation points were marked through each filter and into the agar using a sterile 27g needle and India ink. The filters were first submerged for 5 minutes in denaturing solution, followed by 5 minutes in the neutralizing solution. Following a final rinse of the filters in 2X SSC for 5 minutes, they were dried on Whatman paper for 30 minutes and baked at 80°C for 30 minutes.

The filters were prehybridized, hybridized with the [α - 32 P]dCTP PstI probe and visualized as was previously described. Two positive genomic clones, gDM1 and gDM2, were picked using sterile pasteur pipets and individually stored in 1 mL of SM/1 drop of chloroform.

To recover the plasmid pBARGEM7 DNA, ten-fold serial dilutions (10^{-1} to 10^{-6}) of the gDM1 stock were prepared in 100 μ L aliquots of SM medium. Automatic subcloning (Palazzolo et al., 1990) was performed by infection of 100 μ L of the host *E. coli* BNN132 cells with 10 μ L of positive gDM1 particles (dilutions 10^{-4} to 10^{-6}) for 30 minutes at 30°C in 10 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. BNN132 cells had been prepared as was previously described for the BB4 cells. Five mL of LB broth were added to the gDM1 phage/BNN132 preparation which was then incubated for 1 hour at 30°C. Aliquots (50, 100 and 200 μ L) of the infected culture were plated onto LBA/IPTG/X-gal plates and incubated at 37°C O/N. Positive colonies were picked on the basis of blue/white selection and used to inoculate LBA broth for plasmid DNA extraction as was previously described. Stocks of clones were prepared in 15% glycerol and stored at -70°C.

H. DNA sequencing

All DNA sequencing used the dideoxy, chain termination method of Sanger et al. (1977). Double-stranded DNA templates were prepared for sequencing (1.5-2 μ g in 32 μ L) in microfuge tube by first adding 8 μ L of 2 M NaOH. The contents of the

tube were gently vortexed, briefly centrifuged and incubated at room temperature for 10 minutes. Seven μL of 3M Na-acetate (pH 4.8), 4 μL of dH_2O and 120 μL of absolute EtOH were added to the tube. The DNA solution was mixed and incubated on ice for 15 minutes. The precipitated DNA was collected by centrifuging at 14,000 rpm for 15 minutes. The supernatant was carefully removed and discarded. The DNA pellet was gently washed with 1 mL of ice-cold 70% EtOH and recentrifuged at 14,000 rpm for 10 minutes. The supernatant was discarded and then the pellet was briefly dried under vacuum, and resuspended in 10 μL of dH_2O . Five to ten pmol of the Universal or Reverse primers, 2 μL of annealing buffer (Pharmacia) and dH_2O to a final volume of 14 μL were added to the template. The DNA was gently vortexed, briefly centrifuged and incubated at 65°C for 5 minutes. The tube was quickly transferred to a 37°C water bath and incubated for 10 minutes. The DNA was then incubated for at least 5 minutes, followed by a brief centrifugation.

DNA sequencing reactions were performed according to the Pharmacia Biotech T7 SequencingTM kit instructions. The labeling reaction for each template (14 μL) was prepared by adding 3 μL of Labelling Mix, 1 μL of labelled dNTP [α -³⁵S]dATP, and T7 DNA Polymerase (diluted 1/5 in Enzyme Dilution Buffer). The components were mixed by gentle pipetting and collected at the bottom of the tube by a brief centrifugation and the labeling reaction was incubated at room

temperature for 5 minutes. During this incubation, four microcentrifuge tubes were labelled A, C, G and T for each DNA template. Into each respective tube was dispensed 2.5 μ L of A, C, G or T Mix-Short (sequencing mix). The tubes were then warmed at 37°C for at least 1 minute. When the 5 minute incubation of the labelling reaction was completed, 4.5 μ L of this reaction was transferred into each of the four pre-warmed sequencing mixes. The components were mixed by gentle pipetting and incubated at 37°C for 5 minutes. Five μ L of Stop Solution were added to each tube and gently mixed. The reactions were briefly centrifuged to collect the contents at the bottom of each tube.

The sequencing reactions were analyzed on a 6% acrylamide sequencing gel [33.6 g urea, 11.5 mL 40% acrylamide (38% acrylamide/2% *N,N'*-methylenebisacrylamide), 8 mL 10X TBE (108 g Tris-HCl, 55 g boric acid, 7.6 g EDTA to 1L with dH₂O), 32.8 mL of dH₂O; degased for 15 minutes, 600 μ L 10% APS, 24.5 μ L TEMED] with 1X TBE buffer and under the following conditions: 1500-1900 volts, 60 watts, and 30-45 mA (Bio-Rad Power Supply, model 3000Xi). The samples were loaded onto the gel and electrophoresed for 2.5 hours. For longer read lengths samples were re-applied to new lanes and the gel was run for an additional 2 hours. A solution of 10% acetic acid/10% methanol was used to fix the gel. It was then transferred to a sheet of Whatman paper (13.5 x 15.5 cm²) and dried in a Bio-Rad Gel Dryer, model 583 for 20 minutes at 80°C. The gel was

overlaid with autoradiographic film and stored at room temperature for 24 hours. The film was developed and fixed as was previously described. The DNA sequence was analyzed using the FASTA program (Pearson and Lipman, 1988).

Three synthetic primers were prepared at Carleton University for sequencing purposes and are described in Table 1.

primer	location (nt)	sequence
dm1	61 ← 90	gatagtgttacccttgagggttctagctcga
dm2	361 → 379	ggatcaacctttcaccggcg
dm3	1079 ← 1098	gtcttcttacaccgaacgac

Table 1: Identities, nucleotide locations and directions, and sequences of the synthetic primers.

I. Isolation and analysis of RNA

(i) RNA extraction

RNA extraction from the mycelia of *N. crassa* was carried out following the method of Reinert et al. (1981). Briefly, the lyophilized mycelia from each culture were pulverized with about 1 g of acid washed sea sand and then quickly mixed with 20 ml of a 1:1 mixture of extraction buffer [100 mM Na-acetate (pH 5.0), 1 mM EDTA, 5% SDS] and P:C:I. The slurry was transferred to a PA tube and centrifuged at 10,000 RPM for 10 minutes at 4°C.

The aqueous upper layer was carefully transferred with a bent sterile pasteur pipet to a PP tube containing 60 mL of

predigested pronase (20 mg/mL) (Sigma). The preparation was incubated for one hour at room temperature and then extracted two more times with P:C:I. The RNA was precipitated by adding 2 volumes of absolute EtOH in a new PA tube. The pellet was resuspended in 2 mL of 0.1% DEPC-treated dH₂O at 4°C, followed by the addition of 3 volumes of 4 M Na-Ac, pH 6.0, 10 mM EDTA. The RNA was stored for 10 minutes at 4°C and then centrifuged at 10,000 rpm for 10 minutes at 4°C. The pellet was rinsed in 70% EtOH and again centrifuged for 10 minutes at 4°C. The RNA pellet was resuspended in 2 mL of DEPC-treated dH₂O, containing 0.5% SDS and analyzed at A₂₆₀ to calculate the nucleic acid concentration. The preparation was re-precipitated using 1/13 volume of 4 M Na-Ac (pH 6.0), 10 mM EDTA and 2.5 volumes of absolute EtOH and then centrifuged at 10,000 rpm for 10 minutes at 4°C. The pellet was resuspended to a concentration of 2 mg/mL in DEPC-dH₂O.

(ii) Poly(A+) RNA preparation

Oligo(dT)-cellulose (0.5-1.0 g) was suspended in 0.1 N NaOH. A column of oligo(dT)-cellulose (0.5-1.0 mL of packed volume) was prepared in a Dispocolumn (Bio-Rad) which had been soaked O/N in sterile DEPC-dH₂O. The column was washed with 3 column volumes of sterile DEPC-H₂O. The column was then washed with sterile 1X column-loading buffer [20 mM Tris-HCl (pH 7.6), 0.5 M NaCl, 1 mM EDTA (pH 8.0), to which was added 0.1% sodium lauryl sarcosinate from 10% stock that had been heated to 65°C for 30 minutes] until the pH of the effluent

was less than 8.0.

The RNA (approximately 10 mg) was dissolved in DEPC-treated dH₂O and heated to 65°C for 5 minutes. The solution was quickly cooled to room temperature and an equal amount of 5X column-loading buffer was added to the RNA solution. The solution was applied to the column and the eluate was collected in a sterile tube. The column was flushed with 1X column buffer and the eluate was collected. When all the solution had been eluted, the collected eluate was heated to 65°C for 5 minutes and re-applied to the column. Again the eluate was collected. The column was washed with 8 volumes of 1X column-loading buffer, collecting 1-mL fractions (13). The A₂₆₀ of the first and last fractions was determined. The poly(A⁺) RNA was eluted from the oligo(dT)-cellulose with 3 volumes of sterile, RNAase-free elution buffer [10 mM Tris-HCl (pH 7.6), 1 mM EDTA (pH 8.0), autoclaved and added 0.05% SDS from a stock of 20% SDS made in sterile water]. All the eluate draining from the column was collected and the A₂₆₀ was determined. To the Poly(A⁺) RNA eluted from the oligo(dT)-cellulose column, 3M Na-acetate (pH 5.2) was added to a final concentration of 0.3M. To this solution 2.5 volumes of ice-cold absolute ethanol and then stored on ice for 1 hour. The poly(A⁺) RNA was recovered by centrifugation at 10,000g for 15 minutes at 4°C. The supernatant was carefully discarded and the pellet was washed with 70% ethanol. The pellet was briefly recentrifuged and the supernatant was again removed

and the nucleic acid pellet was air dried. The RNA was redissolved in 400 μ L of DEPC-dH₂O and again the concentration was determined using the A₂₆₀. The polyA⁺ RNA was stored at -20°C.

(iii) Northern analysis

Twenty μ g of RNA, suspended in DEPC-dH₂O, were first lyophilized in the SpeedVac. The RNA samples were then reconstituted in 10 μ L of the RNA reconstitution mix {10% 5X formaldehyde gel-running buffer [0.1 M MOPS (pH 7.0), 40 mM Na-acetate, 5 mM EDTA (pH 8.0)], 17.5% formaldehyde, 50% de-ionized formamide, 22.5% DEPC-H₂O. Also included was 0.5 μ g/20 μ L of EtBr}. The samples were incubated for 15 minutes at 65°C, then quick chilled on ice/5N NaCl. Two μ L of formaldehyde gel-loading buffer [50% glycerol, 1 mM EDTA (pH 8.0), 0.25% bromophenol blue, 0.25% xylene cyanol FF were added to each sample before loading them onto the gel.

The formaldehyde gel [(90 mL): 1.08 g agarose, 55.5 mL DEPC-H₂O, 16.7 mL 5X gel-running buffer, 17.0 mL formaldehyde submerged in 1X gel-running buffer and pre-run at 4V/cm for 15 minutes. Twenty μ g of RNA were loaded per well and run at 60V for 5 hours. Once completed, the gel was soaked in DEPC-dH₂O for 15 minutes with gentle agitation. Approximately 50 mL of 20X SSPE were added to the DEPC-dH₂O and the gentle agitation was continued for another 45 minutes. During this time, one gel-sized sheet of HybondTM-N nylon, 2 gel-sized sheets of Whatman paper, and an 8 cm stack of paper towels were cut.

The nylon was wetted in DEPC-dH₂O and then soaked for more than 5 minutes in 20X SSPE. The Whatman sheets were soaked for more than 5 minutes in 2X SSPE. The transfer of the RNA to the nylon was performed as was previously described for Southern analysis.

The transfer set up was dismantled and the position of the gel wells were marked on the Northern blot filter with a soft pencil. The blot was washed for 5 minutes in 6X SSPE, air dried for 30 minutes and finally baked at 80°C for 30 minutes.

Northern blots were prehybridized for at least 4 hours at 45°C. The volume of prehybridization solution (5X 20 SSPE, 50% deionized formamide, 5X Denhardt's, 20% dH₂O) was calculated as 0.2 mL/cm² of nylon filter. Blots were hybridized for approximately 2 hours at 45 °C prior to the addition of the probe. The volume of hybridization solution [5X 20 SSPE, 50% deionized formamide, 5X Denhardt's, 10% (w/v) 50% dextran sulfate] used was as 0.05 mL/cm² of nylon filter. Northern blots were hybridized with the [α -³²P]dCTP probe (500,000 cpm/mL hybridization solution) for at least 12 hours at 45°C.

Following hybridization, blots were washed twice in 5X SSPE for 5 minutes/wash at 45°C. Blots were then washed in 1X SSPE, 0.1% SDS for 60 minutes at 45°C. The final wash was carried out at room temperature in 0.1X SSPE and 0.1% SDS for 30 minutes. Blots were slightly air dried and exposed to film

as previously described.

(iv) Primer extension analysis of RNA

Attempts were made to determine the location of the transcriptional start site of DM1 using primer extension analysis. The method (McKnight and Kingsbury, 1982) is based on hybridization of the 5' end-labelled dm1 primer to its complementary sequence in RNA. RNA extracted from the *N. crassa* wild type culture grown in VMM (without thiamine) was used for this purpose. Labeling of the 5' end of the dm1 primer with [γ - 32 P]dATP was done according to the method of Sambrook et al. (1989). Extension of the 3' end of dm1 primer by reverse transcriptase in the presence of deoxyribonucleotide triphosphates failed to occur.

J. Analysis of Proteins

(i) Preparation of protein extracts

Proteins were extracted from lyophilized mycelia, crude cell wall preparations and spheroplasts by pulverizing one cube (approximate dry weight of 20 mg) of dry sample in a microcentrifuge tube using a plastic pestle. The powdered mycelia were resuspended in 250 μ L of protein extraction buffer (appendix 3). The sample was incubated on ice for 15 minutes, followed by centrifugation in an Eppendorf Centrifuge model 5415C at 4°C for 4 minutes at 7000 rpm and 15 minutes at 14,000 rpm. The supernatant was removed and its protein concentration was determined using a Hewlett Packard Diode

Array Spectrophotometer model 8452A and the calculation (appendix 4) by Kalb and Bernlohr (1977).

Crude cell wall proteins were extracted using a modified version of the method by Martinez et al. (1991). A cube of lyophilized mycelia was pulverized by a mortar and pestle and the powder then resuspended in 250 μ L of protein extraction buffer (appendix 3) and centrifuged at 1000 rpm for 10 minutes. This step was repeated five times with the cell wall pellet being resuspended following each centrifugation. Proteins and cell wall material were precipitated with 6 volumes of acetone. The sample was centrifuged at 1000 rpm for 10 minutes. The supernatant was removed and the pellet was air dried. Protein extraction buffer was used to resuspend the pellet.

The method of Vollmer and Yanofsky (1986) was used to prepare spheroplasts. This procedure had been carried out by J. Vierula. The protein concentration of the cell wall and spheroplast preparations was determined as was described for the mycelial samples.

(ii) Electrophoretic analysis of proteins

An equal volume of SDS-PAGE buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue) was added to each preparation. This provided a final loading concentration of approximately 20 μ g protein/lane. An acrylamide slab gel (Mini-PROTEAN II Dual Slab Cell SystemTM; Bio-Rad) was used to electrophoretically separate the proteins in the mycelial,

spheroplast and cell wall preparations (Laemmli, 1970). The discontinuous system consisted of a 10% acrylamide resolving gel [3.64 mL dH₂O, 2.5 mL 1.5 M Tris-HCl (pH 8.8), 100 µL 10% (w/v) SDS stock, acrylamide/bis (30% stock), 50 µL 10% APS, 5 µL TEMED (Bio-Rad)] and a 4% acrylamide stacking gel [6.1 mL dH₂O, 2.5 mL 0.5 M Tris-HCl (pH 6.8), 100 µL 10% (w/v) SDS stock, 1.3 mL acrylamide/bis (30% stock), 50 µL 10% APS, 10 µL TEMED].

The samples were heated to 95°C for 5 minutes and loaded onto the gel. Also included was a mid-range molecular weight standard (appendix 5). Electrophoresis was carried out in SDS-PAGE tank buffer (0.025 M Tris-HCl, 0.192 M glycine, 0.1% SDS) at 180 volts for 54 minutes. Once completed, gels not used for the Western analysis were stained using one of the following procedures on a slowly rotating platform and at room temperature.

(a) Coomassie Brilliant Blue R250 (0.25% CBBR-250 in 40% MeOH/10% acetic acid). Gels were immersed in the staining solution for a minimum of 4 hours. Gels were then destained (40% MeOH/10% acetic acid without 0.25% CBBR-250) and fixed for at least 4 hours with the destaining solution being changed 2 or 3 times.

(b) Silver Stain: Silver staining was carried out using the Bio-Rad silver staining kit according to the manufacturers instructions. Briefly, gels were fixed in 10% EtOH/5% acetic acid for 15 minutes with gentle rocking. This step was

repeated. Gels were oxidized for 5 minutes in a 1/10 dilution of Oxidizer ConcentrateTM (Bio-Rad), followed by several washes of dH₂O. Gels were then immersed in a 1/10 dilution of the Silver Reagent ConcentrateTM (Bio-Rad) for 20 minutes, followed by a one minute wash in dH₂O. A 3.2% solution (w/v) of Silver DeveloperTM (Bio-Rad) was applied to the gels in three changes of 30 seconds, 5 minutes and 5 minutes. The reaction was stopped using a 5% acetic acid solution.

All stained gels were dried in a Bio-Rad Gel Dryer model 583 for 2 hours at 80°C.

(iii) Western analysis

Following electrophoresis of the protein samples, the gels were equilibrated for 30 minutes in transfer buffer [0.025 M Tris-HCl, 0.192 M glycine, 20% (v/v) MeOH]. The proteins were transferred using a Mini Transfer Blot Electrophoretic Transfer Cell SystemTM (Bio-Rad) to a nitrocellulose hybridization transfer membrane (HybondTM-ECL; Amersham) (Towbin et al., 1979) at 100 volts for 1 hour. Once completed, the nitrocellulose membrane was stained in 0.2% Ponceau S in 0.3% TCA for 10 minutes, and then destained for 5 minutes in dH₂O.

Using the method for immunoblotting of Young and Davis (1985), the nitrocellulose membrane was blocked in a solution of 5% nonfat milk prepared in TBS and incubated with primary (1/10,000) and secondary antibodies (1/5000) and then visualized as was previously described (isolation of cDNA).

(iv) Stripping protein blots

Membranes used for western analysis were stripped by incubating them in TBS containing 100 mM 2-mercaptoethanol and 2% SDS with gentle shaking for 30 minutes at 50°C. They were washed twice for 15 minutes in large volumes of TBS-T at room temperature. The membranes were then blocked and reprobed as was previously described.

(v) Immunopurification of antibodies

The method of Eroles et al. (1997) was used to purify the *N. crassa* cell wall-associated polyclonal antibodies by adsorbing them against the cDM1 phage lysates. *E. coli* BB4 cells were cultured and harvested as was previously described. Two hundred μ L of BB4 cells and 100 μ L of purified stock of cDM1 phage were combined and incubated at 37°C for 30 minutes. The BB4/cDM1 preparation was mixed with 4 mL of LB/0.7% agarose and plated on solid LB medium. The plate was incubated for 3 hours at 42°C. At that time, the agar was overlayed with a circular nitrocellulose filter which had been saturated with 10 mM IPTG. The plate was incubated at 42°C O/N.

The filter was washed 4 times (5 minutes/wash) in TBS-T with agitation at room temperature. The filter was incubated with 5 mL of the *N. crassa* cell wall antiserum for 40 minutes with agitation at room temperature. The depleted antiserum was removed and saved. The nitrocellulose filter was washed 3 times (5 minutes/wash) in TBS-T with agitation at room

temperature. Four mL of 100 mM glycine (pH 2.8) were used to elute the adsorbed CDM1 antibodies from the filter. The eluted material was neutralized with 3 drops of 3 M Tris/HCl (pH 8.5).

(vi) Deglycosylation with N-glycosidase F

The N-glycosidase F deglycosylation kit (Boehringer Mannheim) was used to test for the existence of asparagine-linked glycan chains on glycoproteins. Three *N. crassa* mycelial samples (labelled 0, 1 and 24) and two control samples of known glycoproteins (labelled 0 and 1) were prepared, with each containing 25 µg/mL of protein. To each tube was added 5 µL of Reduced Denaturation Buffer, followed by incubation at 95°C for five minutes. Ten µL of Reaction Buffer were added to each vial. An additional 10 µL of Reaction Buffer were added to the control and mycelial samples labelled 0. Ten µL of N-glycosidase F were added to the three remaining samples. All five samples were incubated at 37°C.

At one hour post-incubation, the mycelial and control samples labelled 1 were removed and stored at -20°C. Incubation of the three remaining samples was continued at 37°C. At 12 hours post-incubation, another 10 µL of N-glycosidase F were added to the mycelial vial labelled 24. The incubation was continued at 37°C for another 12 hours. The appropriate volume of 2X SDS-PAGE buffer was added to each sample. The samples were heated at 95°C for 5 minutes and 10 µL of each sample were analyzed using SDS-PAGE. The

electrophoresed proteins were transferred to nitrocellulose membranes for western analysis as previously described. The membranes were immunoprobed with the *N. crassa* cell wall antibodies and the cDM1 phage adsorbed antibodies.

K. Expression of cDM1 sequences in bacteria

The 1162 nt *SalI* fragment from pDM1 was ligated into the *SalI* site of pMALTM-c2 plasmid DNA (New England BioLabs). The DNA mixture was quick chilled on ice/5N NaCl. Following a brief centrifugation step, 2 μ L of 10X ligase buffer and 0.5 μ L of T4 ligase (200 units) were added to the DNA mixture which was then incubated at 12 °C O/N. Competent DH5 α F' cells were transformed using the ligation mix and plated on LBA/IPTG/X-gal solid media as described elsewhere in this thesis. Control samples were also prepared by transforming DH5 α F' cells with the pMALTM-c2 vector which did not contain the *SalI* fragment insert. Positive clones (containing the insert) were screened on the basis of blue/white selection.

To screen for the *SalI* insert in the appropriate direction, a 5 mL culture was grown in LBA to a density of approximately 2×10^8 cell/mL (A_{600} of approximately 0.5). A 1 mL aliquot was removed from the 5 mL culture and centrifuged at 14,000 rpm for 2 minutes. The supernatant was discarded and the cells were resuspended in 50 μ L of 1X SDS-PAGE sample buffer. IPTG was added to the remaining culture to a final concentration of 0.3 mM and incubation was continued at 37°C. At 1, 2 and 3 hour intervals, 0.5 mL samples were removed and

centrifuged at 14,000 rpm for 2 minutes. The supernatants were removed and the cells were resuspended in 100 μ L of 1X SDS-PAGE sample buffer.

The samples were placed in a boiling water bath for 5 minutes. Fifteen μ L of each sample was electrophoresed on a 10% SDS-PAGE gel along with a set of mid range molecular weight protein standards (Promega). Proteins in the gel were transferred to nitrocellulose and probed with the *N. crassa* cell wall antibodies as was previously described.

L. Transmission electron microscopy

(i) Sample preparation

An *N. crassa* mycelial culture was prepared by inoculating 45 mL of VMM with conidia (approximately 5×10^8 spores/50 mL of culture medium) resuspended in 5 mL of sterile dH₂O. The cultures were incubated O/N at 25°C with mechanical agitation of 250 rpm.

Eight pieces of *N. crassa* mycelia (approximately 2 mm²) were fixed in 2% glutaraldehyde prepared in 0.1 M cacodylate buffer (pH 7.2). Fixation was continued for 2 hours at room temperature. The pieces were washed twice in 0.1M cacodylate buffer (pH 7.2) for 15 minutes/wash. One half of the tissue was prepared for observing its structural detail. This tissue was post-fixed in 2% OsO₄ in 0.1 M cacodylate buffer for 2 hours at 4°C. Dehydration was done in an increasing ethanol series of 30%, 50%, 70%, 80% and 90% for 5 minutes each; twice in absolute ethanol for 10 minutes each; and, twice in 100%

propylene oxide for 15 minutes each.

O/N infiltration of the tissue was in propylene oxide/Epon 812 (1:1) in the fume hood. The infiltration was again repeated in propylene oxide/epon 812 (1:1) for 5 hours. The tissue was embedded in plastic BEEM capsules in Epon resin (Luft, 1961). Polymerization was at 60°C for 36 hours. The remaining tissue was to be probed with *N. crassa* cell wall antibodies and immunogold labelled. Dehydration of this tissue began with two 15 minute washes in 30% ethanol, followed by 50 minutes each in 50%, 70%, and 90% ethanol. The tissue was infiltrated in two washes of 90% ethanol/LR White (1:1) for 45 minutes each, followed by immersion of the tissue in LR White for approximately 24 hours at 4°C. The tissue was placed in gelatin capsules which were filled with LR White and made airtight with a cover. Polymerization was at 60°C for 36 hours.

(ii) Staining and immunogold labeling

Silver-gold (approximately 70 -80 nm) tissue sections were cut on a Reichert Ultracut E. The Epon and LR White embedded sections to be viewed for their ultrastructure were transferred to 200-mesh copper grids. The LR White sections which were to be immunogold-labelled were transferred to 400-mesh nickel grids.

The ultrastructure sections were stained in 1% uranyl acetate for 10 minutes and then washed with dH₂O. They were post stained in lead citrate, washed in dH₂O and air dried.

The gold-silver sections to be immunolabeled were first incubated for 20 minutes in one drop of 1% BSA in PBS-T and rinsed twice in PBS-T. Grids were incubated for 45 minutes in one of the following preparations: (i) PBS, (ii) preimmune serum, diluted 1:100 in PBS, or (iii) antiserum to crude *N. crassa* cell wall extract, diluted 1:100 in PBS. The specimens were rinsed in PBS-T, three times and incubated in goat antirabbit IgG 10 nm gold conjugated 2° antibody (Bio-Rad), diluted 1:10 in PBS for 45 minutes, and then rinsed four times in PBS-T.

The sections were stained in lead citrate for 5 minutes, rinsed in distilled water, and then allowed to air dry. Post staining was in 2.5% uranyl acetate in 50% methanol for 10 minutes. The sections were rinsed in 50% methanol, air dried, and then examined with a Philips 201C transmission electron microscope operating at 60 kV.

III. Results

A. Characterization of *N. crassa* anti-cell wall antiserum

An immunological screening strategy was chosen to identify DNA sequences encoding proteins associated with the fungal cell wall. Previously, antiserum had been raised against a crude cell wall fraction prepared using the non-denaturing extraction procedure of Martinez et al. (1991). A combination of Western blotting and immunoelectron microscopy was used to analyze this antiserum.

(i) immunoanalysis of *N. crassa* mycelial fractions

SDS-PAGE and Western analysis of *N. crassa* whole cell, spheroplast, and cell wall extracts using the polyclonal anti-cell wall antiserum demonstrated that at least three unique antigens, presumed to be proteins based upon their size and degree of resolution, were present in the wall fraction that were rare or absent in extracts of wall-less spheroplasts. The proteins ranged in size from less than 26.6 kD to larger than 97.4 kD in all three fractions (figure 1a). A number of other proteins are present in all three extracts but the relative abundance of some of them varied considerably as is evidenced by the different band intensities. Surprisingly, the spheroplast extracts also had some unique proteins in addition to sharing many proteins with the whole cell preparation (figure 1b). Although many proteins were common to all three fractions, the antiserum did demonstrate some degree of selectivity because it recognized some proteins

Figure 1. Analysis of the protein content of *N. crassa* mycelial fractions. (a) SDS-PAGE gel and (b) Western analysis by probing with the *N. crassa* anti-cell wall antiserum.

lane M: mid range molecular weight marker.

lane 1: whole cell.

lane 2: spheroplasts

lane 3: cell wall extract.

* indicates unique spheroplast proteins; arrow head indicates unique cell wall proteins.

Figure 2. Incubation of *N. crassa* mycelial proteins and glycoprotein controls with *N*-glycosidase F. The *N*-glycosidase F enzyme (approximately 34 kD) is visible in lanes 2, 4, and 5. (a) SDS-PAGE gel and (b) Western blot probed with *N. crassa* anti-cell wall antiserum.

lane M: molecular weight marker.

lane 1: undigested glycoprotein control.

lane 2: digested glycoprotein control (1 hr).

lane 3: undigested mycelial proteins.

lane 4: digested mycelial proteins (1 hr).

lane 5: digested mycelial proteins (24 hr).

Figure 1

Figure 2

unique to the cell wall fraction. Examples of this selectivity include three proteins of approximately 40, 57 and 70 kD (arrow heads) that were recognized in the mycelial (lane 1) and cell wall (lane 3) samples but not in the spheroplast sample (lane 2). In contrast, a 37 kD and 80 kD protein (*) were recognized in the mycelial and spheroplast preparations but not in the wall extract.

In yeast cells, many cell wall-associated proteins are thought to be mannoproteins (Ballou, 1974). Therefore, cell extracts were treated with the deglycosylating enzyme *N*-glycosidase F to determine if any of the *Neurospora* proteins recognized by the polyclonal antiserum were linked to *N*-glycan chains. There was no evidence of deglycosylation since there was no change in the apparent molecular mass of any *N. crassa* mycelial proteins recognized by the polyclonal antiserum (Figure 2b, lane 4). Extending the conditions by doubling the enzyme and incubating for 24 hours did not alter this finding (lane 5). Evidence of a functional enzyme was seen by the digestion of the control glycoproteins (figure 2a, lane 2), none of which were recognized by the antiserum (figure 2b, lanes 1 and 2).

Since the antiserum recognized so many proteins in the spheroplast and whole cell extracts, immunoelectron microscopy was used to assess whether the antiserum was in fact recognizing cell wall-associated antigens. The hyphal tissue revealed a large nucleus that is bounded by a bilayered

nuclear membrane containing an eccentrically-located ill-defined nucleolus and a matrix of dense material (figure 3a). Several mitochondria are also present and the parallel invaginations of the double membrane, known as cristae, surrounded by a matrix of dense granules are readily observed. The tubular-shaped fragments of endoplasmic reticulum (ER) are more evident at the higher magnification (figure 3b) where the ER appears to be associated with the membrane of a mitochondrion. The cytoplasm is packed with very small granules interspersed with many large electron-opaque bodies that do not possess any apparent internal structure. The double-layered cell membrane is highly convoluted and forms the external border of the cytoplasm next to the cell wall.

The thick wall shows little structural definition and some of its outer surface appears to have been detached from the hyphal cell. The polyclonal antiserum recognized antigenic sites which were predominantly located on the external surface of the hyphal structure (figure 4). Two sites were preferentially labeled, the border between the plasmalemma and the internal surface of the wall and the outer surface of the cell wall which includes wall fragments that had sloughed off the wall surface (a, b, and c). Binding was observed in the middle of the cell wall. Within the cytoplasm, antigenic sites were recognized at the periphery of the electron-opaque bodies (a and b). Further labeling was not associated with any specific organelle other than the

Figure 3. Transmission electron micrographs showing structural detail of *N. crassa* hyphae. (a) Low magnification; bar = 0.5 μm and (b) high magnification; bar = 0.125 μm

Abbreviations used in figures 3 to 5: cm, cell membrane; cw, cell wall; cy, cytoplasm; ER, endoplasmic reticulum; O, opaque body; m, mitochondrion; cr, cristae; N, nucleus; and n, nucleolus.

Figure 3

Figures 4 and 5. Immunogold labeling of *N. crassa* hyphae. Goat anti-rabbit IgG conjugated with 10 nm colloidal gold was used as the secondary antibody. Figures 4a, b, and c were probed with *N. crassa* cell wall polyclonal antiserum; bar = 0.25 μm . Figure 5a is a PBS control; bar = 0.5 μm . Figure 5b is a pre-immune serum control; bar = 0.25 μm . Black arrowheads indicate the presence of gold spheres.

Figure 4

Figure 5

small cytoplasmic granules. PBS and pre-immune serum negative controls showed background levels of immunolabeling (figures 5a and b, respectively).

B. Isolation and analysis of DM1 cDNA and gDNA

(i) screening the Lambda Zap I⁺ library

The polyclonal antiserum against crude cell wall extracts of *N. crassa* was used to probe a Lambda Zap I⁺ expression library of *N. crassa* mycelial cDNA in an attempt to identify clones that expressed cell wall components. Three hundred plaques were screened which resulted in the isolation of five positive clones identified as cDM1 through cDM5 (figure 6a). One additional clone (cDM6) was included from an unrelated screening of 300 plaques. High titred, non-purified stocks of clones cDM2 through cDM6 were further probed with the *Pst*I fragment from cDM1 to determine if they were identical to cDM1 or if they were unrelated cDNAs that had also been recognized by the *N. crassa* cell wall antibodies (figure 6c). It is likely that clones cDM2, 3, 4, and 5 are identical to cDM1 whereas cDM6 may be expressing an unrelated cDNA. Purification of the phage stocks and preliminary sequencing would be necessary to completely define the identity of these clones.

One of these positive clones, cDM1 was selected for further study. A pure culture of cDM1-expressing phage was prepared so that the cDNA could be extracted for characterization purposes (figure 6b).

Figure 6: Screening the Lambda Zap I⁺ expression library of *N. crassa* mycelial cDNA.

(a) initial screening (10^{-5} dilution) and isolation of five positive clones (cDM1 to cDM5) expressing fusion proteins recognized by the *N. crassa* anti-cell wall antiserum.

(b) final screening of a pure phage stock of the cDM1 clone.

(c) preliminary characterization of high titred, non-purified stocks of cDM2 to cDM6 by probing with the [α -³²P]dCTP labeled *Pst*I fragment from cDM1.

Figure 6

(ii) insert size and deleted subclones

To determine the size of the DM1 insert the cDNA was digested with *PvuII* since it was known that two such sites exist on either side of the pBSKS⁺ polylinker. An 1876 bp fragment that included the 445 bp polylinker was generated giving cDM1 an insert of approximately 1431 bp (figure 7a).

Deleted subclones were prepared to facilitate sequencing of the cDNA insert using exonuclease III. Digestion with *HindIII* provided a recessed 3' end close to the insert from which an increasing number of nucleotides were removed by prolonged exonuclease III digestion. This results in a truncated insert (figure 7b). Digestion with *KpnI* protected the more displaced 3' end by converting it into a 3' overhang.

(iii) screening the Lambda BARGEM7 genomic library

To isolate the gene that corresponds to the cDNA clone, an [α -³²P]dCTP-labeled *PstI* fragment of cDM1 was used to screen an *N. crassa* genomic DNA library in Lambda BARGEM7 (Pall and Bruneli, 1994). The initial screening resulted in two positive clones, gDM1 and gDM2 that were found on separate plates. Only gDM1 is shown (figure 8a) and this clone was used for all subsequent research. A pure culture of gDM1 phage was prepared so that the gDNA could be used for sequencing purposes (figure 8b).

Figure 7a: Agarose gel electrophoresis used to analyze 6 positive DH5 α F' colonies and determine the size of the cDM1 insert.

lanes 1 and 10: Lambda *Hind*III marker.

lane 3: pBSKS⁺/*Pvu*II. Fragment 1 is 2513 nt, fragment 2 (arrow head) is 445 nt.

lanes 4 to 9: DM1/*Pvu*II. Fragment 3 is approximately 2513 nt, fragment 4 is 1876 nt.

Figure 7b: Agarose gel electrophoresis used to analyze the cDNA from the Exonuclease III digestion reaction prior to transformation into competent *E. coli* cells for the preparation of deleted subclones.

lanes 1 and 10: Lambda/*Hind*III marker.

lane 2: undigested cDNA insert in pBSKS⁺.

lane 3: 0.5 minutes post-digestion.

lane 4: 2 minutes post-digestion.

lane 6: 4 minutes post-digestion.

lane 7: 7 minutes post-digestion.

lane 8 (sample 15): 9.5 minutes post-digestion.

Figure 7

Figure 8. The Lambda BARGEM7 expression library of *N. crassa* genomic DNA was screened using an [α -P³²] dCTP-labeled probe of the *Pst*I fragment from cDM1.

(a) initial screening (10^{-4} dilution) and isolation of one positive clone (gDM1).

(b) final screening of a pure phage stock of the gDM1 clone.

a

b

Figure 8

(iv) restriction map of cDM1

The restriction map of cDM1 displays the endonuclease restriction sites within the gene and within the polylinker of pBSKS⁺ that were used for cloning and probing purposes (figure 9). These sites include *EagI* at nucleotide 519, *PstI* at nucleotide 1024 and *SalI* at nucleotide 278. The ORF is 1026 nt in length and is located between nucleotides 110 and 1135. cDM1 is inserted into the *EcoRI* site of pBSKS⁺.

(v) sequence analysis

The cDM1 clone was sequenced to determine the identity of the cDNA that, when expressed as a fusion protein, was recognized by polyclonal antiserum raised against a crude cell wall extract of *N. crassa*. The constructs used for sequencing were the cDNA and gDNA inserts, deleted cDNA subclones, and restriction fragment cDNA and gDNA subclones, and universal and three synthetic primers (table 1) prepared from within the gene.

Sequencing of the cDNA revealed an insert of 1412 nt (figure 10) with a possible translational start site (ATG) located at nucleotide 110 encoding an ORF of 1026 nt. The ORF was terminated by the stop codon TGA at nucleotide 1136. A putative polyadenylation signal sequence (AGTAAA) was identified between nucleotides 1345 and 1350. An 18 nt polyA tail is found 43 nt downstream of the polyadenylation signal and 256 nt downstream of the last codon. A 59 nt intron was located between nucleotides 316 and 317 of the ORF. Features

Figure 9. Restriction map of cDM1 in the *EcoRI* site of the polylinker of pBSKS⁺. Fragments that were used for cloning or probing purposes were created by digesting the insert with *EagI*, *PstI*, or *SalI*. The ORF is located between nucleotides 110 and 1135 of the insert. Deleted subclones were prepared using the *HindIII* and *KpnI* sites of pBSKS⁺.

Figure 10: Nucleotide and deduced amino acid sequence of DM1. The nucleotide sequence is positively-numbered from the 5' to the 3' end of the cDM1. Putative translational ATG start site and TGA termination site are capitalized and are in bold, and the polyadenylation signal is underlined. Two predicted N-glycosylation sites (X-Asn-X-Thr/Ser) are in bold. Nucleotides located upstream of the cDNA and those encoding the intron (between nucleotides 316 and 317) are represented by lower case letters. A putative CPC1 protein binding site in the upstream region has been underlined. Potential CAAT and TATA elements are shadowed and bolded, respectively, and the putative transcriptional +1 sequence consensus TCATCANC is double underlined. 10 nt at the 5' end of the cDNA that were not present in the genomic sequence are italicized. The 5', lariat, and 3' signal sequences of the intron have all been bolded.

-229

tgagaagtatggccgagttcttttgagaaggagtgagtcaagatacggggggaatcggat
gaccagctcgtctatattggcaatggcgtcatgcaaatcactcatgacccgatatcggg
gaggtaggatggaagaggtatataactcaaagcaccagcatattgaggagtaaccttgcc
ttttgctcccaaacaacaactcgacatctcgctcttgactttgaatcaa (C)⁺¹¹

+1 cDNA +11

CTGCCATCGTCAACCTGCTATACCTTTGGTACGCCGCCAGCGTCCGAAGAGCCATGAAAT 60

+110 121

CTATCACAATGGGAACTCCAAGATCGAGCTTTGCGTTCGTTGCGCCAACATGTCTACCGAT
MetSerThrAsp

AAGATAACTTTCTCGCCAACTGGCAGCTACGCCGTACCATGCACCGCTTTACCTTGCC 181
LysIleThrPheLeuAlaAsnTrpHisAlaThrProTyrHisAlaProLeuTyrLeuAla

CAGGCCAAAGGCTTCTTCAAAGAACAGGGCATCCAGGTGGCTCTCCTGGAGCCAACGAAC 241
GlnAlaLysGlyPhePheLysGluGlnGlyIleGlnValAlaLeuLeuGluProThrAsn

CCCAGCGACGTAACCGAAATCATCGGCAGCGGCAAAGTCGACCTCGGTTTCAAGGCCATG 301
ProSerAspValThrGluIleIleGlySerGlyLysValAspLeuGlyPheLysAlaMet

316*

ATTCACACTCTTGCTgtaagattcgccattctcagccctcttaagatgggtcaaggctgat
IleHisThrLeuAla

*317

ccttcaccaacagGCCAAAGCCCGTAACTACCCTGTCCTCTCGATTGGCAGCCTTTTGAT 364
AlaLysAlaArgAsnTyrProValLeuSerIleGlySerLeuLeuAsp

GAACCTTTCACCGGCGTCTACCTCAAGGAATCCGGAATCACGACCGACTTTAGGTCC 424
GluProPheThrGlyValValTyrLeuLysGluSerGlyIleThrThrAspPheArgSer

CTCAAGGGCAAGCGTATCGGCTACGTCGGCGAGTTTGGCAAGATTCAGATCGATGAGCTC 484
LeuLysGlyLysArgIleGlyTyrValGlyGluPheGlyLysIleGlnIleAspGluLeu

ACTTCGCACTACGGCCTGACTCCCGCCGATTACACGGCCGTCCGCTGCGGCATGAACGTC 544
ThrSerHisTyrGlyLeuThrProAlaAspTyrThrAlaValArgCysGlyMetAsnVal

TCCAAGGCCATTATCAAGGGGGAGATTGACGCCGGTATCGCGCTCGAAAACGTCCAGATG 604
SerLysAlaIleIleLysGlyGluIleAspAlaGlyIleAlaLeuGluAsnValGlnMet

664
GTTGAGCTGGAGGAGTGGCTTGCTTCCCAGGTCCGGGACAAGGCCGACGTCCAGATGCTT
ValGluLeuGluGluTrpLeuAlaSerGlnValArgAspLysAlaAspValGlnMetLeu

724
CGCATTGACGAGCTCGCCGAACCTCGGATGCTGCTGCTTCTGCTCAATCCTCTACATTGGC
ArgIleAspGluLeuAlaGluLeuGlyCysCysCysPheCysSerIleLeuTyrIleGly

784
AACGAGACCTTCATCTCGCAGAACCCAGACAAGGTTCCGGGACTTTATGCGGGCGGTAAAG
AsnGluThrPheIleSerGlnAsnProAspLysValArgAspPheMetArgAlaValLys

844
AAGGCAACTGACTTTGTCCTGGCCAACCCGGACGCTGGGTGGAAGGAGTACGTTGACTTC
LysAlaThrAspPheValLeuAlaAsnProAspAlaGlyTrpLysGluTyrValAspPhe

904
AAGCCTGTCATGGGCACTGAGCTCAACAGGAAGATCTTTGAGAGGACGTACGCATACTTC
LysProValMetGlyThrGluLeuAsnArgLysIlePheGluArgThrTyrAlaTyrPhe

964
TCTGAGGACCTCAAGAACGTCGAGCGCGACTGGTCCAAGGTCACCAAATATGGTCAACGT
SerGluAspLeuLysAsnValGluArgAspTrpSerLysValThrLysTyrGlyGlnArg

1024
CTCGGCGTTCTGAGCCCAGACTTCACCCCCAACTACACCAACGAGTTTCTTGACTGGCTC
LeuGlyValLeuSerProAspPheThrProAsnTyrThrAsnGluPheLeuAspTrpLeu

1084
TGCAGCCCCGAATCCAGTGACCCTACTGGCGACCAGAAGCGCATGGTTGAGCTCCAGAAG
CysSerProGluSerSerAspProThrGlyAspGlnLysArgMetValGluLeuGlnLys

1135* 1144
AAGTGGCTTGCTGCGGTGGCTTCCACCGCTTGCCCCTTGGGATCAAGGCTTGATTGGTG
AsnValAlaCysCysGlyGlyPheHisArgLeuProLeuGlyIleLysAla *

1204
GGACGGGGTTGTCTTTGCGAATGGCAGAGGGCAACCAAGCTCGGCTTCATAGTCTGGGAT

1264
CTCGTTGCACAACCATTGGGTTTTGGTGGAAAGAAGAGGCTCGTAATCTTGATGTCTTTT

1324
TCGACTCAGTTCCAGATGAGAGTATCGTGTGGATGATCGATGAGGGCCTCAGGTGTAGCT

1384
AGCGAAGGCGTCTGCGTCAGTAGTAAACGTCTGTCTCTCCAGTCTAATTGACGTACGCTC

1412
TTACACCGTTAAAAAAAAAAAAAAAAAAAA

of the intron include a 5' signal (T^gtaag), a lariat signal (gctgat), and a 3' signal (aacag^G).

Several regulatory features were observed upstream of the ATG start codon including a CAAT box (caaat beginning at -135 nt), a TATA box (tatataa at nucleotide -91), and a putative +1 transcriptional initiation sequence (catc at nucleotide -25). One copy of the sequence gagtca was identified between nucleotides -195 and -190 which is identical to an inverted binding site (TGACTC) for the cross pathway control 1 (CPC1) regulatory protein. This element is present in the 5' region of several *N. crassa* genes that respond to amino acid limitation or imbalance through the cross-pathway control system (Orbach et al., 1990). Finally, sequencing the genomic clone revealed an apparent 10 nt artifact at the 5' end of the cDNA insert which was not found in the gDNA.

(vi) regulatory sequence comparison between DM1 and *N. crassa*.

The sequences of the DM1 clone that are putatively involved in transcriptional and translational regulation were compared with previously determined consensus sequences from other *N. crassa* genes in tables 2 (a) and (b) (Bruchez et al., 1993). In the 5' region, there was complete agreement between the TATA and CAAT boxes of DM1 and *N. crassa*. Only moderate agreement was observed with the +1 sequence consensus, a feature known to be located in close proximity to the transcriptional start site of several *N. crassa* genes (Bruchez

et al., 1993). The distance between the 3' end of the TATA box and the +1 sequence consensus of *nmt1* is greater than what is typical of other *N. crassa* genes which suggests that the putative +1 initiation sequence consensus may have been identified incorrectly. Without the primer extension results, the transcriptional start site and promoter region were deduced from sequence data alone.

A single nucleotide change was the difference between the polyadenylation signals from the two gene sources. The termination codon, TGA, used by DM1 is seen in only 27% of the other *N. crassa* genes. The single 59 nt intron in DM1 is typical of *N. crassa* genes. Over 80% of *N. crassa* protein-encoding genes have introns (Edelmann and Staben, 1994). The sequences of 5' and 3' splice sites and lariat formation are in good agreement with the consensus sequences. The noncoding and coding regions of DM1 had GC contents of 47.5 and 58.3%, respectively. Both values are just slightly less for these sequences than was calculated by Edelmann and Staben, (1994).

Tables 2 [(a) and (b)]: Comparison of consensus regulatory sequences involved in transcription and translation between DM1 and *N. crassa* (Bruchez et al., 1993). The subscripted values indicate the percent occurrence of the particular nucleotide. T or G indicate a conserved absence of the particular nt.

(a)

Feature	<i>N. crassa</i>	DM1
CAAT box	CAAAT	CAAAT
TATA box	TATATAA	TATATAA
+1 sequence consensus	TCATCANC	ACATCTCG
polyadenylation signal	AATAAA	AGTAAA
stop codon (% frequency)	TAA (62%)	TGA (27%)
prevalence of introns	1 or 2	1
average intron length	63 nt	59 nt

(b)

Gene Source	Feature/Sequence
	translational start site
<i>N. crassa</i>	C ₅₇ NNNC ₇₇ A ₈₁ (A ₄₄ /C ₄₃)T ₃ A ₉₉ T ₁₀₀ G ₉₉ G ₅₁ C ₅₃
DM1	G CGC C A A C A T G T C
	intron: 5' signal
<i>N. crassa</i>	G ₅₁ ^G ₉₉ T ₉₉ (A ₇₇ /G ₁₇)(A ₅₀ /C ₂₃)G ₉₄ (T ₇₀ /C ₁₅)
DM1	T ^G T A A G A
	intron: lariat
<i>N. crassa</i>	(G ₄₅ /A ₃₇)C ₉₄ T ₉₄ (A ₄₈ /G ₄₀)A ₉₃ C ₈₂
DM1	A C T G A T
	intron: 3' signal
<i>N. crassa</i>	G ₄ (A ₅₆ /T ₂₀)(T ₆₂ /C ₃₃)A ₁₀₀ G ₁₀₀ ^G ₄₀
DM1	A A C A G G

(vii) putative translation product and comparison

The ORF of DM1 encoded a protein of 342 amino acids with a predicted molecular mass of 38.3 kD. The putative product was analyzed using the FASTA program (figure 13) which revealed strong sequence homology (in decreasing order) to the *nm1* (no message in thiamine) gene of *Aspergillus parasiticus* (Cary and Bhatnagar, 1995), *Schizosaccharomyces pombe* (Maundrell, 1990), and *Saccharomyces cerevisiae* (Van Dyck et al., 1995) (table 3). *N. crassa* and *A. parasiticus* also share the same number of amino acids in their putative protein products.

Figure 11: Comparison and alignment of the deduced amino acid sequences of the *NMT1* protein of *N. crassa* (Nc) and the *nmtI* genes of *A. parasiticus* (Ap) (accession number P42882), *S. pombe* (Sp) (accession number P36597), and *S. cerevisiae* (Sc) (accession number P42883). Identical residues between the four genes have been bolded. Dashed lines were used for alignment purposes.

1

Nc MSTDKITFLANWHATPYHAPLYLAQAKGFFKEQGIQVALLEPTNPSDVTE
 Ap MSTDKITFLTNWHATPYHAPLYLAQSKGYFKEEGLKVALLEPNPDSVTE
 Sp MSTNKITFLTNWEATPYHLPIFLAQTRGYVEREGIEVAILEPTNPSDVTA
 Sc MSTDKITFLLN-QPTPYNIPFLAQTKGYFKEQGLDMAILEPTNPSDVTE

51

Nc IIGSGKVDLGFKAMIH TLAAKARNYPVLSIGSLLDEPFTGVVYLKESGIT
 Ap IIGSGKVDMGFKAMIH TLAAKARNFPVTSIGSLLDEPFTGVVYLKDSGIT
 Sp LIGSGKVDMGLKAMIH TLAAKARGYPVTSFGSLLNEPFTGLITLKGNGI-
 Sc LIGSGKVDMGLKAMINTLAARKARGFPVTSVASLIDEPFTGVLYLKGSGIT

101

Nc TDFRSLKGKRIGYVGEFGKIQIDELTSHYGLTPADYTAVRCGMNVSKAII
 Ap EDFRSLKGKIGYNGEFGKIQIDELTKYYGMTADDYTAVRCGMNVTKAII
 Sp NDFKDIKGKRIGYVGEFGKIQLDDLC SKFGLSPSDYTAIRCGMNIAPAI
 Sc EDFQSLKGKIGYVGEFGKIQIGELTKHYGMKPEDYTAVRCGMNVAKYII

151

Nc KGEIDAGIALENVQMVELEEWLASQVRDKADVQMLRIDELAE LGCCCFCS
 Ap RGDIDAGIGLENVQMVELAEWLASQNRPRDDVQIVRIDQLAE LGCCCFCS
 Sp NGEIDGGIGIECMQQVELERWCVSQGRPRSDVQMLRIDRLANLGCCCFCT
 Sc EGKIDAGIGIECMQQVELEEYLAKQGRPASDAKMLRIDKLACLGCCCFCT

201

Nc ILYIGNETFISQNPDKVRDFMRAVKKATDFVLANPDAGWKEYVDFKPVMG
 Ap ILYIANDAF LAANPEKVQKFMRAVKRATDYVLAEPAAAFEEYVDMKPIMG
 Sp ILYIAHDEFIAKHPDKIKAF LRAIHSATLDMLKDPVQTYKEYIHFKREMG
 Sc VLYICNDEFIKKNPEKYRKFLKA IKKATDYVLADPVKA-KEYIDFKPRLN

251

Nc TELNRKIFERTYAYFSED LKNVERDWSKVTKYGQRLGVLSPDFTP NYTNE
 Ap TPVNRKIFERSFAYFSRDLKNVSRDWAKVTNYGKRLGILDADFQPNYTNO
 Sp SELHREQFERCFAYFSHDISNVPRDWNKVTNYSKRLGIIPQDFEPNCTNG
 Sc NDLSYKQYQRCYAYFSSSLYNVHRD-KKVTGYGKRLAILPPDYVSNYTNE

301

Nc FLDWLCSPDSSDPTGDQKRMVELQKNVACCGGFH---RLPLGIKA-
 Ap YLSWTL DADSTDPLGDQKRMAELQKEVACEGGF---KRLQVASSA-
 Sp YLTWELDPDEKDP MGKQEAIAEIQDEIKQKGGVFSGNSLRYVEPANL-
 Sc YLSPEPEEVSDFLEACQRLMAIHQEKCRQEGTF---KRLALPA-

ORGANISM	# of aa	% IDENTITY	% SIMILARITY
<i>N. crassa</i>	342	NA	NA
<i>A. parasiticus</i>	342	76	85
<i>S. pombe</i>	346	59	70
<i>S. cerevisiae</i>	340	61	71

Table 3: Comparison of the percent identity and similarity of *NMT1* of *N. crassa* and the *NMT1* polypeptides of *A. parasiticus*, *S. pombe*, and *S. cerevisiae* determined by a FASTA search of the amino acid sequences.

(viii) chromosomal localization of *nmt1* using RFLP analysis

In an effort to determine the chromosomal location of the *nmt1* gene of *N. crassa*, the 1035 *Pst*I fragment of cDM1 was used as a probe against DNA from the small set of Metzenberg strains 4411 to 4430 (figure 12). An RFLP was established between the two parental strains (4411 and 4416) with respect to the *nmt1* gene and used in combination with the progeny RFLP to compare with the segregation pattern of known markers. The identical RFLP patterns between *nmt1* and *Fsr-17* to the cross-over point between strains 26 and 27 where *nmt1* then segregates with the *trp-3* locus demonstrates that *nmt1* is located between the two known markers on the right arm of linkage group II (table 4). Appendix 6 shows the recombination event between the Mauriceville and Oak Ridge parental strains that resulted in the RFLP for the *trp-3* (M), *nmt1* (O), and *Fsr-17* (O) loci of strain 26.

----- GENOTYPE -----																				
STRAINS:11		16										26 27				30				
<i>trp-3</i>	(O)	O	O	M	M	(M)	M	O	O	O	M	M	M	O	O	M	O	O	M	M
<i>nmt1</i>	(O)	O	O	M	M	(M)	M	O	O	O	M	M	M	M	O	O	O	O	M	M
<i>Fsr-17</i>	(O)	O	O	M	M	(M)	M	O	O	O	M	M	M	M	O	O	M	O	-	-

Table 4: Segregation of *nmt1* compared with *trp-3* and *Fsr-17*, two known markers on linkage group IIR.

Figure 12: Chromosomal localization of *nmt1* in *N. crassa*. Lanes 1 and 6 were loaded with gDNA from parental strains 4411 (Oak Ridge) and 4416 (Mauriceville), respectively. The remaining lanes contain gDNA from their progeny strains 4412 to 4415 and 4417 to 4430. A polymorphism was detected when the DNAs were digested with *EcoRI* which resulted in Oak Ridge and Mauriceville fragments that were either slightly smaller or slightly larger than the Lambda *HindIII* 23,130 nt fragment, respectively. The segregation pattern of DM1 was compared with the markers for *trp-3* and *Fsr-17*. These two loci have been mapped to the right arm of linkage group II. Overloaded lanes show variation in band migration and shape.

Figure 12

C. Control of *nmt1* expression

(i) thiamine regulation of *nmt1*

The *nmt1* gene of *S. pombe* is highly expressed and tightly regulated by thiamine availability (Maundrell, 1990). Consequently, the *nmt1* promoter has been used in many plasmid expression vector constructs to permit thiamine-mediated control of transcription to be applied to cloned genes (Maundrell, 1993). It was therefore of interest to determine if the expression of *nmt1* could be repressed as had been shown with the homologous *nmt1* genes of *S. pombe* (Maundrell, 1990), *S. cerevisiae* (Van Dyck et al., 1995), and *A. parasiticus* (Cary and Bhagnagar, 1995). The *nmt1* probe hybridized to a transcript that migrated somewhat faster than the 17S ribosomal RNA (1700 nt) which would be of sufficient size to encode the *nmt1* gene (figure 13a). The *nmt1* transcript showed moderate repression with the addition of thiamine to the medium (lanes 2 to 8), however, the transcript continued to be expressed even in the presence of very high levels (0.05 M) of thiamine (figure 13a). The increased intensity of the transcript in lane 4 (0.2 μ M thiamine) is likely an artifact of inconsistent loading as demonstrated by the rRNA content which was used as an internal control (figure 13b). Although not quantitatively analyzed, inhibition of mycelial growth was seen when 0.5 M thiamine was added to the culture medium (data not shown).

Figure 13: Northern analysis of *N. crassa* wild type cultures grown in VMM and VMM supplemented with thiamine. (a) Northern blot probed with the [α - 32 P]dCTP-labeled *Pst*I fragment of *cdm1*. (b) Ribosomal RNA content of the same Northern blot was used as an internal control and assessed by ethidium bromide staining. The larger band is the 26S rRNA (2,900 nt) and the smaller band is the 17S rRNA (1,700 nt).

lane 1: VMM only.

lanes 2 through 8: 0.05, 0.10, 0.20, 0.50, 1.0, 2.0 and 5.0 μ M of thiamine were added to the VMM, respectively.

Figure 13

(ii) Poly(A+) enrichment of the *nmt1* whole RNA

Poly(A+) enriched RNA was prepared in order to confirm that the probe constructed from the *Pst*I fragment of *cdm1* was hybridizing only to the mRNA of *nmt1* especially since the transcript was similar in size to the 17S rRNA. Northern analysis of whole RNA, poly(A-) RNA (unbound ribosomal), and poly(A+) RNA demonstrated that mRNA was more abundant in the enriched sample (lane 3) than in the total RNA sample (lane 1) (figure 14a). The probe hybridized to a transcript in the poly(A+) enriched sample that migrated with the putative *nmt1* transcript in the total RNA sample. A second, slightly larger transcript that was not observed in the other two lanes also hybridized to the probe with a less intense signal. This may be an unspliced transcript or a transcript that begins from an alternate start site. The probe did not hybridize to any transcripts in the poly(A-) sample (lane 2).

The blot was stripped and reprobed with [α -³²P]dCTP *crps-7* (cytoplasmic ribosomal protein small subunit) as an internal control (figure 14b). Ribosomal content was also used as an internal control and showed that there was considerably more rRNA in the whole RNA sample than in poly(A-) (lane 2) and poly(A+) (lane 3) preparations (figure 14c).

Figure 14: Comparison of total, poly(A-) and poly(A+) RNA extracted from an *N. crassa* wild type culture which was grown in VMM. (a) Northern blot probed with the [α - 32 P]dCTP-labelled *Pst*I fragment of *cdm1*. (b) Northern blot probed with the [α - 32 P]dCTP-labelled *crps-7*. (c) Ribosomal RNA content of the same Northern blot was used as an internal control and assessed by ethidium bromide staining. The larger band is the 26S rRNA (2,900 nt) and the smaller band is the 17S rRNA (1,700 nt).

lane 1: total RNA.

lane 2: poly(A-).

lane 3: poly(A+).

Figure 14

(iii) *nmt1* and cross pathway control

Several genes encoding amino acid biosynthetic enzymes in *S. cerevisiae* and *N. crassa* are subject to a cross pathway control regulatory system that increases the expression of these genes under conditions of amino acid starvation (Hinnebusch, 1992). Since these organisms can synthesize all 20 amino acids, cross pathway control cannot simply be induced by culturing the cells on minimal medium. Instead, the ability to synthesize one of the amino acids must be impaired. One common way to achieve this is to include 3-amino-1,2,4-triazole (AT), a competitive inhibitor of the histidine pathway (Luo et al., 1995). A number of genes subject to cross pathway control are repressed by the addition of all amino acids to minimal medium (Penn et al., 1984). The possibility that the *nmt1* gene is under cross pathway control (CPC) was suggested when one inverted copy of the binding site for the *CPC1* regulatory protein was identified in the sequence upstream of the *nmt1* gene of *N. crassa*. *nmt1* is moderately repressed by thiamine as previously described (figure 13a, lane 2), but it is not influenced by the addition of an incomplete supplement of amino acids (figure 15a, lane 3). Like other *cpc* regulated genes, *nmt1* is strongly derepressed in the presence of AT (lane 4), however, when AT is added in combination with either the amino acids (lane 7) or with thiamine (lane 6) or with both (lanes 8, 9, and 10), typical wild type levels (lane 1) of *nmt1* expression were observed.

Figure 15: Northern analysis of RNA extracted from *N. crassa* and used to examine cross pathway control regulation of DM1. (a) Northern blot probed with the [α - 32 P]dCTP-labeled *Pst*I fragment of *cDM1*. (b) Northern blot probed with the [α - 32 P]dCTP-labelled *crps-7*. (c) Ribosomal RNA content of the same Northern blot was used as an internal control and assessed by ethidium bromide staining. The larger band is the 26S rRNA (2,900 nt) and the smaller band is the 17S rRNA (1,700 nt).

N. crassa cultures were grown in VMM and supplemented as follows:

lane 1: VMM.

lane 2: thiamine.

lane 3: amino acids.

lane 4: 3-amino-1,2,4-triazole (18 hours post-inoculation).

lane 5: thiamine, amino acids.

lane 6: thiamine, 3-amino-1,2,4-triazole (18 hours post-inoculation).

lane 7: amino acids, 3-amino-1,2,4-triazole.

lane 8: thiamine, amino acids, 3-amino-1,2,4-triazole (15 hours post-inoculation).

lane 9: thiamine, amino acids, 3-amino-1,2,4-triazole (18 hours post-inoculation).

lane 10: thiamine, amino acid solution, 3-amino-1,2,4-triazole (21 hours post-inoculation).

Figure 15

The same cpc Northern blot was probed with [α - 32 P]dCTP crps-7 as an internal control (figure 15b). With respect to crps-7 RNA content, expression of the *nmt1* RNA is upregulated in the presence of 3-amino-1,2,4-triazole. The increased ribosomal RNA content in lanes 8, 9, and 10 has been exaggerated by the photography (figure 15c).

D. Expression of NMT1 as a fusion protein in *E. coli*

The 1162 bp *SalI* fragment of cDM1 was cloned into the pMAL-c2 vector for the purpose of expressing and purifying the protein produced from the DM1 ORF, and to determine if the antiserum recognized *nmt1* cDNA when expressed as a fusion protein in *E. coli*. The fusion protein consisted of the 42.2 kD MBP and the 34.3 kD ORF (286 amino acids) for a combined molecular mass of 76.5 kD (figure 16a, lane 2). It was identified as a prominent band that migrated closely to the 66.2 kD marker. The increase in band intensity confirmed that the fusion protein was recognized by the polyclonal anti-cell wall antiserum (figure 16b). It was visible in the control sample (lane 1) and showed increased expression in response to IPTG induction (lanes 2, 3, and 4). The antibodies recognized one other protein located between the 31 and 40 kD markers but it was not of the correct size to be the fusion protein and it did not respond to IPTG induction. Its presence did show that the increased expression of the putative fusion protein was in response to the IPTG and was not an artifact of inconsistent loading.

Figure 16: Analysis of the fusion protein expressed by the *SalI* fragment of *CDM1* in the *pMAL-c2* vector. The DNA construct was transformed into competent *E. coli* cells and IPTG was used to induce expression of the clones. The Western blots were probed with the *N. crassa* cell wall antibodies. (a) SDS-PAGE gel and (b) Western blot of a positive fusion clone. (c) SDS-PAGE of positive clone with *SalI* fragment not in proper orientation. (d) SDS-PAGE gel and (e) Western blot of clone with only the *pMAL-c2* vector.

lane M: mid range molecular weight marker.

lane 1: uninduced cells (prior to addition of IPTG).

lane 2: one hour post-IPTG induction.

lane 3: two hours post-IPTG induction.

Lane 4: three hours post-IPTG induction.

Figure 16

A clone with the insert in the wrong orientation was not recognized by the antiserum. Although there were several bands near the 66.2 kD marker (figure 16c, lane 1), none of these proteins displayed increased expression during the 3 hour incubation with IPTG (lanes 2, 3, and 4).

The MBP without the DM1 ORF was induced with IPTG which resulted in the increased expression of a protein that migrated near the 55 kD marker (figure 16d). This was identified as the MBP- β -gal- α fusion product that is known to have a molecular mass of 50.8kD. The antibodies recognized a non-induced protein of approximately 55 kD (figure 16e).

E. Immunoanalysis of *N. crassa* mycelial fractions using cDM1 phage affinity purified antibodies

Affinity-purified antibodies were prepared against the cDM1 fusion protein (figure 6b) and used to reprobe the Western blot (figure 1b) to determine in which fraction the gene product encoded by the cDM1 clone (characterized as *NMT1*) might be located (figure 17b). The purified antiserum recognized only two whole cell proteins (approximately 37 and 39 kD) of which the 37 kD protein had the more intense band (lane 2). A 37 kD protein was also visible in the spheroplast sample (lane 3). In contrast, no proteins in the cell wall extract were recognized by the cDM1-purified antibodies (lane 4). The faint shadow of two proteins that bracketed the 37 kD protein was observed in all three lanes and highlighted the gap where the 37 kD protein was absent in the cell wall lane.

Figure 17: Western analysis to determine in which *N. crassa* mycelial fraction the fusion protein expressed by cDM1 (NMT1) was located. (a) Probing mycelial fractions of *N. crassa* with the anti-cell wall antiserum (figure 1b). (b) Reprobing with the affinity purified antibodies prepared against the fusion protein expressed by cDM1.

lane 1: whole cell.

lane 2: spheroplasts.

lane 3: cell wall extract.

* indicates unique spheroplast proteins; **arrow heads** indicate unique cell wall proteins.

Figure 18: Western analysis to examine if the NMT1 protein was N-glycosylated. (a) Probing with the anti-cell wall antiserum (figure 2b). (b) Reprobing with the affinity purified antibodies prepared against the fusion protein expressed by cDM1.

lane M: mid range molecular weight marker.

lane 1: undigested glycoprotein control.

lane 2: glycoprotein digested with N-glycosidase F (1 hr).

lane 3: undigested mycelial proteins.

lane 4: mycelial proteins digested with N-glycosidase F (1 hr).

lane 5: mycelial proteins digested with N-glycosidase F (24 hr).

Figure 17

Figure 18

The Western blot used for the deglycosylation experiment (figure 2b) was reprobed with the cDM1 phage affinity-purified antibodies to determine if the *NMT1* protein *N*-glycosylated. Only one protein of approximately 37 kD was visualized and since its apparent molecular mass was not altered by *N*-glycosidase F (figure 18b, lanes 3, 4 and 5), it was concluded that the *NMT1* protein was not associated with any *N*-linked glycosyl moieties.

IV. DISCUSSION

The fungal cell wall is a complex, dynamic structure consisting principally of polysaccharides. The cell wall is also thought to include a substantial protein component that has not been characterized. In this thesis, I have described a polyclonal antiserum raised against a crude cell wall extract of *N. crassa* and demonstrated that it recognizes several proteins that are enriched in the cell wall fraction of *N. crassa*. Furthermore, the same antiserum was used to screen an *N. crassa* mycelial cDNA expression library from which six positive clones were isolated.

Analysis of *N. crassa* proteins with anti-cell wall antiserum

To retain proteins non-covalently associated with the cell wall, we used a modified version of the protocol by Martinez et al. (1991) that included mechanical breakage of the cells followed by the separation and washing of the walls in low salt buffer. According to Ruiz-Herrera (1992) most of the proteins extracted by Martinez et al. (1991) were true wall components. However, it is known that cytoplasmic proteins are still able to contaminate the preparations by tightly binding to the cell wall during breakage. Furthermore, the cell wall has been shown to be a sticky structure and cytoplasmic proteins can sometimes be found associated with it following cell lysis (Eroles et al., 1997).

Initial characterization of the antisera revealed a number of antigens that were recognized in both cell fractions

(figure 1b). Some proteins, such as 3-phosphoglycerate kinase (3-PGK) of *C. albicans*, are known to be present at the outer surface of the cell membrane, the cell wall, and in the cytoplasm and were expected in the whole cell, spheroplast (wall-less), and cell wall fractions (Alloush et al., 1997). Therefore, some of the proteins recognized by this antisera against *N. crassa* cell walls may likewise be involved in primary metabolism. Other proteins may be associated more exclusively with the wall such as the *N*-glycosylated cell surface acid phosphatase, PHO4 of *S. pombe* (Schweingruber et al., 1986). Since these proteins are synthesized and modified in the cytoplasm, an appreciable amount may be present in any cytoplasmic fraction.

Three proteins which were enriched in the cell wall fraction were initially considered to be the most potentially interesting. Their absence from the spheroplast sample showed that at least some proteins could be enriched for by co-purification with the cell wall. Unexpectedly, a few antigens were recognized by the antiserum in both the whole cell extract and spheroplast preparations that were missing from the wall extract. It is possible that the original wall extract used for antibody production may have been contaminated with cytoplasmic proteins, possibly because of the adhesive nature of the wall as was previously described. It is important to note that spheroplasts are not ideal protoplasts. They are prepared by digesting the wall with a

complex of enzymes including chitinase and protease (Vollmer and Yanofsky, 1986). Wall removal may not be complete and in addition, once spheroplasts are in an environment where they are permitted to regenerate their walls, all of the enzymes and other proteins required for that process are synthesized and delivered to the cell surface.

It had been suspected that the *N. crassa* mycelial preparations would contain some *N*-glycosylated proteins since many yeast cell wall-associated proteins are complexed with mannosyl groups (Ballou, 1976) that are universally attached through two GlcNAc residues and an *N*-linked asparagine. Native structural glycoproteins are known to elicit a strong antigenic response (Celerin et al., 1995) and the carbohydrate moiety rather than the protein part may be more antigenic (Ruiz-Herrera, 1992). In addition, polysaccharides can induce a strong antibody response in some species (eg. human, mouse) but a poor or nil response in others (eg. rabbit, guinea pig); although the reason for this difference is not known (Klein, 1988). When most glycoproteins are introduced by subcutaneous, intramuscular, or intraperitoneal injection into an animal host, they remain in the system for a period of time before significant levels of antibodies are created. Thus, extensive antigen degradation may result before antibody production is stimulated, particularly for glycosylated antigens whose terminal sugars are readily cleaved (Celerin et al., 1995). In this study, no *N*-glycosylated proteins were

observed following digestion with N-glycosidase F. However, this result may have been partially obscured by the many probe-positive proteins (figure 2b). It is also possible that the *Neurospora* proteins are linked to short chains of carbohydrates whose removal would not be detected using SDS-PAGE minigels.

Immunoscreening cDNA expression library

Eroles et al. (1997) and Alloush et al. (1997) successfully immunoscreened expression libraries in their pursuit of cell wall components in *C. albicans*. Using a similar strategy, we isolated six positive clones by screening a cDNA expression library with a polyclonal antibody preparation against the *N. crassa* cell wall (figures 6a and b). Based on hybridization with cDM1, four of the six clones appear to code for the same gene or related genes (figure 6c). Initial sequence analysis revealed strong identity between the cDM1 clone and the *nmt1* gene in *S. pombe*, an allele of the *thi3* locus of the thiamine biosynthetic pathway (Maundrell, 1990). The *nmt1* gene is expressed at high levels in yeast grown without a thiamine supplement. However, the role of the *nmt1* gene product has still not been determined (Maundrell, 1990). In the yeast *S. pombe*, low levels of thiamine added to culture medium can inhibit sexual agglutination and zygote formation (Schweingruber and Edenharter, 1990). Since a possible interaction between NMT1 and the cell wall could not be ruled out, further characterization of the *nmt1* homologue

from *Neurospora* was initiated.

Fungi are more often heteroauxotrophic for thiamine (vitamin B1) than for any other vitamin (Moore-Landecker, 1990). It is a water soluble vitamin that is common to many organisms and consists of a pyrimidine and a thiazole precursor which originate from independent pathways (appendix 6) (Young, 1986). The two moieties are initially phosphorylated to form 4-amino-5-hydroxymethyl-2-methylpyrimidine and 5-(2-hydroxyethyl)-4-methylthiazole. Condensation of the two components results in the formation of thiamine monophosphate which is eventually phosphorylated to thiamine diphosphate (TDP). TDP serves as a required coenzyme for several enzymes of intermediary metabolism including pyruvate decarboxylase, pyruvate dehydrogenase, and α -ketoglutarate dehydrogenase, all of which catalyze the removal or transfer of aldehyde groups. The thiazole moiety is the active portion of the thiamine molecule (Mathews and van Holde, 1990). Few details are known about the initial formation of the pyrimidine and thiazole precursors of thiamine. Early research has lead to the grouping of auxotrophic mutants according to their thiamine requirements ie. thiamine or pyrimidine plus thiazole correct the auxotrophy; thiamine is required but the organism cannot grow in the presence of the two precursors, either thiamine or one of the precursors is required for growth, or neither thiamine nor the precursors result in growth of the organism (Young,

1986).

The metabolic pathway for thiamine synthesis in *N. crassa* was proposed by Tatum and Bell (1946). In 1982, Perkins et al. reviewed the five thiamine auxotrophic mutants that had been isolated in *N. crassa*. The *thi1* gene, which is located on the right arm of linkage group I, is thought to be involved in the utilization of thiamine since *thi1* mutants are able to grow successfully in the presence of thiamine or the precursors pyrimidine plus thiazole (Eberhart and Tatum, 1961). The *thi3* locus has been mapped to the left arm of linkage group VII and it has been suggested that this gene is associated with the thiazole pathway (Tatum and Bell, 1946) since these mutants can use either thiamine or thiazole. In contrast, *thi5* appears to function as part of the pyrimidine pathway because either thiamine or the pyrimidine precursor are required for growth by the organism (Perkins, 1972). *thi5* is located on the right arm of linkage group IV. *thi2* (linkage group IIIR) and *thi4* (to the right of the centromere on linkage group III) auxotrophs need the complete thiamine molecule for growth (Tatum and Bell, 1946). In addition, the level of thiamine in these two mutants is found to be low in mycelia and therefore it has been proposed that thiamine synthesis might have been blocked (Eberhart and Tatum, 1959). These data suggest that products of the *thi2* and *thi4* genes might function in the phosphorylation and/or condensation of the pyrimidine and the thiazole precursors.

Recently, the *thi4* gene of *N. crassa* was cloned by functional complementation of a *thi4* mutant (Akiyama and Nakashima, 1996). The *thi4* gene encodes a protein of 538 amino acids which is similar to two proteins encoded by *S. cerevisiae* but whose function is unknown. Restriction fragment length polymorphism analysis (Metzenberg et al., 1984) confirmed that the *thi4* locus is located near the centromere of linkage group III.

It had been anticipated that *nmt1* might be allelic to the *thi5* locus which is involved in pyrimidine biosynthesis, as is the case with the *nmt1* gene of *S. pombe*. RFLP analysis mapped *nmt1* of *N. crassa* to the right arm of linkage group II between the *trp3* and *Fsr17* marker genes (appendix 7) and this result was compared to the five known thiamine loci of *Neurospora*. The *thi5* locus is located on the right arm of chromosome IV and in fact none of the thiamine loci have been mapped to linkage group II. This result does not preclude the possibility that *nmt1* of *N. crassa* is involved in the pyrimidine pathway though it must be required at some other step. According to Van Dyck et al. (1995), no particular motif or feature of the sequence has helped to assess the exact role of yeast NMT1 in thiamine biosynthesis.

***nmt1* expression and thiamine**

Since *nmt1* was first isolated in *S. pombe* (Maundrell, 1990) homologous genes have been identified in *A. parasiticus* (Cary et al., 1995) and *S. cerevisiae* (Van Dyck et al., 1995; Hather et al., unpublished). Amino acid alignment and comparison have shown that although they are all fungi, the two mold genes are more closely related to each other than is the *nmt1* gene of *N. crassa* to the two yeast counterparts (table 3). The common characteristic of *nmt1* in the other three organisms is that it is tightly regulated and completely repressed by thiamine. Furthermore, the expression of all cloned genes involved in the biosynthesis of thiamine in *S. pombe* and *S. cerevisiae* is repressed in the presence of thiamine (Zurlinden and Schweingruber, 1992). In *S. pombe*, thiamine-repressed genes are thought to be under the control of a Cys-6 zinc finger-type transcription factor encoded by the *thi1* locus (Frankhauser and Schweingruber, 1994).

We have found the expression of *nmt1* in *N. crassa* to be only moderately repressed by thiamine at concentrations up to 100,000 times greater than was required to completely inhibit *nmt1* in the other organisms (figure 13a). It appears that the regulation of thiamine biosynthesis in *N. crassa* is different from that in yeast because in addition to the *nmt1* result, expression of the *thi4* gene of *N. crassa* has also been shown to be constitutive in the presence of thiamine (Akiyama and Nakashima, 1996). Since the *S. pombe nmt1* gene is highly

expressed, its promoter region has been used to construct a series of inducible expression vectors for fission yeast (Maundrell, 1990). When fused to the bacterial chloramphenicol acetyltransferase (*cat*) reporter gene, and under thiamine deprivation, the *nmt1* promoter has been shown to be 6-7 times more active than the strong *adh* promoter of *S. pombe* and about 30 times more active than the SV40 promoter which also functions well in this organism (Basi et al., 1993). The presence of thiamine in the medium results in complete downregulation of the *nmt1* promoter as was evidenced by an estimated 80-fold decrease of CAT activity. Thiamine uptake rates are inversely regulated by the intracellular thiamine pools (Schweingruber et al., 1991). If this uptake is tested when glucose is omitted from minimal medium, or in the presence of a metabolic inhibitor such as arsenate, transport rates are reduced. These results suggest that active metabolism and probably energy generation is a prerequisite for the occurrence of thiamine transport.

Tommasino and Maundrell (1991) showed that when *S. pombe* cells were grown in minimal medium, they actively synthesized thiamine and maintained an internal concentration of 10 pmoles/ 10^7 cells. Thiamine added to culture medium is rapidly sequestered by the cells and the internal concentration of thiamine increases almost 1000-fold before the transport mechanism shuts down (Tommasino and Maundrell, 1991). In the fission yeast the transcriptional activity of the *nmt1* gene is

rapidly repressed as the internal concentration increases. Above the threshold of 50 pmoles/ 10^7 cells, the *nmt1* promoter continues to be repressed. *In vivo* synthesized thiamine is present intracellularly mostly as TDP (Schweingruber et al., 1991) but large amounts of thiamine can be stored in its unphosphorylated form inside *S. pombe* cells (Schweingruber et al., 1991).

When the active form is required, thiamine pyrophosphokinase (TPK) phosphorylates the intracellular thiamine to thiamine pyrophosphate (Frankhauser et al., 1995). Thiamine has been shown to inhibit mating and zygote development (Schweingruber and Edenharter, 1990), however, when TPK mutants are grown in supplemented media, they mate constitutively and are slow growers with aberrant morphology. Akiyama and Nakashima (1996) reported that wild type mycelial growth in *N. crassa* fell to 40% with the addition of 0.01 nM of thiamine to the culture medium. In contrast to the result of Akiyama and Nakashima (1996), no inhibition of mycelial growth below 0.5 M thiamine was observed during my own experiments. Akiyama and Nakashima did not disclose how thiamine was prepared or when it was added to the growth medium. Since these steps can be performed several different ways, this may be the basis for such conflicting growth inhibition results.

Cross pathway control regulation of *nmt1*

Starvation for single amino acids has been shown to

increase the synthesis of enzymes for many amino acid biosynthetic pathways (Ebbole et al., 1991) and in *N. crassa* this general activation has been termed cross pathway control (CPC). CPC1 is the principal regulatory protein required for cross pathway control in *N. crassa* and it shares considerable sequence homology and function with GCN4 of *S. cerevisiae* (Ebbole et al., 1991). Both are DNA binding proteins that recognize the sequence, TGACTC, an element that is located upstream of several genes regulated by cross pathway or general amino acid control (Ebbole et al., 1991). Four copies of this element are found upstream of the CPC-controlled *arg2* gene of *N. crassa* (Orbach et al., 1990). The presence of an inverted copy of this binding site upstream of *nmt1* prompted me to examine the effect of histidine limitation on *nmt1* expression. Cells were exposed to 3-amino-1,2,4-triazole (AT), a competitive inhibitor of imidazole glycerol phosphate dehydratase, an enzyme of the histidine biosynthetic pathway which is encoded by the *his3* gene (Luo et al., 1995). Expression of *nmt1* was significantly upregulated in the presence of the inhibitor demonstrating that the cross pathway control system participates in the regulation of this putative thiamine biosynthetic gene (figure 15a).

A number of genes subject to the general control in *S. cerevisiae* are also repressed several fold by the addition of all amino acids to minimal medium (Hinnebusch, 1992). Furthermore, it has been shown that the addition of histidine

abolishes AT derepression of *N. crassa* CPC1-controlled arginine enzymes yet exogenously added arginine does not influence AT derepression (Fink, 1981). Since histidine was included in the amino acid supplement used in my experiment, this result suggests that histidine is responsible for downregulation of *nmt1* to typical wild type levels from the highly derepressed state caused by the single amino acid limitation. Without the addition of all amino acids, it is not known if *nmt1* can be completely repressed like so many of the *S. cerevisiae* genes. If it is not, this may be another example of how thiamine biosynthesis in *N. crassa* differs from the yeast pathway as was shown earlier by the incomplete repression of *nmt1* and the constitutive expression of the *thi4* (Akiyama and Nakashima, 1996) in the presence of thiamine. Surprisingly, addition of exogenous thiamine negated the derepression by AT. Thiamine, in its active form as thiamine pyrophosphate, is a cofactor for several enzymes of the citric acid cycle from which histidine is also derived (Mathews and van Holde, 1990). If histidine limitation results in the upregulation of tricarboxylic acid cycle enzymes, then, in this instance, enough thiamine may have been sequestered and stored by the *Neurospora* cells to satisfy the TPP cofactor requirement. This would suggest that the *nmt1* promoter is able to override the binding of CPC1 and the cross pathway control response to single amino acid limitation.

Expression of *nmt1* as a fusion protein in *E. coli*

SDS-PAGE analysis showed the *SalI* fragment from cDM1 (*nmt1*) was in the correct orientation to be IPTG-induced and expressed as a functional fusion protein in *E. coli* (figure 16a). Since post-translational modification of bacterial proteins does not occur (Old and Primrose, 1992), Western analysis confirmed that the polyclonal antiserum recognized NMT1 as a proteinaceous antigen and that an antigenic site is within the *SalI* fragment (figure 16b).

Analysis of *N. crassa* NMT1 with affinity-purified antibodies

Western analysis of the *N. crassa* mycelial preparation demonstrated that the only protein recognized by the cDM1 phage affinity purified antiserum did not show any change in its molecular mass following digestion with the *N*-glycosidase F. This result indicated that the NMT1 protein is not associated with large *N*-glycosyl groups even though three putative Asn glycosylation sites were identified in the amino acid sequence. The presence of *O*-linked complexes has not been investigated.

Western analysis of the *N. crassa* mycelial fractions showed that the gene product encoded by the cDM1 clone (*nmt1*) was present in the mycelial and spheroplast fractions. This result, coupled with the absence of this protein from a new cell wall preparation, suggests that NMT1 is a cytoplasmic protein. To confirm and further define the site of NMT1, the immunoelectron microscopy would have to be repeated using the

affinity purified antibodies. The immunogold labeling with the polyclonal antiserum recognized several antigenic sites including the periphery of the electron-opaque cytoplasmic bodies and some not associated with any specific organelles.

The putative 342 aa NMT1 ORF of *N. crassa* that is expressed by the DM1 clone was expected to encode a polypeptide of approximately 38 kD. This result corresponds closely with the 37 kD protein detected in whole cell and spheroplast extracts that was not *N*-glycosylated and probed positive with the cDM1 phage affinity purified antibodies.

V. CONCLUSIONS

1. Polyclonal antiserum prepared against a crude cell wall extract of *N. crassa* recognized 10-15 prominent proteins including 3 which appear to co-purify with the cell wall fraction. Transmission electron microscopy and immunogold labeling confirmed that the polyclonal antibodies bound preferentially to antigenic sites on the outer surface of the cell wall and the inner border between the wall and the plasmalemma. Binding of the antibodies was limited in the middle of the wall, along the periphery of the electron-opaque bodies, and in the cytoplasm unassociated with any specific organelles.
2. The anti-cell wall antiserum was used to detect cDNA clones in an expression library. One clone selected for further study encodes an ORF of 1029 bp that has sequence homology to the tightly regulated *nmt1* genes of *S. pombe*, *A. parasiticus*, and *S. cerevisiae*. In these three organisms, *nmt1* is completely thiamine-repressible which contrasts the regulation of the *nmt1* gene of *N. crassa*.
3. It was also shown that *nmt1* expression in *N. crassa* is regulated by the cross pathway control system since single amino acid limitation using the histidine inhibitor, 3-amino-1,2,4-triazole, resulted in derepression of the *nmt1* gene. This has not been reported for any other *nmt1* homologue

described to date.

4. The polyclonal antiserum recognizes NMT1 as a proteinaceous antigen expressed in bacteria. The protein expressed by the cDM1 clone is approximately 37 kD, is not *N*-glycosylated, and is not likely associated with the *N. crassa* cell wall.

Appendix 1.

Wall and hyphal growth

(Trinci, 1978)

Appendix 2. Lambda/*Hind*III standard fragment sizes (nt):
23,130; 9,416, 6,557; 4,361; 2,322; 2,027; 564; and 125.

Appendix 3. Protein extraction buffer:

10 mM Tris-HCl (pH 7.6)

1 mM EDTA

One mL of these two first components was added to:

1 mM PMSF	5 μ L of 200 mM
1 mM Benzamidine	15 μ L of 100 mg/mL (64 mM)
1 mM Eaminocaproic acid	13 μ L of 100 mg/mL (76 mM)
2 mM DTT	20 μ L of 1 M
1 μ g/mL pepstatin	10 μ L of 1 mg/mL
1 μ g/mL leupeptin	10 μ L of 1 mg/mL
1 μ g/mL TPCK	10 μ L of 1 mg/mL
1 μ g/mL aprotinin	2 μ L of 5 mg/mL

To a final volume of 10 mL with dH₂O.

Appendix 4. Protein concentration calculation (Kalb and Bernlohr, 1977):

$$(183 \times \text{Abs}_{230}) - (75.3 \times \text{Abs}_{260}) = \mu\text{g/mL protein.}$$

Appendix 5. Mid-range molecular weight standard fragment sizes (kD): 97.4, 66.2, 55.0, 42.7, 40.0, and 31.0 (Promega) or 97.4, 66.2, 39.2 and 26.6 (New England Biolabs).

Appendix 6.

Appendix 7.

(Young, 1986)

Appendix 8.

ADDITIONAL LOCI IN LINKAGE GROUP II

<i>acc-9</i>	Between <i>nuc-2</i> (2%) and <i>arg-12</i> (3%) [72a]	<i>Fir-54</i>	Between <i>Fir-32</i> and <i>arg-5</i> [56, 55]
<i>acc-5</i>	Between <i>arg-5</i> (6%) and <i>pe</i> (9%) [68]	<i>Fir-55</i>	Between <i>arg-5</i> & <i>prg</i> [56]; <i>Gya-4</i> & <i>pma-1</i> [55]
<i>acu-5</i>	Linked to <i>arg-5</i> (6%) and <i>aro-3</i> (7%)	<i>gfp-2</i>	Between <i>T(AS176)</i> and <i>T(NM177)</i>
<i>acu-8</i>	Between <i>trp-3</i> (8%) and <i>un-15</i> (3%) [51b]	<i>l3, l14</i>	Between <i>Fir-55</i> and <i>Fir-3</i> [53]
<i>alc-1</i>	Linked to <i>pe</i> (10%)	<i>hbs</i>	Between <i>Fir-54</i> and <i>can-6</i> [53]
<i>aod-2</i>	Linked to <i>arg-5</i> (7%) and <i>thr-3</i> (16%)	<i>het-1</i>	Right of <i>fl</i> (25%)
<i>atp-2</i>	Near <i>arg-5</i> [6a, 55]	<i>hup-1</i>	Near <i>Tel-III</i> [40, 55]
<i>blt-4</i>	Near <i>Fir-34</i> [61c, 55]	<i>lp</i>	Right of <i>thr-3</i> (10%)
<i>blt-7</i>	Between <i>Fir-3</i> and <i>Fir-32</i> [61c, 55]	<i>leu-6</i>	Right of <i>Fir-17</i> [4, 14, 55]
<i>cgg-2</i>	Between <i>Fir-3</i> and <i>Fir-34</i> [49, 55]	<i>mcm</i>	Linked to <i>ro-7</i> (1%) [50a]
<i>cdr-1</i>	Left of <i>arg-12</i> (35%) [47a]	<i>muv-7</i>	Between <i>arg-5</i> (8%; 12%) and <i>nuc-2</i> (11%)
<i>cir-1</i>	Near <i>arg-5</i> [6, 55]	<i>muv-24</i>	Left of <i>pyr-4</i> (8%) [34, 74]
<i>col-10</i>	At or near <i>pl</i>	<i>muv-27</i>	Right of <i>nuc-2</i> (16%) [30]
<i>con-6</i>	Between <i>hbs</i> and <i>Fir-55</i> [5, 53]	<i>nuc</i>	One or more loci right of <i>arg-12</i> [22]
<i>(cox-5)</i>	Between <i>arg-5</i> and <i>Fir-55</i> . Allelic to <i>Gya-4</i> [72]	<i>nub-5</i>	Linked to <i>trp-3</i> (30%); near <i>T(4637)</i>
<i>cpd-4</i>	Right of <i>arg-12</i> (7%) [30]	<i>nub-9</i>	Linked to <i>arg-5</i> [39]
<i>cwl-1</i>	Linked to <i>arg-5</i> (3%)	<i>(pcon)</i>	Probably allelic to <i>nuc-2</i> (0/854)
<i>cwl-2</i>	Right of <i>arg-12</i> (10%), between <i>un-20</i> (4%) and <i>fl</i> (9%)	<i>pma-1</i>	Between <i>arg-5</i> , <i>Fir-55</i> and <i>l3, l14</i> [77a, 55]
<i>Gya-4</i>	Between <i>arg-5</i> and <i>Fir-55</i> . Allelic to <i>cox-5</i> [72]	<i>png</i>	Linked to <i>pyr-4</i> (0/207) [16]
<i>Gyb-3</i>	Left of <i>ro-3</i> (9%)	<i>(ro-9)</i>	Probably allelic to <i>da</i> [62]
<i>Gyr-12</i>	Between <i>thr-3</i> (30%) and <i>trp-3</i> (18%)	<i>scr</i>	Linked to <i>arg-12</i> (2%), probably to the left
<i>da</i>	Linked to <i>thr-3</i> (3%); right of <i>T(NM149)</i>	<i>spco-14</i>	Linked to <i>arg-5</i> (7%)
<i>das</i>	Probably near <i>acc-1</i> [20a]	<i>su((mi-1))-3</i>	Linked to <i>fl</i> (31%)
<i>en(am)-2</i>	Near <i>pe</i>	<i>su((mi-1))-4</i>	Linked to <i>fl</i> (22%) and <i>arg-5</i> (40%)
<i>(fs-2)</i>	Tentative linkage to <i>cur-5</i> (14/48)	<i>su((mi-1))-10</i>	Linked to <i>arg-5</i> (29%) and <i>fl</i> (24%)
<i>Fsp-1</i>	Right of <i>pe</i> (4%)	<i>usg</i>	Between <i>T(NM149)</i> and <i>arg-5</i> (6%; 14%)
<i>Fir-3</i>	Between <i>vma-2</i> and <i>cgg-2</i> [55]	<i>tu</i>	Between <i>pe</i> (8%) and <i>fl</i> (9%)
<i>Fir-17</i>	Right of <i>trp-3</i> near <i>leu-6</i> [56, 55]	<i>tu-1</i>	Linked to <i>pyr-4</i> (31%)
<i>Fir-21</i>	Between <i>arg-12</i> and <i>trp-3</i> [55]	<i>tu-24</i>	Between <i>T(AM10)</i> breakpoints [37a]
<i>Fir-32</i>	Between <i>Fir-52</i> and <i>cir-1</i> , <i>arg-5</i> [56, 55]	<i>ure-3</i>	Between <i>arg-12</i> (7%; 12%) and <i>un-20</i> (8%)
<i>Fir-34</i>	Between <i>arg-12</i> and <i>trp-3</i> . Right of <i>cgg-2</i> [56, 55]	<i>vma-2</i>	Between <i>l3, l14</i> and <i>Fir-3</i> [8, 55]
<i>Fir-52</i>	Between <i>pyr-4</i> and <i>Fir-32</i> [56, 55]	<i>ylo-3</i>	Allelic to <i>fl</i> . Renamed <i>fl'</i>
		<i>xdlh-1</i>	Linked to <i>pe</i> (14%)

(Metzenberg et al., 1984)

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