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Cloning of the *pmb* gene encoding a basic amino acid transport protein in *Neurospora crassa*

Han, Hyo-Young, Ph.D.

University of Hawaii, 1993

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CLONING OF THE *pmb* GENE ENCODING A BASIC AMINO ACID TRANSPORT PROTEIN IN *Neurospora crassa*

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

BIOMEDICAL SCIENCES (GENETICS)

AUGUST 1993

By

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ABSTRACT

Neurospora crassa is known to possess at least six genetically and biochemically distinct amino acid transport systems; three constitutive amino acid permeases and three specialized amino acid permeases. The constitutive permeases include a neutral amino acid-specific system (N system), a basic amino acid-specific system (B system), and a general system (G system) that can transport neutral, basic, and acidic amino acids.

We have cloned the *pmb* gene which codes for a component of the B system. A *Neurospora crassa* triple mutant (*pmn:pmb:his-2*) was constructed from previously described mutants. This strain is suitable for use as a host strain in a transformation procedure for selecting DNA from the wild type *pmb* locus. Protoplasts of the *pmn:pmb:his-2* strain were prepared and transformed by sib selection with DNA fragments from cosmid library pools of wild type *Neurospora crassa.* One cosmid (pMOcosX-X7:5E) complemented the *pmb* mutant allele and was confirmed to be tightly linked to RFLP (Restriction Fragment Length Polymorphism) markers flanking the *pmb* locus on linkage group IV. A subclone of the pMOcosX-X7:5E cosmid, pB22-22 (3.2Kb), complemented the *pmb* mutant allele and was mapped with restriction enzymes. The subclone hybridizes to RNA transcripts 3.5Kb in size on Northern blot experiments. The subclone pB22-22 has been sequenced and the *pmb* portion of the sequence containing a promoter initiation site has been analysed; it

has two CAAT motifs at -166 and -155 when positions are given in base pairs (bp) from the A(+1) of the proposed translational start: a TATA motif is located at -123. There are two CT-rich regions; one is from -117 to -106 (12bp length) and the other is from -50 to -23 (28bp length) containing 83% of and 75% of pyrimidine bases each.

The promoter site of the *pmb* locus contains sequences for binding motifs of CPC-1 (cross-pathway control protein) at upstream and downstream of the translation start point. This suggests that the *pmb* gene is regulated by the *cpc-1* gene.

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CHAPTER 1
INTRODUCTION

1.1 FEATURES OF *NEUROSPORA*

1.1.1 WHAT IS *NEUROSPORA* ? (25, 46)

Neurospora was first named and described by Shear and Dodge (1927). *Neurospora* is a eukaryotic, heterotrophic organism with an absorptive mode of nutrition. *Neurospora* cells contain normal mitochondria, an endoplasmic reticulum with ribosomes, nuclei with typical nuclear membranes, nucleoli, and various inclusions, such as ergosterol crystals, oil droplets, and glycogen bodies. *Neurospora* is both multicellular and multinucleate, and cells are bounded by a double-layered plasma membrane, and a strong cell wall composed of glucose polymers, a peptide-polysaccharide complex, chitin, and polygalactosamine. The *Neurospora* species is found in tropical or subtropical areas growing on trees and cellulosic plant remains. Recently, it has found another habitat as a laboratory organism because of its simple nutrient requirements and its straight forward biochemistry and genetics.

Neurospora has a number of properties which make it important both scientifically and economically. Its haploid genomes, rapid life cycles, cell differentiation, highly regulated metabolic pathways and efficient secretion of proteins make *Neurospora* attractive as a model system for basic biological research of eukaryotic organisms. Since the initial development of recombinant DNA technology in bacteria in

the early 1970s, the approach and techniques of molecular biology enable us to approach fundamental questions about many aspects of fungal biology, by providing the way to directed manipulation of fungal metabolism. The general economic importance of fungi is a reflection of their diverse life forms and metabolic virtuosity.

All fungal strains are inherently unstable as a result of the natural process of spontaneous mutation. While some strains are more stable than others, the effects of genetic instability can be mitigated by regular re-isolation and by reducing the number of subcultures between re-isolation steps to a necessary minimum.

1.1.2 TAXONOMY (24)

The filamentous fungus *Neurospora crassa* is a member of the fungal class Ascomycetes. As an ascomycete, *Neurospora* is related to yeasts, and as a fungus, *Neurospora* is more distantly related to mushrooms. Recently, in recognition of the unique nutritional and morphological attributes of fungi, taxonomists have elevated their taxonomic ranking to a separate kingdom, the fifth kingdom. Fungi are classified in the subkingdom Eumycota of the kingdom Fungi. Eumycota are eukaryotic, uni- to multicellular heterotrophic organisms with absorptive nutrition.

1.1.3 LIFE CYCLE (13, 25, 46)

Reproduction of *Neurospora* is either asexual or sexual. *Neurospora* grows as a branched tubular filament known as hypha

that originates from the germination of a single reproductive spore. The hypha grows at the tip and becomes multicellular by incomplete cross-walls, or septa, which have pores about 0.5um in diameter in their centers. The pores allow cytoplasm to flow along the hyphae, carrying nuclei, mitochondria, and other inclusions for some distance, usually in the direction of growth. In most hyphae there are more than one nucleus in each cell (i.e., between successive septa), ranging from about three to as many as 100 in certain unusual strains. Nuclei divide most actively near hyphal tips, and it appears that chromosomes remain in groups comprising entire sets during the division cycle. Such grouping may prevent chromosome loss during division in rapidly flowing cytoplasm. *Neurospora* has three spore forms, two asexual (microconidia and macroconidia) and one sexual (ascospores). Under appropriate conditions, the vegetative mycelium gives rise to a reproductive mycelium that supports the production of reproductive spores. The type of sporulation as well as the morphology of the spores and spore-bearing structures are key characteristics in fungus identification.

Similar to *Sacharomyces cerevisiaes*, *N. crassa* has two mating types, designated A and a. A normal strain of either mating type can produce male gametes (macro- or micro-conidia) and female gametes (ascogonia, enclosed in a protective sheath, the whole known as a protoperithecium). Macroconidia are used to inoculate vegetative cultures, and they serve as the fertilizing (male) parent in sexual crosses. Microconidia are formed late in growth by a process quite

distinct from macroconidial formation. Microconidia are smaller, almost always uninucleate, and have poor viability. Gametic fusion is accomplished by spreading conidia of one mating type over the surface of a culture of the other mating type which has protoperithecia. Prior to nuclear fusion, parental nuclei divide many times as a network of ascogenous hyphae develops in the growing perithecium. At the termini of this hyphal system, nuclei of the two mating types associate in pairs in the ascus initials, where nuclear fusion occurs. The resulting diploid nucleus is immediately resolved into haploid products by two meiotic divisions and one mitotic division, which take place in the elongating ascus initial. The final product of each meiotic event is an ascus, containing eight ascospores. From each ascospore, a haploid vegetative culture is obtained. *N. crassa* ascospores can be stored in sterile water sealed in small vials without appearent loss of viability for at least a year at room temperature and 18 months at 4 degrees. A convenient feature of the *Neurospora* sexual system is that dormant ascospores must be treated at 60 degree temperature for germination. The activation treatment kills parental cells and other contaminants at the same time and this allows crosses to be analyzed and selected at any time in unambiguously pure form after they are mature.

1.1.4 *NEUROSPORA* CULTURE

Filamentous fungi are able to grow on a wide variety of carbon and nitrogen sources. *Neurospora* can use acetate, succinate, glycerol,

glucose, and other monosaccharides, and a number of oligo- and poly-sacchrides as carbon sources. Nitrogen can be supplied as nitrate, nitrite, ammonium, and amino acids. Besides carbon and nitrogen, *Neurospora* requires only a few simple salts, trace elements, and a single vitamin, biotin, for vigorous growth. In addition, a number of species can carry out specific modifications of, for example, aromatic compounds and steroids in so-called bioconversion processes. The chemical diversity of the natural environment (i.e., soil and organic waste) in which these organisms live and have to compete with other (micro) organisms probably underlies their extraordinary metabolic diversity.

Despite its multicellular vegetative condition, when grown in submerged culture, filamentous fungi exhibit the growth pattern quite similar to those of unicellular microorganisms that reproduce by binary fission. The lag phase represents a period during which the fungal cells or spores adapt to a new environment. Adaptation includes formation of enzymes and intermediates to support resumption of growth. The exponential phase of fungal growth is characterized by a significant increase in cell mass. A reduction in the specific growth rate occurs when the microfungus begins to experience an unfavorable growth environment such as the limitation of a required nutrient, the development of an adverse pH value, or the accumulation of end products of metabolism that are inhibitory. These fungi exhibit different morphological forms, ranging from dispersed mycelial filaments to intricately interwoven,

mycelial masses referred to as pellets. The particular form exhibited is determined not only by the fungal species, but also by the nature of the inoculum as well as the chemical (medium constituents) and physical (pH, temperature, etc.) culturing conditions.

There are several methods for the measurement of growth in *Neurospora*. These are 1) measurement of the rate of mycelial elongation in race tubes, 2) measurement of the amount of growth in stationary or shaken liquid culture after selected time intervals, 3) measurement of the doubling time in logarithmically growing culture. Growth in stationary culture usually appears at the surface of the medium within a day, and growth up the walls of the flask should be submerged by swirling the flasks twice each day. Growth of conidia in the form of a mat is usually complete within 3-4 days For most strains, a slow loss of weight follows the attainment of maximal weight. Moreover, the rate of growth changes with time. Thus, while no difference in dry weight will be noted between a wild type and a leaky mutant if they are incubated long enough, the leaky mutant may not have initiated visible growth by the time that of wild type is maximal.

1.1.5 BYPRODUCTS OF FILAMENTOUS FUNGI (46)

In addition to their scientific importance, despite the fact that fungi secrete only a limited range of proteins naturally, filamentous fungi can be engineered genetically to secrete significant amount of recombinant proteins. The economic importance can be illustrated

by the large variety of products that are made by filamentous fungi, such as organic acids (e.g. gluconic acid and citric acid), antibiotics (e.g. penicillin and cephalosporin) and numerous industrial enzymes (e.g. glucoamylase and lipase). Filamentous fungi are also used as food (mushroom), food additives (e.g. the meat extender Quorn) and condiments (e.g. soy sauce).

1.2 A BRIEF HISTORY OF *NEUROSPORA* RESEARCH.

1.2.1 PRE-RECOMBINANT DNA PERIOD (46, 47)

A number of filamentous fungi have been used for genetic research as general genetic models, because the fungal genetic apparatus (such as nucleus, chromosomes, and genes) is typically eukaryotic, and fungal meiosis, including recombination, is essentially identical to that of plants and animals.

Fungal genetics has been mostly laboratory bound since its inception. Most genetic investigations depend directly or indirectly on information from crosses, and among individuals within the same local population, thousands of nucleotide differences are known to exist among *Neurospora crassa* strains. Almost all the varients used in genetic, biochemical, and molecular studies have been obtained as single-gene mutants that were induced and selected in the laboratory. Most of our information on genetic polymorphisms in fungi has come from isozyme differences.

The classic studies of genetics and biochemistry by Lindegren (1931), Winge (1942), and Beadle and Tatum (1941) demonstrated both the utility and advantages of fungi and, in part, directed their development into the analysis of cellular function. B. McClintock and J. R. Singleton showed in the 1940s that chromosome morphology and behavior during meiosis and mitosis in the ascus resemble those in higher eucaryotes. The first genetic evidence of chromosome rearrangements was obtained in this period, and translocations were confirmed and described cytologically. The publication of the one gene-one enzyme hypothesis with *Neurospora crassa* in 1941 by Beadle and Tatum introduced many of the basic techniques of microbial genetics, which later became usual in the bacteria *Escherichia coli.*

Auxotrophic mutants were used to elucidate biosynthetic pathways. Complementation between alleles was demonstrated both in vivo and in vitro. Temperature-sensitive conditional mutants that were irreparable by supplementation were obtained. Studies of meiosis in the ascus provided important information about genetic recombination and its control because each ascus contains all the haploid nuclei resulting from a single meiotic division. Gene conversion was demonstrated, and its important characteristics were delineated. Meiotic recombination within genes was shown to be polarized. Genes that regulate the frequency of locally specific recombination events within and between other genes were discovered and characterized. The complete meiotic karyotype was

reconstructed in three dimensions for the synaptonemal complex, with its associated recombination nodules.

1.2.2 MODERN MOLECULAR GENETIC PERIOD (4, 47)

During the 1980s, modern molecular genetic techniques have been applied to many fungal species, especially those that have a good background of classical genetics such as *Neurospora crassa* and *Aspergillus nidulans.* Gene cloning is now commonplace, and the genes for many unique fungal products have been sequenced, expressed, and engineered. Numerous plasmids for fungal transformation and genomic libraries have been constructed and are readily available for a number of fungal species. The basic components of these plasmids are; a gene that can be used for selection of fungal transformants, bacterial plasmid sequences that can be used for selection and propagation of the plasmid in *E. coli.* The number of different selective markers available for fungi is now fairly large, and these selective genes often can be used in many different species, thus providing substantial flexibility in designing plasmids for specific purposes.

Mishra and Tatum (1973) first reported transformation of a filamentous fungus: in these experiments, the investigators treated an inositol-requiring *N. crassa* strain with DNA from a wild-type strain and obtained inositol-independent colonies. Although it seems likely that these colonies indeed arose by transformation, at the time molecular techniques were lacking to prove that the strains had

incorporated exogenously added DNA. Direct demonstration of fungal transformation came from the experiments of Case et al (1980) with *N. crassa.*

To date, the technology for introducing vectors into fungal cells has been restricted primarily to systems based on polyethylene glycol promotion of DNA uptake into protoplasts. Methods utilizing plasmids for recombinant DNA techniques in fungal experiments are similar to those developed for *E. coli.* Plasmids can be removed from the cell, segments removed by restriction enzymes, and a new piece of foreign DNA inserted. The restructured circular plasmids can be used to transform other cells. The DNA may become integrated into one of the chromosomes and the course of events whether homologous integration or non-homologous integration depends on the nature of the new DNA and the strain used.

With virtually all filamentous fungi, when transformation occurs by integration most nonabortive transformants are mitotically stable. In a practical test of the mitotic stability of fungal transformants, Finkelstein et al (1989) subjected a number of *A. niger* transformants, carrying extra copies of the glucoamylase gene, to eight serial conidial transfers in the absence of selective pressure. At the end of this period, no modification could be detected in either gene dosage or in enzyme yield. The implication of such mitotic stability is that recombinant filamentous fungi can be grown at production scale without the need to apply selective pressure to maintain the transformed phenotype (Skatrud et al. 1989).

The primary value of *Neurospora* in research at present is that *Neurospora* is eukaryotic, can be handled as easily as bacteria, and thus provide a valuable basis of comparison between prokaryotes and eukaryotes in molecular biology.

1.3 CHROMOSOME MAPPING OF *NEUROSPORA*

1.3.1 CLASSICAL GENETIC METHOD (13, 25, 78)

The DNA content of *Neurospora* is 27,000 kilobases which reveals a haploid genome that is about 10 times larger than that of *E. coli* (Krumlauf, R., Marzluf, G. A. 1979 Biochemistry 18, 3705-3713). *Neurospora* has seven cytologically distinct chromosomes having left and right arms connected through the centromere. The genetic counterparts of these are referred to as linkage groups I-VII, and represented as formal maps. *Neurospora* chromosomes are large enough during ascus development to permit conventional analysis by light microscopy. Shear and Dodge (1927) showed that mating type is determined by a single pair of alleles that show 4:4 segregation among unordered asci which were shot from the perithecia. Morphological differences of spontaneous origin were soon discovered and shown by Dodge to segregate in Mendelian fashion. The linear array of ascospores in the ascus was shown cytologically by M. S. Wilcox to reflect events in meiosis, and genes were shown by Wilcox and by Dodge to segregate sometimes at the first and sometimes at the second division of meiosis. C. C. Lindegren

proposed that second division segregations, as reflected in ascospore order, measured the frequency of crossing-over in the segment between a gene locus and its centromere. Lindegren discovered linked genes and constructed the first linkage maps. The predominantly auxotrophic mutants obtained by G. W. Beadle, E. L. Tatum, and their associates, beginning in 1941, were used to construct more complete maps.

The *Neurospora* genetic maps have been estimated to total about 1000 map units (centimorgans). This gives on average 27 kilobases per map unit. Molecular characterization of *Neurospora* chromosomal DNA is mostly unique-sequence, but with 2% foldback and 8% repetitive sequences. Most of the repetition is accounted for by approximately 200 ribosomal RNA genes, arranged tandemly in the nucleolus organizer region. 5S ribosomal RNA genes are not clustered but are dispersed through the genome as single copies. The apparent paucity of extraneous DNA may be partly due to natural selection for individuals that grow rapidly.

1.3.2 MOLECULAR GENETIC METHOD (25, 39)

Mapping has not been limited to conventional gene markers or to conventional methods based on crossing over frequencies. At the DNA level, Restriction Fragment Length Polymorphisms (RFLP) appear to be ubiquitous in *Neurospora crassa* strains from nature. Any cloned DNA fragment can be genetically mapped if a restriction

fragment length difference is found in the homologous genomic DNA (Metzenberg, Stevens, Selker, Morzycka-Wroblewska, 1984).

Two RFLP mapping kits of *Neurospora crassa* are available. To produce RFLP mapping kits a cross of a laboratory strain with a nominally "Oak Ridge" genetic background and carrying several conventional markers (FGSC#4411, *al-2;nuc-2,arg-12;cot-1;inl-a* or FGSC #4488, *multicent-2-a, un-2*) was made to a wild-collected strain (Mauriceville-1c-A, p538, FGSC#4416) which has not been inbred with laboratory strains. Such a cross is marked not only by the conventional markers, but by thousands of nucleotide differences scattered throughout the genome. The resulting restriction fragment differences can be used as markers for determining linkages of cloned DNA segments in the genetic maps. The random progeny spores, presumably all from different asci, were isolated from ordered tetrads and cultures were prepared from them. These progeny cultures were sorted for these markers by nutrition, color and morphology so that about half the progeny included in the experiment would be of each allele. The allele assignments are then compared with the allele segregation of markers that have already been mapped and ultimately deposited in the Fungal Genetics Stock Center (FGSC).

1.4 FUNGAL MEMBRANES.(23, 26, 65)

1.4.1 MEMBRANE TRANSPORT IN FUNGI

Underlying the rather simple-looking structure of membranes is the vast diversity of chemical constituents indicative of the diverse roles these membranes serve in the lives of cells. Fungi are heterotrophic and enzymes are released from the hyphae on to the substrate for the external hydrolysis of potential nutrients. Solubilized materials are subsequently absorbed, by transport through the membrane, and used for the metabolic processes involved in growth and development. The passage of materials into, and out of, fungal hyphae is mediated by the selective permeability of the plasma membrane which lies on the inner surface of the cell wall and is a highly effective barrier to many molecules. Sugars, amino acids, ions and vitamins pass through the membrane down an electrochemical gradient, but are aided by carrier proteins. These carriers, also known as permeases, act as regulators for both the uptake and release of molecules and are located on the plasma membrane. These are generally highly specific and there are different carriers for different groups of molecules (facilitated diffusion). The rate of transport of a given polar substance through a particular kind of membrane protein is determined by the number of copies of the protein present in each unit area of membrane and the turnover number for transport through each copy. Differences in the numbers of different types of transport proteins reflect differences in the expression of the various corresponding genes. Moreover,

localization of transport proteins to the several kinds of bilayers within a particular cell (e.g., plasma membrane, mitochondria, nuclei) is directed by signal sequences that are genetically determined. Genes thus play a crucial role in the regulation of membrane transport.

1.4.2 MEMBRANE PROTEINS

Proteins and lipids are the major components of biological membranes. While the structural and dynamic properties of lipids are quite well characterised, the understanding of the structure and function of membrane proteins is at a much more primitive stage. Proteins associated with biological membranes can be classified into two groups: peripheral or extrinsic proteins, associated with the membrane surface but not penetrating into the hydrophobic core of the lipid bilayer, and integral or intrinsic proteins which are membrane spanning. Extrinsic proteins are more loosely attached to the cell membrane. They can be removed by treatment with solutions of low ionic strength, often containing EDTA (Ethylene Diamine Tetraacetic Acid), to chelate divalent cations. The peripheral membrane proteins have amino acid compositions and properties similar to those of the soluble proteins normally found in the cytoplasm. Most extrinsic proteins seem to be held to the membrane by being bonded to the intrinsic proteins. Intrinsic proteins are firmly bound to the membranes and can be removed only by treatment with detergents, suggesting that, within the lipid matrix,

they are bound to the lipid hydrocarbon chains. The detergent, in fact, replaces the hydrocarbon of the lipid. The amino acid composition of these integral proteins appears to include a large number of apolar residues (amphipathic). Examples of such proteins include the ATPases, glucose transporter, ion channels, light-transducing proteins, cytochrome oxidases and several families of membrane spanning receptors. Some of these proteins, like glycophorin, span the membrane, extending all the way across it, from one aqueous face to the other. Some, like the proteins involved in transmembrane transport of their substrates, extend only halfway across the bilayer, embedded either in the cytoplasmic face or in the extracellular face of the membrane. Others span it many times as the polypeptide chain of the protein winds back and forth between the two faces of the membrane. These different architectural motifs are associated with different functions of the proteins. A protein destined to be an intrinsic membrane protein bears, in addition to its signal squence, a sequence or sequences consisting of a 20-25 long chain of hydrophobic residues, which will become and remain embedded in the membrane lipids. A protein that spans the membrane once always appears to have its N-terminal signal sequence outside the membrane, a direct consequence of this mode of synthesis where the membrane-spanning sequence appears after a sequence of membrane-leaving residues.

1.4.3 SPHEROPLASTS

Fungi like plants and bacteria have rigid, mainly polysaccharide cell walls that give a characteristic morphology to the cells and provide support and protection to the enclosed protoplast. The wall is composed largely of polysaccharides, with some protein and lipid. The two polymers (chitin and cellulose) form the fibrils that make the rigid component of most fungal walls. The rigid cell wall can be partially or totally removed by enzymatic digestion, leaving a protoplast that, provided conditions are suitably stabilized with an osmoticum, will remain intact. The term protoplast is normally used to describe a naked cell completely devoid of cell wall residues, while spheroplast is used when some wall material could be present. In general, the criteria used for the absence of wall material are osmotic fragility and formation of a spherical shape. The regeneration of protoplasts to normal cells capable of reproduction is a prerequisite for experiments employing genome manipulation in fungal cells. It has been proven that the protoplast of any fungal species, supposing it contains a nucleus, is capable not only of regenerating the missing cell wall as an active biostructure, but it also can repair all other alterations, producing a complete and functional cell.

Protoplasts have had a major impact on two recent advances in the methodology of fungal genetics: in the mid 1970s through fusion techniques and in the last decade through their use in transformation-mediated recombination. Transformation has been reported for a wide range of filamentous fungal species, and the

general characteristics of these systems are very similar. Firstly, it is necessary to remove the permeability barrier represented by the cell wall either by treatment with lithium acetate (Dhawale et al., 1984) or, more commonly, by enzymic digestion with crude extracts of snail gut (Case et al., 1979) or a *Trichoderma* extract commercially available as Novozym 234 (Novo Biolabs Ltd. Bagsvaerd, Denmark, Binninger et al., 1987). Then, DNA is added to the protoplasts suspension in the presence of calcium chloride. The addition of a solution of polyethylene glycol results in the uptake of the DNA. The treated protoplasts are then plated on a medium that permits the regeneration of cells that have acquired the transforming DNA by recombination. Transformation of *Neurospora crassa* generally results in nonhomologous, multiple integration of transforming sequences at random sites as well as homologous integration in the genome.

1.5 AMINO ACID TRANSPORT PROTEINS.IN FUNGI (20, 30, 49, 74)

The specificities of the biological activities of the cell membranes reside primarily in their protein constituents. The nature and types of proteins associated with the various membranes appear to vary widely, reflecting differences in the functional activities of the membranes. Proteins can be covalently modified in a number of ways. The most complex and evolved modification is glycosylation.

Most of the integral membrane proteins including amino acid transport proteins are glycoproteins. The carbohydrate residues found on glycoproteins are exposed only to the external surface of the plasma membranes. This asymmetric disposition of the carbohydrate moieties reflects the functional anisotrophy of the two membrane surfaces. The cell surface carbohydrates have a role in cell adhesion, cell-cell interaction, and receptor function-processes which are involved in external communication. The carbohydrate moiety of glycoproteins generally amounts to about 20%, of the total molecular mass but in extreme cases can be 90%. (30)

Nitrogen is essential for the biosynthesis of complex molecules in cells, such as amino acids, proteins, nucleic acids and some vitamins. Most fungal species can use inorganic nitrogen sources as well as organic nitrogen-containing compounds (proteins and amino acids) for growth. There are very specific carrier systems allowing amino acids to be effectively transported from environment. Much of the understanding of membrane transport has been derived from the study of transport mutants in microorganisms. The study of genetic mutants which ablate a single transport system can define the organization of membrane transport functions. A number of genes encoding membrane proteins that are known to promote the transport of specific amino acids have been isolated, sequenced, and partially characterized. Each provides a regulated pathway for movement of specific substances through the membrane.

Neurospora crassa is known to possess at least six genetically and biochemically distinct amino acid transport systems; three constitutive amino acid permeases and three specialized amino acid permeases. The constitutive permeases include a neutral amino acid-specific system (N system), a basic amino acid-specific system (B system), and a general system (G system) that can transport neutral, basic, and acidic amino acids. Three additional systems which are inducible have been proposed. These are systems for the transport of: the imino acid proline, the acidic amino acids during nitrogen limitation, and, the sulfur-containing amino acid methionine during sulfur starvation (Wolfinbarger, 1980; DeBusk, Ogilvie, 1984).

1.6 WHAT IS THE *pmb* LOCUS? (68, 77)

The *pmb* locus is a gene coding for a component of the basic amino acid transport system (B system) which is one of the three major constitutive amino acid transport systems of *Neurospora crassa*.. The carrier system for basic amino acids was shown by Baurle and Garner(1964) to be specific for transport of arginine, lysine, and arginine analogue canavanine sulphate. The *pmb* mutants were selected as resistant to canavanine which is toxic to the cells. Several mutants lacking basic amino acid permease activity have been isolated and characterized (Woodward, C. K., Read, C. P., Woodward, V. W., 1967, Wolfinbarger, 1971; Perkins, Radford.,Newmeyer, Bjorkman, 1982). Roess and DeBusk(1968) examined basic amino

acid transport in greater detail and reported a mutant, *Cr-10* which was deficient in transport of basic amino acids by the basic amino acid transport system. Wolfinbarger and DeBusk(1972) further evaluated the *Cr-10* mutant, renaming it *pmb*. Thwaites and Pendyala(1969) isolated a further mutant, *bat*, as deficient in basic amino acid transport. Each of these mutants, *Cr-10, pmb*, and *bat* was separately isoated and identified in several laboratories, and they are presumed to be allelic mutants lacking *pmb* gene product activity. The *pmb* mutation was located on the right arm of chromosome IV. This basic amino acid transport system was shown to be highly active in mycelia as compared to a low activity in conidia. Active transport results in the accumulation of arginine and lysine against a concentration gradient. The presence of a mutation at the *pmb* locus reduces the total arginine accumulated by approximately 45-50%. The remaining 50-55% arginine transport in *pmb* mutant has been attributed to the general transport system. Stuart and DeBusk described that *pmb* locus controls a glycoprotein involved in transport of basic amino acids, indicating that *pmb* transport system contains a glycoprotein (Stuart, W. D. and DeBusk, A.G. (1971) Arch. Biochem. Biophys. 144, 512-518). Transport activity in *N. crassa* conidia seems to be quite analogous in many respects to that activity present in other organisms. Analogous systems to the reported basic amino acid transport system are known in *E. coli*, yeast, Ehrlich cells, and mammalian intestine cells.

Future reports may well indicate an even wider distribution of systems analogous to the *pmb* transport system.

1.7 THE CURRENT RESEARCH OF THE BASIC AMINO ACID TRANSPORT PROTEIN

Investigation of the molecular mechanism of transport across biological membranes involves the complete physicochemical description of each transport protein. Identifying such motion requires describing in atomic detail the dynamic, three-dimensional structure of the molecule, the reactions catalyzed by these enzymes and their regulation by transcriptional, translational, and posttranslational mechanisms.

In filamentous fungi genes can often be identified by the interesting properties of strains carrying their mutant alleles. Thus, there is frequently an opportunity to clone a gene that has been detected by mutation. This cloning can be accomplished in many species by genetic complementation of mutations with recombinant DNA clones containing wild-type DNA sequences. Since 1985, in our laboratory, a research project directed towards cloning amino acid transport related genes from *Neurospora crassa* has been developed to establish the non-mammalian model system of amino acid transport through the cell membrane. The first work cloned and characterized the *mtr* gene sequence of *Neurospora crassa* . This gene codes for a membrane protein involved in transporting neutral

aliphatic and aromatic amino acids (N system) (Stuart, Koo, 1988). Stuart and Koo used the *mtr* sequence to predict the amino acid sequence of the gene product. The most recent data suggests that the gene product contains twelve transmembrane helixes and also include an amino acid octomer reported to be conserved in RNA binding sites in many species. In current research, we expand this work into another major transport system. We describe the procedure of identifying and cloning of the *pmb* gene which codes for a component of the basic amino acid transport system (B system) from an ordered cosmid library of genomic DNA of *Neurospora crassa* by complementation of *pmb* mutants. We have been cloned the *pmb* gene by transforming *pmb* mutant cells with DNAs of pMO cosX cosmid library. The cosmid which complements the *pmb* mutant allele and was confirmed to be tightly linked to RFLPs flanking the *pmb* locus on linkage group IV has been subcloned, partially sequenced, and analyzed. The cloned *pmb* DNA fragments can be used as probes in hybridizing with genomic DNA from other organisms for identifying the homology between two organisms.

CHAPTER 2
MATERIALS AND METHODS

The genetic and microbiological procedures of handling *Neurospora crassa* is following the methods described in the Genetic and Microbiological Research Techniques for *Neurospora crassa* (Davis, Serres, 1970). The procedures for constructing multiple mutant strains of *Neurospora crassa*, for preparation of spheroplasts, for DNA preparation from pMOcosX cosmid library and for transformation with exogenous DNA sequences from an ordered library were done as described in several papers (Perkins, Radford, Newmeyer, Bjorkman,1982; Vollmer, Yanofsky, 1986; Stuart, Koo, Vollmer, 1988). The general procedures for carrying out the DNA blotting and hybridization experiments have followed the standard methods in Molecular Cloning, A Laboratory Manual (Sambrook, Fritsch, Maniatis, 1989). For hybridization, nonradioactive DNA labeling and detection kit was purchased from Boehringer Mannheim. For sequencing, non-radioactive Uniplex DNA sequencing kit was purchased from Millipore.

NEUROSPORA TRANSFORMATION

A number of filamentous fungal genes have been cloned by the generally applicable techniques such as differential hybridization (Hynes et al., 1983; Lockington et al., 1985), use of synthetic probes based on protein sequence data (Kinnaird et al., 1982; Boel et al.,

1984), or use of antibodies raised against the purified protein (Viebrock et al., 1982; Shoemaker et al., 1983). Since the above approaches are limited in their application, much effort has been put into the development of methods for isolating genes by transformation of a filamentous fungus with DNA from gene libraries. Such systems enable the identification and cloning of any gene for which there is a discernible phenotype by relief of a mutation or a change in the appearance of the fungus. A transformant with the appropriate phenotype is expected to contain the selected DNA integrated into the genome.

Despite the fact that there are reports of successful recovery of transforming sequences from *N. crassa* by using an *E. coli* strain which is deficient in the two methylcytosine systems (Orbach, 1988; Nelson, Morelli, Carattoli, Romano, Macino,1989), the recovery of freely replicating plasmids from *Neurospora crassa* is currently neither faithful nor frequent enough to make this a practical strategy for use in gene cloning. Commom complications include plasmid rearrangements, recovery of plasmids not containing the desired sequence, and cotransformation, i.e., more than one plasmid type can integrate in the same nucleus during transformation. The problems encountered with DNA rearrangements in recovered clones are, in part, because the bacterial recipient cells used restrict methylated DNA, thereby, especially sequences introduced by transformation, are frequently methylated. To overcome this problem, Akins and Lambowitz (1985) developed a procedure termed sib selection. This

alternative to direct selection and recovery involves use of ordered collections of genomic clones constructed with drug resistance cosmids. DNA from divided pools of clones is tested for its ability to complement a mutation until a single complementing clone is identified. Due to the high transformation frequencies achievable with *N. crassa* drug resistance plasmids, only small amounts of DNA (around 1 ug each time) are needed to conduct these screens. Once DNA has been isolated from the many subcollections required for this approach, the method is straight forward and efficient. For any clone isolated by mutation complementation, the cloned DNA sequence is presumed to correspond to the target gene.

pMOcosX LIBRARY

The recombinant plasmid clone bank containing inserts of *Neurospora crassa* wild type DNA sequences in the pMOcosX vector was constructed by Orbach and Sachs. In the recombinants, the genes for hygromycin resistance and ampicillin resistance were incorporated into the parental vector as dominant selectable markers for fungi (hygromycin resistance) and for *E. coli* (ampicillin resistance). The pMOcosX cosmid genomic library is composed of 50 plates (96 colonies in each plate; 50 X 96 = 4800 colonies). This configuration has greatly facilitated the use of the library for the rapid cloning of genes in sib selection and colony hybridization procedures. (75)

VECTORS

pTZ vectors were used for genetic manipulation such as subcloning of a gene by complementation of a mutation with restriction enzyme fragments, and amplifying the gene for restriction mapping and sequencing. pTZ18R and pTZ19R are small (2.87kb), high copy number *E. coli* plasmids, versatile cloning vectors. They contain a gene encoding resistance to ampicillin as a selective marker for additional selection for plasmid containing colonies. The pTZ plasmids DNA encoding the N-terminal alpha peptide of the beta galactosidase gene carries a 54 base pair multiple cloning site polylinker with many restriction endonucleases from pUC vectors to facilitate cloning while still retaining blue/white colony screening. DH5alpha MCR™ strain was used as a competent cell which provides alpha-complementation of the beta-galactosidase gene of the pTZ vectors.

NON-RADIOACTIVE METHODS

Non-radioactive DNA labeling and detection

Most of the hybridization experiments have traditionally relied on a radioactively labeled DNA hybridization probe. As an alternative, a nonradioactive DNA labeling and detection method was developed. The standard procedure in the nonradioactive DNA labeling and detection method used consists of three parts. 1) The probes are labeled with a nucleotide analog, digoxigenin-11-dUTP, by the random primed labeling technique. 2) The digoxigenin-labeled

probes are hybridized to immobilized DNA. 3) The digoxigenin-labeled probes are detected with an antibody-enzyme conjugate, anti-digoxigenin-alkaline phosphatase. The location of the antibody-antigen conjugate is visualized by an enzyme-linked color reaction.

Non-radioactive sequencing

Sequencing was done by using a Uniplex DNA Sequencing Kit which is the basic product for Sanger dideoxy DNA sequencing procedures in which a biotinylated primer is annealed to the template prior to the sequencing reactions. The biotinylated primers provide the sites for the chemiluminescent reactions for detection on X-ray film. A standard sequencing gel is run and then the DNA sequence pattern is transferred and bound to a membrane. In the detection process, the membrane is incubated with a solution containing streptavidin, which binds to the biotinylated primer. After washing, a solution containing a biotin/alkaline phosphatase complex is added; this complex binds to the streptavidin resulting in the creation of a conjugate between the alkaline phosphatase and the DNA on the membrane. Finally the chemiluminescent substrate (a dioxetane compound) is added; the alkaline phosphatase catalyzes the light reaction by cleavage of a phosphate group, resulting in destabilizing the substrate which then emits photons as it decomposes. The emitted light is detected by exposing the membrane to an X-ray film.

2.1 COMMON REAGENTS USED FOR EXPERIMENTS

E. coli culture LB medium (1L): Bacto-Tryptone 8.0g, Bacto-Yeast Extract 5.0g, Sodium chloride 2.5g, Sodium hydroxide drops (-->pH 7.5), Bacto-Difco Agar 15g, 25mg/ml Ampicillin 2ml

Neurospora culture conidial growth medium (100ml): Vogel's 50X 2ml, Sucrose 2.0g, Bacto-Difco Agar 1.5g

Neurospora culture colony formation plating bottom agar medium (1L): Vogel's 50X 20ml, Bacto-Difco Agar 15g, 10X FIGS 100ml, plus any supplements needed for specific conditions

Neurospora culture colony formation regeneration top agar medium (1L): Vogel's 50X 20ml, sorbitol182g, Bacto-Difco Agar 28g, 10XFIGS 100ml

50X Vogel's stock solution: dissolve the ingredients below in order in 750ml of distilled water. Ingredients; Sodium citrate 150g, Potasium phosphate 250g, ammonium nitrogen 100g, Magnesium sulfate 10g, Calcium chloride 5g, Trace element 5ml, Biotin solution 2.5ml, bring volume to 1.0 liter and add 2ml chloroform

10X FIGS carbon source (1L): Sorbose 200g, Fructose 5g, Glucose 5g, Inositol 2g

Trace element stock solution: dissolve the ingredients below in order in 95ml of distilled water. Ingredients: Citric Acid $1H_2O$ 5.00g, $ZnSO_4$ $7H_2O$ 5.00g, $Fe(NH_4)_2(SO_4)_2$ $6H_2O$ 1.00g, $CuSO_4 \cdot 5H_2O$ 0.25g, $MnSO_4$ $1H_2O$ 0.05g, H_3BO_3 anhydrous 0.05g, Na_2MoO_4 $2H_2O$ 0.05g. Bring volume to 100ml and add 1ml chloroform.

Biotin solution: dissolve 5mg of biotin in 100ml of 50% ethanol and store at 4 degrees. Prepare every 6 months.

2.2 SELECTION OF NEW STRAINS

2.2.1 CROSSING

We used the genetic construction of strains by crossing for selection of *pmb* transport defective mutant before using this strain for transformation. A strain defective in basic amino acid transport system (#1683-*bat um* 535 "A"), was crossed with a double mutant strain defective in neutral amino acid transport and histidine production (*mtr6r:his-2* "a") on Synthetic Crossing (SC) media containing histidine.

2.2.1.1 Materials

Synthetic Crossing (SC) medium (1L): Vogel's 50X 20ml, K_2HPO_4 0.7g, KH_2HPO_4 0.5g, sucrose 2%, Bacto-Difco Agar 2%, trace elements 0.1 ml, Histidine 2%

2.2.1.2 Methods

0.2ml of suspension of the first parental conidia was transferred onto 8ml of SC slants containing histidine in a 18X150mm tube as a female parent, using cotton or sponge plugs. Incubate for a week in the dark at room temperature (25 degrees). 0.2ml of conidial suspension of second parent was spread over the surface of the

female culture. At approximately 10 days after fertilization, ascospores were collected by picking up ejected ascospores on the wall of the tube opposite the agar surface with a wet loop and suspended into a drop of water.

2.2.2 ISOLATION OF THE *pmn:pmb:his-2* STRAIN

A *Neurospora crassa* strain suitable for detecting transformation at the *pmb* locus was isolated from the crossing of two existing mutant strains.

2.2.2.1 Materials

Neurospora colonial medium containing Para-Fluoro-Phenylalanine (PFPA) and canavanine (25ml): 50X Vogel's medium 0.5ml, Bacto-Difco-Agar 0.375g, 100mg/ml Histidine 125ul, 5mg/ml Canavanine 1ml, 5mg/ml PFPA 0.5ml, 10X FIGS 2.5ml

Conidial growth medium containing canavanine (25ml): 50X Vogel's 0.5ml, Sucrose 0.5g, 100mg/ml Histidine 125 ul, 5mg/ml Canavanine 0.5ml

Conidial growth medium containing histidine (25ml): 50X Vogel's 0.5ml, Sucrose 0.5g, 100mg/ml Histidine 125 ul

2.2.2.2 Methods

Ten days after fertilization the ascospores were picked up by a loop formed from 70% platinum and 30% iridium wire with a 0.02 inch diameter from a cross tube and suspended in 1ml of water. The

ascospore suspension was incubated at 60 degrees for 30 minutes. The germinating spores were distributed evenly over a plate of 25 ml of FIGS medium containing PFPA and canavanine using a sterile glass spreader flamed in 95% ethanol. Avoid spreading the spore suspension to the extreme edge of the plate and scratching the surface. The germinating progenies were selected for both resistance to PFPA and canavanine .

A plug of cells from each colony was cored with the tip of a sterile, disposable pipette and transferred onto slants of minimal N media and minimal plus histidine media. The selected *pmn:pmb:his-2* strain was confirmed by showing 100% growth in 1X Vogel's conidial growth liquid media and in 1X Vogel's media containing histidine at 30 degrees for 2-3 days.

2.3 GENERATION OF SPHEROPLASTS (52, 55, 75)

2.3.1 Materials

Conidial medium containing histidine (25ml): 50X Vogel's 0.5ml,
Sucrose 0.375g, 100mg/ml Histidine 125ul, Bacto-Agar 0.375g

Novozym 234

1M sorbitol

1M STC (1M sorbitol, 50mM Tris-HCl pH8.0, 50mM CaCl2)

40% polyethylene glycole 4000

50mM Tris-HCl pH8.0/ 50mM $CaCl_2$

Dimethyl sulfoxide (DMSO)

2.3.2 Methods

The conidia of *pmn:pmb:his-2* strain were transfered on minimal N medium containing histidine with flamed inoculation loops or wires, cooled and wetted in the medium to which the transfer is to be made. Incubate at 30 degrees for 2 days, and then move to 25 degrees in well-lighted conditions for 3-5 more days. Spheroplasts were prepared according to the procedure described in Vollmer, Yanofsky, 1986.

Conidia were harvested from 7-day-old cultures (2 days at 30 degrees, 5 days at 25 degrees) which had been grown on complete medium containing histidine. Conidia were suspended by carefully introducing 10-15 ml of 1X Vogel's media containing 1.5% sucrose and histidine to the growth flask, which is plugged and swirled. After airborne conidia were allowed to settle, the conidial suspension was filtered through one or two layers of sterile cheesecloth, held as a bag with a rubber band in the mouth of a 125ml flask (approximately 10ml of conidial suspension). The conidia were washed twice with sterile water, and the conidial densities were measured directly with a spectrophotometer at 530nm by diluting the filtered conidial suspension in 10-, 100-, or 1000-fold steps in sterile water. Conidia were germinated at 30 degrees for 4-6 hours in flasks containing 150ml of 1X Vogel's minimal N medium with 1.5% sucrose and histidine and were shaken at 150-200 rpm. Germinated conidia were harvested at room temperature in a swinging bucket centrifuge for 5 minutes at 1500-2000 rpm, and

washed 3 times with 30 ml of sterile double distilled water. The germinated conidia were suspended in 10ml of 1M sorbitol and were placed in a 250 ml flask containing 50 mg of solid Novozym 234. After carrying out the cell wall digestion for a 1 hour incubation at 30 degrees with gentle agitation at 100 rpm, the spheroplasts were centrifuged for 5 minutes at 500 rpm, washed twice in 50 ml of 1M sorbitol and once in 50ml of 1M STC. The spheroplasts were brought up to a 16 ml volume with 1M STC and 4ml of 40% polyethylene glycole 4000. 4ml of 50mM Tris-HCl pH8.0/ 50mM $CaCl_2$ and 200ul of dimethyl sulfoxide were then added. 1ml of suspension contains, nominally, 10^8 spheroplasts. Spheroplasts were stored at this stage at -80 degree in 1ml of aliquots.

2.4 PLASMID DNA EXTRACTION (19)

DNAs were extracted by the rapid isolation of plasmid DNA procedure which was developed by Vollmer and Yanofsky and modified by Stuart and Koo.

2.4.1 Materials

2XYT medium: 2% tryptone, 1% yeast extract, 0.5 % NaCl, 0.2% glucose, 50ug/ml ampicillin

GTE solution: 50mM glucose, 25mM Tris-HCl pH8.0, 10 mM NaEDTA

Lysozyme: 10mg/1ml GTE

0.2 N NaOH/1% $NaDodSO_4$

"5M" KOAc (14.6ml): 9ml 5M KOAc, 4.23ml water, 1.37ml glacial HOAc

TE: 10 mM Tris-HCl pH8.0, 1mM EDTA

7.5M Ammonium acetate

5M LiCl

2.4.2 Methods

The colonies on each vertical row of eight wells of the library were individually streaked onto 100-mm petri dishes containing 25ml of 2XYT LB medium containing 50ug/ml concentration of ampicillin. The petri dishes were incubated overnight and the cells on the plate were resuspended in 1ml of water and amplified in the 40ml of liquid 2XYT medium containing 50ug/ml concentration of ampicillin. 40ml of late-log or stationary phase bacterial culture in 2XYT medium was harvested by centrifugation for 10 minutes at 4000 rpm in a Sorval and the pellet was resuspended in 1ml of GTE solution. The suspension was transferred to a 30ml corex tube and incubated at room temperature for 5 minutes. 100ul of Lysozyme was added and the mixture was placed on ice for 20 minutes. 2ml of freshly prepared 0.2 N NaOH/ 1% NaDodSO4 was added and mixed gently into the cell suspension and the mixture was returned to ice for 2-5 minutes. Acidification was achieved by addition of 1.5ml of 0 degree "5M" KOAc and mixing vigorously on voltex. After an additional 10 minutes incubation on ice, the precipitated material was removed by centrifugation at 10,000rpm for 5 minutes at 4

degrees. Transfer 4.5ml of supernatant to a fresh 15ml corex tube. Nucleic acids were precipitated by the addition of 9ml of -20 degree ethanol to the supernatant, mixed, and incubated on ice for 2-5 minutes, then centrifuged for 5 minutes. The pellet was dissolved in 250ul of TE, mixed with an equal volume of 5M LiCl, and placed on ice for 10 minutes. After centrifugation for 5 minutes at 4 degrees, transfer supernatant to a fresh tube. To remove precipitated material, the DNA was recovered by treatment of the supernatant with 1ml of -20 degree ethanol, mixing, incubation on ice for 5 minutes and centrifugation. The pellet was dissolved in 50ul of TE. Plasmid DNA was precipitated by adding 25ul of 7.5M ammonium acetate with 150ul ethanol, mixing, putting on ice, and spinning for 5 minutes. The pellet of cosmid DNA from this procedure was dissolved in 100ul of TE, resulting in a final concentration of 0.1ug per ul of TE. The 12 portions of each of the eight-clone pools were used to assemble fifty 96-clone pools that represented the cosmids contained in each microtiter dish.

2.5 TRANSFORMATION BY SIB-SELECTION

The transformation procedure of *Neurospora crassa* with cosmid DNAs was adapted from the method described by Vollmer and Yanofsky, 1986.

2.5.1 Materials

50 mM spermidine

STC: 1M sorbitol, 50mM Tris-HCl pH8.0, 50mM $CaCl_2$

Heparin: 5mg/ml in STC

PTC: 40% polyethylene glycole 4000, 50mM Tris-HCl pH8.0, 50mM $CaCl_2$

Neurospora culture colonial bottom agar medium (1L): Vogel's 50X 20ml, Bacto-Difco Agar 15g, 10X FIGS 100ml, 0.5mg/ml Histidine, 5mg/ml Phenylalanine, 200 ug/ml Hygromycin

Neurospora culture colonial regeneration top agar medium (1L): Vogel's 50X 20ml, sorbitol182g, Bacto-Difco Agar 28g, 10XFIGS 100ml

2.5.2 Methods

Transformation were carried out at ratios of 0.2 - 5.0ug of DNA per 10^7 spheroplasts. The highest ratios of DNA to protoplasts were used with the first-round sib banks to obtain higher numbers of transformants. The first rounds of the sib selections were generally carried out with 5ug of DNA. The second rounds were carried out with 1ug of DNA, and subsequent rounds were carried out with 0.2 ug of DNA.

5ul of cosmid DNAs were mixed with 2ul of 50mM spermidine, 5ul of heparin and 50ul of spheroplasts (10^7 cells). After incubation on ice for 30 minutes the transformed cells were mixed with 1ml of PTC and incubated at room temperature for 20 minutes. After incubation

with DNA, the spheroplasts were suspended in 14ml of minimal regeneration medium, and the cells were plated on minimal media supplemented with 1X Histidine, 10X Phenylalanine and 200ug/ml concentration of hygromycin. The plates were incubated for 3 days at 30 degrees. The transformants were further tested for growth on minimal media to eliminate the *his*$^{+}$ class.

2.6 RFLP SOUTHERN HYBRIDIZATION.

2.6.1 PREPARATION OF GENOMIC DNA

2.6.1.1 Materials

RFLP Mapping Kits: purchased from FGSC (Fungal Genetics Stock.Center, Kansas City)

1X Vogel's medium with supplements (100ml): Vogel's 50X 2ml, Sucrose 2.0g, Bacto-Difco Agar 1.5g1mM L-Arginine, 50ug/ml Inositol, 1mM L-Citrulline, 2ug/ml Thiamine HCl, 1mM Uridine, 1mM L-Lysine, 2ug/ml Nicotinamide, Aryl sulfatase

Lysis buffer: 50mM Tris-Cl pH8.0, 2%SDS, 62.5mM EDTA

5M KoAc

2.6.1.2 Methods

Each strain from the RFLP mapping kit was incubated at 25 degrees in 100ml of 1X Vogel's medium with supplements in a 250ml flask. Cells were harvested by filtration with Whatman No.1 filter circles and rinsed 2-3 times with double distilled water. Cells

were collected and frozen at -80 degrees. Grind the frozen cells with sand, put cells and sand mixture in a 30ml corex tube and add 5ml of lysis buffer to it. Incubate the mixture for 60 minutes at room temperature with gentle mixing. Centrifuge the mixture at 3,000 rpm at 4 degrees for 10 minutes. Decant supernatant in a 15ml corex tube and add 3.3ml of 5M KoAc. Mix the solution by inversion and place on ice for 30 minutes. Centrifuge at 10,000g at 4 degrees for 10 minutes. Transfer the supernatant in a 30ml corex tube, add an equal volume of isopropanol, and put the mixture at -20 degree overnight. After centrifugation at 10,000g at 4 degrees for 10 minutes, dissolve the pellet with 1.5ml of TE and transferred the nucleic acid solution in a 15ml corex tube. Perform phenol-chloroform extraction procedure which is followed by ammoniumacetate-ethanol precipitation. After rinsing with 70% ethanol, dissolve the pellet with 1ml of TE. For RNA digestion, mix the nucleotide solution with 20ul of 500ug/ml RNAse A and incubate the mixture at 37 degrees for 60 minutes. Perform phenol-chloroform extraction, followed by twice of chloroform extraction After ammoniumacetate-ethanol precipitation, rinse the DNA pellet twice with 70% ethanol and dissolve the pellet with 1ml of TE. Store the DNA solution at 4 degrees.

2.6.2 DNA TRANSFER AND DETECTION

2.6.2.1 Production of probes

Each cloned plasmid DNA is labeled with Klenow polymerase by random-primed incorporation of DIG-dUTP (digoxigenin-labeled deoxyuridine triphosphate) according to the supplier's protocol.

2.6.2.1.1 Materials

Genius labeling and detection kit: purchased from Boehringer Mannheim Biochemicals)

Gene Clean Kit: purchased from Bio 101

2.6.2.1.2 Methods

The cloned plasmid DNA (2ug) was purified by using Gene Clean Kit. Suspend the pellet in 14ul water, spin it for 30 seconds, and take the supernatant. Denature the DNA by heating at 95 degrees for 10 minutes, and place it on ice for 10 minutes. Add 2ul of hexanucleotide mixture primer, 2ul of dNTP labeling mixture containing DIG-dUTP, and 2ul of Klenow enzyme. Mix gently and incubate at 37 degrees for 3-4 hours. Purify the DNA by using Gene Clean Kit. Suspend the pellet in 50ul of TE, spin it for 30 seconds, and take the supernatant. Store the DNA solution at -20 degree.

2.6.2.2 Southern blot transfer

2.6.2.2.1 Materials

10X TBE buffer for electrophoresis (1L): Tris base 108g, Boric acid 55g, 0.5M EDTA (pH 8.0) 40ml

1XTBE with ethidium bromide (EtBr): 50ul of 10mg/ml EtBr in 1L of 1X TBE

0.1M HCl

1.5M NaCl/ 0.5M NaOH

2.5M Ammonium acetate

Magna Nylon Transfer Membrane, 0.45 micron: purchased from Micron Separations inc., Westboro, MA

6XSSPE: 900mM NaCl, 60mM $NaH_2PO_4H_2O$, 6mM EDTA pH7.4

2.6.2.2.2 Methods

2ug each of the prepared genomic DNAs of *Neurospora crassa* strains from 18- or 38-segregation RFLP Kit were digested with a restriction enzyme which shows a usable RFLP overnight at an appropriate temperature. The digested genomic DNA fragments were electrophoresed in the 0.8% agarose gel (15X20cm) for 20 hours at 1.5 voltage per cm of a gel in 1XTBE buffer with ethidium bromide. Transfer the DNA digests on agarose gel electrophoresis as follows. Depurinate the DNA in the gel in 500ml of 0.1M HCl for 45-60minutes on shaker. Denaturate the DNAs in 500ml of 1.5M NaCl/ 0.5M NaOH for 30-45 minutes on shaker. Neutralize the DNAs in 500ml of 2.5M ammonium acetate for 30-60 minutes at 4 degrees.

Transfer the DNA to the nylon blotting membrane with 2L of 1M ammonium acetate at 4 degrees overnight. Soak the membrane in 100ml of 6X SSPE for 10-15 minutes. Crosslink the DNA on membrane in UV Stratalinker™1800 at $120X10^3$ujoules, and use the transfer in hybridization experiments with labeled probes.

2.6.2.3 Hybridization

2.6.2.3.1 Materials

Hybridization solution: 5XSSC, 50% (v/v) formamide, 0.1% (w/v) N-laurylsarcosine, 0.02% (w/v) SDS, 5.0% (w/v) blocking reagent

Wash solution I: 2XSSC/0.1% (w/v) SDS

Wash solution II: 0.1XSSC/0.1% (w/v)SDS

2.6.2.3.2 Methods

The filters are prehybridized in a sealed plastic bag with 20ml of hybridization solution per $100cm^2$ of filter at 37 degrees for at least an hour. Redistribute the solution over the filter from time to time. Discard the prehybridization solution and replace with 2.5ml hybridization solution containing freshly denatured DNA per $100cm^2$ of filter paper. Incubate the filters for at least 6 hours at 42 degrees, redistributing the solution occasionally. Wash filters twice for 5 minutes each at room temperature with 50ml of 2XSSC/ 0.1%SDS per $100cm^2$ of filter and twice for 15 minutes each at 68 degrees with 50ml of 0.1XSSC/ 0.1%SDS per $100cm^2$ of filter. Filters can be used

directly for detection of hybridized DNA or air-dried and stored for later detection.

2.6.2.4 Detection of probes

2.6.2.4.1 Materials

Buffer1: 100mM Tris-HCl, 150mM NaCl, pH 7.5

Buffer2: 0.5% (w/v) blocking reagent in buffer I

Buffer3: 100mM Tris-HCl, 100mM NaCl, 50mM $MgCl_2$, pH 9.5

Buffer4: 10mM Tris-HCl, 1mM EDTA, pH 8.0

Color solution: 45ul NBT (Nitroblue Tetrazolium Salt) solution, 35ul X-phosphate (5-bromo-4-chloro-3-indolyl phosphate) solution, 10ml of buffer3

Antibody-conjugate <DIG>-AP: anti-digoxigenin alkaline phosphatase conjugate

2.6.2.4.2 Methods

Wash filters briefly for a minute in buffer1. Incubate the filters for 30 minutes in 100ml of buffer2 per $100cm^2$ at room temperature by shaking. Wash filters for a minute with buffer1. Incubate filters for 30 minutes in 20ml of diluted antibody-conjugate solution (150mu/ml buffer1) at room temperature by shaking. Remove unbound antibody-conjugate by washing twice for 15 minutes each with 100ml buffer1. Equilibrate membranes for 2 minutes with 20ml of buffer3. Incubate the filter in the dark with 10ml of freshly prepared color solution sealed in a plastic bag. When the desired

spots or bands are detected, stop the reaction by washing the membrane for 5 minutes with 50ml of buffer4. The filter may be dried at room temperature. The color can be restored by wetting the membrane with buffer4. Compare the RFLP pattern of the probe DNA detected by enzyme immunoassay to RFLP segregation data and localize the probe DNA on a linkage group.

2.7 SUBCLONING

The selected cosmid clone which has been identified to have the required gene has a large insert (averageis 40 kb). Thereby, it is necessary to locate the gene within the insert, which might be just a few kilobases. To localize the gene in a cosmid, aliquots of plasmids were digested separately with restriction enzymes. These digests were tested for the ability to transform the strain to prototrophy. The active fragments were purified and cloned into a vector.

2.7.1 PREPARATION OF VECTOR AND INSERT DNA

2.7.1.1 Materials

pTZ18R or pTZ19R: available at several companies including SIGMA

CIAP (Calf Intestinal Alkaline Phosphatase)

CIAP 10X buffer: 500mM Tris-HCl pH9.0, 10mM $MgCl_2$, 1mM $ZnCl_2$, 10mM spermidine

7.5M Ammonium acetate.

2.7.1.2 Methods

A sufficient amount of DNA should be prepared to allow for control reactions for the digestion, ligation, and transformation steps. 5ug of plasmid or insert DNA was digested with an appropriate restriction enzyme by incubation at the appropriate temperature for 1-3 hours. The DNA was treated with CIAP by adding the following components directly to the digested DNA: 10ul of CIAP 10X buffer, CIAP 0.01u/pm ends, deionized water to final volume 100ul. Incubate for 60 minutes at 37 degrees. To stop the reaction, add 2ul of 0.5MEDTA. Add 1 volume of TE- saturated phenolchloroform, vortex for 1 minute, and centrifuge at 12,000X g for 2 minutes. Transfer the upper, aqueous phase to a fresh tube and add 1 volume of chloroform:isoamyl alcohol(24:1). Voltex for 1 minute and centrifuge as in a former step. Repeat this step. Transfer upper aqueous phase to a fresh tube. Add 0.5 volume of 7.5M ammonium acetate. Add 2 volumes of ethanol and leave at -70 degree for 30 minutes. Collect the DNA pellet by centrifugation at 12,000X g for 5 minutes. Carefully pour off the supernatant, wash the pellet with 1ml of 70% ethanol, dry briefly in a vaccum desiccator. Resuspend the pellet in 15-20 ul of nuclease-free water. Check that the reaction has gone to completion by electrophoresis of a sample on a 0.8% agarose minigel. To ensure that the correct restriction fragment is cloned, the insert DNA can be eluted and purified from the gel after electrophoresis.

2.7.2 PREPARATION OF COMPETENT CELLS

These competent cells are prepared using GIBCO BRL's patented modification of the procedure of Hanahan.

2.7.2.1 Materials

Library Efficiency DH5alpha MCR™ Competent Cells (GIBCO BRL, 8289SA)

Trituration buffer:

80% glycerol

LB medium

2.7.2.2 Methods

Streak the bacterial strain on selective 1XYT plates overnight. Inoculate 25ml of LB medium with a single colony and incubate at 37 degrees overnight with vigorous shaking. Shake the culture at 150-200 rpm at 37 degrees until reaching stationary stage, chill the cells in ice water for 2 hours and collect by centrifugation at 2,500X g for 15-20 minutes at 4 degrees. Resuspend the cells in 10-20 ml of ice-cold trituration buffer and dilute to 500ml with the same solution. Incubate the cells on ice for 45 minutes. Centrifuge the cells at 1,800X g for 10 minutes and gently resuspend in 50ml of ice-cold trituration buffer. Pool the cells and add 80% glycerol dropwise with gentle swirling to a final concentration of 15% (v/v). Aliquot the cells in 0.2-1.0 ml quantities, freeze on dry ice, and store at -80 degree.

2.7.3 LIGATION

2.7.3.1 Materials

T4 DNA ligase

Ligase 10X buffer:

2.7.3.2 Methods

Set up a ligation reaction by adding 100ng of vector DNA, 17ng of insert DNA, 1 unit of T4 DNA ligase, 1ul of ligase 10X buffer, and deionized water to final volume of 10ul. Ligation reactions should be performed according to the incubation temperature and time; 4 degrees, overnight or 15 degrees 4-6 hours or 25 degrees 1hour. Following the ligation reaction, transform the plasmid DNA into competent cells of an appropriate host strain.

2.7.4 TRANSFORMATION

To obtain maximum transformation efficiency, the experimental DNA must be free of phenol, ethanol, protein and detergents.

2.7.4.1 Materials

DMSO (dimethyl sulfoxide)

S.O.C. medium: 2% Bactotryptone, 0.5% Yeast extract, 10mM NaCl, 2.5mM KCl, 20mM $MgCl_2$ $MgSO_4$, 20mM Glucose

LB medium

2.7.4.2 Methods

Prepare a dry ice/ ethanol bath and maintain at -70 degree. Remove competent cells from -70 degree freezer, thaw on ice. Add 3ul of DMSO to the competent cells, mix briefly, and add 10-20ng of plasmid DNA. Incubate the cells on ice for 30 minutes. A heat shock at 42 degrees for 1-2 minutes after the incubation on ice has been reported to increase transformation efficiency. Following a heat shock, cool the tube on ice for 1 minute. Add 2ml of S.O.C. medium and shake gently at 37 degrees for 1 hour to allow the cells to recover. The cells can be plated directly or concentrated before plating. To concentrate the cells, centrifuge the culture for 1 minute in a microcentrifuge. Pour off the supernatant and resuspend the cells in each tube in 100-200ul of S.O.C. medium. Plate the cells on an LB plate containing 50ug/ml ampicillin and incubate at 37 degrees for 12-14 hours.

2.7.5 SELECTION FOR TRANSFORMANTS BY BLUE-WHITE COLOR SCREENING

2.7.5.1 Materials

LB plates containing 50ug/ml ampicillin.

0.1M Isopropyl-B-D-thiogalactoside (IPTG) stock solution (50ml):
1.2g IPTG, filter-sterilize, store at 4 degrees.

5-bromo-4-chloro-3-indolyl beta-D-galoctopyranoside (X-gal) stock solution: 50mg per ml of N,N' dimethyl formamide, filter-sterilize, store at 4 degrees

2.7.5.2 Methods

Plate transformed cells on LB plates containing 50ug/ml ampicillin, 0.5mM IPTG, and 40ug/ml X-Gal. Incubate the plates overnight at 37 degrees. An alternative to preparing plates containing IPTG and X-Gal is to spread plates with 20 ul of 50mg/ml X-Gal and 100ul of 100mM IPTG and allow these components to absorb for 30 minutes at 37 degrees prior to plating cells.

2.8 RESTRICTION MAPPING

To construct a restriction map in a subcloned DNA various enzymes that recognize 6 bp sequences (cut on average once every 4096 bp) were used.

2.8.1 Materials

Class II Restricton Enzymes

Restricton Enzyme Buffers

2.8.2 Methods

For analytical purposes the following mixture was used in a 1.5ml microcentrifuge tube: 8ul of DNA solution, 1ul of 10X buffer, 1ul of enzyme. The amount of DNA was in the range 0.1 to 1ug. The tube is then vortexed, briefly spun in a microfuge and incubated at appropriate temperature. Restriction enzyme digests are analyzed on agarose gels with size marker DNA. For double digest a buffer

providing reaction conditions that are amenable to both restriction endonucleases was selected. When no single buffer could be found to satisfy the buffer requirements of both enzymes, the reactions were done sequentially. First, cleaved with the restriction endonuclease that requires the lower salt reaction conditions, then adjust the salt concentration of the reaction by using a small volume of a concentrated salt solution to approximate the reaction conditions of the second restriction endonuclease. Added the second enzyme and incubated to complete the second reaction. Incubated the reaction at 65 degrees for 20 minutes to inactivate the majority of restriction endonucleases that have an optimal incubation temperature of 37 degree.

2.9 NORTHERN BLOT (19, 39, 79)

2.9.1 ISOLATION OF POLY(A)+ RNA BY OLIGO (dT) CELLULOSE

The following protocol describes a method for isolation of poly (A)+RNA from total RNA by binding to oligo (dT) cellulose. Oligo (dT) beads bind poly $(A)^+$ mRNA at a ratio of 0.5mg RNA per 0.3g resin.

2.9.1.1 Oligo (dT) cellulose preparation

2.9.1.1.1 Materials

Oligo (dT)- cellulose (new England Bio Labs 1401)

Binding 2X buffer: 0.02 M Tris-HCl pH7.5, 1M NaCl, 2mM EDTA, 1.0% SDS

Wash buffer: 0.01M Tris-HCl pH 7.5, 0.1 M NaCl, 1mM EDTA
Elution buffer: 0.01M Tris-HCl pH7.5, 1mM EDTA
0.05% Sodium azide

2.9.1.1.2 Methods

Add 2ml of binding 1X buffer to 0.6g of oligo (dT)- cellulose. Add 2ml of 0.1N NaOH to the resin and mix gently for a few minutes. Centrifuge at 1,500xg for 2 minutes. Centrifugation greater than 1,500xg may result in damage to the oligo (dT) beads. Remove supernatant and discard. Repeat this procedure 8-10 times. Add 2ml of binding 1x buffer to the resin and mix gently. Centrifuge at 1,500xg for 2 minutes. Remove the supernatant and discard. Repeat the procedure 8-10 times. Resuspend gently in 2ml of binding 1x buffer. Transfer 1ml of the slurry to each of 2 microcentrifuge tubes. One of these may be stored, for future use, at 4 degrees with the addition of 0.5ml of binding 1x buffer and 0.05% sodium azide.

2.9.1.2 RNA extraction and purification

2.9.1.2.1 Materials

Denaturing solution: 25g guanidine thiocyanate (4M final) per 33ml CSB buffer

CSB buffer: 42mM Sodium citrate, 0.83% N-lauryl sarcosine, o.2 mM Beta-mercaptoethanol.

2.9.1.2.2 Methods

Prepare fungal cultures in sterile 125ml flask in 25ml of liquid media. After several days, harvest the cultures by using Buchner funnels covered with Whatman No.1 filter circles (a typical yield is 1-2 grams of mycelia per flask). Transfer the mycelia to a mortar and pestle, freeze them and grind them with sands to a fine powder. Transfer the mycelial powder to a homogenizing flask containing 12ml of denaturing solution pre-cooled on ice for 5 minutes for tissue disruption. Add 1.2 ml of 2M sodium acetate, pH 4.0 and mix thoughly by inversion. Add 12ml of phenol:chloroform:isoamyl alcohol mixture, mix by inversion and shake vigorously for 10 seconds. Chill on ice for 15 minutes. Transfer this mixture to a 50ml thick-walled polypropylene tube and centrifuge at 10,000xg for 20 minutes at 4 degrees. Carefully remove the top aqueous phase which will contain the RNA and transfer it to a fresh tube. DNA and proteins will remain in the organic phase and at the interface. Add an equal volume of isopropanol and incubate the sample at -20 degree for at least 30 minutes to precipitate the RNA. Pellet the RNA by centrifugation at 10,000xg for 15 minutes at 4 degree. Resuspend the RNA pellet in 5ml of denaturing solution and vortex until the RNA is dissolved. Add an equal volume of isopropanol and precipitate the RNA as described in step, above. Pellet the RNA by centrifugation at 10,000xg for 15 minutes at 4 degree. Wash the pellet with ice-cold 75% ethanol and centrifuge as above. A minimum of 10ml of 75% ethanol should be used. Dry the pellet in a

vacuum desiccator for 15-20 minutes. Resuspend the RNA in 1-3 ml of RNAse-free deionized water and store at -20 degree.

2.9.1.3 mRNA isolation

2.9.1.3.1 Materials

Binding 2x buffer: 1M NaCl, 20mM Tris-Cl pH7.5, 2.0mM EDTA 1% SDS

Wash buffer: 0.01M Tris-Cl pH7.5, 0.1M NaCl, 1mM EDTA

Elution buffer: 0.01M Tris-Cl pH7.5, 1mM EDTA

2.9.1.3.2 Methods

Add 1 volume of binding 2x buffer to 100-300ul of the total RNA prepared. Heat the mixture for 10 minutes at 65 degrees and then allow to cool to room temperature. Add the RNA mixture to the 1ml slurry of oligo (dT) in binding 1x buffer. Shake gently for 15 minutes at room temperature to allow the RNA to bind to the resin. Centrifuge at 1,500xg for 5 minutes and discard the supernatant. Add 500ul of wash buffer to the resin. Shake gently for a few seconds. Centrifuge at 1,500xg for 2 minutes. Discard the supernatant. Repeat this procedure 3 more times. Add 200 ul of elution buffer to the resin and shake gently for a few minutes. Centrifuge at 1,500xg for 5 minutes. Transfer the supernatant to a fresh tube. Repeat this procedure 2 more times, saving the supernatants each time. Add 0.1 volume of 3M sodium acetate and 2.5 volumes of ethanol to the pooled supernatants. Mix and leave at

-20 degree overnight. Centrifuge at 12,000xg for 15 minutes. Wash the RNA pellet with 1ml of 70 % ethanol. Dry the pellet under vacuum and resuspend in an appropriate volume of TE buffer. Store at -70 degree.

2.9.2 NORTHERN BLOTTING TRANSFER

2.9.2.1 RNA preparation

2.9.2.1.1 Materials

10X gel buffer: 60% formaldehyde

Loading dye: 50% (v/v) glycerol, 1mM EDTA, 0.4% (w/v) Bromphenol blue, 0.4% (w/v) Xylene Cyanol.

2.9.2.1.2 Methods

The frozen RNA in water at -70 degree was thawed on ice. 5ug (4.5ul) of thawed RNA was incubated for 15 minutes at 55 degrees in a mixture of 2ul of 10X gel buffer, 3.5ul of formaldehyde, 10ul of formamide, and 2ul of loading buffer.

2.9.2.2 Gel preparation

2.9.2.2.1 Materials

10X MOPS electrophoresis buffer: 400mM MOPS, 100mM sodium acetate, 10mM EDTA

RNA marker 0.3-7.4 kb (molecular SIGMA biology R-7885 Lot 62H-0160)

2.9.2.2.2 Methods

30ml of 1.5% formaldehyde gel is made of 22ml of 2% agarose in water which is melted, 3ml of 10X gel buffer, and 5ml of formaldehyde. Electrophoresis was done at 60-80 voltage in 1X electrophoresis buffer. RNA marker sample solutions were prepared for electrophoresis by mixing following ingredients: 10ul of 1mg/ml RNA marker, 2.5ul 10X MOPS electrophoresis buffer, 3.5ul 37% formaldehyde, and 10ul Deionized formamide. The sample solution was incubated at 65 degrees for 10 minutes and immediately cooled on ice. 2ul of loading dye was added to the sample solution. All 29ul of the RNA marker sample solution was run with appropriate RNA samples on a 10mm thick denaturing (formaldehyde) agarose gel. Electrophoresis was performed in a mini submarine -type apparatus at 4.5V/cm in 1X MOPS electrophoresis buffer. The gel was stained in 5ug/ml ethidium bromide for 15 minutes and destained 1 hour with shaking in water. Nine bands were resolved as size markers which were used for identifying the sample RNA message size.

2.9.2.3 Transfer

2.9.2.3.1 Materials

0.05N NaOH/0.15M NaCl

0.1M tris pH 7.5/0.15 M NaCl

Transfer buffer: 10X SSC

PosiBlot Pressure Blotter and Pressure Control Station: Stratagene, La jolla, CA 92037

2.9.2.3.2 Methods

Following electrophoresis, stain a gel in 5ug/ml ethidium bromide in water for 15 minutes, destain in water for an hour and photograph the gel. Denature the RNAs by treating a gel in 0.05N NaOH/ 0.15M NaCl solution for 30 minutes to 1 hour. Neutralization was done by pouring off the denaturing solution and replacing 0.1M tris pH 7.5/ 0.15 M NaCl solution for 30 minutes to 1 hour. Pre-wet the membrane by first soaking in water for 20 minutes and then soak for an additional 10 minutes in transfer buffer. PosiBlot was used for i hour.

2.9.2.4 Hybridization and detection

These procedures were performed in the same way as done for Southern transfer procedure except prewetting a membrane in 5XSSC for 5-10 minutes before prehybridization.

2.10 SEQUENCING AND DNA ANALYSIS

2.10.1 NESTED DELETION CLONING

2.10.1.1 Materials

10X ExoIII buffer: 660mM TrisCl pH8.0, 66mM MgCl2

10X S1 buffer: 5ml of5M NaCl, 1.1ml of 3M KOAc pH4.5, 5ml of glycerol, 20ul of 1M ZnSO4

S1 mixture: 172ul of water, 27ul of 10X buffer, 60 units of S1 nuclease

S1 stop mixture: 0.3M TrisCl pH7.6, 0.05M EDTA pH8.0

Klenow mixture: 20ul of water, 6ul of 1M MgCl2, 3ul of 0.1MTrisCl pH7.6, 3units of Klenow enzyme

Ligase mixture: 650ul of water, 100ul of 10X t4 ligase buffer, 250ul of 30 % w/v PEG 8000, 5 units of T4 ligase

2.10.1.2 Methods

10ug of purified DNA was cut with two enzymes the cutting sites of which are not adjacent: for pTZ18R vector insert *NcoI* as a 5' overhang cutting enzyme and *SphI* as a 3' overhang cutting enzyme were used: for pTZ19R vector insert *XbaI* and *KpnI* were used for each cutting. It was ensured that the DNA was linear by running a mini gel. The DNA was purified by phenol-chloroform extraction, transferred to fresh tubes, and added 3M NaOAc (pH5.2) to 0.3M concentration. After EtOH precipitation, the pellet was dissolved in 60ul of 1X ExoIII buffer. The DNA solution was incubated for 5 minutes at 37 degrees. In the meantime, prepare 25 0.5ml tubes on ice containing 7.5ul of S1 mixture in each tube. 2.5ul of solution is transferred to the first tube containing 7.5ul of S1 mixture. Mix the rest of the DNA solution (57.5ul) with 600 units of ExonucleaseIII, voltex and put on 37 degrees waterbath. In 45 seconds remove 2.5ul of the mixture to the second tube. Continue to the last tube and incubate all the tubes at 30 degrees for 30 minutes. Add 1ul of S1 stop mixture to each and incubate for 10 minutes at 70 degrees. Run a mini gel with 2ul of each sample. Pool the samples of same size,

add 1ul of Klenow mixture to each 10ul sample, and incubate at 37 degrees for 5 minutes. To each 10 ul sample add 1ul of dNTPs mixture (0.5mM concentration each) and incubate them for 15 minutes at room temperature or for 5 minutes at 37 degrees. Add 40 ul ligase mixture to each sample, mix them , and incubate at room temperature for 2 hours. Transform competent cells with equal volume of sample as described before and spread on ampicillin supplied 2X YT medium plate. After overnight incubation at 37 degrees select colonies for sequencing that differ in size by 150-200bp.

2.10.2 DNA SEQUENCING

2.10.2.1 DNA preparation

2.10.2.1.1 Materials

The UniPlex Sequencing Kit purchased from Millipore

2.10.2.1.2 Methods

DNA is denatured by mixing with equal volume of solution (0.4N NaOH, .4mM EDTA) and incubating for 5 minutes at 85 degrees. Neutralize the DNA by adding 2 ul of 2.5M NH_4OAc and voltexing the mixture. After EtOH precipitation dissolve it in 8ul of water. In a 0.5ml tube mix the 8ul DNA solution with 1ul of M13 primer and 1ul of 10X buffer and incubate for 20 minutes at 42 degrees. Prepare 4 tubes (A,C,G,T: add 3ul of dNTP/ddNTP mixture in each tube. Add 2ul of extension mixture and 1ul (5units) of Klenow enzyme to DNA

samples and incubate at 42 degrees for 5 minutes. Transfer 3ul of sample to each of the 4 tubes. Incubate 10 more minutes at 42 degrees and add 3ul of stop solution/loading dye to each tube. Store at -20 degree until loading.

2.10.2.2 Electrophoresis of sequencing gel

2.10.2.2.1 Materials

6% Acrylamide gel

1X TBE buffer

2.10.2.2.2 Methods

After incubating the DNA sample for 2 minutes at 80 degree load half of the sample in each well of 6% acrylamide gel in 1X TBE buffer. Electrophoreses the sequencing gel at 40 voltage per 1cm of gel until bromophenol blue dye is run off the gel. Transfer the separated DNA in the gel onto nylon filter paper by Southern blotting. Crosslink the DNA to the filter paper at $33X10^3$ uJoule and store the filter paper at room temperature in a plastic bag until the detection procedure is performed.

2.10.2.3 Detection of DNA

2.10.2.3.1 Materials

The UniPlex Detection Kit purchased from Millipore

2.10.2.3.2 Methods

Add 0.1ml blocking solution per cm^2 of the membrane in a plastic bag. After 5 minutes incubation drain it. Add $0.05ml/cm^2$ streptavidin solution, incubate for 5 minutes and drain it. Wash it in $0.5ml/cm^2$ of wash solution I for 10minutes. After draining repeat washing procedure. Incubate the membrane in $0.05ml/cm^2$ of biotinylated alkalin phosphatase solution for 5 minutes. Wash twice with $0.5ml/cm^2$ of wash solution II for 10 minutes each. Incubate the filter membrane with $0.025ml/cm^2$ of 1X Lumigen PPD reagent for 5 minutes. After draining keep the membrane in a sealed plastic bag. Expose the membrane in a bag to X-ray film for 10 minutes. Develop the film and identify the sequence of the sample on a light box.

2.10.3 DNA ANALYSIS

Analysis of sequence data and amino acid sequence prediction were carried out with software package DNA strider™ 1.0 which was written by Christian Marck. DNA sequences for protein coding regions by looking for an initiator codon in frame with a chain termination codon (ORF) were searched. DNA sequence alignments was performed with Wisconsin program using the GenEMBL DNA databases to search for homologous sequences. Computer graphics visualization of deduced secondary structure of the polypeptide was made using TEKPLT01 software package by translating a cloned DNA

sequences theoretically into an amino acid sequence and predicting secondary and tertiary structures of the polypeptide.

2.11 OVERLAPPING CLONING

2.11.1 RESTRICTION MAPPING AROUND THE SUBCLONE DNA

2.11.1.1 Materials

Class II Restriction Enzymes

Genius labeling and detection kit: purchased from Boehringer Mannheim Biochemicals)

Nylon Blotting Membrane: purchased from Micron Separations inc., Westboro, MA

2.11.1.2 Methods

The cloned cosmid insert DNA was mapped by digestion with restriction enzymes followed by separation of the DNA fragments by gel electrophoresis and Southern blotting (the method of DNA digestion with restriction enzymes is same as described in restriction mapping of cosmid insert section). DNA transferred to a membrane were hybridized with the labelled subclone DNA as a probe (the methods of DNA labeling and detection are same as described in RFLP Southern Blotting section). Sizes of detected bands were estimated relative to *Hind III* lambda DNA size markers. According to the sizes of bands detected in hybridization experiments, a preliminary restriction map around subclone insert was constructed.

2.11.2 OVERLAPPING SUBCLONES

2.11.2.1 Materials

pTZ18R: available at several companies including SIGMA

CIAP (Calf Intestinal Alkaline Phosphatase)

CIAP 10X buffer: 500mM Tris-HCl pH9.0, 10mM $MgCl_2$, 1mM $ZnCl_2$, 10mM spermidine

7.5M Ammonium acetate.

LB plates containing 50ug/ml ampicillin.

0.1M Isopropyl-B-D-thiogalactoside (IPTG) stock solution (50ml): 1.2g IPTG, filter-sterilize, store at 4 degrees.

5-bromo-4-chloro-3-indolyl beta-D-galoctopyranoside (X-gal) stock solution: 50mg per ml of N,N' dimethyl formamide, filter-sterilize, store at 4 degrees

Genius labeling and detection kit: purchased from Boehringer Mannheim Biochemicals)

Nylon Blotting Membrane: purchased from Micron Separations inc., Westboro, MA

2.11.2.2 Methods

The method used here is same as described in subcloning section and RFLP Southern transfer section. A DNA fragment partially overlaps the subclone insert fragment was inserted into the pTZ18R vector and used for transformation of *E. coli* competent cells. Insert bearing colonies were picked on blue-white screening plates and inoculated in 2XYT medium for amplyfication. The plasmid DNA was

extracted, cut with *BamH1* and DNA fragments were separated by gel electrophoresis. The DNA fragments were transferred by Southern blotting and hybridized with the 1.82Kb *Pst1* +*PvuII* fragment as a probe to confirm that the clones have an insert of the overlapping fragment in the pTZ18R vector.

2.11.3 THE EXTENT OF THE *pmb* LOCUS

2.11.3.1 Materials

50 mM spermidine

STC: 1M sorbitol, 50mM Tris-HCl pH8.0, 50mM $CaCl_2$

Heparin: 5mg/ml in STC

PTC: 40% polyethylene glycole 4000, 50mM Tris-HCl pH8.0, 50mM $CaCl_2$

Neurospora culture colonial bottom agar medium (1L): Vogel's 50X 20ml, Bacto-Difco Agar 15g, 10X FIGS 100ml, 0.5mg/ml Histidine, 5mg/ml Phenylalanine, 200 ug/ml Hygromycin

Neurospora culture colonial regeneration top agar medium (1L): Vogel's 50X 20ml, sorbitol182g, Bacto-Difco Agar 28g, 10XFIGS 100ml

2.11.3.2 Methods

The procedure is same as described in transformation section. Transformation of *pmn:pmb:his-2* strain was done with the X7:5E cosmid DNA, the 13Kb *Sal1* fragment and the 3.2 Kb *Pst1* fragment to identify the extent of the *pmb* locus in the X7:5E cosmid insert.

0.5ug of DNA was used for transformation with X7:5E. For transformation with the 13Kb *SalI* fragment and the 3.2 Kb *PstI* fragment, 1ug each of X7:5E DNA was used for fragment purification. The colonies were counted on Histidine plus 10X phenylalanine plates in four days after transformation.

CHAPTER 3
RESULTS

3.1 SELECTION OF *pmn:pmb:his-2* STRAIN

A *pmn:pmb:his-2* strain which can be used as a host cell for transformation with wild type library DNA was produced by crossing *pmb* strain to *pmn:his-2 strain.* The *pmn:pmb:his-2* strain was identified by selection for growth on histidine plates containing PFPA and canavanine in media followed by testing for growth on minimal and minimal plus histidine plates. The *pmn:pmb:his-2* strain grew on selection media and on minimal plus histidine media, but did not grow on minimal alone.

3.1.1 SENSITIVITY OF *NEUROSPORA* TO AMINO ACID ANALOGS PFPA AND CANAVANINE

3.1.1.1 Sensitivity to PFPA

A conidial suspension was incubated in a conidial growth N medum containing histidine and PFPA. Conidia were filtered, dried and weighed.

In the data of Table 1, wild type strain *74a* shows sensitivity to PFPA at concentration 50ug/ml and decreased the growth rate abruptly. *mtr10r* and *mtr6r* strains which are mutants defective in N system were resistant to PFPA at concentration of up to 200ug/ml.

#1683 bat um535 and *#2275 bat cam-37(r)* which have a wild type N system showed very little growth in the presence of PFPA at

50ug/ml concentration. A 50ug/ml concentration of PFPA was identified as suitable in experiments for selection of a strain defective in N system.

Table 1 Sensitivity test of *Neurospora* to amino acid analogue PFPA

PFPA(ug/ml)	*74a*	*#1683*	*#2275*	*mtr6r*	*mtr10r*
0	100%	100%	100%	100%	100%
50	0.13	0.12	0.21	84.90	98.40
100	0.08	0.10	0.12	80.80	94.30
150	0	0	0	78.40	89.60
200	0	0	0	75.00	83.90

* The percentage of dry weight of each strain relative to control (0ug/ml PFPA). The conidial suspension of each strain was incubated in liquid minimal N medium containing histidine (500ug/ml) and PFPA. At stationary stage (after 4 days) conidia were filtered, dried, and weighed. *74a* is a wild-type strain. *#1683* and *#2275* are *pmb*$^-$ strains. *mtr6R* and *mtr10R* are *pmn:his-2* strains. The dryweight of each strain grown in media without PFPA is considered to be 100%.

3.1.1.2 Sensitivity to canavanine

Table 2 and Figure 1 show the sensitivity of *Neurospora* strains to amino acid analogue canavanine. A wild type strain *74a* shows high sensitivity to canavanine and decreases the growth rate rapidly in the media containing over 100ug/ml concentration of canavanine. *#1683 (pmb)* and *mtr6r (pmn:his-2)* are strains used as parents in the cross for selecting *pmn:pmb:his-2* strain. The *#1683* strain is resistant to canavanine and shows over 75% growth at 200ug/ml concentration of canavanine. The *mtr6r* strain which is *pmb* + is quite sensitive to canavanine. According to the data 200ug/ml concentration of canavanine is suitable to use in experiments for selection of a strain which is defective in B system.

Table 2. Sensitivity test of *Neurospora* to amino acid analogue canavanine

canavanine (ug/ml)	*74a*	*#1683*	*mtr6r*
0	100%	100%	100%
100	58.7	73.2	27.9
200	28.1	75.7	10.4
300	20.2	74.7	9.1
400	15.6	59.6	5.1
500	9.2	64.4	4.9

* The percentage of dry weight of each strain relative to control (0ug/ml canavanine). The conidial suspension of each strain was incubated in liquid conidial growth N media containing histidine (500ug/ml) and canavanine. At stationary stage conidia were filtered, dried, and weighed. *74a* is a wild-type strain. *#1683* is a *pmb*⁻ strain. *mtr6R* is a *pmn:his-2* strain. The dryweight of each strain grown in media without canavanine is considered to be 100%.

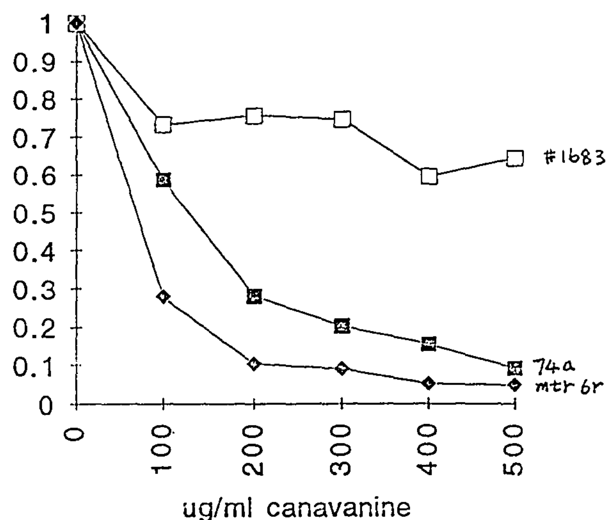


Figure 1. Sensitivity test of *Neurospora* to amino acid analogue canavanine

3.1.2 *pmn:pmb:his-2* STRAIN

A *pmn:pmb:his-2* strain was selected among the progeny colonies from crosses between *pmb* and *pmn:his-2* parental strains on plates containing 50ug/ml PFPA, 200ug/ml canavanine, and 500ug/ml histidine in media, followed by culturing on 1X Vogel's cololial media with or without histidine for discarding a *pmn:pmb* strain. The *pmn:pmb:his-2* strain was selected from colonies which grow only on histidine containing media.

The *pmn:pmb:his-2* strain was verified as a host strain in transformation experiments by culturing in 1X Histidine plus 10X Phenylalanine media. The data of Table 3 show very little growth (1.3%) of the *pmn:pmb:his-2* strain in histidine plus phenylalanine media. Thereby, this data confirm that the selected *pmn:pmb:his-2* strain is useful as a host strain in transformation experiments for selection of transformants with pmb^+ DNA fragments.

Table 3. Dry weight of the *pmn:pmb:his-2* strain

Strains	Minimal+His	Minimal+His+Phe
74a	0.0231g	0.0229g
pmn:pmb:his-2	0.0237g	0.0003g

* *74a* is a wild type strain and used as a control. Minimal+His. media are 1X Vogel's conidial growth media containing 0.5mg/ml histidine. Minimal+His.+Phe. media are 1X Vogel's conidial growth media containing 0.5mg/ml histidine and 5mg/ml phenylalanine.

3.2 CLONING OF THE *pmb* LOCUS

Tests of osmotic stability showed that more than 99% of the conidia were converted to spheroplasts. Between 60% and 100% of the spheroplasts were capable of regenerating to form viable colonies. The competent spheroplasts were stored frozen in the transformation buffer at -70 degree. Such spheroplasts showed no decrease in transformation frequency for at least 1 month and usually much longer. The use of frozen spheroplasts conserves Novozym 234 and facilitates the multiple transformation steps required for the sib selection procedure.

3.2.1 TRANSFORMATION EFFICIENCY OF SPHEROPLASTS

For purposes of computing transformation efficiency of spheroplasts of the *pmn:pmb:his-2* strain we defined stable transformants as those which are capable of growing in slants with selection media after being picked from the initial selection plates. In different transformations, between 50 and 100% of the colonies picked from plates continued to grow in slants. The experiments for testing transformation efficiency of *pmn:pmb:his-2* spheroplasts were performed with his^+ and mtr^+ DNAs. Table 4 shows the transformation efficiency of *pmn:pmb:his-2* strain with the genes already cloned as exogenous DNA sequences in the transformation experiments by former researcheres. The pSV50-6:11E clone contains a wild-type *his-2* gene sequence and the pSV50-5:4H clone has a wild-type *mtr* gene sequence (Stuart, Koo, and Vollmer, 1988).

In each experiment, 1ug of DNA was used for transformation of 10^7 spheroplasts of the *pmbn:pmb:his-2* strain. In the experiments with the 6:11E DNA sequence, minimal medium with no supplements was used for selection. In the experiments with the 5:4H DNA sequence, histidine plus 10X arginine medium was used for selection. The number of transformants was variable, and transformation efficiencies of *pmbn:pmb:his-2* strain with *his-2*$^+$ and *mtr*$^+$ DNA ranged from 40 to 80 transformants per ug of exogenous DNA. The average number was 55 per ug of DNA.

Table 4. Transformation efficiency test of *pmn:pmb:his-2* strain with *his-2*$^+$ and *mtr*$^+$ DNA

	DNA sequences	Transformants
Experiment 1	6:11E	49
Experiment 2	6:11E	64
Experiment 3	6:11E	42
Experiment 4	6:11E	60
Experiment 5	6:11E	83
Experiment 6	5:4 H	40
Experiment 7	5:4 H	48

* pSV50-6:11E cosmid contains the *his-2*$^+$ gene sequence. pSV50-5:4H contains the *mtr*$^+$ gene sequence. In each transformation experiment 1ug of DNA and 10^7 spheroplasts of *pmn:pmb:his-2* strain were used. In the experiments with 6:11E DNA sequence, minimal colony formation medium was used as a bottom agar. In the experiments with 5:4H DNA sequence, minimal medium containing histidine plus 10X arginine was used.

3.2.2 SIB SELECTION

When the triple mutant strain was transformed with the DNA sequences of pSV50 cosmid vector library, the cosmid DNAs rescuing the *pmb* phenotype did not map to linkage group IV by RFLP mapping (11:1H, linkage group III, *trp-1*) (17:10A, linkage group I, *nit-2*). A screen of a second *Neurospora* cosmid library (pMOcosX) was performed using the technique of sib-selection. 50 pools of DNA, each containing 96 different cosmid clones of *Neurospora* genomic wild-type DNA, were tested for their overall frequencies in transforming *pmn:pmb:his-2* spheroplasts. Among 50 pools positively transforming pools (X7, X9, X11, X23, X24, G10, G21) were divided into 12 subpools of 8 cosmids each and the transformation repeated. Finally all subpools which demonstrated transformation of the *pmb* locus were divided into the individual cosmids and the transformation repeated a third time.

Table 5 and Table 6 shows the number of transformants obtained by using cosmid DNAs. In Table 6 the number of transformants recovered with X7:5E ranged from 20 to 50 per ug of input DNA. The average number of transformants is 36 per 1ug of X7:5E DNA and make it a good candidate as a clone containing wild-type *pmb* locus.

Table 5. Transformation of *pmn:pmb:his-2* spheroplasts by uptake of cosmid DNAs

Cosmids	Transformants
No DNA	0
X7:5E	37
X7:10F	11
X9:7C	15
X9:7H	17
X11:12A	30
X11:12B	18
X11:12C	14
X23:9A	9
X23:9C	33
X24:7D	17
X24:7E	11
G10:8F	14
G21:3C	16
G21:9D	40

* *pmn:pmb:his-2* spheroplasts were plated on histidine:phenylalanine =1:10 colonial medium after transformation procedure with cosmid DNAs. The medium contains 0.5mg/ml histidine, 5mg/ml phenylalanine, 200ug/ml hygromycin. 1ug of DNA and 10^7spheroplasts were used per plate in each experiment.

Table 6. Transformation of *pmn:pmb:his-2* spheroplasts by uptake of X7:5E cosmid DNA

	Transformants
Experiment 1	37
Experiment 2	50
Experiment 3	45
Experiment 4	24
Experiment 5	23

* *pmn:pmb:his-2* spheroplasts were plated on histidine: phenylalanine=1:10 colonial medium after transformation procedure with X7:5E DNA. The medium contains 0.5mg/ml histidine, 5mg/ml phenylalanine, 200ug/ml hygromycin. 1ug of X7:5E DNA and 10^7 spheroplasts were used per plate in each experiment.

3.3 RFLP SOUTHERN HYBRIDIZATION

RFLP mapping kits have been developed to map DNA fragments onto the seven linkage groups of *Neurospora crassa* with many polymeric differences. The segregation pattern in one linkage group is different from that of other linkage group. The pattern also differs within a linkage group depending on the distance between markers. The allele assignment of a cloned gene is used to confirm the identity of the putative clone by comparing with the allele segregation data of markers that have already been mapped (Metzenberg, R.L. and J. Grotelueschen Fungal Genetics Newsletter 36, June 1989 51-57).

Procedures for gel electrophoretic analysis of DNA and Southern hybridization were used for RFLP mapping. 0.8% agarose gels were used for electrophoresis to separate DNA isolated from the RFLP kits and digested with *EcoR1*. Southern blots were hybridized with selected recombinant plasmids that had been labeled by random priming method with Digoxigenin-dUTP.

3.3.1 RFLP MAPPING OF X7:5E

Table 7 shows 18 segregants RFLP pattern of X7:5E: X7:5E DNA is tightly linked to the genes *nit-3* and *Fsr-4* on linkage group IV. For more accurate information about the location of X7:5E, 38 segregants RFLP technique was adopted to map X7:5E clone. The 38 segregants RFLP pattern of X7:5E is shown in Table 8: X7:5E DNA is tightly linked to *Fsr-4* position on the linkage group IV. From the 18- and 38-segregants RFLP patterns, the X7:5E clone is confirmed to map to the *pmb* locus on linkage group IV.

Table 7. RFLP of X7:5E 18 Segregants

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
(O)	M	M	M	M	(M)	M	M	O	M	O	O	O	M	O	O	O	M	M	O	*nit-3*
(O)	M	M	M	M	(M)	M	M	O	M	O	O	O	M	M	O	M	M	M	O	X7:5E
(O)	O	M	M	M	(M)	M	M	O	O	-	O	M	M	M	O	M	M	M	M	*Fsr-4*

* 10 ug of the genomic DNA of each of the 18 segregants (#4411-#4430) was digested with 20 units of *EcoR1* enzyme for 16 hours at 37 degrees. The digested DNAs were loaded into 0.8 % agarose gel and electrophoresed for 20 hours at 1.5 voltage per cm of agarose gel in 1XTBE buffer containing 500ug/L concentration of ethidium bromide. The DNAs in the gel were transfered to a nylon sheet by Southern blotting, and hybridized with X7:5E probe. The segregants of *nit-3* and *Fsr-4* were picked up from data in Fungal Genetics Newsletter 36,june 1989.

Table 8. RFLP of X7:5E 38 Segregants

AA 1 4	BB 67	CC 14	DD 57	EE 13	EE 57	FF 13	GG 14	HH 57	I I 68	JJ 14	KK 14	LL 14	MM 58	NN 23	OO 24	PP 14	QQ 24	RR 14	
MO	MM	MO	OO	MO	OM	OM	OO	-M	OM	OO	OM	MM	OM	OO	MO	MO	MO	MM	*00034*
MO	MO	MO	OO	MO	OM	OM	OO	OM	OM	OO	OM	OM	OM	OO	MO	OM	MM	MM	X7:5E
MO	MO	MO	OM	MO	OM	OM	OM	OO	OM	OO	OM	OM	OM	O-	MO	OM	MM	MO	*Fsr4*

* 10ug of the genomic DNA of each of the 38 segregants (#4450-#4488) was digested with 20 units of *EcoR1* enzyme for 16 hours at 37 degrees. The digested DNAs were loaded into 0.8 % agarose gel and electrophoresed for 20 hours at 1.5 voltage per cm of agarose gel in 1X TBE buffer containing 500ug/L concentration of ethidium bromide. The DNAs in the gel were transfered to a nylon sheet by Southern blotting, and hybridized with X7:5E probe. The segregants of *00034* and *Fsr-4* were picked up from data in Fungal Genetics Newsletter 1992.

3.3.2 RFLP MAPPING OF OTHER COSMIDS

Table 9 shows the list of cosmids which also complement *pmb* mutant phenotype but do not map to *pmb* locus. When these cosmid DNAs were cross-hybridized with X7:5E DNA sequence as a probe, only the vector sequence was detected, indicating that there is no common homologous sequence except vector DNA between X7:5E and any of these clones. Therefore, it is presumed that X7:5E contains the unique functional *pmb* locus in the library.

Table 9. RFLP mapping of other candidate cosmids

cosmids	RFLP mapping
X9:7C, X11:12B	LGI, al-2 met-6
G21:9D	LGII, arg-12
X9:7H	LGIV, Tel IVL
G21:3C, G10:8F	LGV, con4-a
X24:7E, X7:10F	LGV, inl
X11:12A, X23:9C	LGVI, Bml con-3
X24:7D, X11:12C, X23:9A	LGVII, un-10

* Each cosmid was mapped by RFLP analysis. Each cosmid DNA was labeled with Digoxigenin-dUTP and used as a probe in Southern hybridization procedure for RFLP mapping.

3.3.3 GENOTYPE OF TRANSFORMANTS WITH X7:5E DNA

The transformants with X7:5E which were picked from a plate with histidine plus 10X phenylalanine selective medium were further analysed to confirm their genotype(s) by the measurement of the growth in liquid culture (Table 10). Neither the *pmn:pmb:his-2* strain nor the transformants grew in minimal media, indicating that the transformants retained the *his-2* genotype. The *pmn:pmb:his-2* strain grows fully in media containing histidine and canavanine, but does not grow well in media containing histidine and 10X phenylalanine. Conversely, the transformants grew fully in histidine plus 10X phenylalanine media and did not grow well in canavanine media, indicating that the genotype of transformants is pmb^+ (*pmn:his-2*). These data prove that the transformants contain the wild-type *pmb* gene which is presumed to originate from X7:5E cosmid DNA.

Table 10. Genotype testing of the transformants with X7:5E

	Minimal	Min+His+Phe	Min+His+Can
pmn:pmb:his-2	0	3.4mg	32.9mg
Transformant 1	0	28.6	2.8
Transformant 2	0	29.9	3.1
Transformant 3	0	34.9	2.2
Transformant 4	0	30.8	2.1

* 200ul of conidial suspension of *pmn:pmb:his-2* strain and each transformants was cultured in liquid conidial growth media 1) minimal N medium 2).minimal medium containing 0.5mg/ml histidine plus 5mg/ml phenylalanine 3) minimal medium containing histidine plus 1mg/ml canavanine. The filtered dried conidia were weighed out to the nearest 0.1 mg.

3.4 SUBCLONING OF THE *pmb* GENE

To localize the specific region of the *pmb* locus from the X7:5E insert, X7:5E DNA was digested into smaller size pieces with different restriction enzymes and using them in transformation procedures. Transformation efficiency with the digested DNA fragments were inspected (Table 11). The transformation efficiency with *PstI* cut DNA yielded highest and about 80% efficiency of that with uncut X7:5E DNA. The individual *PstI* cut fragments were separated, isolated and purified from agarose gels. Each fragment was cloned into pTZ18R vector and used in transformation in testing for the ability to rescue the *pmb* mutant phenotpe (Table 12). 3.2Kb *PstI* subclone had the highest transformation frequency. The transformants were further cultured in liquid media. Table 13 shows the dry weight of cultures. According to the data of Table 13 the transformants with 3.2Kb fragment shows pmb^{+} genotype indicating that 3.2Kb fragment is presumed to harbor pmb^{+} locus.

Table 11. Transformation efficiency

X7:5E	Transformants
uncut X7:5E	34
PstI cut	27
NsiI cut	23
SacI cut	18
BamHI cut	19
no DNA	4

* *pmn:pmb:his-2* spheroplasts were plated on histidine: phenylalanine=1:10 colonial medium after transformation with X7:5E DNA. The medium contains 0.5mg/ml histidine and 5mg/ml phenylalanine.

Table 12. Transformation efficiency

PstI cut X7:5E	Transformants
uncut X7:5E	39
10.0Kb fragment	5
4.5Kb fragment	3
3.2Kb fragment	18
2.5Kb fragment	2
0.8Kb fragment	1
no DNA	2

* *pmn:pmb:his-2* spheroplasts were plated on histidine: phenylalanine=1:10 colonial medium after transformation with DNA. The medium contains 0.5mg/ml histidine and 5mg/ml phenylalanine.

Table 13. Dry weight of *PstI* cut transformants

	Minimal	His+Phe	His+Can
No DNA	0	0.1mg	4.1mg
X7:5E	0	3.8	0.3
0.65Kb	0	0.2	3.9
0.70Kb	0	0.3	3.4
0.80Kb	0	0.4	3.2
3.20Kb	0	2.5	0.4
4.50Kb	0	0.5	3.3
10.00Kb	0	0.4	2.9

* The X7:5E cosmid DNA was digested with *PstI* and electrophoresed in 0.8% agarose gel overnight at 20 voltage. Each DNA band was cut, purified, recombined into pTZ18 vector. After amplification DNAs were used for transformation of *pmn:pmb:his-2* strain on histidine plus 10X phenylalanine plates. The transformants were innoculated in 25ml of liquid conidial growth media 1) minimal N medium 2).minimal medium containing 0.5mg/ml histidine plus 5mg/ml phenylalanine 3) minimal medium containing histidine plus 1mg/ml canavanine. The filtered dried conidia were weighed out to the nearest 0.1 mg.

3.5 RESTRICTION MAPPING

Restriction mapping was done by digestion of a subclone pB22-22 harboring the 3.2Kb *Pst1* fragment in pTZ18R vector with restriction enzymes whose restriction sites are located inside the polylinker of pTZ18R vector: *SacI, BamHI, HindIII, XbaI, NcoI, PvuII, PstI, ApaI, BglII, HpaI, XhoI, HindII, SmaI, SphI, SalI, KpnI, NsiI, ClaI, EcoRV, AccI,* and *EcoRI.* The 3.2 Kb insert has restriction sites of *PvuII, SacI, BamH1*, and *NcoI. Nco1* site is located about 200 bps away from *Pst1* end. The position of BamH1 restriction site is right next to *Nco1* site. Sac1 site is 340 bps away from *Pst1* end. *PvuII* restriction site is located in the right half of the insert: it is about 1470 bps away from *Sac1* site.

Figure 2. Restriction map of pB22-22 subclone

* Restriction mapping was performed in standard methods. 3.2 Kb insert has restriction sites for *PvuII, SacI, BamH1, NcoI.* There is no restriction site for the enzymes *ApaI, BglII, HpaI, XhoI, HindII, SmaI, XbaI, HindIII, SphI, SalI, KpnI, NsiI, ClaI, EcoRV, AccI*, and *EcoRI* in pB22-22 subclone insert. H:*HindIII*, Ps:*PstI*, B:*BamHI*, N:*NcoI*, S:*SacI*, Pv:*PvuII*, X:*XbaI*

3.6 NORTHERN BLOT

The rRNA (28S, 18S, and 5S) molecules are easily distinguished. The mRNA molecules are seen as a smear that extends broad range.

The pB22-22 subclone DNA fragment was labeled and hybridized to *Neurospora* poly(A)+ RNA which was previously separated by agarose gel electrophoresis and blotted onto the nylon sheets. pB22-22 hybridized to a 3.5Kb fragment on the Northern blot (Figure 3). The size of *pmb* message is presumed to be 3.5 kb.

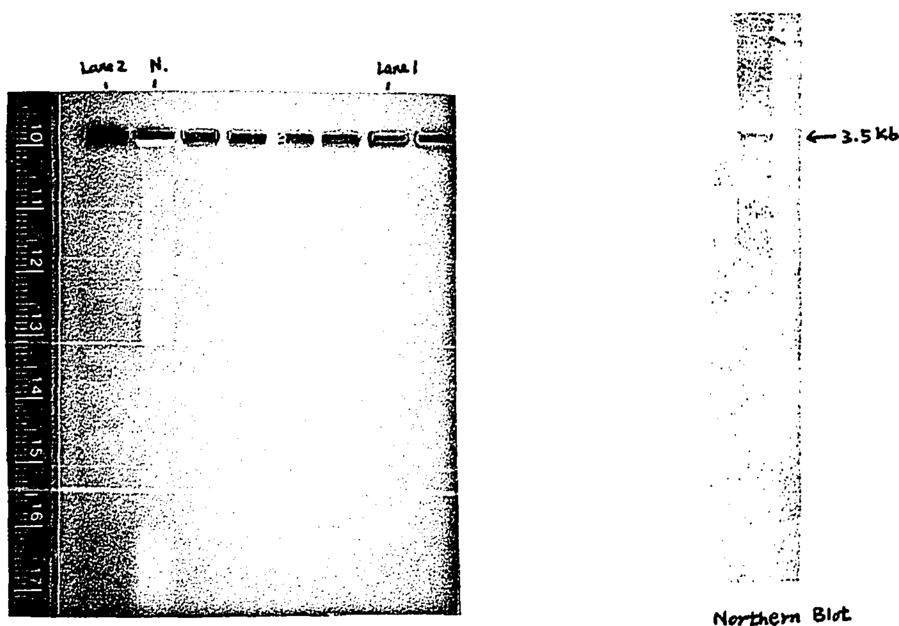


Figure 3. Total RNA obtained from *Neurospora crassa* and Northern Blot

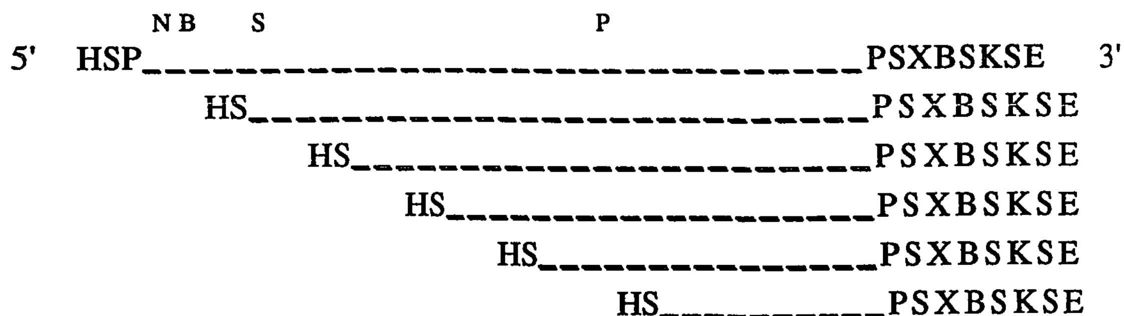
* Lane 1, RNA molecular weight markers Lane (0.24-9.5 Kb RNA ladder, GIBCO BRL). Lane 2, RNA Marker (0.3-7.4Kb, SIGMA)

3.7 SEQUENCING

3.7.1 CREATION OF DELETION SUBCLONES

The subclones containing 3.2Kb *PstI* cut insert in pTZ 18R plasmid or pTZ 19R plasmid were used for generation of nested sets of deletions with ExonucleaseIII. The ExonucleaseIII digested DNAs showed a continuous ladder with 150-200 bp intervals after separation by electrophoresis. After amplifying colonies differing in size by 150-200bp were selected for sequencing. Figure 4 and Figure 5 show the set of deletion clones that are used for sequencing.

The 3.2Kb fragment in pTZ18R vector (subclone pB22-22)



The 3.2Kb fragment in pTZ19R vector (subclone pB22-30)

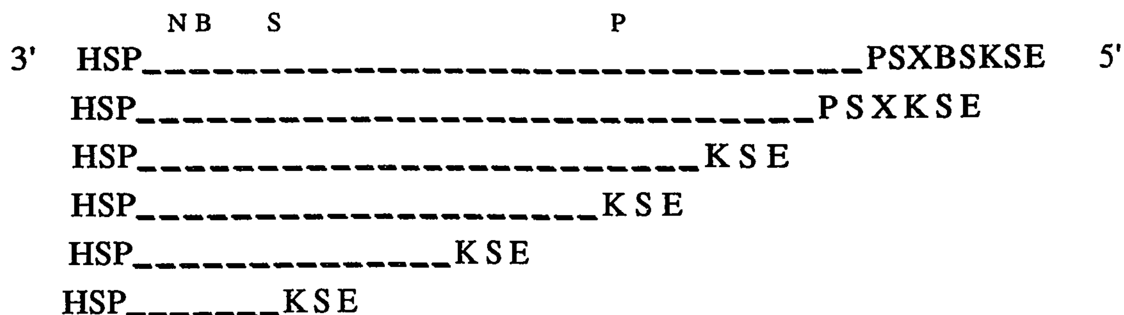


Figure 4. Nested set of deletion cloning

* 10ug of purified DNA was cut with two enzymes: for pTZ18R vector insert *NcoI* as a 5' overhang cutting enzyme and *SphI* as a 3' overhang cutting enzyme were used: for pTZ19R vector insert *XbaI* and *KpnI* were used for each cutting. The DNA solution is incubated with *ExonucleaseIII.* A mini gel was run with 2ul of each sample. The samples of same size were pooled, amplified, and colonies were selected differing in size by 150-200bp for sequencing.

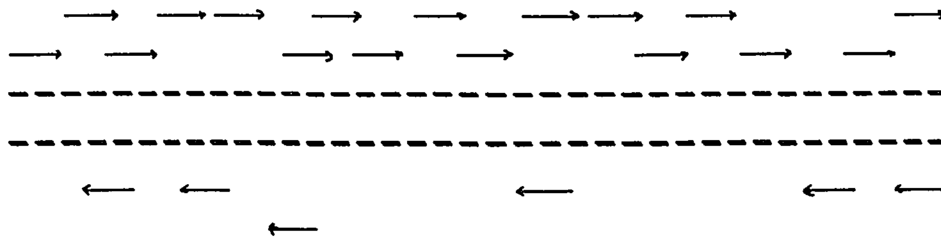


Figure 5. Sets of nested deletions used for sequencing

* Thick lines indicate double strands of 3.2Kb insert in subclones pB22-22 and pB22-30. Arrows show the positions covered by deletion clones. The size of arrows is not accurate.

3.7.2 DNA SEQUENCES

Nested deletion subclones (33 subclones) were used for sequencing of 3.2 Kb *PstI* cut insert. Figure 6 shows the partial nucleotide sequence of *pmb* locus from 3.2Kb insert, including upstream of translation start site of *pmb* locus.

```
1   /   1                                   31  /   11
acg tga ctc ata cga tag tac gat aga gtc ggc tga agg ttc gtg cta cag ccc agc ttg
thr OPA leu ile arg AMB tyr asp arg val gly OPA arg phe val leu gln pro ser leu
61  /   21                                  91  /   31
acg aag cct aca cga aat gag ata cct aca gag cta tga gga agc gcc att ttc gct att
thr lys pro thr arg asn glu ile pro thr glu leu OPA gly ser ala ile phe ala ile
121 /   41                                  151 /   51
atc tat ttg ata acg tgt acg cta tag tga tac gtc tgt acg ctg cac cag cga cga cga
ile tyr leu ile thr cys thr leu AMB OPA tyr val cys thr leu his gln arg arg arg
181 /   61                                  211 /   71
cag tca gtg aca gat gtg atc gta ttt ctc tta cgc atc tgt cgt att tca caa cgc ata
gln ser val thr asp val ile val phe leu leu arg ile cys arg ile ser gln arg ile
241 /   81                                  271 /   91
tgg tgC ACT CTC AGT ACA ATC TGC TCT GAT GCC GCA TAG TTA AGC CAC TAT ACA CTC CCT
trp cys thr leu ser thr ile cys ser asp ala ala AMB leu ser his tyr thr leu pro
301 /  101                                  331 /  111
ATC GCC AAG CGC AGG ATC GCA ACA GAG AGC GCA CGA GGC AGC TGC ACG GGG AAA CGC TGG
ile ala lys arg arg ile ala thr glu ser ala arg gly ser cys thr gly lys arg trp
361 /  121                                  391 /  131
ATC TTA TAG TCC TTC GGT TTC GCC ACT CTG ACT TGA GCG TCG ATT TTT GTG ATG CTC GCA
ile leu AMB ser phe gly phe ala thr leu thr OPA ala ser ile phe val met leu ala
421 /  141                                  451 /  151
GGG CGA GCT ATG GAA GCT AGC TTT ATT AGT ACT TCC ATG CTT TAC TCT TAC GTC TTG ACT
gly arg ala met glu ala ser phe ile ser thr ser met leu tyr ser tyr val leu thr
```

Figure 6. Nucleotide sequence and deduced amino acid sequence of *pmb* locus

*Sequencing was done by Sanger's standard chain termination method with non-radioactive material. In the promoter region there are two CAAT sites(-166, -155), one TATA motif(-123) and two CT-rich regions(-117-->-106, -50-->-23). The designated sites of restriction enzymes in *pmb* locus are *PvuII* (-73), *SacI* (1404), *BamHI* (1537), *NcoI* (1541) and *PstI* (1744).

```
481  /  161                              511  /  171
CAT CTC CCT TGC TGC TCG CAC AAT AAG ACG CAC ATG AGT CAC TCG GTG GAG TCG ATC CAT
his leu pro cys cys ser his asn lys thr his met ser his ser val glu ser ile his
541  /  181                              571  /  191
GCG CTG CAT GGT TGG GGA CGC GCT TGT CGC ACA CTT CCA TCT GAC CTT TCA GAA GGT TTC
ala leu his gly trp gly arg ala cys arg thr leu pro ser asp leu ser glu gly phe
601  /  201                              631  /  211
GTG GTG GGC AAG GAC CAA CCG GT* GCG GCC GTG CGT GGG TGC CTC GCC CGG CAC TGC CAG
val val gly lys asp gln pro --- ala ala val arg gly cys leu ala arg his cys gln
661  /  221                              691  /  231
GGC CAC TGC CGG CAT CCA ACT ATC TGC TCT GTA ACG CAT GTA GCG TTC TGT GTC ATG ATG
gly his cys arg his pro thr ile cys ser val thr his val ala phe cys val met met
721  /  241                              751  /  251
CTC T*A GGG ACT GTC ATG GGT ATG ATC GTA CAA AGT GCT CAC ATC GGT ATG ATG ATG ACA
leu --- gly thr val met gly met ile val gln ser ala his ile gly met met met thr
781  /  261                              811  /  271
TGC CGG TAC TGG ACG TGT GAG GGT AAA CAA CTG GCG GTA TGG *TG CGG CGG TGC GTC GAT
cys arg tyr trp thr cys glu gly lys gln leu ala val trp --- arg arg cys val asp
841  /  281                              871  /  291
CGG CAG ATC CTA GTC ATG CAG CGC TCG TCA GTC AGA TGT AGT AGT CCA GCA GTA GCC AGC
arg gln ile leu val met gln arg ser ser val arg cys ser ser pro ala val ala ser
901  /  301                              931  /  311
AGC ATC TCG ATG CAG ATC CGG ACG ATA ATG GAG CAG GCG CTG ACC TTC CCG TGT TCC ACG
ser ile ser met gln ile arg thr ile met glu gln ala leu thr phe pro cys ser thr
961  /  321                              991  /  331
ACT TTA CGA AAC ACG GAT CTA CGT CGT CCC TAC GTG ATT CAT TCT GCT ACA cgt agg caa
thr leu arg asn thr asp leu arg arg pro tyr val ile his ser ala thr arg arg gln
1021 /  341                              1051 /  351
ccg cag cta gcc ggt ctc acg aca aga gcg atc atg cgc aac tgc cag GAA CCC ACG CTG
pro gln leu ala gly leu thr thr arg ala ile met arg asn cys gln glu pro thr leu
1081 /  361                              1111 /  371
GCC GAG ATG *CG TGC GGT CGC TGG AGA TGG CGG AGC GAT GGA TAT GTT CTG TGT TCG CAT
ala glu met --- cys gly arg trp arg trp arg ser asp gly tyr val leu cys ser his
1141 /  381                              1171 /  391
CAC AGT CTC GAC ATG ATT GCT CCA ATT CTT GAT GTA ATC GTT ACG CGC TTC CAT TCA GGT
his ser leu asp met ile ala pro ile leu asp val ile val thr arg phe his ser gly
1201 /  401                              1231 /  411
CGA GGT GGC CCG GCT CCA TGC ACC TTC CAG CTC GGT AAG GCA CCC CGC CAG CCT AGC CGG
arg gly gly pro ala pro cys thr phe gln leu gly lys ala pro arg gln pro ser arg
1261 /  421                              1291 /  431
TTC CTC ACG AAC ACC AGC ACG ATC ATG CCG CCA CCC GTG CCA GGA CCA ACG CTG CCG AGA
phe leu thr asn thr ser thr ile met pro pro pro val pro gly pro thr leu pro arg
```

Figure 6. (continued) Nucleotide sequence and deduced amino acid sequence of *pmb* locus

```
1321 /  441                                  1351 /  451
TGC GCT CGT GAC GAC TGC TGG GCA GAT GGC GAC GGA TGG ATA TGT CTC AGG TGT GCG CAT
cys ala arg asp asp cys trp ala asp gly asp gly trp ile cys leu arg cys ala his
1381 /  461                                  1411 /  471
CAC GAG TCT CGA CAT GAT GCT CAT CTG ACT GTG ATC GTA GCA GTC CTC ATA GCA TTA AAT
his glu ser arg his asp ala his leu ser val ile val ala val leu ile ala leu asn
1441 /  481                                  1471 /  491
CGC CGT GAC GAT CAG CGT CCA ATG ATC GAA GTT AGG CTG GTA AGA GCC GGA GCG AAT CCT
arg arg asp asp gln arg pro met ile glu val arg leu val arg ala gly ala asn pro
1501 /  501                                  1531 /  511
GGA AGC TGT CCT GAT GGT CGT CAC TAC CTG CCT GGA CAC CAT GCC TGC ACG GGC ATC CGA
gly ser cys pro asp gly arg his tyr leu pro gly his his ala cys thr gly ile arg
1561 /  521                                  1591 /  531
TGC GCG AGC GAG AGA TCA TAT GGA GCA ACG AGA GAT CAT ATG GAG CAT CAG CTC CGT CGC
cys ala ser glu arg ser tyr gly ala thr arg asp his met glu his gln leu arg arg
1621 /  541                                  1651 /  551
GGA GTG CTG GAG CTG CTC AGC CAT CCC ATC GAT GTG ATG GTA ACG TTC TTT GTG GGC CGT
gly val leu glu leu leu ser his pro ile asp val met val thr phe phe val gly arg
1681 /  561                                  1711 /  571
TGC *AT TTG GGG ATC GTC TTT CCG CCC TTG GTT CTG TCT CCG TGC CTG TCC TCC AGA CCT
cys --- leu gly ile val phe pro pro leu val leu ser pro cys leu ser ser arg arg
1741 /  581                                  1771 /  591
CCA TTC TTG TGT GCT CCA TCA CGT GA* CTC GTC TCC ATT GGC TCT TGG CAA ATC GGC AGC
pro phe leu cys ala pro ser arg --- leu val ser ile gly ser trp gln ile gly ser
1801 /  601                                  1831 /  611
GGG GCT GAA TGG TCG AGC TCT ACA GAA TAC CGC GCG CAT CCA TAT GTT AGT TCT GCA ATT
gly ala glu trp ser ser ser thr glu tyr arg ala his pro tyr val ser ser ala ile
1861 /  621                                  1891 /  631
TTC TTG TAT CGG TGC TGT GAC TCA TAC TCC CCC TTT GGC TGG CCT TGC GCA CCA ATA AGA
phe leu tyr arg cys cys asp ser tyr ser pro phe gly trp pro cys ala pro ile arg
1921 /  641                                  1951 /  651
ACG CAC AGT GAA ATC TGC GGT GGG AGT GGA TCC ATG GGC TCC ATG CTG GGA CGG CAC TCT
thr his ser glu ile cys gly gly ser gly ser met gly ser met leu gly arg his ser
1981 /  661                                  2011 /  671
CGA CAT CAT CTC ACC TTC AGA GGA ACT CGG ATG GAT GAT CCA TGC GCT CCA TGC TGG TAC
arg his his leu thr phe arg gly thr arg met asp asp pro cys ala pro cys trp tyr
2041 /  681                                  2071 /  691
TGC TGC ACT GTC GCA CAC TTC CAT CTG ACC TTC AGA AGG TTC GTG TGG CAA GGA CCA ACC
cys cys thr val ala his phe his leu thr phe arg arg phe val trp gln gly pro thr
2101 /  701                                  2131 /  711
GGT CGC GGC GTC GTG GTG CCT TCG CCC GGC ACT GCC AGG GCC ACT GCA CCC ATG
gly arg gly val val val pro ser pro gly thr ala arg ala thr ala pro met
```

Figure 6. (continued) Nucleotide sequence and deduced amino acid sequence of *pmb* locus

3.7.3 SEQUENCE ANALYSIS

Analysis of sequence data and amino acid sequence prediction were performed with the DNA strider™ 1.0 program written by Christian Marck. Nucleotide sequence comparison was done with Wisconsin program in GenEMBL (European Molecular Biology Laboratory) Data Library. Computer graphics visualization of deduced secondary structure of the polypeptide was made using TEKPLT01 software package.

Full restriction map of partial *pmb* sequence is in Appendix. Enzyme names are listed above the double stranded DNA sequence with the positions of the recognition sequence listed below symetrically.

3.7.3.1 Initiation of transcription

The *pmb* promoter sequence has the promoter elements conserved in higher eukaryotic promoter regions, CAAT, TATA and in fungi the CT block. The promoter region of *pmb* gene is identified to locate in the middle of the subclone. An open reading frame extends 1.7Kb to the end of the subclone, indicating that the subclone contains a partial sequence of the *pmb* gene. Because the transcriptional start point (tsp) has not been determined, positions are given in base pairs (bp) from the A(+1) of the translational start. The promoter region has two CAAT motif at positions -166-->-163 and -155-->-152, one TATA box at -123-->-120, and two CT rich pyrimidine blocks. One CT block is located at -117-->-106 (12bp

length) and contains 83% pyrimidine bases. The other one is at -50-->-23 (28bp length) and contains 75% pyrimidine bases.

3.7.3.2 Initiation and termination of translation

The -3 position in *pmb* sequence is G: at which a purine is nearly always present (97%) in filamentous fungi (Kozak, 1987).

The 1.7Kb sequence from the *pmb* promoter site to the end of the clone contains a long open reading frame and no significant polyadenylation signal is present in the sequence, indicating that the *pmb* locus extends beyond pB22-22 subclone insert.

3.7.3.3 Consensus sequences

Nucleotide sequence was entered into computer data bases so that homologous regions or controlling site similarities could be picked up from a variety of sequences. Table 14 shows sizes and identity rates of consensus sequences between the *pmb* sequence and other gene sequences. The overlapping size ranges from 30bp to 260bp and the identity rate is from 55% to 94%. *N. crassa cpc-1* gene encodes a transcriptional activator that is the major cross-pathway control regulator of amino acid biosynthetic genes. The overlapping size between *pmb* sequence and *cpc-1* sequence is 186bp and the homology between them is 89.2%.

Table 14. Consensus sequences

pmb sequence	compared genes	size of overlap
120-220(R)	Streptomyces *Rnase P*	101 bp (58.4%)
140-200	*N. crassa qa-2*	52 (94.2)
150-260	Mouse GDP-1	115 (60)
160-200(R)	Human *mRNA*	31 (74.2)
290-320(R)	*O. cuniculus mRNA*	32 (81.3)
300-450	*E. coli osmC*	154 (55.8)
500-540	*E. coli sucB*	35 (77.1)
660-720(R)	*X. laevis SeCys-tRNA*	56 (82.1)
660-900(R)	Human Cytomegalovirus	260 (56.9)
780-860	Mouse integ. site for CMV	59 (84.7)
1240-1320	Mycobacterium AT103	70 (65.7)
1380-1430(R)	Schistosoma mansoni GST	50 (68)
1400-1585	*N. crassa cpc-1*	186 (89.2)
1510-1630(R)	Mouse amelogenin	131 (60.3)

* Nucleotide sequence comparison was done with the Wisconsin program by using GenEMBL (European Molecular Biology Laboratory) Data Library. The numbers in the first row indicate the locations of consensus sequence in *pmb* locus. The percentages in the third row indicate the identity of consensus sequences. R: reverse strand

3.7.3.4 Secondary structure

Computer graphics visualization of deduced secondary structure of the polypeptide of the partial PMB protein was made using TEKPLT01 software package (Figure 7). The two-dimensional conformation of a protein molecule is determined using its amino acid sequences by predicting alpha helices, beta strands and turns of each sequences separately by two standard prediction algorithms and averaged at homologous sequences' position.

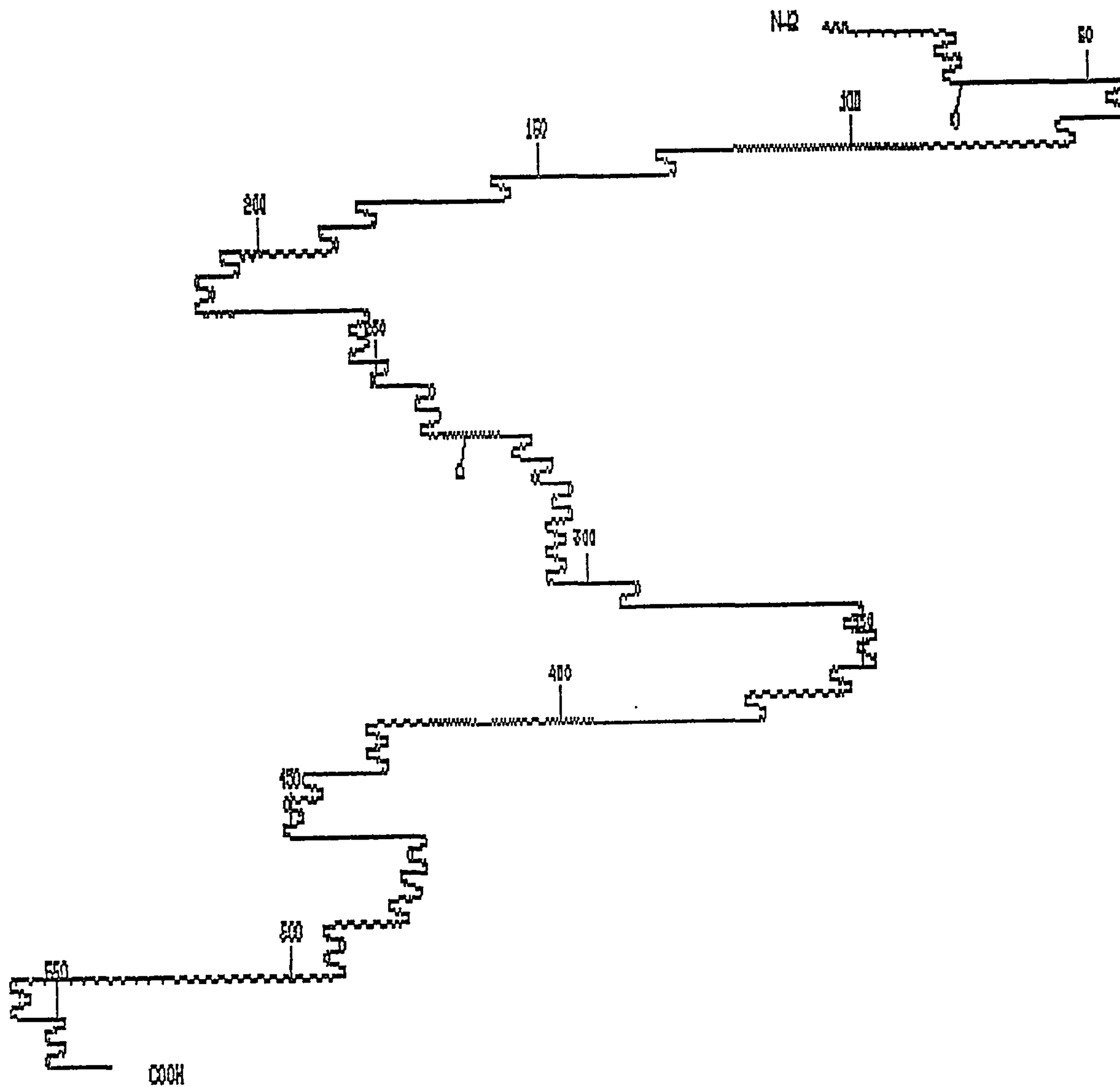


Figure 7. Secondary structure of a portion of PMB protein

* Deduced amino acid sequence from 1.7 Kb nucleotide sequence was inputted for getting secondary structure using TEKPLT01 program. The arrow indicates the location of consensus sequence with CPC-1 protein.

3.8 OVERLAPPING CLONES

3.8.1 RESTRICTION MAPPING AROUND THE pB22-22 SUBCLONE

The X7:5E cosmid insert DNA was mapped by digestion with restriction enzymes followed by separation of the DNA fragments by gel electrophoresis. DNA was transferred to a membrane by the Southern blotting procedure and hybridized with the labelled pB22-22 subclone as a probe. Table 15 shows the sizes of bands detected in hybridization experiments with the *pmb* subclone. According to the data of Table 15, a preliminary restriction map around the pB22-22 subclone insert was constructed (Figure 8).

Table 15. DNA fragments

Enzymes	Detected bands (Kb)	
Pst1	3.20	
BamH1	1.80	3.65
Sal1	13.00	
Sac1	3.85	19.00
EcoR1	4.20	
Nsi1	15.00	
Nco1	4.40	5.30
Nsi1 +Sac1	2.00	3.85
Nsi1 +Pst1	3.20	
BamH1 +Sac1	1.80	3.52
EcoR1 +Pst1	3.20	
BamH1 +Sal1	1.80	3.65
Pst1 +Sal1	3.20	
BamH1 +Nsi1	1.80	3.65
Nsi1 +Sal1	7.00	
EcoR1 +Sal1	4.20	
Pst1 +Sac1	2.86	
EcoR1 +Sac1	3.57	
BamH1 +Pst1	3.00	
EcoR1 +Nsi1	4.20	
Nco1 +Sac1	3.85	4.40

* X7:5E cosmid DNA was digested with *Pst1, BamH1, Sal1, Sac1, EcoR1, Nsi1, Nco1, Nsi1 +Sac1, Nsi1 +Pst1, BamH1 +Sac1, EcoR1 +Pst1, BamH1 +Sal1, Pst1 +Sal1, BamH1 +Nsi1, Nsi1 +Sal1, EcoR1 +Sal1, Pst1 +Sac1, EcoR1 +Sac1, BamH1 +Pst1, EcoR1 +Nsi1*, and *Nco1 +Sac1*. Separated DNA fragments were transferred and hybridized with a labelled pB22-22 probe. Sizes of detected bands were estimated relative to *Hind III* lambda DNA size markers.

```
     Sac    Sal     Nco      Nsi B   E   NB Sac     Pvu          BE   Sac     Sal   Nco          Nsi
-------------------------------------P_______________P---------------------------
          11     4.46     2.54 .12 1.3.29 21     1.48       1.38 .66   .28  1.15   .16         8
```

Figure 8. Restriction map of X7:5E

* Preliminary restriction maps constructed according to data from Table 15.

3.8.2 OVERLAPPING SUBCLONES

A 1.8Kb *BamH1* fragment partially overlaps a 3.2 Kb *Pst1* fragment and extends downstream to the *pmb* locus. It was inserted into the pTZ18R vector and used for transformation of *E. coli* competent cells. Insert bearing colonies were picked on blue-white screening plates and cultured in 2XYT ampicillin media. The plasmid DNAs were extracted, cut with *BamH1* and DNA fragments were separated by gel electrophoresis. The DNA fragments were transferred by Southern blotting and hybridized with the 1.82Kb *Pst1 +PvuII* fragment as a probe to confirm that the clones have an insert of the 1.8Kb *BamH1* fragment in the pTZ18R vector. The detected bands on the lanes of pB-4C, pB-6A and pB-12B clones were sized at 1.8Kb. Therefore, the pB-4C, pB-6A and pB-12B clones were

identified as overlapping clones which contain the 1.8Kb *BamH1* fragment insert in pTZ18R and can be used for further sequencing of the *pmb* locus.

3.8.3 THE EXTENT OF THE *pmb* LOCUS

Transformation of *pmn:pmb:his-2* spheroplasts with X7:5E DNA, a 13Kb *Sal1* fragment, and a 3.2Kb *Pst1* fragment was performed to identify the extent of the *pmb* locus in the X7:5E cosmid insert. 0.5ug of DNA was used for transformation with X7:5E. For transformation with the 13Kb *Sal1* fragment and the 3.2 Kb *Pst1* fragment, 1ug each of X7:5E DNA was used for fragment purification. Table 16 shows the number of colonies counted on Histidine plus 10X phenylalanine plates in four days after transformation. The data suggests that the 13Kb *Sal1* DNA fragment contains the whole *pmb* locus. On the other hand, the 3.2Kb *Pst1* DNA fragment contains only a part of the *pmb* locus.

Table 16. Transformation efficiency

DNAs	Transformants
X7:5E	63
SalI 13Kb	69
PstI 3.2Kb	24
No DNA	3

* Transformation of *pmn:pmb:his-2* strain was done with X7:5E cosmid DNA, *SalI* cutting 13Kb DNA fragments, and *PstI* cutting 3.2Kb DNA fragments. The transformation efficiency of 13Kb sequence is equal to that of X7:5E DNA. On the other hand, the transformation efficiency of3.2Kb sequence is much lower (38%) than that of X7:5E DNA, indicating that 13Kb sequence contains the whole *pmb* locus and 3.2Kb DNA contains a portion of *pmb* locus.

CHAPTER 4
DISCUSSION

4.1 CHARACTERIZATION OF THE *pmb* GENE

A large number of genes of filamentous fungi have now been cloned and information on their primary structure is accumulating. The most extensively studied in this respect are *Neurospora crassa* and *Aspergillus nidulans*. In general, the characteristics of filamentous fungal genes seem very similar. A preliminary determination of the DNA sequence of the *pmb* gene and an analysis of the genomic DNA sequence was conducted in order to predict the primary amino acid sequence of the gene products and to elucidate the primary structure of the proteins.

4.1.1 INITIAL SCREENING FOR THE *pmb* GENE

It is known that there are three constitutive amino acid transport systems in *Neurospora* (Wolfinbarger, 1980; DeBusk, Ogilvie, 1984). Among them, the basic amino acid transport system (B system) is under the genetic control of the *pmb* gene. The basic amino acid transport system is specific for transport of arginine, lysine, histidine and the arginine analogue canavanine sulphate.

A strain mutant at both the *pmb* locus and the *his*-2 locus was constructed by crossing a *pmb*- strain to a *pmn: his*-2 strain. Selection was accomplished by plating the progeny spores on medium containing histidine and the arginine analogue canavanine.

Canavanine is toxic to the cell by incorporating in the protein synthesis instead of arginine and disrupting the protein structure. One selected strain with the genotype *pmn:pmb:his-2* was used as the host strain in transformation experiments.

DNA from a wild type *Neurospora* genomic library was used in transformation experiments as a source of exotrophic DNA. These libraries were composed of 50 microtiter plates, and each plate contains 96 different cosmid clones. Pools of DNA(50) were tested for their overall frequencies in transformation. The positively transforming pools were divided into 12 subpools of 8 cosmids and the transformation was repeated. Finally subpools which demonstrated complementation of the *pmb* mutation were divided into the individual cosmids and the transformation was repeated a third time. This procedure identified 14 cosmid clones which were able to transform the host strain.

4.1.2 LINKAGE ASSIGNMENTS

The *pmb* gene is known to be located on the right arm of linkage group IV (Metzenberg, Stevens, Selker, Morzycka-Wroblewska, 1984). We used two RFLP mapping reference sets to confirm the linkage of the putative clones identified as described in the previous section. The RFLP segregation pattern of the cosmid clone X7:5E using the first reference set ("18") shows that it is tightly linked to the genes *nit-3* and *Fsr-4* on linkage group IV. Using the second reference set ("38"), the segregation pattern of the X7:5E DNA shows

that it is tightly linked to *Fsr-4*, also on linkage group IV. Based on the 18- and 38-segregants RFLP patterns, the X7:5E clone was confirmed to map to the *pmb* locus on linkage group IV. Several other clones also appeared to complement the *pmb* mutation. The transformation efficiencies of the G21:9D, X11:12A, and X23:9C cosmids were as high as the X7:5E cosmid. However, these clones did not map to linkage group IV. G21:9D maps to the *arg-12* locus on LGII, one of the genes of arginine biosynthetic pathway. Cosmid clones X11:12A and X23:9C map to the *Bml* (Benomyl resistant gene) locus on LGVI. The *Bml* locus is tightly linked to the *cpc-1* locus on LGVI.

Because of the correct linkage relationship of X7:5E, further work was done on this clone. The transformants derived from the X7:5E cosmid DNA were cultured in liquid media. The dry weight of strains showed that the *pmn:pmb:his-2* strain grows fully in a medium containing histidine and canavanine, but does not grow well (1.3%) in a medium containing histidine and 10X phenylalanine because the excess phenylalanine in the medium blocks histidine uptake via the G system. These phenotypes result from the fact that histidine is transported via all three major amino acid transport systems (the B, the N, and the G system) and phenylalanine is transported by the N and the G system (Wolfinger, DeBusk, 1971). On the other hand, strains transformed with X7:5E grew fully in a histidine plus 10X phenylalanine medium and did not grow well in a histidine plus canavanine medium, suggesting that the transformed strain is pmb^{+}.

This strongly suggests that the X7:5E cosmid contains the wild type pmb gene.

4.1.3 FINE SCALE LOCALIZATION OF THE *pmb* GENE

The X7:5E cosmid has a 40Kb insert. To localize the specific region containing the *pmb* gene within the X7:5E insert, this cosmid DNA was digested into smaller size pieces with different restriction enzymes. These were used in transformation procedures. The transformation efficiency with *PstI* cut DNA yielded the highest success rate (80%). Among the individual *PstI* cut fragments, a 3.2Kb fragment showed the most positive transformation efficiency. Stable transformants derived from the 3.2Kb fragment showed the *pmb*$^+$ phenotype in liquid culture, indicating that the 3.2Kb fragment is presumed to harbor the *pmb*$^+$ locus. This 3.2Kb fragment also hybridized to transcripts 3.5Kb in size on a Northern Blot, indicating that it makes an mRNA transcript.

The 3.2Kb fragment DNA was cloned using pTZvectors. These vectors were also used for the construction of nested sets of deletions using ExonucleaseIII. The deletion clones were used for DNA sequencing. Analysis of DNA sequence data and amino acid sequence predictions were performed with the DNA strider™ 1.0 program written by Christian Marck. A 1.7Kb sequence extending from the putative *pmb* promoter site to the end of the clone contains a long open reading frame. Also both the transcriptional termination and polyadenylation signals required for cleavage in most fungal genes

were not seen in this region. This, in conjunction with the Northern Blot data indicates that the *pmb* locus extends beyond the 1.7Kb sequenced region.

A 1.8Kb *BamH1* fragment partially overlaps the 3.2 Kb *Pst1* fragment (about 200bp) and extends further downstream. This was also cloned as an insert in the pTZ18R vector as an overlapping clone which can be used for further sequencing of the *pmb* locus.

4.1.4 *pmb* LOCUS GENE STRUCTURE

Sequences close to the AUG translational initiation triplet codon are very conserved in filamentous fungi. The most important position would appear to be the -3 position, at which a purine is nearly always present (97%) (Kozak, 1987). In the case of *pmb* sequence, the -3 position is G.

The fungal genes also show little bias in their use of stop codons (UAA 42%, UAG 28%, UGA 30%) and like other eukaryotes they have a preference (70%) for a purine at the position following the stop codon. Filamentous fungal mRNAs tend to have heterogeneous 3' ends with several apparent polyadenylation sites for cleavage of the primary transcript. The higher eukaryotic polyadenylation signal AATAAA is seen in an intact or degenerate form preceding polyadenylation sites in fungal genes.

The 1.7Kb sequence extending from *pmb* promoter site is a long open reading frame. Both the transcriptional termination and polyadenylation signals required for cleavage in most fungal genes is

not seen in this region of the *pmb* sequence, suggesting that the *pmb* locus extends beyond the 1.7Kb pB22-22 subcloned insert.

Hybridization of labeled DNA to RNA is a strong indication that the DNA corresponds to a gene producing mRNA to be translated into a protein. It also can identify the possible gene product size. To determine that the *pmb* gene codes for a messenger RNA, the DNA fragment in subclone pB22-22 was labeled and hybridized to *Neurospora* poly(A)+ RNA. The size of a putative *pmb* message detected and assessed by a Northern blot is 3.5Kb, suggesting that the size of *pmb* locus is at least 3.5Kb.

Restriction mapping around the pB22-22 subclone (Figure 8) shows that the 13Kb *SalI* fragment encompasses the 3.2Kb *PstI* fragment, extending 8.7Kb further from the end of the 3.2Kb *PstI* fragment. Transformations of *pmn:pmb:his-2* spheroplasts with the X7:5E DNA, the 13Kb *SalI* fragment, and the 3.2Kb *PstI* fragment were performed to identify the extent of the *pmb* locus in the X7:5E cosmid insert. According to the data in Table 16, the transformation efficiency of the 13Kb sequence is equal to that of X7:5E DNA. On the other hand, the transformation efficiency of the 3.2Kb sequence is much lower (38%) than that of the X7:5E DNA, suggesting that the 13Kb *SalI* DNA sequence contains the whole *pmb* locus and the 3.2Kb *PstI* DNA fragment contains a partial *pmb* locus.

4.1.5 PROMOTER SEQUENCE OF THE *pmb* GENE

Initiation of transcription and its regulation have been extensively studied in many organisms, and this is also true for filamentous fungi. The promoter regions of eukaryotes can be described as being composed of the promoter elements, CAAT, TATA and in fungi the CT block, which act as the basic transcriptional signals to determine the start site and efficiency of transcription. Sequences conserved in higher eukaryotic promoters, CAAT and TATA boxes are sometimes evident in filamentous fungal 5'-nontranscribed regions, but there are many instances where they are absent, though functional equivalents may be present.

In higher eukaryotes the conserved sequence GC(C/T)CAATCT is often found around 80bp upstream from the tsp (transcription start points). In the filamentous fungi, sequences similar to this consensus lie 100-200bp from the tsp but some, such as those of *A. oryzae amy* and *T. reesei pgk*, are more than 300bp distant. The conserved sequence TATAAA common in eukaryotic promoters is thought to be involved in binding of a component of the general transcription machinery. Similar motifs can be found in most of the fungal genes. Most fungal TATA motifs are found 50-150bp from the translation initiation site and 40-100bp from the tsp. Pyrimidine-rich tracts or CT-rich regions have been described in the promoters of yeast and filamentous fungal genes often immediately before the major tsp. Deletion analysis of CT boxes in *A. nidulans gpdA* and *oliC* genes has shown them to be important for determining the position of

transcription initiation. CT motifs from 10 to 60bp (average 20bp) can be found in most genes, but some of the positions of these tracts have been shown to occur immediately upstream from the tsp. However, CT-rich regions can also occur between the tsp and translation start as in the amylase genes of *A. niger* or between the CAAT and TATA as in *glaA* of *A. shirousami* or even further upstream as in *A. niger ald A*. The significance of these CT block is therefore unclear.

The characteristics of the promoter region of the *pmb* locus can be described as typical. The promoter region of the *pmb* sequence has two CAAT motifs (-166, -155 site each) and one TATA box at -123 when the positions are given in base pairs(bp) from the A(+1) of the putative translational start. There are two CT rich pyrimidine blocks (-117-->-106, -50-->-23) containing 83% of and 75% of pyrimidine bases each.

4.1.6 CONTROL SEQUENCES

Nucleotide sequence comparisons were done using the Wisconsin program in GenEMBL (European Molecular Biology Laboratory) Data Library. Among homologies found between the *pmb* sequence and other gene sequences, the *cpc-1* gene has significant sequence homology. The overlap between the *pmb* sequence and the *cpc-1* sequence is 186bp, and the DNA sequence homology between them presents 89.2% identical nucleotides. The *Neurospora* cross-pathway control *cpc-1* gene is regulated in response to amino acid starvation

and encodes a transcriptional activator that is the major cross-pathway control regulator of amino acid biosynthetic genes. The CPC1 protein binds most avidly to the DNA fragment containing the hexanucleotide sequence 5'-TGACTC-3'. There are 5'-TGACTC-3' sequences at both upstream (-408-->-403) and downtream (+65-->+70) of the translation start site of *pmb* sequence, suggesting that *cpc-1* may be involved in the regulation of *pmb* gene expression. The strong homology in the extensive region may implicate that these homologous regions are also related to regulatory function of the CPC-1 protein in interaction with the *pmb* sequence.

4.1.7 FUTURE WORK

4.1.7.1 Further sequencing of *pmb* locus

Inside the 13Kb *Sal1* fragment, a 1.8Kb *BamH1* fragment partially overlaps a 3.2 Kb *Pst1* fragment (about 200bp) and extends downstream to the *pmb* locus (Figure 9, restriction map around the pB22-22 subclone). The 1.8Kb *BamH1* fragment was inserted into the pTZ18R vector. The overlapping clones pB-4C, pB-6A and pB-12B were identified to contain the 1.8Kb *BamH1* fragment insert in the pTZ18R vector and can be used for further sequencing of the *pmb* locus. The presence of both a transcriptional termination stop codon and polyadenylation signal AATAAA required for cleavage of the primary transcript preceeding polyadenylation sites will be searched for in this sequence to confirm that we have cloned and sequenced the entire *pmb* gene.

4.1.7.2 The transcriptional start point of the *pmb* gene

Relatively few transcriptional start points have been determined for filamentous fungal genes, but there are frequently a number of possible transcription initiation points, often spread over some distance. There is no evidence of preference for the sequences around these start points. However, in many instances the sites of initiation are in regions that are rich in pyrimidines. Preliminary deletion analysis of the *A. nidulans trpC* promoter has indicated that the TATA box and the pyrimidine-rich tracts may be important in positioning the sites of transcription initiation by the ribosomal subunits (Hamer and Timberlake, 1987). As with mammalian transcripts, any base can be used as a transcript cap site for filamentous fungal genes with a preference of A>U>G>C. The 5' nontranslated mRNA may range from 5bp (*A. niger trpC*) to 305bp (*P.blakesleeanus leu1*) but more commonly is around 30-70bp.

The precise site of initiation of transcription of *pmb* gene can be located by S1 nuclease mapping. This method involves hybridization of RNA with a labelled DNA probe and the subsequent digestion of the overhanging single-stranded DNA with S1 nuclease. The total unlabeled mRNA from a cell can be hybridized to a pure labeled DNA sample that includes all or part of the region of DNA that is transcribed to produce one particular mRNA. The labeled DNA-RNA hybrid is then digested with S1, which removes unpaired nucleic acid strands. The reaction products can be electrophoresed on a DNA sequencing gel alongside a DNA sequencing ladder. The size of the

DNA protected from S1 is then determined from the known size of the sequencing reaction products and, as the 5' end of the DNA has not been degraded, the extent of hybridization to the mRNA is known. An alternative method of mapping the 5' ends of mRNA molecules is primer extension. A synthetic oligonucleotide primer is hybridized to the RNA, is then used together with reverse transcriptase to prime synthesis of a complementary DNA molecule which will extend until it reaches the end of the RNA template. If the same oligonucleotide is used to prime a DNA sequencing reaction which runs alongside the primer-extended fragment on a denaturing acrylamide gel, the position of the base at the 5' end of the mRNA molecule can be read directly from the DNA sequencing reaction.

4.1.7.3 Intervening sequence(s) of the *pmb* gene

The majority of filamentous fungal genes sequenced thus far (68% figured by Gurr et al) contain intervening sequencs, or introns and the sequences at the 5' and 3' splice junctions of introns are highly conserved. The average length is 70bp, with a range in size from 36bp (*A. niger niaD* intron1) to 256bp (*A. niger pacA* intron2), though most are less than 100 bp. All the introns are positioned within the coding sequences of the genes with the exception of the first intron of *T. reesei egl3* which occurs in the 5' non-translated region. Sequences present at the 5' and 3' splice sites are highly conserved and are similar to those observed in yeast (Langford and Gallwitz, 1983) and in higher eukaryotes (Mount, 1982). In addition,

a conserved internal site for lariat formation similar to the yeast conserved internal lariat sequence TACTAAC (Langford and Gallwitz, 1983) is present. According to the data from Orbach et. al. (1986), the consensus sequence at 5' intron splice site in *Neurospora crassa* is GTA(A/C)GT and the consensus sequence at 3' splice sites is CAG. The consensus internal lariat formation sequence is (A/G)CT(A/G)AC(A/T) which is on average 19 bp away from the 3' end of the intron.

The proposed open reading frame of the *pmb* gene may contain one possible intron site at position from +599 to +654 (56bp length). This contains the sequence GTAGGC at 5' intron splice site , the sequence CAG at the 3' intron splice site and the internal lariat formation sequence TCTCACG. Since this does not exactly match the consensus sequences required for accurate intron excision in nuclear genes of *N. crassa* , further study with cDNA cloning and S1 nuclease mapping work will be necessary to determine presence of an intron in this region.

4.2 CLONES THAT COMPLEMENT *pmb* MUTANT PHENOTYPES

Confirmation of clone identity usually involves demonstration that the mutant phenotype is complemented by the cloned fragment to give the expected wild-type phenotype and that the cloned DNA fragment is exclusively linked to the target locus. Table 5 and Table 9 show the cosmid clones which somehow complement *pmb* mutant

phenotypes and yet are not linked to *pmb* locus by RFLP analysis. Among them the G21:9D, X11:12A, and X23:9C cosmids are of interest since their transformation efficiencies are as high as the X7:5E cosmid containing the *pmb* locus. G21:9D cosmid maps *arg-12* locus on LGII: *arg-12* designates one of the genes of arginine biosynthetic pathway. X11:12A and X23:9C cosmids map *Bml* (Benomyl resistant gene) locus on LGVI and the *Bml* locus is tightly linked to *cpc-1* locus on LGVI.

This phenomenon may be explained in several ways. Mechanistically it is generally assumed that translocation of a substrate amino acid across the plasma membrane involves a variety of molecular components (proteins or glycoproteins) which may or may not be directly involved in binding with the transported molecule. Therefore gene products may not interact directly, but may be somewhat functionally related and may affect the phenotype by changing the physiological condition of the cell, perhaps through more efficient transport. For example, one might consider the possibility that binding proteins, rather than being involved in the actual translocation process, may serve to increase the effective concentration of substrate molecules within a restricted binding sphere of a mobile membrane carrier molecule which has a rather low affinity for that substrate molecule. Once the substrate was bound, the membrane carrier would shift orientation towards the membrane interior where, due to its low affinity for the substrate molecule, the latter would simply diffuse into the cell interior. Such

a mechanism could help explain the seemingly rather loose association of the binding protein with the membrane, and provide a convenient means of altering specificity of a given translocation mechanism simply by changing the associated binding proteins, and permit the isolation of a mutant or mutants reduced in transport activity of multiple transport systems.

Another possible explanation for the phenomenon is expression suppressors of various sorts which may be cloned instead of the gene itself. For example, Mann et al. (1988, 1989) inadvertently cloned the *N. crassa pho-4*$^{+}$ gene, encoding a phosphate-repressible phosphate permease, because of the ability of that gene to suppress a mutation in the *nuc-1* regulatory gene. Incubation of conidia with ribonuclease (RNase A) reduced the initial rate of amino acid transport, indicating that glycoprotein subunits of amino acid transport systems in *Neurospora* are sensitive to RNase A. This result suggested that *Neurospora* transport systems are composed, in part, of ribonucleoprotein (RNP) particles or there is a regulatory molecule such as a suppressor of transcription(18, 77).

4.3 CONSENSUS SEQUENCE OF *pmb* LOCUS WITH *cpc-1* SEQUENCE (17, 44)

Computer data base analysis shows consensus sequences picked up from a variety of sequences. Those sequences could indicate homologous regions or controlling site similarities. Table 14 shows

sizes and identity rates of consensus sequences between *pmb* and other gene sequences. Among consensus sequences in the data, the *cpc-1* gene has a significant sequence homology. The overlapping size between *pmb* and *cpc-1* is 186bp and the DNA sequence homology between them presents 89.2% identical nucleotides.

In *N. crassa, Saccharomyces cerevisiae*, and *Aspergillus nidulans* starvation for single amino acid leads to increased synthesis of enzymes for many amino acid biosynthetic pathways. In *Saccharomyces cerevisiae*, this response is termed general control resulting in increased synthesis of GCN4, the polypeptide products of the gene *GCN4*. GCN4 then binds to its specific recognition sequences preceding each regulated gene and activates transcription. In *N. crassa* this global activation of amino acid biosynthetic genes in response to amino acid starvation is referred to as cross-pathway control, or general amino acid control. *N. crassa cpc-1* gene is transcriptionally regulated in response to amino acid starvation and encodes a transcriptional activator that is the major cross-pathway control regulator of amino acid biosynthetic genes. CPC-1 protein binds to target sites in the flanking regions of the associated genes regulated by cross-pathway control and stimulates their transcription. Sequence identity between CPC-1 DNA-binding protein and GCN4 was most pronounced in the DNA-binding and transcriptional activation domains of GCN4. Preliminary DNA sequence analysis of *cpc-1* DNA indicates that it likely encodes cytoplasmic phenylalanyl tRNA synthetase and the hypothesis is that

uncharged tRNA is the signal for the induction of cross-pathway control and cpc-1 expression. It has been shown that the levels of as many as 20% of the total detectable mRNA species in *N. crassa* are influenced directly or indirectly by the presence of a functional *cpc-1* gene in amino acid-starved mycelium. This differential expression of *cpc-1* may also indicate that CPC1 protein plays other roles in addition to activation of genes for amino acid biosynthesis.

The two domains most critical to CPC-1 protein function are its DNA-binding domain and transcription activation domain. CPC1 bound most avidly to the DNA fragment containing the hexanucleotide sequence 5'-TGACTC-3'. There is an exact copy of this hexanucleotide upstream of the *cpc-1* tsp. TGACTC or related sequences are found flanking regions of *Neurospora* genes under cross-pathway control, *arg-2, trp-1, trp-3*, and *his-3.* These sites are located both upstream and downstream of the transcription start sites for these genes. There are 5'-TGACTC-3' sequences both upstream (-408-->-403) and downtream (+65-->+70) of the translation start site of *pmb* sequence, which are required for the positive regulation of the expression under cross-pathway control. Considered together, these data suggest that *cpc-1* may be involved in the regulation of *pmb* gene expression.

The sequence of homology between *pmb* and *cpc-1* bears extensive regions (186bp) and the long stretch of overlapping sequences between them encompasses from +1400 to +1585 of *pmb* sequence and +100 to +285 of *cpc-1 sequence.* The strong similarity

(89.2%) of these regions may implicate that these homologous regions are also related to regulatory function of the CPC-1 protein on *pmb* gene expression in interaction with the *pmb* sequence in addition to the nucleotide binding component 5'-TGACTC-3'.

4.4 CLONING GENES BY SEQUENCE SIMILARITY (18, 46, 52, 59)

Once the genes are cloned, they have in many cases been used as cross-species hybridization probes, usually at low stringency, for Southern blots or filter replicas of clone banks to isolate the equivalent genes from a heterologous organism, since nucleotide sequences of cognate genes of related function are often similar. The DNA segments that reveal sequence conservation are then tested in RNA hybridization for deciding the presence of the gene expression and identifying the possible gene product size. As the number of genes cloned increases, the probability that a gene related to a new target gene will already be available becomes higher and higher. As might be expected, this similarity is roughly related to the phylogenetic relationship of the organisms concerned. The degree of relatedness between two nucleic acid segments is measured by the ability of their component single strands to hybridize under appropriate conditions to form complementary double helices. Calculation of sequence homology is based on the formula for nucleic acids melting temperature on the basis of the G+C content. Stringent

conditions (high temperature, low salt, high formamide concentrations) are used when probe and target DNA are totally or almost totally homologous (greater than 90%). Non-stringent conditions (low temperature, high salt) are chosen in cases of lower homology (70-75% DNA homology). (1, 71)

A number of filamentous fungal genes were isolated by making use of previously cloned genes. Initial success has come when using genes that encode highly conserved proteins over a wide range of species such as ribosomal RNA, histone and tubulin genes. For example, the initial isolation of a fungal beta-tubulin gene was accomplished by probing a *A. nidulans* gene bank with a chicken beta-tubulin gene probe (May et al. 1987). Despite the differences in base composition, yeast genes have proven to be a good resource for the isolation of cognate genes from filamentous fungi. Among the fungal genes so isolated have been a number of genes encoding glycolytic enzymes which are highly conserved. As they have become available, genes from the filamentous fungi have also been used as probes for gene isolation.

The cloned *Neurospora pmb* squence is able to be used as a probe in hybridizing with genomic DNAs from heterologous organisms for detecting the functionally homologous gene(s) on the assumption that specific domains of protein comprising *pmb* transport system will be originated from the same gene sequence conserved between *Neurospora* genome and genomic DNAs of other organisms. The regions of especially high similarity presumably reflect a well

conserved nucleotide motif with common structure and function. This work will contribute toward studying multiple transport systems and identifying transport related gene products in all eukaryotes including mammals, considering the situations that mutations in amino acid accumultion process in mammal cells have proven difficult to obtain and study in a similar manner as in microorganisms and the activities of several genes which control amino acid transport in mammals resemble those of transport genes found in other organisms. For example cystinuria and dibasicaminoaciduria of men mimics a transport mutation affecting basic amino acid in *Neurospora*. The Hartnup mutation of men mimics a transport mutation affecting neutral amino acids in *Neurospora*. (Scriver, 1970; Scriver 1985; Christensen, 1989).

In summary, this dissertation has presented evidence that strongly suggests the presence of the wild-type *pmb* locus in cosmid X7:5E DNA. The *pmb* gene is probably fully contained in a 13Kb *SalI* fragment of the cosmid insert. A smaller subclone, pB22-22 hybridizes to a 3.5Kb poly-A RNA suggesting that the *pmb* gene product could be a protein of approximately 100Kd molecular weight. Strong homology with a specific region of the *Neurospora cpc-1* gene implies that the *pmb* gene may be regulated by the *cpc-1* gene product. This is a surprising and intriguing finding as earlier work failed to reveal any regulation of the *pmb* transport system.

However this new finding is supported by the observation that cosmids tightly linked to the *cpc-1* gene can also rescue the pmb^- phenotype. Further information about the nature of the *pmb* gene may come from the completion of the DNA sequence. This work is in progress.

APPENDIX

FULL RESTRICTION MAP OF 1.7Kb SEQUENCE

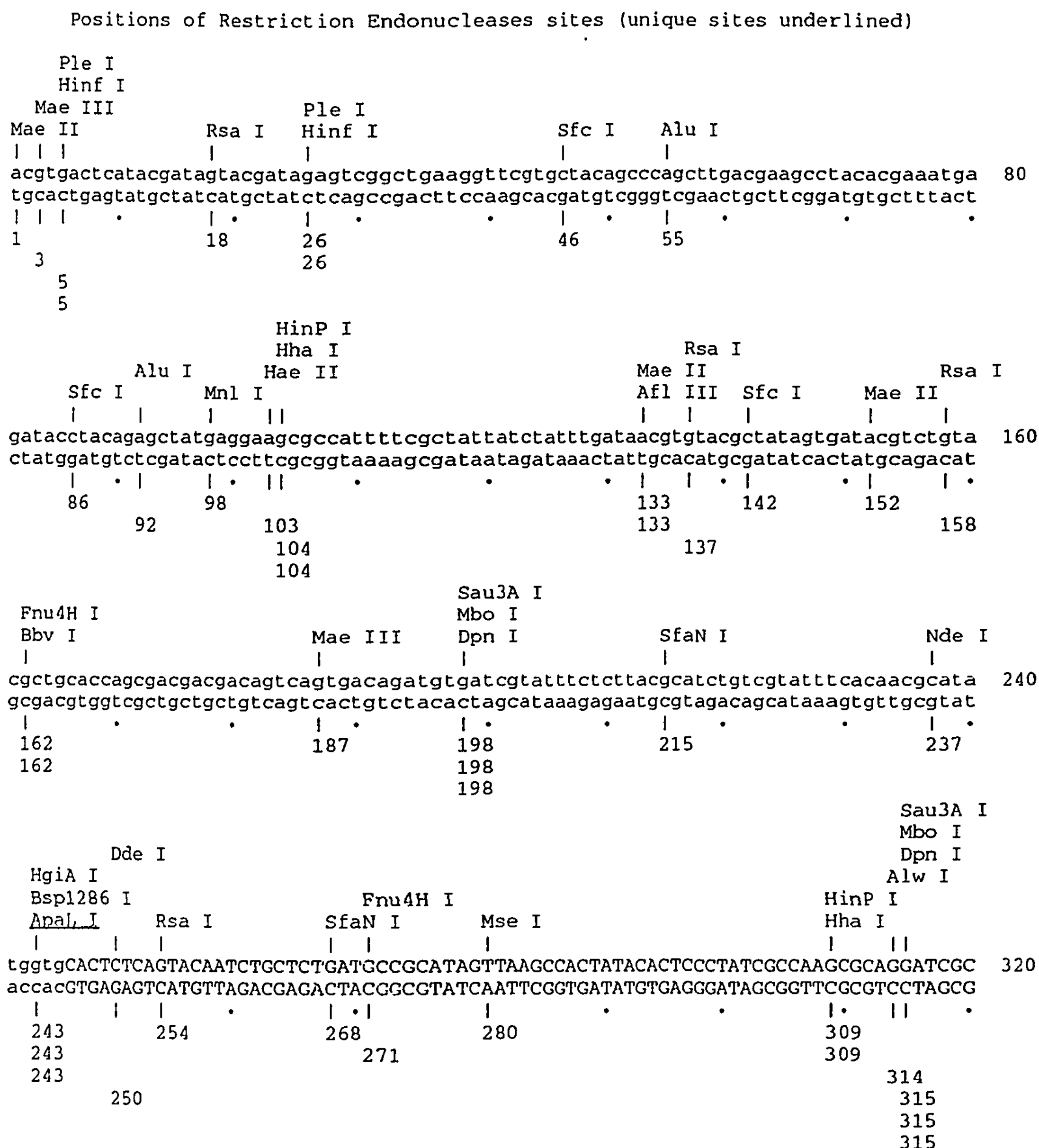


Figure 9. Full restriction map of partial *pmb* sequence

* Enzyme names are listed above the double stranded DNA sequence with the positions of the recognition sequence listed below symetrically.

```
                  Fnu4H I
                  Bbv I
                 Alu I
                Pvu II            Sau3A I
                NspB II           Mbo I
            Mnl I                 Dpn I
       HinP I  Fnu4H I           BstY I                              Taq I
       Hha I   Bbv I             Alw I                      Drd I Hga I
       |    |  ||||              ||                          |     |  |
AACAGAGAGCGCACGAGGCAGCTGCACGGGAAACGCTGGATCTTATAGTCCTTCGGTTTCGCCACTCTGACTTGAGCGT  400
TTGTCTCTCGCGTGCTCCGTCGACGTGCCCCTTTGCGACCTAGAATATCAGGAAGCCAAAGCGGTGAGACTGAACTCGCA
       |.   |  ||||       .      ||        .        .       |     |  |
       329    338                 359                         390   397
       329    338                 359                                 400
            335                   360
               339                360
               339                360
                340
                 341
                 341

                                 Alu I
                                Mae I                             Ple I
                               Nhe I      Rsa I                   Hinf I
         SfaN I       Alu I   Alu I      Sca I  Nla III     Mae II
         |            |       ||| |      ||     |           |     |
CGATTTTTGTGATGCTCGCAGGGCGAGCTATGGAAGCTAGCTTTATTAGTACTTCCATGCTTTACTCTTACGTCTTGACT  480
GCTAAAAACACTACGAGCGTCCCGCTCGATACCTTCGATCGAAATAATCATGAAGGTACGAAATGAGAATGCAGAACTGA
        .|       .    |   .   ||| |.     ||.    |   .       |     |  .
         411          426     435        448    456         470
                               436        449                     477
                               437                                477
                                 439

                                              Sau3A I
                                              Mbo I   Fnu4H I
                                  Dra III     Dpn I   Bbv I          HinP I
                                Mae III       Alw I HinP I           Hha I
                               Ple I        Taq I   Hha I           BstU I
         Fnu4H I            Nla III       Ple I   Nla III         Hga I
         Bbv I         Hga I  Hinf I      Hinf I Xcm I    Nla III Drd I
         |             |    |  | | |      |  | | ||  | |  |       | ||
CATCTCCCTTGCTGCTCGCACAATAAGACGCACATGAGTCACTCGGTGGAGTCGATCCATGCGCTGCATGGTTGGGGACG  560
GTAGAGGGAACGACGAGCGTGTTATTCTGCGTGTACTCAGTGAGCCACCTCAGCTAGGTACGCGACGTACCAACCCCTGC
        .|       .     |  . |  | | |      |. | | || .| |  |  .    | ||
         491           507     516        529    537      547     557
         491                513           529     538             557
                               516          532     541             559
                                518           534   541              560
                                  520         534     543            560
                                              534     543
                                              534
```

Figure 9. (continued) Full restriction map of partial *pmb* sequence

```
                                                                          Gdi II
                                                                          Ecl XI
                                                                   Msp I  Eaq I
                                                                   Hpa II    Bsi YI
                                                                  Cfr10 I  Hae III
                                                                  Age I   Eae I
                                                            Sau96 I       Bsi EI      Nla IV
                                                            Ava II       Fnu4H I      Ban I
                                                            |     ||     ||| |        |
CGCTTGTCGCACACTTCCATCTGACCTTTCAGAAGGTTTCGTGGTGGGCAAGGACCAACCGGT*GCGGCCGTGCGTGGGT  640
GCGAACAGCGTGTGAAGGTAGACTGGAAAGTCTTCCAAAGCACCACCCGTTCCTGGTTGGCCA*CGCCGGCACGCACCCA
         .         .         .         .         . |     ||.     ||| |.        | .
                                                    612          625          638
                                                    612           626         638
                                                          618     626
                                                          618      627
                                                           619       629
                                                           619    626
                                                                  626
                                                                  626

                      Bsi YI
     Msp I          Hae III
      Hpa II       Sau96 I
     ScrF I      Sec I       Fok I
     Nci I       ScrF I    Msp I   SfaN I
     Bcn I       EcoR II   Hpa II                      Nla III
Mnl I            BstN I   Nae I                       NspH I                 SfaN I
Bsi YI           Apy I    Cfr10 I                     Nsp7524 I            Nla III
                                                  Mae III                 BspH I
|     ||         |  || |   ||  ||                  |     ||                 ||  |
GCCTCGCCCCGGCACTGCCAGGGCCACTGCCGGCATCCAACTATCTGCTCTGTAACGCATGTAGCGTTCTGTGTCATGATG  720
CGGAGCGGGGCCGTGACGGTCCCGGTGACGGCCGTAGGTTGATAGACGAGACATTGCGTACATCGCAAGACACAGTACTAC
|     || .       |  || |   ||. ||        .         .|     ||  .        .  ||  | .
642              657       668                      691                 713
642              657       668                            696            714
     647         657        669                           696               717
     647         657        669                            697
     647         657          672
      648           660        673
      648            661
                       663

                                                                    Msp I
                                                                    Hpa II
                                                                    Bsi YI
                       Rsa I                                       Cfr10 I
                    Sau3A I                                     Nla III         Mnl I
                    Mbo I       HgiA I                         NspH I  Rsa I  Mae II
            Nla III Dpn I       Bsp1286 I                      Nsp7524 I     Afl III
            |        |   |       |                             ||   ||  |    |     |
CTCT*AGGGACTGTCATGGGTATGATCGTACAAAGTGCTCACATCGGTATGATGATGACATGCCGGTACTGGACGTGTGA  800
GAGA*TCCCTGACAGTACCCATACTAGCATGTTTCACGAGTGTAGCCATACTACTACTGTACGGCCATGACCTGCACACT
         .  |     .  |   | .     |        .         .      ||.  ||  |   .  |     |.
           735       744         755                       778             793
                     744         755                       778     786     793
                     744                                    779                 799
                         748                                  782
                                                               783
                                                               783
                                                               783
```

Figure 9. (continued) Full restriction map of partial *pmb* sequence

```
                                      Sau3A I                HinP I
                                      Mbo I      Mae I       Hha I
                                      Dpn I   Sau3A I       Hae II
                                     Pvu I    Mbo I         Eco47 III
                                     Bsi EI   Dpn I       Fnu4H I
                                 Hga I        Alw I       Bbv I
                         Fnu4H I    Taq I    BstY I    Nla III
                         |       |  |||      ||   |    |  | ||
GGGTAAACAACTGGCGGTATGG*TGCGGCGGTGCGTCGATCGGCAGATCCTAGTCATGCAGCGCTCGTCAGTCAGATGTA  880
CCCATTTGTTGACCGCCATACC*ACGCCGCCACGCAGCTAGCCGTCTAGGATCAGTACGTCGCGAGCAGTCAGTCTACAT
          .         .    |     . |  ||| .    ||   |    |  | ||          .      .
                         825        836      845       855
                                 833          846         858
                                     837      846         858
                                     837      846           860
                                      838     846           860
                                      838         850        861
                                      838                    861

                                      Msp I
                                      Hpa II
                                   Sau3A I
                                   Mbo I
                                   Dpn I
                             SfaN I  Mro I                 HinP I
                      SfaN I       Alw I                   Hha I
                   Fnu4H I    Bsa BI  BspM II             Hae II
                   Bbv I    Taq I  BstY I                AlwN I
                   |   |    |  |   ||  ||                |  ||
GTAGTCCAGCAGTAGCCAGCAGCATCTCGATGCAGATCCGGACGATAATGGAGCAGGCGCTGACCTTCCCGTGTTCCACG  960
CATCAGGTCGTCATCGGTCGTCGTAGAGCTACGTCTAGGCCTGCTATTACCTCGTCCGCGACTGGAAGGCGCACAAGGTGC
          .        |.  |    |  |.  ||  || .          .  |  ||  .         .      .
                   899      907    914                   934
                   899        909    917                   936
                      902          915                      937
                              909    917                    937
                                   915
                                   915
                                   915
                                     918
                                     918

                                                                            Msp I
                     Mae II                                                 Hpa II
                 Sau3A I                                                Mae I
                 Mbo I                 Tfi I                           Nhe I
                 Dpn I                 Hinf I          Mae II         Alu I
                BstY I             Mae II            Bsa AI         Fnu4H I
                Alw I              Bsa AI           Afl III         Bbv I  Cfr10 I
                ||    |            ||    |          |||             | |||  ||
ACTTTACGAAACACGGATCTACGTCGTCCCTACGTGATTCATTCTGCTACAcgtaggcaaccgcagctagccggtctcac  1040
TGAAATGCTTTGTGCCTAGATGCAGCAGGGATGCACTAAGTAAGACGATGTgcatccgttggcgtcgatcggccagagtg
          .     ||   .|           .||    |    .     |||          . | |||  ||      .
                975                991              1009            1023   1030
                975                  992             1010           1023
                 976                     996          1011             1025
                 976                     996                            1026
                 976                                                     1027
                     981                                                    1031
                                                                            1031
```

Figure 9. (continued) Full restriction map of partial *pmb* sequence

```
               HinP I
               Hha I
              Fsp I
              Avi II       Nla IV
            Nla III     ScrF I
         Sau3A I        EcoR II       Gdi II
         Mbo I          BstN I        Eae I
         Dpn I          Apy I  Bsi YI  Hae III        Bsi EI
         |  | ||        |  |   |      ||              |
gacaagagcgatcatgcgcaactgccagGAACCCACGCTGGCCGAGATG*CGTGCGGTCGCTGGAGATGGCGGAGCGATG  1120
ctgttctcgctagtacgcgttgacggtcCTTGGGTGCGACCGGCTCTAC*GCACGCCAGCGACCTCTACCGCCTCGCTAC
         |  | ||   .    |  | . |      ||          .   |     .        .         .
         1050           1065  1072  1080           1095
         1050           1065       1079
         1050           1065       1079
            1053        1065
              1055         1068
              1055
               1056
               1056

                                                                HinP I
                                                                Hha I
                              Nla III                          BstU I
                SfaN I     Taq I                           Mae III             Taq I
                |          |   |                           |   ||              |
GATATGTTCTGTGTTCGCATCACAGTCTCGACATGATTGCTCCAATTCTTGATGTAATCGTTACGCGCTTCCATTCAGGT  1200
CTATACAAGACACAAGCGTAGTGTCAGAGCTGTACTAACGAGGTTAAGAACTACATTAGCAATGCGCGAAGGTAAGTCCA
         .      |  .       | . |       .        . .        |   ||    .         |
                1137       1148                            1180                1200
                               1152                            1184
                                                                1185
                                                                1185

           Nla IV
         Msp I
         Hpa II
        ScrF I                                            Nla IV
        Nci I                                           Msp I
      Sau96 I                                           Hpa II
      Hae III                         Nla IV           Cfr10 I
 Mnl I  Bcn I   Nla III     Alu I     Ban I         Mae I     Mnl I
 |    | || |    |           |         |             |  || |   |
CGAGGTGGCCCGGCTCCATGCACCTTCCAGCTCGGTAAGGCACCCCGCCAGCCTAGCCGGTTCCTCACGAACACCAGCAC  1280
GCTCCACCGGGCCGAGGTACGTGGAAGGTCGAGCCATTCCGTGGGGCGGTCGGATCGGCCAAGGAGTGCTTGTGGTCGTG
 |    | || |    |  .        |.        |.          .  |  || |.  |      .         .
 1202   1209    1217        1229      1239          1253      1263
      1207                            1239             1256
      1207                                              1257
        1209                                            1257
        1209                                              1259
         1210
         1210
           1212
```

Figure 9. (continued) Full restriction map of partial *pmb* sequence

```
                     Sau96 I
      Fnu4H I     ScrF I
   Nla III        EcoR II
Sau3A I           BstN I                HinP I
Mbo I             Apy I      Fnu4H I    Hha I
Dpn I        Bsi YI  Ava II  Bbv I   SfaN I   Mae III                    Fok I
|  |  |      |    |  |       |       |  |     |                          |
GATCATGCCGCCACCCGTGCCAGGACCAACGCTGCCGAGATGCGCTCGTGACGACTGCTGGGCAGATGGCGACGGATGGA  1360
CTAGTACGGCGGTGGGCACGGTCCTGGTTGCGACGGCTCTACGCGAGCACTGCTGACGACCCGTCTACCGCTGCCTACCT
|  |  |  .   |    |  |      .|       |. |     |.           .         .   |     .
1281         1295    1303    1311    1319     1328                       1354
1281              1300       1311       1322
1281              1300                  1322
   1284           1300
      1287        1300
                     1303
               SfaN I
             HinP I
             Hha I         Taq I  SfaN I           Sau3A I
            Fsp I     Ple I       Bsa BI           Mbo I
     Dde I  Avi II    Hinf I   Nla III     Dde I  Dpn I       Mnl I      Mse I
     |      || |      |    |   |  |         |      |           |          |
TATGTCTCAGGTGTGCGCATCACGAGTCTCGACATGATGCTCATCTGAGTGTGATCGTAGCAGTCCTCATAGCATTAAAT  1440
ATACAGAGTCCACACGCGTAGTGCTCAGAGCTGTACTACGAGTAGACTCACACTAGCATCGTCAGGAGTATCGTAATTTA
     |  .   || |  .   |    |.  |  |    .    |    . |       .   |    .     |    .
     1366   1374      1384     1393         1405   1413        1425       1435
            1374      1384        1396             1413
             1375          1389   1396             1413
             1375
               1377
                                                        ScrF I
                                                        EcoR II
        Sau3A I        Taq I                            BstN I
        Mbo I        Sau3A I                        Tfi I
        Dpn I        Mbo I                  Msp I   Hinf I    Alu I
    Mae III  Hga I   Dpn I                  Hpa II     Apy I                 Mae III
    |   |    |        | |                    |      |   |     |                |
CGCCGTGACGATCAGCGTCCAATGATCGAAGTTAGGCTGGTAAGAGCCGGAGCGAATCCTGGAAGCTGTCCTGATGGTCG  1520
GCGGCACTGCTAGTCGCAGGTTACTAGCTTCAATCCGACCATTCTCGGCCTCGCTTAGGACCTTCGACAGGACTACCAGC
    |   |    |    .   | |   .          .         |  .   |   |.    |      .         |
    1445     1455     1464                   1487       1498                1520
        1450          1464                   1487   1494      1504
        1450          1464                          1494
        1450            1466
                                                        1498
                                                        1498
                                                        1498
         ScrF I
         EcoR II                         BstU I        Nde I                Nde I
         BstN I                     SfaN I         Sau3A I              Sau3A I
         Apy I        Bsi YI    Fok I   HinP I     Mbo I                Mbo I
     BspM I       Nla III      SfaN I   Hha I      Dpn I                Dpn I
     |   |        |   |        ||    |  ||          |  |                 |  |
TCACTACCTGCCTGGACACCATGCCTGCACGGGCATCCGATGCGCGAGCGAGAGATCATATGGAGCAACGAGAGATCATA  1600
AGTGATGGACGGACCTGTGGTACGGACGTGCCCGTAGGCTACGCGCTCGCTCTCTAGTATACCTCGTTGCTCTCTAGTAT
     |  .|        |   |     .  ||    |. ||      .   |  | .          .   |  |  .
     1526         1540         1553     1562        1574                 1594
         1531         1544      1554    1562        1574                 1594
         1531                       1559            1574                 1594
         1531                            1563          1577                 1597
         1531
```

Figure 9. (continued) Full restriction map of partial *pmb* sequence

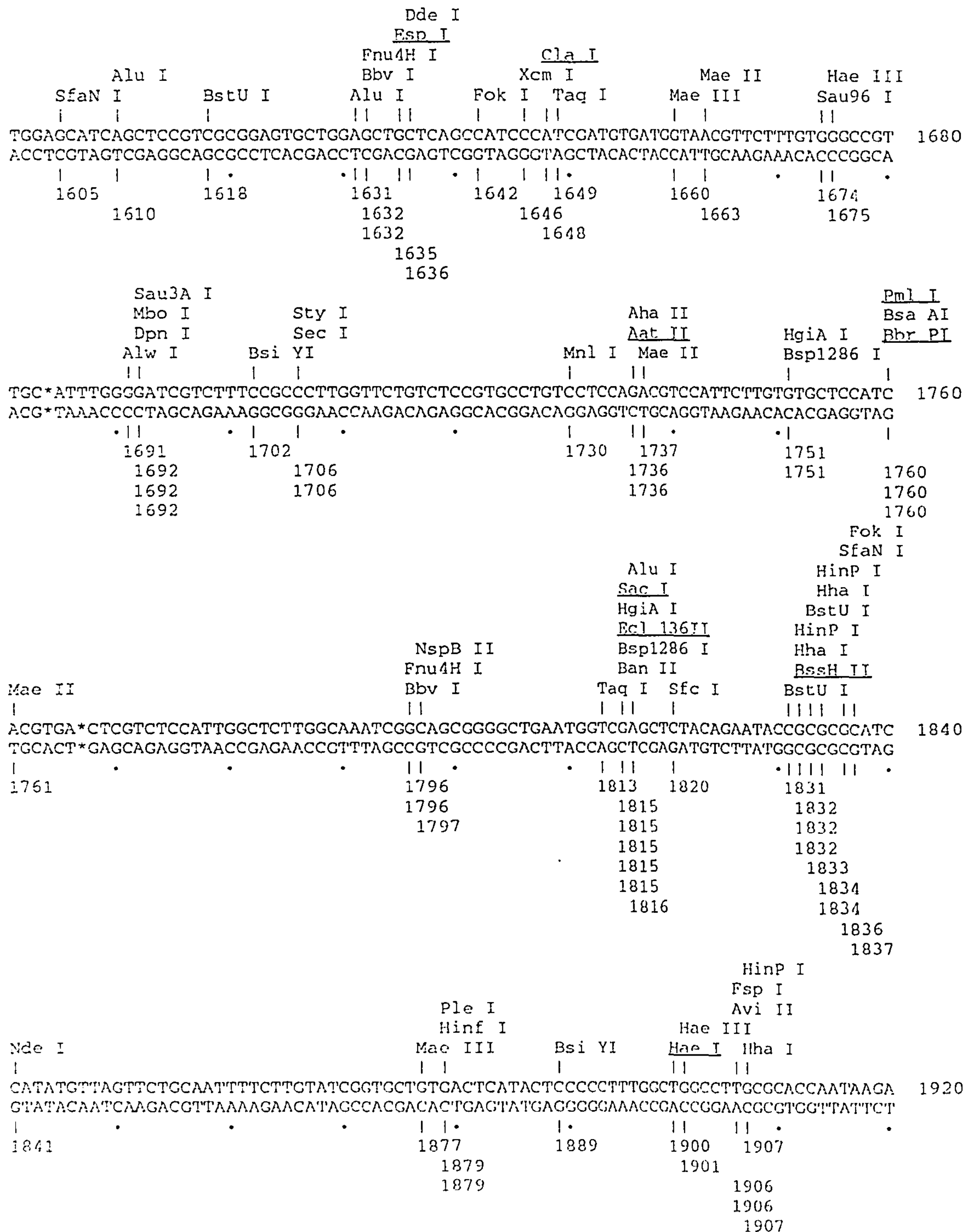


Figure 9. (continued) Full restriction map of partial *pmb* sequence

```
                                Nla III
                               Sty I
                               Sec I
                               Nco I
                               Dsa I
                            Sau3A I
                            Mbo I
                            Dpn I
                            Alw I        Nla III
                           Nla IV       Bsi YI
                           BstY I   Nla IV
                           BamH I  Bsp1286 I                                   Mnl I
                           Alw I   Ban II                  Taq I         Bsi YI
                           ||  ||  ||   ||                 |             |     |
ACGCACAGTGAAATCTGCGGTGGGAGTGGATCCATGGGCTCCATGCTGGGACGGCACTCTCGACATCATCTGACCTTCAG  2000
TGCGTGTCACTTTAGACGCCACCCTCACCTAGGTACCCGAGGTACGACCCTGCCGTGAGAGCTGTAGTAGACTGGAAGTC
         .         .       ||. ||  ||  .||       .         |         .   |     |
                           1948    1956                    1980          1994
                           1948    1956                                        2000
                           1948     1957
                           1948             1961
                            1949             1962
                            1949
                            1949
                            1949
                               1952
                               1952
                               1952
                               1952
                                1953
                Sau3A I
                Mbo I
                Dpn I  HinP I            Fnu4H I
            Fok I   Nla III  Nla III     Bbv I
        Fok I   Alw I  Hha I        Rsa I                          EcoN I
        |   |   |   |  |     |      |    |                          |
AGGAACTCGGATGGATGATCCATGCGCTCCATGCTGGTACTGCTGCACTGTCGCACACTTCCATCTGACCTTCAGAAGGT  2080
TCCTTGAGCCTACCTACTAGGTACGCGAGGTACGACCATGACGACGTGACAGCGTGTGAAGGTAGACTGGAAGTCTTCCA
        |.  |   |  .|  |     |      |  . |       .         .        |.         .
        2009    2017   2024         2037                            2069
            2013    2021     2030        2042
                2017   2024              2042
                2017
                2017
                  Msp I
                  Hpa II                                  Hae III
                  Bsi YI                                 Sau96 I
                 Cfr10 I                     Msp I    Sec I
                 Age I    Aha II             Hpa II   ScrF I
            Drd I       Fnu4H I             ScrF I    EcoR II
           Sau96 I     BstU I     Nla IV    Nci I     BstN I
           Ava II  Bsi EI  Hga I  Ban I     Bcn I     Apy I           Nla III
           ||    |||   || ||      |         ||        |  ||           |
TCGTGTGGCAAGGACCAACCGGTCGCGGCGTCGTGGTGCCTTCGCCCGGCACTGCCAGGGCCACTGCACCCATGC  2155
AGCACACCGTTCCTGGTTGGCCAGCGCCGCAGCACCACGGAAGCGGGCCGTGACGGTCCCGGTGACGTGGGTACG
         . ||    |||   || || .    |    .    ||   .    |  ||.         .|
           2092    2100    2108   2115      2125      2135            2151
           2092        2104       2115      2125      2135
            2093        2105                2125      2135
                 2098     2107               2126     2135
                 2098                        2126     2135
                  2099                                   2138
                  2099                                    2139
                  2099
```

Figure 9. (continued) Full restriction map of partial *pmb* sequence

Restriction Endonucleases site usage

Aat II	1	BspM I	1	HinC II	-	Pml I	1
Acc I	-	BspM II	1	HinD III	-	PpuM I	-
Afl II	-	BssH II	1	Hinf I	9	Pst I	-
Afl III	3	BstB I	-	HinP I	16	Pvu I	1
Age I	2	BstE II	-	Hpa I	-	Pvu II	1
Aha II	2	BstN I	6	Hpa II	11	Rsa I	8
Alu I	12	BstU I	7	Hph I	-	Rsr II	-
Alw I	10	BstX I	-	Kas I	-	Sac I	1
AlwN I	1	BstY I	5	Kpn I	-	Sac II	-
Apa I	-	Bsu36 I	-	Mae I	4	Sal I	-
ApaL I	1	Cfr10 I	6	Mae II	11	Sau I	-
Apo I	-	Cla I	1	Mae III	10	Sau3A I	19
Apy I	6	Dde I	4	Mbo I	19	Sau96 I	7
Asc I	-	Dpn I	19	Mbo II	-	Sca I	1
Ase I	-	Dra I	-	Mlu I	-	ScrF I	9
Asp718	-	Dra III	1	Mnl I	9	Sec I	4
Ava I	-	Drd I	3	Mro I	1	SfaN I	15
Ava II	3	Dsa I	1	Mse I	2	Sfc I	4
Avi II	3	Eae I	2	Msp I	11	Sfi I	-
Avr II	-	Eag I	1	Mun I	-	Sfu I	-
Bal I	-	Ecl 136II	1	Nae I	1	Sgr AI	-
BamH I	1	Ecl XI	1	Nar I	-	Sma I	-
Ban I	3	Eco47 III	1	Nci I	3	SnaB I	-
Ban II	2	EcoN I	1	Nco I	1	Spe I	-
Bbe I	-	EcoO109 I	-	Nde I	4	Sph I	-
Bbr PI	1	EcoR I	-	Nhe I	2	Spl I	-
Bbv I	12	EcoR II	6	Nla III	20	Ssp I	-
Bbv II	-	EcoR V	-	Nla IV	8	Stu I	-
Bcl I	-	Esp I	1	Not I	-	Sty I	2
Bcn I	3	Fnu4H I	17	Nru I	-	Taq I	11
Bgl I	-	Fok I	7	Nsi I	-	Tfi I	2
Bgl II	-	Fsp I	3	Nsp7524 I	2	Tth111 I	-
Bsa AI	3	Gdi II	2	NspB II	2	Tth111 II	-
Bsa BI	2	Hae I	1	NspH I	2	Xba I	-
Bsi EI	4	Hae II	3	Pac I	-	Xca I	-
Bsi YI	12	Hae III	7	PaeR7 I	-	Xcm I	2
Bsm I	-	Hga I	6	PflM I	-	Xho I	-
Bsp1286 I	5	HgiA I	4	Ple I	7	Xma I	-
BspH I	1	Hha I	16	Pme I	-	Xmn I	-

Figure 9. (continued) Full restriction map of partial *pmb* sequence

Enzyme	Site		Use	Site position (Fragment length) Fragment order			
Aat II	gacgt/c		1	1(1735)	1	1736(420)	2
AlwN I	cagnnn/ctg		1	1(933)	2	934(1222)	1
ApaL I	g/tgcac		1	1(242)	2	243(1913)	1
BamH I	g/gatcc		1	1(1947)	1	1948(208)	2
Bbr PI	cac/gtg		1	1(1759)	1	1760(396)	2
BspH I	t/catga		1	1(712)	2	713(1443)	1
BspM I	acctgc	4/8	1	1(1525)	1	1526(630)	2
BspM II	t/ccgga		1	1(916)	2	917(1239)	1
BssH II	g/cgcgc		1	1(1831)	1	1832(324)	2
Cla I	at/cgat		1	1(1647)	1	1648(508)	2
Dra III	cacnnn/gtg		1	1(519)	2	520(1636)	1
Dsa I	c/crygg		1	1(1951)	1	1952(204)	2
Eag I	c/ggccg		1	1(625)	2	626(1530)	1
Ecl 136II	gag/ctc		1	1(1814)	1	1815(341)	2
Ecl XI	c/ggccg		1	1(625)	2	626(1530)	1
Eco47 III	agc/gct		1	1(859)	2	860(1296)	1
EcoN I	cctnn/nnnagg		1	1(2068)	1	2069(87)	2
Esp I	gc/tnagc		1	1(1634)	1	1635(521)	2
Hae I	wgg/ccw		1	1(1899)	1	1900(256)	2
Mro I	t/ccgga		1	1(916)	2	917(1239)	1
Nae I	gcc/ggc		1	1(667)	2	668(1488)	1
Nco I	c/catgg		1	1(1951)	1	1952(204)	2
Pml I	cac/gtg		1	1(1759)	1	1760(396)	2
Pvu I	cgat/cg		1	1(836)	2	837(1319)	1
Pvu II	cag/ctg		1	1(338)	2	339(1817)	1
Sac I	gagct/c		1	1(1814)	1	1815(341)	2
Sca I	agt/act		1	1(447)	2	448(1708)	1

No Sites found for the following Restriction Endonucleases

Enzyme	Site		Enzyme	Site		Enzyme	Site	
Acc I	gt/mkac		EcoR I	g/aattc		Rsr II	cg/gwccg	
Afl II	c/ttaag		EcoR V	gat/atc		Sac II	ccgc/gg	
Apa I	gggcc/c		HinC II	gty/rac		Sal I	g/tcgac	
Apo I	r/aatty		HinD III	a/agctt		Sau I	cc/tnagg	
Asc I	gg/cgcgcc		Hpa I	gtt/aac		Sfi I	ggccnnnn/nggcc	
Ase I	at/taat		Hph I	ggtga	8/7	Sfu I	tt/cgaa	
Asp718	g/gtacc		Kas I	g/gcgcc		Sgr AI	cr/ccggyg	
Ava I	c/ycgrg		Kpn I	ggtac/c		Sma I	ccc/ggg	
Avr II	c/ctagg		Mbo II	gaaga	8/7	SnaB I	tac/gta	
Bal I	tgg/cca		Mlu I	a/cgcgt		Spe I	a/ctagt	
Bbe I	ggcgc/c		Mun I	c/aattg		Sph I	gcatg/c	
Bbv II	gaagac	2/6	Nar I	gg/cgcc		Spl I	c/gtacg	
Bcl I	t/gatca		Not I	gc/ggccgc		Ssp I	aat/att	
Bgl I	gccnnnn/nggc		Nru I	tcg/cga		Stu I	agg/cct	
Bgl II	a/gatct		Nsi I	atgca/t		Tth111 I	gacn/nngtc	
Bsm I	gaatgc	1/-1	Pac I	ttaat/taa		Tth111 II	caarca	11/9
BstB I	tt/cgaa		PaeR7 I	c/tcgag		Xba I	t/ctaga	
BstE II	g/gtnacc		PflM I	ccannnn/ntgg		Xca I	gta/tac	
BstX I	ccannnnn/ntgg		Pme I	gttt/aaac		Xho I	c/tcgag	
Bsu36 I	cc/tnagg		PpuM I	rg/gwccy		Xma I	c/ccggg	
Dra I	ttt/aaa		Pst I	ctgca/g		Xmn I	gaann/nnttc	
Eco0109 I	rg/gnccy							

Figure 9. (continued) Full restriction map of partial *pmb* sequence

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