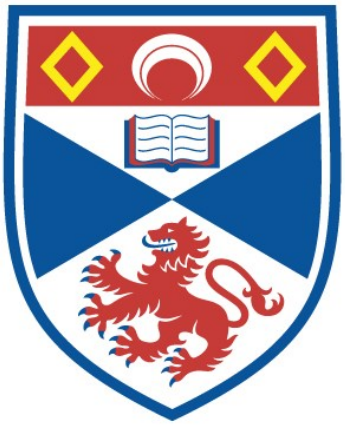
THE ISOLATION AND CHARACTERIZATION OF CNX GENES FROM THE FILAMENTOUS FUNGUS "ASPERGILLUS NIDULANS"

Jacqueline Smith

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1998

THE ISOLATION AND CHARACTERIZATION OF *cnx* GENES FROM THE FILAMENTOUS FUNGUS *Aspergillus nidulans*

JACQUELINE SMITH B.Sc. Hons. (St. Andrews University)

For the degree of Doctor of Philosophy

Submitted January 1998

Plant Sciences Laboratory,

Sir Harold Mitchell Building,

University of St. Andrews,

FIFE, KY16 9TH.

SCOTLAND, UK.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

D21

DEDICATION

This thesis is dedicated to Mum, Dad, Lorna, Alan, Aunt Ina, Uncle Alex and Colin.

I, Jacqueline Smith, hereby certify that this thesis, which is approximately 30,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

Date 18/1/98 Signature of candidate

I was admitted as a research student in October 1992 and as a candidate for the degree of Doctor of Philosophy in July 1993 ; the higher study for which this is a record was carried out in the University of St. Andrews between 1992 and 1995.

Date .....18/1/98..... Signature of candidate

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of Doctor of Philosophy in the University of St. Andrews and that the candidate is qualified to submit this thesis in application for that degree.

Date .....18/1/98..... Signature of Supervisor

In submitting this thesis to the University of St. Andrews I understand that I am giving permission for it to be made available for use in accordance with the University Library for the time being in force, subject to any copyright vested in the work not being affected thereby. I also understand that the title and abstract will be published, and that a copy of the work may be made and supplied to any bona fide library or research worker.

Date .....18/1/98..... Signature of candidate

ABBREVIATIONS

A_{260} - absorbance at 260nm.

A_{280} - absorbance at 280nm.

ATP - adenosine triphosphate.

bp - base pairs.

BPB:NR - bromophenol blue nitrate reductase activity.

BSA - bovine serum albumin.

Ci - Curies.

CR:NR - cytochrome C nitrate reductase activity.

°C - degrees Celsius.

D - Daltons.

d-ATP - 2' - deoxy-adenosine 5' - triphosphate.

d-CTP - 2' - deoxy-cytidine 5' - triphosphate.

d-GTP - 2' - deoxy-guanosine 5' - triphosphate.

dH_2O - distilled water.

d-TTP - 2' - deoxy-thymidine 5' - triphosphate.

DNA - deoxy - ribonucleic acid.

DTT - dithiothreitol.

EDTA - ethylenediamine tetra-acetic acid.

e.g. - *exempli gratia.*

et al. - *et alia* (and others).

EtBr - ethidium bromide.

FAD - flavin adenine dinucleotide.

Fig. - figure.

FMN - flavin mono-nucleotide.

g - grams.

GMP - guanosine monophosphate.

GTP - guanosine triphospate.

Gu - guanine.

hr - hours.

kb - kilobase pairs.

kD - kiloDaltons.

L - litres.

L.B. - Luria broth.

μCi - micro Curies.

μg - micrograms.

μl - microlitres.

M - molar.

mg - milligrams.

MGD - mono guanine dinucleotide.

min -minutes.

ml - millilitres.

mM - millimolar.

Mo - molybdenum.

Mo-co - molybdenum cofactor.

MOPS - 3-(N- Morpholino) propane- sulphonic acid.

MVH:NR - methyl viologen nitrate reductase activity.

mRNA - messenger RNA.

MW - molecular weight.

NADPH - nicotinamide adenine dinucleotide phosphate.

N.B. - *noto bene* (note well).

ng - nanograms.

NiR - nitrite reductase.

nm - nanometres.

nM - nanomolar.

NR - nitrate reductase.

O.D. - optical density.

O/N - overnight.

ORF - open reading frame.

PABA - para-aminobenzoic acid.

PAGE - polyacrylamide gel electrophoresis.

PEG - polyethylene glycol.

p.s.i. - pounds per square inch.

PVP - polyvinyl - pyrrolidone.

rpm - revolutions per minute.

r.t. - room temperature.

s - seconds.

SDS - sodium dodecyl sulphate.

SDW - sterile distilled water.

soln. - solution.

TEMED - N,N,N',N' - tetramethylethylenediamine.

Tris. - tris(hydroxymethyl)methylamine.

t.s. - temperature - sensitive.

U.V. - ultra violet.

w.t. - wild - type.

< - less than.

> - greater than.

~ - about.

Gene Symbols of Fungal Mutants used in this Thesis:

bi - biotin utilization.

cnx - cofactor for nitrate reductase and xanthine dehydrogenase.

fw - fawn spore colour.

ivo - ivory spore colour.

paba - PABA utilization.

prn - proline utilization.

pyro - pyridoxine utilization.

y - yellow.

Chemical Elements - symbols as by convention.

Amino acid single letter code - as by convention.

Amino acid three letter code - as by convention.

ACKNOWLEDGEMENTS

I would like to thank Jim Kinghorn for his help and encouragement during the three years of my postgraduate studies, as well as Shiela Unkles for the work carried out in helping to characterize the *cnx*ABC locus. I must also mention Takashi Kawaguchi to whom I am indebted for his ever-patient help with computer analyses. Thanks also to Ian Armitt for preparing the DNA oligonucleotides used in the sequencing reactions and also to all personnel at the Photographic Unit, University of St. Andrews for the photographs presented in this thesis.

CONTENTS

CHAPTER 3 - ISOLATION and CHARACTERIZATION OF THE *cnx*ABC LOCUS OF *A. nidulans*

ABSTRACT

The filamentous fungus *Aspergillus nidulans* relies on a good source of nitrogen for healthy growth. One of the most important sources of this nitrogen is from nitrate. *A. nidulans* can assimilate the nitrogen from nitrate via the action of its nitrate reductase enzyme. This enzyme requires a molybdenum - pterin containing cofactor for its activity. In *A. nidulans*, there are six loci known to be involved in synthesis of the Mo - cofactor - the so-called *cnx* genes. This work reports on the isolation and characterization of the *cnx*ABC locus.

*cnx*ABC was found to consist of one gene containing an intron which separates two domains which encode one bi - functional protein. The two regions, namely *cnx*A and *cnx*C (each of which are able to phenotypically repair *cnx*A and *cnx*C fungal mutants respectively) show a high degree of homology with the two protein products of *moa*A and *moa*C of *Escherichia coli*, as well as with two proteins encoded by genes isolated from *Arabidopsis thaliana* -- *cnx*2 and *cnx*3. Nitrate is also shown to act as an inducer of *cnx*ABC mRNA synthesis. If *cnx*ABC plays a similar role to its homologues, it has its function at the early stages of Mo-pterin biosynthesis, ie. conversion of guanosine into precursor Z, which is then converted to the molybdopterin which is incorporated into the Mo-cofactor.

Mo-co is a universal molecule which is found in various enzymes throughout the species. Research into the synthesis of this cofactor will provide results which will prove to have important consequences in the understanding of the action of various important enzymes within many species.

CHAPTER 1

INTRODUCTION

1.1. GENERAL BACKGROUND

The ascomycetous filamentous fungus *Aspergillus nidulans* has proved itself to be a very useful experimental model. It is an ideal organism in which to study the genetics of a eukaryotic system. Over the past 50 years, a wealth of knowledge has been built-up using this organism as a genetical experimental host. However, during more recent times, the main experimental thrust, in general, has moved away from classical genetical analyses and towards molecular biological investigations.

A. nidulans provides us with a relatively small genome which can be relatively easily studied in comparison with most other eukaryotic systems, having a size of only 31,000 kb (distributed over eight separate linkage groups) (Brody and Carbon, 1989). It has only basic nutritional requirements (and is therefore inexpensive for laboratory use) and displays a short life-cycle, enabling genetical results to be obtained in a short period of time. Mutant isolation and characterization have also proved to be relatively simple. *A. nidulans* poses no known health problems and is regarded as safe to handle - a fact which has been acknowledged by the General Society for Microbiology, as the organism has now been given GRAS status (Generally Regarded As Safe). There is also the added benefit in that there are no moral or ethical dilemmas accompanying the experimental use of *Aspergillus*.

The life cycle of the organism is shown in Fig. 1.1. A very useful feature of the growth of *A. nidulans* is that it displays sexual, asexual and parasexual cycles - all of which have been exploited in the laboratory for genetical studies. Successful growth can be easily obtained, as long as the organism is provided with a carbon source, nitrogen source, water and oxygen. In common with most organisms, trace amounts of sulphur, phosphorus, metals and vitamins are also required. However, light is not a requirement. Incubation at an optimal temperature of 37°C is usually employed.

The asexual cycle is complete in two days under these conditions, whereas the sexual cycle takes 4-14 days depending somewhat upon the strain of *A. nidulans* being used. The main growth state is haploid, and the asexual conidia contain only one nucleus. With regard to the sexual cycle, *A. nidulans* is homothallic, ie. all stages are undergone by only one mycelium. Wild-type conidial phenotype changes from white to yellow to green as asexual growth progresses.

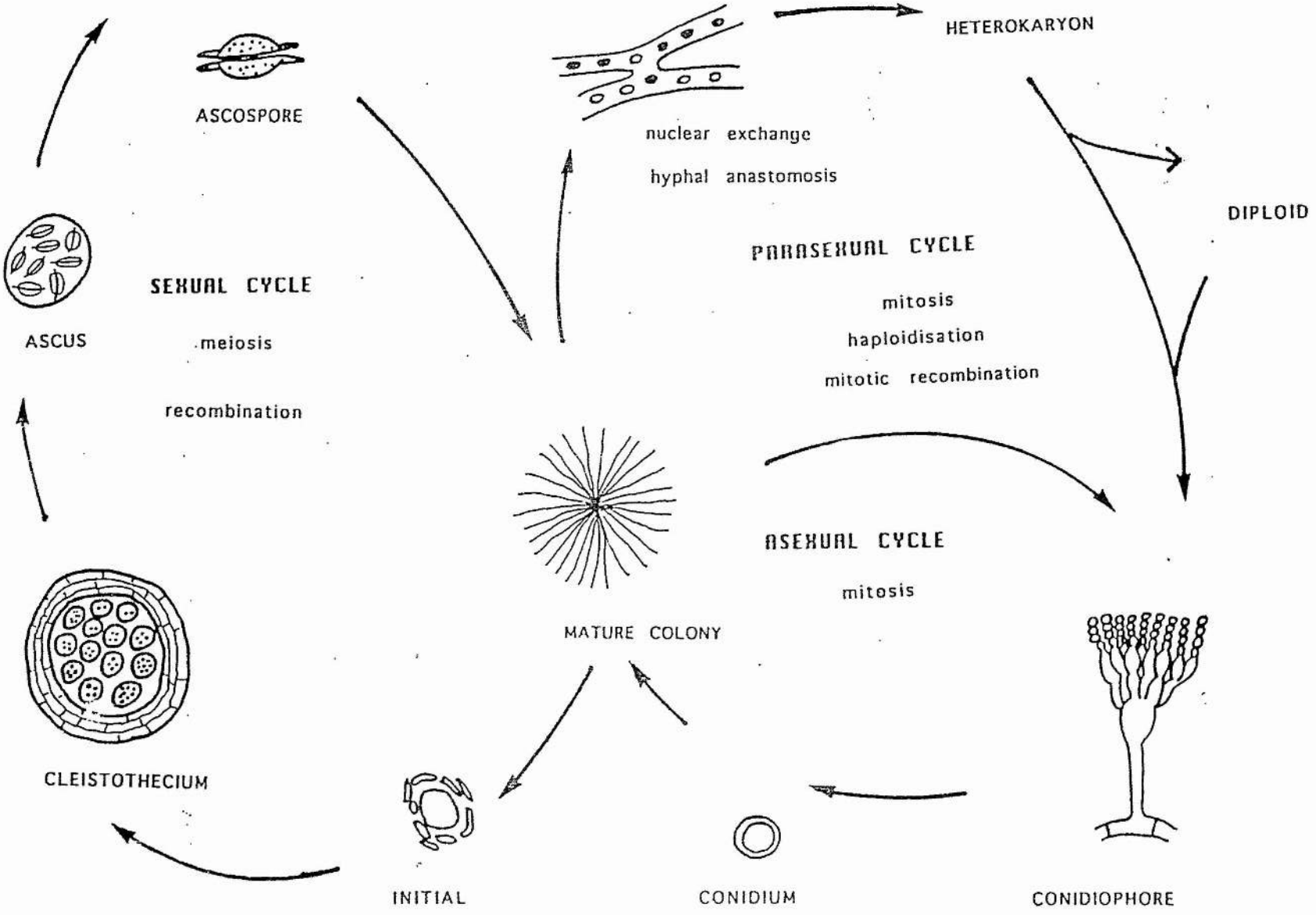
A. nidulans provides us with a good system for studying gene regulation - with some regulatory mechanisms being confined to a specific reaction and others being involved in a host of biochemical pathways within the organism. Gene clusters are also a feature. although they are not organized into genetic operons as in certain prokaryotic organisms eg. *Escherichia coli* (reviewed by Arst, 1983).

As experimental techniques progress, and more information is gathered, a fuller and wider picture of gene structure in *A. nidulans* is being seen. To date, more than 100 genes have been isolated and many of these have also been sequenced. Work is currently in progress to determine the physical positions of genes with respect to each other, along each of the 8 chromosomes. It is hoped to map the whole *A.*

Fig. 1.1.

Representation of the reproductive cycles which occur in *A. nidulans*. This organism exhibits a sexual, asexual and a parasexual growth pattern, as depicted (Reviewed by and taken from Martinelli, 1994).

The Life Cycle of Aspergillus nidulans.



nidulans genome, as 'contig' maps are constructed from data obtained from cloning work which identifies which genes lie within which clones in genomic libraries (see Martinelli and Kinghorn, 1994 and references therein).

Genes from *A. nidulans* can now be isolated relatively easily due to the fact that efficient transformation systems have been developed. This is mainly due to the availability of a wide number of genetic mutants which have been isolated. Various techniques can be employed to facilitate the uptake of DNA into the *A. nidulans* cells, including the use of lithium acetate, electroporation, and 'biolistics' - where DNA is mechanically fired through the fungal cell wall. However, the most common method of fungal transformation is the use of protoplasts, with the uptake of DNA mediated by the presence of PEG and $CaCl_2$ (for genetic transformation reviews, see Lurquin and Kleinhofs, 1982; Tilburn *et al.*, 1983; Johnstone, 1985; Mishra, 1985; Hynes, 1986; Fincham, 1989; Timberlake and Marshall, 1989; Peberdy, 1991; May, 1992).

A number of selective markers are available for use in *A. nidulans* which can be easily traced phenotypically - nutritional selective markers, such as *arg*B, *trp*C, *prn* utilization genes etc. and dominant selectable markers (usually conferring antibiotic resistance eg. hygromycin (Punt *et al.*, 1987), phleomycin (Austin *et al.*, 1990; Mattern *et al.*, 1988). Cotransformation, where two different molecules of DNA are inserted into the host genome, is also a useful tool in genetic studies of *A. nidulans*. This is used when the fragment of DNA under study has no easily selectable marker, so the selection is for the marker contained on the second fragment of DNA (Wernars *et al.*, 1987).

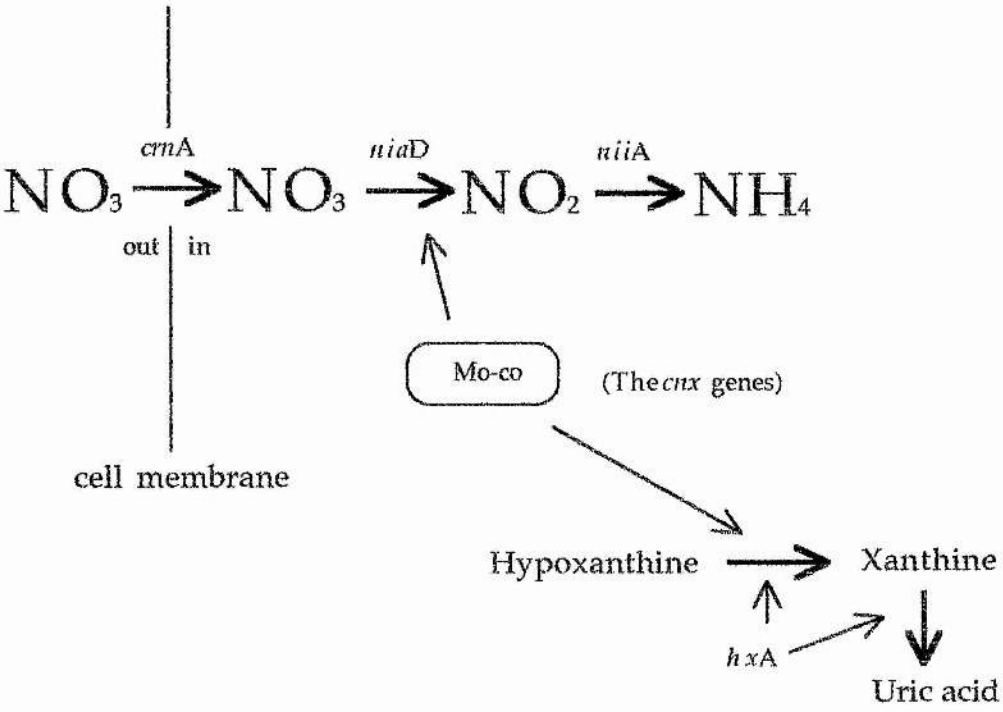
Much work is being carried out on *A. nidulans*, not only to help us understand the genetic make-up which may also lead to an

understanding of systems in other eukaryotic organisms, but because it is also a potentially important industrial organism. *A. nidulans* has already been shown to be able to express heterologous proteins such as human plasminogen activator and human interleukin-6 (Carrez *et al.*, 1990). There is much scope for the fungal production of medically and industrially important compounds. (For a review see Kinghorn and Unkles, 1992).

1.2. NITRATE ASSIMILATION AND ITS REGULATION

One of the main growth requirements of *A. nidulans* is a good source of nitrogen. In this regard, *A. nidulans* is able to utilize a wide variety of nitrogen sources. These include nitrate, nitrite, hypoxanthine, urea and, also, amino acids. One of the favoured sources of inorganic nitrogen is ammonium. Ammonium ions are produced from nitrate. Nitrate ions enter the cell and are processed by what is known as the nitrate assimilation pathway (Pateman *et al.*, 1964; Tomsett and Cove, 1979) (Fig. 1.2.). Briefly, nitrate enters the cell probably *via* the action of a permease; reduction to nitrite; conversion to ammonium ions. Such ammonium ions are further incorporated into glutamate and then glutamine which, in turn, is involved in transamination reactions. The organization and regulation of the nitrate assimilation pathway have been well studied and much is known about the genes and the gene products which are involved in this process (for a review see Wray and Kinghorn, 1989 and references therein).

The permease thought to be involved in nitrate uptake is inferred, from the amino acid sequence (Unkles *et al.*, 1991), to be a 52 kD membrane-spanning transporter protein which is encoded by the gene, *crn*A. Mutants in *crn*A display wild-type growth when nitrate or nitrite



*crn*A - encodes a permease

*nia*D - encodes nitrate reductase

*nii*A -encodes nitrite reductase

*hx*A - encodes purine hydroxylase I

Fig. 1.2. The Nitrate Assimilation Pathway

The nitrate assimilation pathway of *A. nidulans*. Nitrate is taken up into the cell by the action of a permease, encoded by the *crn*A gene. Nitrate is converted to nitrite by the *nia*D gene product - the nitrate reductase enzyme. Nitrite is reduced to ammonium by the *nii*A - encoded nitrite reductase. The *hx*A gene encodes purine hydroxylase I which also requires Mo-co for its function in the creation of uric acid.

are used as sole nitrogen sources, although the kinetics of nitrate uptake in *crn*A mutants are altered (Brownlee and Arst, 1983).

The enzyme responsible for the reduction of nitrate to nitrite in *A. nidulans*, is NADPH-nitrate reductase (Fig. 1.3.). This molecule has been shown to be a homodimer (Cooley and Tomsett, 1985) with total molecular weight of 182 kD (Minagawa and Yoshimoto, 1982). Each subunit contains a flavin-adenine dinucleotide (FAD) and haem prosthetic group. Active enzyme is only formed in the presence of a molybdenum - containing cofactor (Mo-co) (Cove and Coddington, 1965). NADPH is converted to $NADP^+$ as nitrate is reduced. A description of the Mo-co will be given in greater detail in section 1.3. The nitrate reductase enzyme is encoded by a gene designated *nia*D. Mutations in the *nia*D gene lead to the inability to utilize nitrate as sole nitrogen source, while nitrite and ammonium can still be used normally (Pateman *et al.*, 1964).

*nii*A is the third structural gene involved directly in the nitrate assimilation pathway of *A. nidulans* (Cove and Pateman, 1963; Pateman *et al.*, 1967). The *nii*A gene product is the nitrite reductase enzyme which is responsible for the formation of ammonium ions from nitrite. This nitrite reductase molecule has been determined to be approximately 123 kD and to be NADP-dependent (Johnstone *et al.*, 1990). There appears to be marked homology with the *E. coli* protein, which is also NADP-dependent. *nii*A mutants are unable to use either nitrate or nitrite as sole nitrogen sources, although the ability to utilize ammonium is still maintained (Pateman *et al.*, 1964).

These three genes are found to be genetically tightly linked as a gene cluster on chromosome VIII, in the order *crn*A-*nii*A-*nia*D (Tomsett and Cove, 1979). The *nii*A gene is interrupted by seven introns and the

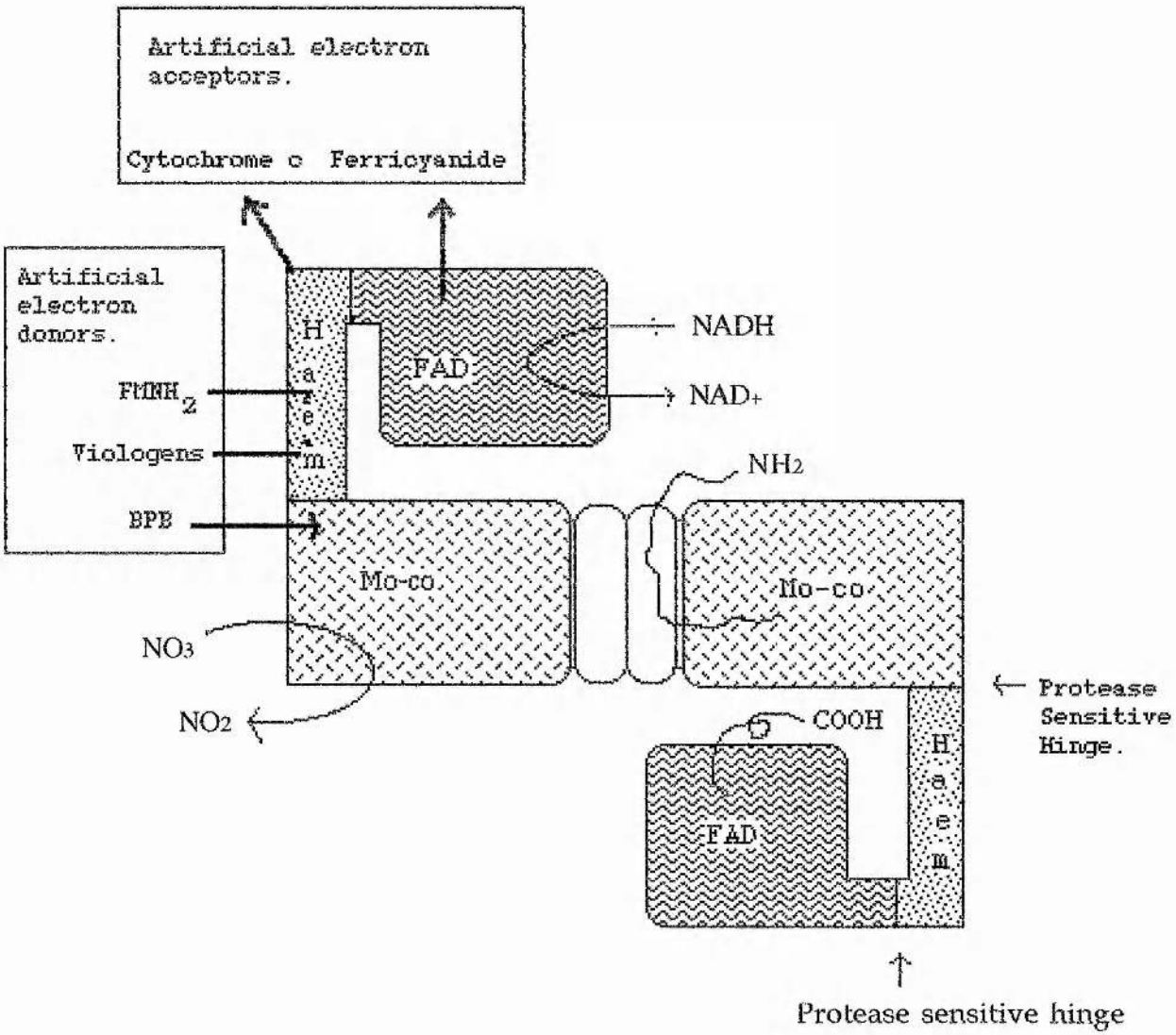


Fig. 1.3. Hypothetical Model of the Structure of the Nitrate Reductase Protein

The proposed structure of the NR molecule. This is the model for the enzyme from higher plants. The basic configuration is probably similar to that found in *Aspergillus nidulans* - the only difference being that the electron donor in the higher plant enzyme is NADH, whereas it is NADPH in fungi. (Taken from Pelsey and Caboche, 1992). The three prosthetic groups of the molecule are shown ie. FAD, haem and Mo-co. Electron donor and acceptor sites are shown.

*nia*D gene six. These two genes have been shown to be divergently transcribed from a 1267 bp intergenic region (Johnstone *et al.*, 1990)

The conversion of ammonium to glutamate is catalysed by glutamate dehydrogenase activity and the further production of glutamine by glutamine synthetase. No more detail will be given on these steps as they are not concerned in this study.

The nitrate assimilation pathway in *A. nidulans* is subject to at least two different kinds of regulation - pathway-specific induction and a more general system of nitrogen metabolite repression (for a review, see Scazzocchio and Arst, 1989). These two activities are catalysed by two genes - *nir*A (Pateman and Cove, 1967) and *are*A respectively (Arst and Cove, 1973)).

The gene *nir*A is responsible for the pathway specific induction of *nii*A, *nia*D and *crn*A by nitrate. It has a positive-acting regulatory role, ie. the gene product switches on structural gene expression. In the absence of ammonium (repressor of the pathway) and in the presence of nitrate, the levels of the nitrate reductase and nitrite reductase enzymes dramatically increase. When the synthesis of enzymes involved in nitrogen utilization are limited by the action of ammonium, it is known as ammonium repression, or nitrogen metabolite repression (Arst and Cove, 1973; Wiame *et al.*, 1985). Furthermore, circumstantial evidence suggests that the nitrate reductase molecule itself regulates the transcription of *nia*D and *nii*A genes (Hawker *et al.*, 1992) and of *crn*A (Unkles *et al.*, 1991). There are two main kinds of mutation which can occur in the *nir*A gene. *nir*A$^-$ mutants show phenotypic growth as in *nii*A mutants ie. they cannot utilize nitrate or nitrite as sole nitrogen sources, but can use ammonium. Induction by nitrate does not occur. These mutants can be distinguished from *nii*A mutants by the excretion

of nitrite. *nir*A$^-$ mutants do not excrete nitrite, in contrast to *nii*A mutants (Cove, 1979). The mutants *nir*A^c show constitutive expression of nitrate reductase, even in the absence of nitrate (Rand and Arst, 1978). There is also a third, albeit much rarer, class of *nir*A mutant, designated *nir*A^d, These mutants again show constitutive expression of nitrate reductase and nitrite reductase, but also exhibit ammonium derepression (Rand and Arst, 1978).

Nitrogen metabolite repression of the pathway in *A. nidulans* is mediated by the wide-domain regulatory gene, *are*A. This is, again, a positive-acting gene which enables favoured nitrogen sources such as ammonium and glutamine to be used in preference to alternative nitrogen sources by repressing the synthesis of enzymes involved in the utilization of these other nitrogenous compounds. The *are*A gene product is required for the expression of structural genes whose gene products are susceptible to repression by ammonium or glutamine. The extracellular ammonium concentration affects the level of nitrate reductase, while the intracellular ammonium concentration regulates the rate of ammonium uptake (Pateman *et al.*, 1973).

Two types of *are*A mutant have been identified - the most common designated *are*A^r. These mutants can only utilize ammonium as a nitrogen source. *are*A - controlled enzymes are found at very low or undetectable levels (Rand and Arst, 1977). A rarer class of mutant, designated *are*A^d, can also be isolated. Ammonium derepression results, with all the nitrogen metabolite-repressible enzymes being synthesized even in the presence of ammonium. Phenotypic growth of *are*A^d mutants is as wild-type (Arst and Cove, 1973).

The AREA protein (76 kD) is a DNA-binding protein as it was shown to contain several putative DNA-binding motifs. For instance, the

sequence of the protein is rich in SPXX and TPXX regions, (X - any nucleotide) and a zinc-finger consensus sequence is also noted -

- $Cys\text{-}X_2\text{-}Cys\text{-}X_{17}\text{-}Cys\text{-}X_2\text{-}Cys$- (Caddick *et al.*, 1986).

It has been postulated that, in the regulation of the nitrate assimilation pathway, the *nir*A and *are*A gene products interact in some way. It has been suggested that the NIRA protein contains two different 'domains' - one where $nirA^{-}/nirA^{c}$ mutations occur, and one site where derepression occurs ($nirA^{d}$). In the presence of ammonium, the AREA protein could bind to the NIRA protein at this 'derepression' site, thus inactivating it (Scazzocchio and Arst, 1989).

1.3. THE MOLYBDENUM COFACTOR

It was in the early 1960's, whilst working on a series of *A. nidulans* mutants unable to grow on nitrate or hypoxanthine, which lacked both nitrate reductase and xanthine dehydrogenase activities, that Pateman *et al.* (1964) first postulated the existence of a cofactor which was common to both these enzymes. The synthesis of this cofactor requires six gene loci, designated *cnx*ABC, *cnx*E, *cnx*F, *cnx*G, *cnx*H (Pateman *et al.*, 1964) and *cnx*J (Arst *et al.*, 1982). The gene designation *cnx* reflects the cofactor for nitrate reductase and xanthine dehydrogenase.

A decade later, Nason and colleagues discovered that *nit-1* mutants of *Neurospora crassa* - *nit*-1 mutants having the same phenotype as *cnx* mutants, (unable to utilise nitrate or hypoxanthine as sole nitrogen source) could be reconstituted (gain a wild-type phenotype) *in vitro* by the addition of a suitable source of molybdenum cofactor (Nason *et al.*, 1970). Examples of 'suitable sources' include xanthine oxidase from bovine milk, aldehyde oxidase from rat liver, xanthine dehydrogenase from chicken liver and nitrate reductases from *E. coli* and

from foxtail (Ketchum *et al.*, 1970; Nason *et al.*, 1971). It was suggested, therefore, that all these molybdo-enzymes contained a universal cofactor, which apparently functioned in a similar manner across the species.

Later, however, Shah and Brill isolated a cofactor from the enzyme nitrogenase which was found to contain not only molybdenum, but also iron, and was unable to reconstitute *nit*-1 mutants of *N. crassa* (Shah and Brill, 1977). It therefore appears that the cofactor of nitrogenase (designated FeMo-co) is different to other molybdenum cofactor (Mo-co), which is universal to all other known molybdo-enzymes. The *nit*-1 reconstitution system was the first one used as an assay for the presence of molybdenum cofactor. It is a very useful laboratory assay system, as it is relatively simple and relatively inexpensive to carry out in practice. However, it is also a non-quantitative measure of molybdenum cofactor, since many of the inactive nitrate reductase molecules are degraded under *in vitro* conditions (Coughlan, 1980 and references therein).

A further assay system was developed and this involved the sulphite oxidase enzyme from rat liver. Inactive sulphite oxidase is synthesized in rats fed a molybdenum - deficient diet but which is supplemented with tungsten (Johnson *et al.*, 1974). Tungsten is an analogue of molybdenum and can insert into the cofactor in place of the molybdenum, thus creating an inactive cofactor. Sulphite oxidase isolated from these rats was found to contain two populations of molecule - some 30% contained the inactive tungsten-cofactor, whilst the other 70% contained no cofactor at all. Addition of Mo-co from a variety of sources was found to be able to reconstitute sulphite oxidase activity in the 'empty' molecules but not in those which already contained W-co (Johnson *et al.*, 1977). From experiments with cycloheximide, which prevents protein synthesis, it is seen that this reactivation is due to re-

insertion of Mo into the cofactor and not to synthesis of new cofactor. This system provides a quantitative assay of Mo-co activity, but it is not however used in practice, mainly because it is expensive and time consuming as well as the fact that it is sensitive to many of the solvents used and much inhibition is seen within the system (Coughlan, 1980 and references therein).

Sulphite oxidase has been isolated from a variety of sources and examined in some detail. It is the enzyme which catalyzes the conversion of sulphite to sulphate. It is generally composed of a homodimer of subunits of MW 55 kD, each containing Mo-pterin, cytochrome b and haem. The molybdopterin is the site of sulphite oxidation and reducing equivalents are transferred to cytochrome c *via* the cytochrome b_{557} prosthetic group. Rat liver sulphite oxidase contains a unique cis-dithiolene group which acts as a ligand to the Mo atom. Mo and haem are in a 1:1 ratio (Johnson *et al.*, 1977).

The expression of active rat sulphite oxidase in *E. coli* was the first demonstration of a eukaryotic Mo-protein being expressed in a prokaryotic system (Barber and Neame, 1990; Garrett and Rajagopalan, 1994).

Although Mo-co is found in a number of enzymes, such as sulphite oxidase, purine hydroxylase and nicotine dehydrogenase, it is as part of the nitrate reductase system in which most of the available information has been gathered. What exactly is the function of this cofactor which appears to play such a vital role in such biochemical pathways as those involving the above enzymes? The function and processes in which Mo-co is involved is still largely unclear. It is thought to play an important part in the stabilization of the various enzymes in which it is incorporated (Nason *et al.*, 1970; MacDonald *et al.*, 1974).

Additionally, it is known that the cofactor is necessary for the dimerization of the subunits of the nitrate reductase protein in *A. nidulans*, although the Mo atom itself does not actually take part in this action. However, it is thought that the presence of the metal is vital for the maintenance of subunit interaction. It does seem evident though that the main function of the Mo is as part of the electron transport chains catalyzing the various enzyme reactions with which it is involved. The *A. nidulans* nitrate reductase molecule exerts its action via one FAD group, one haem iron and one Mo atom. The FAD is reduced by NADPH and re-oxidised by nitrate. Mo-co is also seen to contain a cytochrome b_{557} activity. It is postulated that the Mo acts as the terminal electron donor in nitrate reductase. The electron transport sequence is thought to be -

$$NADPH \longrightarrow SH \longrightarrow FAD \longrightarrow Fe \longrightarrow Mo \longrightarrow NO_3.$$

With the Mo liganded to nitrate, reduction to Mo (oxidation state IV) may occur, with rapid oxidation to Mo (oxidation state VI) and the concomitant release of nitrite (Hewitt, 1974).

The pathway of electron flow varies from organism to organism and from enzyme system to enzyme system. Nitrate reductase of *Neurospora crassa* comprises two haem components (Garrett and Nason, 1969) while the *E. coli* molecule comprises some forty Fe-S centres (Taniguchi and Itagaki, 1960). The kinetics of the nitrate reductase reaction in *A. nidulans* are of random order, (MacDonald, 1969) and various hypotheses for the reaction mechanism have been presented, as an alternative to the Mo-NO_3 liganding theory. For example, reactions could be as follows -

1). $Mo(V) + NO_3 + H^+ - Mo(VI) + NO_2 + OH^-$

$= 2NO_2 = N_2O_4 = NO_3 + NO^+$

$= Mo(V) + NO^+ - MO(VI) + NO$

= $NO^+ + OH^-(H_2O) - NO_2 + H^+(2H^+)$ (Guymon and Spence, 1966).

OR,

2). $FMNH_2 + Mo(VI) = FMN + Mo(IV) + 2H^+$

= $\underline{Mo(VI0 = Mo(IV) = 2Mo(V)_2 \text{ (dimer)}}$

Sum $FMNH_2 + 2Mo(VI) = FMN + 2Mo(V) - [Mo(V)]_2 + 2H^+$ (Coughlan, 1980 and reviews therein).

The cellular location of the various molybdo-enzymes varies, depending upon the enzyme and the organism in question. *A. nidulans* nitrate reductase is a cytoplasmic protein, whereas it is found as a membrane-bound species in *E. coli* where it functions under anaerobic conditions, utilising nitrate as the terminal electron acceptor instead of oxygen (Stouthamer, 1976). Rat liver sulphite oxidase, another Mo-containing enzyme is found in the intermembrane space of mitochondria (Garrett and Rajagopalan, 1994). Expression of molybdo-enzymes seems to be different between organisms. Synthesis of the *A. nidulans, N. crassa* and *E. coli* Mo-co is constitutive whereas in other organisms it is inducible and/or repressible in the presence of certain ions. In the nitrate reductase system of *Aspergillus* and *E.coli* (Amy and Rajagopalan, 1979), tungsten can insert into the cofactor in place of the molybdenum, however resulting in an inactive enzyme. Uniquely, tungsten can substitute molybdenum, to give active formate dehydrogenase in *Clostridium thermoaceticum* (Ljungdahl and Andreeson, 1975).

Mo-co which is not incorporated in the holoenzyme in the cell is thought to be attached to a low MW 'carrier' protein. Mo-co is very unstable and oxygen - labile, and so it appears that this 'carrier' molecule protects the Mo-co from the destabilizing effects of oxygen. A 40 kD protein has been identified in *E.coli* which is loosely bound to Mo-co and can easily dissociate (Amy and Rajagopalan, 1979). It is not known how

dissociation occurs, but it is not by a proteolytic mechanism. Acid treatment also fails to cause dissociation. Mo-co is fairly stable in the pH range 3-11, with activity diminishing rapidly at pH < 3 (Amy and Rajagopalan, 1979). In the green alga *Chlamydomonas reinhardtii*, Mo-co is found tightly bound to a 50 kD carrier protein (Aguilar *et al.*, 1991). As will be discussed later in this thesis, it is thought that the product of one of the *cnx* genes of *A. nidulans* is responsible for the synthesis of the appropriate carrier molecule.

Mo-co is an association of molybdenum with a pterin molecule, and has been found to be weak in the absence of the anchoring ligands. (Fig. 1.4). The ligands around Mo are disrupted upon denaturation of the molecule, thus leaving Mo in a position so as to be easily removed from the pterin. Mo can be easily displaced by eg. mercury, arsenite and sulph-hydryl reagents (Kramer *et al.*, 1987). The extreme lability of the Mo-co renders it very difficult to analyze. The structure of Mo-co from sulphite oxidase and xanthine oxidase was determined *in vitro via* two fluorescent derivatives which form upon oxidation. Studies on these two derivatives, designated form'A' and form'B' (Fig.1.4), led to the elucidation of the chemical nature and structure of the universal Mo-co (Kramer *et al.*, 1987).

Examination of form 'A' led to the conclusion that Mo-co was based upon a pterin structure which contained a 4C side chain with a phosphate group. The absence of any sulphur or iodine was also demonstrated. Form 'B' highlighted the presence of S in the pterin (Johnson *et al.*, 1984). The structural conclusions which were drawn from these studies were as follows -

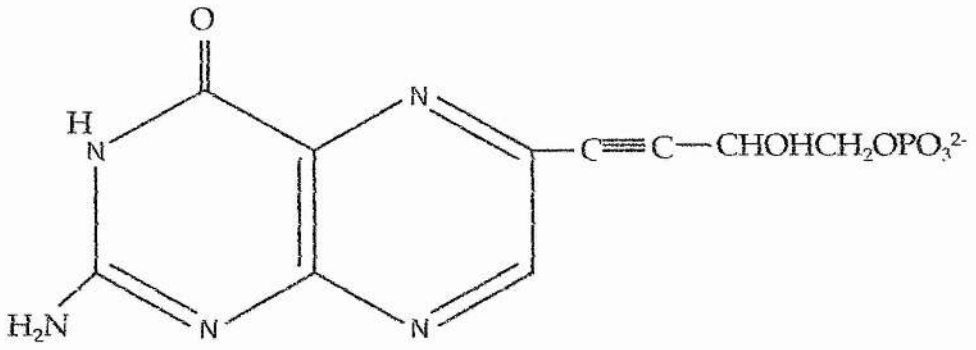
Mo-co contains -

- a 6 alkyl pterin with a 4C side chain.

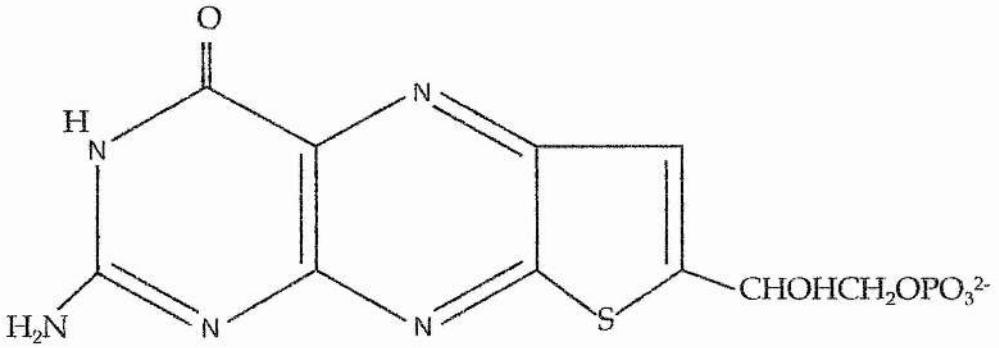
Fig. 1.4. The Structure of Mo-co as Determined from its Derivatives

The structure of Form 'A' and Form 'B' Mo-co derivatives which were used in deducing the actual structure of the molybdenum cofactor molecule, also shown here (Kramer *et al.*, 1987). These compounds are formed *in vitro,* enabling the elucidation of the Mo-co structure. Mo-co itself cannot be readily examined *in vitro*.. Physical analyses on the structures resulted in the deduced form of the cofactor.

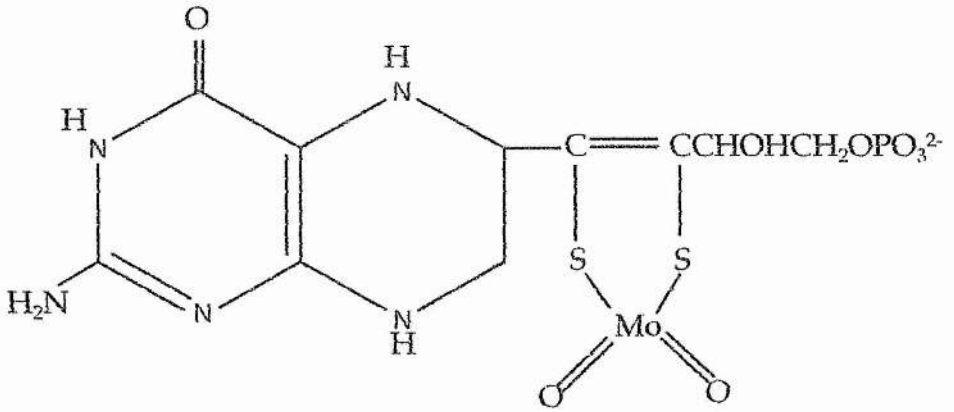
Mo-co DERIVATIVE - FORM 'A'.



Mo-co DERIVATIVE - FORM 'B'.



MOLYBDENUM COFACTOR.



- a terminal glycol function linked to a phosphate ester.
- sulphur atoms on C^1 and C^2, which are thought to act as ligands to the Mo atom.
- a double bond between C^1 and C^2 (Kramer *et al.*, 1987).

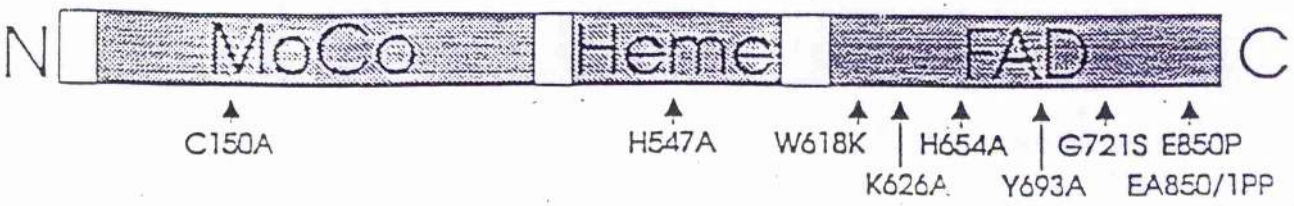
Johnson and colleagues published the deduced structure of Mo-co in 1984. See Fig. 1.4 (For a review of Mo-co chemistry refer to Rajagopalan and Johnson, 1992).

1.4. THE MOLYBDENUM COFACTOR OF NITRATE REDUCTASE

The DNA sequence of the gene, *nia*D, encoding the *A. nidulans* NR enzyme has been analysed, and site-directed mutagenesis carried out at specific sites within the haem, FAD and Mo-co domains, in order to establish the importance of various amino acids in maintaining function (Garde *et al.*, 1995).

Mutation *niaDc150A*, generated by *in vitro* means, substituted alanine for Cys^{150}, a conserved cysteine in the Mo-co domain which is thought to participate in liganding the Mo atom. Another mutation, *nia*DH547A, substituted alanine for His^{547} - a conserved residue which is thought to play a role in haem binding. Also, various other mutations were also created in the FAD domain. The *nia*Dc150A mutation completely abolishes NR, BPB:NR and MVH:NR activities, whilst the *nia*DH547A mutant exhibits no NR or MVH:NR (haem-requiring) activity, although BPB:NR activity (Mo-co requiring) is unchanged.

This indicates a critical catalytic role for His^{547} in the haem domain and for Cys^{150} in Mo-co interaction. It is also thought that Cys^{150} may be involved in subunit dimerization. Studies further imply important roles for His^{654}, Lys^{626} and Trp^{618} within the FAD domain. The results presented in Fig. 1.5 summarise the positions of the



	DNA sequence	Amino acid sequence
wild-type	CTA GTA TGT GCA CCG	L V C A G
niaDC150A	''' ''' GC' ''' '''	' ' A ' '
wild-type	GAG GGA CAC CCC GGC	E G H P G
niaDH547A	''' ''' GC' ''' '''	' ' A ' '
wild-type	AAA GCC TGG ACA AAA	K A W T K
niaDW618K	''' ''' AA' ''' '''	' ' K ' '
wild-type	ACG AAG AAA ACA TCT	T K K T S
niaDK626A	''' ''' GC' ''' '''	' ' A ' '
wild-type	GGC CAG CAC CTG ATG	G Q H L M
niaDH654A	''' ''' GC' ''' '''	' ' A ' '
wild-type	AAA ATC TAC GCC GAG	K I Y A E
niaDY693A	''' ''' GG' ''' '''	' ' A ' '
wild-type	TGC AAG GGT CCT ACT	C K G P T
niaDG721S	''' ''' A'' ''' '''	' ' S ' '
wild-type	GGG CCT GAG GCG ATG	G P E A M
niaDE850P	''' ''' CC' ''' '''	' ' P ' '
wild-type	GGG CCT GAG GCG ATG	G P E A M
niaDEA850/1PP	''' ''' CC' C'' '''	' ' P P '

Fig. 1.5. Site - Directed Mutagenesis of Nitrate Reductase

The position of the various mutations created within the Mo-co, haem and FAD regions of the nitrate reductase sequence of *Aspergillus nidulans*, and the DNA (and amino acid) changes which resulted in the mutation shown. (Taken from Garde *et. al.*, 1995).

mutations within the various domains and a summary of the consequent amino acid changes.

Parallel mutagenesis work has also been carried out in order to study the haem domain of the nitrate reductase of *N. crassa*. The crystal structure of bovine cytochrome b5 was used to predict the tertiary structure of the haem domain of the nitrate reductase molecule, as both proteins share considerable homology. Such studies revealed that two ligands - His^{675} and His^{652} - appeared to be involved in binding the haem iron. Ser^{676} and Ile^{659} are also implicated in playing a vital role in the maintenance of a stable tertiary structure of the protein (Okamoto and Marzluf, 1993).

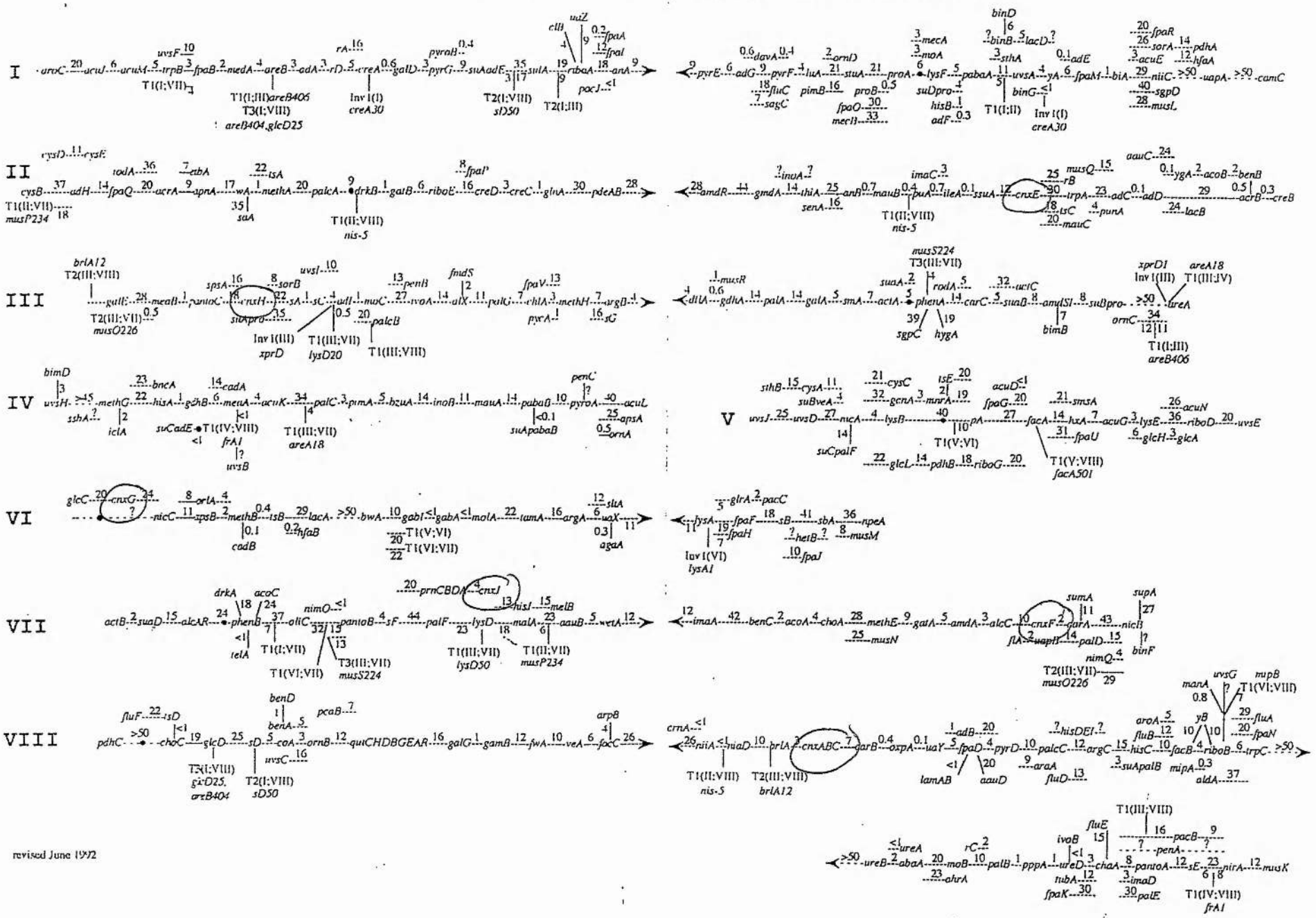
1.5. THE *cnx* GENES

The *cnx* genes which are necessary for the biosynthesis of the molybdenum cofactor in *A. nidulans* are not found as part of an operon (Cove and Pateman, 1963) - a system which is commonly found in bacteria with genes of related function. Nor are the genes tightly linked, or even found close together within the *A. nidulans* genome. The six known loci, designated *cnx*ABC, *cnx*E, *cnx*F, *cnx*G, *cnx*H and *cnx*J, are found scattered over several of the eight host chromosomes. A well-defined genetic linkage map has been 'built up', over the decades, for this organism, (Fig.1.6) with genetic recombination studies giving an insight into the estimated distance between the genes along each chromosome. The *cnx*ABC locus has been genetically assigned to chromosome VIII, while *cnx*E locates on chromosome II, *cnx*F on chromosome VII, *cnx*G on chromosome VI, *cnx*H on chromosome III and *cnx*J also mapping to chromosome VII. Although many genes in *A. nidulans* have now been

Fig. 1.6.

The genetic map of *Aspergillus nidulans*, showing the eight linkage groups, indicating where genes lie within each chromosome. The *cnx* genes are circled. The map units are centiMorgans. (Clutterbuck, 1993).

Linkage Map of *Aspergillus nidulans*



cloned and sequenced, there is very little information known regarding the *cnx* loci at the molecular level.

The *cnx*ABC locus comprises three complementation groups. Mutations in *cnx*A and *cnx*C can phenotypically complement each other, resulting in growth on medium containing nitrate as sole nitrogen source, but neither group shows complementation with *cnx*B (Cove and Pateman, 1963). It was not clear whether *cnx*ABC consists of two closely linked genes (*cnx*A and *cnx*C) or whether it is one gene showing intra-cistronic complementation (Scazzocchio, 1974), *i.e.* one gene providing a gene product with more than one function which can exhibit a wild-type phenotype when there is at least one functional copy of each part of the gene present. No possible role for the *cnx*ABC gene product(s) has been suggested. It is only known, due to the *cnx*ABC mutant growth phenotype, that it is somehow involved in biosynthesis of the molybdenum cofactor.

Growth of *cnx*E mutants on nitrate or hypoxanthine as sole nitrogen source can be partially restored by supplementation of the medium with 33 mM molybdate. The *A. nidulans* nitrate reductase molecule has a sedimentation coefficient of 7.6 S. Some of the *cnx* mutants exhibit a 4.5 S species and others display both a 4.5 S and a 7.6 S compound. *cnx*E mutants, however, have been shown to contain only the 7.6 S form (MacDonald *et al.*, 1974). It has therefore been postulated that *cnx*E mutants contain only 'empty' cofactor. This would also supplement the observation that the mutation is molybdenum - repairable. This phenomenon is seen in all *cnx*E mutants, indicating that this is a locus effect and not an allelic one. Expression of nitrate reductase enzyme within different *cnx*E mutants also varies. *cnx*E-13 shows constitutive expression, whereas synthesis in *cnx*E-14 is inducible.

Constitutive *cnx*E mutants show molybdenum - repairability of chlorate resistance (Arst *et al.*, 1970).

Phenotypic temperature - sensitive mutants of *cnx*E have been isolated, which have a nitrate reductase half-life indistinguishable from that of the wild-type, indicating that the *cnx*E gene product confers a catalytic activity as opposed to being an actual structural component of the molybdenum cofactor (MacDonald and Cove, 1974).

Nitrate reductase may also catalyse the reduction of chlorate to chlorite (Cove, 1976a). Wild-type *Aspergillus* strains are sensitive to chlorate whereas *cnx* mutants exhibit chlorate resistance. It was once thought that the toxic effects of chlorate were due to its conversion to chlorite by nitrate reductase. It is now seen, however, that this is not primarily the cause, but is only part of a more complicated scenario. Cove analysed various nitrate reductase mutants under various conditions and proposed a few theories as to what may or may not be involved in chlorate toxicity (Cove, 1976a and 1976b).

Most mutants lacking NR activity are chlorate resistant, whilst some others are chlorate sensitive. The toxicity of chlorate on wild-type varies, depending upon the nitrogen source used, as does the concentration of chlorate required to bring about inhibition. Chlorate resistance in *nia*D and *cnx* mutants is correlated to their reduced ability to utilize nitrogen sources. So, does the role of *nia*D and the cofactor vary, depending upon the nitrogen source used?

Two types of *cnx* mutant are seen - those which show inducible synthesis of NiR and those which show constitutive expression of NiR. Mutants which show inducible NiR are chlorate sensitive, whilst those which have constitutive NiR are chlorate resistant. However, Cove (1976a) showed that chlorate resistance is not due to the constitutive

synthesis of NiR. He also examined the role of the pentose phosphate pathway and concluded that it was not involved in chlorate toxicity. It is noted, however, that chlorate toxicity on some nitrogen sources can be alleviated by carbon catabolite de-repression, and it is known that the NR molecule and the *nir*A gene product are involved in the process of chlorate toxicity.

With regard to *cnx*F, the role of its gene product is also unknown. Again, phenotypic temperature - sensitive mutants at this locus have been identified (MacDonald and Cove, 1974). Apart from being known to be involved in the biosynthesis of the active molybdenum cofactor, no details are available as to the possible function of *cnx*G. It is hypothesized that the *cnx*H gene product is an integral peptide component of the nitrate reductase protein. Temperature - sensitive mutants of *cnx*H which have a nitrate reductase half-life considerably lower than wild-type have been identified, suggesting the peptide nature of the resultant molecule (MacDonald and Cove, 1974). It has been proposed that the *cnx*H protein is the putative carrier molecule for Mo-co (MacDonald and Cove, 1974; Scazzocchio, 1974). Mutants in *cnx*H which are temperature - sensitive on nitrate are seen to be still fully mutant for growth on hypoxanthine, at both 25°C and at 37°C, indicating a more stringent requirement for cofactor in xanthine dehydrogenase than is called for in nitrate reductase (MacDonald and Cove, 1974).

A further *cnx* locus in *A. nidulans*, designated *cnx*J, is thought to encode a regulatory function of the Mo-co system. *cnx*J mutants are able to grow on nitrate as sole nitrogen source; the *cnx*J phenotype only being selected for on medium containing tungsten. 20 mM tungstate has been shown to inhibit growth of *cnx*J mutants while still enabling growth of wild-type strains. (w.t. is inhibited by 33 mM tungstate). The presence of

tungstate will lead to the production of inactive cofactor in Mo-deficient conditions. This evidence suggests that the *cnx*J gene product encodes a Mo-regulatory or Mo-uptake system (Arst *et al.*, 1982), as the concentration of available molybdenum is obviously impaired in *cnx*J mutants.

1.6. THE PURINE HYDROXYLASES

The enzyme xanthine dehydrogenase is now called purine hydroxylase (PH) and has been shown to exist in two forms - PHI and PHII. PHI utilizes xanthine as a substrate, but not nicotinic acid, whereas PHII can use nicotinic acid but not xanthine (Lewis *et al.*, 1978). Both enzymes are able to hydroxylate hypoxanthine. *hxA* is the structural gene for PHI and is found on chromosome V, while *hxnS* encodes the structural component of PHII, and is located on chromosome VI. Both enzymes are subject to nitrogen metabolite repression and are induced in medium containing purines. PHII is specifically repressed by nitrate. It has been suggested that the PHII core can be recycled to assemble PHI (Scazzocchio, 1973). PHII has been shown to have a pI of 5.7 and to contain 0.96 Mo per FAD and 4.25 Fe per FAD (Mehra and Coughlan, 1984). A class of mutant, designated *hxB*, shows loss of both PHI and PHII activities, with no effect on the nitrate reductase system. NADH dehydrogenase activity is also conserved, indicating that the *hxB* mutation probably does not affect the FAD site (Scazzocchio, 1973).

The *hxA* gene which codes for purine hydroxylase I of *A. nidulans* has been cloned and sequenced. The inferred PHI protein is a 304 kD homodimer, with each subunit containing two Fe-S centres, FAD and Mo. The sequence of various purine hydroxylases have been compared and a conserved sequence has been postulated to be the

putative molybdenum cofactor binding domain. This sequence comparison is shown in Fig. 1.7. The cysteine residue marked with an asterix is the conserved residue found to be so critical in studies by Garde *et al.*, 1995. (Refer to Fig. 1.5. for the mutational work done on this residue).

In *A. nidulans*, a conserved motif - ERXXXH - is found just downstream of the putative Mo-co binding domain. It is thought to be involved in determining substrate specificity (Glatigny and Scazzocchio, 1995). Studies on *Nicotiana plumbaginifolia* nitrate reductase mutants highlighted four residues within the postulated Mo-co binding domain which are critical for function (Meyer *et al.*, 1995).

1.7. GENES INVOLVED IN OTHER ORGANISMS

The nitrate reductase / molybdenum cofactor system has also been examined in another fungus, ie. *Neurospora crassa* (Tomsett and Garrett, 1980; Kramer *et al.*, 1984; Heck and Ninnemann, 1995). Knowledge of the genetics of the *N. crassa* mechanism may help shed some light on the function of the equivalent genes in *A. nidulans*. Many features of the nitrate reductase enzyme are common to both organisms: the molecules contain more than one subunit; FAD, haem and Mo are all necessary for activity; a cytochrome b_{557} function is present and the transfer of electrons is thought to be as follows -

NADPH-FAD-cytochrome b_{557}-Mo-NO_3 (Nason *et al.*, 1970).

The *N. crassa* enzyme is also induced by nitrate, and synthesis and regulation of the components of the enzyme are controlled by nine known gene loci, similar to the case in *A. nidulans*. The homologous genes have been identified and designated *nit*. The *nit*-1 phenotype is the same as that of an *Aspergillus cnx* gene ie. unable to utilize nitrate or

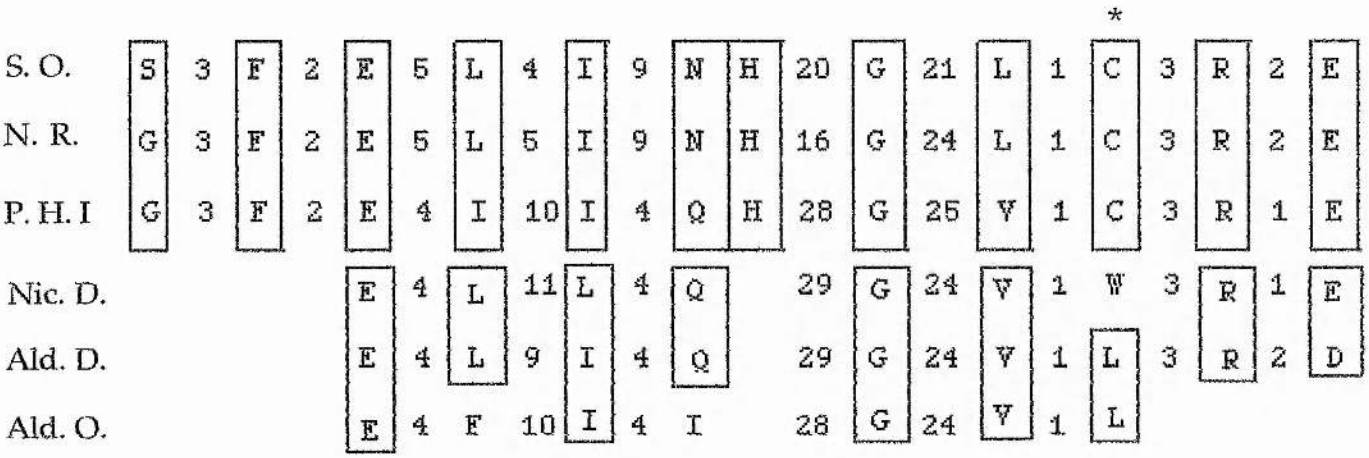


Fig. 1.7. The Putative Mo-co Binding Domain

Consensus sequences of conserved amino acids thought to constitute the Mo-co binding domain of various ezymes. The numbers refer to the residues found between conserved amino acids.

S. O. - sulphite oxidase (rat and chicken)

N. R. - nitrate reductase (ten different sources)

P. H.I - xanthine dehydrogenase (A. nidulans)

Nic. D. - nicotine dehydrogenase (prokaryotic)

Ald. D. - aldehyde dehydrogenase (prokaryotic)

Ald. O. - aldehyde oxidoreductase (prokaryotic)

Taken from Glatigny and Scazzocchio, 1995. For details see references therein.

hypoxanthine as sole nitrogen sources. *nit*-2 has been shown to be a wide-domain regulatory gene which acts upon the nitrate reductase system, and can, in fact, complement an *are*A mutant from *A. nidulans*, suggesting that *nit*-2 is analogous to this global gene (Fu and Marzluf, 1990). *nit*-3 is the structural gene for the *N. crassa* nitrate reductase molecule, rendering it equivalent to the *nia*D gene (Fu and Marzluf, 1987). The *nit*-4/5 locus is a homologue of *nir*A, the pathway - specific regulator necessary for nitrate induction. Finally, it is seen that the nitrite reductase molecule is encoded by *nit*-6, the similar gene to *nii*A (Exley *et al.*, 1993). Three loci *nit*-7, *nit*-8 and *nit*-9 (consisting of A, B, and C genes) have also recently been identified (Tomsett and Garrett, 1980).

Another molybdenum cofactor system which has been extensively examined is that in *E. coli*. Chlorate resistant mutants defective in most if not all molybdo-enzyme activities have been isolated (Dubourdieu *et al.*, 1976). At least seven gene loci are known to be involved. These have been designated *chl*A, *chl*B, *chl*C, *chl*D, *chl*E, *chl*G and *chl*N (Miller and Amy, 1983).

The *chl*A gene product is known to be involved in the synthesis of the molybdopterin moiety of the cofactor (Johnson and Rajagopalan, 1987). Expression of *chl*A is subject to aerobic repression and also to repression by the Mo-co itself. The anaerobic enhancement of *chl*A gene synthesis is independent of the *fnr* gene product which is a transcriptional regulator under anaerobic conditions. (Baker and Boxer, 1991). In *chl*A mutants, active Mo-co is prevented from being incorporated into the cell membrane (Amy, 1981).

*chl*B mutants are the only Mo-enzyme mutants in *E. coli* which can complement *nit*-1 mutants *in vitro*. These mutants contain functional Mo-co. The defect lies in the final maturation step in the

biosynthetic pathway common to *E. coli* Mo-enzymes. The *chl*B gene product is thought to be involved in the insertion of Mo-co into the membrane. Mo-co in *E. coli* also contains a GMP moiety attached to the Mo-pterin to form molybdopterin guanine dinucleotide (MGD). Mutants in *chl*B synthesize Mo-pterin, but lack MGD, indicating that the *chl*B protein is involved in MGD biosynthesis (Johnson *et al.*, 1991). Mutants in *chl*B fail to synthesize a protein, designated protein FA, which is expressed in all other *chl* mutants, suggesting that protein FA is the product of the *chl*B gene (Low *et al.*, 1988).

It has also been shown that a low MW, heat-stable substance distinct from Mo-co is also necessary for nitrate reductase activity. This molecule is thought to be GTP. Factor X, an unidentified protein component is also indispensable for *E. coli.* nitrate reductase activity (Santini *et al.*, 1992).

[The *E. coli* Mo-co contains an MGD component. Other molybdo-enzymes in other organisms also contain dinucleotide variants. eg. carbon monoxide dehydrogenase from *Pseudomonas carboxydoflava* contains molybdopterin cytosine dinucleotide (MCD), and formylmethanofuran dehydrogenase from *methanobacterium thermoautotrophicum* contains molybdopterin adenine dinucleotide (MAD) and molybdopterin hypoxanthine dinucleotide (MHD)] (Joshi and Rajagopalan, 1994).]

The *chl*C locus, now called the *nar* locus, was shown to consist of an operon of three genes - *nar*CHI which encode the three subunits of the NR molecule. Just upstream is another operon - *nar*LXK which codes for proteins involved in the regulation of the *nar*CHI operon. This regulation is mediated via anaerobiosis and by nitrate (Stewart and Parales, 1988).

A *chl*D mutation can be repaired by growth on 500 μM molybdate, this being indicative of the *chl*D gene product having a role in Mo-transport. Expression of the ChlD protein is reduced by Mo concentrations >10 nM, and enhanced by Mo-deprivation, further vindicating the suggestion of a Mo-uptake function. *chl*D appears to be analogous to *cnx*E in *A. nidulans* (Glaser and DeMoss, 1971).
Note that the differences in molybdate concentration required for repair of the mutant phenotype in the two organisms - 500 μM in *E. coli* and 33 mM in *A. nidulans*.

After sequence analysis of the *chl*D region, Johann and Hinton (1987) found an ORF which coded for a protein which showed characteristics of being a hydrophobic inner membrane transport molecule.

It is thought that *chl*E is also involved in synthesis of the Mo-pterin and that the gene product has a role in activation of *chl*A (Johnson and Rajagopalan, 1987). The function of *chl*G is still unclear, although it is noted that *chl*G mutants are unable to synthesize molybdopterin.

The *chl* loci (A to G) are now sometimes refered to as *moa* to *mog* respectively. Table 1.1. shows the old and new designations of these gene loci. Further analysis of the *moa*A locus shows that it is actually an operon consisting of five tightly linked genes, designated *moa*A to *moa*E. A special so-called 'converting factor' (Pitterle and Rajagopalan, 1989) which is involved in the biosynthesis of the Mo-pterin part of the Mo-co, is made up from two protein subunits of 10 kD and 16 kD (Pitterle and Rajagopalan, 1993). These two subunits are encoded by *moa*E and *moa*D respectively. Converting factor works in conjunction with precursor Z to form Mo-pterin (Pitterle *et al.*, 1993). The gene product of another chlorate resistance locus, designated *chl*N, is concerned with creating a

Old Name	New Designation	Gene Product(s)	Function
*chl*A	*moa* (A-E)	MOA(A,B,C,D,E)	Synthesis of converting factor
*chl*B	*mob*A	MOBA	Encodes protein FA
	*mob*B	MOBB	Encodes GTP binding protein
*chl*C	*nar* (C,H and I)	NAR(C,H,I)	Encode NR subunits
*chl*D	*mod* (A-D and R)	MOD(A,B,C,D,R)	Mo transport
*chl*E	*moe*A	MOEA	Mo-pterin synthesis
*chl*G	*mog*	MOG	Incorporation of Mo
*chl*N	*moe*B	MOEB	Creates active sulphur on small subunit of converting factor

Table 1.1. Recent *E. coli* Gene Nomenclature

Table showing the recent names assigned to the *chl* loci of *E.coli*. The function of the gene products is also shown. As can be seen, many of the 'genes' are now known to be operons consisting of several genes.

reactive sulphur on the small subunit of the converting factor. See Fig. 1.8.

The *mod* locus has also been fully sequenced and is seen to be an operon consisting of four genes - *mod*ABCD (Maupin-Furlow *et al.*, 1995). This is the Mo-specific *E. coli* transport system, which is regulated by Mo availability (Rech *et al.*, 1995).

A further locus has also been identified; that of *mol*R, which is thought to be involved in the regulation of Mo-co synthesis (Rivers *et al.*, 1993). *mol*R mutants are also Mo-repairable for growth on nitrate and are therefore also possibly defective in Mo transport (Lee *et al.*, 1990). Mo-uptake mutants, distinct from *mod* and *mol*R have also been isolated (Hemschemeier *et al.*, 1991). Three independent transport systems are found for Mo uptake in *E. coli* - the *mod* system, the sulphate system (*cys*TWA) which can be used when the *mod* transporter is unavailable, and an undefined non-specific mechanism which is active under conditions of high molybdate concentration (Rosentel *et al.*, 1995).

The fact that similar systems, necessary for the synthesis of the universal Mo-co are in operation, is further suggested by the isolation of *cnx* analogues in the plants *Arabidopsis thaliana* (La Brie *et al.*, 1992; Pelsy and Caboche, 1992; Hoff *et al.*, 1995) and *Nicotiana. plumbaganafolia* (Pelsy and Caboche, 1992), and in the fruit-fly *Drosophila melanogaster* (Kamdar *et al.*, 1994). It is known that genes responsible for the synthesis of Mo-co are found right through the species, including humans. Mo-co is a crucial molecule which plays a very important role in the functioning of many enzymes such as sulphite oxidase and xanthine dehydrogenase. Just how indispensable active Mo-co is, can be demonstrated when a baby is born with a genetic defect in the Mo-co system. High levels of brain damage are seen with severe malformities, along with other disabilities

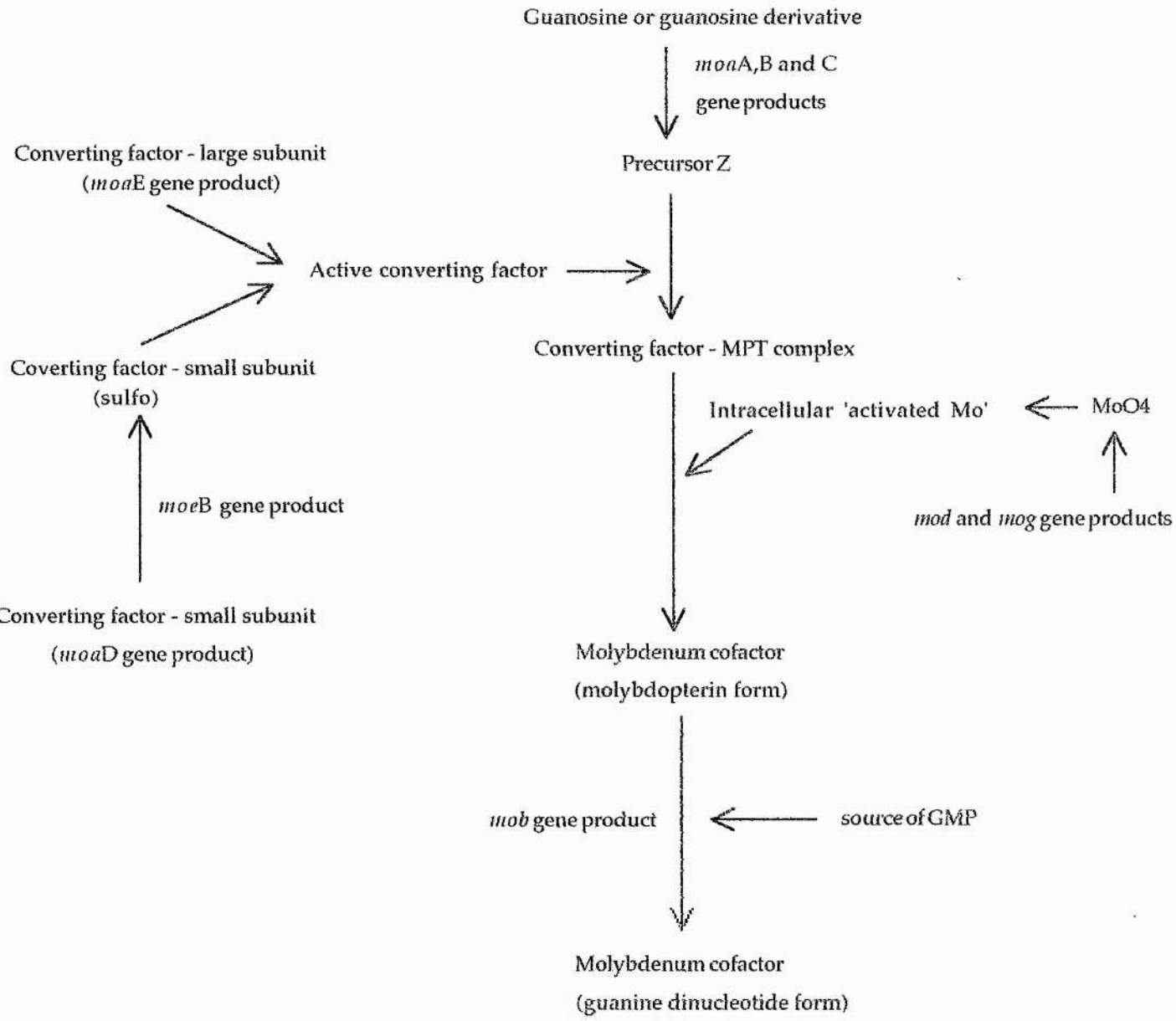


Fig. 1.8. The Molybdenum Cofactor Genes of *E. coli*

Schematic representation of the roles of the gene products (formerly *chl* gene products) in the biosynthesis of the molybdenum cofactor in *Escherichia coli*. (Taken from Johnson and Rajagopalan, 1987).

such as blindness (Rupar *et al.*, 1996). Death often results (Arnold *et al.*, 1991; Slot *et al.*, 1993).

Obviously, the more which is learnt about this system at the basal level, the more information can be gathered on the processes in higher organisms.

Aspergillus provides us with an excellent model organism to use in these studies, being an organism which is most amenable to experimentation, while at the same time, allowing us a glimpse into the biochemical machinery of a eukaryotic organism.

1.8. THE NITROGENASE COFACTOR

All known molybdoenzymes contain a unique molybdenum-containing cofactor, as described previously. One exception to this is the enzyme nitrogenase, which utilizes a molybdenum cofactor which also contains iron (the FeMo-co) (Shah and Brill, 1977 and for a review see Kim and Rees, 1994). Nitrogenase catalyzes the reduction of N_2 to NH_3 - a process which is only carried out by a relatively small number of nitrogen - fixing micro-organisms, termed diazotrophs. The stoichiometry of the overall reaction is -

$$N_2 + 8H^+ + 8e^- + 16MgATP \longrightarrow 2NH_3 + H_2 + 16\ MgADP + 16Pi$$

(Simpson and Burris, 1984).

The nitrogenase molecule is composed of two distinct proteins - the Fe protein (also known as dinitrogen reductase) and the MoFe protein (dinitrogenase) (Ma *et al.*, 1994). The Fe protein is a homodimer with molecular weight of around 60 kD - each subunit having a MgATP binding site. This protein is responsible for the transfer of electrons to the MoFe protein with the concomitant hydrolysis of MgATP (Georgiadis *et al.*, 1992). The MoFe protein is an $\alpha_2\beta_2$ tetramer (MW. 240 kD) which

binds the N_2 and contains the active site of the molecule. FeMo-co also forms part of the active site (Kim and Rees, 1992). The Fe protein contains a bound Fe_4S_4 cluster, and the MoFe protein two of these clusters bound by two Cys thiols (the P-cluster pair) (Chan *et al.*, 1993). The presence of a homocitrate molecule is also important to the activity of nitrogenase. It acts as a ligand to the FeMo-co and although its precise role is unknown, it is thought to have a function either in electron transfer or in mediation of protons to reduced intermediates (Kim and Rees, 1992).

In the bacterium *Klebsiella pneumoniae* there are known to be at least 17 genes spanning 7 or 8 operons and 23 kb of DNA which are involved in nitrogen fixation (the *nif* system) (MacNeil *et al.*, 1978). *nif*D and *nif*K are the structural genes which encode the MoFe protein of nitrogenase, while *nif*H is the structural gene for the Fe protein, as well as playing an essential role in FeMo-co biosynthesis (Filler *et al.*, 1986). These three genes form one of the *nif* operons. Some other genes involved in nitrogenase synthesis include, *nif*B (Shah *et al.*, 1994) *nif*N, *nif*E and *nif*V - all of which have been found to be essential for FeMo-co activity (Roberts *et al.*, 1978). *nif* Q is another gene which is thought to be involved in Mo-metabolism - incorporating Mo into the cofactor during low molybdenum concentrations (Imperial *et al.*, 1984).

K. pneumoniae also has *mol* genes, equivalent to those in *E. coli*. It is noted however, that these *mol* mutants can use hypoxanthine as a sole N-source, indicating a Mo-co independent hypoxanthine dehydrogenase in this organism (Garzon *et al.*, 1992).

Mg^{2+} is the most effective ion in substrate reduction while other divalent cations appear to function to varying degrees eg. Mn^{2+} and Fe^{2+}. Co^{2+} and Cu^{2+} have been shown to be inhibitors of FeMo-co synthesis *in vitro* (Chatterjee *et al.*, 1994).

One of the most studied nitrogenase systems is that of *Azotobacter vinelandii* - an organism which synthesizes three different forms of the enzyme, depending upon environmental conditions (Bishop and Joerger, 1990).

Nitrogenase-1 is the common and well-characterized FeMo-co-containing enzyme. Nitrogenase-2 is a homologue which contains vanadium in place of the Mo in the cofactor. It is encoded by the *vnf* system. Nitrogenase-3 or 'alternative' nitrogenase (the *anf* system) appears to contain only Fe in its cofactor, and has no requirement for either Mo or V (Eady *et al.*, 1988). The bacterium *A. vinelandii* is unique in that it is the only organism, thus far, seen to synthesize three different forms of nitrogenase. It should be noted, however, that *A. chroococcum* also contains the vanadium enzyme (Eady *et al.*, 1988) and that *Rhodobacter capsulatus* was recently discovered to have an alternative Fe-only nitrogenase (Gollan *et al.*, 1993). Four genes - *mod*A, B, C, and D encode the Mo transport system of *R. capsulatus*, and two of these, *mod*C and *mod*B were found to be homologous to *chl*D and *chl*J of *E. coli* respectively (Wang *et al.*, 1993).

The Mo-nitrogenase is synthesized in Mo-sufficiency, while the V-enzyme is produced under Mo-deficient conditions in the presence of vanadium. When these two metals are absent, the alternative nitrogenase is synthesized. In *A. vinelandii*, the dinitrogen reductase components of all three enzymes are homodimers. Dinitrogenase of the Mo-enzyme is a tetramer of MW of around 240 kD, whereas the other two dinitrogenase molecules are composed of a hexameric structure of the stoichiometry - $\alpha_2\beta_2\delta_2$ (Joerger *et al.*, 1989).

The structural genes for nitrogenase-1 are *nif*HDK as previously mentioned, while two separate operons are responsible for the synthesis

of the subunits of nitrogenase-2 - *vnfHorfFd* and *vnf*DGK. The genes encoding the alternative nitrogenase are *anfHDGKorf1orf2*. Expression of the *nif*H, *vnf*H and *anf*H genes dictates synthesis of the dinitrogen reductase subunits, and *nif*DK, *vnf*DK and *anf*DK control biosynthesis of the α and β subunits of dinitrogenase. The δ-subunits found in the V-nitrogenase and the Fe-only enzyme are thought to be encoded by *vnf*G and *anf*G respectively (Wolfinger and Bishop, 1991 and references therein).

The gene clusters *nif* ENX and *vnf* ENX are found next to their respective structural gene operons, *nif* HDKTY and *vnfHorfFd*. These genes are involved in cofactor biosynthesis and it would appear that *vnf* ENX is involved in synthesis of cofactor for nitrogenase 2 and 3, since there does not appear to be an *anf* ENX system in *A. vinelandii*. NifE and *Nif*N can also substitute for *Vnf* E and *Vnf* N when these proteins are unavailable (Wolfinger and Bishop, 1991). Three other genes, designated *nif* U, *nif* S and *nif* V have also been shown to be required for activity in all three nitrogenases (Kennedy and Dean, 1992). Four ORF's are known to encode genes involved in the Mo transport system of *A. vinelandii* - ORF's 3 and 4 being homologous to the *chl*D and *chl*J gene products of *E. coli* respectively (Luque *et al.*, 1993).

A small, globular, acidic protein, designated NifW has been isolated. It has three highly conserved regions although there appear to be no metal or nucleotide binding sites. The conserved areas are -

- a serine and a glutamate in SAE.
- an arginine and a histidine in RLHI.
- two lysines in EKVFKVF.

*nif*W mutants show no difference in their Fe protein, but do exhibit considerably reduced activity of the MoFe protein. Studies indicate that

the *nifW* mutant gives differential inhibition of different substrates and it has been postulated that *nifW* is involved in homocitrate processing (Kim and Burgess, 1994). The whole *nif* area of *A. vinelandii* (shown to contain at least 20 genes) was sequenced by Jacobson *et al.* (1989) and was shown to have the same sequential arrangement as the *nif* genes of *K. pneumoniae.*

Analysis of the *cnx* genes of *A. nidulans* will show, *a priori,* what similarities/differences exist in the genetics of FeMo-co and the Mo-co of nitrate reductase, with regard to biosynthesis and regulation.

1.9. AIMS OF THE PROJECT

The *cnx* loci of *Aspergillus nidulans* are involved in the synthesis of a molybdenum - containing cofactor which plays an important role within this organism and many others. This project set out to:

a). - isolate, and clone by genetic transformation, the *cnx*ABC locus on chromosome VIII of *A.nidulans.*

b). - determine by sequence analysis, how many genes are contained within the locus and identify what their role may be in the Mo-co biosynthetic pathway.

c). - study regulation of *cnx*ABC by Northern analysis.

d). - if time permitted, to isolate and clone the *cnx*J locus on chromosome VII of A.nidulans, by complementation of mutant mycelia on tungstate - containing medium by means of genetic transformation.

CHAPTER 2

MATERIALS AND METHODS

2.1. CHEMICALS

All chemicals used were obtained from BDH or Sigma and were of the pure biochemical or AnalaR grade wherever possible. Constituents of fungal and bacterial media were purchased from Lab M, Difco or Gibco. Amersham plc and MSI provided the nylon membranes used in hybridization experiments, while the autoradiographic film was supplied by Kodak plc. DNA - modifying enzymes, restriction endonucleases and the relevant buffers were from NBL and Pharmacia. Novozym-234 was supplied by Novobiolabs Incorporated and ^{32}P-dCTP was obtained from ICN.

2.2. STRAINS

2.2.1. Bacterial

Competent cells for the purpose of bacterial transformation were prepared from the *Escherichia coli* strain DH5α (F^-, *end*A1, *hsd*R17, *sup*E44, *thi*-1, *rec*A1, *gyr*A96, *rel*A1, ø80R, *lac*ZDM15.)

The bacterial strain used for propagation of the λ - phage bank was MRA [LE392] - (Δ(*mcr*A)183, Δ(*mcr*CB-*hsd*SMR-*mrr*)173, *end*A1, *sup*E44, *thi*-1, *gyr*A96, *rel*A1, *lac*).

2.2.2. Fungal

A. nidulans gene symbols are used according to Clutterbuck, 1973. Dr. John Clutterbuck (University of Glasgow) provided the *A. nidulans*

*bi*A1 strain which was used throughout this work as the 'wild-type' strain. Experiments were carried out with the following mutant strains -

*cnx*A Mutants

G831 *y*A2 *pyro*A4 *cnx*A5.

(Supplied by Dr. John Clutterbuck, University of Glasgow).

α8 *bi*A1 *cnx*A9.

(Supplied by Mr. Adrian Simpson, University of Cambridge).

*cnx*B Mutant

GO55 *bi*A1 *cnx*B11

(Supplied by Dr. John Clutterbuck, University of Glasgow).

*cnx*C Mutant

G832 *y*A2 *pyro*A4 *cnx*C3

(Supplied by Dr. John Clutterbuck, University of Glasgow).

*cnx*J Mutant

2672 *fw*A *paba*A1 *prn*301 *cnx*J1

(Supplied by Dr. D. Cove, Leeds University).

2.3. MAINTENANCE OF CULTURES

2.3.1. Bacterial

After culturing on Luria agar plates, bacterial strains were stored for the short term at 4°C. Longer term storage was achieved by culturing in Luria broth + 20% glycerol and maintaining at -70°C.

2.3.2. Fungal

A. nidulans cultures were grown on complete medium plates (with appropriate supplements) and incubated at 37°C. Stocks could be maintained on these plates for several weeks at 4°C. Long term storage of

fungal strains was carried out by preparation of silica gel cultures (Roberts, 1969). These cultures were again kept at 4°C.

2.4. PREPARATION OF SILICA STOCKS

2 ml of sterile 5% skimmed milk is poured over a sporulating fungal slope and agitated to detach the spores. The 2 ml are then transferred to another tube and vortexed. 0.5 ml of this suspension is then added to a sterile silica vial (pre-cooled on ice), vortexed strongly and replaced on ice for 10 min. This is then left at room temperature for 7 days, the silica vortexed to ensure it flows freely and the viability is then tested and stocks stored at 4°C.

2.5. MEDIA

Unless otherwise indicated, solid media were prepared by addition of 1.2% agar to the relevant liquid media. All media and solutions were sterilized prior to use by autoclaving at 15 psi for 15 min.

2.5.1. Nitrogen Sources

Ammonium or nitrate was added to the pre-cooled (55°C) medium to a final concentration of 10 mM from sterile 1 M stock solutions of ammonium tartrate and sodium nitrate respectively.

2.5.2. Solutions

Salts Solution

26 g KCl

26 g $MgSO_4$

76 g KH_2PO_4

1L with dH_2O.

Trace Elements Solution

1.1 g $Mo_7O_{24}.4H_2O$

11.1 g H_3BO_4

1.6 g $CoCl_2.6H_2O$

1.6 g $CuSO_4.5H_2O$

50 g EDTA

5 g $FeSO_4.7H_2O$

5 g $MnCl_2.4H_2O$

22 g $ZnSO_4.7H_2O$

1L with dH_2O; boiled with stirring; cooled to 60°C; pH 6.5-6.8 with 5 M KOH. Stored in the dark at 4°C.

Vitamin Solution

2.5 g biotin

2.5 g nicotinic acid

0.8 g para amino benzoic acid (PABA)

1 g pyridoxine hydrochloride

2 g pantothenate

2.5 g riboflavin

1.5 g aneurine

20 g choline hydrochloride

1L with dH_2O.

2.5.3. Media for Bacterial Culture

(Described in Sambrook, *et al.*, 1989).

Luria Broth

10 g NaCl

10 g tryptone

5 g yeast extract

1L with dH_2O; pH 7.0 with 5 M NaOH.

For selection, 50 μg/ml ampicillin or 25 μg/ml kanamycin was added after pre-cooling the medium to 55°C. The antibiotic used depending on the resistance marker in the plasmid in question.

2.5.4. Media for *Aspergillus* Culture

Growth of *A. nidulans* was carried out on complete medium, the recipe for which is based on that described by Cove (1966) and Pontecorvo *et al.*, (1953).

Complete Medium

10 g glucose

50 ml salts soln.

1 ml trace elements soln.

1 ml vitamin soln.

2 g peptone

1 g yeast extract

1 g casein hydrolysate

1L with dH_2O; pH 6.5 with 5 M NaOH.

Selective growth conditions were produced on minimal medium (lacking a nitrogen source).

Minimal Medium

10 g glucose

50 ml salts soln.

1 ml trace elements soln.

1L with dH_2O; pH 6.5 with NaOH.

2.5.5. Supplements

Filter sterilized and stored as concentrated solutions at 4°C. Added to pre-cooled medium (55°C) as required.

Supplement	Stock concentration (g per 100ml).	Amount added to 100ml medium
adenine	7.5	1 ml
arginine hydrochloride	4.2	1 ml
biotin	0.1	1 ml
choline hydrochloride	2.0	1 ml
methionine	0.5	1 ml
putrescine	0.2	1 ml
pyridoxine hydrochloride	0.5	1 ml
riboflavin	0.8	1 ml
uridine	24.42	1 ml
L-proline	11.15	1 ml
PABA	0.14	0.5 ml

2.6. FUNGAL SPORE COLLECTION

Sterile saline tween was poured over a sporulating fungal plate. Conidia were scraped from the plate using a sterile glass rod. Spores harvested from fungal plates were resuspended in saline tween soln and stored at 4°C until required for addition to liquid medium.

Saline Tween

0.1% Tween 80

0.9 g NaCl

1L with dH_2O.

2.7. PREPARATION OF PROTOPLASTS AND *Aspergillus* GENETIC TRANSFORMATION

The fungal transformation method used was based upon that reported by Andrianopoulos and Hynes, 1988.

2.7.1. Transformation Solutions and Media

STC

1.2 M sorbitol

10 mM Tris-HCl pH 7.5

10 mM $CaCl_2$

Trapping buffer

0.6 M sorbitol

100 mM Tris-HCl pH 7

Osmotic medium

1.2 M $MgSO_4$

10 mM sodium orthophosphate pH 7

Adjusted to pH 5.8 with 0.2 M Na_2HPO_4, and filter sterilized.

2.7.2. Protoplast Preparation

Day before transformation. - The spore suspension was added to 200 ml minimal medium with 10 mM NH_4 as nitrogen source and any necessary supplements. O/N incubation at 30°C.

Transformation day - Mycelia were harvested in muslin cloth and washed with cold $MgSO_4$ (500 ml, 0.6 M). The cells were then resuspended in 5 ml of cold osmotic medium in a sterile conical flask. 50 mg of Novozym 234 in 2 ml osmotic medium is then added, and incubated on ice for 5 min. 12 mg BSA in 1 ml osmotic medium is then added. This mixture was then incubated at 30°C for 90 min with shaking. Protoplast incubation was stopped by standing flask on ice. The flask was swirled vigorously to release protoplasts. Equal volumes were added to the bottom of two pre-cooled Corex tubes. This was then overlaid with equal volumes of cold trapping buffer, which was allowed to run very slowly down the side of the tube. Protoplasts form as a bushy band at the liquid interface after centrifugation for 20 min at 5,000 rpm, 4°C, in a Sorvall RC-5HB4 rotor.

2.7.3. *Aspergillus nidulans* Transformation

Protoplast bands were carefully removed with a Pasteur pipette and were pooled into a third pre-cooled Corex tube. The protoplasts were washed with STC and centrifuged (Sorval) (HB-4 rotor, 5 min, 7,000 rpm, 4°C). This was repeated twice. The protoplast pellet was resuspended in 100 µl STC. 50 µl protoplasts were added to the DNA soln. (Typically - 10 µg DNA; 10 µl 2 x STC; 30 µl 1 x STC). 25µl of 60% PEG 8000 was added to the mixture and gently mixed. This was incubated on ice for 30 min. A further 1 ml PEG (60%) was added and the tube rolled gently to mix. Incubation at r.t. for 30 min. 5 ml STC was then added to the tube and centrifuged 4,500 rpm, 5 min (swing-out bench top). The supernatant was discarded and the pellet gently resuspended in 300 µl 1x STC. 100 µl was then spread onto each of three selection plates (minimal medium + 1.2 M sorbitol + NO_3 as N source). 10 µl of protoplasts were added to 990 µl

SDW and 100 µl of this was plated out as a control. 10 µl of protoplasts were also added to 990 µl STC and a dilution series made by adding 100 µl of this to another 900 µl STC. 100 µl of this was added to a further 900 µl STC and so on. These dilutions were also plated out as controls, on non-selective plates (NH_4 as nitrogen source).

2.7.4. Calculation of Protoplast Number

The number of protoplasts in 4 of the smallest squares on a haemocytometer were counted and averaged, thus-

$$\frac{\text{total no. of protoplasts}}{4} \times 4 \times 10^6$$

(undiluted sample).

This gives number of protoplasts/ml. A minimum of 10^8 protoplasts/ml were used in each transformation.

The haemocytometer used for these calculations was an Improved Neubauer, depth - 0.1mm (1/400mm^2). (Weber Scientific international Ltd.)

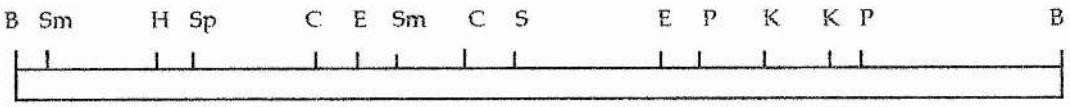
2.8. PLASMIDS AND COSMIDS

2.8.1. Plasmids

pILJ421 - Contains the *A. nidulans brl*A gene (Boylan *et al.*, 1987). Fig. 2.1. Plasmid supplied by Dr. J.A. Clutterbuck, University of Glasgow.

pHELP- Contains the *A. nidulans* autonomously-replicating AMA-1 sequence (Gems *et al.*, 1991; Gems *et al.*, 1993; Clutterbuck *et al.*, 1993). Fig. 2.2. Supplied by Dr. J.A. Clutterbuck (University of Glasgow, Scotland)

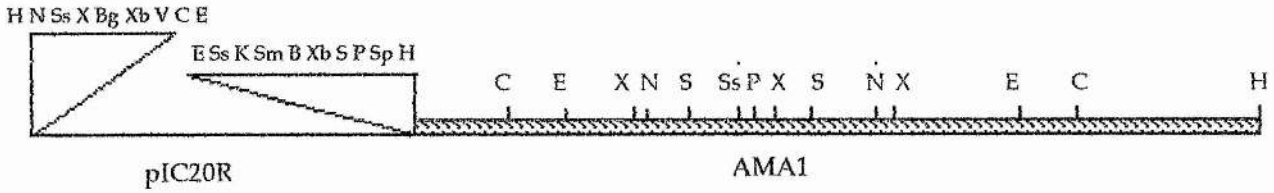
pAN229 - Contains the *A. nidulans prn*A gene of the proline utilization gene cluster (Arst and MacDonald, 1975). Fig. 2.3. Plasmid supplied by Dr. C. Scazzocchio, Orsay University, France.



B - *Bam*HI; Sm - *Sma*I; H - *Hind*III; Sp - *Sph*I; C - *Cla*I
E - *Eco*RI; S - *Sal*I; P - *Pst*I; K - *Kpn*I

Fig. 2.1. The *brl*A - Containing Plasmid

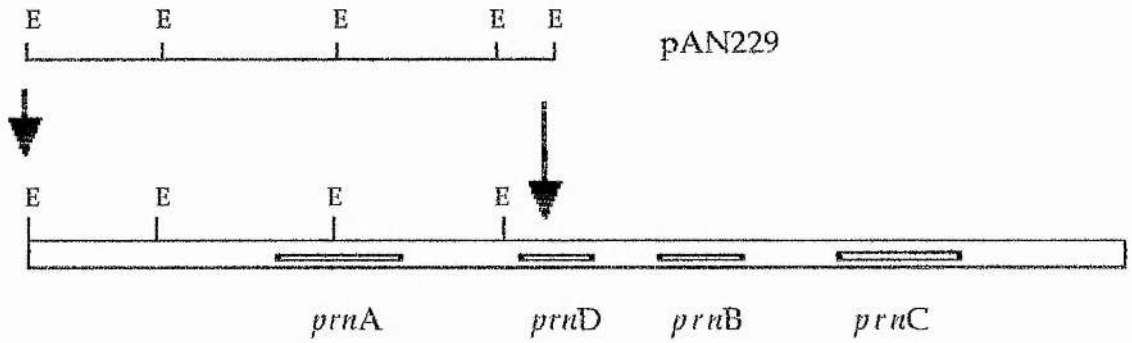
The 4.8kb *Bam*HI fragment on plasmid pILJ421 which contains the *brl*A gene of *Aspergillus nidulans*. The pILJ421 plasmid was isolated by Clutterbuck and colleagues from a bank of *Sau*3A fragments cloned into the *Bam*HI site of pILJ16. (Johnstone *et. al.*, 1985).



H - *Hin*dIII; N - *Nru*I; Ss - *Sst*I; X - *Xho*I; Bg - *Bgl*II; Xb - *Xba*I; P - *Pst*I; V - *Eco*RV; C - *Cla*I; E - *Eco*RI; K - *Kpn*I; Sm - *Sma*I; B - *Bam*HI.

Fig. 2.2. The pHelp Plasmid

The *A. nidulans* autonomously replicating pHelp plasmid (Gems and Clutterbuck, 1993) which contains the *Aspergillus nidulans* AMA1 sequence (Aleksenko *et al.*, 1997). This plasmid is a useful tool for increasing the frequency of transformation when co-transformed along with the DNA of interest.



*prn*A - regulatory gene
*prn*D - encodes proline oxidase
*prn*B - encodes proline permease
*prn*C - encodes pyrroline - 5-carboxylate dehydrogenase

Fig. 2.3. The pAN229 Plasmid

This diagram shows the pAN229 plasmid in relation to the section of *Aspergillus nidulans* chromosome VII DNA which contains the proline utilization gene cluster. pAN229 contains the *prn*A gene which, from classical genetical analyses, is thought to be 3 centiMorgans away from the *cnx*J gene. E - *Eco*RI restriction site

2.8.2. Cosmids

The chromosome-specific *A. nidulans* cosmid library was constructed as detailed in Brody *et al.*, 1991. It has enabled genes to be mapped to specific chromosomes (Timberlake *et al.*, 1985). The clones were constructed in two separate vectors, namely pWE15 which confers ampicillin resistance (Wahl *et al.*, 1987) and pLORIST2 which carries a kanamycin resistance marker (Fig. 2.4.) (Gibson *et al.*, 1987).

2.9. PREPARATION OF COMPETENT *E. COLI* CELLS

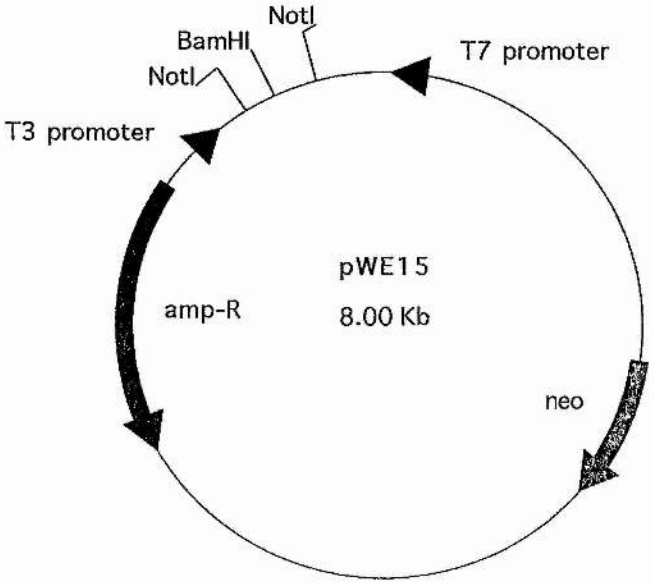
1 ml O/N culture of *E. coli* cells (DH5α strain) was used to inoculate 200 ml Luria broth, which is grown at 37°C until the O.D. was around 0.25. The cells were pelleted by centrifugation at 3000 rpm, 10 min, 4°C (GS-3 rotor). They were then gently resuspended in 100 ml cold 100 mM $MgCl_2$; 5 mM Tris-HCl pH 7.4. The cells were again centrifuged at 3000 rpm, 5 min, 4°C (GS-3 rotor). The pellet was gently resuspended in 100 ml cold 100 mM $CaCl_2$; 5 mM Tris-HCl pH 7.4, and the suspension left to stand on ice for 20-60 min. After a final centrifugation at 3000 rpm, 5 min, 4°C (GS-3 rotor), the pellet was resuspended in 1 ml 100 mM $CaCl_2$; 5 mM Tris-HCl pH 7.4; 14% glycerol, dispensed in 100µl aliquots into pre-cooled microfuge tubes and frozen immediately at -70°C. This method is based on that described in Sambrook *et al.* (1989).

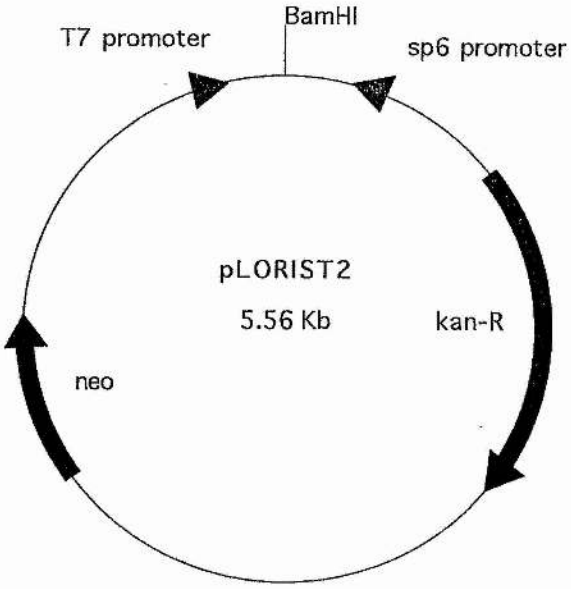
2.10. TRANSFORMATION OF COMPETENT *E. COLI* CELLS

Aliquots of competent *E. coli* cells were thawed on ice and transformation carried out as described (based upon the method described in Cohen *et al.*, 1972).

Fig.2.4. pWE15 and pLORIST2

pWE15 and LORIST2; vectors used in the construction of the *Aspergillus nidulans* cosmid library. pWE15 (Wahl *et al.*, 1987) contains the ampicillin resistance gene, whilst the LORIST2 vector (Gibson *et al.*, 1987) carries the kanamycin resistance gene as a selection marker. Both vectors also have the neomycin gene. Promoters around the cloning site are shown - T3 and T7 in the case of pWE15 and T7 and sp6 in pLORIST2.


NotI
BamHI
NotI
T7 promoter
T3 promoter
pWE15
8.00 Kb
amp-R
neo


BamHI
T7 promoter
sp6 promoter
pLORIST2
5.56 Kb
kan-R
neo

50 ng DNA was added to 100 µl *E. coli* competent cells and the contents mixed gently and left on ice for 30 min. The tube was then placed in a water bath at 42°C for 90 s and immediately transferred to ice for 1-2 min. 900 µl Luria broth was added and the mixture incubated at 37°C for 45 min, with shaking. This incubation allows the bacterial cells to recover and to express the antibiotic resistance marker. Typically 50 µl of transformed cells are plated onto Luria agar + ampicillin (50 µg/ml) plates and incubated O/N at 37°C.

2.11. PHENOL:CHLOROFORM EXTRACTION AND ETHANOL PRECIPITATION OF DNA

Proteins were removed from samples by addition of phenol:chloroform soln. and subsequent precipitation by ethanol. The phenol:chloroform reagent contains constituents in a 1:1 ratio, where the 'phenol' has been equilibrated with 100 mM Tris-HCl pH 8 and then with 10 mM Tris-HCl until pH 7.5. The soln. also contains 0.8% hydroxyquinoline. The 'chloroform' is a 24:1 mixture of chloroform:iso-amyl alcohol.

An equal volume of phenol:chloroform is added to the DNA sample and vortexed to produce an emulsion. This is then microfuged at 14,000 rpm for 2 min. The upper, aqueous layer is removed to a clean microfuge tube and a further volume of phenol:chloroform added and the process repeated. The resultant aqueous layer is then combined with an equal volume of chloroform:iso-amyl alcohol, microfuged at 14,000 rpm for 2 min and the upper, aqueous layer removed to a clean microfuge tube. 0.1 volumes of 3 M sodium acetate pH 5.2 and 2 volumes of cold 96% ethanol were added and the mixture left at -70°C for 30 min. Microfugation at 14,000 rpm for 20 min pelleted the precipitate. After

washing with 70% ethanol, the pellet was dried and resuspended in a suitable volume of TE.

2.12. 'DROP DIALYSIS' OF DNA SAMPLES

30-100 ml dialysis buffer (double distilled water or TE) is poured into a petri dish and a 25 mm Millipore membrane (Type VS, pore size 0.025 mm - VSWP02500 - Millipore, Inc.) is floated on top. The membrane is allowed to wet (~5 min) and a few µl of DNA are pipetted onto the membrane. Dialysis is left to continue for 1-4 hr. DNA is retrieved with a micro-pipette and the concentration is estimated by running on an agarose gel alongside a known amount of λ-*Hin*dIII DNA. (Silhavy *et al.*, 1984).

2.13. PLASMID DNA PREPARATIONS

2.13.1. Small Scale Plasmid Preparation

Based upon the method described in Birnboim and Doly, 1979.

Bacteria are grown O/N in 10 ml LB liquid medium containing antibiotic. The cells were harvested by microfuging at 8,000 rpm, 1 min. The pellet was then resuspended in 200 µl Soln I (see section 2.13.3) and left 5 min at room temperature, after which, 400 µl Soln II (see section 2.13.3) were added and the mixture left on ice for 5 min. 300 µl 7.5 M NH_4Ac (pH 7.8) was added and mixed. The tube was maintained on ice for 10 min. After centrifugation at 10,000 rpm for 3 min, the supernatant was removed to a clean microfuge tube and 0.6 volumes isopropanol added. An incubation at r.t. for 10 min, precipitated the DNA. The DNA was pelleted by microfugation at 14,000 rpm for 10 min, washed with 70% ethanol and dried. Resuspension was in a suitable volume of TE.

2.13.2. Large Scale Plasmid Preparation

200 ml Luria broth with antibiotic was inoculated and the culture grown O/N at 37°C, with orbital shaking. The culture was then centrifuged for 10 min at 5000 rpm, 4°C (Sorvall GS3 rotor) and the pellet resuspended in 4 ml sol.ª 1 (see section 2.13.3). This was incubated at r.t. for 5 min., and 8 ml of freshly prepared sol.ª 2 (see section 2.13.3) then added, and left to incubate on ice for 5 min. Thereafter, 6 ml of ice-cold sol.ª 3 (see section 2.13.3) was added and incubated on ice for 10 min. The mixture was then centrifuged for 40 min at 12000 rpm, 4°C (Sorvall, SS34 rotor). An equal volume of isopropanol was added to the aqueous phase and left to incubate at r.t. for 15 min. The mixture was again centrifuged for 20 min at 12000 rpm, 18°C (Sorvall SS34 rotor). The resulting pellet was washed with 70% ethanol, dried and resuspended in 4 ml TE. RNAse was then added to a final concentration of 100 µg/ml and an incubation at 37°C then followed. 880 µl of 5 M NaCl and 800 µl of 1 M MOPS (pH 7) were added. A Qiagen tip-500 (Diagen) was equilibrated with 10 ml QBT buffer (see section 2.13.3) and allowed to empty by gravity flow. Supernatant was then added to the column and allowed to enter by gravity flow. The column was then washed with 3 x 10 ml of QC buffer (see section 2.13.3). DNA was then eluted into a clean tube by addition of 15 ml of QF (see 2.13.3). 0.7 volumes of isopropanol (r.t.) precipitated the DNA. Finally, centrifuge at 12,000 rpm, 4°C, for 30 min (Sorvall SS34 rotor), wash with 70% ethanol, dry, and resuspend in 200 µl TE.

2.13.3. DNA Preparation Solutions

SOLUTION 1

50 mM glucose; 25 mM Tris-HCl pH 8; 10 mM EDTA.

SOLUTION 2

2 M NaOH; 10% SDS; H_2O.

SOLUTION 3
3 M KAc pH 4.8.

TE
10 mM Tris; 1 mM EDTA.

QBT
1 M NaCl
50 mM MOPS (pH 7)
15% ethanol
SDW

QC
1 M NaCl
50 mM MOPS (pH 7)
15% ethanol
SDW

QF
1.25 M NaCl
50 mM MOPS (pH 8.2)
15% ethanol
SDW

2.14. EXAMINATION OF DNA PLASMID INSERTS BY A QUICK 'COMPLETE LYSIS' METHOD

Based upon the method described in Barnes, 1977.

A bacterial colony was picked off and resuspended in 20 µl SDW. 20 µl cracking buffer was added (200 mM NaOH; 0.5% SDS; 0.6 M sucrose; 0.02% bromocresol green). The total mixture was then loaded onto an agarose gel (not submerged), allowed to run in to the gel, and electrophoresis continued after more buffer was added to the gel tank.

2.15. PREPARATION OF FUNGAL CHROMOSOMAL DNA

2.15.1. Modified Nucleon II Method

This was carried out as in the method using NucleonII kit (Scotlab) and modified by Shiela Unkles.

300-400 mg (pressed wet weight) fungal mycelia were ground to a fine powder in liquid N_2. The resulting powder was suspended in 2 ml Nucleon reagent B in a 15 ml polypropylene tube. 1 µl 10 mg/ml RNAse A was added and the mixture incubated at 37°C, 30 min. 1.5 ml 5 M sodium perchlorate was added and the tube left to mix 100 rpm, r.t., 15 min. The mixture was then incubated at 65°C for 25 min and inverted once or twice during incubation. 5.5 ml cold chloroform was added and rotary mixed, r.t., 10 min. This was followed by centrifugation at 800 x g, 1 min, whereafter, 800 µl Nucleon Silica Suspension was added and this was then centrifuged 1400 x g, 3 min. The upper, aqueous layer was removed to a clean tube and 1 volume of 70% ethanol added. The mixture was gently inverted until thread-like DNA precipitated, whereupon it is spooled out with a sterile Pasteur pipette. The DNA was washed in 70% ethanol, dried and finally resuspended in an appropriate volume of TE.

2.15.2. Rapid Fungal DNA Mini-preparation

This was carried out according to the method of Leach *et al.*, (1986).

10 ml minimal medium was inoculated with a loopful of fungal conidia and incubated 16-30 hr. Mycelium was harvested and washed with SDW and then lyophilised. This was done by freezing the mycelium in liquid nitrogen and vacuum drying O/N, followed by mechanically dicing the dried material. 0.7 ml LETS buffer (0.1 M LiCl; 10 mM EDTA; 10 mM Tris-HCl pH 8; 0.5% SDS.) was added and the mixture suspended by

vortexing at top speed for 2 min. 1 ml of phenol:chloroform: isoamyl alcohol (25:24:1) was added, followed by vortexing for 20 s. The suspension was microfuged at 3,000 rpm for 5 min. 500 µl of the upper aqueous layer was transferred to a sterile microfuge tube and 1 ml 100% ethanol added. The DNA was precipitated by leaving on dry-ice for 15 min. The mixture was then microfuged at 14,000 rpm for 15 min. The supernatant was discarded and the pellet washed with 70% ethanol, allowed to dry and then resuspended in 40 µl TE.

2.16. ANALYSIS OF FUNGAL TRANSFORMANTS BY THE POLYMERASE CHAIN REACTION

The presence of bacterial sequences in putative fungal transformants was confirmed by the ability to amplify any such sequences by use of the polymerase chain reaction.

Ampicillin or kanamycin DNA primers were used depending upon the clone used to transform the fungal mutant.

PCR reactions were set up as follows -

Template DNA - 5 µl; 10 X reaction buffer (20 mM Tris pH 7.4; 0.1 mM EDTA; 1 mM DTT; 100 mM KCl; 0.1% Triton X-100; 160 µg/ml BSA; 50% glycerol) - 10 µl; 20 mM primer 1 - 5 µl; 20 mM primer 2 - 5 µl; 2 mM dNTP - 5 µl; 2.5 units *Taq* polymerase, in a final volume of 100 µl.

Amplification was carried out as follows, in a PCR reactor (Hybaid) -

94°C - 2 min

50°C - 20 s } 1 cycle

72°C - 30 s

94°C - 20 s

50°C - 20 s } 25-30 cycles

72°C - 30 s

2.17. CALCULATION OF DNA CONCENTRATION

The concentration and purity of plasmid DNA samples was determined spectrophotometrically by measuring the optical density at 260 nm and 280 nm. An $A_{260} = 1$ corresponds to approximately 50 µg/ml double stranded DNA. 1 µl DNA sample was added to 999 µl dH_2O and the O.D.'s measured against a water blank.

The A_{260} X dilution X 50 µg/ml gives the concentration of your DNA sample in µg/ml.

The ratio of A_{260}/A_{280} gives a measure of DNA purity. A ratio of 1.8-2.0 is indicative of a good clean preparation. All measurements were recorded in triplicate and the mean taken.

2.18. DNA ELECTROPHORESIS

The method used was as described in Sambrook *et al.*, (1989).

Horizontal 0.8% agarose gels containing 0.5 µg/ml EtBr were prepared and run in TAE buffer (40 mM Tris-acetate; 1 mM EDTA). *Hin*dIII - digested lambda phage DNA was used as a molecular weight marker, with bands corresponding to 23 kb, 9.6 kb, 6.5 kb, 4.5 kb, 2.3 kb, 2.0 kb and 0.5 kb. 0.1 volume of gel loading buffer (30% sucrose; 100 mM EDTA; bromophenol blue crystals) was added to DNA samples and electrophoresis carried out until the dye band had migrated to near the bottom of the gel. DNA (in the presence of ethidium bromide) was then visualised under a UV trans-illuminator (Ultra Violet Products) and

photographed using a Polaroid MP-4 land camera and Polaroid type 667 film (Polaroid plc).

2.19. DIGESTION OF DNA BY RESTRICTION ENDONUCLEASE ENZYMES

DNA samples were incubated at 37°C in the presence of the appropriate restriction enzyme (2-5 fold excess) in the appropriate commercial enzyme dilution buffer. Digestions were usually left O/N to react, although incubation for 3-4 hr suffices. DNA to be used in further procedures was phenol:chloroform and chloroform extracted and acetate/ethanol precipitated as previously detailed in section 2.11.

2.20. ISOLATION OF DNA FRAGMENTS FROM AGAROSE GELS

2.20.1. Extraction of DNA from Restriction Fragments

After electrophoresing the digested DNA sample sufficiently to ensure adequate separation of the desired fragment, the band in question was excised from the gel. This agarose plug was then placed in a small microfuge tube which had a hole pierced in the bottom and which was pre-plugged with siliconized glass-wool. The tube was then placed in a larger microfuge tube and centrifuged (bench-top) 6,000 rpm, 10 min. The resultant soln. which collected in the larger tube was then phenol:chloroform and chloroform extracted and acetate/ethanol precipitated as previously described in section 2.10.

2.20.2. 'Gene Clean' Method

The procedure was carried out according to manufacturer's instructions (Bio 101, California). Use of this kit provides a higher yield

and better degree of purity of DNA as compared to ethanol precipitation methods.

2.21. LIGATION OF DNA FRAGMENTS

Ligations were carried out using the 'Ready-to-Go pUC18 *Bam*HI/BAP + Ligase' kit (Pharmacia). The procedure was carried out as stated in the manufacturers instructions.

2.22. ^{32}P - LABELLING OF DNA

DNA probes were labelled as detailed in Sambrook *et al.*, 1989.

Typically, 25 ng denatured DNA were labelled with 50 μCi of a-^{32}P-dCTP by the action of Klenow enzyme in the presence of reaction buffer and BSA (37°C for 45 min). DNA was separated from unincorporated nucleotides by passage through a Nick column (Pharmacia), and eluted with TE. Probe was then boiled for 3min and quenched on ice before being added to filters for hybridization.

2.23. ISOLATION OF RNA

2.23.1. Isolation of Total RNA

Four grams of fungal mycelia were ground to a fine powder in liquid nitrogen, and added to 12 ml GuSCN + 1.2 ml mercaptoethanol. This was stirred vigorously, homogenized and then added to 60 ml 4 M LiCl. The mixture was left at r.t. 20 min and then at 4°C, O/N. Centrifugation at 2,500 rpm for 5 min, 4°C (Sorval, HB-4 rotor) was performed, and the supernatant recentrifuged at 8,000 rpm for 90 min, 4°C (HB-4 rotor). The resulting pellet was resuspended in 40 ml 3 M LiCl, and once again centrifuged at 11,000 rpm for 60 min, 4°C. The pellet produced was resuspended in 2 ml TESDS and extracted with an equal

volume $CHCl_3$:IAA. The phenol phase was then back extracted with 1 ml TESDS. The aqueous phase was then extracted with $CHCl_3$:IAA until no interface was visible, and finally extracted once more with $CHCl_3$:IAA.

0.1 volumes sodium acetate (3 M, pH 5.2) was added along with 2 volumes ethanol (96%, -20°C). The RNA was allowed to precipitate at -20°C, O/N. The mixture was then centrifuged at 12,000 rpm for 15 min, 4°C and the pellet washed with 90% ethanol and allowed to dry. RNA was resuspended in a suitable volume of DEP-H_2O.

2.23.2 Isolation of messenger RNA

This was carried out using an mRNA purification kit (Pharmacia) and used according to the manufacturer's instructions.

2.23.3. RNA Solutions

All solutions were made with DEP - treated water, and autoclaved and all glassware was siliconized prior to use.

GuSCN

5 M guanidine isothiocyanate (Fluka)

10 mM EDTA

50 mM Tris-HCl (pH 7.5)

The mixture was dissolved at 50°C, filtered and stored at -20°C. 0.1 volume β - mercaptoethanol was added prior to use.

TESDS

1M Tris-HCl (pH7.5) - 1ml

0.5M EDTA (pH8) - 0.2ml

10% SDS - 1ml

DEP-H_2O - 97.8ml

Agarose Gel for RNA Analysis

Agarose - 0.3 g

100X MOPS - 0.3 µl

10mg/ml EtBr - 0.6 µl

H_2O - 28.1 ml

After melting and cooling to 50°C, 1.62 ml formaldehyde was added. Before loading, 5 µl RNA solution was heated to 95°C for 5 min and made up to 20 µl with RNA loading buffer.

RNA Loading Buffer

7.2 ml deionized formamide

1.6 ml 10 X MOPS

2.6 ml formaldehyde

1.8 ml DEP-H_2O

1 ml 80% glycerol

0.8 ml saturated bromophenol blue

2.23.4. Measurement of RNA Concentration

The concentration and purity of RNA was measured spectrophotometrically as for DNA samples (see section 2.17). An A_{260} X dilution X 40 µg/ml gives the concentration of the RNA sample in µg/ml.

2.24. PREPARATION OF COSMID BANK FILTERS

2.24.1. Solutions Required

Neutralizing Solution

1.5 M NaCl; 0.5 M Tris-HCl pH 7.2; 1 mM EDTA.

Denaturing Solution

1.5 M NaCl; 0.5 M NaOH.

2.24.2. Preparation of Filters

Individual cosmid clones were inoculated onto LB with ampicillin (100 μg/ml) or LB + kanamycin (50 μg/ml) plates and incubated O/N. Plates were then left at 4°C for 2-3 hr. Nylon membrane was placed over the bacterial colonies (1.5 min) and lifted off. The membrane was then washed in the following sol^{ns}.

- 10% SDS for 3 min
- denaturing sol^{n} for 10 min
- 1 M Tris-HCl pH 8 for 5 min

(this was repeated)

- neutralizing sol^{n} for 5 min.

After drying for 1 hr at r.t. the filter was placed under UV for 4.5 min to 'fix' the DNA to the membrane.

2.25. SOUTHERN BLOTTING AND HYBRIDIZATION

2.25.1. Stock Solutions

20 X SSPE

3 M NaCl; 0.2 M NaH_2PO_4; 25 mM EDTA pH 7.4.

20 X SSC

3 M NaCl; 0.3 M trisodium citrate pH 7.

Pre-hybridization Solution

5 x SSPE; 6% PEG 6000; 0.5% dried skimmed milk (Marvel); 1% SDS; 0.1% $Na_4P_2O_7$; 250 mg/ml denatured herring sperm DNA.

2. 25.2 Transfer of DNA

After electrophoresis of DNA samples, unused areas of the agarose gel were trimmed away and the gel was treated in the following sol^ns.

- 0.25 M HCl for 15 min, to depurinate.
- denaturing buffer for 30 min (as described in section 2.24.)
- neutralization buffer for 15min (as described in section 2.24.)

rinsing with dH_2O between each stage. DNA was then blotted to a nylon membrane in 10 x SSC sol^n. by the capillary method as described in Sambrook *et al.* (1989). DNA was fixed to the dry filter by exposure to UV for 4.5 min.

2.25.3. Hybridization

All pre-hybridization, hybridization and washes were carried out in a water bath at 65°C, with orbital shaking. Nylon membranes were soaked in pre-hybridization sol^n. for ~5 hr when radio-labelled probe was added to the same sol^n., and incubation continued O/N. Following hybridization, unincorporated probe was removed from the filters by washing twice (20 min periods) in 2 x SSC; 0.5% SDS and twice (20 min periods) in 0.5% SSC.

2.25.4 Autoradiography

After washing in the above solutions, the filters were sealed in plastic bags and exposed to autoradiographic film (Fuji RX) in an autoradiography cassette with intensifying screens. The cassette was then stored at -70°C. Exposure was usually O/N, or longer if a stronger signal was required. Upon defrosting the cassette, the film was removed and processed in a Fuji RGII X-ray film developer.

N.B. If a membrane was to be re-probed, it was first 'stripped' by boiling in 0.1% SDS.

2.26. ELECTROPHORETIC SEPARATION AND NORTHERN BLOTTING OF RNA

This was carried out as described in section 11.4 of Davis *et al.*, 1986.

The radiolabelled probing of filters was carried out essentially as in Southern blotting. Hybridization was carried out at 42°C in 50% deionized formamide; 30% 20 X SSPE; 5% 100 X Denhardt's solution; 5% herring sperm DNA (5 mg/ml); 1% SDS.

[Denhardt's solution - 2% gelatine; 2% PVP; 2% Ficoll 400; 1% SDS.]

High stringency washing was done to 0.2 X SSC; 0.1%SDS; 0.1% sodium pyrophosphate.

2.27. SCREENING OF λ PHAGE BANK.

2.27.1 Stock Solutions

SM Buffer.

100 mM NaCl; 10 mM $MgSO_4$; 50 mM Tris-HCl pH 7.5; 0.001% gelatine

TB Broth.

5 g NaCl; 10 g bactotryptone; pH 7.4 with NaOH

After autoclaving - Add - sterile $MgSO_4$ to 10 mM and sterile maltose to 0.2%.

2.27.2 Cell Infection

10 ml TB broth was inoculated with a loopful of LE392 bacterial cells, and incubated at 37°C for 3-4 hr. The cells were then pelleted by centrifugation at 2,000 rpm for 5 min and resuspended in 3 ml 10 mM $MgSO_4$. Eight 150 µl aliquots were then prepared . To each aliquot, 10 µl of a λ-bank dilution (calibrated to give 10,000 plaques/10 ml) were added. The eight aliquots were then incubated at 37°C with slow shaking for 15 min. The infected cells were then added to pre-cooled top agar (48°C) which was overlaid on top of eight NZY plates. The plates were incubated at 37°C for 8 hr (or until plates were covered in small sized plaques).

2.27.3 Preparation of Filters

After incubation, plates were left for 2-3 hr at 4°C. DNA was then fixed to nylon membrane as follows -

- 1min exposure to bacterial plate, the orientation being marked by needle punctures.
- 30 s in denaturing solution.
- 30 s in neutralization solution.
-washed for 5 min in 2 x SSC.

The filters were allowed to dry for 1hr and then exposed to UV for 4.5 min. The filters could then be used following the standard Southern hybridization procedure.

N.B. A duplicate set of filters were made for each plate - these being exposed to the bacterial cells for 3min.

Washing Solutions.

Washing was carried out (also at 65°C) twice (20 min periods) in 5 X SSC; 0.1% SDS; 0.1% sodium pyrophosphate., and twice (20 min periods) in 2 x SSC; 0.1% SDS; 0.1% sodium pyrophosphate.

Autoradiography.

Carried out as for a standard Southern hybridization, but exposing filters to Kodak XAR5 film.

Subsequent Screens.

Autoradiographic film was orientated over filters and original bacterial plates. The area around any positive signals (which also showed up on duplicate filters) was removed from plates with a sterile toothpick, ensuring all the top agar (containing 10-20 plaques) was collected. This top agar was placed in 100 µl SM buffer + 5 µl chloroform, the mixture vortexed for 20 s and then centrifuged 14,000 rpm for 2 min. The resultant supernatant was then used to prepare 1/500 and 1/1000 dilutions. These dilutions were used to infect bacterial cells as before and screening carried out as before.

Screening was continued until single plaques could be isolated, whereupon a final screen was carried out, resulting in every plaque giving a positive hybridization signal. Usually four screenings were required.

2.28 PREPARATION OF λ DNA.

Three plaques were suspended in 200 µl SM buffer and left at r.t. for 2 hr. 400 µl of an O/N LE392 bacterial culture were then added and the plaques resuspended. Incubation at 37°C was carried out for 1 hr. The mixture was then added to 40 ml pre warmed (37°C) LBCM medium (10 g tryptone; 5 g yeast extract; 10 g NaCl; 1 g casamino acids; 2 g $MgSO_4$, made

up to 1L with dH_2O, pH 7.5) and shaken at 37°C for around 6-7 hr. When cell lysis is complete, 0.8 ml chloroform was added to the flask and this was left to incubate for a further 15-30 min. Upon transfer to centrifuge tubes, the mixture was centrifuged for 20 min at 11,000 rpm, 4°C (Sorvall, SS-34 rotor). Resulting supernatant was transferred to a clean tube and pelleted by centrifugation at 19,000 rpm for 3 hr, 4°C (Sorvall, SS-34 rotor). The supernatant was discarded and the tubes allowed to drain. The pellet was resuspended in 400 µl SM buffer and transferred to microfuge tubes. The following were then added - 10 mg RNAse A; 10 ml 10% SDS; 50 ml 2 M Tris/200 mM EDTA pH 8.5. The resulting solution was incubated at 65°C for 15-30 min. 50 ml 5 M KAc was then added and the tubes left on ice, 20-30 min. Microfugation at 14,000 rpm for 10 min followed. The supernatant was removed (in the cold room) and extracted with chloroform 1-2 times and then with phenol:chloroform, 1-2 times. A final extraction with chloroform was then done (vortexing followed each extraction). The aqueous phases for each preparation were then pooled into a 2 ml microfuge tube. DNA was then precipitated by addition of 0.1 volume 3 M NaAc + 1 volume isopropanol. The tubes were then centrifuged briefly (2 min at 5,000 rpm) and the pellet washed in 70% EtOH and dried. DNA was resuspended in 250 µl TE.

2.29. DNA SEQUENCING

DNA sequencing was carried out using the dideoxy - mediated chain termination method as described by Sanger *et al.*, 1977.

Once subcloned into pUC19, insert DNA was sequenced by means of a Sequenase Version 2.0 kit (United States Biochemical Corporation), with reactions done as described in the manufacturers instructions. DNA was radiolabelled with a - ^{35}S α- dATP (ICN). Primers used in the

sequencing reactions were synthesized in the Biochemistry Department, St. Andrews University.

3 μl samples were loaded onto a 6% denaturing polyacrylamide gel and electrophoresed in TBE buffer for an appropriate length of time at 2000V (50°C). The gel was then dried under vacuum at 80°C for 2 hr (BioRad, Model583) and exposed to Kodak X-OMAT film at r.t. for 24 hr.

Solutions Used

10 X TBE Buffer

0.89 M Tris-borate

0.89 M boric acid

0.02 M EDTA pH 8

6% Denaturing Gel

40% polyacrylamide (Scotlab) - 75 ml

Urea - 230 g

10 X TBE - 50 ml

dH_2O - to 500 ml

To 60 ml of the above polyacrylamide solution were added 60 μl 25% ammonium persulphate and 60 μl TEMED (GibcoBRL).

2.30. DNA SEQUENCE ANALYSIS

Sequence data was stored and manipulated on computer via the VWGCG programme (Daresbury, U.K). Sequence analyses were carried out using the BLAST programme (National Centre for Biotechnology Information) available on the Internet (Netscape Navigator 2.0). Genetic alignments were done via the Genetyx-Mac Homology programme.

CHAPTER 3

ISOLATION and CHARACTERIZATION OF THE *cnx*ABC LOCUS OF *A. nidulans*

3.1. INTRODUCTION

This chapter focuses on the isolation and characterization of the *A. nidulans cnx*ABC locus. From previous genetic analysis, it has been suggested that the *brl*A gene is only 3 centiMorgans distant from the *cnx*ABC gene(s) on chromosome VIII. Taking advantage of the fact that the *brl*A gene has already been characterized, this DNA was used as a starting point from which to 'walk' to *cnx*ABC - containing clones within a chromosome - specific *A. nidulans* cosmid bank (Brody *et al.*, 1991).

3.2. SCREENING OF THE CHROMOSOME VIII COSMID LIBRARY WITH *brl*A

With the implication that the *A. nidulans brl*A gene is close to the *cnx*ABC locus, it was expected that cosmids selected from a genomic library using *brl*A as a probe, may also contain the gene(s) under study. A 4.8 kb *Bam*HI fragment taken from the pILJ421 plasmid (Johnstone *et al.*, 1985), which contains the *brl*A gene, was used to screen the chromosome VIII cosmids in high - stringency hybridizations. Five hundred and ninety one cosmids were screened - 263 pLORIST2 clones and 328 pWE15 clones. Two cosmids gave a strongly positive signal. These two cosmids, W6E8 and L28G11, designated 84F6 and 83B12 respectively (the cosmids were renamed for ease of identification due to the way they were stored in 12 X 12 freezer racks in the laboratory in St. Andrews), were investigated further. Clone 84F6 is constructed in the pWE15 vector

which confers ampicillin resistance, while the 83B12 clone is within the pLorist2 vector which contains the kanamycin resistance gene (Fig. 2.3.).

3.3. FUNGAL TRANSFORMATION OF *cnx*A, *cnx*B and *cnx*C MUTANTS

*A. nidulans cnx*ABC mutants are unable to grow on minimal medium containing nitrate, due to their lack of an active nitrate reductase enzyme or on hypoxanthine as sole nitrogen source, due to their lack of an active xanthine dehydrogenase. Each of the *A. nidulans cnx*A5, *cnx*B11 and *cnx*C3 mutants were tested for complementation independently with the two cosmid clones. Both cosmids 84F6 and 83B12 were independently transformed into each mutant to determine if they were repaired for growth on nitrate, thus indicating the presence of a fully functional *cnx*ABC locus within the cosmid. 5 μg DNA was used in each experiment, and transformants were selected for growth on MM containing 10 mM NO_3.

The experiments were carried out several times, with putative *cnx*A5 and *cnx*B11 transformants observed. No growth was seen on the *cnx*C3 selection plates. The data presented in Table 3.1. shows typical complementation frequencies. These initial results indicated that the two cosmids contained the *cnx*AB gene(s), but not the *cnx*C.

3.4. SUBCLONING OF THE 84F6 COSMID

All further work was carried out using only the 84F6 cosmid. After still being able to transform the *cnx*A5 and *cnx*B11 mutants with total *Bam*HI - digested 84F6 DNA (see Table 3.2. for typical transformation frequencies), subclones were made from each of three larger *Bam*HI fragments. 84F6 DNA digested with *Bam*HI is shown in Fig. 3.1.

pSTA84 - 15 kb *Bam*HI fragment ligated into pUC18

Table 3.1.: 'Transformation of *Aspergillus cnx*A5, *cnx*B11 and *cnx*C mutants with the cosmids 84F6 and 83B12'.

	Representative number of NO_3 utilising transformants/µg DNA		
DNA	*cnx*A5 mutant	*cnx*B11 mutant	*cnx*C3 mutant
Cosmid 84F6	5-10	3-10	0
Cosmid 83B12	3-11	2-8	0
No DNA added	0	0	0

Table 3.2.: 'Transformation of *Aspergillus cnx*A5 and *cnx*B11 mutants with *Bam*HI - digested 84F6 DNA'.

	Representative number of NO_3 utilising transformants/µg DNA	
DNA	*cnx*A5 mutant	*cnx*B11 mutant
Cosmid 84F6	9-11	16-20
*Bam*HI digested 84F6	8-11	15-19
No DNA added	0	0

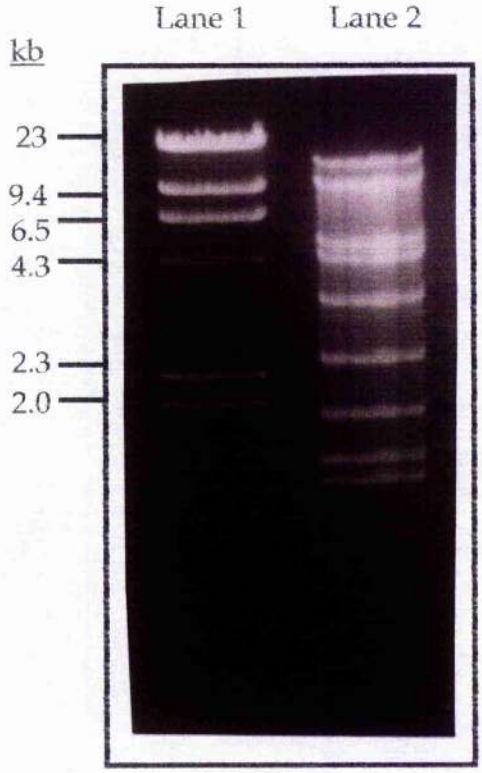


Fig. 3.1. *Bam*HI Digest of Cosmid 84F6 DNA

Lane 1: *Hin*dIII digested lambda DNA. Lane 2: Cosmid 84F6 DNA digested with *Bam*HI. Nine restriction endonuclease fragments are seen - 15 kb, 10 kb, 5.2 kb, 4.5 kb, 3.5 kb, 2.6 kb, 2 kb, 1.6 kb and 1.4 kb.

pSTA83 - 3.5 kb *Bam*HI fragment ligated into pUC18

pSTA82 - 10 kb *Bam*HI fragment ligated into pUC18

The sizes of the other *Bam*HI fragments were as follows - 5.2 kb, 4.5 kb, 2.6 kb, 2 kb, 1.6 kb and 1.4 kb, with most of these smaller bands considered less likely to contain an entire gene.

Each of the subclones (together with cosmid 84F6 as a positive control) were transformed into the *cnx*A5 and *cnx*B11 mutants. The results of these experiments were, however, somewhat uncertain. In some experiments, no transformants were seen on any plate, including the 84F6 cosmid control, and sometimes fungal growth was seen on all plates. The results in Table 3.3. are typical transformation frequencies.

3.5. CHROMOSOME WALKING FROM THE 84F6 COSMID

During the uncertainty of the 84F6 cosmid subclone transformation results, it was decided to carry out a 'chromosome walking' experiment from the 84F6 cosmid in order to identify a cosmid which contained the *cnx*C gene.

Cosmid 84F6 was digested with *Hae*III which does not cut the promoter region and *in vitro* transcription to radiolabelled RNA was carried out using both the T3 and T7 phage RNA polymerase. Only the eukaryotic sequences adjacent to the promoter were transcribed The RNA was used to probe the cosmid bank. Hybridizations showed seven further cosmids. These cosmids were L14E8; L31G2; L30B1; W3E1; W6H1; W28F4 and W26G8, designated 81G12; 83F1; 83D5; 84D1; 84F9; 87B1 and 86G10 respectively. Fig. 3.2. shows each of the cosmids digested with *Eco*RI. It is clear that the cosmids contain overlapping DNA fragments.

Table 3.3.: 'Transformation of *Aspergillus cnx*A5 and *cnx*B11 mutants with 84F6 subclones'.

		Representative number of NO_3 utilising transformants/µg DNA	
DNA		*cnx*A5 mutant	*cnx*B11 mutant
84F6	1	0	10
	2	0	10
	3	>100	0
pSTA82	1	3	9
	2	0	10
	3	>100	0
pSTA83	1	3	4
	2	1	20
	3	>100	0
pSTA84	1	3	5
	2	0	10
	3	>100	0
No DNA added		0-10	0-10

Table 3.4.: 'Transformation of *Aspergillus cnx*C3 mutant with cosmids found by chromosome walking'.

	Representative number of NO_3 utilising transformants/µg DNA
Cosmid DNA	*cnx*C3 mutant
84F6	0
81G12	0
83F1	0
83D5	0
84D1	0
84F9	0
87B1	0
86G10	>300
No DNA added	0

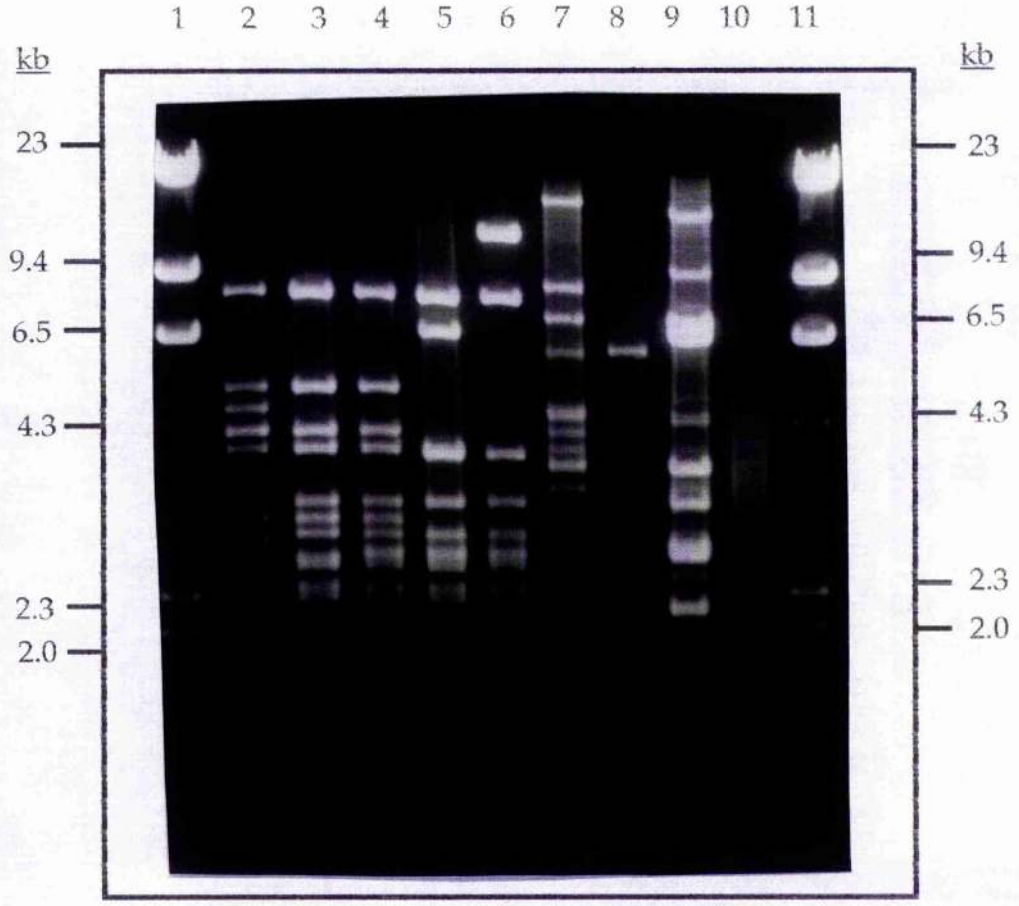


Fig.3.2. *Eco*RI Digests of Chromosome Walking Cosmids

*Eco*RI digests of the cosmids identified by chromosome walking from 84F6. As can be seen, they share many common bands, indicating that the cosmids are overlapping.

Lane 1 -*Hin*dIII digested lambda DNA Lane 2 - 84F6

Lane 3 - 84F9 Lane 4 - 84D1

Lane 5 - 87B1 Lane 6 - 86G10

Lane 7 - 83B12 Lane 8 -83D1

Lane 9 - 81G12 Lane 10 - 100bp ladder

Lane 11 - *Hin*dIII digested lambda DNA

3.6. COMPLEMENTATION OF *cnx*A5, *cnx*B11 and *cnx*C3 MUTANTS WITH THE COSMIDS FOUND BY CHROMOSOME WALKING

Each of the cosmids identified by chromosome walking were isolated, and the DNA used to transform the *cnx*C3 mutant. Only cosmid 86G10 provided us with potential transformants which grew on medium containing nitrate as sole nitrogen source. No growth was seen with any of the other cosmids tested - 81G12, 83F1, 83D5, 84D1, 84F9 or 87B1. This complementation experiment was repeated several times - each time, the 86G10 being the only cosmid to show positive complementation. The data in Table 3.4. shows typical transformation results.

This clone was then used to transform the *cnx*A5 and *cnx*B11 mutants. These mutants were also repaired for growth on nitrate. It therefore appeared that cosmid 86G10 comprised the whole *cnx*ABC locus. A map of where the cosmids lie in relation to one another is shown in Fig. 3.3. This was calculated after probing a series of partial *Eco*RI digests with cosmid fragments. All further experiments were carried out using cosmid 86G10 DNA. After each transformation experiment, putative transformants were tested as being real transformants by their ability to have bacterial vector sequences amplified by a PCR reaction. (See section 2.16 for details).

3.7. DIGESTION OF THE 86G10 COSMID

DNA from cosmid 86G10 was digested with each of the restriction enzymes - *Bam*HI, *Eco*RI, *Hin*dIII and *Sal*I. Total digested DNA was then used to try and retransform each of the *cnx*A5, *cnx*B11 and *cnx*C3 mutants. *Eco*RI - digested DNA was able to complement all three mutants. Table 3.5. presents typical transformation frequencies. Fig. 3.4 shows transformation of the *cnx*C3 mutant with 86G10 DNA. The

Fig. 3.4. Transformation of the *Aspergillus nidulans cnx*C Mutant

Plates showing fungal transformants. The *Aspergillus nidulans cnx*C mutant was transformed with 5 μg of cosmid DNA. The selection medium for transformant growth is minimal medium plus 10mM nitrate.

Plate 1 - 86G10 cosmid DNA. Plate 2 - 84F9 cosmid DNA (control).
Plate 3 - *Bam*HI digested 86G10 DNA. Plate 4 - *Eco*RI digested 86G10 DNA.
Plate 5 - *Hin*dIII digested 86G10 DNA. Plate 6 - *Sal*I digested 86G10 DNA.
Plate 7 - pUC18 DNA (control). Plate 8 - No DNA added (control).

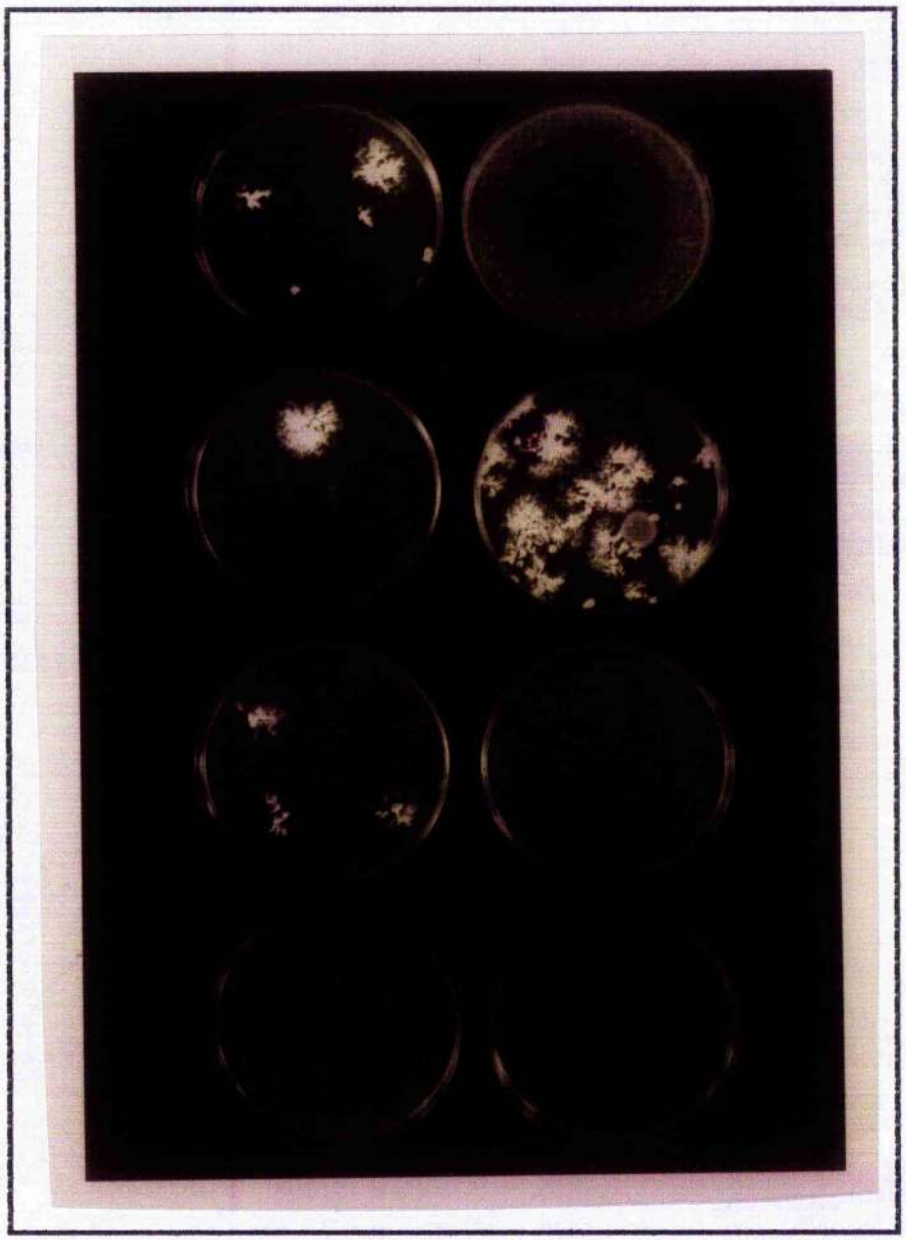


Fig. 3.3.

Restriction map of how the cosmids found by chromosome walking from cosmid 84F6 lie in relation to one another. Only cosmid 86G10 encompasses the entire *cnx*ABC locus. This is reflected in the transformation results, with only cosmid 86G10 able to return the *Aspergillus* mutants *cnx*A5, *cnx*B11 and *cnx*C3 to a wild-type phenotype for growth on nitrate. In this instance, it would seem that 3 cM equates to 50 - 60 kb.

Cosmids Revealed by Chromosome Walking

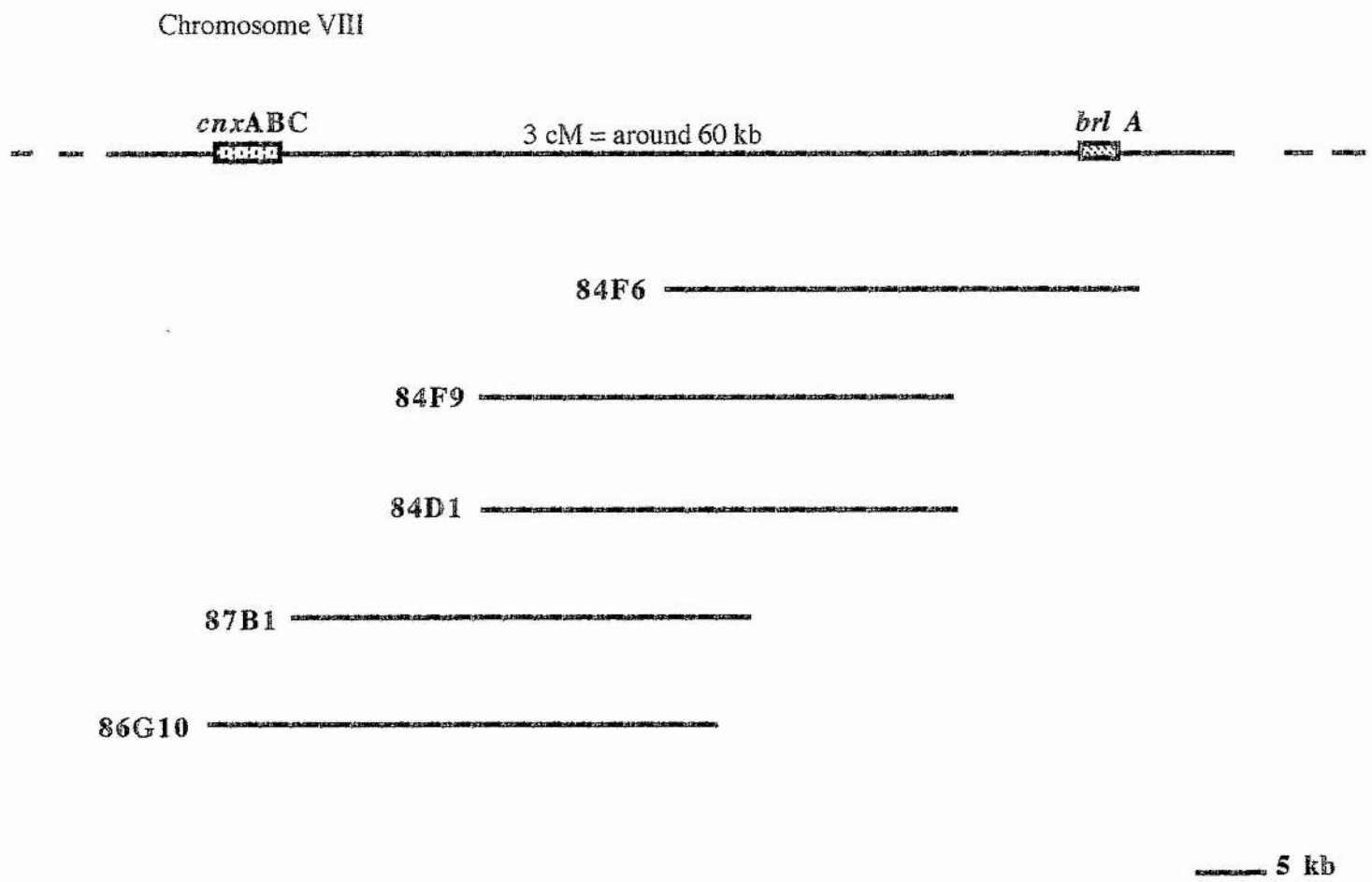


Table 3.5.: 'Transformation of *Aspergillus cnx*A5, *cnx*B11 and *cnx*C3 mutants with restricted 86G10 cosmid DNA'.

	Representative number of NO_3 utilising transformants/µg DNA		
DNA	*cnx*A5 mutant	*cnx*B11 mutant	*cnx*C3 mutant
Cosmid 86G10	50-150	70-120	80-300
Cosmid 84F9	0	0	0
*Bam*HI digested DNA	0	0	1-2
*Eco*RI digested DNA	28-85	>100	50-55
*Hin*dIII digested DNA	3-10	0	0
*Sal*I digested DNA	n/d	n/d	0-3
No DNA added	0	0	0

Table 3.6.: 'Transformation of *Aspergillus cnx*A5, *cnx*B11 and *cnx*C3 mutants with 86G10 subclones'.

	Representative number of NO_3 utilising transformants/µg DNA		
DNA	*cnx*A5 mutant	*cnx*B11 mutant	*cnx*C3 mutant
pSTA502	100-200	200-300	>1000
pSTA502+pHelp	>2000	>800	>1000
pSTA503	0	0	0
pSTA504	2-5	3-7	80-100
pSTA505	0	0	0
pSTA506	10-12	5-10	70-100
pSTA506+pHelp	11-15	80-100	>300
No DNA added	0	0	0

mutant phenotype has been restored to wild-type growth on medium containing nitrate as sole nitrogen source.

Partial digests were carried out and a detailed restriction map of the 86G10 area of DNA was drawn up.

3.8. SUBCLONING OF THE 86G10 COSMID

By studying the restriction map of the area, various subclones were constructed - each fragment having been inserted into the pUC19 vector.

pSTA502 - 6.8 kb *Hin*dIII - *Eco*RI fragment.

pSTA503 - 2.9 kb *Pst*I fragment.

pSTA504 - 3 kb *Bam*HI - *Eco*RI fragment.

pSTA505 - 3.3 kb *Xba*I - *Bam*HI fragment.

pSTA506 - 3.9 kb *Pst*I - *Eco*RI fragment.

pSTA507 - 1.2 kb *Hin*dIII - *Eco*RI fragment.

Subclones shown in Fig. 3.5. were individually transformed into each of the *cnx*A5, *cnx*B11 and *cnx*C3 mutants. pSTA502 complemented all three mutants at a very high frequency. However, pSTA505 was not found to give rise to transformants in any of the mutants tested. pSTA506, on the other hand, was able to complement all three mutants, although at a reduced frequency. (These experiments were carried out as co-transformations in the presence of the pHelp plasmid in a ratio of 4:1, pHelp:DNA). The results presented in Table 3.6. are representative transformation frequencies obtained in these experiments.

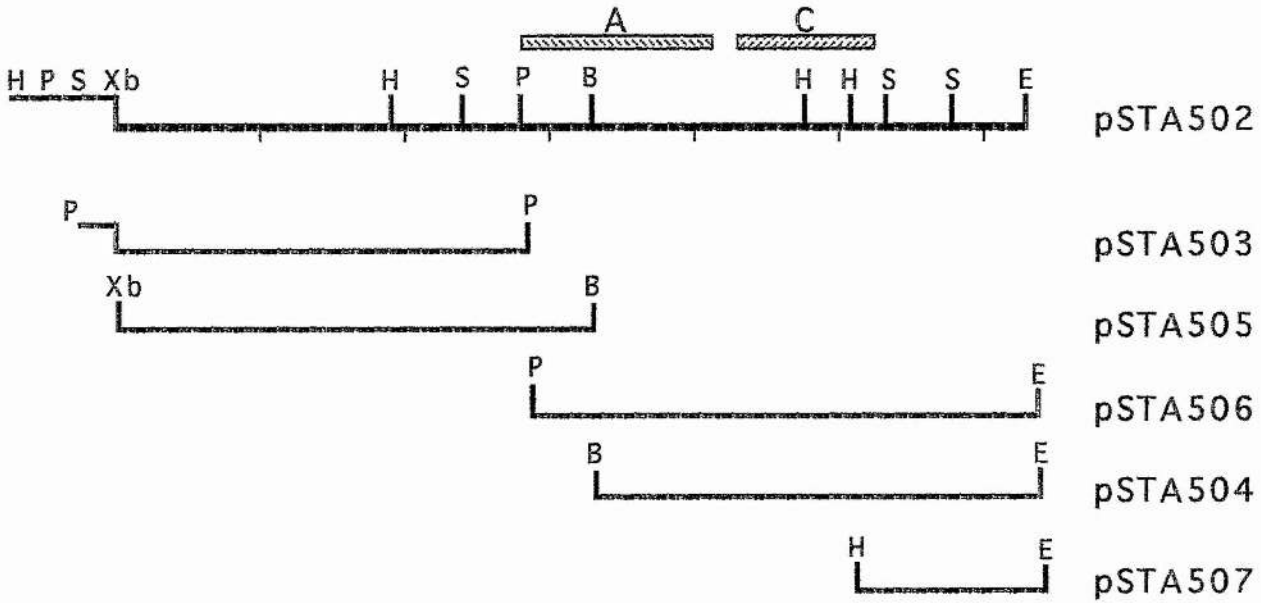


Fig. 3.5. Subclones Derived from Cosmid 86G10

Map of the plasmid subclone pSTA502 and the smaller subclones therein. The position of the *cnx*A and *cnx*C genes is marked. The restriction endonuclease fragments are inserted into pUC18.

3.9. SEQUENCING OF THE *cnx*ABC LOCUS

Both DNA strands of subclone pSTA506 and part of subclone pSTA505 were sequenced by the di-deoxy chain termination method, initially using the -40 universal forward primer (GTCGTGACTGGGAAAC) and universal reverse primer (TTCACACACAGGAAACAG) (United States Biochemicals). Subsequent reactions were carried out with DNA primers synthesized at St. Andrews University. The sequencing strategy was as shown in Fig. 3.6., with a list of the primers used, set out in Fig. 3.7. Two open reading frames of 448 amino acids and 256 amino acids were noted. The results presented in Fig. 3.8. show the nucleotide and amino acid sequence which encompasses these two open reading frames.

3.10. DATABASE SEARCHES

The nucleotide sequence of each ORF was analysed on computer, and using the BLASTX programme (Altschul *et al.*, 1990), peptide sequences which show homology with the sequence in question were discovered. (The BLASTX programme uses the nucleotide sequence to search databases in all six reading frames for any possible matches).

The protein section which is encoded by DNA which retransforms the *Aspergillus cnx*A5 mutant, shows homology with the MOAA protein of *E. coli* and with the CNX2 protein of the plant *Arabidopsis thaliana* (Fig. 3.9.).

Further protein sequence which is encoded by DNA which retransforms the *Aspergillus cnx*C3 mutant, shows homology with the MOAC protein of *E. coli* and with the CNX3 protein of *Arabidopsis thaliana* (Fig. 3.10.).

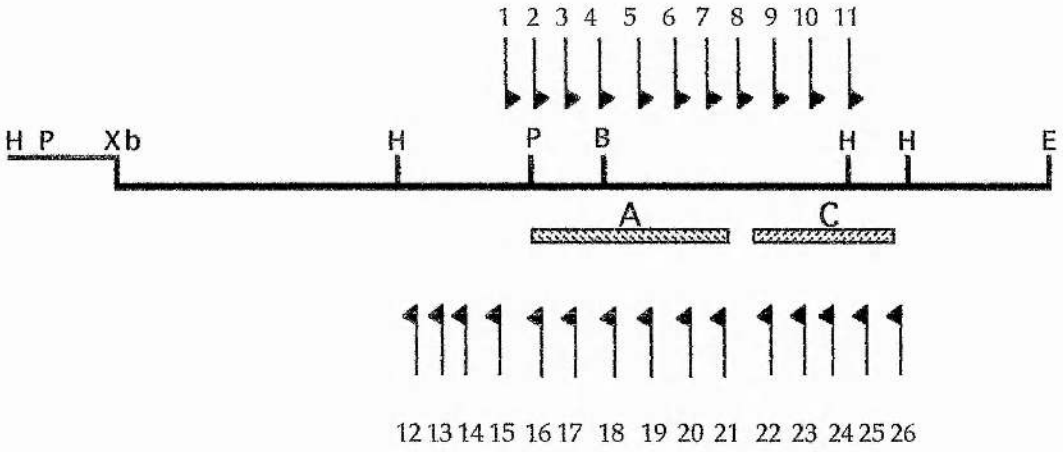


Fig. 3.6. Sequencing Strategy of the pSTA502 Subclone

Outline of the sequencing strategy used in characterization of the *cnx*ABC locus within plasmid subclone pSTA502. Sequencing was carried out on both strands of DNA. A full list of primers used is described in Fig. 3.7.

H - *Hin*dIII; P - *Pst*I; Xb - *Xba*I; E - *Eco*RI; B - *Bam*HI

1). ABCP1 - 5' - CGTTGTCGAGCAGAATC
2). 506R1 - 5' - CATCGTAAGCTCGAGCC
3). ABCP3 - 5' - GTAAATCTCAGTCTGGAC
4). 506R2 - 5' - CAATTGTGTTGTGATGC
5). 506R3 - 5' - TGGATTCCAAGGCCGAG
6). ABCP5 - 5' - TGAGGCAGCTCGAACAG
7). 506R4 - 5' - CTTATTGGTGGGTAGGC
8). 506R8 - 5' - TTGTATACATGATTCAC
9). 506R5 - 5' - CTTTACCATCGCGAACG
10). ABCP7 - 5' - ATATGACGCTGATTGATG
11). 506R6 - 5' - ATCGCTCGTATTGCCGG
12). 505F6 - 5' - CGGACGGCATGTTCGGA
13). 505F5 - 5' - GGATATTGAAATATGAC
14). 505F4 - 5' - GTTCCCGAGCGGTTGAT
15). 505F3 - 5' - CGATTAGTAATTGCAGT
16). 505F2 - 5' - CGCAAGTGGAAGTTCAG
17). 505F1 - 5' - CCTGACGTCGCCGGAGA
18). ABCP2 - 5' GCGGTTATGAAGAGCATA
19). Rcomp2 - 5' - CGAGCATGACGCATAAC
20). ABCP4 - 5' - CTTGACGTTATTGGCATG
21). Rcomp1 - 5' - GCCTACCCACCAATAAG
22). Rcomp3 - 5' - CGTTCGCGATGGTAAAG
23). ABCP6 - 5' - CTTCTCCAACCGACGTC
24). Rcomp4 - 5' - CCGGCAATACGAGCGAT
25). Rcomp5 - 5' - CAAGAGCGGGCATTGGG
26). 506F5 - 5' - CCTGGATGTGTATGACA

Fig. 3.7. Sequence of Primers

List of the primers used in sequencing the pSTA502 subclone. Primers represent both directions of sequencing although they are listed as reading on one DNA strand only, except for Rcomp1, Rcomp3 and Rcomp4 which are the reverse complement sequences of 506R4, 506R5 and 506R6 respectively.

Fig. 3.8. Sequence of the *cnx*ABC Locus

Nucleotide and amino acid sequence of the region of *Aspergillus nidulans* genomic DNA which transforms *cnx*A5, *cnx*B11 and *cnx*C3 mutants to wild-type. Two open reading frames are seen. 1115 bp upstream of the first ORF is also shown. The intron splice sites are shown underlined in bold (positions 1543 and 1738 respectively), while a typical internal intron motif often found in filamentous fungi is shown underlined. Upstream of the coding region, potential TATA and CAAT boxes are seen at positions -241, -315, -446 and -509 respectively. Candidate binding sites for the AREA protein (WGATAR/YTATCW) are underlined as is a putative NIRA binding site at position -146.

```
-1115 TTTGACCTCAAATATCGTGTGGCAAGGAAAGATCATATAAAGACGTCCAAAAAACCGTTT

-1055 AACATAAAGCTTTATAAACTAAGAACTAGGCTTTAGTTATGTCTCTGCTCAAAGAGCGGT

 -995 TTGAGCTACTATCTCGATCAATACTGTTTGTACTTTATGCTGCGTTTCGGGTAGGGTTAA

 -935 ACAGACAACATCGGACGGCATGTTCGGAGCGACGTCACGACGGCGCCCAGAAGAATAGTT

 -875 GGTATCTTGACGTCTTGAAAATCCCATATTTCCGCCTTTGAAAATGTGTTCGCGGTAATA

 -815 CAGCCCTTGGTCTATATCTGGGTCGCAACGTATGCTCGTCAGAAAGGCACAGGCTGCGGG

 -755 CTTGGCGGGCAATGTGAGAACCCCCATGGCAAGCAGAGGAATGATCTGAGGGATTAGATG

 -695 TCATGAAGATTAATCGCTACCTCAAATAGTGGCCGGATATTGAAATATGACGACAAATGC

 -635 ACTTCATGTTCGAGGCATACAGTATGGCCATTGGTTCCTTGGATGAAGCACGCGGGGACA

 -575 TTCGATGATTCCTCGCGCCCTCTGTTTGTGGAGCCATATGACACTAACAAATCACTACAG

 -515 CAGAGATCAATATTAGACTCGAAGCTGACGCGACGGAGGCATCAGACTAAACCTGATCCG

 -455 CGAACTGCACAATTACCAAAGAAGCTATCCAACGAAGTCGACCACGGTTGTTCCCGAGCG

 -395 GTTGATCAGATGCCCTCAAGAAACGGGCGTTGATGCAGTATCAGACAAGAAAACAATCAG

 -335 AGGGCGACACCCAAGCCTAATTTAAATATAGAGTCCATCAGCGTTCTGTTGGGAAATGTC

 -275 AGCATATTGTCTAATATCAGTTGAACATATCTGCTATAATCTTGCTTTTTTAGCGTACGT

 -215 CAGTGCACCGACAGCTATCTTATCGATTAGTAATTGCAGTACTAAGTCAGGTGATGGAGG

 -155 CGACCGAACCCGCGGAGAACAGCGACGATAAGAAATTCCTTATTATCCCATCCTTCCATC

  -95 GTTTGCCTTGGTTTTAGTTGTTAGTGCTTTGTTCCCTCGGGTGATGAAGCGCGTACTTGG

  -35 GATCTGGCCGTTGTCGAGCAGAATCCCTGCAGGAGATGACTGAGCCCATGCTATTTAAGC
                                                M  T  E  P  M  L  F  K  R    9

   26 GGGGCGCAGCTCAAGCGACTTCGTCCTTTTCTCGATTTCCCCGCATCCATACTCGAACTC
        G  A  A  Q  A  T  S  S  F  S  R  F  P  R  I  H  T  R  T  R    29

   86 GACTCCCGACGTGGCGCCGGTTGAGCCCTTCGCCTTCGCGATATGTCACTACCGGTTTGA
        L  P  T  W  R  R  L  S  P  S  P  S  R  Y  V  T  T  G  L  N    49

  146 ACCCGCAAGTGGAAGTTCAGGATGAACCAACTAGGTCTCCATCTCCTACACCGACATCGT
        P  Q  V  E  V  Q  D  E  P  T  R  S  P  S  P  T  P  T  S  S    69

  206 CGACTCGCTGGAACGCGCTGAAAACAGCAAAGCCGTTCTCGGCGTTTCTGACAGACACAT
        T  R  W  N  A  L  K  T  A  K  P  F  S  A  F  L  T  D  T  F    89

  266 TCAACCGCCAGCATGATTACCTTAGGATCAGTGTCACGGAGCGCTGCAATCTGCGCTGTC
        N  R  Q  H  D  Y  L  R  I  S  V  T  E  R  C  N  L  R  C  L   109

  326 TATATTGTATGCCTGAAGAAGGCGTACCGCTATCCCCTCCCGCGCACGTCCTGACGTCGC
        Y  C  M  P  E  E  G  V  P  L  S  P  P  A  H  V  L  T  S  P   129
```

386 CGGAGATTGTGTACCTTTCGTCGCTCTTCGTCTCGCAGGGCGTGACCAAGATCCGTCTGA
E I V Y L S S L F V S Q G V T K I R L T 149

446 CTGGCGGAGAACCGACTGTTCGGAAAGACATCGTTCCTCTGATGCAGTCAATTGGAGAAT
G G E P T V R K D I V P L M Q S I G E L 169

506 TACGACATCACGGACTCCGAGAATTATGCTTGACAACCAATGGCATTTCACTCCATCGTA
R H H G L R E L C L T T N G I S L H R K 189

566 AGCTCGAGCCTATGGTGGAGGCTGGGTTGACTGGGGTAAATCTCAGTCTGGACACTTTGG
L E P M V E A G L T G V N L S L D T L D 209

626 ATCCGTTCCAGTTTCAAATTATGACGAGGAGGAAGGGCTTTGATGCGGTTATGAAGAGCA
P F Q F Q I M T R R K G F D A V M K S I 229

686 TAGACCGCATCCAGGAGCTGAATAAGATGGGAGCTGGGATAAAGCTCAAGATCAATTGTG
D R I Q E L N K M G A G I K L K I N C V 249

946 TTGTGATGCGGGGCCTCAACGAACGCGAGATTATTCCGTTTGTCGAAATGGGGCGTGATA
V M R G L N E R E I I P F V E M G R D S 269

1006 GCCCCATCGAAGTGCGGTTCATTGAGTATATGCCATTTGATGGCAATAAGTGGAGTAAGG
P I E V R F I E Y M P F D G N K W S K G 289

1066 GAAAAATGGTTTCCTATCAGGAGATGCTGGCCCTTATTCGGGAGAAGTATCCAACATTGG
K M V S Y Q E M L A L I R E K Y P T L E 309

1126 AGAAGGTGGTGGATCATAAGAATGACACGAGCAAAACTTATCGCATTCCTGGATTCCAAG
K V V D H K N D T S K T Y R I P G F Q G 329

1186 GCCGAGTTGGCTTTATCACGAGCATGACGCATAACTTCTGCGGCACTTGCAACCGCCTTC
R V G F I T S M T H N F C G T C N R L R 349

1246 GCATTACGTGCGATGGGAATCTTAAAGTCTGCCTATTTGGAAACTCGGAAGTCTCGCTGC
I T C D G N L K V C L F G N S E V S L R 369

1306 GTGATATAATCCGACAACAGAATAATGGCGAGCCCATTGACGAGACTGCGCTGCAAGAGC
D I I R Q Q N N G E P I D E T A L Q E L 389

1366 TAGGTCTCCTTGAGGCAGCTCGAACAGCAGCCCGCGTTCATGACGAAGGTGGAGTAGTCA
G L L E A A R T A A R V H D E G G V V S 409

1426 GTCAAAGAGAAAGAGAGCTTCTTGACGTTATTGGCATGGCAGTGAAGCGGAAAAAGGCCA
Q R E R E L L D V I G M A V K R K K A K 429

1486 AGCATGCCGGCATGGGAGAGTTAGAGAACATGAAGAACCGGCCGATGATCCTTATTG**gtg**
H A G M G E L E N M K N R P M I L I D 447

1546 **ggt**aggcactttccaccacctatatatatgactgcgacgagagactatgatcgatgagtt

1606 gtatacatgattcacttggtttttttttctttttttttttttccttccttttctttttctt

1667 gacgcgggcgttcaaatcacattcttgtatatacggttatgcatgacactgagagatgct
449

1727 gataggcggtt**cag**ATAAAACGAGCGATGCCCAGAGAAATATAAGGTACTTCGCCTCCAT
K T S D A Q R N I R Y F A S M 469

```
1787 GTCGTCAATGATGTCCAAGGGACAAGTAATGAACGTATCGACGCATTCACTCGGTCTCGG
      S  S  M  M  S  K  G  Q  V  M  N  V  S  T  H  S  L  G  L  G    489

1847 GATGCCAATGGCTACCCAAGTCCGACTTTACCATCGCGAACGTACCACAACTTCGTGCGA
      M  P  M  A  T  Q  V  R  L  Y  H  R  E  R  T  T  T  S  C  E    509

1907 GGAACCCAGCAACAAGGACTCAAAATTCGCTTCGCTCCCTACATCCGACGACCCCGATCT
      E  P  S  N  K  D  S  K  F  A  S  L  P  T  S  D  D  P  D  L    529

1967 GCCTCATCTCAATCGCTCGCAAAACGTCCATATGACGCTGATTGATGAAAAGCCCATTTC
      P  H  L  N  R  S  Q  N  V  H  M  T  L  I  D  E  K  P  I  S    549

2027 AAAACGCCTCGCAACAGCCACCTGTCACGTCCGCTTCTCCAACCGACGTCCCTGGGAACT
      K  R  L  A  T  A  T  C  H  V  R  F  S  N  R  R  P  W  E  L    569

2087 CCTCAGACAAGGCCCCGGCAGCCGCAAAGGCGACGTATTTGGCATCGCTCGTATTGCCGG
      L  R  Q  G  P  G  S  R  K  G  D  V  F  G  I  A  R  I  A  G    589

2147 TATCACCGCCGCGAAGAAGACGCCGGATATTGTACCGCTTTGTCATCCTGGGCTGGGGTT
      I  T  A  A  K  K  T  P  D  I  V  P  L  C  H  P  G  L  G  L    609

2207 GACGGGGGTGGAGGTGGATGTGAAGCTTCTTGACCCGTCCGCCGATGACGCTGAGATGAA
      T  G  V  E  V  D  V  K  L  L  D  P  S  A  D  D  A  E  M  K    629

2267 GCATGGGGCAATGCATGTCACGGCCACGGTGGGCTGTGTTGGAAGAACGGGGGTGGAGAT
      H  G  A  M  H  V  T  A  T  V  G  C  V  G  R  T  G  V  E  M    649

2327 GGAAGCCATGACGGCGACGATGGGGGCTGCATTGACGGTGTATGATATGTTGAAAGCGGT
      E  A  M  T  A  T  M  G  A  A  L  T  V  Y  D  M  L  K  A  V    669

2387 TGACAAGGGGATGGTGATTGGGGGAGTGAAACTGCTAGAGAAGATGGGGGGCAAGAGCGG
      D  K  G  M  V  I  G  G  V  K  L  L  E  K  M  G  G  K  S  G    689

2447 GCATTGGGTAAGAGAGGAGAACGTGAAGGATGAGTAGATGTGGTTGACACACTGGGTTGA
      H  W  V  R  E  E  N  V  K  D  E  *                            700

2507 GTCTGTACCTGTACCTATTTACCTGGATGTGTATGACAACGGGACCTGGCGGT
```

Fig. 3.9. Homology of the CNXA Amino Acid Sequence with the *E.coli* MOAA Protein and with the *A.thaliana* CNX2 Protein

Alignments opposite show how the CNXA amino acid sequence compares to those of MOAA - *E.coli* (Oshima et al., 1996) and CNX2 - *A.thaliana* (Hoff et al., 1995). There is a 31.8% similarity between CNXA and MOAA and 56.9% with CNX2. Asterisks below sequences denote identity and dots similarity.

```
 61' SPSPTPTSSTRWNALKTAKPFSAFLTDTFNRQHDYLRISVTERCNLRCLYCMPEEGVPLS  cnxA
                                ***.*.*.  ***.*.*. **.** **.*..  * .
  1"                        MASQLTDAFARKFYYLRLSITDVCNFRCTYCLPDGYKPSG  moaA

121' PPAH-VLTSPEIVYLSSLFVSQGVTKIRLTGGEPTVRKDIVPLMQSIGELRHHGLRELCL  cnxA
   ...   **  **  ... *.. *..*.*******..*.*.. ..... *  ....*.. .
 41" VTNKGFLTVDEIRRVTRAFARLGTEKVRLTGGEPSLRRDFTDIIAAVRE--NDAIRQIAV  moaA

180' TTNGISLHRKLEPMVEAGLTGVNLSLDTLDPFQFQIMTRRKGFDAVMKSIDRIQELNKMG  cnxA
     **** .*.*....  .*****.*.*.*.**. **. .* .. *..** .**     . ..
 99" TTNGYRLERDVASWRDAGLTGINVSVDSLDARQFHAITGQDKFNQVMAGID-----AAFE  moaA

240' AGI-KLKINCVVMRGLNEREIIPFVEMGRDSPIEVRFIEYMPF-DGNKWSKGKMVSYQEM  cnxA
     **. *.*.* *.**..*.... .*..  ...**..**** *  .*.. . . .* * .
154" AGFEKVKVNTVLMRDVNHHQLDTFLNWIQHRPIQLRFIELMETGEGSELFRKHHISGQVL  moaA

298' LALIREKYPTLEKVVDHKNDTSKTYRIPGFQGRVGFITSMTHNFCGTCNRLRITCDGNLK  cnxA
     . . .  .... .......... *...* .*.* . ...**.******... *.*.
214" RDELLRR-GWIHQLRQRSDGPAQVFCHPDYAGEIGLIMPYEKDFCATCNRLRVSSIGKLH  moaA

358' VCLFGNSEVSLRDIIRQQNNGEPIDETALQELGLLEAARTAARVHDEGGVVSQRERELLD  cnxA
     .****...*.***.. .... .....   ..*
273" LCLFGEGGVNLRDLLEDDTQQQALEARISAALREKKQTHFLHQNNTGITQNLSYIGG      moaA
```

```
  1' MTEPMLFKRGAAQATSSFSRFPRIHTRTRLPTWRRLSPSPSRYVTTGLNPQVEVQDEPTR  cnxA

  1"                        MRRCFSKITDCHLGFKNSNFLLVGSEVGSGSVTRTITTTTS  cnx2

 61' SPSPTPTSSTRWNALKTAKPFSAFLTDTFNRQHDYLRISVTERCNLRCLYCMPEEGVPLS  cnxA
                              .* *..*.*.*.* *.*****.******** ****.*** *.
 42" ERLFSSSYAAHQVDQIKDNPVSDMLIDKFGRLHTYLRISLTERCNLRCQYCMPSEGVELT  cnx2

121' PPAHVLTSPEIVYLSSLFVSQGVTKIRLTGGEPTVRKDIVPLMQSIGELRHHGLRELCLT  cnxA
     * ...*. .*** *..****.**.***************  .  ...*.  **..* .*
102" PKPQLLSQSEIVRLAGLFVSAGVNKIRLTGGEPTVRKDIEEICLQLSSLK--GLKNLAIT  cnx2

181' TNGISLHRKLEPMVEAGLTGVNLSLDTLDPFQFQIMTRRKGFDAVMKSIDRIQELNKMGA  cnxA
     ****.* .**  .. * **...*.***** * .*...***** * ******   **    .
160" TNGITLAKKLPRLKECGLDSLNISLDTLVPAKFEFLTRRKGHDRVMKSIDTAIEL----G  cnx2

241' GIKLKINCVVMRGLNEREIIPFVEMGRDSPIEVRFIEYMPFDGNKWSKGKMVSYQEMLAL  cnxA
       .*.***.*****. **  ***..**.**.*****.****** *.  *.*.*.*....
216" YNPVKVNCVIMRGLNDDEICDFVELTRDKPINVRFIEFMPFDGNVWNVKKLVPYAEVMDK  cnx2

301' IREKYPTLEKVVDHKNDTSKTYRIPGFQGRVGFITSMTHNFCGTCNRLRITCDGNLKVCL  cnxA
     . ...*..... ** ..*.*.. * *  *.*.******..**..*****.  ***.****
276" VVKRFPSIKRMQDHPTETAKNFTIDGHCGSVSFITSMTEHFCAGCNRLRLLADGNFKVCL  cnx2

361' FGNSEVSLRDIIRQQNNGEPIDETALQELGLLEAARTAARVHDEGGVVSQRERELLDVIG  cnxA
     ** *******  .*  ...*.. *                    .
336" FGPSEVSLRDPLRSGADDEALREIIGAAVKRKKAAHAGMLDIAKTANRPMIHIGG       cnx2
```

Fig. 3.10. Homology of the *cnx*C Amino Acid Sequence with the *E.coli moa*C Protein and with the *A.thaliana cnx*3 Protein

The alignments opposite show how the CNXC amino acid sequence compares to those of MOAC - *E.coli* (Oshima et al., 1996) and CNX3 - *A.thaliana* (Hoff et al., 1995). There is a 42.9% similarity between CNXC and MOAC and 39.6% with CNX3. Asterisks denote residue identity and dots similarity.

```
 61' DSKFASLPTSDDPDLPHLNRSQNVHMTLIDEKPISKRLATATCHVRFSNRRPWELLRQGP   cnxC
                ..*.*.* . ..**. ...*. . * * *.  * . .. . ... .*.
  1"           MSQLTHINAAGEAHMVNVSAKAETVREARAEAFVTM-RSETLAMIIDGR   moaC

121' GSRKGDVFGIARIAGITAAKKTPDIVPLCHPGLGLTGVEVDVKLLDPSADDAEMKHGAMH   cnxC
   .*****..****** ***.* *..***** * *.   *.*.* . .*... .     .
 49" -HHKGDVFATARIAGIQAAKRTWDLIPLCHP-LMLSK--VEVNLQAERAQSGRI-----E   moaC

181' VTATVGCVGRTGVEMEAMTATMGAALTVYDMLKAVDKGMVIGGVKLLEKMGGKSGHWVRE   cnxC
.   ..  *.*******.**.  ****.*** ***.*.**** *.**.* *****..  *
100" TLCRLT--GKTGVEMEALTAASVAALTIYDMCKAVQKDMVIGPVRLLAKSGGKSGDFKVE   moaC

241' ENVKDE                                                        cnxC
     ..
158" ADD                                                           moaC
```

```
  1'                                          KTSDAQRNIRYFASMSSM   cnxC

  1" MISTLRRAVFLRRFPAVVSPIKRAFSSRIDDEFDPQIMNINELNQEMQSIFGQEPSPDGP   cnx3

 19' MSKGQVMNVSTHSLGLGMPMATQVRLYHRERTTTSCEEPSNKDSKFASLPTSDDPDLPHL   cnxC
                                    ...* *... *.  ...*.. .** ..*.*.
 61" GTMDFSELKSSKIEPLRSKNIDFRQQIEYHKSTHSSKNDSQAIEQYAKV-ASDMSKLTHV   cnx3

 79' NRSQNVHMTLIDEKPISKRLATATCHVRFSNRRPWELLRQGPGSRKGDVFGIARIAGITA   cnxC
. . ...*. ...*  *** * * *.* .. .* ..*. ... . ****.*.*.****..
120" GIAGEAQMVDVSSKDNSKRTALACCKVILG-KRVFDLVLANQMG-KGDVLGVAKIAGING   cnx3

139' AKKTPDIVPLCHPGLGLTGVEVDVKLLDPSADDAEMKHGAMHVTATVGCVGRTGVEMEAM   cnxC
**.*....**** ...** * **.. *.*       .. .........*.*.********
178" AKQTSSLIPLCH-NIALTHVRVDLR-LNP-------EDFSVDIEGEASCTGKTGVEMEAM   cnx3

199' TATMGAALTVYDMLKAVDKGMVIGGVKLLEKMGGKSGHWVREENVKDE               cnxC
**.  *.****** **..*.. *..*.*  * ***** * *
229" TAVSVAGLTVYDMCKAASKDISITDVRLERKTGGKSGSWSRL                     cnx3
```

3.11. THE *cnx*ABC LOCUS CONSISTS OF ONE GENE

From the completed DNA sequence, a 198 bp non-coding region of DNA is seen between two open reading frames. This would indicate that there are either two separate genes or that there is one gene interrupted by an intron. Two primers bridging this area - 506R2 and Rcomp3 - were used in PCR reactions using both DNA and RNA. The DNA and RNA products are shown in Fig. 3.11. The difference in size of the products indicates that there is indeed an untranscribed region of around 190 bp in length. By analysis of the sequence, no promoter region for the second ORF is seen. Typical intron boundaries are however noted - GTGGGT - at the end of ORF1 and - CAG - just previous to ORF2. An internal intron sequence is also seen - CACTGAG - (shown underlined in Fig. 3.8.) (Gurr *et al.*, 1988).

This therefore indicates that the *cnx*ABC locus consists of only one gene which has one intron within its sequence. Indeed, the 'intron -less' PCR product (Fig. 3.11.) has subsequently been sequenced and the position of the intron verified (Unkles *et al.*, in press).

3.12. NORTHERN BLOT OF pSTA502

The pSTA502 subclone was used to probe mRNA prepared from mycelia grown in medium with ammonium as sole nitrogen source and from mycelia grown in medium containing nitrate as sole nitrogen source. Fig. 3.12. shows that in each case three, perhaps four, messages are seen. The signals in the lane containing RNA prepared from mycelia grown in nitrate medium are more intense than those on RNA prepared from mycelia grown in ammonium medium, indicating a modest nitrate induction of *cnx*ABC RNA synthesis.

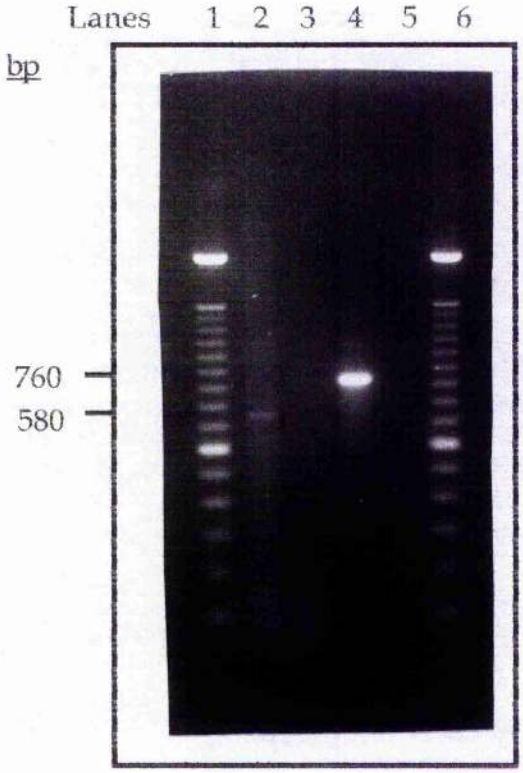


Fig. 3.11. Demonstration of the Presence of an Intron

The primers 506R2 and Rcomp3 were used in PCR reactions using both RNA and DNA, so that the sizes of the two products can be compared directly. From the photograph it can be seen that the RNA PCR product is around 570bp while the DNA product is around 760bp. This would imply that there is an intron of around 190bp within the *cnx*ABC locus.

Lanes 1 and 6 - 100bp ladder

Lane 2 - RNA + reverse transcriptase

Lane 3 - RNA - reverse transcriptase

Lane 4 - pSTA502 DNA

Lane 5 - no DNA or RNA

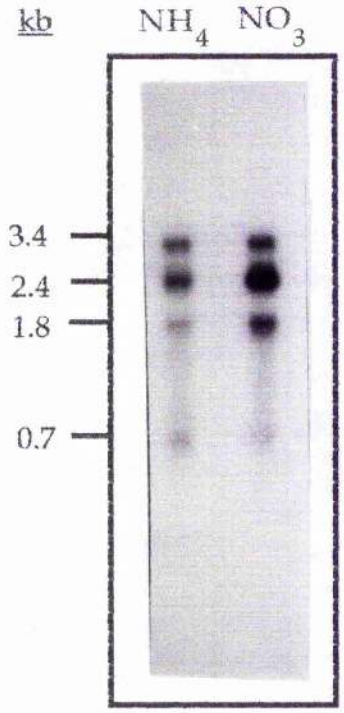


Fig. 3.12. Hybridization of pSTA502 to *Aspergillus* w.t. RNA

Messenger RNA prepared from w.t. mycelia grown in medium with ammonium as sole nitrogen source and from medium with nitrate as sole nitrogen source was probed with pSTA502. At least three messages can be seen.

3.13. NITRATE INDUCTION

To investigate this further, mRNA was prepared from wild-type mycelia grown in medium containing ammonium as sole nitrogen source and from mycelia grown in medium containing nitrate as sole nitrogen source. The 0.6 kb *Bam*HI-*Pst*I fragment of pSTA502 (*cnx*A) and the 0.35 kb *Hin*dIII fragment of pSTA502 (*cnx*C) were both used to probe RNA prepared under the two different growth conditions. In the first case, a 2.4 kb fragment is seen and in the latter, two messages - 2.4 kb and 1.8 kb are highlighted. In both cases the nitrate signals are stronger than the ammonium. From the Northern blot shown in Fig. 3.13., nitrate would indeed seem to be involved in an induction of the system.

Using a gene, which was not involved in the nitrate assimilation pathway in any way, as a control, the *act*A gene was also used as a probe under the same conditions. Only one message is seen in each case and no nitrate induction is visualised (Fig. 3.14.).

3.14. ARE THERE MULTIPLE COPIES OF *cnx*C?

From the result of the Northern blot in Fig. 3.13., it should be considered that there may be more than one transcript of the *cnx*C gene. To investigate this further, a Southern hybridization was carried out. Wild-type DNA was probed with the *Pst*I-*Bam*HI fragment of pSTA502 (*cnx*A) and with a PCR fragment generated from the primers 506R4 and 506F5 (*cnx*C). Each probe was hybridized to four different digests - *Eco*RI; *Eco*RI/*Pst*I; *Bam*HI/*Xba*I and *Nru*I. In every case, only one band gave a signal in each digest, corresponding to the size of fragment expected from the restriction map. It would therefore appear that there is indeed only one copy of the *cnx*ABC locus (Fig. 3.15.). It should be noted that the Northern blotting experiment as shown in Fig. 3.13. has subsequently

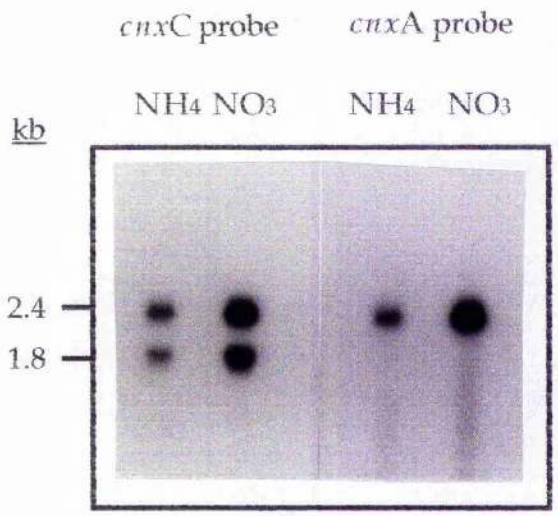


Fig. 3.13. Nitrate Induction of *cnx*A/*cnx*C RNA Synthesis

Messenger RNA was prepared from wild-type mycelium grown in medium with ammonium as sole nitrogen source. The first two lanes in the above photo were probed with the DNA fragment containing *cnx*C and the last two lanes were hybridized against a *cnx*A probe. Nitrate appears to be a slight inducer of RNA synthesis.

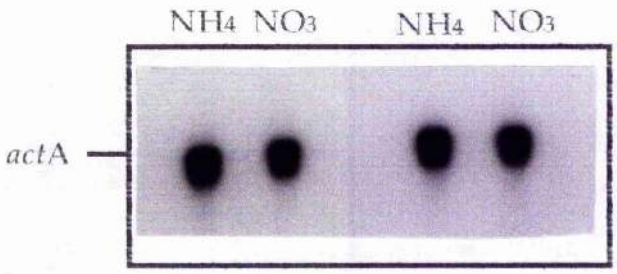


Fig. 3.14. Nitrate Induction (Control)

Northern blot with mRNA prepared from mycelia as in Fig. 3.12. The *act*A gene was used to probe the mRNA, thus providing a control. The intensity of hybridization appears to be the same with nitrate as with ammonium.

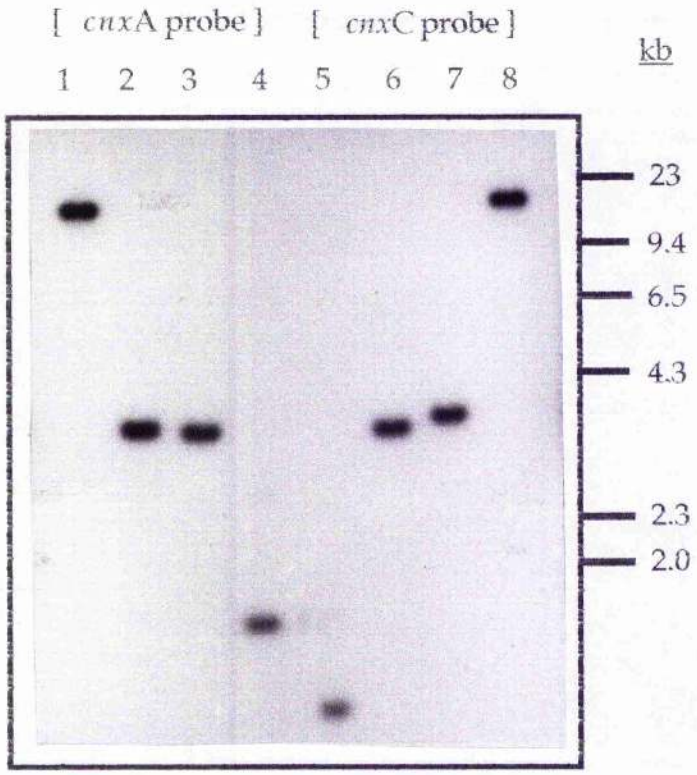


Fig. 3. 15. Hybridization of *cnx*A and *cnx*C Genes to Wild-type DNA

5µg w.t. DNA was digested and used in a Southern blot probed with the *Pst*I-*Bam*HI fragment of pSTA502 which encompasses the *cnx*A gene and with a PCR fragment which covers the *cnx*C gene. (Primers used - 506R4 and 506F5). Lanes 1 and 8 - *Eco*RI digested DNA. Lanes 2 and 7 - *Eco*RI/*Pst*I. Lanes 3 and 6 - *Bam*HI/*Xba*I. Lanes 4 and 5 - *Nru*I.
*Hin*dIII digested lambda DNA was used as a size marker.

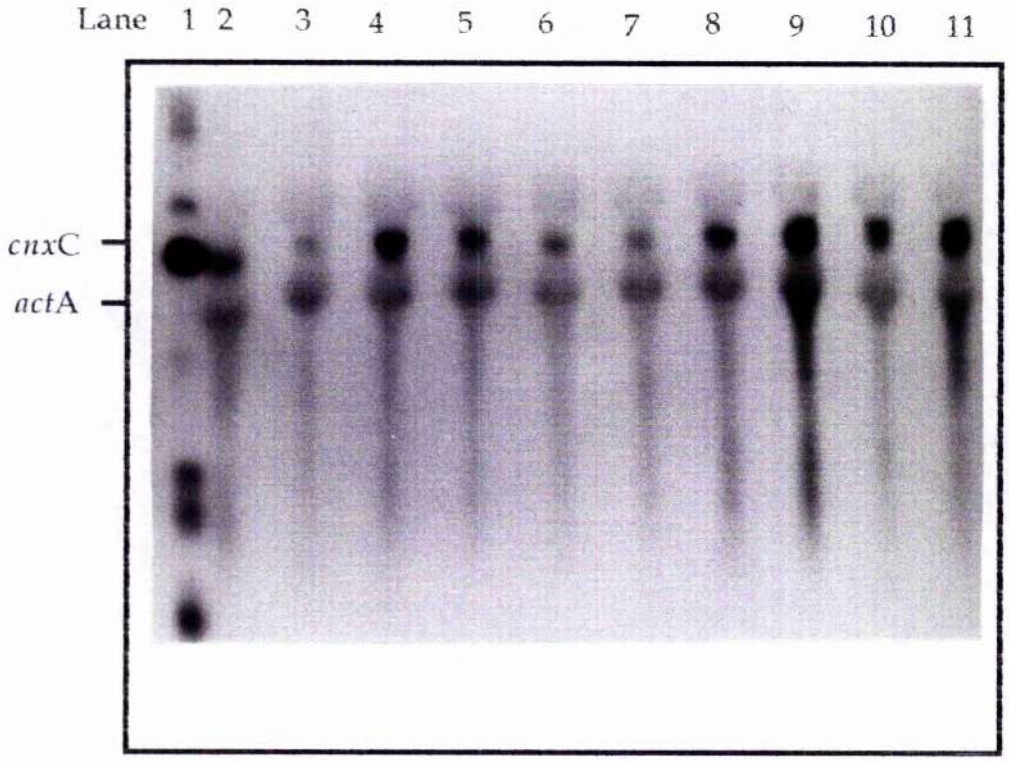


Fig. 3.16. Effect of Different Nitrogen Sources on RNA Synthesis

Total RNA was prepared from wild-type mycelia grown in different nitrogen sources and was probed with the 0.35 kb *Hin*dIII fragment of pSTA502 (Fig. 3.5) and with the *act*A gene (Fidel *et al.*, 1988) from *Aspergillus nidulans*.

Lane 1 - size markers

Nitrogen sources (10mM final concentration) were as follows:

Lane 2 - uric acid Lane 3 - ammonium

Lane 4 - nitrate Lane 5 - nitrite

Lane 6 - adenine Lane 7 - glutamate

Total RNA in lanes 8-11 was prepared from mycelia grown in ammonium for 16hr at 30°C and then transferred to nitrate for 5hr.

Lane 8 - wild-type mycelia Lane 9 - *cnx*A5 mutant mycelia

Lane 10 - *cnx*B11 mutant mycelia

Lane 11 - *cnx*C3 mutant mycelia.

been repeated in order to verify the result. In every following experiment tried, the 1.8 kb band has not been reproducible. It would therefore appear that in the experiment shown in Fig. 3.13., this second band is some kind of artefact, and there is indeed only one transcript of 2.4 kb in size.

3.15. EFFECT OF DIFFERENT NITROGEN SOURCES ON RNA SYNTHESIS

Total RNA prepared from wild-type mycelia grown in medium containing different nitrogen sources was Northern blotted and probed with the 0.35 kb *Hin*dIII fragment of pSTA502. This is a fragment contained within the *cnx*C gene. Mycelia from each of the *cnx*ABC mutants was also used to prepare RNA which was also probed with the *cnx*C fragment. The *act*A gene was used as a control in the same experiment. Nitrogen sources used were uric acid, ammonium, nitrate, nitrite, adenine and glutamate - all at a concentration of 10 mM. The mutant mycelia were grown in ammonium - containing medium and transferred to nitrate medium. Once again, the only noticeable effect that is seen from Fig. 3. 16. is a nitrate induction of RNA synthesis.

3.16. SUMMARY

This chapter reports on the isolation of a 6.8 kb clone which is able to restore *Aspergillus nidulans cnx*A5, *cnx*B11 and *cnx*C3 mutants to the wild-type phenotype for growth on nitrate as a sole nitrogen source. The nucleotide and amino acid sequence of the *cnx*ABC locus is presented and homology with proteins from *E.coli* and *A.thaliana* shown. Initial regulation studies have been done, and by means of Northern blots, a modest induction of *cnx*ABC RNA by nitrate is

indicated. An insight into the possible structure of the locus is now possible.

CHAPTER 4

THE ISOLATION AND CHARACTERIZATION OF THE *cnx*J GENE OF *Aspergillus nidulans*

4.1. INTRODUCTION

This chapter is concerned with the identification of *A.nidulans* clones found to contain the previously uncharacterized *cnx*J gene. Initial clones were obtained by exploiting the fact that *cnx*J was known to lie fairly close to the previously cloned *prn* gene cluster on chromosome VII. (Arst and MacDonald, 1975; Hull *et al.*, 1989). The cluster comprises *prn*A which is a regulatory gene mediating proline induction, *prn*D encoding proline oxidase, *prn*B coding for proline permease (Sophianopoulou and Scazzocchio, 1989) and *prn*C coding for pyrroline 5-carboxylate dehydrogenase. A 2 kb *Hin*dIII fragment, from a plasmid designated pAN229 which contains the *prn*A gene, was used as a hybridization probe with which to identify putative *cnx*J - containing clones (Fig. 2.3.).

4.2. SCREENING OF THE *A.nidulans* COSMID LIBRARY WITH pAN229

In an attempt to find DNA clones which contained the *cnx*J gene, a 2 kb *Hin*dIII fragment of pAN229 was used as a probe in the hybridization of an *A.nidulans* cosmid bank. All the chromosome VII clones were screened at high stringency. Four hundred and sixty eight cosmids were screened - 203 pLORIST clones and 265 pWE15 clones. No cosmids were found to hybridize to the pAN229 probe. It therefore appeared that the

DNA of interest was not represented within this library. Using a similar strategy, an *A. nidulans* λ-phage bank was subsequently utilised.

4.3. SCREENING OF THE *A.nidulans* λ-PHAGE BANK WITH pAN229

The 2 kb *Hin*dIII fragment of pAN229 was used in a hybridization of 65,000 plaques from the λ-phage library (Prepared by V. Appleyard, University of St. Andrews) using a Lambda DashII /*Bam*HI vector kit - Stratagene. Twenty-nine positively - hybridizing clones were subjected to further screening. 1:1000 dilutions of these plaques were reprobed a further twice. The secondary screen identified seventeen clones on the basis of hybridization on duplicate filters. Thirteen purified clones were obtained after a tertiary screen.

4.4. DNA PREPARATION OF SIX λ CLONES

From the thirteen positively - hybridizing clones obtained from the λ bank, six were chosen at random and DNA prepared from them. This was done as described in Chapter 2. The six clones identified were designated 1.1; 1.2; 2.3; 5.1; 5.2 and 8.2.

4.5. DIGESTION AND SOUTHERN BLOTTING OF SIX λ CLONES

DNA from each of the six lambda clones was digested with each of *Bgl*II, *Hin*dIII, *Pst*I, *Sal*I and *Xho*I restriction enzymes. Restriction patterns were compared for the presence of common bands between the clones. Indeed, many similarities are seen, although there are also differences amongst the clones (Fig. 4.1a and 4.1b).

After electrophoresis, the gels were Southern blotted to nylon membranes. *Hin*dIII - digested pAN229 was then used as a probe against these digestions in subsequent hybridizations. From the positively -

Fig. 4.1a. Restriction Digests of λ Clones

Restriction digests of three putative *cnxJ* - containing λ - clones. *Bgl*II, *Hin*dIII, *Pst*I, SalI, and *Xho*I were the enzymes used in this analysis.
Lanes 1 and 19 - *Hin*dIII - digested λ DNA MWt. markers. Lanes 2 and 18 - pGEM MWt. markers. Lane 3 - λ clone 1.1 digested with *Bgl*II.
Lane 4 - λ clone 1.1 digested with *Hin*dIII. Lane 5 - λ clone 1.1 digested with *Pst*I. Lane 6 - λ clone 1.1 digested with *Sal*I. Lane 7 - λ clone 1.1 digested with *Xho*I. Lane 8 - λ clone 1.2 digested with *Bgl*II. Lane 9 - λ clone 1.2 digested with *Hin*dIII. Lane 10 - λ clone 1.2 digested with *Pst*I. Lane 11 - λ clone 1.2 digested with *Sal*I. Lane 12 - λ clone 1.2 digested with *Xho*I. Lane 13 - λ clone 2.3 digested with *Bgl*II. Lane 14 - λ clone 2.3 digested with *Hin*dIII. Lane 15 - λ clone 2.3 digested with *Pst*I. Lane 16 - λ clone 2.3 digested with *Sal*I. Lane 17 - λ clone 2.3 digested with *Xho*I.

Fig. 4.1b. Restriction Digests of λ Clones

Restriction digests of three putative *cnxJ* - containing λ - clones. *Bgl*II, *Hin*dIII, *Pst*I, *Sal*I and *Xho*I were the enzymes used in this analysis.
Lanes 1 and 19 - *Hin*dIII - digested λ DNA MWt. markers. Lanes 2 and 18 - pGEM MWt. markers. Lane 3 - λ clone 5.1 digested with *Bgl*II. Lane 4 - λ clone 5.1 digested with *Hin*dIII. Lane 5 - λ clone 5.1 digested with *Pst*I. Lane 6 - λ clone 5.1 digested with *Sal*I. Lane 7 - λ clone 5.1 digested with *Xho*I. Lane 8 - λ clone 5.2 digested with *Bgl*II. Lane 9 - λ clone 5.2 digested with *Hin*dIII. Lane 10 - λ clone 5.2 digested with *Pst*I. Lane 11 - λ clone 5.2 digested with *Sal*I. Lane 12 - λ clone 5.2 digested with *Xho*I. Lane 13 - λ clone 8.2 digested with *Bgl*II. Lane 14 - λ clone 8.2 digested with *Hin*dIII. Lane 15 - λ clone 8.2 digested with *Pst*I. Lane 16 - λ clone 8.2 digested with *Sal*I. Lane 17 - λ clone 8.2 digested with *Xho*I.

hybridizing bands which resulted, it was possible to determine roughly where each clone lay relative to the *prn* region, and whether it was likely to contain the *cnx*J gene or not (Fig. 4.2a and 4.2b).

4.6. MAPPING OF SIX CLONES RELATIVE TO THE PROLINE UTILIZATION GENES

From Southern blot analysis, it was possible to position the six clones along chromosome VII. Their position was mapped according to the presence of certain restriction fragments. When these bands are compared to restriction fragments as shown in Fig. 4.3, their geography can be determined.

Clone 1.1 - 2 kb *Hin*dIII; 4.6 kb *Bgl*II; 4.3 kb *Sal*I.

Clone 1.2 - 6.5 kb *Sal*I; 6 kb *Xho*I.

Clone 2.3 - 12 kb *Sal*I; 3 kb *Sal*I.

Clone 5.1 - 4.6 kb *Bgl*II; 5 kb *Sal*I.

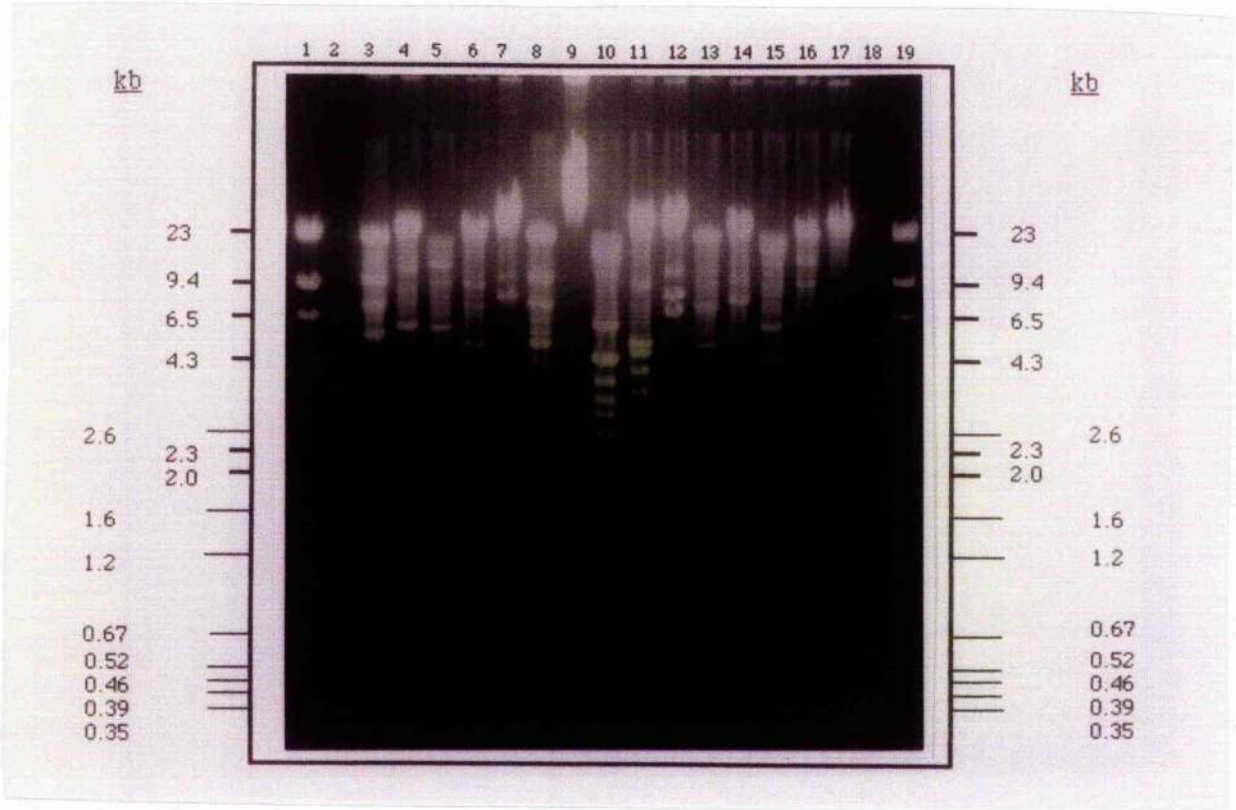
Clone 5.2 - Truncated 2 kb *Hin*dIII (1.5 kb).

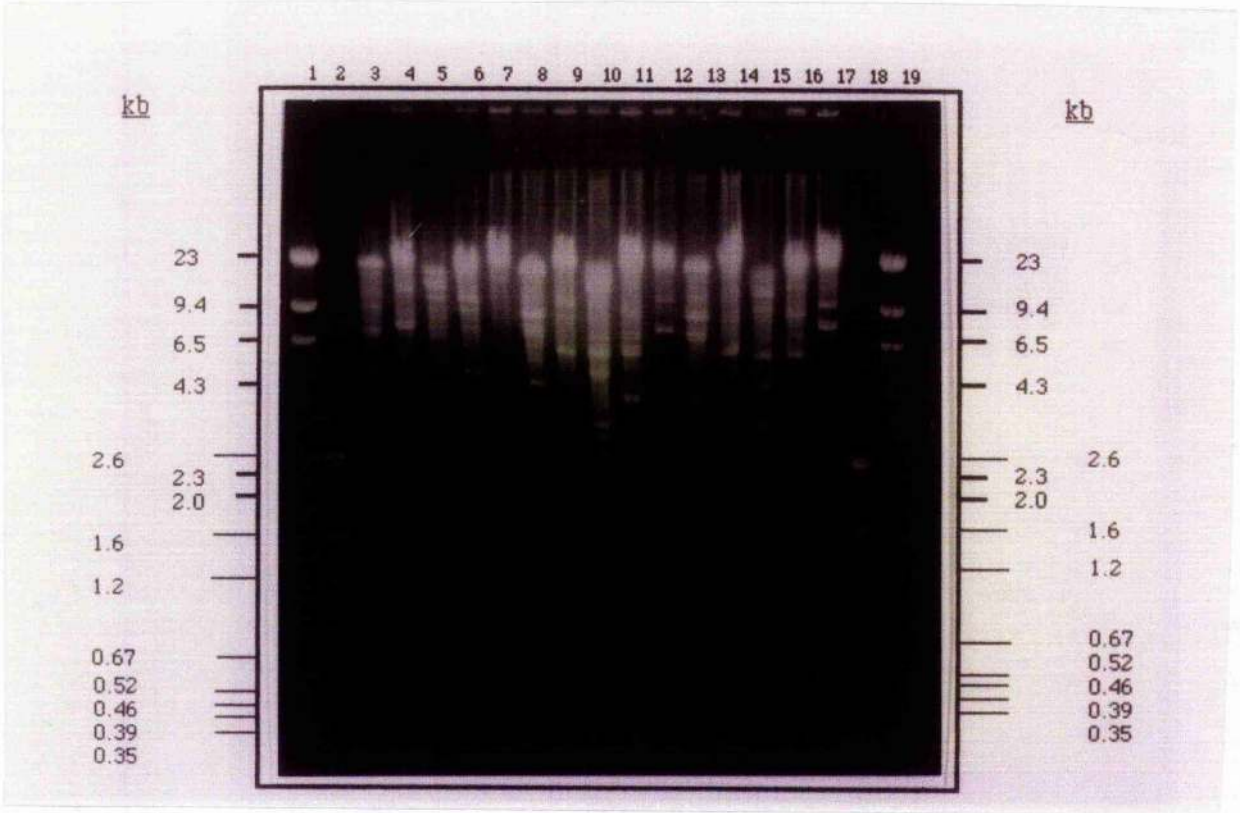
Clone 8.2 - Unclear from blot.

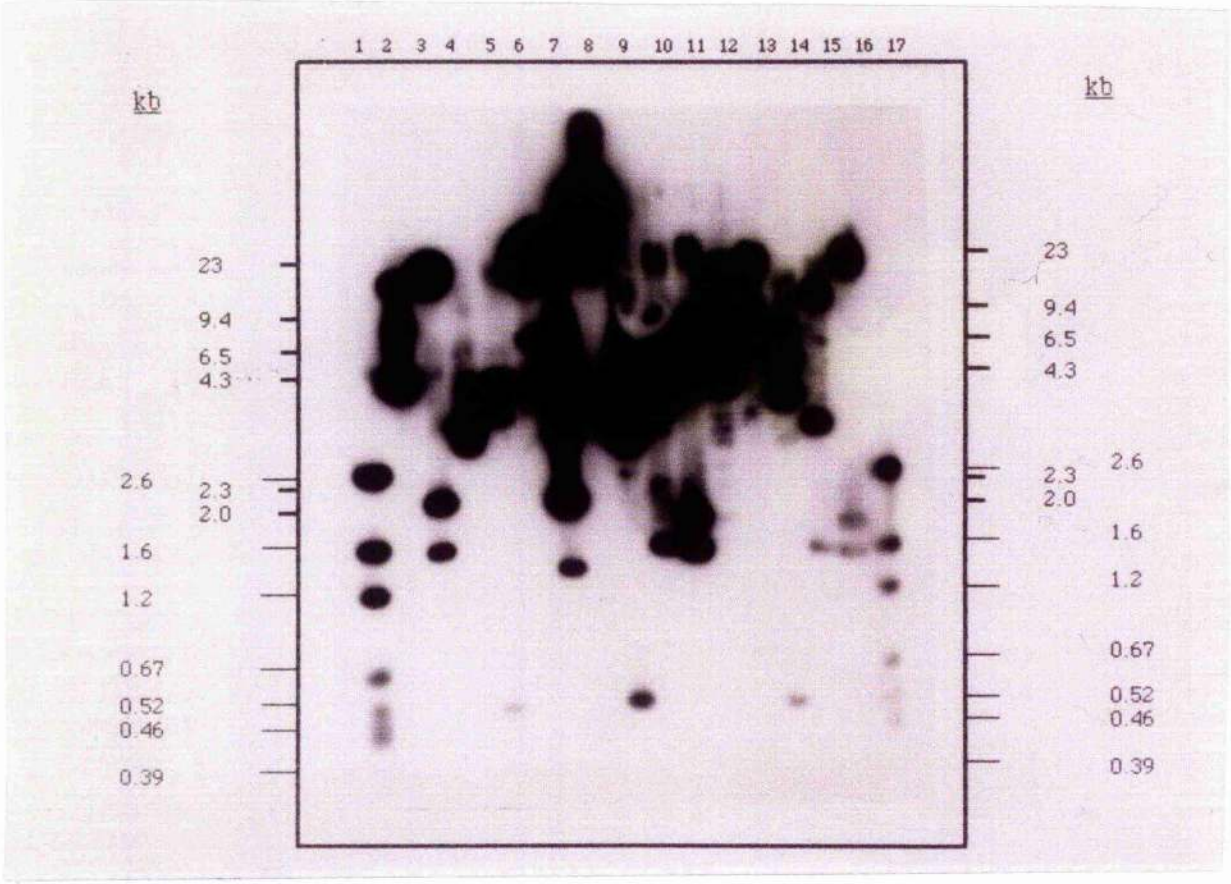
From this it was decided that clones 1.1 and 5.2 were most likely to contain *cnx*J, as they both extend away from the proline region. (Fig. 4.4.).

4.7. TRANSFORMATION OF AN *Aspergillus nidulans cnx*J MUTANT

In an attempt to prove the existence of the *cnx*J gene on either/both of the two λ clones, namely 1.1 and 5.2, fungal transformations were performed. This is where exogenous DNA is taken up by fungal protoplasts and integrates into the host genome. The *cnx*J mutant is unable to grow in the presence of tungstate (WO_4), unlike the wild-type which can tolerate WO_4 concentrations up to 33 mM (Arst *et al.*, 1982). Transformants were selected for on minimal medium containing 10 mM NO_3 and 30 mM


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
kb
kb
23
9.4
6.5
4.3
2.6
2.3
2.0
1.6
1.2
0.67
0.52
0.46
0.39
0.35


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
kb
kb
23
9.4
6.5
4.3
2.6
2.3
2.0
1.6
1.2
0.67
0.52
0.46
0.39
0.35


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
kb
23
9.4
6.5
4.3
2.6
2.3
2.0
1.6
1.2
0.67
0.52
0.46
0.39
kb
23
9.4
6.5
4.3
2.6
2.3
2.0
1.6
1.2
0.67
0.52
0.46
0.39

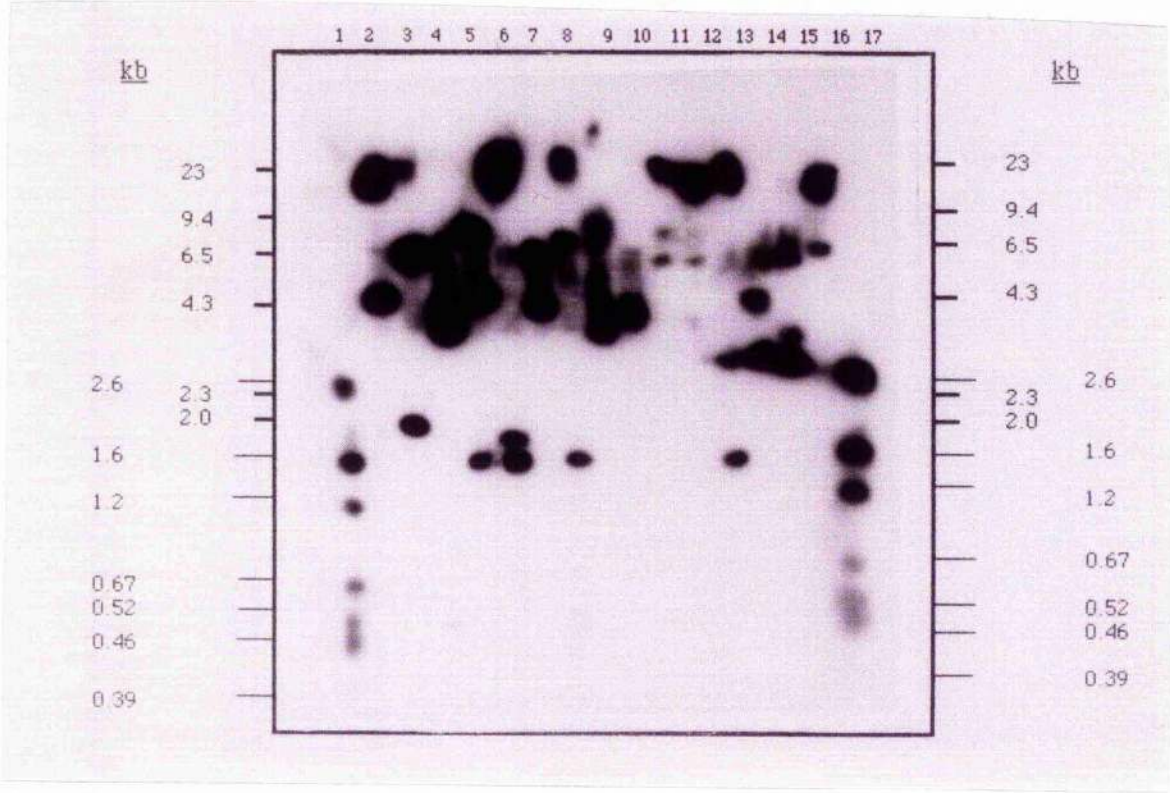

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
kb
23
9.4
6.5
4.3
2.6
2.3
2.0
1.6
1.2
0.67
0.52
0.46
0.39
kb
23
9.4
6.5
4.3
2.6
2.3
2.0
1.6
1.2
0.67
0.52
0.46
0.39

Fig. 4.2a. pAN229 Southern Blot

Southern blot of three putative *cnx*J - containing λ - clones digested with various restriction enzymes and probed with *Hin*dIII - digested pAN229 plasmid. Lanes 1 and 17 - pGEM MWt. markers. Lane 2 - λ clone 1.1 digested with *Bgl*II. Lane 3 - λ clone 1.1 digested with *Hin*dIII. Lane 4 - λ clone 1.1 digested with *Pst*I. Lane 5 - λ clone 1.1 digested with *Sal*I. Lane 6 - λ clone 1.1 digested with *Xho*I. Lane 7 - λ clone 1.2 digested with *Bgl*II. Lane 8 - λ clone 1.2 digested with *Hin*dIII. Lane 9 - λ clone 1.2 digested with *Pst*I. Lane 10 - λ clone 1.2 digested with *Sal*I. Lane 11 - λ clone 1.2 digested with *Xho*I. Lane 12 - λ clone 2.3 digested with *Bgl*II. Lane 13 - λ clone 2.3 digested with *Hin*dIII. Lane 14 - λ clone 2.3 digested with PstI. Lane 15 - λ clone 2.3 digested with *Sal*I. Lane 16 - λ clone 2.3 digested with *Xho*I.

Fig. 4.2b. pAN229 Southern Blot

Southern blot of three putative *cnx*J - containing λ - clones digested with various restriction enzymes and probed with *Hin*dIII - digested pAN229 plasmid. Lanes 1 and 17 - pGEM MWt. markers. Lane 2 - λ clone 5.1 digested with *Bgl*II. Lane 3 - λ clone 5.1 digested with *Hin*dIII. Lane 4 - λ clone 5.1 digested with *Pst*I. Lane 5 - λ clone 5.1 digested with *Sal*I. Lane 6 - λ clone 5.1 digested with *Xho*I. Lane 7 - λ clone 5.2 digested with *Bgl*II. Lane 8 - λ clone 5.2 digested with *Hin*dIII. Lane 9 - λ clone 5.2 digested with *Pst*I. Lane 10 - λ clone 5.2 digested with *Sal*I. Lane 11 - λ clone 5.2 digested with *Xho*I. Lane 12 - λ clone 8.2 digested with *Bgl*II. Lane 13 - λ clone 8.2 digested with *Hin*dIII. Lane 14 - λ clone 8.2 digested with PstI. Lane 15 - λ clone 8.2 digested with SalI. Lane 16 - λ clone 8.2 digested with *Xho*I.

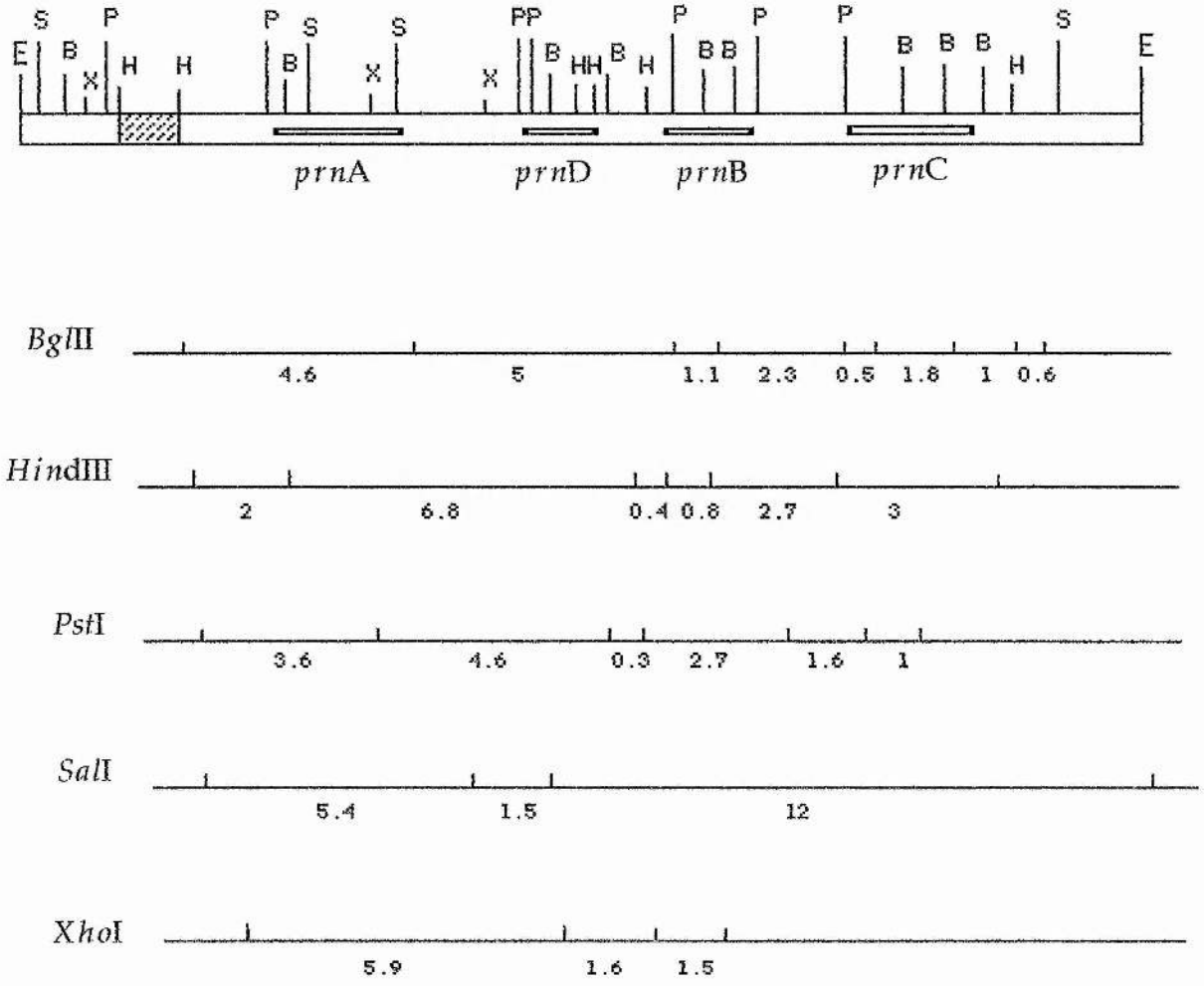
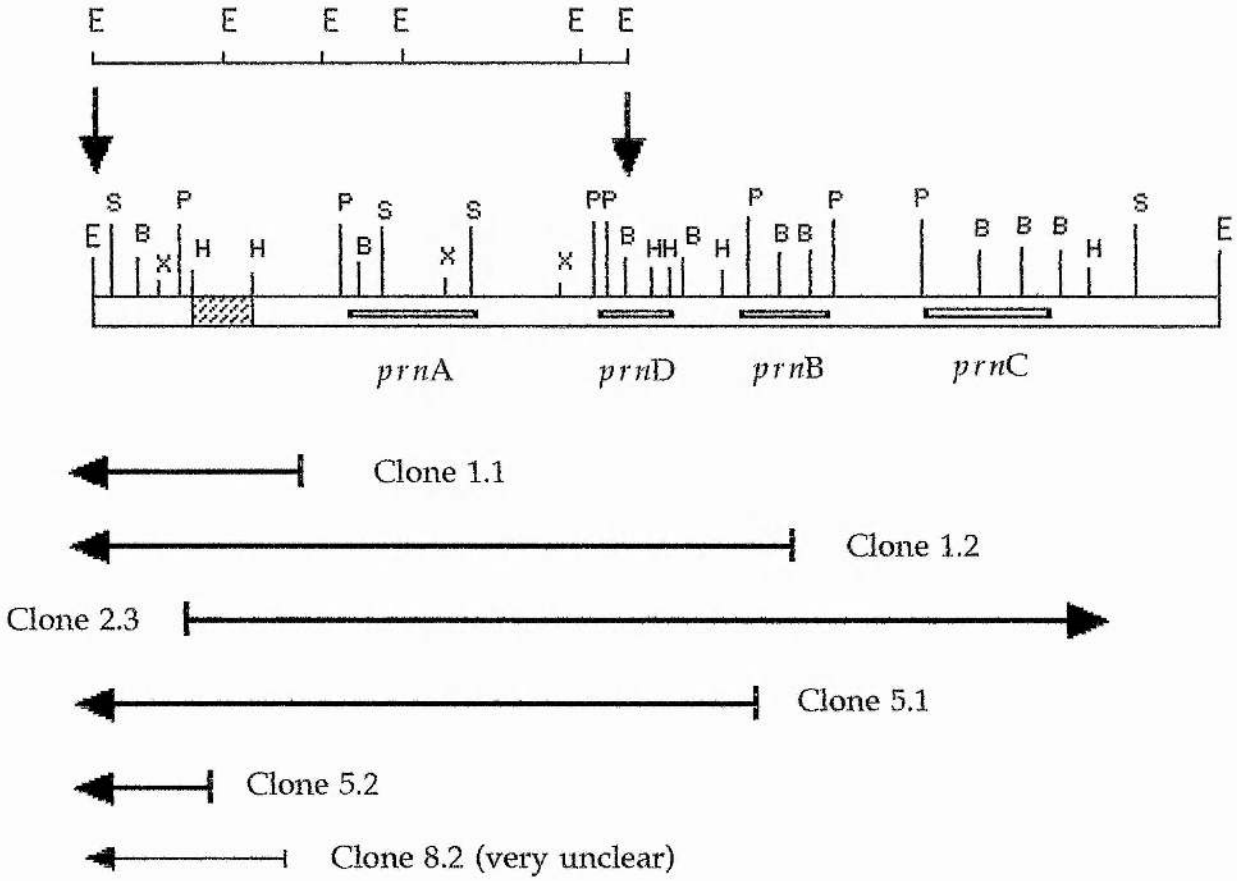


Fig. 4.3. Mapping of Lambda Clones

The restriction fragments (kb) found in the area of the proline gene cluster. From analysis of which of these fragments appear to be present in the six lambda clones, the position of each of the six clones could be determined. The shaded area represents the 2 kb *Hin*dIII fragment used as a hybridization probe. (Not to scale). B - *Bgl*II; E - *Eco*RI; H - *Hin*dIII; P - *Pst*I; S - *Sal*I; X - *Xho*I.



Restriction sites:

B - *Bgl*II; E - *Eco*RI; H - *Hin*dIII; P - *Pst*I; S - *Sal*I; X - *Xho*I

Fig. 4.4. Mapping of Lambda Clones

Diagram showing the pAN229 plasmid in relation to the section of *A. nidulans* chromosome VII DNA which contains the proline utilization gene cluster. From the results of Southern blots, the approximate position of the six lambda clones, relative to the proline genes, has been calculated. (Not to scale).

WO_4. The *cnx*J1 mutant has a very 'leaky' phenotype however, and much background growth is seen, making it quite difficult to distinguish transformant colonies from background growth. 30 mM WO_4 was used as this was the concentration found to give the best differentiation - reducing some of the background growth whilst still being non-toxic to any transformed cells.

5 μg λ DNA was used in each experiment. Both the 1.1 and the 5.2 clones were transformed into the mutant, along with pUC13 which was used as a negative control. The experiment was repeated four times and eight putative transformants were isolated: four from the 1.1 clone and four from 5.2, indicating that both clones may in fact contain the *cnx*J gene.

In order to locate the position of the gene more precisely within the λ clone, various digests were done. (All future work was carried out on the 5.2 clone alone). Clone 5.2 DNA was digested with *Xba*I, *Eco*RI, *Hin*dIII and with *Sal*I. This digested DNA (5 μg) was then used to try and retransform the *cnx*J mutant. Putative transformants (3-4) were only produced in the transformation with *Sal*I digested DNA. Total 5.2 DNA was also transformed into the mutant as a control, and also producing 3-4 putative transformants.

It therefore appeared that *Sal*I - digested DNA was still able to complement the fungal mutant, indicating that the *cnx*J gene was contained wholly within a *Sal*I fragment of the 5.2 clone. Digestion with the *Sal*I restriction enzyme results in the production of seven fragments, of sizes - 20 kb, 9 kb, 6 kb, 4 kb, 2.5 kb, 2.3 kb and 0.7 kb. The 20 kb and 9 kb fragments are the λ DNA arms of the vector, whilst the remaining 15.5 kb constitutes the *Aspergillus nidulans* DNA insert. DNA from the individual *Sal*I fragments was prepared and used to retransform the *cnx*J mutant. The only sample which produced transformant colonies, was

DNA from the 2.3 kb band. This fragment was subcloned into a pUC vector (section 4.10) and 5 μg of this subclone, designated pSTA500, used to transform the mutant. Ten putative transformants were isolated and it was now assumed that the *cnx*J gene lay within the 2.3 kb *Sal*I fragment isolated from the λ clone designated 5.2.

4.8. POSITIVE IDENTIFICATION OF PUTATIVE *cnx*J TRANSFORMANTS

As a quick and relatively easy means of testing transformants to see if they are indeed real transformants, a technique using the polymerase chain reaction was developed (S.E. Unkles). Primers designed to recognize λ DNA sequences were used in a PCR reaction with transformed DNA to amplify any λ DNA which would be present in a transformant. For pSTA500 subclone transformants, primers to amplify ampicillin sequences were used (present in the pUC vector).

Primers: λ1 - GTTCTCAATTTCAGCATCC -

λ2 - GACAGGTGCTGAAAGCG -

Amp1 - CTGTGACTGGTGAGTAC -

Amp2 - CAACATTTCCGTGTCGC - (Fig. 4.5.)

Putative transformants were also plated onto minimal medium + 10 mM NO_3 and with 30 mM WO_4, to ensure that they could grow in the presence of tungstate. All transformants tested did indeed grow on the selective medium (Fig. 4.6.).

DNA from all six lambda clones initially isolated, was also used to transform the 2672 *fw*A *pabaA1 prn*301 *cnx*J1 mutant in the respect of it also being a *prn*301 mutant. Selection was on minimal medium + 10 mM proline as a nitrogen source. Only cells transformed in the proline gene, will be able to utilize proline as a nitrogen source. This experiment was

Fig.4.5. PCR Amplification of Transformant DNA

The presence of λ DNA sequences in putative fungal transformants was shown by the polymerase chain reaction.

A). Lane 1 - 100bp ladder.

Lanes 2-10 - DNA from putative *prn* transformants:

Lane 2 - Mutant transformed with the 1.1 λ clone.

Lanes 3 and 4 - Mutant transformed with the 2.3 λ clone.

Lane 5 - Mutant transformed with the 5.2 λ clone.

Lanes 6,7,8 and 9 - Mutant transformed with the 5.1 λ clone.

Lane 10 - Mutant transformed with the 8.2 λ clone.

Lanes 11-18 - DNA from putative *cnxJ* transformants:

Lanes 11,12,17 and 18 - Mutant transformed with the 1.1 λ clone.

Lanes 13,14,15 and 16 - Mutant transformed with the 5.2 λ clone.

Lane 19 - DNA from transformant containing no λ sequences.

Lane 20 - DNA from the w.t.

Lane 21 - SDW control.

Lane 22 - λ DNA.

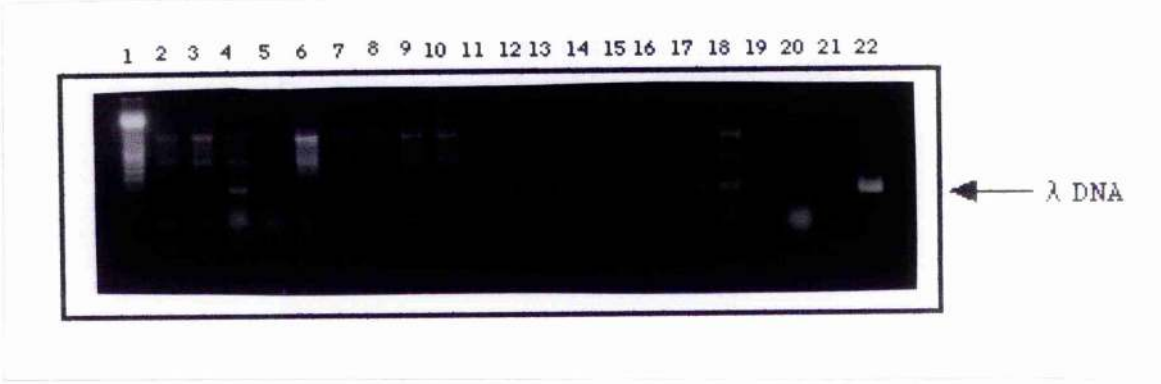
B). Lane 1 - 100bp ladder.

Lanes 2-8 - DNA from putative pSTA500 transformants.

Lane 9 - w.t. DNA.

Lane10 - SDW control.

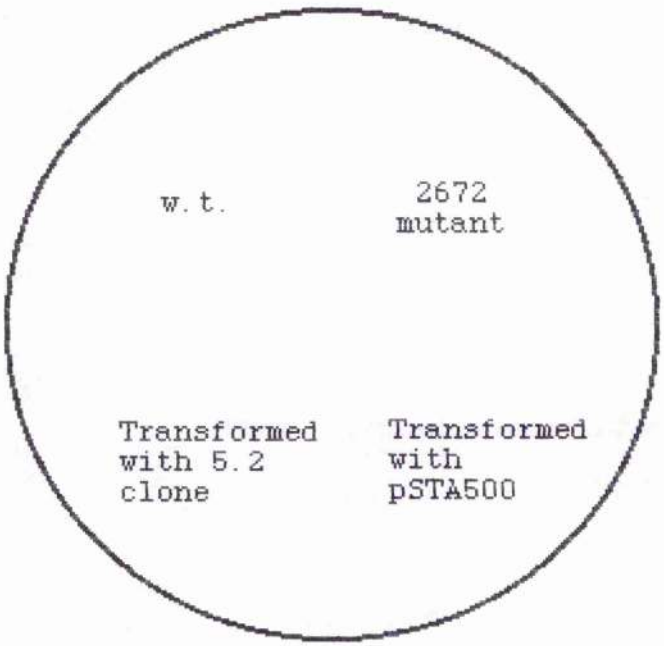
Lane 11 - λ DNA.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
λ DNA


1 2 3 4 5 6 7 8 9 10 11
λ DNA

Fig. 4.6. Growth of Transformants on Tungstate - Containing Medium

A plate showing growth of the transformed 2672 *fw*A *paba*A1 *prn*301 *cnx*J1 mutant strain, repaired in the *cnx*J gene. The *bi*A1 strain was used as the wild-type. Growth is shown on MM containing 10 mM nitrate and 30 mM tungstate.



done to see which of the clones contained proline genes. Eight putative transformants were isolated and these tested by PCR amplification. The results of this are seen in Fig. 4.5.

The one colony produced from transformation with clone 5.2 did not amplify, implying it to be a contaminant, reinforcing the earlier supposition that the 5.2 clone did not contain the proline genes and extended towards the *cnx*J gene (for reference see Fig. 4.4.).

4.9. NORTHERN BLOTS

Messenger RNA was prepared from 2672 *fw*A *paba*A1 *prn*301 *cnxJ*1 mycelia grown in the presence of 10 mM NH_4 and of 10 mM NO_3 as sole nitrogen sources. This mRNA was then used in Northern blotting experiments as shown in Fig. 4.7. The mRNA was probed with radio labelled 5.2 DNA and the filters washed in 2 X SSC solution at 42°C. After O/N exposure to auto radiographic film, a positively - hybridizing band of 1.4 kb could be seen. Subsequent Northern blots were done, using individual *Sal*I fragments of the λ clone as probes. The 2.3 kb *Sal*I fragment showed positive hybridization to a 1.4 kb transcript, thus providing more evidence that the *cnx*J gene was contained within this restriction fragment. Hybridization to the nitrate induced mRNA and to the ammonium induced mRNA is seen to be of equivalent intensity, indicating that the *cnx*J gene is not under nitrate regulation. (Any slight differences are due to variations in the amount of mRNA used in the experiment).

4.10. SUMMARY

This chapter reports on the isolation of a 2.3 kb DNA fragment which appears to contain the *Aspergillus cnx*J gene. Taking advantage of the known proximity of the proline gene cluster to the *cnx*J gene and

Fig. 4.7. Northern Blots

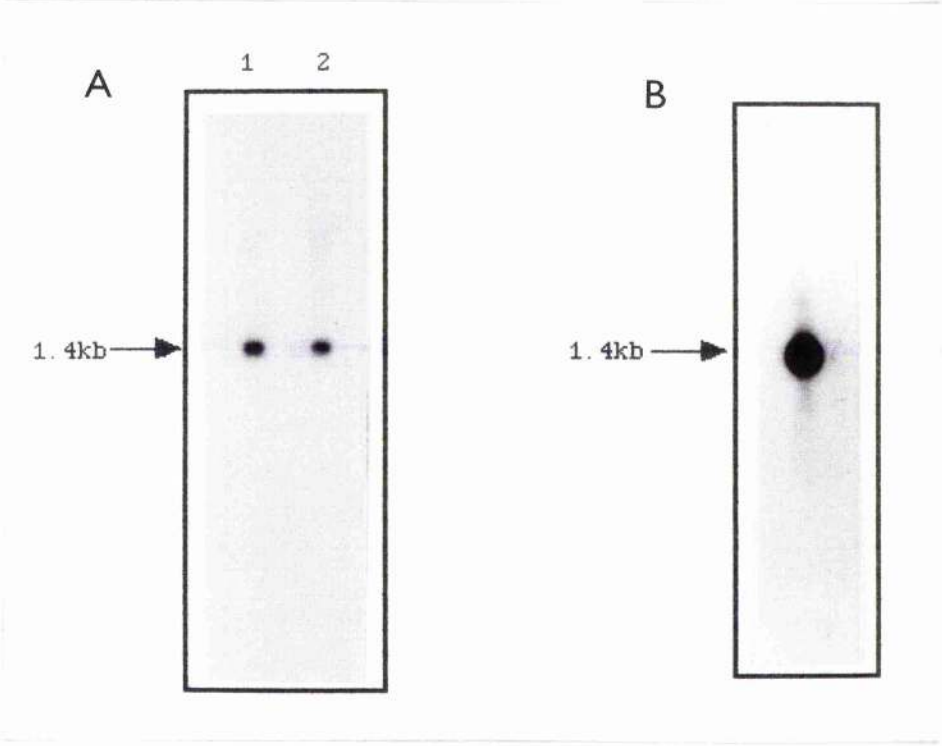
Mycelia from the fungal mutant 2672 *fw*A *paba*A1 *prn*301 *cnx*J1 was grown 12h, 30°C in liquid minimal medium supplemented with 10 mM NO_3 and with 10 mM NH_4 as nitrogen sources. mRNA was prepared from mycelia grown under these two sets of conditions,and blotted to nylon membrane after electrophoresis on a 1% agarose gel.

A). mRNA probed with the 5.2 λ-clone.

Lane 1 - mRNA from NH_4-grown mycelia.

Lane 2 - mRNA from NO_3-grown mycelia.

B). mRNA probed with the 2.3kb *Sal*I fragment of the 5.2 λ-clone. Only mRNA prepared from NH_4-grown mycelia was used. A 1.4kb transcript is seen in both blots.



subsequently using restriction mapping and genetic transformation, a clone from a λ phage library was identified. DNA sequencing and further genetic and regulatory analyses can now be carried out to determine the exact role of the *cnx*J gene product.

CHAPTER 5

DISCUSSION

5.1. THE *cnx*ABC LOCUS

5.1.1. Isolation of the *cnx*ABC Locus

The *cnx*ABC locus of *Aspergillus nidulans* is one of six loci involved in the synthesis of a molybdenum cofactor which is essential for the function of the fungal molybdoenzymes. The locus is located on chromosome VIII of the *A. nidulans* genome and, through genetic linkage studies, is thought to be close to the previously characterized *brl*A gene (3cM).

Starting from a cosmid known to contain this gene, 'chromosome walking' experiments were carried out, identifying a cosmid which was found to be able to transform all three *cnx*ABC mutants - *cnx*A5; *cnx*B11 and *cnx*C3 - to wild-type phenotype for growth on medium containing nitrate as the sole nitrogen source. The distance from the *brl*A gene to the *cnx*ABC locus proved to be 65 kb, allowing us to equate genetic map distance to physical distance. Although recombination is not equal over the whole genome, it can be estimated that, in this instance at least, in this region of chromosome VIII, 3 cM equals approximately 65 kb.

Transformation of *Aspergillus nidulans cnx*A5, *cnx*B11 and *cnx*C3 mutants proved to be not as straightforward as was first anticipated. Preparation of suitable numbers of viable protoplasts, transformation with the optimal amount of DNA and avoiding contamination all proved to be critical factors in obtaining reproducible

genetic transformation results. Even when the experiments are working well, variable numbers of fungal transformants are obtained. Fluctuations in frequency of transformation are observed when the same DNA is used in different mutants and even when the same fragment of DNA is used to transform the same mutant in different experiments. This can be explained by the fact that there are different means by which the foreign DNA establishes itself within the mutant host cell.

Recombinant DNA can insert into the genome in the exact place where the functional gene would have been. This is known as homologous recombination and involves a single crossover event. If the DNA recombines with the host genome in a random manner, at no specific locus, it is known as heterologous recombination - again involving one crossover. If two crossovers take place, gene replacement can occur, with the foreign gene taking the place of the mutated host gene in a two-step process. For reviews see Mishra, 1985 and May, 1992.

5.1.2. Sequence Analysis of the *cnx*ABC Locus

The region within the 84F6 cosmid, essential for a *cnx*ABC$^+$ phenotype, was identified and the DNA sequence of the resulting subclone determined. Analysis of the sequence showed there to be two open reading frames in this region - the first being 448 amino acids in length and the second, 246. The region containing the first ORF transforms the *cnx*A5 mutation whilst DNA encompassing the second ORF is able to transform *cnx*C3 mutants to a wild-type phenotype for growth on nitrate. The 5' end of the non-coding sequence is - GTGGGT - and the 3' site is - CAG - showing that this gene contains the intron splice sites of - 5' - GT - and - AG - 3', as is seen in yeast, plants and vertebrates (Gurr *et al.*, 1988). There is also, however, a CT rich region present within

this region, which could be acting as a promoter region for transcription of a second gene. (Gurr *et al.*, 1988). Putative 'CAAT' and 'TATA' boxes are marked on the sequence in the region upstream of the gene(s), as are candidate binding sites for the *are*A protein- WGATAR/YTATCW. A possible NIRA site - CCGCGG - is also indicated.

The nucleotide sequence of each of the two ORF's was analyzed using the BLASTX programme. The first amino acid sequence, CNXA, was found to show, 33.4% similarity to the MOAA amino acid sequence of *E. coli* and 54.8% homology with the CNX2 protein of *A. thaliana*. MOAA in *E. coli* is known to be involved in the conversion of guanosine-X into 'precursor Z' which then forms the molybdopterin component of the cofactor. It is therefore assumed that CNXA has a similar role in the early stages of Mo-co biosynthesis in *A. nidulans*.

The sequence of the *cnx*C region shows 42.9% similarity to the MOAC amino acid sequence of *E. coli* and 39.6% homology with the CNX3 protein of *A. thaliana*. *moa*C works in conjunction with *moa*A in *E. coli* to form the Mo-pterin moiety.

5.1.3. Regulation

Upon hybridization of wild-type RNA with the pSTA502 subclone (which contains the complete *cnx*ABC locus), four signals were initially seen. They were 3.4 kb, 2.4 kb, 1.8 kb and 0.7 kb in size. At first sight, this could have indicated that there were indeed three separate genes within the *cnx*ABC locus (plus one other transcript wholly or partially contained within the pSTA502 subclone). However, after analysis of the sequence data, it can be seen that the messages relate to the following:

- The 2.4 kb RNA represents the entire *cnx*ABC gene, encompassing both the *cnx*A and *cnx*C regions.
- The 1.8 kb RNA and 0.7kb RNA are not reproducible.
- The 3.4 kb message does not appear to be involved in the *cnx*ABC system and can be assumed to be another gene in close proximity to the *cnx*ABC locus.

Due to the presence of the 2.4 kb message, it would therefore seem that the *cnx*ABC locus is indeed only one gene containing a 198 bp intron.

At first, when the *cnx*C - containing 0.35 kb *Hin*dIII fragment of pSTA502 was used to probe wild-type RNA, it was thought there may be two copies of the *cnx*C region, since both the 2.4 kb and 1.8 kb messages were seen upon hybridization. However, Southern analysis proved that there was indeed only one copy of the gene, with discreet single bands being highlighted with various digests of wild-type DNA.

Northern blot experiments had to be done using mRNA as opposed to total RNA since the *cnx* messages appear to be expressed at fairly low levels.

From Northern blots using mRNA from mycelia grown under various nitrogen conditions, it is seen that, to some extent, nitrate acts as an inducer of *cnx*ABC RNA biosynthesis. Examination of the sequence data also indicates the presence of a putative NIRA protein binding site, thus implying a role for this molecule in the system.

The level of RNA synthesis under the influence of different nitrogen sources was also tested. The initial experiments presented here, showed no real indication of any major regulatory or nutritional effect, except again for a slight nitrate induction. The regulatory mechanisms

which act on the *cnx*ABC gene still have to be discovered and investigated further.

5.1.4. The *cnx*ABC Mutations

One original theory as to the structure of the *cnx*ABC locus was that *cnx*A and *cnx*C were individual genes and that the *cnx*B mutation represented a deletion of both the *cnx*A and *cnx*C genes. However, during transformation experiments using the *cnx*B11 mutant, a low level of reversion was seen (1 in 10^8 protoplasts) which indicated that *cnx*B could not be associated with a deletion.

Recent work done on the *cnx*ABC mutants, *cnx*A9, *cnx*B11 and *cnx*C3, has highlighted the sequence anomalies causing each of the three mutations. It was shown that the *cnx*A9 mutation was due to a 9 bp insertion at the 1236 bp position as shown in Fig. 3.8. The mutation *cnx*B11 is a 13 bp duplication at position 962 as shown in Fig. 3.8., while *cnx*C3 represents a 4 bp deletion at the start of the *cnx*C coding region at nucleotide 1766 as depicted in Fig. 3.8. (S.E. Unkles, unpublished). See Fig. 5.1.

From this work it can be seen that *cnx*B is not a gene *per se* but the mutation represents a mis-reading of sequence occurring in the *cnx*A region of the gene and which also disrupts *cnx*C. Therefore, both *cnx*A and *cnx*C activities are disrupted. The *cnx*A9 insertion leaves the rest of the sequence in frame, thus leaving CNXC actively intact. The *cnx*C3 deletion means that the *cnx*C mutant has an intact *cnx*A gene product before the mutation is met which results in a faulty *cnx*C gene product. The duplication at *cnx*B11 will result in no CNXA or CNXC protein. It is therefore indicated that the *cnx*ABC locus is one gene which produces a protein which has both *cnx*A and *cnx*C activities. There is no *cnx*B gene.

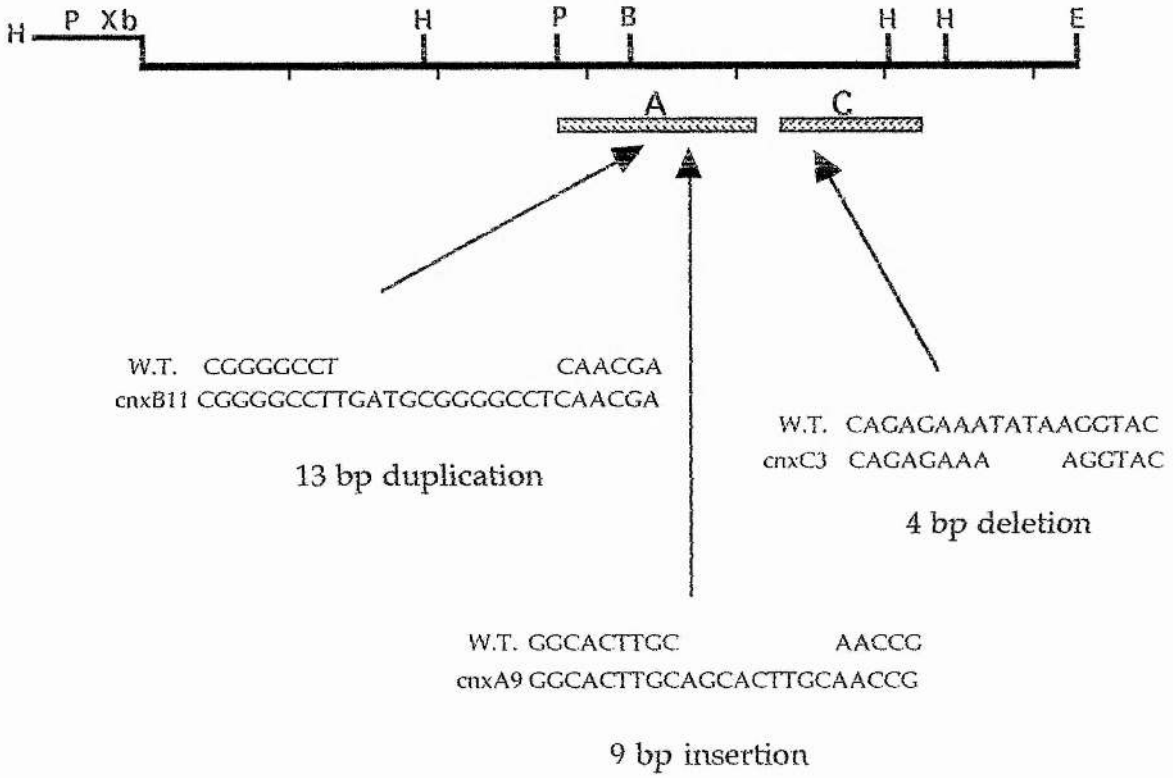


Fig. 5.1. Sequence of Representative *cnx*A, B and C Mutations

Sequences which give rise to the *cnx*A, B and C phenotypes. *cnx*A5 is due to a 13 bp duplication of sequence. *cnx*B11 has a 4 bp deletion within the '*cnx*A' coding region and *cnx*C3 is due to a 9 bp insertion in the '*cnx*C ' coding region.

5.1.5. Organization of the *cnx*ABC Locus

The *cnx*ABC locus of *Aspergillus nidulans* is considered to be one gene which contains an intron separating two coding regions specifying *cnx*A and *cnx*C activities. This gives rise to two possibilities as to how the protein products are translated. Firstly, the 2.4 kb mRNA transcribed from this locus could specify one protein which has a bifunctional role ie. it has both *cnx*A and *cnx*C activities. This is a fairly unusual situation in *A. nidulans* with very few examples of such a protein. For example, the AROM protein is a pentafunctional protein with multi-functional domains (Moore and Hawkins, 1993), but this aside, there are no other documented cases of such organization in *A. nidulans.*

In *A.thaliana*, however, this is how the *cnx*1 gene is arranged. A domain at the N-terminal of the protein is homologous to the *E.coli* protein MOEA whilst the C-terminal domain shows homology to two *E.coli* Mo-co proteins - MOAB and MOGA. The CNX1 protein is also homologous to the Cinnamon protein in *Drosophila*. It is thought to be involved in the final stages of Mo-co biosynthesis, inserting molybdenum into the pterin (Stallmeyer *et al.*, 1995; Schwarz *et al.*, 1997).

Another possibility as to how the two protein functions are conferred by one gene is that the mRNA could undergo alternative splicing to produce smaller mRNA's which produce two separate proteins which have *cnx*A and *cnx*C activity respectively. There are putative intron splice sites upstream of the *cnx*A ORF at positions -82 and -1. Consensus donor splice sites in *Aspergillus* and in other eukaryotic organisms have been analysed and are documented (Rogozin and Milanesi, 1997). In conjunction with the internal intron, the mRNA

splice products could be as shown in Fig. 5.2. This is unlikely, however, as the 2.4 kb RNA transcript is the only one which is reproducible.

From the data thus far obtained, ie. the sequence and mutational analyses, it would appear fairly conclusive that the *cnx*ABC locus is one gene, 2.4 kb in length which produces one transcript interrupted by an intron. The translation product is one bi-functional protein which has two active domains homologous to the *E. coli* proteins MOAA and MOAC. Studies with *cnx*A and *cnx*C mutants on nitrate - containing medium show complementation with one another, but at a level slightly lower than that of wild-type. This is again indicative of one gene showing intragenic complementation as opposed to there being two separate genes. Indeed, chemical analysis of *cnx*A, *cnx*B and *cnx*C mutants supports this notion. These have undetectable levels of precursor Z compared with the wild-type (Heck, unpublished). The CNXABC protein probably has a dimeric structure. The intron lies within the non-homologous region of DNA between the *cnx*A and *cnx*C domains. Being homologous to the MOAA and MOAC proteins, CNXABC can be seen to have a role in the early stages of the Mo-co biosynthetic pathway, as depicted in Fig. 5.3.

5.1.6. Comparative Analyses

Now that the molecular structure of the *cnx*ABC locus has been established, it can be compared to genes involved in Mo-co biosynthesis from other organisms. As has been shown, *cnx*ABC in *Aspergillus nidulans* is one gene which encodes a bi-functional protein with two distinct domains. The N-terminal half of the protein, which has CNXA activity, shows homology with the *E. coli* MOAA protein and with CNX2 of *A. thaliana,* whereas the CNXC domain is similar to MOAC and CNX3

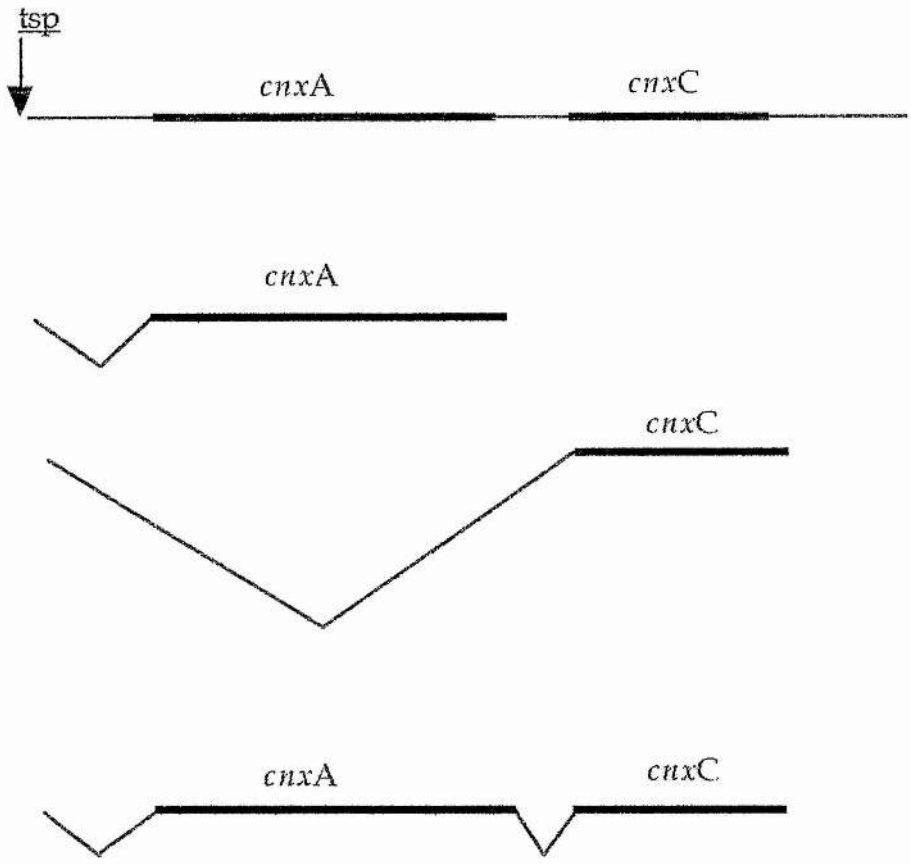
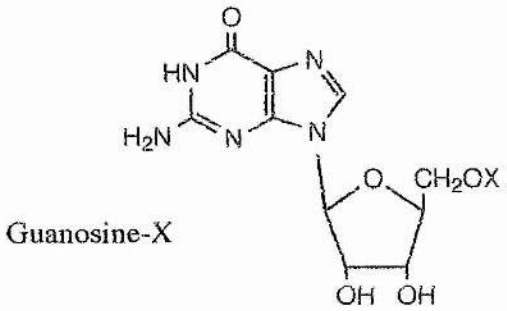


Fig. 5.2. Possible Alternative Splicing Strategies Within the *cnx*ABC Locus

Schematic representation of how the *cnx*ABC transcriptional unit may be spliced in different ways to produce either two independent protein products from one gene or how a bifunctional protein could be produced from the full-length RNA. tsp - transcriptional start point.

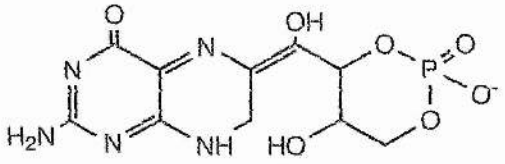
Fig. 5.3. The Mo-co Biosynthetic Pathway

The biochemical pathway showing the synthesis of molybdenum cofactor from a guanosine derivative. The role of the *cnx*ABC gene product of *A. nidulans* is shown to be that of forming precursor Z from the guanosine derivative.

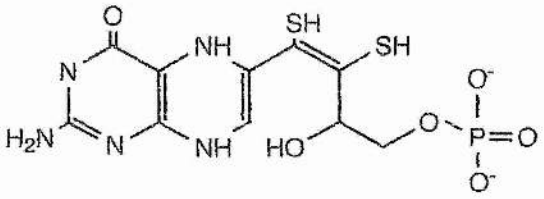


Guanosine-X

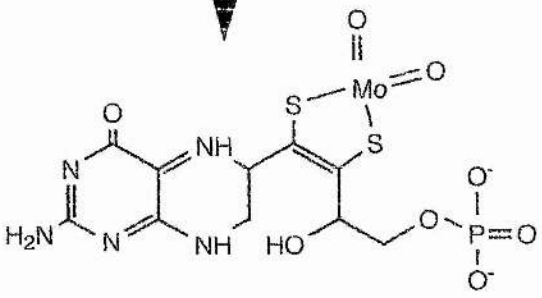
*crnx*ABC



Precursor Z



Molybdopterin



Molybdenum cofactor

proteins as shown in Fig. 5.4. The one CNXABC protein carries out the function of two proteins in the bacteria and the plant species. It is seen that there are fewer molybdenum cofactor biosynthetic genes in *Aspergillus* than there are in *E. coli.*

To date, no *A. nidulans cnx* gene equivalent to *moa*B has been isolated. It is likely that *moa*B has a regulatory role in *E. coli,* and as the regulatory systems involved in bacteria and fungi are different, it is maybe not too surprising that a '*cnx*B' gene with a role similar to *moa*B has not been identified. Alternatively, it may be that not all *cnx* genes have been identified in *A.nidulans.* The MOAB protein shows homology to another *E. coli* protein involved in molybdenum cofactor biosynthesis - MOG. Similarity is also shown to the *Drosophila melanogaster* protein CINNAMON and to the rat neuro-protein, gephyrin. The *E. coli* MOE protein also shares homology with the C-terminal domain of CINNAMON and of gephyrin (See Fig. 5.4.). Here again, is an example of where two bacterial genes are required when only one gene is needed in the eukaryotic system.

The structure of these genes can give us an insight into the evolutionary mechanisms which are occurring throughout the species. The tendency would appear to be towards fewer genes to carry out particular functions as you go up the evolutionary scale from bacteria to plants to fungi to insects to mammals. It is more economical for the cell to have fewer genes to transcribe and to have proteins which can exercise a variety of functions. It will be interesting to consider this point further when more genetic information becomes available for a wider variety of species.

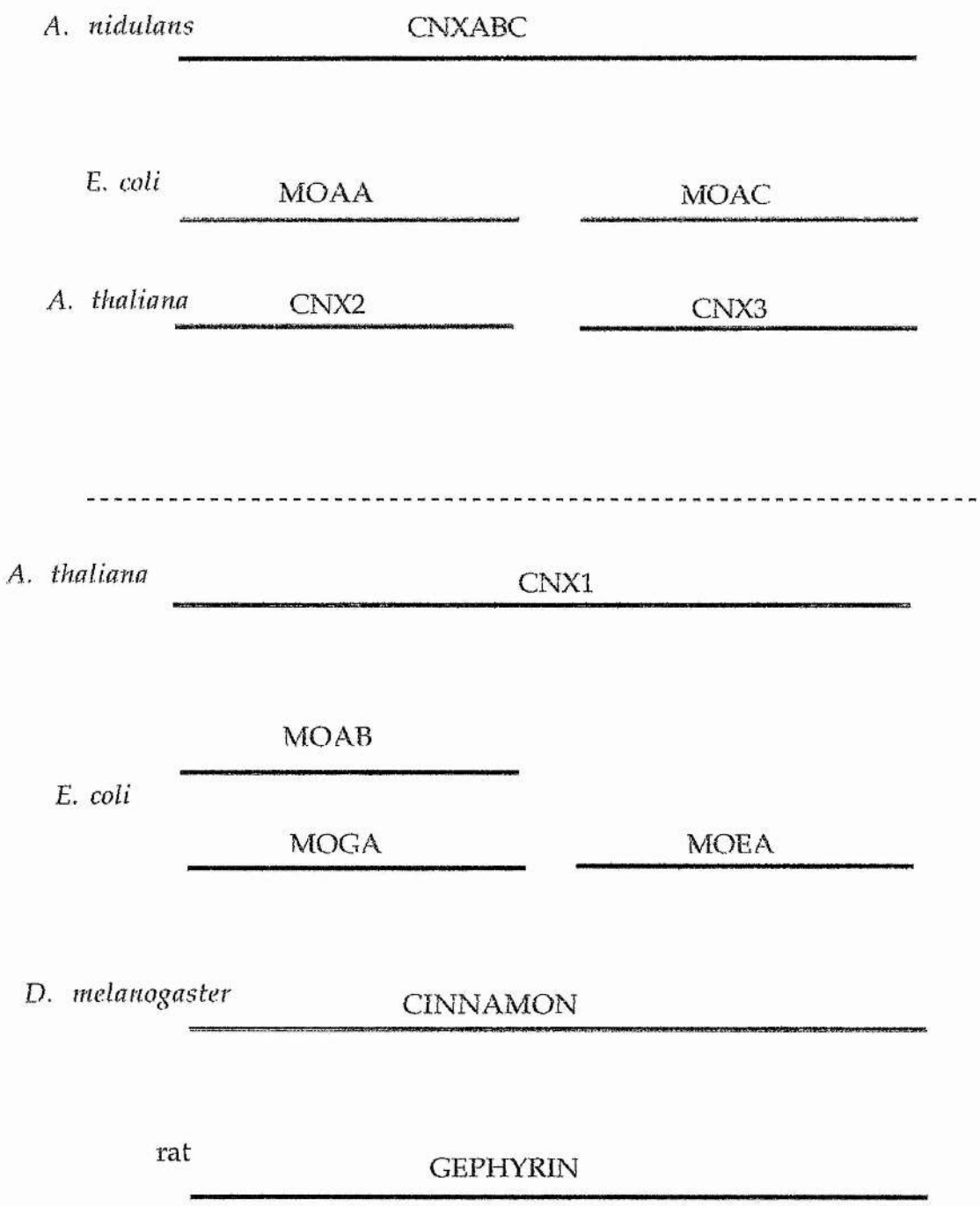


Fig. 5.4. Protein Comparisons

The homologous proteins of CNXABC in *E. coli* and *A. thaliana*. In each case, two proteins carry out the functions of the one *Aspergillus* protein. The diagram also shows how the MOAB protein of *E. coli* (with no known homologue in *A. nidulans*) shares regions of homology with proteins in other species.

5.2. THE *cnx*J LOCUS

A 2.3 kb DNA fragment has been isolated which, by genetic transformation, is seen to restore the phenotype of *cnx*J to wild-type ie. the mutant has been repaired for growth on tungstate - containing medium. However, the phenotype is somewhat difficult and the lack of unequivocal evidence led us to leave further study of this locus in the meantime. The nucleotide and amino acid sequence should be analysed in order to predict the structure and function of the gene product. This along with regulation studies, will help determine the role of the *cnx*J gene in the molybdenum-cofactor biosynthetic pathway. Whether the *cnx*J protein is a regulator of the pathway (Arst *et al.*, 1982) or possibly a Mo transporter, or indeed, whether it has an entirely different function, can now be established.

5.3. FURTHER WORK

There still remains much work which can be done on the *cnx* gene system of *Aspergillus*. It will be important to isolate and characterize the other *cnx* genes, as has been done with *cnx*ABC ie. *cnx*E, *cnx*F, *cnx*G and *cnx*H Mutational analysis work could also be done in order to see if any further *cnx* mutations can be isolated.

When more information is gathered on the *cnx* genes *per se*, the regulation mechanisms should be examined in detail. A wider range of nitrogen sources could be used in Northern blots of wild-type RNA as well as with RNA prepared from the mutants. Regulatory mutants like *are*A$^-$ and *nir*A$^-$ could also be probed with *cnx* genes under various growth conditions.

As Mo-co is a universal molecule and there appear to be parallels between the genes involved in its biosynthesis in different species,

complementation studies using genes from different organisms could be attempted. If DNA from one organism is able to transform a mutant from another species, the degree of homology and conservation maintained throughout evolution can be evaluated.

REFERENCES

Aguilar, M.R, Cardenas, J, and Fernandez, E. 'Regulation of Molybdenum Cofactor Species in the Green Alga *Chlamydomonas reinhardtii*.' (1991). Biochimica et Biophysica Acta. 1073, 463-469.

Aleksenko, A, Nikolaev, I, Vinetsky, Y, and Clutterbuck, A.J. 'Gene Expression from Replicating Plasmids in *Aspergillus nidulans*.' (1997). Molecular and General Genetics. 253, 242-246.

Altschul, S.F, Gish, W, Miller, W, Myers, E.W, and Lipman, D.J. 'Basic Local Alignment Search Tool.' (1990). Journal of Molecular Biology. 215, 403-410.

Amy, N.K, 'Identification of the Molybdenum Cofactor in Chlorate Resistant Mutants of *Escherichia coli*.' (1981). Journal of Bacteriology. 148, 274-282.

Amy, N.K, and Rajagopalan, K.V. 'Characterization of Molybdenum Cofactor from *Escherichia coli*.' (1979). Journal of Bacteriology. 140, 114-124.

Andrianopoulos, A, and Hynes, M.J. 'Cloning and Analysis of the Positively Acting Regulatory Gene, *amdr*, from *Aspergillus nidulans*.' (1988). Molecular and Cellular Biology. 8, 3532-3541.

Arnold, G.L, Greene, C.L, and Goodman, S.I. 'Molybdenum Cofactor Defect - an Under-recognized Inborn Error of Metabolism.' (1991). American Journal of Human Genetics. 49, 93.

Arst, H.N, Jr., MacDonald, D.W, and Cove, D.J. 'Molybdate Metabolism in *Aspergillus nidulans*.' (1970). Molecular and General Genetics. 108, 129-145.

Arst, H.N, Jr., MacDonald, D.W, and Cove, D.J. 'Molybdate Metabolism in *Aspergillus nidulans*.' (1970). Molecular and General Genetics. 108, 146-153.

Arst, H.N, Jr.,and Cove, D.J. 'Nitrogen Metabolite Repression in *Aspergillus nidulans*.' (1973). Molecular and General Genetics. 126, 111-141.

Arst, H.N, and MacDonald, D.W. 'A Gene Cluster in *Aspergillus nidulans* with an Internally Located cis-acting Regulatory Region.' (1975). Nature. 254, 26-31.

Arst, H.N, Jr., Tollervey, D.W, and Sealy-Lewis, H.M. 'A Possible Regulatory Gene for the Molybdenum-Containing Cofactor in *Aspergillus nidulans*.' (1982). Journal of General Microbiology. 128, 1083-1093.

Arst, H.N, Jr. (1983). 'Fungal Systems.' in "Eukaryotic Genes: Their Structure, Activity and Regulation." Maclean, N, Gregory, S.G, and Flavell R.A. (Eds.). London: Butterworths.

Austin, B, Hall, R.M, and Tyler, B.M. 'Optimized Vectors and Selection for Transformation of *Neurospora crassa* and *Aspergillus nidulans* to Bleomycin and Phleomycin Resistance.' (1990). Gene. 93, 157-162.

Baker, K.P, and Boxer, D.H. 'Regulation of the *chl*A Locus of *Escherichia coli* K12: Involvement of Molybdenum Cofactor.' (1991). Molecular Microbiology. 5, 901-907.

Barber, M.J, and Neame, P.J. 'A Conserved Cysteine in Molybdenum Oxotransferases.' (1990). The Journal of Biological Chemistry. 265, 20912-20915.

Barnes, W.M, 'Plasmid Detection and Sizing in Single Colony Lysates.' (1977). Science. 195, 393.

Birnboim, H.C, and Doly, J. 'A Rapid Alkaline Extraction Procedure for Screening Recombinant Plasmid DNA.' (1979). Nucleic Acid Research. 7, 1513.

Bishop, P.E, and Joerger, R.D. 'Genetics and Molecular Biology of Alternative Nitrogen Fixation Systems.' (1990). Annual Review of Plant Physiology and Plant Molecular Biology. 41, 109-125.

Boylan, M.T, Mirabito, P.M, Willett, C.E, Zimmerman, C.R, and Timberlake, W.E. 'Isolation and Physical Characterisation of

Three Essential Conidiation Genes from *Aspergillus nidulans.*' (1987). Molecular and Cellular Biology. 7, 3113-3118.

Brody, H, and Carbon, J. 'Electrophoretic Karyotype of *Aspergillus nidulans.*' (1989). Proceedings of the National Academy of Science (USA). 86, 6260-6263.

Brody, H, Griffith, J, Cuticchia, A.J, Arnold, J, and Timberlake, W.E. 'Chromosome-specific Recombinant DNA Libraries from the Fungus *Aspergillus nidulans.*' (1991). Nucleic Acids Research 19, 3105-3109.

Brownlee, A.G. and Arst, H,N. Jr. 'Nitrate Uptake in *Aspergillus nidulans* and Involvement of the Third Gene of the Nitrate Assimilation Gene Cluster.' (1983). Journal of Bacteriology 155, 1138-1146.

Caddick, M.X, Arst, H.N. Jr., Taylor, L.H, Johnson, R.I, and Brownlee, A.G. 'Cloning of the Regulatory Gene *are*A Mediating Nitrogen Metabolite Repression in *Aspergillus nidulans.*' (1986). The EMBO Journal. 5, 1087-1090.

Carrez, D, Janssens, W, Degrave, P, van den Hondel, C.A.M.J.J, Kinghorn, J.R, Fiers, W, and Contreras, W. 'Heterologous Gene Expression by Filamentous Fungi: Secretion of Human Interleukin-6 by *Aspergillus nidulans.*' (1990). Gene. 94, 147-154.

Chan, M.K, Kim, J, and Rees, D.C. 'The Nitrogenase FeMo-Cofactor and P-Cluster Pair: 2.2A Resolution Structures.' (1993). Science, 260, 792-794.

Chatterjee, R, Allen, R,M, Shah, V.K, and Ludden, P.W. 'Nucleotide and Divalent Cation Specificity of *In vitro* Iron-Molybdenum Cofactor Synthesis.' (1994). Journal of Bacteriology. 176, 2747-2750.

Clutterbuck, A.J. 'Gene Symbols in *Aspergillus nidulans*.' (1973). Genetical Research Cambridge. 21, 291-296.

Clutterbuck, A.J. '*Aspergillus nidulans* nuclear genes.' In: "Genetic Maps 5; Locus Maps of Complex Genomes, vol.3: Lower Eukaryotes." (1993). Ed. S.J. O'Brien. Cold Spring Harbor Laboratory, New York. pp 3.71-3.84.

Cohen, S.N, Chang, A.C.Y, and Hsu, L. 'Non-Chromosomal Antibiotic Resistance in Bacteria: Genetic Transformation of *Escherichia coli* by R-Factor DNA.' (1972). Proceedings of the National Academy of Science (USA). 69, 2110.

Cooley, R.N, and Tomsett, A.B. 'Determination of the Subunit Size of NADPH Nitrate Reductase from *Aspergillus nidulans*.' (1985). Biochimica et Biophysica Acta. 831, 89-93.

Coughlan, M. (1980). "Molybdenum and Molybdenum-Containing Enzymes." Pergamon Press. Oxford.

Cove, D.J. 'The Induction and Repression of Nitrate Reductase in the Fungus *Aspergillus nidulans*.' (1966). Biochimica et Biophysica Acta. 113, 51-56.

Cove, D.J. 'Chlorate Toxicity in *Aspergillus nidulans*.' (1976a). Molecular and General Genetics. 146, 147-159.

Cove, D.J. 'Chlorate Toxicity in *Aspergillus nidulans*: The Selection and Characterization of Chlorate Resistant Mutants.' (1976b). Heredity. 36, 191-203.

Cove, D.J. 'Genetic Studies of Nitrate Assimilation in *Aspergillus nidulans*.' (1979). Biological Reviews. 54 , 291-327.

Cove, D.J, and Coddington, A. 'Purification of Nitrate Reductase and Cytochrome C Reductase from *Aspergillus nidulans*.' (1965). Biochimica et Biophysica Acta. 110, 312-318.

Cove, D.J, and Pateman, J.A. 'Independently Segregating Loci Concerned with Nitrate Reductase Activity in *Aspergillus nidulans*.' (1963). Nature. 168 , 262-263.

Davis, L.G, Dibner, M.D, and Battey, J.F. (1986). 'Basic Methods in Molecular Biology'. Elsevier. New York.

Dubourdieu, M, Andrade, E, and Puig,J. 'Molybdenum and Chlorate Resistant Mutants in *Escherichia coli* K12.' (1976). Biochemical and Biophysical Research Communications. 70, 766-773.

Eady, R.R, Robson, R.L, Pau, R.N, Woodley, P, Lowe, D.J, Miller, R.W, Thorneley, R.N.F, Smith, B.E, Gormal, C, Fisher, K, Eldridge, M, and Bergstorm, J. 'The Vanadium Nitrogenase of *Azotobacter chroococcum.*'(1988). In H. Bothe, F.J. de Bruijn and W.E. Newton. (Eds.) "Nitrogen Fixation:100 Years After." Gustav Fischer. Verlag. Stuttgart, Germany. p81-86.

Exley, G.E, Colandene, J.D, and Garrett, R.H. 'Molecular Cloning, Characterization and Nucleotide Sequence of *nit*-6, the Structural Gene for Nitrite Reductase in *Neurospora crassa.*' (1993). Journal of Bacteriology. 175, 2379-2392.

Feinberg, A.P, and Vogelstein, A. 'Technique for Radiolabelling DNA Restriction Endonuclease Fragments to High Specific Activity.' (1983). Biological and Analytical Biochemistry. 132, p6.

Fidel, S, doonan, J.H, and Morris, N.R. '*Aspergillus nidulans* Contains a Single Actin Gene which has Unique Intron Locations and Encodes a Gamma Actin.' (1988). Gene. 70, 283-293.

Filler, W.A, Kemp, R.M, Ng, J.C, Hawkes, T.R, Dixon, R.A, and Smith, B.E. 'The *nif*H Gene Product is Required for the Synthesis or Stability of the Iron-Molybdenum Cofactor of Nitrogenase from

Klebsiella pneumoniae.' (1986). European Journal of Biochemistry. 160, 371-377.

Fincham, J.R.S. 'Transformation in Fungi.' (1989). - Microbiology Reviews 53, 148-170.

Fu, Y-H and Marzluf, G.A. 'Molecular Cloning and Analysis of the Regulation of *nit*-3, the Structural Gene for Nitrate Reductase in *Neurospora crassa.*' (1987). Proceedings of the National Academy of Science (USA). 84, 8243-8247.

Fu, Y-H and Marzluf, G.A. '*nit*-2, the Major Nitrogen Regulatory Gene of *Neurospora crassa,* Encodes a Protein with a Putative Zinc Finger Binding Domain.' (1990). Molecular and Cellular Biology. 10, 1056-1065.

Garde, J, Kinghorn, J.R, and Tomsett, A.B. 'Site-directed Mutagenesis of Nitrate Reductase from *Aspergillus nidulans*: Identification of Some Essential and Some Non-Essential Amino Acids Among Conserved Residues.' (1995). Journal of Biological Chemistry. 270, 6644-6650.

Garrett, R.H, and Nason, A. 'Further Purification and Properties of *Neurospora* Nitrate Reductase.' (1969). Journal of Biological Chemistry. 244, 2870-2882.

Garrett, R.M, and Rajagopalan, K.V. 'Molecular Cloning of Rat Liver Sulphite Oxidase.' (1994). The Journal of Biological Chemistry. 269, 272-276.

Garzon, A, Li, J, Flores, A, Casadesus, J, and Stewart, V. 'Molybdenum Cofactor (Chlorate Resistant) Mutants of *Klebsiella pneumoniae* M5al Can Use Hypoxanthine as the Sole Nitrogen Source.' (1992). Journal of Bacteriology. 174, 6298-6302.

Gems, D.H, and Clutterbuck, A.J. 'Cotransformation with Autonomously Replicating Helper Plasmids Facilitates Gene Cloning from an *Aspergillus nidulans* Gene Library.' (1993). Current Genetics. 24, 520-524.

Gems, D, Johnstone, I.L, and Clutterbuck, A.J. 'An Autonomously Replicating Plasmid Transforms *Aspergillus nidulans* at High Frequency.' (1991). Gene. 98, 61-67.

Georgiadis, M.M, Komiya, H, Chakrabarti, P, Woo, D, Kornuc, J.J, and Rees, D.C. 'Crystallographic Structure of the Nitrogenase Iron Protein from *Azotobacter vinelandii*.' (1992). Science. 257, 1653-1659.

Gibson, T.J, Coulson, A.R, Sulston, J.E, and Little, P.F.R. 'Lorist2 - A Cosmid with Transcriptional Terminators Insulating Vector Genes from Interference by Promoters within the Insert: Effect on DNA Yield and Cloned Insert Frequency.' (1987). Gene. 53, 275-281.

Glaser, J.H, and DeMoss, J.A. 'Phenotypic Restoration by Molybdate of Nitrate Reductase Activity in *chlD* Mutants of *Escherichia coli.*' (1971). Journal of Bacteriology. 108, 854-860.

Glatigny, A, and Scazzocchio, C. 'Cloning and Molecular Characterization of *hxA*, the Gene Coding for the Xanthine Dehydrogenase (Purine Hydroxylase I) of *Aspergillus nidulans.*' (1995). Journal of Biological Chemistry. 270, 3534-3550.

Gollan, U, Schneider, K, Muller, A, Schuddekopf, K, and Klipp, W. 'Detection of *in vivo* Incorporation of a Metal Cluster into a Protein. The FeMo Cofactor is Inserted into the FeFe Protein of the Alternative Nitrogenase of *Rhodobacter capsulatus.*' (1993). European Journal of Biochemistry. 215, 25-35.

Gurr, S.J, Unkles, S.E, Kinghorn, J.R. (1988). 'The Structure and Organization of Nuclear Genes of Filamentous Fungi.' In "Gene Structure in Eukaryotic Microbes." Ed. - Kinghorn, J.R. IRL Press. Oxford. p 93-139

Guymon, E.P, and Spence, J.T. 'The Reduction of Molybdate by Mo(V).' (1966). Journal of Physical Chemistry.' 70, 1964-1969.

Hawker, K.L, Montague, P, and Kinghorn, J.R. 'Nitrate Reductase and Nitrite Reductase Transcript Levels in Various Mutants of *Aspergillus nidulans*: Confirmation of Autogenous Regulation.' (1992). Molecular and General Genetics. 231, 485-488.

Heck, I.S, and Ninnemann, H. 'Molybdenum Cofactor Biosynthesis in *Neurospora crassa*: Biochemical Characterization of Pleiotropic Molybdoenzyme Mutants *nit*-7, *nit*-8, *nit*9A, B and C.' (1995). Photochemistry and Photobiology. 61, 54-60.

Hemschemeier, S, Grund, M, Keuntje, B, and Eichenlaub, R. 'Isolation of *Escherichia coli* Mutants Defective in Uptake of Molybdate.' (1991). Journal of Bacteriology. 173, 6499-6506.

Hewitt, E.J. In MTP International Review of Science. (1974). 11, 201-245.

Hoff, T, Schnorr, K.M, Meyer, C, and Caboche, M. 'Isolation of Two *Arabidopsis* cDNAs Involved in Early Steps of Molybdenum Cofactor Biosynthesis by Functional Complementation of *Escherichia coli* Mutants.' (1995). Journal of Biological Chemistry. 270, 6100-6107.

Hull, E.P, Green, P.M, Arst, H.N. Jr., and Scazzocchio, C. 'Cloning and Physical Characterisation of the L-proline Catabolism Gene Cluster of *A. nidulans*.' (1989). Molecular Microbiology. 3, 553-559.

Hynes, M.J. 'Transformation of Filamentous Fungi.' (1986). Experimental Mycology. 10, 1-8.

Imperial, J, Ugalde, R.A, Shah, V.K, and Brill, W.J. 'Role of the *nif*Q Gene Product in the Incorporation of Molybdenum into Nitrogenase

in *Klebsiella pneumoniae.*' (1984). Journal of Bacteriology. 158, 187-194.

Jacobson, M.R, Brigle, K.E, Bennet, L.T, Setterquist, R.A, Wilson, M.S, Cash, V.L, Beynon, J, Newton, W.E, and Dean, D.R. 'Physical and Genetic Map of the Major *nif* Gene Cluster from *Azotobacter vinelandii.*' (1989). Journal of Bacteriology. 171, 1017-1027.

Joerger, R.D, Jacobson, M.R, Premakumar, R, Wolfinger, E.D, and Bishop, P.E. 'Nucleotide Sequence and Mutational Analysis of Structural Genes (*anf*HDGK) for the Second Alternative Nitrogenase from *Azotobacter vinelandii.*' (1989). Journal of Bacteriology. 171, 1075-1086.

Johann, S, and Hinton, S.M. 'Cloning and Nucleotide Sequence of the *chl*D Locus.' (1987). Journal of Bacteriology. 169, 1911-1916.

Johnson, J.L, Cohen, H.J, and Rajagopalan, K.V. 'Molecular Basis of the Biological Function of Molybdenum. Molybdenum-free Sulphite Oxidase from Rat Livers of Tungsten-Treated Rats.' (1974). Journal of Biological Chemistry. 249, 5046-5055.

Johnson, J.L, Hainline, B.E, Rajagopalan, K.V, and Arison, B.H. 'The Pterin Component of the Mo-Cofactor - Structural Characterisation of Two Fluorescent Derivatives.' (1984). Journal of Biological Chemistry. 259, 5414-5422.

Johnson, J.L, Indermaur, L.W, and Rajagopalan, K.V. 'Molybdenum Cofactor Biosynthesis in *Escherichia coli*.' (1991). Journal of Biological Chemistry. 266, 12140-12145.

Johnson, J.L, Jones, H.P, and Rajagopalan, K.V. '*In vitro* Reconstitution of Demolybdo-sulphite oxidase by a Molybdenum Cofactor from Rat Liver and Other Sources.' (1977). Journal of Biological Chemistry. 252, 4994-5003.

Johnson, M.E, and Rajagopalan, K.V. 'Involvement of *chl*A, E, M, and N Loci in *Escherichia coli* Molybdoprotein Biosynthesis.' (1987). Journal of Bacteriology. 169, 117-125.

Johnstone, I.L. 'Transformation of *Aspergillus nidulans*.' (1985). Microbiological Sciences. 2, 307-311.

Johnstone, I.L, Hughes, S.G, and Clutterbuck, A.J. 'Cloning an *Aspergillus nidulans* Developmental Gene by Transformation.' (1985). EMBO Journal. 4, 1307-1311.

Johnstone, I.L, McCabe, P.C, Greaves, P, Gurr, S.J, Cole, G.E, Brow, M.A.D, Unkles, S.E, Clutterbuck, A.J, Kinghorn, J.R, and Innis, M.A. 'Isolation and Characterisation of the *crn*A-*nii*A-*nia*D Gene Cluster for Nitrate Assimilation in *Aspergillus nidulans*.' (1990). Gene. 90, 181-192.

Joshi, M.S, and Rajagopalan, K.V. 'Specific Incorporation of Molybdopterin in Xanthine Dehydrogenase of *Pseudomonas*

aeruginosa.' (1994). Archives of Biochemistry and Biophysics. 308, 331-334.

Kamdar, K.P, Shelton, M.E, and Finnerty, V. 'The *Drosophila* Molybdenum Cofactor Gene, *cinnamon*, is Homologous to Three *Escherichia coli* Cofactor Proteins and to the Rat Protein Gephyrin.' (1994). Genetics. 137, 791-801.

Kennedy, C, and Dean, D. 'The *nifU*, *nifS* and *nifV* Gene Products are Required for Activity of all Three Nitrogenases of *Azotobacter vinelandii*.' (1992). Molecular and General Genetics. 231, 494-498.

Ketchum, P.A, Cambier, H.Y, Frazier, W.A.III., Madansky, C.H, and Nason, A. '*In vitro* Assembly of Assimilatory Nitrate Reductase from Protein Subunits of a *Neurospora* Mutant and the Xanthine Oxidising or Aldehyde Oxidase Systems of Higher Animals.' (1970). Proceedings of the National Academy of Science (USA). 66, 1016-1023.

Kim, J, and Rees, D.C. 'Crystallographic Structure and Functional Implications of the Nitrogenase Molybdenum-Iron Protein from *Azotobacter vinelandii*,' (1992). Nature. 360, 553-560.

Kim, J, and Rees, D.C. 'Nitrogenase and Biological Nitrogen Fixation.' (1994). Biochemistry. 33, 389-397.

Kim, S, and Burgess, B.K. 'Purification and Characterization of Nitrogenase from a *nifW* Strain of *Azotobacter vinelandii*.' (1994). The Journal of Biological Chemistry. 269, 4215-4220.

Kinghorn, J.R, and Unkles, S.E. 'Molecular Genetics and Expression of Foreign Proteins.' (1992). In "Biotechnology Handbooks." Eds. Atkinson, T. and Sherwood, R.F. *Aspergillus* Ed. - Smith, J.E. Vol. 7. Plenum Press.

Kramer, S, Hageman, R.V, and Rajagopalan, K.V. '*In vitro* Reconstitution of Nitrate Reductase Activity of the *Neurospora crassa* Mutant *nit*-1: Specific Incorporation of Molybdopterin.' (1984). Archives of Biochemistry and Biophysics. 233, 821-829.

Kramer, S.P, Johnson, J.L, Ribeiro, A.A, Millington, D.S, and Rajagopalan, K.V. 'The Structure of the Molybdenum Cofactor - Characterization of di-(carboxamidomethyl)molybdopterin from sulphite oxidase and xanthine oxidase.' (1987). Journal of Biological Chemistry. 262, 16357-16363.

La Brie, S.T, Wilkinson, J.Q, Tsay, Y-F, Feldmann, K.A, and Crawford, N.M. 'Identification of Two Tungstate-Sensitive Molybdenum Cofactor Mutants, *chl*2 and *chl*7, of *Arabidopsis thaliana*.' (1992). Molecular and General Genetics. 233, 169-176.

Leach, J, Finkelstein, D.B, and Rambosek J.A. 'Rapid Mini-prep of DNA from Filamentous Fungi.' (1986). Neurospora Newsletter. 33, 32-33.

Lee, J.H, Wendt, J.C, and Shanmugam, K.T. 'Identification of a New Gene, *m o l*R, Essential for Utilization of Molybdate by *Escherichia coli*.' (1990). Journal of Bacteriology. 172, 2079-2087.

Lewis, N.J, Hurt, P, Sealy-Lewis, H.M, and Scazzocchio, C. 'The Genetic Control of the Molybdoflavoproteins in *Aspergillus nidulans*_ IV. A Comparison Between Purine hydroxylase I and II.' (1978). European Journal of Biochemistry. 91, 311-316.

Ljungdahl, L.G, and Andreesen, J.R. 'Tungsten, a Component of Active Formate Dehydrogenase from *Clostridium thermoaceticum*.' (1975). FEBS Letters. 54, 279-282.

Low, D.C, Pommier, J, Giordano, G, and Boxer, D.H. 'Biosynthesis of Molybdo-enzymes in *E. coli*: *chl*B is the Only Chlorate Resistance Locus Required for Protein FA Activity.' (1988). FEMS Microbiology Letters. 49, 331-336.

Luque, F, Mitchenall, L.A, Chapman, M, Christine, R, and Pau, R.N. 'Characterization of Genes Involved in Molybdenum Transport in *Azotobacter vinelandii*.' (1993). Molecular Microbiology. 7, 447-459.

Lurquin, P.F, and Kleinhofs, A. "Genetic Engineering in Eukaryotes. NATO ASI Series - Series A." (1982).Life Sciences. 61, Plenum Press. p.1-5.

Ma, L, Gavini, N, Liu, H.I, Hedman, B, Hodgson, K.O, and Burgess, B.K. 'Large Scale Isolation and Characterization of the Molybdenum-Iron Cluster from Nitrogenase.' (1994). Journal of Biological Chemistry. 269, 18007-18015.

MacDonald, D.W. 'Ph.D. Thesis. University of East Anglia, UK. 'Nitrate and Cytochrome-C Reductase from Wild-type and Mutant Strains of *Aspergillus nidulans*.' (1969).

MacDonald, D.W, and Cove, D.J. 'Studies on Temperature Sensitive Mutants Affecting the Assimilatory Nitrate Reductase of *Aspergillus nidulans*.' (1974). European Journal of Biochemistry. 47 , 107-110.

MacDonald, D.W, and Cove, D.J, and Coddington, A. 'Cytochrome c Reductase from Wild-type and Mutant Strains of *Aspergillus nidulans*.' (1974). Molecular and General Genetics. 128, 187-199.

MacNeil, T, MacNeil, D, Roberts, G.P, Supiano, M.A, and Brill, W.J. 'Fine-structure Mapping and Complementation Analysis of *nif* (Nitrogen Fixation) Genes in *Klebsiella pneumoniae*.' (1978). Journal of Bacteriology. 136, 253-266.

Martinelli, S.D, and Kinghorn, J.R. "Aspergillus: 50 Years On." (1994). Progress in Industrial Microbiology. Vol. 29. Elsevier. Amsterdam.

Mattern, I.E, Punt, P.J, and van den Hondel, C.A.M.J.J. 'A Vector of *Aspergillus* Transformation Conferring Phleomycin Resistance.' (1988). Fungal Genetics Newsletters. 35, p25.

Maupin-Furlow, J-A, Rosentel, J.K, Lee, J-H, Deppenmeier, U, Gunsalus, R.P, and Shanmugam, K.T. 'Genetic Analysis of the *modABCD* (Molybdate Transport) Operon of *Escherichia coli*.' (1995). Journal of Bacteriology. 177, 4851-4856.

May, G. (1992). 'Fungal Technology.' in "Applied Molecular Genetics of Filamentous Fungi." Eds.- Kinghorn, J.R, and Turner, G. Blackie Press, Glasgow.

Mehra, K.R, and Coughlan, M.P. 'Purification and Properties of Purine Hydroxylase II from *Aspergillus nidulans*.' (1984). Archives of Biochemistry and Biophysics. 229, 585-595.

Meyer, C, Gonneau, M, Caboche, M, and Rouzé, P. 'Identification by Mutational Analysis of Four Critical Residues in the Molybdenum Cofactor Domain of Eukaryotic Nitrate Reductase'. (1995). FEBS Letters. 370, 197-202.

Miller, J.B, and Amy, N.K. 'Molybdenum Cofactor in Chlorate-resistant and Nitrate Reductase-Deficient Insertion Mutants of *Escherichia coli*.' (1983). Journal of Bacteriology. 155, 793-801.

Minagawa, N, and Yoshimoto, A. 'Purification and Characterization of the Assimilatory NADPH-Nitrate Reductase of *Aspergillus nidulans*.' (1982). Journal of Biochemistry. 91, 761-774.

Mishra, N.C. 'Gene Transfer in Fungi.' (1985). Advances in Genetics. 23. Academic Press. p.73-173

Moore, J.D, and Hawkins, A.R. 'Overproduction of, and Interaction within, Bifunctional Domains from the Amino and Carboxy Termini of the Pentafunctional AROM Protein of *Aspergillus nidulans*.' (1993). Molecular and General Genetics, 240, 92-102.

Nason, A, Antoine, A.D, Ketchum, P.A, Frazier, W.A. III, and Lee, D.K. 'Formation of Assimilatory Nitrate Reductase by *in vitro* Inter-cistronic Complementation in *Neurospora crassa*.' (1970). Proceedings of the National Academy of Science (USA). 65, 137-144.

Nason, A, Lee, K-Y, Pan, S-S, Ketchum, P.A, Lamberti, A, and Derries, J. '*In vitro* Formation of Assimilatory Reduced Nicotinamide Adenine Dinucleotide Phosphate: Nitrate Reductase from a *Neurospora* Mutant and a Component of Molybdenum Enzymes.' (1971). Proceedings of the National Academy of Science (USA). 68, 3242-3246.

Okamoto, P.M, and Marzluf, G.A. 'Nitrate Reductase of *Neurospora crassa*: the Functional Role of Individual Amino Acids in the Haem Domain As Examined by Site-Directed Mutagenesis.' (1993). Molecular and General Genetics. 240, 221-230.

Oshima, T, Aiba, H, Baba, T, Fujita, K, Hayashi, K, Honjo, A, Ikemoto, K, Inada, T, Itoh, T, Kajihara, M, Kanai, K, Kashimoto, K, Kimura, S, Kitigawa, M, Makino, K, Masuda, S, Miki, T, Mizobuchi, K, Mori, H, Motomura, K, Nakamura, Y, Nashimoto, H, Nishio, Y, Saito, N, Sampei, G, Seki, Y, Tagami, H, Takemoto, K, Wada, C, Yamamoto, Y, Yano, M, and Horiuchi, T. 'A 718kb DNA Sequence of the *Escherichia coli* K12 Genome Corresponding to the 12.7-28.0 min Region on the Linkage Map. (1996). DNA Research. 3, 137-155.

Pateman, J.A, and Cove, D.J. 'Regulation of Nitrate Reduction in *Aspergillus nidulans*.' (1967). Nature. 215, 1234-1237.

Pateman, A.J, Cove, D.J, Rever, B.M, and Roberts, D.B. 'A Common Cofactor for Nitrate Reductase and Xanthine Dehydrogenase which also Regulates the Synthesis of Nitrate Reductase.' (1964). Nature. 201, 58-60.

Pateman, J.A, Kinghorn, J.R, Dunn, and Forbes, E. 'Ammonium Regulation in *Aspergillus nidulans*.' (1973). Journal of Bacteriology. 114, 943-950.

Pateman, J.A, Rever, B.M, and Cove, D.J. 'Genetical and Biochemical Studies of Nitrate Assimilation in *Aspergillus nidulans*.' (1967). Biochemical Journal. 104, 103-111.

Peberdy, J.F. 'Fungal Protoplasts', in "More Gene Manipulations in Fungi."(1991). Academic Press Inc. p307-317.

Pelsy, F, and Caboche, M. 'Molecular Genetics of Nitrate Reductase in Higher Plants.' (1992). Advances in Genetics. 30, 1-40.

Pitterle, D.M, Johnson, J.L, and Rajagopalan, K.V. '*In vitro* Synthesis of Molybdopterin from Precursor Z Using Purified Converting Factor. Role of Protein Bound Sulphur in Formation of the Dithiolene.' (1993). The Journal of Biological Chemistry. 268, 13506-13509.

Pitterle, D.M, and Rajagopalan, K.V. 'Two Proteins Encoded at the *chl*A Locus Constitute the Converting Factor of *Escherichia coli chl*A1.' (1989). Journal of Bacteriology. 171, 3373-3378.

Pitterle, D.M, and Rajagopalan, K.V. 'The Biosynthesis of Molybdopterin in *Escherichia coli*. Purification and Characterization of the Converting Factor.' (1993). Journal of Biological Chemistry. 268, 13499-13505.

Pontecorvo, G, Roper, J.A, Hemmons, L.H, MacDonald, K.D, and Bufton, A.W.J. 'The Genetics of *Aspergillus nidulans*.' (1953). Advances in Genetics. 5, 141-238.

Punt, P.J, Oliver, R.P, Dingemanse, M.A, Pouwels, P.H, and van den Hondel, C.A.M.J.J. 'Transformation of *Aspergillus* Based on the Hygromycin- B Resistance Marker from *E. coli.*' (1987). Gene. 56, 117-124.

Rajagopalan, K.V, and Johnson, J.L. 'The Pterin Molybdenum Cofactors.' (1992). Journal of Biological Chemistry. 267, 10199-10202.

Rand, K.N, and Arst, H.N. 'A Mutation in *Aspergillus nidulans* Which Affects the Regulation of Nitrite Reductase and is Tightly Linked to its Structural Gene.' (1977). Molecular and General Genetics. 155, 67-75.

Rand, K.N, and Arst, H.N. 'Mutations in *nir*A Gene of *Aspergillus nidulans* and Nitrogen Metabolism.' (1978). Nature. 272, 732-734.

Rech, S, Deppenmeier, U, and Gunsalus, R.P. 'Regulation of the Molybdate Transport Operon, *mod*ABCD, of *Escherichia coli* in Response to Molybdate Availability.' (1995). Journal of Bacteriology. 177, 1023-1029.

Rivers, S.L, McNairn, E, Blasco, F, Giordano, G, and Boxer, D.H. 'Molecular-Genetic Analysis of the *moa* Operon of *Escherichia coli* K12 Required for Molybdenum Cofactor Biosynthesis.' (1993). Molecular Microbiology. 8, 1071-1081.

Roberts, C.F. 'Isolation of Multiple Aromatic Mutants in *Aspergillus nidulans*,' (1969). *Aspergillus* Newsletter. 10, 19-20.

Roberts, G.P, MacNeil, T, MacNeil, D, and Brill, W.J. 'Regulation and Characterization of Protein Products Coded by the *nif* (Nitrogen Fixation) Genes of *Klebsiella pneumoniae*.' (1978). Journal of Bacteriology. 136, 267-279.

Rogozin, I.B, and Milanesi, L. 'Analysis of Donor Splice Sites in Different Eukaryotic Organisms.' (1997). Journal of Molecular Evolution, 45, 50-59.

Rosentel, J.K, Healy, F, Maupin-Furlow, J-A, Lee,J-H, and Shanmugam, K.T. 'Molybdate and Regulation of *mod* (Molybdate Transport), *fdh*F and *hyc* (Formate Dehydrogenlyase) Operons in *Escherichia coli*.' (1995). Journal of Bacteriology. 177, 4857-4864.

Rupar, C.A, Gillett, J, Gordon, B.A, Ramsay, D.A, Johnson, J.L, Garrettt, R.M, Rajagopalan, K.V, Jung, J.H, Bacheyie, G.S and Sellers, A.R. 'Isolated Sulphite Oxidase Deficiency.' (1996). Neuropaediatrics. 27, 299-304.

Sambrook, J, Fritsch, E.F, and Maniatis, T. 'Molecular Cloning- a Laboratory Manual.'- 2nd Ed. (1989). 1, Section 3:20. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Sanger, F, Nicklen, C, and Coulson, A.R. 'DNA Sequencing with Chain Terminating Inhibitors.' (1977). Proceedings of the National Academy of Science (USA). 74, 5463-

Santini, C-L, Iobbi-Nivol, C, Romane, C, Boxer, D.H, and Giordano, G. 'Molybdoenzyme Biosynthesis in *Escherichia coli*: *in vitro* Activation of Purified Nitrate Reductase from a *chl*B Mutant.' (1992). Journal of Bacteriology. 174, 7934-7940.

Scazzocchio, C. 'The Genetic Control of Molybdoflavins in *Aspergillus* nidulans.' (1973). European Journal of Biochemistry. 36, 428-445.

Scazzocchio, C. 'The Genetic Determination of the Molybdoflavoenzymes in *A. nidulans*.' (1974). Journal of the Less-Common Metals. 36, 461-464.

Scazzocchio, C, and Arst, H.N. Jr., "Molecular and Genetic Aspects of Nitrate Reductase." (1989). Eds.- Wray, J.L, and Kinghorn, J.R. Oxford Scientific Publications. Oxford.

Schwarz, G, Boxer, D.H, and Mendel, R.R. 'Molybdenum Cofactor Biosynthesis. The Plant Protein CNX1 Binds Molybdopterin with High Affinity.' (1997). Journal of Biological Chemistry. 272, 26811-26814.

Shah, V.K, Allen, J.R, Spangler, N,J, and Ludden,P.W. '*In vitro* Synthesis of the Iron-Molybdenum Cofactor of Nitrogenase. Purification

and Characterization of NifB Cofactor, the Product of NifB Protein.' (1994). Journal of Biological Chemistry. 269, 1154-1158.

Shah, V.K, and Brill, W.J. 'Isolation of an Iron-Molybdenum Cofactor from Nitrogenase.' (1977). Proceedings of the National Academy of Science (USA). 74, 3249-3253.

Silhavy, T.J, Berman, M.L, and Enquist, L.W. (1984). 'Experiments in Gene Fusions.' CSH Press.

Simpson, F.B, and Burris, R.H. 'A Nitrogen Pressure of 50 Atmospheres Does Not Prevent Evolution of Hydrogen by Nitrogenase.' (1984). Science. 224, 1095-1097.

Slot, H.M.J, Overwegplandsoen, W.C.G, Bakker, H.G, Abeling, N.G.G.M, Tamminga, P, Barth, P.G, and Vangennip, A.H. 'Molybdenum Cofactor Deficiency - An Easily Missed Cause of Neonatal Convulsions'. (1993). Neuropaediatrics. 24, 139-142.

Sophianopoulou, V, and Scazzocchio, C. 'The Proline Transport Protein of *Aspergillus nidulans* is Very Similar to Amino Acid Transporters of *Saccharomyces cerevisiae*.' (1989). Molecular Microbiology. 3, 705-714.

Stallmeyer, B, Nerlich, A, Schiemann, J, Brinkmann, H and Mendel, R.R. 'Molybdenum Cofactor Biosynthesis - The *Arabidopsis thaliana* cDNA *cnx*1 Encodes a Multifunctional Two-Domain Protein

Homologous to a Mammalian Neuroprotein Cinnamon and Three *Escherichia coli* Proteins.' (1995). Plant Journal, 8, 751-762.

Stewart, V, and Parales Jr., J. 'Identification and Expression of Genes *nar*L and *nar*X of the *nar* (Nitrate Reductase) Locus in *Escherichia coli* K12.' (1988). Journal of Bacteriology. 170, 1589-1597.

Stouthamer, A.H. 'Biochemistry and Genetics of Nitrate Reductase in Bacteria.' (1976). Advances in Microbial Physiology. 14, 315-375.

Taniguchi, S, and Itagaki, E. 'Nitrate Reductase of Nitrate Respiration Type from *Escherichia coli*. Solubilization and Purification of the Particulate System with Molecular Characterization as a Metallo-protein.' (1960). Biochimie et Biophysica Acta. 44, 263-279.

Tilburn, J, Scazzocchio, C, Taylor, G.G, Zabicky-Zissman, J.H, Lockington, R.A, and Davies, R.W. 'Transformation by Integration in *A. nidulans*.' (1983). Gene 26, 205-221.

Timberlake, W.E, Boylan, M.T, Cooley, M.B, Mirabito, P.M, O'Hara, E.B, and Willett, C.E. 'Rapid Identification of Mutation-Complementing Restriction Fragments from *Aspergillus nidulans* Cosmids.' (1985). Experimental Mycology. 9, 351-355.

Timberlake, W.E, and Marshall, M.A. 'Genetic Engineering of Filamentous Fungi.' (1989). Science. 244, 1313-1317.

Tomsett, A.B, and Cove, D.J. 'Deletion Mapping of the *nii*A-*nia*D Gene Region of *Aspergillus nidulans*.' (1979). Genetical Research. 34, 19-32.

Tomsett, A.B, and Garrett, R.H. 'The Isolation and Charaterization of Mutants Defective in Nitrate Assimilation in *Neurospora crassa*.' (1980). Genetics. 95, 649-660.

Unkles, S.E, Hawker, K.L, Grieve, C, Campbell, E.I, Montague, P, and Kinghorn, J.R. '*crn*A Encodes a Nitrate Transporter in *Aspergillus nidulans*.' (1991). Proceedings of the National Academy of Science (USA). 88, 204-208.

Unkles, S.E, Smith, J, Kanan, G.J.M.M, Millar, L.J, Heck, I.S, Boxer, D.H, and Kinghorn, J.R. 'The *Aspergillus nidulans cnx*ABC Locus is a Single Gene Encoding Two Catalytic Domains Required for Synthesis of Precursor Z, an Intermediate in Molybdenum Cofactor Biosynthesis.' (1997). Journal of Biological Chemistry. In Press.

Wahl G.M, Lewis, K.A, Ruiz, J.C, Rothenberg, B, Zhao, J, and Evans, G.A. 'Cosmid Vectors for Rapid Genomic Walking, Restriction Mapping, and Gene Transfer.' (1987). Proceedings of the National Academy of Science (USA). 84, 2160-2164.

Wang, G, Angermuller, S, and Klipp, W. 'Characterization of *Rhodobacter capsulatus* Genes Encoding a Molybdenum

Transport System and Putative Molybdenum - Pterin - Binding Proteins.' (1993). Journal of Bacteriology. 175, 3031-3042.

Wernars, K, Goosen, T, Wennekes, B.M.J, Swart, K, van den Hondel, C.A.M.J.J, and van den Broek, H.W.J. 'Cotransformation of *Aspergillus nidulans*: a Tool for Replacing Fungal Genes.' (1987). Molecular and General Genetics. 209, 71-77.

Wiame, J.M, Grenson, M, and Arst, H.N. Jr. ' Nitrogen Catabolite Repression in Yeasts and Filamentous Fungi.' (1985). Advances in Microbial Physiology. 26, 1-88.

Wolfinger, E.D, and Bishop, P.E. 'Nucleotide Sequence and Mutational Analysis of the *vnf*ENX Region of *Azotobacter vinelandii*.' (1991). Journal of Bacteriology. 173, 7565-7572.

Wray, J.L, and Kinghorn, J.R. "Molecular and Genetic Aspects of Nitrate Assimilation." (1989). Oxford Scientific Publications. Oxford.