Mutational analysis of *creA*, the mediator of carbon catabolite repression in *Aspergillus nidulans*.

Robert Shroff, B.Sc.(Hons)

A thesis submitted for the degree of
Doctor of Philosophy
in
The University of Adelaide
Department of Genetics
(Faculty of Science)

December, 1997

This thesis is printed on archival paper

TABLE OF CONTENTS

ABSTRACT *v*
DECLARATION *vii*
ACKNOWLEDGEMENTS *viii*
LIST OF FIGURES *ix*
LIST OF TABLES *xi*

CHAPTER 1 INTRODUCTION **1**

1.1 - CARBON CATABOLITE REPRESSION IN THE YEAST *SACCHAROMYCES CEREVISIAE* **3**
1.1.1 - The galactose utilisation genes, *GAL* 6
1.1.2 - The sucrose utilisation genes, *SUC* 7
1.1.3 - Genes required for glucose repression 8
1.1.4 - Genes required for glucose derepression 14
1.1.5 - A model for Mig1p dependent glucose repression in *S. cerevisiae* 16
1.1.6 - Other glucose regulatory mechanisms 16
1.2 - CARBON CATABOLITE REPRESSION IN *ASPERGILLUS NIDULANS* **19**
1.2.1 - Mutations affecting carbon catabolite repression 19
1.2.2 - *creA* 21
1.2.3 - *creB* and *creC* 27
1.2.4 - *creD* 28
1.3 - SYSTEMS IN *ASPERGILLUS NIDULANS* WHICH ARE REGULATED BY CARBON SOURCE 29
1.3.1 - Regulation of *amdS* 29
1.3.2 - Regulation of the *prn* cluster 33
1.3.3 - Regulation of *alcA* 34
1.3.4 - Regulation of *ipnA* 35
1.4 - CYS_2-HIS_2 ZINC FINGER DNA BINDING DOMAIN **36**
1.4.1 - X-ray crystallographic analysis of Zif268 38
1.4.2 - Mutations within the DNA binding domain of WT1 38
1.5 - AIMS AND OBJECTIVES **40**

CHAPTER 2 MATERIALS AND METHODS **42**

2.1 - MATERIALS **43**
2.1.1 - Buffers 43
2.1.2 - Solutions 44
2.1.3 - Growth Media 45
2.1.4 - *Aspergillus nidulans* Strains 47
2.1.5 - *Escherichia coli* Strains 48
2.1.6 - Olignucleotides 49
2.1.7 - Plasmids 49
2.2 - METHODS **49**
2.2.1 - Manipulation of *A. nidulans* 49
2.2.2 - Nucleic acid analysis 50
2.2.3 - DNA Sequencing 50
2.2.4 - Polymerase chain reaction 50
2.2.5 - Single stranded conformation polymorphism 50
2.2.6 - Alcohol dehydrogenase (ADH, EC 1.1.1.1) enzyme assays 51

CHAPTER 3 PHENOTYPIC ANALYSIS OF *CREA* MUTANTS 52

3.1 - MORPHOLOGY AND LIFE CYCLE OF STRAINS CONTAINING *CREA* MUTANT ALLELES 54
3.1.1 - Morphology on solid media 54
3.1.2 - Morphology in liquid media 58
3.1.3 - Effect on the formation of cleistothecia 61
3.2 - TEMPERATURE SENSITIVITY 63
3.3 GROWTH IN REPRESSING CONDITIONS 66
3.3.1 - Genes involved in the utilisation of acetamide, proline, GABA and β-alanine 66
3.3.2 - Genes involved in the utilisation of lactose and starch 69
3.3.3 - Genes involved in the utilisation of ethanol 72
3.4 - GROWTH IN DEREPRESSING CONDITIONS 76
3.5 - SUMMARY 77

CHAPTER 4 MOLECULAR ANALYSIS OF *CREA* MUTANTS 78

4.1 - ANALYSIS OF *CREA* HOMOLOGUES 79
4.2 - PCR-SSCP ANALYSIS OF *CREA* MUTANTS 86
4.3 - *CREA* ALLELES PREDICTED TO PRODUCE TRUNCATED POLYPEPTIDES 94
4.3.1 - *creA*303 and *creA*304 96
4.3.2 - *creA*305 97
4.3.3 - *creA*322 98
4.3.4 - Other alleles that result in a truncated CreA polypeptide 100
4.4 - *CREA* ALLELES WITH MISSENSE MUTATIONS IN THE DNA BINDING DOMAIN 101
4.4.1 - *creA*311 and *creA*334 104
4.4.2 - *creA*331 105
4.4.3 - *creA*1 106
4.4.4 - *creA*204 and *creA*225 107
4.4.5 - *creA*306 108
4.5 - SUMMARY 110

CHAPTER 5 PHENOTYPIC ANALYSIS OF *CREA* NULL ALLELES 112

5.1 - CONSTRUCTION OF STRAINS CONTAINING *CREA* DISRUPTION ALLELES 114
5.1.1 - A *creA* null allele 115
5.1.2 - A partially disrupted *creA* allele 115
5.2 - PHENOTYPIC ANALYSIS OF *CREA* DISRUPTED STRAINS 115
5.2.1 - Morphology of creA disrupted strains 117
5.2.2 - Effect of *creA* gene disruption on gene expression 120
5.3 - WHAT CAUSES THE LEAKY LETHAL PHENOTYPE? 124
5.4 - SUMMARY 125

CHAPTER 6 PRELIMINARY ANALYSIS OF THE INTERNAL REGIONS OF CREA 127

6.1 - *RGR1* DOMAIN SWAP 129
6.2 - A INTERNAL DELETION OF THE *CREA* GENE SPANNING AMINO ACIDS 207-365 131
6.2.1 - Analysis of pANC4ΔRV10 transformed strains in repressing growth conditions 135

6.2.2 - Analysis of pANC4ΔRV10 transformed strains in derepressing growth conditions 141
6.3 - SUMMARY **146**

CHAPTER 7 SELECTION OF MUTANTS THAT SUPPRESS *CREA* ALLELES **148**

7.1 - SUPPRESSORS OF THE *CREA*306 MUTANT ALLELE **150**
7.1.1 - Suppressors with a greatly improved growth rate and conidiation 152
7.1.2 - Suppressors with a partially improved growth rate and conidiation 158
7.1.3 - *su*(*creA*306)9730 163
7.1.4 - Suppressors with an improved growth rate that are aconidial 165
7.2 - SUPPRESSORS OF THE *CREA*Δ4 MUTANT ALLELE **165**
7.3 - SUMMARY **167**

CHAPTER 8 CONCLUSIONS **170**

8.1 - FUNCTIONAL REGIONS WITHIN CREA **172**
8.2 - COMPARISON WITH MIG1P **176**
8.3 - IDENTIFICATION OF CREA INTERACTING PROTEINS **178**

REFERENCES **180**

APPENDIX **197**

Addendum

page 47, Table 2.1 - the following entries should be included.

Strain	Genotype	Reference
SA20 X	*yA*1, *riboB*2, *adE*20, *suA*1*adE*20, *creA*220	Shroff *et al.* (1996)
SA20	*yA*1, *riboB*2, *adE*20, *suA*1*adE*20, *areA*217, *creA*220	Shroff *et al.* (1996)
SA21 X	*yA*1, *riboB*2, *adE*20, *suA*1*adE*20, *creA*221	Shroff *et al.* (1996)
SA21	*yA*1, *riboB*2, *adE*20, *suA*1*adE*20, *areA*217, *creA*221	Shroff *et al.* (1996)
SA25 X	*yA*1, *riboB*2, *adE*20, *suA*1*adE*20, *creA*225	Shroff *et al.* (1996)
SA25	*yA*1, *riboB*2, *adE*20, *suA*1*adE*20, *areA*217, *creA*225	Shroff *et al.* (1996)

page 160, Figure 7.5 - the following should be added to the figure legend.

Note - on plate (a) the colony on the bottom row, position two is *creA*$^+$ and on plate (b) the colony on the bottom row, position two is *creA*Δ4.

ABSTRACT

Carbon catabolite repression is a wide domain regulatory system that in the presence of glucose acts to prevent the expression of numerous enzymes that are involved in the utilisation of more complex carbon sources. In *Aspergillus nidulans* the process of carbon catabolite repression is mediated by CreA. *creA* has been cloned (Dowzer and Kelly 1989) and theoretical translation indicated the presence of potentially functional domains within the protein. The most notable of these was a classical Cys_2-His_2 zinc finger DNA binding domain.

This thesis presents a study undertaken with the aim of defining functionally important regions within the CreA protein. The approach used to achieve this aim has been to characterise a bank of *creA* mutant alleles. This characterisation involved the phenotypic analysis of strains containing each *creA* allele and determining the basis of the mutation at the DNA level. Functional regions of the protein are determined from the comparison of the site and nature of each mutation with the resulting phenotype. Using this approach the *creA* mutant alleles analysed can be broadly grouped into two categories. Seven *creA* mutant alleles are the result of missense mutations within the DNA binding domain, providing clear evidence that the zinc finger DNA binding domain is functional *in vivo*. The remaining mutant alleles consist of mutations that are predicted to result in truncated CreA mutant polypeptides. The smallest truncation is predicted to occur in CreA322. Strains containing this mutation have significant levels of derepression of carbon catabolite regulated systems. Therefore the final 80 amino acids must contain a region that mediates repression. CreA303 is predicted to be the most severely truncated polypeptide, terminating after the first 69 residues. Strains containing *creA*303 have extreme pleiotropic phenotypes. However these strains are viable, which conflicts with previous data from a strain with a deletion of a region of the genome including *creA* (Dowzer and Kelly 1991). This resulted in a reassessment of the phenotype of a *creA* null allele, which was shown to reflect the phenotype of the most extreme alleles in this study, *creA*303 and *creA*304.

Strains transformed with a construct that has an in frame internal deletion of amino acids 207-365 were made. Transformants with 3-5 copies of this construct were able to fully complement a *creA*Δ strain in repressing conditions, suggesting that the C-terminal region required for repression was contained in the last 51 amino acids. The same transformants had an impaired ability to grow on sole carbon sources that are non-repressing. This suggests that the deleted region is required for the expression of some carbon catabolite repressed genes in non-repressing conditions.

The most phenotypically extreme allele in this study is *creA*306. Surprisingly *creA*306 is the result of a missense mutation within the DNA binding domain. This resulted in the hypothesis that CreA306 is a full length polypeptide with little or no ability to bind DNA and that the extreme phenotype is the result of the titration of potential CreA interacting proteins. Based on this hypothesis, *creA*306 was the ideal candidate for use in a screen for suppression of *creA*$^-$ mutations with the goal of identifying genes that encode CreA interacting proteins. Suppressor mutants were selected on the basis of improved growth rate and conidiation resulting in the identification of mutants that grew at very strong and at intermediate levels by comparison to a *creA*306 strain. Mutations that restore *creA*306 growth to,or near, wild type levels were all shown to

be the result of reversions or allelic mutations that changed the residue substituted in the original *creA*306 mutation. The mutations analysed that had intermediate growth rates compared to *creA*306 were all the result of extragenic mutations. One of these mutations was shown to be very closely linked and probably allelic to *creB*15. This is the first evidence of a direct functional link between CreA and CreB. The remaining mutants analysed from this group all had phenotype similar to *creA*Δ strains. Six of them were tested by meiotic analysis and shown to be very tightly linked to each other. They are excellent candidates for a gene that encodes a CreA interacting protein.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and that, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

The *creA*Δ strains analysed in Chapter 5 were constructed by S. M. O'Connor.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Robert Shroff
23rd December, 1997

ACKNOWLEDGEMENTS

I would like to thank Dr. Joan Kelly, an endless source of advice and encouragement throughout my time in the Genetics Department and for the opportunity to learn so much from her. Most importantly I would like to acknowledge her friendship. It is mainly Joan's influence that has made this such a satisfying and rewarding (I hope!) experience. Joan I have the greatest amount of respect for you. Thank you for everything.

I am extremely grateful to Dr. Robin Lockington. Firstly for being such a good friend and of course for teaching me so much. I have always enjoyed the lengthy afternoon-tea discussions. There can't be too many world problems we have left unsolved. Also, thank you for your input into the preparation of this thesis. It has been invaluable and it is very much appreciated.

Throughout my time in the Genetics Department it has been my privilege to work with a large number of people. It is the environment they have created that makes it such an enjoyable place to work. It would be impossible to name everyone individually, but I am going to try! Thank you to Michael, Chris, Steve, Mathew, Celia, Sandie, Darren, Jo, Nicole, Tricia, Guy, Mi-Hye, Shuliang and of course Doug (you have to respect someone who can get a job just by taking his pants off and can dodge the law with a graph!). The pastures that you all currently inhabit may be greener but I would be surprised if they are as much fun. To the 'Murphys', Rachael (head Murphy, but only by birth!), Richard, Dave, John and Sharon (even if you do support the world's worst football team!) thank you for all the fun times, they mean a lot to me. Thank you to Sue, Rosemary, Tamzin and Nigel (my only regret is that I won't be able to give you a hard time when you are writing up), all of the "fruitflies" (there really are too many of you to name) and Fiona (the patient and caring one, yeah right!). When I started this research I was employed in the teaching side of the department. I would like to thank the members of academic staff for helping me so much. With your help it at least looked like I knew what I was doing. Also, thank you to Doug (we must have a round of golf!), Roger, Sharon, Michelle, Paul, Sonia, Jane, Melanie, Michelle and Sandra. I apologise to all of those people I've neglected.

To all of my friends away from the department thank you for reminding me that there is life outside of the Fisher Building.

Finally, I would like to acknowledge the support of my family. Most of all to my parents, who have never failed to be there when I needed them, thank you for the love, support and encouragement even though (or maybe, especially because) I don't think you have ever quite understood the obsession.

LIST OF FIGURES

Chapter 1

Figure 1.1 - Life cycle of *A. nidulans* 4
Figure 1.2 - Schematic of the functional domains in Mig1p. 12
Figure 1.3a - Regulatory pathway which results in glucose repression. 17
Figure 1.3b - Model for glucose repression in *S. cerevisiae* 18
Figure 1.4 - Schematic representing potential domains in CreA. 23
Figure 1.5 - Comparison of the polypeptide sequenecs of the DNA binding domain of CreA and a number of proteins that contain Cys2-His2 zinc fingers 24
Figure 1.6 - Consensus CreA binding site based on published *in vitro* DNA footprinting data 25
Figure 1.7 - Comparison of similar region between CreA and Rgr1p 27
Figure 1.8 - Control of the *amdS* gene. 30
Figure 1.9 - Genetic organisation and control of the proline utilisation system. 33
Figure 1.10 - Genetic organisation and control of the ethanol utilisation system. 35

Chapter 3

Figure 3.1 - Growth rate and conidiation of strains grown on 1% complete medium 55
Figure 3.2 - Microscopic examination (400X) of mycelial samples grown in carbon rich liquid medium 59
Figure 3.3 - Microscopic examination (100X) of mycelial samples grown in carbon rich liquid medium 60
Figure 3.4 - Microscopic examination of mycelial samples grown in carbon limiting liquid medium 62
Figure 3.5 - Formation of abortive cleistothecial initials 64
Figure 3.6 - Suppression of *areA*217 68
Figure 3.7 - Expression levels of β-galactosidase and α-amylases in repressing conditions 71
Figure 3.8 - Sensitivity of *creA* strains to allyl alcohol 74

Chapter 4

Figure 4.1 - CLUSTALW analysis of CreA homologues 82
Figure 4.2 - Match-Box analysis of CreA homologues 83
Figure 4.3 - Summary of conserved region identified in the CreA protein 85
Figure 4.4 - Strategy 1 for the production of DNA fragments used for SSCP analysis 87
Figure 4.5 - Strategy 2 for the production of DNA fragments used for SSCP analysis 88
Figure 4.6 - Example of a typical SSCP analysis of fragment PS2 89
Figure 4.7 - Summary of SSCP results 90
Figure 4.8 - Sequence analysis of *creA*1, *creA*204 and *creA*225 93
Figure 4.9 - *creA* mutant alleles predicted to produce truncated polypeptides 95
Figure 4.10 - Missense mutations within the Cys_2-His_2 zinc finger DNA binding domain 102
Figure 4.11 - Critical residues in the CreA Cys_2-His_2 zinc finger DNA binding domain 103

Chapter 5
Figure 5.1 - *creA* disruption constructs 116
Figure 5.2 - Growth rate and conidiation of *creA* disrupted strains grown on 1% complete medium 118
Figure 5.3 - Suppression of *areA*217 and level of α-amylase expression in *creA*Δ strains 122
Figure 5.4 - Sensitivity of *creA*Δ strains to allyl alcohol 123

Chapter 6
Figure 6.1 - Construction of pANC4RGR1a 130
Figure 6.2 - Construction of pANC4ΔRV10 133
Figure 6.3 - Identification of pANC4ΔRV10 cotransformed strains 134
Figure 6.4 - Estimation of the number of copies of pANC4ΔRV10 in cotransformed strains. 136
Figure 6.5 - Growth rate and conidiation of pANC4ΔRV10 cotransformed strains grown on 1% complete medium 138
Figure 6.6 - Growth of pANC4ΔRV10 cotransformed strains in repressing conditions 140
Figure 6.7 - Growth of pANC4ΔRV10 cotransformed strains on media with non-repressing carbon sources 143
Figure 6.8 - Growth of pANC4ΔRV10 cotransformed strains in derepressing conditions 145

Chapter 7
Figure 7.1 - Basic model for the process of carbon catabolite repression. 149
Figure 7.2 - Growth rate and conidiation of allelic suppressors of *creA*306 154
Figure 7.3 - Growth of allelic suppressors of *creA*306 in repressing conditions 155
Figure 7.4 - DNA sequence changes in allelic suppresor mutations of the *creA*306 mutant allele 157
Figure 7.5 - Growth rate and conidiation of extragenic suppressors of *creA*306 grown on 1% complete medium 160
Figure 7.6 - Growth of extragenic suppressors of *creA*306 in repressing conditions 162

Chapter 8
Figure 8.1 - Functional regions within CreA. 175
Figure 8.2 - Comparison of functional regions of CreA from *A. nidulans* and Mig1p from *S. cerevisiae.* 177

LIST OF TABLES

Chapter 1
Table 1.1- Point mutations within the DNA binding domain of WT1. 39

Chapter 2
Table 2.1 - *A. nidulans* strains used in this study. 47
Table 2.2 - Strains of *E. coli* used in this study. 48
Table 2.3 - Oligonucleotides used in this study. 49
Table 2.4 - Plasmids used in this study. 49

Chapter 3
Table 3.1 - Measurement of growth rate and degree of conidiation of strains containing *creA* mutant alleles. 57
Table 3.2 - Measurement of growth rate of strains containing *creA* mutant alleles at different temperatures 65
Table 3.3 - Measurement of growth rate of strains containing *creA* mutant alleles in an *areA*217 background. 67
Table 3.4 - Levels of expression of β-galactosidase and α-amylase from strains containing *creA* mutant alleles. 70
Table 3.5 - Sensitivity of strains containing *creA* alleles to varying concentrations of allyl alcohol. 73
Table 3.6 - ADH1 activity (nM product/(mg soluble protein/minute)) from strains containing selected *creA* alleles. 76

Chapter 4
Table 4.1 - DNA sequence change and predicted CreA polypeptides. 92

Chapter 5
Table 5.1 - Measurement of growth rate and degree of conidiation of strains containing *creA* mutant alleles. 119
Table 5.2 - Growth of *creA*Δ strains on media containing 1% D-glucose and a range of different carbon sources. 121

Chapter 6
Table 6.1 - Growth of pANC4ΔRV10 transformants in repressing conditions. 137
Table 6.2 - Growth of pANC4ΔRV10 transformants in derepressing conditions. 142

Chapter 7
Table 7.1 - Phenotypic analysis of allelic suppressors of the *creA*306 mutation 153
Table 7.2 - Phenotypic analysis of extragenic suppressors of the *creA*306 mutation 159

Chapter 1

Introduction

LIBRARY UNIVERSITY OF ADELAIDE

As is the case for most organisms, the chief source of energy for *Aspergillus nidulans* is the oxidation of glucose. When glucose is available to the organism it will be used preferentially over other carbon sources. In the absence of glucose *A. nidulans* is able to utilise a wide range of alternative carbon sources. Numerous proteins are required to catabolise these alternative carbon sources. However, it is energetically inefficient to produce all of these proteins unless the fungus is growing in an environment that lacks glucose, the preferred carbon source, and a specific alternative carbon source is present. To prevent this potential waste of energy, the expression of the genes that encode the proteins required to utilise alternative carbon sources is tightly regulated. The transcriptional regulation of these genes can often be divided into two mechanisms. The first is a pathway specific regulatory mechanism that acts in response to a specific inducer. The second form of regulation is by wide domain systems which act in response to the general growth environment.

Carbon catabolite repression, or glucose repression, is an example of a wide domain regulatory system. In the presence of glucose carbon catabolite repression acts to prevent the expression of a range of enzymes and permeases that are required for the utilisation of alternative carbon sources. By repressing expression of these genes in the presence of glucose the organism does not waste energy producing proteins that are not required.

Carbon catabolite repression has been identified in numerous fungi and bacteria. Amongst the simple eukaryotes carbon catabolite repression has been most extensively studied in the yeast *Saccharomyces cerevisiae* and the ascomycete fungus

A. nidulans. In *A. nidulans* carbon catabolite repression is mediated by the product of the *creA* gene. This study aims to characterise CreA and determine its role in the process of carbon catabolite repression in *A. nidulans*.

A. nidulans is an ideal organism for the study of gene regulation and, in particular, carbon catabolite repression. The organism grows on simple defined liquid and solid media and is able to utilise a wide range of compounds as carbon sources. Many aspects of the *A. nidulans* life cycle make it suitable for genetic analysis (figure 1.1). *A. nidulans* has both an asexual and a sexual cycle. Hyphae readily form heterokaryons from which diploid strains can be isolated. Numerous genes have been identified and mapped resulting in a comprehensive linkage map being produced for *A. nidulans* (Clutterbuck 1997).

1.1 - Carbon catabolite repression in the yeast *Saccharomyces cerevisiae*

The most extensive analysis of a eukaryotic carbon catabolite repression regulatory system has been carried out in the yeast, *S. cerevisiae*. *S. cerevisiae* has evolved to utilise glucose quite differently to most other organisms. In repressing conditions, such as high levels of glucose or fructose, yeast metabolises fermentable carbon sources through the glycolytic pathway. In other organisms pyruvate formed from glycolysis is further metabolised by the Krebs cycle. This does not occur in yeast since many mitochondrial functions are repressed by glucose. Glucose repression of mitochondrial functions is a specialised development and is a reflection of the purely fermentative metabolism of glucose by yeast. In yeast, pyruvate is decarboxylated and

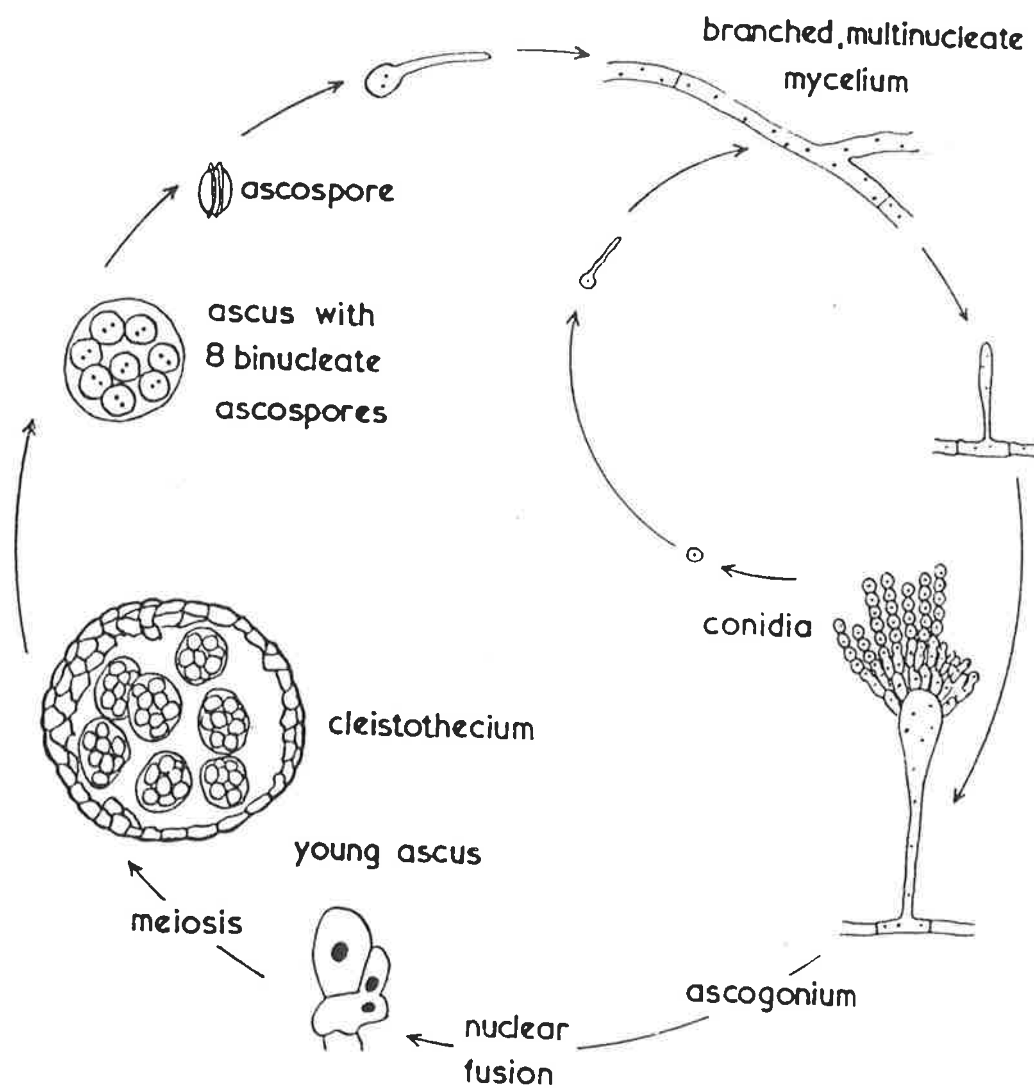


Figure 1.1 - Life cycle of *A. nidulans*
Taken from Fincham and Day (1963).

the resulting acetaldehyde is reduced to ethanol to regenerate the NAD that is used in glycolysis. Therefore, the glucose is not fully metabolised, producing significantly less ATP than during oxidative growth. The carbon is not permanently lost to the cell. When glucose is depleted mitochondrial enzymes are derepressed and ethanol is metabolised. *S. cerevisiae* can also utilise many di- and trisaccharides such as lactose, sucrose, maltose and raffinose. These sugars are broken down into their component monosaccharides and enter the glycolytic pathway as hexose phosphates and can then be metabolised in the same way as glucose.

Yeast can also utilise numerous non-fermentable carbon sources, such as ethanol and acetate which are converted to acetyl-CoA. To obtain energy acetyl-CoA feeds into the Krebs cycle with ATP being obtained via respiration. The cell also requires hexose phosphates for biosynthetic reactions. In the absence of glucose they are produced via gluconeogenesis.

Depending on the carbon source available to the organism it will continually switch between fermentative and gluconeogenic growth. The genes encoding many of the enzymes required for these processes are transcriptionally repressed in the presence of glucose. In yeast glucose repressible genes can be divided into three distinct groups (Ronne 1995).

a) Fructose-1,6-bisphosphatase and PEP carboxylase are unique to gluconeogenesis. These two enzymes are repressed by glucose, thus preventing glycolysis and gluconeogenesis functioning simultaneously and depleting cellular ATP levels.

b) Glucose repressible mitochondrial enzymes involved in the Krebs cycle and respiration.

c) Enzymes that are involved in the uptake and metabolism of more complex carbon sources eg. galactose and sucrose. Some of these systems are regulated at two levels where carbon catabolite repression has to interact with other regulatory mechanisms. The galactose and maltose utilisation systems have specific induction pathways as well as being glucose repressed. Alternatively, the sucrose utilisation system is only regulated by glucose repression.

1.1.1- The galactose utilisation genes, *GAL*

The *GAL* genes are a set of enzyme encoding and regulatory genes that enable yeast cells to utilise galactose as a carbon source. The *GAL* genes are strongly regulated at the level of transcription.

The *GAL* structural genes are *GAL1, -2, -7* and *-10* and *MEL1*. These genes code for products which transport galactose into the cell and catabolise galactose to the glycolytic substrate glucose-1-phosphate. The *GAL* genes have three major regulated states. They are repressed by the presence of glucose, derepressed in the absence of glucose and induced in the absence of glucose and the presence of galactose. The activation system is very powerful with transcription induced 100-1000 fold when galactose is the sole carbon source. Conversely, these structural genes are not expressed at a detectable level in the presence of glucose.

There are three regulatory genes that control the induction pathway of the *GAL* structural genes. Gal4p is required for the galactose induction of expression of the structural *GAL* genes. Gal80p binds to and masks the Gal4p transcriptional activation function in media lacking galactose. Gal3p mediates galactose induced release of Gal80p inhibition of Gal4p.

The presence of glucose triggers the glucose repression system. Glucose repression acts in a number of ways to repress the galactose utilisation system. *GAL4* is moderately repressed in the presence of glucose. The absence of Gal4p results in no transcription of the *GAL* structural genes. This transcriptional repression is mediated by the globally acting glucose repression mechanism (Griggs and Johnston 1991) and is effected by Mig1p (Nehlin *et al.* 1991). *GAL80* is not repressed in the presence of glucose (Shimada and Fukasawa 1985). *GAL3* is completely glucose repressed (Bajwa *et al.* 1984).

The *GAL1* and *GAL2* structural genes are also strongly glucose repressed in a Mig1p dependent fashion (Johnston 1987). *GAL7* and *GAL10* are also strongly glucose repressed but in a Mig1p independent fashion (Flick and Johnston 1992).

1.1.2 - The sucrose utilisation genes, *SUC*

The utilisation of sucrose and raffinose is mediated by the *SUC* gene family. Each *SUC* gene codes for an intracellular and extracellular form of invertase (Carlson and Botstein 1983). Invertase acts to hydrolyse sucrose. Invertase activity is not induced

by sucrose but is reduced over 100 fold by the presence of glucose (Perlman and Halvorson 1981). Glucose regulation of *SUC2* occurs at the transcriptional level. An upstream regulatory region, subsequently shown to contain Mig1p binding sites, is sufficient for glucose regulated gene expression (Sarokin and Carlson 1985). Numerous mutants affecting the regulation of genes required for the utilisation of a number of carbon sources have been selected. Mutants selected for their effects on *SUC2* have been particularly useful for the study of glucose repression. As there is no induction mechanism required for *SUC2* expression the majority of mutants that increase or decrease *SUC2* expression disrupt some aspect of glucose repression.

1.1.3 - Genes required for glucose repression

1.1.3.1 - *HXK2*

HXK2 (*HEX1*, *GLR1*) mutants were isolated by their growth on media containing sucrose and the glucose analogue 2-deoxyglucose (Zimmermann and Scheel 1977). The mutation results in derepression of invertase, malate dehydrogenase, maltase, succinate dehydrogenase and NADH-dehydrogenase. They result in reduced hexose phosphorylating activity but the activity of all other glycolytic enzymes appears to be normal (Entian and Zimmermann 1980). *HXK2* encodes hexokinase PII (Lobo and Maitra 1977). The mechanism by which Hxk2p affects glucose repression is unknown. Residual phosphorylation activity of Hxk2p is correlated with the level of glucose repression and is dependent on the presence of Hxk2p. The level of phosphorylation activity modulated by Hxk1p or Glk1p does not correlate with glucose repression

(Ma *et al.* 1989a,b; Rose *et al.* 1991). This indicates that glucose repression is not only associated with the phosphorylation activity of hexokinase PII, the presence of hexokinase PII protein is necessary to give the signal for glucose repression. Hexokinase PII may act as the initial sensor for glucose repression, possibly by phosphorylating a regulatory protein or small molecule. Hexokinase PII is phosphorylated *in vivo* and the extent of labelling varies with the amount of glucose in the growth medium (Vojtek and Fraenkel 1990). Hxt2p also affects hexose transport (Bisson and Fraenkel 1984). The signal may in fact be a combination of glucose transport coupled to its phosphorylation by Hxt2p.

1.1.3.2 - *REG1*

reg1 (*HEX1*) mutants were selected by resistance to 2-deoxyglucose in the presence of sucrose (Niederacher and Entian 1987). Mutations in *REG1* result in higher invertase, galactokinase, maltose uptake and hexokinase PII activity than in wild type cells. Even though *reg1* mutants have a higher level of maltose uptake they are unable to grow on maltose as a sole carbon source. Deletion of *REG1* resulted in over accumulation of glycogen, slow growth and enlarged cell size suggesting that *REG1* has a role in carbohydrate storage. *REG1* has been cloned and encodes a 113 kDa protein containing a putative nuclear localisation signal. Tu and Carlson (1995) showed that Reg1p interacts physically with Glc7p (see Section 1.1.3.4), a type 1 protein phosphatase catalytic subunit. The two proteins act together to negatively regulate glucose repression. Reg1p may act as a phosphatase regulatory subunit that targets Glc7p to proteins involved in the glucose repression pathway.

1.1.3.3 - *GRR1*

Mutations in *GRR1* (*CAT80*) allow expression of invertase, maltase, galactokinase and cytochrome C in the presence of glucose (Bailey and Woodward 1984). *SUC2* expression is glucose inducible in *grr1* mutants. *grr1* mutants also have morphological effects. They have a reduced rate of growth compared to wild type, they produce elongated cells and fail to sporulate when homozygous (Flick and Johnston 1991). *GRR1* is predicted to encode a protein of 1151 amino acids and is asparagine and glutamine rich. It also contains twelve leucine rich repeats that may mediate protein-protein interactions. Transcription of *GRR1* is not glucose regulated. The mode of action is unknown but it is suggested that its function is required for the generation or transduction of the intracellular signal that causes glucose repression. The altered regulatory responses to glucose seen in *grr1* mutants may be the result of glucose transport defects (Ozcan *et al.* 1994; Vallier *et al.* 1994).

1.1.3.4 - *GLC7*

Mutations in *GLC7* (*CID1*) were selected for growth on sucrose in the presence of 2-deoxyglucose. *glc7* mutants cause glucose insensitive expression of invertase and maltase. Homozygous diploid strains have sporulation defects. *GLC7* has been cloned and codes for a protein phosphatase type 1 and is an essential gene (Tu and Carlson 1995). Genetic analysis suggests that this protein acts, in conjunction with Reg1p,

antagonistically to the Snf1p protein kinase in glucose repression (Tu and Carlson 1995).

1.1.3.5 - *MIG1*

MIG1 was isolated by screening a yeast library for genes that, when present in multiple copies, repressed the *GAL1* promoter in inducing conditions. Multiple copies of *MIG1* also repress *SUC2* expression (Nehlin and Ronne 1990). *mig1* mutations partially relieve repression of *SUC2, GAL1* and *GAL4*. A role for *MIG1* as an activator of transcription has been shown in a *ssn6* mutant strain (Treitel and Carlson 1995). *MIG1* is epistatic to *SNF1* (Vallier and Carlson 1994). *MIG1* has been cloned and encodes a protein of 504 amino acids that has two Cys_2-His_2 zinc fingers in the amino terminal region. *In vitro* DNase 1 footprinting has defined the Mig1p DNA binding site in the promoters of *SUC2* (Nehlin and Ronne 1990) and *GAL1* and *GAL4* (Nehlin *et al.* 1991) as WWWWSYGGGG.

MIG1 homologues have been cloned from two *Kluyveromyces sp.* (Cassart *et al.* 1997). Comparison of the homologous proteins has identified a number of conserved regions. Deletion mapping analysis of *MIG1* has identified functional roles for some of these conserved domains. In addition to the previously identified DNA binding domain, an effector domain required for transcriptional repression and two regions designated R1 and R2 which mediate inhibition of Mig1p activity in the absence of glucose, have been identified. A potential nuclear localisation domain has also been identified (Ostling *et al.* 1996). Nuclear localisation of Mig1p is dependent on the

presence of glucose (Devit *et al.* 1997). A spatial representation of these domains is shown in figure 1.2.

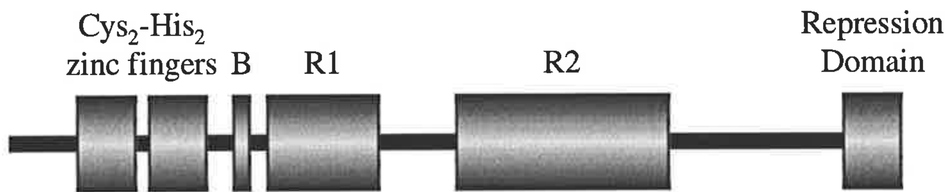


Figure 1.2 - Schematic of the functional domains in Mig1p.
This schematic is adapted from Ostling *et al.* (1996). The B domain represents a potential nuclear localisation domain. The R1 and R2 domains are required for the inhibition of Mig1p function in derepressing conditions.

1.1.3.5 - The Tup1p/Ssn6p repression complex

tup1 (*aar1, aer1, amm1, cyc9, sfl2, umr7*) mutants have been selected by numerous quite different screens. This is consistent with the vast array of pleiotropic phenotypes which result from *tup1* mutations. *tup1* mutations result in the constitutive expression of numerous glucose repressible enzymes including invertase and maltase (Trumbly 1986). *tup1* strains result in flocculence (Stark *et al.* 1980), elevated levels of cytochrome C (Rothstein and Sherman 1980); and mating and sporulation defects (Lemontt *et al.* 1980). None of these phenotypes have any obvious relationship to the glucose repression system. *TUP1* has been cloned (Mackay 1983; Fujita *et al.* 1990). It encodes a 713 amino acid protein. The C-terminal third of the protein contains six WD-40 repeats, which may be involved in protein-protein interactions (Neer *et al.* 1994; Komachi and Johnston 1997).

Mutations within *ssn6* (*cyc8*) exhibit the same range of phenotypes as *tup1* strains (Schultz and Carlson 1987). *SSN6* encodes a protein of 107 kDa in mass (Schultz and Carlson 1987; Trumbly 1988). Near its amino terminus Ssn6p has ten repeats of the tetratricopeptide repeat (Hirano *et al.* 1990; Sikorsky *et al.* 1990). Large deletions of this region abolish Ssn6p function, while smaller deletions result in a subset of phenotypes (Schulz *et al.* 1990).

These two genes appear to be functionally related. They result in the same pleiotropic array of phenotypes and have been isolated in the same mutant screens (Rothstein and Sherman 1980; Trumbly 1986). Ssn6p and Tup1p associate in a high molecular weight complex (Williams *et al.* 1991). The complex is composed of one Ssn6p subunit and four Tup1p subunits (Varnasi *et al.* 1996). Overexpression of *SSN6* and *TUP1* reduces *SUC2* transcription in the absence of glucose confirming the genetic evidence that these proteins act to repress transcription (Schultz and Carlson 1987; Williams and Trumbly 1990). However neither protein is able to directly bind DNA and they must therefore act by interacting with a DNA binding protein, Mig1p in the case of glucose repression (Keleher *et al.* 1992; Trietel and Carlson 1995). By utilising different DNA binding proteins this complex also acts to repress transcription of genes regulated by cell type (Mukai *et al.* 1987), oxygen (Zitomer and Lowrey 1992) and DNA damage (Elledge *et al.* 1993).

A functional dissection of these two proteins suggests that Tup1p functions to repress transcription and that Ssn6p links the repressor complex to pathway specific DNA binding proteins (Tzamarias and Struhl 1994). Distinct combinations of TPR motifs

are required for Ssn6p to interact with specific DNA binding proteins (Tzamarias and Struhl 1995).

1.1.3.6 - *RGR1*

The *rgr1* mutation was selected as causing overexpression of a reporter gene placed under the control of the *SUC2* promoter (Sakai *et al.* 1988). The mutation is pleiotropic affecting *SUC2* transcription levels, reduced amounts of reserved carbohydrates, temperature sensitive growth, defects in sporulation and cell morphology. *RGR1* has been cloned and encodes a 1082 amino acid hydrophobic protein that is essential for growth (Sakai *et al.* 1988; Sakai *et al.* 1990). Rgr1p has subsequently been shown to have a more regulatory role (Stillman *et al.* 1994; Covitz *et al.* 1994) acting as part of the mediator/holoenzyme complex acting directly upon RNA polymerase to control transcription (Li *et al.* 1995).

1.1.4 - Genes required for glucose derepression

1.1.4.1 - *SNF1*

snf1 (*cat1, ccr1*) mutants were selected by their inability to ferment sucrose and raffinose (Carlson *et al.* 1981). Mutations in *snf1* result in strains that are unable to utilise fermentable and non-fermentable carbon sources other than glucose (Celezna and Carlson 1986). Snf1p acts at the level of transcription, failing to derepress *SUC2* mRNA production (Carlson and Botstein 1982). This is not a general effect since Snf1p has no effect on a number of non glucose repressed genes (Carlson *et al.* 1981). *SNF1* is also involved in sporulation, glycogen storage, thermotolerance and peroxisome biosynthesis (Thompson-Jaeger *et al.* 1991; Schuller and Entian *et al.*

1992). *SNF1* is constitutively expressed and encodes a protein of 633 amino acids that is a serine/threonine protein kinase (Celezna and Carlson *et al.* 1986). The protein kinase domain is required for Snf1p function (Celezna and Carlson 1989).

1.1.4.2 - *SNF4*

snf4 mutants were selected in a screen similar to that used to isolate *snf1*. They have the same pleiotropic phenotypes as *snf1* and lack Snf1p catalytic activity *in vitro* (Niegerborn and Carlson 1984). *SNF4* is expressed constitutively and encodes a 322 amino acid protein with no obvious functional domains.

The Snf1p kinase complexes with Snf4p. Genetic and biochemical evidence suggests that Snf4p activates the Snf1p kinase (Celezna and Carlson 1989; Celezna *et al.* 1991). During glucose starvation Snf1p kinase activity increases, and this increase requires Snf4p (Woods *et al.* 1994). Two hybrid analysis suggests that the Snf1p/Snf4p interaction is regulated by glucose, where high glucose inhibits interaction and limiting glucose promotes interaction. When glucose is limiting, Snf4p binds to the Snf1p regulatory domain, counteracting an autoinhibitory interaction between the Snf1p kinase domain and the regulatory domain (Jiang and Carlson 1996). Co-immunoprecipitation data suggests that Snf1p and Snf4p are always complexed. Therefore glucose may regulate conformation of the complex (Jiang and Carlson 1997). The Snf1p/Snf4p interaction is also regulated via Reg1p and Glc7p (Jiang and Carson 1996).

1.1.5 - A model for Mig1p dependent glucose repression in *S. cerevisiae*

Glucose is transported into the cell by hexose transport systems, encoded by *HXT* genes, which are regulated by *GRR1*. The actual signal that the cell is in a high glucose environment is unknown, however it is most likely that the signalling molecule is an early intermediate of glycolysis. The glucose is phosphorylated by one of the three glucose-phosphorylating enzymes Hxk1p, Hxk2p and Glk1p. Hxk2p appears to have an additional role in the signalling process, via Reg1p and Glc7p, to inactivate the Snf1p/Snf4p complex (figure 1.3a)

The Snf1p/Snf4p complex regulates Mig1p function. In the presence of glucose Mig1p/Tup1p/Ssn6p form a complex that binds to the promoters of glucose repressible genes to inhibit transcription. The mechanism of repression is unknown, possible mechanisms include general effects on chromatin structure and specific interactions with the basal transcriptional machinery. In glucose limiting conditions the Snf1p/Snf4p complex is active and phosphorylates the R1 and R2 regions of Mig1p. Phosphorylation of Mig1p results in the Mig1p protein being transported from the nucleus to the cytoplasm, resulting in derepression of glucose repressible genes (figure 1.3b).

1.1.6 - Other glucose regulatory mechanisms

Other regulatory mechanisms have been identified which respond to the presence of glucose. While all of the glucose regulatory systems are different, they are all likely to respond to the same glucose monitoring and signalling pathways.

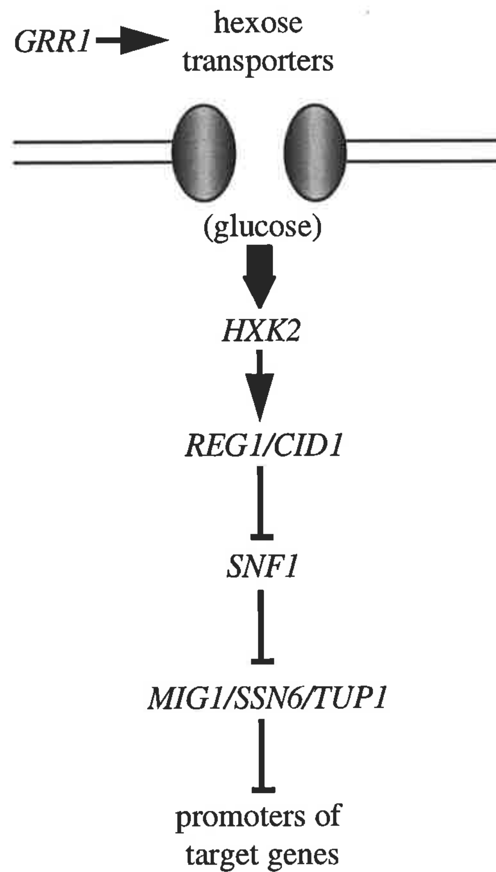


Figure 1.3a - Regulatory pathway which results in glucose repression.

Hu *et al.* (1995) identified a Mig1p independent glucose repression system. It is not known how this system functions but it may act by inhibiting a general transcriptional activation complex.

Upon addition of glucose to a culture grown in glucose limiting conditions certain enzymes are rapidly inactivated. This inactivation occurs via a proteolytic mechanism. This process is called glucose inactivation and has been reviewed by Holzer 1989.

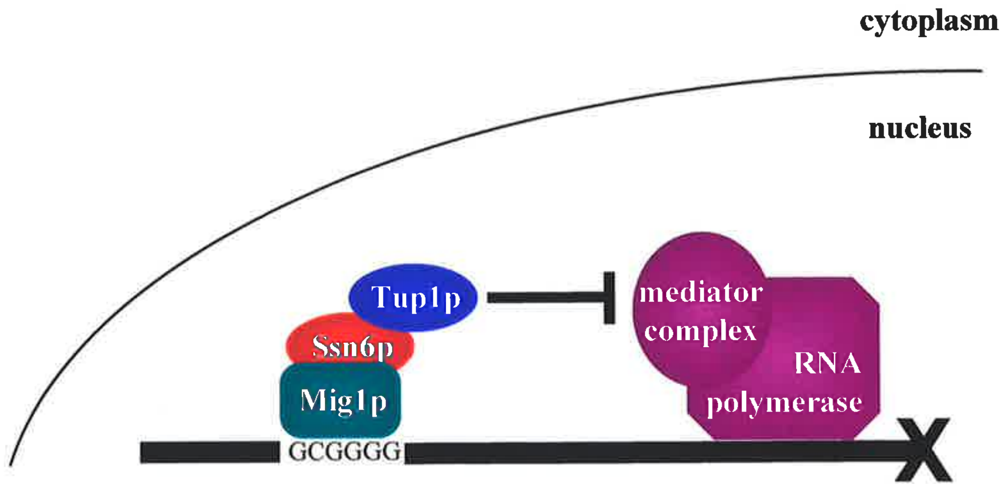
Glucose can also trigger induction of transcription. Several glycolytic enzymes and some ribosomal proteins are induced when a growing culture is shifted from a non-

Figure 1.3b - Model for glucose repression in *S. cerevisiae*

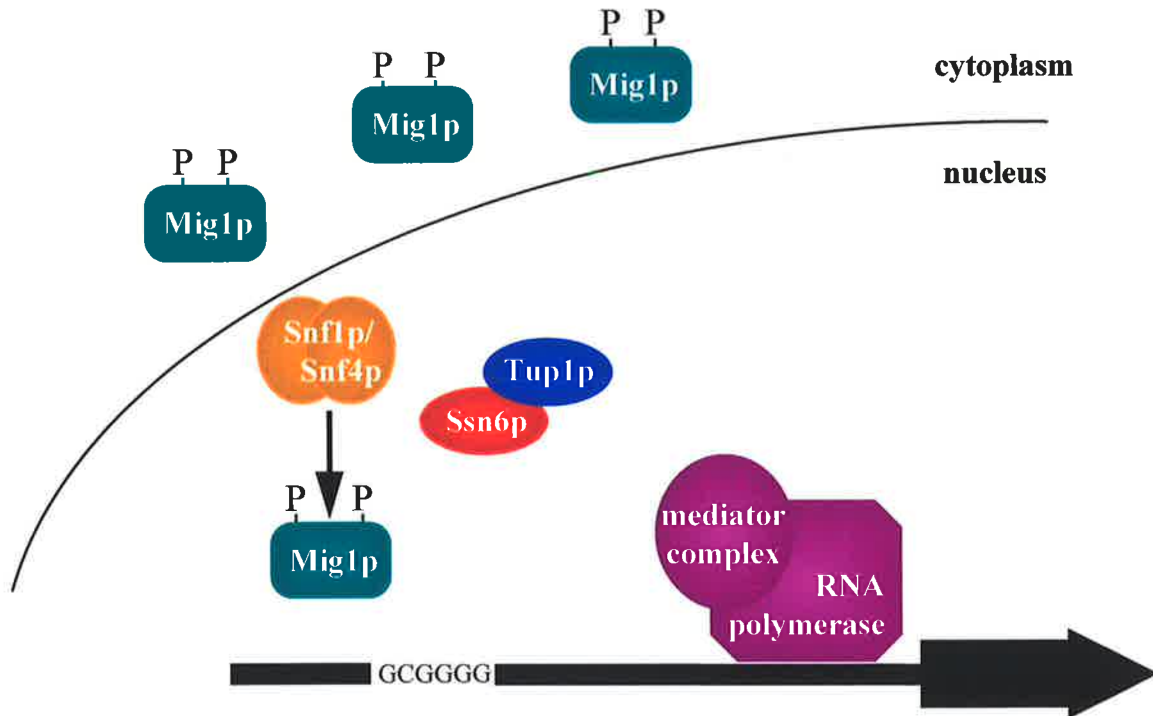
(i) In the presence of a repressing carbon source, such as glucose, Mig1p binds to the Tup1p/Ssn6p repressor complex and recruits this complex to the promoters of glucose repressible genes. The mechanism used by the Tup1p/Ssn6p complex is unknown but it may act by affecting the chromatin structure of the promoter and preventing the transcription machinery from functioning.

(ii) In derepressing conditions the Snf1p/Snf4p protein kinase complex is activated by Reg1p/Glc7p. The activated complex phosphorylates Mig1p. The phosphorylated Mig1p proteins are actively transported out of the nucleus releasing the promoter from glucose repression.

(i) Repressing Conditions



(ii) Derepressing Conditions



fermentable carbon source to glucose (Entian *et al.* 1984; Donovan and Pearson 1986; Herruer *et al.* 1989).

1.2 - Carbon catabolite repression in *A. nidulans*

Unlike *S. cerevisiae*, *A. nidulans* metabolises glucose via the mitochondrial pathways used by most eukaryotic organisms. *A. nidulans* is able to utilise a wide range of compounds as carbon sources. However, the expression of the genes required for the use of many of these carbon sources is dependent on the absence of glucose. The expression of these genes is regulated by carbon catabolite repression. Of eukaryotic organisms that obtain energy via the oxidation of glucose, *A. nidulans* has the most extensively studied carbon catabolite repression gene regulatory system. Mutational analyses in *A. nidulans* has resulted in the identification of four loci which result in the disruption of the carbon catabolite repression process.

1.2.1 - Mutations affecting carbon catabolite repression

A number of different approaches have been used to select mutations that affect the carbon catabolite repression system. The most commonly utilised selection protocol has been to select mutants that suppress an *areA* loss of function allele. However a number of other selections have also been used to isolate mutants.

Compounds such as acetamide and proline can supply both a carbon and a nitrogen source to the cell. Aside from pathway specific regulation, the genes required to break down these compounds are under the control of the global regulatory systems carbon catabolite repression and nitrogen metabolite repression. Lifting either repression mechanism will allow transcription to occur. Nitrogen metabolite repression is

analogous to carbon catabolite repression. Nitrogen metabolite repression acts to repress the genes which code for the proteins required to break down compounds that supply a nitrogen source. Nitrogen metabolite repression is mediated by the positively acting regulatory protein AreA. *areA* loss of function mutants are unable to activate transcription of nitrogen metabolite repression regulated genes. As a result *areA* loss of function strains are only able to grow on media that contain ammonium or glutamine as a nitrogen source when glucose is the carbon source. However when grown on media that contains acetamide or proline as the sole carbon and nitrogen sources *areA* loss of function strains can grow. This is because carbon catabolite repression is lifted in the absence of glucose, resulting in the expression of the genes required to break down these compounds. This feature can be used to select mutants which result in the derepression of carbon catabolite repression regulated genes. In carbon repressing conditions (glucose or sucrose) with acetamide or proline as the nitrogen source *areA* loss of function mutants are unable to grow. However a mutation that results in derepression of carbon catabolite repression regulated genes will allow growth. The majority of carbon catabolite repression mutations have been selected using this method of selection. These mutations occur in three different loci, designated *creA, creB* and *creC*. The *creA* mutations constitute the majority of mutants selected by this method (Arst and Cove 1973; Bailey and Arst 1975; Bailey 1976 Arst and Bailey 1977; Hynes and Kelly 1977; Kelly and Hynes 1977).

Pyruvate dehydrogenase, PdhA, is required to convert pyruvate to acetyl-CoA (Romano and Kornberg 1968). Acetyl-CoA can be supplied from other sources. Any compound that can be broken down to acetate can supply a source of acetyl-CoA. Ethanol and acetamide are compounds that can be broken down to acetate. The

expression of the proteins required to breakdown these compounds are glucose repressible. Therefore *pdhA*⁻ strains are able to grow on medium that has ethanol as the sole carbon source. They cannot grow on medium that contains ethanol and glucose due to carbon catabolite repression of the ethanol utilisation genes. Carbon catabolite repression mutations can be selected as *pdhA*⁻ strains that are able to grow on medium that contains ethanol and glucose. A number of mutants, all allelic to *creA*, have been selected using this screen (Bailey and Arst 1975; Bailey 1976).

creA mutants have also been selected by suppressing the requirement for acetate created by a pyruvate carboxylase, *pycA*, mutation on complete media (Shroff *et al.* 1997). A *creA* allele has also been selected by resistance to the toxicity caused by growth of a *frA*1 strain on 1% D-mannitol (Arst *et al.* 1990).

The fourth locus identified which disrupts the normal carbon catabolite repression regulatory process is *creD*. *creD*34 was isolated as a suppressor of the *creC*27 allele. *creC*27 is sensitive to fluoroacetamide in the presence of a repressing carbon source. *creD*34 was a spontaneous mutation selected for resistance of a *creC*27 strain to fluoroacetamide in carbon repressing conditions (Kelly and Hynes 1977).

No carbon catabolite repression mutants that lead to a permanently repressed phenotype (or a failure to derepress) have been isolated (Bailey and Arst 1975; Arst and Bailey 1977; Kelly and Hynes 1977; Bailey 1976).

1.2.2 - *creA*

creA is on chromosome I and is tightly linked to *galD* (Arst and Cove 1973; Hynes and Kelly 1977). Strains containing *creA* alleles result in the partial derepression of

genes that are regulated by carbon catabolite repression. All *creA* alleles tested are recessive in heterozygous diploids. Since the most common class of *creA* mutants are recessive, and result in derepression, it is likely that *creA* is a negatively acting repressor of carbon catabolite repression (Arst and Bailey 1977). Repression of *alcA* via *creA* has been shown to occur at the level of transcription (Lockington *et al.* 1987).

creA has been cloned by complementation of function (Dowzer and Kelly 1989). Alcohol dehydrogenase I is required for the breakdown of ethanol. Allyl alcohol can act as a substrate for alcohol dehydrogenase I. In this reaction allyl alcohol is converted to the toxic compound acrolein. Expression of alcohol dehydrogenase I is regulated by carbon catabolite repression. Therefore unlike wild type strains, *creA*$^-$ strains result in sensitivity to allyl alcohol on media containing glucose. *creA*$^+$ was cloned by transforming a genomic library made from a *creA*$^+$ strain into a *creA204* strain and selecting on medium containing 2.5mM allyl alcohol and a repressing carbon source. Strains containing multiple copies of the *creA*$^+$ gene grew better than wild type on media containing sucrose and allyl alcohol, indicating tighter repression (Dowzer and Kelly 1989).

Sequencing of the rescued clone, two genomic clones and two cDNA clones was carried out (Dowzer and Kelly 1989). A single open reading frame of 416 amino acids was identified. This open reading frame has a number of features consistent with CreA acting as a regulatory protein. The most obvious functional domain consisted of two Cys_2-His_2 zinc fingers that have been found to function as DNA binding domains in other proteins. The open reading frame also had an alanine rich region, and acidic

region and frequent S(T)PXX motifs (Dowzer and Kelly 1991). A spatial representation of these regions is shown in figure 1.4.

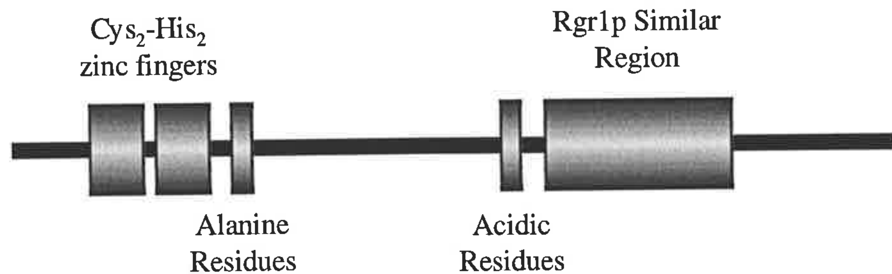


Figure 1.4 - Schematic representing potential domains in CreA.
Schematic adapted from Dowzer and Kelly (1991).

The Cys_2-His_2 zinc finger has a very high degree of similarity to a domain in the yeast protein Mig1p, the protein that mediates glucose repression in *S. cerevisiae*. There is also a high degree of similarity to the DNA binding domains of numerous other proteins (figure 1.5). Included in this group of proteins is the mouse early growth response protein Zif268. X-ray crystallographic studies have been carried out on the DNA binding properties of Zif268 (Pavletich and Pabo 1991; Elrod-Erickson *et al.* 1996). *In vitro* DNA binding studies have been performed on the 5' regions of a number of CreA regulated genes, and by CASTing (cyclic amplification and selection of targets) experiments (Kulmberg *et al.* 1993; Cubero and Scazzocchio 1994; Espeso and Peñalva 1994; Chamalaun-Hussey 1996). The resulting CreA binding site consensus sequence is SYGGRG (figure 1.6). Considering the similarities between the binding domains of the two proteins it is not surprising that the binding sites between CreA and Mig1p are very similar. However the CreA binding site does not have the A/T rich sequence that is immediately five prime of the Mig1p binding site. It has been

(i) *creA* zinc finger 1

Protein																							
CreA	C			P	L	C	E	R	A	F	H	R	L	E	H	Q	T	R	H	I	R	T	H
CreA (*A. niger)*	C			P	L	C	E	R	A	F	H	R	L	E	H	Q	T	R	H	I	R	T	H
Mig1p	C			P	I	C	H	R	A	F	H	R	L	E	H	Q	T	R	H	M	R	I	H
AmdA	C			E	Y	C	N	R	S	F	A	R	L	E	H	L	Q	R	H	L	R	T	H
AmdX	C			T	T	C	G	R	S	F	A	R	L	E	H	L	K	R	H	E	R	S	H
BrlA	C	K	E	P	G	C	N	G	R	F	K	R	Q	E	H	L	K	R	H	M	K	S	H
Adr1p	C			E	V	C	T	R	A	F	A	R	Q	E	H	L	K	R	H	Y	R	S	H
Zif268	C	P	V	E	S	C	D	R	R	F	S	R	S	D	E	L	T	R	H	I	R	I	H
WT1	C	A	Y	P	Q	C	N	K	R	Y	F	K	L	S	H	L	Q	M	H	S	R	K	H
PacC	C	Q	W	G	S	C	R	T	T	T	V	K	R	D	H	I	T	S	H	I	R	V	H
TFIIIA	C	S	F	A	D	C	G	A	A	Y	N	K	N	W	K	L	Q	A	H	L	C	K	H

(ii) H-C linker

Protein							
CreA	T	G	E	K	P	H	A
CreA (*A. niger)*	T	G	E	K	P	H	A
Mig1p	T	G	E	K	P	H	A
AmdA	T	K	E	K	P	F	S
AmdX	T	K	E	K	P	F	E
BrlA	S	K	E	K	P	H	V
Adr1p	T	N	E	K	P	Y	P
Zif268	T	G	Q	K	V	F	Q
WT1	T	G	E	K	P	Y	Q
PacC	V	P	L	K	P	H	K
TFIIIA	T	G	E	K	P	F	P

(iii) *creA* zinc finger 2

Protein																								
CreA	C	Q	F	P	G	C	S	K	R	F	S	R	S	D	E	L	T	R	H	S	R	I		H
CreA (*A. niger)*	C	Q	F	P	G	C	T	K	R	F	S	R	S	D	E	L	T	R	H	S	R	I		H
Mig1p	C	D	F	P	G	C	V	K	R	F	S	R	S	D	E	L	T	R	H	R	R	I		H
AmdA	C	D	I			C	S	K	S	F	A	R	S	D	L	L	V	R	H	E	R	L	V	H
AmdX	C	P	D			C	S	R	C	F	A	R	R	D	L	L	L	R	H	Q	Q	K	L	H
BrlA	C	W	V	P	G	C	H	R	A	F	S	R	S	D	N	L	N	A	H	Y	T	K	T	H
Adr1p	C	G	L			C	N	R	C	F	T	R	R	D	L	L	I	R	H	A	Q	K	I	H
Zif268	C	D	I			C	G	R	K	F	A	R	S	D	E	R	H	R	H	T	K	I		H
WT1	C	D	F	K	D	C	E	R	R	F	F	R	S	D	Q	L	K	R	H	Q	R	R		H
PacC	C	D	F			C	G	K	A	F	K	R	P	Q	D	L	K	K	H	V	K	T		H
TFIIIA	C	K	E	E	G	C	E	K	G	F	T	S	L	H	H	L	T	R	H	S	L	T		H

Figure 1.5 - Comparison of the polypeptide sequences of the DNA binding domain of CreA and a number of proteins that contain Cys2-His2 zinc fingers

Zinc coordinating residues are highlighted in blue. Residues predicted to interact with the DNA binding site are highlighted in green. The remaining conserved residues are highlighted in yellow.

CreA from *A. nidulans* (Dowzer and Kelly 1991), CreA from *A. niger* (Drysdale *et al.* 1993), Mig1p from *S. cerevisiae* (Nehlin and Ronne 1990), AmdA from *A. nidulans* (Lints *et al*. 1995), AmdX from *A. nidulans* (Murphy *et al.* 1997), BrlA from *A. nidulans* (Adams *et al*. 1988), Adr1 from *S. cerevisiae* (Hartshorne *et al*. 1986), Zif268 from *M. musculus* (Christy *et al*. 1988), WT1 from *H. sapiens* (Call *et al*. 1990), PacC from *A. nidulans* (Tilburn *et al*. 1995), TFIIIA from *X. laevis (*Miller *et al.* 1985)

amdS -96 / -90					T	G	A	G	G	G	G
amdS -88 / -82					T	G	C	G	G	G	G
facB -1357 / -1351					G	C	C	G	G	G	G
facB -1219 / -1213					T	C	C	G	G	G	G
alcR -97 / -103					T	C	T	G	G	G	G
alcR -120 / -126					T	G	C	G	G	G	G
alcR -224 / -230					T	G	T	G	G	G	G
alcR -657 / -651					T	G	C	G	G	G	G
alcA -155 / -161					T	A	C	G	G	G	G
alcA -303 / -297					G	G	C	G	G	G	G
prnB -304 / -310					T	C	T	G	G	G	G
prnB -318 / -312					T	G	C	G	G	A	G
creA consensus					T	S	Y	G	G	R	G
MIG1 consensus	W	W	W	W	T	S	Y	G	G	G	G

Figure 1.6 - Consensus CreA binding site based on published *in vitro* DNA footprinting data

Nucleotides that have been shaded do not fit the consensus sequence. The data has been collected from the following references: *alcA, alcR* - *Kulmberg et al.* (1993), *prnB* - Cubero and Scazzocchio (1994), amdS, facB - Chamalaun-Hussey (1996), Mig1p - Nehlin *et al*. (1991).

suggested that it is not the Cys_2-His_2 zinc fingers that bind the A/T sequence (Lundin *et al.* 1994). CreA has nine consecutive alanine residues directly after the DNA binding domain. Alanine rich regions have been commonly found in transcriptional repressor proteins (Hanna-Rose and Hansen 1996). A deletion of the alanine rich region of Kruppel results in a decreased ability of the protein to act as a repressor (Licht *et al.* 1990). Further analysis shows that the alanine rich region of Kruppel has a structural role in the protein by stabilising the repression domain (Licht *et al.* 1994).

Regions rich in acidic residues are commonly found in transcriptional activator proteins (Ma and Ptashne 1987). If this domain is functional in *A. nidulans* it suggests a positive role for CreA. This would be consistent with a transcriptional activation role suggested for the yeast protein Mig1p (Trietel and Carlson 1995).

Northern analysis indicated that *creA* is autoregulated. *creA* mRNA levels are higher in cultures grown in non-repressing carbon sources than in glucose grown cultures. This differential expression is lost in strains that contain *creA* mutant alleles. *creA* mutant strains have high levels of *creA* message in glucose grown cultures as well as cultures grown in non-repressing conditions (Shroff *et al.* 1996).

The *creA* homologue has been cloned from *A. niger* (Drysdale *et al.* 1993). A 45 amino acid sequence in the C-terminal half of CreA is identical between the two species. Database searches with this sequence identified a degree of similarity (81%) with a region of the *S. cerevisiae* protein Rgr1p (figure 1.7). Rgr1p was originally identified as having a role in glucose repression, but has recently been shown to have

a more general role in transcriptional regulation (Sakai *et al.* 1988; Stillman *et al.* 1994; Covitz *et al.* 1994; Li *et al.* 1995).

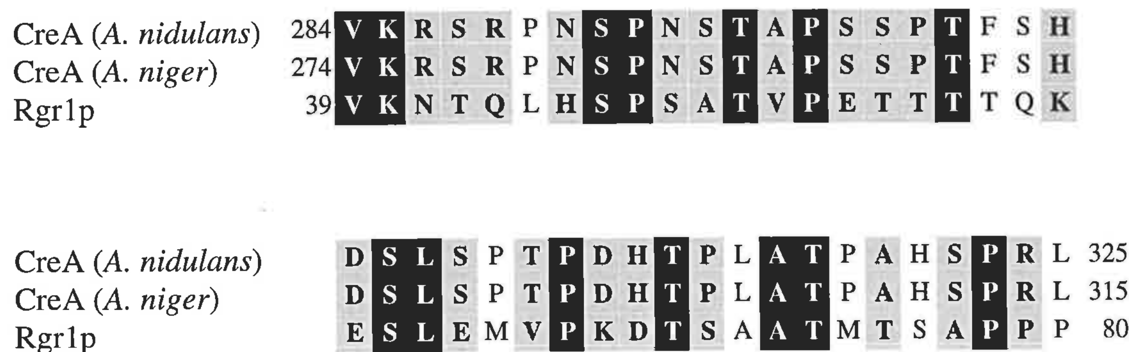


Figure 1.7 - Comparison of similar region between CreA and Rgr1p
A region of 45 amino acids is invariant between CreA in *A. nidulans* (Dowzer and Kelly 1991) and *A. niger* (Drysdale *et al.* 1993). This region has a high degree of similarity to a region of the Rgr1p protein from *S. cerevisiae*. Residues shaded black are identical and residues with similar properties are shaded grey.

1.2.3 - *creB* and *creC*

The *creB* and *creC* loci both map to chromosome II, however the two genes are not linked. *creB* is closely linked to *acoB* and *acrB* near the end of chromosome II. *creB* was shown to be allelic to a *molB* mutant that confers resistance to molybdate (Arst 1981). *creC* is tightly linked to *glnA* and *creD* within close proximity to the centromere on chromosome II (Hynes and Kelly 1977).

Strains containing *creB* or *creC* mutations are derepressed for the expression of a number of carbon catabolite repression regulated genes, such as alcohol dehydrogenase I and acetamidase. However these strains grow poorly on media that contains L-proline, D-quinate or D-glucuronate as sole carbon sources. This may

reflect a failure to derepress the genes involved in the utilisation of these substrates (Hynes and Kelly 1977; Kelly and Hynes 1977).

All tested alleles of *creB* and *creC* are recessive to wild type in diploid strains. Generally, *creB* mutations lead to more extreme phenotypes than *creC*. *creB* and *creC* double mutations are not additive. In most growth conditions *creA*204 is epistatic to either *creB* or *creC*, except on media that contains L-proline as the carbon source where *creB* and *creC* are epistatic to *creA*204 (Hynes and Kelly 1977; Kelly and Hynes 1977). It is unlikely that either *creB* or *creC* encodes a DNA binding protein. It is more likely that CreB and CreC affect gene regulation by indirect mechanisms, or possibly by interacting with CreA. No direct link between CreA and either CreB or CreC has been established.

1.2.4 - *creD*

*creD*34 maps to chromosome II, three map units from *creC* on the centromeric side. Strains containing *creD*34 are affected in acetamide utilisation in the presence, but not the absence, of a repressing carbon source. *creD*34 strains are more resistant than wild type to fluoroacetate and fluooacetamide in carbon repressing conditions. The presence of *creD*34 reversed the high sensitivity of *creA*, *creB* and *creC* strains to allyl alcohol in the presence of glucose. *creD*34 is recessive to wild type. The mechanism by which *creD* affects carbon catabolite repression is unknown. *creD* may encode a protein that interacts with CreA (Kelly and Hynes 1977).

1.3 - Systems in *Aspergillus nidulans* which are regulated by carbon source

The regulation of genes which encode the proteins used for the catabolism of various carbon sources is complex. Most of these systems are regulated by pathway specific induction mechanisms and the global regulatory mechanism carbon catabolite repression. The best studied example of a system regulated in this fashion is the ethanol utilisation system. Other compounds, such as acetamide and proline, are able to supply both carbon and nitrogen sources. The regulation of the systems required for the use of these compounds is regulated by pathway specific induction pathways and carbon catabolite repression. They are also regulated by a second global regulatory system nitrogen metabolite repression.

1.3.1 - Regulation of *amdS*

The *amdS* gene encodes acetamidase. Acetamidase is required by the organism for the utilisation of acetamide as a carbon or a nitrogen source. Acetamidase hydrolyses acetamide to acetate, which can be used as a carbon source, and ammonium, which can be used as a nitrogen source. The regulation of *amdS* is controlled by at least seven trans acting factors. Five of them are pathway specific and two are part of global regulatory mechanisms. Specific sites have been identified within the *amdS* promoter region that critically affect the response of pathway specific inducers (figure 1.8).

A CCAAT sequence in the promoter of *amdS* is important in setting the level of *amdS* gene expression. A protein is present in nuclear extracts, AnCF (*A. nidulans* CCAAT binding factor), that binds this sequence. Specific mutations within the CCAAT

sequence, *in vitro* generated deletions, and the promoter mutation *amdI*93 which deletes the CCAAT sequence, greatly reduces the basal levels of expression of *amdS*::*lacZ* fusion reporter systems (Hynes 1980; Littlejohn and Hynes 1992; Richardson *et al.* 1992; Hynes 1994). AnCF may be related to the *S. cerevisiae* HAP complex (Bonnefoy *et al.* 1995).

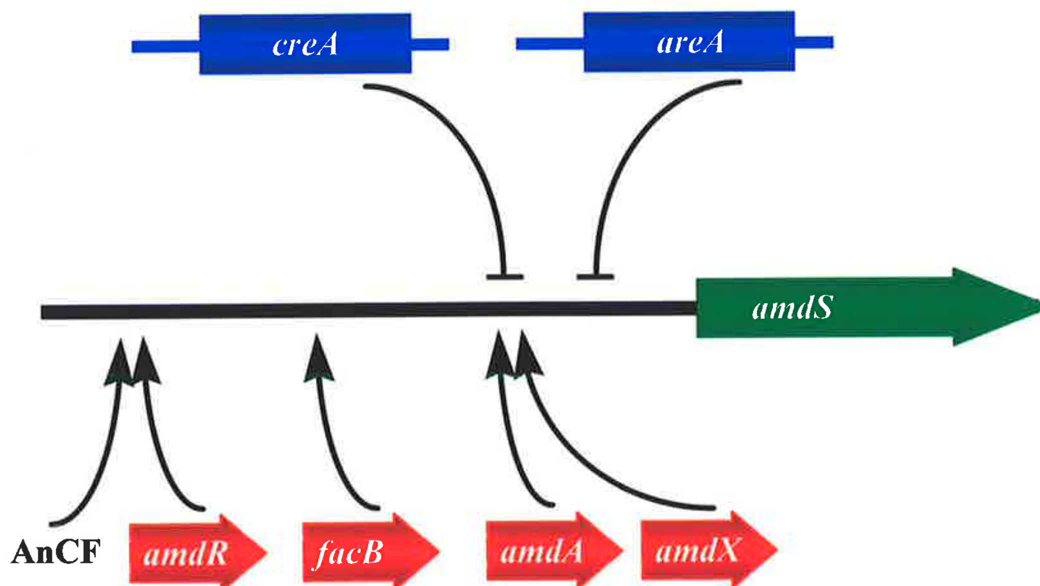


Figure 1.8 - Control of the *amdS* gene.
Genes involved in pathway specific control are shown in red and global regulatory pathways are shown in blue. Pointed arrows represent induction control circuits and closed arrows indicate repression control circuits. Adapted from Davis *et al.* (1993).

amdR is a positive activator of transcription which responds to ω-amino acids such as β-alanine and γ-amino butyric acid. In the presence of ω-amino acids AmdR activates transcription of genes that encode enzymes for ω-amino acid metabolism, some lactams and *amdS* (Arst 1976; Arst *et al.* 1979). A deletion of 30bp approximately 160 bp upstream of the *amdS* transcription start point, *amdI*93, eliminates *amdS* induction by ω-amino acids (Hynes 1978). *amdR* has been cloned. The protein

contains a Cys_6 zinc finger and has been shown to bind within the 30 bp region deleted in *amdI93* (Andrianopoulos and Hynes 1990).

facB is necessary for acetate induction of *amdS* and several other proteins required for the utilisation of acetate as a sole carbon source. A single base change in *amdI9* approximately 200 bp upstream of the *amdS* transcription start point causes an enhancement of *amdS* expression. This enhanced expression is lost in *facB*$^-$ backgrounds (Hynes 1975; Hynes 1977). Titration analysis is consistent with FacB binding to this region of the *amdS* promoter (Kelly and Hynes 1987). *facB* has been cloned and sequenced (Katz and Hynes 1989; Todd *et al.* 1996). *In vitro* binding and footprinting studies have confirmed that FacB binds the region of the *amdS* promoter mutated in the *amdI9* mutation (Todd 1995).

AmdA and AmdX appear to encode transcriptional activators of *amdS*. AmdA may induce *amdS* in response to acetate, independent of FacB. The *amdI66* mutation has a 17 bp insertion 100 bp upstream of the *amdS* start point. This mutation causes a great increase in *amdS* expression which is eliminated in *amdA*$^-$ mutants (Hynes 1982). The *amdA* gene has been cloned. The polypeptide contains two Cys_2-His_2 zinc fingers of a similar type to CreA. The AmdA binding site lies between -75 and -100 bp upstream of the *amdS* start point of transcription (Lints *et al.* 1995). AmdX is a weak positive activator of *amdS*. AmdX acts via an unknown inducer. AmdX also has two Cys_2-His_2 zinc fingers and binds the same site as AmdA (Murphy *et al.* 1997).

areA acts positively to activate transcription of *amdS*, but only on growth media low in ammonium. AreA binds a GATAA sequence (Peters and Caddick 1994). Mutation of a potential GATAA like sequence in the *amdS* promoter significantly reduced

amdS expression in nitrogen limiting conditions. A degree of repression is still observed in these mutations, and in a strain with a deletion of the five prime sequence upstream from base pair -117, suggesting that other sequences are also involved in nitrogen repression (Hynes *et al.* 1988; Davis *et al.* 1993). The requirement for *areA* mediated gene expression is bypassed in carbon derepressing conditions.

creA has a dual role in the regulation of *amdS*. Carbon catabolite repression directly acts on the *amdS* promoter, but in addition one of the regulatory genes, *facB*, is also carbon catabolite repressed. This combination of direct and indirect regulation is not uncommon in global regulatory systems. It is also seen in the regulation of *alcA* in *A. nidulans* (Lockington *et al.* 1987) and *GAL4* in *S. cerevisiae* (Giniger *et al.* 1985). CreA has a Cys_2-His_2 zinc finger DNA binding domain very similar to AmdA and AmdX. All three of these proteins compete for binding sites which overlap. Therefore there may be numerous mechanisms by which CreA controls *amdS* expression. CreA regulates *amdS* expression by controlling expression of the pathway specific regulatory protein *facB*. CreA has the potential to directly regulate *amdS* by two quite different mechanisms. The overlap of binding sites between CreA, AmdA and AmdX may suggest competition for binding site occupancy by these factors. In addition, CreA can repress transcription induced by acetate via FacB and by ω-amino acids via AmdR. This suggest that CreA must form part of an active repression mechanism that can affect transcription induced by regulatory proteins that bind the *amdS* promoter at quite different sites. In nitrogen and carbon repressing conditions both global regulatory systems act to prevent transcription, regardless of the presence of inducers. Lifting either one of these global regulatory systems results in *amdS* transcription.

1.3.2 - Regulation of the *prn* cluster

Proline is catabolised to glutamate and can supply both nitrogen and carbon to the cell. All five genes required for this process are clustered together in the genome (Arst and MacDonald 1975). These genes consist of three structural genes *prnB*, proline permease, *prnC*, L-Δ-pyrroline-5-carboxylase dehydrognase and *prnD*, proline oxidase. *prnA* encodes a pathway specific transcriptional activator which induces the structural genes in the presence of proline (Jones *et al.* 1981). A fifth gene, *prnX*, is also present in the cluster. Its expression is proline regulated but its function is unknown (Gavrias 1993). The spatial arrangement of this cluster is shown in figure 1.9

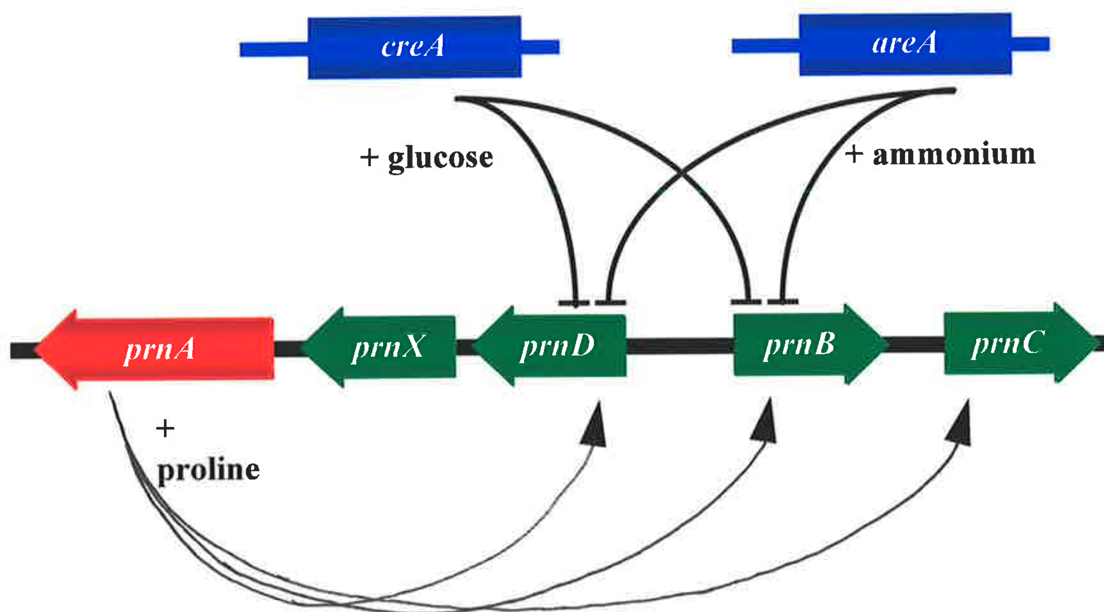


Figure 1.9 - Genetic organisation and control of the proline utilisation system. Genes involved in pathway specific control are shown in red and global regulatory pathways are shown in blue. Pointed arrows represent induction control circuits and closed arrows indicate repression control circuits. Adapted from Scazzocchio *et al.* (1995).

Aside from pathway specific regulation, the *prn* structural genes are also regulated by nitrogen metabolite repression and carbon catabolite repression. The prime target of these global regulatory systems is *prnB* since the permease is the limiting step (Arst and Cove 1973; Arst *et al.* 1980). A number of *prn* derepressed mutants occur in the intergenic region between *prnB* and *prnD* (Sophianopoulou *et al.* 1993). These mutations have been shown to occur in CreA binding sites. In addition to global regulatory mechanisms it has recently been proposed that a second positively acting element, ADA, also regulates *prnB*. *prnB* transcription can be activated either by AreA or by the ADA element. The ADA element's activity or binding would be prevented by CreA in carbon repressing conditions (Gonzalez *et al.* 1997). This system may be analogous to the competition for binding sites model proposed between CreA, AmdA and AmdX in *amdS* regulation.

1.3.3 - Regulation of *alcA*

Alcohol dehydrogenase I, *alcA*, is required for the utilisation of ethanol. *alcA* expression is regulated by the pathway specific positively acting inducer, AlcR, and carbon catabolite repression, figure 1.10 (Pateman *et al.* 1983; Sealy-Lewis and Lockington 1984; Lockington *et al.* 1987). Carbon catabolite repression acts in two ways to repress *alcA* transcription. The presence of glucose completely represses the expression of *alcR* (Lockington *et al.* 1987). Secondly, CreA acts to directly repress *alcA* (Mathieu and Felenbok 1994). CreA binding sites and AlcR binding sites have been characterised in both the *alcA* and *alcR* promoters. Some CreA binding sites are very near to or overlapping AlcR binding sites (Kulmberg *et al.* 1992; Kulmberg *et al.* 1993). This has resulted in a double lock competition model being proposed for the

control of *alcA* regulation. The double lock mechanism refers to carbon catabolite repression acting on both the regulatory and structural genes. It is proposed that CreA acts by competing with AlcR for binding sites (Scazzocchio *et al*. 1995).

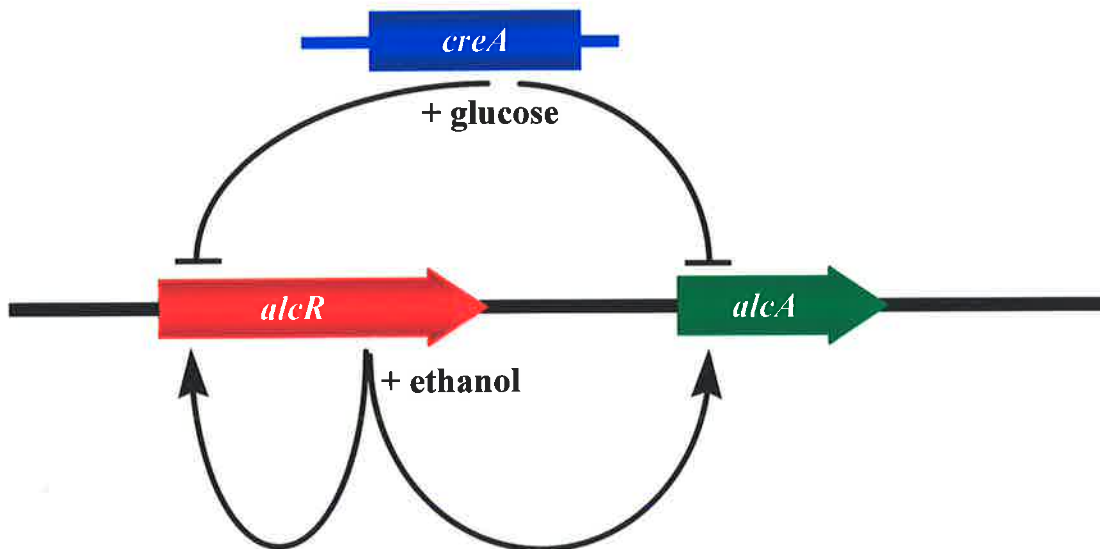


Figure 1.10 - Genetic organisation and control of the ethanol utilisation system. Genes involved in pathway specific control are shown in red and global regulatory pathways are shown in blue. Pointed arrows represent positive control circuits and closed arrows indicate negative control circuits. Adapted from Fillinger and Felenbok (1996).

1.3.4 - Regulation of *ipnA*

In *A. nidulans* the process of penicillin biosynthesis is regulated by carbon source, the level produced is low in the presence of glucose. IpnA, isopenicillin-N-synthase, is regulated at the level of transcription and is regulated in response to the carbon source present (Espeso and Penalva 1992). Although *ipnA* is regulated by carbon source, none of the *creA* mutants tested result in full derepression in glucose media (Espeso and Penalva 1992). *In vitro* CreA binding sites have been mapped in the *ipnA*

promoter. A precise deletion of this CreA binding site has no affect on the transcription levels of an *ipnA* reporter gene, suggesting that these sites are not functional *in vivo*. Loss of function mutants in *creB* or *creC* elevate the levels of *ipnA* transcription. However it has been shown that this is due to the indirect affects on pH and carbon source levels in the growth media rather than a direct effect on *ipnA* transcription (Espeso *et al.* 1995). These results strongly suggest that the presence of a second form of carbon catabolite repression in *A. nidulans* which is independent of the *cre* genes previously identified.

1.4 - Cys_2-His_2 zinc finger DNA binding domain

The zinc finger motif was initially inferred from an analysis of the amino acid sequence of the transcription factor TF111A from *Xenopus laevis*. This factor was isolated from the oocytes and acts to regulate transcription of ribosomal 5S RNA (Miller *et al.* 1985). The 344 amino acid polypeptide chain of TF111A contains nine repeated sequences of approximately thirty residues each. These repeated sequences are not identical. However, each contains two cysteine residues at the amino terminal end and two histidine residues at the carboxyl end. The second of the two cysteine residues and the first histidine residue of each repeat are separated by twelve residues. These twelve residues contain two invariant hydrophobic side chains. The transcriptional activity of the protein is dependent on the presence of zinc ions. The cysteine and histidine residues coordinate a zinc atom. The loop that forms between the second cysteine and first histidine residues forms the DNA binding region. Each of the nine repeats in TF111A is therefore termed a zinc finger.

This class of Cys_2-His_2 zinc fingers has been identified in over 200 proteins. Comparisons of these proteins has resulted in Cys_2-His_2 zinc fingers being defined by the following consensus sequence X_3-Cys-$X_{2\text{-}4}$-Cys-X_{12}-His-$X_{3\text{-}4}$-His-X_4 where X is any amino acid proteins (Blumberg *et al.* 1987). The zinc finger is a motif that is repeated in tandem to recognise DNA sequences of different lengths. Amongst the proteins with Cys_2-His_2 zinc fingers that have been identified the number of zinc fingers varies from a single zinc finger up to 34. CreA has two zinc fingers.

Although each zinc finger is different they are based on a similar framework and each interacts with a small number of base pairs (2 or 3 bp). The strength of the interaction can be varied by changes in the sequence of both the protein and the DNA recognition sequence. These changes allow for a high level of specificity in recognition, and this modular design offers a large number of combinatorial possibilities for specific recognition of DNA. There is a large amount of variation in the DNA recognition sequences bound by these proteins. Some protein such as hunchback, from *D. melanogaster*, bind an A/T rich recognition sequence (Zuo *et al.* 1991). Alternatively, zinc fingers that utilise arginine residues for base recognition tend to bind G/C rich recognition sequences, CreA falls into this class (Pavletich and Pabo 1991). Other proteins that have Cys_2-His_2 zinc fingers of the same type as CreA are Adr1p from *S. cerevisiae* (Bemis and Denis 1988); Kruppel (Stanojevic *et al.* 1989) from *D. melanogaster* ; the mammalian transcription factor Sp1 (Gidoni *et al.* 1984); the Wilm's tumour protein WT1 (Call *et al.* 1990) and the mouse early growth response factor Zif268 (Christy *et al.* 1988).

1.4.1 - X-ray crystallographic analysis of Zif268

To understand how zinc finger domains achieve site specific DNA recognition, X-ray crystallographic analysis has been used on three different proteins that contain Cys_2-His_2 zinc fingers. One of the proteins that has been studied by X-ray crystallography is Zif268 (Pavletich and Pabo 1991; Elrod-Erickson *et al.* 1996). Zif268 has three zinc fingers. The first and third of these zinc fingers have a very high degree of similarity to CreA. The three zinc fingers are arranged in a semicircular structure that fits into the major groove of the DNA helix in an antiparallel fashion. Each finger consists of an antiparallel β-sheet and an α-helix, which are held together by a zinc ion and a number of hydrophobic residues. The two cysteine residues are found within the β-sheet and the two histidines are found within the α-helix (Pavletich and Pabo 1991). The α-helix of each finger sits directly within the DNA helix major groove. Residues from the N-terminal portion of each finger make their primary contacts with three base pairs within the DNA recognition sequence, accounting for the nine base pair recognition sequence that Zif268 binds. The β-sheet is on the back of the helix away from the nucleotide pairs. The first β-strand does not make any contacts with the DNA, and the second β-strand contacts the sugar phosphate backbone along one strand of the DNA. Most of the contacts with the DNA are made with the G-rich strand.

1.4.2 - Mutations within the DNA binding domain of WT1

Wilm's tumour is a malignancy of the kidneys. A tumour suppressor gene, *WT1*, has been isolated and has been shown to be associated with this disease (Call *et al.* 1990; Gessler *et al.* 1990). WT1 is expressed during early development within the kidneys, gonads, spleen and lining of the intestine (Pritchard-Jones *et al.* 1990). The WT1

polypeptide has a number of features consistent with it acting as a transcriptional regulator, including four Cys_2-His_2 zinc fingers in the C-terminal region of the protein. These zinc fingers of the same type as those found in CreA. Numerous mutations within *WT1* have been characterised, including many point mutations within the DNA binding domain (table 1.1).

Mutations in *WT1* have been associated with WAGR syndrome and Denys-Drash syndrome (DDS). The zinc finger contains 92% of *WT1* mutations in DDS patients indicating a strong bias between the zinc finger mutations and classical DDS. In all cases of DDS the mutations result in reduced affinity for DNA binding and in most cases are predicted to result in the total inactivation of DNA binding. Many DDS mutations have an apparent dominant negative affect. This is thought to be due to the involvement of the remaining N-terminal portion of the protein in WT1 homodimerisation, without being able to participate in transcriptional regulation. Thus association between wild type and DDS mutant proteins may remove the wild type protein from the DNA (Englert *et al.* 1995).

Table 1.1- Point mutations within the DNA binding domain of WT1. The number of examples each mutation is in parentheses.

Mutation	Number Recorded	Region of Zinc Finger	Clinical Effect[1]
C330Y	1	zinc coordinating residue	DDS
C355Y	1	zinc coordinating residue	DDS
C360G	1	zinc coordinating residue	DDS
C360Y	1	zinc coordinating residue	DDS

R362stop	5	β-sheet of second zinc finger	DDS (3); WT (1); other tumour (1)
R366H	3	DNA interacting residue	DDS
R366C	1	DNA interacting residue	WT
H373Q	1	zinc coordinating residue	DDS
H373Y	1	zinc coordinating residue	WT
H377R	1	zinc coordinating residue	DDS
C385Y	1	zinc coordinating residue	other tumour
R390X	3	DNA interacting residue	WT
R394W	21	DNA interacting residue	DDS (19); WT (1); other tumour (1)
R394P	3	DNA interacting residue	DDS (1); WT1 (1); other tumour (1)
D396N	5	recognition helix, structural role	DDS
D396G	1	recognition helix, structural role	DDS
L398P	1	conserved hydrophobic residue	DDS
H401Y	1	zinc coordinating residue	DDS

Note - (1) DDS - Denys-Drash Syndrome; WT - Wilm's Tumour

(2) Data collected from the following references. Akasaka *et al.* (1993); Baird *et al.* (1992a); Baird *et al.* (1992b); Baird and Cowell (1993); Bruening *et al.* (1992); Call *et al.* (1990); Clarkson *et al.* (1993); Coppes *et al.* (1992); Coppes *et al.* (1993); Devriendt *et al.* (1995); Gessler *et al.* (1994); Kikuchi e*t al.* (1995); King-Underwood *et al.* (1996); Kosters *et al.* (1995); Little *et al.* (1992); Little *et al.* (1993); Nordenskjold *et al.* (1995); Pelletier *et al.* (1991); Poulat *et al.* (1993); Pritchard-Jones *et al.* (1994); Quek *et al.* (1993); Sakai *et al.* (1993); Tsuda *et al.*(1993); Varanasi *et al.* (1994); Webb *et al.* (1995).

1.5 - Aims and Objectives

creA encodes a protein which is a negative repressor of transcription. It is central to the process of carbon catabolite repression in *A. nidulans*. CreA acts to repress all carbon catabolite repression regulated genes in the presence of a repressing carbon sources such as glucose. Cloning of *creA* from *A. nidulans* and *A. niger* resulted in the identification of a number of potential functional regions within the protein. These regions include a potential DNA binding domain and a region with a high degree of

similarity to the Rgr1p protein from *S. cerevisiae*. At the initiation of this study there was no evidence to show that either of these regions, or any other regions within the protein, were functional. Subsequent work has defined both *in vitro* and *in vivo* CreA binding sites.

The objective of this study was to determine the role of CreA in the process of carbon catabolite repression. The main approach used to meet this objective was the analyses of *creA*$^-$ mutant alleles. This involved the phenotypic analysis of strains containing each of these alleles and determining the nature of each mutation at the molecular level. Functional regions within CreA were defined based on correlations between the site and nature of each mutation within the gene and the resulting phenotype from that mutation. The second approach was to initiate a screen for mutations which suppressed *creA*$^-$ mutant alleles. The aim of this approach was to identify genes that encode proteins that interact with CreA, or act in the same pathway as CreA.

Chapter 2

Materials and Methods

2.1 - Materials

General Reagents: General chemicals and growth media were of laboratory grade.

Enzymes: Restriction enzymes were purchased from Boehringer-Mannheim, New England Biolabs, Progen Industries and Promega Corporation. DNA ligase was purchased from Boehringer-Mannheim. Taq polymerase and the Klenow fragment of *E. coli* DNA polymerase I was purchased from Bresatec Pty. Ltd. Novozyme was purchased from InterSpex. All enzymes were used following the manufacturers instructions.

Molecular Weight Markers: All DNA molecular weight markers were purchased from Bresatec Pty. Ltd. or Progen Industries.

Nucleotides: Nucleotide stocks were purchased from either Boehringer-Mannheim or Pharmacia.

Isotopes: α-^{32}P-dATP was purchased from Amersham and Bresatec Pty. Ltd.

2.1.1 - Buffers

10xLoad Buffer for agarose gels: 0.25% bromophenol blue, 0.25% xylene cyanol FF, 25% ficoll (type 400)

10 x Oligolabelling buffer: 0.5 M Tris-HCl pH 6.9, 0.1 M $MgSO_4$, 1mM DTT, and 0.6mM each of dCTP, dGTP, and dTTP.

10 x Stop Buffer: 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 2% SDS

2.1.2 - Solutions

1 x SSC: 0.15 M NaCl, 0.015 M $Na_3C_6H_5O_7.2H_2O$, pH 7.2

1 x SSPE: 0.18 M NaCl, 10 mM NaH2PO4, 1 mM EDTA, pH7.4

1 x TAE: 40 mM Tris base, 20 mM NaAc, 2 mM EDTA, pH 7.8 with glacial acetic acid

10 x TBE: 1 M Tris base, 0.9 M H3BO4, 0.2 M EDTA, pH 8.3

1 x TE: 10 mM Tris-HCl pH 8.0, 1 mM EDTA

Aspergillus trace element solution: (per litre): 40 mg $Na_2B_4O_7$, 400 mg $CuSO_4$, 1 g $FePO_4$, 600 mg $MnSO_4.H_2O$, 800 mg $Na_2MoO_4.2H_2O$, 8 g $ZnSO_4.7H_2O$ and 2ml $CHCl_3$ as a preservative.

Aspergillus vitamin solution: (per litre): 40 mg *p*-aminobenzoic acid, 50 mg thiamine HCl, 1 mg D-biotin, 400 mg inositol, 100 mg nicotinic acid, 200 mg calcium D-pantothenate, 100 mg riboflavin, 50 mg pyridoxine and 2ml $CHCl_3$ as a preservative.

Aspergillus salt solution: (per litre): 26 g KCl, 26 g MgSO4.7H2O, 76 g KH2PO4, 50 ml *Aspergillus* trace element solution and 2ml as a preservative. The pH of this solution should be 6.5.

2.1.3 - Growth Media

Bacterial and Phage Media,

L-Broth: 1% NaCl, 0.5% yeast extract, 1% tryptone, pH 7.2. L-agar plates were solidified with 1.5% class I agar. When required Ampicillin was added to 100µg/ml.

TB Broth: 1% tryptone, 0.5% NaCl. TB-agar plates were solidified with 1.5% class I agar and top agar plates were solidified with 0.7% class I agar.

Aspergillus growth media,

Carbon free medium: 2% *Aspergillus* salt solution, pH 6.5. Plates were solidified with either 1% or 2.2% class I agar.

Complete medium: 1% D-glucose, 0.2% peptone, 0.15% casein hydrolysate, 0.1% yeast extract, 10 mM ammonium tartrate, 2% *Aspergillus* salt solution, 1% *Aspergillus* vitamin solution, 25 µg/ml riboflavin, pH 6.5. Plates were solidified with either 1% or 2.2% class I agar.

Protoplast medium: 1 M sucrose, 1% D-glucose, 2% *Aspergillus* salt solution, pH 7.0 and solidified with 1% class I agar.

Unless otherwise stated all media had a carbon source added to a final level of 1% or 50 mM and a nitrogen source was added to a final level of 10 mM.

Supplements: where required growth supplements were added to a final concentration of,

D-biotin	0.01 µg/ml
nicotinic acid	1.0 µg/ml
pyridoxine sulphate	0.5 µg/ml
p-aminobenzoic acid	50 µg/ml
riboflavin	2.5 µg/ml
sodium thiosulphate	0.1 %

2.1.4 - *Aspergillus nidulans* Strains

The strains of *A. nidulans* used in this study are presented in table 2.1.

Table 2.1 - *A. nidulans* strains used in this study.

Strain	Genotype	Reference
MH2	*biA*1, *niiA*4	Hynes (1975)
Wild type	*yA*1, *riboB*2, *adE*20, *suA*1*adE*20	Shroff *et al.* (1996)
H17A12	*yA*1, *riboB*2, *adE*20, *suA*1*adE*20, *areA*217	Hynes (1975)
1070X	*yA*1, *riboB*2, *adE*20, *suA*1*adE*20, *creA*1	Arst and Cove (1973)
1070A	*yA*1, *riboB*2, *adE*20, *suA*1*adE*20, *areA*217,*creA*1	Shroff *et al.* (1996)
SA4 X	*yA*1, *riboB*2, *adE*20, *suA*1*adE*20, *creA*204	Shroff *et al.* (1996)
SA4	*yA*1, *riboB*2, *adE*20, *suA*1*adE*20, *areA*217,*creA*204	Hynes and Kelly (1977)
SA18 X	*yA*1, *riboB*2, *adE*20, *suA*1*adE*20, *creA*218	Shroff *et al.* (1996)
SA18	*yA*1, *riboB*2, *adE*20, *suA*1*adE*20, *areA*217, *creA*218	Shroff *et al.* (1996)
30	*biA*1, *niA*4, *creA*30	Arst *et al.* (1990)
30A	*biA*1, *riboB*2, *areA*217, *creA*30	Shroff *et al.* (1996)
H17CR1	*yA*1, *riboB*2, *adE*20, *suA*1*adE*20, *areA*217, *creA*301	Shroff *et al.* (1997)
H17CR2	*yA*1, *riboB*2, *adE*20, *suA*1*adE*20, *areA*217, *creA*302	Shroff *et al.* (1997)
H17CR3	*yA*1, *riboB*2, *adE*20, *suA*1*adE*20, *areA*217, *creA*303	Shroff *et al.* (1997)
H17CR3X	*yA*1, *riboB*2, *adE*20, *suA*1*adE*20, *creA*303	Shroff *et al.* (1997)
H17CR4	*yA*1, *riboB*2, *adE*20, *suA*1*adE*20, *areA*217, *creA*304	Shroff *et al.* (1997)
H17CR4X	*yA*1, *riboB*2, *adE*20, *suA*1*adE*20, *creA*304	Shroff *et al.* (1997)
H17CR5	*yA*1, *riboB*2, *adE*20, *suA*1*adE*20, *areA*217, *creA*305	Shroff *et al.* (1997)
H17CR6	*yA*1, *riboB*2, *adE*20, *suA*1*adE*20, *areA*217, *creA*306	Shroff *et al.* (1997)
H17CR6X	*yA*1, *riboB*2, *adE*20, *suA*1*adE*20, *creA*306	Shroff *et al.* (1997)
H17CR7	*yA*1, *riboB*2, *adE*20, *suA*1*adE*20, *areA*217, *creA*307	Shroff *et al.* (1997)

MJH1918	*yA*1, *riboB*2, *adE*20, *suA*1*adE*20, *areA*217, *creA*311	Shroff *et al.* (1997)
MJH1923	*yA*1, *riboB*2, *adE*20, *suA*1*adE*20, *areA*217, *creA*322	Shroff *et al.* (1997)
MJH1923X	*yA*1, *riboB*2, *adE*20, *suA*1*adE*20, *creA*322	Shroff *et al.* (1997)
MJH1926	*yA*1, *riboB*2, *adE*20, *suA*1*adE*20, *areA*217, *creA*331	Shroff *et al.* (1997)
MJH1928	*yA*1, *riboB*2, *adE*20, *suA*1*adE*20, *areA*217, *creA*334	Shroff *et al.* (1997)
*creA*Δ4	*yA*2, *paba*A1, *areA*217, *creA*Δ4	Shroff *et al.* (1997)
*creA*Δ21	*yA*2, *paba*A1, *areA*217, *creA*Δ21	Shroff *et al.* (1997)
*creA*Δ99	*yA*2, *paba*A1, *areA*217, *creA*Δ99	Shroff *et al.* (1997)
*creA*Δ4X	*yA*2, *pabaA*1, *riboB*2, *creA*Δ4	S. M. O'Connor unpublished
MJH532	*biA*1, *creB15*	Kelly and Hynes (1977)
MJH747	*biA*1, *creC*27, *niiA*4	Kelly and Hynes (1977)
A461	*biA*1, *proA*1, *phenA*2, *malA*1, *riboB*2	FGSC
MSF	*yA*1, *adE*20, *suA*1*adE*20, *AcrA*1, *galE*1, *pyroA*4, *facA*303, *sB*3, *nicB*8, *riboB*2	Kafer 1961
MSF-RL	*biA*1, *AcrA*1, *galE*1, *pyroA*4, *facA*303, *sB*3, *nicB*8, *niiA*4	R. A. Lockington unpublished

Note - (1) Gene symbols are described in Clutterbuck (1993).
(2) FCSC - Fungal Genetics Stock Center
(3) all strains are *veA*1 mutants

2.1.5 - *Escherichia coli* Strains

The strains of *E. coli* used in this study are presented in table 2.2.

Table 2.2 - Strains of *E. coli* used in this study.

Strain	Genotype	Reference
DH5α	F⁻, φ80d *lacZ*ΔM15, *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17, (r_K^-, m_K^+), *supE*44, *relA*1, *deoR*, Δ(*lacZYA-argF*)U169	Hanahan (1983)
JM101	*supE, thi*Δ*(lac-proAB)*, F'[*traD*36, *proAB*$^+$, *lacI*9^q, *lacZ*M15]	Messing (1979)

2.1.6 - Oligonucleotides

The oligonucleotides used in this study are presented in table 2.3.

Table 2.3 - Oligonucleotides used in this study.

Oligonucleotide	Nucleotide Sequence
323	5' - ACAGACTGGCTCCTGAAG - 3'
349	5' - TTGCTGGGAGGGGGCATC - 3'
350	5' - GCAACCAGGATCGTCAGT - 3'
576	5' - TCCCCAAGCAGCAGGCGATCTGGAT - 3'
590	5' - CAAGACTTGTGTGTGAAGGCAAGG - 3'
724	5' - GCGTCATCAGGCATGGATAACGAGATGCA - 3'
725	5' - CTCAGGGCGAGACATGATTGCCTGGGT - 3'
863	5' - GCTCGAATCTGAGCCCCTAC - 3'
864	5' - TTCGCTGTGCACCCTCAG - 3'
S2	5' - CCAACTCGACTGCTCCTT - 3'
S3	5' - GGAGTCGTGGGAGAAGGT - 3'

2.1.7 - Plasmids

The plasmids used in this study are presented in table 2.4

Table 2.4 - Plasmids used in this study.

Plasmid	**Insert**	**Reference**
pANC4	2.3 kb *BamHI-XbaI* genomic clone of *creA*$^+$ in pUC19.	Dowzer and Kelly (1989)
pPL3	*riboB*$^+$	Oakley *et al.* 1987
pANC4RGR1a	473 bp *EcoRV* deletion in pANC4 and in frame fusion of Rgr1p similar region from *S. cerevisiae*	this study
pANC4ΔRV10	473 bp in frame *EcoRV* deletion in pANC4	this study

2.2 - Methods

2.2.3 - Manipulation of *A. nidulans*

Growth testing and meiotic analyses were performed using the methods described by Cove (1966). Haploidisation of diploid strains was done by the method of Hastie (1970) using 1 µl of 0.075% benlate per ml of media. Transformation experiments were carried out using the method of Tilburn *et al.* (1983).

2.2.2- Nucleic Acid Analysis

General molecular biology protocols used are outlined in Sambrook *et al.* (1993). DNA probes were radioactively labelled using the random oligonucleotide primer method described by Hodgson and Fisk (1987). DNA hybridisations were carried out using the conditions described by Church and Gilbert (1984).

2.2.3- DNA Sequencing

PCR products were purified using the Wizard PCR purification system (Promega) before being directly sequenced. DNA sequencing was carried out using the *f*mol sequencing kit (Promega) or by using the dye terminator kit (Applied Biosystems-Perkin Elmer). Dye terminator reactions were electrophoresed on an ABI373 or ABI 377 automated sequencing machine at the Institute of Medical and Veterinary Science sequencing facility.

2.2.4- Polymerase chain reaction (PCR)

PCR amplification of 100ng of genomic DNA were performed in a final reaction volume of 25 μl. Annealing temperatures ranged between 55°C and 60°C. When the samples were to be used for SSCP analysis the reaction mixture had 1 μCi of α-^{32}PdATP added. Unless otherwise stated the standard cycling conditions used were, 1 min. @ 95°C; 0.5 min. @ 55°C; 1 min. @ 72°C.

2.2.5- Single stranded conformation polymorphism (SSCP)

PCR products of 500 bp or less were analysed directly using the SSCP technique. Larger PCR products were digested with appropriate restriction enzymes before being

used in an SSCP analysis. The SSCP protocol used was a modified from Peletier *et al.* (1991). Denatured products were separated on a 6% polyacrylamide-bis-acrylamide (29:1) gel containing 5% glycerol and electrophoresed at 3W for 18 h.

2.2.6- Alcohol dehydrogenase (ADH, EC 1.1.1.1) enzyme assays

Culture media (200ml) were inoculated with $2x10^8$ conidia and incubated as described below. Mycelia were harvested and washed, patted dry, and ground with sand in 1 ml per 100 g of wet weight mycelia in a solution of 100 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$ and 1mM EDTA. The sand and debris were pelleted by centrifugation and the supernatant was assayed for ADH activity as in Creaser *et al.* (1985). The growth conditions used were;

Repressing conditions: 22 h at 25°C in media containing 1% D-glucose(w/v).

Nonrepressing conditions: 22h at 25°C in media containing 1% D-fructose(v/v).

Repressing, induced conditions: 27h at 25°C in media containing 0.1% D-glucose and 1% ethanol(v/v).

Nonrepressing, induced conditions: 27 h at 25°C in media containing 1% D-fructose and 1% ethanol(v/v).

Chapter 3

Phenotypic Analysis of *creA* Mutants

A large bank of *creA* mutants has been selected using numerous screens. The majority of alleles used in this study were selected by suppression of an *areA* loss of function allele. AreA is a positively acting regulatory protein that activates genes under the control of nitrogen metabolite repression. *areA* loss of function alleles, such as *areA*217, grow poorly on D-glucose media containing nitrogen sources other than ammonium. However on media that only contains compounds that can be used as both nitrogen and carbon sources, such as acetamide and proline, *areA* loss of function strains are able to grow. These strains are able to grow because lifting carbon repression allows expression of the genes which code for proteins required to utilise these compounds. Mutations that mimic carbon catabolite derepressed conditions can be selected as suppressors of an *areA* loss of function strain on media that contains D-glucose and a nitrogen source that can also act as a carbon source. *creA*1 was selected as a suppressor of the *areA*1 loss of function allele on media containing D-glucose and proline (Arst and Cove, 1973). Except for the two alleles outlined below, all other alleles were selected as suppressors of the *areA*217 loss of function allele on media containing sucrose and acetamide (Hynes and Kelly 1977; Shroff *et al.* 1996; Shroff *et al.* 1997). The two exceptions to this are *creA*30 and *creA*401. *CreA*30 was selected as a suppressor of *frA*1 (Arst *et al.* 1990). The *creA*401 allele was selected in a screen for suppressors of the requirement for acetate by a *pyc*A (pyruvate carboxylase) mutation on complete medium. The mutation must allow deregulation of endogenous acetate production (Shroff *et al.* 1997).

Previous analyses of *creA* mutants have demonstrated that strains containing *creA* alleles have very heterogenous phenotypes (Bailey and Arst 1975; Arst and Bailey

1977). Therefore the *creA* mutants used in this study were phenotype tested to determine their range of phenotypes.

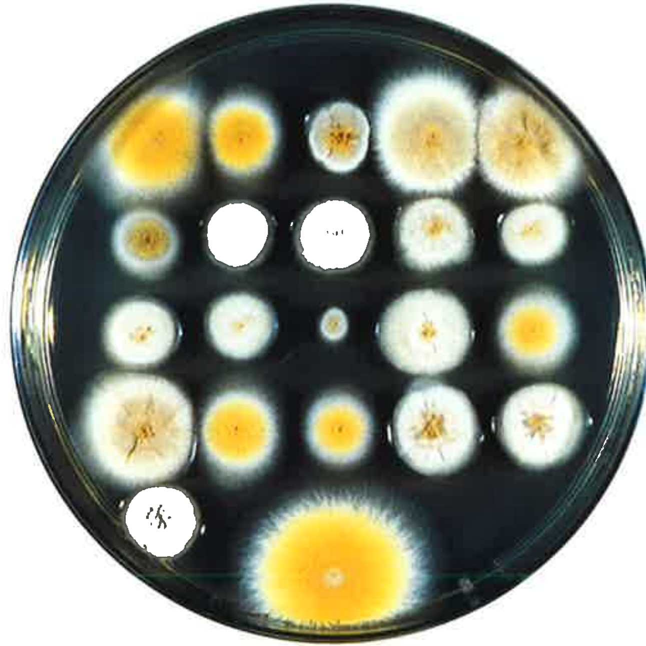
3.1 - Morphology and life cycle of strains containing *creA* mutant alleles

3.1.1 - Morphology on solid media

Morphology on solid media is defined by the rate of growth and the degree of conidiation of a colony. Strains containing the *creA* alleles had a reduced growth rate when compared to wild type strains. The decreased growth rate observed by a strain that contained one of the *creA* mutant alleles is evident on all carbon repressing media. For consistency and comparability the range of altered growth rates has always been scored on complete medium (figure 3.1and table 3.1). Complete medium is a nutrient rich complex growth medium that contains glucose, peptone, casein hydrolysate, yeast extract, ammonium and a full range of salts, trace elements and vitamins. Strains containing *creA*306 had the slowest growth rate of all of the *creA* mutant alleles. *creA*306 containing strains form very small compact colonies, considerably smaller than wild type colonies. Severe effects on growth rate were also observed in strains containing the following alleles (in decreasing order of severity) *creA*303, -304, -305, -225, -30, -302 and -218. While these alleles lead to the greatest decrease in growth rate, all alleles had some effect resulting in growth rates slower than wild type.

Figure 3.1 - Growth rate and conidiation of strains grown on 1% complete medium

The strains were grown at 37°C for 48 hours on 1% complete media. The genotype of each strain is *yA*1, *adE*20, *su*(*adE*20)*A*1, *riboB*2, *areA*217. The genotype of each strain with regard to the *creA* locus is given in the key below the plate. The growth rate and degree of conidiation was estimated for each colony and is summarised in table 3.1.



creA1 *creA204* *creA218* *creA220* *creA221*

creA225 *creA30* *creA301* *creA302* *creA303*

creA304 *creA305* *creA306* *creA307* *creA311*

creA322 *creA331* *creA334* *creAΔ4* *creAΔ21*

creAΔ99

creA$^+$

The fact that all alleles resulted in a decreased growth rates may indicate a link between the carbon status of the cell and the cell cycle. There may be a direct relationship between carbon catabolite repression and the regulation of the rate of the cell cycle. However, a more likely explanation is that the decreased growth rate is a more general effect caused by the derepression of some or all carbon repressible systems. An energy deficit will result from this. More energy is used transcribing and translating these derepressed genes than is gained from the catabolism of any carbon source present in the growth medium. This idea is supported by the correlation between alleles that result in the highest levels of expression of CreA regulated genes in the presence of glucose and those that have the slowest rates of growth.

In addition to reducing growth rates, strains containing a number of the mutant alleles also resulted in significantly reduced levels of conidiation on complete medium. Strains containing the following alleles had the most reduced levels of conidiation (in decreasing order of severity) *creA*303, -304, -305, -30, -306, -225, -218, -301, -302 and -307 (figure 3.1 and table 3.1). Strains containing alleles that had the most severe effect on conidiation had a characteristic morphology. They produced a small region of conidiation in the center of the growing colony (figure 3.1). The decreased conidiation phenotype is only clearly observed on complete medium. The phenotype is very difficult to score on minimal medium and is not observed on medium that contains derepressing carbon sources. Strains containing *creA*1, -204, -311, -331 and -334 formed colonies that have a reduced growth rate but very little, if any, effect on the ability of the colony to conidiate. This physical effect is limited to the amount of

conidia produced by a colony of a certain size and does not appear to have any effect on the timing of conidiation.

Table 3.1 - Measurement of growth rate and degree of conidiation of strains containing *creA* mutant alleles.

Strain [1]	Size [2]	Conidiation [2]
creA$^+$	+++++	+++++
*creA*1	++++	+++++
*creA*30	+++	++
*creA*204	+++	+++++
*creA*218	+++	+++
*creA*220	++++	++
*creA*221	++++	++
*creA*225	++	++++
*creA*301	+++	++
*creA*302	+++	++
*creA*303	+++	++
*creA*304	+++	++
*creA*305	+++	++
*creA*306	+	+
*creA*307	++++	++
*creA*311	+++	++++
*creA*322	++++	++
*creA*331	+++	+++++
*creA*334	+++	++++

Note: (1) all strains are in an *areA*217 background.
(2) denotes increasing rate of growth or degree of conidiation -; +; ++; +++; ++++; +++++.
(3) all strains grown on 1% complete medium.

Decreased conidiation is an allele specific phenotype for *creA*, indicating that a specific defect within the protein is necessary to affect the regulation of the conidiation process. It is therefore highly likely that *creA* is directly involved in the regulation of conidiation in *A. nidulans*. The mechanism through which CreA may regulate this pathway is unknown. In addition there have been few studies

investigating the relationship between CreA and the regulation the genes that control the conidiation process. The effect of *creA* mutations on the regulation of these genes has not been directly tested. Alternatively, the decreased conidiation may be an indirect effect of *creA* mutations altering the internal carbon homeostatic controls that may be involved in signalling to the conidiation regulatory system.

3.1.2 - Morphology in liquid media

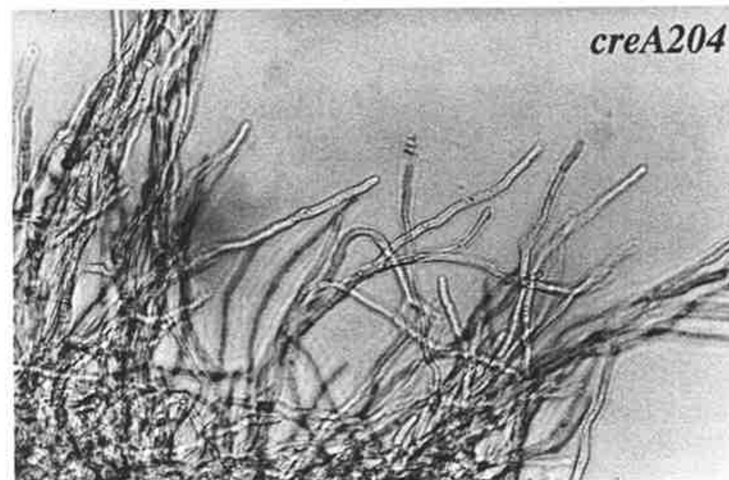
Strains that contained particular *creA* mutant alleles had a very characteristic morphology when grown in liquid media. To characterise this trait a selection of *creA* alleles was studied. When grown in a liquid medium rich in carbon sources, containing 1% D-glucose and 1% ethanol, strains containing *creA*30, -218, -220 and -221 grew with the mycelial mass being much more diffuse and not forming tight clumps as seen in wild type. When observed by high powered light microscopy, small dense bodies were observed within the mycelia (figure 3.2). The number of dense bodies observed was quite variable between the different alleles but directly correlated with the degree of diffuse mycelial growth observed.

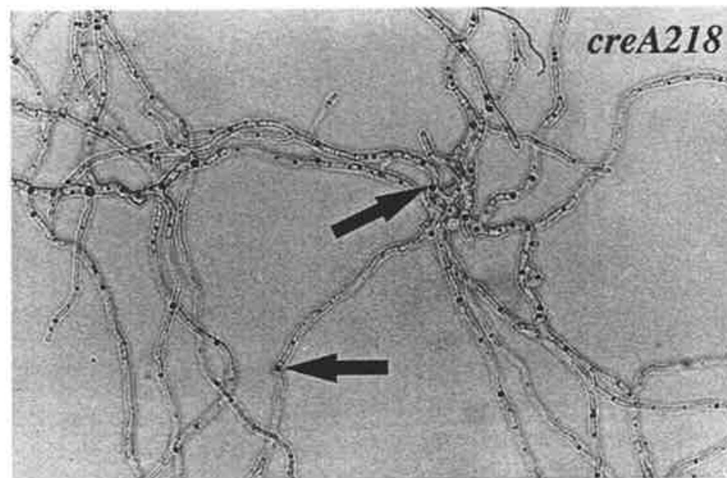
A strain containing *creA*218 showed the most severe effect. This strain resulted in no clumping of mycelia and had the highest observed occurrence of dense bodies within the mycelia (figure 3.2 and figure 3.3). In decreasing order of severity, strains containing *creA*221, -303, -304, -30, -305 and -220 also demonstrated this phenotype. This growth characteristic was not seen in strains containing *creA*1, -204 and -225 which had a morphology similar to wild type in carbon rich liquid growth media.

Figure 3.2 - Microscopic examination (400X) of mycelial samples grown in carbon rich liquid medium

Strains containing the *creA* alleles were grown for 21 hours at 37°C on a shaking table in liquid media containing 1% D-glucose and 1% ethanol as the carbon source and 10 mM ammonium tartrate as the nitrogen source. Samples were examined by light microscopy at a magnification of 400X. Examples of three *creA* alleles are shown in comparison to a wild type strain. There are no carbohydrate storage bodies visible in the wild type or *creA*204 strains. Many carbohydrate storage bodies are visible in the samples of *creA*218 and *creA*221 strains. They appear as small dark bodies (examples are marked with black arrows) within the mycelia.

wild type


creA204


creA218

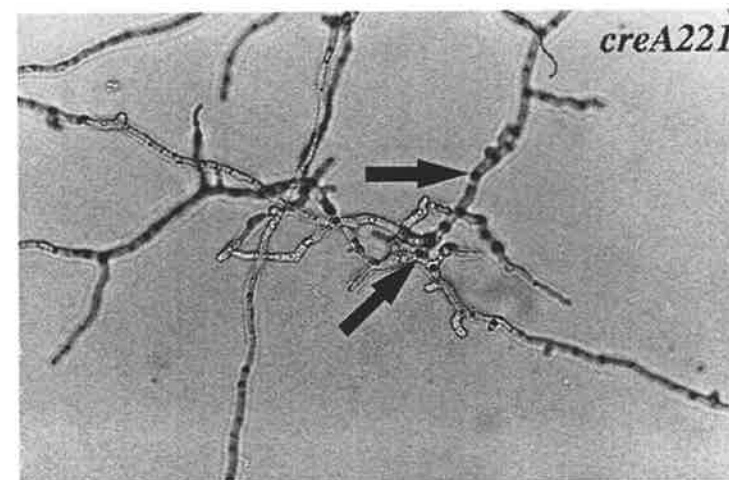
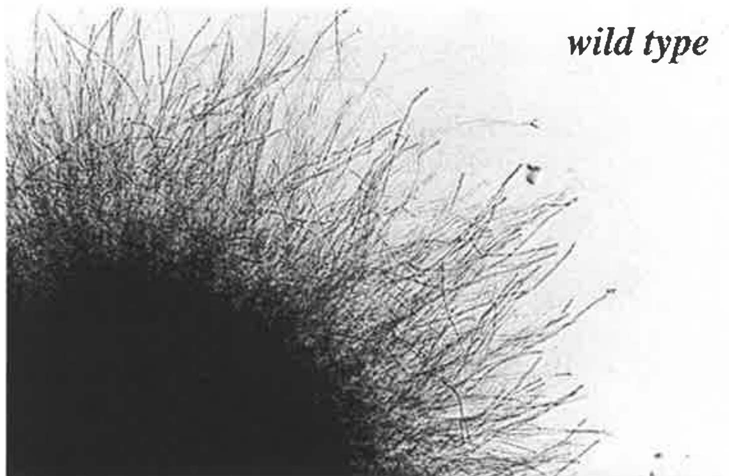
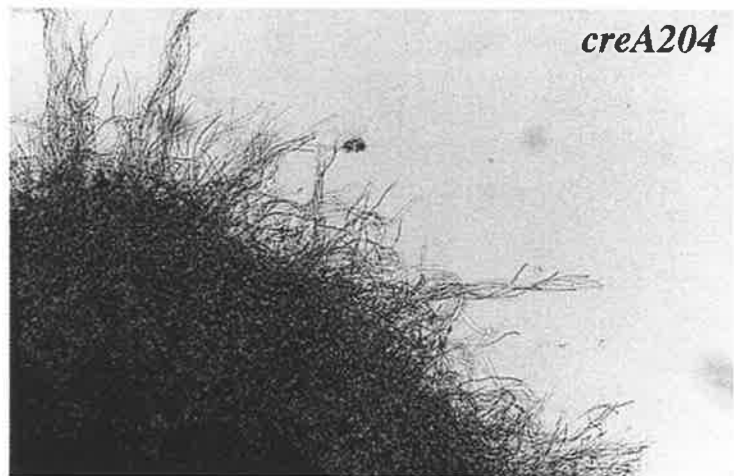
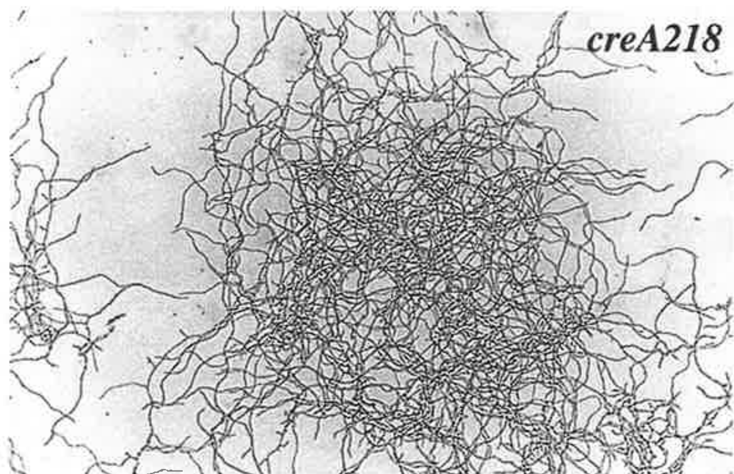
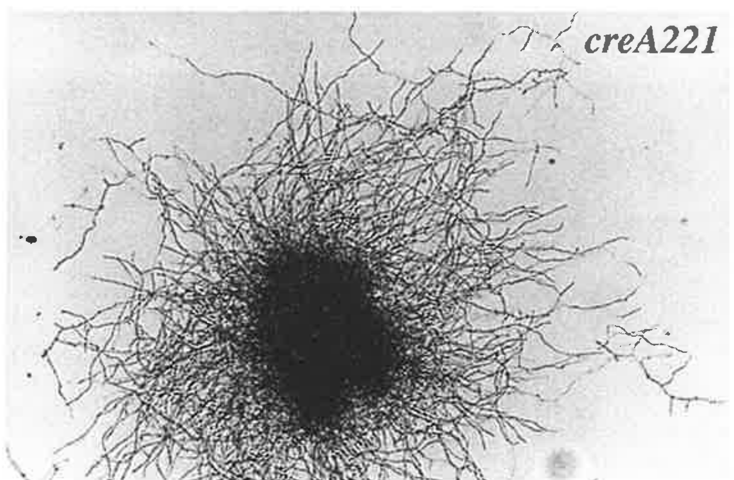

creA221

Figure 3.3 - Microscopic examination (100X) of mycelial samples grown in carbon rich liquid medium
Strains containing the *creA* alleles were grown for 21 hours at 37°C on a shaking table in liquid media containing 1% D-glucose and 1% ethanol as the carbon source and 10 mM ammonium tartrate as the nitrogen source. Samples were examined by light microscopy at a magnification of 100X. Examples of three *creA* alleles are shown in comparison to a wild type strain. In wild type strain the mycelia clump tightly together. This is also observed in the *creA*204 sample. Strains containing large quantities of carbohydrate storage products do not clump tightly and have very diffuse mycelia. Mycelia from the *creA*218 strain does not clump together and grows as a diffuse mycelial mass in these growth conditions.


wild type


creA204


creA218


creA221

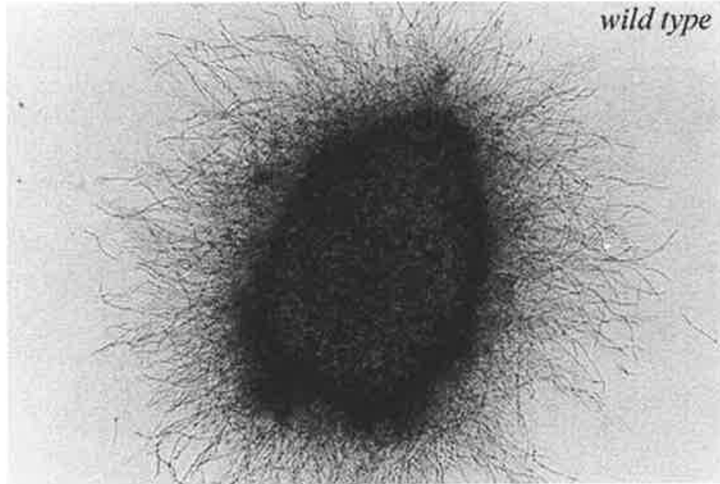
Since this phenotypic effect was seen in media rich in carbon source, 1% D-glucose and 1% ethanol, the same strains were tested in liquid media with limiting supply of carbon source, 0.1% D-fructose. When carbon source is limiting strains containing *creA*218, -221, -303, -304, -30, -305 and -220 no longer demonstrate this gross morphological change (figure 3.4). The mycelial mass forms tight clumps, although often not to the same degree as wild type, and the small dense bodies previously observed within the mycelia are no longer present.

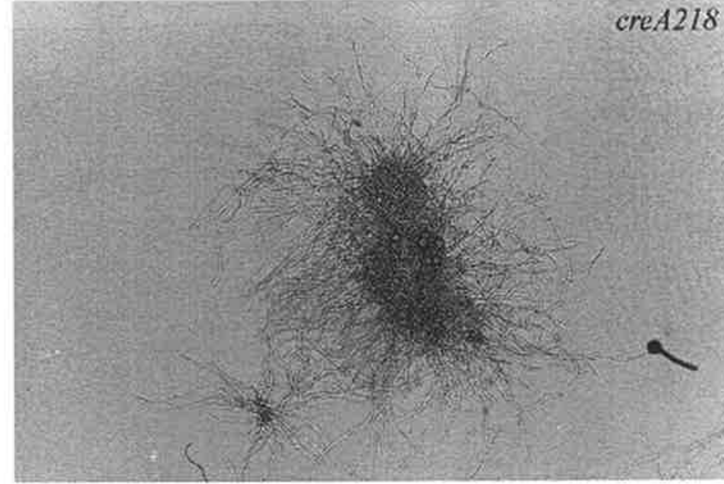
This difference between growth media suggests that the presence of these small dense bodies and associated gross morphological changes are linked to the storage of carbohydrates within the cell. Consequently CreA may be directly involved in the regulation of genes involved in the production of carbohydrates which the cell can store, such as glycogen.

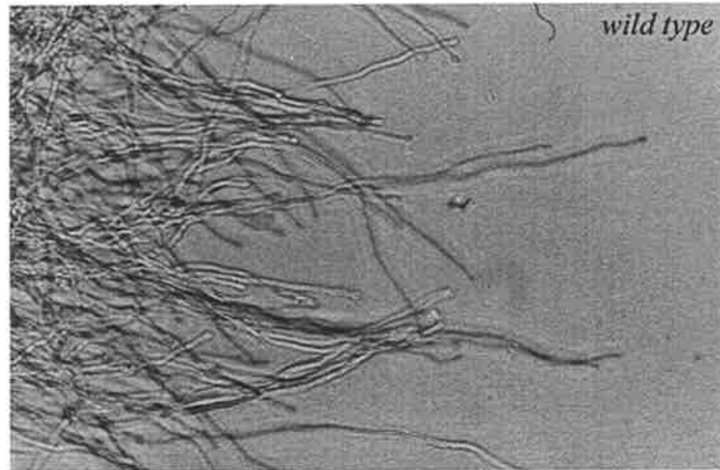
3.1.3 - Effect on the formation of cleistothecia

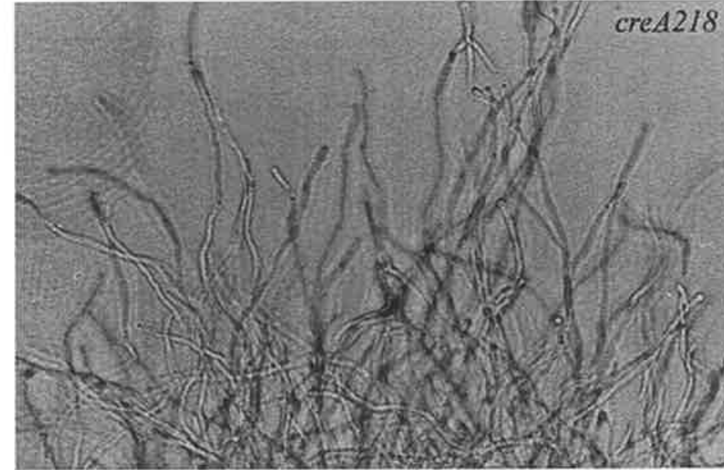
In the asexual growth cycle at 37°C conidia germinate to form hyphae which elongate upon and just under the agar surface radiating out to form the growing colony. Behind the growing hyphae conidiophores develop which carry chains of conidia. Conidia are first detected at approximately 18 hours after germination and the process of conidiation continues until approximately 40 hours after germination. It is after this process that the sexual cycle can be observed. The first indication is the presence of Hulle cells close to the center of the colony. The Hulle cells surround cleistothecial initials which appear at approximately 50 hours after germination.

Figure 3.4 - Microscopic examination of mycelial samples grown in carbon limiting liquid medium
A strains containing the *creA*218 allele and a wild type strain were grown for 21 hours at 37°C on a shaking table in liquid media containing 0.1% D-fructose as the carbon source and 10 mM ammonium tartrate as the nitrogen source. Samples were examined by light microscopy at a magnification of 100X (top panels) and 400X (bottom panels). *creA*218 is shown since it demonstrated the most extreme phenotype in carbon rich medium. In carbon limiting media the mycelia from a *creA*218 strain have some degree of clumping, although not to the same degree as a wild type strain (100X, top panel) and have no visible carbohydrate storage bodies (400X, bottom panels). Similar results were observed from strains containing other *creA* alleles with carbohydrate storage defects.


wild type


creA218


wild type


creA218

The cleistothecia expand to contain mature, viable ascospores (Champe *et al.* 1994). However the laboratory strains used carry velvet, *veA*1, mutations which increase conidia production 20-fold and have a 5-fold reduction in cleistothecia production. Therefore the laboratory strains have a reduced sexual cycle. Laboratory strains are grown on thick solid culture media under partially anaerobic conditions to favour cleistothecium formation.

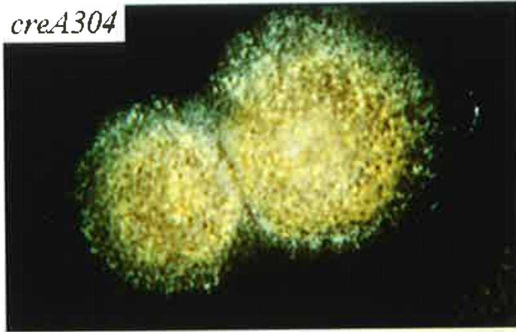
Strains containing *creA*303, - 304, -305, -30, 301, -218, -302, -307, -221 and -220 (in decreasing order of severity) all produced large numbers of abortive cleistothecial initials when grown at 37°C on solid 1% complete media in aerobic conditions (figure 3.5). The cleistothecial initials do not mature. None of the other *creA* mutant alleles have this phenotype. This suggests that CreA may play a direct or indirect role in the signalling pathway of the sexual cycle. Strains containing the alleles that produce the highest numbers of cleistothecial initials have the lowest levels of conidiation when grown on 1% complete media.

3.2 - Temperature sensitivity

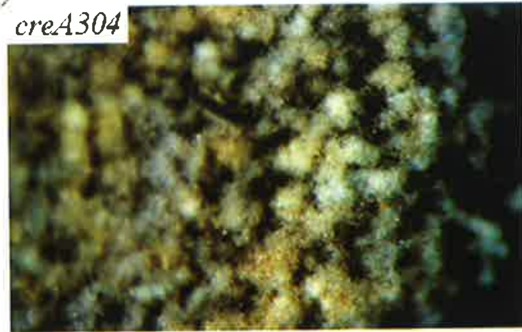
Strains containing all of the *creA* mutant alleles were tested for their ability to grow at temperatures above (42°C) and below (25°C) the optimal growth temperature (37°C). All strains grew at a slower rate at 25°C. However, this was solely due to the lower temperature and no observable differences were seen between the *creA* alleles. At 42°C, all strains grew at a slower rate than the optimal temperature. In addition, there were clear differences in growth rate between strains that contained *creA*1, -204, -225, -311, -331 and -334, and the remaining alleles (table 3.2). These alleles resulted

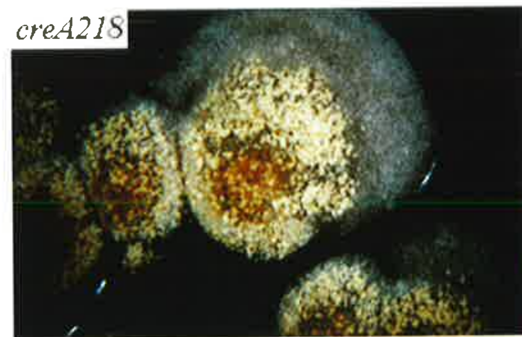
Figure 3.5 - Formation of abortive cleistothecial initials
Strains containing a number of the *creA* mutant alleles formed abortive clestothecial initials. Strains containing four alleles (*creA*304, *creA*305, *creA*218 and *creA*220) are shown microscopically at (a) low power, 10X and (b) higher power, 50X.

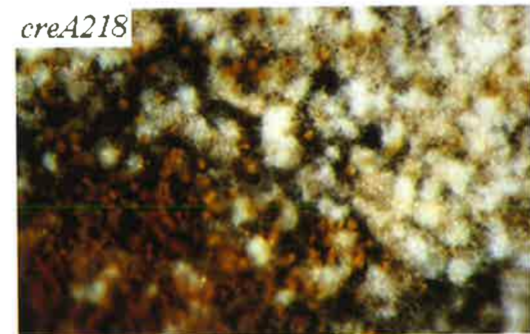
a)

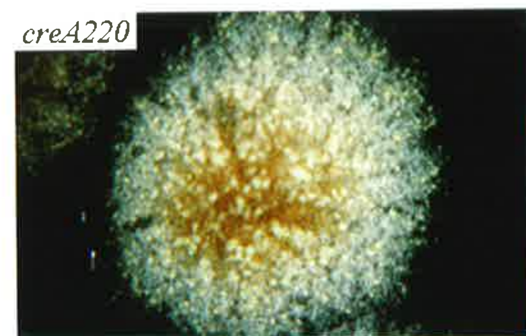


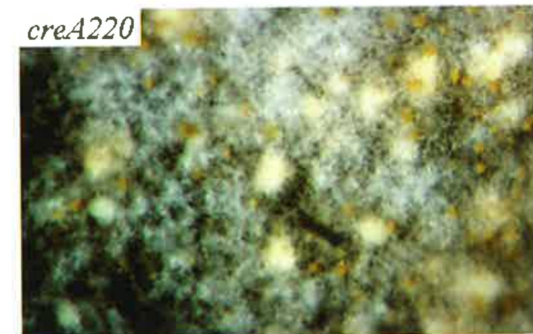
b)











in temperature sensitive growth, which could not be accounted for by the temperature change alone as measured by comparing wild type at the two temperatures.

Table 3.2 - Measurement of growth rate of strains containing *creA* mutant alleles at different temperatures

Strain (1)	25°C (2)	37°C (2)	42°C (2)
creA$^+$	+++	+++++	++++
*creA*1	+++	+++++	+++
*creA*30	+++	+++++	+++
*creA*204	+++	+++++	+
*creA*218	+++	+++++	++++
*creA*220	+++	+++++	++++
*creA*221	+++	+++++	++++
*creA*225	+++	+++++	+
*creA*301	+++	+++++	++++
*creA*302	+++	+++++	++++
*creA*303	+++	+++++	++++
*creA*304	+++	+++++	++++
*creA*305	+++	+++++	++++
*creA*306	+++	+++++	++++
*creA*307	+++	+++++	++++
*creA*311	+++	+++++	+++
*creA*322	+++	+++++	++++
*creA*331	+++	+++++	++
*creA*334	+++	+++++	+++

Note: (1) all strains are in an *areA*217 background.
(2) denotes increasing rate of growth -; +; ++; +++; ++++; +++++. Strains were not scored to take account of the different rates of growth between the different alleles. Optimal growth was scored at 37°C and growth at other temperature was scored relative to this.
(3) at all temperatures strains were grown on minimal medium containing 1% D-glucose with 10 mM ammonium tartrate as the nitrogen source.

Temperature sensitivity was only observed at higher temperatures. There are a number of possible explanations that could account for this phenotype. It is possible that some of the *creA* mutants may produce proteins that are less stable at the higher temperature, resulting in poorer growth at higher temperature. The higher

temperature may also may also destabilise intracellular interactions. If a mutation resulted in a reduced affinity for a DNA/protein or protein/protein interaction this effect may be amplified at higher temperatures leading to poorer growth.

3.3 - Growth in repressing conditions

The *creA* alleles are the result of screens that selected for the derepression of genes regulated by carbon catabolite repression. The level of derepression of a number of *creA* regulated systems can be estimated by simple growth testing of strains containing the *creA* mutant alleles.

3.3.1 - Genes involved in the utilisation of acetamide, proline, GABA and β-alanine

Acetamide, proline, γ-amino butyric acid (GABA) and β-alanine are compounds that can act as both carbon and nitrogen sources. Subsequently, the genes which code for the proteins required for the utilisation of these compounds are regulated by both carbon catabolite repression and nitrogen metabolite repression. The level of expression of these genes can be estimated by the degree to which a strain containing the *areA*217 loss of function allele is suppressed by a *creA* allele on media containing a repressing level of D-glucose (1%) and any of these compounds as the nitrogen source. There is a direct correlation between the rate of growth and conidiation with the level of expression of the genes required to break down these compounds. Since the strains contain an *areA* loss of function allele the level of expression is set by the

degree to which the *creA* allele leads to derepression of transcription of these genes. The results of these growth tests are presented in figure 3.6 and summarised in table 3.3. The data in table 3.3 was scored as an interaction of the effect on both mycelial and conidial densities, related to colony size, conidiation and density on minimal medium.

Table 3.3 - Measurement of growth rate of strains containing *creA* mutant alleles in an *areA*217 background.

Strain [(1)]	1% D-glucose and 10mM Ammonium tartrate [(2)]	1% D-glucose + 10 mM Acetamide [(2)]	1% D-glucose + 10 mM L-proline [(2)]	1% D-glucose + 10 mM GABA [(2)]	1%D-glucose +10mM β-alanine [(2)]
creA$^+$	+++++	+	+	+	+
*creA*1	++++	++	+++	+	+
*creA*30	+++	++	++	+	+
*creA*204	++	++++	+++	++	+
*creA*218	+++	+++	+++	+++	++
*creA*220	++++	++++	+++++	++++	+++
*creA*221	++++	++++	+++++	++++	+
*creA*225	+++	++	++	++	+
*creA*301	+++	+	++	+	+
*creA*302	+++	++	+++	++	+
*creA*303	++	+++	++	++	++
*creA*304	++	+++	++	++	++
*creA*305	++	+++	++	++	+
*creA*306	++	++++	++++	+++	+
*creA*307	+++	++	+++++	+++	+
*creA*311	++	++++	++++	+++	+
*creA*322	+++	++	+++	+++	+
*creA*331	++	+++	++++	+++	+
*creA*334	++	++	++	+++	+

Note: (1) all strains are in an *areA*217 background.
(2) denotes increasing growth -; +; ++; +++; ++++; +++++.

Figure 3.6 - Suppression of *areA*217

Strains containing *creA* mutant alleles were grown for 48 hours at 37°C on 1% minimal media containing: (a) 1% D-glucose and 10 mM ammonium tartrate (synthetic complete)

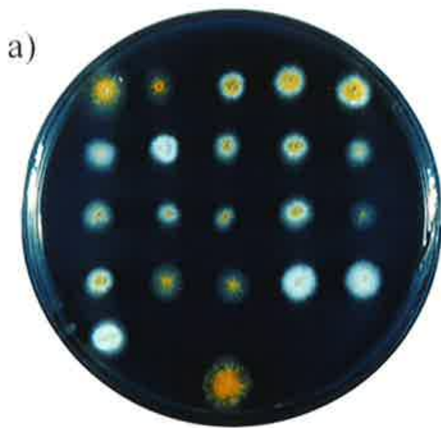
(b) 1% D-glucose and 10 mM acetamide

(c) 1% D-glucose and 10 mM L-proline

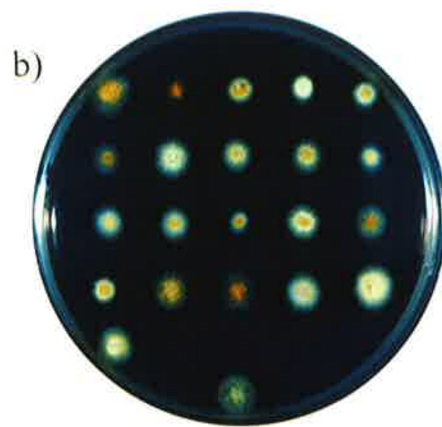
(d) 1% D-glucose and 10mM γ-amino butyric acid

The genotype of each colony is *yA*1, *adE*20, *su*(*adE*20)*A*1, *riboB*2, *areA*217 and contains the *creA* allele shown in (e). The *creA*$^+$ *areA*217 strain is unable to use compounds other than ammonium as a nitrogen source and therefore grow very poorly on plates (b), (c) and (d). A mutation in the *creA* gene is able to suppress this phenotype. The degree of suppression has been estimated and is presented in table 3.3. Suppression is estimated from the amount of growth with respect to the wild type control and compared to the reduced growth rate of each *creA* mutant strain on synthetic complete medium (a).

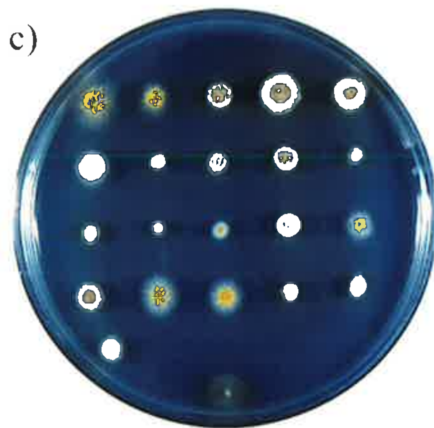
a)



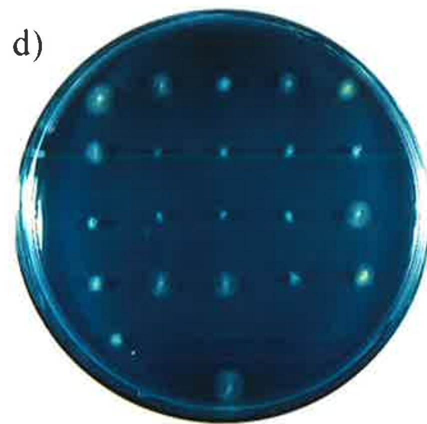
b)



c)



d)



e)

creA1	*creA204*	*creA218*	*creA220*	*creA221*
creA225	*creA30*	*creA301*	*creA302*	*creA303*
creA304	*creA305*	*creA306*	*creA307*	*creA311*
creA322	*creA331*	*creA334*	*creA*Δ*4*	*creA*Δ*21*
*creA*Δ*99*				

creA$^+$

All of the alleles suppress *areA*217 on at least one of the compounds. In addition, all alleles demonstrated some degree of suppression on D-glucose medium containing either acetamide or proline as the nitrogen source. All alleles except for *creA*1, -30 and -301 suppressed the *areA*217 phenotype on media containing 1% D-glucose and GABA. On media containing D-glucose and β-alanine the strains that suppressed *areA*217 to the highest levels contained *creA*303, -304, -218, -220 and -311 (in decreasing order of severity).

Overall, strains containing *creA*306, -304, -303, -305, -220, -311, -334, -331 and -218 result in the consistently highest levels of the enzymes required for the breakdown of either acetamide, proline, GABA and β-alanine.

3.3.2 - Genes involved in the utilisation of Lactose and Starch

The level of expression of the genes whose products are required for the breakdown of lactose (β-galactosidase) and starch (α-amylases) can be estimated by direct plate testing.

To estimate the levels of β-galactosidase expression strains containing the *creA* mutant alleles were grown on media which contained 1% D-glucose, 0.5% lactose and 100μg/ml X-gal. The level of β-galactosidase was indicated by the intensity of blue colouration produced by each colony. In the presence of D-glucose, strains which contained *creA*303, -304, -204, -225, -306 and -301 (in decreasing order of

severity) demonstrated significantly higher levels of β-galactosidase activity when compared to wild type (figure 3.7a and table 3.4).

Table 3.4 - Levels of expression of β-galactosidase and α-amylase from strains containing *creA* mutant alleles.

Strain (1)	1% D-Glucose + 0.5% Lactose + X-gal (2)	1% D-Glucose + 0.5% Starch (2)
creA+	-	-
*creA*1	-	W
*creA*30	-	S
*creA*204	S	S
*creA*218	-	I
*creA*220	-	I
*creA*221	-	I
*creA*225	-	I
*creA*301	-	I
*creA*302	-	I
*creA*303	S	S
*creA*304	S	S
*creA*305	-	S
*creA*306	I	S
*creA*307	-	I
*creA*311	-	I
*creA*322	W	I
*creA*331	-	W
*creA*334	-	I

Note: (1) all strains are in an *areA217* background.
(2) amount of blue colouration: -, no colouration; W, weak; I, intermediate; S, Strong
(3) size of clear halo: -, no halo; W, weak; I, intermediate; S, Strong

Levels of α-amylase expression were determined by growing strains containing *creA* mutant alleles on medium that contained 1% D-glucose and 0.5% starch. Colonies which are able to utilise starch as a carbon source produce extracellular α-amylases.

Figure 3.7 - Expression levels of β-galactosidase and α-amylases in repressing conditions

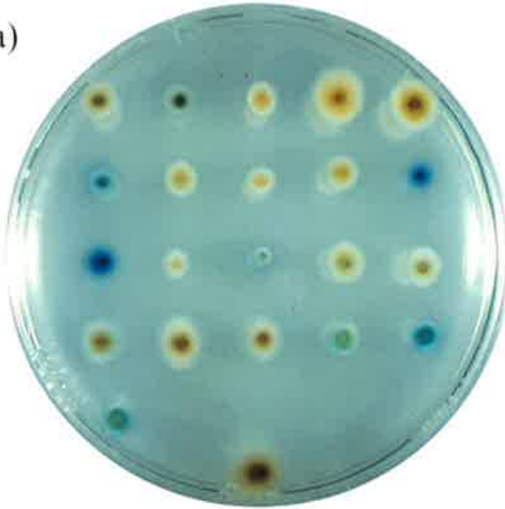
Strains containing *creA* mutant alleles were grown for 48 hours at 37°C on 1% minimal media containing:

(a) 1% D-glucose, 0.5% lactose, 10 mM ammonium tartrate and 100 µg/ml X-gal. The level of β-galactosidase expression is estimated from the degree of blue colouration in the medium which is the result of X-gal acting as a substrate for the enzyme. The genotype of each colony with respect to the *creA* locus is shown.

(b) 1% D-glucose, 1% starch and 10 mM ammonium tartrate. After the strains were grown for 48 hours the plates were flooded with a dilute iodine solution which stains the remaining strach blue. The level of α-amylase expression is estimated from the size of the cleared zone of starch compared to the size of the colony. The genotype of each colony with respect to the *creA* locus is shown.

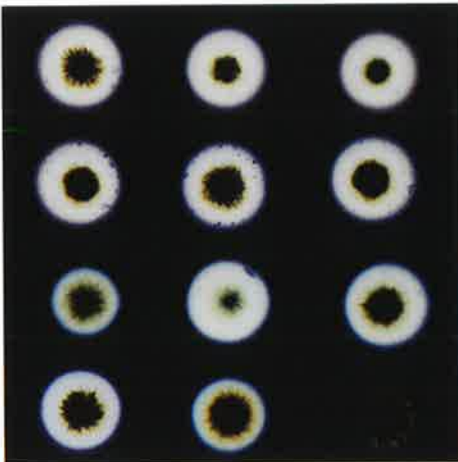
The results of these test are summarised in table 3.4.

a)



creA1	*creA204*	*creA218*	*creA220*	*creA221*
creA225	*creA30*	*creA301*	*creA302*	*creA303*
creA304	*creA305*	*creA306*	*creA307*	*creA311*
creA322	*creA331*	*creA334*	*creAΔ4*	*creAΔ21*
creAΔ99				
		creA^{+}		

b)



creA303	*creA304*	*creA306*
creAΔ4	*creAΔ21*	*creAΔ99*
creA1	*creA204*	*creA30*
creA218	*creA322*	*creA*$^{+}$

The amount of extracellular α-amylase can be determined by flooding the plate with an iodine solution, which stains any starch remaining in the plate blue. The level of α-amylase expression is indicated by the size of the zone of cleared starch around the colony. The larger the size of the zone of cleared starch the higher the level of α-amylase expressed by that particular strain. Strains containing *creA*306, -303, -304, -305, -204 and -218 (in decreasing order of severity) resulted in the highest levels of α-amylase expression (figure 3.7b and table 3.4).

3.3.3 - Genes involved in the utilisation of ethanol

Ethanol is converted to acetaldehyde by the catabolic enzyme alcohol dehydrogenase (ADH1). In *A. nidulans* ADH1 is coded for by the gene *alcA*. The expression of this gene has been shown to be tightly controlled by carbon catabolite repression (Lockington *et al.* 1985). Allyl alcohol can also act as a substrate for ADH1. In this reaction allyl alcohol is converted to acrolein, which is a toxic compound. This reaction enables a sensitive plate test to determine levels of ADH1 activity in any strain. Strains containing *creA* mutant alleles were grown on media containing 1% D-glucose and varying levels of allyl alcohol. In repressing conditions the level of *alcA* expression resulting from the presence of a *creA* mutation will determine the level of allyl alcohol that the strain will be sensitive to. A wild type strain is not sensitive to allyl alcohol in the presence of 1% D-glucose because *alcA* is not transcribed due to carbon catabolite repression.

Strains containing *creA*220 and *creA*331 were able to grow well on media containing 25mM allyl alcohol, although they were not as resistant as wild type. The remaining mutant alleles lead to significant levels of *alcA* expression in the presence of D-glucose. Strains containing the remaining *creA* alleles were extremely sensitive to allyl alcohol concentration of 2.5mM or below (figure 3.8 and table 3.5).

Table 3.5 - Sensitivity of strains containing *creA* alleles to varying concentrations of allyl alcohol.

Strain [1]	1% D-glucose + 0.1 mM Allyl Alcohol[2]	1% D-glucose + 0.5 mM Allyl Alcohol[2]	1% D-glucose + 2.5 mM Allyl Alcohol[2]	1% D-Glucose + 25.0 mM Allyl Alcohol[2]
creA$^+$	+++++	+++++	+++++	++++
*creA*1	+++	+++	+	-
*creA*30	++	-	-	-
*creA*204	++	-	-	-
*creA*218	++++	-	-	-
*creA*220	++++	++++	+++	++
*creA*221	++++	++	-	-
*creA*225	+	-	-	-
*creA*301	+++	-	-	-
*creA*302	+++	-	-	-
*creA*303	+	-	-	-
*creA*304	++	-	-	-
*creA*305	++	-	-	-
*creA*306	+	-	-	-
*creA*307	+++	-	-	-
*creA*311	+++	-	-	-
*creA*322	+++	-	-	-
*creA*331	++++	++++	+++	++
*creA*334	+++	-	-	-

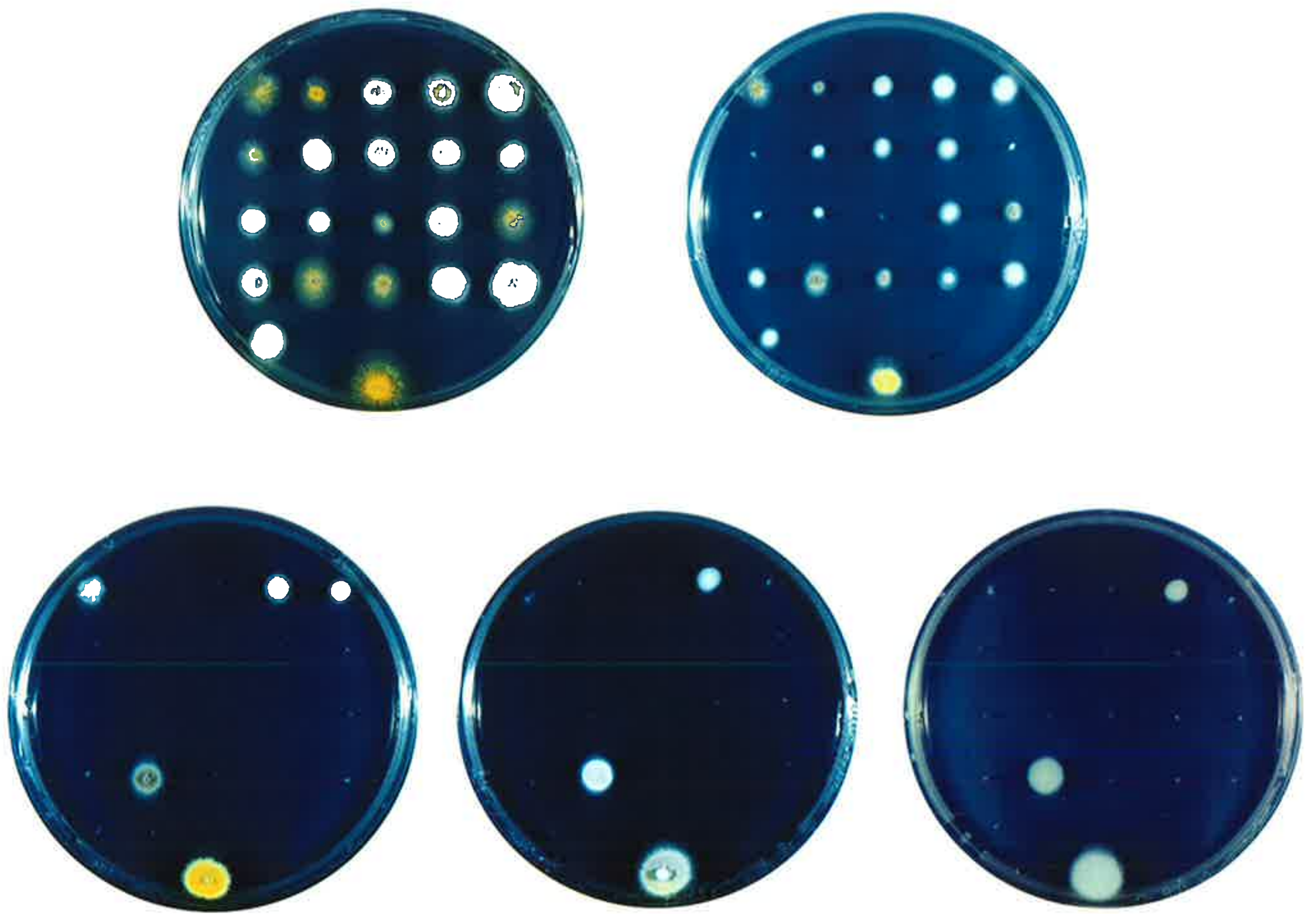
Note: (1) all strains are in an *areA*217 background.
(2) denotes increasing rate of growth -; +; ++; +++; ++++; +++++.
(3) scores between plates are comparable.

Figure 3.8 - Sensitivity of *creA* strains to allyl alcohol

Strains containing *creA* mutant alleles were grown for 48 hours at 37°C on 1% minimal media containing with 1% D-glucose as the carbon source and 10 mM ammonium tartrate as the nitrogen source. Each plate contained different concentrations of allyl alcohol:

(a) 0 mM allyl alcohol
(b) 0.1 mM allyl alcohol
(c) 0.5 mM allyl alcohol
(d) 2.5 mM allyl alcohol
(e) 25.0 mM allyl alcohol

Allyl alcohol can act as a substrate for alcohol dehydrogenase I (ADHI) and is broken down to the toxic compound acrolein. Wild type strains are resistant to allyl alcohol on glucose containing media because ADHI is carbon catabolite repressed. The degree of sensitivity of strains containing *creA* mutant alleles is depends on the level of ADHI expression. The genotype of each colony with respect to the *creA* locus is shown. The results of these tests are summarised in table 3.5.



creA1 *creA204* *creA218* *creA220* *creA221*

creA225 *creA30* *creA301* *creA302* *creA303*

creA304 *creA305* *creA306* *creA307* *creA311*

creA322 *creA331* *creA334* *creAΔ4* *creAΔ21*

creAΔ99

creA^{+}

Variations in levels of sensitivity were observed at very low concentration of allyl alcohol (0.1mM). Strains containing *creA*306, -304 and -225 were very sensitive to 0.1mM allyl alcohol in the presence of 1% D-glucose, indicating that they result in the highest levels of *alcA* expression.

To further investigate the ethanol utilisation system ADH1 enzyme activity assays were performed on strains containing selected *creA* mutant alleles (table 3.6). All of the strains tested had elevated levels of enzyme activity in all growth conditions tested, regardless of the presence of exogenously added inducer. The allyl alcohol plate tests reflect the levels of ADH1 in the presence of D-glucose and the absence of inducer, suggesting that allyl alcohol may only be a poor inducer of *alcA* or unable to induce expression at all. The general trends seen on allyl alcohol plate testing were also observed in the ADH1 assays. For example, strains containing *creA*220 consistently lead to much lower level of ADH1 than strains containing the other alleles. At the other end of the scale, *creA*204 containing strains were very sensitive to low concentrations of allyl alcohol and consistently produced the most ADH1 activity. Aside from resulting in clear derepression the *creA* mutant alleles also resulted in significantly elevated levels of expression, which has also been reported by Mathieu and Felenbok (1994).

Both *areA*217 and *areA*$^+$ strains containing the *creA*204 and *creA*220 mutant alleles were assayed for ADH activity. Similar results were obtained in both genetic backgrounds, indicating that the results were not influenced by the presence of the *areA*217 allele.

Table 3.6 - ADH1 activity (nM product/(mg soluble protein/minute)) from strains containing selected *creA* alleles.

Strains [1]	1% D-glucose [2]	0.1% D-fructose[3]	1% D-glucose + 1% ethanol [4]	0.1% D-fructose + 1% ethanol [5]
creA^{+}	10	30	5	190
*creA*1	75	145	85	640
*creA*204	190	180	1800	1810
*creA*225	260	160	2200	2680
*creA*218[6]	125	60	1005	1830
*creA*220	30	65	785	1140
*creA*221	80	90	1810	2580
*creA*30	180	155	1020	1690

Note: (1) All strains are in an *areA217* background.

(2) Repressing conditions: 22 hours at 25°C in media containing 1% (w/v) D-glucose.

(3) Derepressing conditions: 22 hours at 25°C in media containing 0.1% (w/v) D-fructose.

(4) Repressing, induced conditions: 27 hours at 25°C in media containing 1% (w/v) D-glucose and 1% (v/v) ethanol.

(5) Derepressing, induced conditions: 27 hours at 25°C in media containing 0.1% (w/v) D-fructose and 1% (v/v) ethanol.

(6) Due to an extremely poor rate of growth strains containing the *creA218* allele were grown for 25 hours in non-induced conditions and 31 hours in induced conditions to obtain a mycelial mass comparable to other strains.

(7) Ammonium tartrate (10mM) was used as the nitrogen source.

3.4 - Growth in derepressing conditions

Strains containing the mutant *creA* alleles were tested on growth media containing a wide variety of sole carbon sources at non-repressing levels. No significant differences in growth were seen between strains containing a *creA* mutant allele when compared to wild type. Since the selection protocols used to select these mutant alleles screens for a derepressed phenotype it is unlikely that any obvious phenotype would be visible when grown on derepressing carbon sources.

3.5 - Summary

creA mutants are pleiotropic. *creA* containing strains have clear phenotypic effects on morphology and rate of growth, initiation of the sexual cycle, carbohydrate storage mechanisms and the regulation of genes that produce products involved in the catabolism of carbon sources. Strains containing different *creA* alleles are extremely heterogeneous. As is clearly seen in the results presented, each allele produces quite specific phenotypes. These range from the most extreme allele, *creA*306 that results in an extremely weak growth rate and, in the presence of glucose, very high levels of expression of carbon repressible systems to alleles that have much less severe phenotypes, such as *creA*331 and *creA*334. The alleles used in this study were all selected as loss of CreA function. Clearly the heterogeneous phenotypes observed indicates that not all alleles result in a complete loss of CreA function, with the degree of function retained depending on the specific mutation in each allele. The heterogeneous phenotypes observed also argues against the majority of mutations resulting in the production of unstable polypeptides which result in the loss of functional CreA protein.

Chapter 4

Molecular Analysis of *creA* Mutants

The phenotypic analysis of strains containing various *creA* alleles demonstrated very heterogeneous phenotypes amongst them. In an attempt to define functional domains within the CreA protein the mutant alleles were characterised at the molecular level. This analysis included comparing homologues of the *creA* gene from other fungal species for highly conserved regions within the protein, and the determination of the nature of the mutation for each allele. The aim of this approach was to define functional domains by correlating the position of individual mutations within the protein with the phenotypic analysis carried out on strains containing that allele.

4.1 - Analysis of *creA* homologues

creA was cloned by complementation of the *creA*204 allele on a medium containing 1% D-glucose and 2.5mM allyl alcohol. Sequencing of the gene and translation of the putative open reading frame gave a predicted polypeptide sequence of 416 amino acids (Dowzer and Kelly 1989). The sequence had a number of features consistent with CreA having a role as a transcriptional repressor. The most prominent of these was a classical Cys_2-His_2 zinc finger DNA binding domain. Comparison to genomic databases showed a very high degree of similarity between the DNA binding domains of CreA and Mig1p, which is the regulatory protein that mediates carbon catabolite repression in *S. cerevisiae*. Other features identified were a stretch of nine consecutive alanine residues and frequent STPXX motifs (Dowzer and Kelly 1989, 1991). Alanine rich regions have been identified in a number of proteins that are transcriptional repressers such as Tup1p and Kruppel (Hanna-Rose *et al.* 1997). STPXX motifs have

been found in disproportionately high numbers in transcriptional regulatory proteins (Suzuki 1989).

In a further attempt to define functional domains in CreA, the functionally homologous gene was cloned from the related fungus *Aspergillus niger*. Comparison of the two polypeptide sequences identified a stretch of 45 invariant amino acids in the C-terminal half of the gene. This region has a significant degree of similarity to the *S. cerevisiae* protein Rgr1p, which has been shown to be involved with the regulation of carbon catabolite repression (Sakai *et al.* 1988).

During the course of this study many homologues of the *creA* gene had been isolated from a number of organisms including; *Trichoderma reesei, Trichoderma harzianum, Neurospora crassa, Metarhizium anisopilae, Sclerotinium sclerotinia* and *Gibberella fujikkuroi* (Drysdale *et al.* 1993; Ilmen *et al.* 1996; Reymond-Cotton *et al.* 1996; Screen *et al.* 1997; Strauss *et al.* 1996; Takashima *et al.* 1996, B. Tudzynski unpublished, B. Tyler unpublished). The analysis of a number of *creA* homologues enabled a more detailed study of the polypeptide sequences in order to identify any conserved domains. The polypeptide sequences of the CreA homologues were compared using the computer alignment software ClustalW (Thompson, Higgins and Gibson 1994) and Match-Box (Depiereux and Feytmans 1992). These programs compare the polypeptide sequences for regions of identity and similarity. ClustalW creates a multiple sequence alignment from a group of sequences using progressive pairwise alignments. The program does a series of progressive, pairwise alignments between sequences and clusters of sequences to generate the final alignment. Match-

Box performs a simultaneous alignment. Match-Box analyses sequences in two steps. The first step analyses the global similarity between the whole set of sequences. The second step matches the most similar nine residue segments in a scan of the whole set of sequences. The similarity between segments is defined by a scoring matrix and is limited by a statistical threshold. Only complete matches are retained. A complete match is a set of segments, selected in all the sequences, in which each segment is similar to all the other segments. Four steps of matching are performed successively with a decreasing statistical significance in order to fix reliable anchor points at the beginning and to enlarge them in the next steps. The program aims to identify regions within the protein that have highly conserved physiochemical profiles.

The highest regions of conservation were the previously identified Cys_2-His_2 zinc finger binding domain and the Rgr1p similar domain (figure 4.1). However, a number of other regions are conserved between the homologues (figure 4.1 and figure 4.2). There are eleven regions within the protein that show a high degree of conservation. These regions will be referred to as conserved "boxes" ie. box 1 through to box 11. Box 2 corresponds to the Cys_2-His_2 zinc finger DNA binding domain (box 2) and box 7 corresponds to the region with similarity to the Rgr1p protein of *S. cerevisiae*.

The remaining conserved regions correspond to previously undefined sections of the protein. Of these regions the most highly conserved are box 1, box 4, box 9 and box 10. Other than the DNA binding domain and the Rgr1p similar region there is no

Figure 4.1 - CLUSTALW analysis of CreA homologues
CreA homologues were analysed using the multiple sequence alignment software CLUSTALW. Identical residues within the proteins are highlighted in blue. Similar residues are highlighted in yellow. In positions where the most (approximately 75% or greater) but not all residues are similar a pale shade of yellow has been used. The CreA homologues compared are from the following strains; *A. nidulans* (Dowzer and Kelly 1989), *A. niger* (Drysdale *et al.* 1993), *S. Sclerotinia* (Screen *et al.* 1996), *N. crassa* (B. Tyler unpublished), *T. reesei* (Ilmen *et al.* 1996), *T. harzianum* (Ilmen *et al.* 1996), *M. anisopilae* (Reymond-Cotton *et al.* 1997), *G. fujikkuroi* (B. Tudzynski unpublished).

```
A. nidulans     M P Q P G S S V D F S N L L N P Q N N - - - - - - - - - - - - - T A I P A E V S N - - - - - - - A T A S A T M A S G A S L
A. niger        M P P P A S S V D F S N L L N P Q N N S T D S T P S T P V D S S K T P S T P S S - - - - - - - T Q S N S N M A S S V S L
S. sclerotinia  M Q R A S S A V D F N S L L N P Q - S T Q E R E - - - - - H Q A A R Q K L A L I Q - - - - - - Q Q Q Q H Q R E A E M A A
N. crassa       M Q R V Q S A V D F S N L L N P S E S T A E K R - - - - - D H S G S P R Q Q T A Q P Q Q Q Q Q Q P Q P E A D M A T V G L
T. reesei       M Q R A Q S A V D F S N L L N P T - S A A G Q - - - - - - - - D S - - - - - - - - - - - - - - - - - - - - - G A M S T A A V T V
T. harzianum    M Q R A Q S A V D F S N L L N P T - S A A G Q - - - - - - - - D S D A E - - - - - - - - - - - - - Q G S G A M S T A A V T V
M. anisopilae   M Q R S Q S A V D F T N L L N P S - A Q A D H - - - - - - - - N S - R S R - - - - - - - - - - - - Q A D A A M A S A S V T V
G. fujikkuroi   M Q R A Q S A V D F S N L L N P T - V P A D K - - - - - - - - E S E K P H - - - - - - - - - - - - Q G D V E M A T A A V T V

A. nidulans     L P P M V K G A R P A A E E A R Q D L P R P Y K C P L C E R A F H R L E H Q T R H I R T H T G E K P H A C Q F P G C S K
A. niger        L P P L M K G A R P A T E E V R Q D L P R P Y K C P L C D R A F H R L E H Q T R H I R T H T G E K P H A C Q F P G C T K
S. sclerotinia  I S M M P A M V G G H H G D D R Q D L P R P Y K C P L C E K A F H R L E H Q T R H I R T H T G E K P H A C L F P G C T K
N. crassa       L R P N G P L P G A Q A T E P A N E L P R P Y K C P L C D K A F H R L E H Q T R H I R T H T G E K P H A C Q F P G C S K
T. reesei       I K P N G P I P G T Q S I E T A N E L P R P Y K C P L C D K A F H R L E H Q T R H I R T H T G E K P H A C Q F P G C S K
T. harzianum    I K P N G P I P G A Q S T E A A N E L P R P Y K C P L C E K A F H R L E H Q T R H I R T H T G E K P H A C Q F P G C S K
M. anisopilae   I K P N G P L P G A Q A S D T T N E L P R P Y K C P L C D K A F H R L E H Q T R H I R T H T G E K P H A C Q F P G C S K
G. fujikkuroi   I K P N G P L P G V Q N S E N S N E L P R P Y K C P L C D K A F H R L E H Q T R H I R T H T G E K P H A C Q F P G C S K

A. nidulans     R F S R S D E L T R H S R I H N N P N S R R G N - - K A Q H L A A A A A A A A A N Q D G S A M A N N A G S M M P P P S K
A. niger        R F S R S D E L T R H S R I H N N P N S R R R N - - K A Q H L A A A A A A A A G - Q D - N A M A N T A S A M M P P P S K
S. sclerotinia  R F S R S D E L T R H S R I H N N P N S R R S N - - - - K T Q Q A P - - - - - - Q M G V P M H S E S M A T M M P P P N K
N. crassa       K F S R S D E L T R H S R I H S N P N S R R G N - - - - K G Q Q Q Q Q H P L V H N H G L Q P - - - - - D M M P P P - G P
T. reesei       K F S R S D E L T R H S R I H S N P N S R R G N - - - - K G Q Q - - - Q H Q L H H Q G M P H P M H V D G L M H P P A A P
T. harzianum    K F S R S D E L T R H S R I H S N P N S R R G N - - - - K G Q Q Q H - Q Q H L H H Q G L P H H M H V D G M M P P P - V P
M. anisopilae   K F S R S D E L T R H S R I H N N P N S R R G N - K G Q H H H H Q H - H H H H H H Q G L P P H M H H E G M M A P P P A P
G. fujikkuroi   K F S R S D E L T R H S R I H N N P N S R R G N K A A Q A H Q Q Q Q - H Q M H Q Q Q G L P P H M M P D G M M A P P P A P

A. nidulans     P I T R S A P V S Q V G S P D I S P P H S F S N Y A N H - M R S N L S P Y S R T - - - - S E R A S S G - - - M D I N L L
A. niger        P M T R S A P V S Q V G S P D I S P P H S F S N Y A S H - M R S N L G P Y A R K - - - - G D E A S S G - - - M E L Y L L
S. sclerotinia  N I T R S A P P S A I G S P N V S P P H S Y T S Y S S N - H L S S L N P Y G R S L G - - G S P N N G Q A P L T D I N M L
N. crassa       K A I R S A P P T A M S S P N V S P P H S Y S P Y N F A - - P S G L N P Y S H S R S S A G S Q S G P - - - - - - D I S L L
T. reesei       K A I R S A P P S T L V S P N V S P P H S Y S S F V M P - - H G P I S H Y G R - - - - - - G - - - N - - - - - - - D I T M L
T. harzianum    K A I R S A P T S T L V S P N V S P P H S Y S S F V M P - - Q T P M A H Y N R - - - - - - G - - - N - - - - - - - D I T M L
M. anisopilae   K T I R S A P T S T L A S P N V S P P H S F A S F A Q H P H P P M H P Y S R - - - - - - G - - - G - - - - - - - D I S M L
G. fujikkuroi   K T I R S A P G S A L A S P N V S P P H S Y S T F A L - - - P V S A V H Y N R - - - - - - G - - - G - - - - - - - D I S M L
```

```
A. nidulans     A T A A S Q V E R D E S F G F R S G Q R S H H - - - - M Y G P R H G - - - - S R G - L P S L S A Y A I S H S M S R S H S
A. niger        A T A A S Q V E R D E H F D F H A G P R N H H - - - - L F S S R H H - - - - G S G R L P L L A A Y A I T H N M S R S H S
S. sclerotinia  A T A A T Q V E R D S S T T A N H Y S Q A R H - - Q P Y Y S H S N H - - - N S R T H L P S L Q A Y A M T R A Y S H E E -
N. crassa       A R A A G Q V E R D G A A - - - H H H F Q P R - - F Q F Y G N T L H A A T A S R N Q L P G L Q A Y H M S R S H S H E D -
T. reesei       A K A A N Q I E R E T L S G G P S N H N S R H - - H P Y F G Q G V P - - - G S R G H P - S L S S Y H M A R A H S N D E -
T. harzianum    A K A A N Q I E R E T L S G G P S N H N S R H - - H P Y F G Q G L P - - - N S R G H P P S L S S Y H M A R S H S N D D -
M. anisopilae   A K A A T Q V E R E T L T - A P P S H S A R H - - - - Y Y G H S M H - - - S S R G H P N G L S S Y H M A R S H S S E D -
G. fujikkuroi   A K A A T Q V E R E T L T - A P P H H S N N H R H H P Y F G H G M H - - - S S R G H L P T L S S Y H M G R S H S N E D P

A. nidulans     H E D E D S Y A S H R V K R S R P N S P N S T A P S S P T F S H D S L S P T P D H T P L A T P A H S P R L K P L S P S E
A. niger        P E D D D - G Y S H R V K R S R P N S P N S T A P S S P T F S H D S L S P T P D H T P L A T P A H S P R L R P L G S S D
S. sclerotinia  - D D H Y A - - H R H A K R S R P N S P M S T A P S S P T F S H D S L S P T P D H T P L A T P A H S P R L R P Y G G - G
N. crassa       H D D H Y G Q S Y R H A K R S R P N S P N S T A P S S P T F S H D S L S P T P D H T P L A T P A H S P R L R P H P - - G
T. reesei       - D D H Y H G S L R H A K R S R P N S P N S T A P S S P T F S H D S L S P T P D H T P I A T P A H S P R L R P F S - - G
T. harzianum    - D D H Y S - S M R H A K R S R P N S P N S T A P S S P T F S H D S L S P T P D H T P I A T P A H S P R L R P F S - - G
M. anisopilae   H D D H Y N G - M R H A K R S R P N S P N S T A P S S P T F S H D S L S P T P D H T P I A T P A H S P R L R P F S - - G
G. fujikkuroi   S D D H Y S G A M R H A K R S R P N S P N S T A P S S P T F S H D S L S P T P D H T P I A T P A H S P R L R P F S T - G

A. nidulans     L H L P S I R H L S L - - - H H T P A L A P M E P Q A E G P N Y Y N - - - - - - - P N Q P H V G P S I S D I M S R P E G
A. niger        L H L P S I R H L S L - - - H H T P A L A P M E P Q P E G P N Y Y S - - - - - - - P S Q G H H G P S I S D I M S K P D G
S. sclerotinia  Y D L P G I R N L S L - - H H - T P A L A P M E P Q H L D G Q Y H A T S T T T T A T S A P R M G L T I S D I M S R T D G
N. crassa       L E L P P F R N L S L G Q Q H T T P A L T P L E P A L D G - Q F S L P Q T P P P A P R S S - - G M S L T D I I S R P D G
T. reesei       Y E L P S L R N L S L - Q H N T T P A L A P M E P H L D A P Q F H P - Q L Q A N T T R S P - - G M S L T D I I S R P D G
T. harzianum    Y E L P S L R N L S L - Q H N T T P A L A P M E P H L D A P Q F P P - Q L Q A N N N R S P - - G M S L T D I I S R P D G
M. anisopilae   Y E L P S I R N L S L - H H N T T P A L A P M E P H L D A P Q F P P - - - Q L N A P R S N - - G M S L T D I I S R P D G
G. fujikkuroi   Y E L P S L R N L S L - Q H N T T P A L A P M E P H L E Q N Q F Q Q G S A P T T Q P R P T - - G M S L T D I I S R P D G

A. nidulans     A Q R K L P I P Q V P K V A V Q D M L N P - S G - - F T S V S S S T A N S V A G G D L A E R F -
A. niger        T Q R K L P V P Q V P K V A V Q D M L N P G S G - - F S S V H S S T A N S V A G G D L A E R F -
S. sclerotinia  S T R K L P V P Q - A P V A V Q D L S S P G E I G F N T S G Q S S T T G S V A G N D L A D R M I
N. crassa       T Q R K L P V P - - - K V A V Q D L L G P A D G - F N P S V R N S S S T S L S G A E M M D R L -
T. reesei       S Q R K L P V P Q V P K V A V Q D L L S - - D G V F P N S G R S S T T G S L A G G D L M D R M -
T. harzianum    S H R K L P V P Q V P K V A V Q D L L S - - D G V F P N S G R S S T A G S L A G G D L M D R M -
M. anisopilae   A Q R K L P V P Q V P K V A V Q D L L S - - D S G Y S N S G R S S T A G S L A G G D L M D R V -
G. fujikkuroi   S Q R K L P V P Q V P K V A V Q D L L S - - D N G F S H S G R S S G T S S L A G G D L M D R M -
```

Figure 4.2 - Match-Box analysis of CreA homologues
CreA homologues were analysed using the multiple sequence alignment software Match-Box. The program identifies conserved regions between the proteins and assigns a degree of significance to each conserved region. The eleven boxes identified by this analysis are highlighted in this figure and figure 4.3. The colours used to highlight the conserved regions are in decreasing order of significance blue, red and yellow. The CreA homologues compared are from the following strains; *A. nidulans* (Dowzer and Kelly 1989), *A. niger* (Drysdale *et al.* 1993), *S. Sclerotinia* (Screen *et al.* 1996), *N. crassa* (B. Tyler unpublished), *T. reesei* (Ilmen *et al.* 1996), *T. harzianum* (Ilmen *et al.* 1996), *M. anisopilae* (Reymond-Cotton *et al.* 1997), *G. fujikkuroi* (B. Tudzynski unpublished).

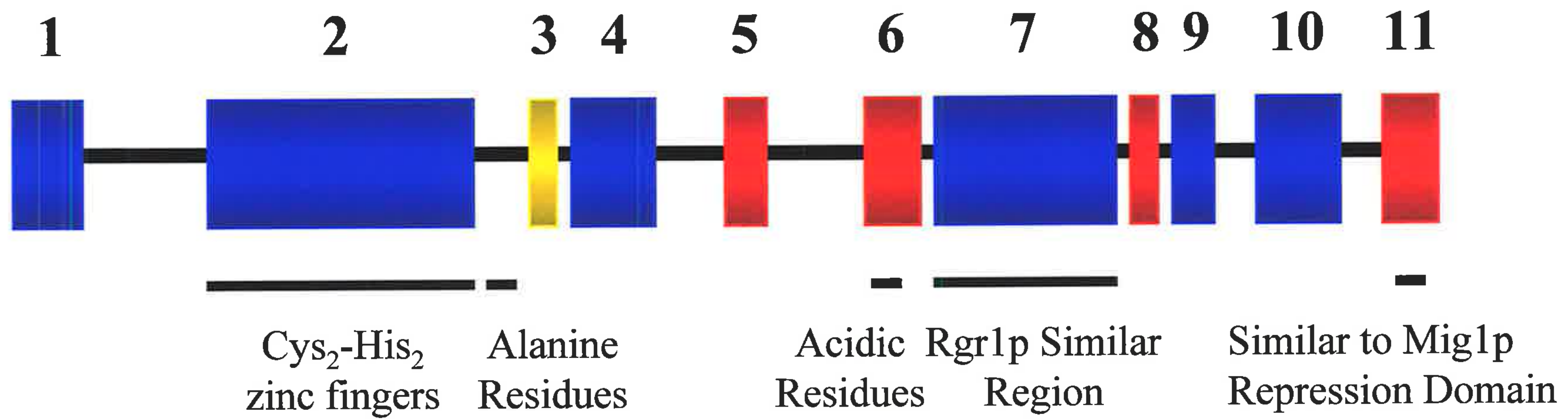
```
A. nidulans     MPQPGSSVDFSNLLNPQNNTAIPAEVSNATASATMASGASLLPPMVKGARPAAEEA--------------
A. niger        MPPPASSVDFSNLLNPQNNSTDSTPSTPVDSSKTPSTPSSTQSNSNMASSVSLLPPLMKGARPATEEV--
S. sclerotinia  MQRVQSAVDFSNLLNPSESTAEKRDHSGSPRQQTAQPQQQQQQPQPEADMATVGLLRPNGPLPGAQATEP
N. crassa       MQRAQSAVDFSNLLNPTSAAGQDSGAMSTAAVTVIKPNGPIPGTQSIET---------------------
T. reesei       MQRAQSAVDFSNLLNPTSAAGQDSDAEQGSGAMSTAAVTVIKPNGPIPGAQSTEA---------------
T. harzianum    MQRSQSAVDFTNLLNPSAQADHNSRSRQADAAMASASVTVIKPNGPLPGAQASDT---------------
M. anisopilae   MQRASSAVDFNSLLNPQSTQEREHQAARQKLALIQQQQQHQREAEMAAISMMPAMVGGHHGDD-------
G. fujikkuroi   MQRAQSAVDFSNLLNPTVPADKESEKPHQGDVEMATAAVTVIKPNGPLPGVQNSEN--------------

A. nidulans     RQDLPRPYKCPLCERAFHRLEHQTRHIRTHTGEKPHACQFPGCSKRFSRSDELTRHSRIHNNPNSRRGNK
A. niger        RQDLPRPYKCPLCDRAFHRLEHQTRHIRTHTGEKPHACQFPGCTKRFSRSDELTRHSRIHNNPNSRRRNK
S. sclerotinia  ANELPRPYKCPLCDKAFHRLEHQTRHIRTHTGEKPHACQFPGCSKKFSRSDELTRHSRIHSNPNSRRGNK
N. crassa       ANELPRPYKCPLCDKAFHRLEHQTRHIRTHTGEKPHACQFPGCSKKFSRSDELTRHSRIHSNPNSRRGNK
T. reesei       ANELPRPYKCPLCEKAFHRLEHQTRHIRTHTGEKPHACQFPGCSKKFSRSDELTRHSRIHSNPNSRRGNK
T. harzianum    TNELPRPYKCPLCDKAFHRLEHQTRHIRTHTGEKPHACQFPGCSKKFSRSDELTRHSRIHNNPNSRRGNK
M. anisopilae   RQDLPRPYKCPLCEKAFHRLEHQTRHIRTHTGEKPHACLFPGCTKRFSRSDELTRHSRIHNNPNSRRSNK
G. fujikkuroi   SNELPRPYKCPLCDKAFHRLEHQTRHIRTHTGEKPHACQFPGCSKKFSRSDELTRHSRIHNNPNSRRGNK

A. nidulans     AQHLAAAAAAAAANQDGSAMANN-AGSMMPPPSK-PITRSAPVSQVGSPDISPPHSFSNYANHMRSNLSP
A. niger        AQHLAAAAAAAAGQDNAMANT---ASAMMPPPSK-PMTRSAPVSQVGSPDISPPHSFSNYASHMRSNLGP
S. sclerotinia  GQQQQQHPLVHNHGL---------QPDMMPPPGP-KAIRSAPPTAMSSPNVSPPHSYSPYNFAPSGLNPY
N. crassa       GQQQHQLHHQGMPHPMH-------VDGLMHPPAAPKAIRSAPPSTLVSPNVSPPHSYSSFVMPHGPISHY
T. reesei       GQQQHQQHLHHQGLPHHMH-----VDGMMPPPVP-KAIRSAPTSTLVSPNVSPPHSYSSFVMPQTPMAHY
T. harzianum    GQHHHHQHHHHHHQGLPPHMHH-EGMMAPPPAP-KTIRSAPTSTLASPNVSPPHSFASFAQHPHPPPMH
M. anisopilae   TQQAPQMGVPMHSES---------MATMMPPPNK-NITRSAPPSAIGSPNVSPPHSYTSYSSNHLSSLNP
G. fujikkuroi   AAQAHQQQQHQMHQQQGLPPHMMPDGMMAPPPAP-KTIRSAPGSALASPNVSPPHSYSTFALPVSAVHYN

A. nidulans     YSRTSERASSGM-----DINLLATAASQVERDESFGFRSGQRSHHMYGPRHGSRGLPSLSAYAI-SHSMS
A. niger        YARKGDEASSGM-----ELYLLATAASQVERDEHFDFHAGPRNHHLFSSRHHGSGRLPLLAAYAITHNMS
S. sclerotinia  SHSRSSAGSQSGP----DISLLARAAGQVERDGAAHHHFQPRFQFYGNTLHAATASRNQLPGLQ-AYHMS
N. crassa       GRGN-------------DITMLAKAANQIERETLSGGPSNHNSRHHPYFGQGVPGSRGHPSLS--SYHMA
T. reesei       NRGN-------------DITMLAKAANQIERETLSGGPSNHNSRHHPYFGQGLPNSRGHPPSLS-SYHMA
T. harzianum    PYSRGG-----------DISMLAKAATQVERETLTAPPSHSARHYYGHSMHSSRGHPNGLS----SYHMA
M. anisopilae   YGRSLGGSPNNGQAPLTDINMLATAATQVERDSSTTANHYSQARHQPYYSHSNHNSRTHLPSLQ-AYAMT
G. fujikkuroi   RGG--------------DISMLAKAATQVERETLTAPPHHSNNHRHHPYFGHGMHSSRGHLPTLSSYHMG
```

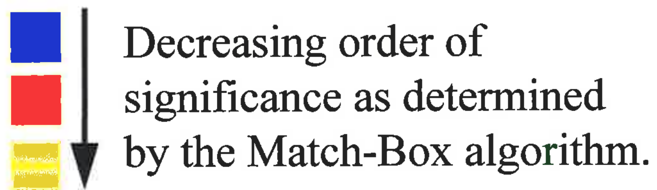
```
A. nidulans     RSHSHEDEDSY ASH---- RVKRSRPNSPNSTAPSSPTFSHDSLSPTPDHTPLATPAHSPRLKPLS PS ELH
A. niger        RSHSPEDDDGY SH----- RVKRSRPNSPNSTAPSSPTFSHDSLSPTPDHTPLATPAHSPRLRPLG SS DLH
S. sclerotinia  RSHSHEDHDDH YGQSYR- HAKRSRPNSPNSTAPSSPTFSHDSLSPTPDHTPLATPAHSPRLRPHP -- GLE
N. crassa       RAHSNDEDDHY HGSLR-- HAKRSRPNSPNSTAPSSPTFSHDSLSPTPDHTPIATPAHSPRLRPFS -- GYE
T. reesei       RSHSNDDDDHY SSMR--- HAKRSRPNSPNSTAPSSPTFSHDSLSPTPDHTPIATPAHSPRLRPFS -- GYE
T. harzianum    RSHSSEDHDDH YNGMR-- HAKRSRPNSPNSTAPSSPTFSHDSLSPTPDHTPIATPAHSPRLRPFS -- GYE
M. anisopilae   RAYSHEEDDHY AHR---- HAKRSRPNSPMSTAPSSPTFSHDSLSPTPDHTPLATPAHSPRLRPYG G- GYD
G. fujikkuroi   RSHSNEDPSDD HYSGAMR HAKRSRPNSPNSTAPSSPTFSHDSLSPTPDHTPIATPAHSPRLRPFS T- GYE

A. nidulans     LPSIRHLSLHH --- TPALAPMEPQAEGP NYYNPNQP------- HVGPSISDIMSRPEGAQRKLPIPQVPK
A. niger        LPSIRHLSLHH --- TPALAPMEPQPEGP NYYSPSQG------- HHGPSISDIMSKPDGTQRKLPVPQVPK
S. sclerotinia  LPPFRNLSLGQ QHT TPALTPLEPALDGQ FSLPQTPPPAPR--- SSGMSLTDIISRPDGTQRKLPVPKVAV
N. crassa       LPSLRNLSLQH NT- TPALAPMEPHLDAP QFHPQLQANTTR--- SPGMSLTDIISRPDGSQRKLPVPQVPK
T. reesei       LPSLRNLSLQH NT- TPALAPMEPHLDAP QFPPQLQANNNR--- SPGMSLTDIISRPDGSHRKLPVPQVPK
T. harzianum    LPSIRNLSLHH NT- TPALAPMEPHLDAP QFPPQLNAPR----- SNGMSLTDIISRPDGAQRKLPVPQVPK
M. anisopilae   LPGIRNLSLHH --- TPALAPMEPQHLDG QYHATSTTTTATSAP RMGLTISDIMSRTDGSTRKLPVPQAPV
G. fujikkuroi   LPSLRNLSLQH NT- TPALAPMEPHLEQN QFQQGSAPTTQPR-- PTGMSLTDIISRPDGSQRKLPVPQVPK

A. nidulans     VAV QDMLNPSGFTSVS-- SSTANSVAGGDLAERF -
A. niger        VAV QDMLNPGSGFSSVH- SSTANSVAGGDLAERF -
S. sclerotinia  QDL LGPADGFNPSVR--- NSSSTSLSGAEMMDRL -
N. crassa       VAV QDLLSDGVFPNSGR- SSTTGSLAGGDLMDRM -
T. reesei       VAV QDLLSDGVFPNSGR- SSTAGSLAGGDLMDRM -
T. harzianum    VAV QDLLSDSGYSNSGR- SSTAGSLAGGDLMDRV -
M. anisopilae   AVQ DLSSPGEIGFNTSGQ SSTTGSVAGNDLADRM I
G. fujikkuroi   VAV QDLLSDNGFSHSGR- SSGTSSLAGGDLMDRM -
```

similarity between these conserved regions and any sequences within the databases. Box 1 contains a high proportion of hydrophobic residues and contains characteristic serine/threonine phosphorylation and asparagine glycosylation sites. Box 4 is highly conserved between the *creA* homologues and is characterised by a high proportion of proline residues. Boxes 5, 6 and 8 all contain potential serine/threonine phosphorylation sites. In addition box 6 contains the region previously identified as having a region of acidic residues. Boxes 9 and 10 have a high proportion of hydrophobic residues. Box 10 also contains a small region which has some similarity to a region in Mig1p which has been shown to required for Mig1p mediated transcriptional repression (Ostling *et al.* 1996). A spatial representation of these conserved regions is shown in figure 4.3.

Comparison of CreA homologues from the two *Aspergillus* species had identified a region consisting of consecutive alanine residues located immediately after the DNA binding domain. Alanine rich regions have been identified in a number of repressor proteins. However alanine rich regions were not present in the CreA homologues from other species. In place of the alanine rich regions the other CreA homologues have stretches of either glutamine or histidine residues. It is not known if these different residues will retain similar functional properties. This region is not identified as a conserved region of the proteins by either CLUSALW or Match-Box analysis.

Figure 4.3 - Summary of conserved region identified in the CreA protein
The eleven boxes identified by Match-Box analysis (figure 4.3) are shown. The colours represent the significance of the aligned sequences. In decreasing order of significance the colours used are blue, red and yellow. Below the protein schematic are solid lines representing regions of the protein previously identified as potential functional domains. With the exception of the region rich in acidic residues they correspond to boxes which have been assigned the highest degree of significance.


1
2
3
4
5
6
7
8
9
10
11
Cys2-His2 zinc fingers
Alanine Residues
Acidic Residues
Rgr1p Similar Region
Similar to Mig1p Repression Domain

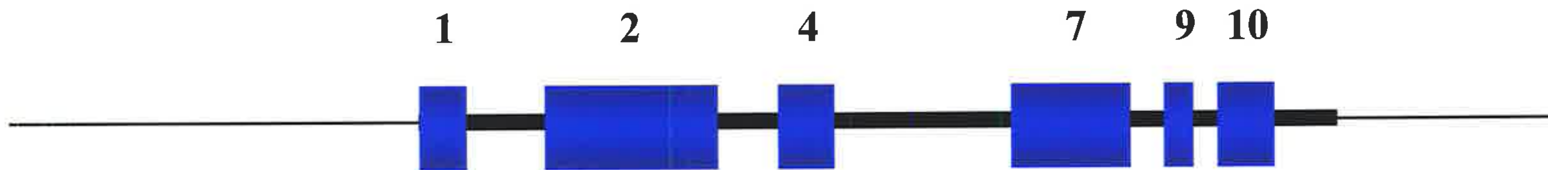

Decreasing order of significance as determined by the Match-Box algorithm.

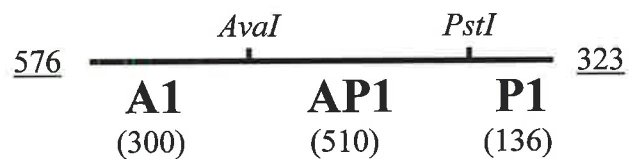
4.2 - PCR-SSCP analysis of *creA* mutants

Single strand conformation polymorphism (SSCP) is a rapid and sensitive method for the detection of base changes in given sequences of genomic DNA (Orita *et al.* 1989). Sequences of interest are amplified and radioactively labelled by the polymerase chain reaction (PCR). The amplified DNA fragments are then denatured and separated by nondenaturing polyacrylamide gel electrophoresis. In nondenaturing conditions, single stranded DNA takes up a folded conformation that is stabilised by intrastrand interactions. Therefore the conformation of a DNA fragment is sequence specific. Fragments of different conformation migrate through the polyacrylamide matrix at different rates. This enables sequence differences to be identified down to the sensitivity of a single base change (Orita *et al.* 1989). PCR-SSCP is a technique that has been widely used to locate the position of mutations in numerous systems.

Two strategies were developed to divide the *creA* gene into fragments suitable for SSCP analysis. The first strategy was to amplify the *creA* gene and promoter as two overlapping fragments. These fragments were then restriction digested with *Pst1* and *EcoRV*. Restriction digested fragments were 550bp or less and spanned all regions of the gene including part of the promoter (figure 4.4). The second strategy was to develop a bank of oligonucleotide primers that enabled the *creA* coding sequence to be amplified in a series of overlapping fragments, which could be directly analysed by SSCP (figure 4.5). A combination of both strategies was used to cover the entire length of the coding and promoter regions. With both strategies a number of steps were taken to minimise the possibility of a false positive result due an incorporation

Figure 4.4 - Strategy 1 for the production of DNA fragments used for SSCP analysis
Two sets of oligonucleotides, 576-323 and 350-590, were used to PCR amplify overlapping DNA fragments which spanned the *creA* gene including 5' and 3' flanking sequences. The fragments were digested with restriction endonucleases to produce fragments of the appropriate size for SSCP analysis. The size of each fragment in base pairs is shown in brackets below each fragment. The position of each of these fragments is shown with respect to the *creA* gene. The coding sequence shows the positions of the most highly conserved boxes, 1, 2, 4, 7, 9 and 10 of CreA (see figure 4.3).


1
2
4
7
9
10


AvaI
PstI
576
323
A1
(300)
AP1
(510)
P1
(136)

PstI
PstI
350
590
PS1
(156)
PS2
(247)

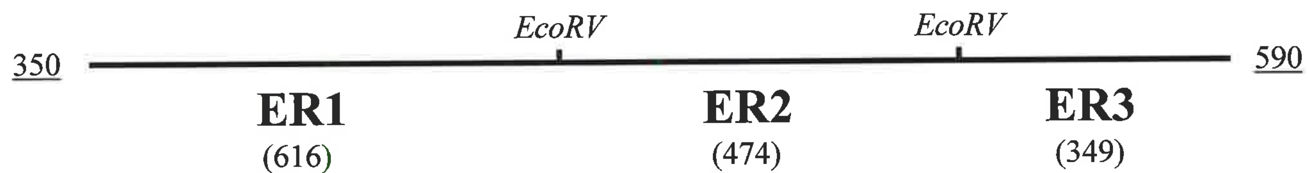
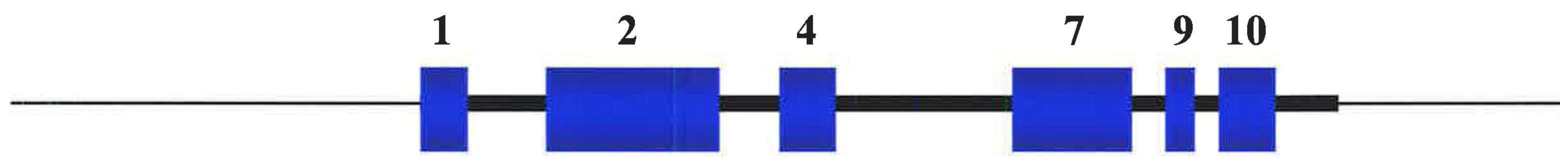

EcoRV
EcoRV
350
590
ER1
(616)
ER2
(474)
ER3
(349)

Figure 4.5 - Strategy 2 for the production of DNA fragments used for SSCP analysis

Sets of oligonucleotides were used to PCR amplify overlapping DNA fragments which spanned the coding regions of the *creA* gene. The fragments generated were directly used for SSCP analysis. The size of each fragment in base pairs is shown in brackets below each fragment. The position of each of these fragments is shown with respect to the *creA* gene. The coding sequence shows the positions of the most highly conserved boxes, 1, 2, 4, 7, 9 and 10 of CreA (see figure 4.3).



350A

350 —— 349
(442)

382A

382 —— 349
(288)

382B

382 —— 348
(638)

863A

863 —— S3
(322)

863B

863 —— 864
(561)

S2A

S2 —— 864
(281)

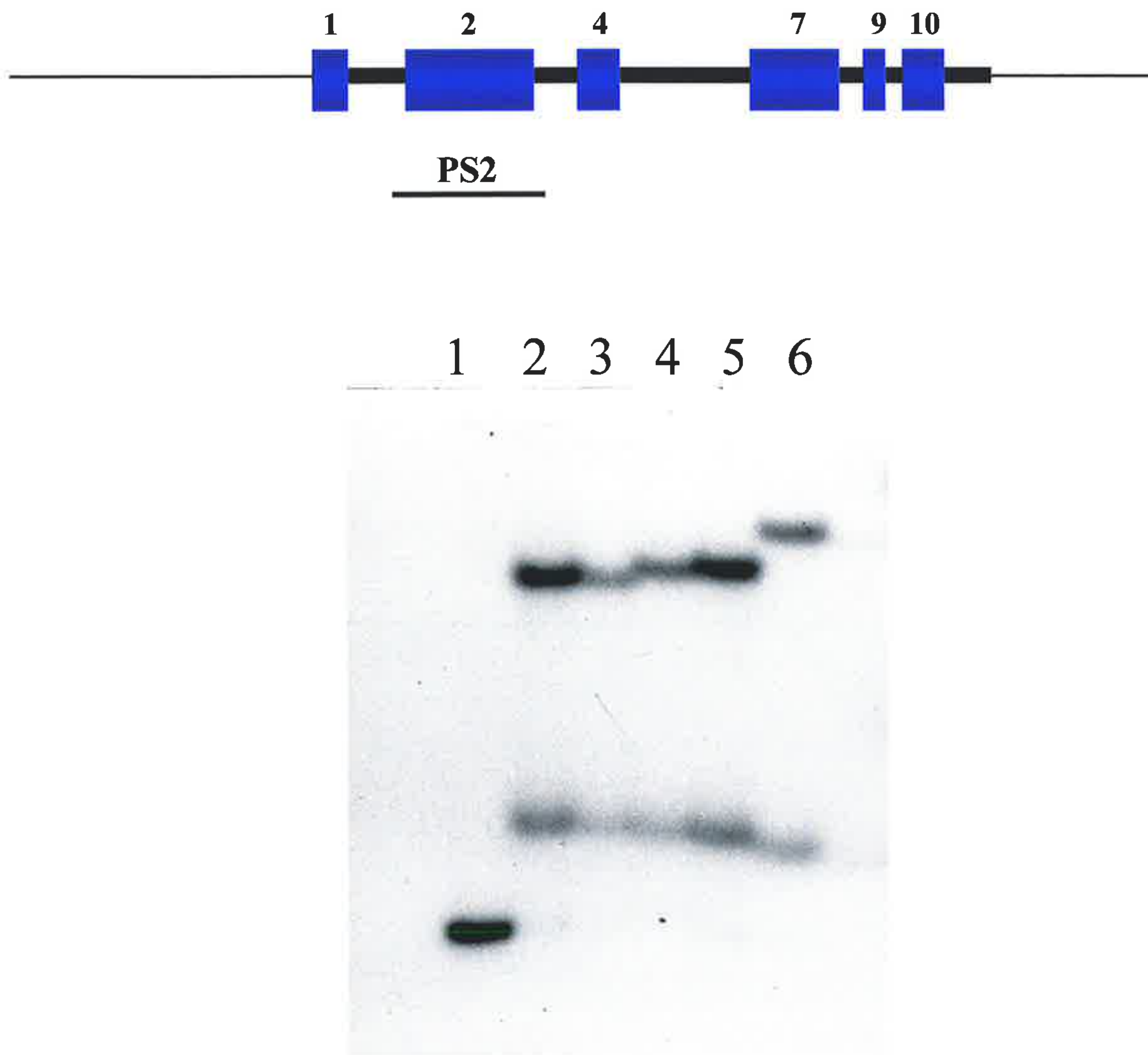
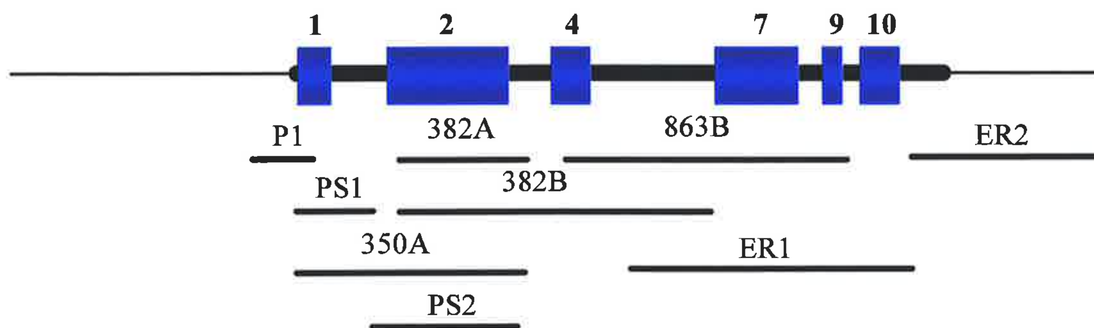


Figure 4.6 - Example of typical SSCP analysis of fragment PS2
Fragment PS2 spans the DNA binding domain. Each track contains 1- wild type non-denatured; 2- wild type; 3-*creA*220; 4- *creA*204; 5- *creA*225; 6- *creA*1. Positive band shifts can be observed in tracks 4, 5 and 6 where the fragments have migrated to different positions in the gel compared to a wild type control (track 2).



Allele	P1	PS1	350A	PS2	382A	382B	863B	ER1	ER2
creA$^+$	✗	✗	✗	✗	✗	✗	✗	✗	✗
*creA*1	✗	✗	✓	✓	✓	✓	✗	✗	✗
*creA*204	✗	✗	✓	✓	✓	✓	✗	✗	✗
*creA*218	✗	✗	✗	✗	✗	✗	✓	✓	✗
*creA*220	✗	✗	✗	✗	✗	✗	✓	✓	✗
*creA*221	✗	✗	✗	✗	✗	✗	✓	✓	✗
*creA*225	✗	✗	✓	✓	✓	✓	✗	✗	✗
*creA*301	✗	✗	✓	✗	nd	✓	✗	✗	✗
*creA*302	✗	✗	✗	✗	nd	✓	✗	✗	✗
*creA*303	✗	✗	✓	✓	nd	✓	✗	✗	✗
*creA*304	✗	✗	✓	✓	nd	✓	✗	✗	✗
*creA*305	✗	✗	✓	✓	nd	✓	✗	✗	✗
*creA*306	✗	✗	✓	✓	nd	✓	✗	✗	✗
*creA*307	✗	✗	✗	✗	nd	✓	✓	✓	✗
*creA*311	✗	✗	✓	✓	nd	✓	✗	✗	✗
*creA*322	✗	✓	✓	✗	nd	✗	✓	✓	✗
*creA*331	✗	✗	✓	✓	nd	✓	✗	✗	✗
*creA*334	✗	✗	✓	✓	nd	✓	✗	✗	✗
*creA*RL	✗	✗	✗	✗	nd	✓	✗	✗	✗

Figure 4.7 - Summary of SSCP results
A summary of results of each mutant allele for the fragments most commonly used in SSCP analysis. ✓ denotes a positive result; ✗ denotes a negative result; nd - not determined.

error during PCR amplification. The major steps taken were to begin all amplification reactions with a minimum of 100ng of genomic DNA and to never undergo more than 25 rounds of amplification. Different *creA* alleles gave positive SSCP results along the entire length of the of the *creA* coding region. An example of SSCP results for the PS2 fragment is shown in figure 4.6. The largest cluster of mutations was in fragments that contained the Cys_2-His_2 zinc finger DNA binding domain (figure 4.7). These alleles were *creA*1, - 204, -
225, -303, -304, -305, -306, -311, -331 and -334. The remaining alleles had mutations dispersed along the entire length of the of the gene (figure 4.7). All DNA fragments that had a positive SSCP result were directly sequenced. Corresponding mutations were identified in all positive SSCP fragments. The DNA sequence changes for all of the *creA* mutant alleles are summarised in table 4.1, and examples of sequencing results for a selection of alleles are shown in figure 4.8.
In order to determine that the system developed to locate the mutation and determine the altered DNA sequence was accurate three alleles, *creA*204, -218 and -221 were sequenced in their entirety. The only DNA sequence changes that differed from the known wild type sequence were those identified by the PCR-SSCP analysis.

The *creA* mutant alleles can be divided into two broad groups. The first group consists of alleles that have either frameshift or nonsense mutations that are predicted to truncate the CreA polypeptide. The remaining *creA* alleles all have missense mutations within the DNA binding domain.

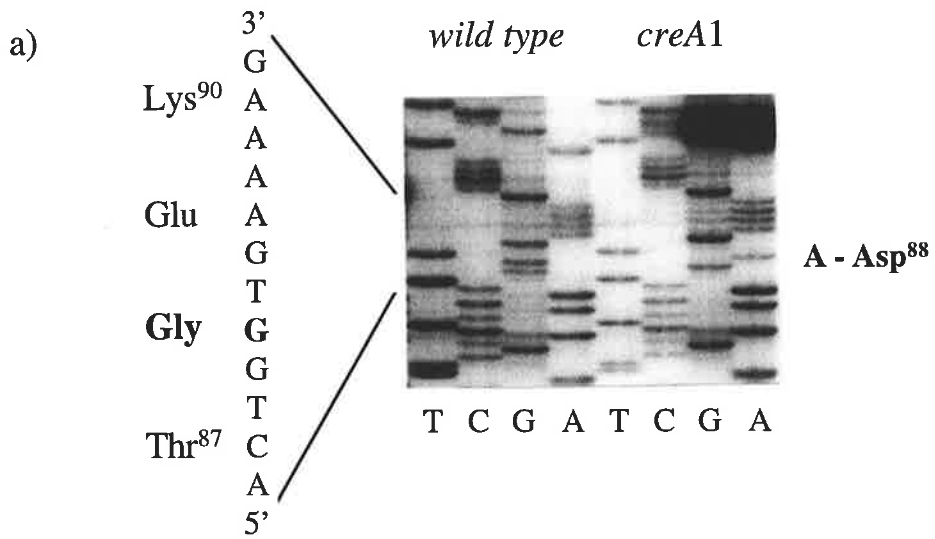
Table 4.1 - DNA sequence change and predicted CreA polypeptides.

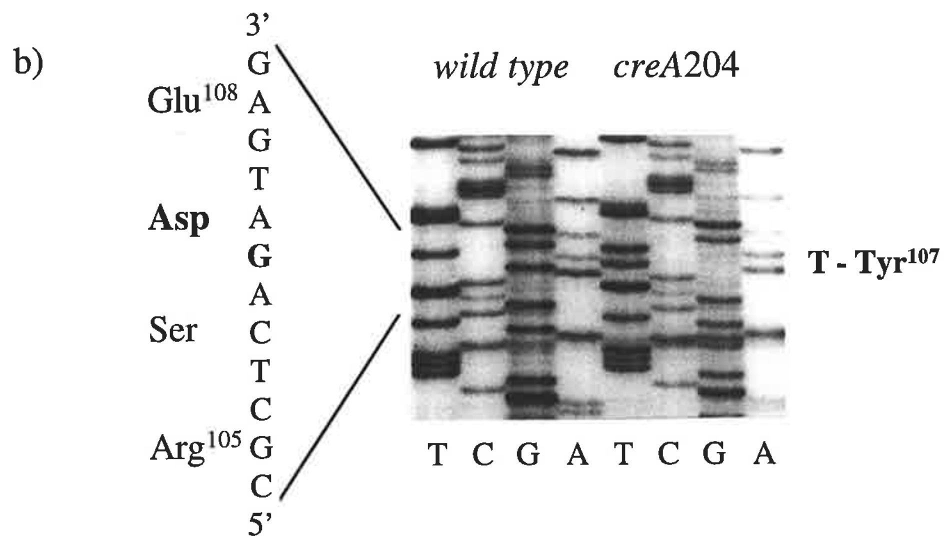
Allele	Mutation	Change to Amino Acid Sequence
*creA*1	G→A at bp 264	G→D at aa 88
*creA*30	pericentric inversion - breakpoint at bp 391	*wt* until aa130 + RDGAALVWLGPLGLLGSIR*
*creA*204	G→T at bp 320	D→Y at aa 107
*creA*218	ΔG at bp 731	*wt* until aa 244 + DFLLFQPTPSPTA*
*creA*220	C→A at bp 813	S→termination codon at aa 271
*creA*221	7bp insertion 'GCGTCAT' between bp 808-809	*wt* until aa 271 + CVTSRQAFKT*
*creA*225	G→A at bp 320	D→N at aa 107
*creA*301	ΔG at bp 424	*wt* until aa 141 +MVARWRTTLDQ*
*creA*302	seven bp Δ bp 522 - 529	wt until aa 174 + PRTLSPTMPTTCARI*
*creA*303	G→T at bp 208	E→termination codon at aa 70
*creA*304	four bp Δ bp 337-340 'TCGC'	*wt* until aa 112 +ESITTPTQDVETRLNTWRQ PPQLQLRTKMVARWRTTLD Q*
*creA*305	'A' inserted between bp 364-365	*wt* until aa 121 +KTWKQGSTPGGSRRSCSCE PRW*
*creA*306	T→C at bp 327	L→P at aa 109
*creA*307	four bp Δ at bp 723-726 'GCAG'	*wt* until aa 240 +AGDFLLFQPTPSPTA*
*creA*311	G→A at bp 243	R→K at aa 81
*creA*322	(i) C→T at bp 108 A→G at bp 112 (ii) ΔC at bp 1010 or 1011	(i) A→V at aa 36 S→S at aa 37 (ii) wt until aa 336 +RLSLQWSPRPRDPIIITRTNL MLAQA*
*creA*331	C→T at bp 251	R→C at aa 84
*creA*334	G→A at bp 243	R→K at aa 81
*creA*401	ΔC at bp 533	*wt* until aa 177 +RTLSPTMPTTCARI*

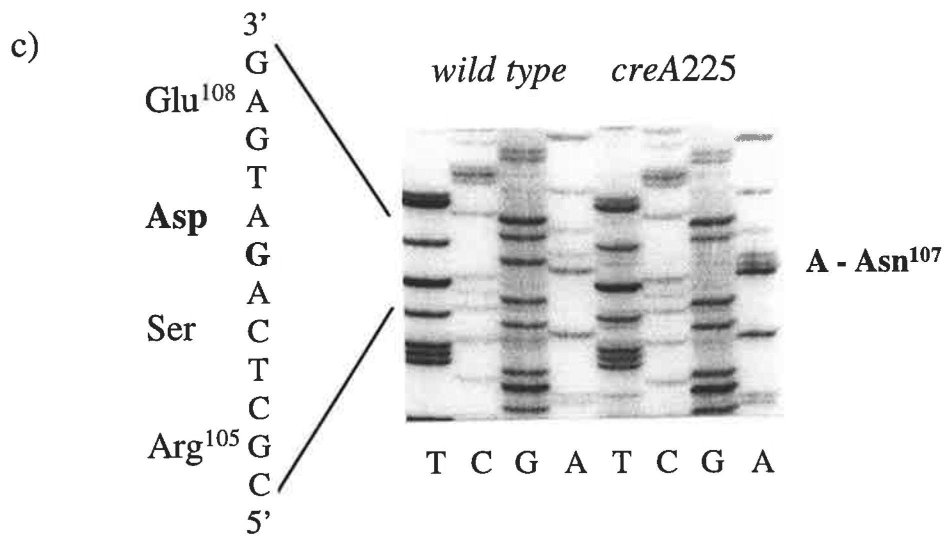
Note: (1) Nucleotide and protein sequence numbering are based on the sequence published in Dowzer and Kelly (1991).
(2) * denotes a termination codon.

Figure 4.8 - Sequence analysis of *creA*1, *creA*204 and *creA*225

(a) *creA*1, (b) *creA*204 and (c) *creA*225 had positive SSCP results in a region of the gene which contained the DNA binding domain (see figure 4.6). Fragment 350A from wild type and the mutant strains were PCR amplified and DNA sequenced. The results of these sequencing reactions are presented including the predicted change in the polypeptide sequence. The coding sequence shows the positions of the most highly conserved boxes, 1, 2, 4, 7, 9 and 10 of CreA(see figure 4.3).


a)
3'
G
Lys90 A
A
A
Glu A
G
T
Gly G
G
T
Thr87 C
A
5'
wild type
creA1
T C G A T C G A
A - Asp88


b)
3'
G
Glu108 A
G
T
Asp A
G
A
Ser C
T
C
Arg105 G
C
5'
wild type
creA204
T C G A T C G A
T - Tyr107


c)
3'
G
Glu108 A
G
T
Asp A
G
A
Ser C
T
C
Arg105 G
C
5'
wild type
creA225
T C G A T C G A
A - Asn107

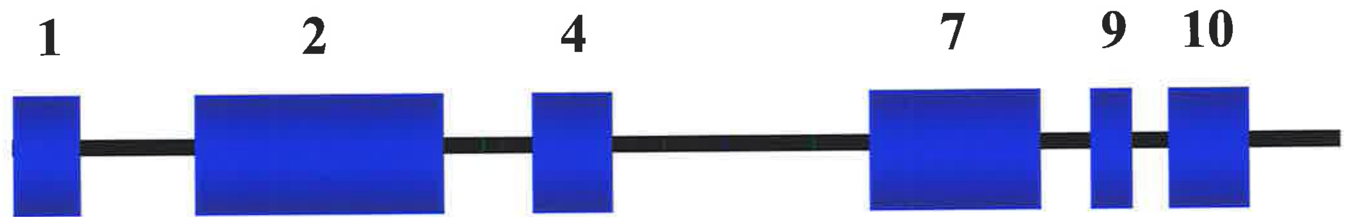
4.3 - *creA* alleles predicted to produce truncated polypeptides

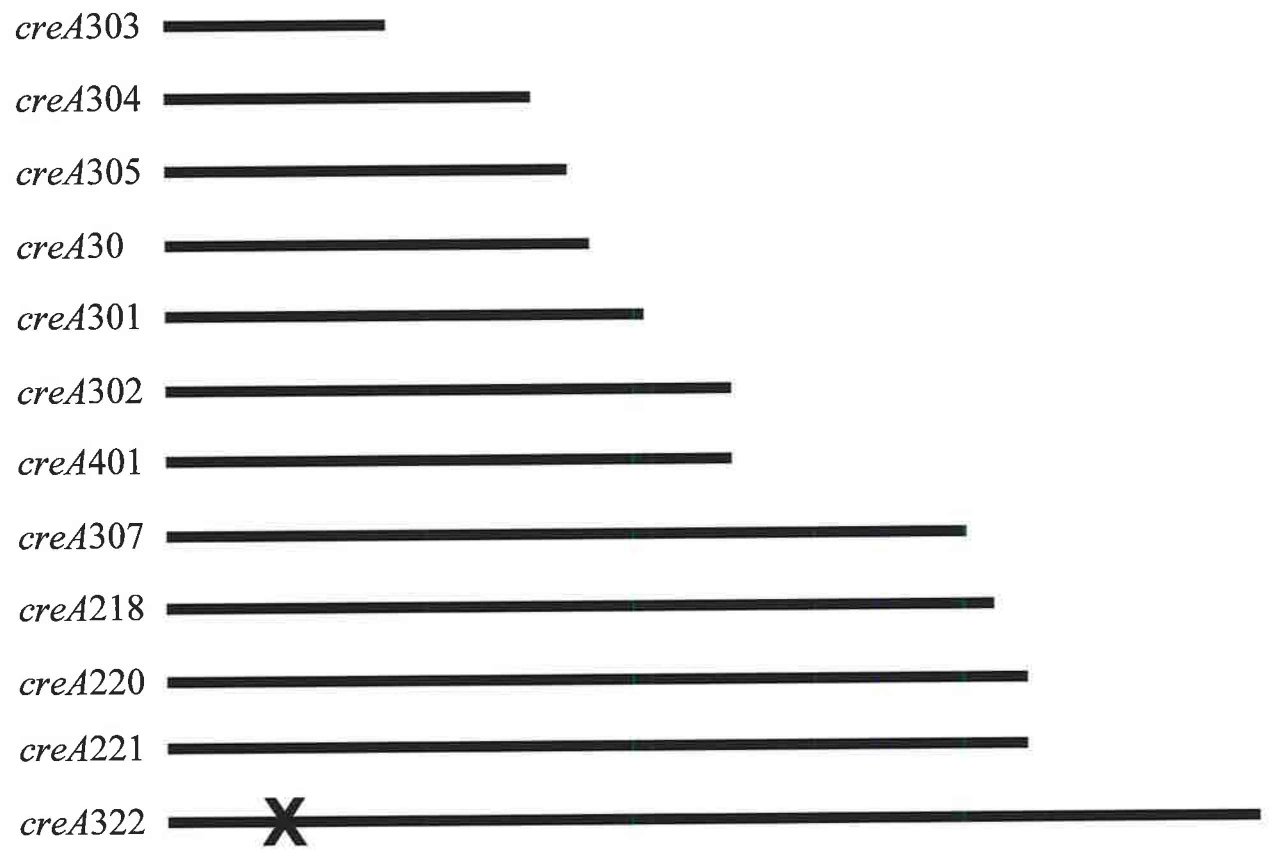
Twelve *creA* alleles had either frameshift or nonsense mutations which are predicted to result in truncated forms of the CreA polypeptide. These alleles had positive SSCP results which spanned the entire length of the gene. The range of truncations of the CreA polypeptide is summarised in figure 4.9. The most extremely truncated polypeptide is produced from the *creA*303 allele and it only retains the first 68 amino acids. Conversely, *creA*322 has a mutation which alters the reading frame after amino acid 336, only losing the C-terminal 80 amino acids.

All frameshift mutations continue in their new reading frame, producing short, novel polypeptide sequences which soon terminate. The longest predicted novel sequence is 39 amino acids and the shortest ten amino acids. None of these novel polypeptide sequences had any similarity to known sequences within the protein databases.

*creA*30 has been genetically characterised and shown to be the result of a pericentric inversion where one of the breakpoints occurs within the *creA* gene (Arst *et al.* 1990). This allele was sequenced to position the breakpoint within the gene and determine if any other open reading frame had been fused to *creA*. The breakpoint occurred at base pair 391. This positions the breakpoint after the DNA binding domain and just before the beginning of the alanine repeat region. The reading frame of CreA30 only continued past the breakpoint for a further 19 amino acids before terminating. These 19 amino acids had no similarity to any sequences in the protein database. It is

Figure 4.9 - *creA* mutant alleles predicted to produce truncated polypeptides
Schematic showing the predicted length of the polypeptides from *creA* mutant alleles which have frameshift or nonsense mutations. The point of truncation is shown relative to the conserved regions of CreA. *creA*322 has point mutations in the N-terminus of the protein. The position of these point mutations is shown by a black cross. The coding sequence shows the positions of the most highly conserved boxes, 1, 2, 4, 7, 9 and 10 of CreA (see figure 4.3).


1
2
4
7
9
10


*creA*303
*creA*304
*creA*305
*creA*30
*creA*301
*creA*302
*creA*401
*creA*307
*creA*218
*creA*220
*creA*221
*creA*322

therefore unlikely that the pericentric inversion has fused *creA* to any other gene in the genome. This effectively places *creA*30 together with other mutations which lead to a truncated polypeptide.

4.3.1 - *creA*303 and *creA*304

A nonsense mutation at base pair 208 results in the CreA303 polypeptide being terminated at amino acid 68. *creA*304 has a four base pair deletion, removing nucleotides 337-340. This deletion results in a frameshift after amino acid 112. The predicted polypeptides from these two alleles are the most extremely truncated proteins in this study. Both polypeptides are truncated within the DNA binding domain. CreA303 truncates the polypeptide early within the first zinc finger, effectively removing the entire DNA binding domain. CreA304 results in the loss of both zinc coordinating histidine residues of the second zinc finger. Without these two histidine residues the second zinc finger cannot coordinate a zinc ion and will not form. The only conserved domains retained by the two predicted proteins is the box one region at the N-terminal region of the protein. In addition CreA304 retains the first of the two zinc fingers. However since this class of DNA binding domain requires at least two zinc fingers to bind DNA it is very unlikely that CreA304 retains any ability to bind DNA and thus it is very doubtful that these two mutant alleles could produce any functional CreA protein.

Strains containing *creA*303 and *creA*304 have an extreme phenotype. They have a slow rate of growth and conidiate very poorly. They also result in high levels of

expression of all carbon repressible genes tested. This extreme phenotype is consistent with the suggestion that *creA*303 and *creA*304 fail to produce any functional protein.

A similar mutation has been identified in Cre1, the *T. reesei* homologue of *creA*. This mutation truncated the polypeptide within the DNA binding domain resulting in a predicted polypeptide of 95 amino acids, predicted to contain only the first of the two zinc fingers. Strains containing this mutation result in very high levels of cellobiose hydrolase I in the presence of glucose (Ilmen *et al.* 1996) .

4.3.2 - *creA*305

*creA*305 has a single base inserted between bases 364 and 365. The predicted CreA305 polypeptide shifts reading frame after amino acid 121. This positions the frameshift point directly after the DNA binding domain. The difference between the polypeptides formed by CreA305 and CreA303/CreA304 is that CreA305 has the potential to form an intact DNA binding domain.

Phenotypically strains containing *creA*305 are very similar to *creA*303 and *creA*304 containing strains. They are morphologically extreme and have high levels of expression of *alcA, amdS, prnD* and α-amylases. However, unlike *creA*303 and *creA*304, strains containing *creA*305 have no detectable levels of β-galactosidase activity. This suggests that the *creA*305 allele produces a protein that retains some function at the β-galactosidase promoter, and thus does not cause a total loss of CreA function.

4.3.3 - *creA*322

*creA*322 has two regions within the gene which contain mutations. The N-terminal region of the gene has two point mutations. One of these mutations is synonymous, while the other is a missense mutation. The missense mutation is a C→T transition at base pair 108. This results in an alanine to valine substitution at amino acid 36. In addition to the N-terminal mutations, *creA*322 has a single base deletion resulting in a shifted reading frame after amino acid 336. These mutations represent the most N-terminal and most C-terminal mutations amongst the *creA* alleles analysed in this study.

Strains containing *creA*322 have weak to moderate phenotypes for most of the systems tested. These strains have significant levels of expression of the genes required for the breakdown of acetamide, proline, ethanol, starch and lactose, but the level of expression is not as high as has been observed in strains containing many of the other alleles. This phenotypic effect is likely to be the result of both mutations, but it is unlikely that each mutation makes an equal contribution towards the phenotype.

The alanine to valine substitution does not occur in any well conserved region within the CreA protein. Additionally, an alanine to valine substitution is a relatively conservative change. Both residues have hydrophobic properties and there is only a

small difference in their sizes. As a result, this mutation is predicted to contribute a very small proportion of the overall *creA*322 phenotype.

The C-terminal mutation results in a frameshift after amino acid 336, resulting in a predicted polypeptide that does not contain the final 80 amino acids of the normal CreA protein. If this mutation is the major contribution to the phenotype of strains containing *creA*322 then the missing 80 amino acids must define a region of the protein required by CreA to repress transcription. The frameshift occurs within the conserved box nine region, and boxes ten and eleven are not present. The presence of a region necessary for repression in this region of CreA is supported by the results of Scazzocchio *et al.* (1995). They described another *creA* allele which resulted in clear derepression of the proline utilisation genes and was shown to be the result of the loss of the C-terminal portion of the protein from residue 361. This mutation would also result in the loss of boxes ten and eleven. However, unlike the mutation characterised by Scazzocchio *et al.* (1995) strains containing the *creA322* allele also lead to derepression of genes involved in the metabolism of compounds which only supply a carbon source to the cell. The repression domain must be either one or a combination of these two conserved regions. Included in box ten is a small region which has sequence similarity to Mig1p which has been shown to be required for repression of glucose repressible systems in *S. cerevisiae* (Ostling *et al.* 1996).

4.3.4 - Other alleles that result in a truncated CreA polypeptide

The remaining alleles which are predicted to form truncated polypeptides cover a large proportion of CreA (figure 4.9). *creA*30 and *creA*301 are predicted to produce polypeptides which retain box one and the DNA binding domain. CreA30 produces a frameshift just before the alanine rich region and CreA301 terminates just after the nine consecutive alanine residues. Strains containing *creA*30 have a more extreme phenotype than strains containing *creA*301. This may be due to the loss of the alanine repeats in CreA30 or may reflect a slightly less stable polypeptide.

CreA302 and CreA401 shift reading frame within the conserved box four region and CreA307 and CreA218 frameshift between box five and box six. The final two mutations are *creA*220 and *creA*221. The CreA220 and CreA221 proteins are predicted to produce polypeptides which terminate or frameshift at the first residue of the Rgr1p similar region. These two polypeptides retain the first six conserved regions of the protein and have lost the 145 C-terminal residues, which includes the Rgr1p similar region and the potential repression domain present in the final 80 amino acids.

Strains containing these alleles have quite mixed phenotypes. There is no obvious correlations between a given phenotype and loss of specific conserved domains. However due to the lack of an effective antibody for CreA, this analysis is complicated by not knowing the relative stability of each predicted polypeptide. It is very unlikely that all truncated polypeptides are unstable, due to the large range of phenotypes observed from strains containing these alleles. Determining the stability of

these polypeptides will no doubt enable some function to be assigned to some of the CreA protein's central conserved regions.

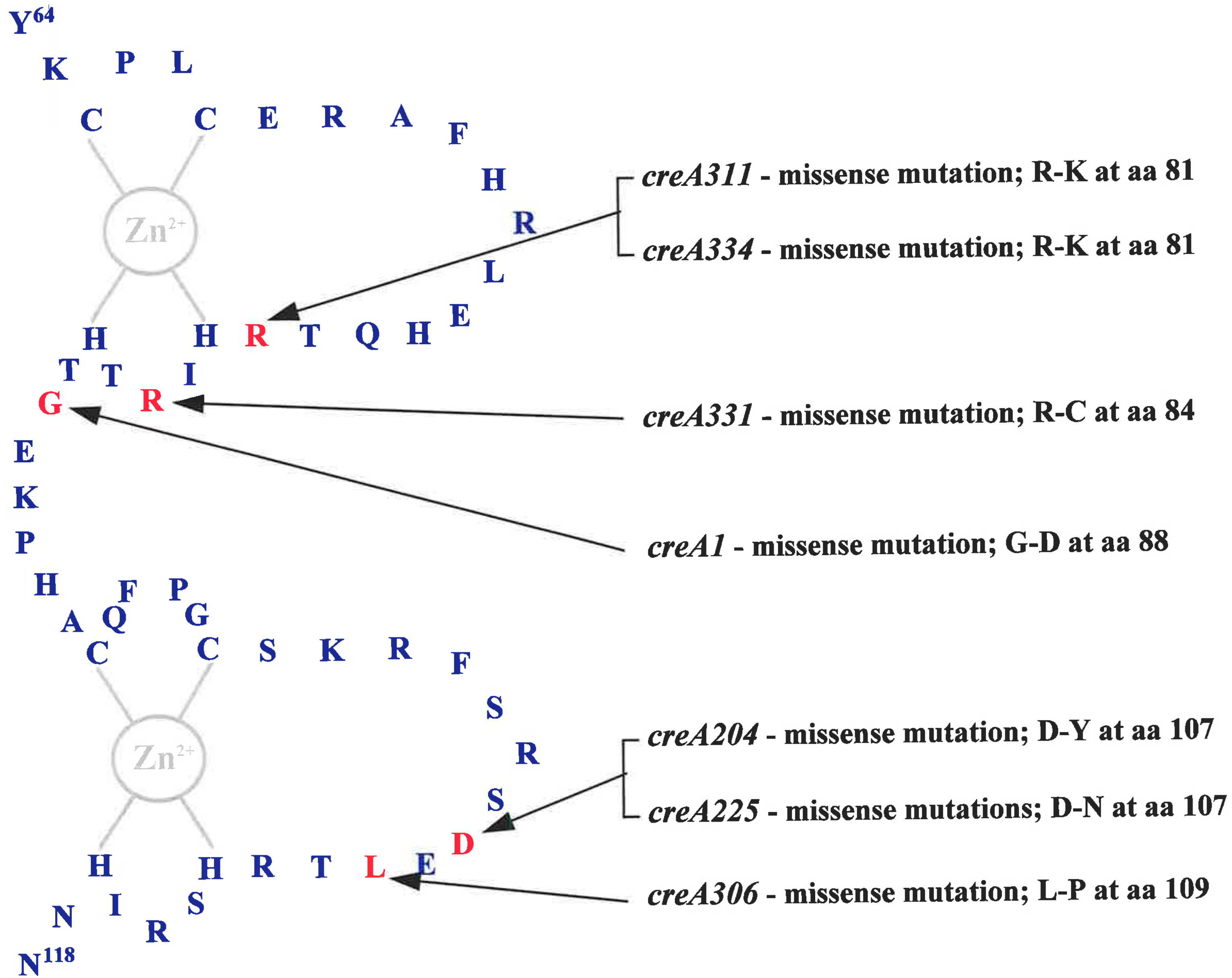
4.4 - *creA* alleles with missense mutations in the DNA binding domain

Ten *creA* alleles produced positive SSCP results for fragments that spanned the Cys_2-His_2 DNA binding domain. One of these alleles, *creA*305, was due to a frameshift mutation just past the DNA binding domain. *creA*303 and *creA*304 were due to a nonsense mutation and a frameshift mutation within the DNA binding domain. These three alleles are predicted to result in a truncated polypeptide and were discussed in the previous section. The remaining alleles have missense mutations within the DNA binding domain. The relative position of each mutation within the DNA binding domain is shown in figure 4.10.

This class of zinc finger DNA binding domain has been extensively characterised by both Nuclear Magnetic Resonance (Párraga *et al.* 1988) and X-ray crystallography studies (Pavletich and Pabo 1991; Elrod-Erickson *et al.* 1996). Based on these studies it is possible to make predictions about the roles played by certain amino acid residues in the process of CreA binding its DNA target (figure 4.11). Numerous residues contact the DNA binding site. The majority of these interactions stabilise the structure by interacting with the phosphodiester backbone of the DNA molecule. A small number of these residues within the α-helix directly bind to specific nucleotides. It is these interactions that determine the specificity of the protein-DNA

Figure 4.10 - Missense mutations within the Cys_2-His_2 zinc finger DNA binding domain

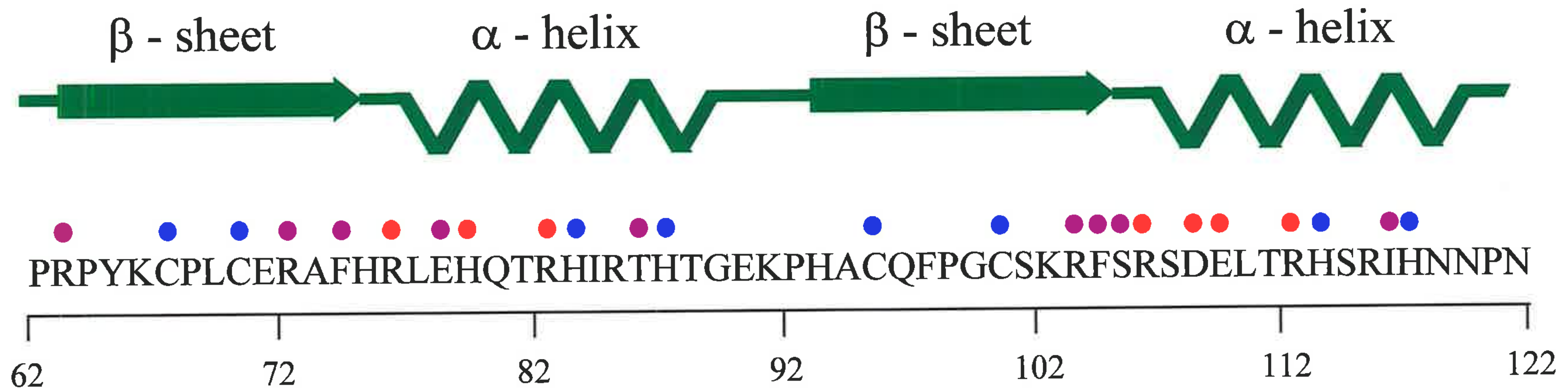
Seven *creA* mutant allele had missense mutations within the DNA binding domain. The position of each mutation is shown by a red residue. All seven mutations occur in the α-helix of the first or second of the two zinc fingers.



Y64
K P L
C C E R A F
H
R
L
E
H R T Q H
T T I
G R
E
K
P
H F P
A Q G
C C S K R F
S
R
S
D
H H R T L E
N I S
R
N118
Zn2+
Zn2+
creA311 - missense mutation; R-K at aa 81
creA334 - missense mutation; R-K at aa 81
creA331 - missense mutation; R-C at aa 84
creA1 - missense mutation; G-D at aa 88
creA204 - missense mutation; D-Y at aa 107
creA225 - missense mutations; D-N at aa 107
creA306 - missense mutation; L-P at aa 109

Figure 4.11 - Critical residues in the CreA Cys_2-His_2 zinc finger DNA binding domain

The critical residues within the CreA DNA binding domain have been predicted based on X-ray crystallography data from the Zif268-DNA interaction (Pavletich and Pabo 1990). Zinc coordinating residues are identified by a blue dot. Residue predicted to form direct hydrogen bonds with a nucleotide in the DNA recognition sequence are identified by a red dot. The residues with a purple dot are predicted to form stabilising hydrogen bonds with the phosphodiester backbone of the DNA strand.



zinc coordinating residue

residue makes contact with base in recognition sequence

residue contacts the phosphodiester backbone

interaction and define the sequence of the DNA binding site. Based on these predictions the effect of each missense mutation on DNA binding can be estimated.

4.4.1 - *creA*311 and *creA*334

The mutant alleles *creA*311 and *creA*334 have the identical mutation, a G→A transition at base pair 243. This mutation results in an arginine to lysine substitution at amino acid 81. An arginine residue at this position forms a pair of hydrogen bonds with a guanine base in the DNA recognition sequence and also makes water mediated contacts with a neighbouring base. Substitution of this residue with the amino acid lysine is a relatively conservative change. Lysine is a slightly smaller residue than arginine, but like arginine it is positively charged. It may still be able to form hydrogen bonds with the DNA recognition sequence. $Arginine_{81}$ occurs at position six along the recognition α-helix of the first zinc finger. Of the residues that specifically bind a nucleotide in the recognition sequence, this position is situated the furthest from the DNA strand (Suzuki 1994). The presence of a smaller residue at this position and subsequent decreased ability to form hydrogen bonds with the DNA recognition sequence is expected to lower the DNA binding affinity of the mutated zinc finger. However, this change is not expected to lead to a total loss of DNA binding and the mutated protein may still be able to function at many CreA regulated promoters.

Strains containing *creA*311 and *creA*334 do not have extreme phenotypes. They have an intermediate growth rate and conidiate normally. In carbon repressing conditions strains containing these alleles lead to relatively low levels of expression for all of the carbon regulated systems tested. The weak phenotype observed for these strains

correlates with the prediction that these zinc finger mutations will only have a minor effect on the ability of the altered protein to bind DNA.

4.4.2 - *creA*331

A C→T transition in the *creA*331 allele results in an arginine to cysteine substitution at amino acid 84. This corresponds to the ninth residue of the α-helix of the first zinc finger. This residue of the zinc finger has a structural role. An arginine residue in this position forms a hydrogen bond with a glycine residue within the H-C link, terminating the α-helix of the first zinc finger. This structure acts to stabilise the second of the two zinc coordinating histidine residues. $Arginine_{84}$ also hydrogen bonds to a region between the β-sheet and the α-helix of the adjacent zinc finger, adding to the stability of the overall binding domain. Substituting a cysteine residue at this position is predicted to disrupt the DNA recognition helix of the first zinc finger and prevent the hydrogen bonding that occurs between the two zinc fingers.

While the mutation in *creA*331 is expected to reduce the stability of the DNA binding domain, it is very difficult to predict the degree to which this change will affect DNA binding affinity. Interestingly, the *creA*331 mutation leads to very heterogeneous phenotypes. An *areA*217 strain containing *creA*331 has quite strong growth on media containing 1% D-glucose and either acetamide, proline or GABA, indicating high levels of expression of the genes required to break down these compounds. However, *creA*331 containing strains are resistant to allyl alcohol levels of 25mM and above. This is the highest levels of allyl alcohol that any *creA* alleles are resistant to, suggesting very low levels of alcohol dehydrogenase I are being produced from this

strain. *creA*331 strains also produce low levels of α-amylase and no detectable levels of β-galactosidase in repressing conditions.

It can be concluded that this mutation leads to a much greater reduction of DNA binding affinity for the promoters of genes which code for proteins that breakdown compounds that can act as both carbon and nitrogen sources. Conversely, the CreA331 mutant protein functions almost as a wild type protein in systems which breakdown compounds that only supply a carbon source. This effect may be coincidental, and may not continue to be observed as more systems are tested. Alternatively, it may reflect that CreA functions quite differently on different promoters.

4.4.3 - *creA*1

*creA*1 has a missense mutation within the H-C link region between the two zinc fingers. This region controls the orientation and spacing of the two adjacent fingers. The H-C link is highly conserved with the consensus sequence TGEKPF/Y. A G→A transition in *creA*1 results in the conserved glycine residue being substituted for an aspartic acid residue at amino acid 88. Glycine in the H-C link forms a hydrogen bond with an $arginine_{84}$ at position nine of the α-helix, stabilising the second of the two zinc coordinating histidine residues of the first zinc finger. Loss of glycine in this position will disrupt the α-helical conformation by preventing its termination at the normal point, disrupting the normal orientation and spacing of the two zinc fingers.

Strains containing *creA*1 are morphologically very similar to wild type, with normal conidiation and only a slightly decreased growth rate. These strains have an

intermediate level of derepression for most of the systems tested. The highest level of expression was observed from the proline utilisation system. Since *creA*1 was selected by suppression of *areA*1 on media containing glucose and proline, a reasonably high level of proline oxidase expression is not unexpected. *creA*1 containing strains produce no detectable levels of β-galactosidase activity and quite low levels of α-amylase activity in repressing conditions. By correlating the phenotypic results and the predicted effect of the *creA*1 mutation it is concluded that the CreA1 mutant polypeptide has a general decrease of DNA binding affinity at most carbon repressible promoters.

4.4.4 - *creA*204 and *creA*225

*creA*204 and *creA*225 are both due to missense mutations in the same base pair within the second of the two zinc fingers. *creA*204 has a G→T transversion resulting in a tyrosine residue being substituted for the wild type aspartic acid at amino acid 107. *creA*225 is the result of a G→A transition that results in an aspartic $acid_{107}$ to asparagine substitution. The wild type aspartic acid residue in this position is highly conserved in Cys_2-His_2 zinc fingers of this type. Mutations of this residue have been characterised on numerous occasions in the Wilm's tumour protein (Pelletier *et al.* 1991). The aspartic acid residue is located at position two of the recognition α-helix and forms a pair of hydrogen bonds with an arginine residue which directly precedes the α-helix. This arginine bonds to a guanine nucleotide in the DNA recognition sequence. The hydrogen bonds formed between the aspartic acid and arginine residues help to orientate the arginine side chain and therefore increase the specificity of the arginine-guanine interaction. In addition, the coupled arginine-aspartic acid residue

pair makes water mediated contacts with the cytosine, which is the complementary base to the guanine nucleotide directly bound by $arginine_{107}$. This interaction will most likely act to ensure that the coupled arginine-aspartic acid residues bind very tightly and specifically to the G/C base pair. A mutation of this aspartic acid is expected to disrupt the α-helix of the second zinc finger and prevent the arginine-guanine interaction and reduce the overall DNA binding affinity of the protein.

Since both alleles are due to different missense mutations at the same position it is possible to directly compare their effect on DNA binding affinity by comparison of their phenotypic effects. In most tests the strains containing *creA*225 had a more extreme phenotype than *creA*204 containing strains. The main exception to this is β-galactosidase activity that is very high in strains containing *creA*204 but could not be detected in *creA*225 containing strains. This suggests that the CreA225 mutation leads to a more general decrease in DNA binding affinity, while the aspartic acid to tyrosine substitution has more specific effects dependent on the specific system being regulated.

4.4.5 - *creA*306

The *creA*306 allele has a missense mutation within the recognition α-helix of the second zinc finger. A T→C transition results in a leucine to proline substitution at amino acid 109. The leucine residue is the fourth residue of the α-helix and forms part of the conserved hydrophobic core that acts to stabilise the zinc finger structure. A proline substitution at this position is predicted to totally disrupt the α-helical structure of the second zinc finger. The side chains of residues in an α-helix project out from the helix and do not interfere with the helical structure, except for proline

where the last atom of the side chain is bonded to the main chain nitrogen atom forming a ring structure. The ring structure prevents the nitrogen atom from participating in hydrogen bonding and also provides some degree of steric hindrance to the α-helical conformation. Proline residues usually produce a significant bend in the helix. Therefore, this mutation is expected to totally destroy the binding properties of the second of the two zinc fingers.

Strains containing *creA*306 had the most extreme phenotype of all alleles tested in this study. These strains have a greatly reduced growth rate and lead to very high levels of expression from almost all carbon repressible systems tested. Surprisingly, the *creA*306 mutation does not result in complete loss of the CreA protein as might have been expected considering the results from *creA*303 and *creA304*. Instead *creA*306 has a missense mutation in the recognition helix of the second zinc finger. There are two potential explanations that could explain how a point mutation in the DNA binding domain would lead to the most extreme phenotypic effects.

The mutation could result in a gain of function if the CreA306 polypeptide is able to bind promoters not normally regulated by CreA and interfere with their normal transcription pattern. If this occurs the extreme phenotype demonstrated by strains containing *creA*306 would be the result of these inappropriately regulated genes. Under this hypothesis the *creA*306 allele may be expected to be dominant. Diploids were constructed that were heterozygous *creA*$^{+}$/*creA*306. These diploids have a wild type morphology, suggesting that *creA*306 is not dominant with respect to the effects on growth rate and conidiation observed for *creA*306 strains. Therefore this explanation is less likely to account for the phenotype of *creA*306 containing strains.

The nature of the mutation, a leucine to proline substitution, is most likely to destroy the α-helix of the second zinc finger and prevent it binding DNA rather than altering its DNA binding specificity.

The more likely explanation is that *creA*306 produces a polypeptide that is full length but with very little or no affinity for DNA binding. As a result this mutation produces a mutated CreA protein that is unable to bind DNA, but can still undergo any normal protein-protein interactions. *creA* is autoregulated. Since *creA* mutations lead to higher expression levels of themselves, *creA*306 will produce more mutant protein and titrate any CreA interacting proteins. Therefore, the extreme phenotype observed from *creA*306 containing strains would be due to the titration of CreA interacting proteins.

4.5 - Summary

All of the *creA* mutations could be categorised into two broad groups.

The largest group consisted of alleles which have either frameshift or nonsense mutations. The polypeptides predicted from these mutants are all truncated in comparison to wild type. Strains containing this group of alleles have greatly reduced conidiation on complete media. Defects in carbohydrate storage and the abnormal formation of cleistothecia were specific to strains containing these alleles.

The most truncated polypeptides are CreA303 and CreA304. Strains containing these two alleles have the most extreme phenotypes of all alleles in this group. The predicted CreA303 and CreA304 polypeptides are unlikely to retain any ability to bind DNA and are the most likely alleles to reflect a complete loss of CreA function. The

least truncated polypeptide is formed from *creA*322. CreA322 retains all the wild type sequence except the last 80 amino acids. In the presence of glucose strains containing *creA*322 result in significant levels of expression of carbon repressible systems. This provides strong evidence that the C-terminal 80 amino acids contain a region, most likely the conserved box ten and eleven regions, required for CreA to repress transcription.

The second category of *creA* mutants all had missense mutations within the Cys_2-His_2 DNA binding domain. With the possible exception of *creA*306, all of the mutants are predicted to produce proteins which retain some degree of function. Based on studies done on other proteins which contain the same class of zinc finger it was possible to rationalise the phenotype of these alleles with the predicted effect on DNA binding affinity. Mutations within the recognition α-helix lead to some decrease in DNA binding affinity and in some cases may also alter DNA binding specificity. A direct correlation exists between decreased binding affinity and severity of phenotype.

Surprisingly, the allele that had the most extreme phenotype was the result of a missense mutation in the DNA binding domain. CreA306 has a leucine to proline substitution within the recognition helix of the second zinc finger. This mutation is predicted to result in little or no capability for DNA binding. Therefore, this allele is the only mutation which produces a full length protein with no ability to bind the normal CreA recognition sequence. However CreA306 would still be available to participate in normal protein-protein interactions. The subsequent titration of interacting proteins could be the basis of the extreme phenotype associated with *creA*306.

Chapter 5

Phenotypic Analysis of *creA* Null Alleles

To define the phenotype of a *creA* null allele a strain was constructed that had the *creA* gene and surrounding sequences replaced by the *riboB*$^+$ gene. Diploid strains heterozygous for this deletion were phenotypically normal. In a haploidisation analysis only *creA*$^+$ haploids were recovered from a diploid heterozygous for the deleted region, indicating that this *creA* deletion results in either an extremely reduced viability or lethality. The deletion allele could also be recovered as a heterokaryon. In a heterokaryon it was possible to differentiate conidia containing either *creA*$^+$ or the deletion of the *creA* region. Microscopic observation of conidia containing a deletion of the *creA* region showed that a conidium germinates and a germ tube extends briefly before growth is halted. This phenotype was referred to a leaky lethal phenotype (Dowzer and Kelly 1991).

Two mutant alleles analysed in this study, *creA*303 and *creA*304, are predicted to produce very little of the CreA protein. They result in truncated polypeptides, where CreA303 only retains the first 68 amino acids and CreA304 has the first 112 amino acids of the wild type polypeptide. These altered polypeptides are both truncated within the DNA binding domain and are not expected to retain any ability for DNA binding. The only conserved region of the protein retained by these two mutants is the box one region.

There are clear phenotypic differences between the *creA* deletion strains constructed by Dowzer and Kelly (1991) and strains containing either *creA*303 or *creA*304. There are two alternative hypotheses that could account for the phenotypic differences between these strains. These two hypotheses are;

a) The N-terminus of the CreA protein is essential for function. This hypothesis implies that in repressing conditions CreA can retain partial function without the DNA binding domain. If this hypothesis is correct it may demonstrate a functional role for the conserved region at the very N-terminal end of the protein, the box one region.

b) The disruption of the *creA* region constructed by Dowzer and Kelly is not an accurate reflection of the true *creA* null allele phenotype.

To differentiate between these two hypotheses a reanalysis of the phenotype of a *creA* null allele was undertaken.

5.1 - Construction of strains containing *creA* disruption alleles

The deletion of the *creA* region described in Dowzer and Kelly (1989) included the coding region in addition to 1 kb 5'and 1.2 kb 3' of the coding sequence. Strains containing two new *creA* disruption alleles were constructed by S. M. O'Connor. Both new *creA* disruption alleles were created by more precise replacement of the *creA* gene with the $argB^+$ gene. Two strategies were developed to create the new *creA* replacement strains. One strategy produces a completely null *creA* allele and the second was designed to mimic the *creA*303 and *creA*304 mutant alleles.

5.1.1 - A *creA* null allele

The first gene replacement was designed to totally prevent any CreA production but not disrupt any sequences 3' of the gene. The *argB*$^+$ gene was inserted at the same position 5' of the gene as the Dowzer and Kelly 1991 deletion construct and terminated in the middle of the *creA* coding region (figure 5.1). Strains containing this construct were obtained with the *argB*$^+$ gene inserted in either orientation; *creA*Δ4 and *creA*Δ21.

5.1.2 - A partially disrupted *creA* allele

The second strategy was designed to mimic the *creA*303 and *creA*304 mutant alleles. In this construct the region of the gene coding for the DNA binding domain was deleted and relaced by the *argB*$^+$ gene (figure 5.1). Therefore this construct retains the promoter and the initial coding sequence. It is predicted that this construct will produce a wild type polypeptide sequence up to amino acid 45. After amino acid 45 the *creA* gene is disrupted by the *argB*$^+$ sequence. The strain *creA*Δ99 contains this construct.

5.2 - Phenotypic analysis of *creA* disrupted strains

Since viable disrupted strains were obtained it is clear that disruption of the *creA* region constructed by Dowzer and Kelly (1991) did not represent a true *creA* null allele phenotype. Phenotypic analysis of the new *creA* disrupted strains was carried

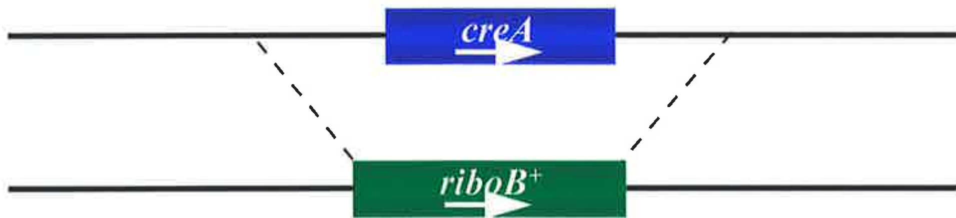
Figure 5.1 - *creA* disruption constructs
Schematic showing the changes in the genome between wild type and the *creA* disrupted strains.

(a) The disruption strain constructed by Dowzer and Kelly (1991). The *creA* gene and flanking regions have been replaced by the *riboB*$^+$ gene. One kilobase of flanking DNA 5' of the *creA* coding region, including the promoter, has been deleted and 1.2 kb of DNA 3' of the transcription termination point has been deleted.

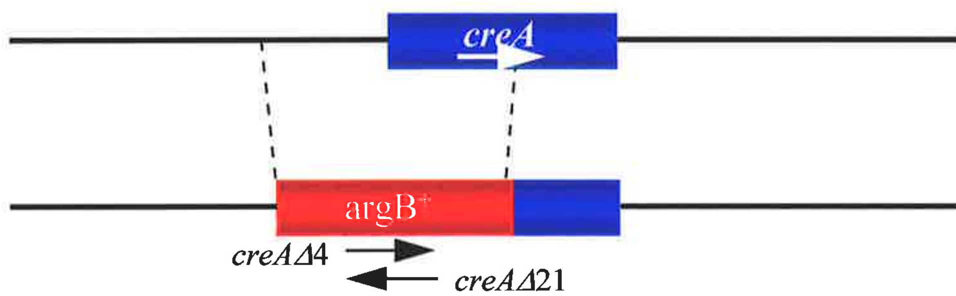
(b) *creA*Δ4 and *creA*Δ21 strains constructed by S. M. O'Connor. These strains have the *argB*$^+$ gene inserted into the *creA* locus. The 5' insertion point is the same as the Dowzer and Kelly (1991) disrupted strain. The 3' insertion point is in the middle of the *creA* coding region. This creates a *creA* null allele. *creA*Δ4 and *creA*Δ21 differ by having the *argB*$^+$ gene in opposite orientations.

(c) *creA*Δ99 strain constructed by S. M. O' Connor. This partial disruption of *creA* was constructed to mimic the *creA*303 and *creA*304 alleles. The *argB*$^+$ gene has been inserted into the *creA* coding region replacing the DNA binding domain. This allele produces a wild type polypeptide up to amino acid 45.

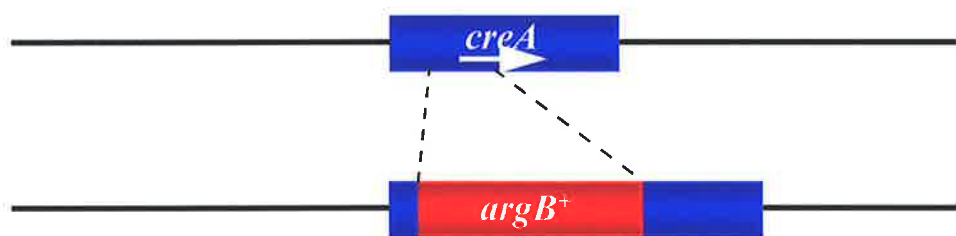
a)



b) *creA*Δ4 and *creA*Δ21



c) *creA*Δ99



out to differentiate between the two hypotheses that were formulated to explain the phenotypic differences observed between the Dowzer and Kelly (1991) deletion strain and strains containing *creA*303 and *creA*304.

Strains containing *creA*Δ4, *creA*Δ21 and *creA*Δ99 are viable, but like other *creA* alleles have pleiotropic effects.

5.2.1 - Morphology of *creA* disrupted strains

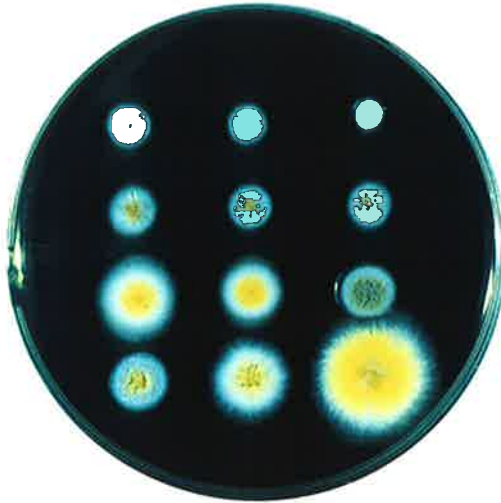
As expected, strains containing *creA*Δ99 had the same morphology on 1% complete medium as strains containing *creA*303 and *creA*304. Strains *creA*Δ4 and *creA*Δ21, containing a null allele of *creA*, also had a similar morphology to *creA*303 and *creA*304 containing strains (figure 5.2 and table 5.1). On 1% complete medium *creA* disrupted strains have a significantly decreased growth rate and conidiate very poorly. Their growth rate is slightly stronger than that of *creA*303 and *creA*304 containing strains. This is probably due to differences in genetic background. This is supported by the fact that a difference in growth rate is seen between *creA*Δ99 strains and *creA*303 and *creA*304. When the *creA* disrupted strains are allowed to grow for 3-4 days they begin to conidiate in the center of the colony. This may reflect the changing supply of carbon source within the growth media. As the colony grows it uses the glucose available within the growth media. As the colony continues to grow the media becomes depleted of glucose until it eventually switches to a derepressing level. This suggests that on 1% complete medium the loss of $creA^{+}$ prevents the

Figure 5.2 - Growth rate and conidiation of *creA* disrupted strains grown on 1% complete medium

(a) The strains were grown at 37°C for 48 hours on 1% complete media. The genotype of each *creA*Δ disruption strain is *creA*Δ, *yA*2, *pabaA*1, *areA*217. The genotype of the remaining strains is *creA*, *yA*1, *adE*20, *su*(*adE*20)*A*1, *riboB*2, *areA*217. The growth rate and degree of conidiation was estimated for each colony and is summarised in table 5.1. The genotype of each colony with respect to the *creA* locus is shown.

(b) The strains were grown at 37°C for 72 hours on 1% complete media. The genotype of each *creA*Δ disruption strain is *creA*Δ, *yA*2, *pabaA*1, *areA*217. The genotype of the remaining strains is *creA*, *yA*1, *adE*20, *su*(*adE*20)*A*1, *riboB*2, *areA*217. The strains were allowed to grow for a longer period of time to develop the full morphological phenotype of these strains. The growth rate and degree of conidiation was estimated for each colony and is summarised in table 5.1. The genotype of each colony with respect to the *creA* locus is shown.

a)



creA303 *creA304* *creA306*

*creA*Δ*4* *creA*Δ*21* *creA*Δ*99*

creA1 *creA204* *creA30*

creA218 *creA322* *creA*$^{+}$

b)

creA303 *creA304* *creA306*

*creA*Δ*4* *creA*Δ*21* *creA*Δ*99*

conidiation process from initiating, but this may be overcome in derepressing conditions.

Table 5.1 - Measurement of growth rate and degree of conidiation of strains containing *creA* mutant alleles.

Strain[1]	Size[2]	Conidiation[2]
creA$^+$	+++++	+++++
*creA*303	++	++
*creA*304	++	++
*creA*Δ4	+++	++
*creA*Δ21	+++	++
*creA*Δ99	+++	++

Note: (1) strains containing *creA*303 and *creA*304 are in a *yA*1, *areA*217, *riboB*1 background and *creA* disrupted strains are in a *yA2, pabaA1, areA217* background.
(2) denotes increasing rate of growth or degree of conidiation -; +; ++; +++; ++++; +++++.
(3) all strains grown on 1% complete media.

As with the mutants predicted to truncate the CreA polypeptide, the *creA* disrupted strains produce abnormal cleistothecia when grown aerobically on complete medium. Cleistothecia do not normally form in this growth condition. Mature cleistothecia do not form and they do not contain viable ascospores. Therefore the loss of *creA*$^+$ results in a signal that triggers some aspect of the sexual cycle. Again, this effect is only observed after at least 3-4 days growth and may be associated with a change from repressing to derepressing conditions.

creA disrupted strains have carbohydrate storage defects similar to those identified in strains that have *creA* mutants predicted to produce truncated polypeptides. However it is an intermediate phenotype. When grown in liquid media rich in carbon a moderate degree of dense bodies are visible within the mycelial mass and the hyphae do not clump as tightly as in wild type strains.

5.2.2 - Effect of *creA* gene disruption on gene expression

Strains containing *creA*Δ4, *creA*Δ21 and *creA*Δ99 were tested to determine the levels of expression of a number of carbon repressible systems. The systems tested were the genes coding for proteins required for the utilisation of acetamide, cellulose, ethanol, lactose, proline and starch. The results are shown in figures 5.3 and 5.4 and in table 5.2. When grown on media containing glucose, strains containing *creA* gene disruptions resulted in high levels of expression of all systems tested. There were no significant differences between strains that contain the *creA* null alleles, *creA*Δ4 and *creA*Δ21, and strains containing *creA*Δ99. However, there were some differences between the *creA* disrupted strains and strains containing *creA*303 and *creA*304. The level of ADH1 expression was relatively high in the *creA* disrupted strains but was lower than in *creA*303 and *creA*304 containing strains which were more sensitive to 0.1 mM allyl alcohol. The *creA* disrupted strains had a higher level of acetamidase expression, than *creA*303 and *creA*304 containing strains. The differences observed between these two strains is most likely due to differences in the genetic backgrounds between the strains. This is supported by the fact that there is no differences observed between the strains containing *creA*Δ4, *creA*Δ21 or *creA*Δ99.

Since most of the phenotypic tests show no significance differences it is highly likely that the phenotypes of strains containing *creA*303 and *creA*304 reflect the phenotype of a *creA* null allele.

Table 5.2 - Growth of *creA*Δ strains on media containing 1% D-glucose and a range of different carbon sources.

Strain[1]	1% D-glucose[2]	1% D-glucose + 10mM Acetamide[2]	1% D-glucose + 10mM Proline[2]	1% D-glucose + 10mM γ-amino butyric acid[2]	1% D-glucose + 10mM β-alanine[2]	1% D-glucose + 0.1 mM Allyl Alcohol[2]	1% D-glucose + 2.5 mM Allyl Alcohol[2]	1% D-glucose + 0.5% lactose + 200 μg/ml X-gal[3]	1% D-glucose + 1% Starch[3]	1% D-glucose + 1% Cellulose[3]
creA$^+$	+++++	+	+	+	+	+++++	+++++	-	-	-
*creA*303	++	+++	++	++	++	+	-	S	S	S
*creA*304	++	+++	++	++	++	++	-	S	S	S
*creA*Δ4	+++	++++	++	++	++	+++	-	S	S	S
*creA*Δ21	+++	++++	++	++	++	+++	-	S	S	S
*creA*Δ99	+++	++++	++	++	++	+++	-	S	S	S

Note: (1) strains containing *creA*303 and *creA*304 are in a *yA*1, *areA*217, *riboB*1 background and *creA* disrupted strains are in a *yA2, pabaA1, areA217* background.
(2) denotes increasing rate of growth and degree of conidiation -; +; ++; +++; ++++; +++++.
(3) size of clear halo or blue colouration: -, none; W, weak; I, intermediate; S, Strong

Figure 5.3 - Suppression of *areA*217 and level of α-amylase expression in *creA*Δ strains

Strains containing *creA* mutant alleles were grown for 48 hours at 37°C on 1% minimal media containing: (a) 1% D-glucose and 10 mM ammonium tartrate (synthetic complete)

(b) 1% D-glucose and 10 mM acetamide

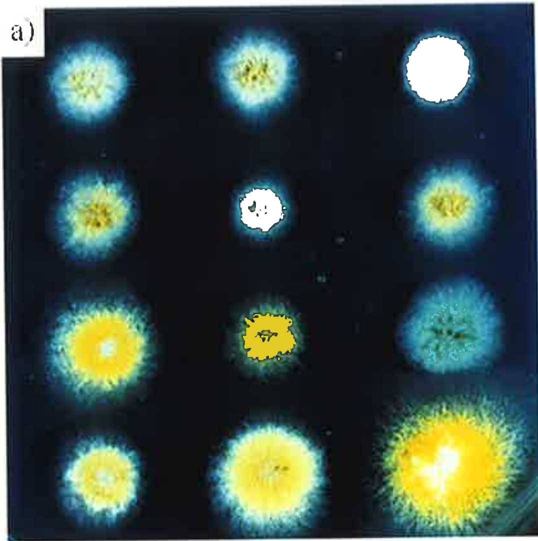
(c) 1% D-glucose and 10 mM L-proline

(d) 1% D-glucose and 10mM β-alanine

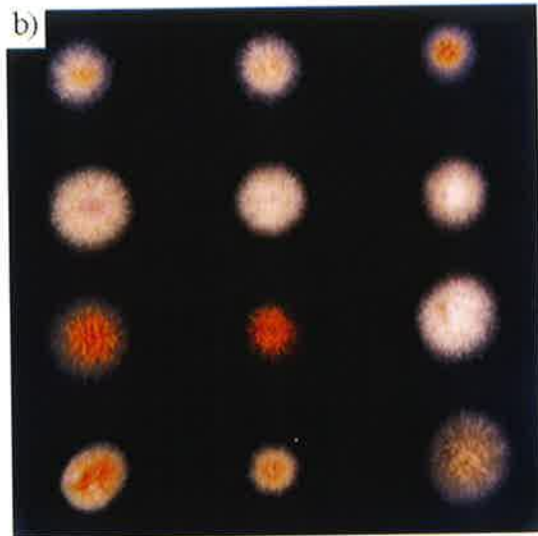
The genotype of each *creA*Δ disruption strain is *creA*Δ, *yA*2, *pabaA*1, *areA*217. The genotype of the remaining strains is is *yA*1, *adE*20, *su*(*adE*20)*A*1, *riboB*2, *areA*217 and contains the *creA* allele shown in (f). The $creA^{+}$ *areA*217 strain is unable to use compounds other than ammonium as a nitrogen source and therefore grow very poorly on plates (b), (c) and (d). A mutation in the *creA* gene is able to suppress this phenotype. The degree of suppression has been estimated and is presented in table 5.2. Suppression is estimated from the amount of growth with respect to the wild type control and compared to the reduced growth rate of each *creA* mutant strain on synthetic complete medium (a).

(e) 1% D-glucose, 1% starch and 10 mM ammonium tartrate. After the strains were grown for 48 hours the plates were flooded with a dilute iodine solution which stains the remaining starch blue. The level of α-amylase expression is estimated from the size of the cleared zone of starch compared to the size of the colony. The genotype of each colony with respect to the *creA* locus is shown (f). The levels of α-amylase expression are summarised in table 5.2.

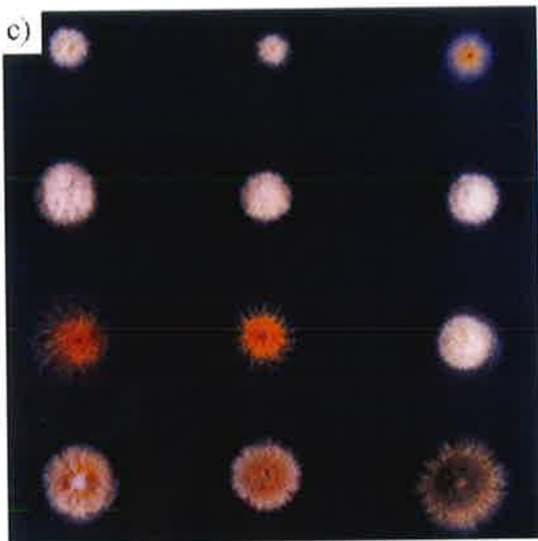
a)



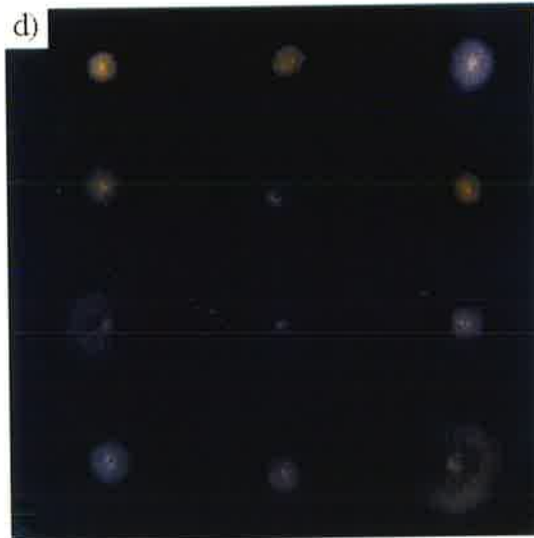
b)



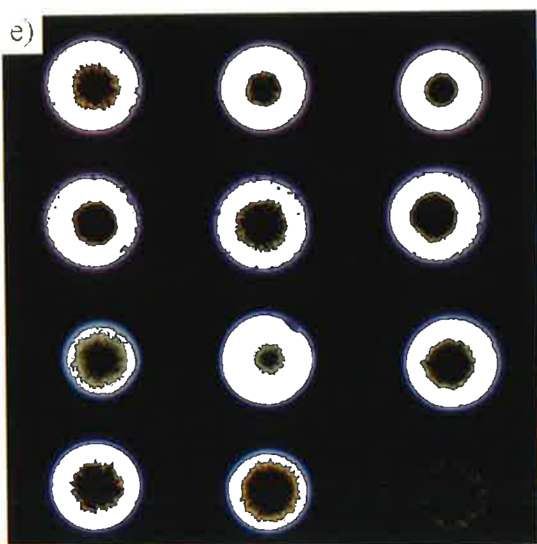
c)



d)



e)



f)

creA303 *creA304* *creA306*

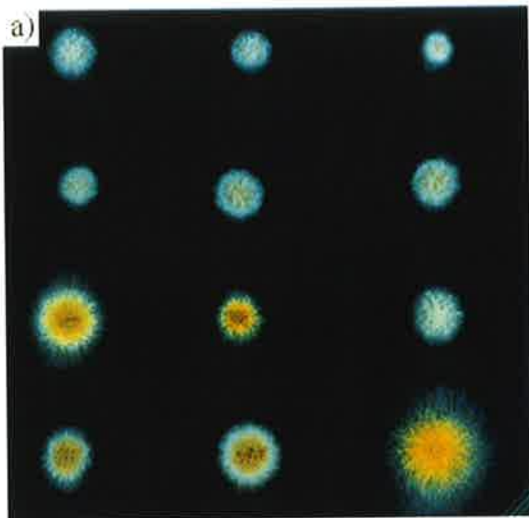
*creA*Δ*4* *creA*Δ*21* *creA*Δ*99*

creA1 *creA204* *creA30*

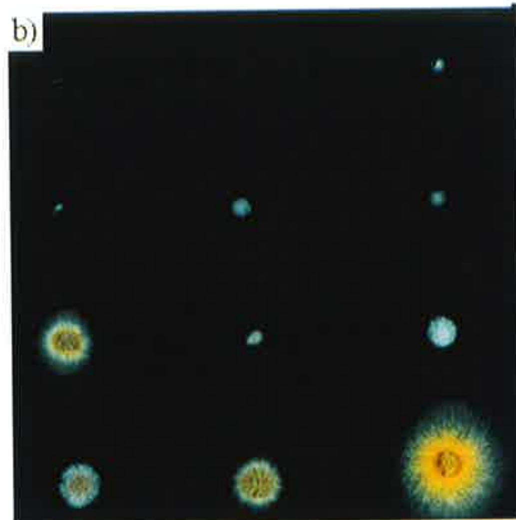
creA218 *creA322* *creA*$^{+}$

Figure 5.4 - Sensitivity of *creA*Δ strains to allyl alcohol
Strains containing *creA* mutant alleles were grown for 48 hours at 37°C on 1% minimal media containing with 1% D-glucose as the carbon source and 10 mM ammonium tartrate as the nitrogen source. Each plate contained different concentrations of allyl alcohol:
(a) 0 mM allyl alcohol
(b) 0.1 mM allyl alcohol
(c) 0.5 mM allyl alcohol
(d) 2.5 mM allyl alcohol
Allyl alcohol can act as a substrate for alcohol dehydrogenase I (ADHI) and is broken down to the toxic compound acrolein. Wild type strains are resistant to allyl alcohol on glucose containing media because ADHI is carbon catabolite repressed. The degree of sensitivity of strains containing *creA* mutant alleles is depends on the level of ADHI expression. The genotype of each colony with respect to the *creA* locus is shown (e). The results of these tests are summarised in table 5.2.

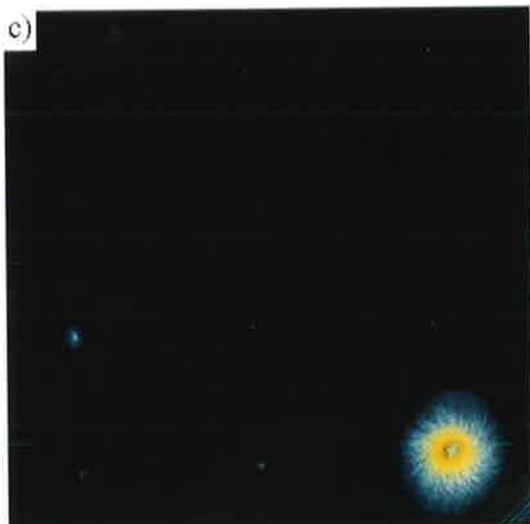
a)



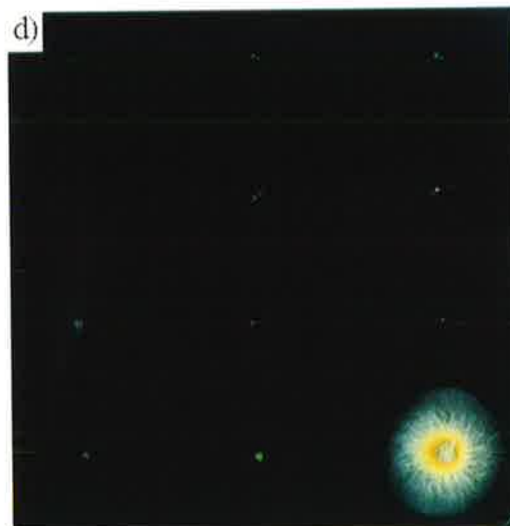
b)



c)



d)



e)

creA303	*creA304*	*creA306*
creAΔ4	*creAΔ21*	*creAΔ99*
creA1	*creA204*	*creA30*
creA218	*creA322*	*creA*$^{+}$

5.3 - What causes the leaky lethal phenotype?

As a *creA* null allele is viable, it remains to be explained why the disruption strain constructed by Dowzer and Kelly 1991 had a leaky lethal phenotype. The *creA*Δ4, *creA*Δ21 and the Dowzer and Kelly construct disrupt the *creA* locus at the same position 5' of the gene. The difference between the constructs is at the 3' end of the gene. In the *creA*Δ4 and *creA*Δ21 constructs the disrupted region of the locus ends in the middle of the coding region of *creA*. In the Dowzer and Kelly construct the disrupted region ends 1.2 kb downstream of the *creA* coding region (figure 5.1). Of this 1.2 kb the majority, approximately 1 kb, had been sequenced and showed no significant features. Subsequent sequencing further 3' of the *creA* gene has identified a large open reading frame. This open reading frame has no similarity to any sequences in the databases (Olesnicky, O'Connor and Kelly pers. comm.). At this time it is not known if the Dowzer and Kelly (1991) disruption construct affects this open reading frame. The *creA* disruption end point is very close to this open reading frame, and further analysis is required to precisely locate the 3' end of the putative gene. It is probable that the deletion will disrupt the three prime untranslated region of any transcript produced from this open reading frame. If this disrupted transcript results in its decreased stability then it is likely that the loss of this gene generates the leaky lethal phenotype observed. *gal*D encodes a galactose-1-P-uridyl transferase and is very closely linked to the *creA* locus. However sequence analysis of the open reading frame shows no similarity to a galactose-1-P-uridyl transferase and therefore is an unlikely candidate.

It is possible that the leaky lethal phenotype is due to the compounded effect of losing both *creA* and the unidentified open reading frame. Suppressors of the leaky lethal phenotype appear quite commonly (Kelly pers. comm.). Interestingly these suppressors have a morphology on 1% complete media similar to wild type and not like the *creA* null phenotype that may have been expected. This may imply an association between *creA* and the unidentified gene. Further analysis is required to determine if such an association exists.

5.4 - Summary

Previous experiments had defined the phenotype of a *creA* disrupted strain as leaky lethal. The leaky lethal phenotype is extreme. Spores cease growth shortly after germination (Dowzer and Kelly 1991). This observation conflicted with results from the most severely truncated mutants, *creA*303 and *creA*304, which were viable. This conflict suggested either the presence of an extremely important domain in the N-terminal region of CreA or alternatively it indicated that the initial analysis of a *creA* disrupted strain did not accurately reflect the true phenotype of a *creA* null allele. To address this two more precise disruptions of *creA* were constructed by S. M. O'Connor. Strains with these disruptions have a phenotype very similar to strains containing *creA*303 and *creA*304. This strongly suggests that *creA*303 and *creA*304 are phenotypically the equivalent of a *creA* null allele. The leaky lethal phenotype observed in the earlier work appears to be the result of disrupting a closely neighbouring gene 3' of the *creA* gene. The close presence of a gene which is required for survival may account for having very few *creA* null alleles from the mutant screens. Any large deletion of this region of the genome would not be viable.

Therefore null alleles could only result from specific mutations, such as *creA*303 and *creA*304, which prevent the production of a functional polypeptide.

Chapter 6

Preliminary Analysis of the Internal Regions of CreA

The analysis of *creA* mutants has clearly demonstrated that the DNA binding domain is functional *in vivo* and indicated the presence of a repression domain in the C-terminal end of the protein. However the series of *creA* mutants predicted to form truncated polypeptides gave no clear information regarding the function of conserved regions in the internal part of the protein.

Analysis of the polypeptide sequences of *creA* homologues showed that some regions of the protein are very highly conserved. The most highly conserved regions are boxes 4, 7 and 9. In addition boxes 3,5,6 and 8 show some similarity between the CreA homologues (see Chapter 4.1). Database searches have resulted in little information regarding the function of these regions. However the high degree of conservation of these regions between distantly related fungal species strongly suggests that they are crucial to the function of the protein.

Previous analysis had indicated that a deletion of the *creA* region resulted in strains that were not viable (Dowzer and Kelly 1991). More precise disruptions of the *creA* locus have demonstrated that a *creA* null allele is viable. Strains containing a *creA* null allele have an extreme phenotype. They have significantly reduced growth rates, conidiate poorly and result in high levels of expression of carbon catabolite repression regulated genes in the presence of glucose. Since a *creA*Δ strain is viable, it is possible to construct internal deletions of the *creA* gene and transform these constructs into a *creA*Δ strain and determine the affect on CreA function.

6.1 - *RGR1* domain swap

Box 7 has similarity to a region of the Rgr1p protein of *Saccharomyces cerevisiae*. This region is very highly conserved between *creA* homologues. There is 81% similarity (33% identity) between the corresponding regions of *Aspergillus nidulans* and *S. cerevisiae* (Drysdale *et al.* 1993). The region has a number of copies of the S/TPXX motif which are conserved in their order and spacing (figure 1.7). There is no similarity between any other regions of Rgr1p and CreA. Although there is a high degree of similarity it is not known if these two regions are functionally homologous.

To test if these regions are functionally equivalent a domain swap construct was made (figure 6.1). An internal deletion of 473 bp was made in the *creA* gene, in pANC4. This deletion removes boxes 5,6,7,8 and 9 of the polypeptide. A region of 302 bp, from base pair 415 to base pair 717, which spans the region of similarity with CreA was PCR amplified from the *S. cerevisiae* genome and fused, in frame, into the position deleted from *creA*. This construct is referred to as pANC4RGR1a. pANC4RGR1a was transformed into the *creA* mutant strains SA4 and SA20. *creA*204 has a missense mutation in the second zinc finger of the DNA binding domain. It is predicted that this mutation reduces the affinity of DNA binding by the CreA204 polypeptide. The hybrid protein has an intact DNA binding domain and is expected to successfully compete for DNA binding against the mutant CreA204 mutant protein. If the domain swap construct encodes a complementing functional hybrid protein then it would be expected to complement function of the mutant strain.

Figure 6.1 - Construction of pANC4RGR1a

(a) Oligonucleotides 724 and 725 were made to PCR amplify the region within *RGR1* which has similarity to CreA. The 474 bp *EcoRV* site was removed from pANC4 and the *RGR1* PCR amplified fragment was liagated into these sites. The fusion was sequenced to ensure that the reading frame was intact and that there were no PCR induced error in the sequence.

(b) The resulting polypeptide produced from pANC4RGR1a showing the conserved regions that have been deleted from CreA and the position of the Rgr1p in frame fusion. The coding sequence shows the positions of the conserved boxes within CreA (see figure 4.3).

a)

EcoRV
EcoRV
BamHI
XbaI
pANC4

RGR1
724
725

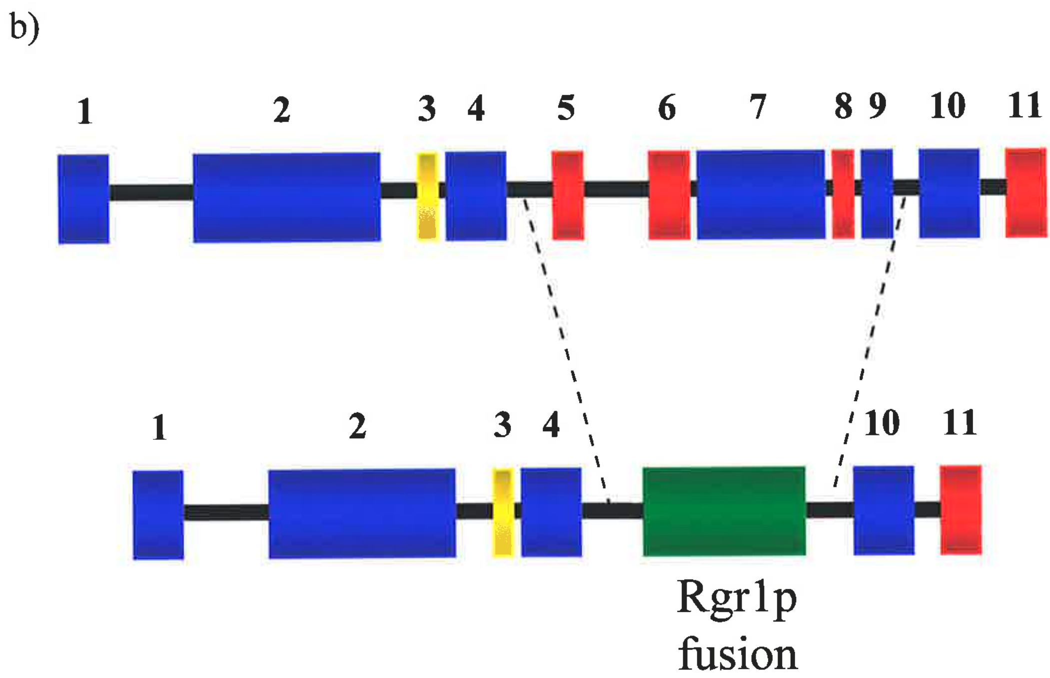
EcoRV restriction digest

PCR amplification across Rgrp1 similar region

Ligation

XbaI
BamHI
pANC4Rgr1a

b)



A deletion strain was not chosen for this experiment since a hybrid protein with an intact zinc finger would be expected to improve the phenotype of a deletion strain regardless of the functionality of the region swapped into the construct.

Transformants were obtained by cotransformation of pANC4RGR1a and pPL3, which contains the *riboB*$^+$ gene. *riboB*$^+$ transformants were selected and southern analysis was used to determine which transformants were cotransformed with the pANC4RGR1a construct. Positive transformants were phenotype tested in both repressing and derepressing conditions. In all conditions tested all pANC4RGR1a transformants had the same phenotype as the SA4 or SA20 host strains. Since the hybrid protein was unable to complement the *creA*204 or *creA*220 mutant phenotype it is likely that the similar domains in CreA and Rgr1p are not functionally homologous. However the regions flanking the Rgr1p similar domain have also been deleted in this construct, and it is possible that the flanking regions are required for the Rgr1p similar domain to function.

6.2 - A internal deletion of the *creA* gene spanning amino acids 207-365

The Rgr1p similar domain is not functionally homologous between the *A. nidulans* CreA and *S. cerevisiae* Rgr1p proteins. However the high degree of conservation between the *creA* homologues implies that it is important for the function of the CreA protein.

An in frame internal deletion of the protein was constructed in order to determine if the conserved internal regions of the protein are required for function. The internal deletion consisted of a 473 bp (158 amino acids) *EcoRV* fragment which was removed from the *creA* containing plasmid to produce a construct referred to as pANC4ΔRV10. Included in the deleted region are five of the conserved regions within the protein, boxes 5,6,7,8 and 9. The resultant polypeptide retains the first 207 amino acids and the final 51 C-terminal amino acids. Therefore the predicted polypeptide retains the conserved regions boxes 1-4, that includes the DNA binding domain, and boxes 10 and 11 that are likely to include the repression domain identified by analysis of *creA* mutants (figure 6.2).

The pANC4ΔRV10 construct was transformed into a strain containing a *creA*Δ allele (*yA*2 *creA*Δ4 *pabaA*1; *riboB*2). Transformants were obtained by cotransformation with pANC4ΔRV10 and pPL3 ($riboB^{+}$) and selecting for complementation of the *riboB*2 mutation. $riboB^{+}$ transformants were screened for cotransformation by PCR analysis. DNA amplification using oligonucleotides 350 and 590 spans the region that includes the region deleted in pANC4ΔRV10. Amplification of $creA^{+}$ produces a fragment of 1438 bp. All cotransformed strains have an amplified fragment of 965 bp that has the 473 bp deleted from it. TMΔ4::ΔRV4, -10, -19, -20 and -23 were all found to be cotransformed with the pANC4ΔRV10 plasmid sequence. TMΔ4::ΔRV21 was not cotransformed with pANC4ΔRV10 (figure 6.3).

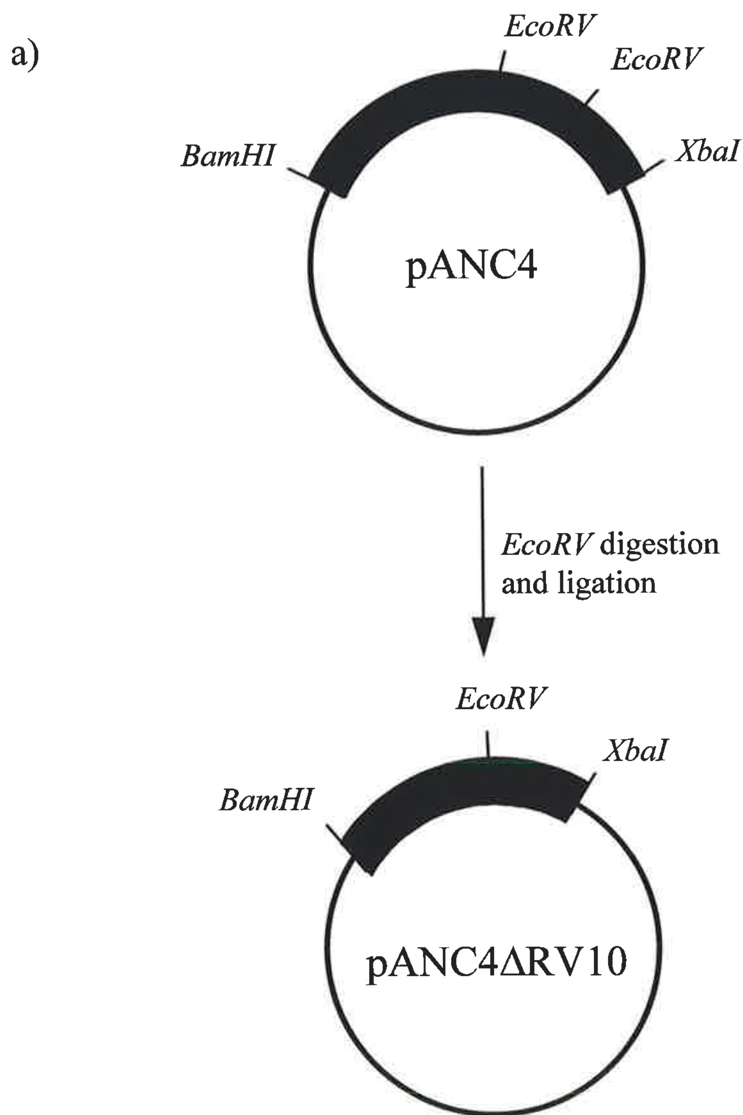
The number of copies of the pANC4ΔRV10 transformed sequence present in each strain was determined by southern analysis of genomic DNA samples digested with

Figure 6.2 - Construction of pANC4ΔRV10

(a) pANC4 was digested with the restriction endonuclease *EcoRV*. The 474 bp *EcoRV* fragment was removed and the remaining plasmid religated. The fusion was sequenced to ensure that the reading frame was intact.

(b) The resulting polypeptide produced from pANC4ΔRV10 showing the conserved regions that have been deleted from CreA. The reading frame is left intact by this deletion. The coding sequence shows the positions of the conserved boxes within CreA (see figure 4.3).

a)



b)

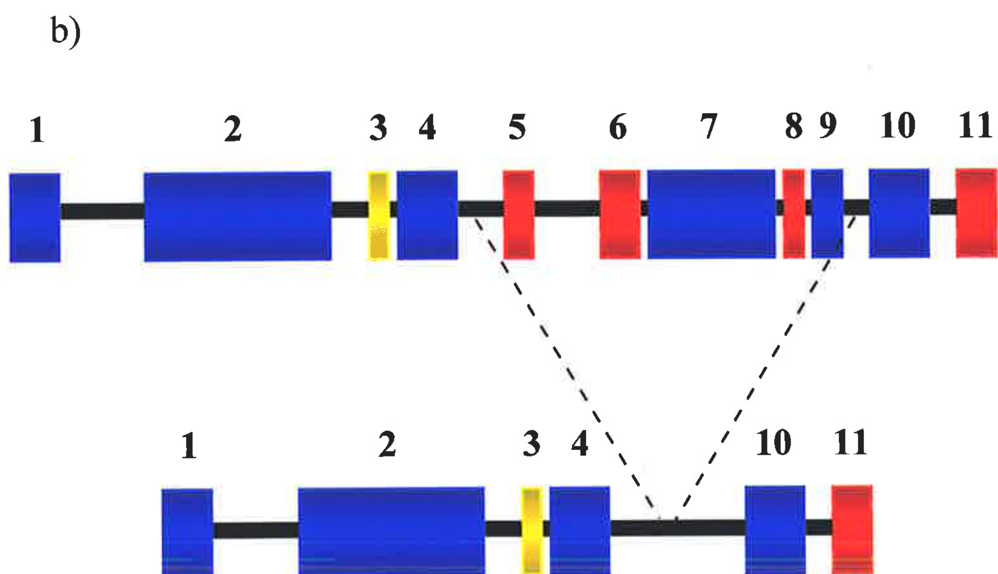


Figure 6.3 - Identification of pANC4ΔRV10 cotransformed strains

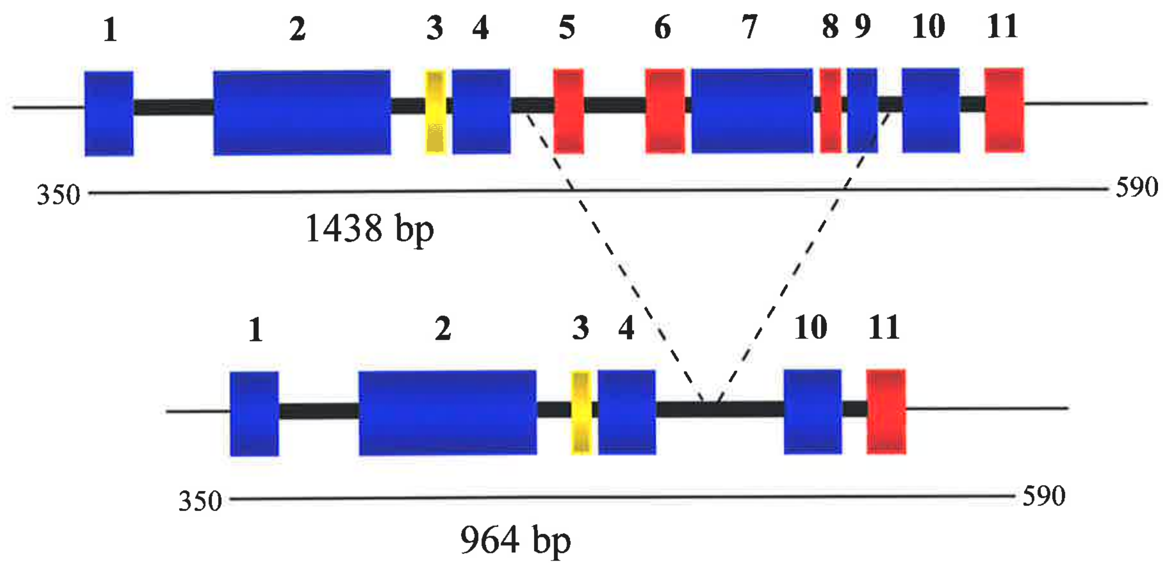
(a) *riboB*$^+$ transformed strains were anlysed by PCR to identify strains which were cotransformed with pANC4ΔRV10. Oligonucleotides 350 and 590 amplifies across the coding region of *creA*$^+$ producing a 1438 bp fragment. Any transformant which contains copies of pANCΔRV10 will result in the amplification of a fragment of 964 bp, due to the deletion of the 474 bp *EcoRV* fragment.

(b) Gel electrophoresis of PCR products from *riboB*$^+$ transformants. Each track contains a sample of PCR products amplfied using oligonucleotides 350 and 590 from genomic DNA samples purified from; 2 - *creA*$^+$, 3 - *creA*Δ4, 4 - TMΔ4::ΔRV4, 5 - TMΔ4::ΔRV6, 6 - TMΔ4::ΔRV10, 7 - TMΔ4::ΔRV19, 8 - TMΔ4::ΔRV20, 9 - TMΔ4::ΔRV21, 10 - TMΔ4::ΔRV23.

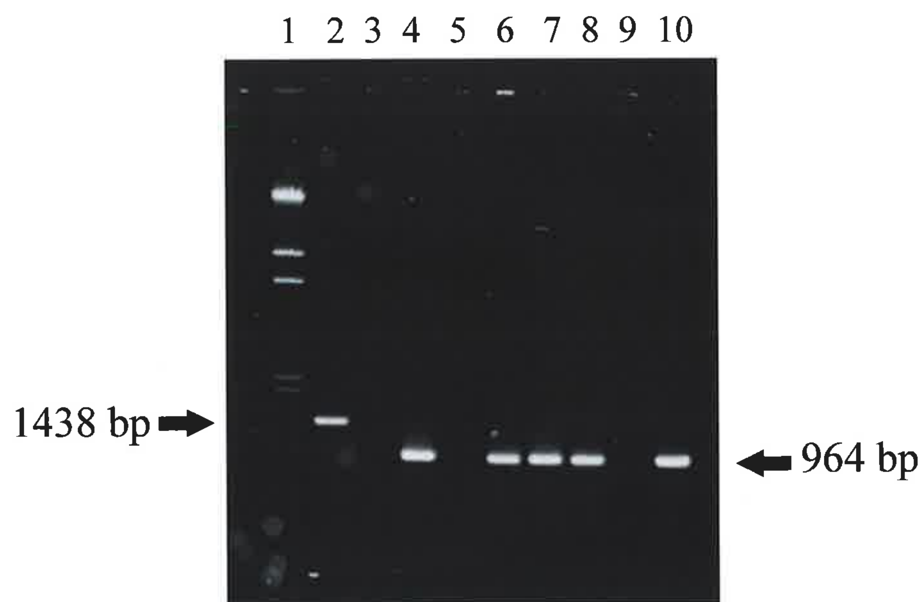
Track 1 contains a sample of λ DNA restricted with *HindIII* and used as DNA size markers. The size of these fragments in decreasing order are; 23.1 kb, 9.4 kb, 6.6 kb, 4.4 kb, 2.3 kb, 2.0 kb and 0.5 kb.

Strains cotransfromed with pANC4ΔRV10 amplify a 964 bp product. *creA*Δ strain and strains not cotransformed with pANC4ΔRV10 did not produce a PCR product.

a)



b)

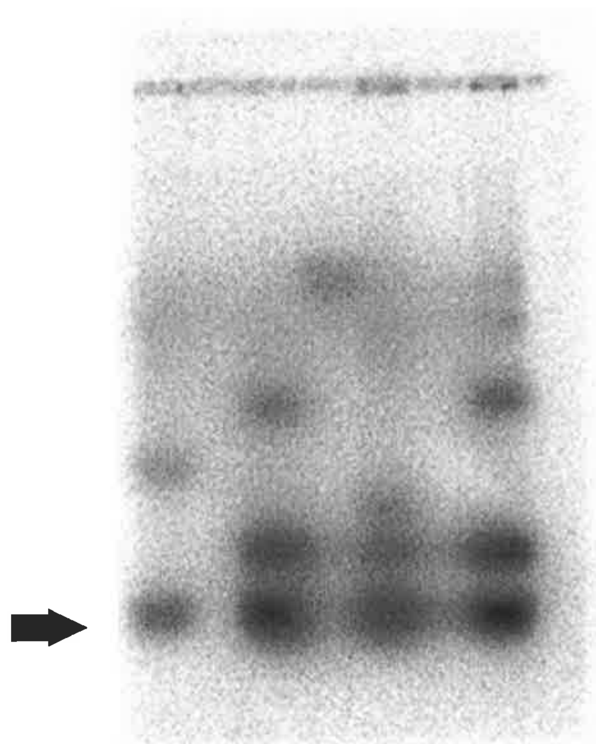


the restriction enzyme *PstI*. *PstI* was chosen because it restricts the *creA* gene internally in a region that is removed in the *creA*Δ allele but is not disrupted in the pANC4ΔRV10 sequence. A 247 bp fragment spanning the DNA binding domain was used as the target for estimating the number of copies of the gene present in each strain. The copy number was estimated from the intensity of hybridisation and standardised against a *creA*$^{+}$ strain using a Fuji 1000 phosphorimaging system. The transformants analysed had up to five copies of the transforming sequence inserted in the genome (figure 6.4).

6.2.1 - Analysis of pANC4ΔRV10 transformed strains in repressing growth conditions

TMΔ4::ΔRV4, -10, -19, -20, -21 and -23 were phenotype tested on media containing 1% D-glucose, repressing conditions. The morphological phenotype was determined by growth on 1% complete media and 1% synthetic complete media. All strains which were transformed with pANC4ΔRV10 demonstrated some degree of complementation. Transformants with 1-2 copies of pANC4ΔRV10 partially complemented the morphology of the *creA*Δ4 host strain. Strains with 3-5 copies of the transforming plasmid appeared to fully complement the morphological phenotype of *creA*Δ strains.

TMΔ4::ΔRV4, -19 and -23 grew and conidiated at similar rates to a *creA*$^{+}$ strain (figure 6.5 and table 6.1). The level of derepression of enzymes involved in ethanol and starch utilisation was determined for these transformants. TMΔ4::ΔRV10 and -20



	Hybridisation Intensity[(1)]	Copy Number
creA$^+$	21	1
TMΔ::ΔRV4	93	4-5
TMΔ::ΔRV10	36	2
TMΔ::ΔRV19	67	3
TMΔ::ΔRV20	38	2
TMΔ::ΔRV23	112	5-6

Note - (1) Hybridisation intensity is calculated in arbitrary units determined using a FUJI 1000 phosphorimager. They refer to the amount of hybridisation intensity in the 247 bp *PstI* fragment (marked by an arrow). Track 2 contains DNA from a *creA*Δ4 strain and was used as background for the calculation of other samples.

Figure 6.4 - Estimation of the number of copies of pANC4ΔRV10 in cotransformed strains.
Each track contains: 1 - *creA*$^+$; 2 - *creA*Δ4; 3 - TMΔ4::ΔRV4; 4 - TMΔ4::ΔRV10; 5 - TMΔ4::ΔRV19; 6 - TMΔ4::ΔRV20; 7 - TMΔ4::ΔRV23
The southern was hybridised with a PCR fragment (oligonucleotides 349-350) which spans the DNA binding domain. Hybridisation intensity for each track was calculated from a 247 bp *PstI* fragment (marked by an arrow) which spans the DNA binding domain.

Table 6.1 - Growth of pANC4ΔRV10 transformants in repressing conditions.

Strains[1]	No. of copies of transforming sequence	Growth rate on 1% Complete Media[2]	Conidiation on 1% Complete Media[2]	1% D-glucose[2]	1% D-glucose + 0.5 mM Allyl Alcohol[2]	1% D-glucose + 2.5 mM Allyl Alcohol[2]	1% D-glucose + 25.0 mM Allyl Alcohol[2]	1% D-glucose + 1% Starch[3]
creA$^+$	na[4]	+++++	+++++	+++++	+++++	+++++	+++++	-
*creA*Δ4	na[4]	+	+	++	-	-	-	S
TMΔ4:: ΔRV4	3-5	+++++	+++++	+++++	+++++	+++++	+++++	-
TMΔ4:: ΔRV10	1-2	+++	++++	++++	++	++	-	W
TMΔ4:: ΔRV19	3-5	+++++	+++++	+++++	+++++	+++++	+++++	-
TMΔ4:: ΔRV20	1-2	+++	++++	++++	++	++	-	W
TMΔ4:: ΔRV21	0	+	+	++	-	-	-	S
TMΔ4:: ΔRV23	3-5	+++++	+++++	+++++	+++++	+++++	+++++	-

Note: (1) all strains are in a *yA*2, *pabaA*1, *riboB*2 background.
(2) denotes increasing rates of growth or conidiation -; +; ++; +++; ++++; +++++.
(3) size of cleared halo -, no colouration; W, weak; I, intermediate; S, strong.
(4) na - not applicable
(5) all growth conditions have 10 mM Ammonium tartrate as the nitrogen source.

Figure 6.5 - Growth rate and conidiation of pANC4ΔRV10 cotransformed strains grown on 1% complete medium

The strains were grown at 37°C for 48 hours on 1% complete media. Each strain is identified in the key below the plate. The growth rate and degree of conidiation was estimated for each colony and is summarised in table 6.1.

TMΔ4::ΔRV4   TMΔ4::ΔRV10   TMΔ4::ΔRV19

TMΔ4::ΔRV20   TMΔ4::ΔRV21   TMΔ4::ΔRV23

creAΔ4   *creA*$^{+}$

showed partial complementation of the derepression phenotype exhibited by the *creA*Δ4 strain. TMΔ4::ΔRV10 and -20 resulted in moderate levels of expression of alcohol dehydrogenase I and α-amylases. These levels of expression are significantly lower than *creA*Δ strains, but higher than $creA^+$ strains (figure 6.6 and table 6.1). Therefore, in repressing conditions, 1-2 copies of the transforming sequence resulted in partial complementation of all *creA*Δ phenotypes tested.

TMΔ::RV4, -19 and -23 have no detectable levels of alcohol dehydrogenase I or α-amylase expression in the presence of glucose (figure 6.6 and table 6.1). These three transformants all have 3-5 copies of the transforming sequence. It appears that, in repressing conditions, 3-5 copies of the transforming sequence is sufficient to fully complement a *creA*Δ4 strain.

pANC4ΔRV10 has over a third of the internal region of CreA removed. Clearly this deleted region is not required for CreA to act as a repressor of transcription. Analysis of *creA* mutant alleles that truncate the CreA polypeptide isolated the repression domain to the 80 C-terminal amino acids. The pANC4ΔRV10 encoded polypeptide only has the final 51 amino acids. Therefore since this construct is able to complement a *creA*Δ strain in repressing conditions the repression domain of CreA must be located within these 51 amino acids.

Multiple copies of pANC4ΔRV4 were required to achieve full complementation of a *creA*Δ strain. There are a number of possible reasons for this. The protein encoded by pANC4ΔRV10 is not wild type. The structure taken up by the mutant protein may be

Figure 6.6 - Growth of pANC4ΔRV10 cotransformed strains in repressing conditions

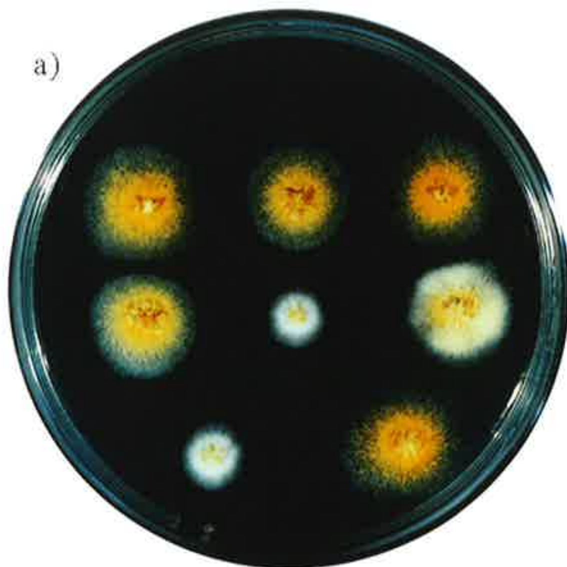
pANC4ΔRV10 cotransformed strains were grown for 48 hours at 37°C on 1% minimal media containing:

(a) 1% D-glucose and 10 mM ammonium tartrate (synthetic complete)
(b) 1% D-glucose, 1% starch and 10 mM ammonium tartrate
(c) 1% D-glucose, 2.5 mM allyl alcohol and 10 mM ammonium tartrate
(d) 1% D-glucose, 25 mM allyl alcohol and 10 mM ammonium tartrate

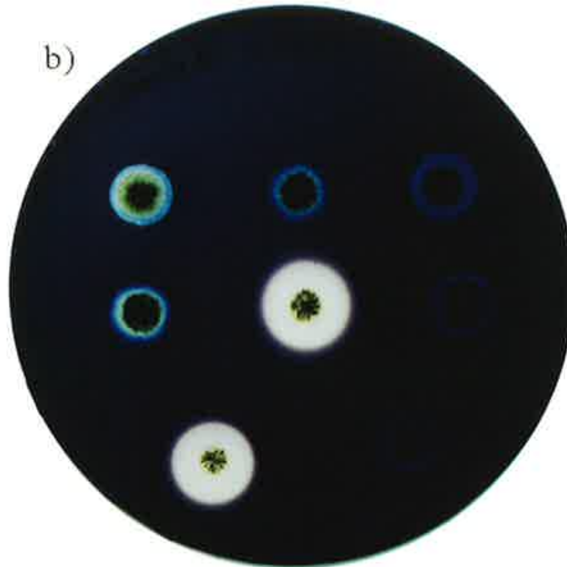
Each strain is identified in the key (e).

The levels of α-amylase expression and resistance to allyl alcohol are summarised in table 6.1.

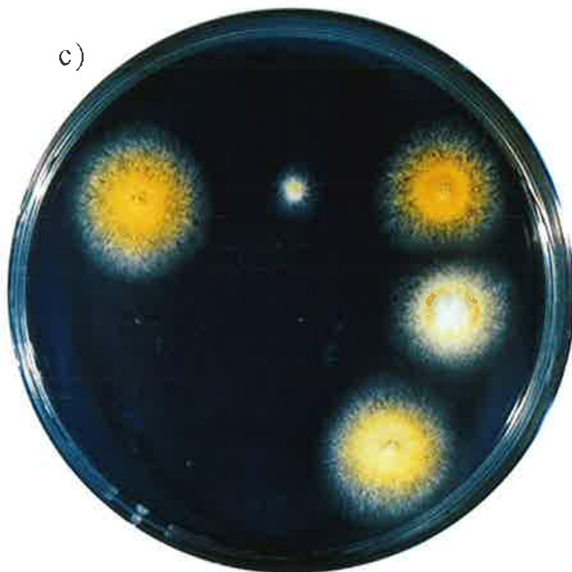
a)



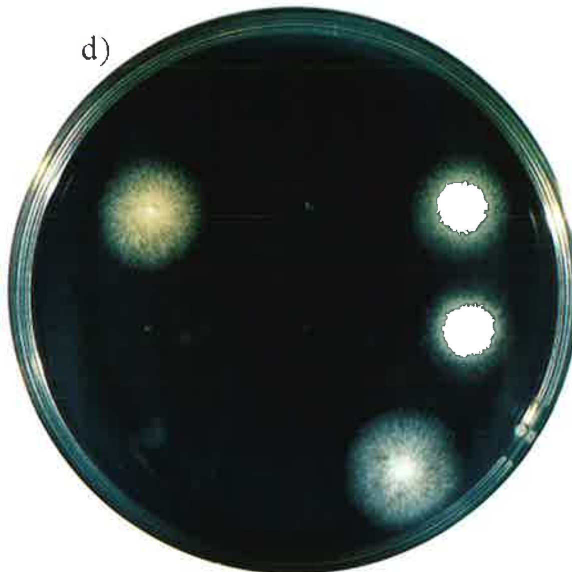
b)



c)



d)



e)

TMΔ4::ΔRV4 TMΔ4::ΔRV10 TMΔ4::ΔRV19

TMΔ4::ΔRV20 TMΔ4::ΔRV21 TMΔ4::ΔRV23

creAΔ4 *creA*$^+$

less stable than the wild type sequence and require higher levels of expression to have the same effect. Also, differential expression can result from the transforming sequences integrating at varying sites within the genome. This may result in extra copies being required to achieve expression patterns similar to wild type.

6.2.2 - Analysis of pANC4ΔRV10 transformed strains in derepressing growth conditions

The same group of transformants were tested in media that contain derepressing carbon sources. TMΔ4::ΔRV10 and -20 partially complemented *creA*Δ4 in repressing conditions. The transformants had a phenotype that corresponds to a weak *creA* mutant allele. These strains often displayed the strongest levels of growth on media with derepressing carbon sources (figure 6.7 and table 6.2). In addition to derepression of enzyme activity strains containing *creA* mutants often result in elevated levels of enzyme activity (see Chapter 3.3.3 and Mathieu and Felenbok 1994). If these two transformants are acting as partial *creA* mutants the improved growth on various carbon sources may be due to elevated levels of enzyme activity often associated with *creA* mutant alleles. This hypothesis is not supported by the phenotypic analysis of strains containing other *creA* mutants. No phenotype was observed from these strains when grown under derepressing conditions. However the *creA* mutant alleles previously analysed had more extreme morphological phenotypes. Therefore it is possible that subtle phenotypic affects were not observed because they were masked by the decreased growth rate associated with these alleles. Due to their

Table 6.2 - Growth of pANC4ΔRV10 transformants in derepressing conditions.

Strain	50 mM Acetamide	50 mM L-proline	1% Ethanol	1% Cellobiose	1% Starch	1% Quinate	1% Glycerol + 0.5mM Allyl Alcohol	1% Glycerol + 1.0 mM Allyl Alcohol	1% Glycerol + 2.5mM Allyl Alcohol
creA^{+}	++++	++++	+++++	+++++	++++	+++++	++	++	+
*creA*Δ4	++++	+++	++	++++	+++	+++	-	-	-
TMΔ4:: ΔRV4	++	+	++	+++	++	++++	+++++	+++++	+++++
TMΔ4:: ΔRV10	++++	+++++	+++	+++++	++++	+++++	+	+	+
TMΔ4:: ΔRV19	++	++	+++++	++++	+++	+++++	++++	++++	++++
TMΔ4:: ΔRV20	+++++	+++++	+++	+++++	+++++	+++++	+	+	-
TMΔ4:: ΔRV21	++++	+++	++	++++	++++	+++	-	-	-
TMΔ4:: ΔRV23	++	+	+++	++++	+++	++++	++++	++++	++++

Note: (1) all strains are in a *yA*2, *pabaA*1, *riboB*2 background.
(2) denotes increasing rates of growth or conidiation -; +; ++; +++; ++++; +++++.
(3) all growth conditions have 10 mM Ammonium orthophosphate as the nitrogen source.

Figure 6.7 - Growth of pANC4ΔRV10 cotransformed strains on media with non-repressing carbon sources

pANC4ΔRV10 cotransformed strains were grown for 48 hours at 37°C on media containing:

(a) 1% starch and 10 mM ammonium orthophosphate.

(b) 1% cellobiose and 10 mM ammonium orthophosphate.

(c) 50 mM acetamide and 10 mM ammonium orthophosphate.

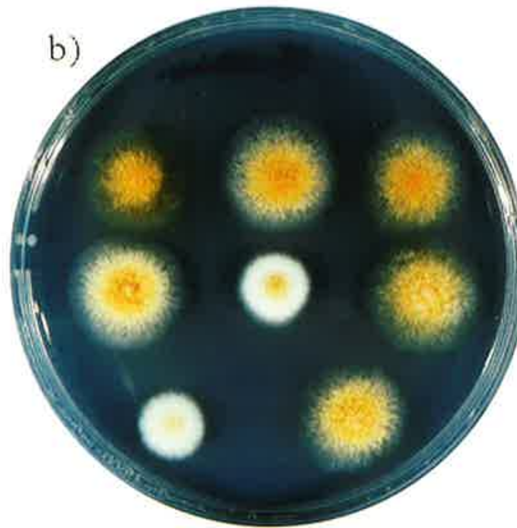
(d) 50 mM L-proline and 10 mM ammonium orthophosphate.

Each strain is identified in the key (e).

The level of growth of each strain is summarised in table 6.2.

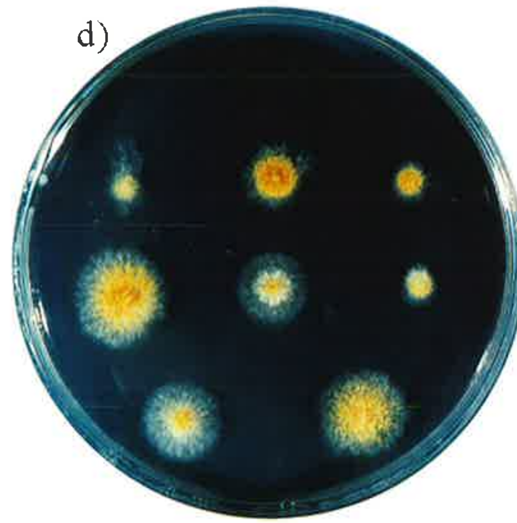
a)

b)



c)

d)



e)

TMΔ4::ΔRV4 TMΔ4::ΔRV10 TMΔ4::ΔRV19

TMΔ4::ΔRV20 TMΔ4::ΔRV21 TMΔ4::ΔRV23

creAΔ4 *creA*$^{+}$

relatively higher growth rates TMΔ4::ΔRV10 and -20 may be the first examples where a phenotype in derepressing growth conditions has been identified.

Transformants with 3-5 copies of pANC4ΔRV10 fully complemented *creA*Δ4 strains in repressing conditions. However, on media containing derepressing carbon sources, TMΔ4::ΔRV4, -19 and -23 were the poorest growing strains. TMΔ4::ΔRV4 was generally the poorest growing strain of this group (figure 6.7 and table 6.2). pANC4ΔRV10 transformed strains were grown on media containing 1% glycerol and varying concentrations of allyl alcohol (figure 6.8 and table 6.2). Media containing 1% glycerol are carbon derepressing and also lead to the induction of alcohol dehydrogenase I expression. Expression of alcohol dehydrogenase I results in toxicity due to the presence of allyl alcohol in the media. Therefore *creA*$^{+}$ strains are sensitive to the allyl alcohol in the growth media and grow poorly. *creA*Δ4 strains are also sensitive to the allyl alcohol in this media because they lead to derepression of *alcA* in all growth conditions. TMΔ4::ΔRV4, -19 and -23 were much more resistant to allyl alcohol than *creA*$^{+}$ strains in the presence of 1% glycerol. TMΔ4::ΔRV10 and -20 were very sensitive to allyl alcohol in these growth conditions, again having a phenotype equivalent to a weak *creA* mutant allele.

pANC4ΔRV10 has an internal deletion of 158 amino acids. Strains transformed with a high number of copies of this plasmid grew poorly on media containing derepressing carbon sources. Therefore it can be concluded that the 158 amino acids are required for the expression of carbon catabolite repression regulated genes in derepressing conditions. CreA may have to be inactivated to switch between the repressing and

Figure 6.8 - Growth of pANC4ΔRV10 cotransformed strains in derepressing conditions

pANC4ΔRV10 cotransformed strains were grown for 48 hours at 37°C on media containing:

(a) 1% Glycerol and 10 mM ammonium orthophosphate.

(b) 1% Glycerol, 0.5 mM allyl alcohol and 10 mM ammonium orthophosphate.

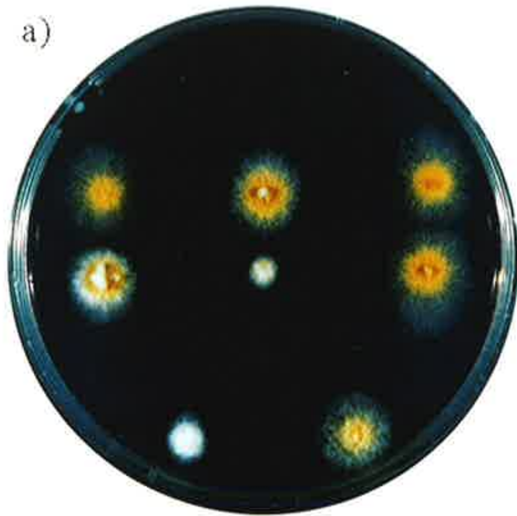
(c) 1% Glycerol, 1.0 mM allyl alcohol and 10 mM ammonium orthophosphate.

(d) 1% Glycerol, 2.5 mM allyl alcohol and 10 mM ammonium orthophosphate.

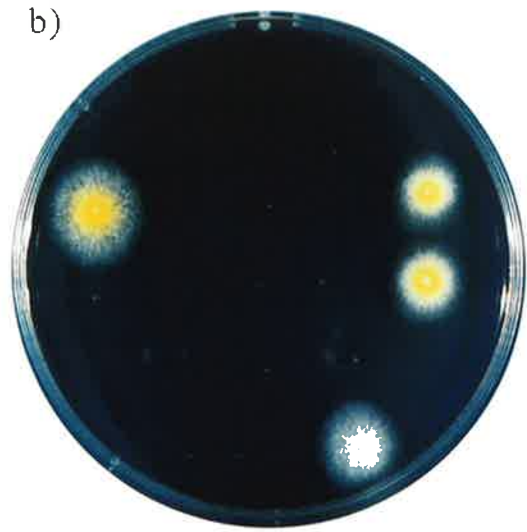
Each strain is identified in the key (e).

1% glycerol is a non-repressing carbon source and induces ADHI expression. Therefore a *creA*$^{+}$ strain is sensitive to growth on allyl alcohol on media that contains 1% glycerol as the sole carbon source. TMΔ4::ΔRV4, -ΔRV19 and ΔRV23 grew at increased rates compared to wild type indicating lower levels of ADHI expression. TMΔ4::ΔRV10 and -ΔRV20 din not grow at any of the allyl alcohol concentrations used suggesting that these strains are producing ADHI at much higher levels than wild type.

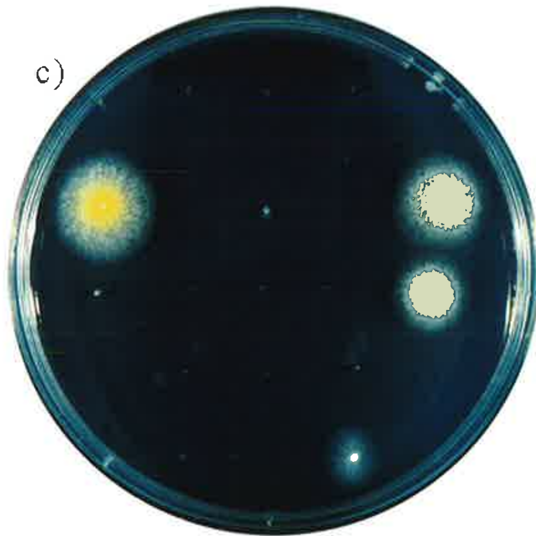
a)



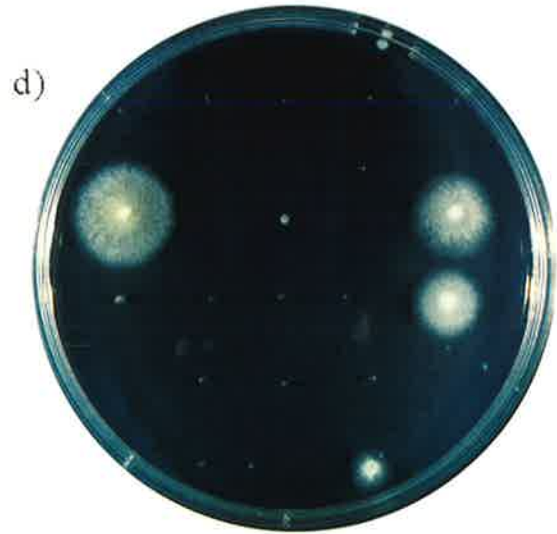
b)



c)



d)



e)

TMΔ4::ΔRV4	TMΔ4::ΔRV10	TMΔ4::ΔRV19
TMΔ4::ΔRV20	TMΔ4::ΔRV21	TMΔ4::ΔRV23
	creAΔ4	*creA*$^{+}$

derepressing states. If this model is correct then a region within the deleted 158 amino acids may be necessary for this change. Within the deleted region are a number of potential serine/threonine protein kinase phosphorylation sites, which may indicate that phosphorylation is required to switch between the two functional states.

6.3 Summary

Analysis of *creA* mutant alleles did not result in any information concerning the function of the conserved regions within CreA. To try and identify the function of these regions of the protein, constructs were made that disrupt these regions of *creA*$^+$. These constructs were then transformed into a *creA*Δ strain.

The box 7 region of CreA is very highly conserved between all *creA* homologues. This region has similarity to a corresponding region of the yeast protein Rgr1p. This region was swapped into the *creA* gene, pANC4RGR1a. This construct was transformed into strains containing either *creA*204 or *creA*220 mutant alleles. This construct did not complement either mutant allele suggesting that although the two regions are similar they are not functionally homologous.

An internal deletion of *creA* was constructed, pANC4ΔRV10. This deletion construct was transformed into a strain containing a deletion of the *creA* locus. Two classes of transformants were isolated. Transformants with 1-2 copies of the transforming sequence partially complemented the *creA*Δ4 allele. These transformants had a phenotype similar to a weak *creA* mutant allele but demonstrated stronger growth

than wild type strains on sole non-repressing carbon sources. A similar effect was not observed from strains transformed with a construct that had the yeast Rgr1p similar domain replacing the region in *creA*$^+$, pANC4RGR1a. This may indicate partial function of the yeast domain which had been swapped in to *creA*$^+$. No other phenotype could be identified from strains transformed with pANC4RGR1a. The second class of pANC4ΔRV10 transformants had three or more copies of the transforming sequence. When grown in repressing conditions these transformants fully complemented every *creA*Δ4 phenotype tested. Therefore the repression domain within CreA can be isolated to the final 51 C-terminal amino acids. Also the internal region of the protein does not appear to be required for CreA to act as a repressor of transcription. However when grown in derepressing conditions these transformants had low levels of expression of carbon catabolite repression regulated genes. Therefore the internal region of the gene has a role in allowing an increase in the level of expression of carbon catabolite repression regulated genes in derepressing conditions. The deleted region of the protein may be required for CreA to switch between repressing and derepressing states. When the region is deleted the resulting protein remains a repressor that cannot switch into the derepressing state, allowing transcription to occur.

Chapter 7

Selection of Mutants that Suppress *creA* Alleles

The process of carbon catabolite repression is complex and must consist of a number of stages. The cell must have a glucose uptake system. The levels of glucose available to the cell must be monitored. As the cell monitors the glucose levels available to it a signal transduction process must exist to feed into the carbon catabolite repression system and trigger carbon catabolite repression or derepression (figure 7.1).

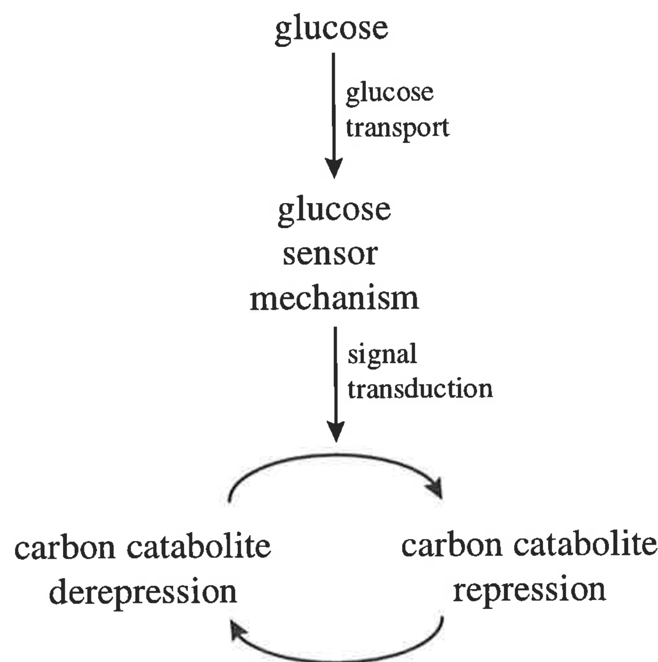


Figure 7.1 - Basic model for the process of carbon catabolite repression.

It is possible to formulate a model where most of these aspects of carbon catabolite repression are mediated by CreA. However the data are inconsistent with this idea. Therefore it is most likely that the process of carbon catabolite repression in *Aspergillus nidulans* involves a number of proteins. These proteins could act upstream of CreA or in conjunction with CreA to effect repression of carbon catabolite repression regulated genes. Three other loci, *creB*, *creC* and *creD*, have

already been identified which are predicted to have a role in the process of carbon catabolite repression (Hynes and Kelly 1977; Kelly and Hynes 1977). At this point the function of these three genes is unknown. There is also no direct evidence of a direct relationship between CreA and CreB, CreC or CreD. Suppressors of *creA* mutant alleles were isolated in an attempt to identify genes that encode proteins that function in the glucose signal transduction pathway or proteins that act directly with CreA to effect carbon catabolite repression.

7.1 - Suppressors of the *creA*306 mutant allele

To date screens for suppressors of *creA* mutant alleles have been undertaken by suppressing the *creA*$^-$ phenotype that leads to derepressed levels of expression of carbon catabolite repression regulated systems in the presence of glucose. For example *creA*$^-$ mutants result in derepression of *alcA*, resulting in sensitivity to allyl alcohol in glucose containing media. Suppressors of *creA*$^-$ can be isolated by selecting *creA*$^-$ strains that are resistant to allyl alcohol in the presence of glucose. However previous efforts to select suppressors by this method have been unsuccessful (Bailey 1976). One of the problems with this approach is that the vast majority of mutants selected are the result of loss of function mutations in the alcohol dehydrogenase gene. Although these mutations are easily identified by their failure to grow on ethanol and normal growth on other carbon sources it greatly increases the numbers that need to be screened.

Instead of using approaches that were previously unsuccessful it was decided to screen for suppressors of *creA*⁻ alleles based on changes in their morphological phenotype. Strains containing the most extreme *creA*⁻ alleles have very distinct morphological phenotypes. On 1% complete media they have greatly reduced growth rates and conidiate very poorly by comparison to *creA*⁺ strains.

Strains containing *creA*306 have the most extreme morphology of all of the *creA*⁻ alleles. These strains also result in consistently high levels of elevation and derepression of carbon catabolite repression regulated systems. The phenotype of *creA*306 containing strains is even more extreme than strains that have the *creA* locus deleted. The *creA*306 mutant is predicted to encode a full length CreA polypeptide with no DNA binding affinity. It is proposed that the extreme phenotype exhibited by *creA*306 strains is due to the CreA306 polypeptide binding CreA interacting proteins and titrating them, preventing their action on the regulation of non-carbon catabolite repressed systems. If this hypothesis is correct it makes *creA*306 the ideal candidate to use in a screen for suppressor mutants.

Suppressors of a *creA*306 strain (H17CR6) were screened by growth of both UV-exposed and non UV-exposed spores on 1% complete media. Suppressors were selected as sectors that either grew at a faster rate than *creA*306 strains and/or sectors that conidiate better than *creA*306 strains. Eighty mutants were selected that suppressed the *creA*306 morphology. Fifty six of the eighty mutants were from spores exposed to UV-light. The remaining suppressor mutations (24/80) were

spontaneously occurring mutations. Based on their altered morphology on 1% complete medium the eighty suppressor mutations can be divided into four groups.

7.1.1 - Suppressors with a greatly improved growth rate and conidiation

Seven suppressors of *creA*306 had growth rates and a degree of conidiation very similar to *creA*$^+$ strains on 1% complete media. The suppressors were designated *su*(*creA*306)5, -6, -9701, -9702, -9703, -9704 and -9705. There was a subtle difference in growth rate between these suppressors. Strains containing *su*(*creA*306)5, -6 and -9702 grew at a slightly slower rate than the other four suppressors in this group which grew at a rate very similar to wild type (figure 7.2 and table 7.1). All seven of these suppressors conidiated at similar levels to a *creA*$^+$ strain.

The levels of expression of acetamidase, α-amylase, alcohol dehydrogenase 1 and proline oxidase in the presence of glucose were estimated using plate tests. Strains containing *su*(*creA*306)9701, -9703, -9704 and -9705 had similar levels of expression of the tested enzymes as a *creA*$^+$ strain. When compared to a *creA*306 strain the remaining three suppressors in this group had significantly reduced levels of expression of all enzymes tested (figure 7.3 and table 7.1). However this reduced expression is not to the levels observed in a *creA*$^+$ strain. All seven suppressors in this group have pleiotropic effects, reducing the severity of morphological phenotypes as well as reducing or stopping the derepressed phenotype associated with the *creA*306 allele. This suggests that these seven suppressor mutations are ideal candidates for mutations that affect the process of carbon catabolite repression.

Table 7.1 - Phenotypic analysis of allelic suppressors of the *creA*306 mutation

Strain[1]	Growth rate on 1% Complete Media[2]	Conidiation on 1% Complete Media[2]	1% D-glucose[2]	1% D-glucose + 10 mM acetamide[2]	1% D-glucose + 10 mM proline[2]	1% D-glucose + 2.5 mM allyl alcohol[2]	1% D-glucose + 25 mM allyl alcohol[2]	1% D-glucose + 1% starch[3]
creA$^+$	+++++	+++++	+++++	-	-	+++++	+++++	-
*creA*306	+	+	++	+++++	+++++	-	-	S
su(*creA*306)5	++++	+++++	+++++	++	++	+++	++	W
su(*creA*306)6	++++	+++++	+++++	++	++	+++	++	W
su(*creA*306)9701	+++++	+++++	+++++	-	-	+++++	+++++	-
su(*creA*306)9702	++++	+++++	+++++	++	++	+++	++	W
su(*creA*306)9703	+++++	+++++	+++++	-	-	+++++	+++++	-
su(*creA*306)9704	+++++	+++++	+++++	-	-	+++++	+++++	-
su(*creA*306)9705	+++++	+++++	+++++	-	-	+++++	+++++	-

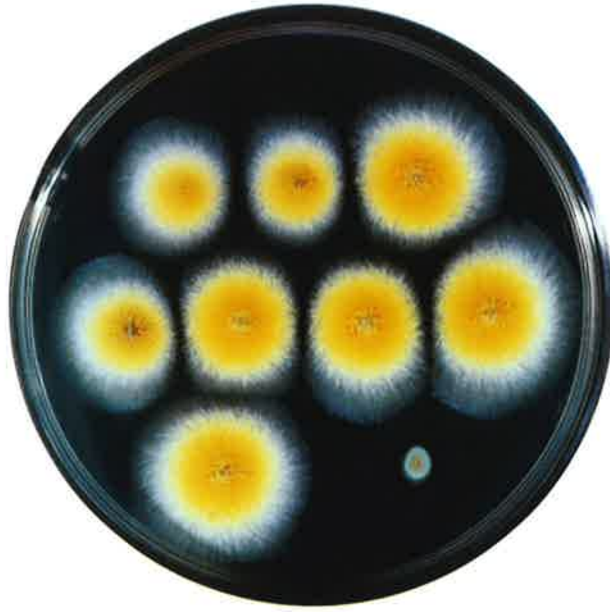
Note: (1) strains are in an *areA*217background

(2) denotes increasing rate of growth or degree of conidiation -; +; ++; +++; ++++; +++++.

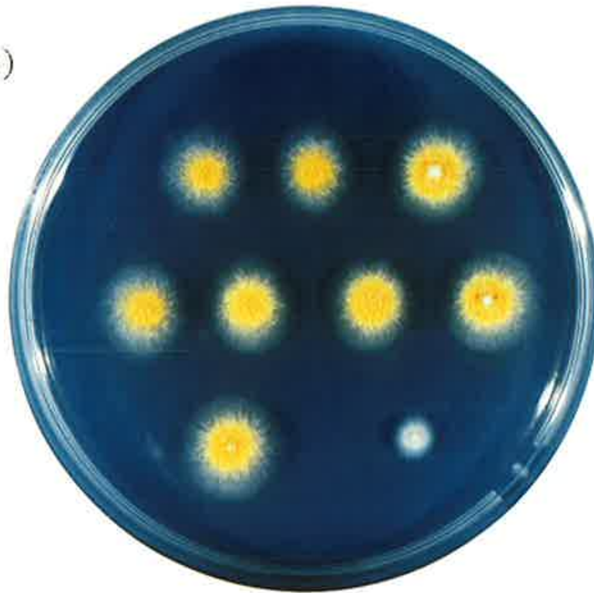
(3) size of clear halo or blue colouration: -, none; W, weak; I, intermediate; S, Strong

Figure 7.2 - Growth rate and conidiation of allelic suppressors of *creA*306
(a) The strains were grown at 37°C for 48 hours on 1% complete medium. Each strain is identified in the key below the plates. The growth rate and degree of conidiation were estimated for each colony and is summarised in table 7.1.
(b)The strains were grown at 37°C for 48 hours on minimal medium containing 1% D-glucose and 10 mM ammonium tartrate. Each strain is identified in the key below the plates.

a)



b)

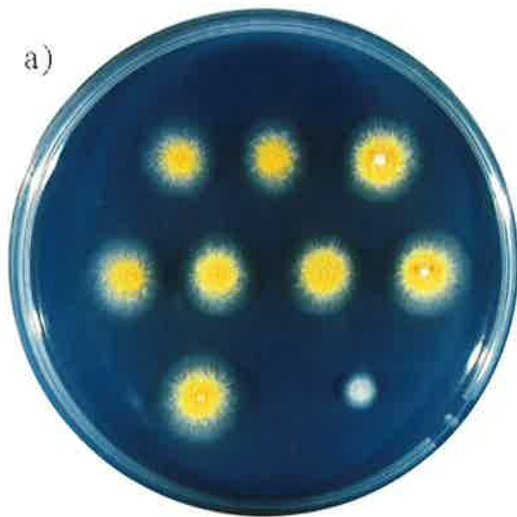


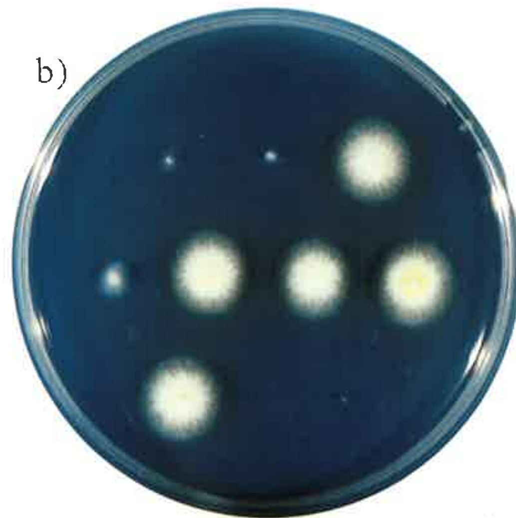
su(creA306)5 *su(creA306)6* *su(creA306)9701*

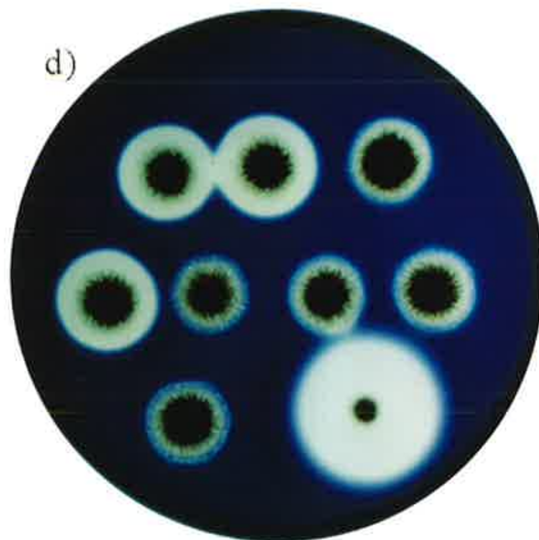
su(creA306)9702 *su(creA306)9703* *su(creA306)9704* *su(creA306)9705*

creA^{+} *creA306*

Figure 7.3 - Growth of allelic suppressors of *creA*306 in repressing conditions
Allelic suppressors of *creA*306 were grown for 48 hours at 37°C on 1% minimal media containing:
(a) 1% D-glucose and 10 mM ammonium tartrate (synthetic complete)
(b) 1% D-glucose, 2.5 mM allyl alcohol and 10 mM ammonium tartrate
(c) 1% D-glucose, 25 mM allyl alcohol and 10 mM ammonium tartrate
(d) 1% D-glucose, 1% starch and 10 mM ammonium tartrate
Each strain is identified in the key (e).
The levels of resistance to allyl alcohol and α-amylase expression are summarised in table 7.1.







e)

su(creA306)5 *su(creA306)6* *su(creA306)9701*

su(creA306)9702 *su(creA306)9703* *su(creA306)9704* *su(creA306)9705*

creA^{+} *creA306*

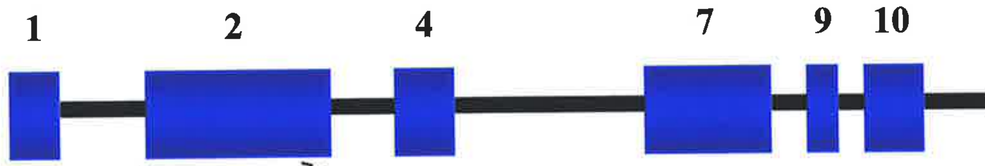
The seven suppressor strains were outcrossed to a *creA*$^+$ strain (*biA*1; *niiA*4) to determine if the new mutations were allelic to the *creA* locus. For each cross in excess of 500 progeny were screened. In all seven crosses there were no occurrences of the cross segregating *creA*306 progeny. Therefore all seven mutations must be allelic or very tightly linked to *creA*. To determine the site of the new mutation in each suppressor mutation a PCR-SSCP approach was used. All seven suppressor mutations were located within the Cys_2-His_2 DNA binding domain of *creA*.

DNA sequence analysis showed that *su*(*creA*306)9701, -9703, -9704 and -9705 were all reversion to the wild type sequence. This is consistent with the wild type phenotype observed from strains containing these suppressors. The remaining three suppressors all had a second site point mutation within the DNA binding domain. All three suppressors have the same mutation, a C$\rightarrow$T transition at bp 362 immediately preceding the site of the original *creA*306 mutation. This base change results in a proline to serine substitution at amino acid 109 (figure 7.4).

The original leucine to proline substitution in CreA306 is predicted to totally disrupt the second zinc finger and prevent the ability of CreA306 to bind DNA. A change to serine in this position is much more conservative. Serine is a smaller residue than proline and does not prevent formation of the α-helix as the proline residue of CreA306 is expected to do. This is supported by the phenotypic data. The allelic suppressors of *creA*306 all have the phenotype of a weak *creA*$^-$ allele. These strains result in low to moderate levels of expression of most carbon catabolite repressed genes in the presence of glucose. Of particular interest is the morphological phenotype

Figure 7.4 - DNA sequence changes in allelic suppresor mutations of the *creA*306 mutant allele

Schematic showing the position and nature of each allelic mutation which suppresses the phenotype of *creA*306. Four of the mutations are reversions to the wild type sequence. The remaining three, *su*(*creA*306)5, -6 and -9702 had a second mutation adjacent to the original *creA*306. The resulting amino acid changes are shown. The coding sequence shows the positions of the most highly conserved boxes, 1, 2, 4, 7, 9 and 10 of CreA (see figure 4.3).



PRPYKCPLCERAFHRLEHQTRHIRTHTGEKPHACQFPGCSKRFSRSDE**L**TRHSRIH

wild type	gatgag**ctt**acccgg	L
*creA*306	gatgag**cct**acccgg	L ► P
su(*creA*306)5	gatgag**tct**acccgg	P ► S
su(*creA*306)6	gatgag**tct**acccgg	P ► S
su(*creA*306)9701	gatgag**ctt**acccgg	P ► L
su(*creA*306)9702	gatgag**tct**acccgg	P ► S
su(*creA*306)9703	gatgag**ctt**acccgg	P ► L
su(*creA*306)9704	gatgag**ctt**acccgg	P ► L
su(*creA*306)9705	gatgag**ctt**acccgg	P ► L

of these suppressor strains. They have a slightly reduced growth rate compared to wild type strains, but there appears to be no reduction in the level of conidiation in these strains. No other *creA*⁻ mutant allele has a similar morphological phenotype. All other *creA*⁻ alleles in this study resulted in a reduction in the degree of conidiation and significantly reduced growth rates. *creA*⁻ mutations could effect conidiation indirectly. Derepression of all carbon catabolite repression regulated genes creates an energy deficit within the cell and this wasted energy may, in turn disrupt the conidiation process. Alternatively CreA may have a direct role in regulating the process of conidiation. There is evidence that the conidiation process responds to a low nutrient environment in *fluG* mutant strains (Lee and Adams 1996). Strains carrying different *creA*⁻ mutant alleles that have missense mutations within the DNA binding domain result in a wide range of effects on the ability of these strains to conidiate. These effects range from minimal conidiation in *creA*306 and *creA*225 strains to no effect in *su*(*creA*306)5, -6 and -9702 strains. Therefore the normal conidiation is correlated with higher predicted affinity of DNA binding by CreA. This strongly suggests a more direct role for CreA in the control of the conidiation process.

7.1.2 - Suppressors with a partially improved growth rate and conidiation

The most common class of suppressors (48/80) were selected as sectors that had an increased growth rate and a slightly improved level of conidiation. This group of suppressors have a morphology very similar to that observed from strains containing *creA*Δ alleles (figure 7.5 and table 7.2). The level of derepression of the carbon

Table 7.2 - Phenotypic analysis of extragenic suppressors of the *creA*306 mutation

Strain[1]	Growth rate on 1% Complete Media [2]	Conidiation on 1% Complete Media [2]	1% D-glucose[2]	1% D-glucose + 10 mM acetamide[2]	1% D-glucose + 10 mM proline[2]	1% D-glucose + 2.5 mM allyl alcohol[2]	1% D-glucose + 25 mM allyl alcohol[2]	1% D-glucose + 1% Starch[3]
creA+	+++++	+++++	+++++	-	-	+++++	+++++	-
*creA*306	+	+	++	+++++	+++++	-	-	S
*creA*Δ4	+++	++	+++	++++	++	-	-	S
su(*creA*306)9711	++++	++	+++	++++	++	-	-	S
su(*creA*306)9712	++++	++	+++	++++	++	-	-	S
su(*creA*306)9713	+++	++	+++	++++	++	-	-	S
su(*creA*306)9716	++++	++	+++	++++	++	-	-	S
su(*creA*306)9739	++++	++	+++	++++	++	-	-	S
su(*creA*306)9730	+++	++	+++	++++	-	-	-	S

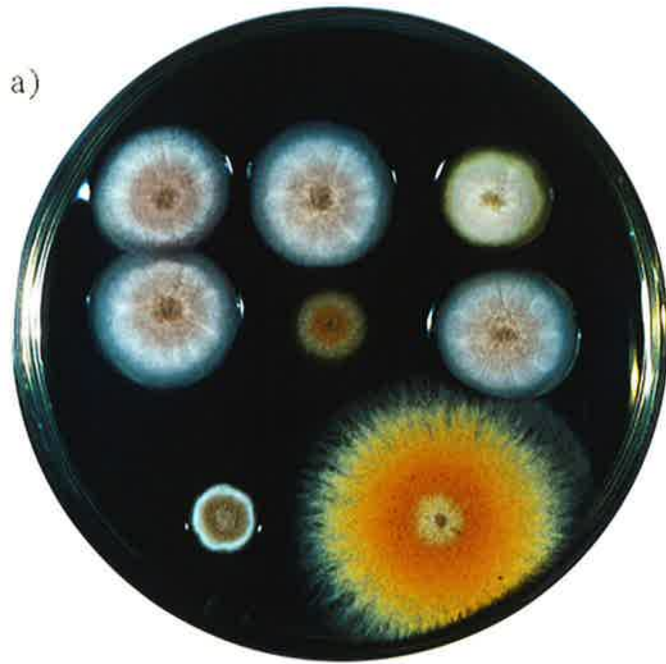
Note: (1) strains are in an *areA*217background
(2) denotes increasing rate of growth or degree of conidiation -; +; ++; +++; ++++; +++++.
(3) size of clear halo or blue colouration: -, none; W, weak; I, intermediate; S, Strong

Figure 7.5 - Growth rate and conidiation of extragenic suppressors of *creA*306 grown on 1% complete medium

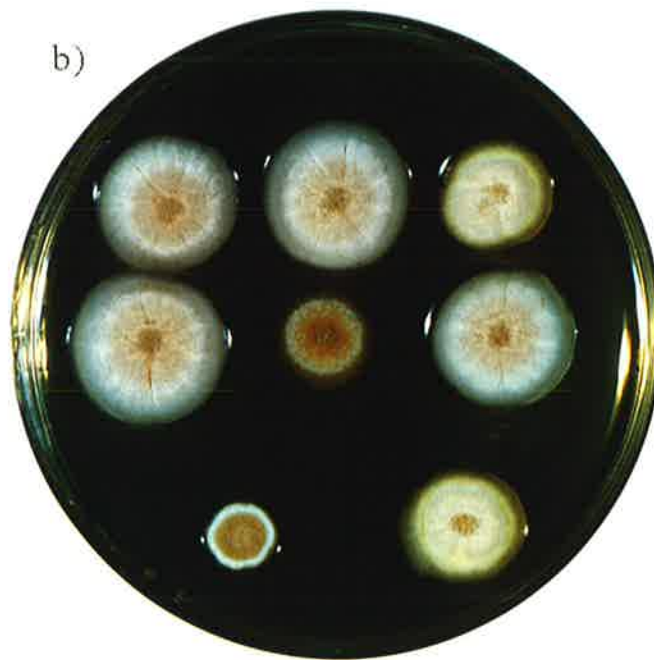
(a) The strains were grown at 37°C for 48 hours on 1% complete medium. Each strain is identified in the key below the plates.

(b)The strains were grown at 37°C for 96 hours on 1% complete medium. Each strain is identified in the key below the plate. The growth rate and degree of conidiation were estimated for each colony and is summarised in table 7.2.

a)



b)



su(creA306)9711 *su(creA306)9712* *su(creA306)9713*

su (creA306)9716 *su (creA306)9730* *su (creA306)9739*

creA306 *creA*$^{+}$*/creA*Δ*4*

catabolite repression regulated systems tested was also very similar to *creA*Δ strains (figure 7.6 and table 7.2). There are two explanations which could account for this group of suppressors having a similar phenotype to *creA*Δ strains. A mutation of the *creA* gene that results in a total loss of function would be expected to give this result. An alternative explanation is that this group of suppressors has mutations that disrupt a protein that interacts with CreA. If the loss of a CreA interacting protein removes the titrating effect of the *creA*306 mutation then the resulting strain would be expected to have a *creA*Δ phenotype. To differentiate between these two possibilities ten suppressor strains from this group (*su*(*creA*306)2, -7, -8, -11, -13, -9711, -9712, -9713, -9730 and -9739) were outcrossed to a wild type strain (*biA*1; *niiA*4). The suppressor mutation could only be scored in the cross when it was present in a *creA*306 background. In all crosses the progeny segregated $creA^{+}$, *creA*306 and *su*(*creA*306) phenotypes. Therefore all ten suppressor mutations were extragenic to *creA*, indicating that a gene encoding a CreA interacting protein has been mutated.

su(*creA*306)7 was crossed to five other suppressor strains from this group (*su*(*creA*306)2, -8, -11, -9711 and -9712). In each cross over 300 progeny were scored and only the suppressor phenotype segregated amongst the progeny. Therefore all six of these mutants affect the same gene or very tightly linked genes.

Diploids were constructed between three of the suppressors, *su*(*creA*306)2, -7 and -8, and a multi-marked strain (MSF-RL) to map the suppressor mutations by haploidisation analysis. Due to technical reasons this analysis was unsuccessful.

Figure 7.6 - Growth of extragenic suppressors of *creA*306 in repressing conditions

Extragenic suppressors of *creA*306 were grown for 48 hours at 37°C on 1% minimal media containing:

(a) 1% D-glucose and 10 mM acetamide

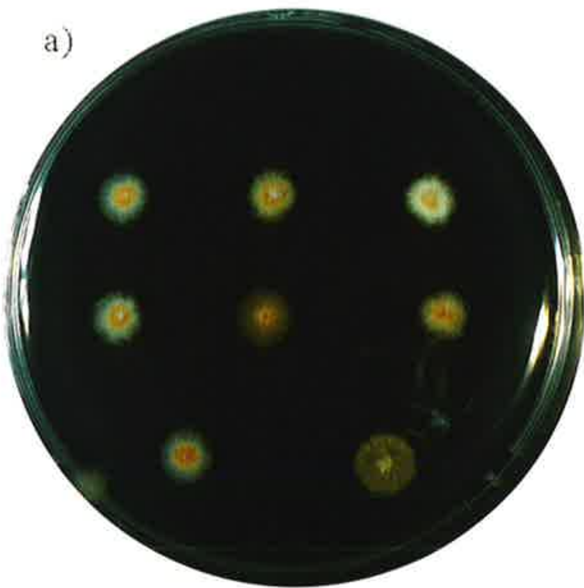
(b) 1% D-glucose and 10 mM L-proline

(c) 1% D-glucose, 0.1 mM allyl alcohol and 10 mM ammonium tartrate

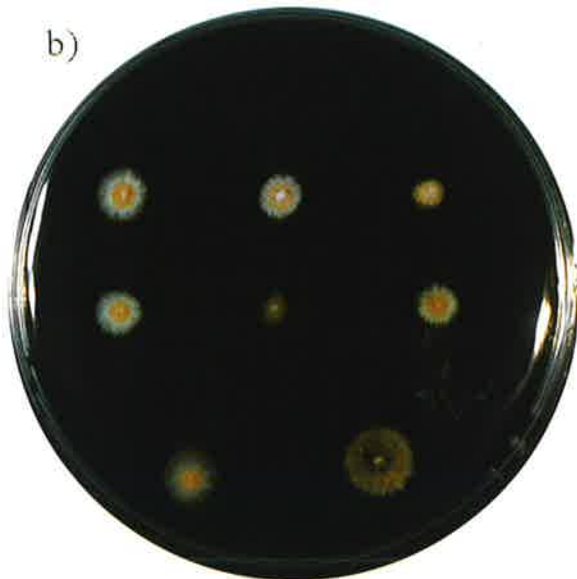
Each strain is identified in the key (d).

The levels of suppression of *areA*217 and resistance to allyl alcohol are summarised in table 7.2.

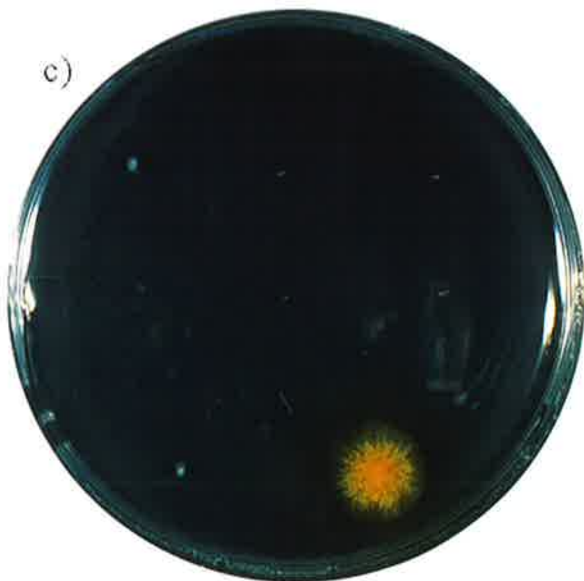
a)



b)



c)



d)

su(creA306)9711	*su(creA306)9712*	*su(creA306)9713*
su (creA306)9716	*su (creA306)9730*	*su (creA306)9739*
creA306	$creA^{+}$	

Haploid sectors with the suppressor phenotype could not be isolated. The phenotype of the suppressor mutation could only be identified in a *creA*306 background. The *creA*306 *su*(*creA*306) double mutant grows and conidiates very poorly by comparison to *creA*$^{+}$ strains. In an haploidisation analysis haploid sectors containing the *creA*306 *su*(*creA*306) double mutant were over grown by *creA*$^{+}$ sectors and therefore could not be isolated.

7.1.3 - *su*(*creA*306)9730

One suppressor, *su*(*creA*306)9730, was selected on 1% complete medium as having a slightly increased growth rate, but it had little or no improvement of its ability to conidiate (figure 7.5 and table 7.2). There were no obvious changes in the level of expression of carbon catabolite repression regulated systems compared to *creA*306 strains, except for growth on glucose and proline. *su*(*creA*306)9730 was unable to utilise proline as a nitrogen source on media containing 1% D-glucose and 10mM L-proline (figure 7.6 and table 7.2). *su*(*creA*306)9730 was also unable to utilise proline as a carbon source on media containing 50mM L-proline and 10mM ammonium tartrate. When this suppressor strain was outcrossed to a wild type strain (*biA*1; *niiA*4) amongst the 300 progeny scored were equal numbers of *creA*306 and *su*(*creA*306) colonies. The remaining colonies all had a *creA*$^{+}$ phenotype. From the results of the cross clearly *su*(*creA*306)9730 is not linked to the *creA* locus. Amongst the progeny with a *creA*$^{+}$ morphology half were unable to utilise proline as either a carbon or a nitrogen source. These colonies must correspond to the *su*(*creA*306)9730 mutation in a *creA*$^{+}$ background.

A diploid was constructed between the suppressor *su(creA*306)9730 and MSF. The *su(creA*306)9730 mutation was recessive in this heterozygous diploid since the diploid strain was able to utilise proline as a carbon and a nitrogen source. Haploidisation analysis resulted with the suppressor mutation cosegregating with *acrA*1. *acrA* is located on chromosome II and therefore the suppressor mutation must also be located on chromosome II. *creB* and *creC* mutant alleles are two loci previously identified as having a role in the process of carbon catabolite repression. Both *creB* and *creC* map to chromosome II. Strains containing either *creB* or *creC* are unable to utilise proline as a carbon or a nitrogen source (Hynes and Kelly 1977; Kelly and Hynes 1977). It was therefore quite likely that the *su(creA*306)9730 mutation is allelic to either *creB* or *creC*. To check this crosses were set between *su(creA*306)9730 and strains containing mutations in *creB* and *creC* respectively. Half of the progeny from the cross between *su(creA*306)9730 and a *creC*27 containing strain were able to utilise proline and half were not able to utilise proline as either a carbon or a nitrogen source. Therefore the suppressor mutation and *creC* assort independently. When the suppressor mutation was crossed to a strain containing *creB*15, the vast majority of progeny were unable to utilise proline. Only three progeny were recovered from over 1000 tested that were able to utilise proline suggesting that *su(creA*306)9730 and *creB* are very tightly linked and probably allelic.

Selecting a *creB* allele in a screen for suppressors of a *creA*$^-$ allele establishes a direct link between the two genes. It remains to be determined if the two proteins directly interact with each other or if they act as part of the same pathway.

7.1.4 - Suppressors with a improved growth rate that are aconidial

The remaining suppressor mutations (24/80) all have an increased growth rate compared to the original *creA*306 strain. The degree to which the growth rate increases varies amongst this group. The suppressors in this group form aerial hyphae and form few if any conidia. They have a morphology similar to previously described aconidial (*aco*⁻) and fluffy (*flu*⁻) mutants. It is possible that these mutations directly effect the conidiation process, establishing a link between CreA and the proteins involved in the conidiation process. Alternatively these suppressors may be the result of a general increase in growth rate that is unrelated to the process of carbon catabolite repression.

7.2 - Suppressors of the *creA*Δ4 mutant allele

*creA*Δ strains have a very poor growth rate and on complete media only conidiate in the center of the growing colony. In an initial screen 13 spontaneous suppressors of a *creA*Δ4 (*creA*Δ4 *yA*2; *pabaA*1; *riboB*2) were selected by improved growth rates. The 13 suppressors selected could be divided into two groups based on their morphology on 1% complete medium. Two suppressors, *su*(*creA*Δ4)12 and -14, had significantly improved growth rates and conidiation. *su*(*creA*Δ4)12 and -14 had similar levels of expression of carbon catabolite repression regulated genes as the original *creA*Δ strain except for α-amylase expression that was considerably lower. The second group, *su*(*creA*Δ4)1, -2, -3, -5, -6, -7, -8, -9, -10, -11 and -13, consisted of suppressors that conidiate poorly and have a slightly increased growth rate compared to a *creA*Δ

strain. There were no obvious differences between the level of expression of carbon catabolite repressed systems in this second group of suppressors and *creA*Δ strains.

Examples from both groups, *su*(*creA*Δ4)2, -7, -12 and -14, were outcrossed to a *creA*$^+$ strain (*biA*1; *niA*4). In all four crosses the progeny segregated *creA*$^+$, *creA*Δ4 and *su*(*creA*Δ4) phenotypes suggesting that all four mutations are extragenic to *creA*. The suppressor phenotypes could only be identified in *creA*Δ4 backgrounds. Diploid strains were constructed between these four suppressor strains and MSF-RL. In an haploidisation analysis sectors with the suppressor mutation could only be identified from the diploid constructed with the *su*(*creA*Δ4)14 strain. In the haploidisation experiment *su*(*creA*Δ4)14 cosegregated with *nicB*. Therefore the suppressor mutation maps to chromosome VII. To position the mutation on chromosome VII crosses were set between *su*(*creA*Δ4)14 and two genes on chromosome seven, *alcA* and *nicB*. The *su*(*creA*Δ4)14 mutation was not linked to either *alcA* or *nicB*.

During phenotype testing of *su*(*creA*Δ4)14 strains it was noticed that they were unable to utilise maltose as a carbon source. A previously identified mutation, *malA*, is also unable to utilise maltose and maps to chromosome VII. No recombinants were found amongst 143 progeny from a cross between a *su*(*creA*Δ4)14 strain and a *malA* strain (A461). Therefore the suppressor is either allelic or very closely linked to *malA*. *malA* is thought to be a positive activator of maltase and maltose permease expression. It is unclear why a mutation in *malA* would suppress a *creA*Δ strain. One possibility is that the phenotype of a *creA*Δ4 strain is the result an energy penalty caused by derepression of all carbon catabolite repressed genes. By removing the expression of the maltose utilisation genes this energy deficit may be lessened,

resulting in the improved phenotype. It is also possible that although *malA* was isolated as affecting expression of maltose utilisation genes, it may have a more general regulatory role including an involvement in carbon catabolite repression. Further analysis of *malA* is required to determine exactly what role it may play in the regulation of gene expression.

7.3 - Summary

Suppressor mutations of *creA* alleles were selected to isolate genes which encode proteins that act with CreA in the process of carbon catabolite repression. Strains containing *creA*306 and *creA*Δ4 have an extreme morphological phenotype. This means they have a greatly reduced growth rate and conidiate very poorly. Their growth on 1% complete media is so poor that it is possible to select suppressors of the *creA* alleles by increased growth rate. The major disadvantage of this approach is that it is difficult to separate mutations which result in an increased growth rate but are unrelated to the process of carbon catabolite repression.

Four classes of mutations were isolated as suppressors of a *creA*306 strain. One group (7/80) consisted of allelic mutations. There were examples of reversions to the wild type sequence and also of a second site mutation within the DNA binding domain. This missense mutation changes the same amino acid as the original *creA*306 mutation. Through the two rounds of mutation amino acid 109 has changed from leucine to proline to serine. A serine in this position can only occur through two base changes and therefore is very unlikely to have occurred in any of the original screens for *creA* mutants. This *creA* allele is of particular interest because unlike any other

creA⁻ mutant alleles it does not result in a reduction of conidiation and has minimal effects on the growth rate while still leading to some derepression.

The remaining three groups were all the result of extragenic mutations. The largest group (48/80) resulted in a change from a *creA*306 phenotype to a *creA*Δ-like phenotype. Ten of these mutations tested were all extragenic. No complete loss of function *creA*⁻ alleles were found although it is possible that some of the remaining untested alleles in this group could be *creA*⁻ null mutations. Six of the suppressors were found to be tightly linked or allelic. At this stage it is not known if any other loci are present in this group. A single mutation was isolated and found to be tightly linked or allelic to *creB*. This establishes a link between the function of these two genes. Based on the similarity between *creB* and *creC* mutants it is likely that *creC* mutants can also suppress a *creA*306 strain. To determine the relationship between these three genes an analysis of double mutants formed from different *creA*⁻ alleles and either *creB* or *creC* alleles should be undertaken. The final (24/80) group consisted of suppressors that were aconidial. It is unknown if they have any significance to the process of carbon catabolite repression.

A preliminary screen isolated 13 suppressors of a *creA*Δ4 strain. One of these mutations was studied in detail and found to be tightly linked or allelic to *malA*. *malA* is a positive regulator of the maltose utilisation genes. It is not known if the loss of *malA* leads to an increased growth rate by an indirect mechanism or by a direct role in carbon catabolite repression.

Neither of the screens for suppressor mutants, especially the screen for suppressors of *creA*Δ4, have been performed to a saturation point. Since mutants of interest have been isolated using this approach a more intensive screen should now be carried out.

Chapter 8

Conclusions

Aspergillus nidulans is able to utilise a wide variety of carbon sources in addition to glucose, its preferred carbon source. When glucose is not present the organism is able to break down many other compounds to supply a carbon source eg ethanol, starch and cellulose. There are many genes required which produce enzymes that act to transport these compounds into the cell and break them down into useable components. The expression of these genes is controlled by a complex regulatory mechanism, carbon catabolite repression. Carbon catabolite repression functions to prevent the expression of the large range of enzymes required for the utilisation of less favourable carbon sources when a more readily utilised carbon source is present. In *A. nidulans* this process is controlled by the product of the *creA* gene. Genetic analysis shows that CreA is a wide domain, negatively acting repressor of transcription. The *creA* gene has been cloned from both *A. nidulans* and *Aspergillus niger*. Sequence analysis highlighted a number of potential functional domains within the protein. The protein has a Cys_2-His_2 zinc finger DNA binding domain with a high level of similarity to the DNA binding domain of Mig1p that mediates glucose repression in *Saccharomyces cerevisiae*. CreA also contains an alanine repeat and an acidic region, all of which are characteristic of regulatory proteins. There is also a stretch of 45 amino acids that are identical in *A. nidulans* and *A. niger* that show significant similarity to a region of Rgr1p, a protein involved in glucose repression in *S. cerevisiae*. Using a number of different strategies many *creA* mutations have been selected. All of these alleles have a phenotype of derepression of the CreA regulated genes in repressing conditions. Conversely mutants with a phenotype of failure to derepress have never been isolated despite numerous attempts.

8.1 - Functional regions within CreA

The major aim of this study was to phenotypically characterise a large bank of *creA* mutant alleles and determine the nature of each mutation in order to further define functional domains within the CreA polypeptide. Twenty *creA* alleles were used in this study.

Strains containing the *creA* mutant alleles showed varying phenotypes. These mutations result in increased levels of expression of carbon catabolite repressed genes in the presence of glucose. They also cause decreased rates of growth and conidiation and some alleles also have defects in carbohydrate storage and the formation of cleistothecia. These phenotypes indicated a role for carbon catabolite repression, either directly or indirectly, in the processes of conidiation carbohydrate storage as well as the sexual and asexual cell cycles.

The allele that resulted in the most extreme phenotype was *creA*306. Other alleles, such as *creA*303, -304, -305 and -30 also have extreme phenotypes, but they are not as phenotypically extreme as *creA*306. The remaining alleles result in varying phenotypes. *creA*1 containing strains had the weakest morphological phenotype of any of this group of alleles. Since mutations leading to derepression were isolated at reasonably high frequencies, and mutations that fail to derepress were never isolated, it is likely that the derepression is due to a loss of CreA function. However the range of phenotypes amongst these alleles suggests that most of the mutations do not lead to a total loss of CreA function.

The *creA* alleles were analysed at the molecular level to determine the site and nature of each mutation within the gene. The mutations could be divided into two broad groups. The first group consists of missense mutations within the DNA binding domain. Included in this group is *creA*306. *creA*306 has a leucine to proline substitution in the recognition helix of the second zinc finger and is predicted to completely abolish DNA binding affinity of the CreA306 polypeptide. It is expected that CreA306 retains the ability to undergo any potential interactions with other proteins. Since *creA* is autoregulated the mutation would also lead to a higher level of expression of *creA*306. This higher expression and the ability to still bind other interacting proteins may result in the titration of the interacting proteins and the prevention of their normal function. The titration of these proteins is predicted to be the cause of the extreme phenotype observed for *creA*306 strains. The other alleles with missense mutations in the DNA binding domain are all expected to result in a reduction of DNA binding affinity. It is unlikely that any of these mutations produces a polypeptide that has totally lost all DNA binding function. Based on the analysis of the Cys_2-His_2 zinc fingers of other proteins, the severity of the phenotype of each of these alleles correlates with the predicted reduction of the ability of the protein to bind DNA.

All of the remaining *creA* mutant alleles analysed in this study have nonsense or frameshift mutations and are predicted to produce truncated polypeptides. The sites of each of the truncations span the length of the CreA protein. The *creA*322 allele results in significant pleiotropic phenotypes. CreA322 has the last 80 C-terminal amino acids

removed. Therefore the last 80 amino acids of CreA must contain a region that is absolutely required for the protein to function as a transcriptional repressor. This was also supported by the phenotype of the *in vitro* constructed internal deletion of *creA*, as discussed below.

The alleles predicted to produce the most truncated polypeptides are *creA*303 and *creA*304. Both of these alleles are predicted to produce polypeptides that truncate within the DNA binding domain. Therefore the CreA303 and CreA304 polypeptides only constitute the N-terminal region of the protein up to the DNA binding domain. Previous analysis of a strain that contained a deletion of a region of the genome that included *creA*$^+$ resulted in the phenotype of the strain being described as leaky lethal. Strains containing precise disruptions of the *creA*$^+$ locus have been constructed. Analysis of these strains showed that they were phenotypically similar to *creA*303 and *creA*304, suggesting that *creA*303 and *creA*304 are total loss of function alleles. The leaky lethal phenotype described previously was presumably the result of disrupting *creA* and a gene very closely linked to *creA*.

Strains containing *creA*Δ alleles were used as transformation parent strains to test the function of constructs that were constructed with deletions within *creA*. An internal, in frame deletion of 474 bp was constructed which removed amino acids 207-365 of CreA. Strains that contained 3-5 copies of this construct were able to fully complement a *creA*Δ strain in repressing conditions. Therefore the region deleted from CreA is not required for the protein to repress transcription. The protein produced from this deletion only retains the final 51 amino acids of the C-terminal end

of CreA. This final 51 amino acids is enough to allow the altered protein to act as a repressor of transcription and therefore must contain the repression domain identified by analysis of *creA* mutant alleles. These strains grew poorly by comparison to a wild type strain when grown on some non-repressing sole carbon sources. This suggests that the deleted region is required for the derepression of many carbon catabolite repression regulated genes in non-repressing conditions.

The functional regions within CreA identified in this study are summarised in figure 8.1. A more refined, site directed mutational analysis is now required to further define these functional regions within CreA.

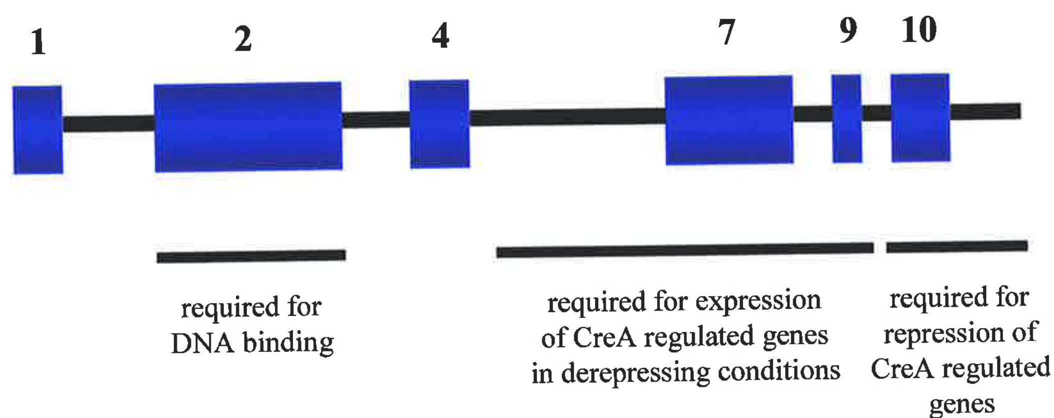


Figure 8.1 - Functional regions within CreA.
Schematic showing the positions of functional regions identified within CreA and the corresponding positions of the most conserved regions of the protein.

8.2 - Comparison with Mig1p

In the process of glucose repression in *S. cerevisiae* Mig1p plays the analogous role to CreA in carbon catabolite repression in *A. nidulans*. At this stage the exact relationship between these two systems is not known. It is likely that they have evolved from the same ancestral system, however there are a number of significant differences between the two systems in these organisms.

Yeast is a highly specialised organism that preferentially utilises fermentative metabolism of glucose and as part of this adaptation its mitochondrial functions are glucose repressible. In the study of carbon catabolite repression in *A. nidulans*, aside from strains containing pANC4ΔRV10, no mutations have been identified which result in a failure to derepress transcription in non-repressing conditions. In *S. cerevisiae* mutations have been identified in a number of loci, such as *snf1*, which have this phenotype. This suggests that there are differences in the mechanism for lifting CreA/Mig1p repression in derepressing conditions or, alternatively that there is some degree of redundancy in the *A. nidulans* system. Homologues of *creA* have been identified in a number of fungal systems including *Schizosaccharomyces pombe*. However despite the entire sequence of *S. cerevisiae* being determined there is no obvious homologue of CreA other than Mig1p.

However, outside the DNA binding domain, there is very little sequence similarity between CreA and Mig1p. There are however, striking similarities in the spatial arrangement of the functional regions within the two proteins (figure 8.2). This conserved spatial arrangement and high level of sequence conservation between the

DNA binding domains of the two proteins strongly suggests that CreA and Mig1p are ancestrally related. The degree of functional conservation between the two proteins and the processes of carbon catabolite repression in *A. nidulans* and glucose repression in *S. cerevisiae* remains to be determined. As most other fungi studied have clear CreA homologues, it is likely that the process used by *A. nidulans* is more highly conserved amongst fungal species and that the changes seen in *S. cerevisiae* reflect its adaptation to its specialised growth conditions.

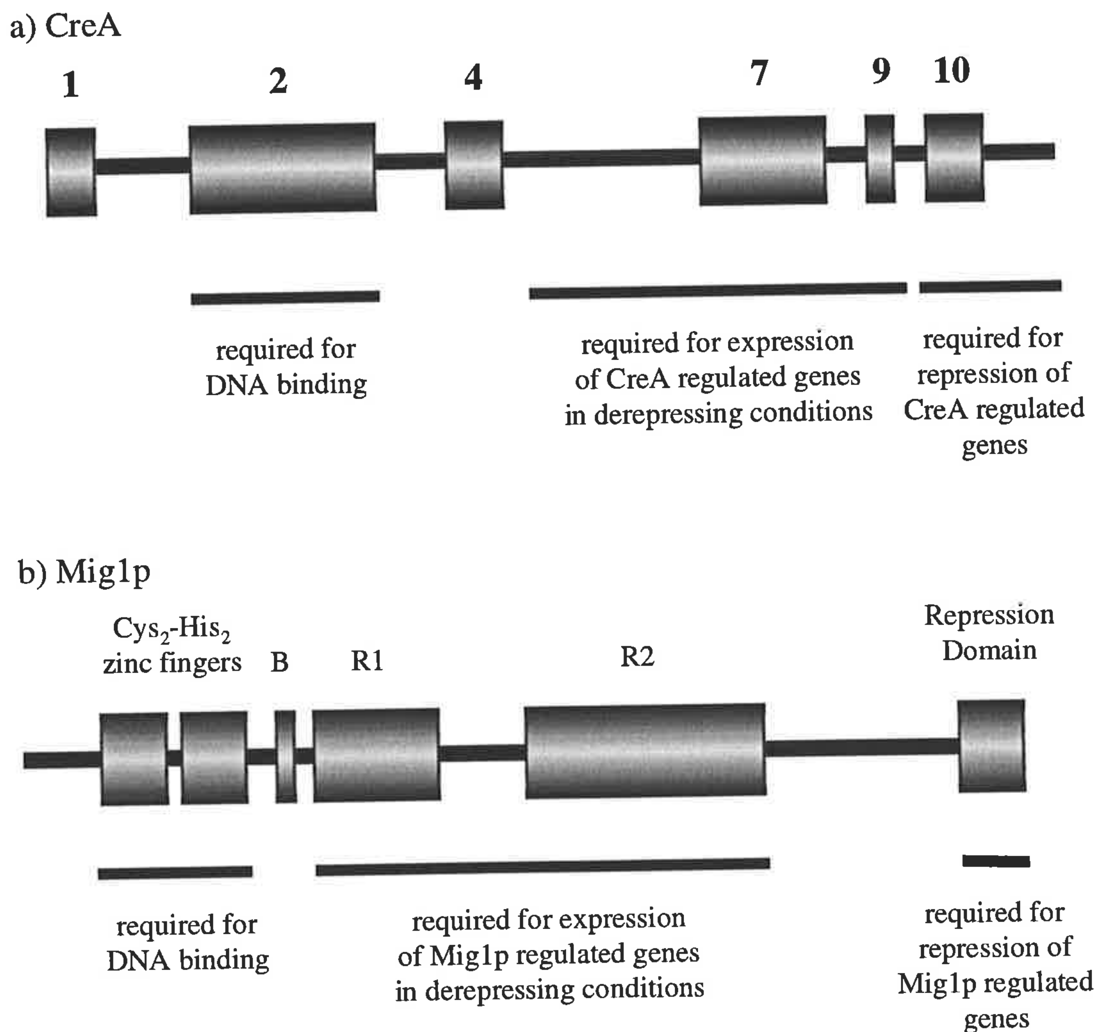


Figure 8.2 - Comparison of functional regions of CreA from *A. nidulans* and Mig1p from *S. cerevisiae*.
a) Functional regions within CreA as defined by this study. b) Functional regions within Mig1p as defined by Ostling *et al.* 1996.

8.3 - Identification of CreA interacting proteins

Screens for suppressors of *creA* alleles were carried out in an attempt to isolate mutations in genes that encode proteins which function in the same pathway or directly interact with CreA.

CreA306 is a full length polypeptide that is unable to bind DNA but still undergo any protein-protein interactions. As a result *creA*306 strains have the most extreme phenotype of any *creA* mutant alleles, including null alleles. Therefore the strain was an ideal candidate for a suppressor screen. Eighty mutants that partially or fully suppressed the effects of *creA*306 were selected on the basis of improved growth rate and conidiation.

Seven of these mutations restored growth and conidiation to levels similar to wild type strains. These mutations were allelic. Four of them were reversions back to the wild type *creA*$^+$ sequence. The remaining three were second site allelic mutations. The missense mutation is adjacent to the original *creA* mutation and changes the same amino acid. This residue changes from a proline to serine. Clearly a serine in this position is not as detrimental to CreA function as a proline residue. This strain has the phenotype of a weak *creA* mutant however unlike all other *creA* alleles this strains with this mutation do not result in reduced conidiation. This further suggests a role for CreA in the process of conidiation.

The remaining suppressors of *creA*306 that were analysed were all extragenic. One of these mutations, *su*(*creA*306)9730, was shown to be allelic or very tightly linked to *creB*. *creB* is a locus previously identified as having a role in carbon catabolite repression in *A. nidulans*. However this is the first direct functional link demonstrated for these two genes.

The remaining extragenic suppressors analysed resulted in strains which have a phenotype similar to *creA*Δ strains. Six of these mutations were tested by sexual analysis. All of the six mutations occur at one locus or are very tightly linked, no recombinants were identified from crosses between these strains. Therefore it is highly likely that these suppressor mutations occur within the same gene. This altered phenotype may be the result of a mutation within a gene that encodes a CreA interacting protein therefore preventing the extreme phenotype of *creA*306 strains. Identification and analysis of these potential interacting proteins will enable a more complete understanding of the process of carbon catabolite repression in *A. nidulans*.

References

Adams TH, Boylan MT, Timberlake WE (1988) *brlA* is necessary and sufficient to direct conidiophore development in *Aspergillus nidulans. Cell* **54**: 353-362

Akasaka Y, Kikuchi H, Nagai T, Hiraoka N, Kato S, Hata J (1993) A point mutation found in the WT1 gene in a sporadic Wilm's tumour without genitourinary abnormalities is identical with the most frequent point mutation in Denys-Drash syndrome. *FEBS* **317**: 39-43

Andrianopoulous A, Hynes MJ (1988) Cloning and analysis of the positively acting regulatory gene *amdR* from *Aspergillus nidulans. Molecular and Cellular Biology* **8**: 3532-3541

Andrianopoulous A, Hynes MJ (1990) Sequence and functional analysis of the positively acting regulatory gene *amdR* from *Aspergillus nidulans. Molecular and Cellular Biology* **10**: 3194-3202

Arst HN (1976) Integrator gene in *Aspergillus nidulans. Nature* **262**: 231-234

Arst HN (1981) Aspects of the control of gene expression in fungi. *Symposium of the Society of General Microbiology* **31**: 131-160

Arst HN, Bailey CR (1977) The regulation of carbon metabolism in *Aspergillus nidulans*. In: Smith JE, Pateman JA (eds) *Genetics and Physiology of Aspergillus nidulans.* Academic Press, London, pp 131-146

Arst HN, Cove DJ (1973) Nitrogen metabolite repression in *Aspergillus nidulans. Molecular and General Genetics* **126**: 111-141

Arst HN, MacDonald DW (1975) A gene cluster in *Aspergillus nidulans* with an internally located cis-acting regulatory region. *Nature* **254**: 26-31

Arst HN, MacDonald DW, Jones SA (1980) Regulation of proline transport in *Aspergillus nidulans. Journal of General Microbiology* **116**: 285-294

Arst HN, Penfold HA, Bailey CR (1979) Lactam utilisation in *Aspergillus nidulans*: evidence for a fourth gene under the control of the integrator gene *intA*. *Molecular and General Genetics* **166**: 312-327

Arst HN, Tollervey D, Dowzer CEA, Kelly JM (1990) An inversion truncating the *creA* gene of *Aspergillus nidulans* results in carbon catabolite repression. *Molecular Microbiology* **4**: 851-854

Bailey CR (1976) Carbon catbolite repression in *Aspergillus nidulans*., University of Canbridge

Bailey CR, Arst HN (1975) Carbon catabolite repression in *Aspergillus nidulans. European Journal of Biochemistry* **51**: 573-577

Bailey RB, Woodword A (1984) Isolation and characterisation of a pleiotropic glucose repression resistant mutant of *Saccharomyces cerevisiae*. *Molecular and General Genetics* **193**: 507

Baird P, Cowell J (1993) A novel zinc finger mutation in a patient with Denys-Drash syndrome. *Human Molecular Genetics* **2**: 301-305

Baird P, Groves N, Haber D, Housman D, Cowell J (1992a) Identification of mutations in the WT1 gene in tumours from patients with the WAGR syndrome. *Oncogene* **7**: 2141-2149

Baird P, Santos A, Groves N, Jadresic L, Cowell J (1992b) Constitutional mutations in the WT1 gene in patients with Denys-Drash syndrome. *Human Molecular Genetics* **1**: 301-305

Bajwa W, Meyhack B, Rudolph H, Schweingruber AM, Hinnen A (1984) Structural analysis of the two tandemly repeated acid phosphatase genes in yeast. *Nucleic Acids Research* **12**: 7721

Bemis LT, Denis CL (1988) Identification of functional regions in the yeast transcriptional activator Adr1p. *Molecular and Cellular Biology* **8**: 2125-3131

Bisson LF, Fraenkel DG (1984) Expression of kinase-dependent glucose uptake in *Saccharomyces cerevisiae*. *Journal of Bacteriology* **159**: 1013

Blumberg H, Eisen A, Sledziewski A, Bader D, Young ET (1987) Two zinc fingers of the yeast regulatory protein shown by genetics evidence to be for its function. *Nature* **328**: 443-445

Bonnefoy N, Copley J, Hynes MJ, Davis MA (1995) Yeast proteins can activate expression through regulatory sequences of the *amdS* gene of *Aspergillus nidulans*. *Molecular and General Genetics* **246**: 223-227

Bruening W, Bardeesy N, Silverman B, Cohn R, Machin G, Aronson A, Houseman D (1992) Germline intronic and exonic mutations in the Wilm's tumour gene (WT1) affecting urogenital development. *Nature Genetics* **1**: 144-148

Call KM, Glaser T, Ito CY, Buckler AJ, Pelletier J, Haber DA, Rose EA, Kral A, Yeger H, Lewis WH, Jones C, Housman DE (1990) Isolation and characterisation of a zinc finger polypeptide gene at the human chromosome 11 WIlm's Timour locus. *Cell* **60**: 509-520

Carlson M, Botstein D (1982) Two differentially regulated mRNAs with different 5' ends encode secreted and intracellular forms of yeast invertase. *Cell* **28**: 145

Carlson M, Botstein D (1983) Organisation of the *SUC* gene family in *Saccharomyces*. *Molecular and Cellular Biology* **3**: 351

Carlson M, Osmond BC, Botstein D (1981) Mutants of yeast defective in sucrose utilisation. *Genetics* **98**: 25

Cassart JP, Georis I, Ostling J, Ronne H, Vandenhaute J (1997) The Mig1 repressor from *Kluveromyces lactis*: cloning, sequencing and functional analysis in *Saccharomyces cerevisiae*. *FEBS Letters*

Celezna JL, Carlson M (1986) A yeast gene that is essential for for release from glucose repression encodes a protein kinase. *Science* **233**: 1175

Celezna JL, Carlson M (1989) Mutational analysis of the *Saccharomyces cerevisiae* Snf1 protein kinase and evidence for interaction with the Snf4 protein. *Molecular and Cellular Biology* **9**: 5034

Celezna JL, Eng FJ, Carlson M (1989) Molecular analysis of the *SNF4* gene of *Saccharomyces cerevisiae*: Evidence for physical association of the Snf4 protein with the Snf1 protein kinase. *Molecular and Cellular Biology* **9**: 5045

Chamalaun-Hussey ND (1996) Characterisation of DNA - binding by the CreA protein of *Aspergillus nidulans*., University of Adelaide

Champe SP, Nagle DL, Yager LN (1994) Sexual Sporulation. In: Martinelli SD, Kinghorn JR (eds) *Aspergillus: 50 years on*, **vol 29**. Elsevier, Amsterdam, London, New York, Tokyo, pp 851

Christy BA, Lau LF, Nathans D (1988) A gene activated in mouse 3T3 cells by serum growth factors encodes a protein with "zinc finger" sequences. *Procedings of the National Academy of Sciences* **85**: 7857-7861

Church GM, Gilbert W (1984) Genomic sequencing. *Procedings of the National Academy of Sciences* **81**: 1991-1995

Clarkson P, Davies H, Williams D, Chaundhary R, Hughes I, Patterson M (1993) Mutational screening of the Wilm's tumour gene, WT1, in males with genital abnormalities. *Journal of Genetical Medicine* **30**: 767-772

Clutterbuck AJ (1993) *Aspergillus nidulans*. In: O'Brien SJ (ed) *Genetic Maps: Locus maps of complex genomes.*, 6th. edn. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, Plainview, N. Y., pp 3.71-3.84

Clutterbuck AJ (1997) The validity of the *Aspergillus nidulans* linkage map. *Fungal Genetics and Biology* **21**: 267-277

Coppes M, Liefers G, Higuchi M, Zinn A, Balfe J, Williams B (1992) Inherited WT1 mutation In Denys-Drash syndrome. *Cancer Research* **52**: 6125-6128

Coppes M, Liefers G, Paul P, Yeger H, Williams B (1993) Homozygous somatic WT1 point mutations in sporadic unilateral Wilm's tumour. *Procedings of the National Academy of Sciences* **90**: 1416-1419

Corvitz PA, Song W, Mitchell AP (1994) Requirement for *RGR1* and *SIN4* in Rme1 dependent repression in *Saccharomyces cerevisiae*. *Genetics* **138**: 577-586

Cove DJ (1966) The induction and repression of nitrate reductase in the fungus *Aspergillus nidulans*. *Biochimica et Biophysica Acta* **113**: 51-56

Creaser EH, Porter RL, Britt KA, Pateman JA, Doy CH (1985) Purification and preliminary characterisation of alcohol dehydrogenase in *Aspergillus nidulans*. *Journal of Biochemistry* **225**: 449-454

Cubero B, Scazzocchio C (1994) Two different adjacent and divergent zinc finger DNA binding sites are necessary for CreA-mediated carbon catabolite repression in the proline gene cluster of *Aspergillus nidulans*. *The EMBO Journal* **13**: 407-415

Davis M, Kelly JM, Hynes MJ (1993) Fungal catabolic gene regulation: molecular genetic analysis of the *amdS* gene of *Aspergillus nidulans*. *Genetica* **90**: 133-145

Depiereux E, Feytmans E (1992) MATCH-Box: A fundamentally new algorithm for the simultaneous alignment of several protein sequences. *Computer Applications in the Biosciences* **8**: 501-509

DeVit MJ, Waddle JA, Johnston M (1997) Regulated nuclear translocation of the Mig1 glucose repressor. *Molecular Biology of the Cell* **8**: 1603-1618

Devriendt K, Deloof E, Moerman P, Legius E, Vanhole C, Zegher Fd, Proesmans W (1995) Diaphragmatic Hernia in Denys-Drash syndrome. *American Journal of Medical Genetics* **57**: 97-101

Donovan DM, Pearson NJ (1986) Transcriptional regulation of ribosomal proteins during a nutritional upshift in *Saccharomyces cerrevisiae*. *Molecular and Cellular Biology* **6**: 2429

Dowzer CEA, Kelly JM (1989) Cloning of *creA* from *Aspergillus nidulans*: a gene involved in carbon catabolite repression. *Current Genetics* **15**: 457-459

Dowzer CEA, Kelly JM (1991) Analysis of the *creA* gene, a regulator of carbon catabolite repression in *Aspergillus nidulans*. *Molecular and Cellular Biology* **11**: 5701-5709

Drysdale MR, Kolze SE, Kelly JM (1993) The *Aspergillus niger* carbon catabolite repressor gene, *creA*. *Gene* **130**: 241-245

Elledge SJ, Zhou Z, Allen JB, Navas TA (1993) DNA damage and cell cycle regulation of ribonucleotide reductase. *BioEssays* **15**: 333-339

Elrod-Erickson M, Rould MA, Nekludova L, Pabo CO (1996) Zif268 protein - DNA complex refined at 1.6A: a model system for understanding zinc finger - DNA interactions. *Structure* **4**: 1171-1180

Englert C, Vidal M, Maheswaran S, Ge YM, Ezzell RM, Isselbacher KJ, Haber DA (1995) Truncated WT1 mutants alter the subnuclear localisation of the wild type protein. *Procedings of the National Academy of Sciences* **92**: 11960-11964

Entian KD, Koetzki E, Frohlich KU, Mecke D (1984) Cloning of hexokinase isoenzyme PI from *Saccharomyces cerevisiae*: PI transformants confirm the role of hexokinase PII for glucose repression in yeast. *Molecular and General Genetics* **198**: 50

Entian KD, Zimmermann FK (1980a) Glycolytic enzymes and intermediates in carbon catabolite repression mutants of *Saccharomyces cerevisiae. Molecular and General Genetics* **177**: 345

Entian KD, Zimmermann FK (1980b) Glycolytic enzymes and intermediates in carbon catbolite repression mutants of *Saccharomyces cerevisiae. Journal of Biological Chemistry*

Espeso EA, Fernandez-Canon JM, Penalva MA (1995) Carbon regulation of penicillin biosynthesis in *Aspergillus nidulans*: A minor effect of mutations in *creB* and *creC. FEMS Microbiological Letters* **126**: 63-68

Espeso EA, Penalva MA (1992) Carbon catabolite repression can account for the temporal pattern of expression of a penicillin biosynthetic gene in *Aspergillus nidulans. Molecular Microbiology* **6**: 1457-1465

Fillinger S, Felenbok B (1996) A newly identified gene cluster in *Aspergillus nidulans* comprises five novel genes localised in the *alc* region that are controlled both by the specific transactivator AlcR and the general carbon-catabolite repressor CreA. *Molecular Microbiology* **20**: 475-488

Fincham JRS, Day PR (1963) Fungal Genetics. Blackwell Scientific Publications, Oxford

Flick JS, Johnston M (1991) *GRR1* of *Saccharomyces cerevisiae* is required for glucose repression and encodes a protein with leucine-rich repeats. *Molecular and Cellular Biology* **11**: 5101

Flick JS, Johnston M (1992) Analysis of URS_G -mediated glucose repression of the *GAL1* promoter of *Saccharomyces cerevisiae. Genetics* **130**: 295

Fujita A, Matsumoto S, Kuhara S, Misumi Y, Kobayashi H (1990) Cloning of the yeast *SFL2* gene: Its disruption results in pleiotropic phenotype characteristic of *tup1* mutants. *Gene* **89**: 93

Gavrias V, Cubero B, Cazelle B, Sophianopoulou V, Scazzocchio C (1993) The proline utilisation gene cluster of *Aspergillus nidulans*. In: Powell KA, Peberdy J, Renwick A (eds) *The Genus Aspergillus*, vol FEMS Symposia No. 69. Plenum Press, New York and London, pp 225-232

Gessler M, Konig A, Arden K, Grundy P, Orkin SH, Sallan S, Peter C (1994) Infrequent mutations of the WT1 genein 77 Wilms' timours. *Human Mutation* **3**: 212-222

Gessler M, Poustka A, Cavenee W, Neve RL, Orkin SH, Bruns GAP (1990) Homozygous deletion in Wilm's tumour of a gene identified by chromosome jumping. *Nature* **343**: 774-778

Gidoni D, Dynan WS, Tjian R (1984) Multiple specific contacts between a mammalian transcription factor and its cognate promoters. *Nature* **312**: 409-413

Giniger E, Varnum SM, Ptashne M (1985) Specific DNA binding of Gal4, a positive regulatory protein of yeast. *Cell* **40**: 767

Gonzales R, Gavrias V, Gomez D, Scazzocchio C, Cubero B (1997) The integration of nitrogen and carbon catabolite repression in *Aspergillus nidulans* requires the GATA factor AreA and an additional positive-acting element, ADA. *The EMBO Journal* **16**

Griggs DW, Johnston M (1991) Regulated expression of the *GAL4* activator genein yeast provides a sensitive genetic switch for glucose repression. *Procedings of the National Academy of Sciences* **88**: 8597

Hanahan (1983) Studies of transformation of *Escherichia coli* with plasmids. *Journal of Molecular Biology* **161**: 557

Hanna-Rose W, Licht JD, Hansen U (1997) Two evolutionarily conserved repression domains in the *Drosophila* Kruppel protein differ in activator specificity. *Molecular and Cellular Biology* **17**: 4820-4829

Hastie AC (1970) Benlate induced instability of *Aspergillus* diploids. *Nature* **226**: 771

Herruer MH, Mager WH, Doorenbosch TM, Wessels PLM, Wassenaar TM, Planta RJ (1989) The extended promoter of the gene encoding ribosomal protein S33 in yeast consists of multiple protein binding elements. *Nucleic Acids Research* **17**: 7427

Hirano T, Kinoshita N, Morikawa K, Yanagida M (1990) Snap helix with knob and hole: essential repeats in *S. pombe* nuclear protein *nuc2*$^{+}$. *Cell* **60**: 319

Hodgson CP, Fisk RZ (1987) Hybridisation probe size control: optimised oligolabelling. *Nucleic Acids Research* **15**: 6295

Hu Z, Nehlin JO, Ronne H, Michels CA (1995) *MIG1* dependent and *MIG1* independent glucose regulation of *MAL* gene expression in *Saccharomyces cerevisiae*. *Current Genetics* **28**: 258-266

Hynes MJ (1975) A cis dominant regulatory mutation affecting enzyme induction in the eukaryote *Aspergillus nidulans*. *Nature* **253**: 210-212

Hynes MJ (1977) Induction of the acetamidase of *Aspergillus nidulans* by acetate metabolism. *Journal of Bacteriology* **131**: 770-775

Hynes MJ (1978) Multiple independent control mechanisms affecting the acetamidase of *Aspergillus nidulans*. *Molecular and General Genetics* **161**: 59-65

Hynes MJ (1980) A mutation adjacent to gene *amdS*, defining the site of action of positive control gene *amdR* in *Aspergillus nidulans*. *Journal of Bacteriology* **142**: 400-406

Hynes MJ (1982) A cis-dominant mutation in *Aspergillus nidulans* affecting the expression of the *amdS* gene in the presence of mutations in the unlinked gene, *amdA*. *Genetics* **102**: 139-147

Hynes MJ, Corrick CM, Kelly JM, Littlejohn TG (1988) Identification of the sites of action for regulatory genes controlling the *amdS* gene of *Aspergillus nidulans*. *Molecular and Cellular Biology* **8**: 2589-2596

Hynes MJ, Kelly JM (1977) Pleiotropic mutants of *Aspergillus nidulans* altered in carbon metabolism. *Molecular and General Genetics* **150**: 193-204

Ilmen M, Thrane C, Pentilla M (1996) The glucose repressor gene *cre1* of *Trichoderma*: Isolation and expression of a full-length and a truncated mutant form. *Molecular and General Genetics* **251**: 451-460

Jiang R, Carlson M (1996) Glucose regulates protein interactions within the yeast Snf1 protein kinase complex. *Genes and Development* **10**: 3105-3115

Jiang R, Carlson M (1997) The Snf1 protein kinase and its activating subunit , Snf4, interact with distinct domains of the Sip1/Sip2/Gal83 component in the kinase complx. *Molecular and Cellular Biology* **17**: 2099-2106

Johnston M (1987) A model fungal gene regulatory mechanism: The *GAL* genes of *Saccharomyces cerevisiae*. *Microbiological Reviews* **51**: 458

Jones SA, Arst HN, MacDonald DW (1981) Genes role in the *prn* cluster of *Aspergillus nidulans*. *Current Genetics* **3**: 49-56

Kafer E (1961) The process of spontaneous recombination in vegetative nuclei of *Aspergillus nidulans*. *Genetics* **45**: 1581-1609

Katz ME, Hynes MJ (1989) Isolation and analysis of of the acetate regulatory gene, *facB*, from *Aspergillus nidulans*. *Molecular and Cellular Biology* **9**: 5696-5701

Keleher CA, Redd MJ, Schultz J, Carlson M, Johnson AD (1992) Ssn6-Tup1 is a general repressor of transcription in yeast. *Cell* **68**: 709

Kelly JM, Hynes MJ (1977) Increased and decreased sensitivity to carbon catabolite repression of enzymes of acetate metabolism in mutants in *Aspergillus nidulans*. *Molecular and General Genetics* **156**: 87-92

Kelly JM, Hynes MJ (1987) Multiple copies of the *amdS* gene of *Aspergillus nidulans* cause titration of trans-acting regulatory proteins. *Current Genetics* **11**: 21-31

Kikuchi H, Akasaka Y, Kurosawa Y, Yoneyama H, Kato S, J H (1995) A critical mutation in both WT1 alleles is not sufficient to cause Wilms' tumour. *FEBS Letters* **360**: 26-28

King-Underwood L, Renshaw J, Pritchard-Jones K (1996a) Mutations in the Wilms' tumour gene WT1 in leukaemias. *Blood*

King-Underwood L, Renshaw J, Pritchard-Jones K (1996b) Mutations in the Wilms' tumour gene WT1 in leukaemias. *Blood* **87**: 2171-2179

Komachi K, Johnson AD (1997) Residues in the WD repeats of TUP1 required for the interaction with alpha-2. *Molecular and Cellular Biology* **17**: 6023-6028

Kosters R, Adams V, Hassam S, Schafer R, Schmid M, Moos R, Briner J (1995) Mutational analysis of the tumour suppressor gene WT1 - detection of a homozygous point mutation in sporadic unilateral Wilms' tumour. *International Journal of Oncology* **7**: 1103-1107

Kulmberg P, Judewicz N, Mathieu M, Sequeval D, Felenbok B (1992) Specific binding sites for the activator protein, AlcR, in the *alcA* promoter of the ethanol regulon of *Aspergillus nidulans*. *Journal of Biological Chemistry* **267**: 21146-21153

Kulmberg P, Mathieu M, Dowzer CEA, Kelly JM, Felenbok B (1993) Specific binding sites in the *alcR* and *alcA* promoters of the ethanol regulon for the CreA repressor mediating carbon catabolite repression in *Aspergillus nidulans*. *Molecular Microbiology* **7**: 847-857

Lee BN, Adams TH (1996) FluG and FlbA function independently to initiate conidiophore development in *Aspergillus nidulans* through *brlA*-beta activation. *The EMBO Journal* **15**: 299-309

Lemontt JF, Fugit DR, MacKay VL (1980) Pleiotropic mutations at the *TUP1* locus that affect the expression of mating-type-dependent functions in *Saccharomycs cerevisiae*. *Genetics* **94**

Li Y, Bjorklund S, Jiang YW, Kim Y-J, Lane WS, Stillman DJ, Kornberg RD (1995) Yeast global transcriptional regulators Sin4 and Rgr1 are components of mediator complex / RNA polymerase II holoenzyme. *Procedings of the National Academy of Sciences* **92**: 10864-10868

Licht JD, Grossel MJ, Figge J, Hansen UM (1990) *Drosophila Kruppel* protein is a transcriptional repressor. *Nature* **346**: 76-79

Licht JD, Hanna-Rose W, Reddy JC, English MA, Ro M, Grossel M, Shankovich R, Hansen U (1994) Mapping and mutagenesis of the amino-terminal transcriptional repression domain of the *Drosophila Kruppel* protein. *Molecular and Cellular Biology* **14**: 4057-4066

Lints R, Davis MA, Hynes MJ (1995a) The positively acting *amdA* gene of *Aspergillus nidulans* encodes a protein with two C2H2 zinc-finger motifs. *Molecular Microbiology* **15**: 965-975

Little M, Prosser J, Condie A, Smith P, Heyningen Vv, Hastie N (1992) Zinc finger mutations within the WT1 gene in Wilms' tumour gene in Wilms' tumour patients. *Procedings of the National Academy of Sciences* **89**: 4791-4795

Little M, Williamson K, Mannens M, Kelsey A, Gosden C, Hastie N, Heyningen Vv (1993) Evidence that WT1 mutations in Denys-Drash syndrome may act in a dominant negative fashion. *Human Molecular Genetics* **2**: 259-264

Littlejohn TG, Hynes MJ (1992) Analysis of the site of action of the *amdR* product for regulation of the *amdS* gene of *Aspergilus nidulans*. *Molecular and General Genetics* **235**: 81-88

Lobo Z, Maitra PK (1977) Genetics of yeast hexokinase. *Genetics* **86**: 727

Lockington RA, Scazzocchio C, Sequeval D, Mathieu M, Felenbok B (1987) Regulation of *alcR*, the positive regulatory gene of the ethanol utilisation regulon of *Aspergillus nidulans*. *Molecular Microbiology* **1**: 275-281

Lockington RA, Sealy-Lewis HN, Scazzocchio C, Davies RW (1985) Regulation of *alcR*, the positive regulatory gene of the ethanol utilisation regulon of *Aspergillus nidulans*. *Gene* **33**: 137-149

Lundin M, Nehlin JO, Ronne H (1994) Importance of a flanking AT-rich region in target site recognition by the GC box-binding zinc finger protein Mig1. *Molecular and Cellular Biology* **14**: 1979-1985

Ma H, Bloom LM, Walsh CT, Botstein D (1989a) The residual phosphorylation activity of hexokinase II mutants is correlated with glucose repression in *Saccharomyces cerevisiae. Molecular and Cellular Biology* **9**: 5643

Ma H, Bloom LM, Zhu Z, Walsh CT, Botstein D (1989b) Isolation and characterisation of mutations in *HXK2* gene of *Saccharomyces cerevisiae. Molecular and Cellular Biology* **9**: 5630

Ma J, Patashne M (1987) Deletion analysis of Gal4 defines two transcriptional activating segments. *Cell* **48**: 847

MacKay VL (1983) Cloning of yeast *STE* genes in two micron vectors. *Methods in Enzymology* **101**: 325-343

Mathieu M, Felenbok B (1994) The *Aspergillus nidulans* CreA protein mediates glucose repression of the ethanol regulon at various levels through competition with the AlcR specific transactivator. *The EMBO Journal* **13**: 4022-4027

Messing J (1979) A multipurpose cloning system based on a single-stranded DNA bacteriophage M13. *Recombinant DNA Technology Bulletin* **2**: 3999-4010

Miller J, McLachlan AD, Klug A (1985) Repetitive zinc-binding domains in the protein transcription factor IIIA from *Xenopus* oocytes. *The EMBO Journal* **4**: 1609-1614

Mukai Y, Harashima S, Oshima Y (1991) AAR/TUP1 protein, with a structure similar to that of the β-subunit of G proteins, is required for **α1**-α2 and α2 repression in cell type control of *Saccharomyces cerevisiae. Molecular and Cellular Biology* **11**: 3773

Murphy RL, Andrianopoulous A, Davis MA, Hynes MJ (1997) Identification of *amdX*, a new Cys_2-His_2 (C2H2) zinc-finger gene involved in the regulation of the *amdS*of *Aspergillus nidulans. Molecular Microbiology* **23**: 591-602

Neer EJ, Schmidt CJ, Nambudripad R, Smith TF (1994) The ancient regulatory-protein family of WD-repeat proteins. *Nature* **371**: 297-300

Nehlin J, Ronne H (1990) Yeast Mig1p repressor is related to the mammalian early growth response and Wilms' tumour finger proteins. *The EMBO Journal* **9**: 2891-2898

Nehlin JO, Carlberg M, Ronne H (1991) Control of yeast *GAL* genes by Mig1p repressor: a transcriptional cascade in the glucose response. *The EMBO Journal* **10**: 3373-3377

Niederacher D, Entian KD (1987) Isolation and characterisation of the regulatory *HEX2* gene necessary for glucose repression in yeast. *Molecular and General Genetics* **206**

Niegeborn L, Carlson M (1984) Genes effecting the regulation of *SUC2* gene expression by glucose repression in *Saccharomyces cerevisiae*. *Genetics* **108**: 845

Nordenskjold A, Friedman E, Sandstedt B, Soderhall S, Anvret M (1995) Absence of mutation in the WT1 gene with XY gonadal dysgenesis. *Human Genetics* **96**: 102-104

Oakley CE, Well CF, Kretz PL, Oakley BR (1987) Cloning of the *riboB* locus of *Aspergillus nidulans*. *Gene* **53**: 293-298

Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T (1989) Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Procedings of the National Academy of Sciences* **86**: 2766-2770

Ostling J, Carlberg M, Ronne H (1996) Functional domains in the Mig1 repressor. *Molecular and Cellular Biology* **16**: 753-761

Ozcan S, Schulte F, Friedel K, Weber A, Ciriacy M (1994) Glucose uptake and metabolism in *grr1/ cat8* mutants of *Saccharomyces cerevisiae*. *Journal of Biochemistry* **224**: 605-611

Parraga G, Horvath SJ, Eisen A, Taylor WE, Hood L, Young ET, Klevit RT (1988) Zinc-dependent structure of a single-factor domain of yeast Adr1p. *Science* **241**: 1489-1492

Pateman JH, Doy CH, Olson JE, Norris U, Creaser EH, Hynes M (1983) Regulation of alcohol dehydrogenase (ADH) and Aldehyde dehydrogenase (AldDH) in *Aspergillus nidulans*. *Procedings of the Royal Society of London* **B217**: 243-264

Pavletich NP, Pabo CO (1991) Zinc finger-DNA recognition: crystal structure of Zif268 - DNA complex at 2.1 A. *Science* **252**: 809-817

Pelletier J, Bruening W, Kashtan C, Mauer S, Manivel J, Stiegel J, Houghton D (1991) Germline mutations in the Wilms' tumour suppressor gene are associated with abnormal urogenital development in Denys-Drash syndrome. *Cell* **67**: 437-447

Perlman D, Halvorson HO (1981) Distinct repressible mRNAs for cytoplasmic and secreted yeast invertase are encoded by a single gene. *Cell* **25**: 525

Peters DG, Caddick MX (1994) Direct analysis of native and chimeric GATA specific DNA binding proteins from *Aspergillus nidulans*. *Nucleic Acids Research* **22**: 5164-5172

Poulat E, Morin D, Konig A, Brun P, Giltway J, Sultan C, Dumas R (1993) Distinct molecular origins for Denys-Drash and Frasier syndromes. *Human Genetics* **91**: 285-286

Pritchard-Jones K, Fleming S, Davidson D, Bickmore W, Porteus D, Gosden C, Bard J (1990) The candidate Wilm's tumour gene is involved in genitourinary development. *Nature* **346**: 194-197

Pritchard-Jones K, Renshaw J, King-Underwood L (1994) The Wilms' tumour gene (WT1) is mutated in a secondary leukaemia in a WAGR patient. *Human Molecular Genetics* **3**: 1633-1637

Quek H, Show V, tock E (1993) The 3rd zinc finger of the WT1 gene is mutated in Wilms' tumour but nit in a broad range of other urogenital tumours. *Anticancer Research* **13**: 1575-1580

Reymond-Cotton P, Fraissinet-Tachet L, Fevre M (1996) Expression of the *Sclerotinia sclerotiorum* polygalacturonase *pg1* gene: possible involvement of CreA in glucose catabolite repression. *Current Genetics* **30**: 240-245

Richardson IB, Katz ME, Hynes MJ (1992) Molecular characterisation of the *lam* locusand sequences involoved in regulation by the AmdR protein of *Aspergillus nidulans*. *Molecular and Cellular Biology* **12**: 337-346

Romano AH, Kornberg HL (1968) Regulation of sugar utilisation by *Aspergillus nidulans*. *Biochimica et Biophysica Acta* **158**: 491-493

Ronne H (1995) Glucose repression in fungi. *Trends in Genetics* **11**: 12-17

Rose M, Albig W, Entian KD (1991) Glucose repression in *Saccharomyces cerevisiae* is directly associated with hexose phosphorylation by hexokinases PI and PII. *European Journal of Biochemistry* **199**: 511

Rothstein RJ, Sherman F (1980) Genes affecting the expression of cytochrome c in yeast: Genetic mapping and genetic interactions. *Genetics* **94**: 871

Sakai A, Shimizu Y, Hishinuma F (1988) Isolation and characterisation of mutants which show an oversecretion phenotype in *Saccharomyces cerevisiae*. *Genetics* **119**: 499

Sakai A, Shimizu Y, Kondou S, Chibazakura T, Hishinuma F (1990) Structure and molecular analysis of *RGR1*, a gene required for glucose repression of *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* **10**: 4130

Sakai A, Tadokoro K, Yanagisawa H, Nagafuchi S, Hoshikawa N, Suzuki T, Kohsaka T (1993) A novel mutation of the WT1 gene (A tumour suppressor gene for Wilms' tumour) in a patient with Denys-Drash syndrome. *Human Molecular Genetics* **2**: 1969-1970

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual., 2nd. edn. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, Plainview, N. Y.

Sarokin L, Carlson M (1985) Upstream region of required for the regulated expression of of the glucose repressible *SUC2* gene of *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* **42750**

Scazzocchio C, Gavrias V, Cubero B, Panozza C, Mathieu M, Felenbok B (1995) Carbon catabolite repression in *Aspergillus nidulans*: a review. *Canadian Journal of Botany* **73(Supplement 1)**: S160-S166

Schuller HJ, Entian KD (1991) Extragenic suppressor of yeast glucose derepression mutants leading to constitutive synthesis of several glucose repressible enzymes. *Journal of Bacteriology* **173**: 2045-2052

Schultz J, Carlson M (1987) Molecular analysis of *SSN6*, a gene functionally related to the *SNF1* protein kinase of *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* **7**: 3637

Schultz J, Marshall-Carlson L, Carlson M (1989) The N-terminal tetratricopeptide region is the functional domain of SSN6, a nuclear phosphoprotein of *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* **10**: 4744

Screen S, Bailey A, Charnley K, Cooper R, Clarkson J (1997) Carbon regulation of the cuticle degrading enzyme PR1 from *Metarhizium anisopilae* may involve a transacting DNA binding protein CRR1, a functional equivalent of the *Aspergillus nidulans* CreA protein. *Current Genetics* **31**: 511-518

Sealy-Lewis HM, Lockington RA (1984) Regulation of two alcohol dehydrogenases in *Aspergillus nidulans*. *Current Genetics* **8**: 253-259

Shimada H, Fukasawa T (1985) Controlled transcription of the yeast regulatory gene *GAL80*. *Gene* **39**: 1

Shroff RA, Lockington RA, Kelly JM (1996) Analysis of mutations in the *creA* gene involved in carbon catabolite repression in *Aspergillus nidulans*. *Canadian Journal of Microbiology* **42**: 950-959

Shroff RA, O'Connor SM, Hynes MJ, Lockington RA, Kelly JM (1997) Null alleles of *creA*, the regulator of carbon catabolite repression in *Aspergillus nidulans*. *Fungal Genetics and Biology* **22**: 28-38

Sikorsky RS, Boguski MS, Goebl M, Hieter P (1990) A repeating amino acid motif in *CDC23* defines a family of proteins and a new relationship among genes required for mitosis and RNA synthesis. *Cell* **60**: 307

Sophianopoulou V, Suarez V, Diallinas T, Scazzocchio C (1993) Operator derepressed mutations in the proline utilisation gene cluster of *Aspergillus nidulans. Molecular and General Genetics* **236**: 209-213

Stanojevic D, Small S, Levine M (1991) Regulation of a segmentation stripe by overlapping activators and repressors in the *Drosophila* embryo. *Science* **254**: 1385-1387

Stark HC, Fugit D, Mowshowitz DB (1980) Pleiotropic properties of a yeast mutant insensitive to catabolite repression. *Genetics* **94**: 921

Stillman DJ, Dorland S, Yu Y (1994) Epistasis analysis of suppressor mutations that allow *HO* expression in the absence of the yeast *SWI5* transcriptional activator. *Genetics* **136**: 781-788

Strauss J, Mach RL, Zeilinger S, Hartler G, Stoffler G, Wolschek M, Kubicek C (1995) Cre1, the carbon catabolite repressor protein from *Trichoderma reesei. FEBS Letters* **376**: 103-107

Suzuki M (1989) SPXX, a frequent sequence motif in genes encoding regulatory proteins. *Journal of Molecular Biology* **207**: 61-84

Suzuki M, Gerstein M, Yagi N (1994) Stereochemical basis of DNA recognition by Zn fingers. *Nucleic Acids Research* **22**: 3397-3405

Takashima S, Nakamura A, Iikura H, Masaki H, Uozumi T (1996) Clonong of a gene encoding a putative carbon catabolite repressor from *Trichoderma reesei. Biosciences Biotechnology and Biochemistry* **60**: 173-176

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673-4680

Thompson-Jaeger S, Francois J, Gaughran JP, Tatchell K (1991) Deletion of *SNF1* affects the nutrient response of yeast and resembles mutations which activate the adenylate cyclase pathway. *Genetics* **129**: 697-706

Tilburn J, Sarkar S, Widdick DA, Espeso EA, Orejas M, Mungroo J, Penalva M, Arst HN (1995) The *Aspergillus* PacC zinc finger transcription factor mediates regulation of both acid- and alkaline-expressed genes by ambient pH. *The EMBO Journal.* **14**: 779-790

Tilburn J, Scazzocchio C, Taylor G, Zabicky-Zissman J, Lockington RA, Davies RW (1983) Transformation by integration in *Aspergillus nidulans*. *Gene* **26**: 205-221

Todd RB (1995) Analysis of the *facB* gene of *Aspergillus nidulans*., University of Melbourne

Todd RB, Murphy RL, Martin HM, Sharp JA, M. A D, Katz ME, Hynes MJ (1996) The acetate regulatory gene *facB* of *Aspergillus nidulans* encodes a $Zn(II)_2Cys_6$ transcriptional activator. *Molecular and General Genetics* **254**: 495-504

Treitel MA, Carlson M (1995) Repression by Ssn6-Tup1 is directed by Mig1, a repressor / activator protein. *Procedings of the National Academy of Sciences* **92**: 3132-3136

Trumbly RJ (1986) Isolation of *Saccharomyces cerevisiae* mutants constitutive for invertase synthesis. *Journal of Bacteriology* **166**: 1123

Trumbly RJ (1988) Cloning and characterisation of the *CYC8* genemediating glucose repression in yeast. *Gene* **73**: 97

Tsuda M, Sakiyama T, Kitagawa T, Watanabe S, Takahashi S, Kawaguchi H (1993) Molecular analysis of two Japanese cases of Denys-Drash syndrome. *Journal of Inherited Metabolic Disease* **16**: 876-880

Tu J, Varlson M (1995) Reg1p binds to protein phosphotase type I and regulates glucose repression in *Saccharomyces cerevisiae*. *The EMBO Journal* **14**: 5939-5946

Tzamarias D, Struhl K (1994) Functional dissection of the yeast Cyc8-Tup1 transcriptional corepressor complex. *Nature* **369**: 758-761

Tzamarias D, Struhl K (1995) Distinct TPR motifs of Cyc8 are involved in recruiting the Cyc8-Tup1 corepressor complex to differentially regulated promoters. *Genes and Development* **9**: 821-831

Vallier LG, Carlson M (1994) Synergistic release from glucose reprssion by *mig1* and *ssn* mutations in *Saccharomyces cerevisiae*. *Genetics* **137**: 49-54

Varanasi R, Bardeesy N, Ghahremani M, Petruzzi M, Nowak N, Adam M, Grundy P (1994) Fine structure analysis of the WT1 gene in sporadic Wilms' tumour. *Procedings of the National Academy of Sciences* **91**: 3554-3558

Varanasi U, Klis M, Mikesell PB, Trumbly RJ (1996) The Cyc8 (Ssn6) - Tup1 corepressor complex is composed of one Cyc8 and four Tup1 subunits. *Molecular and Cellular Biology* **16**: 6707-6714

Vojtek AB, Fraenkel DG (1990) Phosphorylation of yeast hexokainases. *European Journal of Biochemistry* **190**: 371

Webb N, Lewis M, Willamson K, Heyningen Vv, Bruce J, Lendon M, Postlethwaite R (1995) Do children with diffuse mesangial sclerosis in association with mutations of the Wilms' tumour suppressor gene (WT1) require bilateral nephrectomy? *Pediatric Nephrology* **9**: 252-253

Williams FE, Trumbly RJ (1990) Characterisation of *TUP1*, a mediator of glucose repression in *Saccharomyces cerevisiae. Molecular and Cellular Biology* **10**: 6500

Williams FE, Varanasi U, Trumbly RJ (1991) The CYC8 and TUP1 proteins involved in glucose repression in *Saccharomyces cerevisiae* are associated in a protein complex. *Molecular and Cellular Biology* **11**: 3307

Woods A, Munday MR, Scott J, Yang X, Carlson M, Carling D (1994) Yeast Snf1 is functionally related to mammalian AMP-activated protein kinase and regulates acetyl-CoA carboxylase *in vivo. Journal of Biological Chemistry* **30**: 19509-19515

Zimmermann FK, Scheel L (1977) Mutants of *Saccharomyces cerevisiae* resistant to carbon catabolite repression. *Molecular and General Genetics* **154**: 75

Zitomer RS, C. V L (1992) Regulation of gene expression by oxygen in *Saccharomyces cerevisiae. Microbiological Reviews* **56**: 1-11

Zuo P, Stanojevic D, Colgan J, Han K, Levine M, Manley JL (1991) Activation and repression of transcription by the gap proteins hunchback and kruppel in cultured *Drosophila* cells. *Genes and Development* **5**: 254-264

Appendix

Two articles have been published from this work. They are,

Shroff RA, Lockington RA, Kelly JM (1996) Analysis of mutations in the *creA* gene involved in carbon catabolite repression in *Aspergillus nidulans*. *Canadian Journal of Microbiology* **42**: 950-959

Shroff RA, O'Connor SM, Hynes MJ, Lockington RA, Kelly JM (1997) Null alleles of *creA*, the regulator of carbon catabolite repression in *Aspergillus nidulans*. *Fungal Genetics and Biology* **22**: 28-38

Shroff, R.A., Lockington, R.A., and Kelly, J.M., (1996) Analysis of mutations in the *creA* gene involved in carbon catabolite repression in *Aspergillus nidulans. Canadian Journal of Microbiology, v. 42 (9), pp. 950-959.*

NOTE:
This publication is included in the print copy
of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

http://dx.doi.org/10.1139/m96-122

Shroff, R.A., O'Connor, S.M., Hynes, M.J., Lockington, R.A., and Kelly, J.M., (1997) Null alleles of *creA,* the regulator of carbon catabolite repression in *Aspergillus nidulans.*
Fungal Genetics and Biology, v. 22 (1), pp. 28-38.

NOTE:
This publication is included in the print copy
of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

http://dx.doi.org/10.1006/fgbi.1997.0989