Molecular analysis of genes involved in carbon catabolite repression in *Aspergillus nidulans*

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Erratum:

p17 (after final line) involved in mouse early growth response, has allowed the prediction of which residues are involved in binding (Pavletich and Pabo, 1991).

p42 (line 17) Novozyme was used at a concentration of 0.1mg per gram of dry mycelia.

p42 (end section 2.2.1) The liquid media used for protoplast preparation was ANM.

p55 and Fig. 3.3 The removal of the region encoding amino acids 47-124 was achieved using PCR.

p58, Fig. 3.4 The total amount of genomic DNA loaded in each lane in Part A and C is approximately 15 micrograms, and in part B approximately 10 micrograms.

p104, Fig. 4.4 The sizes of the molecular weight markers shown on the left hand side of part A are incorrectly aligned - the sizes of the bands making the doublet are 42 and 40kDa respectively, followed by a 31kDa band and a 21kDa band.

p133, Fig. 5.1 The parental origin of the mutant strains is given in the Legend to Figure 5.2 on page 135.

p146, Fig. 5.7 In figures A, B and C, the first two colonies of each row are duplicates inoculated with the double mutant strain listed at the left hand side.

p150, Fig. 5.11 The yellow strain designated Wild Type is H17A12.

p155, Fig. 5.11 The first track in parts B and C labelled λ contains the molecular weight marker λ digested with Hind111.

Additions to the references:

Southern, E. M. (1975) Detection of Specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.,* **98**:503.

Upshall, A. (1986) Molecular analysis of the *argB* gene of *Aspergillus nidulans*. *Molecular and General Genetics,* **204**:349-354.

Bailey (1976), Chamalaun Hussey, (1996), and Shroff (1997), are PhD theses.

Abstract

Carbon catabolite repression is a system of regulation of gene expression such that, in the presence of glucose, the expression of genes required to utilise carbon sources other than glucose is repressed. This system benefits the cell by ensuring that glucose is used in preference to other carbon sources, thus limiting unnecessary expenditure of energy. Carbon catabolite repression has been studied in both prokaryotes and in simple eukaryotes. In eukaryotes, carbon catabolite repression has been studied extensively in *Saccharomyces cerevisiae* and many elements involved in the regulation, from membrane bound glucose sensors to the DNA binding proteins, have been described in this organism.

Much is also known about carbon catabolite repression in *Aspergillus nidulans* from genetic analysis and a number of genes have been identified. CreA, a zinc finger DNA binding protein, binds to the promoters of both structural genes and positively acting regulatory genes, leading to repression. Many *creA* alleles are available for study and they all show partial derepression of some enzymes usually subject to carbon catabolite repression. Many also have a compact morphology on complete medium.

A CreA null strain has been described which has a lethal phenotype (Dowzer and Kelly, 1991). However, recent analysis of some extreme *creA* alleles has suggested that a CreA-null strain may be viable (Shroff, *et al.* 1997). In this thesis the construction of a viable CreA null strain is outlined, and the phenotype of this strain is compared with the extreme alleles that led to its construction. An explanation for the apparent lethality of the original CreA null strain has been proposed.

Antibodies are essential for investigating aspects of regulation both by and of, CreA, but they have been difficult to obtain (Chamalaun-Hussey, 1996). Two approaches have been used in this thesis to detect native CreA with antibodies. The first approach

has been to raise polyclonal antibodies to two fusion proteins containing GST and two regions of CreA. The second approach has been to construct a strain of *A. nidulans* which expresses MRGS.His_6-tagged CreA which can be recognised by commercially produced, monoclonal antibodies to the tag. Neither approach has allowed detection of CreA, and it has been concluded that CreA is either present at very low levels in the cell, or is very unstable under the various extraction conditions used. These properties make it difficult to detect CreA with antibodies.

Strains which show altered response to carbon catabolite repression have been selected in many fungal species as 2-deoxy-D-glucose resistant mutants. In *S. cerevisiae*, several loci have been identified which are involved in carbon catabolite repression. Consequently, mutants of *A. nidulans* which are resistant on medium containing 2-deoxy-D-glucose and a non-repressing carbon source, have been selected and analysed to identify more loci involved in carbon catabolite repression. Fourteen strains have been selected and the analyses are presented in this thesis. They are all either allelic or very closely linked to *sorA*. The role of SorA in carbon catabolite repression in *A. nidulans* is not yet clear.

Declaration

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

The major part of the sequencing of the gene at the 3' end of *creA*, as described in Chapter 3 (Figures 3.14 and 3.15), was done by N. Olesnicky.

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

Susan O'Connor

February 19th, 1999

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List of Abbreviations

ANM: *Aspergillus* nitrogen free media
APS: ammonium peroxidosulphate
bp: base pair
BSA: bovine serum albumen
°C: degrees Celsius
cDNA: DNA complementary to mRNA
dH2O: distilled water
DNA: deoxyribonucleic acid
DTT: 1,4-dithiothreitol
EDTA: disodium salt of ethylenediaminetetraacetic acid
g: gram
GST: glutathione-S-transferase
GFP: green fluorescent protein
HCl: hydrochloric acid
His: histidine
IPTG: isopropyl-b-D-thiogalactopyranoside
kb: kilobase
kDa: kiloDalton
mCi: microCurie
mg: microgram
ml: microlitre
mg: milligram
ml: millilitre
mM: millimolar
M: Mole
mRNA: messenger ribonucleic acid
NAD: b-nicotinamide-adenine dinucleotide
NaOH: sodium hydroxide
d-NTP's: 2'-deoxynucleoside-triphosphate
^{32}P-a-dATP: 2'-deoxyadenosine-triphosphate
L: litre
PAGE: poly acrylamide gel electrophoresis
PEG: polyethylene glycol
PCR: polymerase chain reaction
3' RACE: random amplification of cDNA ends
rpm: revolutions per minute
RT: reverse transcriptase
SDS: sodium dodecyl sulphate
TEMED: N,N,N',N',-tetramethylethylenediamine
Tris: Trizma Base(tris(hydroxymethyl)aminomethane
UV: ultraviolet
X-gal: 5'-bromo-4-chloro-3-indolyl-b-D-galactopyranoside
V: volt
v/v: volume per volume
w/v: weight per volume

Chapter 1 Introduction

The regulation of gene expression is an essential function in all living organisms and the maintenance of differentiation, the stages of development and the provision of energy and food for the cell, are examples of precisely controlled gene expression. To effect this control, cells must be able to detect stimuli and transmit a signal to the regulatory mechanisms acting at the DNA level, which respond by activating or repressing genes or groups of genes as required.

Among the mechanisms employed to regulate gene expression are those acting at the level of transcription or translation, including post transcriptional or translational modifications. Examples of such modifications include altered mRNA stability or structure, altered chromatin structure and protein phosphorylation. One of the most commonly employed methods of regulation occurs at the level of initiation of gene transcription, and this is commonly regulated by repression so that genes are not transcribed unless required. Repression may occur not only at the promoters of structural genes but also at the promoters of positively acting regulatory genes to ensure tight control. Activation of transcription also occurs at several levels and often requires induction, activation and either relief from repression or the ability to override repression mechanisms.

Carbon catabolite repression is a system which is regulated by transcriptional control. Here, the transcription of many genes is repressed to ensure that cells only use the most favourable carbon sources available to them. When simple hexoses are present, the enzymes and permeases necessary to utilise other carbon sources are not expressed because their genes have been repressed in response to the glucose/sucrose stimulus in the environment. Carbon catabolite repression, which is also known as glucose repression, has been studied in many organisms. Glucose repression of the lactose operon in *Eschericia coli* is an example of transcriptional regulation in a prokaryote and is regulated by cyclic AMP (cAMP) and the catabolite activator protein (CAP). Levels

of cAMP are low when glucose levels are high and vice versa. As the level of glucose in the cell decreases, the amount of cAMP increases to a level where, together with CAP, it activates transcription of the operon.

Glucose repression in eukaryotes has been extensively studied in *Saccharomyces cerevisiae* and many genes have been identified in the complex regulatory cascade that comprises glucose repression in this species, including those encoding putative glucose sensor molecules. In *Aspergillus nidulans* glucose repression and its main effector, the wide domain repressor protein CreA, have also been well studied. In these two species, the regulatory controls imposed by glucose repression have been revealed by mutational analyses. Mutants which have lost their ability to repress the enzymes and permeases usually subject to carbon catabolite repression also commonly exhibit other defects including poor sporulation and severely altered colony morphology. These mutant phenotypes indicate that the maintenance of carbon catabolite repression is required for the normal function of many cellular activities.

The elements of glucose repression in *S. cerevisiae* and *A. nidulans* are discussed in detail in this chapter. Information from research in other fungal species such as *Neurospora crassa*, *Trichoderma reesii* and *Aspergillus niger,* has been added to provide an overview of the regulatory events which occur in response to the presence of glucose in the cellular environment.

1.1 Carbon Catabolite Repression

1.1.1 Carbon catabolite repression in *Saccharomyces cerevisiae*

In the yeast, *S. cerevisiae*, carbon catabolite repression has been shown to occur at the level of transcription as mRNA levels for genes subject to repression are reduced in the presence of glucose (Carlson and Botstein, 1982).

Well studied systems which are repressed by glucose, include the *GAL* genes which are required for the metabolism of galactose and melibiose, the *MAL* genes which are

necessary for the utilisation of maltose, and *SUC2* which encodes invertase which is required for the metabolism of sucrose, raffinose and other disaccharides. The *MAL* and *GAL* genes are inducible while the expression of the *SUC2* is constitutive. However all three systems are repressed by glucose.

Glucose repression of the *GAL* gene family operates at two levels. The gene involved in the induction mechanism, *GAL4*, which encodes the transcription activating protein Gal4p, is repressed by glucose, as are the genes encoding permeases and enzymes required for the utilisation of galactose and melibiose (*GAL1, GAL2, GAL7, GAL10*, and *MEL1*). A similar situation is found for the *MAL* gene family. Glucose repression affects both *MAL63* which encodes the transcriptional activator Mal63p, which is involved in induction by maltose, and the structural genes *MAL61* and *MAL62* which encode maltose permease and maltase respectively.

Many of the genes which are involved in carbon catabolite repression in *S. cerevisiae* have been identified as mutations which lead to either derepression in the presence of glucose, or failure to derepress when glucose levels are low.

1.1.1.1 *MIG1*

MIG1 encodes the repressor protein, Mig1p, which was first identified as a multi copy inhibitor of *GAL1* in derepressing conditions (Nehlin and Ronne, 1990). Subsequently, other genes, *GAL4*, *SUC2* , *MAL61, MAL62* and *MAL63* were shown to be similarly repressed, and strains carrying a *MIG1* deletion, mutated *MIG1* or mutated Mig1p binding sites, showed relief of glucose repression (Nehlin and Ronne, 1990; Nehlin *et al.* 1991; Lundin *et al.* 1994; Hu *et al.* 1995). This evidence suggested that Mig1p is a negatively acting regulatory protein.

Mig1p is a Cys_2His_2, zinc finger DNA binding protein which binds to the sequence 5' A/T A/T A/T C/G C/T G G G G 3' (Lundin *et al.* 1994). Binding by Mig1p alone does

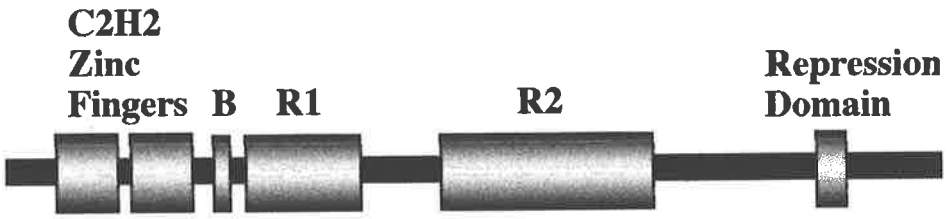
not cause repression as was shown when *lexA-MIG1* fusion proteins repressed target genes bearing *lexA* operators in an *SSN6-TUP1* dependant manner. In mutant *TUP1* or *SSN6* backgrounds, *LexA-MIG1* activated, rather than repressed, the transcription of target genes (Keleher *et al.* 1992; Tzamarias and Struhl, 1994). Yeast 2-hybrid screens showed a direct interaction between Ssn6p and Mig1p and indicated that Ssn6p provides the link between Tup1p and Mig1p (Treitel and Carlson, 1995). LexA-Ssn6 needs Tup1p to repress but LexA-Tup1 can repress independently of Ssn6p (Tzamarias and Struhl, 1994).

An extensive range of *MIG1* deletions have been analysed for their ability to repress target promoters in the presence and absence of glucose, with the result that, in addition to the zinc finger DNA binding domain, three other functional domains have been identified. The repression domain is comprised of the last 24 amino acids of the carboxy terminus and is necessary for repression to occur, and two internal regulatory domains, R1 and R2, are essential for relief from repression. In addition, a potential nuclear localisation domain which is very similar to the nuclear localisation signal of a yeast transcription factor Swi5, has been identified in the region following the zinc fingers and called the B (basic) domain (Figure 1.1) (Cassart *et al.* 1995; Ostling *et al.* 1996). However, the nuclear localisation signal in Mig1p has been further defined by a series of deletion constructs fused to GFP, and the region required for nuclear localisation in response to glucose levels is thought to occur between amino acids 261-400, rather than in the B domain identified by Ostling *et al.* (1996). The constructs have also identified this region as required for nuclear export (DeVit *et al.* 1997).

Experiments using a Mig1::GFP fusion protein have demonstrated that Mig1p is located in the nucleus when glucose is present and in the cytoplasm when glucose is unavailable (DeVit *et al.* 1997). Immunodetection of western blots with antibodies to Mig1p has

Figure 1.1 The functional domains of Mig1p from *S. cerevisiae.*

Adapted from Ostling *et al.* (1996) and reproduced from Shroff (1997). 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.


C2H2
Zinc
Fingers
B
R1
R2
Repression
Domain

shown that Mig1p is phosphorylated in the absence of glucose but not when glucose is present. This evidence suggests a role for phosphorylation in the regulation of the Mig1p response to glucose, and correlates with the fact that the Snf1p kinase is required for release of glucose repression by Mig1p, and that in *SNF1* mutants, Mig1p is constitutively located in the nucleus (Celenza and Carlson, 1986; Vallier and Carlson, 1994; DeVit *et al.* 1997).

A second repressor, Mig2p, has been identified. It has Cys_2His_2 zinc fingers with 78% identity to the Mig1p zinc fingers, and also binds to the promoter of *SUC2* and represses invertase expression in the presence of glucose (Lutfiyya and Johnston, 1996). While Mig1p alone causes full repression of *SUC2*, a deletion of Mig1p does not produce full derepression due to the effect of Mig2p. A deletion of Ssn6p causes total derepression of *SUC2* which suggests that Mig2p, as well as Mig1p, can recruit Ssn6/Tup1.

1.1.1.2 *SSN6* and *TUP1*

Screens for mutants with defects in carbon catabolite repression identified two genes, *SSN6* and *TUP1*, which appeared to be functionally related because mutations in either one caused some similar phenotypes including constitutive expression of glucose repressible enzymes, sporulation and mating defects, abnormal cell morphology and flocculence (Rothstein and Sherman, 1980; Trumbly, 1986).

Experiments have shown that these proteins associate to function - both co-migrate on non-denaturing western blots and co-precipitate with antibodies directed against only one of the proteins (Williams *et al.* 1991). Analysis of the high molecular weight Tup1p/Ssn6p complex has demonstrated that 1 unit of Ssn6p associates with 4 units of Tup1p (Varanasi *et al.* 1996). The complex represses the expression of genes regulated by a variety of signals other than glucose, including mating type, DNA damage and oxygen (Keleher *et al.* 1992; Treitel and Carlson, 1995; Komachi *et al.* 1994; Zhou and

Elledge, 1992; Balasubrahmanian *et al.* 1993). While specific genes are regulated by the *SSN6-TUP1* complex, neither *TUP1* nor *SSN6* appears to bind DNA. It has been proposed that the complex is recruited to promoters by specific DNA binding proteins, and for carbon catabolite repression, Mig1p is the DNA binding protein which has been shown to bind directly to Ssn6p and recruit *SSN6-TUP1* to the UAS of *SUC2* (Keleher *et al.* 1992).

The first seventy two amino acids at the N-terminal of Tup1p are the site of interaction with Ssn6p (Tzamarias and Struhl, 1994). The carboxy terminal of Tup1p contains six copies of a WD repeat sequence (Williams and Trumbly, 1990) and Ssn6p contains 10 copies of a 34 amino acid TPR (tetratricopeptide repeat) motif (Schultz *et al.* 1990). Both motifs have been implicated in protein-protein interactions. The specificity of the Ssn6/Tup1p repressor complex for the structurally dissimilar DNA binding proteins by which it is recruited, may be permitted by various combinations of these WD and TPR repeat sequences (Tzamarias and Struhl, 1995). This has been demonstrated by introducing single mutations into different WD repeats and producing a varied pattern of derepression for genes regulated by the Ssn6p/Tup1p complex (Carrico and Zittomer, 1998).

1.1.1.2.1 Tup1 Homologues

The *rco-1* gene from *N. crassa* was isolated when a mutant strain was found to express *con-10* during mycelial growth when it is normally expressed during conidiation. The sequence of *rco-1* suggests that it is a homologue of *TUP1* from *S. cerevisiae.* It has seven WD repeats with 68% identity to those of Tup1p, and the eighty N-terminal residues are proposed to form an α-helix in keeping with the seventy-two N-terminal residues of Tup1p, which form the interaction domain with Ssn6p. Rco-1p has a proline-rich region which is represented in Tup1p by a string of glutamines and alanines (Yamashiro *et al.* 1996). However, there is no evidence that *rco-1* acts as a repressor in

response to glucose, and its functional similarity to Tup1p is as a cell type-specific repressor (Lee and Ebbole, 1998).

1.1.1.3 *SNF1*

The gene *SNF1* encodes a serine/threonine protein kinase and is absolutely required for release from glucose repression (Celenza and Carlson, 1986). *SNF1* was identified in several different screens when mutants were unable to grow on glycerol or maltose, on ethanol, or on sucrose or raffinose (Zimmermann and Scheel, 1977; Ciriacy, 1977; Entian and Zimmermann, 1982; Neigeborn and Carlson, 1984).

The mammalian homologue of Snf1p, AMP-activated protein kinase (AMPK), has been identified and its activity is repressed in the presence of glucose (Carling *et al.* 1994). Snf1p is also inactivated in a glucose rich environment by a complex pattern of regulation. The protein has a regulatory domain and a kinase domain which are proposed to bind to each other to inhibit Snf1p function when glucose levels are high. In a low glucose environment, an activating subunit, Snf4p, binds to the regulatory domain of Snf1p to activate the protein (Jiang and Carlson, 1996). As glucose levels rise a complex made up of protein phosphatase 1 (PP1) and Reg1p, is proposed to bind to the kinase domain of Snf1p to inactivate it (Lundin *et al.* 1998) (Figure 1.2).

Two serine residues within Mig1p are targeted by Snf1p. Experiments using a Mig1-VP16 fusion protein have shown that when these sites are mutated, Snf1p can no longer phosphorylate Mig1p in low glucose conditions (Ostling and Ronne, 1998). These sites correlate with the region of Mig1p already mentioned (Section 1.1.2.1) which when mutated, causes it to be constitutively located in the nucleus, and further implicates Snf1p in the regulation of Mig1p and glucose repression in *S. cerevisiae* (DeVit *et al.* 1997).

Figure 1.2 Regulation of the protein kinase, Snf1p.

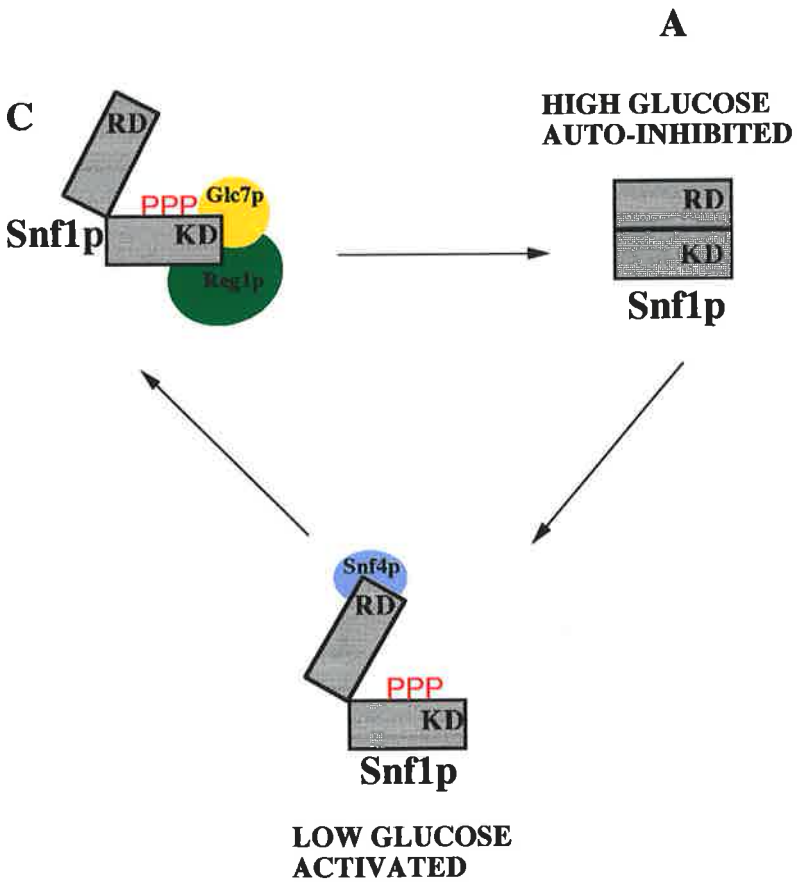
A: The activity of Snf1p is auto-inhibited in conditions of high glucose when the regulatory and kinase domains of the protein bind to each other.

B: When glucose levels are low, the kinase domain of Snf1p is phosphorylated (possibly autophosphorylated) and the domains no longer bind to each other. An activating sub-unit, Snf4p binds to the regulatory domain of Snf1p and in this form, Snf1p is an active kinase.

C: As glucose levels rise in the cell, Reg1p is proposed to recruit Glc7p (protein phosphatase 1) to the kinase domain of Snf1p, effect dephosphorylation and thus allow the kinase and regulatory domains to bind to each other and return Snf1p to an inactive state.

Dephosphorylation of the kinase domain is proposed to weaken the association between Snf4p and the regulatory domain, thus allowing the regulatory domain to bind to the kinase domain and inactivate Snf1p.

Drawn from: Jiang and Carlson, 1996; Jiang and Carlson, 1997; Gancedo, 1998.


A
HIGH GLUCOSE
AUTO-INHIBITED
RD
KD
Snf1p
C
RD
PPP
Glc7p
Snf1p
KD
Reg1p
Snf4p
RD
PPP
KD
Snf1p
LOW GLUCOSE
ACTIVATED
B

1.1.1.4 *REG1, GLC7, HXK2,* and *GRR1*

REG1, HXK2, and *GRR1* were isolated as 2-deoxy-D-glucose resistant mutants and are also known as *HEX2, HEX1* and *CAT80* respectively (Zimmermann and Scheel, 1977; Neideracher and Entian, 1987; Entian and Zimmermann, 1980). In these mutants, invertase, maltase and malate dehydrogenase were expressed in the presence of glucose, and some hexokinase activities were altered. However, none of the proteins encoded by these genes appeared to bind directly to promoters to repress transcription.

A yeast two hybrid screen has demonstrated that Reg1p interacts with Glc7p (which forms the catalytic subunit of protein phosphatase type 1) and the two proteins can be co-immunoprecipitated which suggests that they form a complex (Tu and Carlson, 1995). A proposed role for Reg1p/Glc7p is to dephosphorylate Mig1p in conditions of high glucose and therefore enable Mig1p mediated repression to occur (DeVit *et al.* 1997). As mentioned in Section 1.1.1.3, Reg1p has been shown to bind to the kinase domain of Snf1p to effect inactivation by recruiting Glc7p.

It has also been proposed that Grr1p targets Reg1p for degradation by recognising PEST sequences within the protein (Li and Johnston, 1997). In this way, Grr1p could inactivate the Reg1p/Glc7 complex (by degrading Reg1p). As a result, Snf1p would be in its active form and Mig1p would be in a phosphorylated form, be located in the cytoplasm and not be active as a repressor (Figure 1.3). Mutants in *GRR1* have glucose transport defects and are unable to activate the *HXT1-4* genes (hexose transporter genes). Grr1p also has a role in the regulation of Rgt1p and this is discussed in Section 1.2.1.2

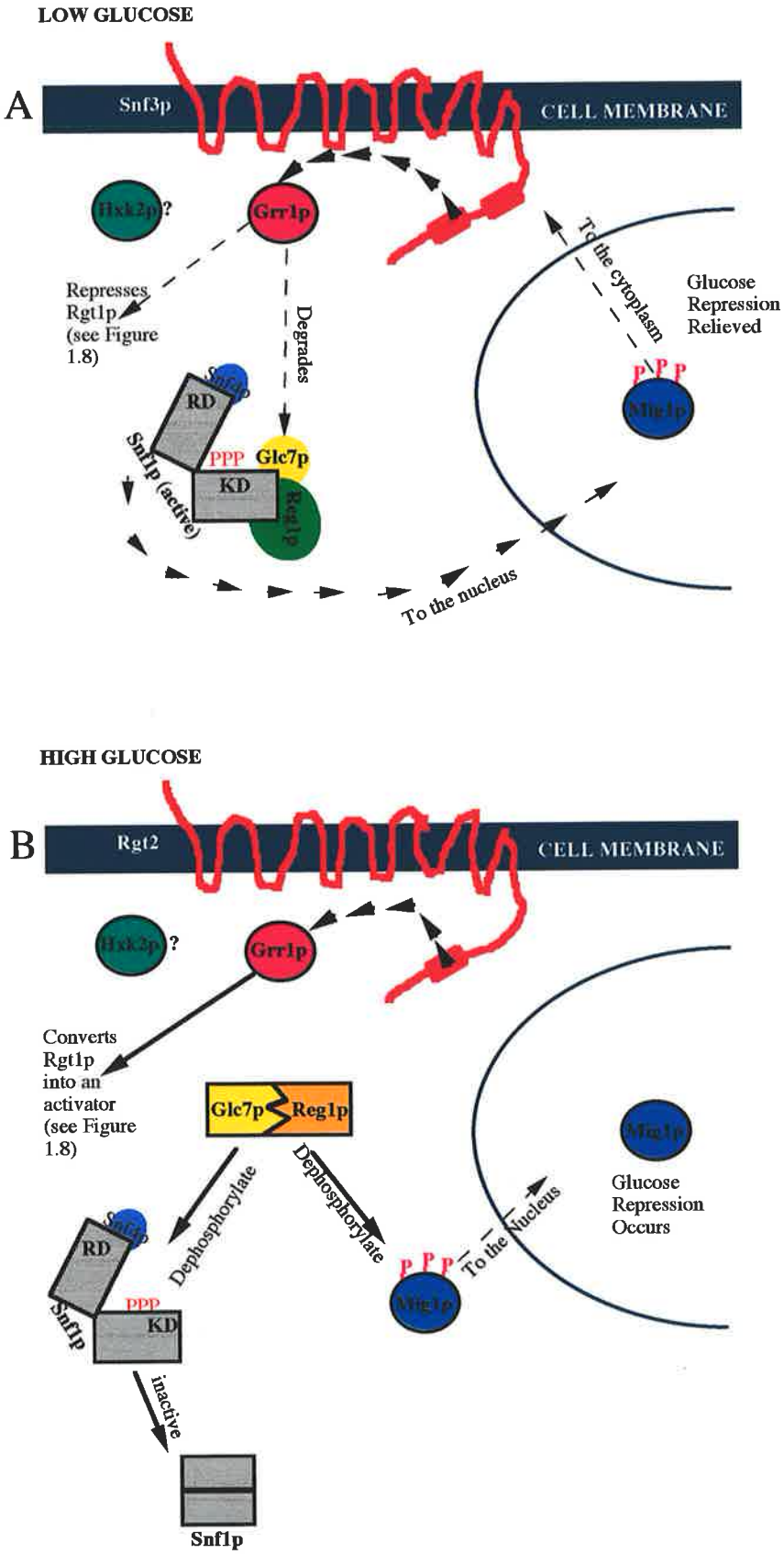
The derepressed phenotype of *HXK2* mutants indicates a regulatory role for the product of this gene, which encodes hexokinase isoenzyme P-II (Lobo and Maitra, 1977). This enzyme has two sites of phosphorylation and *in vivo* experiments have shown that the site at serine-14 is increasingly phosphorylated as glucose levels are

Figure 1.3 Elements of the signalling pathway in *S. cerevisiae* which respond to high or low glucose levels in the cellular environment and lead to glucose repression or derepression.

A: In conditions of low glucose, the membrane bound glucose sensor Snf3p (Section 1.2.1.2), transmits an intracellular signal via a 25 amino acid sequence in its long C-terminal tail, to possibly Grr1p which degrades Reg1p and thus inactivates the Reg1p/Glc7p complex. This allows the kinase domain of Snf1p to remain phosphorylated and with other factors, such as Snf4p, Snf1p becomes an active kinase, and phosphorylates Mig1p which results in relief from carbon catabolite repression.

B: Rgt2p has a long C-terminal tail with the same 25 amino acid residues found in the tail of Snf3p, and is thought to transmit the intracellular signal via Grr1p. When glucose levels are high Grr1p converts the repressor Rgt1p into an activator (Vallier *et al.* 1994). The Reg1p/Glc7p complex is active and can dephosphorylate Snf1 and Mig1 with the result that Snf1 is inactivated and Mig1p is located in the nucleus and effects glucose repression. Snf3p is repressed by high glucose levels but basal expression levels are proposed to be sufficient to activate some Hxt1p expression (low affinity glucose transporter).

Drawn from: Erickson and Johnston, 1994; DeVit *et al.* 1997; Li and Johnston, 1997; Lundin *et al.* 1998; Ostling and Ronne, 1998.


LOW GLUCOSE
A
Snf3p
CELL MEMBRANE
Hxk2p?
Grr1p
Represses Rgt1p (see Figure 1.8)
Degrades
To the cytoplasm
Glucose Repression Relieved
RD
Snf1p
PPP
Glc7p
KD
Reg1p
Snf1p (active)
Mig1p
To the nucleus
HIGH GLUCOSE
B
Rgt2
CELL MEMBRANE
Hxk2p?
Grr1p
Converts Rgt1p into an activator (see Figure 1.8)
Glc7p
Reg1p
Dephosphorylate
Dephosphorylate
Mig1p
Glucose Repression Occurs
To the Nucleus
RD
Snf1p
PPP
KD
inactive
Snf1p

depleted (Kriegel *et al.* 1994). Substitution of the serine-14 with glutamine, has demonstrated a relationship between phosphorylation at serine-14 and the existence of hexokinase molecules as monomers. In this form, they have a higher affinity for glucose. It is possible that the phosphorylation status of Hxk2p could provide an intracellular signal of glucose status (Behlke *et al.* 1998). In another experiment, disruption of the amino acids 7-16, has shown this region to be necessary for nuclear localisation of Hxk2p and has indicated that phosphorylation also plays a role in nuclear localisation of the protein (Herrero *et al.* 1998). The ability of Hxk2p to phosphorylate glucose and fructose has been found to correlate with the repression of invertase and maltase being triggered by these substrates. This fact combined with its role as a regulatory protein, implies that Hxk2p may also be involved in glucose sensing or signalling (Rose *et al.* 1991). Reg1p, Glc7p and Grr1p mutants lack the ability to dephosphorylate the Hxk2p monomers which suggests that they are involved in the dephosphorylation of this protein. An Hxk2p mutant which has the phosphorylation site mutated, shows loss of glucose repression indicating that phosphorylation is necessary for the glucose repression signal to occur (Randez-Gil *et al.* 1998). Glucose sensing is discussed in more detail in the section on glucose transport.

1.1.1.5 *RGR1*

The involvement of *RGR1* in glucose repression was shown when mutations in the gene led to overexpression of a reporter gene under the control of the *SUC2* promoter (Sakai *et al.* 1988). Mutations in *RGR1* cause derepression of a number of genes subject to glucose repression, and produce an abnormal cell morphology. Rgr1p acts as a negative transcriptional regulator of *HO* and *IME1*, but is also required for the full transcriptional activation of other genes, for example, *HIS4* and *CTS1* (Jiang and Stillman, 1992). Recent work has demonstrated that Rgr1p interacts with the mediator and RNA polymeraseII holoenzyme as part of a subcomplex which is proposed to control transcription by RNA polymerase. This would explain why Rgr1p has been seen in both activating and repressing roles (Li *et al.* 1995). Sin4p regulates the transcription of

HIS4, CTS1, HO and *IME1* in a similar way to Rgr1p, and Sin4p and Rgr1p have been shown to associate *in vivo* (Jiang *et al.* 1995). These two proteins appear to function together in the transcriptional regulation of a range of genes and are not exclusively concerned with carbon catabolite repression.

1.1.2 Carbon Catabolite Repression in *Aspergillus nidulans*

The *creA* gene from *A. nidulans* has been the focus of research into carbon catabolite repression in this species. Mutations in the *creA* gene have been selected in a number of screens, and they lead to reduced repression of many systems normally subject to carbon catabolite repression, and in diploid strains the mutant allele is recessive to the wild type allele for its effects (Arst and Cove, 1973; Dowzer and Kelly, 1991). This evidence suggests that *creA* encodes a wide domain, negatively acting repressor protein. Various *creA* alleles show no clear hierarchy in their derepression of enzymes in the presence of glucose, which further indicates a broad regulatory role for CreA, and also implies that not all of the alleles have completely lost CreA function. *creA* was first identified as a suppressor mutation in a strain bearing the *areA^r-1* mutation (Arst and Cove, 1973).

1.1.2.1 Identification of *creA* as a suppressor of *areA^r-1*

Strains bearing the *areA^r-1* mutation lack the transcriptional activator AreA, which is necessary for the expression of genes encoding the enzymes required to use nitrogen sources other than ammonium, for example, expression of acetamidase for the utilisation of acetamide (Arst and Cove, 1969). These strains can only grow on ammonium. However, as acetamide and proline are carbon sources as well as nitrogen sources, the enzymes required for their utilisation are also regulated by the levels of glucose in the cell. If acetamide is the only carbon/nitrogen source available, an *areA^r-1* strain will grow because glucose repression is not active and acetamide is metabolised to provide a carbon source and, coincidentally, a nitrogen source. In the presence of glucose, the strain will not be able to grow however, because glucose repression prevents the

Figure 1.4 CreA and AreA both regulate genes which express enzymes required for the utilisation of substrates that provide a carbon and nitrogen source.

This figure shows the activity of CreA and AreA on the 5' region of *amdS*. Strains of four different genotypes are depicted inoculated onto medium containing acetamide, which is both a carbon and nitrogen source, and the repressing carbon source glucose. The product of the *areA* gene is represented by a blue box. AreA is required to activate the transcription of *amdS* in the absence of ammonium under carbon repressing conditions, to enable the strain to utilise acetamide as a nitrogen source. The product of the *creA* gene is represented by a red circle. CreA represses the transcription of genes required to use other carbon sources, when glucose is available. Thus, *amdS* expression is repressed by CreA when glucose is present.

A: Growth on this medium of a strain bearing wild type CreA and AreA, indicates that activation of *amdS* by AreA overrides repression by CreA.

B: In the absence of AreA, *amdS* expression is repressed by CreA and this strain cannot grow.

C: When CreA repression is not occurring in the presence of glucose due to a mutant *creA* in the strain, *amdS* expression is not repressed by CreA and is activated by AreA, which results in growth.

D: In an *areA* mutant strain, *amdS* expression is not activated by AreA but if the strain also contains a *creA* mutation, then loss of repression by CreA will allow expression of *amdS* and the strain will grow.

	strain	medium	5' region *amdS*	*amdS* expression	growth
A	*areA*$^{+}$, *creA*$^{+}$	acetamide and glucose		yes	+
B	*areA*$^{-}$, *creA*$^{+}$	acetamide and glucose		no	-
C	*areA*$^{+}$, *creA*$^{-}$	acetamide and glucose		yes	+
D	*areA*$^{-}$, *creA*$^{-}$	acetamide and glucose		yes	+

metabolism of acetamide (Figure 1.4). A suppressor mutation was identified (*creA*^{d}*-1*) which allowed growth of the *areA*^{r}*-1* strain on proline and glucose. Further analysis with a double mutant strain containing *creA*^{d}*-1* and *pdhA-1* (lacking pyruvate dehydrogenase) showed that *creA*^{d}*-1* also suppressed *pdhA-1* as the double mutant was able to utilise ethanol in the presence of glucose. Glucose uptake in *creA*^{d}*-1* strains was shown to be normal and it was concluded that *creA*^{d}*-1* caused loss of carbon catabolite repression (Arst and Cove, 1973). The *creA* locus was mapped to chromosome I (Arst and Cove, 1973) and subsequent work has shown that it is tightly linked to *galD* (Hynes and Kelly, 1977).

1.1.2.1 Cloning *creA*

creA from *A. nidulans* was cloned by complementation of a mutant allele, *creA204* (Dowzer and Kelly, 1989). When the sequence of *creA* was determined, it was found to encode a 416 amino acid protein with two zinc fingers of the Cys_2His_2 class that had 84% identity to the zinc finger region of Mig1p from *S. cerevisiae.* As discussed in the previous section, Mig1p is a repressor protein involved in carbon catabolite repression.

creA was also cloned from *A. niger* using cross hybridisation to the *A. nidulans* gene and when the two sequences were compared, the zinc finger regions differed in only two amino acids. The proteins also contained a string of alanine residues (eight for *A. niger* and nine for *A. nidulans*) and an identical region of 42 amino acids was present in both genes (Drysdale *et al.* 1993). This region is referred to as the *RGR1* similar region because it has 34/42 amino acids which are similar or identical to a region of Rgr1p from *S. cerevisiae* (Figure 1.5). However, when this region of the *S. cerevisiae* protein was substituted into CreA, it was not functional (Shroff *et al.* 1996).

1.1.2.3 Functional domains of CreA

The functional domains within CreA have been identified by protein sequence comparison with the functionally similar Mig1p, data base searches for protein motifs,

Figure 1.5 A comparison of protein sequences within CreA from *A. nidulans* and *A. niger* with Rgr1p from *S. cerevisiae.*

The 42 amino acid region which is identical in CreA from *A. nidulans* (Dowzer and Kelly, 1989) and *A. niger* (Drysdale *et al.* 1993) and similar to a region of Rgr1p from *S. cerevisiae* (Sakai *et al.* 1988). Identical residues between all three proteins are shown in dark blue, similar residues in light blue and unrelated residues are in grey.with zinc fingers in the same class as CreA, has identified which amino acids are critical for finger stability and thus, DNA binding ability, but the mutations in most of the CreA zinc finger alleles do not disrupt these critical amino acids (Pavletich and Pabo, 1991). Another indication that these alleles may still bind DNA is that they do not show the highest levels of derepression.

CreA (*A. nidulans*)	284	V	K	R	S	R	P	N	S	P	N	S	T	A	P	S	S	P	T	F	S	H
CreA (*A. niger*)	274	V	K	R	S	R	P	N	S	P	N	S	T	A	P	S	S	P	T	F	S	H
Rgr1p (*S. cerevisiae*)	39	V	K	N	T	Q	L	H	S	P	S	A	T	V	P	E	T	T	T	T	Q	K

CreA (*A. nidulans*)	D	S	L	S	P	T	P	D	H	T	P	L	A	T	P	A	H	S	P	325
CreA (*A. niger*)	D	S	L	S	P	T	P	D	H	T	P	L	A	T	P	A	H	S	P	315
Rgr1p (*S. cerevisiae*)	E	S	L	E	M	V	P	K	D	T	S	A	A	T	M	T	S	A	P	80

mutant analyses, and most recently by protein sequence comparison with the several CreA homologues which have been cloned from other fungal species.

1.1.2.3.1 Mutant Analyses

Several *creA* mutants have been selected and analysed. They fall into two broad categories: those which have mutations affecting the zinc finger DNA binding domain and those which are predicted to make truncated proteins. All of these mutations result in derepression of a range of enzymes usually repressed by glucose, and most have a compact morphology on complete medium. However, the mutations are pleiotropic with respect to the amount of derepression they show, the range of enzymes affected, and in the extent to which they affect colony morphology.

1.1.2.3.1.1 Alleles predicted to produce truncated proteins

Analysis of the class of mutants predicted to truncate the protein has indicated that the region required for repression is located within the eighty amino acids at the C-terminal end of CreA. The allele *creA322* is predicted to produce a protein which lacks only this region and yet it shows significant derepression in the presence of D-glucose, of the genes necessary to utilise starch, proline, acetamide and ethanol (Shroff *et al.* 1997). A strain containing this allele also exhibits a decreased growth rate on complete medium compared with $creA^{+}$ strains. The repression domain (effector domain) identified in Mig1p from *S. cerevisiae*, is also located at the C-terminus, and there is some similarity in the sequences of these two proteins in this region (Ostling *et al.* 1996).

1.1.2.3.1.2 Alleles with mutations in the zinc finger region

The mutated zinc fingers produced by these alleles are predicted to have an altered affinity for their binding site, and they all exhibit a derepressed phenotype for at least some systems. However, most of these mutants are still predicted to bind DNA because they do not have an identical phenotype and therefore cannot all be loss-of-function alleles (Shroff *et al.* 1996; 1997). Analysis of the crystal structure of Zif268, a protein

Three alleles, *creA303*, *creA304* and *creA306* are predicted to have no DNA binding ability either because they have mutations in amino acids identified as critical for binding, or they produce proteins which are truncated before or within the finger. These alleles would be predicted to have no functional CreA and thus would be predicted to be loss of function alleles. They are the most derepressed and they have the poorest growth and sporulation (Shroff *et al.* 1997). However, their phenotype is not the same as that described by Dowzer and Kelly (1991), and *creA303*, *creA304* and *creA306* are the subject of Chapter 3.

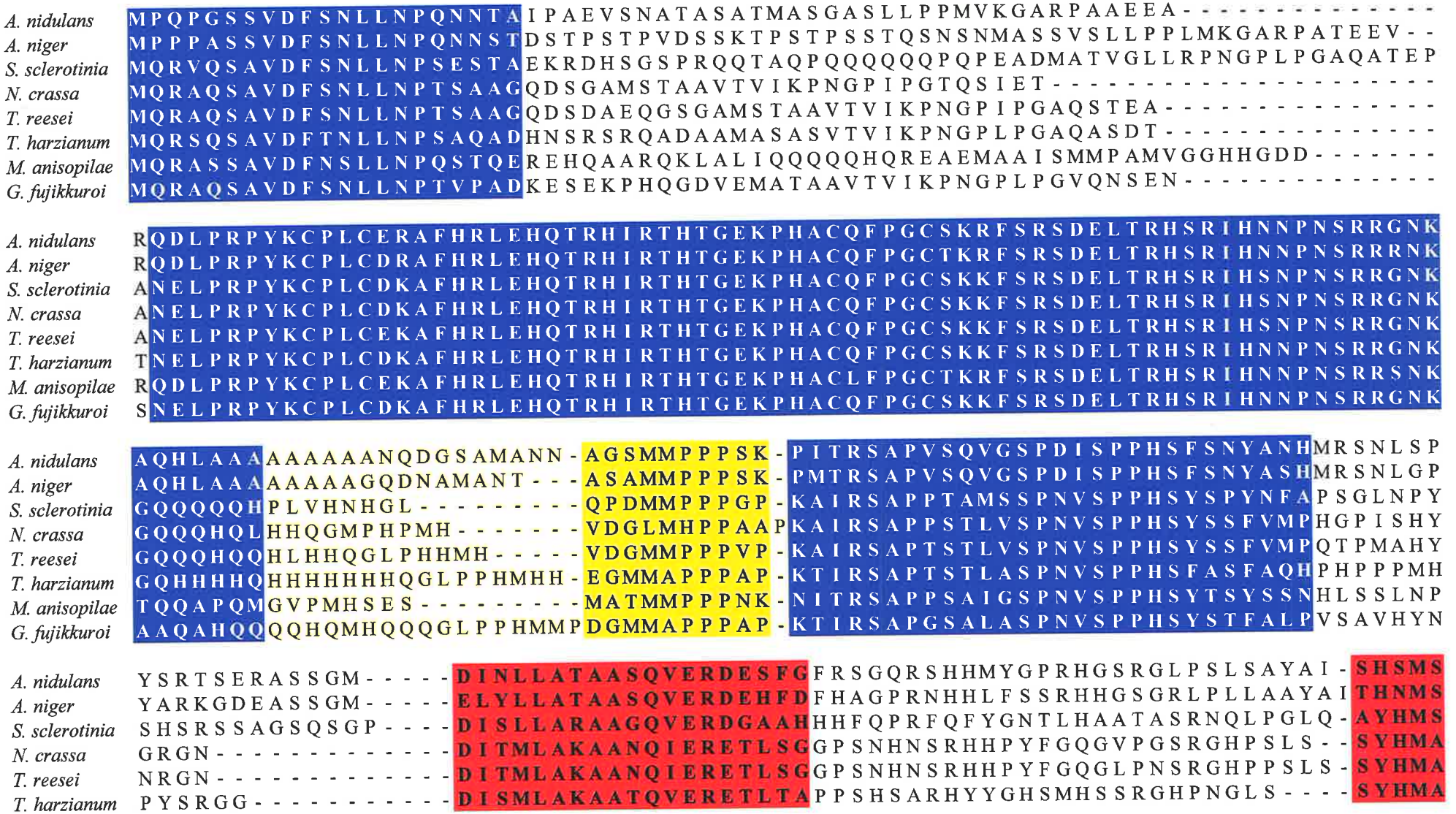
1.1.2.3.2 Comparison of the protein sequence of CreA with CreA homologues

CreA homologues have been cloned from several other fungal species: *T. reesei, T. harzianum, Neurospora crassa, Metarhizium anisopilae, Sclerotinium schlerotinia, Gibberella fujikkuroi* (Ilmen *et al.* 1996; B. Tyler, unpublished; Reymond-Cotton *et al.* 1996; Screen *et al.* 1997; B. Tudzinski, unpublished). An alignment of their protein sequences using the Match-Box programme (Depiereux and Feytmans, 1992) has indicated regions of high identity which are likely to be functionally important (Shroff, 1997) (Figure 1.6, Figure 1.7). Some of these regions have already been identified from comparisons with Mig1p from *S. cerevisiae* (zinc finger region, C-terminal repression domain) and CreA from *A. niger (*Rgr1p-similar region and alanine rich region) and from motif analyses of the protein (STPXX motifs, acidic region) (Dowzer and Kelly, 1989).

The striking similarities between the homologues has confirmed the importance of some regions such as the zinc finger region (Box 2); the sequence similar to the Basic domain of Mig1p (at the C-terminal end of the finger region) (Box2); the *RGR1* similar region (Box7), the acidic region (Box 6) and the Mig1p-similar repression domain (Box 10); and identified some which had not previously been recognised, for example the regions labelled Box 1, 4, and 9 in Figure1.7. The functional significance of the sequences highlighted in Boxes 1, 4 and 9, cannot be determined from data base searches but Box 1 is rich in hydrophobic residues and contains serine/threonine phosphorylation sites

Figure 1.6 Match-Box analysis of CreA homologues

(This figure has been reproduced from Shroff, 1997 where it appears as Figure 4.2). CreA homologues were analysed using the multiple sequence alignment software Match-Box. The programme 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. 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
A. niger
S. sclerotinia
N. crassa
T. reesei
T. harzianum
M. anisopilae
G. fujikkuroi
MPQPGSSVDFSNLLNPQNNTAIPAEVSNATASATMASGASLLPPMVKGARPAAEEA
MPPPASSVDFSNLLNPQNNSTDSTPSTPVDSSKTPSTPSSTQSNSNMASSVSLLPPLMKGARPATEEV
MQRVQSAVDFSNLLNPSESTAEKRDHSGSPRQQTAQPQQQQQQPQPEADMATVGLLRPNGPLPGAQATEP
MQRAQSAVDFSNLLNPTSAAGQDSGAMSTAAVTVIKPNGPIPGTQSIET
MQRAQSAVDFSNLLNPTSAAGQDSDAEQGSGAMSTAAVTVIKPNGPIPGAQSTEA
MQRSQSAVDFTNLLNPSAQADHNSRSRQADAAMASASVTVIKPNGPLPGAQASDT
MQRASSAVDFNSLLNPQSTQEREHQAARQKLALIQQQQQHQREAEMAAISMMPAMVGGHHGDD
MQRAQSAVDFSNLLNPTVPADKESEKPHQGDVEMATAAVTVIKPNGPLPGVQNSEN
RQDLPRPYKCPLCERAFHRLEHQTRHIRTHTGEKPHACQFPGCSKRFSRSDELTRHSRIHNNPNSRRGNK
RQDLPRPYKCPLCDRAFHRLEHQTRHIRTHTGEKPHACQFPGCTKRFSRSDELTRHSRIHNNPNSRRRNK
ANELPRPYKCPLCDKAFHRLEHQTRHIRTHTGEKPHACQFPGCSKKFSRSDELTRHSRIHSNPNSRRGNK
ANELPRPYKCPLCDKAFHRLEHQTRHIRTHTGEKPHACQFPGCSKKFSRSDELTRHSRIHSNPNSRRGNK
ANELPRPYKCPLCEKAFHRLEHQTRHIRTHTGEKPHACQFPGCSKKFSRSDELTRHSRIHSNPNSRRGNK
TNELPRPYKCPLCDKAFHRLEHQTRHIRTHTGEKPHACQFPGCSKKFSRSDELTRHSRIHNNPNSRRGNK
RQDLPRPYKCPLCEKAFHRLEHQTRHIRTHTGEKPHACLFPGCTKRFSRSDELTRHSRIHNNPNSRRSNK
SNELPRPYKCPLCDKAFHRLEHQTRHIRTHTGEKPHACQFPGCSKKFSRSDELTRHSRIHNNPNSRRGNK
AQHLAAAAAAAAANQDGSAMANN-AGSMMPPPSK-PITRSAPVSQVGSPDISPPHSFSNYANHMRSNLSP
AQHLAAAAAAAAGQDNAMANT---ASAMMPPPSK-PMTRSAPVSQVGSPDISPPHSFSNYASHMRSNLGP
GQQQQQHPLVHNHGL---------QPDMMPPPGP-KAIRSAPPTAMSSPNVSPPHSYSPYNFAPSGLNPY
GQQQHQLHHQGMPHPMH-------VDGLMHPPAAPKAIRSAPPSTLVSPNVSPPHSYSSFVMPHGPISHY
GQQQHQQHLHHQGLPHHMH-----VDGMMPPPVP-KAIRSAPTSTLVSPNVSPPHSYSSFVMPQTPMAHY
GQHHHHQHHHHHHHHQGLPPHMHH-EGMMAPPPAP-KTIRSAPTSTLASPNVSPPHSFASFAQHPHPPPMH
TQQAPQMGVPMHSES---------MATMMPPPNK-NITRSAPPSAIGSPNVSPPHSYTSYSSNHLSSLNP
AAQAHQQQQHQMHQQQGLPPHMMPDGMMAPPPAP-KTIRSAPGSALASPNVSPPHSYSTFALPVSAVHYN
YSRTSERASSGM-----DINLLATAASQVERDESFGFRSGQRSHHMYGPRHGSRGLPSLSAYAI-SHSMS
YARKGDEASSGM-----ELYLLATAASQVERDEHFDFHAGPRNHHLFSSRHHGSGRLPLLAAYAITHNMS
SHSRSSAGSQSGP----DISLLARAAGQVERDGAAHHHFQPRFQFYGNTLHAATASRNQLPGLQ-AYHMS
GRGN-------------DITMLAKAANQIERETLSGGPSNHNSRHHPYFGQGVPGSRGHPSLS--SYHMA
NRGN-------------DITMLAKAANQIERETLSGGPSNHNSRHHPYFGQGLPNSRGHPPSLS-SYHMA
PYSRGG-----------DISMLAKAATQVERETLTAPPSHSARHYYGHSMHSSRGHPNGLS----SYHMA

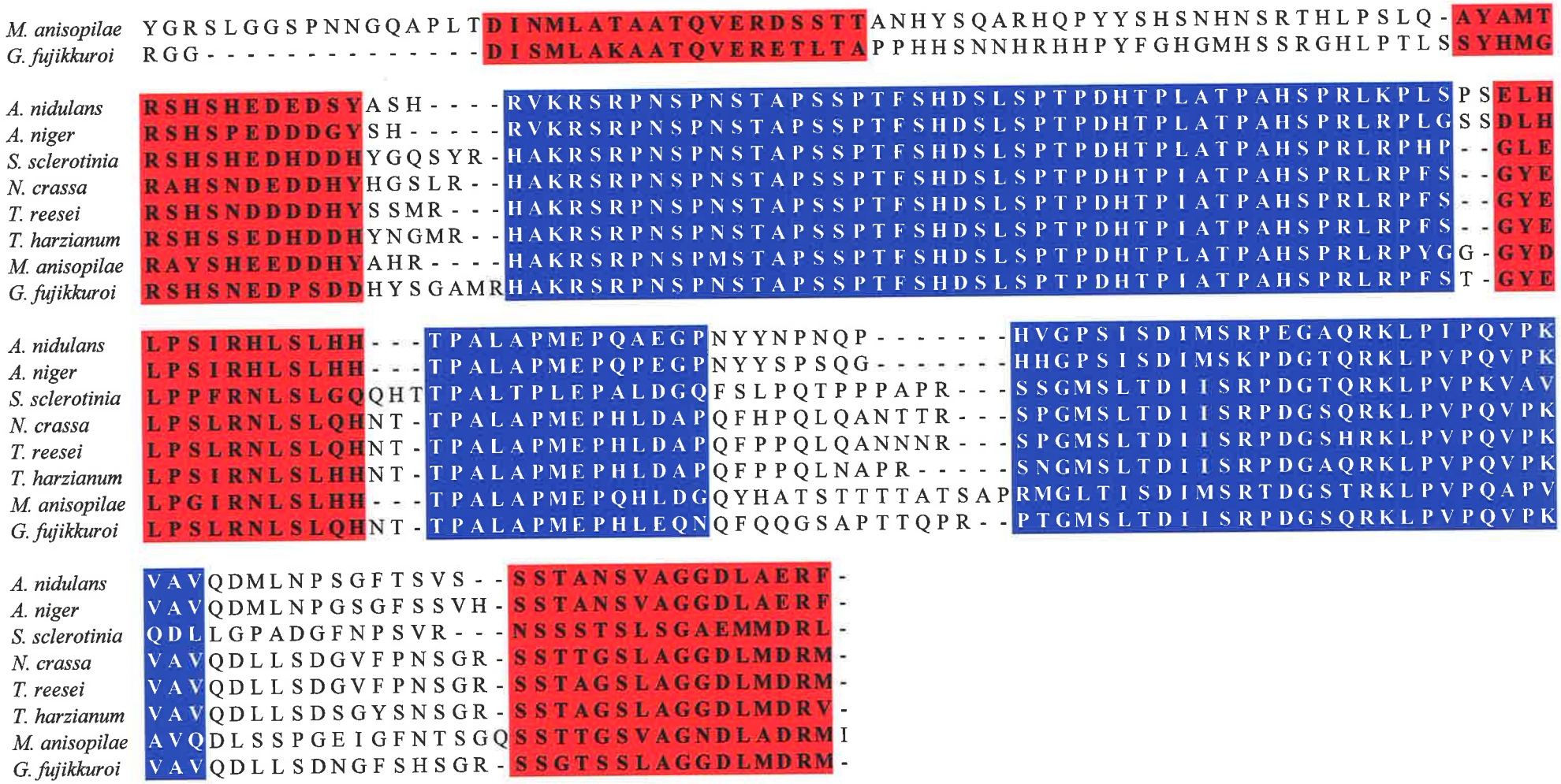
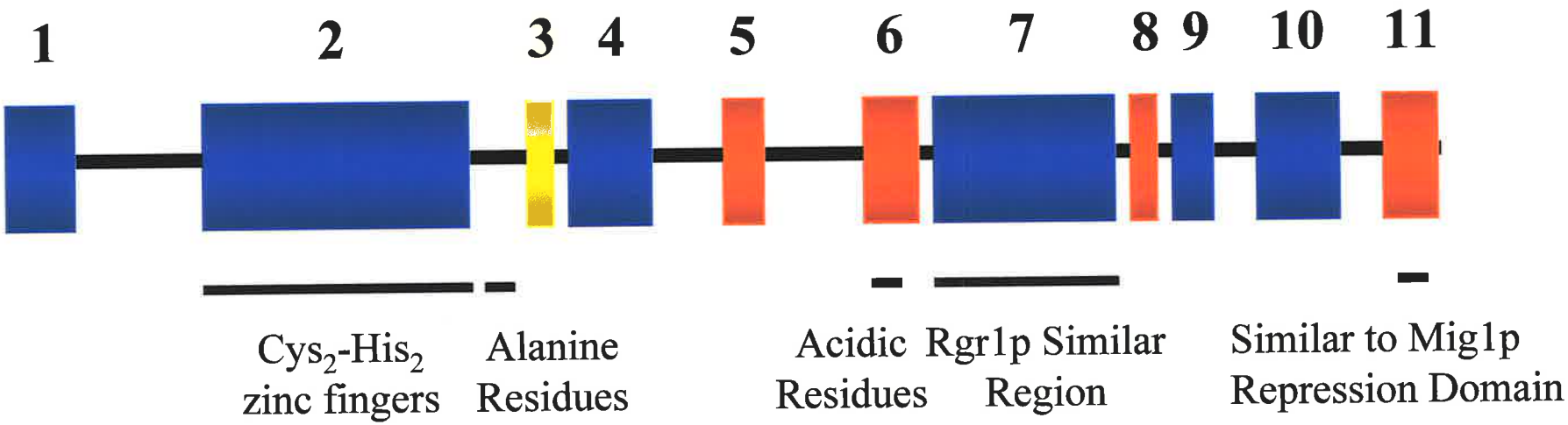
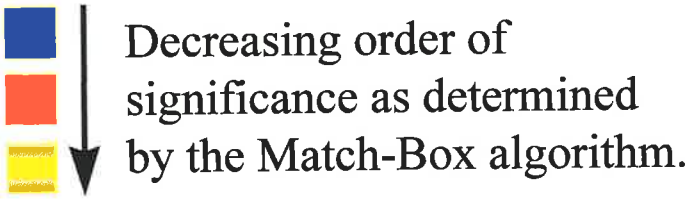

M. anisopilae YGRSLGGSPNNGQAPLTDINMLATAATQVERDSSTTANHYSQARHQPYYSHSNHNSRTHLPSLQ-AYAMT
G. fujikkuroi RGG---------------DISMLAKAATQVERETLTAPPHHSNNHRHHPYFGHGMHSSRGHLPTLSSYHMG
A. nidulans RSHSHEDEDSYASH----RVKRSRPNSPNSTAPSSPTFSHDSLSPTPDHTPLATPAHSPRLKPLSPSELH
A. niger RSHSPEDDDGYSH-----RVKRSRPNSPNSTAPSSPTFSHDSLSPTPDHTPLATPAHSPRLRPLGSSDLH
S. sclerotinia RSHSHEDHDDHYGQSYR-HAKRSRPNSPNSTAPSSPTFSHDSLSPTPDHTPLATPAHSPRLRPHP--GLE
N. crassa RAHSNDEDDHYHGSLR--HAKRSRPNSPNSTAPSSPTFSHDSLSPTPDHTPIATPAHSPRLRPFS--GYE
T. reesei RSHSNDDDDHYSSMR---HAKRSRPNSPNSTAPSSPTFSHDSLSPTPDHTPIATPAHSPRLRPFS--GYE
T. harzianum RSHSSEDHDDHYNGMR--HAKRSRPNSPNSTAPSSPTFSHDSLSPTPDHTPIATPAHSPRLRPFS--GYE
M. anisopilae RAYSHEEDDHYAHR----HAKRSRPNSPMSTAPSSPTFSHDSLSPTPDHTPLATPAHSPRLRPYGG-GYD
G. fujikkuroi RSHSNEDPSDDHYSGAMRHAKRSRPNSPNSTAPSSPTFSHDSLSPTPDHTPIATPAHSPRLRPFST-GYE
A. nidulans LPSIRHLSLHH---TPALAPMEPQAEGPNYYNPNQP-------HVGPSISDIMSRPEGAQRKLPIPQVPK
A. niger LPSIRHLSLHH---TPALAPMEPQPEGPNYYSPSQG-------HHGPSISDIMSKPDGTQRKLPVPQVPK
S. sclerotinia LPPFRNLSLGQQHTTPALTPLEPALDGQFSLPQTPPPAPR---SSGMSLTDIISRPDGTQRKLPVPKVAV
N. crassa LPSLRNLSLQHNT-TPALAPMEPHLDAPQFHPQLQANTTR---SPGMSLTDIISRPDGSQRKLPVPQVPK
T. reesei LPSLRNLSLQHNT-TPALAPMEPHLDAPQFPPQLQANNNR---SPGMSLTDIISRPDGSHRKLPVPQVPK
T. harzianum LPSIRNLSLHHNT-TPALAPMEPHLDAPQFPPQLNAPR-----SNGMSLTDIISRPDGAQRKLPVPQVPK
M. anisopilae LPGIRNLSLHH---TPALAPMEPQHLDGQYHATSTTTTATSAPRMGLTISDIMSRTDGSTRKLPVPQAPV
G. fujikkuroi LPSLRNLSLQHNT-TPALAPMEPHLEQNQFQQGSAPTTQPR--PTGMSLTDIISRPDGSQRKLPVPQVPK
A. nidulans VAVQDMLNPSGFTSVS--SSTANSVAGGDLAERF-
A. niger VAVQDMLNPGSGFSSVH-SSTANSVAGGDLAERF-
S. sclerotinia QDLLGPADGFNPSVR---NSSSTSLSGAEMMDRL-
N. crassa VAVQDLLSDGVFPNSGR-SSTTGSLAGGDLMDRM-
T. reesei VAVQDLLSDGVFPNSGR-SSTAGSLAGGDLMDRM-
T. harzianum VAVQDLLSDSGYSNSGR-SSTAGSLAGGDLMDRV-
M. anisopilae AVQDLSSPGEIGFNTSGQSSTTGSVAGNDLADRMI
G. fujikkuroi VAVQDLLSDNGFSHSGR-SSGTSSLAGGDLMDRM-

Figure 1.7 Summary of conserved regions identified in the CreA protein.

(This figure has been reproduced from Shroff, 1997 where it appears as Figure 4.3). The eleven boxes identified by Match-Box analysis (Figure 1.6) 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.

which suggest a role for this region in the regulation of nuclear localisation, if CreA is regulated in a similar fashion to Mig1p (by the phosphorylation of these residues). Box 4 is a proline rich region and Box 9, like Box 1, is also rich in hydrophobic residues. The string of alanine residues common to CreA from *A. niger* and *A. nidulans* is not found in the other proteins included in the Match-Box alignment and therefore this region has not been identified, but at the same position in these other proteins a string of glutamines or hisitidines is found, indicating possible structural functionality.

A CreA construct has been made with an internal deletion of 158 amino acids which removes Boxes 5-9 and so removes the acidic region and the Rgr1p-similar region (Figure 1.7). This has been transformed into the CreA-null strain to determine the function of this highly conserved region. Strains transformed with three or more copies of the deletion construct, pANC4ΔRV10, can exert carbon catabolite repression but grow very poorly on derepressing carbon sources (Shroff, 1997). This result implies that sequences within the deleted region are required for relief from carbon catabolite repression.

1.1.2.4 Binding Sites for CreA

The ethanol regulon of *A. nidulans* involves two structural genes, *alcA* encoding alcohol dehydrogenase I, and *aldA* encoding aldehyde dehydrogenase. The induction of these two genes requires the positively acting regulatory gene, *alcR*. DNase1 protection analysis of the *alcA* and *alcR* promoters has shown that CreA binds to the sequence:

5' C/G C/T G G G G 3'

(Kulmberg *et al.* 1993). This sequence is similar to the binding site for the *MIG1* protein in *S. cerevisiae* in the genes subject to Mig1p control. *In vitro* analysis has defined the sequence recognised by CreA in the 5' region of several genes as 5' S Y G G R G 3'. These genes include *ipnA*, the *prn* cluster, *amdS* and *facB* (Espeso and Penalva, 1994;

Cubero and Scazzochio, 1994; Chamelaun-Hussey, 1996). The binding sequence for *cre1* from *T. reesei* has been determined as 5'-GCGGAG-3', using a GST::*cre1* fusion protein, and as would be predicted, it is very similar to the site for CreA in *A. nidulans* (Strauss *et al.* 1995) and deletion of these sequences in the promoter of the *cbh1* gene which encodes cellobiohydrolase, has resulted in expression of this enzyme in the presence of glucose (Pentillä *et al.* 1993).

1.1.2.5 Regulation of *creA*

Analysis of mRNA prepared from mycelium grown in glucose (repressing) or arabinose (derepressing) cultures has shown that levels of *creA* mRNA are higher in derepressing conditions. When *creA*$^{+}$ strains are compared with strains containing *creA204* under these conditions, mRNA levels are higher in the derepressed mutant strain than in wild type (Dowzer and Kelly, 1991). These results, combined with the fact that *creA* consensus binding sites have been identified in the 5' region of *creA,* imply that *creA* is auto regulated (Shroff *et al.* 1996). The *cre1* genes from *T. reesei* and *T. harzianum* contain *cre1* consensus binding sites in their promoters, and observations of mRNA levels in wild type and mutant *T. reesei* strains grown with and without glucose, indicate that, like *creA*, *cre1* may be auto regulated (Ilmen *et al.* 1996).

1.1.2.6 A Null Allele

A previously described null strain, which was constructed by disrupting *creA* and some adjacent 3' sequence, indicated that the loss of *creA* was lethal (Dowzer and Kelly, 1991). However, the phenotype of the alleles, *creA303* and *creA304* raises questions about this interpretation, and Chapter 3 of this thesis discusses the phenotype of a more precise null allele in detail.

1.1.2.7 Other Genes affecting Carbon Catabolite Repression in *A. nidulans.*

1.1.2.7.1 *creB* and *creC*

creB15 and *creC27* were selected as suppressors of an *areA217* strain on medium containing glucose and acetamide. They were distinguished from *creA* alleles by complementation and recombination analysis. Further genetic and phenotypic analysis provided evidence for two new loci which mapped to linkage group II and were not allelic to each other (Hynes and Kelly, 1977).

creB15 and *creC27* share a very similar range of phenotypes although *creB15* has a more extreme phenotype than *creC27*. There are three aspects in which the mutants are altered. First, the expression of several genes is derepressed in the presence of glucose in strains containing these alleles as indicated by the suppression of the effects of *areA217* on glucose and acetamide medium, which implies that *amdS* is being expressed, and by sensitivity to allyl alcohol in the presence of glucose due to the expression of *alcA* (Hynes and Kelly, 1977). Second, strains containing these alleles grow poorly on many sole carbon sources including L-proline, D-quinate, D-galactose and D-lactose, which may be due to a failure to derepress the enzymes required for their utilisation, as direct measurements of glucose and 2-deoxy-D-glucose uptake have indicated that neither the *creB15* nor *creC27* mutations lead to reduced sugar uptake. Growth on carbon sources such as glucose, sucrose, xylose, glycerol, ethanol and acetate is not affected by the mutations (Hynes and Kelly, 1977; Arst, 1981). Third, *creB15* and *creC27* containing strains are more resistant than wild type strains to 33mM molybdate as determined by growth on medium containing molybdate. A mutant which was selected for resistance to molybdate, *molB-35* (Arst *et al.* 1970; Arst and Cove, 1970) was subsequently shown to be allelic to *creB15*. The *creB15* and *creC27* mutant alleles are recessive to their wild type alleles in heterozygous diploids. When *creB15* and *creC27* are together in a double mutant strain, the two mutant phenotypes are not additive, and the phenotype of the double is no more extreme than *creB15* (Hynes and Kelly, 1977).

Double mutant strains between either *creB15* or *creC27* with *creA204,* have the compact morphology of a strain containing *creA204*, and they show the amount of derepression of a *creA204* strain implying that *creA204* is epistatic to *creB15/creC27* for derepression. However, the presence of *creA204* in a double mutant strain with either *creB15* or *creC27* cannot repair the poor growth seen on L-proline and D-quinate in *creB15* or *creC27* containing strains.

1.1.2.7.2 *creD*

The *creD34* mutation was identified as a suppressor of the phenotype caused by the *creC27* mutation on medium containing glucose and fluoracetamide, and phenotypic analysis of the *creD34* mutation in a $creC^+$ background showed that it was more resistant than wild type strains on this medium, and also on glucose and fluoracetate, indicating tighter repression (Kelly and Hynes, 1977). The effect of the *creD34* mutation was examined in double mutants with alleles which are sensitive on these media; *creA204*, *creB15* and *creC27.* The double mutants were resistant to fluoracetamide and fluoracetate in the presence of glucose due to the tight repression provided by *creD34* (Kelly and Hynes, 1977). *creD34* also allowed growth when in combination with the *creB15* or *creC27* mutations, on medium containing glucose and allyl alcohol, but not in combination with *creA204*. However enzyme assay data revealed some increased repression of alcohol dehydrogenase I in the *creD34;creA204* double mutant strain when compared with the *creA204* containing strain. The failure-to-derepress phenotype conferred by the *creC27* and *creB15* mutations on some carbon sources such as D-quinate, was not completely relieved in double mutants with *creD34*.

Strains carrying the *creD34* allele are resistant to acriflavine but they are sensitive to 33mM molybdate which is the reverse of the effects of the *creB15* and *creB27* alleles. Double mutants strains bearing the alleles *creB15* or *creC27* with *creD34* , have not been examined on media containing molybdate or acriflavine. However, double mutant strains containing *creD34* and one of several *creA* alleles (*creA1, creA220, creA204,*

creA218, creA304 and creA322), have been examined for their resistance to acriflavine. With the exception of *creA204*, strains carrying any one of these *creA* alleles are sensitive to acriflavine and only the double mutant strain bearing the *creA204* and *creD34* alleles shows resistance to acriflavine. The growth of this double mutant is slightly better than for the *creA204* strain (Silson and Kelly, unpublished).

Genetic analyses have indicated that *creD* is closely linked to *creC* on chromosome II and that *creD34* is recessive to $creD^{+}$ in a diploid strain (Kelly and Hynes, 1977). Double mutants have been constructed between *creD34* and *creB15* with a *creA* null allele, and their phenotypes are discussed in detail in Chapter 3 of this thesis.

1.2 Glucose Transport and Signalling

The presence of glucose in the extracellular environment also induces the expression of many genes. These genes are required for the transport of glucose into the cell, for glucose utilisation, and for the intracellular signalling pathway which is necessary for all glucose related functions to occur. In *S. cerevisiae* and *N. crassa*, mutants which have been selected for their loss of carbon catabolite repression phenotype, have included genes involved in these various pathways, indicating the importance of the glucose transport and signalling machinery for glucose repression.

1.2.1 Glucose transport and signalling in *Saccharomyces cerevisiae*

Glucose transporters in *S. cerevisiae* share the structural feature of 12 membrane spanning domains which was first characterised in the extensively studied glucose transporter, the facilitated diffusion carrier of the human erythrocyte (Bisson *et al.* 1993; Mueckler *et al.* 1985). In this section, the regulation of three major hexose transporters is described, and two proteins are discussed which have twelve membrane spanning domains and function weakly as glucose transporters, but which also regulate other hexose transporters and are proposed to receive the glucose signal.

1.2.1.1 Hexose Transporter (*HXT*)Genes

Studies of the hexose transporter (*HXT*) genes, *HXT1*, *HXT2* and *HXT4* have shown that glucose transport is regulated by glucose levels. The high affinity transporters *HXT2* and *HXT4* are induced when glucose levels are low and repressed in conditions of high glucose, while the low affinity transporter *HXT1* is induced when glucose levels are high (Bisson, 1988).

MIG1, *SSN6* and *TUP1* are involved in the regulation of these transporters. *MIG1* mutants show constitutive expression of the high affinity transporters, *HXT2* and *HXT4* and in *SSN6* deletion mutants, all three transporters are constitutively expressed (Ozcan and Johnston, 1995). Mig1p has been shown to bind to the promoter and recruit Tup1p/Ssn6p, to repress the expression of *HXT2* and *HXT4* when glucose levels are high while a different repressor Rgt1p, binds to repress *HXT1*, *HXT2* and *HXT4* expression when there is no glucose available (Figure 1.8) (Ozcan and Johnston, 1995; Ozcan and Johnston, 1996). However, when glucose levels are low, Rgt1p is necessary for the full activation of Hxt1p expression and the conversion of Rgt1p into an activator is effected by Grr1p. (Ozcan *et al.* 1996)1.2.1.2 *SNF3* and *RGT2*

Snf3p and Rgt2p each have the twelve membrane spanning domains which, because of their similarity to other glucose transporters, implies that they too are glucose transporters. However, experimental evidence shows that neither protein can transport sufficient amounts of glucose into a cell to restore growth in an *HXT*-null strain, and Rgt2p/Snf3p double mutants do not show any glucose induced expression of the *HXT* genes. Conversely, overexpression of the glucose transporters *HXT1* and *HXT2* does not restore *RGT2/SNF3* mutants (Ozcan *et al.* 1998). These results suggest that glucose transport is not the major function of these molecules and they have been proposed to act as membrane bound glucose sensors. There has been considerable interest in the uncharacteristically long carboxy-termini found in both proteins, for example, in *HXT1* the C-terminus is 26 amino acids long, but for Snf3p and Rgt2p, the C-termini are 342 and 217 amino acids long. It has been proposed that both proteins sense glucose status

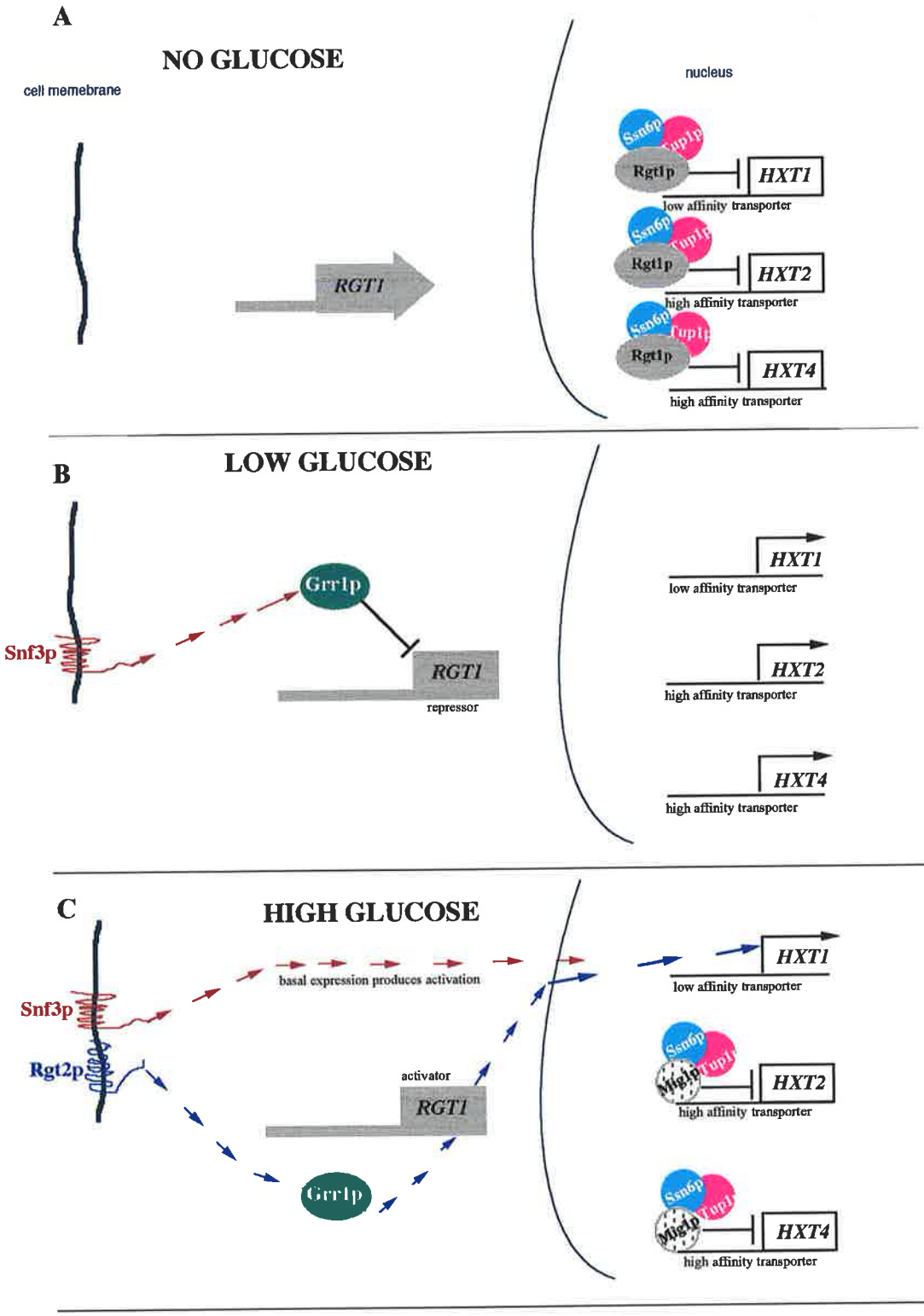
Figure 1.8 Glucose induces the expression of hexose transporter genes.

A: In the absence of glucose, the repressor Rgt1p recruits the Ssn6p/Tup1p complex to the promoters of *HXT1, HXT2* and *HXT4*

B: When glucose levels are low, Snf3p is necessary for the induction of the high affinity transporters Hxt2p and Hxt4p. Induction occurs through the action of Grr1p, which represses the repressor Rgt1p.

C: When glucose levels are high, Rgt2p is required for full expression of the low affinity transporter, Hxt1p, while the high affinity transporters are repressed by glucose repression (Mig1p/Ssn6p and Tup1p). Grr1p has been proposed to convert Rgt1p into an activator of gene expression. Basal levels of Snf3p are also necessary for full Hxt1p induction.

Drawn from: Ozcan and Johnston, 1995; Ozcan *et al.* 1996a; Ozcan *et al.* 1996 b.


A
NO GLUCOSE
cell membrane
nucleus
RGT1
Ssn6p
Tup1p
Rgt1p
HXT1
low affinity transporter
HXT2
high affinity transporter
HXT4
high affinity transporter
B
LOW GLUCOSE
Snf3p
Grr1p
RGT1
repressor
HXT1
low affinity transporter
HXT2
high affinity transporter
HXT4
high affinity transporter
C
HIGH GLUCOSE
Snf3p
Rgt2p
basal expression produces activation
activator
RGT1
Grr1p
HXT1
low affinity transporter
Mig1p
HXT2
high affinity transporter
HXT4
high affinity transporter

perhaps by binding glucose to an extracellular region of the transmembrane domain, and then generating an intracellular signal via a conserved, 25 amino acid sequence found in the carboxy terminus of both proteins. When this sequence is deleted from either protein, it loses its signalling function and when it is transferred to the carboxy terminus of an *HXT* gene, it confers a signalling role to that gene (Ozcan *et al.* 1998).

Experimental evidence has shown that *SNF3* is repressed by high concentrations of glucose but when glucose levels are low, it regulates the expression of the high affinity transporters, *HXT2* and *HXT4* by repressing the repressor, Rgt1p which allows the expression of *HXT2* and *HXT4* (Ozcan and Johnston, 1995). *SNF3* mutants are unable to grow on raffinose or sucrose, or on low levels of glucose and have lost the ability to take up glucose when levels are low (Neigeborn and Carlson, 1984; Neigeborn *et al.* 1986; Bisson *et al.* 1987). Surprisingly, Snf3p is also required for the full induction of Hxt1p when glucose levels are high, even though *SNF3* is repressed in these conditions, and it is thought that basal levels of Snf3p expression are sufficient for this (Ozcan *et al.* 1998).

RGT2 was identified as a dominant suppressor of a *SNF3* mutant (Marshall-Carlson *et al.* 1991) and Rgt2p is necessary for the induction of *HXT1* in high glucose conditions (Ozcan *et al.* 1996b). Another protein, Grr1p is also required for maximum expression of *HXT1* and is proposed to alter the repressor, Rgt1p into an activator of *HXT1* expression in high glucose conditions. When glucose levels are low, and in response to a signal generated by Snf3p, Grr1p represses the repressor Rgt1p to allow the expression of all three *HXT* genes (Flick and Johnston, 1991; Ozcan *et al.* 1994; Ozcan and Johnston, 1995) (Figure 1.8). The intracellular signals generated by the C-terminal tails of Snf3p and Rgt2p are transmitted by as yet unidentified proteins. Possible candidates are Hxk2p and Grr1p (Section 1.1.2.5), which have multiple roles and appear to function in both high and low glucose conditions.

1.2.2 Glucose transport and signalling in *Neurospora crassa*

As is the case with *S. cerevisiae*, *N. crassa* has two glucose transport systems, one is a high affinity, active transport system and the other is a low affinity, facilitated diffusion system. The low affinity transporters are expressed constitutively in repressing and derepressing conditions while the high affinity transporters are repressed by glucose and expressed when glucose levels are low or during growth on derepressing carbon sources. The number of loci contributing to these systems is not known (Schneider and Wiley, 1971).

Recently, an *rco-3* mutation has been described which allows conidiation in submerged cultures, results in resistance to 2-deoxy-D-glucose and sorbose, and leads to loss of glucose repression for *qa-2* and *con-10*. The sequence of *rco-3* suggests that it encodes a glucose transporter with twelve membrane spanning domains that share 37% identity to *SNF3* and 35% identity to *HXT1* from *S. cerevisiae*. Analysis of glucose uptake has revealed that both high and low-affinity glucose transport is affected in the mutant and implies that a regulator of transport may be affected. The pleiotropic phenotype of this mutant has led to the proposal that, like Snf3 and Rgt2, *rco-3* may be a sensor of glucose in the cell. However, *rco-3* lacks the long, C-terminal region of the *S. cerevisiae* proteins and the repeat sequence motif necessary for their function (Madi *et al.* 1997). A protein with a long C-terminus containing the repeat sequence important for Snf3p and Rgt2p function, has recently been isolated from the yeast, *K. lactis*. While the function of this protein has not been established, its possession of these two structural features suggests that *Rco3* is not the *N. crassa* equivalent of Snf3p and Rgt2p (Ozcan *et al.* 1998).

1.2.3 Glucose transport and signalling in *A. nidulans*

Studies of the transport systems in *A. nidulans* for D-glucose, D-galactose and D-fructose, have shown that transport is constitutive, carrier-mediated and, except for fructose, is subject to competitive inhibition. Sugar transport requires energy and occurs

across a concentration gradient. Analysis of competitive inhibition for each system showed that there were three distinct and fairly specific carriers involved. The D-glucose carrier could also transport D-galactose, D-xylose, D-glucose and D-mannose, and the galactose carrier could also transport D-fructose, L-arabinose, D-glucose and D-mannose. The D-fructose transporter was not inhibited by any of the sugars tested (D-galactose, D-xylose, D-glucose and D-mannose, D-fructose, L-arabinose, D-arabinose, L-sorbose, sucrose, mannitol) (Mark and Romano, 1971). The specific affinities of various sugars for particular transporters are similar in *A. nidulans*, *S. cerevisiae* and the human erythrocyte which suggests that transport systems are highly conserved across species (Cirillo, 1968).

1.3 Selection of Mutants Resistant to 2-deoxy-D-glucose

2-deoxy-D-glucose is a glucose analogue which, like D-glucose, provides the signal for carbon catabolite repression but has an inhibitory effect on growth and fermentation in *S. cerevisiae*. This effect is not related to defects in transport of various sugars or in their phosphorylation, but does seem to require phosphorylation of 2-deoxy-D-glucose (Heredia *et al.* 1963). Strains with mutations which allow growth on medium containing 2-deoxy-D-glucose and a derepressing carbon source such as quinate or raffinose, may be altered in their response to carbon catabolite repression as growth may be due to derepression in the presence of 2-deoxy-D-glucose, of the enzymes required for quinate or raffinose utilisation. 2-deoxy-D-glucose has been used to select mutants in a number of species. Many of the resistant strains have pleiotropic phenotypes which include altered response to glucose and derepression of enzymes usually subject to carbon catabolite repression.

1.3.1 Metabolism of 2-deoxy-D-glucose in *Saccharomyces cerevisiae.*

In *S. cerevisiae*, 2-deoxy-D-glucose is phosphorylated by hexokinases and is able to activate glucose repression (Gancedo and Gancedo, 1986). The effect of 2-deoxy-D-glucose on metabolism has been studied in *S. cerevisiae* by NMR spectroscopy and

biochemical analyses (Herve *et al.* 1992). When D-glucose and 2-deoxy-D-glucose are simultaneously added to cultures of *S. cerevisiae*, both are phosphorylated indifferently by hexokinase to glucose-6-phosphate and 2-deoxy-D-glucose-6-phosphate respectively. Glucose-6-phosphate is rapidly metabolised further to carbon dioxide and water whereas 2-deoxy-D-glucose-6-phosphate is not further metabolised. After 100 minutes, over 64% of the D-glucose is converted to glucose-6-phosphate (or further) whereas only 10% of the 2-deoxy-D-glucose has been phosphorylated. The 10% conversion to 2-deoxy-D-glucose-6-phosphate occurs in the first 30 minutes after which the level does not change. This suggests that an equilibrium concentration of 2-deoxy-D-glucose-6-phosphate has formed by around 30 minutes. The inhibition of growth by 2-deoxy-D-glucose is thought to be a consequence of the intracellular accumulation of 2-deoxy-D-glucose-6-phosphate (Martin and Heredia, 1977). In *S. cerevisiae*, the accumulation of glucose-6-phosphate has an inhibitory effect on hexokinase and the consumption of glucose is regulated by the rate of breakdown of glucose-6-phosphate. However, 2-deoxy-D-glucose-6-phosphate has not been shown to inhibit hexokinase in *S. cerevisiae* (Herve *et al.* 1992).

A 2-deoxy-D-glucose resistant mutant has been selected which expresses a specific phosphatase, 2-deoxy-D-glucose-6-phosphate phosphatase, that prevents the intracellular accumulation of 2-deoxy-D-glucose-6-phosphate and is thought to confer the resistant phenotype (Heredia and Sols, 1967). Isolation and characterisation of the phosphatase showed its only substrates to be 2-deoxy-D-glucose-6-phosphate and to a lesser extent, fructose-1-phosphate. Levels of the phosphatase in wild type yeast were found to be 10-20 times lower than in 2-deoxy-D-glucose resistant mutants. The phosphatase was therefore thought to have a physiological function in wild type strains on an as yet unidentified substrate (Martin and Heredia, 1977).

An *S. cerevisiae* gene which expresses the 2-deoxy-D-glucose-6-phosphate phosphatase has recently been cloned and characterised from the 2-deoxy-D-glucose resistant mutant.

Strains transformed with one copy of the phosphatase are resistant to 2-deoxy-D-glucose indicating that this allele is dominant. Disruption of the wild type version of this gene has no discernible effect on the strain (Sanz *et al.* 1994).

Further experiments with the 2-deoxy-D-glucose resistant strain have shown that the signal for glucose repression is no longer produced by 2-deoxy-D-glucose as the enzymes required for the utilisation of maltose and raffinose are derepressed in the presence of 2-deoxy-D-glucose. Glucose is still able to repress those enzymes however. It has been suggested that dephosphorylation of 2-deoxy-D-glucose-6-phosphate may cause loss of the repressing signal in 2-deoxy-D-glucose resistant strains - there is a direct correlation between high levels of phosphatase, low levels of 2-deoxy-D-glucose-6-phosphate, resistance to 2-deoxy-D-glucose and loss of carbon catabolite repression (Randez-Gil *et al*, 1995).

1.3.2 2-deoxy-D-glucose resistant mutants in *Saccharomyces cerevisiae*

Strains which lack functional Mig1p are resistant to 2-deoxy-D-glucose but *MIG1* mutants were not originally isolated on medium containing 2-deoxy-D-glucose, and of the many 2-deoxy-D-glucose resistant mutants which have been isolated in *S. cerevisiae*, none are allelic to any of the major repressors known to regulate glucose repression. Nevertheless, mutants which have a role in carbon catabolite repression have been identified on medium containing 2-deoxy-D-glucose and galactose and include *HEX1/HXK2; HEX2/REG1;* and *GRR1/CAT80* (Zimmermann and Scheel, 1977; Entian *et al.* 1977; Entian and Zimmermann, 1980; Bailey and Woodward, 1984). These genes have been discussed in Section 1.1.1.4.

1.3.3 2-deoxy-D-glucose resistant mutants in *Neurospora crassa*

Several 2-deoxy-D-glucose resistant mutants have been isolated on medium containing 2-deoxy-D-glucose and fructose (Klingmueller, 1967; Allen *et al.* 1989). As with *S.*

cerevisiae; the mutants map to several loci and have pleiotropic phenotypes. Four loci have been studied in detail. The alleles *dgr-1*, *dgr-2* and *dgr-3* lack the constitutively expressed, low affinity glucose transport system while *dgr-4* has elevated expression. The expression of the high affinity glucose transporters is constitutive in all mutants but ranges from medium to high. In other words, the regulation of glucose transport is severely disrupted in these mutants. The enzymes, invertase and glucose-amylase are also found to be expressed in the presence of glucose. The fact that both transport systems are altered in their expression coupled with the loss of glucose repression seen for some other enzymes, suggests that these loci may have a general regulatory role in carbon catabolite repression (Allen *et al.* 1989). The *dgr-3 m*utants were found to be allelic to two sorbose resistant alleles. In *N. crassa,* it has been shown that the glucose transport systems also transport 2-deoxy-D-glucose and L-sorbose (Neville *et al.* 1971; Scarborough, 1970).

1.3.4 2-deoxy-D-glucose resistant mutants in *Aspergillus nidulans*

Two mutations, (*sorA2* and *sorB11*) have been identified which confer sorbose resistance to strains of *A. nidulans* (Elorza and Arst, 1971). Strains carrying the *sorA2* mutation are also resistant to 2-deoxy-D-glucose and are able to utilise all of the carbon sources tested including D-glucose. Experimental evidence shows that D-glucose uptake is 70% of wild type levels in *sorA2* mutants and the uptake of L-sorbose, at 10% of wild type levels, is greatly impaired. The uptake system for L-sorbose is assumed to also carry 2-deoxy-D-glucose which would explain the cross resistance of *sorA2* mutants to 2-deoxy-D-glucose (Elorza and Arst, 1971). Mutants called *sum*$^{-}$ (sugar uptake mutants), have been described and their phenotype includes allowing a *pdhA1* strain to grow on ethanol and 1% D-glucose plus 1% sucrose, suppression of *areA*^{r}*-1* on acetamide and 1% D-glucose, resistance to 2-deoxy-D-glucose and L-sorbose, and inability to grow on glucose (Bailey, 1976).

1.3.5 2-deoxy-D-glucose resistant mutants in other systems

A class of 2-deoxy-D-glucose resistant mutants in *A. niger* grow poorly on glucose, have reduced hexokinase formation, and have a reduced rate of citric acid accumulation. An impaired response to the glucose signal has been proposed to explain this phenotype but the poor growth on glucose indicates that a transport defect may occur in these strains (Steinbock *et al.* 1993). Another strain, selected for resistance to 2-deoxy-D-glucose accumulates high levels of citric acid at a much faster rate than wild type strains. This mutant is not defective in glucose uptake and appears to have lost carbon catabolite repression (Parvez *et al.* 1998).

Mutants which are altered for carbon catabolite repression on medium containing glucose and lactose, glucose and starch, and glucose and ally alcohol, were originally selected on medium containing D-quinate and 2-deoxy-D-glucose. SSCP analysis indicated that they were not *creA* mutants and, as they grew well on many carbon sources including glucose, the glucose transport system was assumed to be functioning (O'Connor, 1994). *GRR1* and *HXK2* and *REG1* mutants in *S. cerevisiae* show loss of carbon repression for those enzymes and for cytochrome oxidase (*GRR1*), succinate dehydrogenase *(HXK2* and *REG1*), and hexokinase activity, none of which have been assessed in these mutants (Lobo and Maitra, 1977).

In *S. pombe* selection for resistance to 2-deoxy-D-glucose has identified a glucose transporter with the classic structural feature of transporters from many species - twelve transmembrane domains. These mutants have other defects - in mating and in the induction and repression of glyoxylate shunt pathway enzymes. The length of the C-terminal of this transporter or the presence of a motif with similarity to that found in the C-terminus of Snf3p or Rgt2p, has not been reported (Milbradt and Hofer, 1994; Mehta *et al.* 1998). Two other yeast species, *Pichia pinus* and *Kluyveromyces lactis*, have produced 2-deoxy-D-glucose resistant mutants with glucose transport defects and loss of carbon catabolite repression (Alamae and Simisker, 1994; Weirich *et al.* 1997).

1.3.6 A summary of 2-deoxy-D-glucose mutants selected in yeast and fungal species.

Selection on 2-deoxy-D-glucose has identified many mutants with defects in glucose transport. These mutants appear to be altered in carbon catabolite repression and grow poorly on glucose, and other substrates which share the same transporters, such as fructose and mannose. Other classes of mutants have been identified however which are derepressed for some enzymes and yet grow well on glucose, and are either not affected in glucose transport, or have lost regulation of glucose transporters. Some of these mutants in *S. cerevisiae* have been assigned to loci eg., *REG1, GRR1, HXK2* and are involved in the pathway between sensing the presence of glucose in the extracellular environment and the transduction of the glucose signal to genes necessary for glucose transport, metabolism and repression.

1.4 The aims and objectives of this study.

The first aim of this work was to reanalyse the effects of the absence of CreA in the cell. Although a CreA null strain had been constructed and was shown to be lethal (Dowzer and Kelly, 1991), new evidence provided by the molecular analysis of extreme *creA* alleles suggested that the absence of CreA may not be lethal to the cell.

Previous research into the function of CreA has used the *creA204* allele as a representative allele. However, molecular analysis of this allele has led to the suggestion that it most likely retains some DNA binding activity. Therefore, if a deletion of *creA* was in fact viable, then the deletion strain so generated would provide a useful tool as a recipient strain in the analysis of *creA* deletion constructs in functionality studies. These experiments are outlined in Chapter 3.

Raising antibodies for the detection of CreA was the second aim of this work. Research into CreA and carbon catabolite repression is at the point where the detection of CreA is required to address issues such as the size and stability of the proteins produced by

the bank of *creA* alleles available; the localization of CreA in response to glucose levels in the cell; and the modification of CreA in various growth conditions. Two methods were used to detect CreA. The first method involved raising antibodies to GST::CreA fusion proteins in rabbits, and the second method made use of the CreA null strain which was transformed with a his-tagged *creA* construct so that tagged CreA could be detected with purchased antibodies to the tag. These experiments are outlined in Chapter 4.

The third aim of this work was to identify new loci involved in carbon catabolite repression other than those which have already been identified (*creA, creB, creC* and *creD*) by using different genetic selection methods. Selection for resistance to the glucose analogue 2-deoxy-D-glucose in medium containing a derepressing carbon source, has been used successfully to identify mutants affected in carbon catabolite repression in *S. cerevisiae* and *N. crassa* and this method was also employed with *A. nidulans.* The analysis of mutants obtained in this way is described in Chapter 5.

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Buffers and stock solutions

Aspergillus Salt solution: (1L) 26g KCl, 26g $MgSO_4$, 76g KH_2PO_4, 50ml *Aspergillus* trace element solution, 2ml $CHCl_3$.

Aspergillus vitamin solution: (1L) 40mg p-amino benzoic acid, 50mg thiamine, 1mg biotin, 400mg inositol, 100mg nicotinic acid, 200mg calcium D-pantothenate, 100mg riboflavin, 50mg pyridoxine, 2ml CH_3Cl.

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

Osmotic medium: 1.2M $MgSO_4$ made up in 0.01M Na_2HPO_4/NaH_2PO_4, pH 5.8 with 0.2M Na_2HPO_4.

Protoplast Wash: 1.2M sorbitol, 10mM Tris-HCl pH7.5.

Trapping Buffer: 0.6M sorbitol, 100mM Tris-HCl pH7.0.

Stop Buffer: 10mM Tris, 1mM EDTA, 2% (w/v) SDS.

10x oligolabelling buffer: 0.5M Tris-HCl pH6.9, 0.1M $MgSO_4$, 1mM DTT, 0.6mM of each of dCTP, dGTP and dTTP.

60% PEG: 60% polyethylene glycol 4,000, 10mM Tris-HCl pH7.5, 10mM $CaCl_2$

10x Loading buffer for agarose gels: 0.25% bromophenol blue, 0.25% xylene cyanol FF, 25% ficoll (type 400).

2.1.2 1 x Reagents

1 x SSC: 0.15M NaCl, 15M $Na_3C_6H_7.2H_2O$, pH 7.2.

1 x SSPE: 018M NaCl, 10mM NaH_2PO_4, 1mM EDTA, pH 7.4.

1 x STC: 1.2M sorbitol, 10mM Tris-HCl pH 7.5, 10mM $CaCl_2$.

1 x TAE: 40mM Tris, 20mM CH_3COONa, 2mM EDTA pH 7.8.

1 x TBE: 89mM Tris, 89mM H_3B04, 2mM EDTA, pH 8.4

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

2.1.3 Media

2.1.3.1 Liquid media

Carbon-free: (c-free): 2% (v/v) salt solution, pH6.5 with 1M NaOH.

ANM: 2% (v/v) salt solution, 1% (w/v) D-glucose, pH 6.5 with 1M NaOH.

Luria Broth: 1% (w/v) NaCl, 0.5% (w/v) yeast extract, 1% (w/v) tryptone, pH7.5

2.1.3.2 Solid media

Solid *Aspergillus* media

Complete Media: 1% (w/v) D-glucose, 0.2% (w/v) peptone, 0.15% (w/v) casein hydrolysate, 2% (v/v) salt solution, 0.1% (w/v) vitamin solution, 25mg/ml riboflavin, 1% or 2.2% (w/v) Oxoid class 3 agar, pH 6.5 with 1M NaOH.

Carbon-free: (c-free): 2% (v/v) salt solution, 1% or 2.2% Oxoid class 1 agar, pH6.5 with 1M NaOH. Unless stated otherwise, carbon sources were added at a final concentration of 50mM.

Aspergillus Nitrogen-free Media: 2% (v/v) salt solution, 1% (w/v) D-glucose, 1% or 2.2% Oxoid class 1 agar, pH 6.5 with 1M NaOH. Nitrogen sources were added to a final concentration of 10mM unless stated otherwise.

Protoplast Media: 1M sucrose, 1%(w/v) D-glucose, 2% (v/v) salt solution, pH 7.0 with 1M NaOH. Underlay: 1% (w/v) bacteriological agar No.1. Overlay: 0.25% (w/v) bacteriological agar No.1. Nitrogen sources were added to a final concentration of 10mM unless stated otherwise.

L-agar: 1% (w/v) tryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract, 1.5% Oxoid class 1 agar, pH 7.0 with 1M NaOH.

Supplements:

Growth supplements: growth supplements were added to the final concentrations listed below (when required).

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

ampicillin: final concentration - 100µg/ml

IPTG: final concentration - 0.5mM.

x-gal: final concentration - 80µg/ml

2.1.4 Strains:

2.1.4.1 *Aspergillus nidulans* strains and genotypes

Strain	Genotype	Reference
Wild Type	*biA1; niiA4*	Cove, 1966
H17A12	*yA1 adE20 suA1adE20; areA217; riboB2*	Hynes (1975)
SA4	*yA1 adE20 suA1adE20 creA204; areA217; riboB2*	Hynes and Kelly (1977)
SA18	*yA1 adE20 suA1adE20 creA218; areA217; riboB2*	Shroff *et al.* (1996)
creAΔ21	*yA1 creAΔ7 pabaA1*	Shroff *et al.* (1997); This thesis
creAΔ4	*yA1 creAΔ37 pabaA1*	Shroff *et al.* (1996); This thesis
creAΔ99	*yA1 creAΔ3 pabaA1*	Shroff *et al.* (1997); This thesis
H17CR3	*yA1 adE20 suA1adE20 creA303; areA217; riboB2*	Shroff *et al.* (1997)
H17CR4	*yA1 adE20 suA1adE20 creA304; areA217; riboB2*	Shroff *et al.* (1997)
H17CR6	*yA1 adE20 suA1adE20 creA306; areA217; riboB2*	Shroff *et al.* (1997)
C26-1-1-10	*yA2 pabaA1; argB2*	Upshall (1986)
MSF	*yA1 adE20 suA1adE20; acrA1; galE1; pyroA4; facA303; sB3; nicB8; riboB2*	Kafer (1961)
sorA2	*biA1 sorA2*	Elorza and Arst (1971)
sorB11	*yA1 sorB11; cnx9*	Elorza and Arst (1971)
MJH532	*biA1; creB15*	Hynes and Kelly (1977)
MJH747	*biA1; creC27; niiA4*	Hynes and Kelly (1977)
MJH1000	*creD34; niiA4*	Kelly and Hynes (1977)

2.1.5 Plasmids

Plasmid	Insert	Reference
pGEX-*creA*(Zf)	621bp Nco1 fragment from within *creA* of *A. nidulans*	Kulmberg *et al.* (1993)
pGEX-*creA*(Rgr)	474bp EcoRV fragment from within *creA* of *A. nidulans*	Chamalaun-Hussey (1996)
pANC4	BamH1-Xba1 fragment containing *creA* from *A. nidulans*	Dowzer and Kelly, 1989
pANC7	EcoR1-fragment containing *creA* and surrounding sequences from *A. nidulans*	Dowzer and Kelly, (1991)
pANC8	pANC7 with the BamH1 Xba1 fragment replaced with the *riboB*$^+$ gene	Dowzer and Kelly, (1991)
pANC7ΔXΔZf	pANC7 with the Xba1 site destroyed and the zinc finger region replaced with a short polylinker	Shroff *et al.* (1997)
pANC4ΔRV10	pANC4 with an EcoRV fragment within *creA*, deleted.	Shroff (1997)
pPL3	*riboB*+ from *A. nidulans*	Oakley *et al.* (1987)
pMOO6	*argB*+ from *A. nidulans*	Upshall (1986)
pANCΔ7 and pANCΔ3	EcoR1-Sac1 fragment containing *creA* and surrounding sequences, in which the BamH1-Nde1 fragment within *creA* has been replaced by the *argB*$^+$ gene.	Shroff *et al.* (1997); This thesis
pANCΔ3	pANC4ΔRV10 with the *argB*+ gene inserted into the Xba1 site of the polylinker inserted within the zinc finger region.	Shroff *et al.* (1997); This thesis
pFH2	*rco-3* from *N. crassa*	Madi *et al.* (1997)
pQE16	murine DHFR.His_6	Qiagen (1997)

2.1.6 Oligonucleotides

1. His 1 5' ATG GGA TCC TCT CAT GAA CCT CTC AGC CAA 3'

2. His 2 5' AGA GGA TCC CAT CAC CAT CAC CAT CAC TAA TCC GGC C 3'

3. 590 5' CAA GAC TTG TGT GTG AAG GCA AGG 3'

2.2 **Methods**

2.2.1 Growth conditions for *Aspergillus* mycelia

Conidia were produced by criss-crossing plates of 2.2% complete medium with an inoculum of spores and incubating them for 2-3 days at 37°C. The conidia were scraped, vortexed in 0.01% sterile tween and added to 800ml flasks of supplemented liquid media for protoplast preparations, and 20ml flasks of supplemented liquid media for DNA preparations. The cultures were incubated overnight at 37°C with shaking.

2.2.2 Preparation of *Aspergillus* protoplasts

Mycelium from an 800ml overnight culture of the desired strain, was harvested through sterile muslin and washed with 0.6M $MgSO_4$ before being pressed dry with paper towelling. The weight of the semi-dry mycelia was recorded. The mycelium was resuspended in osmotic medium (5ml per gram of mycelia) in a 150ml conical flask and Novozyme was added at a concentration of 100µl per gram of mycelia. The flask was left on ice for five minutes and then a 12mg/ml solution of BSA was added at a ratio of 250µl/g. The flask was incubated, shaking, at 37°C until the surface of the solution became milky due to released protoplasts, after approximately 1 hour. The contents of the flask were transferred to a 30 ml corex tube, overlayed with an equal volume of trapping buffer and centrifuged at 2500 RPM for twenty minutes in a bench top centrifuge. The band of protoplasts, which formed at the interface of the two solutions, was extracted using a pasteur pipette and transferred to a 15ml corex tube. To this, an equal volume of protoplast wash was added. The protoplasts were centrifuged for

fifteen minutes at 2500 RPM in a bench top centrifuge and the pellet was resuspended in 200µl of 1xSTC and kept on ice.

2.2.3 Transformation of protoplasts

5µg of each plasmid was added for co-transformations, to an aliquot of protoplasts (50-100µl) in a 1.5ml eppendorf tube. An aliquot of protoplasts to which no DNA had been added was used as a control. 25µl of 60% PEG was mixed in by gentle inversion before the tube was left on ice for 20 minutes. The tube was then filled with 60% PEG, gently mixed well and left at room temperature for 5 minutes. The cells were collected by centrifuging for 5 minutes in a microfuge. The supernatant was removed with a pasteur pipette and the pellet was gently resuspended in 100µl 1xSTC. Dilutions of 10^{-2}, 10^{-3} and 10^{-4} of the control protoplasts were made in both distilled H_2O and 1xSTC. Both test and control protoplast suspensions were gently mixed into 3ml of molten protoplast medium overlay and poured onto 20ml solid protoplast medium underlay plates. Following solidification of the overlay, the plates were incubated at 37°C (following the method of Tilburn *et al.* (1983)).

2.2.4 A sexual cross in *Aspergillus nidulans*

The two strains to be crossed were inoculated 2mm apart on 1% complete medium and incubated for two days at 37°C. Small blocks of agar were removed from the intersection where the two strains had grown together and placed onto 5ml plates of selective media which allowed only the progeny of the cross to survive. The small plates were incubated for two days at 37°C and then taped with Parafilm and left in the incubator for seven days. The products of the sexual cross were in the spherical bodies which formed during this time, called cleistothecia. The cleistothecia were rolled on 3% water agar plates to clean them and then each one was squashed in 200µl of dH_2O in an eppendorf. A 10µl aliquot from each cleistothecia was placed on complete medium and the rest was placed at 4°C. Cleistothecia which produced two-coloured progeny were the result of a cross. 100µl of each crossed sample were vortexed in 1ml of dH_2O and

then plated out onto complete medium. Master colonies were picked from these plates and then replica plated onto a variety of selective media to establish their genotype.

2.2.5 Haploidisation of diploid strains in *Aspergillus nidulans*

Haploidisation of diploid strains was done following the method of Hastie (1970). A 0.075% working solution of benlate was used at concentrations ranging from 60-100µl per 100mls of media (15-25µl per plate).

2.2.6 Mini preparation of nucleic acids from *Aspergillus* mycelia

Mycelia from a culture grown overnight in 20ml of liquid media at 37°C, shaken, were harvested on sterile paper towelling. The semi-dried mycelium was placed in a 1.5ml eppendorf tube with 1ml of 20mM EDTA, shaken well and harvested on sterile paper towelling. The semi-dried mycelium was placed in a 1.5ml eppendorf tube containing 1ml of absolute alcohol, shaken well and harvested on sterile paper towelling. The mycelium was placed in a 1.5ml eppendorf tube and dried under vacuum for 5-10 minutes. When the pellet was totally dry, a small amount of acid washed sand was added to the tube and the mycelium was ground to a fine powder using a blue tip which had been rounded off using a flame. Two to three volumes of 10mM EDTA, 20mM Tris-HCl, 1% SDS, pH7.5 and 100mg/ml proteinase K (proteinase K added fresh) were added. The powdered mycelium was thoroughly mixed in with this solution. The tube was heated to 65°C for 5 minutes and then incubated at 37°C for one hour. Mycelial debris and sand were pelleted by spinning in the microfuge for 2 minutes. The supernatant was carefully removed with a yellow tip and transferred to a fresh eppendorf. The supernatant was extracted with an equal volume of phenol/chloroform by shaking the tube and spinning quickly to separate the phases. The supernatant was removed to a fresh tube, extracted with chloroform and then removed to a fresh tube. To this tube was added 1/2 volume of 3M Potassium acetate (pH7-8). A pellet was precipitated by spinning for 1 minute in a microcentrifuge and the supernatant was transferred to a fresh eppendorf. An equal volume of isopropanol was added, inverted

several times and spun in a microcentrifuge for 1 minute to precipitate the DNA. The supernatant was removed with a yellow tip and the damp pellet was dissolved in 100ml of dH_2O. An equal volume of 7.5M ammonium acetate was added and mixed in well, followed by 500ml of absolute ethanol to precipitate the DNA. After shaking, the DNA was precipitated by spinning for 1 minute in a microfuge. The supernatant was removed, the pellet was washed in 500ml of absolute ethanol and then dried under vacuum for approximately 5 minutes. The pellet was resuspended in sterile, distilled H_2O.

2.2.7 DNA restrictions

DNA was incubated with the appropriate restriction enzyme(s) and buffers, with H_20 making up the required volume, according to the manufacturers' instructions (Boehringer-Mannheim, Promega Corporation).

2.2.8 Southern transfer

Southern blotting was performed using the alkali transfer method described in Sambrook *et al.* (1989), based on the technique devised by Southern (1975).

2.2 9 Isolation of DNA from agarose gels.

The protocol supplied with the BRESAclean Kit from Bresatec was followed to purify DNA from agarose using a batch purification method.

2.2.10 Oligolabelling and Hybridisation

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).

2.2.11 Stripping membranes of hybridised, radioactive, single-stranded DNA.

Bound DNA was removed from the membrane by washing it three times in a boiling solution of 0.1% SSC, 0.1% SDS. The membrane was shaken gently and constantly for each 5' wash. If the membrane still recorded a signal on the Geiger counter, more washes were done. The 'silent' filter was exposed overnight to x-ray film to ensure that there was no residual signal.

2.2.12 Sequencing

Automated sequencing was performed using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit produced by the Perkin-Elmer Corporation.

2.2.13 Protein work

2.2.13.1 Preparation of total protein extracts from *A. nidulans*

Mycelium was harvested from 50ml cultures grown overnight at 37°C. The mycelium was pressed dry between sheets of paper towelling and weighed to determine the amount of buffer to add to the ground mycelium. 1µl of 0.2M phosphate buffer was added for every milligram of mycelium. Complete™ protease inhibitor was added to the buffer at the recommended concentration (Boehringer). The mycelium was ground under liquid nitrogen and then spun at 12000rpm for 20' at 4°C in a Sorval. The supernatant was removed and respun at 12000rpm for 20' at 4°C in a Sorval. Following this spin, the supernatant was stored in 1.5ml microcentrifuge tubes in aliquots of 100µl at -80°C. 5-7µl of protein was loaded in each well when using the Hoeffer Mighty Small apparatus.

2.2.13 2 Purification of 6xHis-tagged proteins using Ni-NTA spin kits

The protocol supplied by Qiagen was followed to purify the proteins under native or denaturing conditions (Ni-NTA Spin Kit-For purification of 6xHis-tagged proteins, August 1996, pages 6-8).

2.2.13.3 Expression and purification of GST fusion proteins

This was done using the procedures in: GST Gene Fusion System, Third Edition, Revision 1. from Pharmacia Biotech as detailed below-

Procedure 4. - Preparation of competent cells and transformation with vector. Page 8

Procedure 9. - Screening of pGex recombinants for fusion protein expression. Page 13

Procedure 12 - Batch purification of fusion proteins using bulk glutathione sepharose 4B. Page 16.

2.2.13.4 Cleavage of GST fusion proteins with thrombin

Procedure 15, page 18 in GST Gene Fusion System, Third Edition, Revision 1. from Pharmacia Biotech

2.2.13.5 SDS-PAGE gels

5 ml of Stacker (5% acrylamide)

30% (29.1) acrylamide solution from Biorad	0.83mls
water	3.4mls
1M Tris/HCl pH 6.8	0.63mls
10% SDS	0.05mls
TEMED	0.005mls
APS (10% solution, freshly prepared)	0.05mls

15mls of Separator (10% acrylamide)

30% (29.1) acrylamide solution from Biorad	5mls
water	4mls
1M Tris/HCl pH 8.8	5.7mls
10% SDS	0.15mls
TEMED	0.006mls
APS (10% solution, freshly prepared)	0.15mls

(Quantities are for a 1x10ml gel for the Hoeffer Mighty Small Apparatus)

Tris/glycine electrophoresis buffer

500mls of 5x stock:

Tris (250mM)	15.14g
Glycine (2.5mM, pH 8.3)	93.84g
SDS (10%)	50mls
dH_20	to 500mls

10mls of 2x Load Buffer:

1M Tris/HCl pH6.8	1ml
10% SDS	4mls
100% glycerol	2mls
bromophenol Blue	20mg
dH_2O	1ml

Freeze in 800µl aliquots.

Add 200µl of 1M DTT to an 800µl aliquot before using.

Running Conditions: 150v for 1-1.5 hours.

2.2.13.6 Western Transfer

Western transfer was performed with a tank system following the instructions and using the reagents as stated in the Immobilon-P Transfer Membrane User Guide from MILLIPORE, pages 6-9. The proteins were transferred overnight at 11v.

2.2.13.7 Western Blot

Western blotting was performed using the protocol supplied by Qiagen - Western and Colony Blot Protocols, May 1997. All solutions used were as described in this protocol.

2.2.13.8 Immunodetection

ECL detection was used to visualise the proteins on the western and was performed following Steps 9-12 on page 21 of the ECL Western Blotting Protocols produced by Amersham Life Science.

2.2.14 Activity gels (alcohol dehydrogenase 1 activity).

2.2.14.1 Protein preparations

0.3g of ground, frozen mycelium was added to 3ml of extraction buffer (1/25 dilution of Tris-glycine stock). This was then centrifuged for 20 minutes at 12000rpm in a Sorval and the supernatant was retained.

2.2.16.2 Non-denaturing polyacrylamide gels

2.5ml acrylamide (30% (29:1) BIORAD)

5.9ml H_2O

1ml Tris-glycine stock (0.25M:30.3g Tris, 142.3g glycine)

0.6ml 2% fresh ammonium persulphate

6µl TEMED

(Quantities are for a 1x10ml gel for the Hoeffer Mighty Small Apparatus)

10x Load Buffer: 0.01g bromophenol blue in 20% sucrose

Running Buffer:1/10 dilution of the Tris-glycine stock solution.

Running conditions: 80v for 3.5 hours.

2.2.14.3 Staining for ADH Activity

NAD 0.2mg/ml

PMS 0.01mg/ml (from 1mg/ml solution in water)

INT tetrazolium 0.15 mg/ml (2-(4-iodophenol)-3-(4-nitrophenol)-5-phenyltetrazolium chloride)

100mM ethanol (or other desired alcohol) in 0.1M orthophosphate buffer pH 8.2

PMS solution, ethanol and solid NAD were added to 40mls of INT solution. Gels were stained overnight, covered (H. Sealy-Lewis, pers. com.).

2.2.15 Isolation of Lambda DNA

Plaques were picked into 200µl of SM buffer and the phage were eluted for at least 1 hour. 300µl of KW251 cells from a freshly grown overnight culture were added and incubated at 37°C for 1 hour. The cells, agarose plugs and phage were added to 40mls of prewarmed L-broth + 0.2% maltose + 10mM MgSO4 and grown overnight, shaking at 200rpm/37°C. 800µl of chloroform were added to the overnight culture and incubated shaking at 37°C for 30'. The culture was split into 2 Oakridge tubes and the bacterial debris was pelleted at 11000rpm at 4°C for 20'. The supernatant was decanted into an Oakridge tube, 20µl of 1mg/ml DNase1, and 20µl of 10mg/ml RNaseA were added and the tube was incubated at 37°C for 60'. 1M solid sodium chloride and 10% solid PEG8000, were added, dissolved completely and incubated on ice for 60'. The phage particles were pelleted by spinning at 9500rpm at 4°C for 20', then the pellet was drained and thoroughly resuspended in 500µl of SM buffer. 5µl of 0.5M EDTA and 5µl of 10% SDS were added and the tube was incubated at 68°C for 20'. A phenol chloroform extraction was performed and an equal volume of isopropanol was added. The tube was incubated at -20°C for 10' and the DNA was pelleted by spinning at 14000rpm for 10'. The pellet was drained, resuspended in 100µl of TE buffer and the DNA was reprecipitated with 7.5M ammonium acetate and 100% ethanol, washed with 70% ethanol, dried and resuspended in 50µl of water or TE.

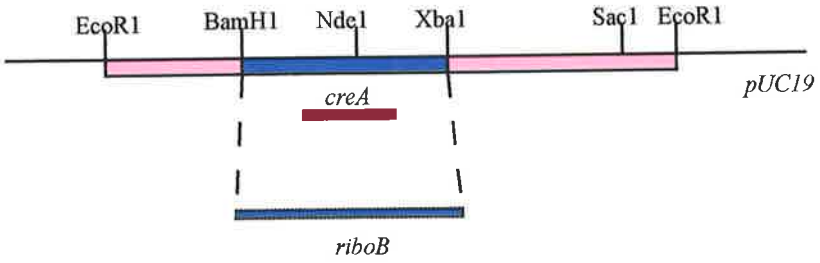
Chapter 3 Disruption of the *creA* gene in *Aspergillus nidulans*

When the *creA* gene from *Aspergillus nidulans* was initially characterised, a strain lacking *creA* was constructed using the plasmid, pANC8 (Figure 3.1). In this plasmid, the entire coding region of *creA*, as well as 1.5 kb of 3' flanking sequence was deleted and replaced with the *riboB*$^+$ gene from *A.nidulans*. pANC8 was used to transform a strain of *A. nidulans* that was *riboB2*. Transformants which had a gene replacement at the *creA* locus were only recovered in a diploid. When haploidised, the diploids segregated only haploids of a single colour. Since the colour marker *yA* , and *creA* are both on chromosome I, this indicated that the replaced allele of *creA* was not being recovered in a haploid. A haploid strain was transformed with pANC8 and a heterokaryon between the gene replacement strain and the parent haploid was recovered. This heterokaryon would not streak out to produce single colonies but microscopic examination revealed that the conidia germinated and formed a germ tube. The limited viability of this strain was termed "leaky lethal" and the disruption of *creA* was thought to be responsible for this phenotype (Dowzer and Kelly, 1991).

Subsequently, many *creA* mutant alleles have been isolated and analysed at both the phenotypic and the molecular levels (Shroff *et al.* 1996, 1997). Molecular analysis of two alleles, *creA303* and *creA304* has indicated that both are predicted to produce severely truncated proteins which have little or no function. The putative protein produced by *creA303* would contain only the first 68 amino acids and terminates in the first zinc finger, thus abolishing DNA binding and removing all of the proposed functional domains of the protein such as the Rgr1p similar domain, the Mig1p repression domain, the alanine rich region and the acidic region (Dowzer and Kelly, 1991, Drysdale *et al.* 1993, Shroff *et al.* 1997). The putative protein from *creA304* contains the first 112 amino acids, followed by 39 novel amino acids, and then terminates. This protein is disrupted in the second zinc finger and, as in the case of

Figure 3.1: pANC8

The construct pANC8 used to make the "leaky-lethal' *creA*-null strain, *creA*::pANC8 (Dowzer, 1991). To make this construct, a BamH1-Xba1 fragment was removed from the plasmid pANC7 (which contains a 7.3 kb fragment of genomic DNA from *A. nidulans* which includes *creA,* cloned into pUC19*),* and replaced with the *riboB*$^{+}$ gene.



pANC8

creA303, is predicted to be unable to bind DNA and lacks the other functional domains of *creA*.

Although *creA303* and *creA304* grow very poorly on complete and synthetic media and are very derepressed in the presence of D-glucose for many systems subject to carbon catabolite repression, they are viable and bear no resemblance to the "leaky lethal" phenotype of the *creAΔ*::pANC8 null strain. Their existence suggested that a *creA* null strain may not be lethal and led to the work presented in this chapter.

Three main issues were investigated. The first was whether the lethality of the *creAΔ*::pANC8 null strain was caused by the disruption of an essential gene in the region 3' of *creA* since 1.5 kb of sequence in this region was deleted in addition to *creA*. The second issue for consideration was whether the first 68 amino acids of *creA*, which are present in *creA303* and *creA304* but not in the *creAΔ*::pANC8 null strain, may be the reason for the difference in viability between the strains. Finally, the alleles *creA*303 and *creA304* may contain an extragenic mutation which suppresses the "leaky lethal" phenotype characteristic of loss-of-function *creA* alleles. This is a possibility because both alleles were selected as suppressors of the *areA*217 mutation after spores of the strain H17A12 had been mutagenised with the powerful mutagen, N-methyl-N^1-nitro-nitrosoguanidine (NTG).

Two constructs were designed to address these issues.

3.1 Construction of a precise *creA*-null strain

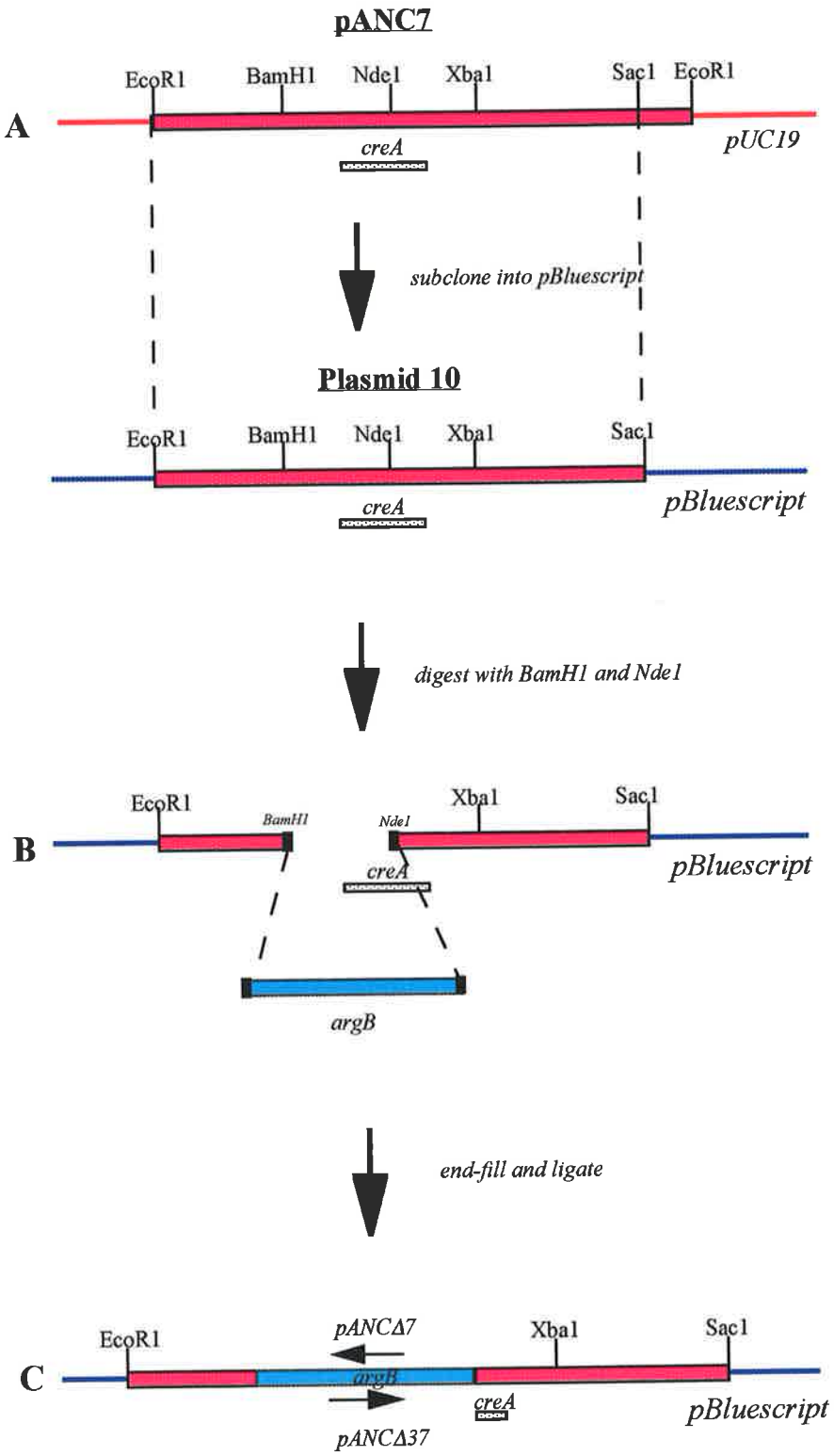
A plasmid was designed and constructed to precisely disrupt *creA* but not any adjacent sequence hence avoiding the possibility of disrupting an adjacent gene. The steps taken to make this plasmid are outlined in Figure 3.2. Two variations of the construct were obtained, with the *argB*$^+$ gene inserted in the same orientation as *creA* in pANCΔ37; and in the opposite orientation in pANCΔ7. The difference

Figure 3.2: pANCΔ7 and pANCΔ37

A: An EcoR1-Sac1 fragment containing *creA* and surrounding sequences, was removed from the plasmid pANC7 and subcloned into pBluescript to take advantage of the lack of an Nde1 site in this vector. The subclone was called Plasmid 10.

B: A 1.5kb BamH1-Nde1 fragment was removed from Plasmid 10, the remaining DNA was end-filled, and replaced with an end-filled 3.3kb Xba1 fragment containing the *argB*$^+$ gene from *A. nidulans.*

C: Plasmids were obtained with *argB*$^+$ inserted in both orientations. pANCΔ7 has *argB*$^+$ in the opposite orientation to *creA* while pANCΔ37 has *argB*$^+$ in the same orientation as *creA.*



pANCΔ7 and pANCΔ37

between pANC8 and the constructs pANCΔ7 and pANCΔ37 (Figure 3.2) is the region between the Nde1 and Xba1 sites which is deleted in pANC8 but not in pANCΔ7 and pANCΔ37. Within this region lies 1.5kb of sequence which may contain another gene. The sequence which has been deleted in the new constructs consists of 670 base pairs before the ATG and the first 830 base pairs of the CreA coding region. This has been replaced with a 3.3kb Xba1 fragment containing the *argB*$^+$ gene.

Both plasmids were used to transform the *A. nidulans* strain C26-1-1-10 which carries the *argB2* mutation, and arginine-independent transformants were selected and inoculated to plates of complete medium. Most *creA* mutant alleles have a distinct, compact morphology on complete medium and it was anticipated that if a homologous recombination had occurred at the *creA* locus to produce a *creA*-disrupted strain, then this strain would be easy to identify from other transformants by its morphology. The strain *creAΔ4* resulted from transformation with linearised pANCΔ37 and the strain *creAΔ21* resulted from transformation with circular pANCΔ7.

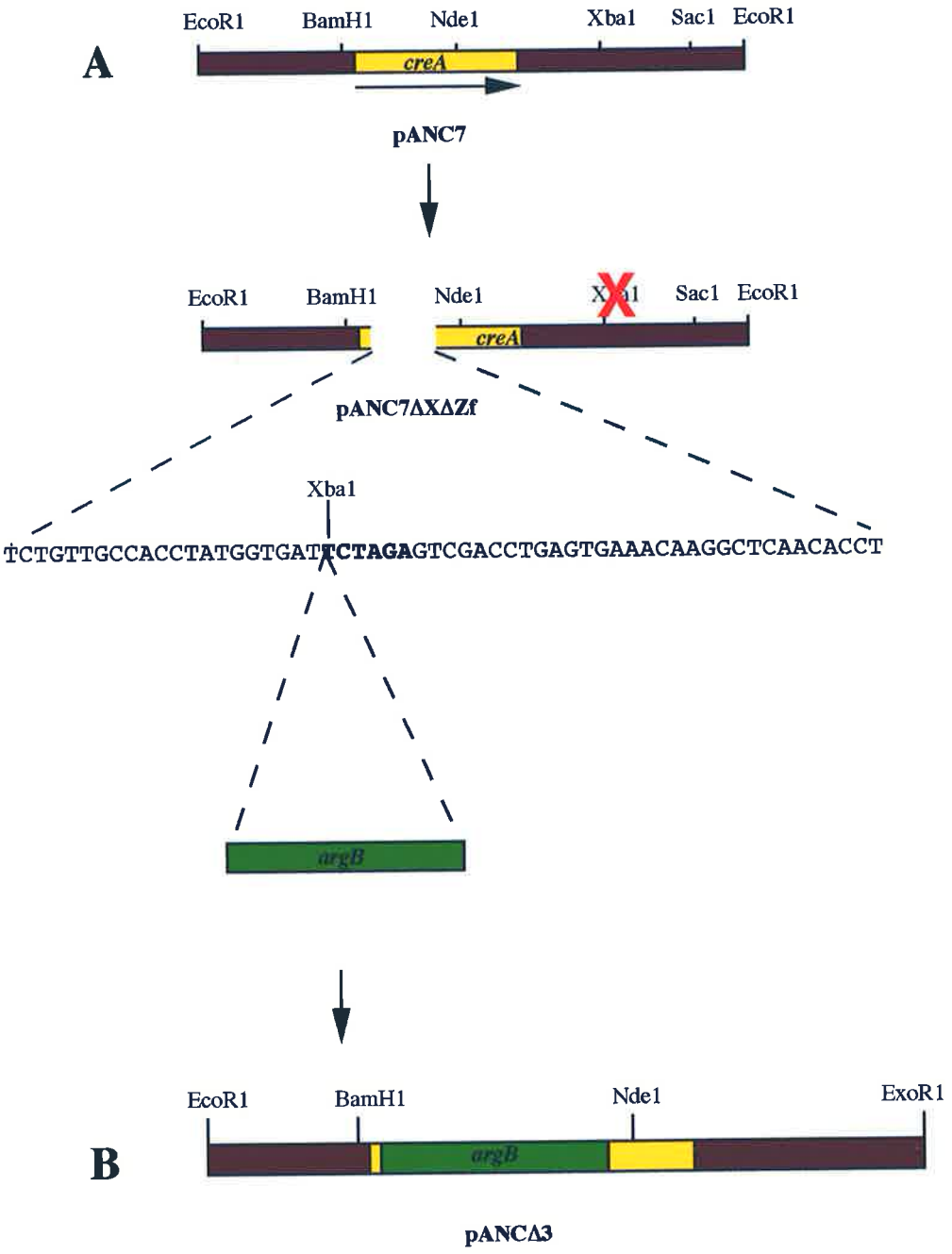
3.2 Construction of a strain similar to *creA303* at the *creA* locus.

In order to make a strain containing a *creA* allele similar to *creA303*, the construct pANCΔ3 was made using the plasmid pANCΔXΔZf as the starting point as outlined in Figure 3.3 (Shroff *et al.* 1997). A strain resulting from a homologous recombination event between pANCΔ3 and the *creA* locus was predicted to produce a protein containing the first 48 amino acids of *creA* and have no zinc finger domain or any of the proposed functional domains of *creA* after this region. This strain would be similar to *creA303* which produces a putative protein of the first 68 amino acids and is disrupted in the first zinc finger. pANCΔ3 was transformed into the *argB2* strain, C26-1-1-10 and arginine-independent transformants were selected and tested on complete medium for the characteristic *creA*-allele morphology. The

Figure 3.3: pANCΔ3

A: An Xba1 site in pANC7 was destroyed and the region encoding amino acids 47 to 124, which includes the zinc finger region of *creA*, was removed and replaced with sequences which contained an Xba1 site. This yielded the plasmid, pANCΔXΔZf (Shroff *et al.* 1997).

B: A 3.3kb Xba1 fragment containing the *argB+ gene* from *A. nidulans* was cloned into the Xba1 site to create the plasmid pANCΔ3.


A
EcoR1
BamH1
Nde1
Xba1
Sac1
EcoR1
creA
pANC7
EcoR1
BamH1
Nde1
Xba1
Sac1
EcoR1
creA
pANC7ΔXΔZf
Xba1
TCTGTTGCCACCTATGGTGATTCTAGAGTCGACCTGAGTGAAACAAGGCTCAACACCT
argB
B
EcoR1
BamH1
Nde1
ExoR1
argB
pANCΔ3

strain *creAΔ99* was selected in this way and had very compact growth on complete medium, similar to *creA303.*

3.3 Evidence for integration of the deletion constructs at the *creA* locus

Integration of the constructs pANCΔ7, pANCΔ37 and pANCΔ3 at the *creA* locus in the strains *creAΔ4 creAΔ21* and *creAΔ99* respectively, was confirmed by Southern analysis. Genomic DNA from the strains *creAΔ4 creAΔ21* and *creAΔ99* and the parent $creA^+$ strain was digested with EcoR1, and with EcoR1 plus Sac 1. Digested DNA was electrophoresed on a 0.8% agarose gel and transferred to a nylon membrane by Southern transfer. The DNA on the membrane was hybridised overnight with the ^{32}P-labelled 6.3 kb EcoR1 - Sac 1 fragment of pANC7 containing $creA^+$ and surrounding sequences, and then exposed to X-ray film. EcoR1 cuts outside the $creA^+$ gene and produces a genomic DNA fragment of 7.3 kb. The EcoR1 digests produced bands of 9.1 kb in the strains transformed with the constructs pANCΔ7 and pANCΔ37. These plasmids have had 1.5kb removed from within this region and 3.3kb inserted, which has resulted in the distance between the two EcoR1 sites being 1800 bases longer than in the wild type *creA* strain. pANCΔ3 has had 231 bases removed and 3300 bases inserted into this region to produce an EcoR1 fragment in the strain, *creAΔ*99 of 10.3 kb (Figure 3.4A).

The null strains, *creAΔ*4, *creAΔ*21 and *creAΔ*99, have $argB^+$ at the *creA* locus, which contains an internal Sac1 site. Digestion with EcoR1/Sac 1 yielded a single 6.3 kb fragment from $creA^+$ genomic DNA but produced two fragments in the three null strains. pANCΔ7 and pANCΔ37 produce fragments of different sizes because $argB^+$ is in opposite orientation in these strains (Figure 3.4B). DNA from a strain containing wild type *creA* and from *creAΔ*4 , was digested with EcoR1 and hybridised with the BamH1-Nde1 fragment which had been removed in the deletion strain. There were no hybridising sequences seen in the deletion strain which confirmed that the plasmids had integrated at the *creA* locus (Figure 3.4C).

Figure 3.4: Southern analysis of genomic DNA from strains containing *creA*$^{\pm}$ and *creA*-null alleles.

A:

Lane 1- Molecular weight marker, Lambda/Hind111

Lane 2- *creA*$^{+}$ strain

Lane 3- creAΔ4

Lane 4- creAΔ21

Lane 5- creAΔ99

The DNA in this panel has been digested with EcoR1 and probed with an EcoR1-Xba1 fragment from pANC7 containing *creA* and surrounding sequences.

B:

Lane 1- *creA*$^{+}$ strain

Lane 2- creAΔ4

Lane 3- creAΔ21

Lane 4- creAΔ99

The DNA in this panel has been digested with EcoR1 plus Sac1, and probed with an EcoR1-Xba1 fragment from pANC7 containing *creA* and surrounding sequences.

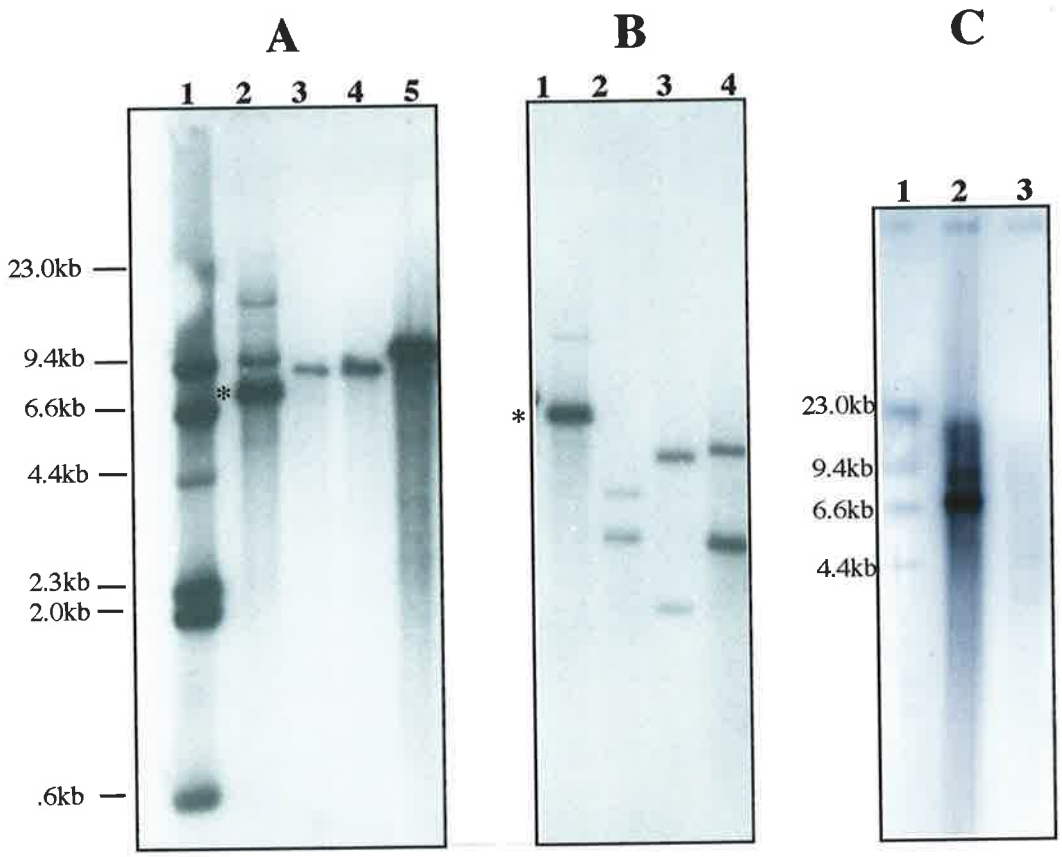
C:

Lane 1- Molecular weight marker, Lambda/Hind111

Lane 2- *creA*$^{+}$ strain

Lane 3- *creAΔ4*

The DNA in this panel has been digested with EcoR1, and probed with the BamH1-Nde1 fragment from *creA* which was removed in the *creA* deletion constructs pANCΔ7 (strain *creAΔ21*) and pANCΔ37 (strain *creAΔ4*) and therefore should not be present in the deletion strain.


A
1 2 3 4 5
23.0kb
9.4kb
6.6kb
4.4kb
2.3kb
2.0kb
.6kb
*
B
1 2 3 4
*
C
1 2 3
23.0kb
9.4kb
6.6kb
4.4kb

3.4 Confirmation of the gene replacement event at the *creA* locus by crossing the null strains, *creAΔ4*, *creAΔ21* and *creAΔ99*, to *creA204*

As further evidence for the gene replacement event at the *creA* locus, the strains were crossed to *creA204* and the progeny were plated onto complete medium. If wild-type *creA* was present in any of the strains, the *creA*$^+$ progeny would be obvious by their morphology on complete medium. Each cross was plated onto five plates of complete media and all the progeny had the characteristic, *creA*-like compact morphology. There were two classes of compact morphology however with the null alleles producing a much more severe phenotype than *creA204*.

3.5 Phenotypic analysis of *creAΔ4*, *creAΔ21* and *creAΔ99*.

The phenotypes of the strains constructed were analysed by growth testing and histochemical staining. These tests allowed deductions to be made about the effects of the gene replacements on strain morphology, the repression of different systems in the presence of D-glucose, and on the absolute levels of some enzymes. The systems that were chosen for investigation were subject to carbon catabolite repression and thus regulated by *creA*. Evidence for loss of, or reduced repression in the presence of D-glucose was assessed as this was an expected feature of *creAΔ4*, *creAΔ21* and *creAΔ99*. The two putative null alleles, *creA303* and *creA304*, and also *creA306* and *creA*$^+$ were included in the analysis for comparison. Figure 3.5A shows the putative proteins which could be produced by each allele - none of which are thought to bind DNA - and the functional domains which they may contain. *creA306* is the most extreme *creA* allele in terms of morphology and loss of repression. On complete or synthetic medium, *creA306* results in very small, compact colonies and slow sporulation (Figure 3.5B). Molecular analysis has revealed that *creA306* is due to a mutation in the second zinc finger, which is proposed to completely disrupt DNA binding, but a full length protein is still predicted. This is the only *in vivo* selected allele with these properties. The severe phenotype of this allele has been attributed to the ability of the full-length, possibly

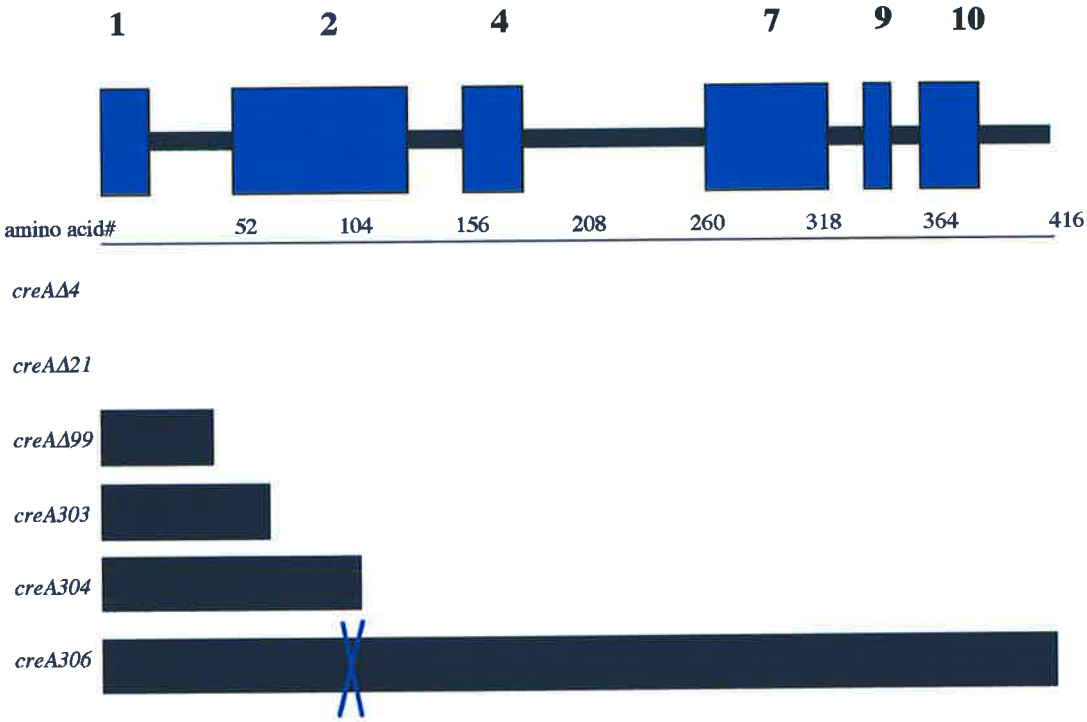
Figure 3.5 A molecular and phenotypic summary of the *creA* alleles used in this study

A: This diagram shows the functional domains of wild type CreA (Section 1.1.2.3.2) and aligned below are the putative proteins produced by the alleles used in this study. The cross on the protein produced by *creA306* represents the mutation which disrupts the zinc finger and abolishes binding.

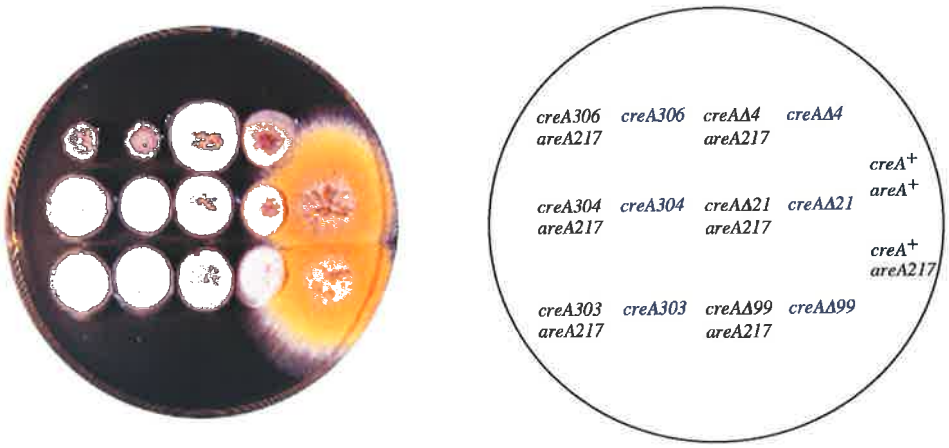
B: The strains were inoculated onto complete medium and incubated for 72 hours at 37°C. For wild type strains, 48 hours were sufficient for sporulation to occur, but the null alleles developed more slowly. The strains are present in both an *areA*$^{+}$ and an *areA217* background.

On complete medium, the strains bearing *creA* null alleles have an extreme morphology and only sporulate in the centre of the colony which allows for ready identification of strains bearing *creA* null alleles. The more extreme morphology of *creA306*, even when compared with the null alleles, can be seen in this figure.

A


1
2
4
7
9
10
amino acid#
52
104
156
208
260
318
364
416
creAΔ4
creAΔ21
creAΔ99
creA303
creA304
creA306

B


creA306 areA217
creA306
creAΔ4 areA217
creAΔ4
creA+ areA+
creA304 areA217
creA304
creAΔ21 areA217
creAΔ21
creA+ areA217
creA303 areA217
creA303
creAΔ99 areA217
creAΔ99

nuclear localised protein to titrate proteins that interact with CreA (Shroff *et al.* 1977). *creA306* was included in the comparative analyses with the null alleles to determine if it had a generally more extreme phenotype than strains which do not produce any CreA.

3.5.1 Alcohol dehydrogenase I

Alcohol dehydrogenase I is encoded by the *alcA* gene and its function is to convert ethanol to acetaldehyde (Pateman *et al.* 1983; Sealy-Lewis and Lockington, 1984). The expression of *alcA* in repressing conditions can be assessed on solid medium containing D-glucose and allyl alcohol. If *alcA* is expressed, alcohol dehydrogenase I is produced and acts on allyl alcohol to produce the toxic substance acrolein, which kills the strain. Strains with a wild type copy of *creA* can grow on high levels of allyl alcohol (25-50mM) in the presence of D-glucose because repression of *alcA* by CreA is very tight. This plate test is a sensitive indicator of *alcA* expression and levels as low as 0.1mM can be used to detect derepression in extreme *creA* alleles (Lockington *et al.* 1985). *creA306*, and all of the *creA* null-alleles, were sensitive to allyl alcohol concentrations as low as 0.1mM in the presence of D-glucose which indicated that the *alcA* gene was derepressed in these strains (Figure 3.6).

Growth testing on medium containing allyl alcohol and glucose does not show whether the expression of the *alcA* gene is elevated in strains bearing extreme *creA* alleles, or whether alcohol dehydrogenase 1 is expressed in the absence of any inducer, in these strains. In order to assess the levels of ADH1 in these strains, protein extracts were analysed for alcohol dehydrogenase I activity in the absence of inducer in both repressing (1% D-glucose) and derepressing (1% fructose) conditions (Figure 3.7). The three new null strains, *creAΔ4, creAΔ21* and *creAΔ99* had high levels of enzyme activity in the presence of 1% D-glucose (Figure 3.7B) and even higher levels when grown in 1% fructose (Figure 3.7D). The extreme allele, *creA306* had similarly high levels in both conditions and the *creA*$^{+}$ strain showed

Figure 3.6 *creA*-null alleles on medium containing allyl alcohol and 1% D-glucose.

A: 0.1mM allyl alcohol, 1% D-glucose, ammonium tartrate and supplements.

B: 0.5mM allyl alcohol, 1% D-glucose, ammonium tartrate and supplements.

A

B

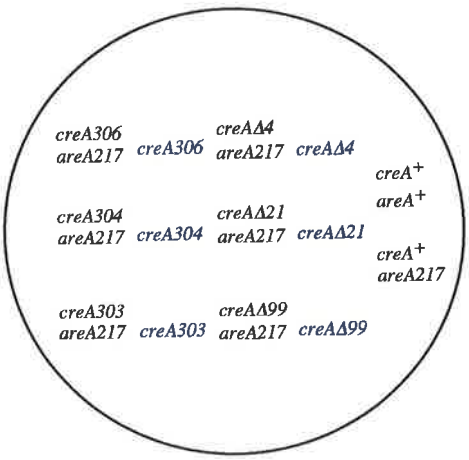

creA306
areA217
creA306
creAΔ4
areA217
creAΔ4
creA+
areA+
creA304
areA217
creA304
creAΔ21
areA217
creAΔ21
creA+
areA217
creA303
areA217
creA303
creAΔ99
areA217
creAΔ99

Figure 3.7 Alcohol dehydrogenase 1 activity in strains contating *creA* null alleles

The proteins were prepared in non-denaturing conditions, run on non-denaturing gels and stained for enzyme activity (Section 2.2.14) or with Coomassie blue.

A. Coomassie blue stained gel

B. activity gel loaded with proteins prepared from mycelium grown overnight in 1% glucose.

C. Coomassie blue stained gel

D. activity gel loaded with proteins prepared from mycelium grown overnight in 1% fructose.

Lane1- *creAΔ4*

Lane 2- *creAΔ4*; *areA217*

Lane 3- *creAΔ21*

Lane 4- *creAΔ21; areA217*

Lane 5-*creAΔ99*

Lane 6-*creAΔ99; areA217*

Lane 7-*creA303*

Lane 8-*creA303*; *areA217*

Lane 9-*creA304*

Lane 10-*creA304*; *areA217*

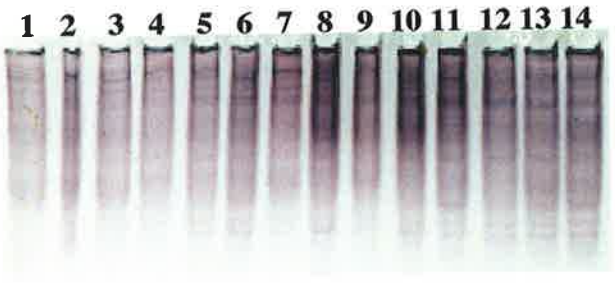
Lane 11-*creA306*

Lane 12-*creA306; areA217*

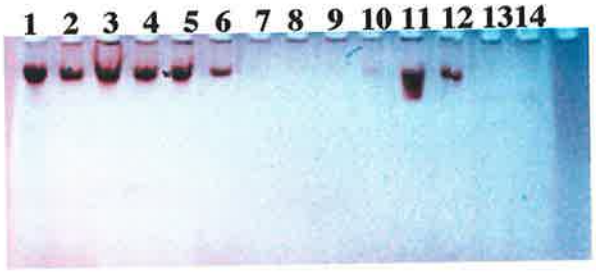
Lane 13-$creA^{+}$

Lane 14- $creA^{+}$; *areA217*

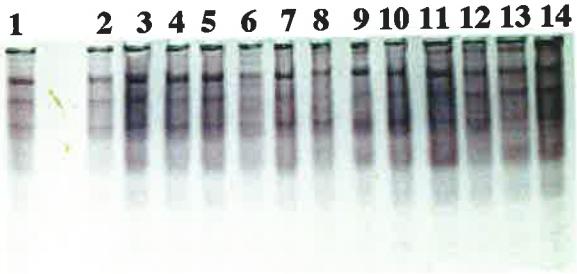
A


1 2 3 4 5 6 7 8 9 10 11 12 13 14

B


1 2 3 4 5 6 7 8 9 10 11 12 13 14

C


1 2 3 4 5 6 7 8 9 10 11 12 13 14

D


1 2 3 4 5 6 7 8 9 10 11 12 13 14

very little ADH1 activity in 1% fructose and no detectable activity in 1% glucose. *creA303* and *creA304* differed from the other null alleles by having very low ADH1 activity when grown in 1% D-glucose and less activity than the other *creA* alleles in 1% fructose, particularly *creA303*. However, on medium containing 0.1mM or 0.5mM allyl alcohol and 1% D-glucose, all of the strains were dead except the wild type *creA* strains (Figure 3.6). This result indicated that *creA303* and *creA304* were as sensitive to low concentrations of allyl alcohol in the presence of 1% D-glucose as the other strains and yet they expressed significantly lower levels of ADH1 in non-induced conditions. This suggests that either allyl alcohol induces *alcA* expression, or that very low ADH1 activity is sufficient to produce toxic levels of acrolein on allyl alcohol.

All strains were assessed in an *areA*$^{+}$ and an *areA217* background. When grown in 1% glucose and in an *areA217* background, *creAΔ*4, *creAΔ*99 and *creA306* showed slightly reduced levels of ADH1 activity. This effect was not clearly seen for *creAΔ21*.

3.5.2 α-amylases

α-amylases are secreted by *A. nidulans* into the surrounding medium in the presence of starch. They break down starch molecules into glucose. On solid medium containing starch as the sole carbon source, the extra-cellular α-amylases produced by *A. nidulans* digest the starch in the surrounding area. This can be visualised by staining the medium with an iodine solution which reacts with the starch in the plate to form a deep blue colour (Shroff *et al.* 1997). Around each colony, the area from which the starch has been digested does not stain and appears as a 'halo' of clear agar. The size of this halo is an indication of the amount of α-amylase secreted by the colony.

Figure 3.8 Expression of α-amylases in strains containing *creA* null alleles.

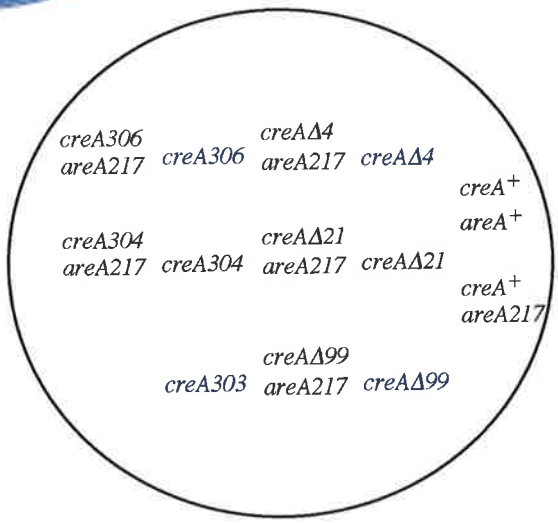
A: 1% D-glucose and 0.5% starch plus 10mM ammonium tartrate and appropriate supplements.

B: 1% D-glucose and 0.5% starch stained with iodine solution plus 10mM ammonium tartrate and appropriate supplements.

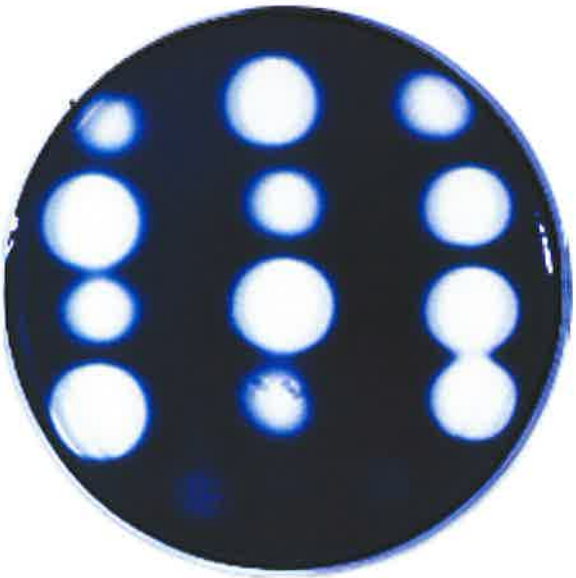
C: 0.5% starch medium stained with iodine solution.

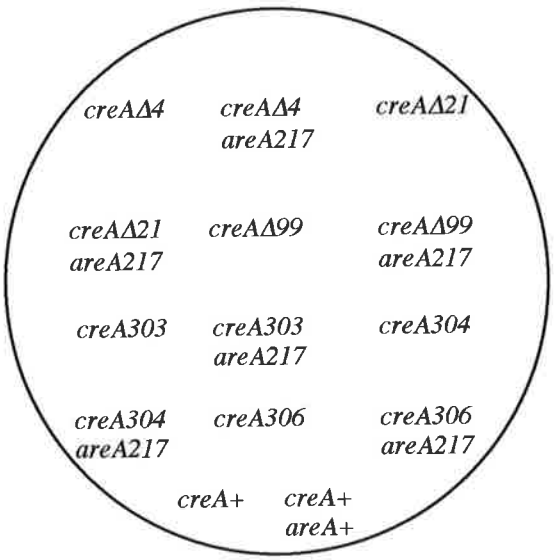
A

B


creA306 areA217
creA306
creAΔ4 areA217
creAΔ4
creA+ areA+
creA304 areA217
creA304
creAΔ21 areA217
creAΔ21
creA+ areA217
creA303
creAΔ99 areA217
creAΔ99

C




creAΔ4
creAΔ4 areA217
creAΔ21
creAΔ21 areA217
creAΔ99
creAΔ99 areA217
creA303
creA303 areA217
creA304
creA304 areA217
creA306
creA306 areA217
creA+
creA+ areA+

Strains containing *creA*$^+$ do not clear starch in medium which also contains D-glucose because α-amylase expression is repressed. This test is useful for visualising the extent to which α-amylases are derepressed in the presence of D-glucose, in *creA* alleles (Figure 3.8). All of the strains showed significant levels of derepression in the presence of D-glucose except the wild type strains which showed much less clearing. When incubated for 48 hours, the wild type colonies typically do not produce any clearing on this medium, however, in order to grow the *creA* alleles, the strains were incubated for 72 hours. Therefore, the *creA*$^+$ strains were expressing some α-amylases due to the gradual depletion of glucose in the medium (Figure 3.8, B).

To determine whether α-amylases are expressed by the strains containing *creA* alleles, in the absence of starch, the strains were grown overnight in liquid culture containing 1% D-glucose. 20μl drop of supernatant from each culture was placed onto solid 0.5% starch medium and incubated overnight at 37°C. The medium was stained with iodine to show α-amylase activity (Figure 3.8C). All of the strains except for the *creA*$^+$ strains, produced a clear area on the plate and were therefore producing α-amylases in the absence of inducer and in the presence of D-glucose. The strains carrying the *areA217* allele consistently produced a larger clearing than those with *areA*$^+$, except in the case of *creA304* which produced equal amounts of clearing in both *areA*$^+$ and *areA217* backgrounds.

3.5.3 β-galactosidase

A. nidulans produces the enzyme β-galactosidase which converts lactose to galactose and glucose. The gene encoding this enzyme is subject to carbon catabolite repression and is not expressed in the presence of D-glucose in a *creA*$^+$ strain. The expression of β-galactosidase by a colony growing on solid medium can be detected by the inclusion of the chromogenic substrate, 6-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal) in the agar. β-galactosidase metabolises X-Gal to produce a

bright blue compound - the more β-galactosidase expressed, the brighter and more intense the blue colour.

Using X-Gal as an indicator of β-galactosidase activity, the strains were examined for derepression on solid media containing D-glucose and lactose (results not shown), and for elevated levels of enzyme on lactose only (Figure 3.9). On medium containing 1% D-glucose and 0.5% lactose, the wild type *creA* strains did not produce any blue colour, but all of the strains in a mutant/null *creA* background turned blue, indicating that the expression of β-galactosidase was derepressed in these strains in the presence of glucose. Differences in colony size and β-galactosidase expression between strains with and without *areA217,* were not seen on this medium (results not shown). On 0.5% lactose medium, only the constructed *creA* null strains were tested in an *areA217* background and once again there was little difference in colony size between the $areA^+$ and *areA217* bearing strains with the same *creA* allele. When grown on medium containing lactose and X-gal, the three *creA*-null strains, *creAΔ4, creAΔ21* and *creAΔ99,* produced a much deeper blue colour in an $areA^+$ background than in an *areA217* background. The $creA^+$ strain did not produce any blue colour (Figure 3.9B).

3.5.4 Suppression of *areA217*

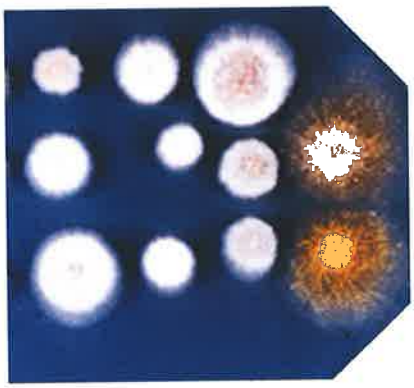
Substrates which can be utilised as carbon sources and nitrogen sources are regulated by both *creA* and *areA* (Arst *et al.* 1973). Strains containing *areA217* can not grow on nitrogen sources other than ammonium because they lack the activator, AreA. If an *areA217* strain also carries a mutation in *creA*, it will be derepressed in the presence of D-glucose, for enzymes normally subject to carbon catabolite repression, including those which breakdown substrates to produce both carbon and nitrogen sources. There is some variation in the amount by which *creA* alleles suppress *areA217* on medium containing D-glucose and a nitrogen source which can also be utilised as a carbon source (Shroff *et al.* 1996, 1997) and therefore it was of

Figure 3.9 Expression of β-galactosidase in strains containing *creA* null alleles.

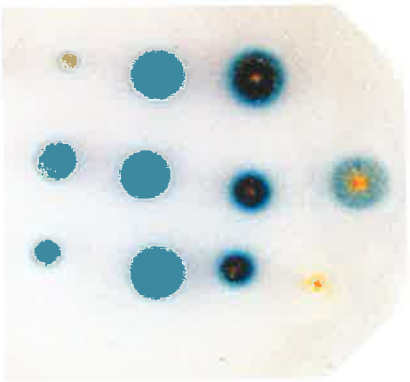
A: 0.5% lactose, plus 10mM ammonium tartrate and appropriate supplements.

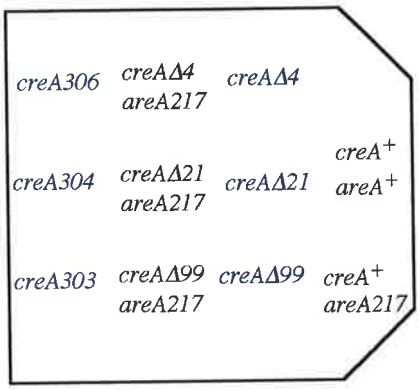
B: 0.5% lactose plus 100µl X-Gal solution (20mg per ml), plus 10mM ammonium tartrate and appropriate supplements.

A



B




creA306
creAΔ4 areA217
creAΔ4
creA304
creAΔ21 areA217
creAΔ21
creA+ areA+
creA303
creAΔ99 areA217
creAΔ99
creA+ areA217

interest to see how strongly the *creA* null alleles would suppress the *areA217* mutation on media containing acetamide and D-glucose or proline and D-glucose. The *creA* null strains, *creA306* and a *creA*$^+$ strain, were tested in *areA*$^+$ and *areA217* backgrounds so that the extent to which each *creA* allele suppressed *areA217* could be compared to how well the strain grew with a fully functional *areA* gene. For all of the strains tested, the *creA* alleles allowed strains which also carried the *areA217* mutation to grow on acetamide or proline in the presence of 1% D-glucose. However, none of the strains grew as well as those carrying the *areA*$^+$ allele suggesting that acetamidase is not fully derepressed in the absence of CreA and that AreA is required for full activation of this gene (results not shown).

3.5.5 A summary of the phenotype of strains containing the *creA* null-allele in an *areA217* or *areA*$^{\pm}$ background.

The involvement of *areA* in the regulation of genes encoding enzymes required for the utilisation of substrates which provide carbon and nitrogen sources, has been reported for many enzymes. One example is extra-cellular proteases, where the *areA217* loss-of-function mutation causes reduced expression of the proteases and the *areA102* "up-mutation" increases their expression (Hynes and Kelly, 1977). There is some evidence to suggest that *are*A may also regulate genes encoding enzymes required for the utilisation of substances which only provide a carbon source, for example in the absence of glucose and in the presence of ammonium, a similar effect has been observed for the expression of cellulases. The *areA102* and *xprD1* alleles increase cellulase expression and the *areA217* loss-of-function allele causes a significant reduction in cellulase expression (R.Lockington, pers. com.). Growth testing of *creA* null strains (presented in this chapter) has also shown differences in the expression of certain enzymes between *areA*$^+$ strains and strains bearing the *areA217* allele, in both repressing and derepressing conditions. Differences were observed between these strains irrespective of their CreA status, for example both *creA306* (which is predicted to produce a full length CreA) and

creAΔ4 (a CreA null strain) had higher levels of α-amylases in an *areA217* background than in an *areA*$^+$ background when grown in repressing conditions (glucose and ammonium tartrate) (Figure 3.8, C). Conversely, when grown in derepressing conditions (0.05% lactose), *creA* deletion strains produced higher levels of β-galactosidase in an *areA*$^+$ background than in an *areA217* background. Analysis of the *creA* null strains in *areA*$^+$ and *areA217* backgrounds suggests that AreA is involved in the regulation of genes encoding enzymes required to utilise carbon sources which do not also provide a nitrogen source.

3.5.6 Suppression of *creAΔ4*

A suppressor of *creAΔ4* has been isolated which allows much more vigorous growth and conidiation on complete medium. Genetic analysis has indicated that this suppressor mutation is either allelic to or closely linked to *malA,* which is proposed to be a positive activator of maltose permease and maltase expression (Shroff, 1997). Perhaps the growth and conidiation defects seen in strains bearing *creA* alleles, result from the derepression of so many enzymes that an energy imbalance causes a failure to grow and conidiate normally. When in a double mutant strain with *creAΔ4,* the mild suppression provided by the *creD34* allele has some affect on colony size and sporulation and a mutation in a regulatory gene such as *malA* has a greater effect.

3.6 Phenotypic analysis of double mutants between *creAΔ4* and *creB15* or *creD34*

creB, *creC* and *creD* are three other genes which appear to be involved in carbon catabolite repression. Mutants have been identified in each gene and their analysis has shown that, like *creA* mutants, they are pleiotropic, they affect utilisation of various carbon sources, *creB* and *creC* mutations can suppress the *areA217* mutation in certain conditions, and they alter sensitivity to toxic substances like allyl alcohol or fluoracetate in the presence of a repressing carbon source (Hynes and Kelly, 1977; Kelly and Hynes, 1977).

The *creB15* and *creC*27 alleles show loss of, or reduction in carbon catabolite repression of many enzymes such as alcohol dehydrogenase 1, acetamidase, isocitrate lyase, extra cellular proteases and acetyl-CoA-synthase. For other systems however, reduced levels of enzyme in these mutants results in very poor growth, for example, when D-quinate, lactose, L-arabinose and D-glucuronate are used as sole carbon sources. Both mutations strongly suppress the effects of *creA217* on medium containing glucose and acetamide, but not on medium containing glucose and L-proline.

Possible genetic interactions between CreB and CreC with CreA have been examined in the double mutant strains, *creB15;creA204* and *creC27;creA204*. On complete medium, the *creB15* and *creC27* mutations result in only slight morphological alteration in contrast to *creA204* but the double mutants show as compact morphology as *creA204* alone (Hynes and Kelly, 1977). Where both single mutants show reduced carbon catabolite repression (for example, *alcA*, *amdS*), the double mutants are also very derepressed. Where *creB15* and *creC27* grow poorly, in conditions generally thought of as derepressing, such as on 0.5% D-quinate, the presence of *creA204* does not allow stronger growth.

The *creD34* mutation results in increased carbon catabolite repression for a number of systems (Kelly and Hynes, 1977). For this reason, strains with the *creD34* mutation are even more strongly resistant on media containing glucose in the presence of allyl alcohol or fluoracetamide. Possible genetic interactions between CreD with CreB or CreC or CreA has also been examined in the double mutants *creD34;creB15, creD34;creC27,* and *creD34;creA204. creD34* is able to suppress the sensitivity of the *creA204*, *creB15* and *creC27* mutations to fluoracetate or fluoracetamide in the presence of glucose in double mutants. However, on 1% D-glucose and 2.5mM allyl alcohol, only the *creB15* and *creC27* mutant phenotypes

are suppressed. The poor growth of *creB15* and *creC27* mutants on L-proline and L-glutamate is reduced further by the presence of *creD34* which also reduces the wild type levels of growth seen in *creA204* strains. These results suggest that *creD* is epistatic to *creA*, *creB* and *creC*. The *creD34* strain has normal morphology on complete medium but the double mutant strains with *creA204* have a *creA204*-like morphology.

To provide more insight into the interactions between the *cre* loci, double mutants were made between the *creA* null allele, *creAΔ4* with *creB15*, and *creD34*.

3.6.1 Generation of the double mutant strain, *creAΔ4;creD34*.

The *creAΔ4* strain was crossed to a strain containing the *creD34* mutation and master plates were made from the progeny of a crossed cleistothecium. From the results of previous work (Hynes and Kelly, 1977; Kelly and Hynes, 1977) it was assumed that the *creD34* mutation would not suppress the extremely compact morphology which is characteristic of *creA*-null alleles and that the double mutant would have compact morphology on complete medium. Therefore, only these colonies were selected for further analysis.

Candidate *creAΔ4;creD34* double mutant strains were identified by replica plating to complete media containing various concentrations of acriflavine. Strains bearing the *creD34* mutation are resistant to acriflavine and strains bearing the *creAΔ4* mutation are sensitive. In addition, they were replicated to medium containing 1% D-glucose and 0.5% starch. A *creD34* strain does not produce any clearing after iodine treatment due to increased sensitivity to glucose repression whereas the *creAΔ4* strain produces a large halo around each colony. The *creAΔ4* morphology progeny did not fall into two defined classes on either medium, as would be expected if the phenotype of the double mutant was intermediate between the two parental phenotypes. Thus, several progeny were chosen and outcrossed to

determine whether both *creD34* and *creAΔ4* were segregating, and in this way a *creAΔ4;creD34* double mutant was identified.

3.6.2 Generation of a *creAΔ4; creB15* double mutant strain

A strain containing *creB15* was crossed to a strain containing *creAΔ4* and master plates of progeny with the very compact *creAΔ4* morphology were inoculated onto minimal medium containing 50mM D-quinate as the carbon source, and onto minimal medium containing 1% D-glucose and 0.5% starch. Strains bearing the *creB15* allele grew weakly on D-quinate and in double mutants between *creB15* and *creA204*, the same phenotype on D-quinate was observed (Hynes and Kelly, 1977). Weaker growth on D-quinate was looked for as a sign of the presence of *creB15*. Derepression on starch in the presence of D-glucose is a phenotype of both alleles and although the *creB15;creA204* double mutant has not been tested on this medium, (Hynes and Kelly, 1977), it was possible that in combination with *creAΔ4* greater derepression may be seen for some colonies compared with the parent *creAΔ4* strain, thus indicating the presence of *creB15*. Double mutant strains were not reliably identifiable so once again, colonies having a *creAΔ4* morphology were outcrossed. A *creB15;creAΔ4* double mutant was recovered in this way.

3.6.3 Growth testing to assess the phenotypes of *creAΔ4;creB15*, and *creAΔ4;creD34*.

The double mutant strains were assessed for growth on various media to determine the effect of the *creB15* and *creD34* mutations in a *creA*-null background. The media allowed the assessment of loss of carbon catabolite repression, increased carbon catabolite repression, resistance to various toxic substances and growth on various carbon sources.

3.6.3.1 Complete medium and minimal medium

The appearance of the strains on complete medium highlights the severely reduced growth and sporulation of CreA-null strains compared with $CreA^+$ strains (Figure 3.10A). The *creAΔ4;creD34* strain forms a larger colony than *creAΔ4* although the amount of sporulation is the same, while the *creAΔ4;creB15* strain is smaller and barely sporulating. This result supports the suggestion that the extreme morphology on complete medium of strains bearing *creA* alleles, in particular the CreA-null strain, may result from the partial or complete derepression of all the systems normally repressed by CreA. When the CreA null allele is in a strain with the *creD34* allele, which causes tighter carbon catabolite repression, the strain shows better growth. When the CreA null allele is in a strain with *creB15* which shows reduced carbon catabolite repression, the strain grows more poorly.

On minimal medium, the differences in growth between $CreA^+$ and $CreA^-$ strains is much less pronounced (Figure 3.10B). Strains containing the *creB15* and *creC27* alleles are quite weak on this medium with the *creB15*-containing strain showing weaker growth. *creD34* grows as well the wild type strain. The *creAΔ4* strain and the *creAΔ4;creD34* double mutant strains grow equally well but the *creAΔ4;creB15* double mutant strain shows very weak growth.

3.6.3.2 Allyl alcohol

The strains were inoculated onto medium containing 1% D-glucose and 0.1mM or 0.5mM allyl alcohol. Strains containing *creAΔ4, creB15* or *creC27* are sensitive to these concentrations of allyl alcohol, but a strain containing *creD34* is not. Both of the double mutant strains were sensitive on these media indicating that, as with a *creD34;creA204* double mutant strain, the *creD34* allele did not suppress the sensitivity of the *creAΔ4* allele in a double mutant (Figure 3.10, C and D).

Figure 3.10 *creAΔ4; creB15* and *creAΔ4; creD34* double mutant strains on complete medium, minimal medium and 1% D-glucose plus allyl alcohol.

A: Complete medium (incubated at 37°C for 72 hours).

B: Minimal medium (incubated at 37°C for 48 hours) plus 10mM ammonium tartrate and appropriate supplements.

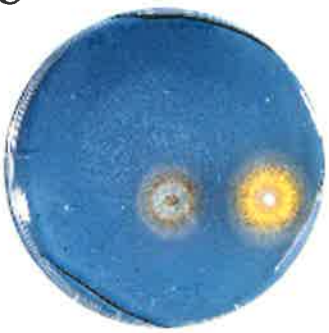
C: 1% D-glucose plus 0.1mM allyl alcohol (incubated at 37°C for 48 hours) plus 10mM ammonium tartrate and appropriate supplements.

D: 1% D-glucose plus 0.5mM allyl alcohol (incubated at 37°C for 48 hours) plus 10mM ammonium tartrate and appropriate supplements.

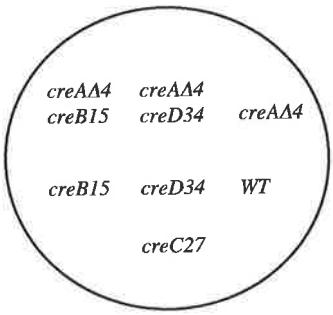
A

B

C



D


creAΔ4
creB15
creAΔ4
creD34
creAΔ4
creB15
creD34
WT
creC27

3.6.3.3 Molybdate and acriflavine resistance.

Strains containing the alleles *creB15* or *creC27* are resistant to molybdate when compared with wild type strains and with *creD34* strains (Figure 3.11, A), whereas acriflavine resistance is a phenotype of strains containing the *creD34* allele (Figure 3.11, B). The *creAΔ4* strain is sensitive to both of these substances and the double mutant strains were also sensitive.

3.6.3.4 D-quinate and lactose.

Strains containing the alleles *creB15* or *creC27*, grow poorly on several carbon sources including quinate, lactose, arabinose and proline (Hynes and Kelly, 1977). The double mutant strains were tested on medium containing 50mM D-quinate and 0.5% lactose and the *creB15;creAΔ4* strain barely grew on either medium although both the *creAΔ4* strain and the *creAΔ4;creD34* double mutant strain grew well (Figure 3.11, C and D).

3.6.3.5 Starch

The expression of α-amylases was examined on medium containing 1% D-glucose and 0.5% starch, and on 0.5% starch alone. On starch medium, the *creD34* strain expressed lower levels of α-amylases when compared to the wild type strain or the derepressed alleles, *creB15, creC27* and *creAΔ4* (Figure 3.12B). Strains containing the *creAΔ4* allele produced the largest areas of clearing after treatment with iodine solution and the presence of the *creD34* allele did not appear to suppress α-amylase expression in the double mutant strain (Figure 3.12, B and D). In fact this strain grew better, and produced a larger area of clearing than the strain bearing *creAΔ4* alone. The *creAΔ4;creB15* double mutant strain did not grow on starch or starch plus glucose medium, although a faint circle of clearing can be seen at the inoculation point for this strain on starch and glucose medium (Figure 3.12D).

Figure 3.11 *creAΔ4; creB15* and *creAΔ4; creD34* double mutant strains on medium containing acriflavine, molybdate, quinate and lactose.

A: Minimal medium plus 33mM molybdate plus 10mM ammonium tartrate and appropriate supplements.

B: Complete medium plus 15µl of a 1% acriflavine solution per 20ml plate.

C: 50mM D-quinate plus 10mM ammonium tartrate and appropriate supplements.

D: 0.5% L-lactose plus 10mM ammonium tartrate and appropriate supplements.

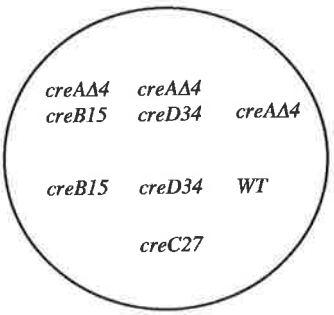
The plates were incubated at 37°C for 48 hours.

A

B

C

D


creAΔ4
creB15
creAΔ4
creD34
creAΔ4
creB15
creD34
WT
creC27

3.6.3.6 A summary of the phenotype of the double mutant strain *creAΔ4;creD34.*

Phenotypic analyses of a strain which contained both the *creD34* mutation and the *creAΔ4* allele, indicated that the presence of *creD34* allowed the strain to grow better on complete medium. However, it did not confer the resistance to acriflavine which has been shown by plate testing for a double mutant strain containing the *creD34;creA204* alleles (Silson, pers. com.) or the suppression of *alcA* expression as shown by enzyme assay in a double mutant strain containing the *creD34;creA204* alleles (Kelly and Hynes, 1977). Two factors may explain this difference. CreA null strains show very high levels of derepression of enzymes usually subject to carbon catabolite repression whereas *creA204* strains are predicted to produce a protein which still has some DNA binding ability and they are therefore less extreme in their derepression. In addition, CreA null strains have a more severe colony morphology than strains containing *creA204* which may also contribute to their increased sensitivity to toxic substances like acriflavine and allyl alcohol.

On glucose plus starch medium, the *creD34*; *creAΔ4* strain formed denser, sporulating colonies (compared to the strain containing only the *creAΔ4* allele which was barely sporulating) and appeared to have higher expression of α-amylases on this medium and on starch alone. The *creD34* mutation partially suppresses the colony size and sporulation defects of the *creAΔ4* mutation but did not significantly suppress the derepressed phenotype of the systems tested. Strains bearing the *creAΔ4* alleles have such an extreme phenotype that any influence of the *creD34* mutation in the same strain may be very subtle and unable to be detected by plate testing.

The analysis of *creD34* in a *creA* null background indicates that the *creD34* mutation cannot suppress the null phenotype whereas it can suppress the phenotype conferred by a partially functional *creA* allele, *creA204*. Therefore,

Figure 3.12 The expression of α-amylases in *creAΔ4; creB15* and *creAΔ4; creD34* double mutant strains.

A: 0.5% starch.

B: 0.5% starch stained with iodine solution.

C: 1% D-glucose plus 0.5% starch.

D: 1% D-glucose plus 0.5% starch stained with iodine solution.

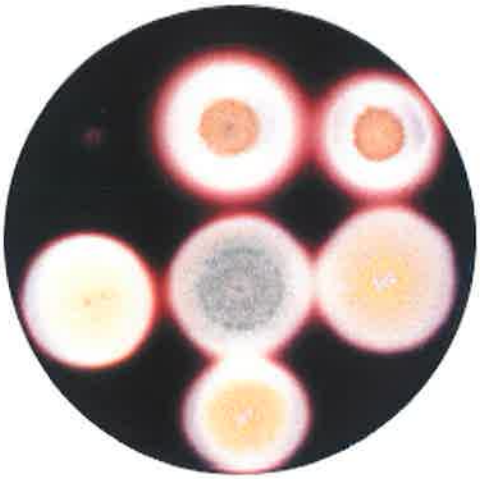
The nitrogen source is 10mM ammonium tartrate and the plates were incubated at 37°C for 48 hours.

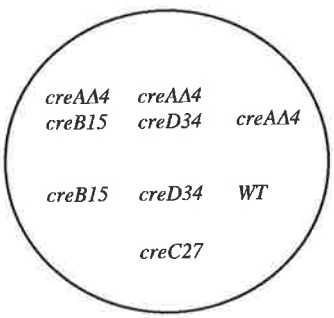
A

B

C

D




creAΔ4
creB15
creAΔ4
creD34
creAΔ4
creB15
creD34
WT
creC27

creD34 may need to interact with CreA, or a component of the CreA repression pathway, to effect its suppression.

3.6.3.7 A summary of the phenotype of the double mutant strain *creAΔ4;creB15*

The strain, *creB15;creAΔ4* was barely able to grow on media other than complete medium, and thus the effect of both mutations together appeared to be detrimental. To determine whether the major components of complete medium, casein hydrolysate and yeast extract, were allowing growth, minimal media was supplemented with each compound. On medium containing either casein hydrolysate or yeast extract, a stronger and more dense colony was produced and since both supplements allowed growth, this suggested that the double mutant strain may lack the ability to synthesise some amino acids (Figure 3.13, A and B). Perhaps the derepression of all the systems regulated by carbon catabolite repression is so extensive in this double mutant, due to the combination of the *creA*-null allele with the derepressed allele *creB15*, that energy levels are depleted to the extent that amino acid synthesis is inhibited or defective. The procedure outlined in Section 3.6.2 which led to the isolation of a *creB15*; *creAΔ4* double mutant was also used in an attempt to isolate a double mutant strain containing the *creC27* and *creAΔ4* alleles. From two independant crosses, a total of twenty *creAΔ4* phenotype progeny were outcrossed, but *creC27* did not segregate. The failure to isolate this double mutant suggested that it may not be viable. Although *creB15* and *creC27* produce similar phenotypes, the slight differences between them may be great enough that a double mutant strain bearing *creB15* and *creAΔ4* is viable while one bearing *creC27*and *creAΔ4* is not.

A suppressor of the morphology conferred by the *creA306* mutation on complete medium has been isolated which allows intermediate growth rather than very

Figure 3.13 The *creB15; creAΔ4* double mutant strain on supplemented minimal media

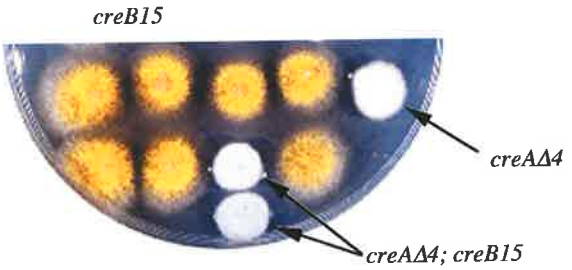
A: Minimal media supplemented with casein hydrolysate (0.15%w/v).

B: Minimal medium supplemented with yeast extract (0.15% w/v).

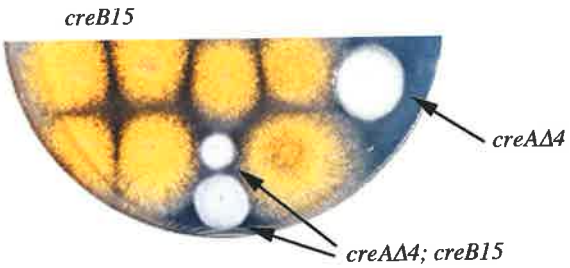
The nitrogen source is 10mM ammonium tartrate.

The plates were incubated at 37°C for 48 hours.

A



B



compact growth. Genetic analysis has shown that this suppressor is allelic to *creB* (Shroff, 1997) which suggests that CreA and CreB may be functionally related.

There are two possible explanations for the growth differences seen between a strain bearing the *creA306* and *creB* alleles compared with a strain bearing the *creAΔ4* and *creB15* alleles. Strains bearing the *creA306* allele are predicted to produce full length CreA which cannot bind DNA. Perhaps some interaction between the *creA306* protein and the mutant *creB* protein reduces the extensive derepression of systems which has been proposed to cause the poor growth seen by a strain carrying the *creB15* and *creAΔ4* alleles. Alternatively the *creB* allele which suppresses *creA306* may not carry the same mutation as *creB15*, and may also suppress *creAΔ4* in a double mutant strain.

3.7 Discussion

The work presented in this chapter shows that a CreA-null strain is viable in contrast to earlier results with *creAΔ*::pANC8. Comparisons between the CreA-null strain and *creA303* and *creA304* show that these strains have very similar morphologies on 1% complete medium and other media, and similar levels of derepression for the systems analysed. A strain has been developed (*creAΔ99*) with a similar mutation at the creA locus to *creA303*. The phenotype of *creAΔ99* confirms that the extreme but viable phenotype of *creA303* is not caused by an extragenic suppressor of the leaky lethal phenotype formerly associated with a CreA-null strain. To explain the leaky lethal phenotype of *creA*::pANC8, the 3' region adjacent to *creA* was explored.

3.8 Investigation of the region 3' of *creA*

As described in the introduction to this chapter the original CreA null strain, CreA::pANC8 had a leaky lethal phenotype (Dowzer and Kelly, 1991) but the CreA-null strains described in this chapter are viable. The significant difference

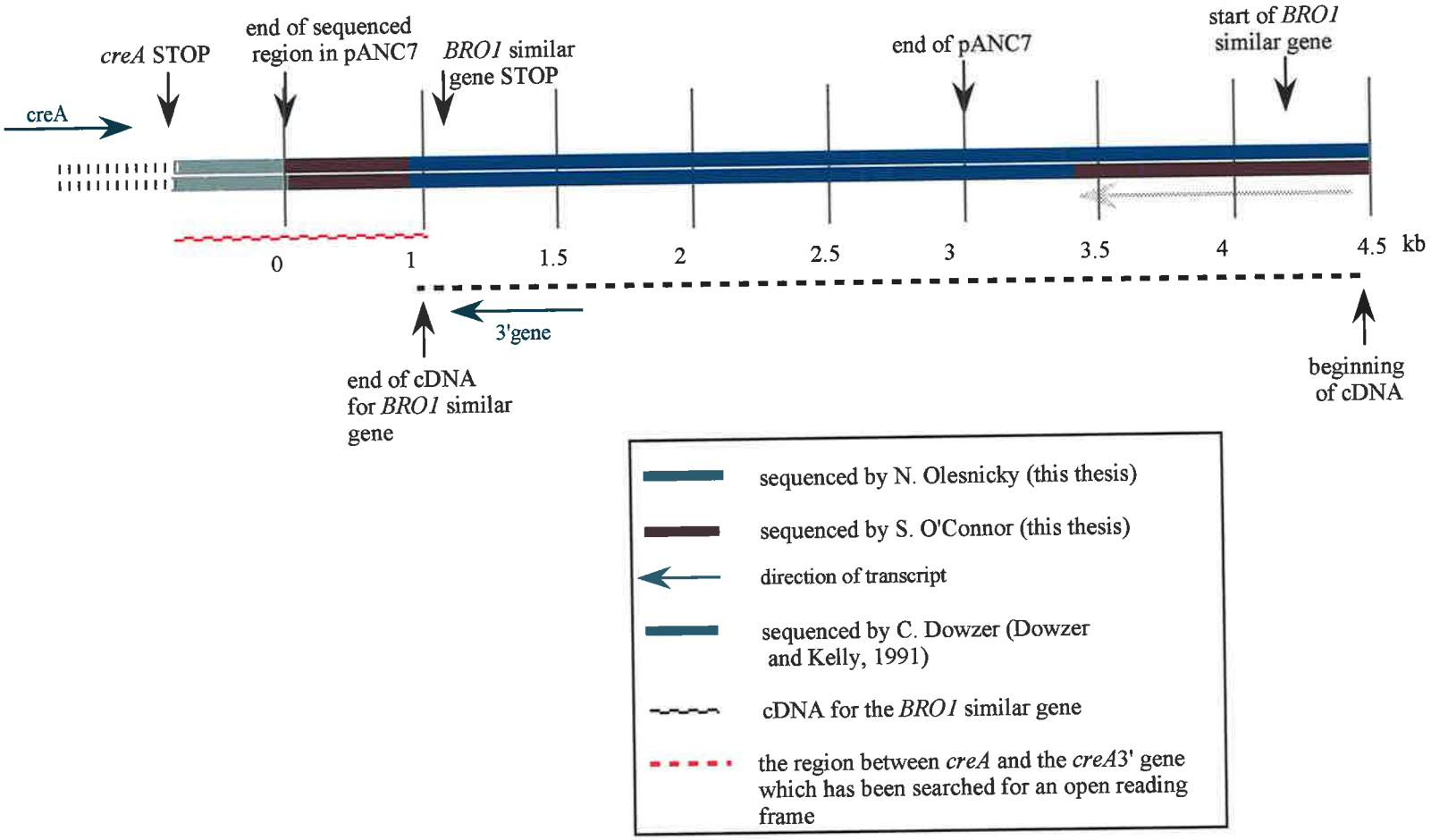
between the two constructs is at the 3' end. In the construct pANC8, 983 base pairs of sequence 3' of the *creA* stop codon have been deleted, which are present in the pANCΔ7/37 constructs. To investigate the possibility that a gene adjacent to the 3' end of *creA* may have been disrupted in the strain transformed with CreA::pANC8, and produced a leaky lethal phenotype, the region 3' of the *creA* stop codon was sequenced using the genomic clone pANC7 which contains 2.7kb of DNA 3' of *creA* (Figures 3.14 and 3.15).

3.8.1 The sequence of the region 3' of *creA*.

Sequencing of the 2.7kb at the 3' end of pANC7 showed that this region contains a large open reading frame running in the opposite direction to *creA*. However, the putative coding region stops 283 bp before the 3' end of the region disrupted by pANC8. To obtain the sequence of the whole gene, and to identify the 3' end of the transcript, a cDNA clone was isolated from a library made with mRNA extracted from mycelium grown in 1% glucose (N. Olesnicky). The cDNA was 3.5 kb in length and provided an extra 1.5 kb of 5' sequence, but lacked a poly-A tail. The 3' end of the cDNA finished 207bp before the end of the region deleted in pANC8 (Figure 3.14). For disruption of this gene to be caused by the deletion in pANC8, the 3' untranslated end must be longer than 300bp.

Conceptual translation of the sequence revealed a gene encoding 884 amino acids (Figure 3.15). Data base searches were performed using the BLASTP programme (Altschul *et al.* 1990), which produced high scoring segment pairs with several proteins, including *AIP1* from *Mus musculus* (Vito *et al.* 1999), a hypothetical 86.6kD protein from *S. pombe*, *BRO1* from *S. cerevisiae* (Nickas and Yaffe, 1997), *palA* from *A. nidulans* (Negrete-Urtasun *et al.* 1997), a putative protein tyrosine phosphatase from *Rattus norvegicus* (Cao *et al.* 1998) and *YNK1* from *C. elegans* (Che *et al.* 1997). Each of these proteins was submitted to the BLASTP programme which identified the others in the 6 highest scoring proteins (Table 3.1). Alignments

Figure 3.14 The sequencing strategy for the gene adjacent to the 3' end of *creA*


creA
creA STOP
end of sequenced region in pANC7
BRO1 similar gene STOP
end of pANC7
start of BRO1 similar gene
0
1
1.5
2
2.5
3
3.5
4
4.5 kb
3'gene
end of cDNA for BRO1 similar gene
beginning of cDNA
sequenced by N. Olesnicky (this thesis)
sequenced by S. O'Connor (this thesis)
direction of transcript
sequenced by C. Dowzer (Dowzer and Kelly, 1991)
cDNA for the BRO1 similar gene
the region between creA and the creA3' gene which has been searched for an open reading frame

Sequences submitted	Sequences producing High Scoring Segment Pairs	Smallest Sum Probability
putative protein produced by the gene at the 3' end of *creA*	*AIP1* (*Mus musculus*)	8.7e-44
	Hypothetical 86.6kD protein (*S. pombe*)	1.3e-41
	BRO1 (*S. cerevisiae*)	1.2e-39
	palA (*A. nidulans*)	2.6e-31
	Protein Tyrosine kinase TD14 *(R. norvegicus)*	4.9e-31
	Hypothetical 98.3kD protein (*C. elegans*)	1.0e-22
AIP1	Hypothetical 98.3kD protein (*C. elegans*)	4.3e-216
	palA (*A. nidulans*)	6.5e-58
	Protein Tyrosine kinase TD14 *(R. norvegicus)*	2.8e-40
	Hypothetical 86.6kD protein (*S. pombe*)	5.8e-21
	BRO1 (*S. cerevisiae*)	3.2e-19
BRO1	Hypothetical 86.6kD protein (*S. pombe*)	1.4e-31
	AIP1 (*Mus musculus*)	3.8e-19
	Hypothetical 98.3kD protein (*C. elegans*)	2.9e-15
	Protein Tyrosine kinase TD14 *(R. norvegicus)*	3.8e-11
	palA (*A. nidulans*)	3.9e-9

Table 3.1: Proteins with similarity to the putative protein produced by the gene at the 3' end of *creA*.
The putative protein sequence of the 3' gene was submitted to the BLAST-P programme in WEBangis. *AIP1* and Bro1p were also submitted to the BLAST-P programme and the five highest scoring proteins for each are shown. (Statistical significance is estimated under the assumption that the equivalent of one complete reading frame of the data base codes for protein and that significant alignments will involve only coding reading frames).

between the proteins showed conservation over most of the protein sequences rather than being confined to a particular motif. Bestfit pairwise alignments were undertaken for the putative protein produced by the gene at the 3' end of *creA* and the three proteins which produced the highest scoring segment pairs (Devereaux *et al.* 1984). Over 845 residues, there was 45.65% similarity and 26.47% identity between the putative protein produced by the gene at the 3' end of *creA* (884aa) and *AIP1* (869aa); over 667 residues there was 47.97% similarity and 26.47% identity between the putative protein produced by the gene at the 3' end of *creA* and an 86.6kD hypothetical protein from *S. pombe* (667aa); and over 780 residues there

was 44.9% similarity and 23.78% identity between the putative protein produced by the gene at the 3' end of *creA* and Bro1p (844aa). The pathways in which these proteins are involved are known for the *AIP1* protein which operates in the apoptosis pathway by associating with the protein produced by *ALG2* (apoptosis linked gene) in a calcium dependent manner (Vito *et al.* 1996); for Bro1p which interacts with components of the Pkc1p-MAP kinase pathway (Nickas and Yaffe, 1997), for the tyrosine phosphatase TD14 which suppresses Ha-ras-mediated transformation, and for *palA* which is one of six genes in a signal transduction pathway that regulates gene expression in a pH dependent manner (Negrete-Urtesun *et al.* 1997). All are involved in regulatory systems which suggests that a family of genes has been identified. Like Bro1p, they may also form part of a MAP-kinase signal transduction pathway. The open reading frame at the 3' end of *creA* will be referred to as the *BRO1*-similar gene for the rest of the chapter.

3.8.2 Expressed Sequence Tags (EST's) from the region 3' of *creA*.

In order to obtain the complete 3' sequence for the *BRO1*-similar gene, the *A. nidulans* EST data base (Altschul *et al.* 1990) was searched (April, 1998 and February, 1999) using the entire sequence spanning *creA* and the *BRO1*-similar gene. Two overlapping EST's, v4eO3a1.fl and g5cO1a1.fl, were present in the database representing a gene running in the opposite direction to *creA*, and one of them (v4e03a1) extended beyond the *creA* stop codon (Figure 3.16). The sequences in this data base have been directionally cloned so that the orientation of genes can be determined. However, if this cDNA is continuous with the *BRO1*-similar gene, then the 3' untranslated region is 1300bp which is excessively long for an *A. nidulans* gene. *creA* is thought to have a long 3' untranslated region of 428bp (Dowzer and Kelly, 1991) as is *areA* with a 3' untranslated region of 539bp (Kudla *et al.* 1990). In addition, the two EST's began at a position only 14 bases from the end of the cDNA clone for *creA*. These two facts raised the question as to whether the EST's may not be in the correct orientation and had actually come from *creA*. To determine whether

Figure 3.15 The sequence of the gene in the region 3' of *creA*

The region from 1-1500 was sequenced by N. Olesnicky in one direction. The region between 1500-3500 was sequenced by N. Olesnicky in both directions. The sequence covered by the 3.5kb cDNA clone extends from 1-3500. The first 1500 bases of this cDNA provided new sequence while the region from 1500-4533 was also contained in pANC7. The intron spans 2031-2088. The 3' region sequence which was deleted in the leaky-lethal strain *creA*Δ::pANC8, extends from 3549. The 1.3kb region between the stop codon of the *creA* 3' gene and the stop codon of *creA* extends from 3233-4533.

```
   1 GAATTCCCTTTTCAGTCCTGCTTTCTTTCCCTTCTCAATCTTTGATTTCATTTCCAGTTT  60

  61 CATCTCCCACAACGTATAATCCATATTACTCTTCGTTCCAAACAACATCTCCCCGCTCTC  120

 121 CTTTTAGGAAGATGGGAGCTCCCCGCTCCACGACGGCAAGATGGTTCAGTCGCCTATGAT  180

 181 ATCATGTCCCCTCAAACAGACCAACGAAATCGACTGGATCCAACCTCTCAAGGATTATAT  240

 241 TCGGCAAAGTTATGGCGAAGACCCTGAGCGCTACAGTCAGGAGTGTGCTACACTCAACCG  300

 301 GTTGCGTCAGGATATGAGGGGTGCCGGCAAGGACAGCGCGACAGGACGAGACCTACTCTA  360

 361 CCGCTACTATGGGCAACTAGAGCTTCTGGATCTCAGGTTCCCTGTGGATGAGCAATCATA  420

 421 TAAAAATATCATTTACCTGGTATGATGCATTCACCCACACGCCGACCTCGCAGTATTCTC  480

 481 TCGCGTTCGAAAAGGCCTCGATTATCTTCAATATATCTGCGGTCCTTTCTTGTCATGCAG  540

 541 CGAACCAGAACCGCGCAGATGATATTGGCTTGAAGCTGCCTACCACAACTTCCAGGCATC  600
                         Y  W  L  E  A  A  Y  H  N  F  Q  A  S  -

 601 CGCCGGCATGTTCACCTACATCAACGAAAACTTCCTCCACGCCCCCTCGACTGATCTCAA  660
       A  G  M  F  T  Y  I  N  E  N  F  L  H  A  P  S  T  D  L  N  -

 661 CCGCGAAACTGTAAAGACTCTCATCAACATCACGCTTGCTCAAGGTCAGGAAGTTTTCCT  720
       R  E  T  V  K  T  L  I  N  I  T  L  A  Q  G  Q  E  V  F  L  -

 721 CGAGAAGCAGATCATGGACCACAAAAAGGCCGGGTTCCTGGCGAAACTCGCCAGCCAGGC  780
       E  K  Q  I  M  D  H  K  K  A  G  F  L  A  K  L  A  S  Q  A  -

 781 TTCATATCTATACGCACAGGCCATTGAAGGGACACAGGAACATGCTAAAGGCATATTCGA  840
       S  Y  L  Y  A  Q  A  I  E  G  T  Q  E  H  A  K  G  I  F  D  -

 841 CAAATCATGGGTTACGCTTCTGCAAGTGAAGTCGGCGCATATGGGCTCCGTGGCCTCGTA  900
       K  S  W  V  T  L  L  Q  V  K  S  A  H  M  G  S  V  A  S  Y  -

 901 TTATCAGGCTCTAGCCGACGGCGAATCAGGTTCGCACGGAGTAACTGTTGCTAGGCTCCA  960
       Y  Q  A  L  A  D  G  E  S  G  S  H  G  V  T  V  A  R  L  Q  -

 961 GCTAGCAGAGAAGCATTCAACGAGTGCGTTGAGTTGGGCTAAGTCGCTCCCTTCGTCAAT 1020
       L  A  E  K  H  S  T  S  A  L  S  W  A  K  S  L  P  S  S  I  -

1021 ATCACCGAACACAAACCTTACATCGGAGGCTGGGCCCAGTCTAGTAAATATCGTGAAGTT 1080
       S  P  N  T  N  L  T  S  E  A  G  P  S  L  V  N  I  V  K  F  -

1081 CCATCTCGCAAACGTGCAGTCACAGCTTGCTACCTTCGTCAAGGATAACGATTTTATTTA 1140
       H  L  A  N  V  Q  S  Q  L  A  T  F  V  K  D  N  D  F  I  Y  -

1141 TCATCAGCCTGTTCCGAGCGAGGCAGGACTGTCTGCGGTATCTAAGCTCCCTGCAGCCAA 1200
       H  Q  P  V  P  S  E  A  G  L  S  A  V  S  K  L  P  A  A  K  -

1201 GGCAATTCCAGTTAGCGAGTTGTATCAGGGCCAAGATATTCAACGGATTATCGGTCCAGA 1260
       A  I  P  V  S  E  L  Y  Q  G  Q  D  I  Q  R  I  I  G  P  D  -

1261 TATCTTCCAGAAACTCGTTCCCATGTCGGTTACGGAGACAGCAAGTCTTTATGATGAGGA 1320
       I  F  Q  K  L  V  P  M  S  V  T  E  T  A  S  L  Y  D  E  E  -

1321 AAAGGCGAAACTGATCCGGGCAGAGACAGAGAAGGTTGAAACCGCCGATGGCGAGATGGC 1380
       K  A  K  L  I  R  A  E  T  E  K  V  E  T  A  D  G  E  M  A  -

1381 AGCAAGCTTAGACTATTTCAAGCTTCCCGGTAGTTTGAACATTTTGAAAGGTGGAATGGA 1440
       A  S  L  D  Y  F  K  L  P  G  S  L  N  I  L  K  G  G  M  D  -

1441 TCAGGAAGTGATGGTCGACGAAGAATTCCAACGTTGGTGTCAGGAACTAGCAGGACATGA 1500
       Q  E  V  M  V  D  E  E  F  Q  R  W  C  Q  E  L  A  G  H  D  -

1501 TTCTTTTGCGAAGGCTTTCGATACCCTTCAAGATCGCAAGTCAGAAGTGCTAGCTACCCT 1560
       S  F  A  K  A  F  D  T  L  Q  D  R  K  S  E  V  L  A  T  L  -
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1561 TGACCAATGCGCCAAGCAGTTAGACTTGGAAGAAAGCGTTTGCGAGAAAATGCGCTCAAA 1620
     D  Q  C  A  K  Q  L  D  L  E  E  S  V  C  E  K  M  R  S  K  -

1621 ATATGGGGCTGATTGGAGCCAGCAGCCCAGTAGTCGATTGAACATGACTCTCCGCAATGA 1680
     Y  G  A  D  W  S  Q  Q  P  S  S  R  L  N  M  T  L  R  N  D  -

1681 TATTCGGACATACCGAGACACGGTGCATGAAGCCAGTGCTAGTGACGCTCAGCTCTCTGC 1740
     I  R  T  Y  R  D  T  V  H  E  A  S  A  S  D  A  Q  L  S  A  -

1741 AACTCTTCGGCAATACGAATCTGACTTTGATGAGATGCGATCGGCAGGAGAAACAGACGA 1800
     T  L  R  Q  Y  E  S  D  F  D  E  M  R  S  A  G  E  T  D  E  -

1801 GGCGGATGTGCTTTTCCAGCGAGCAATGATCAAGGCAGGCTCAAAGCAAGGCAAAACTAA 1860
     A  D  V  L  F  Q  R  A  M  I  K  A  G  S  K  Q  G  K  T  K  -

1816 AAATGGTGTGACGAGCCCTTATTCGGCTACGGAGGGAAGTCTACTAGACGATGTCTATGA 1920
     N  G  V  T  S  P  Y  S  A  T  E  G  S  L  L  D  D  V  Y  D  -

1921 TGATGGCGTTCCCTCTGTGGCAGAGCAGATTGCCAGGGTGGAATCCATTTTGAAGAAACT 1980
     D  G  V  P  S  V  A  E  Q  I  A  R  V  E  S  I  L  K  K  L  -

1981 AAACCTGGTTAAACGGGAGCGAACCCAGGTCTTGAAGGACTTAAAGGAAAAGGTGAATGC 2040
     N  L  V  K  R  E  R  T  Q  V  L  K  D  L  K  E  K

2041 CACCAGTGCATATAGATATTGTTTCAAAGCTCCTAACGGGCGTTCTAGGTGCGCAACGAT 2100
                                                    V  R  N  D  -

2101 GATATATCGAACGTCTTGATACTTAACAAGAAGTCGATTACGGGCCAGGAGAGTCAACTC 2160

     D  I  S  N  V  L  I  L  N  K  K  S  I  T  G  Q  E  S  Q  L  -

2161 TTCGAAGCGGAGTTGGAGAAATTCCACCCTCATCAAATGCGAATAGTTCAGGCCAATCAT 2220
     F  E  A  E  L  E  K  F  H  P  H  Q  M  R  I  V  Q  A  N  H  -

2221 AAGCAAACAGCGTTGATGAAGGAGCTCACGAAAACTTATGGAGATCTATTACAGGATAAG 2280
     K  Q  T  A  L  M  K  E  L  T  K  T  Y  G  D  L  L  Q  D  K  -

2281 CGAGTGAGAGCTGAACAATCCAAATATGAGTCGATAACCCGACAACGCAACTCCGTCATG 2340
     R  V  R  A  E  Q  S  K  Y  E  S  I  T  R  Q  R  N  S  V  M  -

2341 GCCAGATATAAGAAGATATACGACTCCTTCAACAACCTTGGCTCTGGAATCAAGCAGGCT 2400
     A  R  Y  K  K  I  Y  D  S  F  N  N  L  G  S  G  I  K  Q  A  -

2401 CAAACTTTCTACGCTGAGATGACTGAAACTGTTGACAGCCTCAAGAAGAACGTCGACACT 2460
     Q  T  F  Y  A  E  M  T  E  T  V  D  S  L  K  K  N  V  D  T  -

2461 TTCATCAACAATCGCAGATCTGAGGGTGCCCAACTTTTGGGGCAGATCGAACGTGAAAAG 2520
     F  I  N  N  R  R  S  E  G  A  Q  L  L  G  Q  I  E  R  E  K  -

2521 GCAGCCGGAACGTCCGACCACGAAGAGCGCGAGCGAGAAAAGTTGAGGCAGCTCATGGAG 2580
     A  A  G  T  S  D  H  E  E  R  E  R  E  K  L  R  Q  L  M  E  -

2581 CGTCTGTCCACAGAGCCCAAGCCTCCCTCCGTTCCCCCAGGCTCGTCAACTGCCGGCCCA 2640
     R  L  S  T  E  P  K  P  P  S  V  P  P  G  S  S  T  A  G  P  -

2641 GCCAAAGCCAAATCTCCGCCGCCACCTGTCAAAGCACCCGCCTACCCGACAAATATCGCT 2700
     A  K  A  K  S  P  P  P  P  V  K  A  P  A  Y  P  T  N  I  A  -

2701 CCTCCAAAAGCATCACCTCATTTCGCTCCGACCGTTCCACAACAGCACGGCACACCCGTC 2760
     P  P  K  A  S  P  H  F  A  P  T  V  P  Q  Q  H  G  T  P  V  -

2761 TCCCATTCCCCGGCGCCTTACAGCCAGTACGTGCCTCCAGGCGCTGGCGTCTCGTACCTC 2820
     S  H  S  P  A  P  Y  S  Q  Y  V  P  P  G  A  G  V  S  Y  L  -

2821 CCTTCTCAGTCCTTCCAGCAAGGTGCTGCTGCACCGCTTTCCGAGGGCTATAACCCGATG 2880
     P  S  Q  S  F  Q  Q  G  A  A  A  P  L  S  E  G  Y  N  P  M  -

2881 GCATACCCGATCCCCGCATTCATCCATGTCACCTCCCCCAAGTCAGCCGTTCTACTCCCC 2940
     A  Y  P  I  P  A  F  I  H  V  T  S  P  K  S  A  V  L  L  P  -
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2941 AACTCCTACCCCATTCTACACCAGCCCAACGCCCCCCGTTCCTTCGGGTCAATACATGCC 3000
     N  S  Y  P  I  L  H  Q  P  N  A  P  R  S  F  G  S  I  H  A  -

3001 CCAAGGCTATGTTCCCCCTCCGCCACCTCCCCGACCACAACAGCCCACCTACCCACCATC 3060
     P  R  L  C  S  P  S  A  T  S  P  T  T  T  A  H  L  P  T  I  -

3061 AACAGGGCCGTTCCCCTCGGGACCAGGCGGGTATGCTCAGAGCCGACCATACGGAAGCAG 3120
     N  R  A  V  P  L  G  T  R  R  V  C  S  E  P  T  I  R  K  Q  -

3121 CCAGCACCACAAGGCTCATTCACAGTCATCACCGCAAACTGGACCTTCCGTTTCCGCAAA 3180
     P  A  P  Q  G  S  F  T  V  I  T  A  N  W  T  F  R  F  R  K  -

3181 TTCTTCCGATCCTTGGGCCGGGCTGAATGCCTGGAAGTAGGTGGCGTTATCTTTTTTTTT 3240
     F  F  R  S  L  G  R  A  E  C  L  E  V  G  G  V  I  F  F  F  -

3241 TTTTTGCAGATATGGTTGTTTCATTTGACTTAAGTTATGAAGAGTTACGATAGATTTACG 3300
     F  L  Q  I  W  L  F  H  L  T  *

3301 TATGATTGTACCCAGTATTTCATTCTATGTGACATTGGCGTTCATTACTATCCGTGCATC 3360

3361 GCGGCTTTTGTCATTTACTGCGATGCTAAGCCGTTTTTGGACATTGCTCCCCTGTCCACC 3420

3421 GATTTCTCAACGTCCAGGATACCATATCTCCATCTCATCCATTGCCTGTTCTGTCAAAAG 3480

3481 TCCTGCCCCATACATCAAACATCAAAACGTCAAGTTCATCTATCCAATCATAGATGTGAC 3540

3541 TACCTACCTCTAGACTTCGAGTATAATCAGACCGGCATGAGACCTGGCTCCCAGCCGCCG 3600

3601 CCCAAGCTTCCAATGACCTAATCAGCACTCACACATCAACACTCGTCTGAAAGTACCCCA 3660

3661 AGCCTGATGACTTACACGATAAGCAAACATATATCCCAGGCTTCAGGTACTCACCAATCA 3720

3721 ATACGTACGGCTTTCCGCAATACAGAAATACACTGGCAACAATCTTCCCGATTGTGGTCC 3780

3781 GCATCTTCACTGGCTATGTATCTAGTAGGTGCTTATATTTATTGTATCAGTGCCCAGTAT 3840

3841 AGGCCTTCCTTTCGTGCCAGTAAATCATCCATGTAGGCCCTGTAGGGGATTTCTGCAGGA 3900

3901 ATTTCACTGAGAGGTTGCGAACTAAGATATTGAGCAAGTGTCTATAGACTACAGTATCTA 3960

3961 CACTCCAGATGAGATTACTAAGCCTTCTTATCAGTAAGATATAAGATAGATGTACTAACA 4020

4021 AGTTACTGACGGCAGTGGCTGTATAAGCTATGGATATGGAGGATGCTCTGTGGAGACTAT 4080

4081 GCGGGGTACTCCATAGTCGCTAGAAAAATGCAGTCCAGTTGTAGTCCTCATGAGGAGTAT 4140

4141 TCATTTAATACGGAAAGAGATAAATGGTACAGTGTTGGCATTCAAGGAAACTCGTTCTTT 4200

4201 TGTATTTGATTATGAAGTGTAACCTCAAGAAAATAGTAAACTGCTGAGGAGCTTGTCCGA 4260

4261 TAGGAGAGGATCTCGAAAGGAAAGAGATAAAAGCTTGGTGTAATAGGTGTCAAAGAAGAG 4320

4321 AAGTGAATAGAATGAAAACAAGACTTGTGTGTGAAGGCAAGGGATAATCCGTCTATAGGG 4380

4381 GCAACGCCTCGCAAAATAGTGTACTCAAACAGTGTGAAGAATCGTCAAACAACCTAATTC 4440

4441 CGTGACACCAATGATGCCCATGAATCAACCAGAAATGCAAAGGTCTATATCTTTCGTACG 4500

4501 CCTAACAAGAAAACGAAGTTTTTTGGCCGGATTA                           4533

                                *
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a mRNA spanned this region, an attempt was made to amplify between the end of g5c01a1 and the 3' end of the cDNA from the *BRO1*-similar gene, using an RT-PCR approach. A product was obtained from genomic DNA, but not from three different RNA preparations which suggested that these cDNAs are not continuous with the *BRO1* similar gene. The EST's may possibly be the 3' end of *creA*.

3.8.3 Future Work

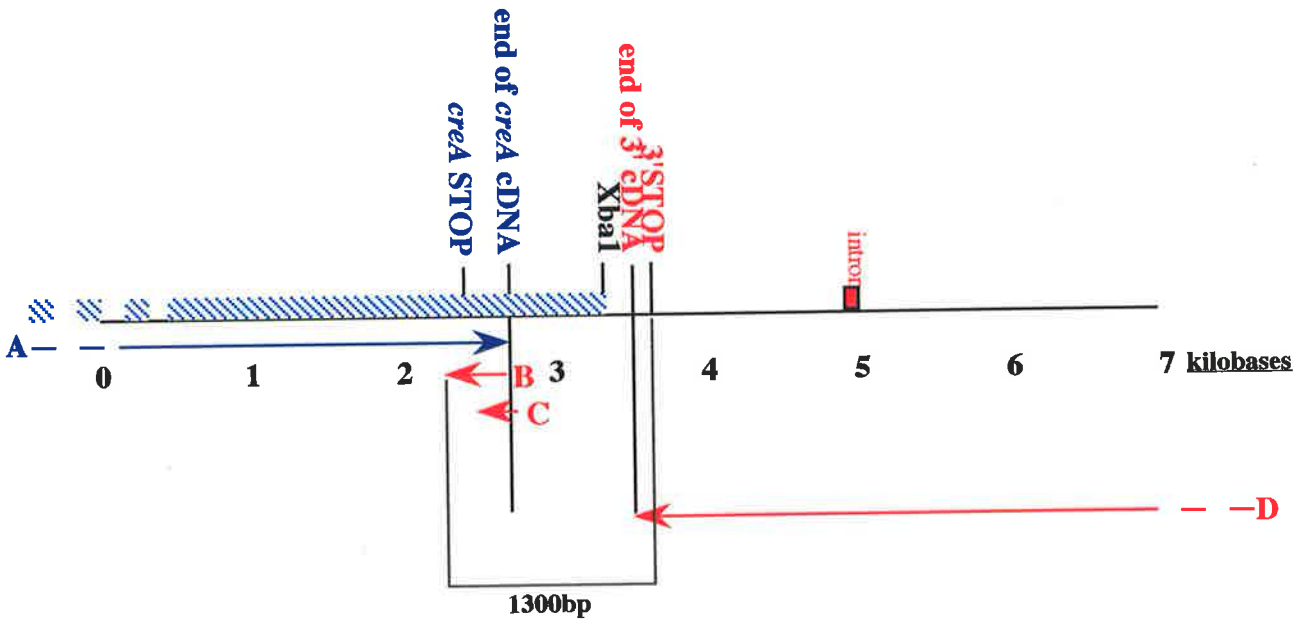
To understand the phenotype of the leaky lethal *creA*::pANC8 strain, it is necessary to determine whether the 3' end of the mRNA for the *BRO1* similar gene extends into the region deleted in pANC8, a distance of 283 base pairs. If so, the disruption of this gene may be lethal, or the disruption of this gene in combination with a disruption of *creA,* may cause lethality. It has been reported that a null mutation in *BRO1* is synthetically lethal with a null mutation in *PKC1, BCK1*, or *MPK1* (three genes which operate in the same MAP (mitogen activated) kinase pathway) (Nickas and Yaffe, 1996) which adds strength to the idea that the combination of disrupted genes may be lethal.

Two approaches are possible to address these issues. The first is the use of 3 prime RACE to determine the 3' end of the mRNA from the *BRO1* similar gene. The second approach would be to make a construct to disrupt the *BRO1* similar gene and transform this construct into both a wild type *creA* strain and a CreA null strain. This would allow an answer to the question of whether disruption of this gene alone causes lethality or if it only occurs in combination with a *creA* null.

Convergently orientated genes with overlapping coding regions have been discovered in *Candida albicans*. While the coding regions overlap by only 13bp, each of the 3' untranslated ends extend much further into the coding region of the opposite gene (Gerads and Ernst, 1998). The possibility that a small gene may lie in the 1300bp between *creA* and the *BRO1* similar gene has been addressed by looking for open

Figure 3.16 The region 3' of *creA*

This diagram shows the region covering the 3' end of *creA* and the 3' end of the *BRO1* homologue, which are adjacent in the *A. nidulans* genome. Two EST's which may be from the *BRO1* homologue are shown as well as the most 3' cDNA clones which have been isolated for both genes. The region 3' of *creA* which was deleted in pANC8, is shown as a blue hatched area.



A. cDNA for *creA*

B. v4e04a1

C. g5c01a1

D. cDNA for 3' gene

reading frames in this region but none have been identified, even considering the possibility of overlapping coding sequences (Figure 3.14, Figure 3.15).

3.9 Summary

The construction of a strain bearing a precise deletion of *creA* has shown that the *creA* protein is not essential for the viability of *A. nidulans*. Comparative analyses between the CreA null strain and strains containing extreme *creA* alleles (*creA303* and *creA304*), has indicated that they are also effectively CreA-nulls.

The phenotype of a CreA null strain has two components. It has a characteristic small and compact colony morphology on complete medium and only conidiates in the centre of the colony. The strain shows both derepressed and constitutive expression of enzymes which are usually repressed by glucose and appears to have elevated levels of enzymes in both induced and uninduced conditions compared to wild type strains and strains bearing other *creA* alleles.

Double mutant strains containing a *creA* null allele and either the *creD34* allele or the *creB15* allele, have provided more information about possible interactions between the products of these genes. The *creAΔ4;creD34* strain is very similar to the *creAΔ4* strain and the effect of the *creD34* mutation can not be detected in a *creAΔ4* background. The *creD34* allele does partially suppress the effects of the *creA204* allele however which suggests that some CreA is required for CreD34 to have an effect.

In a double mutant strain containing the *creB15* allele with the *creA204* allele, the poor growth on various carbon sources such as quinate and lactose, of a strain carrying the *creB15* allele, is also seen for the double mutant. Similarly, but to a greater extent, the double mutant strain *creAΔ4;creB15* is weaker than a CreA null strain and it has been proposed that the presence of both alleles in one strain has an

additive effect which results in a strain which can barely grow on any medium except enriched (complete) medium.

The sequence adjacent to the 3' end of *creA* has been determined in an attempt to explain the phenotype of the 'leaky lethal' null strain *creA*Δ::pANC8. A gene encoding a protein similar to Bro1p from *S. cerevisiae* has been identified in this region and it is possible that the 3' untranslated end of this gene was disrupted in the *creA*Δ::pANC8 strain thus producing the 'leaky lethal' phenotype. Disruption of Bro1p has a synthetic lethal effect when in combination with the disruption of one of several genes in the same pathway and therefore disruption of the gene at the 3' end of *creA* will need to be tested experimentally to see whether it has an effect on the viability of *A. nidulans.*

The CreA null strain has provided a useful molecular tool and deletion constructs have been transformed into the strain with the result that a region of CreA required for relief from carbon catabolite repression has been identified (Shroff, 1997). Protein extracts from the null strain have provided a negative control for western analysis of native CreA using the antibodies raised in rabbits. The strain has also been transformed with copies of an MRGS.His6-tagged *creA* in order to detect *in vivo* expressed CreA protein with antibodies to the tag. These experiments are described in Chapter 4.

Chapter 4 Detection of CreA by specific antibodies

The role of CreA and its mode of action has been predicted from comprehensive analyses of mutant alleles and *creA*-null strains. These studies have indicated that, in the presence of glucose and sucrose, CreA regulates carbon catabolite repression in *A. nidulans*, by binding to and causing repression of the expression of genes required for the utilisation of non-repressing carbon sources. All of the *creA* mutants analysed to date show derepressed expression of at least some genes in the presence of glucose, whether they have disruptions in the DNA binding domain or produce proteins with truncations in the regions beyond the zinc finger (Dowzer and Kelly, 1991; Shroff *et al.* 1996, 1997). The theoretical sizes of the mutant truncated CreA proteins have been predicted from DNA sequence but their stability *in vivo* is not known. In order to know whether the altered peptides are stable, a method of protein detection is required, such as would be afforded by specific polyclonal antibodies.

The regulation of CreA in response to the presence or absence of glucose in the cell is another area which could be investigated with antibodies. Levels of *creA* mRNA are higher when *A. nidulans* is grown in derepressing carbon sources such as arabinose, than when grown in glucose (Dowzer and Kelly, 1991). In a strain carrying the *creA204* allele, mRNA levels are high, whether it is grown in repressing or derepressing conditions, indicating that in this allele the transcription of *creA* is also derepressed implying that *creA* expression is auto regulated. It appears that there is more *creA* mRNA present in mycelia grown in derepressing conditions when it is not required for repression, than in glucose grown mycelia. It is not known whether this mRNA is translated into CreA which is then modified to prevent it from acting as a represser in conditions of low glucose, or whether translation of the mRNA is prevented in these conditions. CreA-specific antibodies would indicate whether pre- or post-translational mechanisms were operating in the cell.

There is some evidence from other organisms that phosphorylation plays a role in the regulation of glucose repression. *In vitro* experiments with *Trichoderma reesei*, have suggested that the CreA homologue Cre1, is phosphorylated in the presence of glucose and requires phosphorylation to be bound to DNA (Mach *et al.* 1995 - poster at the 18th Fungal Genetic Conference at Asilomar). Glucose repression via Mig1p in *S. cerevisiae* has also been shown to involve phosphorylation, but in this case, the protein is phosphorylated in the absence of glucose. Investigation of the role of phosphorylation in CreA regulation is another area where antibodies are required for analysis.

The location of Mig1p in the cell has been demonstrated to be regulated in response to glucose - when glucose is added to glycerol grown cells, cytoplasmic Mig1p is found in the nucleus (DeVit *et al.* 1997). These experiments have been done with GFP-tagged Mig1p. CreA has also been GFP-tagged at the carboxy-terminus but could not be detected when expressed in *A.nidulans* from the *creA* native promoter in this laboratory (R.Shroff, pers. com.). An alternative approach to studying the cellular location of CreA in response to glucose would be to use a fluorescent secondary antibody to a CreA-specific primary antibody.

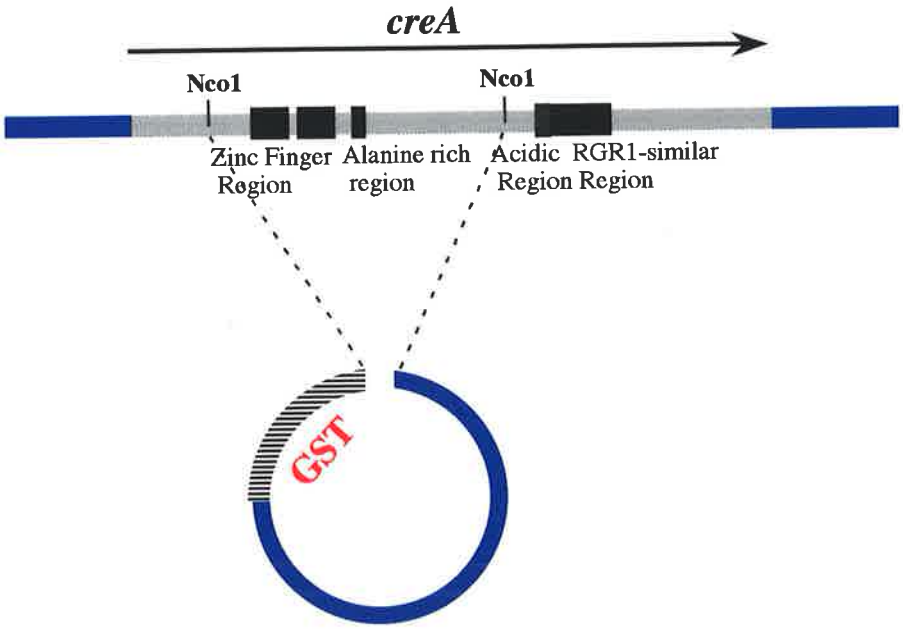
Rapid turnover is another method of regulating proteins like CreA. PEST sequences have been implicated in rapid protein degradation and the first 33 amino acids of CreA from *A. niger* have been assigned a significant PEST score of +9.89 by the PESTfind program (http://www.at.embnet.org/htbin/embnet/PESTfind; Rechsteiner and Rogers, 1996). Within CreA from *A. nidulans* however, no significant PEST sequences have been found. The absence of a strong PEST sequence within CreA does not rule out the possibility that it may still be regulated by turnover as many other peptide motifs have been identified as targets for proteolysis. It remains to be seen whether the activity of CreA in response to glucose is regulated like Mig1p, by

Figure 4.1: GST-fusion protein constructs

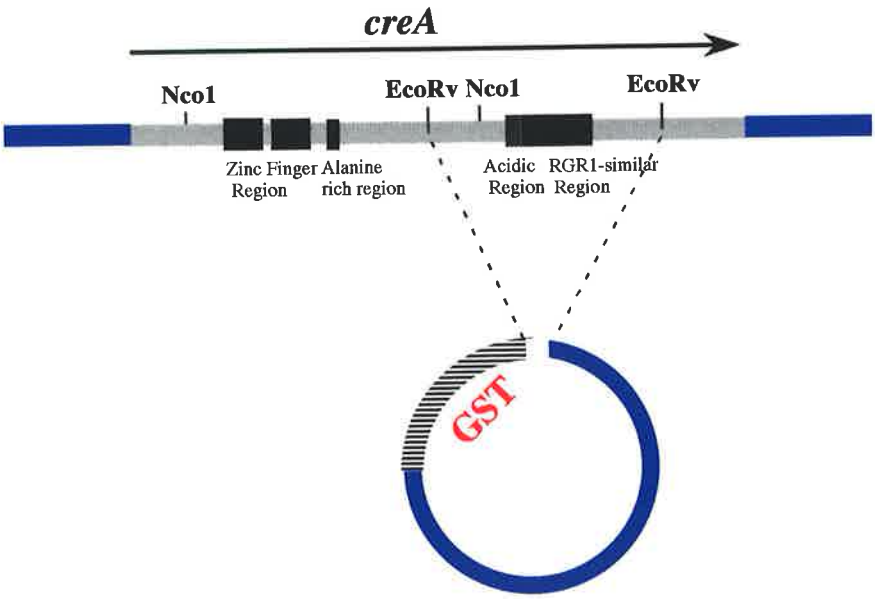
A: To produce the GST::Zf construct, a 617bp Nco1 fragment from *creA* was cloned into the Sma1 site of the expression vector pGEX-2T (Kulmberg *et al.* 1993). The vector pGEX-2T encodes the glutathione S transferase (GST) gene under the control of the *tac* promoter (Smith and Johnson, 1988).

B: The GST::Rgr construct was produced when a 480bp EcoRV fragment from *creA* was cloned into the end-filled EcoR1 site of the pGEX-2T vector (Chamalaun-Hussey, 1996).

A


creA
Nco1
Nco1
Zinc Finger Region
Alanine rich region
Acidic Region
RGR1-similar Region
GST

B


creA
Nco1
EcoRv
Nco1
EcoRv
Zinc Finger Region
Alanine rich region
Acidic Region
RGR1-similar Region
GST

regulation of its localisation which in itself has to have a regulatory mechanism such as phosphorylation or dephosphorylation, or by differential stability or translation.

CreA specific antibodies could also be used to discover whether the protein binds as a monomer or in a multimeric form, and they could be used to co-precipitate CreA and any proteins bound to it *in vivo* which may assist it to effect repression or derepression (as with Tup1p/ Ssn6p in *S. cerevisiae*).

4.1 Previous work

A previous attempt was made to raise antibodies in rabbits to two *E. coli* expressed GST::CreA fusion proteins (Chamalaun-Hussey, 1996). One protein was expressed from a construct which fused glutathione-S-transferase (GST) to a 617bp NcoI fragment from *creA* encoding the zinc finger region (Figure 4.1-A). This protein was predicted to be 52kDa. The other protein was expressed from a construct which fused GST to a 480bp EcoRV fragment encoding the 'Rgr1p-similar' region of *creA* (Figure 4.1, B). This protein was predicted to be 46kDa. The GST portion of each fusion protein is 26kDa and there is a common 34-amino acid portion of CreA between the two constructs (Figure 4.1-B) making it possible that antibodies raised to each fusion protein may recognise both antigens. The adjuvant used was aluminium hydroxide (Imject^{R} alum, Pierce Chemicals).

The antibodies raised against both fusion proteins were able to recognise the antigens they were raised to, and GST alone. When the GST::Rgr fusion protein was cleaved with thrombin to produce bands of 26 kDa (GST) and 20 kDa (Rgr) respectively, both bands were recognised by the GST::Rgr antibody which showed that the antibody was recognising the 'Rgr1p similar region' of CreA, and not only GST (Figure 4.2-A, Lane 6, bands 1 and 2). However, the GST::Zf antibody only detected the GST band implying that the overlap of 34 amino acids was not sufficient for recognition (Figure 4.2-B, Lane 6, band 1) (Chamalaun-Hussey, 1996).

Figure 4.2: Immunodetection of GST::Zf and GST::Rgr fusion proteins with antibodies raised against GST::Zf and GST::Rgr.

Protein extracts were electrophoresed on a 10% SDS-polyacrylamide gel and then transferred to Immobilon P membrane. Immunodetection was performed with:

A: 500µl of serum containing antibodies raised against the GST::Rgr fusion protein

B: 500µl of serum containing antibodies raised against the GST::Zf fusion protein

The protein extracts loaded in each track are:

Lane 1-GST only

Lane 2-column purified GST::Zf fusion protein

Lane 3-column purified GST::Zf fusion protein cleaved with thrombin

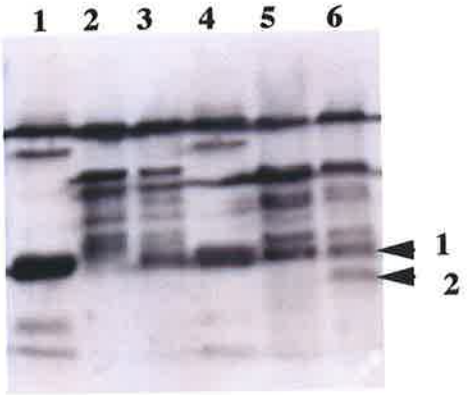
Lane 4-GST only

Lane 5-Column purified GST::Rgr fusion protein

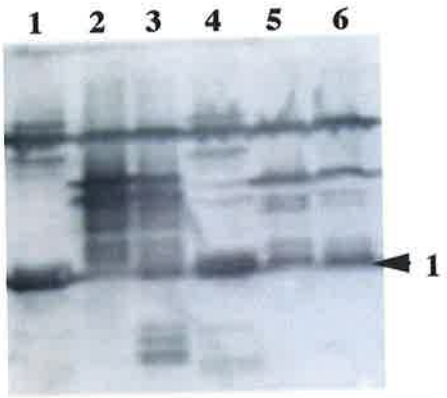
Lane 6-Column purified GST::Rgr fusion protein cleaved with thrombin.

This figure is reproduced from Chamalaun-Hussey (1997), where it appears as Figure 5.2, C and D.

A



B



Cleavage of the GST::Zf protein with thrombin produces two proteins of 26kDa which can not be distinguished on an SDS-PAGE gel (Figure 4.2-A and B, Lane 3). Western analysis of nuclear protein extracts and total protein extracts with these antibodies showed that both cross reacted with numerous proteins and neither were sufficiently specific for CreA to be used for further analysis (Chamalaun-Hussey, 1996). These sera are no longer available.

4.2 Antibody production in rabbits to two GST-CreA fusion proteins.

Antibodies to the same GST::CreA fusion proteins described above were raised again, with procedural modifications designed to improve both antibody production and detection. First, prior to inoculation, samples of blood were obtained from the two rabbits to ascertain whether antibodies to fungal proteins were present in the serum. Some reactivity was seen which suggested that the rabbits had already been exposed to fungal proteins or to GST (Figure 4.3-A and B, Lanes 3-6). The GST-only track gave the strongest response to the GST::Rgr fusion protein (Figure 4.3-A, Lane 6). Second, Freund's Complete Adjuvent (SIGMA) was used with the antigens in place of aluminium hydroxide, (Freund's Incomplete Adjuvent was used with subsequent inoculations) in order to induce a more aggressive immunological response in the hosts. Third, a CreA-null strain was available to provide a CreA null control and enable non-specific cross reactions to be clearly identified.

On three occasions, the rabbits were inoculated with an emulsion of equal volumes of protein and adjuvant, which contained approximately 100µg of fusion protein and ranged in volume from 80-120 µl (Table 4.1). Blood samples were obtained after each inoculation.

Day	Activity	Amount
1 (March 25)	-5ml serum collected from rabbits	
	-first inoculation	100mg of GST::Zf and GST::Rgr in an emulsion with Freund's complete adjuvent
28 (April 22)	-5ml serum collected from rabbits	
	-second inoculation	100mg of each fusion protein in an emulsion with Freund's incomplete adjuvant
82 (June 17)	-5ml serum collected from each rabbit	
	-third inoculation	400mg of each fusion protein in an emulsion with Freund's incomplete adjuvant
126 (July 31)	Final serum collection from rabbits - approximately 30 ml from each.	

Table 4.1 The inoculation schedule used to raise antibodies against the GST::Zf and GST:: Rgr fusion proteins in Rabbits.

Figure 4.3: Detection of fungal proteins with serum from rabbits, prior to their inoculation with fusion protein antigens.

Two 10% SDS-polyacrylamide gels were loaded with duplicate protein extracts, electrophoresed and transferred to Hybond-C extra (Amersham). The membranes were detected with

A: serum from the rabbit subsequently inoculated with the GST::Rgr fusion protein antigen.

B: serum from the rabbit subsequently inoculated with the GST::Zf fusion protein antigen.

Serum was used at a dilution of 1/100 and the Amersham Rabbit Ig, horseradish peroxidase-linked whole antibody (from donkey) was diluted 1/2000. Detection was performed using ECL™ (30 second exposure).

The proteins loaded in each lane are:

Lane 1-total protein extract from the CreA-null strain

Lane 2-total protein extract from a $CreA^{+}$ strain

Lane 3-GST::Rgr fusion protein

Lane 4-GST::Rgr fusion protein cleaved with thrombin

Lane 5-GST::Zf fusion protein

Lane 6-GST only

A

B

4.3 Western analysis and Immunodetection of CreA in *A. nidulans* protein extracts using antibodies raised to the fusion proteins; GST::Zf and GST::Rgr.

In order to detect CreA, the antibodies were tested against total protein extracts from the CreA-null strain, the $CreA^+$ strain, GST::Zf column purified fusion protein, GST::Rgr column purified fusion protein, cleaved GST::Rgr column purified fusion protein and GST alone. The total protein extracts from *A. nidulans* were prepared from mycelia grown overnight in liquid medium containing 1% D-glucose and ammonium tartrate. The mycelia was ground under liquid nitrogen and resuspended in 0.2M phosphate buffer at a ratio of 1ml of buffer per milligram of mycelia. The fusion proteins and GST alone gave strong, clean signals but no protein was detected in the track containing total protein extract from the $CreA^+$ strain. Serum was used at a dilution of 1:1000 (Figure 4.4).

As the fusion proteins were much more concentrated for CreA than the total protein extract, the total protein extracts were prepared in a variety of ways to maximise recovery of CreA so that it may be detected and analysed with the antibodies. Great care was taken to keep the activity of proteases to a minimum and thus prevent the degradation of CreA. This included maintaining the lowest temperature possible for all stages of protein preparation, and using protease inhibitors such as PMSF and Complete™ Protease Inhibitor Cocktail tablets (Boehringer). The buffer added to the ground mycelia was also varied to determine whether certain conditions favoured the recovery of CreA (0.2M Phosphate Buffer, 1x TGE buffer, 1/25x native protein gel buffer, 8M urea elution buffer, 2x loading buffer). Protein extracts were also made from mycelium grown in repressing (glucose) and derepressing conditions (arabinose/fructose) in order to compare CreA produced in the two conditions and also in the event that it may only be detected in one condition rather than another. When equal volumes of total protein extract were loaded onto an SDS-PAGE gel, the samples prepared from mycelia grown in derepressing conditions were consistently

Figure 4.4 Immunodetection with antibodies raised against GST::Zf and GST::Rgr fusion proteins.

Lane 1-molecular weight marker

Lane 2- CreA-null strain

Lane 3-CreA^{+} strain

Lane 4- *creA*$^{+}$ multi copy Transformant

Lane 5-GST::Zf fusion protein

Lane 6-GST::Zf fusion protein cleaved with thrombin

Lane 7-GST::Rgr fusion protein

Lane8-GST::Rgr fusion protein cleaved with thrombin

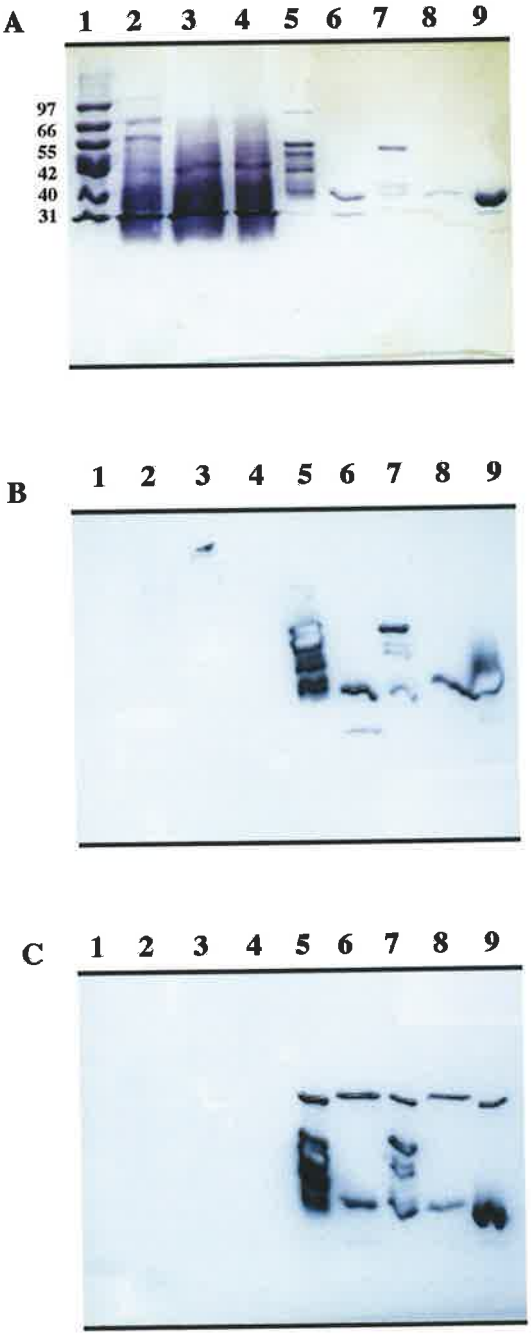
Lane 9- GST only

A: Coomassie Brilliant Blue R-250 stained 12% SDS-polyacrylamide gel

B: Immunodetection performed with anti GST::Zf (final bleed)

C: Immunodetection performed with anti GST::Rgr (final bleed)

The primary antibodies were diluted 1/100 and the secondary antibodies were diluted 1/2000 (Amersham Rabbit Ig, horseradish peroxidase-linked whole antibody (from donkey)) followed by ECL™ detection (exposure time-10 seconds).


A
1 2 3 4 5 6 7 8 9
97
66
55
42
40
31
B
1 2 3 4 5 6 7 8 9
C
1 2 3 4 5 6 7 8 9

more concentrated. An increase in protein concentration was also seen for the CreA-null strain (compared with $CreA^{+}$ strains) regardless of whether it was grown in repressing or derepressing the conditions (result not shown).

Tracks containing the cleaved GST::Rgr fusion protein were expected to produce two major bands (26kDa and 20kDa), representing the GST and RGR portions respectively, on a Coomassie blue stained gel, but only the 26kDa GST band was seen. As the second band may have run to the same position as the dye front on the gel, which was very close to the 20kDa position, a larger gel (10x14cm) was run to increase the possibility of visualising the two bands by allowing smaller proteins to be resolved before the dye front. However, the smaller band still could not be seen on the Coomassie Blue stained gel.

Samples of the GST::Rgr fusion protein were cleaved several times using different concentrations of thrombin and for periods varying from 30 minutes to overnight. In all cases, although cleavage was apparent, the 20kDa Rgr band was not seen on a Coomassie Blue stained gel and was not detected with the antibody raised to the GST::Rgr protein (Figure 4.5, A and B, Lane 6). No proteins were detected in the tracks containing total extracts from wild type CreA strains either, even when a double volume was loaded (Figure 4.5-B, Lanes 3 and 7).

The profile of the cleaved GST::Rgr fusion protein in these experiments is very different from that seen in Figure 4.2, and may be due to degradation of the CreA portion of the protein after digestion with thrombin. Thrombin digestion of the GST::Rgr fusion protein failed to produce a separate Rgr portion, and thus did not give a clear answer as to whether the antibodies recognise CreA.

The three post-inoculation sera from each rabbit showed increasingly strong positive reactions with the fusion-protein antigens and with GST alone (Figure 4.6, Lanes 3-6 for

Figure 4.5: Immunodetection with antibodies raised against the GST::Rgr fusion protein.

A: Coomassie Brilliant Blue R-250 stained 12% SDS-polyacrylamide gel

B: Western analysis- the primary antibody (GST::Rgr-final bleed) used at 1/100 and the secondary antibody used at 1/2000 (Amersham Rabbit Ig, horseradish peroxidase-linked whole antibody (from donkey)) followed by ECL™ detection (exposure time-10 seconds).

Lane1- molecular weight marker

Lane 2-total protein extract from CreA-null strain

Lane 3-total protein extract from $CreA^+$ strain

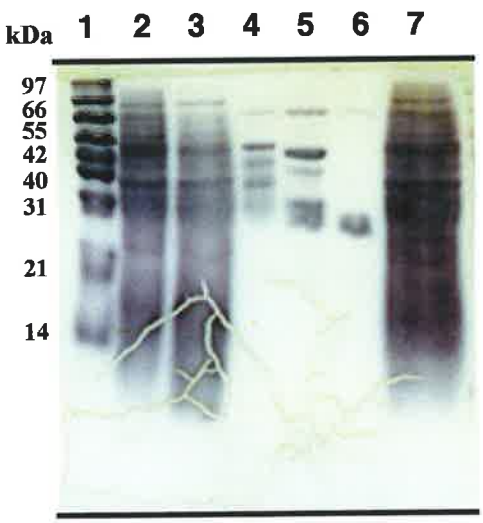
Lane 4--GST::Zf fusion protein

Lane 5-GST::Rgr fusion protein

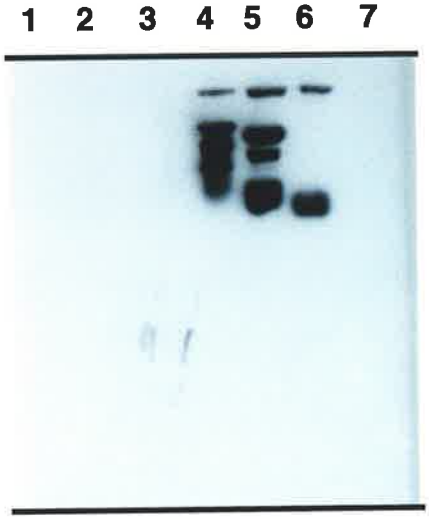
Lane 6-GST::Rgr fusion protein cleaved with thrombin

Lane 7-total protein extract from $CreA^+$ strain (double the volume loaded in Lane 3).

A



B



A-F) but failed to react with any proteins in the tracks containing total protein from the CreA$^+$ strain except when immunodetection was performed with the serum collected after the final inoculation with GST::Zf, at a dilution of 1:100. This serum produced faint signals in the tracks containing total protein from both the CreA null strain and the wild type CreA strain (Figure 4.6, F, Lanes 1 and 2). Further western analysis and immunodetection was performed to determine whether CreA may be detected with this antibody when used at the dilution of 1:100.

A large SDS-polyacrylamide gel (10x14cm) was run to enable more of the whole protein extract from CreA null and CreA$^+$ strains to be loaded. Immunodetection with the antibody raised to the GST::Zf protein, revealed two bands in the lane containing extracts from a CreA$^+$ strain. The signal from the lower band was quite strong. The size of the band was estimated to be approximately 60kDa when compared with GST::Zf at 52kDa and GST::Rgr at 46kDa. This band was larger than that predicted for CreA (52.149kDa, (PEPSTATS-WebANGIS)) but was considered a likely candidate because the actual size of a protein can vary from the predicted one, and because the signal was clean, and absent from the deletion strain (Figure 4.7, Lane 2, Band 1).

To clearly show that CreA was the protein being detected with the GST::ZF antibodies, this serum was used against total protein extracts from several strains. Two strains bore *creA* alleles which are predicted to produce truncated CreA proteins. A strain carrying the *creA303* allele is predicted to make only the first 69 amino acids of CreA due to a mutation in the first zinc finger. This protein would be approximately six times smaller than CreA$^+$. Antibodies raised to the GST::Zf fusion protein are expected to recognise CreA303, (if a stable protein is produced) as there are thirty-five amino acids common to both proteins. The strain bearing the *creA218* allele is also predicted to produce a truncated CreA protein as the result of a mutation just before the acidic region of *creA*. A stable protein produced by this strain would be approximately half the size of CreA$^+$

Figure 4.6: Immunodetection with serum produced after each of three inoculations with either GST::Rgr or GST::Zf fusion proteins.

Duplicate 10% SDS-polyacrylamide gels were loaded with:

Lane 1-total protein extract from CreA null strain

Lane 2 total protein extract from $CreA^{+}$ strain

Lane 3- GST::Rgr fusion protein

Lane 4-GST::Rgr fusion protein cleaved with thrombin

Lane 5- GST::Zf fusion protein

Lane 6-GST only

Immunodetection was performed with serum collected from rabbits

A: after the first inoculation with GST::Rgr

B: after the second inoculation with GST::Rgr

C: after the third inoculation with GST::Rgr

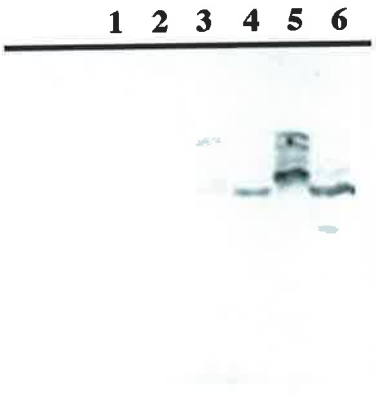
D: after the first inoculation with GST::Zf

E: after the second inoculation with GST::Zf

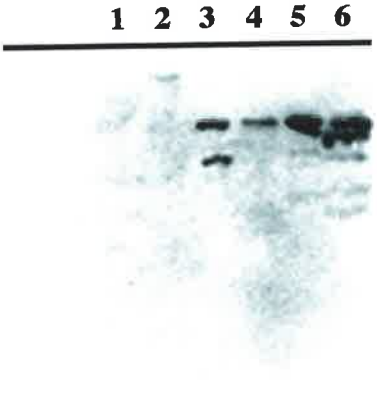
F: after the third inoculation with GST::Zf.

The 1° antibody was diluted 1/100 and the 2° antibody was diluted 1/2000 (Amersham Rabbit Ig, horseradish peroxidase-linked whole antibody (from donkey)) followed by ECL™ detection. Exposure times ranged from 1 second (C, E and F) to 15 seconds (A and D).

A

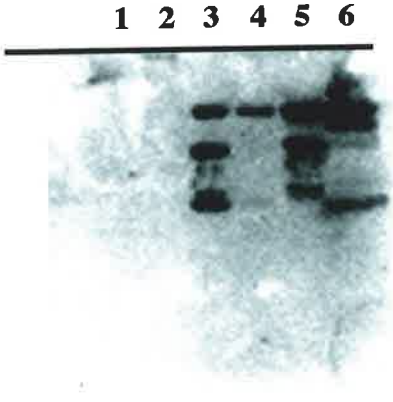


D

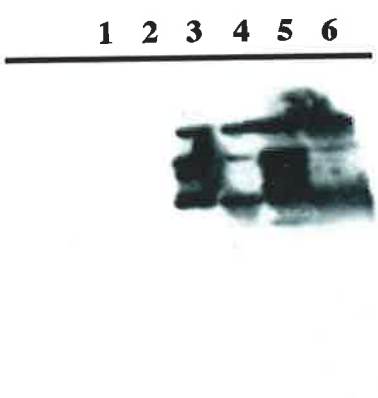


B

E



C



F

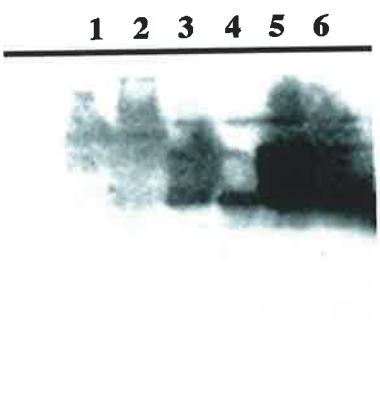


Figure 4.7: Immunodetection of protein extracts from *A. nidulans* with antibodies raised against the GST::Zf fusion protein.

Lane 1-CreA-null strain

Lane 2-CreA^{+} strain

Lane 3-GST::Rgr fusion protein

Lane 4-GST::Rgr fusion protein cleaved with thrombin

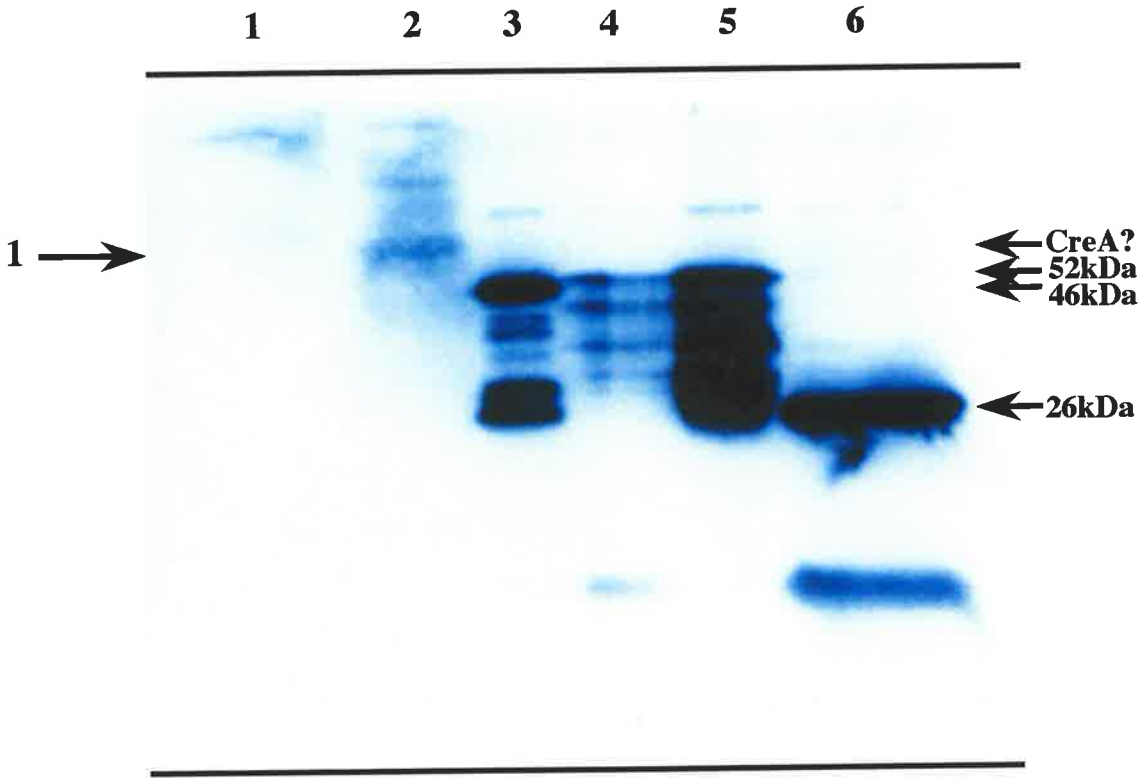
Lane 5- GST::Zf fusion protein

Lane 6- GST only

Primary antibody diluted 1/100 (final bleed)

Secondary antibody diluted 1/2000 (Amersham Rabbit Ig, horseradish peroxidase-linked whole antibody (from donkey))

Exposure time for ECL™ detection was 15 seconds


1
2
3
4
5
6
1
CreA?
52kDa
46kDa
26kDa

and it should also be recognised by the GST::Zf antibodies. Protein extracts were made from two strains, *creA204* (Dowzer and Kelly, 1991; Shroff *et al.* 1996) and TC43E (Dowzer, 1991), which show higher levels of mRNA on northern blots compared with wild type strains, in both repressing and derepressing conditions. The strain bearing the allele *creA204,* is predicted to produce full length CreA but has a mutation in the zinc finger region which affects DNA binding and causes greatly reduced carbon catabolite repression. TC43E is a multi copy *creA* transformant (approximately 15 copies). These strains were used in the event that higher levels of mRNA would result in greater amounts of CreA being produced, and thus lead to a strong signal on a western. Protein extracts from the CreA null strain, which does not produce any CreA, were included in the analysis, as were extracts from $CreA^{+}$ strains grown in glucose or arabinose.

The protein extracts were run on a 10% 10x14cm SDS-polyacrylamide gel and immunodetection was performed with the GST::Zf antibodies (Figure 4.8). On this western, the band which corresponds to Band 1 in Figure 4.7 and may represent CreA, is shown with a 1 and an arrow. As expected, this band is present in the lanes containing extracts from $CreA^{+}$ strains including TC43E, and is absent in lanes 2 and 8 which contain extracts from the CreA-null strain. The Coomassie Blue stained gel showed that less protein was loaded in lane 8 (result not shown). However, in lane 2, which contains protein from the CreA-null strain, some bands are recognised by the antibodies but they are at a different position from Band 1. Band 1 is absent in Lanes 9 and 10 which contain protein extracts from strains bearing the *creA303* and *creA218* alleles respectively. Whether the band is present in lane 11, which contains protein extracts from the *creA204* strain, is unclear. The band is not clear in Lane 12 which contains protein from the same strain and condition as Lane 3, indicating that if the band is CreA, then there are problems with reproducibility of detection. Another band is detected on this western, and has been labelled with an arrow and Band 2. It appears in Lane 2 which contains CreA-null strain proteins, and in several

Figure 4.8: Immunodetection of protein extracts from *A. nidulans* strains bearing a variety of *creA* alleles and grown in repressing or derepressing conditions.

Lane 1-molecular weight marker

Lane 2-CreA-null strain grown in glucose

Lane 3-CreA$^+$ strain grown in glucose

Lane 4-CreA$^+$ strain grown in glucose

Lane 5-multi-copy *creA*$^+$ transformant grown in glucose

Lane 6-CreA$^+$ strain grown in glucose

Lane 7-CreA$^+$ strain grown in arabinose

Lane 8-CreA-null strain grown in glucose

Lane 9-*creA303* strain grown in glucose

Lane 10-*creA218* strain grown in glucose

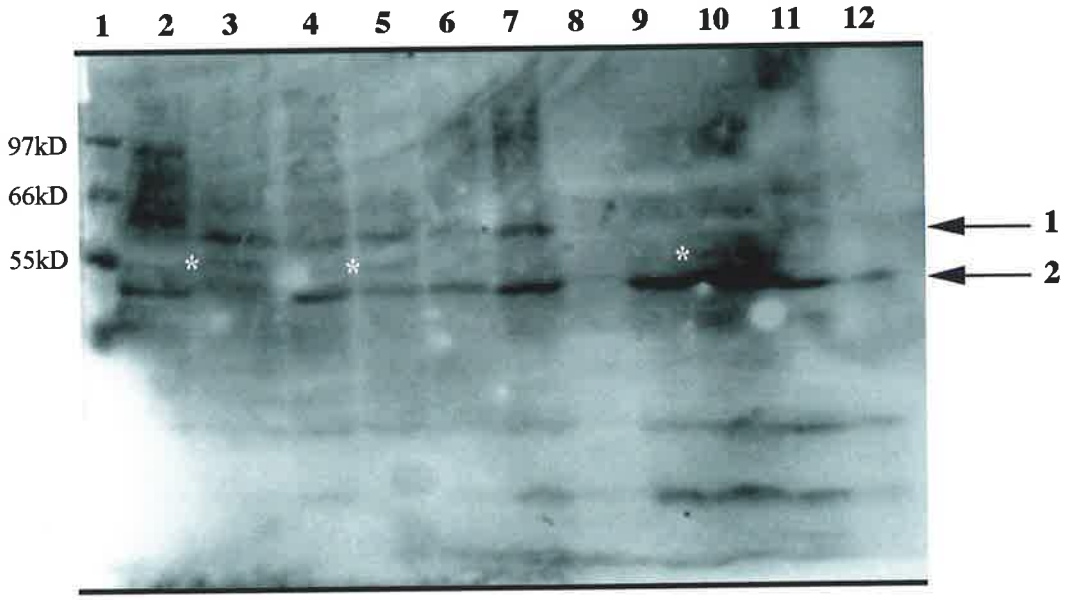
Lane 11-*creA204* strain grown in glucose

Lane 12-CreA$^+$ strain grown in glucose

Primary antibody diluted 1/100 (final bleed)

Secondary antibody diluted 1/2000 (Amersham Rabbit Ig, horseradish peroxidase-linked whole antibody (from donkey))

Exposure time for ECL™ detection was 60 seconds


1 2 3 4 5 6 7 8 9 10 11 12
97kD
66kD
55kD
1
2

lanes containing CreA$^+$ proteins, and in the lanes containing proteins from strains carrying the *creA* alleles, *creA218* and *creA303*. The pattern of presence and absence shown for Band 2 clearly indicates that it does not represent CreA. A faint band appears in lanes 3, 5 and 10 at the 55kDa position and has been indicated with an asterisk. This band is not a candidate for CreA because it is not present in all tracks loaded with extracts from a CreA$^+$ strain and protein from the *creA218* strain should not have a band of this size in common with CreA$^+$ strains.

Thus, the GST::Zf antibodies were not reliably detecting CreA on this western and so there is no conclusive evidence to suggest that the antibodies raised to either the GST::Rgr or the GST::Zf fusion proteins recognise CreA.

4.4 Epitope tagging of CreA

An alternative approach to CreA detection was undertaken using commercially available antibodies to an epitope tag which was expressed with CreA. A construct was made in which ten amino acids (MRGS.His$_6$) were inserted at the C-terminus of CreA immediately before the stop codon. MRGS.His$_6$ was chosen in preference to His$_6$ because the purchased antibody to this tag had greater specificity. The tag was inserted using the polymerase PFU, and an inverse PCR approach (Figure 4.9). Nine plasmids were sequenced using oligonucleotide 590, which primes 180bp 3' of the stop codon, to check that the entire tag and stop codon were present. At least 100bp of the 3' end of the *creA* coding sequence were checked in each plasmid. Six of the plasmids were correct and the other three contained PCR-induced errors which resulted in tandem duplications of the tag sequence.

4.4.1 Transforming *creAΔ4* with epitope-tagged creA.

Since the construct was produced using PCR, and thus there could be errors in the *creA* sequence, three of the plasmids (2, 7 and 8) that contained the correct tag were used to cotransform a *creAΔ4* strain together with pPL3, to determine that they

Figure 4.9: The MRGS.His6 tagged CreA construct.

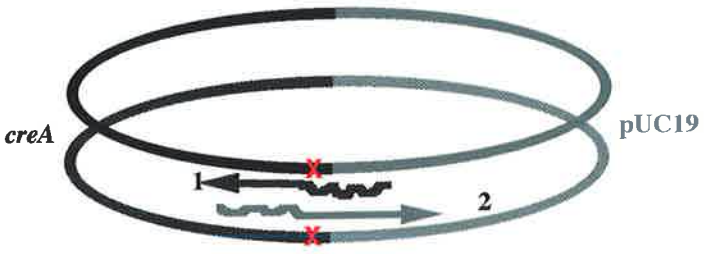
A: The starting point for this construct was the plasmid pANC4 which is pUC19 with a 2.3 kb BamH1-Xba1 insert from *A. nidulans* which contains *creA* (Dowzer and Kelly, 1989). The BamH1 site in pANC4 was destroyed by cutting the plasmid with BamH1, end-filling and religating, to give the plasmid pANC4B. The MRGS.His6 tag was introduced into pANC4B by PCR amplification of the entire plasmid using primers 1 and 2 and PFU polymerase (Clontech).

B: Primer 1 binds to the *creA* sequence immediately before the stop codon and has a tail encoding the 5 amino acids, M.R.G.S.H. The codons used for glycine and serine form a BamH1 site. Primer 2 included the stop codon and has a tail of 9 amino acids - H.H.H.H.H.H.S.G.R. The codons used for glycine and serine also formed a BamH1 site in this tail.

C and **D**: The PCR product was gel purified, cut with BamH1 and religated to form the MRGS.His6 tag immediately before the stop codon. Ligated products were transformed into *E. coli.* Plasmid mini preps were made from 16 transformants and of these, 9 cut with BamH1, which indicated that the tag was present in those plasmids. They were sequenced to check that the entire tag and the stop codon were correct.

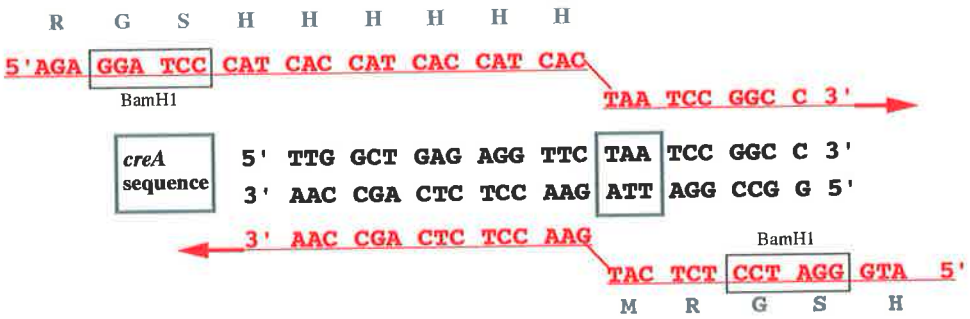
A

pANC4B



Primer 2

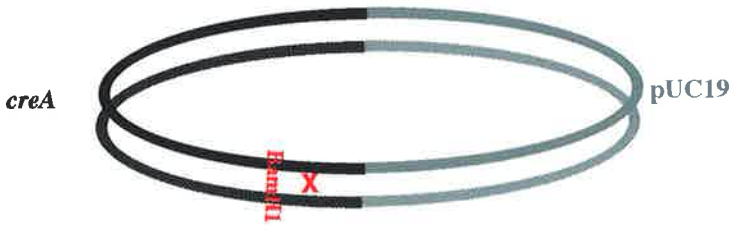
B



Primer 1

C

D



X = Stop Codon

contained functional *creA* genes. Riboflavin independent colonies were selected on medium lacking riboflavin, and then inoculated to complete medium. Strains which had been transformed with a tagged copy of *creA* were expected to show complementation, and thus have a wild type morphology on complete medium rather than the very small, compact morphology of the CreA null strain. Transformants with a different morphology from *creAΔ4* were recovered. Plasmid #2 produced three colonies with a wild type morphology and plasmids #7 and #8 each produced a colony which was morphologically different from *creAΔ4* but not identical to the wild type strain. The strains transformed with #2 and the strain transformed with #8 were analysed further.

4.4.2 Phenotypic analysis of potential, MRGS.His6-tagged CreA strains.

The phenotypes of the strains that were transformed with construct #2 and had wild type morphology on complete medium, were tested further, including assessment of the levels of derepression in the presence of 1% D- glucose, of alcohol dehydrogenase I (using allyl alcohol), glucoamylases (using starch and iodine staining), and β-galactosidase (using X-Gal). This was done by inoculating the transformants to solid medium containing 1% D-glucose plus 2.5mM allyl alcohol, 1% D-glucose plus 0.5% starch, and 1% D-glucose plus 0.5% lactose plus X-gal, and comparing them with a strain containing wild type CreA and the parent strain, *creAΔ4*. The three strains transformed with construct #2 were not derepressed for any of these enzymes in the presence of D-glucose, and thus appeared to be fully complemented on these media. The colonies transformed with construct #7 and #8 were also tested but they were as derepressed as *creAΔ4* in the presence of D-glucose, for the enzymes being assessed (Table 4.2).

4.4.3 Southern analysis of the potential CreA-MRGS.His6 strains.

Southern analysis of the potential CreA-MRGS.His6 strains was performed to confirm the presence of the MRGS.His6 tagged *creA* in each strain, and to check

that it was due to a replacement event and that a single crossover event had not occurred at the *creA* locus in any of the strains. Such an event could produce a strain with an untagged, wild type *creA* and a tagged, disrupted *creA*. This strain would make functional CreA and thus appear as a complemented transformant but would not react with antibodies to the tag in western analyses (Figure 4.10).

Genomic DNA was prepared from the three strains transformed with construct 2, (4, 5, and 8) and the strain transformed with construct 8 (1-8). The DNA was digested in three different ways: with EcoRI, EcoRI plus Xba I, and EcoRI plus BamH1, in order to ascertain whether a single crossover event had occurred. Southern analysis of these strains indicated that they all contained the *creAΔ4* genomic bands, except for strain 8 in which band 5 is not clear (Figure 4.11: Bands 2, 5 and 7). Strain 1.8 produced a strong, *creAΔ4* genomic band for each digest but did not have any bands representing ectopic copies of the construct (Figure 4.11, Bands 3 and 8). Since phenotype testing had shown that a functional CreA was not being expressed in this strain, its altered phenotype compared to *creAΔ4* was attributed to the integration of pPL3.

The strains transformed with plasmid #2 (4, 5, and 8), were all multi-copy transformants. Strains 5 and 8 showed very similar patterns of hybridisation and only varied by one band in the EcoR1 digest and the EcoR1/Xba1 digest (Figure 4.11: Bands A and B). Bands predicted to be produced by ectopic integration of the construct after digestion with EcoR1/Xba1 and EcoR1/BamH1 were present for strains 4, 5 and 8 (Figure 4.11: Bands 3 and 8). As there is only one EcoR1 site in the construct, digestion with EcoR1 would not produce a discrete band, but a range of bands depending on where it had integrated in the genome. If a single crossover event occurred, bands of particular sizes would be predicted to hybridise to the probe, and these are shown in Figure 4.10. The bands predicted to hybridise after an EcoR1/Xba1 digest would be seen at 1166bp (X-X) and 3070 bp (E-X) and 5770bp

Strain	Complete Medium	1% D-glucose and 0.5% starch	1% D-glucose and 1mM allyl alcohol	1% D-glucose and 2.5mM allyl alcohol	1% D-glucose and 0.5% lactose and X-gal
creAΔ4	+	++++	0	0	+
WT	++++	0	+++	+++	0
4 (#2)	++++	0	+++	+++	0
5 (#2)	++++	0	+++	+++	0
8 (#2)	++++	0	+++	+++	0
1.7 (#7)	+	++	(+)	0	0
1.8 (#8)	+	++	0	0	0

Table 4.2: Phenotypes of the MRGS.His6-tagged strains.

Strain The number in brackets after each strain is the number of the transforming his-tagged construct.

Complete Medium: The number of + signs represents the size of each colony.

1% D-glucose and 0.5% starch: The + signs refer to the amount of clearing produced around each colony after the medium has been stained with iodine. 0 indicates that no clearing is present.

1% D-glucose and 1mM allyl alcohol: The + signs indicate the size of each colony. 0 indicates that the colony is not growing.

1% D-glucose and 2.5mM allyl alcohol: The + signs indicate the size of each colony. 0 indicates that the colony is not growing.

1% D-glucose 0.05% lactose and X-Gal: The + sign indicates that the colony is blue on this medium due to the activity of β-galactosidase on X-Gal. 0 means that no blue colour is present.

Figure 4.10: A single cross-over event between the CreA.MRGS.His6 construct and the region of homology in the deletion strain, may produce strains which express untagged, wild-type CreA.

A: A portion of the genome from the *creAΔ4* strain which contains the *creA* disrupted region.

B: The *creA*.MRGS-His6 construct

C: A representation of the genomic DNA which may result from a single crossover event in the region of homology between the *creAΔ4* strain genome and the *creA*.MRGS-His6 construct.

Each diagram is accompanied by dashed lines which represent the bands produced by each digest which are expected to hybridise to the probe. The enzymes which cut each piece of DNA are shown by their first letter at the end of each line.

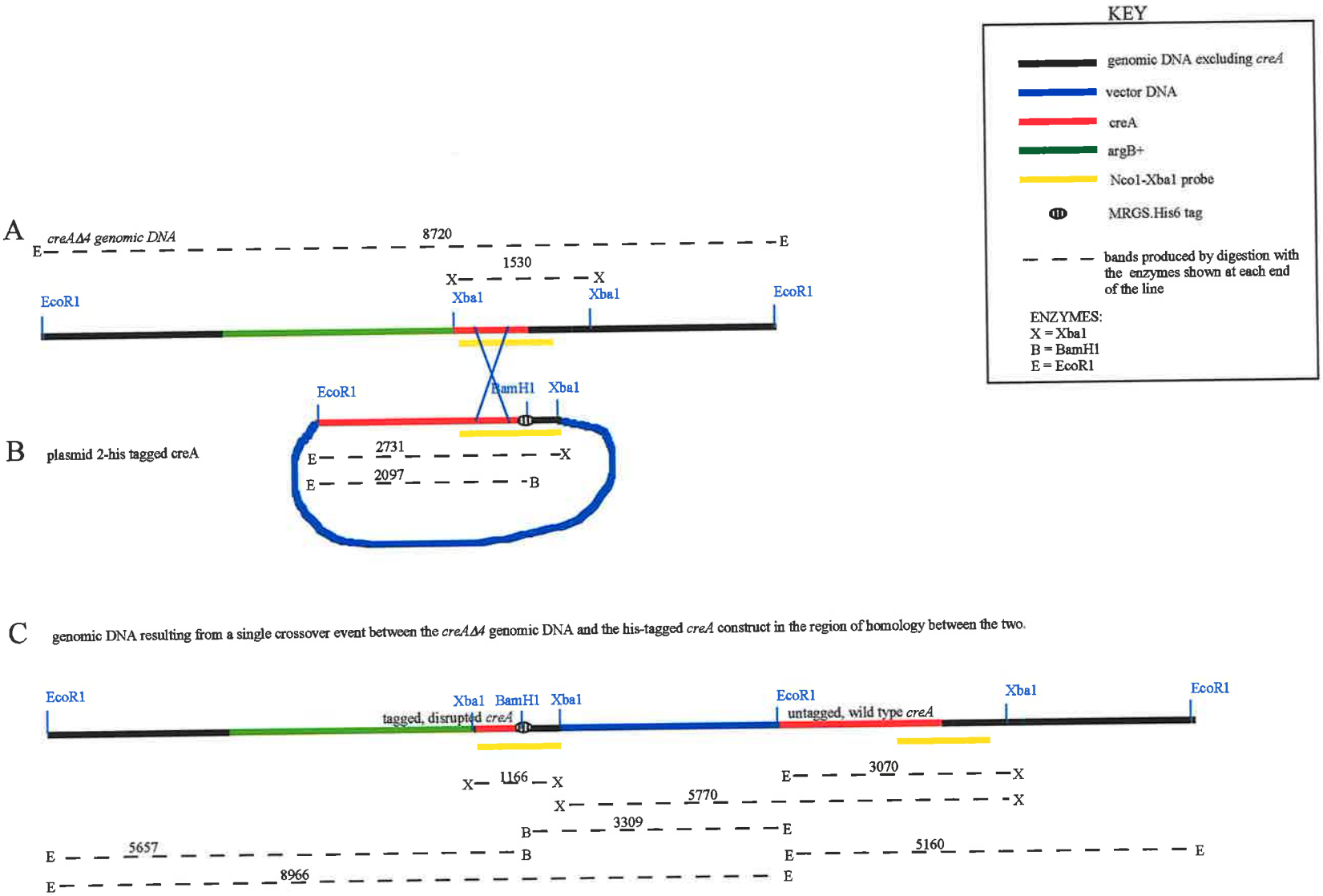

KEY
genomic DNA excluding creA
vector DNA
creA
argB+
Nco1-Xba1 probe
MRGS.His6 tag
bands produced by digestion with the enzymes shown at each end of the line
ENZYMES:
X = Xba1
B = BamH1
E = EcoR1
A
creAΔ4 genomic DNA
E
8720
E
1530
X
X
EcoR1
Xba1
Xba1
EcoR1
EcoR1
BamH1
Xba1
B
plasmid 2-his tagged creA
E
2731
X
E
2097
B
C
genomic DNA resulting from a single crossover event between the creAΔ4 genomic DNA and the his-tagged creA construct in the region of homology between the two.
EcoR1
Xba1
BamH1
Xba1
tagged, disrupted creA
EcoR1
untagged, wild type creA
Xba1
EcoR1
X
1166
X
E
3070
X
X
5770
X
B
3309
E
E
5657
B
E
5160
E
E
8966
E

Figure 4.11: Southern analysis of strains transformed with the *creA*.MRGS.His6 construct.

Genomic DNA from transformed strains was digested with

A: EcoR1 and Xba1

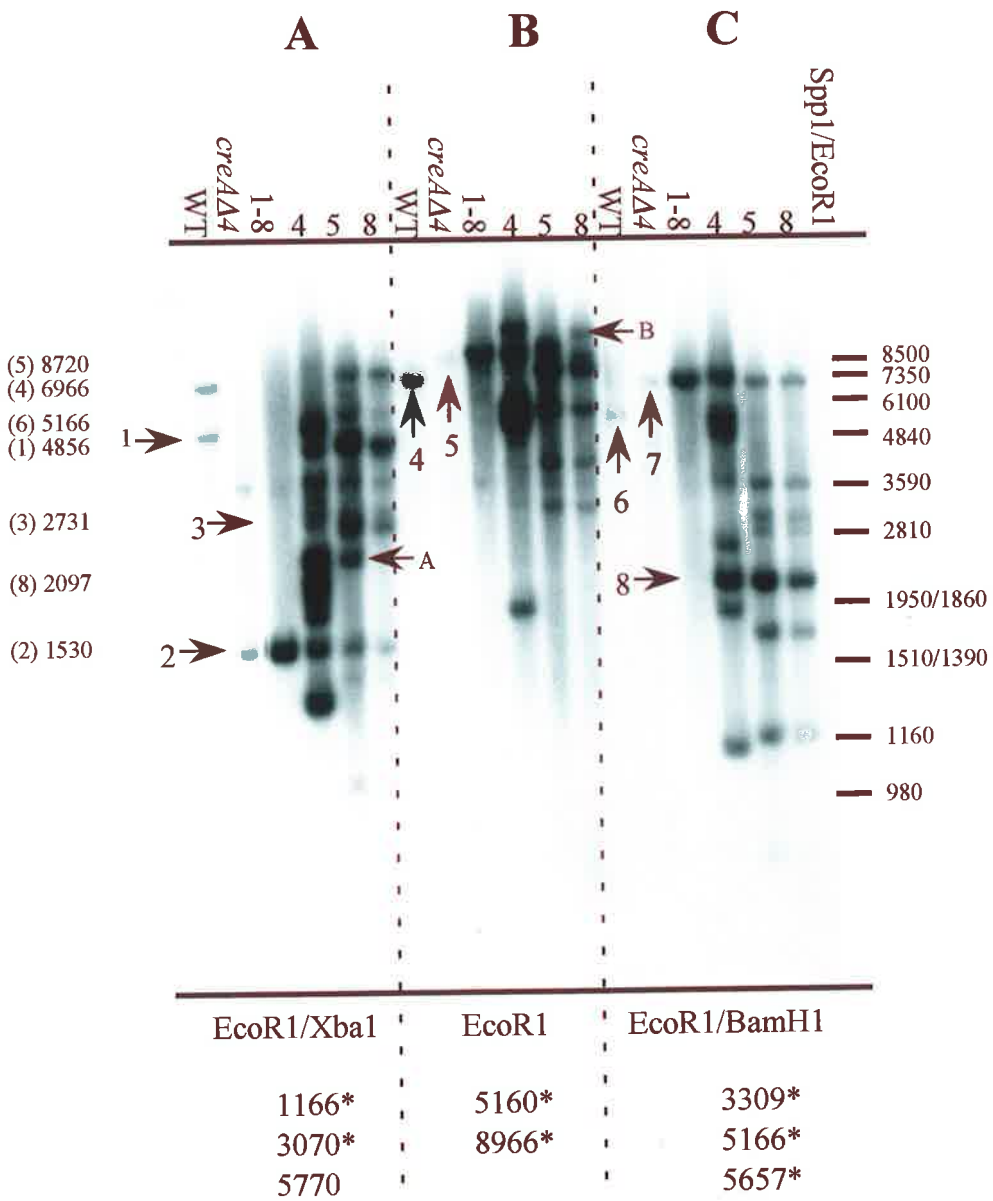
B: EcoR1 only

C: EcoR1 and BamH1

and electrophoresed on a 0.8% aragose gel. The DNA was Southern transferred to Hybond N^+ and hybridised with a ^{32}P-labelled, 1100bp Nco1-Xba1 fragment from pANC4. The strain from which the DNA was prepared is shown above each lane on the figure.

Below each panel are the sizes of the bands which would be produced from each digest if a single crossover event had occurred. The bands marked with an asterisk are not present in any of the strains.

The expected bands of known size, which are discussed in the text, are shown on the left hand side of the figure with their numbers (1-8) and sizes. The bands have been arranged in order of size, for example, band 5 appears first because it is the largest band. These bands have been used in conjunction with the molecular weight marker (on the right of the figure) to estimate the positions of bands which would be produced by a single crossover event.


A
B
C
WT
creAΔ4
1-8
4
5
8
Spp1/EcoR1
(5) 8720
(4) 6966
(6) 5166
(1) 4856
(3) 2731
(8) 2097
(2) 1530
1
2
3
4
5
6
7
8
A
B
8500
7350
6100
4840
3590
2810
1950/1860
1510/1390
1160
980
EcoR1/Xba1
EcoR1
EcoR1/BamH1
1166*
3070*
5770
5160*
8966*
3309*
5166*
5657*

(X-X) (Figure 4.11-A). None of these bands appear to be present in panel A. Digestion with EcoR1 alone (Figure 4.11-B) would produce hybridising bands of 8966bp and 5160bp. For strain 4, the 5160bp band may be present but it is masked by strong signals from a higher band. However, it is definitely absent from the lanes which contain strains 5 and 8. The larger band of 8966bp is not present. There are bands above the *creAΔ4* band, (which is 8720bp and thus 250 bp smaller) but too far above to be 8966bp and so there is no clear evidence to suggest that either of the bands predicted from an EcoR1 digest are present in panel B. The bands expected on a Southern from an EcoR1/BamH1 digest (Figure 4.11-C) would be 5657bp (E-B), 3309bp (B-E) and 5160bp (E-E). The 5657bp and 5166bp bands are not present for the strains 5 and 8 but once again it is difficult to tell with strain 4, because there is a strongly hybridising band in this region. The 3309bp band is not present for strain 4 but may be represented by the bands seen in this region for strains 5 and 8.

While in some cases it is not possible to say that a particular critical band was absent on the Southern, in no case were all of the predicted bands present to suggest that a single crossover event had occurred. Thus Southern analyses indicated that the strains 4, 5 and 8 were transformed with copies of *creA*-MRGS.His6 and could be expected to produce MRGS.His6-tagged protein which would be detected by antibodies specific for the MRGS.His6 tag.

4.5. Western analysis of CreA-MRGS.His6 strains.

Mouse monoclonal antibodies raised against the epitope MRGS.His6 (Qiagen), were used to detect MRGS.His6-tagged CreA. No proteins were detected in extracts from transformants 1.8, 4, 5 and 8 by the antibody (result not shown), and this result was assumed to be due to the concentration of CreA being very low in total protein extracts. The extracts were passed down Ni/NTA spin columns (Qiagen) which bind the His tag and thus purify tagged CreA. Native conditions were used. The eluates from these columns were run on an SDS-PAGE gel, which showed that two major

Figure 4.12: Ni-NTA column purified proteins

Total protein extracts from three strains were purified on Ni/NTA spin columns (Qiagen) under native conditions using 20mM imidazole to bind the proteins to the column and 250mM imidazole to elute the bound proteins. Samples of whole extracts and eluates were loaded onto a 12% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue R-250

Lane 1-molecular weight marker

Lanes 2, 3 and 4 (protein extracts from glucose grown transformant 4)

Lane 2-whole extract

Lane 3-eluate 1

Lane 4- eluate 2

Lanes 5, 6 and 7 (protein extracts from arabinose grown transformant 4)

Lane 5-total extract

Lane 6-eluate 1

Lane 7-eluate 2

Lanes 8, 9 and 10 (protein extracts from glucose grown $CreA^{+}$ strain)

Lane 8-total extract

Lane 9-eluate 1

Lane 10-eluate 2

Lanes 11, 12 and 13 (protein extracts from glucose grown CreA-null strain)

Lane 11-total extract

Lane 12-eluate 1

Lane 13-eluate 2

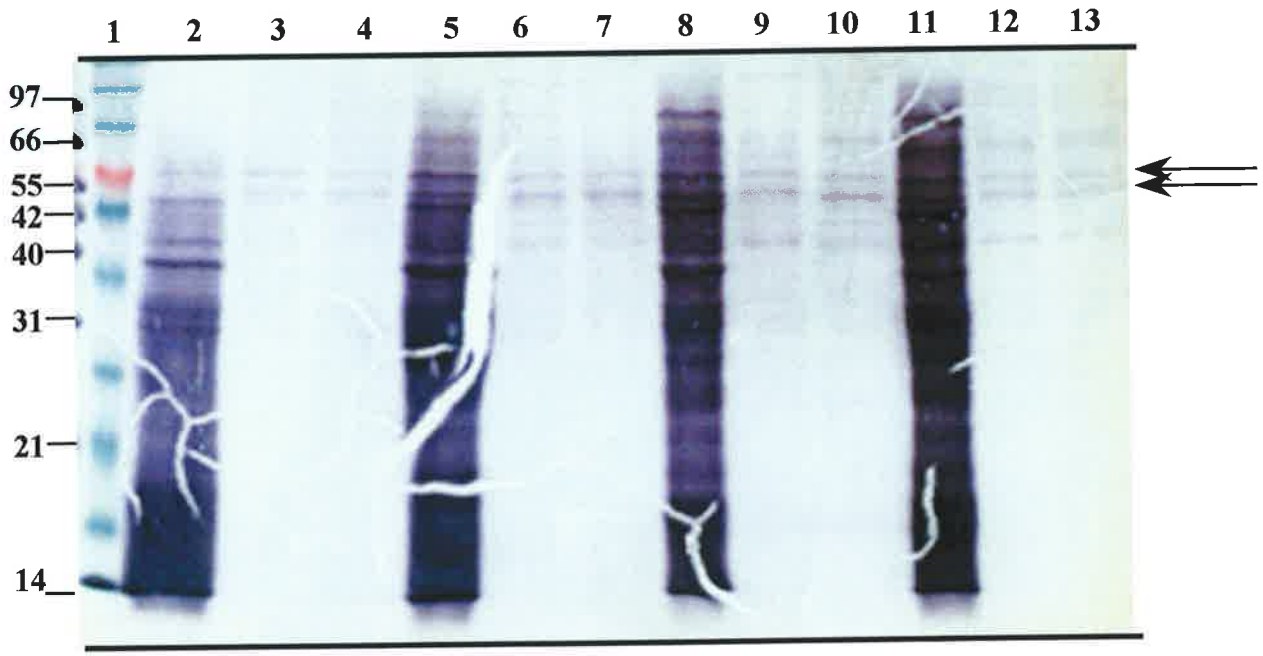

1
2
3
4
5
6
7
8
9
10
11
12
13
97
66
55
42
40
31
21
14

Figure 4.13: Immunodetection of His-tagged proteins using a penta-his antibody.

Total protein extracts were loaded onto a 10% SDS-polyacrylamide gel with several samples of an *E. coli* expressed His_6- tagged murine DHFR protein (Qiagen). The loadings are:

Lanes 1, and 10 -molecular weight markers

Lane 2-strain 1.7

Lane 3- strain 1.8

Lane 4-strain 4

Lane 5-strain 5

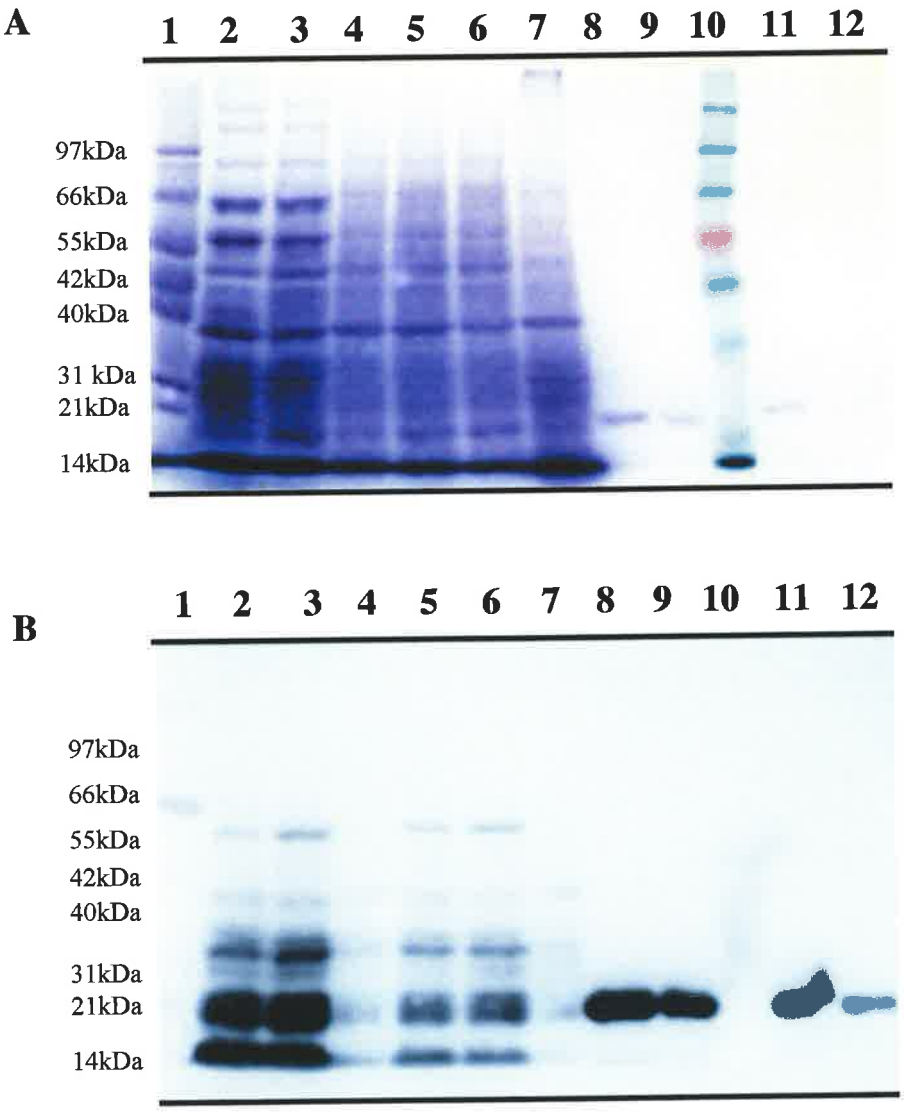
Lane 6-strain 8

Lane 7-$CreA^+$ strain

Lanes 8, 9, 11, and 12- various amounts of DHFR.His_6-(5ul, 2.5ul, 1ul, 1/10 dilution respectively).

A: Coomassie Brilliant Blue R-250 stained SDS-polyacrylamide gel.

B: Immunodetection was performed with a penta-his primary antibody and the Amersham Mouse Ig, horseradish peroxidase-linked whole antibody (from sheep) both diluted 1/2000, followed by ECL™ (Amersham). (Exposure time-30 seconds)


A
1 2 3 4 5 6 7 8 9 10 11 12
97kDa
66kDa
55kDa
42kDa
40kDa
31 kDa
21kDa
14kDa
B
1 2 3 4 5 6 7 8 9 10 11 12
97kDa
66kDa
55kDa
42kDa
40kDa
31kDa
21kDa
14kDa

proteins had been purified in this procedure (Figure 4.12, Arrows). However, the apparently identical proteins were purified from WT extracts which do not contain His-tagged CreA, and from *creAΔ4* extracts which do not contain any CreA (Figure 4.12, Lanes 9/10 and 12/13). The MRGS.His6 antibody did not recognise any proteins from these eluates (result not shown). Column purification was repeated using different concentrations of imidazole to bind the protein to the column under native conditions (20mM, 5mM and 1mM), and denaturing conditions were also used. These variations did not change the profile of the eluates seen on SDS-PAGE gels.

To investigate the possibility that CreA-MRGS.His6 was not being detected in total protein or column purified extracts because the antibodies had lost their activity, a positive control was required. A control protein with the MRGS.His6 epitope was not available and the MRGS.His6 antibodies could not recognise a His6 tag. For this reason, the antibody was changed to a mouse monoclonal Penta-His antibody (Qiagen) which recognises any proteins possessing a string of 4, 5 or 6 histidines and would therefore recognise both the MRGS.His6 tagged CreA and a commercially available His6 tagged protein (His6-tagged murine DHFR protein expressed in M13pREP4 cells from the vector pQE16 (Qiagen)). The positive control protein was purified under native conditions using nickel-NTA spin columns. Total protein extracts from glucose grown His-tagged strains, the $CreA^{+}$ strain and dilutions of the positive control protein, were run on an SDS-PAGE gel, western transferred and probed with the Penta-His antibody (Figure 4.13). The positive control was clearly recognised by the antibody as were proteins in the extracts from the CreA-MRGS.His6 transformed strains 1-7, 1-8, 5, and 8. The absence of a signal from transformant 4 was puzzling, as was the presence of a signal in the tracks containing protein from the transformants 1-7 and 1-8. Southern analysis had indicated that these strains were only transformed with pPL3 and did

not contain copies of his-tagged *creA* (Southern results not shown for transformant 1.7).

Fresh protein extracts were prepared from glucose grown and arabinose grown mycelia of the strains 1-7, 1-8, 4, 5, 8, and *creAΔ4* (the negative control). The protein extracts were purified on Nickel/NTA spin columns (Qiagen) and the eluates were analysed by immunodetection of western blots using the Penta-His antibody. The SDS-PAGE gel photograph (Figure 4.14A) shows that many more proteins were eluted from the columns for all strains although the same elution conditions were used as in Figure 4.12. It can also be seen that, although equal volumes of protein were loaded in each track, the quantity of protein eluted from the arabinose grown strains was consistently higher than that produced by glucose grown strains. This difference must be independent of carbon catabolite derepression because it is seen in protein extracts from the *creAΔ* strain and transformants 1-7, and 1-8.

The antibody reacted strongly with the positive control and with several bands in the protein molecular weight marker, in particular phosphorylase B (97kDa), and L-glutamic dehydrogenase (55kDa). Strongly hybridising bands were also seen in the arabinose grown protein extracts from all strains (except 1-8) including the extract from the deletion strain which again indicated that CreA.MRGS.His_6 was not the protein being purified on the column or recognised by the antibody (Figure 4.14, B-Arrows).

Despite several attempts, western analysis did not produce consistent results to indicate that the either the MRGS.His_6 or the Penta-His antibodies were recognising his-tagged CreA from the strains transformed with the CreA-MRGS.His_6 construct. There were many inconsistencies seen, for example, the antibodies reacted with proteins in tracks that contained total protein from both untagged CreA wild type strains and CreA null strains, and did not react consistently with protein from

Figure 4.14: Immunodetection of column purified proteins using the penta-His antibody (Qiagen).

Proteins were purified on Ni/NTA spin columns using native conditions as described in Figure 4.12, electrophoresed on an SDS-polyacrylamide gel, and transferred to Hybond C-extra. Immunodetection was performed using the penta-His antibody from Qiagen at a dilution of 1/2000 and the Amersham Mouse Ig, horseradish peroxidase-linked whole antibody (from sheep) diluted 1/2000, followed by ECL™. (Exposure time-60 seconds)

A: Coomassie Brilliant Blue R-250 stained SDS-polyacrylamide gel.

B: Immunodetection of western blot.

Loadings are:

Lane 1-molecular weight marker

Lane 2- murine DHFR.His_6 fusion protein expressed in *E. coli* (positive control)

Lane 3-CreA-null strain grown in glucose

Lane 4-CreA-null strain grown in arabinose

Lane 5-CreA.MRGS.His_6 strain 1.7 grown in glucose

Lane 6-CreA.MRGS.His_6 strain 1.7 grown in arabinose

Lane 7-CreA.MRGS.His_6 strain 1.8 grown in glucose

Lane 8-CreA.MRGS.His_6 strain 1.8 grown in arabinose

Lane 9-CreA.MRGS.His_6 strain 4 grown in glucose

Lane 10-CreA.MRGS.His_6 strain 4 grown in arabinose

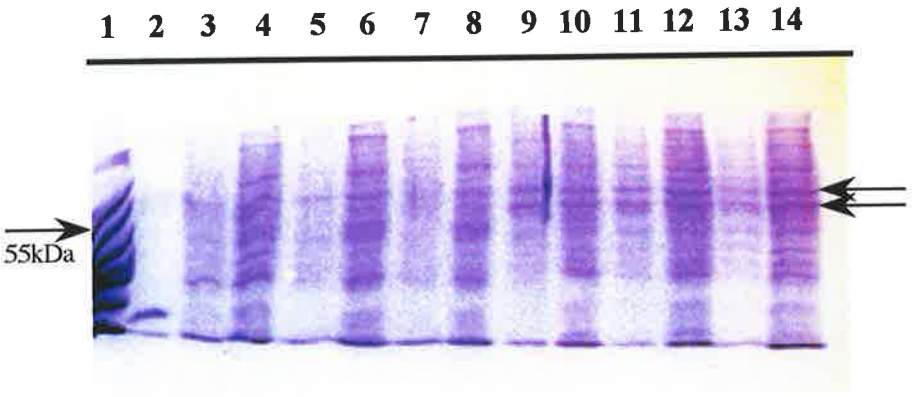
Lane 11-CreA.MRGS.His_6 strain 5 grown in glucose

Lane 12-CreA.MRGS.His_6 strain 5 grown in arabinose

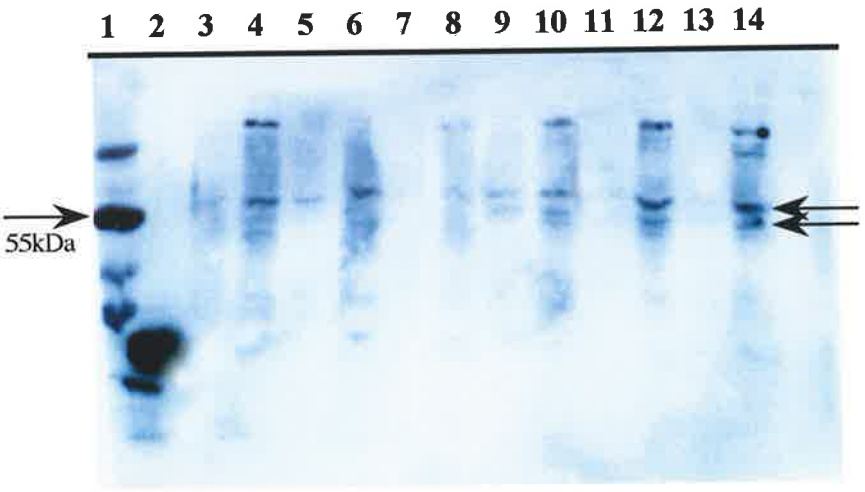
Lane 13-CreA.MRGS.His_6 strain 8 grown in glucose

Lane 14-CreA.MRGS.His_6 strain 8 grown in arabinose

A



B



strains which contained tagged CreA such as 4, 5 and 8. When nickel-NTA spin columns were used to purify CreA-MRGS.His6 from total protein extracts, some of the purified proteins were recognised by the antibodies. However the same result was obtained with proteins purified from the CreA-null strain which showed that the proteins being recognised were not tagged CreA. Attempts were made to purify tagged CreA in both native and denaturing conditions but the results did not improve.

To rule out the possibility that CreA was only being detected faintly and the signal had been overlooked, proteins were freshly prepared from glucose grown and fructose grown mycelia and duplicate SDS-PAGE gels were run. These were western transferred and one was probed with the Penta-His antibody and the other with the GST::Zf antibody (Figure 4.15-A and B). If both antibodies were recognising CreA, there would be similarities between the two westerns - such as bands in common between them, (although protein degradation could change the profile presented by each antibody), or signals present in the same tracks.

The Penta-his antibody detected proteins in tracks 4, 5, 7, and 9 plus the molecular weight marker. The wild type, untagged protein in lane 4, and the marker should not be recognised. The GST::Zf antibodies recognised proteins in all tracks, including the *creAΔ4* strain, excepting those containing the molecular weight marker and the His-tagged positive control in Lane 9 (Figure 4.15-A and B). This experiment showed that proteins in extracts from the fructose grown $CreA^{+}$ strain reacted strongly with both antibodies - and once again indicated that CreA was not being detected.

4.6 Discussion

CreA has not been reliably detected in total protein extracts with the three antibodies used in this thesis. To effect its role as a wide domain regulatory protein, CreA is expected to reside in the nucleus at least in repressing conditions and

Figure 4.15: Duplicate western blots detected with the penta-His antibody and the antibodies raised against GST::Zf

A: Immunodetection was performed with the penta-His 1° antibody and the Amersham Mouse Ig, horseradish peroxidase-linked whole antibody (from sheep), which were diluted 1/2000, followed by ECL™.

B: Immunodetection was performed with anti GST::Zf 1° antibody diluted 1/100 and the Amersham Rabbit Ig, horseradish peroxidase-linked whole antibody (from donkey), which was diluted 1/2000, followed by ECL™. (Exposure time-90 seconds)

The lanes are loaded with:

M-molecular weight marker

Lane 1-CreA-null strain grown in glucose

Lane 2-CreA null strain grown in fructose

Lane 3-CreA+ strain grown in glucose

Lane 4-CreA^{+} strain grown in fructose

Lane 5-CreA.MRGS.His$_6$ transformant 1.8 grown in glucose

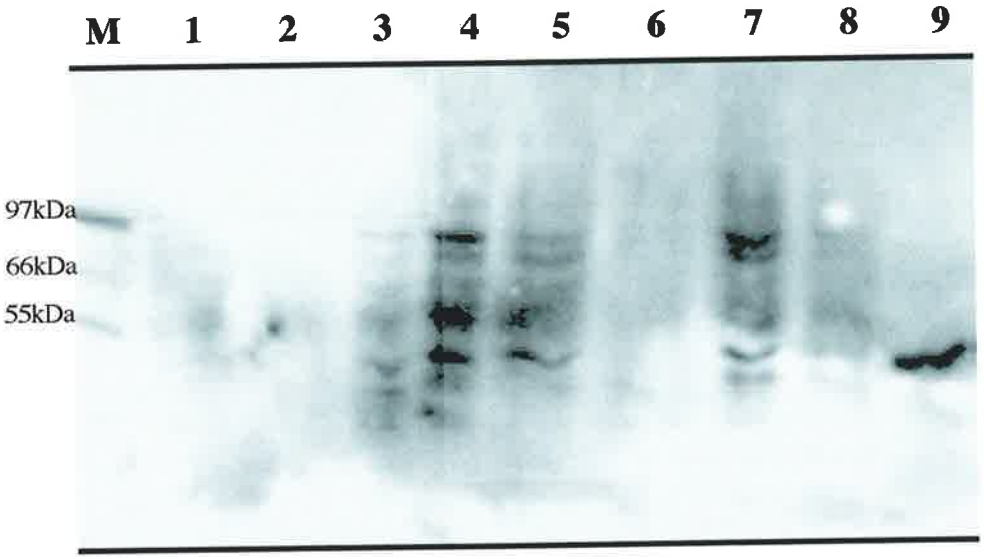
Lane 6-CreA.MRGS.His$_6$ transformant 1.8 grown in fructose

Lane 7-CreA.MRGS.His$_6$ transformant 8 grown in glucose

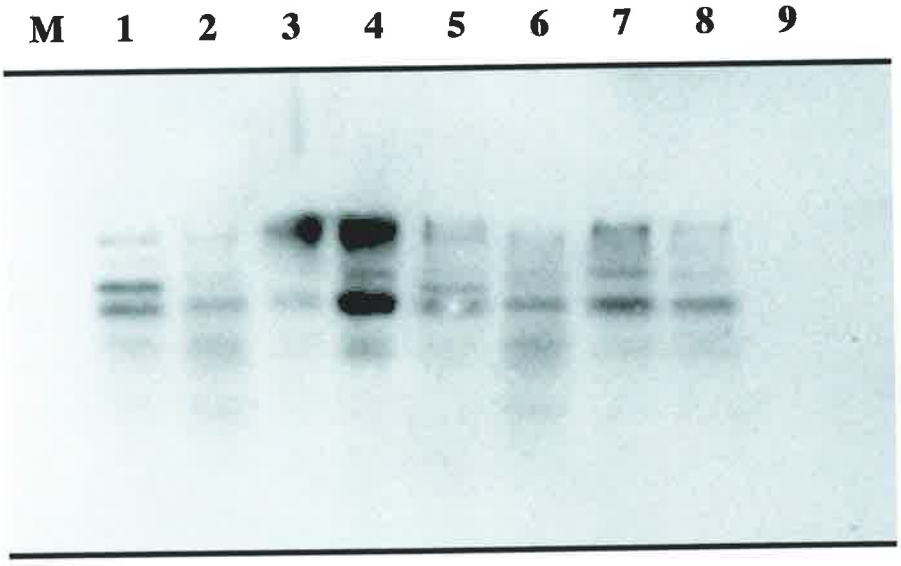
Lane 8-CreA.MRGS.His$_6$ transformant 8 grown in fructose

Lane 9- murine DHFR.His$_6$ fusion protein expressed in *E. coli* (positive control)

A



B



perhaps it could have been detected in nuclear extracts. This was not the case in previous work however (Chamelaun-Hussey, 1996), and experiments with Mig1p from *S. cerevisiae* have since shown that the location of Mig1p in the cell is regulated by carbon source. Mig1p is found in the nucleus when glucose is present and in the cytoplasm when glucose levels are low or depleted, and relocation of the protein occurs within minutes (De Vit *et al.* 1997). If CreA is regulated in a similar way, nuclear protein preparations of CreA may in fact contain very little CreA as a consequence of the low glucose environment induced while harvesting the mycelia.

While CreA was not successfully detected in these experiments, some proteins in both the CreA-null and CreA$^+$ strains reacted with the GST::Zf antibody. Many other zinc finger proteins of the Cys_2-His_2 class have been identified in *A. nidulans* and perhaps some of these were being recognised by the antibody raised to the zinc finger region of CreA. In *S. cerevisiae* there are two zinc finger proteins, Mig1p and Mig2p, with 71% identity in their fingers (Lutfiyya and Johnston, 1996). If there is a protein in *A. nidulans* with zinc fingers like CreA then it would be recognised by the GST::Zf antibody and be present in protein extracts from *creAΔ4* and *creA*$^+$ strains. Mig2p has little similarity to Mig1p, or any other proteins outside the finger region, so if a similar protein exists in *A. nidulans* it would not be recognised by the GST::Rgr antibody.

CreA may be difficult to detect with antibodies because only small quantities of protein are present at any time and/or it is subject to rapid degradation. Although several different protease inhibitors were used when the protein extracts were being prepared (PMSF, Complete™ Inhibitor Protease Cocktail tablets), they may not have been effective for the protease which degrades CreA. Most of the other conditions which may enhance protease activity were avoided by precautions such as: working in the cold at all times, always using freshly prepared protein, adding

protease inhibitor to the grinding buffer, and using a variety of buffers in case one promoted protease activity.

An attempt was made to purify CreA-specific antibodies away from the non-specific fraction based on the assumption that the titre of the CreA-specific antibodies was very low and concentrating them would allow detection of CreA in total protein extracts. The two GST-fusion protein antigens were run in ten lanes on two separate SDS-PAGE gels and then transferred to nitrocellulose. The continuous band of full-length protein on each gel was cut out and incubated overnight with the appropriate serum and then the antibodies were eluted off the strips and used against total protein extracts from $CreA^{+}$ and CreA-null strains. This procedure did not allow detection of CreA.

For the CreA-MRGS.His_6 strains an alternative approach was used. An excess of antibodies was incubated with *creAΔ4* total protein extract in an attempt to cross-react them with the proteins that were not CreA, prior to using them to detect CreA on a western. This procedure did not allow detection of CreA and the cross-reactivity of the antibodies was not significantly reduced either.

4.7 Future Work

Although antibodies to CreA from *A. nidulans* have still not been made, they will provide a useful molecular tool and so alternative approaches to raising antibodies have been considered. The first method is to fuse GST to the entire *creA* protein and then to cleave GST from the protein before using it as an antigen. This will remove the interference provided by antibodies raised against fusion proteins recognising GST.

Polyclonal antibodies to Cre1 from *T. reesei* have been successfully raised in rabbits against a synthetic peptide made of 9 amino acids from the C-terminal region of the

zinc finger domain (Strauss *et al.* 1995). This amino acid sequence is identical to CreA from both *A. niger* and *A. nidulans* and this procedure could be duplicated.

At the same time that the GST::Rgr and GST::Zf fusion proteins were used to raise antibodies to CreA in two rabbits, a third rabbit was inoculated with an unrelated GST fusion protein (Alcohol dehydrogenase III from *A. nidulans*) and subsequently, the serum from this rabbit was used successfully to detect that protein in wild type *A. nidulans* extracts. Therefore, the inoculation regime and the amounts of protein used in each inoculation were adequate for producing antibodies to a specific protein.

Another approach would be to tag the protein with a different epitope at the N-terminus, and at the C-terminus to overcome possible complications such as non-specific binding of zinc finger proteins to the nickel columns, or binding by other histidine rich proteins from the total protein extract. Snf1p from *S. cerevisiae* has a string of 14 consecutive histidines which have been used to purify the protein on nickel columns (Jiang and Carlson, 1997) and it is possible that *A. nidulans* total protein extracts may also contain histidine rich proteins. The use of other protease inhibitors during the preparation of protein extracts should also be considered.

The failure to detect CreA in these experiments was not due to technical problems. Western blots and immunodetections were performed successfully as shown by the reliable detection of GST by the antibodies raised in rabbits against GST-fusion proteins, and detection of the positive control protein by the Penta-his antibody. In addition, most of the westerns had very little background which indicated that the membranes were well blocked, and there was very little non-specific detection by the primary or secondary antibodies.

Two different methods were used to detect CreA. One involved raising antibodies to GST::CreA fusion proteins which could then be used to detect CreA in native extracts, and the other involved detecting native, tagged CreA with purchased antibodies. The fact that two such different approaches failed to allow detection of CreA suggests that CreA has properties which make it difficult to purify from total protein extracts, and to detect with antibodies. These properties are likely to be related to protein stability.

Chapter 5 Analysis of 2-deoxy-D-glucose resistant strains in *Aspergillus nidulans.*

2-deoxy-D-glucose has been used to select mutants affected in carbon catabolite repression in *Saccharomyces cerevisiae*, *Neurospora crassa* and several other fungal species. All the mutants show derepression of some enzymes usually repressed in the presence of 2-deoxy-D-glucose and glucose, and the genes which have been identified in this way can be separated into three broad functional groups.

In the first group, glucose transport is impaired; in the second group, a phosphatase specific for 2-deoxy-D-glucose-6-phosphate is overexpressed; and in the third group, the regulation of glucose transport is defective. Three genes from *S. cerevisiae* which have been identified in this third group are *GRR1, HXK2* and *REG1* . They have been studied extensively and are involved in the regulation of carbon catabolite repression. A proposed role for *HXK2* and *GRR1* is to transmit the signal from membrane bound glucose sensors to intracellular effectors of glucose repression. *REG1* has been shown to interact with the protein kinase, Snf1p and a phosphatase, Glc7p (Figures 1.2 and 1.3).

The role played in glucose repression by loci identified as 2-deoxy-D-glucose resistant mutants in other species, (*N. crassa, S. pombe, Pichia. stipitus, P pinus, K. lactis*, and *A. niger)* has not been established because glucose repression is not as well studied in these organisms as it is in *S. cerevisiae.* Resistance to 2-deoxy-D-glucose is not a phenotype of mutants in *SSN6,* and *TUP1* which are regulators of glucose repression in *S. cerevisiae,* but Mig1p deletion strains are resistant. The mutants which have been selected on 2-deoxy-D-glucose have identified genes which are necessary for glucose repression, but are not involved at the DNA level, in the transcriptional regulation of enzymes required for the utilisation of various carbon sources. In *A. nidulans*, carbon catabolite repression has been studied in detail with much of the work focussed on the repressor CreA. There have been many attempts to isolate mutants affected in carbon catabolite repression in this species and three

other loci, *creB*, *creC* and *creD* have been identified. However, selection of 2-deoxy-D-glucose resistant mutants has not been reported. In order to isolate a different class of mutants involved in carbon catabolite repression, this method has been employed and comprehensive genetic and phenotypic analyses of *A. nidulans* mutants selected for their resistance to 2-deoxy-D-glucose are presented in this chapter.

5.1 Selection of 2-deoxy-D-glucose resistant strains.

Spores from two strains, Wild Type *(biA1; niiA4)* and H17A12 *(yA1 suA1adE20 adE20; areA217; riboB2)* were inoculated onto medium containing 0.01% 2-deoxy-D-glucose, 10mM ammonium tartrate, the appropriate supplements and either 50mM D-quinate or 1% ethanol as the carbon source. The spontaneous resistant colonies which appeared on these plates were purified and all were inoculated onto the selective media (0.01% 2-deoxy-D-glucose with either 50mM D-quinate or 1% ethanol) to determine whether resistance to 2-deoxy-D-glucose was substrate specific. Their morphology was examined on complete and minimal media, and they were inoculated to a range of other media to determine whether they were affected in carbon catabolite repression. On all media, the 2-deoxy-D-glucose resistant strains were compared with the parental strains, Wild Type and H17A12, and with two *A. nidulans* strains which contain the *sorA2* and *sorB11* alleles respectively. The latter strains have been included because they are resistant to another glucose analogue, L-sorbose and the *sorA2* strain is also resistant to 2-deoxy-D-glucose.

5.2 Phenotypic analyses of 2-deoxy-D-glucose resistant strains

5.2.1 Selective media: quinate plus 2-deoxy-D-glucose and ethanol plus 2-deoxy-D-glucose

The mutants were selected on medium containing 0.01% 2-deoxy-D-glucose and 50mM D-quinate except MB2, MB3 and PB1, which were selected on medium containing 0.01% 2-deoxy-D-glucose and 1% ethanol. All strains were inoculated onto both selective media and this showed heterogeneity among the mutants in their

Figure 5.1: 2-deoxy-D-glucose resistant strains growing on medium containing ethanol or quinate, with and without 2-deoxy-D-glucose.

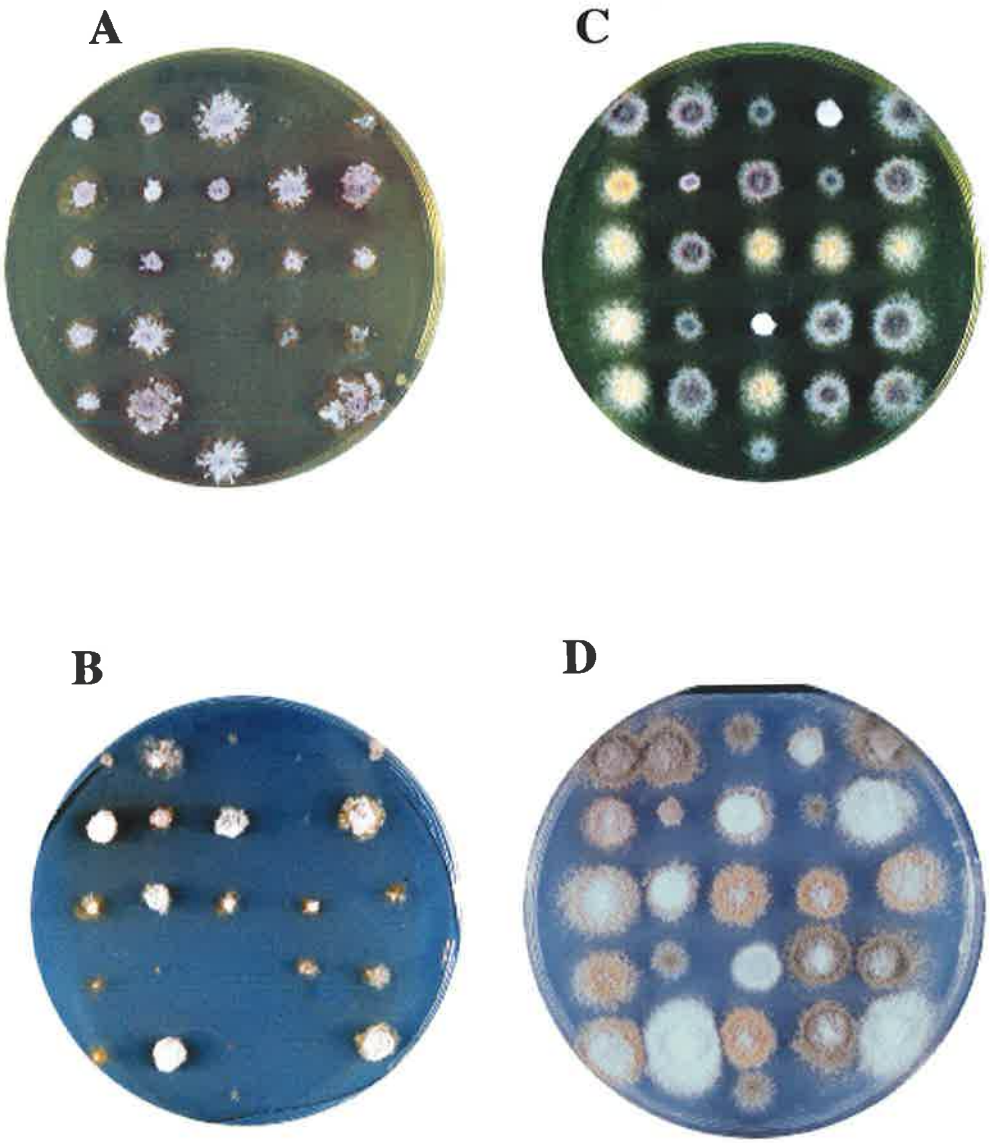
A: 50mM D-quinate and 0.01% 2-deoxy-D-glucose

B: 1% ethanol and 0.01% 2-deoxy-D-glucose

C: 50mM D-quinate

D: 1% ethanol

The medium also contains 10mM ammonium tartrate and the appropriate supplements.



DS15 DS10 DS4 *sorB11* *sorA2*
MS3 MB3 MB2 JK3 DS20
BK2 MB2 YF4 JK1 PB1
MM1 DS4 *sorB11* *sorA2* DS15
PB1 DS20 H17A12 *biA1, niiA4* DS20
JK3

resistance to 2-deoxy-D-glucose (Figure 5.1A and B). There was also some variation between the mutants on each medium, for example, JK3 is weakly resistant on ethanol and 2-deoxy-D-glucose but strong on quinate plus 2-deoxy-D-glucose. The strains which showed the most resistance on medium containing 2-deoxy-D-glucose and quinate were DS20, DS4, and JK3. To test whether resistance to 2-deoxy-D-glucose was related to changes in expression of quinate dehydrogenase or alcohol dehydrogenase 1, the strains were inoculated to medium containing 50mM D-quinate or 1% ethanol as the sole carbon source (Figure 5.1C and D). A comparison of the plates with and without 2-deoxy-D-glucose indicated that there was no obvious relationship between growth on quinate or ethanol and resistance to 2-deoxy-D-glucose.

5.2.2 Complete and minimal medium

On 1% complete medium, there was some morphological variation seen among the mutants. Colonies that were noticeably different were MB2, MB3 and DS4 which were small and compact, and MS3, PB1 and JK3 which were weaker growing colonies (Figure 5.2). On media other than complete, the compact phenotype of MB2, MB3 and DS4 was less noticeable but all the colonies were still smaller than wild type. There was no correlation seen between colony morphology and 2-deoxy-D-glucose resistance, as the weak or compact colonies were not the least or most resistant (see Figure 5.1).

5.2.3 Derepression of enzymes in the presence of 1% D-glucose

Whether carbon catabolite repression was altered in these mutants was examined on media containing 1% D-glucose and various other substrates which showed derepression of enzymes usually subject to carbon catabolite repression. Derepression was assessed in four ways: by staining for secretion of enzymes into

Figure 5.2: 2-deoxy-D-glucose resistant mutants on 1% complete medium

The green mutants were isolated in the strain *biA1; niiA4* and the yellow mutants were isolated in the strain H17A12 *(yA1 suA1adE20 adE20; areA217; riboB2).* Both of these parent strains are included on the plate as well as strains containing the *sorA2* and *sorB11* alleles respectively. The *sorB11* containing strain is aconidial and has an extreme, compact morphology.

DS15 DS10 DS4 *sorB11* *sorA2*

MS3 MB3 MB2 JK3 DS20

BK2 MB2 YF4 JK1 PB1

MM1 DS4 *sorB11* *sorA2* DS15

PB1 DS20 H17A12 *biA1*,*niiA4* DS20

JK3

Figure 5.3: Growth on medium containing starch plus glucose

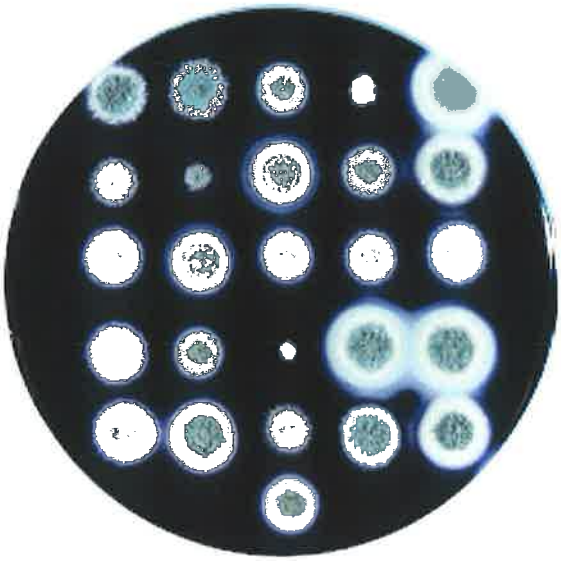
The strains have been inoculated onto medium containing 0.5% starch, 1% D-glucose, (plus 10mM ammonium tartrate and appropriate supplements).

A: 0.5% starch, 1% D-glucose

B: 0.5% starch, 1% D-glucose stained with iodine.

A

B



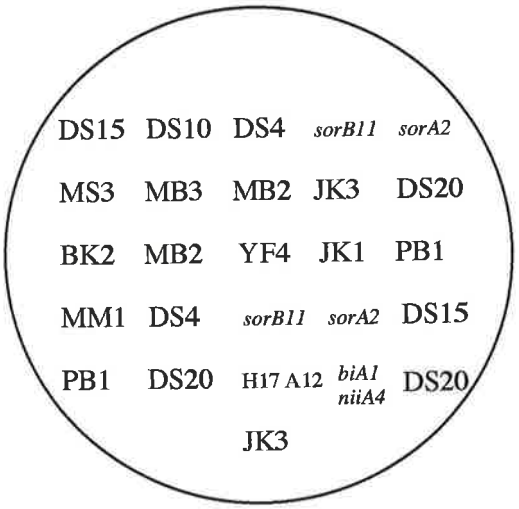

DS15 DS10 DS4 sorB11 sorA2
MS3 MB3 MB2 JK3 DS20
BK2 MB2 YF4 JK1 PB1
MM1 DS4 sorB11 sorA2 DS15
PB1 DS20 H17 A12 biA1 niiA4 DS20
JK3

Figure 5.4: Growth on medium containing allyl alcohol plus glucose.

The strains have been inoculated onto medium containing 2.5mM allyl alcohol and 1% D-glucose (plus 10mM ammonium tartrate and appropriate supplements).

A: 1% Complete medium

B: 2.5mM allyl alcohol and 1% D-glucose

None of the colonies appear to be expressing significant levels of alcohol dehydrogenase 1 in the presence of glucose. MB3 is growing poorly on this medium but similar weak growth is seen for this strain on all other media except 1% complete and media containing 2-deoxy-D-glucose.

DS15 DS10 DS4 *sorB11* *sorA2*

MS3 MB3 MB2 JK3 DS20

BK2 MB2 YF4 JK1 PB1

MM1 DS4 *sorB11* *sorA2* DS15

PB1 DS20 H17A12 *biA1* *niiA4* DS20

JK3

the media surrounding a colony, by staining for enzyme activity produced by a colony, by the use of substrates which require the activity of derepressed enzymes to produce a toxic effect, and by suppression of loss-of-function mutations by the activity of derepressed enzymes. These methods have been outlined in detail in Chapter 3, Section 3.5.1 to 3.5.4.

5.2.3.1 α-amylases

sorA2, MB2, DS20 and DS15 showed significant clearing on medium containing 0.5% starch in the presence of 1% D-glucose, while DS4, JK3 and PB1 showed less clearing. The wild type strains did not clear at all and neither did *sorB11*, MS3, DS10, or MB3. BK2, MM1, YF4 and JK1 appeared to clear a small area at the perimeter of each colony (Figure 5.3). This result suggested that α-amylases were derepressed in some of these strains. The strains, BK2 and MM1, have also been shown to express higher levels of cellulases than wild type strains in induced, derepressed conditions (R. Lockington, pers. com.).

5.2.3.2 β-galactosidase

On medium containing 1% D-glucose, 0.5% lactose and X-gal, DS15, JK1 and DS20 produced faint blue colour indicating altered repression of β-galactosidase in these strains (result not shown).

5.2.3.3 Alcohol dehydrogenase 1

All strains were resistant to 2.5mM allyl alcohol in the presence of 1% D-glucose (Figure 5.4). MB3, which was originally selected on 2-deoxy-D-glucose plus ethanol, grew very poorly on this medium but also showed poor growth on ethanol alone and most other media tested. The other two strains which were originally selected on ethanol and 2-deoxy-D-glucose, MB2 and PB1, did not show poor growth on this medium which supported the idea that their resistance to 2-deoxy-D-glucose and ethanol was not related to expression of *alcA* in the presence of glucose.

Figure 5.5: Growth on medium containing 2-deoxy-D-glucose as the sole carbon source. The strains were inoculated to medium containing 0.01% 2-deoxy-D-glucose plus 10mM ammonium chloride and the appropriate supplements.

DS15 DS10 DS4 *sorB11* *sorA2*

MS3 MB3 MB2 JK3 DS20

BK2 MB2 YF4 JK1 PB1

MM1 DS4 *sorB11* *sorA2* MM1

PB1 DS20 H17A12 *biA1* *niiA4* DS20

JK3

5.2.3.4 suppression of *areA217*

None of the mutants selected in H17A12 strain, which contains the *areA217* mutation, were able to suppress *areA217* on medium containing 1% D-glucose plus acetamide or proline as the nitrogen source indicating that the expression of *amdS* or the *prn* genes was not derepressed in these mutants.

5.3 Growth on 2-deoxy-D-glucose as the sole carbon source

In other fungal species, mutants have been selected on 2-deoxy-D-glucose as the sole carbon source. The *A. nidulans* mutant strains were inoculated to plates containing 2-deoxy-D-glucose and 10mM ammonium tartrate. Nine of the thirteen strains, MB2, DS20, JK3, JK1, DS10, DS15, PB1, BK2 and MB3, were able to grow weakly on this medium, (Figure 5.5). This growth was not stronger than that seen on carbon free medium and thus represents lack of inhibition rather than utilisation.

5.4 2-deoxy-D-glucose resistance in the presence of a variety of carbon sources

The mutants were tested for growth, both with and without 0.01% 2-deoxy-D-glucose, on a variety of carbon sources. The carbon sources used ranged from derepressing (arabinose) to repressing (acetate). The classification of the carbon sources is as in Bailey (1976), and is based on the utilisation of L-proline and acetamide in *areA^r-1* strains, and the degree of ethanol supplementation of *pdhA-1* strains, allowed by each carbon source (Bailey, 1976). The results are shown in Figure 5.6. On medium containing acetate and 2-deoxy-D-glucose, the mutants selected in the strain H17A12, which carries the *areA217* allele, show strong growth but this is the only medium on which this affect is seen. Acetate is a strongly repressing carbon source and most of the alleles are growing on this medium in the presence of 2-deoxy-D-glucose. On medium containing sucrose and 2-deoxy-D-glucose, growth is the same as on medium containing sucrose only, and even the wild type strains and a strain containing *sorB2,* are growing. This result is also seen when the strains are grown on medium with glucose or glucose plus 2-deoxy-D-glucose.

Figure 5.6: Growth on a variety of repressing and derepressing carbon sources, with and without 2-deoxy-D-glucose.

The strains have been inoculated onto medium containing the following carbon sources, arranged in order from derepressing to repressing. The medium in the upper plate of each pair contains 0.01% 2-deoxy-D-glucose, while the medium in the lower plate contains the carbon source only. All media also contains 10mM ammonium tartrate and the appropriate supplements.

1- 1% L-arabinose.

2- 0.5% lactose

3- 1% ethanol

4- 1% raffinose

5- 1% maltose

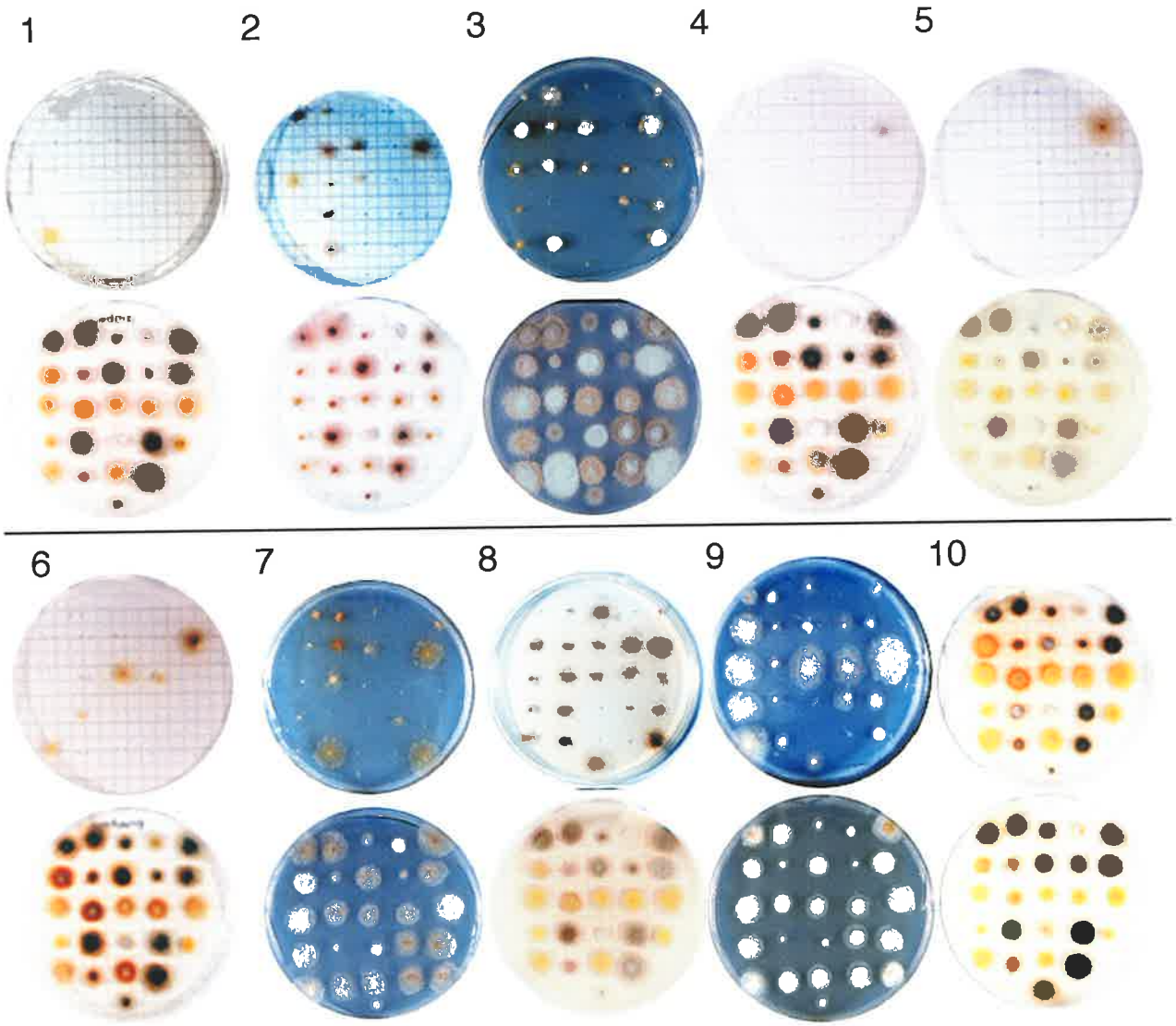
6- 1% fructose

7- 1% galactose

8- 50mM D-quinate

9- 1% acetate

10- 1% sucrose


1
2
3
4
5
6
7
8
9
10

These growth tests suggest that repressing carbon sources allow more resistance to 2-deoxy-D-glucose.

Genetic analysis was undertaken to determine the locus or loci contributing to 2-deoxy-D-glucose resistance in these strains.

5.5 Mapping the 2-deoxy-D-glucose resistant phenotype to a chromosome.

Each of the strains isolated from a *biA1, niiA4* parent was formed into a diploid with the strain MSF which carries a mutation marking each chromosome (Table 5.1).

Linkage Group	Marker
I	*yA1*, yellow conidia
II	*acrA1,* resistant on medium containing acriflavine
III	*galE8*, cannot utilise galactose
IV	*pyroA4*, pyridoxine requiring
V	*facA303*, cannot utilise acetate
VI	*sB3*, thiosulphate requiring
VII	*nicB8*, nicotinic acid requiring
VIII	*riboB2*, riboflavin requiring

Table 5.1: The genotype of the multiply marked *A. nidulans* strain, MSF which was used to form diploids with 2-deoxy-D-glucose resistant alleles.

Haploidisation of each diploid showed that the 2-deoxy-D-glucose resistant phenotype segregated with the colour locus *yA*, as all of the resistant colonies were green, and thus the mutations are located in linkage group I. The mutant strains isolated in the *yA1 suA1adE20 adE20; areA217; riboB2* strain were also made to form diploids with MSF. In this case, there were two markers in common to the two strains, *yA1* on linkage group I and *riboB2* on linkage group V111, so diploids were also formed with a *biA1; niiA4* strain. Haploids from the diploids with MSF indicated that the mutations were not on linkage groups 2-7 while those formed with *biA1; niiA4* segregated 2-deoxy-D-glucose resistance with yellow colour - the *yA1*

locus on linkage group I. These results indicated that all the 2-deoxy-D-glucose resistance mutants mapped to linkage group 1. All of these diploids were tested on medium containing quinate and 2-deoxy-D-glucose to determine whether the alleles conferring 2-deoxy-D-glucose resistance were dominant. Nine strains were able to grow when in a diploid with MSF or *biA1; niiA4* (Table 5.2), indicating dominance.

Diploids formed with MSF or *biA1; niiA4* which are resistant on medium containing 0.01% 2-deoxy-D-glucose and 50mM D-quinate.
Parent strain:H17A12
MM1
BK2
MS3
JK1
Parent strain:*biA1; niiA4*
DS4
DS10
DS15
DS20
JK3

Table 5.2: The 2-deoxy-D-glucose resistant alleles which are dominant in a diploid.

5.6 Are the 2-deoxy-D-glucose resistant mutants closely linked?

The 2-deoxy-D-glucose resistant mutants analysed in this chapter have pleiotropic phenotypes. To determine whether mutations at one or several loci were causing 2-deoxy-D-glucose resistance, the alleles were crossed to each other and fifty progeny from each cross were scored for resistant growth on medium containing 0.01% 2-deoxy-D-glucose plus 1% quinate. If the 2-deoxy-D-glucose resistance of two strains was caused by mutations at different loci, when the strains were crossed to each

other, some of the recombinant progeny would be wild type at both loci and therefore be sensitive on medium containing 0.01% 2-deoxy-D-glucose and 1% quinate. The frequency of this phenotype among the progeny would depend on whether the loci are linked and how closely. All of the progeny scored from each cross were able to grow on this medium which implied that only one gene, or closely linked genes were involved.

5.7 Are mutations at the *creA* locus responsible for resistance to 2-deoxy-D-glucose?

Haploidisation analysis indicated that the 2-deoxy-D-glucose resistant mutants mapped to chromosome I. As the *creA* locus is also on Chromosome I (Clutterbuck, 1993) three of the mutants, DS10, MB2 and BK2, were crossed to the *creA* allele, *creA204*. *creA204* is unable to grow on 2.5mM allyl alcohol and 1% D-glucose, has a compact morphology on 1% complete medium and is not resistant to 2-deoxy-D-glucose. The 2-deoxy-D-glucose resistant alleles are able to grow on 1% D-glucose and allyl alcohol. On complete medium, MB2 forms compact colonies but the other two mutants produce colonies with wild type morphology. The results from these crosses are shown in Table 5.3 and they suggest that there is no linkage between the locus conferring 2-deoxy-D-glucose resistance and *creA*.

Strains containing both the *creA204* mutation and a 2-deoxy-D-glucose resistant mutation were examined on medium containing 0.01% 2-deoxy-D-glucose and 50mM quinate, 50mM quinate alone, 0.5% starch plus 1% D-glucose and 1% complete medium . The compact phenotype of the *creA204* strain on complete medium was the same as the morphology of the double mutants (result not shown). On medium containing 0.01% 2-deoxy-D-glucose and 50mM quinate, resistance to 2-deoxy-D-glucose was the same for the double mutants as for the resistant parents (Figure 5.7A). Strains bearing the *creA204* allele are extremely derepressed for α-amylases on

	MB2 X *creA204*	DS10 X *creA204*	BK2 X *creA204*
Number of progeny scored	**51**	**48**	**52**
Genotypes:			
2-deoxy-D-glucose resistant, *creA*$^+$	17	7	11
2-deoxy-D-glucose sensitive, *creA204*	14	13	21
2-deoxy-D-glucose resistant, *creA204*	10	8	11
2-deoxy-D-glucose sensitive, *creA*$^+$	10	20	9
% Recombinant	**40%**	**›50%**	**40%**

Table 5.3 : The progeny from crosses between three 2-deoxy-D-glucose resistant mutants and *creA204* indicated that the two loci were not linked on Chromosome I.

medium containing 0.5% starch plus 1% D-glucose. MB2 and BK2 are also significantly derepressed on this medium for α-amylases compared to wild type strains but much less so than *creA204*. Figure 5.7B shows that the amount of starch clearing around the double mutant colonies between *creA204* with MB2 or BK2 is greater than for *creA204* alone or for *creA204* with DS10 which is not derepressed for α-amylases. MB2, BK2 and DS10 grew strongly on medium containing 50mM D-quinate but the *creA204* strains were weaker on this medium. The BK2;*creA204* and MB2;*creA204* double mutants showed intermediate levels of growth, and the DS10;*creA204* double was similar to the *creA204* parent (Figure 5.7C). Thus the double mutant phenotypes appear to be additive, and there is no indication of genetic interaction.

Figure 5.7: Growth tests of double mutant strains containing 2-deoxy-D-glucose alleles with the *creA204* allele.

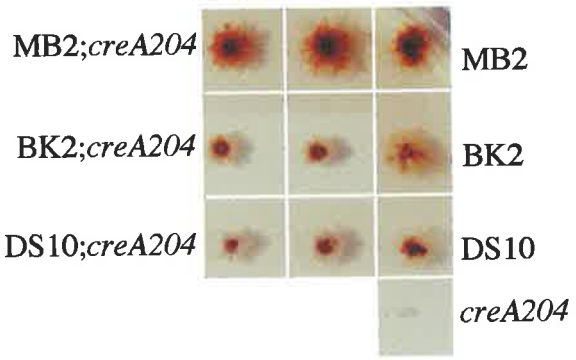
A: 0.01% 2-deoxy-D-glucose , 50mM D-quinate

B: 50mM D-quinate

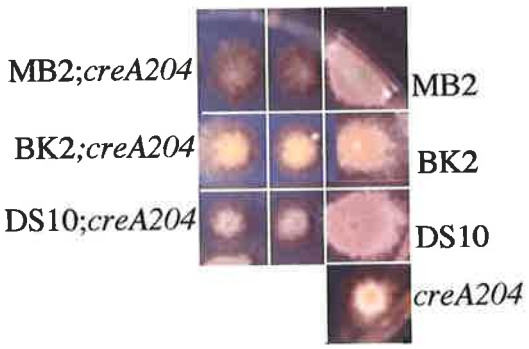
C: 0.5% starch plus 1% D-glucose, stained with iodine solution

All media also contains 10mM ammonium tartrate and the appropriate supplements.

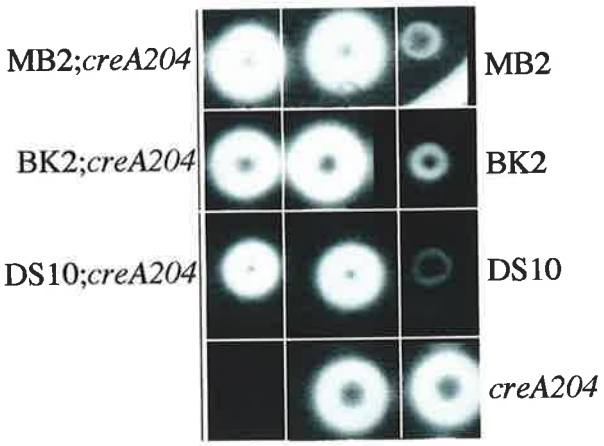
A


MB2;*creA204*
MB2
BK2;*creA204*
BK2
DS10;*creA204*
DS10
creA204

B


MB2;*creA204*
MB2
BK2;*creA204*
BK2
DS10;*creA204*
DS10
creA204

C


MB2;*creA204*
MB2
BK2;*creA204*
BK2
DS10;*creA204*
DS10
creA204

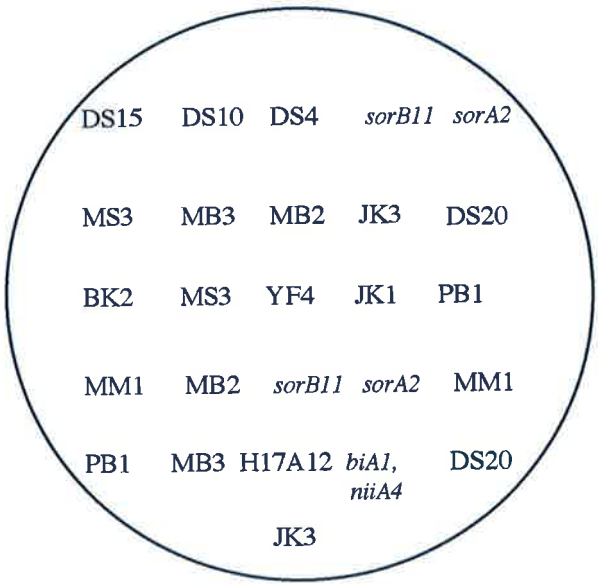
5.8 Are the mutations which confer 2-deoxy-D-glucose resistance occurring at the *sorA* locus?

The cross between *creA204* and the 2-deoxy-D-glucose resistant alleles indicated a recombination frequency of approximately 30% between the 2-deoxy-D-glucose resistant mutants and the colour locus *yA*, which suggested linkage to this locus. *yA* is on the opposite arm of chromosome I to *creA*. On the linkage map of Chromosome I of *A. nidulans* (Clutterbuck, 1993) one of the genes linked to *yA* in the 30 map-unit range, is *sorA*. Mutants in *sorA* are resistant to L-sorbose and also to 2-deoxy-D-glucose (See section 1.3.4) (Elorza and Arst, 1971). A mutant allele, *sorA2* was obtained from the Fungal Genetics Stock Centre (Number 705) and crossed to one of the 2-deoxy-D-glucose resistant alleles, YF4. *sorA2* produces strong resistant colonies on 2-deoxy-D-glucose whereas YF4 produces colonies with weaker resistance. 200 progeny were scored and all were resistant to 2-deoxy-D-glucose. The resistant phenotype of each colony was either strong like the *sorA2* parent, or weak like the YF4 parent. None of the colonies appeared to be stronger than *sorA2* which might happen if mutations in two separate genes were inherited together in one strain to give an additive effect, and none of the colonies were sensitive to 2-deoxy-D-glucose which would be the phenotype of a strain with wild type copies of both *sorA* and the 2-deoxy-D-glucose resistance locus. These results suggested that the 2-deoxy-D-glucose resistant mutants were either very tightly linked, or allelic, to *sorA*.

The alleles were inoculated onto medium containing 0.05% L-sorbose, 5mM urea, and 1% glycerol to test their resistance to L-sorbose (Elorza and Arst, 1971) and also onto medium containing 0.05% L-sorbose, with 10mM ammonium tartrate and 50mM D-quinate, which are the carbon and nitrogen sources used in the 2-deoxy-D-glucose medium. The sorbose-resistant phenotype was very subtle on this medium (Figure 5.8). The two parent strains, *biA1; niiA4* and H17A12, were tolerant of this level of L-sorbose in the medium and, except for *sorA2* which formed the strongest colony, the

Figure 5.8: Growth testing the 2-deoxy-D-glucose resistant alleles for resistance to L-sorbose.

The alleles were inoculated to medium containing 0.05% L-sorbose, 1% glycerol and 5mM urea plus appropriate supplements.


DS15 DS10 DS4 *sorB11* *sorA2*
MS3 MB3 MB2 JK3 DS20
BK2 MS3 YF4 JK1 PB1
MM1 MB2 *sorB11* *sorA2* MM1
PB1 MB3 H17A12 *biA1*, *niiA4* DS20
JK3

mutants showed only slightly stronger growth than the parent strains. The 2-deoxy-D-glucose-resistant phenotype in these mutants, where the presence of traces of 2-deoxy-D-glucose (0.01%) in the medium completely inhibits growth of wild type strains, is much more striking than their sorbose resistance. There is no clear correlation between the degree of resistance to 2-deoxy-D-glucose and L-sorbose. A clear example of this can be seen with DS20 and JK3 which are very resistant to 2-deoxy-D-glucose but are not the strongest colonies on L-sorbose (Figure 5.1A and 5.8).

5.9 Extreme 2-deoxy-D-glucose resistant alleles.

Extremely resistant sectors formed spontaneously on 0.01% 2-deoxy-D-glucose and 50mM quinate from already resistant colonies of MB2. These sectors sporulated well and formed large colonies on all media tested (Figure 5.9).

To determine whether these strains contained a second mutation at the *sorA* locus or a mutation in another gene, one of the purified sectors ($MB2^R$) was crossed to a wild type strain (*biA1; niiA4).* On complete medium the progeny from this cross resembled either the wild type strain or had the enhanced growth of the $MB2^R$ strain.

When tested on medium containing 0.01% 2-deoxy-D-glucose and 50mM quinate, approximately half the progeny were resistant and of these, some were resistant like MB2 and others were extremely resistant like $MB2^R$. The presence of the MB2-like resistant phenotype among the progeny indicated that the mutation causing extreme resistance in $MB2^R$ was segregating from the MB2 mutation. However, as only half of the progeny were resistant to 2-deoxy-D-glucose, it seemed that the second mutation was not able to confer 2-deoxy-D-glucose resistance by itself. There are several examples of the presence of two (or more) mutations in a strain producing increased resistance to 2-deoxy-D-glucose even though one of the mutations does not

Figure 5.9 Extreme 2-deoxy-D-glucose resistant alleles.

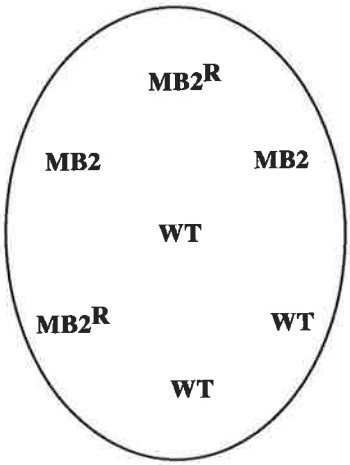
A: 1% Complete medium

B: 50mM D-quinate

C: 50mM D-quinate plus 0.01% 2-deoxy-D-glucose

B and C also contain 10mM ammonium tartrate plus the appropriate supplements.

A



B

C

produce resistance by itself. Double mutants strains carrying the *sorA2* and *sorB11* alleles are more resistant to 2-deoxy-D-glucose than *sorA2* strains alone, even though strains containing *sorB11* are not resistant (Elorza and Arst, 1971). Triple-mutant strains carrying the alleles *malA*$^-$, *sorA*$^-$, and *creB*$^-$, or *creC*$^-$ (Arst, 1981) are extremely resistant to 2-deoxy-D-glucose and grow very poorly on D-glucose as the sole carbon source, even though the presence of just one of the *malA*$^-$, *creB*$^-$ or *creC*$^-$ alleles does not confer 2-deoxy-D-glucose resistance to a strain. MB2^R was inoculated onto medium containing maltose as the sole carbon source to determine whether it may carry a *malA* allele, but growth was not different to that of MB2. MB2^R was also inoculated to medium containing quinate as a sole carbon source and growth was not affected, indicating that it was unlikely to carry a mutation like *creB15* or *creC27*, which results in reduced quinate utilisation. Thus the mutation in MB2^R is unlikely to be in *malA, creB* or *creC.* Mutations which affect both the high and low affinity glucose transport systems, have been shown to result in extremely resistant 2-deoxy-D-glucose strains in *N. crassa* (Allen *et al.* 1989). Hexose transport and uptake in MB2^R remains to be examined.

5.10 Can the *Neurospora crassa rco-3* gene complement *sorA* mutant alleles?

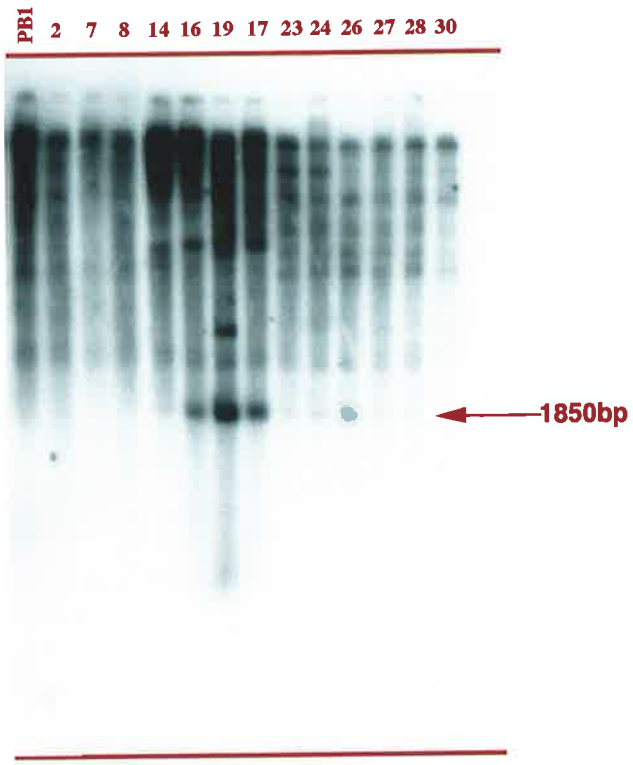
The phenotype of the *sorA* alleles of *A. nidulans* selected for their resistance to 2-deoxy-D-glucose, was similar to an *rco-3* mutant of *N. crassa* (Madi *et al.* 1997). This mutant *rco-3*^{1}, had a pleiotropic phenotype which included resistance to L-sorbose and 2-deoxy-D-glucose, and derepression for functions which are usually repressed by glucose in wild type strains, for example, conidiation in liquid medium containing glucose, xylose, glycerol or fructose. Strains bearing *rco-3*^{1}, were capable of strong growth on glucose, xylose, glycerol and fructose which implied that glucose transport was not affected (Madi *et al.* 1997) but glucose uptake experiments in another mutant, *rco-3*^{2}, showed that glucose uptake was reduced. The protein produced by *rco-3* has the twelve-transmembrane-domains structural feature of glucose transporters from *S. cerevisiae* such as Snf3p, Rgt2p and Hxt2p (Celenza *et*

Figure 5.10: Analysis of strains resulting from the transformation of PB1 with *rco-3* from *N. crassa.*

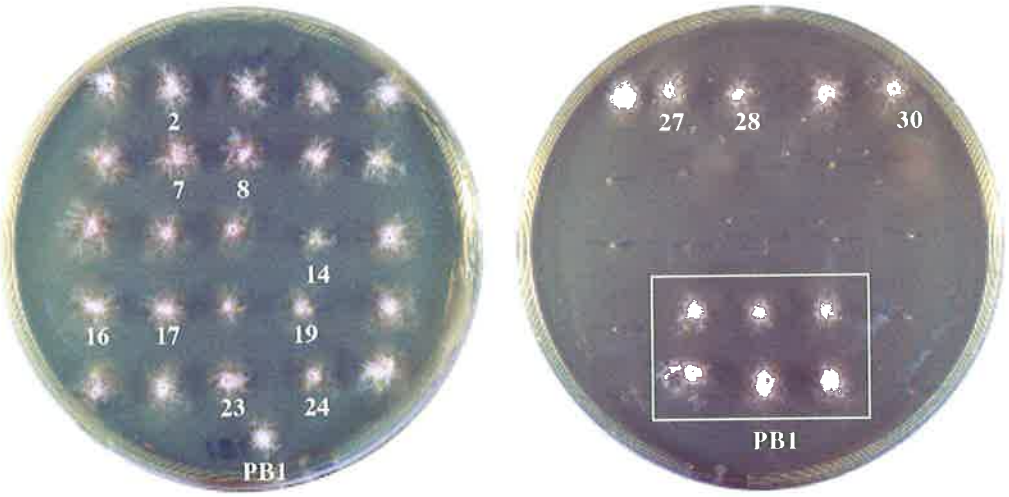
A: The strain PB1 was co-transformed with *rco-3* from *N. crassa* and the *riboB*$^{+}$ gene from *A. nidulans*. Riboflavine independent transformants were selected and genomic DNA was prepared from thirteen transformants. It was digested with Xho1, electrophoresed on a 0.8% agarose gel and Southern transferred to HybondN^{+}. The membrane was hybridised with a ^{32}P-labelled, 1850 bp Xho1 fragment from *rco-3*. The 1850bp band from *rco-3* which hybridises to the probe is shown with an arrow. Transformants 14, 16, 19 and 24 contain the 1850bp band which is contained within the transforming sequence, and thus hybridises strongly to the probe. Transformant 19 has the most copies of *rco-3*, while 16 and 17 also contain many copies. The strains 14, 23, 24, 27, and 28 may also have been transformed at a low frequency judging by the faint band seen at 1850bp.

B: The transformants were inoculated onto two plates containing 0.01% 2-deoxy-D-glucose plus 50mM D-quinate, 10mM ammonium tartrate and the appropriate supplements. The strength of their resistance on this medium was compared with PB1, the parent strain of the transformants.

A


PB1
2
7
8
14
16
19
17
23
24
26
27
28
30
1850bp

B


2
7
8
14
16
17
19
23
24
PB1
27
28
30
PB1

al. 1988; Ozcan *et al.* 1996; Kruckeberg and Bisson, 1991). However, as *rco-3*^{1} and *rco-3*^{2} have different transport phenotypes, there may also be a regulatory role for the product of this gene as has been demonstrated for Snf3p, Rgt2p and Hxt2p. *sorA* mutants are also able to utilise many carbon sources and do not appear to have glucose transport defects. Other *N. crassa* mutants which have phenotypic similarities to the *sorA* alleles of *A. nidulans* are *dgr-1-4,* which have not been cloned (Allen *et al.* 1989). Preliminary investigation has suggested that *rco-3* may be allelic to *dgr-1* and/or *dgr-2* as they all map to the centromere of linkage group I and have defective conidiation in liquid culture. *rco-3* was tested to determine whether it is a functional homologue of *sorA*.

PB1 was chosen to be transformed with *rco-3* to determine whether it could complement a *sorA* allele, because the 2-deoxy-D-glucose resistant phenotype of PB1 is recessive to the wild type allele in a diploid.

A plasmid, pFH$_2$, which contains the *rco-3* gene from *N. crassa* and surrounding sequences including the promoter, was co-transformed into PB1 with pPL3 which contains the *riboB*$^{+}$ gene. PB1 is riboflavine requiring. pPL3 transformants were selected on medium lacking riboflavine and then Southern analysis was used to determine whether the strains also contained copies of the pFH$_2$. Genomic DNA was prepared for Southern analysis from thirteen transformants and they were tested for resistance to 2-deoxy-D-glucose on medium containing 0.01% 2-deoxy-D-glucose and 50mM D-quinate (Figure 5.10).

Genomic DNA was digested with Xho1, electrophoresed on a 0.8% agarose gel, Southern transferred and hybridised with the ^{32}P-labelled 1.85kb Xho1 fragment which contains *rco-3*. Several strains were transformed with copies of *rco-3* but no correlation was seen between the presence of *rco-3* in a transformant and its

resistance to 2-deoxy-D-glucose. It was concluded that *rco-3* from *N. crassa* does not complement *sorA* alleles from *A. nidulans.*

5.11 The isolation of a genomic clone of the *A.nidulans* homologue of *rco-3* from *N. crassa.*

A genomic library made with *A. nidulans* DNA and cloned into lambda gem 11 (M. Katz, 1994), was screened with a 1.85kb Xho1 fragment containing *rco-3* from *N. crassa*. Five strongly hybridising plaques were selected and purified (Figure 5.11, A). DNA was prepared from the Lambda clones, digested with a range of enzymes and Southern analysis was performed to determine whether the clones contained the same or different inserts, and to identify smaller fragments which contained sequences that hybridised to *rco-3* (Figure 5.11, B and C). Southern analysis showed that clones #4-6 probably contained a BamH1 fragment of similar size, and clone #2 was unique. The 2.7kb EcoR1 fragment from clone #2 was subcloned into pBluescript, and sequenced (Figure 5.12, A and B).

Sequencing and conceptual translation revealed that the cloned fragment was not homologous to *rco-3* but contained twenty-nine consecutive glutamines. The fragment hybridised to the *rco-3* probe because there is a region encoding several glutamines (22/31 amino acids are glutamines) at the 3' end of *rco-3.* Both of the codons for glutamine - CAA and CAG - were similarly represented in the DNA sequence of the cloned fragment and *rco-3*. The glutamine rich region of *rco-3* has not been identified as an important functional domain of this protein (Madi *et al.* 1997, Altschul *et al.* 1990).

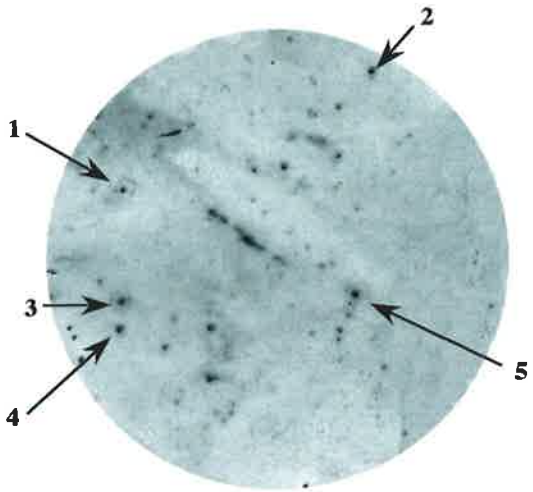
To avoid identifying more glutamine rich proteins and to increase the chance of detecting an *rco-3* homologue, the same library was screened again, but this time with a fragment of *rco-3* from *N. crassa* from which the region encoding the 29 glutamines had been removed (a 2 kb Sma1-Apa1 fragment). The other lambda clones which

Figure 5.11 Screening an *A. nidulans* genomic library for a homologue to *rco-3* from *N. crassa*

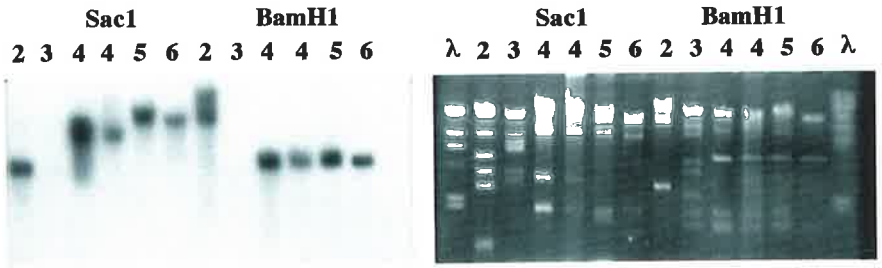
A: An *A. nidulans* lambda genomic library (M. Katz, 1994) was screened with a ^{32}P-labelled 1850bp fragment from *N. crassa* containing the *rco-3* gene. Several strongly hybridising plaques were seen. Five (numbered 1-5) were purified and DNA was prepared.

B and C: DNA from each sample was digested with Sac1, BamH1, EcoR1 and EcoR1plus Sac1, electrophoresed on a 0.8% agarose gel, Southern transferred to Hybond N^+, and hybridised with the ^{32}P-labelled 1850bp fragment from *N. crassa* containing the *rco-3* gene. Autoradiographs of the digests showed that the DNA from lambda #3 did not contain any hybridising bands and that lambdas 4-6 contained similar inserts. 2 and 4 were analysed further.

A


1
2
3
4
5

B


Sac1
BamH1
2 3 4 4 5 6 2 3 4 4 5 6
Sac1
BamH1
λ 2 3 4 4 5 6 2 3 4 4 5 6 λ

C

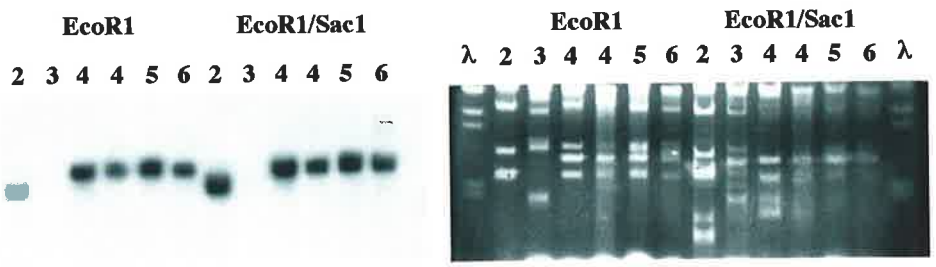

EcoR1
EcoR1/Sac1
2 3 4 4 5 6 2 3 4 4 5 6
EcoR1
EcoR1/Sac1
λ 2 3 4 4 5 6 2 3 4 4 5 6 λ

Figure 5.12: Strategy for sequencing lambda clones

A:

Left: Agarose gel photo Right: Southern

Lane 1- lambda clone 2 digested with EcoR1

Lane 2- lambda clone 2 digested with Sac1

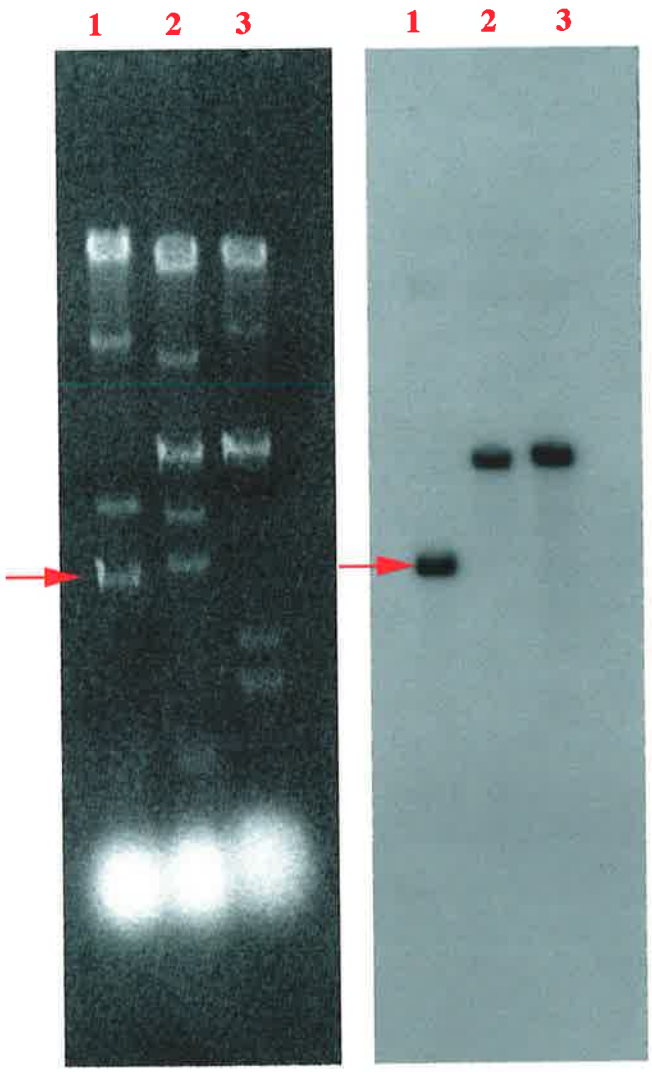
Lane 3- lambda clone 4 digested with BamH1.

Southern analysis showed that a 2.7kb EcoR1 band and a 5.5kb.Sac1 band from #2 contained sequences which hybridised to the ^{32}P-labelled 1850bp fragment from *N. crassa* containing the *rco-3* gene. For #4, a 5.5kb BamH1 band hybridised. The 2.7kb EcoR1 fragment from #2 and the >5.5kb Sac1 band from #4 were subcloned into pBluescript. The EcoR1 fragment from #2 was sequenced.

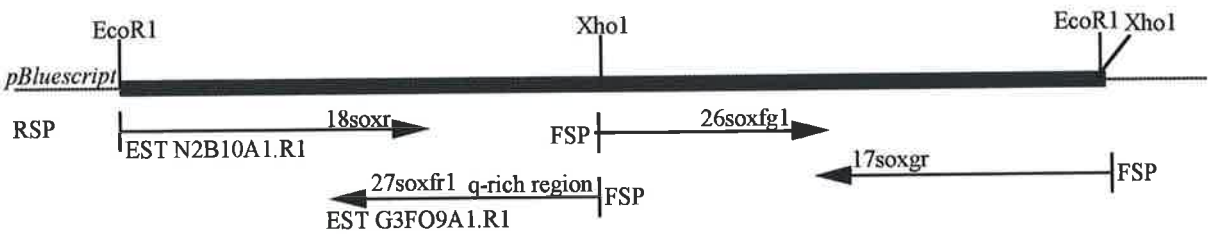
B:

The subcloned EcoR1 fragment was sequenced from either end using the forward (FSP) and reverse (RSP) pUC/M13 primers with pBluescript. The internal regions were sequenced with these primers by deleting the Xho1 fragment from two different EcoR1 subclones which had the insert in opposite orientations. The position of the glutamine rich region is indicated. Two EST's were retrieved from the *A. nidulans* data base in WEBangis and their positions are also shown.

A


1
2
3
1
2
3

B


EcoR1
Xho1
EcoR1 Xho1
pBluescript
RSP
18soxr
EST N2B10A1.R1
FSP
26soxfg1
17soxgr
FSP
27soxfr1 q-rich region
FSP
EST G3FO9A1.R1

hybridised to the original *rco-3* probe (4, 5 and 6) were not subcloned and sequenced. Eleven weakly hybridising plaques were selected and purified. Digested lambda DNA from these eleven plaques was Southern transferred and re hybridised with the ^{32}P-labelled *rco-3* probe, but no bands were seen (Results not shown).

rco-3 was transformed into the *sorA* allele concurrently with the isolation of a genomic clone from *A. nidulans* using a fragment of *rco-3* from *N. crassa* as a probe. The transformation experiments showed that *rco-3* does not complement a *sorA* mutant, and as the 11 weakly hybridising plaques did not contain an *rco-3* homologue, no further attempts were made to identify this gene in the *A. nidulans* library.

5.12 Summary

The 2-deoxy-D-glucose resistant mutants from *A. nidulans* which have been discussed in this chapter, have been assigned to linkage group I by haploidisation analysis and shown by crosses to be either allelic or very closely linked to each other. To determine whether *sorA* on Chromosome I is the locus affected in these mutants, one mutant was crossed to a strain containing the *sorA2* allele and the result indicated that the mutants are allelic or very tightly linked to *sorA*.

Diploids were made between the recessive mutations MB2, MB3, YF4 and PB1 and *sorA2* and analysed for resistance to 2-deoxy-D-glucose. The diploids were resistant which suggested that these mutants and *sorA* were alleles. It has been previously reported that the *sorA1* and *sorA2* alleles are recessive (Arst and Cove, 1971). Linkage analyses suggest that the rest of the 2-deoxy-D-glucose resistant alleles are at least tightly linked to *sorA,* and thus they have been referred to as *sorA* alleles in this thesis.

If all the mutants are affected in the same gene then it is likely that not all are complete loss-of-function alleles because they are so phenotypically variant. The extent of the involvement of the *sorA* locus in carbon catabolite repression is not clear. Some alleles clearly lead to α-amylases showing derepressed expression in the presence of D-glucose, and the expression of β-galactosidase in the presence of D-glucose has been seen for three alleles. However, none of the *sorA* mutants are sensitive to 2.5mM allyl alcohol in the presence of D-glucose and none suppress the effects of *areA217* on medium containing proline or acetamide.

A phosphatase specific for 2-deoxy-D-glucose-6-phosphate is expressed at high levels in some 2-deoxy-D-glucose resistant mutants in *S. cerevisiae* (Sanz *et al.* 1991) and results in low levels of 2-deoxy-D-glucose-6-phosphate in the cell. These mutants show derepression for some enzymes usually subject to carbon catabolite repression such as invertase, maltase and α-glucosidase in the presence of 2-deoxy-D-glucose but, unlike the *sorA* alleles from *A. nidulans*, not in the presence of glucose (Randez-Gil *et al.* 1995). It has been proposed that inhibition of growth on 2-deoxy-D-glucose is caused by 2-deoxy-D-glucose-6-phosphate, and subsequently demonstrated that overexpression of the phosphatase produces the 2-deoxy-D-glucose resistant phenotype. Analysis of the phosphorylation status of 2-deoxy-D-glucose-6-phosphate in the *sorA* mutants or identification of the phosphatase may help to identify the defects in *sorA* alleles from *A. nidulans*.

5.13 Future Work

Some of the mutants identified as 2-deoxy-D-glucose resistant strains in *S. cerevisiae* have subsequently been shown to be involved in the signalling pathway between the sensing of glucose in the extra-cellular environment and transcriptional repression by DNA binding proteins. One of these mutants, Reg1p is a regulatory subunit of the phosphatase, Glc7p which is proposed to act on Mig1p and Snf1p. Over-expression of a phosphatase specific for 2-deoxy-D-glucose-6-phosphate has been implicated in

2-deoxy-D-glucose resistance in *S. cerevisiae.* There appears to be a link between phosphatase activity and resistance to 2-deoxy-D-glucose, and therefore *REG1* may encode a functional homologue of *sorA*. The phenotype of Reg1p mutants is similar to *sorA* alleles. *GRR1* and *HXK2* mutants also have a similar phenotype and are other possible candidates for functional homologues of *sorA*. An analysis of glucose uptake in the 2-deoxy-D-glucose mutants isolated in *A. nidulans* would be relevant because many of the 2-deoxy-D-glucose resistant mutants isolated in other fungal species have shown impaired glucose transport.

The degree of involvement of the *sorA* locus in glucose repression is not clear but there are several lines of evidence to suggest that SorA operates somewhere in this system. SorA mutants are resistant to the glucose analogues L-sorbose and 2-deoxy-D-glucose, and the phenotype of *sorA* mutants on medium containing 2-deoxy-D-glucose varies depending on the type of carbon source (repressing versus derepressing) also present in the medium. Strains carrying *sorA* alleles become less resistant to 2-deoxy-D-glucose in the presence of increasingly derepressing carbon sources. The main evidence which suggests that SorA is involved in glucose repression is the derepression of enzymes usually subject to glucose repression seen in several *sorA* alleles.

Selection of mutants on 2-deoxy-D-glucose has identified several genes in *S. cerevisiae* and *N. crassa* but only *sorA* has been identified in the screen presented in this chapter. As these mutants from other species, which have similar phenotypes to *sorA* alleles, have been assigned to roles in glucose repression, further investigation into the function of SorA could provide more information about carbon catabolite repression in *A. nidulans*.

Chapter 6 Discussion

Carbon catabolite repression has been studied extensively in *S. cerevisiae* but much less research has been done in this area in *A. nidulans*. As the body of knowledge about carbon catabolite repression in *A. nidulans* has increased, the similarities and differences between the two species, with respect to the regulation of this system, have become more apparent. In *A. nidulans*, CreA is the main DNA binding protein that is required to effect repression. It has two C_2H_2 zinc fingers which have 84% similarity to the Mig1p repressor from *S. cerevisiae* and binds to a similar consensus sequence in the promoters of the genes it regulates to the Mig1p binding site (Dowzer and Kelly, 1991; Lundin *et al.* 1994). Outside of the DNA binding domain however, the proteins are not very similar except for a region located at the C-terminus of each protein which mediates repression, and a small group of basic residues which lie adjacent to the second zinc finger (Ostling *et al.* 1996). Alignments between Mig1p from *S. cerevisiae* and the homologous proteins from *K. lactis* and *K. marxianus* show much higher identity across the whole protein than alignments between Mig1p and CreA (Cassart *et al.* 1997). Similarly, CreA has much higher identity with homologues from other filamentous fungi than with Mig1p.

The first aim of this work was to construct a strain bearing a precise CreA deletion, and to analyse its phenotype and compare it with a series of CreA alleles which were thought to also represent functionally null CreA alleles. The viable CreA-null strain which resulted has allowed more comparisons to be made between Mig1p and CreA. Strains bearing null alleles of Mig1p and CreA vary in the amount of derepression seen for enzymes which are usually subject to carbon catabolite repression. The level of expression of genes which are regulated by Mig1p such as *SUC2,* is not fully derepressed in Mig1p null strains, whereas gene expression in CreA null strains is highly derepressed. Recent work has identified a second repressor in *S. cerevisiae*, Mig2p, which is involved in the regulation of several genes (Lutfiyya and Johnston, 1996; Lutfiyya *et al.* 1998), and whose presence was suspected long before it was

identified, due to the 'not fully derepressed' phenotype of *mig1* mutants. Strains carrying deletions of both Mig1p and Mig2p show full derepression of systems regulated by both of these repressors. None of the genes examined are regulated by only Mig1p or Mig2p, which has led to the suggestion that the two proteins can partially substitute for each other (Lutfiyya *et al.* 1998). The phenotype of a Mig2 null strain is very subtle and can only be detected in a Mig1p null or mutant strain (Lutfiyya *et al.* 1996). Null alleles of *MIG1* and *creA* have very different morphologies. Strains bearing the *mig1* allele are morphologically normal, but can be identified by their resistance to 2-deoxy-D-glucose, whereas CreA null strains are sensitive to 2-deoxy-D-glucose, and on complete medium they form very compact, barely sporulating colonies which can be readily distinguished from wild type strains or strains carrying other CreA alleles. The derepressed phenotype of a CreA null strain and its extreme morphology, suggests that proteins which can functionally substitute for CreA are not present in *A. nidulans.* Nevertheless, the CreA null strain has only recently been constructed and subtle suppressors of this strain may be revealed which are the *A. nidulans* equivalent of Mig2p.

The location of Mig1p in the cell is regulated in response to glucose. When glucose is present, Mig1p is found in the nucleus, is not phosphorylated, and glucose repression occurs. In the absence of glucose, Mig1p is located in the cytoplasm, is phosphorylated and glucose repression is relieved (DeVit *et al.* 1997). The location of Mig1p in the cell was shown using GFP-tagged Mig1p, and and its phosphorylation status was shown using antibodies specific for Mig1p (DeVit *et al.* 1997). Another aim of this work was to detect CreA with antibodies in order to address the issue of CreA localisation and regulation in response to glucose (and other aspects of CreA). Two different strategies have been described in this thesis. Visualisation of GFP-tagged CreA has also been attempted (R.A. Shroff, pers. com.). The inability to visualise CreA with antibodies has suggested that the protein has properties which make it difficult to detect, such as being targeted for rapid degradation.

In *S. cerevisaie*, glucose repression occurs at three main levels. Repression of the expression of genes required to utilise other carbon sources has been discussed at length, but two other mechanisms also operate in the cell. These are: accelerated protein degradation, which is known as carbon catabolite inactivation, and inhibition of the activity of enzymes which are specifically related to sugars (Klein *et al.* 1998). Carbon catabolite inactivation provides a means of regulating protein levels so that rapid changes in the glucose status of the cell can be rapidly responded to by the initiation or cessation of protein degradation. An example of this exists in *S. cerevisiae* where recent work has indicated that the repressor Rgt1p, which represses the expression of glucose transporter genes in the absence of glucose, may be modified by ubiquitin and thus targeted for degradation (Li and Johnston, 1997; Bai *et al.* 1996). As mentioned in Chapter 4, CreA from *A. nidulans* does not have any sequences which correspond to a high scoring PEST sequence, and neither does Cre1 from *T. reesei* (PEST sequences provide a signal for protein degradation) but CreA from *A. niger* does. Protein degradation and PEST sequences are not fully characterised and CreA from *A. nidulans* may be degraded by a different protease which targets a feature of the protein not yet identified.

Cre1 from *T. reesei* has been successfully detected in total protein extracts (20mg - 100mg) with antibodies raised against 10mg of a 9 amino acid synthetic polypeptide. It is difficult to explain why CreA cannot be detected in *A. nidulans* when the two proteins share 55.6% similarity and the sequence used to raise the synthetic polypeptide is identical in both (Strauss *et al.* 1995). CreA from *A. nidulans* and *A. niger* are different with respect to PEST sequences, even though the two proteins share 90% similarity, and perhaps the levels of Cre1 in *T. reesei* are regulated in a different way to CreA in *A. nidulans* such that Cre1 can be detected with antibodies and CreA can not. There are other properties which could result in CreA being difficult to detect, such as lack of antigenicity or poor affinity of CreA antibodies for their target, but these

factors would not apply to the MRGS.His6-tagged CreA. It is possible therefore that CreA is not regulated by localisation and phosphorylation as has been demonstrated for Mig1p (DeVit *et al.* 1997).

The third aim of this thesis was to identify more genes in *A. nidulans* which are involved in carbon catabolite repression. Studies in *S. cerevisiae* have revealed three genes which interact with the repressor, Mig1p. The first of these is the protein kinase, Snf1p which is repressed by glucose and is required for release from glucose repression by Mig1p (Jiang and Carlson, 1996, Celenza and Carlson, 1986). Screens for carbon catabolite repression mutants in *A. nidulans* have not identified any homologues of Snf1p however, which implies either that a protein kinase does not have a role in carbon catabolite repression, or that several kinases may be involved rather than one. Homologues of Snf1p have been identified in many species including human (AMP-activated protein kinase, Carling *et al.* 1994), *Arabidopsis* spp. (Alderson *et al.* 1991) and *C. elegans* (Guo and Kemphues, 1995). Southern blots of digested genomic DNA from *A. nidulans* have been probed with the radioactive *SNF1* gene from *S. cerevisiae* and several hybridising bands were present (R.A. Shroff, pers. com.). Thus, there are *SNF1* similar sequences in the *A. nidulans* genome and they may represent a family of protein kinases. One *creA* allele results in a 'failure to derepress' phenotype similar to a *SNF1* mutant strain in *S. cerevisiae* (Shroff, 1997). This allele was produced by transforming the CreA null strain with a construct in which the *RGR1*-like sequence was deleted and the repression domain at the C-terminus of the protein was retained. If a family of protein kinases phosphorylates the sites within this sequence, then a mutation in just one kinase would not have a detectable phenotype thus explaining why a *SNF1* homologue has not been identified.

Another aspect of repression by Mig1p in *S. cerevisiae,* which has not been demonstrated for CreA in *A. nidulans* is the recruitment of the co-repressors, Tup1p and Ssn6p to effect carbon catabolite repression. The sequence of an *A. nidulans*

homologue of *TUP1* is present in the *A. nidulans* EST data base and has been cloned and sequenced (R. Lockington, pers.com.) but outside of the WD40 repeat region, the two proteins are not very similar in sequence, and there is no other information to suggest that they are functionally similar. Strains carrying mutants in either Tup1p or Ssn6p, have high levels of expression of the many genes they repress, and at much higher levels than Mig1p mutant strains (Tzamarias and Struhl, 1994, Redd *et al.* 1997).

A. nidulans strains bearing mutant alleles of *creB* and *creC* also show derepression for some enzymes usually subject to carbon catabolite repression but the levels of derepression are not as high as seen for most *creA* alleles nor is the range of enzymes affected as broad (Hynes and Kelly, 1977). Furthermore, *creB* and *creC* mutant strains have characteristics, such as poor growth on some carbon sources and resistance to molybdate, which have not been described for Tup1p and Ssn6p mutant strains. Phenotypic analyses of a double mutant strain carrying the *creAΔ4* and *creB15* alleles indicate that there is an additive effect of having both *creAΔ4* and *creB15* mutations in the one strain which is more severe than the effect of either allele alone. This suggests that rather than operate as a heteromeric protein, CreB15 and CreAΔ4 operate either in different parts of a single mechanism or in different pathways. It has been proposed that the failure to isolate a double mutant strain bearing *creC27* and *creAΔ4* is the result of a 'too severe to be viable' phenotype, which would indicate that *creC* and *creA* also operate as more than heteromeric proteins or subsequent steps in a single pathway. This evidence also implies that *creB* and *creC* do not produce functional homologues of Tup1p and Ssn6p. Moreover, mutational screens in *A. nidulans* have not produced alleles of any other gene which are carbon catabolite derepressed (which would be possible candidates for Tup1p or Ssn6p homologues), except for the *sorA* alleles described in this thesis.

These *sorA* alleles have a wide range of phenotypes which include colony morphology, the derepression of some glucose repressed enzymes in the presence of glucose which

varies for each allele, and a broad range of resistance to 2-deoxy-D-glucose. Colony morphology extends from compact to small to wild type, and the resistance to 2-deoxy-D-glucose takes two forms - colony size on medium containing 2-deoxy-D-glucose, and the number of carbon sources on which the colonies will grow in the presence of 2-deoxy-D-glucose. This heterogeneity of phenotypes indicates a regulatory role for SorA.

There are three pieces of evidence which suggest that the *sorA* locus may be a part of the carbon catabolite repression system in *A.nidulans*. The first is the derepression of some enzymes in the presence of glucose shown by some *sorA* alleles. The second is the interaction between alleles of *creB* (or *creC*), *malA* and *sorA* which has been reported to produce extremely resistant 2-deoxy-D-glucose strains. *creB* and *creC* have a role in carbon catabolite repression of some genes. *malA* may also play a significant role, as recent evidence suggests that a mutation in *malA* suppresses the morphology of a *creA*-null strain (Shroff, 1997). A mutation in *creB* has also recently been identified as a suppressor of another *creA* allele, *creA306*. A more circumstantial association between *sorA* and carbon catabolite repression is the fact that the alleles studied in this thesis were identified for their resistance to 2-deoxy-D-glucose and many genes from *S. cerevisiae* which are involved in carbon catabolite repression have also been identified in this way.

The screen for 2-deoxy-D-glucose resistant mutants in *A. nidulans* has only identified the *sorA* gene whereas similar screens in *N. crassa* and *S. cerevisiae* have identified several loci. In *S. cerevisiae,* these loci have been well characterised and they have a role in the carbon catabolite repression signalling pathway between the glucose sensors and the repressor proteins. It is possible that *sorA* may encode the functional homologue of one of these genes from *S. cerevisiae* as *sorA* mutant alleles have a phenotype very similar to mutant alleles of *GRR1* and *REG1*.

There are many genes still to be identified in the *A. nidulans* carbon catabolite repression system, not only repressors but elements of the signal transduction pathway and sensors of glucose status in the cell. The function of the *BRO1* similar gene, which lies adjacent to *creA,* has not been shown in *A. nidulans* but in *S. cerevisiae* it functions as part of a signal transduction pathway. If this gene has a similar function in *A. nidulans,* the phenotype of an *A. nidulans* strain which has been disrupted at the *BRO1* similar locus, may indicate which pathway is involved. The leaky-lethal phenotype of the original CreA null strain, which disrupted CreA and possibly the adjacent *BRO1*-similar gene, may be similar to the synthetic lethal phenotype described for Bro1p null strains in which another gene in the same pathway has been disrupted (Nickas and Yaffe, 1997).

Several loci which are involved in carbon catabolite repression have now been identified in *A. nidulans*. Of particular interest are the suppressors of *creA* alleles such as *creB*, *creD* and *malA*. Alleles of *creB*, *creC, creD* and *sorA* all demonstrate an altered response to carbon catabolite repression but the precise nature of their interactions with *creA,* and of their role in carbon catabolite repression is not clear. The phenotypes of alleles of the *TUP1* and *SNF1* homologues as well as the *BRO1*-similar gene, may also provide more insight into carbon catabolite repression in *A. nidulans*.

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