Complementation of the *sor-4* Gene of *Neurospora crassa*

by

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ABSTRACT

The study of catabolite repression in *Neurospora crassa* examines regulation of genetic expression in the presence of various carbon sources. Certain mutant strains of *N. crassa* do not display preferential expression of genes for metabolizing preferred carbon sources. An example is the sorbose resistant *sor-4* strain.

A cotransformation experiment was devised to determine if the gene relieving the *sor-4* mutant strain of catabolite repression was complementary to *cre-1*, a gene thought to be involved in the events leading to catabolite repression. *Neurospora crassa* conidia were converted to spheroplasts in order to render them competent to take up DNA. Conidia from wild type (74A) and mutant (*sor-4*) strains were transformed with both the *cre-1* gene and a gene encoding resistance to Hygromycin B, an antibiotic used as a selectable marker. The transformed colonies were further examined for morphology, and it was determined that the *sor-4* colonies were not converted back to wild type phenotype. Although resistance to the antibiotic was conferred on both wild type and mutant cells, a Southern blot showed that copies of *cre-1* were not integrated into the genome of *sor-4* *Neurospora crassa*. Cotransformation did not give evidence of complementation of the *cre-1* gene and the *sor-4* mutation.

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Special thanks to my parents, whose many sacrifices gave me the opportunity to continue my education at Youngstown State University.

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Introduction

Kingdom Fungi

Fungi are a diverse group of heterotrophic organisms consisting of over 10,000 species. Most fungi exist in soil; only a few species are parasitic. Parasitic fungi receive nourishment from the protoplasm of plants, animals, and humans. Those fungi that are not parasitic exist as saprobes, meaning they feed off of decaying organic matter.

One way in which fungi differ from bacteria is that they are eukaryotic organisms. Eukaryotic organisms are more complex than prokaryotes; they contain membrane-bound organelles and their DNA is housed in a nucleus. Fungi also differ from plants and animals in their cellular composition. All fungi have rigid cell walls containing polysaccharides such as chitin. Also unique to fungi are their cytoplasmic membranes, which are composed of sterols, or steroids containing an attached alcohol.

All species of fungi reproduce asexually (Figure 1). In many species, the asexual spores that undergo budding are called conidia. When conidia begin to germinate, cytoplasmic extensions, or hyphae, are formed. Hyphae resemble long tubes that branch and intertwine; they form a meshwork

called a mycelium. Asexual reproduction is often induced by air and light exposure.

Kingdom Fungi contains four classes of organisms. Class Phycomycota is comprised of lower fungi; a common example is *Rhizopus stolonifer* ("black bread mold"). Class Basidiomycota is the class to which higher fungi such as mushrooms belong. "Imperfect fungi" belong to Class Deuteromycota. They are imperfect because of a lack of sexual reproduction. The fourth class of fungi is the Ascomycota, to which *Neurospora crassa* belong. Ascomycota is the largest class of fungi, characterized by a sac-like structure called an ascus.

Genus *Neurospora*

Neurospora was discovered in 1927 by two botanists, C. Shear and B. Dodge (Alexopolous, et al., 1996). Commonly called "red bread mold", it is a spore-bearing organism which is multicellular and multinucleated (contains more than one nucleus). *Neurospora* undergo both sexual and asexual reproduction. In asexual reproduction, mycelia are induced to sporulate simply by air exposure. This induces a series of morphological changes that culminate in the production of multinucleate asexual spores called conidia (Madi, et al, 1994). Large numbers of conidia are produced

which are identical to the parent. Sexual reproduction can occur following interaction of two different mating types. During sexual reproduction two haploid nuclei fuse, resulting in a diploid cell that undergoes meiosis, thus producing haploid ascospores.

In 1941, biochemical mutants of a filamentous fungus called *Neurospora crassa* were discovered (Alexopolous, et al., 1996). Since then, *N. crassa* has been used as a model system for genetic and biochemical studies in eukaryotic organisms. It can be grown rapidly on simple media, is relatively inexpensive, and has a small eukaryotic genome. The small haploid genome (27,000 kbp) of *N. crassa*, along with extensive knowledge of its metabolism and nutrition, makes the fungus an easy target for genetic manipulation (Vollmer and Yanofsky, 1986). Both sexual and asexual reproduction occur in *Neurospora crassa*; asexual reproduction leads to rapid accumulation of large numbers of conidia, which can be recovered for analysis in the laboratory.

Figure 1: Asexual reproduction in fungi.

Conidia

Budding

Hyphae

Mycelium
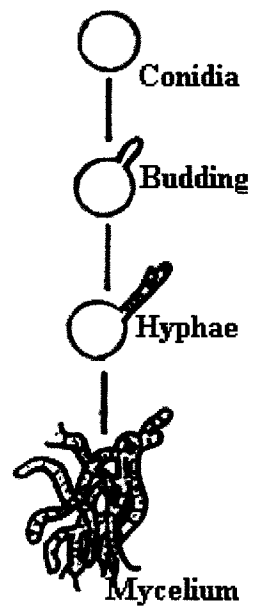
Gene Expression in Fungi

In eukaryotic organisms, there are two types of gene clusters that exist. The more common type of cluster encodes a multienzyme complex or multifunctional polypeptide, while the less frequent type appears to have bacterial operon-like characteristics (Giles, 1978). The quinic acid genes of *Neurospora crassa* represent this second type of gene cluster. Another cluster of genes exists in the yeast *Saccharomyces cerevisiae*, which is used to better understand gene expression and transcriptional control in eukaryotic organisms. This gene cluster is involved in the metabolism of galactose by the cell, thus is termed the *GAL* system.

Galactose Metabolism in Yeast

The *GAL* system of *Saccharomyces cerevisiae* ("baker's yeast") is a set of scattered genes encoding proteins for the utilization of galactose (Figure 2). Galactose is a disaccharide consisting of α-linked galactose and glucose. Expression of the *GAL* genes is subject to glucose repression, a global regulatory mechanism that requires several gene products (Erickson and Johnston, 1993). In *Saccharomyces cerevisiae*, evidence suggests that a common feature of glucose-repressible gene expression is in the use of sequence-specific DNA binding proteins which function to activate and

repress gene expression based on their state of phosphorylation (Wang, et al., 1994; Schultz, et al., 1990).

A transcriptional activator protein, GAL4, controls transcription of the *GAL* genes. Some of the other genes are MEL1, which encodes an α-galactosidase for cleaving the melibose disaccharide; GAL2 encoding a galactose permease which catalyzes entry of galactose into the cell; GAL1 which encodes a galactokinase, GAL7 encoding α-D-galactose-1-phosphate uridyltransferase, GAL10 encoding uridine diphosphoglucose 4-epimerase, and GAL5 encoding phosphoglucomutase, all which together convert galactose into glucose 6-phosphate for use in glycolysis (Kang, et al. 1993).

In the presence of galactose, it is necessary that all GAL genes are expressed. In this condition, the GAL4 activator protein binds to upstream activating sequences (UAS_G) of each *GAL* gene, promoting their transcription. In the absence of galactose, another protein, GAL80, binds to GAL4 and inhibits the activation function of GAL4 (Yun et al. 1991). The addition of galactose relieves GAL80 mediated inhibition (Flick, 1991). Another regulatory protein, GAL3, acts as an inducer. GAL3 binds to GAL80 so that it is unable to deactivate GAL4. The mechanism of this induction remains unknown.

Figure 2: Metabolism of galactose in *Saccharomyces cerevisiae*. The disaccharide melibose is cleaved by α-galactosidase (MEL1) to produce galactose, which enters the cell via help from GAL2, a permease. Once inside the cell, galactose is phosphorylated by GAL1, and the phosphate group is in turn transferred to a glucose molecule. Glucose-1-P is then converted into Glucose-6-P by action of GAL5, a mutase. Glucose-6-P can then be used in glycolysis.
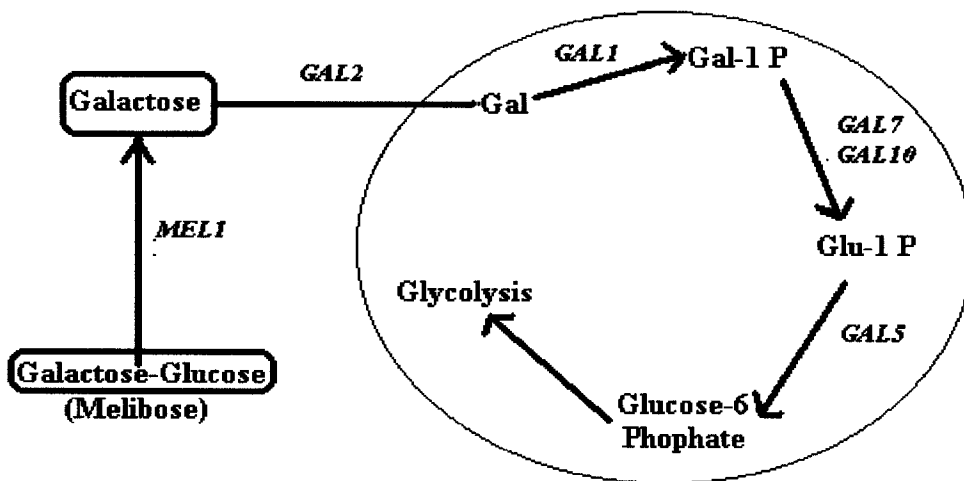
Quinic Acid Gene Cluster

The quinic acid (*qa*) gene cluster consists of seven genes spread over 17.3 kb of DNA on *Neurospora crassa* linkage group VII (Figure 3). The DNA sequence of the entire *qa* gene cluster has been determined and transcripts for each gene have been mapped (Geever, et al., 1989). These seven genes include five structural and two regulatory genes. Structural genes encode proteins which are used in metabolic pathways, while regulatory gene products are utilized in gene expression. The structural genes include *qa*-2, *qa*-3, and *qa*-4, which encode enzymes which catalyze the catabolism of quinic acid. *Qa*-2 encodes catabolic dehydroquinase, *qa*-3 codes for quinate dehydrogenase, and *qa*-4 encodes dehydroshikimate dehydratase. The gene *qa*-y is another structural gene encoding a quinic acid permease. The function of the *qa*-x gene is not fully understood. The two regulatory genes are located at one end of the cluster. *Qa-1F* encodes an activator protein, while *qa*-1S encodes a repressor protein (Giles et al. 1991).

Both genetic and molecular analyses indicate that regulation of the *qa* gene cluster occurs primarily at the level of transcription (Huiet and Giles, 1986). Expression of the *qa* gene cluster represents a positive control system; positive control is mediated by the action of one or more activator

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Figure 3: Transcriptional map of the quinic acid gene cluster. Arrows

represent bidirectional control of transcription. Structural genes are grouped

together, as are regulatory genes. The genes are spread out over 17.3 kb of

DNA. Introns are shown by white lines in QA-X and QA-1S.

Quinic Acid Gene Cluster

QA-X QA-2 QA-4 QA-3 QA-Y QA-1S QA-1F

STRUCTURAL GENES   REGULATORY GENES
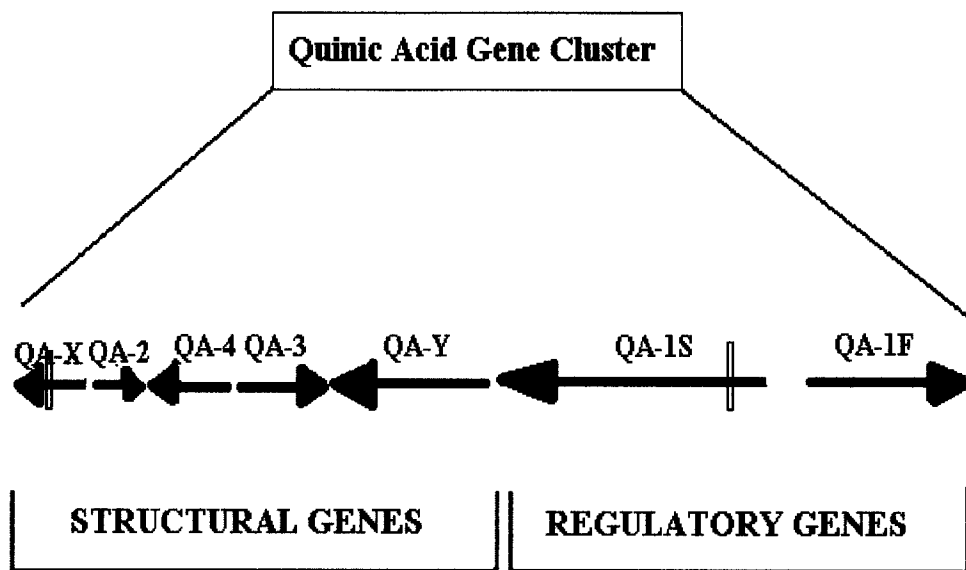
proteins normally required for the initiation of transcription by RNA polymerase (Geever, et al., 1983).

In wild type *Neurospora crassa*, in the absence of the quinic acid inducer, the *qa*-1S repressor protein probably binds the *qa*-1F activator protein. This prevents the action of the activator, and *qa* gene products are produced at very low basal levels (Giles et al. 1991). The addition of quinic acid eliminates the repression, and the activator can initiate its own transcription (autoregulation), as well as synthesis of all other *qa* mRNAs, including the repressor mRNA (Giles et al. 1991). Therefore, without the presence of quinic acid to act as an inducer, expression of *qa* genes does not occur at high levels.

The *qa*-1S gene encodes a repressor protein of 918 amino acids, and has a domain that is believed to interact with the inducer quinic acid. The repressor targets the *qa*-1F activator protein (816 amino acids), therefore is probably not a DNA binding protein. This activator protein positively affects transcription in all *qa* genes, including itself. The activator protein binds to a conserved, symmetrical sixteen base pair sequence that is present 5' to each *qa* gene. A single upstream activating sequence (UAS) in the common 5' regions of *qa*-1S and *qa*-1F suggests bi-directional control of transcription (Giles et al. 1991).

Catabolite Repression

The aim of all cells is to produce energy through glycolysis. When the cell has all the nutrients to produce enough energy, it can turn off expression of unnecessary genes. Catabolite repression is a regulatory system in which an organism displays a preference for glucose as a nutrient source. When glucose is present, the enzymes required for utilization of alternative carbon sources are synthesized at low rates or not at all (Gancedo, 1998). Wild-type *N. crassa* strains grown on quinic acid together with a preferred carbon source such as glucose show a level of induction of the *qa* enzymes about 1% of the levels of induction observed with quinic acid as a sole carbon source (Case et al. 1992). Glucose can affect enzymatic activity in several ways, but its main effect takes place at the transcriptional level. This could occur by two basic mechanisms: glucose interferes directly with transcriptional activators, or facilitates the action of proteins with a negative effect on transcription. The present belief is that in eukaryotes, glucose does not act directly on DNA-binding proteins, but produces signals which are transmitted through a series of proteins to the promoters of corresponding genes (Gancedo 1998). In the presence of glucose, transcription of the *qa* genes occurs only at low levels. In the absence of glucose, if quinic acid is present, *qa* promoters are induced, thus

activating transcription of the *qa* genes. The cell will then produce the necessary enzymes to metabolize quinic acid.

Carbon repression is much better understood in *Saccharomyces cerevisiae* than in filamentous fungi. Therefore, it is used as a model system for understanding how this occurs in *Aspergillus nidulans* and *N. crassa* (Figure 4). In yeast, two proteins, SNF3 and RGT2, appear to act as glucose receptors. The SNF3/RGT2 receptors generate an intracellular glucose signal, suggesting that glucose signaling in yeast is a receptor-mediated process (Ozcan, et al., 1996). The signal which is activated is a complex of two proteins, GRR1 and SKP1. GRR1 is an F-box/leucine-rich repeat protein involved in multiple signaling pathways and acts with SKP1 to target proteins for degradation (Ebbole, 1998; Li and Johnston, 1997). The GRR1/SKP1 signal recognizes and activates another protein, REG1, which has dephosphorylation activity. Once activated, REG1 dephosphorylates a kinase, SNF1, rendering it inactive. The inactivation of the SNF1 kinase prevents it from phosphorylating a DNA binding protein, MIG1. Since MIG1 remains unphosphorylated, it is able to bind to the promoters of glucose repressible genes. MIG1 works in conjunction with SSN6 and TUP1; SSN6 is a TPR protein and TUP1 contains a repeated domain originally identified in the β subunits of G proteins (Keleher, et al. 1992).

Figure 4: Carbon catabolite repression in yeast and filamentous fungi. (Ebbole, 1998) Extracellular glucose is detected by a sensor/receptor, and sends a signal to activate several kinases. They affect Mig1 in yeast, CreA and *cre-1* in filamentous fungi. These proteins have a direct effect on the transcription of glucose repressible genes. (Ebbole, 1998)

Yeast   ○   ○   ○   ○   *Neurospora/Aspergillus*   ○

○   ○ glucose   ○   ○   ○   ○

○   ○   glucose

```
┌─────────────────────┐        ┌─────────────────────┐
│ Glucose receptor/    │        │ Glucose receptor/   │
│ sensor (Snf3/Rgt2)   │        │ sensor (RCO-3)      │
└─────────────────────┘        └─────────────────────┘
```

hexokinase 2

```
( Glucose signal Grr1 : Skp1 )
```

```
┌──────┐
│ Reg1 │
└──────┘

┌──────┐
│ Snf1 │
└──────┘

┌───────────┐
│ Ssn6/Tup1 │
│   Mig1    │
└───────────┘
```

?

sugar transporter activity   **?**   conidiation

```
( glucose signal )

┌───────────┐
│ CREA/cre-1│
└───────────┘
```

Transcription of glucose repressible genes.   *Transcription of glucose repressible genes.*
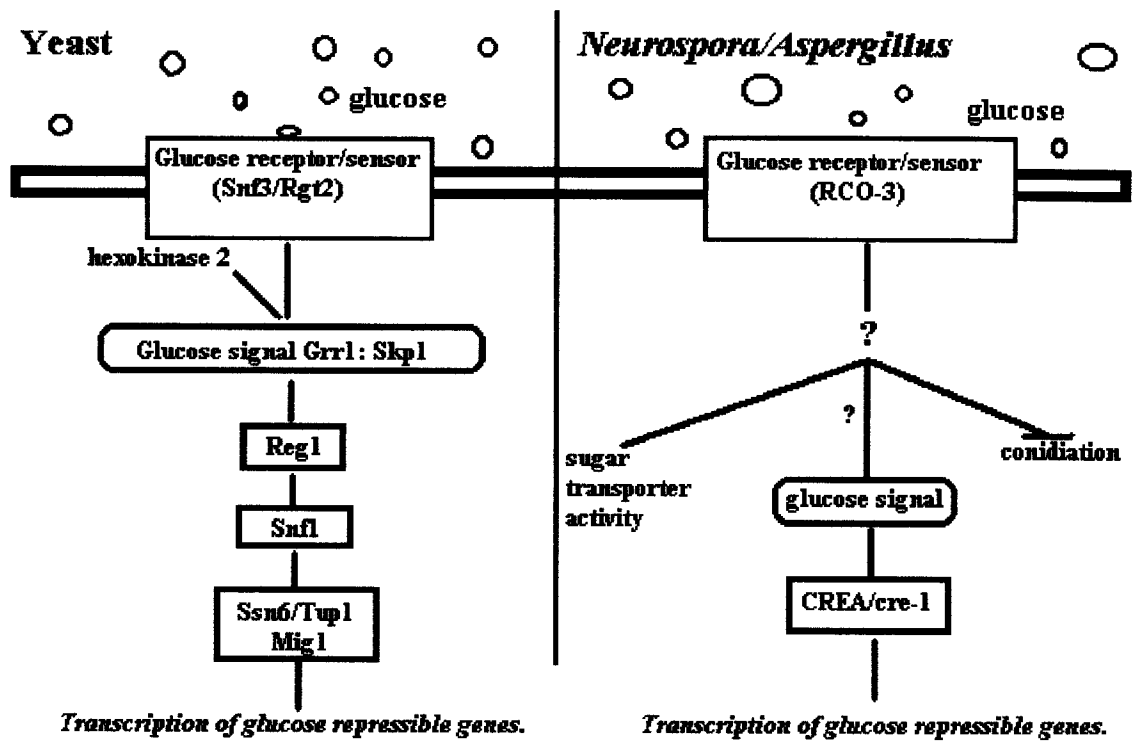
If extracellular glucose is not present, this regulatory pathway is interrupted, and transcription of the particular genes of interest can occur.

In *Neurospora crassa* and *Aspergillus nidulans*, extracellular glucose is sensed by the receptor RCO-3 (regulator of conidiation gene-3), which is homologous to SNF3 of *Saccharomyces cerevisiae*. Evidence suggests that RCO-3 does not function as a low-affinity glucose transporter (Ebbole, 1998; Madi, et al., 1997), but as a glucose sensor which transduces information about extracellular glucose into the cell (Ebbole, 1998).

RCO-1 is another protein which deals with regulation of conidiation in filamentous fungi. The polypeptide sequence predicted from the nucleotide sequence of *rco-1* suggests that RCO-1 is homologous to *Saccharomyces cerevisiae* TUP1, a known protein of the SSN6-TUP1 complex (Yamashiro, et al., 1996; Keheler, et al., 1992). TUP1 represses transcription by a mechanism involving alteration of chromatin structure (Ebbole, 1998; Gancedo, 1998). Despite the homology between RCO-1 and TUP1, it appears that RCO-1 does not play a role in glucose repression in *Neurospora crassa* (Ebbole, 1998).

Sugar transporters/sensors such as RCO-3 likely participate in sending a signal about glucose availability that may not require hexokinase activity (Ebbole, 1998; Ruijter, et al. 1996). This signal affects catabolite

repression through CREA in *Aspergillus* and *cre-1* in *Neurospora*. CREA

has been found to be homologous to MIG1 of *Saccharomyces cerevisiae*.

MIG1 binds to the promoters of genes, repressing their transcription. CREA

and MIG 1 recognize similar DNA sequences (Felenbok and Kelly, 1996).

A gene from *Neurospora crassa* called *cre-1* has been identified as

homologous to CREA and MIG1. However, its role in glucose repression

has not been extensively characterized and a *cre-1* mutant has not been

produced (Ebbole, 1998). Because of its sequence homology to MIG1, it is

speculated that the protein encoded by *cre-1* acts directly on the transcription

of other glucose repressible genes.

Wild type *Neurospora crassa* displays carbon repression of the *qa*

gene cluster in the presence of glucose. However, there are mutations that

have proven to eliminate the carbon repression of the *qa* genes. These

include *sor-4, dgr-1, cri-1, and cri-2*; they are resistant to sugar analogs 2-

deoxyglucose and L-sorbose. Due to their resistance to sugar analogs, these

mutants are able to metabolize quinic acid, even in the presence of preferred

carbon sources.

Sor-4 is of particular interest to this study. Sorbose dramatically

limits the growth characteristics of filamentous fungi and also has been used

to select resistant mutants (Allen, et al. 1989). Wild-type *N. crassa* grows as

colonies on agar media containing sorbose, while resistant mutants, such as *sor-4*, grow in a mycelial fashion. It has been determined that there are at least six unlinked *sor* loci. The biochemical basis for resistance in these strains is not yet clear, but some of the mutants appear to take up sorbose at a reduced rate (Allen, et al., 1989).

Recently, a regulatory gene *cre-1* has been shown to mediate catabolite repression by binding to the promoter regions of glucose sensitive genes. Little is known about the phenotype of *cre-1* and its exact role in catabolite repression. I have attempted to determine if the *cre-1* gene can restore wild-type phenotypes in the *sor-4* mutant strain, thus complementing the existing mutation. This will be accomplished through co-transformation with Hygromycin B as a selectable marker.

Hygromycin B is an antibiotic produced in the bacterium *Streptomyces hygroscopicus*. It is an amino-glycoside that inhibits protein synthesis by causing mistranslation (Staben, et al., 1989). Resistance to this antibiotic will be introduced through the cloned Hygromycin B resistance gene (Figure 5). Integration the gene into the *N. crassa* genome will be monitored by selecting transformants for the tightly linked resistance markers. The presence of the insert region of the plasmid can be verified by genomic Southern analysis (Campbell, et al., 1994)

DNA-mediated transformation has played valuable roles in genetic studies of many organisms and is an inherently interesting phenomenon (Miao, et al., 1995). In the past, transformation of filamentous fungi was limited due to cell wall differences which restricted DNA uptake (Case, et al., 1979). Presently, fungal cells are converted to spheroplasts before transformation, by partially digesting the cell wall with enzymes. However, only a small fraction of the spheroplasts is actually competent for transformation (Grotelueschen and Metzenberg, 1995).

Cotransformation is the simultaneous assimilation of two or more DNA molecules presented to a cell; its frequency is very high in many organisms, including *Neurospora crassa* (Miao, et al., 1995). The phenomenon has been widely used to obtain strains transformed for characters that are difficult or impossible to recover directly by selective plating (Miao, et al., 1995). In this study, we have attempted to employ cotransformation on the sorbose resistant *sor-4* strain of *Neurospora crassa*. The transforming characters are *cre-1* (our gene of interest) and the Hygromycin resistance gene (our selectable marker). If complementation occurs, the *sor-4* strain should be successfully transformed with the *cre-1* gene, and colonies should remain small, as in wild-type strains (Figure 6).

Transformations were also be attempted using wild type (74A) *Neurospora crassa*. Since wild type conidia will grow as confined colonies on sorbose, transformants can be compared in amount of growth and morphology to mutant transformants. Also, the genomic DNA extracted from wild type transformants were digested and used in a Southern blot to compare hybridization by the *cre-1* probe to the hybridization of the probe to mutant *sor-4* DNA.

Figure 5: pBC-hygro plasmid. Chloramphenicol and Hygromycin B

Resistance genes are shown, as well as restriction enzyme sites.

hygromycin (R)

chloramphenicol (R)

pBC-hygro
6.8 kb

lacZ

ApaI
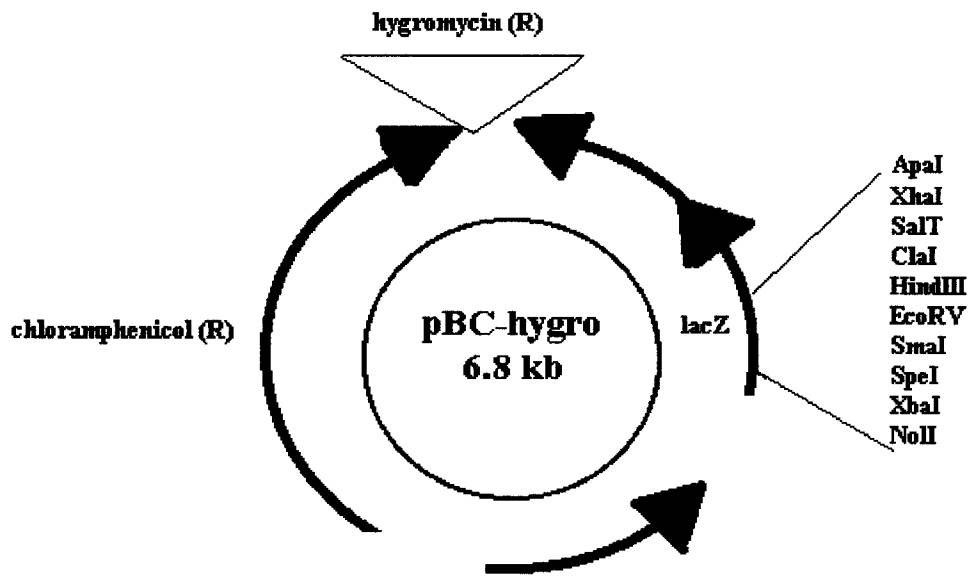XhaI
SalT
ClaI
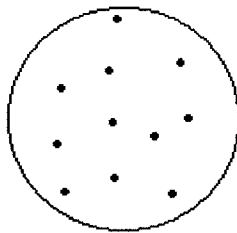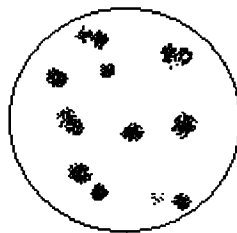HindIII
EcoRV
SmaI
SpeI
XbaI
NotI

Figure 6: Complementation of *cre-1* and *sor-4* would lead to restoration of

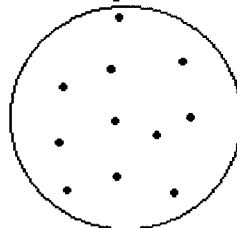the wild type phenotype (flat colonies) in the *sor-4* strain.

74A grown on media
containing sorbose

sor-4 grown on media
containing sorbose

transform with cre-1 DNA

wild type phenotype restored in
successful transformation

Materials and Methods

Strains and Media

Neurospora crassa strains used in this study are listed in Table 1. For production of conidia, *Neurospora crassa* was grown in 25 ml of Horiwitz complete media (Table 2) containing 1.5% agar. Competent conidia were plated on media containing 1X Vogel's minimal media (Vogel, 1956) solidified with 1.5% agar, and containing 5 ml of 2X FIGS (final concentration: .0025% fructose, .025% glucose, .1% sucrose) for every 100ml of media. Regeneration agar (top agar) consisted of 1X Vogel's minimal media, 1M sorbitol, and 5 ml/100 ml of 2X FIGS. Transformants were grown in 1X Vogel's minimal media containing 2% sucrose as the carbon source.

Transformation

Preparing conidia competent for transformation

Hygromycin B sensitive *N. crassa* strain 74A (wild type) were germinated in 50ml Horowitz complete media. Conidia were then grown in .5X Vogel's salts + 2% sucrose, shaking 200 RPM at 37°C for 2-3 hours (in Lab-line® Orbit Environ-shaker). Examination under light microscope

Table 1: Strains used in this study are 74A and a strain containing the *sor-4* mutation.

Strain	Genotype	Source
74A	wild type	Advisor's Stock Collection
sor-4	sorbose resistant	Advisor's Stock Collection

Table 2: Ingredients of Horowitz complete media (1 liter). After

dissolving, media is autoclaved and stored at room temperature.

Horowitz Complete Media

potassium tartate	5.0 g
sodium nitrate	4.0 g
potassium dihydrogen phosphate	1.0 g
magnesium sulfate (7H2O)	0.5 g
sodium chloride	0.1 g
calcium chloride	0.1 g
glycerol	16.0 g
hydrolyzed casein	0.25 g
yeast extract	5.0 g
malt extract	5.0 g
water	1.0 L

using a hemocytometer confirmed germination. When cells reached 75%

germination, the conidia were centrifuged in Sorvall® RC5B Plus

Centrifuge for 10 minutes at 3000 RPM. In the method of Vollmer and

Yanofsky (1986), the conidia were then washed three times in 50ml 1M

sorbitol, and resuspended in 25mg Novozyme 253 dissolved in 10ml 1M

sorbitol. Shaking resumed for 2 hours 100 RPM at 30°C. Spheroplasts were

then washed 3 times in 1M sorbitol, and once in 50ml sorbitol/MOPS/CaCl$_2$

(1M sorbitol, 10mM MOPS, 50mM CaCl$_2$, pH 6.3). These washes occurred

in Beckman® centrifuge at no more than 700RPM. Pellets were

resuspended in 1.2ml sorbitol/MOPS/ CaCl$_2$, 15μl DMSO, 300μl

PEG/MOPS/CaCl$_2$ (40% PEG, 10mM MOPS, 50mM CaCl$_2$), and frozen at

-70°C until further use.

Test for Spheroplasting

Frozen cells were diluted in both 1M sorbitol and sterile H$_2$O. One

hundred μl of each dilution were added to 10ml regeneration agar and plated

on Vogel's + FIGS agar plates. Plates were incubated at 30°C for 48 hours.

Successful spheroplasting was identified by growth only on the plates

containing the 1M sorbitol dilutions.

Co-transformation Procedure

First, 100μl thawed competent 74A or cells containing the *sor-4* mutation were added to 30μl of *cre-1* DNA + 30μl pBC-hygro. Cells (100μl) were also be added to 60μl sterile H_2O (negative control). The cells remained on ice for 30 minutes, then 1ml PEG/MOPS/$CaCl_2$ were added to both tubes, which sat at room temperature for 20 minutes. Next, 100μl of the transformation mix was added to 10ml regeneration agar (containing 200μg/ml Hygromycin B), then plated on Vogel's + Hygromycin B + FIGS plates and incubated at 30°C for at least 48 hours.

Purification of Transformants

Streaking for Isolation

Transformation plates are examined under a dissecting microscope, those colonies which are clearly visible and isolated were removed. Using a sterile needle, colonies were lifted from agar plates, and placed in Horowitz complete slants containing 1.5% agar. The slants were incubated at 30°C. After growth appeared in the slants, a loopful of conidia were streaked onto Vogel's + FIGS plates and incubated at 30°C. After 48 hours, the plates were examined under a dissecting microscope, and again large isolated

colonies were lifted out of the plates and placed into agar slants. This isolation procedure was repeated several times.
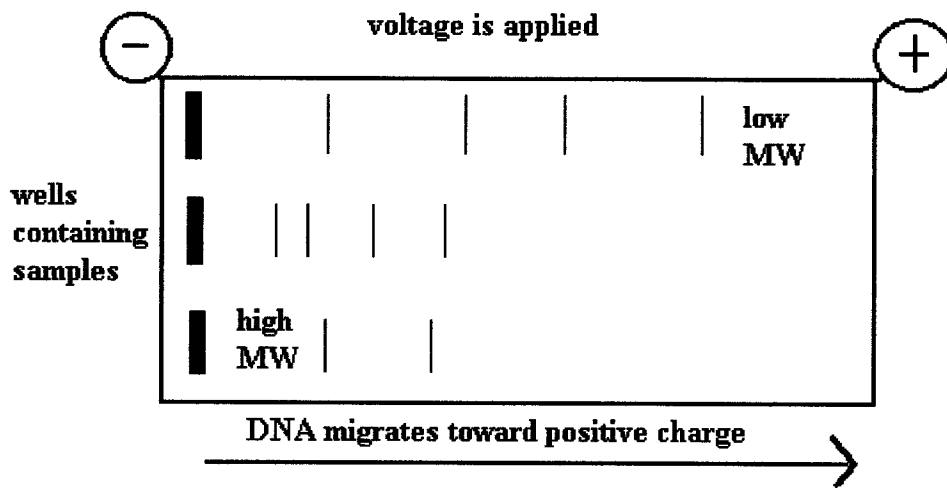
DNA extraction

After isolated colonies have been achieved, they were used to inoculate flasks containing 1X Vogel's salts + 2% sucrose, and incubated for at least 48 hours. The resulting hyphal mats were vacuum filtered, wrapped in foil and frozen at −70°C. One night previous to performing the actual extraction, Samples were placed into sterile 50ml conical tubes and lyophilized (freeze-dried) overnight in Labconco® lyophiliser. The next morning, samples were ground up to a fine powder, and genomic DNA was extracted. The first step involved in this procedure was to add the ground powder to microfuge tubes, and a solution of 500μl 50mM EDTA, 0.2% SDS and 1.5μl DEP were added to each tube. The samples were mixed on Vortex® Jr. Mixer, then placed at 68°C for 20 minutes, and mixed again. The samples were then spun for 5 minutes in Eppendorf® centrifuge 5415C in order to pellet cell debris. The supernatant was transferred to a new tube, and 30μl 8M KOAc was added. The samples were then mixed well, and placed on ice for 5 minutes. The tubes were centrifuged for another 5 minutes in order to pellet the DNA. After the supernatant was removed,

samples were resuspended in 200μl 1X TE (10mM Tris, 1mM EDTA), and placed at 68°C to aid in resuspension. A solution of 3μl RNAse A (10mg/ml) was made in 10mM EDTA and added to the samples (200μl). Phenol m-cresol was added to the samples, which were then mixed, and an equal amount of chloroform was added to each tube. The samples were centrifuged for 5 minutes, the organic layer was removed, and the aqueous layer was transferred to a new tube. This process was repeated one time. To the remaining aqueous layer, 10μl 10M LiCl and 500μl 95% ethanol were added. The samples were mixed well, then centrifuged for 5 minutes. After the supernatant was removed, the tubes were inverted to allow the DNA pellets to dry. After dried, the DNA was resuspended in 100μl 1X TE and heated at 65°C until resuspension occurred. The samples were refrigerated until further use.

Gel Electrophoresis

The extracted DNA was run on a 1% agarose gel to confirm its presence (Figure 7). The gel is composed of .5g agarose and 50ml 1X TPE electrophoresis buffer (.08M Tris-Phosphate, .008M EDTA, .5M EDTA). Making up each lane was 10μl of DNA, 5μl sterile H2O, and 2μl tracking dye. The gel was run at app. 60V for 60 min. After completion, the gel was stained in 50μl ethidium bromide (40mg/ml) for visualization.

Figure 7: Agarose gel electrophoresis. Negatively charged DNA is loaded

onto one end of gel. Electrical current is passed through electrophoresis

chamber, DNA molecules migrate toward positive end. Large molecular

weight molecules are retarded close to wells, small molecular weight

proteins move farther toward positive end.

voltage is applied

wells containing samples

low MW

high MW

DNA migrates toward positive charge

Restriction Digests

Extracted DNA was digested with various restriction enzymes. Enzymes used were *Bam*HI, *Eco*RI, *Hind*III, and *Pst*I. For a small digest, 10μl DNA, 16μl sterile H_2O, 3μl appropriate 10X buffer, and 1μl of the restriction enzyme were added to an Eppendorf® tube and placed on a 37°C heating block overnight. The digested DNA was then run on a 1% agarose gel and stained with ethidium bromide.

Southern Blots

Making of the Probe

A large EcoRI digest was performed using 100μl *cre-1* DNA, 160μl sterile H_2O, 3μl 10X buffer, and 10μl EcoRI. The mixture was heated at 37°C for 5 hours. The digest was then electrophoresed on a 1% agarose gel in 1X TPE buffer. The gel was run at 29V overnight. The gel was then stained and examined under UV light. The band of interest was removed from the gel with a clean razor blade and placed in a dialysis bag. The bag was filled with .5X TAE (20mM Tris acetate, 1mM EDTA), the ends were clamped, and it was placed in an electrophoresis tank. The apparatus was run for 45 minutes, when the polarity was reversed for one minute. The

liquid was then drawn out of the bag using a pipette, and the mixture was passed over an Elutip column. The column had been previously primed by passing 3ml high salt buffer (1M NaCl, 20mM Tris, 1mM EDTA) through it, followed by 3ml low salt buffer (.4M NaCl, 20mM Tris, 1mM EDTA). After the liquid containing DNA was passed through the column, the DNA was eluted with 400μl high salt buffer. The DNA solution was then mixed with 400μl phenol, and centrifuged for 10 minutes. The top layer was drawn off, and placed in a clean microfuge tube. This layer was mixed with 400μl chloroform, and centrifuged for 5 minutes. Again, the top layer was drawn off, and placed in a clean tube. To this tube was added 400μl isopropanol, and it was centrifuged for another 10 minutes. The mixture was washed with 70% ethanol, dried, resuspended in 20μl 1X TE, and frozen for future use.

Pre-Blotting

A restriction digest of the 74A and *sor-4* transformants was performed by adding 3μl 10X buffer, 1μl HindIII, and 16μl sterile H_2O to 10μl of DNA. The digests remained on 37°C heating block overnight. The completed digests were run on a 1% agarose gel. After staining, the gel was placed in 250-400ml .25M HCl and rocked for 15 minutes. The HCl was removed, and the procedure was repeated to depurinate the DNA. The gel

was then transferred to a different tray containing 250-400ml .5M NaOH, 1M NaCl for 15 minutes. This was repeated once to ensure denaturing of DNA. Next, the gel was placed in 250-400ml .5M Tris, 3M NaCl pH 7.4 and rocked for 30 minutes.

Blotting

A piece of Boehringer-Mannheim positively charged nylon membrane was cut to the size of the gel, wet in H_2O, and soaked in 10X SSC (1.5M NaCl, .5M sodium citrate). A blotting sponge of approximately the same size as the gel was placed in a plastic container and saturated in 10X SSC. Then, a gel-sized piece of Whatman® 3MM chromatography paper was placed on top of the sponge. The gel was carefully placed upside down on top of the sponge, and the nylon membrane directly on top of the gel. More chromatography paper was placed on top, along with a large stack of gel-sized paper towels. A glass plate was placed on the stack of towels, with a weight placed on top of the glass (Figure 8). The gel was allowed to blot overnight. The next morning, the membrane was baked at 80°F in Precision® vacuum oven for 60 minutes to crosslink the membrane. The membrane was stored in a cool, dry place away from light.

Figure 8: Apparatus for Southern blotting procedure. Through capillary action, DNA is drawn from agarose gel onto nylon membrane. Paper towels are used to absorb blotting buffer, and a weight is used to keep the apparatus compressed.

5 lb

GLASS PLATE

PAPER TOWELS

BLOTTING PAPER

NYLON MEMBRANE

GEL

BLOTTING PAPER

BLOTTING SPONGE
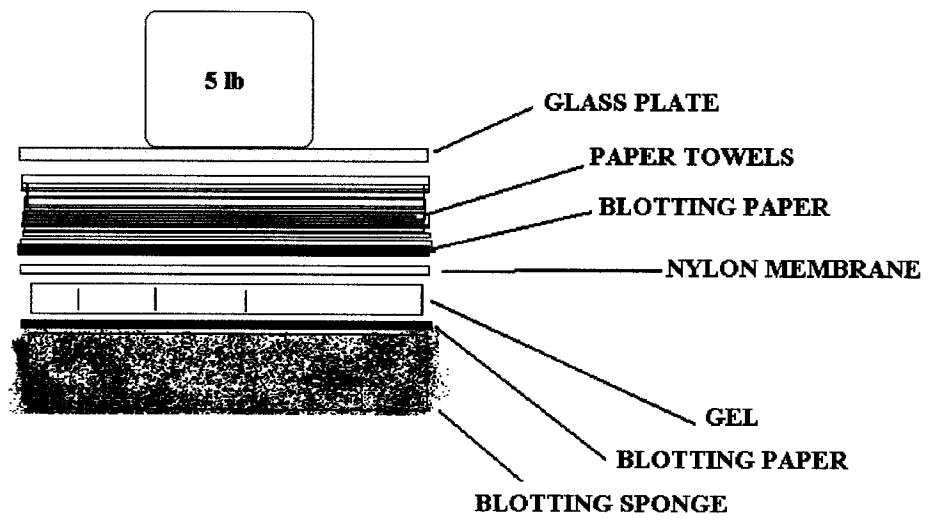
Labeling of Probe

First, 50ng of DNA was added to a solution of 10μl TE buffer + 1μl H20, and denatured on 90° heat block for 5 minutes. Then, 2.5μl 10X buffer, 6μl dNTPs, 2μl dATP, 2.5μl [32P] dATP, and 1μl klenow fragment were added to DNA. This mixture was incubated at room temperature 2-10 hours. While incubating, a spin column was prepared. A small amount of siliconized glass wool was packed into a syringe containing without a needle or plunger. Sephadex G-50 beads were pipetted into column; as liquid dripped out, beads were continually pipetted in until column was packed full with beads. Syringe was then placed in a 15ml conical tube, and spun in IEC® clinical centrifuge for 5 minutes at setting 3. After centrifuging, should have had approximately .9ml of packed beads. Liquid was then removed. The labeled mixture was then loaded onto the spin column, and centrifuged 5 minutes, on the third setting. Radioactivity counts (using Geiger counter) were compared in syringe and conical tube; should have gotten approximately 50% incorporation. Probe was kept in conical tube.

Prehybridization

The stored membrane was rolled up and put into hybridization tube. Then, 30ml of 6X SCP (.6M NaCl, .18M Na_2PO_4)+ 1% sarkosyl was added to tube, and membrane incubated in rolling tube (Techne® Hybridiser HB-

1D) 60°C for 30 minutes. While prehybridizing, prepared hybridization mix by first boiling 12ml 6X SCP with 1% sarkosyl and 150µl 10mg/ml sheared salmon sperm DNA. The probe was then added, and the mixture boiled for 5 more minutes.

Hybridization

Prehybridization mixture was removed from tube, and hybridization/probe mixture was added. Hybridization then took place overnight at 60°C in rotating tube. The next morning, the probe was saved in a new tube, and membrane was washed with 100ml 2X SCP with 1% Sodium Dodecyl Sulfate (SDS), and rotated for 30 minutes at 60°C. The wash was removed, and blot was placed in a tray where it was washed twice with .2X SCP + 1% SDS, fifteen minutes each time. The blots were then wrapped in plastic, and exposed to Konica® X-ray film by autoradiography.

Results

Using strains 74A and a strain containing a *sor-4* mutation, I have

attempted to demonstrate the phenotype of a novel gene, *cre-1*, of

Neurospora crassa. This was accomplished through transformation.

Four types of transformations were attempted in this study (Table 3).

First, 74A was transformed with a plasmid containing the Hygromycin B

resistance gene only. This would determine if the cells were actually

capable of being transformed. Conidia from strain 74A had to first be

converted to spheroplasts in order to become competent for transformation.

The cell walls were partially digested with Novozyme 234. Then, to

determine if this digestion was successful, spheroplasts were diluted in

sorbitol or sterile H_2O. Dilutions were plated on Vogel's media and

incubated for 48 hours. The plates containing cells diluted in sorbitol

showed substantially more growth than those plates containing cells diluted

in H_2O (Figure 9). This confirmed that the cells were spheroplasted, since

cells in sorbitol were able to regenerate their cell walls and grow easily in

solid media. Those cells diluted in H_2O were lysed before they could

regenerate their cell walls. However, some colonies were present due to a

small percentage of the cell walls not being digested. Those cells with

undigested walls should grow in media, but will not be able to be transformed.

After the spheroplasting tests showed positive results, wild type 74A was transformed with the plasmid containing a Hygromycin resistance gene, and transformants were selected on media containing Hygromycin. The negative control, which consisted of competent 74A conidia transformed with sterile H_2O, showed no growth at all; these cells were still sensitive to Hygromycin. These results led to the belief that the wild type cells were indeed competent, and our selection with Hygromycin B was working. We then proceeded with the next transformation.

Another control experiment was performed with Hygromycin B, now with the strain containing the *sor-4* mutation. This transformation would determine if the mutant cells were able to be transformed, as well as to confirm that the correct amount of antibiotic was being used in the media. These cells were first treated and digested with Novozyme, and checked for spheroplasting. The spheroplast tests showed that the cells were competent to undergo transformation; cells diluted in sorbitol were able to grow on solid media, but not those cells which were diluted in H_2O. The transformation proceeded, using only the plasmid containing the Hygromycin resistance gene. A negative control was also used; *sor-4* cells

were transformed with sterile H$_2$O rather than plasmid DNA. This experiment yielded positive results; transformants were present on plates containing Hygromycin. Colonies did not appear on the negative control plates. Hygromycin sensitive *sor-4* conidia had been successfully transformed with the resistance gene, allowing the cells to grow in the presence of the antbiotic.

Cotransformation of wild type 74A *Neurospora crassa* using a plasmid containing the cloned *cre-1* gene and a second plasmid containing the gene for Hygromycin resistance was attempted. Before cotransforming with *cre-1*, the plasmid was digested with *Hin*DIII, *Eco*RI, and *Bam*HI to ensure that the *cre-1* gene was actually present in the plasmid. The resulting digests gave the predicted banding pattern (Figure 10).

Wild type conidia tested positive for spheroplasting and were transformed. After the transformation procedure and incubation period, the results were interpreted. Colonial growth appeared on plates containing Hygromycin; they were small and flat, typical of a wild type colony. The negative control plates were void of growth. Thus, wild type conidia were successfully transformed with the Hygromycin B resistance gene. It was believed that the *cre-1* gene was also incorporated, but this could not be confirmed.

The transformants were then isolated. After the isolation of the transformants was complete, they were further treated and DNA was successfully extracted (Figure 11).

In the final type of transformation, the strain containing the *sor-4* mutation was cotransformed with the two plasmids previously mentioned; one containing the cloned *cre-1* gene and the other containing the Hygromycin resistance gene. The *sor-4* conidia were first made competent by cell wall digestion, this was confirmed by spheroplasting tests in which cells diluted in sorbitol grew much more readily than those diluted in H_2O. The cotransformation of competent *sor-4* conidia was performed using the 2 plasmids, after incubation the plates were examined for growth. Colony growth was apparent on all plates except on the negative control plates. Some transformants appeared small and confined like wild type colonies, others looked large and fluffy like sorbose resistant colonies. This led to the belief that the mutant cells were taking up the Hygromycin resistance, and some may have been taking up the *cre-1* DNA. The transformants were streaked for isolation, and their DNA was successfully extracted (Figure 12) and saved for future use in restriction digests. The cotransformation of *sor-4* *Neurospora crassa* with *cre-1* and Hygromycin resistance was repeated several times, but did not produce the same results.

Another *EcoR*I restriction digest was performed to isolate a fragment containing the *cre-1* gene for use as a probe. The agarose gel showed two bands in the lane containing digested *cre-1* plasmid. This confirmed that the digest was successful, since the same bands were shown in the same location as those appearing in the earlier gel (Figure 10).

The band of interest was physically cut out of gel and placed in a dialysis bag (see Materials and Methods). After passing the remains of the bag through an Elutip column and further extracting the DNA (see Materials and Methods), a small amount was run on an agarose gel (Figure 13). Since the fragment was visible, it was concluded that we had a viable fragment of *cre-1* DNA for use as a probe.

An initial Southern Blot was performed on *sor-4* transformants. DNA was extracted from both the *sor-4* transformants and untransformed 74A (wild type) *Neurospora crassa*. The extracted DNA was digested with *Hind*III. The resulting digest was electrophoresed, then transferred to a membrane for blotting. P^{32} labeled *cre-1* fragment was used as a probe; the resulting autoradiogram showed that only one copy of the *cre-1* gene was contained in wild type DNA and *sor-4* transformants (Figure 14). If cotransformation with the plasmid containing *cre-1* and the plasmid containing the Hygromycin resistance gene had been successful, the labeled

cre-1 probe might have hybridized to 2 copies of the gene in the *sor-4* transformant DNA. On the autoradiogram, only one band is visible in the sor-4 transformant lanes, thus only one copy of the *cre-1* gene is present. This could be due to homologous integration of the *cre-1* DNA. Although the strain containing the *sor-4* mutation was transformed by a plasmid containing a Hygromycin resistance gene, we could not definitely demonstrate uptake of the *cre-1* DNA. Untransformed wild type DNA showed one band on the autoradiogram; this was expected since only one copy of the *cre-1* gene is present in the *Neurospora crassa* genome.

Table 3: Results of various transformations are summarized. Growth is represented by a plus (+) sign and lack of growth is represented by a negative (-) sign. The genes which were attempted to be integrated were *cre-1* and a gene encoding resistance to the antibiotic Hygromycin B.

Strains

Genes	74A	sor-4
Hyg res only	+	+
Hyg res + cre-1	+	+/−

Figure 9: Example diagram of positive test for spheroplasts. Plates of cells diluted in sorbitol show more colonial growth than each respective plate of cells diluted in sterile H_2O.
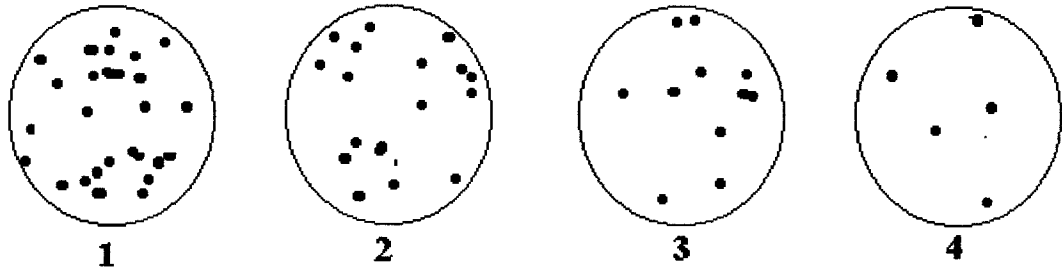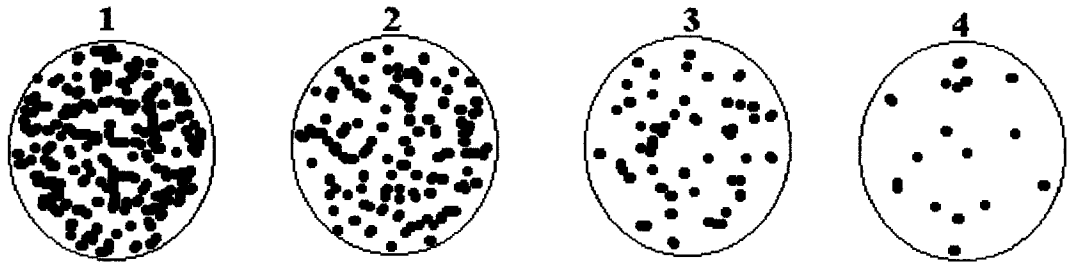
SORBITOL DILUTIONS

Figure 10: Agarose gel electrophoresis of restriction digest of *cre-1* DNA.

Lane 1 represents *BamHI* cut DNA, lane 2 represents *EcoRI*, and lane 3
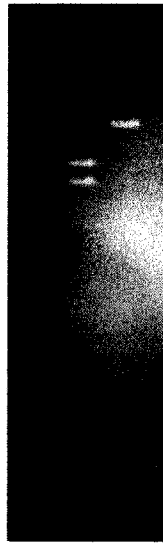
represents *HindIII*.

Figure 11: Agarose gel electrophoresis of DNA extraction of 74A strain of

Neurospora crassa. A strong band is evident in each lane, suggesting that

DNA was in fact extracted from each isolated transformant.
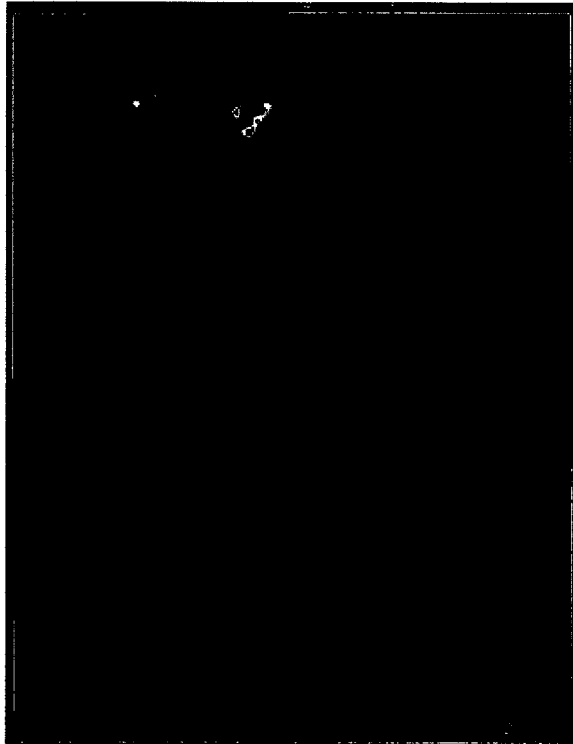
1 2 3 4 5 6 7 8 9 10 11

Figure 12: Agarose gel electrophoresis of DNA extraction of *sor-4*

Neurospora crassa. DNA is represented by a single band in each of 3 lanes.

1   2   3

Figure 13: Agarose gel electrophoresis of *cre-1* DNA fragment. Arrow indicates band in lane 1showing that DNA was present after purification steps.

Figure 14: Autoradiogram of southern blot of *sor-4* DNA probed with *cre-1* fragment. Lane 1 represents wild type *Neurospora crassa*, all other lanes represent the mutant strain. One band is present in each lane, suggesting that only one copy of the *cre-1* gene is present in the genome.

13 11 9 7 wt
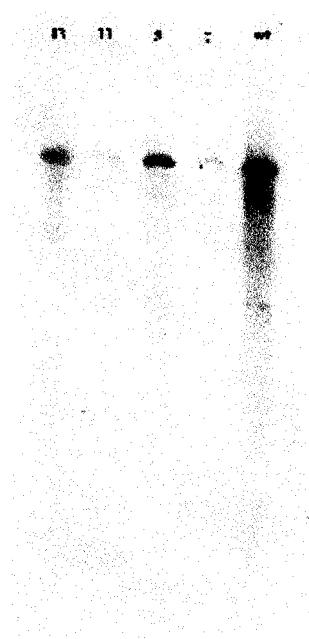
Discussion

Neurospora crassa is a filamentous fungus that is widely used in genetic studies. Mutants of this eukaryotic organism help to elucidate the functions of the various genes contained within its genome. One particular area of interest is the quinic acid gene cluster, which displays carbon catabolite repression in the presence of a preferred carbon source, such as glucose. When glucose is present for the cell to use for energy, the cell turns off expression of the genes required to metabolize quinic acid.

The mechanism by which *Neurospora crassa* undergoes catabolite repression is not fully understood. However, studies involving a novel gene, *cre-1*, have helped demystify the cascade of events leading to transcriptional repression. It has been found that *cre-1* of *Neurospora crassa* is homologous to an important DNA binding protein of *Saccharomyces cerevisiae*, MIG1. Little is known of exactly how *cre-1* works, but because of its homology to MIG1, it is believed to act directly in the repression of certain genes, like those of the quinic acid gene cluster.

Since *cre-1* is not well understood, studies involving the gene will be helpful in uncovering its role in carbon repression. I have attempted to determine if *cre-1* complements an already existing mutation, *sor-4*. The

mutation in the *sor-4* gene has been shown to eliminate carbon repression of the quinic acid gene cluster in *Neurospora crassa* (Asch, unpublished). Since *cre-1* is thought to play a major role in the regulation of glucose repressible genes, we attempted to determine if cre-1 and sor-4 were allelic, actually two forms of the same gene. If the genes complement each other, wild type phenotype would be restored in mutant strain *sor-4*, suggesting that the two are in fact the same gene. Thus far, we have not been able to conclusively demonstrate successful cotransformation of a wild type strain or a strain containing the *sor-4* mutation with the *cre-1* gene. In order to determine if the gene in question, *cre-1*, was in fact part of the plasmid with which we were attempting to transform *sor-4 Neurospora crassa*, the plasmid was sequenced. The data obtained confirmed that this was indeed *cre-1* being used in the cotransformations (Dr. David Asch, personal communication).

While transformation of *Neurospora crassa* conidia is a common experimental procedure, there are some barriers and limitations to its success. For example, in order to break down the cell wall barrier, cells are converted to spheroplasts before transformation is attempted. However, only a small fraction of the spheroplasts is actually competent for

transformation (Grotelueschen and Metzenberg, 1995). This is one possibility for the lack of repeatable results in this particular experiment.

Experiments were run to determine if there might have been procedural error. Transformations with only the plasmid containing the Hygromycin B resistance gene were run to determine if the correct concentration of Hygromycin was being used in the media, as well as to ensure that the transformation protocol was being followed accurately. The Hygromycin resistance gene was in fact integrated into the *Neurospora crassa* genome in both wild type and mutant strains. This was proven through observation that growth occurred on media containing Hygromycin only with conidia transformed with pBC-hygro. The negative control, which was "transformed" with sterile water, showed very little or no growth on plates containing Hygromycin. Therefore, it was concluded that both the procedure and concentrations being used were effective.

There are several other mutations in *Neurospora crassa* which have been shown to eliminate carbon repression of the quinic acid gene clusters. These include deoxyglucose resistant mutations such as *dgr-1*, *cri-1*, and *cri-2* as well as another sorbose resistant mutation, *sor-1*. Future studies may examine these particular genes' roles in catabolite repression of the quinic

acid gene cluster, in order to further clarify the role of *cre-1* in filamentous fungi.

The study of catabolite repression in *Neurospora crassa* is an important aspect of molecular biology, since it deals with gene regulation. An understanding of how genes are turned on and off is essential to comprehending the mechanics of a cell, the simplest form of life. This study was completed with hopes of clarifying the role of a particular gene found in *Neurospora crassa*, in order to demystify the cascade of events which occurs during catabolite repression in filamentous fungi. By hopefully uncovering more details about this phenomenon, the intricate workings of the eukaryotic cell will become more apparent.

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