Site-directed Mutagenesis of the –144 Activator-Binding Site of *qa-1F* in *Neurospora crassa*

by

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Submitted in Partial Fulfillment of the Requirements

For the Degree of

Master of Science

in the

Biological Sciences Program

YOUNGSTOWN STATE UNIVERSITY

July, 2002

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Abstract

The quinic acid cluster of *Neurospora crassa* provides an interesting model for studying genetics. Its unique operon-like organization provides a good opportunity to study the eukaryotic mechanism of activation and repression of the cluster responsible for utilizing quinic acid as a carbon source. The activator protein, qa-1F, elevates levels of transcription by binding at a conserved sequence that occurs at least 14 times within the cluster. The activator, qa-1F, has one of its highest affinities for the activator-binding site that regulates its own transcription. This site occurs at –144 in relationship to the start site of transcription of *qa-1F* . By isolating this sequence using PCR, and cloning into a RF M13mp18 baculovirus vector, the vector will be utilized to produce single-stranded infectious phage particles with our cloned binding site. Once these infectious phage particles are isolated, site-directed mutagenesis will be performed using the kunkel method to change specific conserved bases. These mutants can be transformed back into *Neurospora* where the significance of these changes will be better understood.

Acknowledgements

I would like to thank Dr. Asch for guidance, knowledge and patience, Dr. Walker for the use of his equipment, and Dr. Lorimer for her great protocols and creative troubleshooting. Bruce Levinson and Diana Arnett were also a great help with the sequencing. Most importantly I would like to thank my family for without them none of this could have been possible.

Table of Contents

List of Figures

List of Tables

Introduction

I. Fungi Kingdom

The kingdom fungi consists of three phyla: zygomycota, basidomycota, and ascomycota. These phyla consist of an extremely diverse group of eukaryotic organisms made of over 100,000 different species. Their diversity is represented in the fact that even though most fungi are filamentous, or growing as hyphae or branching filaments, they can also be unicellular growing as yeasts.

II. Ascomycota Phyla

Ascomycota is the largest of the fungi kingdom with almost 2000 genera. The majority of ascomycetes are saphrophytes – those that feed on decaying plant debris. The distinguishing feature of this phylum, is that they have an ascus, a sac-like cell containing spores called ascospores.

III. *Neurospora crassa*

Neurospora crassa, is a heterothallic species, meaning there are two different mating types. There are many reasons why *Neurospora crassa* serves as a good model organism to study heredity. The most important reason, for molecular biologists, is that it can grow on simple media, which makes it inexpensive to grow and easy to handle. *Neurospora* grows quite rapidly since its

genome, which consists of only seven chromosomes and 47 million base pairs, is relatively small for a eukaryotic organism. *Neurospora* also exists in a haploid state during vegetative growth, making it easy to isolate many specific sequences and mutations. It provides a eukaryotic system that can be used to study different aspects of gene control. All the products of meiosis are also easily recovered and analyzed (Alexopolous *et al.* 1996). The *GAL* system of *Saccharomyces cerevisiae,* like *qa* cluster in *Neurospora crassa*, uses multiple gene products to utilize a carbon source. This makes it a good model for understanding the mechanisms of the *qa* cluster and for studying gene regulation in eukaryotes.

IV. *GAL* system of *Saccharomyces cerevisiae*

The *GAL* system of *Saccharomyces cerevisiae* is responsible for metabolizing galactose and serves as a good model for gene regulation in eukaryotes. The *GAL* system uses a coordination of gene products to utilize galactose as a carbon source. The activity of the *GAL* system is mediated by activator and repressor protein interactions. The enzymes encoded by the structural genes are *Gal1* (galactokinase), *Gal7* (galactose-1-phosphate uridylytransferase), *Gal10* (uridine diphosphoglucose-4-epimerase), and *Gal5* (phosphoglucomutase) (Douglas and Hawthorne 1964, 1972. The expression of these genes, except *Gal5*, which is unregulated and always expressed (Bevan and Douglas, 1969), is activated by galactose and repressed by glucose. The *Gal2* gene encodes a permease that allows galactose to enter the cell. The

Mel1 gene product (alpha galactosidase) allows the cell to use the sugar melibiose (disaccharide) in the galactose pathway. This is because it cleaves melibiose into galactose and glucose (Lazo *et al.* 1978). The *Gal3* gene, which also acts to regulate the pathway, appears to encode an enzyme that catalyzes the synthesis of the inducer from galactose. The positive activator is the *GAL4* protein.

The *GAL4* gene encodes a protein 881 amino acids in length that activates the transcription of genes necessary for galactose metabolism. Even though the protein is made in the cytoplasm, it acts in the nucleus. Therefore some specific transport system is necessary which is believed to be located at the N-terminal region of the protein, just outside the DNA-binding domain (Silver *et al.,* 1984). The *GAL4* activator protein has five functional domains: DNA-binding at1-65 residues, dimerization ability at 65-94 residues, and activation of transcription at 148-196 and 768-881 residues (Ma & Ptashne, 1987). The *GAL4* protein activates transcription of the *GAL* genes, and interacts with another regulatory protein, *GAL80. GAL80* acts as a repressor of the *Gal* system by binding *GAL4* at base pairs 851-881, which also lies in one of the transcriptional activation domains of *GAL4* that is located at 768-881 base pairs. The presence of galactose causes the GAL80 protein to disassociate from the GAL4 activating domain allowing it to form the proper dimmer structure to activate elevated transcriptional levels.

The DNA-binding domain of *GAL4* in the first 65 N-terminal amino acids is homologous to other eukaryotic DNA binding proteins (Johnson and

Dover, 1987) and contains six cysteine residues that form a structure called a "cysteine-zinc DNA binding finger". This zinc finger evidence has been seen in mutants of *GAL4* that alter this structure eliminating the DNA binding ability (Johnson and Dover, 1987) This yeast activator also binds at 11 known upstream activating sequences (UAS_{GAL}) that have the conserved 17 bp symmetrical binding site CGGAGGACTGTCCTCCG (Giniger *et al.,* 1985). The residues of the central region of the GAL4 protein are believed to be involved in catabolite repression. This region has been found to be necessary for inhibition by glucose and activation of GAL4 in the absence of glucose (Kang *et al.,* 1993).

The *GAL80* gene encodes a protein 435 amino acids long, that acts to inhibit the GAL4 activator protein (Lue *et al.,* 1987). There are three domains of the GAL80 protein: one that interacts with the GAL4 protein (similar functions as qa-1F), one that interacts with the inducer, and one for targeting to the nucleus. Like GAL4, GAL80 is made in the cytoplasm and acts in the nucleus, thus a specific transport system is necessary. The region of the protein responsible this specific transport is thought to be found in residues 1-109 and 341-405 of the GAL80 protein. The GAL80 protein is thought to interact with the inducer at residues 322-340 (Yun *et al.,* 1991). Evidence of this has been seen in mutations that lie in the inducer-binding domain where the GAL80 protein was unable to recognize the inducer and hence transcription did not occur (Douglas & Hawthorne, 1964).

The activator protein GAL4 regulates transcription of the GAL80 gene. There is an approximate 5-fold increase of *GAL80* transcription in the presence of galactose, which is due the binding of the GAL4 protein at the UAS_{gal80} within the *GAL80* promoter (Baum *et al.,* 1986). This increased transcription of the *GAL80* gene in the presence of the inducer is to prevent saturation of the GAL80 protein by galactose and provide the yeast with an efficient way of quickly shutting down the *GAL* genes.

V. Background of Quinic Acid Gene Cluster

In prokaryotes, the system of replication and regulation of many genes is well understood. Genes of linked function (in prokaryotes) are usually found close together in groups called operons. However, genes of related function in eukaryotes aren't found in proximity with each other, they are dispersed randomly throughout the genome. This is the case with the GAL system of yeast. The *qa* gene cluster of *Neurospora crassa* is an exception to this eukaryotic consistency. The structural and regulatory genes are located in a single cluster that provides a unique opportunity to closely study the regulation of genes in a eukaryotic system.

Twenty-three years ago, H. Rines began the first genetic studies about various *Neurospora crassa* mutants that were unable to utilize quinic acid as a carbon source (Rines 1968). In the early 70's, researchers had identified four

genes associated with the catabolism of quinic acid that were in a tightly-linked cluster. The *qa*-2 gene was the first gene of the cluster to be isolated and cloned (Giles *et al.* 1985). Three of these genes,

qa-2, qa-3 and *qa-4* , were found to code for inducible enzymes responsible for the conversion of quinic acid to protocatechuic acid. Once this data had been provided, a hypothesis was formed. This was that the presence of the inducer, quinic acid, along with the product of a positively-acting regulatory gene (*qa-1*) had regulated the enzymes of the *qa* cluster (Case & Giles 1975).

Various mutants of *qa* genes were studied to determine the function of the three enzymes of the cluster. The first studies of *qa-2* mutants revealed that they lacked catabolic dehydroquinase activity (C-DHQase) (Giles *et al.* 1985). This suggests that the *qa-2* gene product is responsible for the catabolism of dehydroquinic acid to dehydroshikimic acid. In experiments utilizing quinic acid as the sole carbon source two other mutants were identified (Chaleff 1974). The *qa-3* mutants lacked both quinic acid dehydrogenase (QHDase) and shikimic acid dehydrogenase (SDHase) activity. This suggests that this gene product of *qa-3* catalyzes both quinate and shikimate dehydrogenation. Another mutant isolated by Case (Case & Giles 1975), displayed a lack of ability to catalyze the conversion of dehydroshikimic acid to protocatechuic acid. This suggests that the *qa-4* gene codes for the enzyme dehydroshikimic dehydrase (DHS-Dase) (Chaleff 1974).

Considering these early studies, it was thought that a single multimeric protein with two functional domains regulated the *qa* gene cluster. Two types

of *qa-1* mutants were obtained: *qa-1F*$^{-}$ (non-inducible), a pleiotropic mutant displaying a complete lack of activity of all enzymes, and *qa-1*^{C} constitutive mutants, which produced all the enzymes all the time. The first constitutive mutants (*qa-1*^{C}) were obtained (Valone *et al.* 1971) that produced high levels of all three enzymes in the absence of the inducer. It was hypothesized that the constitutive mutants were capable of producing an activator protein capable of initiating transcription, even in the absence of the inducer. In *qa-2* studies, the two types of non-inducible mutants were categorized. The one group showed a (S)low/weak complementation (*qa-1S*), the other showed (F)ast/strong complementation. These two different groups were better characterized when it was found that the multimeric protein was actually two separate regulatory genes, *qa-1F* and *qa-1S* (Geever *et al.* 1989).

VI. Organization of the Quinic Acid Gene Cluster

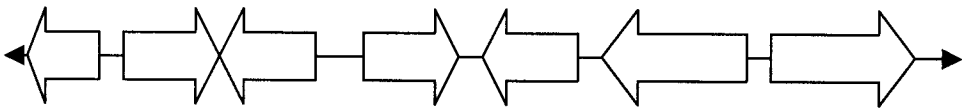
Transformation experiments, S1 mapping, Northern blot analysis, and RNA sequencing have determined the molecular organization of the *qa* gene cluster. The cluster is 17.3 kb long and is located close to the *met-7* locus on the right arm of linkage group VII (Case & Giles 1975). The cluster has five structural genes: *qa-2*, *qa-3*, *qa-4*, *qa-x*, and *qa-y*. The two regulatory genes are *qa-1S* and *qa-1F* (Geever *et al.* 1989). The cluster is organized so that the regulatory genes are located at one end, and the structural genes at the other (Figure 1). Each of the three pairs of genes is divergently transcribed (*qa-*

1S/qa-1F, *qa-4/qa-3*, *qa-x/qa-2*) in pairs. These two features are also found in the *qut* cluster of *Aspergillis nidulans*, despite differences in the order of genes between the two (Grant *et al.* 1988; Hawkins *et al.*1988).

Differences in the regulatory and structural paired genes are largely based on the difference in intergenic distance and in the number of activator-binding sites. Between the regulatory gene pair, this intergenic distance is relatively short (373 bp between inducible start sites). Because of this it is thought that they share a common activator-binding site that mediates induction of both of the *qa-1* genes bidirectionally (Geever *et al.* 1989). To support this theory, transcripts of both genes are inducible to similar extents (Huiet, 1984; Patel & Giles, 1985). However the paired structural genes are separated by a greater distance (1022 bp and 1672 bp). They also have several activator-binding sites with different levels of induction between genes of a pair (Geever *et al.* 1986). The single, unpaired *qa-y* gene separates the structural gene pairs from the regulatory gene pair (Giles *et al.*1985). This gene is thought to encode a permease for quinic acid (Geever *et al.* 1985). This is because of its sequence homology with the *qutD* gene (quinic acid utilizing) of *Aspergillis nidulans*, which is known to be a quinic acid permease (Whittington *et al.* 1987). The transcriptional levels of this gene elevate during induction of the cluster by quinic acid, which is likely because of its supposed permease function.

The paired gene RNA transcripts vary in transcript length (Figure 2). The 3' modifications remain the same regardless of the 5' modifications. These

Figure 1: Organization of the qa gene cluster


qa-x
qa-2
qa-4
qa-3
qa-y
qa-1S
qa-1F

differences are thought to be the result of different levels of induction. The model of gene clustering is thought to have a greater effect on the control of the level of induction rather than on the initiation of induction of the structural genes (Geever *et al.* 1986).

VII. Quinic Acid Metabolism

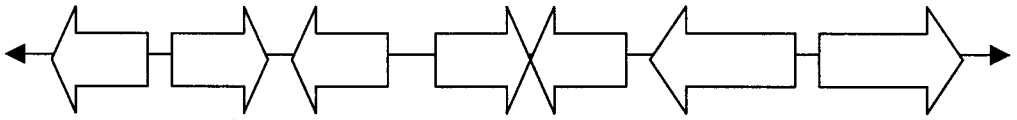
The structural genes – *qa-2, qa-3,* and *qa-4* – code for the enzymes for the first three steps of quinic acid metabolism (quinic acid $\rightarrow\rightarrow\rightarrow$ protocatechuic acid) (Figure 3). *Qa-3* codes for a quinic acid/shikimic acid dehydroquinase, which converts quinic acid and shikimic acid to dehydroquinic acid (DHQ) and dehydroshikimic acid (DHS) respectively. *Qa-2* codes for catabolic dehydroquinase (C-DHQ), which converts dehydroquinic acid (DHQ) to dehydroshikimic acid (DHS). The *qa-4* gene product, DHS dehydrase, coverts the dehydroshikimic acid to protocatechuic acid (PCA), which is further broken down to be utilized as a carbon source (Geever *et al.* 1987).

VIII. Regulation of the *qa* Gene Cluster

The *qa* gene cluster is regulated by two types of control at the level of transcription (Patel *et al.*1981). The first and most prominent mechanism of control is the interaction of the two regulatory genes *qa-1S* and *qa-1F* (Giles *et*

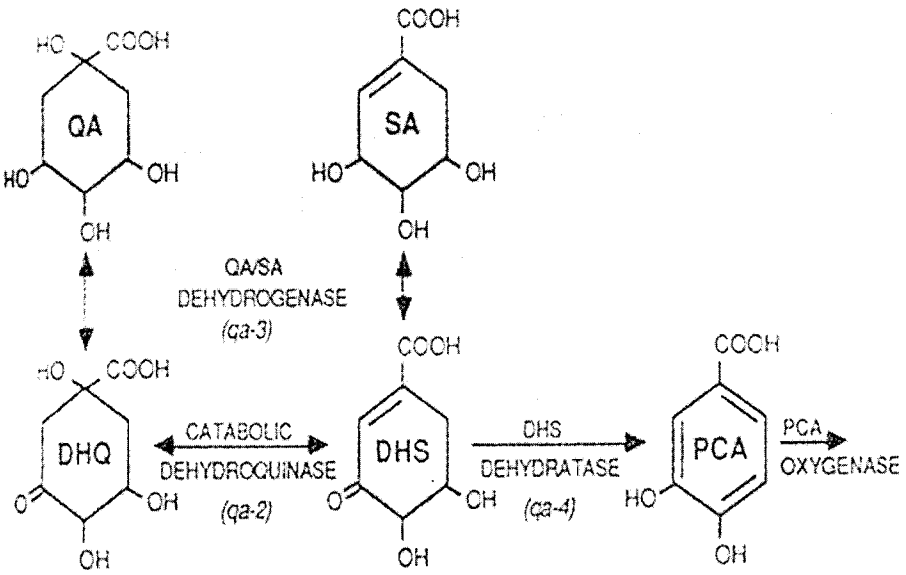
Figure 2: Transcriptional map of the qa gene cluster showing the sizes of the principal
mRNA transcripts plus any minor transcripts

mRNA's



qa-x	*qa-2*	*qa-4*	*qa-3*	*qa-y*	*qa-1S*	*qa-1F*
1.3 kb	1.0 kb	1.5 kb	1.4 kb	2.3 kb	4.1 kb	2.9 kb
1.5 kb	1.2 kb	1.7 kb	1.6 kb		3.4 kb	3.0 kb
					1.25 kb	

Figure 3: Quinic acid metabolism

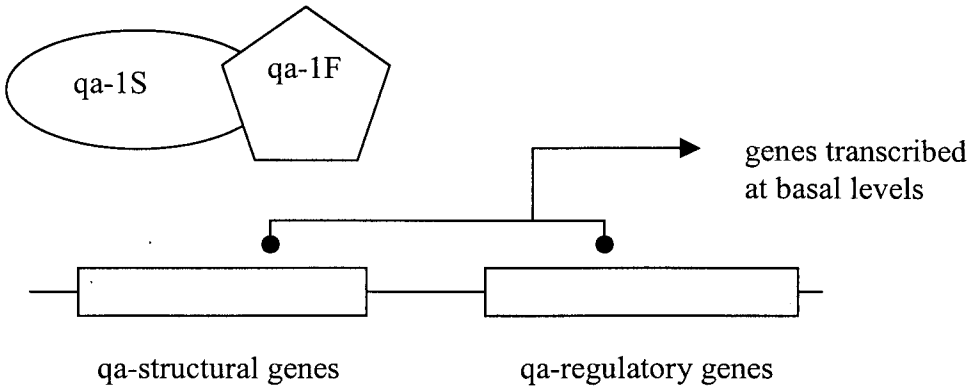

HO
COOH
QA
HO
OH
OH
COOH
SA
HO
OH
OH
QA/SA
DEHYDROGENASE
(qa-3)
HO
COOH
DHQ
O
OH
OH
CATABOLIC
DEHYDROQUINASE
(qa-2)
COOH
DHS
O
OH
OH
DHS
DEHYDRATASE
(qa-4)
COOH
PCA
HO
OH
PCA
OXYGENASE

*al.*1985). The *qa-1S* gene product acts as a repressor, and the *qa-1F* gene product acts as an activator of the cluster and seems to auto-regulate its own transcription (Huiet 1984). The interaction is mediated by the presence/absence of the inducer quinic acid (Figure 4). A secondary level of control is catabolite repression. Here a preferred carbon source, like glucose, will repress *qa* gene expression.

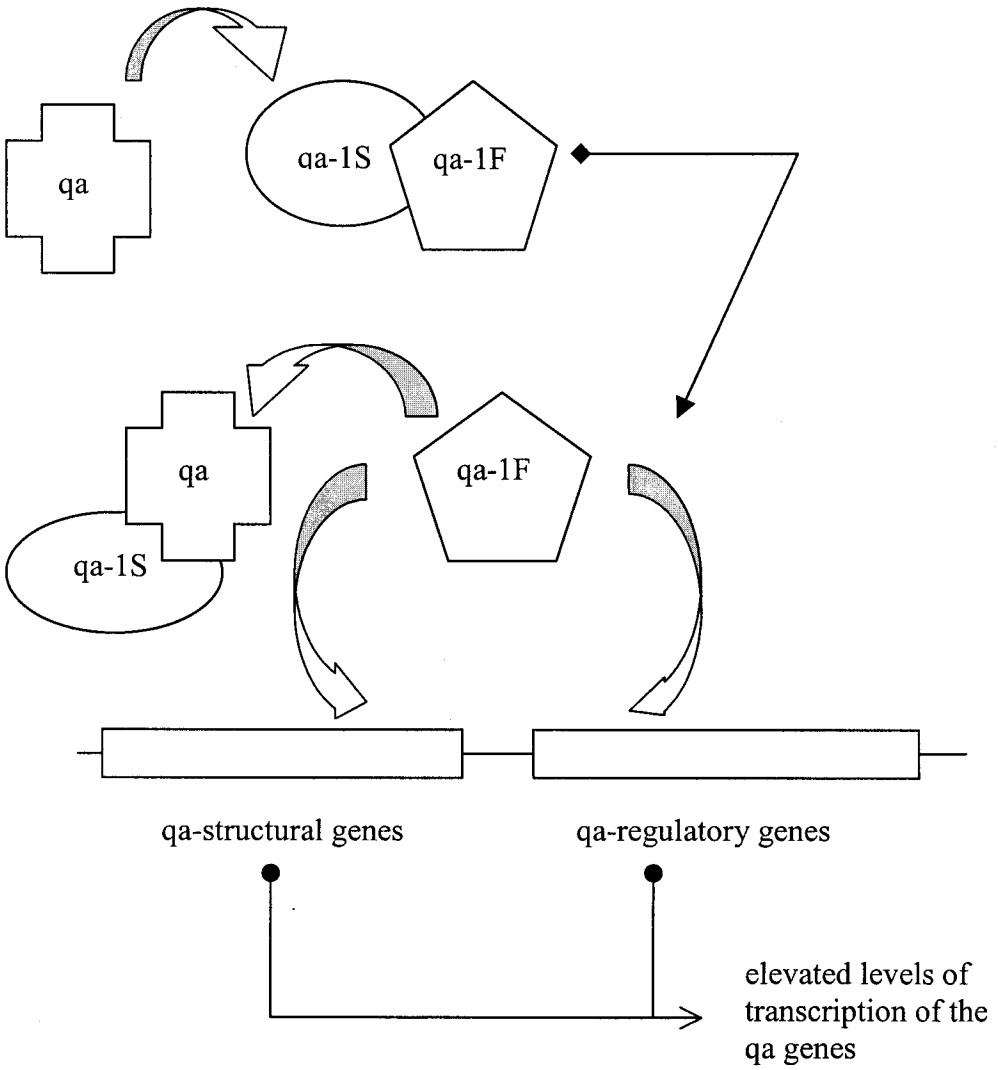
Normally, the *qa-1F* gene codes for an activator protein, whose DNA-binding ability is located at its N-terminus (Case 1992), similar to *GAL4* of *S. cerevisiae* (Keegan 1986), that binds at a conserved 16 bp sequence with little variations. In the current model for regulation in the wild-type (*qa-1S*+ /*qa-1F*+), where both *qa-1* genes are producing functional gene products, the *qa-1S* protein can interact with the *qa-1F* protein to either prevent or permit dimmerization of the activator protein. This dimmerization is necessary to form the structure of *qa-1F* that has the ability to elevate levels of transcription of *qa* genes by binding at the activator-binding sites in the intergenic regions of the paired genes. *Qa-1S* is thought to prevent the increased transcription of *qa-1F* in the absence of an inducer (qa) and the genes are transcribed at basal levels of gene expression. Whether the interaction between these two genes is direct or indirect is not exactly known. Presence of the inducer will release this inhibition of *qa-1F*, by *qa-1S*, and allow the positively-acting regulatory protein to increase the level of all *qa* genes of the cluster. The roles of the regulatory proteins were initially established through studies of mutants. *Qa-1F*$^-$ mutants are thought to produce

Figure 4: Current theory of regulation of the quinic acid gene cluster

Absence of inducer:



Presence of inducer (quinic acid):



defective activators since they are non-inducible in the presence of quinic acid (Giles *et al.* 1985). Deletion experiments have shown Δ*qa-1S* mutants to constitutively express genes of the *qa* cluster, providing evidence that of its repressor function (Case *et al.* 1992).

The *qa-1F* gene encodes an 816 Amino acid, 86kDa activator protein (Giles *et al.* 1985). *Qa-1F* binds the qa gene cluster at 14 different known sites determined by DNase I footprinting experiments (Geever *et al.,* 1987). Using qa-1F produced by a baculovirus overexpression vector was originally done by inserting the gene into the polylinker downstream from a baculovirus polyhedrin promoter (Miller *et al.,*1986) (Baum *et al.,* 1987). The N-terminus of *qa-1F* shares a conserved segment of 28 amino acids that has a six-cysteine motif (Figure 5) (Geever *et al.,*1987 p.348). It shares this motif with *LAC9* (Salmeron & Johnson 1986)from *F.lactis*, *GAL4* (Laughon & Gesteland 1984), *PPRI* (Kammerer *et al.,* 1984) and *ARGRII* (Messenguy *et al.,* 1986) from *S. cerevisiae*. The elevation of transcriptional levels of *qa* gene levels involves binding to a conserved 16 bp sequence 5' - GGRTAARYRYTTATCC –3' (Baum, Geever and Giles 1987) by *qa-1F* (Table 1). The *qa-1F* protein shown to bind to this conserved sequence *in vitro* (Geever *et al.,* 1987). The DNA-binding portion of the *qa-1F* protein is located in the first 183 amino acids at the N-terminus (Case 1992). Similarly, the *GAL4* DNA-binding domain is localized to the first 74 amino acids (Keegan 1986). Both of these regions are composed of an arginine-lysine- rich domain and six cysteines (Laughon 1984), which are homologous to sequences in the that it interacts with the activator to

Figure 5: Conserved amino acid sequences of five fungal activator proteins. Position of first cysteine residue is indicated.

```
                 *     *        *        *     *        *
qa-1F      76  C DQ C RAAREK C DGIQPA C FP C VSQGRS C
LAC9       95  C DA C RKKKWK C SKTVPT C TN C LKYNLD C
GAL4       11  C DI C RLKKLK C SKEKPK C AK C LKNNWE C
PPRI       34  C KR C RLKKIK C DQEFPS C KR C AKLEVP C
ARGRII     21  C WT C RGRKVK C DLRHPH C QR C EKSNLP C
```

Table 1: Sequences of Activator Binding Sites

Gene	Position	Conserved Sequence
Qa-2	-127	GGGGAC **GGGTAA TCGC TTATCC** GCTCGT
Qa-1F-qa-1S	-144	ACAAAA **GGATAA ACAA TTATCC** TCCCAA
Qa-3	-449	ACTGCT **GGGTAA GTAT TTAAGC** GGTAGG
Qa-y	-512	TCCCCC **GGTTAT ACAT TCATCC** TCACCC
Qa-4	-510	ACGGCC **GGCTAA GTGT TTAACA** CGCAAG
Qa-y	-681	CTGGCT **GGGTAA TGGC TTTTCC** GTTCAT
Qa-2-qa-x	-510	TCGGCT **GGATGA GTTT TTAACC** GTTGAT
Qa-3	-264	CCCAAA **GGCAAA ACGC TCATCC** TGAATC
Qa-2	-391	TTTCTT **GGATAA ATCC TAACCC** TTTCTT
Qa-1S	+236	CTGATC **GGATGA GTGA TTCTCC** AGACAT
Qa-3	-70	AAAATG **GGGGAA TAAC TTATAG** CCACGC
Qa-4	-374	CCCACC **CGTTAA TGCT TTATTC** CGCTGA
Qa-y	-412	GCTGCC **GGCTCA ACAC TCATCA** GCTTCA

Consensus Sequence (14 total):

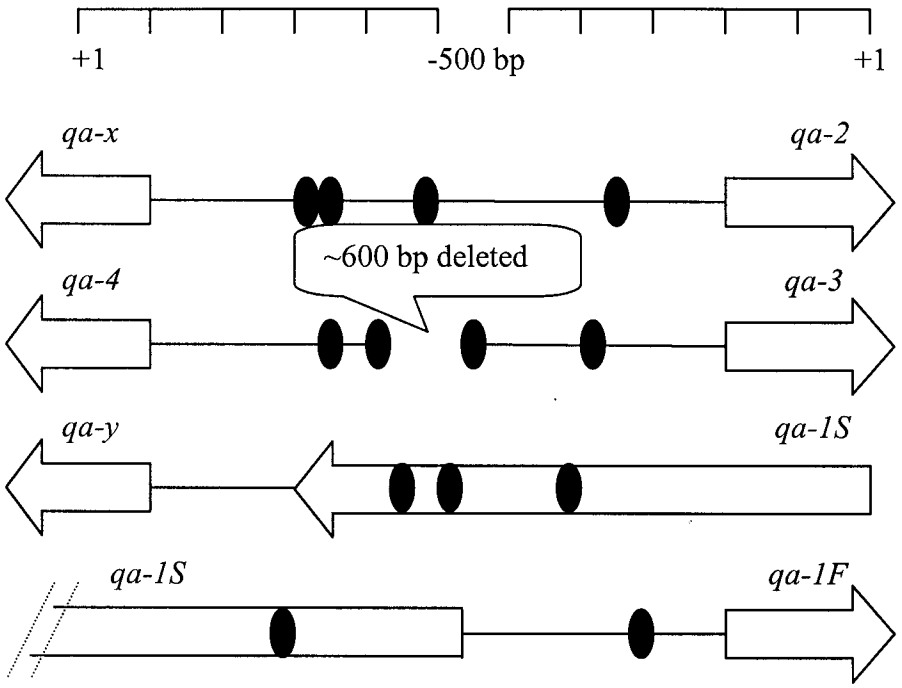
$$\mathbf{G^{13}G^{14}R^{9}T^{12}A^{11}A^{13}R^{10}Y^{11}R^{10}Y^{12}T^{14}T^{9}A^{12}T^{9}C^{11}C^{11}}$$

inhibit its function (Giles *et al.* 1985). The repressor protein is thought to have at least twodomains: one for the inducer, quinic acid (Huiet 1984), and one that interacts with another protein, most likely the activator gene product.

Qa-1F controls the level of induction by binding the DNA at a conserved 16bp sequence at any of the 14 possible sites located in the inter-coding regions of the paired genes (Figure 6). This is responsible for the variation of the 5' end of the transcripts at different levels of induction. It is thought that a combination of binding events *by qa-1F* allows for greater degree of control of induction of the cluster. Presence of *qa-1F* is necessary for autoreguation of its own transcriptional level by binding at the 5'-GGATAAACAATTATCC-3' sequence at –468 base pairs in relation to the start site transcription of *qa-1F.*

The *qa-1F* and *qa-1S* genes have only one binding site in their shared 5'-flanking regions. Binding of *qa-1F* at this site causes induction of *qa-1* genes 40- to 50-fold. The structural genes have at least three binding sites in their 5'-flanking regions. By binding here, *qa-1F* induces the transcription of the structural genes 300- to 1000-fold (Baum *et al.* 1987). Once the inducer is present, it binds to *qa-1S* in the N-terminal region of the protein (Giles *et al.* 1985), inhibition of *qa-1F* by the repressor us released, and the activator will now auto-regulate its own transcription, while increasing the levels of amino termini of lower eukaryotic activator proteins *PPR1* (Kammerer 1984), *ARGRII* and *LAC9* (Messenguy 1986).

Figure 6: Locations of the Activator Binding Sites



Divergent pairs of transcripts (arrows) are aligned by their initiation site.The binding sites are indicated by solid circles. A segment (~600 bp) of the intergenic region between qa-3 and qa-4 has been omitted.

The *qa-1S* gene encodes a 101kDa repressor protein 918 amino acids in length (Geever *et al.* 1987). It has not been proven that *qa-1S* is a DNA-binding protein, yet it is thought induction of the mRNA's of the other *qa* genes. This level of transcriptional control is thought to be possible because of a coordination of various DNA-binding events at the multiple activator-binding sites in the 5'-flanking regions of the induced genes. The elevation of *qa-1S* levels while in the presence of the inducer (*qa*) by *qa-1F* activator protein is hypothesized to provide an ample amount of repressor protein so that when no significant amount of inducer is available, there will be enough repressor protein present so that the transcription of the cluster can be rapidly shut down to a basal level.

Several mutants have been studied for the significance of their activator-binding sites in their 5'-flanking region. The first studies of these mutants began with the cloning of *qa*-2. Some transcriptional mutants, having rearrangements in the 5'-flanking regions of *qa-2*, so only the –127 activator-binding site is retained, express this gene at 15-30% of induced wild-type levels (Geever *et al.* 1986). This suggests that at least one of the missing binding sites at –391 and –510 are necessary for full induction. Another study of transcriptional *qa-2* mutants suggested that multiple binding events are associated with repression of a basal level of transcription instead of induction (Geever *et al.*, 1986;Baum *et al.*,1987). In support of this it was observed that a pair of *GAL4* binding sites were required for effective *GAL80* repression of

basal level transcription, but only one binding-site was necessary for high levels of transcriptional induction (Bram *et al.*, 1986). Binding at an additional site does not increase this level however.

There is some indirect evidence of partial occupation of the activator-binding sites prior to induction. A chromatin analysis of *qa* intergenic sequences showed a distinct class of DNase I-hypersensitive sites (HSSs) dependent of the presence of *qa-1F* (Baum & Giles, 1985, 1986). The localized enhancement of chromatin sensitivity of the HSSs of the induced strain was not seen in the uninduced wild-type strain or in the non-inducible *qa-1S* mutant strains. Also, these localized areas of increased sensitivity in the induced strain corresponded to the activator-binding sites determined by *in vitro* footprinting (Baum *et al.* 1987). These positions of HSSs in induced strains were clearly distinguishable from those basal level HSSs, and inducible HSSs were not found in *qa-1F*$^-$ mutants. These basal levels of HSSs were shared by all strains but the *qa-1S*^{c}, where there are discrete sites of sensitivity, being that the sensitivity was uniform regardless of induction (Baum & Giles, 1985). This information suggests that a codependence of *qa-1S* and *qa-1F* must exist for the formation of inducible HSSs.

IX. Catabolic Repression

Catabolite repression is the second method of regulation of the *qa* gene cluster. While under the influence of catabolite repression, the genes of the *qa*

gene cluster are transcribed at about 10% of the levels that occur when quinic acid is the preferred carbon source. It is suggested that the *qa-x* gene product may play a role in catabolite repression, being that the protein has 31% amino acid homology with *GAM1* of *S. cerevisiae* (Giles *et al.,* 1991). The *GAM1* protein is believed to serve a role in catabolite repression by modifying the *GAL4* activator protein in the GAL system of *S. cerevisiae.*

When sucrose-grown *qa-1S*^{c} strains were shifted from a sucrose to a carbon-free or glycerol medium, the levels of four of the mRNA's only increased 1.5- to 2-fold (Giles *et al.,* 1985). This shows that the effect of catabolite repression on the transcriptional levels of some *qa* genes, like *qa-2, qa-x* and *qa-4* is not significant. However, the effect of catabolite repression on other *qa* genes like *qa-1F, qa-3* and *qa-y* is significant. This may be related to the increased affinity of the *qa-1F* activator protein (Baum *et al.,*1987) for the activator-binding sites of these genes most affected by catabolite repression over those affected less.

X. DNase I Hypersensitive Sites (HSSs) of the *qa* Gene Cluster

At least 14 activator-binding sites have been identified by DNase I footprinting, with at least one site before each *qa* gene. The 5'-flanking areas of the *qa* genes, which range between 100 and 500 bp in length, have been isolated by the creation of restriction fragments for *qa*-y and each of the paired genes. The DNase I footprinting for both wild-type (*qa-1F*$^{+}$) and in activator-

independent (*qa-1F*$^-$) strains were analyzed. In general, it was found that the DNase I HSSs in the uninduced wild type and the activator-independent mutants were much more upstream from the start site of transcription than the HSSs of the induced wild type.

The positions of DNase I HSSs within the *qa-2* and *qa-x* region have been identified, and inducible HSSs within this region are shown to be under the control of the *qa-1F* protein (Baum & Giles 1985). In the uninduced, wild-type chromatin, five DNase I HSSs were located in the 5'-flanking region of *qa-2* centered near positions –430, -330, -165, -85, and +105 relative to the +1 site of transcription (Baum & Giles 1986). Induction of the wild-type (F^+) produced enhanced cleavage at the positions –165 and –85 HSSs (Baum & Giles 1985). In *qa-1F*$^-$ strains, which produce defective activators, the position at –165 HSS is absent, and the cleavage by DNase I at the position –430 HSS is reduced. This suggests that DNase I cleavage at these sites is affected by activator protein binding. Only two HSSs are detectable in the uninduced wild-type near positions -300 and –210 relative to the +1 start site of *qa-x* transcription. Some DNase I digestion can be detected at –475 on autoradiograms when exposed for extended lengths of time. Once induced, additional DNase I cleavage is observed near position –90, and increased cleavage occurred at HSS positions –210, -300, and –475. All of these HSSs are absent in chromatin isolated from induced or uninduced *qa-1F*$^-$ strains (Baum & Giles 1986).

The organization of DNase I HHSs within the intergenic region of *qa-4-qa-3* were similar to that of the *qa-2-qa-x* region. There were three HSSs

observed in the flanking regions of *qa-3* centered near positions –510, -375, and –120 while only one weak HSS was located in the 5'-flanking region of *qa-4* near position –540 relative to the +1 site of transcription of the gene. Once induced, the 5' region of *qa-3* had additional DNase I HSSs near positions –290, -220, and –40 while the 5'region of *qa-4* exhibits additional HSSs near positions –430 and –320. The ~500 bp DNase I-hypersensitive region from position –510 to –40 HSSs of *qa-3* is interrupted by three short DNase I-protected regions (Baum & Giles 1986).

By comparing these two intergenic regions many similarities can be observed. First, the predominant regions of DNase I digestion in uninduced wild-type are in the 5'-flanking regions of *qa-2* and *qa-3*. The position –430 to –330 of *qa-2* and the position –510 to –375 region of *qa-3* extend for at least 100 bp and are interrupted by short regions protected by DNase I digestion. Also, in the uninduced wild-type, both genes displayed generally weaker DNase I digestion closer to their 5' ends. Second, the 5'-flanking regions of *qa-x* and *qa-4* show relatively weak DNase I cleavage in uninduced wild-type but strong DNase I cleavage in induced wild-type. In general, the group of paired structural genes - *qa-2, qa-x, qa-3, qa-4* - have inducible HSSs that tend to be closer to the 5' ends of their respective genes than do the constitutive activator-independent HSSs.

The *qa-y* gene of the *qa* cluster is the only unpaired gene that has no divergently transcribed gene partner. In the uninduced wild-type, three HSSs can be detected near the positions –450, -180, and –90. Once induced,

extensive changes occur within the region extending upstream from the position –450 HSS. In the induced wild-type, additional HSSs are observed near positons-930, -840, -750, -610, and –360 with DNase I cleavage at the broad position –610 HSS extending for ~100 bp. A similar pattern of DNase I-protected regions occurs as mentioned above. In the 400-bp DNase I-hypersensitive region from position –360 to –750 HSSs is interrupted by three short DNase I-protected regions. Unlike the other *qa* structural genes, DNase I cleavage of induced wild-type occurs more distal rather than proximal to the 5' region of the *qa-y* gene.

Both *qa-1S* and *qa-1F* regulatory genes are divergently transcribed from a 370 bp intergenic region. Relative to the +1 site of transcription of *qa-1F*, the DNase I HSSs of the uninduced wild-type chromatin were observed at –730, -490, -360, -220, +50, and +100. The most predominant of these HSSs is the position –220, that extends at least 130 bp (Baum & Giles 1986). Once induced, another HSS can be detected at -120, though some mild cleavege is detectable in the uninduced wild-type. Unlike the *qa* structural genes, the *qa* regulatory genes show very little change in their DNase I HSSs once induced.

In the process of identifying the DNase I HSSs of the *qa* gene cluster, many mutants were studied. The purpose of the mutants was to establish some difference between the DNase I HSSs of the wild-type and mutants in both induced and non-induced conditions. One of the mutants studied was an activator-independent strain ($qa\text{-}2^{ai}$) that allowed transcription of the *qa-2* gene in the absence of a functional *qa-1F* gene product. These mutants were

obtained by ultraviolet irradiation of the *qa-2*$^{+}$ *qa-1F*$^{-}$ *arom-9*$^{-}$ double mutants as revertants that were now able to grow on minimal media. Studying these *qa-2*^{ai} mutants has revealed the identity of a 105 bp region of the 5'-flanking region of *qa-2* that is necessary for its induced level of transcription mediated by *qa-1F* $^{+}$(Geever *et al.,* 1986). This region is flanked by two DNase I hypersensitive regions. One of these regions is only found in *qa-1F* $^{+}$ (inducible) strains, and not in *qa-1F* $^{-}$ (non-inducible) strains (Baum *et al.,*1985). This suggests that the additional binding site is created by direct activator binding and is supported by the finding that cleavage by DNase I at these HSSs is enhanced once it was induced by quinic acid. This data of *qa-2* ^{ai} *qa-1F* $^{+}$ mutants indicate that mutations retaining the upstream binding site were inducible, but those with the sequence deleted were non-inducible (Giles *et al.,* 1985). The mutants' expression of *qa-2*$^{+}$ was constitutive regardless of deletion, point mutation or rearrangement (1-45 % of induced wild-type) (Giles *et al.,* 1985).

XI. Activator Binding Sites

The *qa* structural genes, which are induced 300- to 1000-fold in the presence of quinic acid, have multiple activator-binding sites in their 5'-flanking regions (Baum *et al.,*1987). The positions of these binding sites, and their relationship to the start site of transcription, vary. The degrees of inducibility, that occur at the binding sites in relatively the same positions in

regard to the +1 site of structural *qa* mRNA's, are also unrelated. In the case of *qa-2, qa-3, qa-4,* and *qa-x,* it was generally seen that in the induced wild-type chromatin, activator-binding sites closer to the +1 site of transcription, were being utilized. The three activator-binding sites for *qa-y* reside in the first and second poly(A) tail addition sites of the *qa-1S* gene (Giles *et al.,* 1985). The site that is closer to the site of second poly(A) tail addition, which is closer to +1 site of *qa-y*, is the site observed in the uninduced wild type. Those activator binding sites closer to the first poly(A) addition site, further upstream from the +1 site of *qa-y* transcription, are utilized in induced wild-type. This is indicated by the DNase I HSS of *qa-y* in induced (*qa-1F* $^{+}$) wild-type strains (Baum & Giles 1986). This is the exception to the evidence that activator-binding sites that lie closer to the +1 start site are necessary for higher levels of induction.

To determine the relative binding strengths of the activator binding sites, a competitive binding assay was done on twelve of the sites. Each of the 12 sites was isolated on a separate restriction fragment, which were incubated with equal concentrations with progressively increasing amounts of activator protein. As observed by the disappearance of free DNA as the activator concentration was increased, the -127 binding site of *qa-2* and –144 site of *qa-1S* and *qa-1F* displayed the highest affinity for the activator protein (Baum *et al.,* 1987) (Table 2). The binding strengths were determined by the amount of activator protein necessary for complete binding in the DNA-binding assay. The four binding sites that exhibited the highest affinity for the activator protein were observed in the 5'-flanking regions of the genes of the cluster.

However, the –391 site of *qa-2*, the –391 site of *qa-4*, the –70 site of *qa-3*, and the +236 site of *qa-1S* , displayed incomplete binding at even the highest concentrations of activator extract (Baum *et al.,* 1987). By DNase I footprinting analysis, it was observed that these sites, along with the –412 site of *qa-y*, can have as much as a 20-fold difference in their affinity for the activator protein.

In an effort to determine if this regulatory sequence is enough to promote activator protein binding, several oligonucleotides, that were variants of the conserved 16 bp sequence, were tested for their ability to bind the *qa-1F* gene product. The restriction fragment produced was cloned into polylinkers of plasmids pUC18 and pUC19. It was shown, through DNase I footprinting, that the sequence, without other factors, was sufficient to allow binding of the activator protein (Baum *et al.,* 1987).

A digested PCR product that retains the -144 activator binding site is to be isolated. This restriction fragment with the –144 activator binding site will be cloned into a plasmid and used for site-directed mutagenesis using the Kunkel method. When the mutant strand has served as the template for the new activator-binding sequence, the new mutants will be transformed back into *N. crassa.* The purpose of the mutagenesis is to determine the degree of significance of certain highly conserved base pairs as it applies to the ability of *N. crassa* to regulate quinic acid metabolism. Future applications of this research could include *in vitro* DNaseI footprinting experiments to determine if the mutations were significant enough to affect the binding ability of *qa-1F* at its own regulatory sequence.

Table 2: Relative Binding Strengths of the Activator Binding Sites

Gene Pair	Position	Conserved Sequence	Affinity
Qa-2/qa-x	-127	GGGGAC **GGGTAA TCGC TTATCC** GCTCGT	1.0
Qa-1S/qa-1F	-144	ACAAAA **GGATAA ACAA TTATCC** TCCCAA	0.8
Qa-4/qa-3	-449	ACTGCT **GGGTAA GTAT TTAAGC** GGTAGG	0.5
Qa-y	-512	TCCCCC **GGTTAT ACAT TCATCC** TCACCC	0.4
Qa-4/qa-3	-510	ACGGCC **GGCTAA GTGT TTAACA** CGCAAG	0.25
Qa-y	-681	CTGGCT **GGGTAA TGGC TTTTCC** GTTCAT	0.25
Qa-2/qa-x	-510	TCGGCT **GGATGA GTTT TTAACC** GTTGAT	0.25
Qa-4/qa-3	-264	CCCAAA **GGCAAA ACGC TCATCC** TGAATC	0.15
Qa-2/qa-x	-391	TTTCTT **GGATAA ATCC TAACCC** TTTCTT	<0.1
Qa-2/qa-x	-391	TTTCTT **GGATAA ATCC TAACCC** TTTCTT	<0.1
Qa-1S/qa-1F	+236	CTGATC **GGATGA GTGA TTCTCC** AGACAT	<0.1
Qa-4/qa-3	-70	AAAATG **GGGGAA TAAC TTATAG** CCACGC	<0.1
Qa-4/qa-3	-374	CCCACC **CGTTAA TGCT TTATTC** CGCTGA	<<0.1
Qa-y	-412	GCTGCC **GGCTCA ACAC TCATCA** GCTTCA	<<0.1

This would provide a better understanding of the conserved sequences of activator-binging sites in eukaryotic systems.

Materials and Methods

I. Materials

QIAGEN coloums were purchased from QIAGEN Inc., Chatsworth, CA; Elutip-D columns were purchased from Scleicher & Schuell, Keene, NH; pBluescript was purchased from Stratagene, LaJolla, CA; Polaroid film, yeast extract, sodium chloride, ampiciullan, tetracycline, isopropyl-β-D-thiogalactosidase (IPTG), 5'-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal), dimethyl formamide, calcium chloride, dextrose, Trizma base, ethylenediaminetetraacetic acid-disodium salt (EDTA), sodium hydroxide, sodium dodecyl sulfate (SDS), potassium acetate, 3-N-morpholino-propane sulfonic acid (MOPS), octyl phenoxy polyethoxyethanol (Triton X-100), phenol, cresol, chloroform, sodium acetate, magnesium chloride, glycerol, polyethylene glycol (PEG), and adenosine triphosphate (ATP) were purchased from Sigma Chemical Company, St. Louis, MO; dNTP's were purchased from PE biosystems, Norwalk, CT; sequencing supplies were purchased from Beckman Coulter, Inc., Fullerton, CA; primers were purchased from Integrated DNA technologies, Inc. Coralville, IA; Ethanol was purchased from Aaper Alcohol and Chemical Company, Shelbyville, KY; isopropanol was purchased from Baxter Healthcare Corporation, McGraw Park, IL; restriction endonucleases (*EcoRI* , *BamHI*, *HindIII*, *PstI*, and *SmaI*), RNase A, T4 DNA ligase, pBR322, and M13mp18

were purchased from Boehringer Mannheim, Indianopolis, IN; bacto-tryptone and bacto-agar were purchased from Difco Laboratories, Detroit, MI; agarose was purchased from EM Science, Cherry Hill, NJ; ethidium bromide, 85% phosphoric acid, and acetic acid were purchased from Fisher Scientific, Fairlawn, NJ;

II. Strains and Media

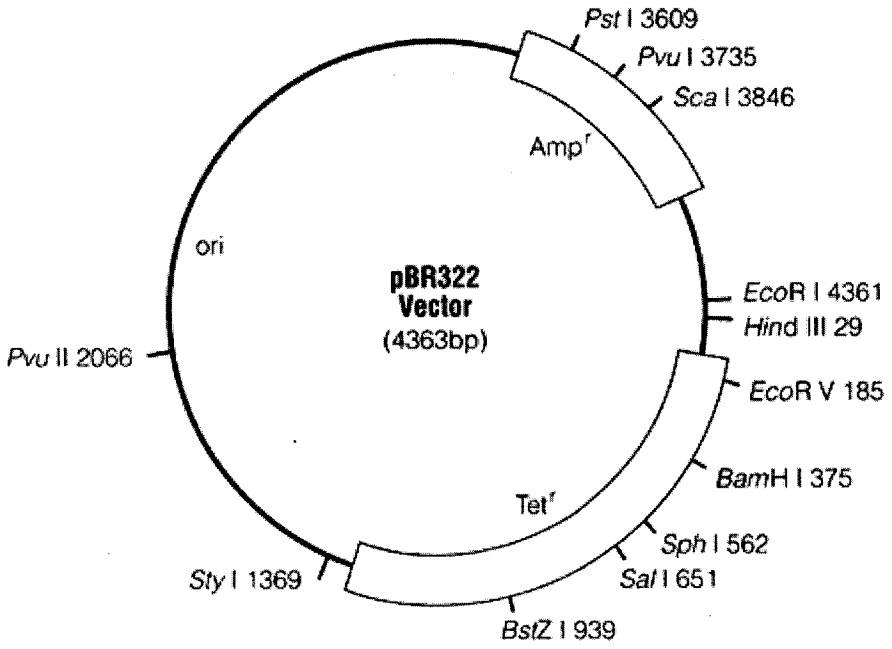
The strain JM101 of *Escerichia coli* used was obtained from the *E. coli* Genetic Stock Center (CGSC) at Yale University. Strain JM101 was used for all experiments involving the pBluescript-based recombinant plasmids and RF M13mp18. Cells were cultured in Luria Broth (LB) (1% bacto-tryptone, 0.5% yeast extract, 1% NaCl). Transformants were selected on Luria Agar with ampicillan [LA100] (Luria broth, agar (1.5%) and ampicillan (100μg/ml)). Blue-white screening was done using 100μl of 200mM IPTG and 50 μl 2% X-Gal in formamide spread on LA100 plates. RF M13mp18 was cultured in 2xYT broth (1.6% bacto-tryptone, 0.5% yeast extract, 0.5% NaCl). Transformants were selected as plaques on YT plates (0.8% bacto-tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% bacto-agar).

III. pBR322

One of the most commonly used vectors is pBR322, a 4363 kb cloning vector (Figure 7). It contains both ampicillan and tetracycline resistance genes to.

Figure 7: Map of pBR322

Figure adapted from www.promega.com


Pst I 3609
Pvu I 3735
Sca I 3846
Amp^r
ori
pBR322
Vector
(4363bp)
EcoR I 4361
Hind III 29
EcoR V 185
BamH I 375
Tet^r
Sph I 562
Sal I 651
Sty I 1369
BstZ I 939
Pvu II 2066

be used for selection. Both of these genes contain unique restriction sites allowing for reverse selection.

IV. pBluescript II KS (+/-) Phagemid

A pUC19 derivative (Figure 8), This phagemid has 2,961 bp and contains an amipicillan resistance gene allowing for antibiotic selection of recombinants. It also contains a potion of the *lacZ* gene, allowing for blue-white selection. Cloning into the multiple cloning site (MCS) disrupts the *lacZ* gene, resulting in white recombinants.

V. M13mp18

M13 is a single-stranded bacteriophage that infects male *E.coli* strains. M13mp18 is an M13 derivative containing a MCS located within a portion of the β-galactosidase gene (Figure 9). Cloning into the MCS disrupts the β-galactosidase gene, allowing for blue-white screening of recombinants.

M13 exists in two different forms-a single-stranded infectious bacteriophage and a double-stranded, intracellular replicative form. The replicative form can be manipulated as a plasmid. The phage coat can be removed from the single-stranded phage to isolate the single-stranded DNA.

Figure 8: Map of Vector pBluescript

Figure adapted from www.stratagene.com

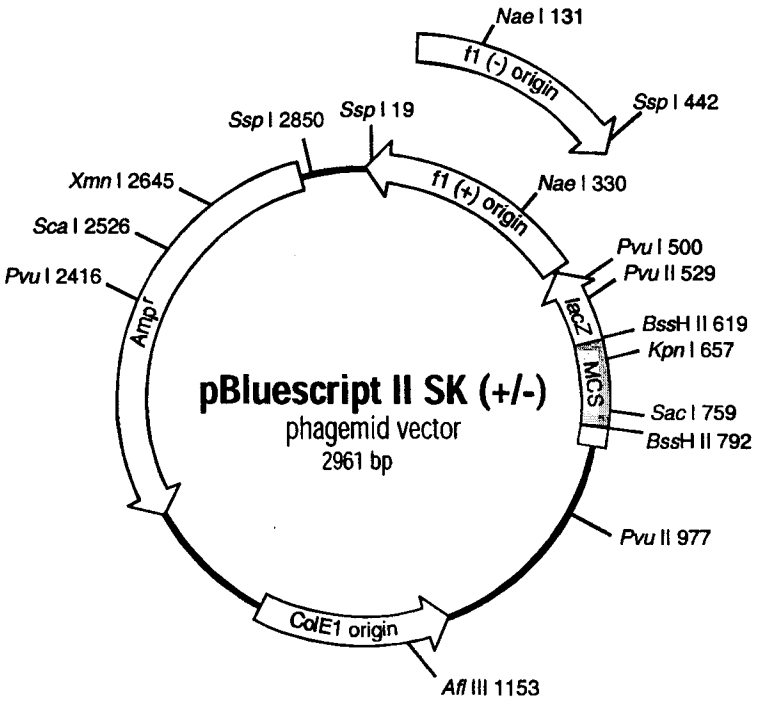
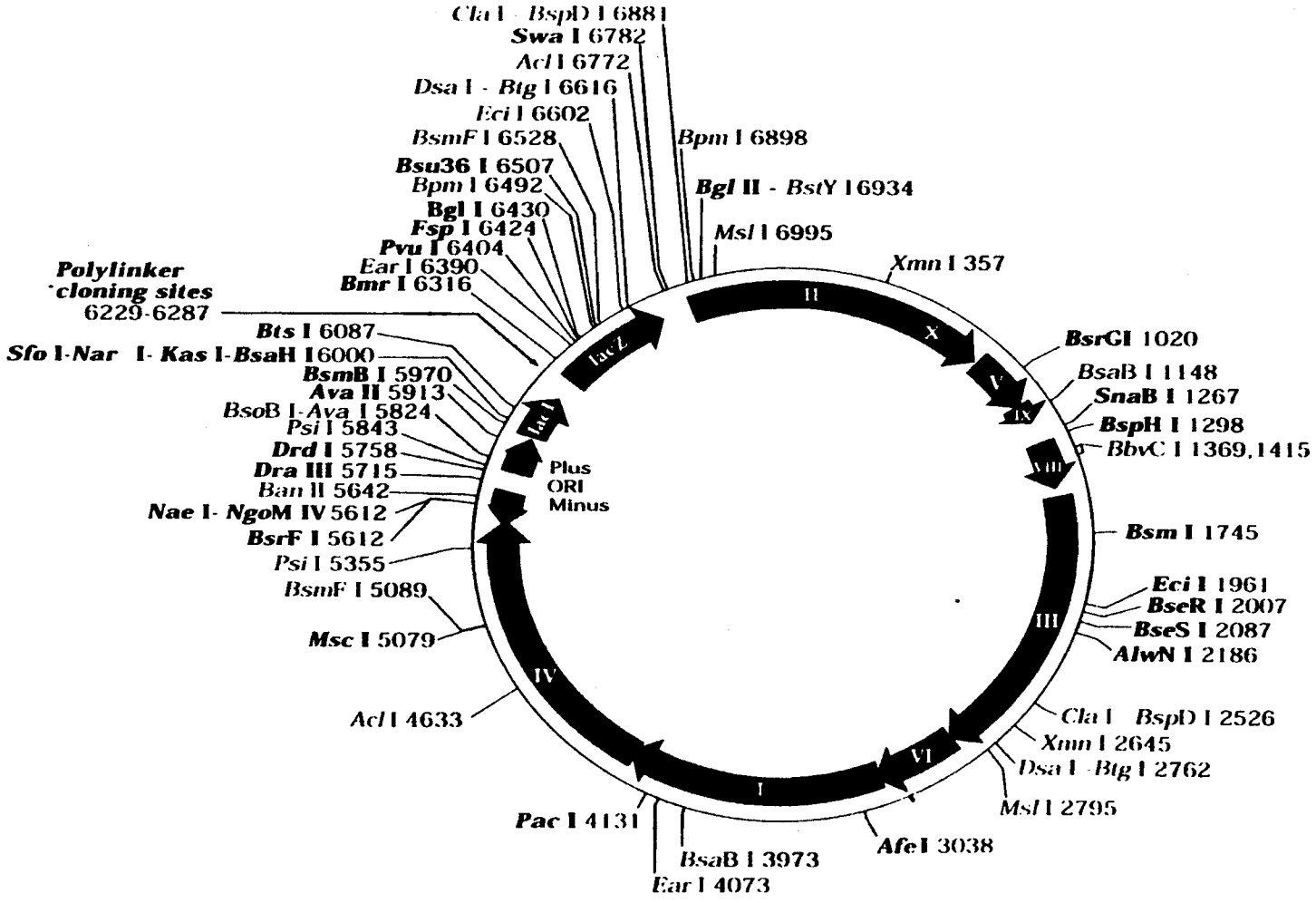

Nae I 131
f1 (-) origin
Ssp I 442
Ssp I 2850
Ssp I 19
Xmn I 2645
f1 (+) origin
Nae I 330
Sca I 2526
Pvu I 500
Pvu I 2416
Pvu II 529
Ampr
lacZ
BssH II 619
Kpn I 657
MCS
pBluescript II SK (+/-)
phagemid vector
2961 bp
Sac I 759
BssH II 792
Pvu II 977
ColE1 origin
Afl III 1153

Figure 9: Map of Vector M13mp18

Figure adapted from Arnett, D. MS Thesis.

Youngstown State University 1999


Cla I - BspD I 6881
Swa I 6782
Acl I 6772
Dsa I - Btg I 6616
Eci I 6602
BsmF I 6528
Bsu36 I 6507
Bpm I 6492
Bgl I 6430
Fsp I 6424
Pvu I 6404
Ear I 6390
Bmr I 6316
Polylinker
cloning sites
6229-6287
Bts I 6087
Sfo I-Nar I- Kas I-BsaH I 6000
BsmB I 5970
Ava II 5913
BsoB I-Ava I 5824
Psi I 5843
Drd I 5758
Dra III 5715
Ban II 5642
Nae I- NgoM IV 5612
BsrF I 5612
Psi I 5355
BsmF I 5089
Msc I 5079
Acl I 4633
Pac I 4131
BsaB I 3973
Ear I 4073
Afe I 3038
Msl I 2795
Dsa I - Btg I 2762
Xmn I 2645
Cla I - BspD I 2526
AlwN I 2186
BseS I 2087
BseR I 2007
Eci I 1961
Bsm I 1745
BbvC I 1369,1415
BspH I 1298
SnaB I 1267
BsaB I 1148
BsrGI 1020
Xmn I 357
Msl I 6995
Bgl II - BstY I 6934
Bpm I 6898
lacZ
lacI
Plus
ORI
Minus
I
II
III
IV
V
VI
VIII
IX
X

VI. Transformation

Fifty milliliters of LB was inoculated with JM101 and grown to an OD of 0.2 to 0.4. After being placed on ice for 5 minutes, the cells were centrifuged at 10,000 x *g* for 10 minutes at 4°C. The pellet was then resuspended in 15 ml of 0.1 M CaCl2 and placed n an ice bath for 30 minutes. The cells were again centrifuged at 10,000 x *g* for 10 minutes at 4°C. The resulting pellet was resuspended in 0.5 ml of 1M CaCl2. Next, 100 µl of these competent cells were dispensed into each of two eppendorf tubes. To one tube, the experimental tube, DNA was added. No DNA was added to the second, control tube. The tubes were placed in an ice bath for 30 minutes, then in an incubator at 37°C for a five minute heat shock. One milliter of LB was added to each tube and the tubes were incubated at 37°C for one hour. Finally, 200µl from each tube was spread onto LA100 plates, which were incubated at 37°C overnight.

VII. Alkaline Plasmid Screen

Colonies were selected from LA100 plates and picked to a 2 ml of LA100 broth and incubated at 37°C overnight. The following day, 1.5 ml of the overnight culture was transferred to an eppendorf tube. The cells were pelleted in a micrcentrifuge and the supernatant was completely removed. The pellet was resuspended in 200 µl of G-buffer (0.05 M dextrose; 0.025 M Tris, pH 8.0; 0.01

M EDTA, pH 8.0). Once well vortexed, 400 µl of denaturing solution (0.2 N NaOH; 1% SDS) was added, followed by gentle mixing. The mixture was placed on ice no more than four minutes before being neutralized by the addition of 300 µl of cold neutralizing solution (3 M KOAc; 2 M HOAc). The tube was then gently mixed and placed in an ice bath for 20 minutes. The mixture was then spun in a micrcentrifuge and spun at 15,000 x *g* for 15 minutes. The pellet was washed with 80% EtOH and allowed to air dry before being resuspended in 50 µl 1x TE buffer (0.01 M tris, pH 8.0; 0.001 M EDTA, pH 8.0). Finally, 10 µl of the sample was run on a 1% agarose gel and the remainder was stored at –20°C.

VIII. Agarose Gel Electrophoresis

All DNA isolated in these experiments was analyzed by gel electrophoresis. DNA was resolved on a 0.8 to 1.0% agarose gel. The buffer used was 1x tris-phosphate EDTA (TPE) buffer [0.08 M Tris, 0.005 M EDTA, 85% phosphoric acid (1.679 mg/ml)]. Gels were stained with ethidium bromide (EtBr) [50 mg/ml] and the DNA was visualized on a transilluminator.

IX. Large Scale Isolation of Plasmid DNA (QIAGEN Preparation)

A colony was picked from an LA100 plate to 50 ml of LA100 broth and grown at 37° C overnight. The cells were centrifuged at 10,000 x *g* for 10 minutes at 4° C. The resulting pellet was resuspended in 7 ml of G-buffer with 70 µl of

RNase A, then 7 ml of denaturing solution was added. After mixing gently, the tube was incubated on ice for 5 minutes. Next, 7 ml of neutralizing solution was added. The tube was immediately mixed and placed on ice for 20 minutes. The mixture was spun at 15,000 x *g* for 30 minutes and the supernatant was transferred to a new tube and recentrifuged at 15,000 x *g* for 30 minutes. The QIAGEN column was equilibrated by applying 7 ml of QBT buffer (0.75 M NaCl, 0.05 M MOPS, 15% EtOH, 0.15% Triton X-100; pH 7.0) and allowing it to gravity flow. The supernatant was then applied to the primed column, allowing the DNA to bind to the resin. The bound plasmid was then washed twice with 15 ml portions of QC buffer (1 M NaCl, 0.05 M MOPS, 15% EtOH; pH 7.0) and eluted with 10 ml of buffer QF (1.25 M NaCl, 0.05 M Tris, 15% EtOH; pH 8.5). The DNA was precipitated by the addition of 7 ml of isopropanol followed by centrifugation at 15,000 x *g* for 30 minutes at 4° C. The pellet was washed twice with ice-cold 70% EtOH and allowed to air dry. The pellet was resuspended in 7 ml 1x TE and divided into six aliquots, five of these were stored at –20° C. From the remaining aliquot, 10 µl of sample was analyzed on a 1.0% agarose gel. The aliquot was then stored at 4° C.

X. Polymerase Chain Reaction (PCR)

The PCR reaction was performed in the PTC-100 programmable thermal cycler. The primers used were #569 (5'-ATC CGG AGA GAT ATG TTG CGA ATC AGG-3') and #770 (5'-CGA GTT AAT AAA TGC CTT GCG GC-3'). The

reaction mixture consisted of: 2 μl of vector, 2 μl of primer #1 (5 mM), 2 μl of primer #2 (5 mM), 3 μl 10x reaction buffer, 3 μl of 10x NTP's, 17 μl of sterile water, and 1 μl of Taq polymerase, for a total of 30 μl. The reaction was mixture was then overlayed with a drop of mineral oil. The first cycle was as follows: 5 minutes at 92.5° C, 2 minutes at 42° C, and 2 minutes at 72° C. The next cycle which was repeated 43x is: 30 seconds at 92° C, 30 seconds at 45° C, and 2 minutes at 72° C. The final cycle was 30 seconds at 92° C, 30 seconds at 42° C, and 10 minutes at 72° C, followed by holding the reaction at 4° C. To check the results of the reaction, a 5 μl sample was run on an 0.8% agarose gel. The reaminder of the reaction was stored at 4° C.

XI. Restriction Digest of Vector and Vector Isolation

Both pBluescript SK(+/-) and RF m13 were digested with restriction enzymes. Into an eppendorf tube was placed 50 μl (1μg/20μl) of Bluescript plasmid 449 or RF m13, 20 μl of 10x buffer, 1 μl of restriction enzymes, and sterile water to a 200 μl total volume. The reaction was incubated at 37° C overnight. Complete digestion was confirmed by running 15 μl of the reaction mixture on a 1.0% agarose gel. If completely digested, the mixture was extracted two times with equal volumes of alkaline-buffered –*m*-cresol and once with an equal volume of chloroform. Two volume of isopropanol were added and the DNA was precipitated by spinning in a microcentrifuge at 15,000 x *g* for 15 minutes. The pellet was washed twice with cold 70% EtOH and allowed to air dry

before being resuspended in 30 µl of 1x TE. The DNA was analyzed again on a 1.0% agarose gel and the cut vector was stored at –20° C.

XII. Restriction Digest of Fragment and Fragment Isolation

Approximately 1 µg of vector was digested. The reaction was incubated overnight at 37° C. The entire reaction mixture was rum on a 1.0% agarose gel and the desired fragment was cut from the gel and placed in a dialysis bag. The bag was filled with 0.5x tris-acetate (TAE) buffer (0.04 M Tris, pH 7.9;0.2 M NaOAC; 0.002 M Na2EDTA, pH 7.9) and placed in an electrophoresis tank filled with the same 0.5x TAE buffer. During the 30 minutes of electrophoresis, a Elutip-D column was primed by first passing 3 ml of high salt buffer (1 M NaCl; 0.02 M Tris; 0.001 M EDTA, pH 7.4) followed by 3 ml of low salt buffer (0.02 M NaCl; 0.02 M Tris; 0.001 M EDTA, pH 7.4). The 0.5x TAE buffer containing the DNA was drawn out of the dialysis bag and passed over the primed Elutip-D column. The DNA bound the resin of the column was eluted by passing 400 µl of high salt buffer and collected in an eppendorf tube. The mixture was then extracted, precipitated, and washed as explained above. The pellet was then resuspended in 20 µl of 1x TE. A 5 µl sample was analyzed on a 1.0% agarose gel to confirm the presence of the isolated fragment. The remaining sample was stored at –20° C.

XIII. Ligation and Transformation of Isolated Digested Vectors and Fragments

The vectors used for the ligations were the digested pBluescript SK (+/-) and RF M13mp18. The ligation mixture consisted of 1 μl of vector, 4 μl of fragment, 4 μl of 5x ligase buffer with PEG, 1 μl 5mM ATP, 9 μl of sterile water and 1 μl of T4 DNA ligase. The reaction was incubated at 14° C overnight. Ten microliters of the ligation mixture were used in the transformation. IPTG and X-Gal were used in the media to allow for blue-white selection of recombinant plasmids. White colonies were selected for alkaline plasmid screening.

XIV. Transformation of Frozen Competant *E. coli* JM101 with RF M13 DNA

Frozen competent *E. coli* strain JM101 was prepared and used for all remaining transformations. JM101 was grown in 250 ml of LB to an OD of 0.48.The cells were chilled on ice for 5 minutes while shaking, then pelleted by spinning for 5 minutes at 5,000 x *g*. The pellet was resuspended in 75 ml of cold 0.1 M MgCl2 then spun to 5,000 x *g*. The resulting pellet was then resuspended in 75 ml of cold 0.1 M CaCl2. After incubating on ice for 30 minutes, the cells were again spun to 5,000 x *g*. The pellet was resuspended in 12.5 ml of 14% glycerol in 0.1 M CaCl2, deispensed into 500 μl aliquots, and frozen at –70° C.

For the transformations, 2 ml of LB was inoculated with JM101 and incubated overnight at 37° C. The next day, 50 ml of LB was inoculated with the 500 μl of the overnight growth and incubated at 37° C for 2 hours. The cells

would serve as lawn cells for the bacteriophage. A tube of frozen JM101 competent cells was thawed and 100 µl was dispensed into each of two eppendorf tubes. To the experimental tube, 10 µl of RF M13 DNA was added, and no DNA was added to the control tube. The cells were placed on ice for 30 minutes, and then heat shocked at 37° C for five minutes. The cells were plated by combining the following: 3 ml of soft YT (0.8% bacto-tryptone, 0.5% yeast extract, 0.5% NaCl, 0.6% bacto-agar), 20 µl IPTG, 50 µl 2% X-Gal, 200 µl uninfected cells, and 30 µl of transformation mix. These mixtures were vortexed gently and poured over YT plates and incubated overnight at 37° C.

XV. Direct Electrophoresis of M13 DNA

The next morning, phages were harvested by picking a plaque with a toothpick and adding it to 2 ml of 2xYT inoculated with 20 µl of an overnight growth of JM101. The cells were incubated overnight at 37° C, and then pelleted in a micrcentrifuge. The supernatants contaning the phage were transferred to eppendorf tubes and stored at 4° C. Direct electrophoresis was done by running 12 µl of supernatant on a 0.8 % agarose gel.

XVI. Sequencing

Sequencing was performed using the Beckman CEQ 2000, a dye terminating sequencer, using the protocol developed by Beckman-Coulter. The dye-terminator reaction mixture consisted of 50-100 fmol of dsDNA (25-50 fmol of ssDNA) with four dye terminators (ddATP, ddGTP, ddCTP, ddTTP), 10x reaction buffer, dNTP mix, polymerase enzyme, a primer, and sterile water to a total volume of 20 μl. The primers used were the standard Beckman –47 pUC19 primer, and the #569/#770 primers for p176. The mixture was place in a thermal cycler on the following program: 96° C for 20 sec, 50° C for 20 sec, and 60° C for four minutes for 45 cycles and then held at 4° C. After the thermal cycling, the reaction was stopped by the addition of stop solution (1.5 M NaOAc, 50mM EDTA) and glycogen. The DNA was precipitated with pure ethanol and washed two times with 75% ethanol. The pellets were vacuum dried for 1 hour and resuspended in deionized formamide. The samples were then loaded into the Beckman CEQ 2000 and run with the desired method.

Results

I. Construction of Plasmid p176

The *qa* cluster of *N. crassa* has unique restriction enzyme sites at the cluster's periphery that allow it to be easily cut and ligated into a plasmid vector. The cluster has three *Pst*I restriction enzyme sites within the cluster. These sites occur within the coding regions of the most lateral genes, *qa-x* and *qa-1F*, at positions 718 and 15416 respectively, and in the middle of the cluster at position 7953, between coding regions for *qa-y* and *qa-3*. The *Pst*I digest yields two fragments that can easily be maintained in plasmids. The two fragments include all of the inter-genic sequences of of the *qa* genes and nearly all of the coding regions (with the exception of *qa-x* and *qa-1F*). The plasmid p176 is a fairly large pBR322 plasmid vector that has a *Pst*I site within its Ampicillan resistance gene. Of the two fragments created *Pst*I retsriction endonuclease digestion, the 7.2 kb insert that retained the -144 activator-binding site of *qa-1F* within the inter-genic region of *qa-1S* and *qa-1F*, was ligated into the pBR322 plasmid and characterized as p176. This subclone was then transformed into *E.coli* JM101. Once transformed, p176 was isolated by a QIAGEN column (Figure 10, Lanes 1 and 2).

Figure 10: Isolation of p176 by QIAGEN column
Lane 1 and 2: Qiagen isolated p176
Lane 3: Lambda size standard, digested with *Hind*III

Lanes 1 2 3

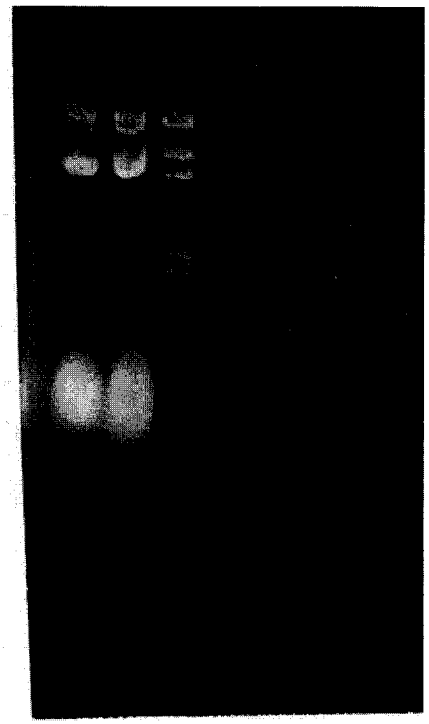
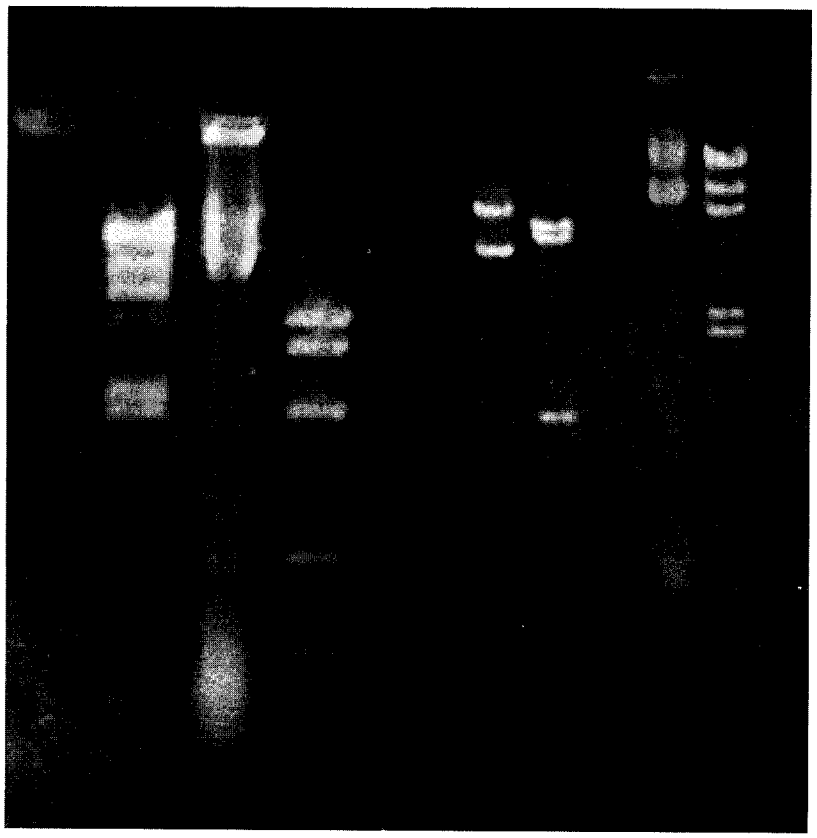


Figure 11: Restriction Digests of p176
Gel 1: Lane 1: *Hind*III digested lambda size standard.
Lane 2: Supercoiled p176.
Lane 3: *Eco*RI/*Pst*I digested p176 (4.9 kb, 3.6 kb, 2.1 kb, .8 kb, .4 kb)
Gel 2: Lane 1: *Pst*I digested p176 (7.5 kb, 4.3 kb).
Lane 2: *Eco*RI digested p176 (5.7 kb, 4.9 kb, 1.2 kb)
Lane 3: Supercoiled p176. Lane 4: *Hind*III digested lambda size standard

Gel 1 Gel 2

Lanes 1 2 3 1 2 3 4



II. Characterization of Plasmid p176

To further characterize the p176 subclone, various restriction digests were performed to produce a restriction map of p176. The first of which was a digest using the restriction endonuclease *Pst*I (Figure 11 Gel 2, lane 1). This digest essentially excises the entire insert to produce a 7.2 kb fragment and confirms the size of the plasmid by leaving the 4.3 kb fragment representing linearized p176. The size of these fragments can be estimated by comparison to a size standard (lambda DNA digested by *Hind*III) (Figure 11, Gel 1, lane 1, Gel 2, lane 4). The size of these bands produced by the digestion are 23.13, 9.42, 6.56, 4.36, 2.32, 2.03, 0.54 kilobases from the top to the bottom of the gel. The *Eco*RI/*Pst*I digest yielded fragments 4.9 kb, 3.6 kb, 2.1 kb, 0.8 kb, and 0.4 kb. The *Eco*RI digest produced fragments of 5.7 kb, 4.9 kb and 1.2 kb in length.

III. Sequencing the p176 clone

The p176 clone was sequenced using a primer from the PCR reaction and a Beckman CEQ 2000 sequencer. The sequencing run shows that the –144 activator-binding site is present (Figure 12). A FASTA search was done to confirm the presence of DNA from the intergenic region of *qa-1S* and *qa-1F* (Figure 13). The restriction enzyme sites for *Eco*RI and *Bam*HI are highlighted in the results of the FASTA search.

Figure 12: Sequencing results of p176 using primer #560 from the PCR reaction. The activator-binding site is italicized.

GTCCCTGTCCCGATCTTTTTTTTTTGTTGTTGTTCATGACCCTCTACGAACC
ATGGCGACGTTGTAGTGGGTGTCTGGGAGTCCGATCATGGCTTGAATGGG
GTTGCGTAGAGGCCGAGGTGGGTGCATAGCGATATCATGAGCGAAGAAG
CTGGATGGCAGTGAATGTTTGGATCCGTCACGGCAGTGGAACCGGTAGT
GAATTCGGTGCGGGGGGAAGCTCGGCTTTTGCTGTGGCATCGATGTACGTC
CAACGAGAATTCGAAGACACTTTCGCCTGTAGATTTCAGGGAAGCATAT
GACTATCGGTAGGTAGTCTTATAGTCCATTGTCGAACCTGTGAGGCAACA
GGACAAACAGGTGTGTTGGAGTTGTGAACGGCGTGAACAGAGGAGAGA
AAATGGAGGCCCAGGTTGGGA***GGATAATTGTTTATCC***TTTTGTGCAGTGA
G

Figure 13: Results of FASTA comparison on sequence data obtained from p176 using primer #569 from the PCR reaction. 'Query' represents the sequence of the PCR fragment and 'gb' represents the database sequence. The -144 activator-binding site is bold, underlined and italicized. The *Eco*RI sites within the sequence are underlined. The *Bam*HI site is in bold print and underlined.

FASTA searches a protein or DNA sequence data bank
version 3.3t02 December 5, 1999
Please cite:
W.R. Pearson & D.J. Lipman PNAS (1988) 85:2444-2448

./tmp/fastaIN22511: 448 nt
.query (genbank-nonst)
vs /bio/db/fasta/genbank/nonst library
searching /bio/db/fasta/genbank/nonst library

3049183011 residues in 1095296 sequences
Expectation_n fit: rho(in(x))= 5.2284+/-0.00039; mu= 21.2808+/-0.028;
Mean_var=96.4321+/-17.561, 0's: 0 Z-trim: 61 B-trim: 8675 in 2/75

The best score is: opt bits E(1111749)

gb:NCQA [X14603] Neurospora crassa qa gene clu (18120) [r] 2200 425 8.5e-118
gb:NCQA1SRA [M13208] N. crassa qa-1S (3526)[f] 1042 772 6.7e-37
gb:NCQA [X14603] Neurospora crassa (18120) [f] 216 151 0.0049
gb:NCQA1SRA [M13208] N. crassa qa-1S [r] 216 158 0.01

>>gb:NCQA [X14603] Neurospora crassa qa gene cluster. (18120 nt)
rev-comp initn: 2195 init1: 2195 opt: 2200 z-score: 2216.5 bits: 424.7 E(): 8.5e-118
99.552% identity in 446 nt overlap (448-3:14818-15262)

```
                                                 440         430         420
query-                                           CTCACTGCACAAAA***GGATAAACAATTATCC***
:::::::::::::::::::::::::::::::
CATGGCGGGTGTTTTCACGTTAACGGAGGTCTCACTGCACAAAA***GGATAAACAATTATCC***
  14790       14800       14810       14820       14830       14840

 410         400         390         380         370         360
q:TCCCAACTTGGGCCTCCATTTTCTCTCCTCTGTTCACGCCGTTCACAACTCCAACACACC
   ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
  TCCCAACTTGGGCCTCCATTTTCTCTCCTCTGTTCACGCCGTTCACAACTCCAACACACC
14850       14860       14870       14880       14890       14900

 350         340         330         320         310         300
TGTTTGTCCTGTTGCCTCACAGGTTCGACAATGGACTATAAGACTACCTACCGATAGTCA
 ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
TGTTTGTCCTGTTGCCTCACAGGTTCGACAATGGACTATAAGACTACCTACCGATCGTCA
14910       14920       14930       14940       14950       14960

 290         280         270         260         250         240
TATGCTTCCCTGAAATCTACAGGCGAAAGTGTCTTCGAATTCTCGTTGGACGTACATCGA
 ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
TATGCTTCCCTGAAATCTACAGGCGAAAGTGTCTTCGAATTCTCGTTGGACGTACATCGA
14970       14980       14990       15000       15010       15020

 230         220         210         200         190         180
TGCCACAGCAAAAGCCGAGCTTCCCCCGCACGAATTCACTACCGGTTCCACTGCCGTGA
 ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
TGCCACAGCAAAAGCCGAGCTTCCCCCGCACGAATTCACTACCGGTTCCACTGCCGTGA
15030       15040       15050       15060       15070       15080

 170         160         150         140         130         120
C**GGATCC**AAACATTCACTGCCATCCAGCTTCTTCGCTCATGATATCGCTATGCACCCACC
 ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
C**GGATCC**AAACATTCACTGCCATCCAGCTTCTTCGCTCATGATATCGCTATGCACCCACC
15090       15100       15110       15120       15130       15140

 110         100          90          80          70          60
TCGGCCTCTACGCAACCCCATTCAAGCCATGATCGGACTCCCAGACACCCACTACAACGT
 ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
TCGGCCTCTACGCAACCCCATTCAAGCCATGATCGGACTCCCAGACACCCACTACAACGT
15150       15160       15170       15180       15190       15200

 50          40          30          20          10
CGCCATGGTTCGTAGAGGGTCATGAACAACAAAAAAAAAAAGATCGGGACAGGGAC
 ::::::::::::::::::::::::::::::::::: : ::::
CGCCATGGTTCGTAGAGGGTCATGAACAACAAAAAAAAAAAGATCGGCA-AGGGCA
15210       15220       15230       15240       15250
```

IV. Amplification of the Intergenic Sequence of *qa-1F* and *qa-1S* including the -144 Activator-binding Site by Polymerase Chain Reaction (PCR)

In order to construct a clone with an insert containing the *qa-1F* - *qa-1S* intergenic region, a fragment had to be generated that would be small enough to be easily maintained in M13.There were no compatible restriction enzyme sites in the intergenic sequence of *qa-1F* / *qa-1S* , and also in the M13mp18 bacteriophage vector, and the pBluescript II SK (+/-) phagemid vector. Because of this, a PCR reaction was designed to create a blunt-ended fragment 773 bp in length that could be ligated into either vector (Figure 14, Lane 1-9). The primers used were 5'-ATC CGG AGA GAT ATG TTG CGA ATC AGG-3' and 5'-CGA GTT AAT AAA TGC CTT GCG GC-3'. The PCR short template product would extend to include bases 15527-15300 of the *qa* gene cluster. The major product of this reaction which contains the *qa-1F* activator-binding site of interest, the 773 bp short template, will be isolated for the purpose of a blunt-ended ligation with the pBluescript and m13mp18 vectors. In addition to the presence of the -144 activator-binding site, the isolated short template from the reaction also contains a *Bam*HI site that does not interrupt the activator-binding site. This would allow for the opportunity of directionally cloning the fragment since both vectors mentioned above also have *Bam*HI sites. Lanes 1-9 of Figure 14 are all samples of the PCR reactions using the same reagents and primers that were mentioned above. The reaction consistantly produced a 773 bp insert that contains the -144 activator-binding site of *qa-1F*.

V. Isolation of the RF M13 mp18 Bacteriophage Vector

M13 is a single-stranded bacteriophage that infects male *E.coli* strains. The bacteriophage M13 mp18, which has a multiple cloning site (MCS) within a portion of the ß-galactosidase gene, was transformed into *E. coli* strain JM101. The location of this MCS allows for blue-white screening of recombinants. The phage was grown on a large scale for the purpose of isolating its double-stranded replicative form. Because of its double-stranded nature, this intracellular replicative form acts as a plasmid when it applies to transformations and cloning. The isolated supercoiled RF M13 mp18 is 7.3 kb in size (Figure 15, Gel 1 Lanes 2-6). The 7249 kb size of RF M13 mp18 was more easily observed when it was linearlized by a *Bam*HI digest (Figure 15, Gel 2 Lane 1). The purpose of isolating the replicative form of m13 mp18 is to utilize its replicative cycle to produce single-stranded copies of our clones that will retain the -144 activator-binding site of *qa-1F* within the 773 bp insert to be used for site-directed mutagenesis

Figure 14: Polymerase Chain Reaction (PCR) amplifying the intergenic sequence of *qa-1F* and *qa-1S*. The short template product is ~800 bp in length.
Lanes 1-9: Samples from the PCR reactions exhibiting the 800 bp short template retaining the -144 activator-binding site.
Lane 10: *Hind*III digested lambda size marker

Lanes 1 2 3 4 5 6 7 8 9 10

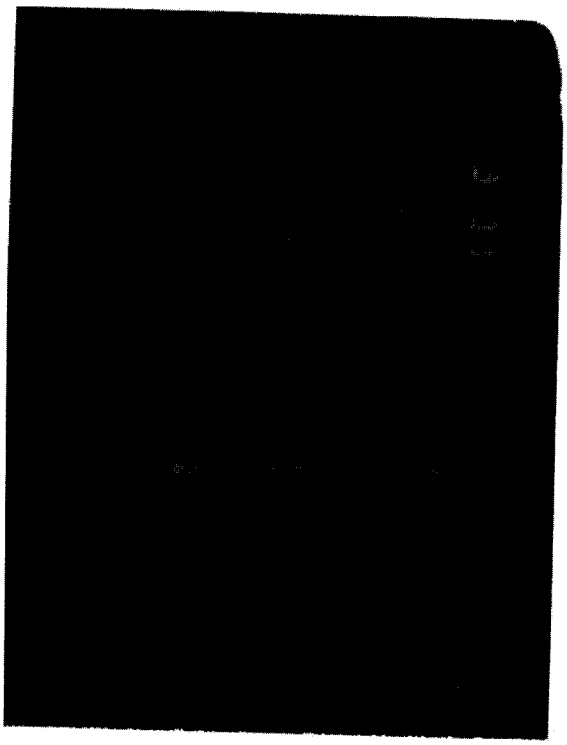
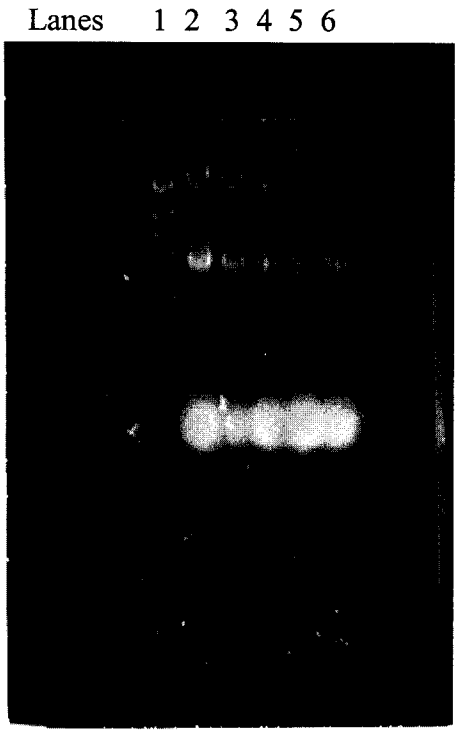
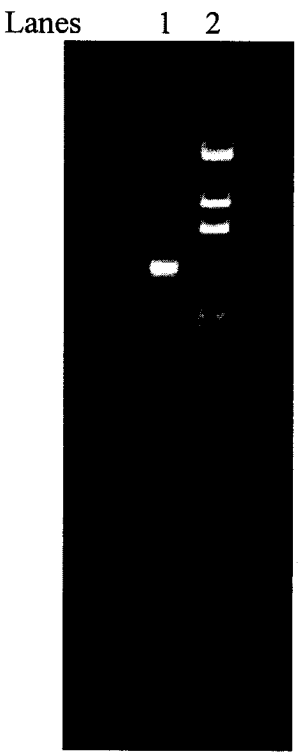


Figure 15: Isolation of RF M13 mp18 Bacteriophage
Gel 1: Lane 1: *Hind*III digeted lambda size standard.
Lanes 2-6: Isolated supercoiled RF M13 mp18 DNA
Gel 2: Lane 1: *Bam*HI digest of RF M13 mp18 (7.3 kb)
Lane 2: *Hind*III digested lambda size standard

Gel 1



Gel 2



V. Ligation of the insert with the -144 Activator-Binding Site of *qa-1F* RF M13mp18 Clone

Various attempts were made to produce the RF M13mp18 clone using different restriction enzymes. The first attempt was one using the restriction enzyme SmaI to make a blunt-ended cut in the M13 vector. This was done under the assumption that the insert that was created by the PCR reaction was a blunt-ended fragment. To increase the chances of a successful ligation, the vector was cipped using an Alkaline Phosphatase enzyme to remove 5' phosphate to prevent recircularization of the vector during ligation. The cloning attempts were unsuccessful, probably due to the difficulty of producing a blunt-ended ligation. The next attempt at ligation into the M13 vector utilized to different enzymes, *Sma*I and *Bam*HI, which would allow the insert to directionally cloned into the vector. There were a few reasons why these two enzymes were chosen. First, by using the double-digest, the ends that would be created in the vector would prevent recircularization during ligation, therefore the cipping procedure would not be necessary (*Sma*I enzyme creates a blunt-end cut, *Bam*HI creates a staggered cut with a 3 base overhang). Second, by using *Bam*HI, the digest would create a sticky-end that would provide some compatible H-bonds for the insert to covalently bond to during ligation. The primers that were used in the PCR reaction were chosen as to include a *Bam*HI site within the insert that does not interrupt the -144 activator-binding site. The one problem with the double digest was that the buffers for both enzymes were too different and therefore the digest had to be extracted twice, after each digest,

which could result in the loss of some of the vector. The ligation attempts were unsuccessful and some recircularization had occurred. The recircularization of the vector shows that there was an incomplete digest by one of the enzymes. This incomplete digest could be the result of either a faulty enzyme or because the positions of the sequences on the vector that the enzymes recognize were virtually right next to each other (not even one base of separation). After failed ligation attempts what is believed to be the real problem with the reaction is that the insert created by the PCR reaction was not a completely blunt-ended molecule. The polymerase enzyme that was used in the reactions was a Taq polymerase. This enzyme leaves a single overhanging adenine base at the ends of the insert. This single overhanging A base was the reason why the ligations using the blunt-ended *Sma*I digest were unsuccessful.

Discussion

The regulation of the levels of transcription of the genes of the *qa* gene cluster is believed to be governed by the qa-1F activator protein. The current model of regulation of the cluster involves the *qa-1F* gene product participating in a coordination of binding events in the 5' flanking regions of the *qa* genes. The DNA-binding activity of the qa-1F activator protein is somewhat regulated by interaction with the qa-1S repressor protein. Whether this interaction is direct or indirect is not known. Binding of the activator protein occurs at a conserved 16 bp sequence, of which at least 14 of these sites have been identified within the cluster. The distinctive palindrome-like activator-binding sequence shares some sequence homology with that of other fungal activators like LAC9 and GAL4 (Keegan 1986) (Messenguy 1986). The focus of this research is to better understand the mechanism of control of transcription of the genes involved with the utilization of quinic acid as a carbon source. By isolating these activator-binding sequences, through site-directed mutagenesis we can establish the conserved bases and their significance in qa-1F's ability to recognize and bind at these sites.

It is believed to be a coordination of binding events by the *qa-1F* gene product at its various activation sites in the 5' flanking regions of the *qa* genes that continuously modifies the level of gene expression of the cluster. It was also observed in the *GAL* system that a pair of *GAL4* binding sites were required for repression of *GAL80* expression to a basal level, and that only one site was required for high levels of induction (Baum *et al.,* 1986). Even in the presence of glucose or

another preferred carbon source, there are still basal levels of *qa* gene expression. A chromatin analysis of the intergenic sequences by DNase I footprinting displays evidence that there are a distinct class of HSSs dependent on the presence of the qa-1F protein (Baum & Giles, 1985, 1986). The positions of these HSSs in the induced strain are distinctly different from those in uninduced, *qa-1S*^{c} or *qa-1F*$^{-}$ strains (Baum & Giles, 1985).

In order to further examine the significance of the sequence of the -144 activator-binding site of *qa-1F,* the sequence had to be isolated and inserted into a suitable vector. The vector that was chosen was the RF M13mp18 vector because of its ability to produce single-stranded copies of our clone. These SS copies would be necessary for the site-directed mutagenesis. Since there were no compatible restriction enzyme sites between the vector and the intergenic sequence of *qa-1F / qa-1S* , another method had to be used to create insert that retained the activator-binding site. Since the RF M13 mp18 vector has a *Sma*I site, a PCR experiment was designed to produce an ~800 base insert that could be easily maintained by the vector. The ligation of this insert, which was assumed to be a blunt-ended molecule, into the RF M13 vector was unsuccessful. In an attempt to increase the chances of successful ligation, the fragment created by the PCR experiment and the vector were further digested with *Bam*HI. The purpose of this was to provide one sticky-end to work with. This ligation was also unsuccessful due to the fact that the Taq polymerase used in the PCR experiment leaves a single, unpaired 'A' base. This unpaired base at the end of the insert prevents any chances of successful blunt-end ligations at the *Sma*I site.

By producing a clone for the site-directed mutagenesis, the significance of the sequence of the -144 activator-binding site can be further examined. After the mutagenesis, the clones can be transformed back into *Neurospora crassa* in an attempt to assay the significance of the sequence changes on the organisms ability to induce levels of *qa-1F* gene expression and subsequently those levels of all the genes of the *qa* cluster. Once this is done and the bases of most significance with regard to activator-binding can be established, these highly conserved bases can be compared to that of other fungal activators like *LAC9* and *GAL4*. This would provide a better understanding as to the exact mechanism of the activator proteins role in catabolite repression in eukaryotes.

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