Site-Directed Mutagenesis of the –127 Activator Binding Site of *qa-2* in *Neurospora crassa*

by

Shelly Dripps

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Shelly Dripps

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Shelly L. Dripps, Student — 4-16-02 Date

Approvals:

Dr. David Asch, Thesis Advisor — 4/16/02 Date

Dr. Gary Walker, Committee Member — 4/16/02 Date

Dr. Robert Leipheimer, Committee Member — 4/16/02 Date

Peter J. Kasvinsky, Dean of Graduate Studies — 4/22/02 Date

ABSTRACT

The quinic acid cluster of *Neurospora crassa* is under two types of regulation. One level, catabolite repression, occurs when transcription of the quinic acid genes is repressed in the absence of a preferred carbon source. The other level deals with regulatory proteins within the *qa* cluster. When the inducer, quinic acid, is not present, the repressor protein binds to the activator protein preventing transcription of the quinic acid genes. Upon introduction of quinic acid, the activator protein is freed and will bind to activator binding sites within the cluster. This will increase transcription levels. There are 14 locations within the quinic acid cluster that bind the activator protein and they share a 16 bp consensus sequence. Studies have been done to determine activator binding protein affinity for each of these sites *in vitro,* however the significance of the particular bases within each site and the *in vivo* significance of the site in general is not known.

The –127 activator binding site of *qa-2* gene was chosen to study because the activator protein has a high affinity for this site. A segment containing this site was subcloned and a site-directed mutagnesis was reformed. Clones were isolated, however DNA sequencing of these clones was unsuccessful. We feel that the M13 primer site may have been deleted.

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INTRODUCTION

I. Kingdom Fungi

Fungi constitute a large group, approximately 69,000 organisms, that are found virtually everywhere (Alexopoulos, 1996). Fungi are probably most significant as agents of decay, but they also play a role in plant diseases, fermentation, antibiotic production, biological control and many other activities, useful or otherwise. Some fungi are capable of causing diseases in humans as well as in plants. Fungal infections can be serious and can result in death (Alexopoulos, 1996).

Fungi are usually filamentous and multicellular. The filaments that constitute the body of most fungi are called hyphae. The body itself is called mycelium and this mycelium is composed of the hyphae. Yeasts, however, exist as single cells that will reproduce by budding or fission (Alexopoulos, 1996).

The kingdom *Fungi* has three major established lineages, *Zygomycota, Basidiomycota*, and *Ascomycota* (Starr, 1997). Other fungi, known as "imperfect fungi", are lumped together but are not a formal taxonomic group (Starr, 1997). Members of the phylum *Zygomycota* produce sexual spores called zygospores (Solomon, 1993). The most common type is the black bread mold. The phylum *Basidiomycetes* consists of the most familiar types of fungi, mushrooms and puff balls. Members belonging to this phylum are also known as club fungi. Members of the *Ascomycota* phylum are often referred to as sac fungi, because their sexual spores are produced in little sacs (Solomon, 1993).

II. Phylum Ascomycota

Approximately 30,000 species of fungi make up the phylum Ascomycota. This group of fungi has often been referred to as sac fungi because during the sexual reproduction phase, the spores are produced in sacs known as asci. The most common types of ascomycetes found in this phylum are yeasts, molds, truffles, morels, and powdery mildews. Asexual reproduction in the ascomycetes includes the production of conidia from specialized hyphae called conidiophores. Sexual reproduction will take place when two hyphae grow together. Eventually this is where the asci will develop. Once an ascus has developed, the two nuclei fuse together and form a diploid nucleus, which will then undergo meiosis resulting in four haploid nuclei. Mitotic division then occurs in all four haploid nuclei, which results in eight haploid nuclei. Each nucleus then develops into an ascospore, resulting in eight haploid ascospores within each ascus (Solomon, 1993). The ascomycete fungi have the characteristics needed to be efficient as a model system an organism. They possess a good genetic map, a culture collection that contains mutant strains, as well as the ability to maintain sufficient research interest (Taylor, 1993).

III. *Neurospora crassa*

Neurospora crassa is a member of the *Ascomycota* phylum that produces long, slender asci. As with other members of this phylum it produces eight

haploid nuclei which will become eight linearly arranged ascospores within a single ascus (Alexopoulos, 1996). Due to this process B.O. Dodge laid the foundation of using *Neurospora* as an almost perfect organism to study the then new field of haploid genetics (Alexopoulos, 1996).

Neurospora crassa is a red bread mold that was discovered by C. L. Shear and B. O. Dodge in 1927. Since then *Neurospora* has become an important tool in genetics research (Alexopoulos, 1996). *Neurospora* is eukaryotic but it differs from other organisms used by geneticists because it is haploid, or has only one set of chromosomes per cell, for most of its life cycle. This is important for research purposes. First, because genetic changes can be easily monitored and second, individual DNA sequences are able to be isolated. *Neurospora* is also often used since it has simple nutritional requirements and short generation times (Perkins, 1992). A number of concepts in different biological fields have originated from the study of *Neurospora crassa* (Mishra, 1991). Perhaps most importantly *Neurospora* was used by Beadle and Tatum in Noble Prize winning research on genetic control of enzymes (Alexopoulos, 1996). It has since been used in a variety of ways to study genetics, including the development of transformation systems in fungi.

Fungi have the ability to adapt for growth in environments where preferred carbon sources are not available. This ability requires special pathways that can overcome this restriction by utilizing diverse carbon metabolites. (Geever et al, 1987). One example of this is the quinate/shikimate pathway of *Neurospora crassa*. (Geever et al, 1987). This pathway involves three enzymatic steps in

which quinic acid and shikimic acids are metabolized to protcatechuic acid. (Geever et al, 1987). In doing so, either quinic acid or shikimic acid can serve as the sole carbon source for growth (Geever et al, 1987).

IV. Background of the Quinic Acid Gene Cluster

Early studies of the quinic acid cluster of Neurospora established the gene order within this cluster (Case and Giles, 1976). Originally it was believed that only four genes were present in the quinic acid cluster. A regulatory protein termed *qa-1* and three other proteins *qa-2, qa-3* and *qa-4*. To identify the gene order was important for determining how this cluster was transcribed, but also to detect mutations in the controlling elements. Case and Giles used four different crosses. One would order the *qa* genes relative to the methionine-7 because it was known that the quinic acid gene cluster was located on the right arm of linkage group VII very close to the methionine-7 locus. The results of that cross indicated that the gene order was *qa-1F qa-3 qa-4 qa-2 met-7*. The next cross was done to order the structural genes relative to the *qa-1* regulatory gene and the results agreed with the previous order. Next, Case and Giles performed a three factor cross involving the mutants of the three structural genes to determine their order relative to each other. Results showed that the order of these three structural genes could only be *qa-3 qa-4 qa-2*. Finally a four factor cross was done utilizing a triple mutant *qa-3 qa-4 qa-2* (Case and Giles, 1976). The results

of this cross reaffirm the results of the previous three crosses establishing the order of the quinic acid gene cluster as *qa-1 qa-3 qa-4 qa-2 met-7*.

Another breakthrough in the study of the quinic acid cluster occurred when Schwiezer and his colleagues were able to clone two of the *qa* genes into a cosmid vehicle pHC79 (Schwiezer et al, 1981). Although *qa-3* and *qa-4* have no analogs in E. coli, earlier experiments showed that the *qa-2* gene was able to be expressed in E. coli (Case et al, 1979). However, in this particular study a larger clone was identified which carries a functional *qa-3* gene. This recombinant plasmid containing the *qa-2* and *qa-3* genes was called pMSK308 (Schwiezer et al, 1981). The *qa-2* gene is functionally expressed in *E. coli* and is functional when transformed back into *Neurospora crassa*. *Qa-3* gene expression is only detected upon reintroduction into *Neurospora crassa* (Schwiezer et al, 1981).

The *qa-2* gene was utilized in early studies on the transformation of *Neurospora crassa* (Case et al, 1979). This transformation system was developed using pVK88, a recombinant *Escherichia coli* plasmid that carried the *qa-2* gene of *Neurospora crassa*. Recipient strains in these transformation studies were *qa-2*$^-$ as well as having an arom9$^-$ mutation so that the strain did not have any catabolic nor biosynthetic dehydroquinase activity. The results of this experiment showed that transformation in *Neurospora crassa* occurred and because of this the possibilities of genetic manipulation of *Neurospora crassa* were significantly increased (Case et al, 1979).

In 1981, studies were done to identify specific *qa*-mRNAs (Patel et al, 1981). To do this the researchers utilized cloned DNA fragments as hybridization

probes. It was found that each *qa* gene is transcribed into a main mRNA transcript. Transcription of *qa-2* resulted in a 1.4 kb transcript, *qa-3* to a 1.9 kb fragment, and *qa-1* to a 4.5 kb fragment. The *qa-4* gene appeared to hybridize to two different mRNAs. It is hypothesized that the primary transcript of 2.6 kb is processed into a "mature" 2.3 kb mRNA (Patel et al, 1981). Two additional transcripts were also found in this set of experiments. A 1.9 kb transcript was found to the left of *qa-2* and this newly found gene was termed *qa-x*. A second new gene was located to the right of *qa-3* with a 3.4 kb transcript and this gene was labeled *qa-y*. Other mRNAs were also identified that flank the gene cluster but that are not under quinic acid or *qa-1* regulation.

The entire *qa* gene cluster was later cloned into other recombinant plasmids (Schwiezer et al, 1981). These plasmids were also developed using the cosmid cloning vehicle pHC79. pMSK331 and pMSK335 were obtained by doing a partial restriction digest of *Neurospora crassa* DNA using the restriction enzyme *EcoRI* and then performing a ligation into pHC79. Once these plasmids were transformed back into *Neurospora crassa* they were both shown to contain the entire quinic acid gene cluster (Schwiezer et al, 1981).

Although the *qa-1* gene was believed to encode a single positively controlled regulatory protein, it was ultimately shown that regulation of the quinic acid gene cluster was actually under the control of two interacting genes (Huiet, 1984). It was previously established that there were two types of noninducible *qa-1* mutants, *qa-1S*$^-$ and *qa-1F*$^-$ which mapped in two nonoverlapping regions at opposite ends of the *qa-1* gene (Huiet, 1984). The hypothesis then was that the

two classes of mutants indicated two separate domains of the regulatory protein. Using Neurospora transformation and DNA•RNA hybridization experiments Huiet was able to show that the *qa-1* region was comprised of two distinct genes. Using mRNA hybridization it was determined that the *qa-1F* region, as determined by the mutations, encoded a 2.9 kb mRNA and this gene was labeled *qa-1F*. The *qa-1S* region encodes a 4.1 kb mRNA, as well as a 3.4 kb mRNA (Huiet, 1984). It is thought that the 3.4 kb mRNA is possibly a degradation or a product of the 4.1 kb mRNA. S1 nuclease mapping of the DNA•RNA hybrids was then performed to determine the direction of transcription of the *qa-1S* and *qa-1F* mRNAs. It was determined that the two genes are divergently transcribed and that the origin of transcription of these genes is initiated within the region between the two transcripts (Huiet, 1984). Due to these results earlier conclusions on the regulation of this cluster needed to be revised. It is now thought that the *qa-1F* gene encodes for a positive activator protein that controls expression of all the qa genes and that the *qa-1S* gene would have a negative role in regulation.

V. Organization of the Quinic Acid Gene Cluster

In prokaryotes, related genes are often found clustered together. In eukaryotes related genes are spread throughout the entire genome. In the eukaryote *Neurospora crassa*, however, the related genes for quinic acid metabolism are found clustered together. Gene clusters can occur in two forms,

appearing as adjacent identical genes or as adjacent related genes. The quinic acid cluster of *Neurospora* belongs to the latter group. The quinic acid cluster is involved in the degradation of quinic and shikimic acid to protocatechuic acid and provides a useful system for studying gene regulation of enzymes in eukaryotes (Case and Giles, 1975).

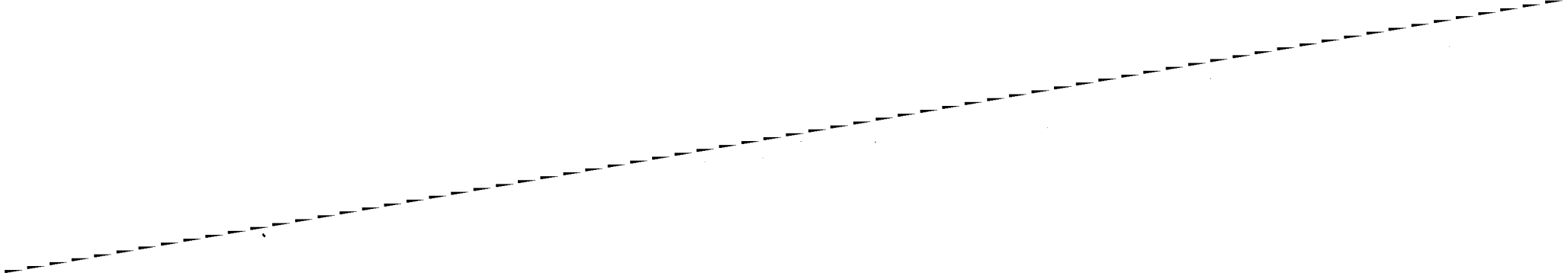
The quinic acid cluster comprises an approximately 17.3-kb segment located on linkage group VII of *Neurospora crassa.* This cluster is comprised of five structural genes and two regulatory genes that control the utilization of quinic acid as a carbon source (Giles et al, 1991). The only unpaired gene, *qa-y*, separates the two structural gene pairs from the regulatory gene pair. The other genes comprising this cluster are paired into twos because they are divergently transcribed from common flanking 5' regions. These pairs consist of *qa-x* and *qa-2*, *qa-4* and *qa-3*, as well as the pair of regulatory genes *qa-1S* and *qa-1F* (Giles et al, 1991). Organization of these genes from distal to proximal end is as follows: *qa-x, qa-2, qa-4, qa-3, qa-y, qa-1S, qa-1F* (Figure 1). Transcriptional sizes of the *qa*-mRNAs can be seen in Figure 2.

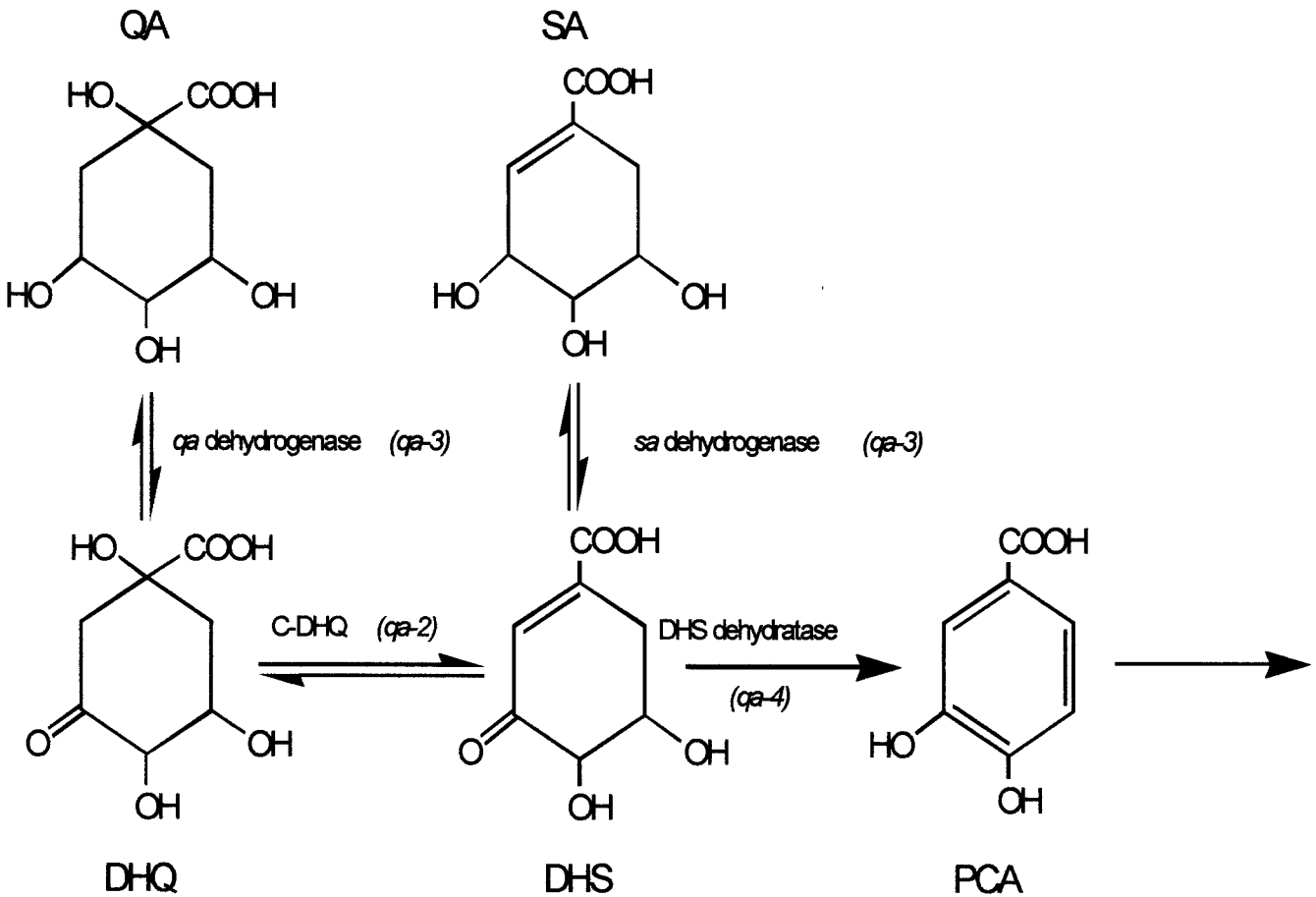
The five structural genes include *qa-2, qa-3, qa-4, qa-x* and *qa-y*. Three of the structural genes encode enzymes that aid in the utilization of quinic acid as the preferred carbon source (Figure 3). *Qa-2* or the catabolic dehydroquinase gene encodes the enzyme 5-dehydroquinate hydrolase. This enzyme is able to convert dehydroquinic acid to dehydroshikimic acid. The *qa-3* gene, quinate dehydrogenase, encodes quinate NAD oxidoreductase, an enzyme that converts quinic acid to dehydroquinic acid. This enzyme can also convert shikimic acid to

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

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

Inducible Structural Genes | Regulatory Genes




QA
SA
HO
COOH
HO
OH
OH
COOH
HO
OH
OH
qa dehydrogenase (qa-3)
sa dehydrogenase (qa-3)
HO
COOH
C-DHQ (qa-2)
COOH
DHS dehydratase
(qa-4)
COOH
O
OH
OH
O
OH
OH
HO
OH
DHQ
DHS
PCA

dehydroshikimic acid. The *qa-4 gene*, dehydroshikimate dehydrase encodes 5-hydroshikimate dehydratase (Case and Giles, 1976). This enzyme converts dehydroshikimic acid to protocatechuic acid, which can be utilized as a carbon source.

The products of the other two structural genes, *qa-y* and *qa-x*, is still somewhat unclear. *Qa-y* is hypothesized to encode for a quinic acid permease and the fifth structural gene, *qa-x*, has a quinic acid inducible RNA with an unknown function (Case et al, 1992).

The other two genes belonging to the quinic acid cluster encode regulatory proteins. The *qa-1F* gene codes for an activator protein, which has been shown to control expression of the other *qa* genes, including itself. It was determined that the product of the *qa-1F* gene is necessary for transcription of the *qa* structural genes (Patel et al, 1981). This is on account of no production of specific *qa* mRNAs in *qa-1F*$^-$ strains, as well as the fact that constitutive *qa-1F* strains had high levels of *qa*-specific mRNAs in the absence of quinic acid (Patel et al, 1981). Evidence supporting that *qa-1F* is an activator protein stems from experiments done by Avalos and colleagues in which disruption of the *qa-1F* gene leads to noninducible transcription of all *qa* genes at low basal levels (Giles et al, 1991).

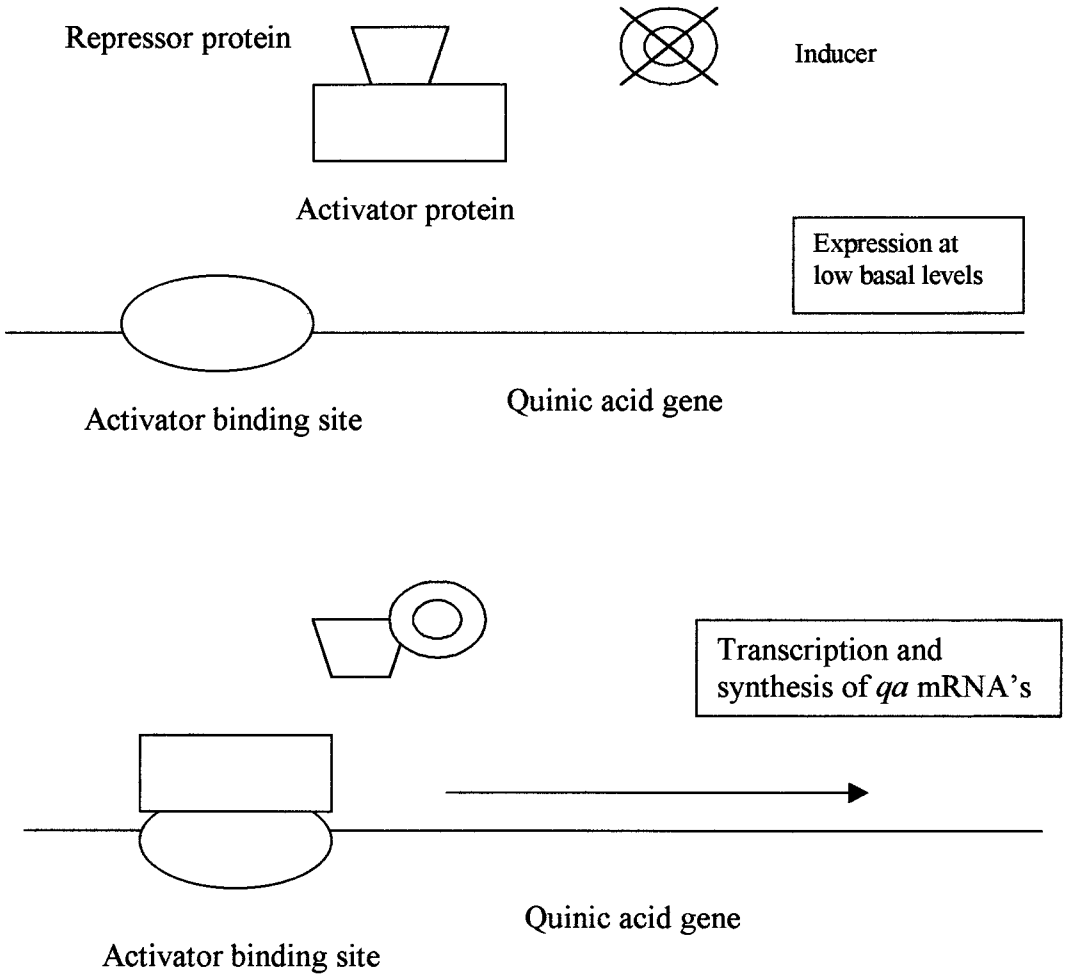
Qa-1S is a repressor-coding gene that appears to block transcription of *qa-1F* in the absence of the inducer (Tyler et al, 1984). This gene encodes related 4.1-kb and 3.4-kb mRNAs (Huiet, 1984). The repressor gene's mutants can be either noninducible for all three quinic acid enzyme activities or constitutive, producing high levels of all three enzymes in the absence of the inducer (Giles et

al, 1985). Supporting evidence that the *qa-1S* gene encodes for a repressor protein comes from work done by Geever and Case. They found that deleting the *qa-1S* gene will cause constitutive transcription of all the *qa* genes at high levels (Giles et al, 1991). These two genes are known to be transcribed constitutively at low levels as well as being subject to regulation by the inducer quinic acid and to autoregulation. (Baum et al, 1986).

VI. Regulation of the Quinic Acid Gene Cluster

Two methods, both at the level of transcription, are utilized in the regulation of this cluster. The first method involves the regulatory proteins of this cluster as well as the inducer, quinic acid. The second method is known as catabolite repression.

In the first level of control, the actions of both *qa-1S* and *qa-1F* along with that of the inducer quinic acid regulate the utilization of quinic acid as a carbon source in Neurospora (Giles et al, 1985). In the absence of the inducer quinic acid, the repressor protein prevents expression of the *qa-1F*$^{+}$ gene by binding to the activator protein. The *qa* gene products are therefore only expressed at low basal levels. Once quinic acid is added, the repressor no longer inhibits *qa-1F*$^{+}$ expression and the activator will begin autoregulation (transcription of itself) along with transcription of all other *qa* mRNAs, including synthesis of the repressor. This is thought to provide enough repressor to turn off the quinic acid system once the inducer levels drop off (Figure 4) (Giles et al, 1985).


Repressor protein
Inducer
Activator protein
Expression at
low basal levels
Activator binding site
Quinic acid gene
Transcription and
synthesis of qa mRNA's
Quinic acid gene
Activator binding site

Evidence to support this theory stem from experiments involving mutants of both the *qa-1F* and the *qa-1S* gene. Two types of *qa-1S* mutants have been identified, a recessive, constitutive mutant (*qa-1S*^{c}) and a semidominant, noninducible type of mutant (*qa-1S*^{i}). In the constitutive mutant with no inducer present, there is constitutive transcription of all the genes. This is thought to be due to inactivation of the repressor (Giles et al, 1985). In the noninducible type of mutant, *qa-1S*^{i}, only basal levels of transcription of the cluster occur, suggesting that these mutants may act as superrepressors that are not sensitive to activation by the inducer (Giles et al, 1985). The mutant of activator gene, *qa*-1F, is also noninducible. Only basal levels of transcription are seen, indicating that a functional activator protein is required for transcription of the quinic acid gene cluster (Giles et al, 1985).

Mechanisms of the repressor protein have also been investigated. It was first thought that the target for the repressor was an operator sequence located 5' to the *qa-1F* gene. When bound, the repressor would then block transcription from this gene thereby repressing expression of the *qa* genes. Addition of quinic acid would then release the repressor, allowing synthesis of the activator protein (Giles et al, 1985). Results by Case et al (unpublished), as well as results of S1 nuclease studies showing that *qa-1*F is not subject to induction have proved this theory wrong. It is now thought, due to indirect evidence, that the target of the repressor protein may be the activator protein (Giles et al, 1985).

A second level of regulation also occurs in the quinic acid system. This type of regulation involves catabolite repression. Transcription of the *qa* genes is

repressed in the presence of a preferred carbon source, such as glucose or sucrose. When grown on quinic acid with a preferred carbon source present, such as glucose or sucrose, *Neurospora crassa* shows *qa* enzyme induction to be only 1% of the level of induction of these enzymes when quinic acid is utilized as the sole carbon source (Case et al, 1992). It is unknown whether this control mechanism is arbitrated through repressor action, through the absence of quinic acid, the inducer or whether it is a combination of both of these things (Geever et al, 1987).

VII. GAL4

One of the most intensively studied systems of gene control in eukaryotes is the regulation of the genes that produce the enzymes responsible for metablolizing galactose, the *GAL* genes, in yeast. (Lewin, 1990) The GAL system serves as a model comparable to the regulation of quinic acid pathway in *Neurospora crassa*. Although most genes in the GAL system are not clustered, some genes in the regulation of galactose are divergently transcribed, *GAL1* and *GAL10*, similar to the genes in the quinic acid system. These genes require the activation of a UAS (upstream activating sequence). The GAL4 protein will bind at the UAS_G to activate a target gene (Lewin, 1990). Similar to the regulation of quinic acid whose genes are induced by quinic acid, the *GAL* genes are induced by the presence of galactose. Galactose binds to the GAL80 protein, which when bound to galactose prevents GAL80 from associating with GAL4. GAL4 if bound to DNA can then activate transcription (Lewin, 1990). With the removal

of the inducer galactose, GAL80 binds to GAL4. In this case GAL4 can still bind to the UAS_G, but will not activate transcription (Lewin, 1990).

The GAL4 regulatory protein is approximately 881 amino acids (Griffith et al, 1993). This protein has three distinct functions: the binding of DNA, the binding of GAL80, and the activation of transcription (Lewin, 1990).

The DNA-binding domain of GAL4 is located within the 98 amino-terminal residues. The N-terminal fragment can bind a 17 bp consensus sequence on DNA but it is not sufficient for activating transcription (Lewin, 1990). The ability to activate transcription lies in the C-terminal end of GAL4. As long as the protein is bound to the DNA and contains the 114 amino acids of the C-terminal end, transcription can be activated (Lewin, 1990). One other region of the protein, located between residues 148 and 196, is also involved in activating transcription (Lewin, 1990). The DNA-binding domain will bring the protein to the right location, and once it is bound to the DNA, the transcription-activating domains will cause initiation of transcription (Lewin, 1990). The binding of GAL80 to GAL4 is suggested to occur in the same region of the major transcription-activating domain (Lewin, 1990). This is because when the 30 C-terminal amino acids of GAL4 are deleted, GAL4 will constitutively activate transcription and is not repressed by GAL80 (Lewin, 1990).

The mechanism of activation used by GAL4 seems to have been conserved between yeast and higher eukaryotes (Lewin 1997). When a yeast UAS_G is inserted near a promoter of a high eukaryote gene, this gene is able to be

activated by GAL4 which means that GAL4 can interact with the transcription factors of higher organisms (Lewin, 1997).

VIII. *Qa-1F*

Qa-1F has many features in common with GAL 4. A protein of 816 amino acids is encoded by the *qa-1F* gene and has a molecular weight of 89kd (Geever et al, 1987). This activator protein was found to have four functional domains (Giles et al, 1991). The DNA-binding domain is located in the first 183 amino acids of the protein (Baum et al, 1987). The second domain is thought to be a dimerization domain. This domain occurs between codons 296 and 562 (Giles et al, 1991). The third region contains mainly acidic residues and is located at the carboxy terminus of the activator (Giles et al, 1991). Since the region is similar to the GAL4 region, which has been implicated in interacting with transcriptional factors, this third region is thought to be a transcriptional activation domain (Giles et al, 1991). The final domain is a region that is presumed to interact with the quinic acid repressor protein (Giles et al, 1991).

IX. Activator Binding Sites

In an experiment performed by Baum and colleagues, this activator protein was expressed using a baculovirus expression vector in an insect cell

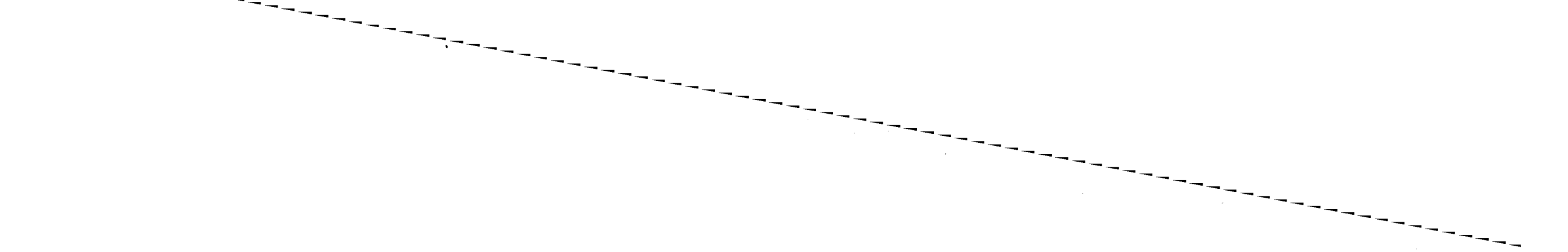
culture. This is a useful vector system because the polyhedrin promoter is exceptionally strong and transcription from this polyhedrin promoter occurs late in infection (Baum et al, 1987). Using this vector system, it was discovered that the activator protein binds to sites within the gene cluster that share a conserved 16 base pair sequence of partial dyad symmetry (Baum et al, 1987). This sequence (GGRTAARYRYTTAYCC) was found to be present at least once 5' to each one of the qa genes (Table1).

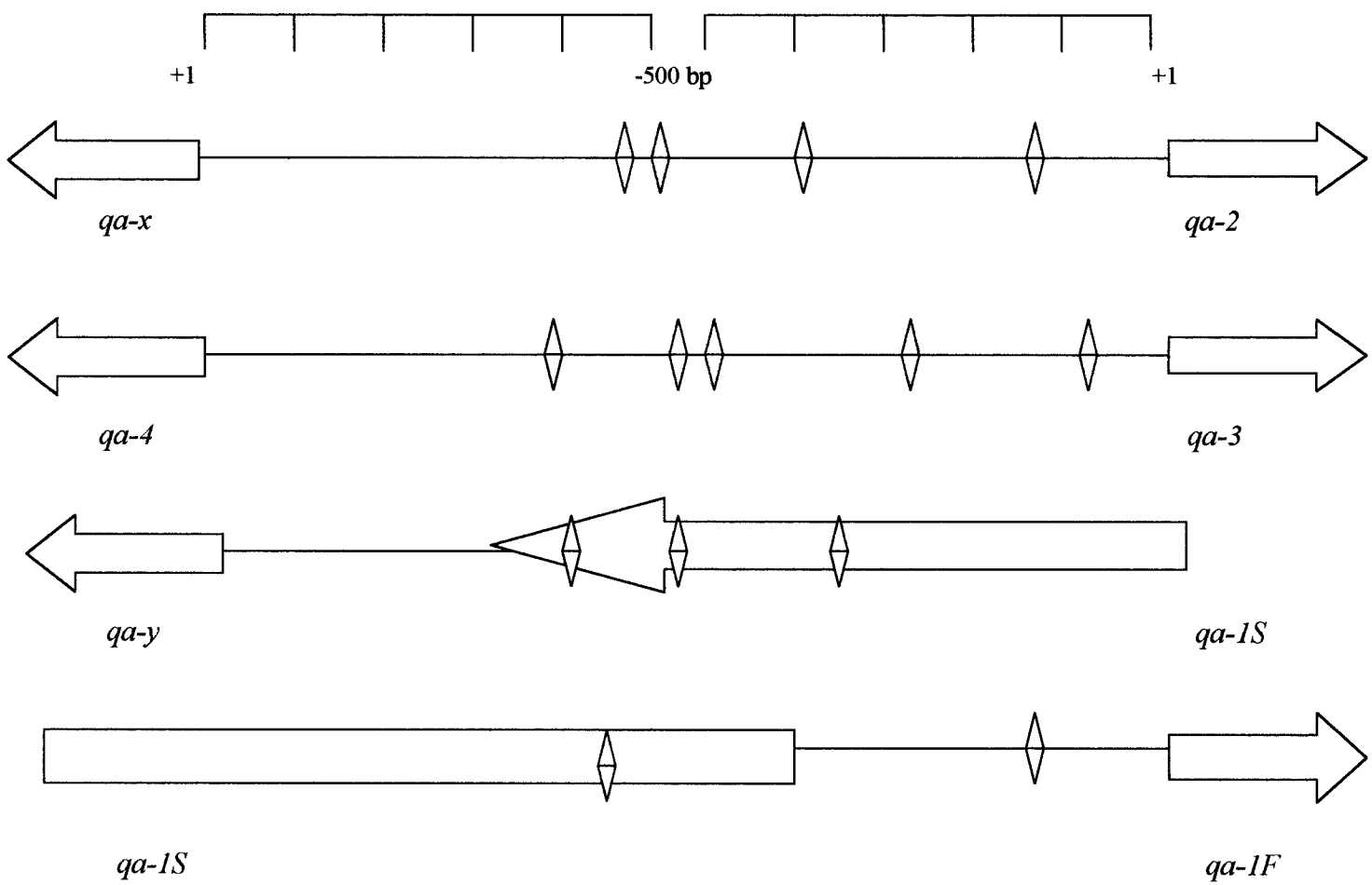
Experiments done using DNase I footprinting analysis found that the regions protected from digestion by the activator protein were typically 21 or 22 base pairs in length and that each one contained the related 16 base pair sequence of partial dyad symmetry. It was also determined that there are 14 locations in the quinic acid cluster that are characterized by this 16 base pair sequence and to which the activator protein binds (Figure 5)(Baum et al, 1987; Geever et al, 1989). This 16 base pair sequence is sufficient for the activator protein to bind (Baum et al, 1987). The quinic acid structural genes have three or more binding sites in their 5' regions and are able to be induced 300- to 1000-fold (Baum et al, 1987). However, the two regulatory genes, *qa-1*F and *qa-1*S have only one binding site in there common 5' flanking region and are only induced 40- to 50-fold (Baum et al, 1987). This could mean that multiple binding sites for the activator allow for greater transcriptional control by *qa-1*F^{+} (Baum et al, 1987).

In a DNA binding assay performed by Baum and his colleagues in 1987 they tested the binding strengths of the activator binding sites by incubating them with increasing amounts of activator extract. It was shown that the –127 binding

Gene pair	Position	Sequence
qa-x/2	-127	GGGTAA TCGC TTATCC
qa-x/2	-510	GGATGA GTTT TTAACC
qa-x/2	-391	GGATAA ATCC TAACCC
qa-x/2	-487	GGCGAA CGTT TTACCC
qa-1S/F	-144	GGATAA ACAA TTATCC
qa-1S/F	+236	GGATGA GTGA TTCTCC
qa-4/3	-449	GGGTAA GTAT TTAAGC
qa-4/3	-510	GGCTAA GTGT TTAACA
qa-4/3	-264	GGCAAA ACGC TCATCC
qa-4/3	-70	GGGGAA TAAC TTATAG
qa-4/3	-374	CGTTAA TGCT TTATTC
qa-y	-512	GGTTAT ACAT TCATCC
qa-y	-681	GGGTAA TGGC TTTTCC
qa-y	-412	GGCTCA ACAC TCATCA

Conserved Sequence: GGRTAA RYRY TTATCC




+1
-500 bp
+1
qa-x
qa-2
qa-4
qa-3
qa-y
qa-1S
qa-1S
qa-1F

site of *qa-2* showed the highest affinity for binding the activator protein (Table 2). Transcriptional mutants that retained only the –127 activator binding site but lost the other two binding sites for *qa-2* were only able to express the *qa-2* gene at 15-30% of normal induced wild-type levels. This is thought to mean that at least one of these sites, located between –391 and –510, is required for fully induced wild-type levels. It is interesting to note that the four binding sites that show the strongest affinity for the activator protein are located in a different 5'-flanking region of the gene cluster (Baum et al, 1987).

It is known that the 16 bp sequence is sufficient for the activator to bind, but what is not yet known is the significance of specific bases of this sequence. Since the –127 binding site of qa-2 showed the highest affinity for binding the activator protein, this site was chosen to study. In order to do this, a site-directed mutagenesis will be performed to mutate a specific base in this binding site. The mutation will be transformed back into *Neurospora crassa* and the rfM13 DNA will be isolated. Once isolated the DNA will be sequenced to confirm the presence of the mutation. This mutation will later be reintroduced into the *quinic acid* cluster of *Neurospora crassa* and the effects of the mutation will be studied.

Gene Pair	Location	Binding Affinity
qa-x/2	-127	1.0
qa-1S/F	-144	0.8
qa-4/3	-449	0.5
qa-y	-512	0.4
qa-x/2	-510	0.25
qa-4/3	-510	0.25
qa-y	-681	0.25
qa-4/3	-264	0.15
qa-x/2	-391	<0.1
qa-4/3	-70	<0.1
qa-1S/F	+236	<0.1
qa-x/2	-487	<<0.1
qa-4/3	-374	<<0.1
qa-y	-412	<<0.1

MATERIALS AND METHODS

I. Materials

Ethanol purchased from Aaper Alcohol and Chemical Company, Shelbyville, KY; isopropanol purchased from Baxter Healthcare Corporation, McGraw Park, IL; sequencing supplies purchased from Beckman Coulter, Inc., Fullerton, CA; the restriction enzymes *HindIII* and *KpnI* , RNase, single-stranded binding protein, T4 DNA ligase, T4 polynucleotide kinase, T7 DNA polymerase, and M13mp18 were purchased from Boehringer Mannheim, Indianapolis, IN; bacto-agar and bacto-tryptone were purchased from Difco Laboratories, Detroit, MI; agarose purchased from EM Science, Cherry Hill, NJ; acetic acid, ethidium bromide and sodium citrate were purchased from Fisher Scientific, Fairlawn, NJ; primers were purchased from Integrated DNA Technologies, Inc., Coralville, IA; Qiagen columns were purchased from QIAGEN, Inc., Chatsworth, CA; elutip columns were purchased from Scleicher & Schuell, Keene, NH; 3-N-morpholino-propanesulfonic acid (MOPS), adenosine triphosphate (ATP), ampicillin, calcium chloride, chloroform, cresol, dextrose, dimethyl formamide, ethylenediaminetetraacetic acid-disodium salt (EDTA), glycerol, magnesium chloride, octyl pheoxy polyethoxyethanol (Triton X-100), phenol, polaroid film, polytheylene glycol (PEG), potassium acetate, sodium acetate, sodium chloride, sodium dodecyl sulfate (SDS), sodium hydroxide, Trizma base and yeast extract were purchased from Sigma Chemical Company, St. Louis, MO; dinucleotide triphosphates (dNTPs) were purchased from PE Biosystems, Norwalk, CT.

II. Media

YT broth was made using 8 grams of bacto-tryptone, 5 grams of yeast extract and 5 grams of sodium chloride per liter. YT plates were made by adding 15 grams of bacto-agar per liter of YT broth. The 2xYT broth was made by mixing sixteen grams of bacto-tryptone, ten grams of yeast extract and ten grams of sodium chloride per liter.

Luria broth was made by combining ten grams of bacto-tryptone, five grams of yeast extract and ten grams of sodium chloride per liter of solution. To make luria broth plates one and a half grams of bacto-agar were added per one hundred milliliters of solution. LA-100 plates were made by adding ampicillin to a final concentration of 100 μg/ml.

III. Strains

JM101, a strain of *Escherichia coli,* was obtained from the *Escherichia coli* Genetic Stock Center at Yale University. Strain JM101 bacterial cells were grown in Luria Broth (as made above).

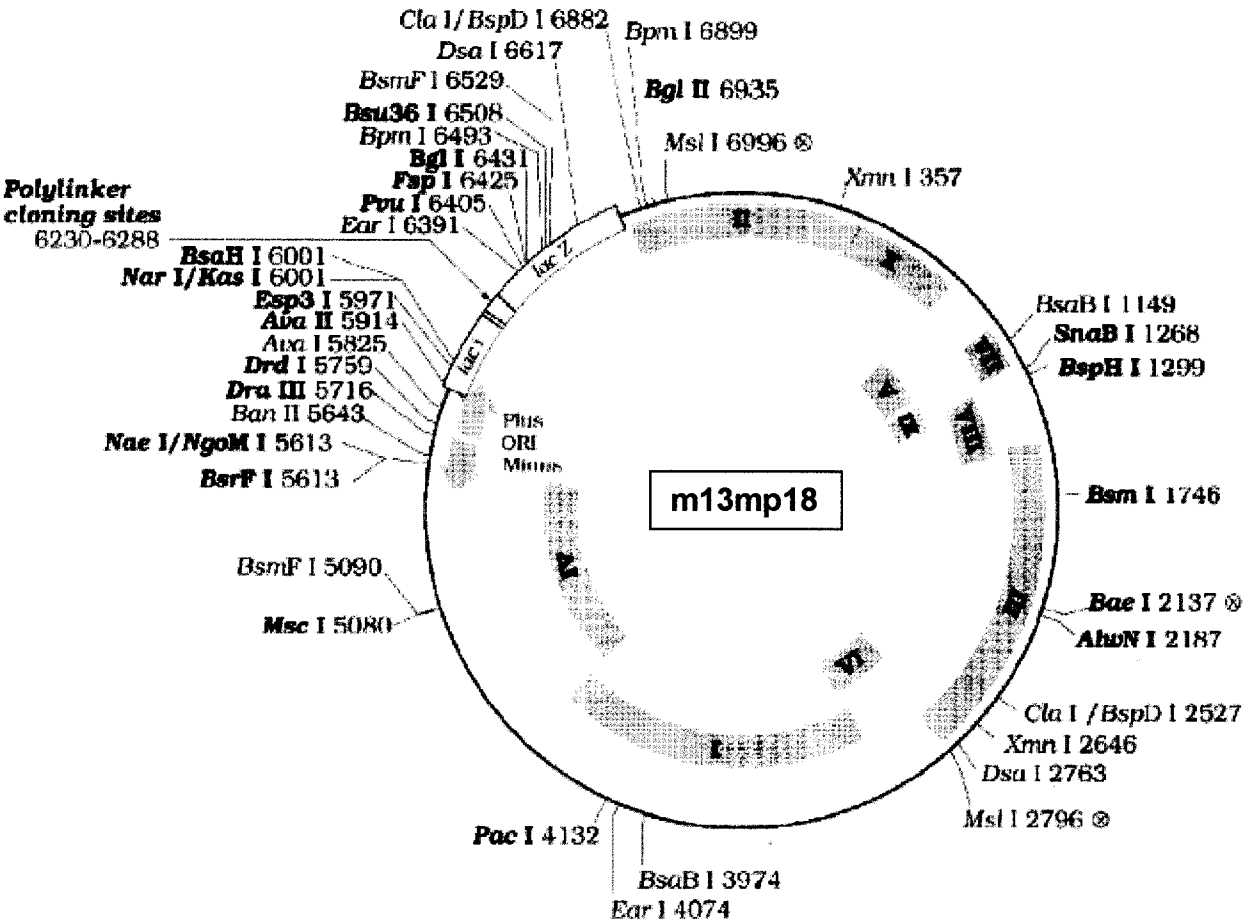
A second strain of bacteria, CJ236, was also purchased from CGSC, the Genetic Stock Center at Yale University. This particular strain of *Escherichia coli* is deficient in two enzymes. The dut gene normally encodes for the enzyme, UTPase, which degrades uracil. Because CJ236 is dut$^-$, there will be an increase in the uptake of uracil into DNA. The ung gene encodes for an enzyme that removes uracil that has been misincorporated into DNA. In an ung$^-$ strain, uracil

will not be removed from DNA. In the case of CJ236, which is both dut$^-$ung$^-$, not only will there be an increase of misincorporation of uracil into DNA, once incorporated there is no mechanism for its removal. CJ236 was grown in 2xYT broth and cultured on YT plates.

IV. M13mp18

M13 is a circular, single-stranded, filamentous bacteriophage that infects *Escherichia coli* host. M13 codes for ten genes and is approximately 6400 bp long. M13 can exist in two different forms. The single-stranded form as well as a double-stranded replicative form. The single-stranded phage will infect a male *E. coli* host. The host (*E. coli*) components will then convert the single-stranded genome to a circular double-stranded genome known as the replicative form of M13.

M13 was utilized because it possesses both a single- and double-stranded form as well as being a useful cloning vector. In the vector used, M13mp18 (Figure 6), the following elements have been cloned into the genome: the operator-proximal region of the lacZ gene to allow for complementation in a host with operator-proximal deletion of the lacZ gene, a gene encoding for the lac repressor (lacI), a lac promoter upstream of the lacZ gene, and a polylinker (multiple cloning site) region inserted several codons into the lacZ gene. Cloning into the multiple cloning site will disrupt the lacZ gene, allowing for blue-white screenings to be performed.


Cla I/BspD I 6882
Bpm I 6899
Dsa I 6617
BsmF I 6529
Bgl II 6935
Bsu36 I 6508
Msl I 6996 ⊗
Bpm I 6493
Bgl I 6431
Xmn I 357
Fsp I 6425
Polylinker cloning sites 6230-6288
Pvu I 6405
Ear I 6391
BsaH I 6001
Nar I/Kas I 6001
BsaB I 1149
Esp3 I 5971
SnaB I 1268
Ava II 5914
BspH I 1299
Ava I 5825
Drd I 5759
Dra III 5716
Ban II 5643
Bsm I 1746
Nae I/NgoM I 5613
BsrF I 5613
m13mp18
BsmF I 5090
Bae I 2137 ⊗
Msc I 5080
AhaN I 2187
Cla I /BspD I 2527
Xmn I 2646
Dsa I 2763
Msl I 2796 ⊗
Pac I 4132
BsaB I 3974
Ear I 4074

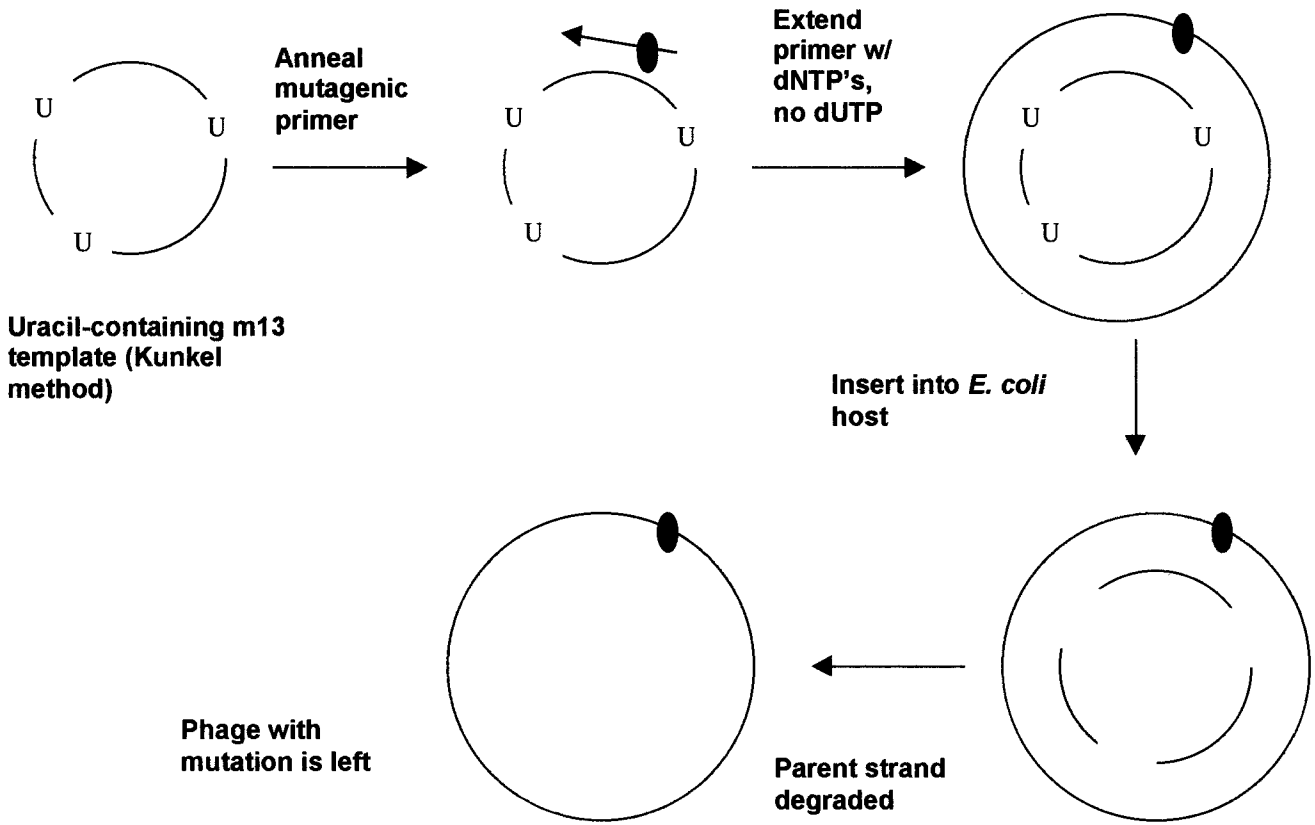
V. Isolation of Uracil-Containing ssM13

In order to isolate the single-stranded form of M13, an overnight culture of a dut$^-$ung$^-$ strain of E. coli, CJ236 was grown in 2 ml of 2xYT broth. The next morning 50 ml of 2xYT broth was inoculated with the 500 µl of overnight growth. This was then grown shaking for 2 hours at 37°C. At this point 40 µl of the wild-type M13 lysate was added. This mixture was grown shaking at 37°C for 6 hours. It was then centrifuged for 30 minutes at 5,000g. Then 35 ml of the lysate was mixed with 9 ml of 5xPEG/NaCl and incubated for one hour on ice. This was then centrifuged for 15 minutes at 5,000g and the pellet resuspended in 5 ml of 1xTE buffer (10 mM Tris, 8.0, 1 mM EDTA, 8.0). It was then vortexed, placed on ice for one hour and centrifuged at 5,000g for 15 minutes. The supernatant was transferred to a new tube and mixed with equal portions of a 25:25 phenol/chloroform mix. This was centrifuged at 4,000g for 5 minutes. The aqueous layer was then transferred to a new tube and 1/10 the volume of 3M NaOAc, with a pH of 5.2, was added. Two volumes of ethanol at 0°C was also added and the entire thing mixed and placed on ice for 30 minutes. This was then centrifuged for 20 minutes at which point 10 ml of 70% ethanol at room temperature was added. The mixture was then vortexed and recentrifuged for 20 minutes. The supernatant was removed and the pellet allowed to dry. It was then resuspended in 200 µl of 1x TE buffer. DNA extractions were done by adding 500 µl of an equal mix of phenol and chloroform to the samples. These were then vortexed and centrifuged for five minutes. This was followed by the addition of

50 µl of 3M NaOAc and 500 µl of isoproponal. The samples were then frozen fifteen minutes and then centrifuged for twenty minutes. The pellets were washed with 75% cold ehtanol and the pellets were resuspended in sterile water or 1x TE buffer.

VI. Site Directed Mutagenesis

The mutagenesis that I performed was taken from a method developed by Kunkel (Figure 7), which utilizes the single-stranded form of the M13 phage. First CJ236, a strain of bacteria that is dut^-, ung^-, was grown. The dut gene encodes for a UTPase that normally degrades uracil. In a dut^- strain there will be an increase in the misincorporation of uracil into DNA. The ung gene encodes an enzyme that removes uracil from DNA. In an ung^- strain the incorporated uracil will not be able to be removed from DNA. This will allow single-stranded M13 to form with uracil in its genetic code. The first step used in this protocol was the phosphorylation of the mutagenic oligonucleotide (which served as a primer for *in vitro* DNA replication). To do this the mutagenic oligonucleotide was incubated with 2 µl T4 polynucleotide kinase buffer, 2 µl T4 polynucleotide kinase and 2 µl 10mM ATP, along with sterile water to 20 µl at 37° C for 60 minutes. This reaction was terminated by adding 1.5 µl 0.2M EDTA and heating to 70° C to denature the enzyme. At this point the five µl of uracil-containing ssM13 (previously grown) was added along with 1.25 µl of 20x SSC (3M NaCl, 0.3M


U
U
U
Anneal mutagenic primer
U
U
U
Extend primer w/ dNTP's, no dUTP
U
U
U
Uracil-containing m13 template (Kunkel method)
Insert into E. coli host
Phage with mutation is left
Parent strand degraded

Na_3 citrate · $2H_2O$, pH of 7.0). The mixture was then mixed and spun for 5 seconds. The mixture was placed in a 500 ml beaker of water that was heated to 70° C and then cooled to room temperature. The mutagenic primer will have annealed to the single-stranded DNA template of the dut⁻ung⁻ strain. This was then spun 5 seconds again and placed on ice. While on ice 20 µl of polymerase mix, 1 µl of T4 ligase, 1 µl T7 polymerase, 2 µl of single-stranded binding protein, 4 µl of 5x ligase buffer, and 72 µl of sterile water was added to the mixture. The polymerase mix contained each of the following nucleotides: dATP, dTTP, dCTP, dGTP, as well as Tris·Cl, DTT, $MgCl_2$ and ATP. No dUTP was added. This new mixture was incubated on ice for 5 minutes, and then incubated at room temperature for 5 minutes. Finally it was incubated at 37° C for 2 hours. During this time the polymerase synthesized the new strand of DNA from the mutagenic primer. Ligase was added to join together the ends once synthesis of the new strand was completed. EDTA was then added again to terminate the reaction. The resulting dsDNA consisted of the original strand with uracil residues and the new strand, which has no uracil but does contain the mutant bases present in the oligonucleotide primer.

VII. Transformation

An overnight culture was started of *E. coli* in 2 ml of 2xYT broth shaking at 37° C. The next day 50 ml of 2xYT was inoculated with 500 µl of the *E. coli* overnight growth. This was then grown for 2 hours shaking at 37°C. A new flask

was then inoculated with 100 µl of the 2-hour growths and this was incubated at 37° C shaking until needed. The original flask was iced 10 minutes and then spun for 10 minutes at 7,000g. The pellet was resuspended in 10 ml of 0.1 M $CaCl_2$ and then incubated on ice for 20 minutes before being centrifuged again. The pellet was again resuspended in 250 µl of $CaCl_2$. The cells were then divided into separate eppendorf tubes. The eppendorf tubes contained 100 µl of the competent cells grown in *E. coli*. Next DNA mix was added to one eppendorf tube containing the *E.coli* cells. Both eppendorf tubes were then placed on ice for 60 minutes after which they were heat-shocked for 5 minutes at 37° C. Next 30 µl of each transformation mix was mixed with 3 ml of soft agar and 200 µl of uninfected cells that will have continued to grow. These were then poured onto separate plates. Three plates would have the ligation mix and one would be a control. The plates were then incubated overnight at 37° C.

VIII. Isolation of rfM13

To isolate the M13 DNA, a JM101 was grown overnight in 2 ml of luria broth. The next day 50 ml of 2xYT broth was inoculated with 500 µl of the overnight growth and this was grown two hours at 37°C while shaking. This was then inoculated with 40 µl of phage lysate. The phage was incubated with JM101 for 6 hours shaking at 37°C and then centrifuged at 10K for ten minutes. The pellet was resuspended in 2 ml of G buffer (50mM dextrose, 25 mM Tris, 8.0, 10 mM EDTA, 8.0) for gentle lysis and 4 ml of denaturing solution (0.2 N NaOH,

1% SDS) to get ssDNA. This solution was then placed on ice for 5 minutes followed by the addition of 3 ml of neutralizing solution (3M KOAc, 2 M HOAc) in order to renature DNA. This mixture was then placed on ice for 20 minutes before being centrifuged again. Then 5.4 ml of isopropanol was added to the supernatant to precipitate the nucleic acid and then the mixture was spun for 30 minutes. The pellet was then dried and resuspended in 500 µl of 1x TE buffer. All DNA was then purified using Qiagen purification or CsCl purification.

IX. Qiagen Purification

A qiagen column was first used to purify the DNA. The column was first primed with 7 ml of QBT buffer (750 mM NaCl, 50 mM MOPS, 7.0, 15% isoproponal, 0.15% Triton X-100), a low salt buffer which allowed the phage DNA to bind, while the degraded RNA, proteins flowed through. DNA samples were then passed through the column. The column was then washed twice with 7 ml of a medium-salt buffer, QC (1.0 M NaCl, 50 mM MOPS, 7.0, 15% isopropanol). This buffer should have removed any contaminants without removing the DNA from the column. Finally the DNA was eluted off the column with 7 ml of a high-salt buffer, QF (1.25 M NaCl, 50 mM Tris-HCl, 15% ethanol). To the eluted DNA, 7 ml of isopropanol was added to precipitate nucleic acids out of the solution. This was then centrifuged for 15 minutes and the pellet washed twice with 10 ml of cold 70% ethanol. This was then spun at

15K for 15 minutes, the supernatant decanted and the pellet resuspended in 1x TE buffer.

X. CsCl Gradients for Purification

First, 15 grams of CsCl were weighed and put into 15 ml centrifuge tubes. The resuspended DNA in 1xTE buffer was then added to these tubes and more 1xTE buffer will be added until there is approximately 13.5 ml of the mixture in the centrifuge tube. The CsCl and DNA will be resuspended by inverting each tube. The concentration of the CsCl was checked by utilizing a light refractometer. Then 1.5 ml of ethidium bromide was then added to each tube and mixed carefully. The CsCl/DNA mix was then carefully transferred to an ultracentrifuge tube and each tube was run at 65K for 24 hours. DNA bands were visible and they were extracted with a syringe. The ethidium was then extracted from the DNA using salt-saturated butanol until clean. The DNA was then precipitated with ethanol before being centrifuged and resuspended in 1ml 1xTE buffer.

XI. Agarose Gel Electrophoresis

All DNA samples were checked by agarose gel electrophoresis. The gel was a 1% agarose gel made by mixing 0.5 grams of agarose with 50 ml of 1x TPE buffer (0.09 M Tris-phophate, 0.002 M EDTA) and microwaving for one minute. The solution is poured into a gel tray and allowed to solidify. Samples were

usually run with 2 µl of bromophenol blue, 5 µl of sample and 10 µl of sterile water. Gels were stained with ethidium bromide and visualized on a transilluminator. Photos were taken with polaroid film.

RESULTS

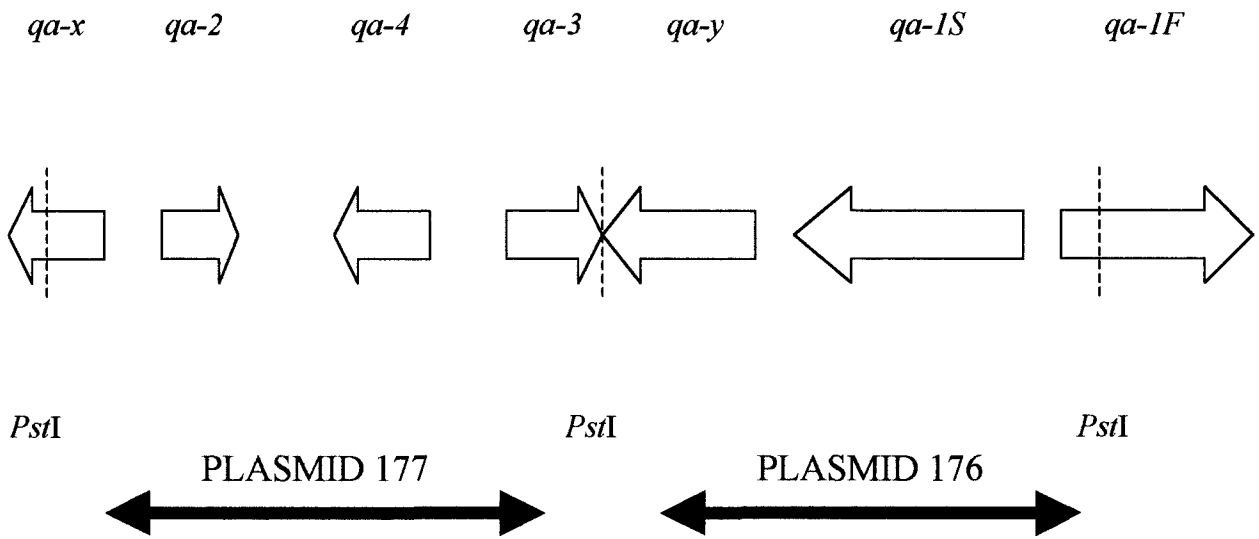
I. Subcloning of the –127 activator binding site

Previously two *Pst*I restriction enzyme fragments containing most of the *qa* gene cluster of *Neurospora crassa* had been cloned into pBR322 in order to form two plasmids, plasmid 176 and plasmid 177 (Figure 8). Plasmid 177 contains *qa-2, qa-4, qa-3* and a portion of the *qa-x* gene. Plasmid 176 contains *qa-y, qa-1S* and a portion of *qa-1F*.

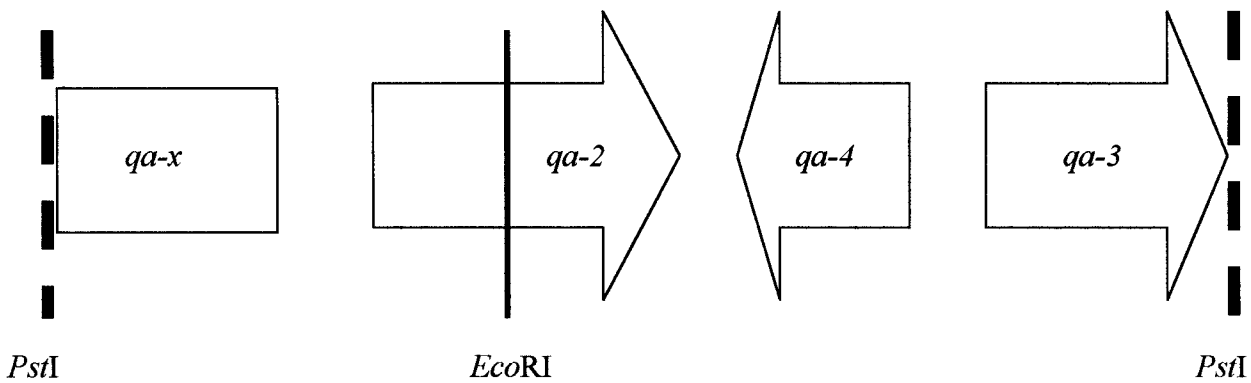
Plasmid 177 contains the fragment we are concerned with and was previously digested further with *Eco*RI and *Pst*I (Figure 9) yielding a 2.5 kb fragment. The resulting fragment was cloned into bluescript and named plasmid #10(EP). Plasmid 10(EP) was then further cut with EcoRI and HindIII, resulting in a 1.8 kb fragment (Figure 10). This fragment was then inserted into bluescript to form plasmid EH. Plasmid EH was then cut with KpnI (Figure 11) and this gave a fragment of approximately 500bp. This fragment contains the activator binding site of interest, the –127 activator binding site of *qa-2* (Figure 12). This fragment was then cloned into M13mp18 and site-directed mutagenesis was performed.

II. Isolation of uracil-containing ssM13

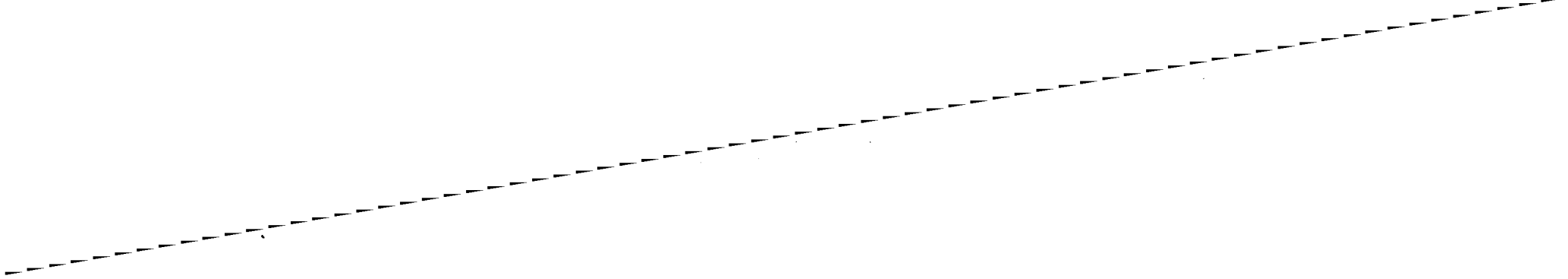
An overnight growth of CJ236 was done in 2 ml of 2xYT broth. The next day 500 μl of that overnight growth was added to 50 ml of 2xYT broth and grown


qa-x
qa-2
qa-4
qa-3
qa-y
qa-1S
qa-1F
PstI
PstI
PstI
PLASMID 177
PLASMID 176

PLASMID 177



PLASMID #10(EP)



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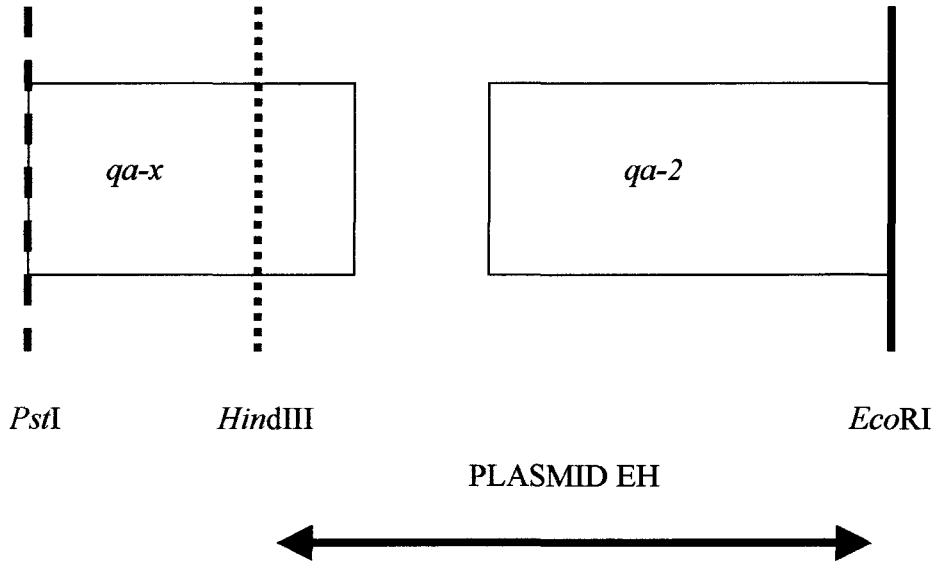
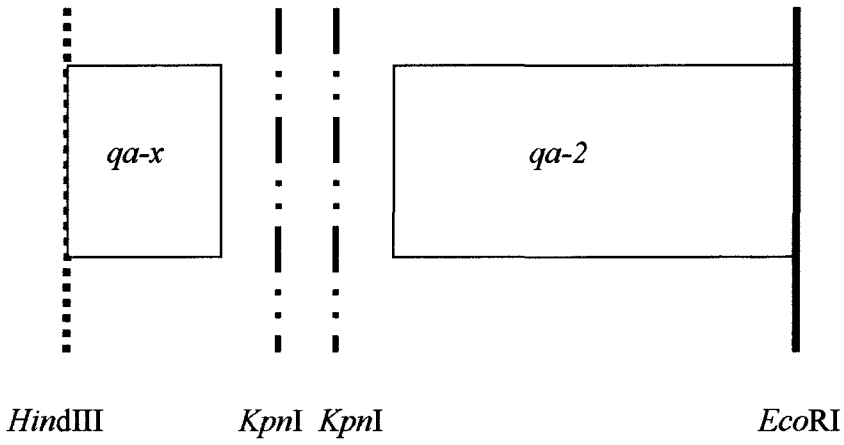
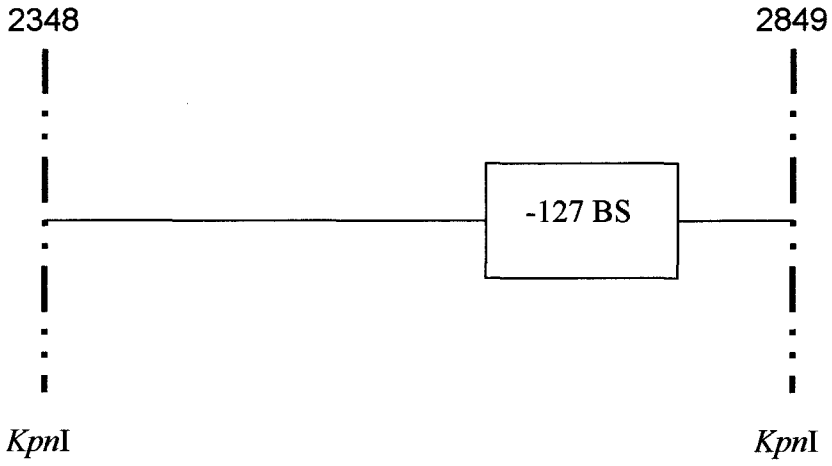


PLASMID EH

PLASMID EP



*Kpn*I fragment

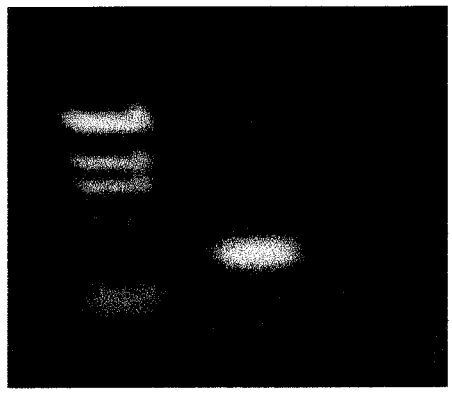


shaking at 37°C for two hours. At this point 40 µl of phage #9, a M13 clone containing the –127 activator binding site, was added to the flask. This was grown shaking for six hours at 37°C. After pelleting the cells, 35 ml of the supernatant containing M13 phage particles was mixed with PEG/NaCl, to remove the phage coats and was incubated for one hour on ice. The solution was spun again and the DNA in the supernatant was transferred to a new tube and a phenol/chloroform solution was added to extract the DNA. The DNA was then precipitated and washed. A gel electrophoresis was then run to check the sample. A *Hin*dIII-cut lambda ladder was run in one lane and 5 µl of the sample containing the ssM13, 2 µl of dye and 12 µl of sterile water were ran in another lane (Figure 13).

III. Site-Directed Mutagenesis

First the mutagenic oligonucleotide was phosphorylated by incubating the mutagenic oligonucleotide (QA-2-1) with diluted T4 kinase, 10x T4 kinase buffer, 10 mM ATP and sterile water. The uracil containing, single-stranded M13 clone was then incubated with he phosphorylated mutagenic oligonucleotide and a 5x polymerase mix containing the dNTPs and ATP, T4 DNA ligase, T7 DNA polymerase, sterile water and single-stranded binding protein (ssbp). This mixture would allow synthesis of a strand of DNA containing our mutation.

1 2



IV. Transformation

An overnight culture of both JM101 and CJ236 were grown. The next day 500 µl of each were added to different flasks containing 50 ml of 2xYT broth. This was grown for two hours. New flasks were inoculated with 100 µl of the two-hour growth and remained incubated until needed. The old flasks were iced before being centrifuged. The pellets were resuspended in 10 ml of 0.1 M CaCl2 and being iced and centrifuged again. The pellets were resuspended in 300 µl of CaCl2. Four eppendorf tubes were labeled and 100 µl of JM101 cells were placed in three of the tubes, two of which contained 10 µl of the mutagenesis mix and one tube which contained nothing. Into the fourth tube, 100 µl of CJ236 cells were placed along with 10 µl of the mutagenesis mix and the four tubes were iced for sixty minutes. The tubes were then heat shocked for five minutes at 37°C and plated. The plates were then incubated overnight at 37°C. The following day the plates were examined for results of a successful mutagenesis.

In the transformation the CJ236 cells were a control. Once plated with the mutagenesis mix and incubated the plate should contain many plaques. This is the plate that contained the dut^- ung^- bacteria. Any phage still capable of replicating will be seen as a plaque.

The JM101 strains are dut^+ ung^+. This means it contains the enzymes to remove uracil from DNA. When the dsDNA from the mutagenesis was transformed into JM101 the uracil-containing strand was degraded and the mutagenic strand replicated. When looking at the three JM101 plates, the one

incubated with no mutagenesis mixture will also be a control. It should contain no plaques. The other two JM101 plates will be indicative of a successful mutagenesis. Any plaques on these plates will be of phages that incorporated the mutation.

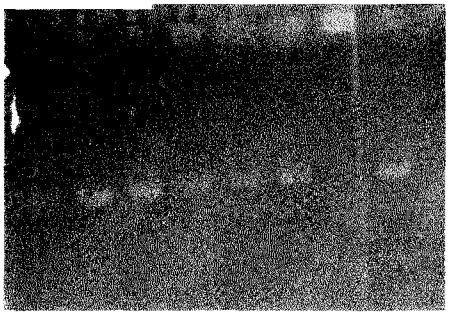
The mutagenesis and transformation were successful as plaques were seen in both plates containing JM101 cells mixed with the mutagenesis and the control plate of JM101 with no mixture had no plaques. The CJ236 control plate with the mutagenesis mixture also worked because many plaques were seen (Table 3). Eight plaques were chosen and each plaque was picked with a toothpick and placed in a tube with 200 µl of overnight growth of JM101 and 2 ml of 2xYT broth. These were then grown overnight shaking at 37°C. The next day the cells were spun down and the lysates were kept. A 1 % agarose gel was run to check results. Each lane contained 20 µl of lysate, 2 µl of dye and 2 µl of 2% SDS-page. DNA was seen in all lanes (Figure 14).

V. Isolation of rfM13

The replicative form of M13 was isolated from both my plaques and plaques from a previous student's site-directed mutagenesis (Diana Arnett's MS thesis). There were 19 phage lysates in which to isolate DNA from. Once the DNA was isolated, all samples were purified using a Qiagen column. After purification a gel check was performed to see if the rfM13 was isolated from each

Cell type	Mutagenesis Mixture	Plaque Number
JM101	-	0
JM101	+	8
JM101	+	2
CJ236	+	+200

1 2 3 4 5 6 7 8

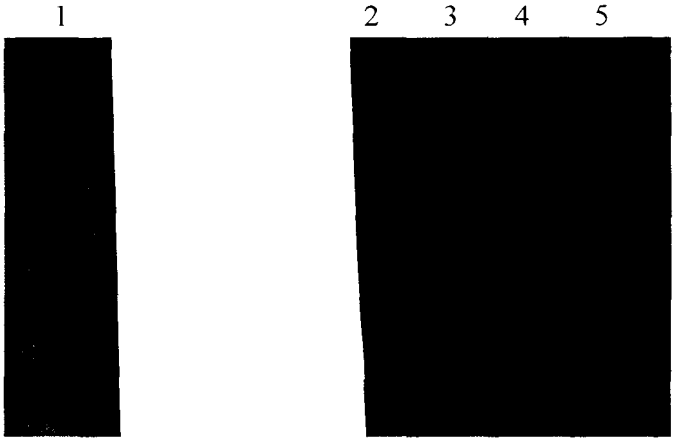


sample. This procedure was performed on phage lysates 1, 3 and 9 (Figure 15A) and repeated with phage lysates 2,4,5 and 6 (Figure 15B), lysates 7, 8, and 10 through 15 (Figure 15C). Although DNA was barely visible on phages 12 and 15, it was clearly seen in all other lanes. Finally these experiments were repeated on phage lysates 16, 17, 18 and 19 (Figure 16). DNA was clearly visible in all lanes except for the lane containing phage 17.

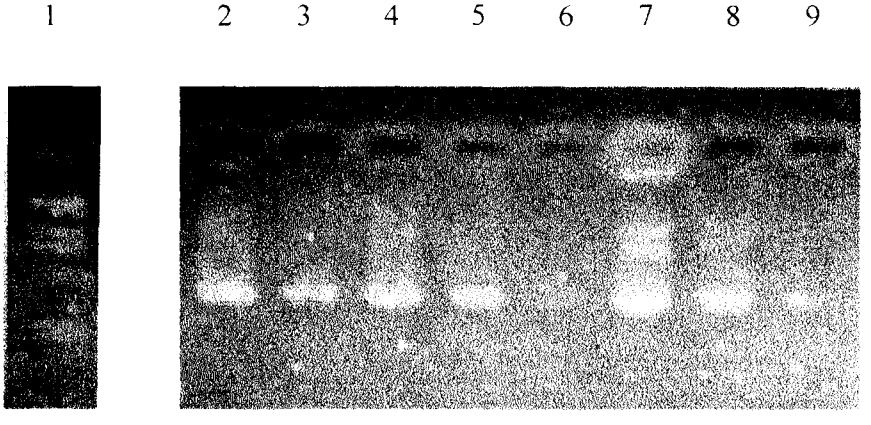
The isolation of the replicative form of the M13 phages from my site-directed mutagenesis was done similiarly to the previous isolations with some modifications. JM101 was grown overnight in 50 ml flasks of 2xYT, shaking at 37°C. In the morning 1 L flasks containing 250 mL of 2xYT were inoculated with 2 mL of overnight growth and 500 µl of the phages. This was grown shaking for six hours at 37°C before being transferred to centrifuge bottles and being centrifuged at 7K for ten minutes. The supernatant was discarded and the pellet was resuspended in 30 ml of G buffer. The solutions were centrifuged again for ten minutes at 8K and the supernatant discarded. The pellets were resuspended in 30 ml of G buffer and placed on ice before adding 60 ml of denaturing solution. This was mixed and iced for fifteen minutes. Then 45 ml of neutralizing solution was added to the mix and the solutions were iced for an additional sixty minutes. The centrifuge bottles were then spun at 9K for twenty minutes. The supernatant was then filtered through gauze into new centrifuge bottles where 81 ml of isopropanol was added. These new solutions were then spun at 9K for thirty minutes and the supernatant was decanted. The bottles then

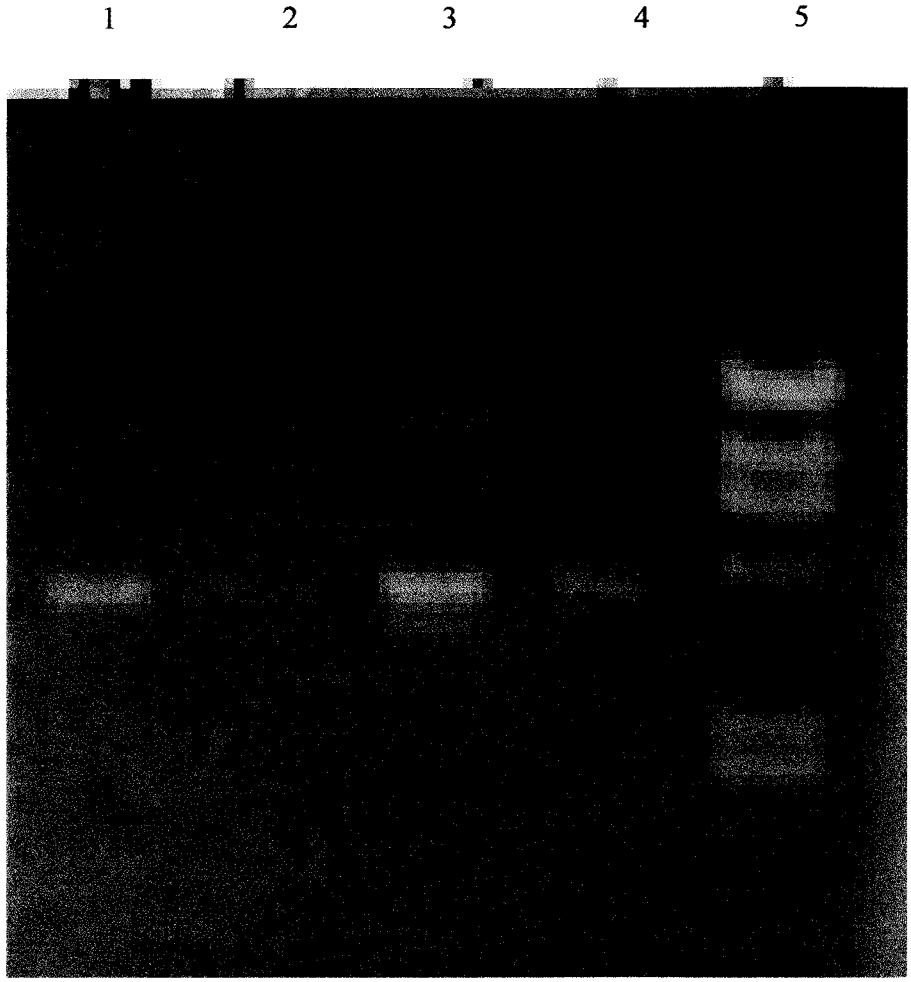
A

B



C



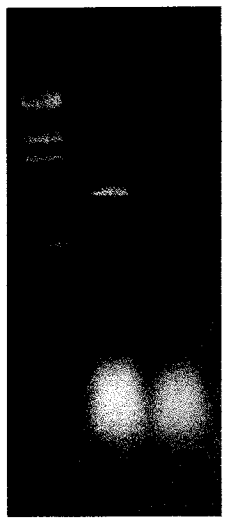

1
2
3
4
5

sat thirty minutes to drain them and lyopholized for thirty minutes. The pellets were then resuspended in 7 ml of 1x TE buffer.

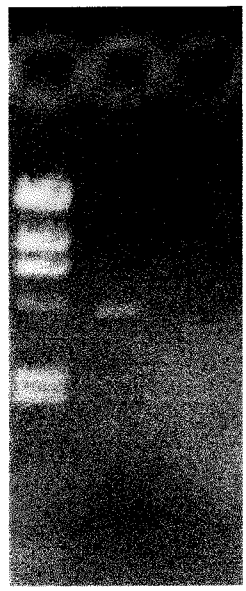
To purify the isolated DNA from my phages, a cesium chloride purification was chosen instead of qiagen purification due to the fact that the previously isolated DNA was unable to be sequenced by the Beckman automated sequencer. Following cesium chloride purification the samples were all resuspended in sterile water before being gel checked on a 1% agarose gel for verification of the presence of rfM13. Each sample was run using 2 µl of dye, 3 µl of sample and 12 µl of 1x TE buffer (Figures 17-19). DNA was not seen from phages 2 and 4 and barely seen from phage 6. DNA was clearly visible from phages 1, 3, and 5, particularly 5b. DNA was also seen from phages 7 and 8. The best samples were chosen to be sequenced by the Beckman automated sequencer.

The samples chosen were given to the operators of the Beckman sequencer to prepare them for sequencing. Once prepared they were run through the sequencer, but were unable to be sequenced. The analysis of the DNA sequences showed nothing on samples run (Figure 20). Due to the fact that nothing appears on the analysis, it may be that the primer didn't even anneal to the DNA in order to begin sequencing. This could indicate a spontaneous deletion or mutation within the targeted primer site.

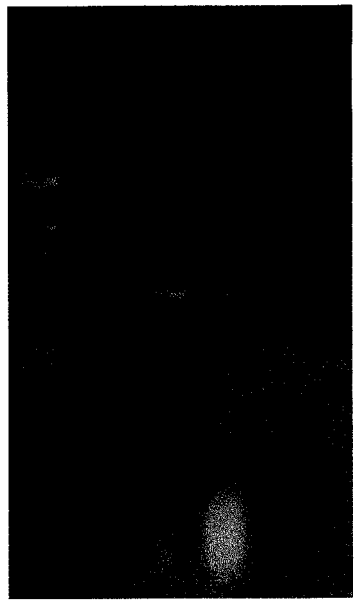
A 1 2 3



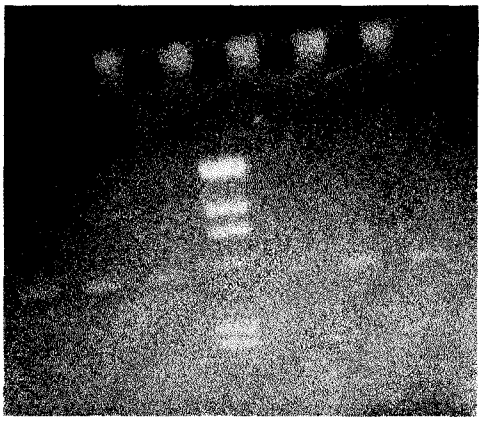
B 1 2 3

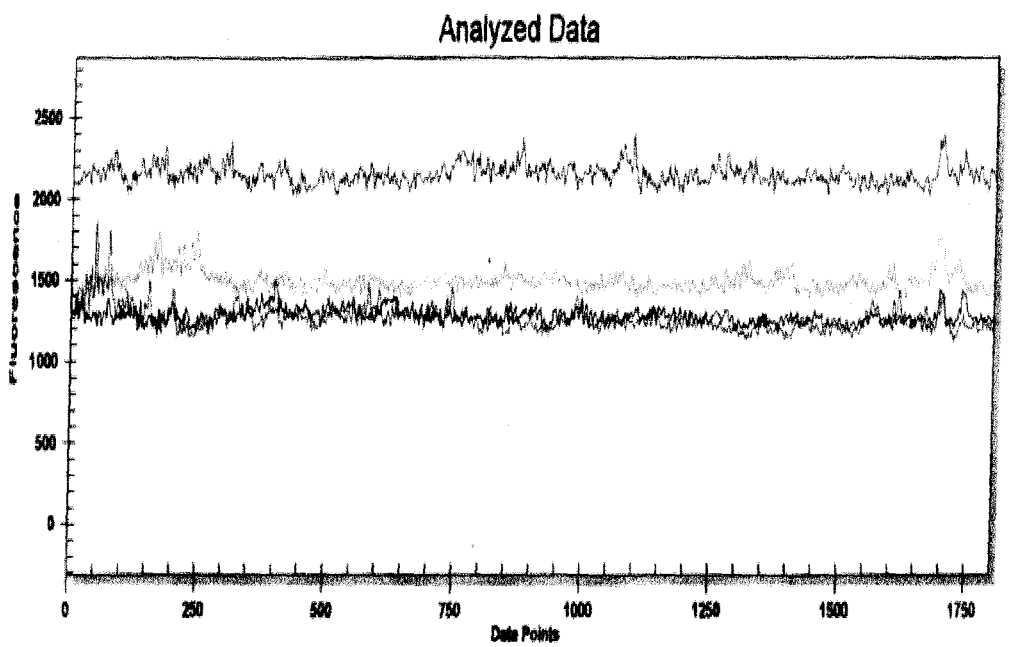


1 2 3 4 5



1 2 3 4 5 6 7




Analyzed Data
Fluorescence
2500
2000
1500
1000
500
0
0
250
500
750
1000
1250
1500
1750
Data Points

DISCUSSION

Much is known about the regulation of the quinic acid gene cluster. We know that there are two levels of regulation. One level being catabolite repression, in which transcription of the quinic acid genes is repressed in the presence of a preferred carbon source. The other involves the interaction of the products of the quinic acid regulatory genes and quinic acid itself. In the absence of the inducer quinic acid, the repressor protein (the product of *qa-1S*) prevents expression of the *qa-1F* gene by binding to its product, the activator protein. This allows the quinic acid genes to be expressed only at low basal levels. Once quinic acid is introduced the repressor will no longer inhibit *qa-1F* expression and the activator will allow transcription of itself as well as all other *qa* genes.

It is also known that the activator protein binds to particular sites in the quinic acid cluster that share a conserved sequence, GGRTAARYRYTTAYCC. The 16 base pair sequence is present in 14 locations within the cluster and at least once 5' to each *qa* gene. Each of the 14 locations have a specific binding affinity for the activator protein, with the –127 activator binding site of *qa-2* having the highest affinity for binding of the activator protein.

What is not known is its significance *in vivo* or the significance of the individual bases of this sequence. We chose to perform a site-directed mutagenesis of a base of the –127 activator binding site of *qa-2* in order to determine its significance. The mutated primer was incubated with a dut$^-$ ung$^-$ strain of *E. coli*, CJ236. In this strain of *E. coli* there is no dut gene, which

encodes for an enzyme that degrades uracil and there is no ung gene which encodes an enzyme that removes uracil from DNA. Therefore uracil was misincorporated into the DNA. The primer was extended with the following phosphorylated nucleotide triphosphates: dATP, dCTP, dGTP, dTTP. No dUTP was added. The resulting double stranded DNA contained the original strand of DNA containing uracil and the newly formed strand containing our mutation and no uracil. The ds-DNA was then inserted into a dut^+ ung^+ *E. coli* host, JM101. This strain of E. coli contains the genes that remove and degrade uracil from DNA, so the original strand containing uracil was preferentially degraded, leaving us our strand with the desired mutation.

The mutagenesis was determined to be successful because only a few plaques grew on the plates that contained the mutagenesis mixture transformed with JM101. If many or no plaques were seen, this would mean that the mutagenesis and transformation were unsuccessful.

When isolating the phages from a previous student's mutagenesis and using a Qiagen purification method, the DNA was unable to be sequenced. We then chose to do a new mutagenesis and to try Cesium Chloride purification instead of the Qiagen purification to see if we would get better results. With cesium chloride purification, nucleic acids and other cellular debris are separated from each other based on the different amounts of ethidium bromide incorporated into the sample and the cesium chloride density gradient. Once the samples were purified the next step was sequencing by the CEQ DNA analysis system. This system separates DNA fragments by capillary electrophoresis. Within this system

each base gives off a different fluorescence letting us know which bases are present in which order.

Results from the sequencing reaction yielded no usable sequence. The flat line suggests that the sequencer was unable to even begin the sequencing. We know DNA was present in the samples but sequencing was unable to be performed. This could be for any number of reasons. Possibly the DNA was contaminated with cesium chloride. If more than 5mM of cesium chloride was still present in the sample following the purification, this would significantly reduce the quality of sequencing results. However, that would not account for the lack of sequencing in the qiagen purified DNA. It is also possible that the primer site within the M13 clone underwent a deletion or mutation itself, disallowing the primer to anneal during sequencing. This would prevent any sequencing from occurring.

Although we are unable to confirm that the mutation occurred, we did isolate M13 phages following the mutagenesis which leads me to believe at least one of the phages contains the mutation even if they won't sequence.

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