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

by

Diana Arnett

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Signature:

8/7/2000
Diana Arnett, Student Date

Approvals:

David Asch 8/7/00
Dr. David Asch, Thesis Advisor Date

8/7/00
Dr. Diana Fagan, Committee Member Date

Gary R. Walker 8/7/00
Dr. Gary Walker, Committee Member Date

8/8/00
Peter J. Kasvinsky, Dean of Graduate Studies Date

ABSTRACT

The quinic acid (*qa*) gene cluster is a positively regulated system. In the absence of the inducer, quinic acid, the repressor protein binds to the activator protein, blocking transcription of the *qa* genes. Addition of quinic acid releases the activator protein, which is then free to bind to its activator binding sites, increasing the levels of transcription of the *qa* genes. The activator binding sites are composed of a 16 base pair (bp) conserved sequence, but the importance of the individual bases in the sites is currently unknown.

To determine the importance of the bases, the DNA containing the activator binding site was cloned and isolated. The –127 binding site of the *qa-2* gene was chosen due to its high binding affinity for the activator protein. A plasmid known to contain the entire *qa* cluster was digested with PstI and the fragment containing the *qa-2-qa-x* intergenic region was cloned into pBR322 to form plasmid 177. This was digested further with the enzymes EcoRI and PstI and the desired fragment cloned into pBluescript to form pEP, which was then digested with EcoRI and HindIII and the appropriate fragment was cloned into pBluescript to form pEH. PEH was finally digested with KpnI to give a 500 bp fragment that was cloned into M13. Using the method described by Kunkel, et al (1991), site-directed mutagenesis was performed on one of the most highly conserved bases in the site. A clone was then sequenced to determine if the mutagenesis was successful. While the clone chosen did not contained the desired mutation, future students will study the remaining prepared to clones to attempt to isolate a mutant.

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INTRODUCTION

I. Kingdom Fungi

The kingdom fungi represents an extremely diverse group of eukaryotic organisms, contained in three phyla: zygomycota, ascomycota, and basidomycota. This group numbers more than 100,000 species with more being described every year. Fungi can be unicellular but most are filamentous, growing as branched filaments or hyphae. Rather than ingesting food, fungi secrete enzymes that break food down and then absorb the nutrients. Being heterotrophic, fungi cannot synthesize their own food from inorganic materials but instead survive as saprobes (feeding on nonliving organic matter) or parasites (feeding on living organic matter) (Alexopoulos, et al 1996).

II. Phylum Ascomycota

Ascomycota is the largest division of the fungal kingdom, with over 30,000 species. The distinguishing morphological characteristic of ascomycetes is the presence of the ascus—a sac-like cell containing spores called ascospores. The two groups of organisms in this phylum most important to molecular biology are the yeasts, such as the *Saccharomycetales*, and the filamentous ascomycetes, which include the genus *Neurospora* (Alexopoulos, et al 1996).

III. *Neurospora crassa*

First described in 1842 as "red-bread mold," *Neurospora* has been a model organism for studies in cellular and molecular scientific experiments since the early 20th century. It began to be used for the study of genetics in the 1920's by Bernard Dodge and C.L. Shear, who performed crosses and studied ascospore production. In the 1940's, Beadle and Tatum used *Neurospora* to propose the one-gene one-enzyme hypothesis—the theory that one gene results in the synthesis of one enzyme. (This has since been modified to one gene-one polypeptide). *Neurospora* also played a pioneering role in studying biochemical mutants and in learning about recombination. (Perkins 1992).

Neurospora has many qualities that make it ideal for molecular biology experiments. First, and probably foremost, it can be grown and maintained inexpensively on relatively simple media, and stocks can be preserved easily by freezing. It also has a relatively small genome size for a eukaryotic organism, with seven chromosomes and 47 million base pairs. Its rapid growth is another positive quality, allowing for the study of multiple generations. In addition, *Neurospora* is easily manipulated by many of the standard microbiological techniques. As it exists primarily in a haploid state (single copy of each gene), genetic changes are readily monitored and individual DNA sequences can be isolated relatively easily (Mishra 1991).

IV. History of the Quinic Acid Gene Cluster

In prokaryotes gene regulation is well understood: each series of genes performing a single function are regulated together in operons. On the other hand, most genes responsible for any given pathway in eukaryotes appear to be randomly spread throughout the entire genome of the organism. However, in *Neurospora crassa* all of the structural and regulatory genes required for quinic acid utilization appear to be located in a single cluster, providing an excellent opportunity to study gene regulation.

The *quinic acid (qa)* gene cluster in *N. crassa* has been studied extensively, since mutants unable to use quinic acid as a carbon source were discovered in the 1960s. In the early 1970s, research established the presence of four genes in the cluster involved with quinic acid metabolism. Three of these genes (*qa-2, qa-3,* and *qa-4*) encoded inducible enzymes responsible for the catabolism of quinic acid to protocatechuic acid while the fourth gene (*qa-1*) was believed to serve in a regulatory role—in concert with the inducer quinic acid as a positive regulator of gene expression (Chaleff 1974).

Early studies identified *qa-2* mutants that lacked catabolic dehydroquinase (C-DHQase) activity, providing evidence that the *qa-2* gene product is responsible for the catabolism of dehydroquinic acid to dehydroshikimic acid (Giles, et al 1985). *Qa-3* mutants were isolated that did not display either quinic acid dehydrogenase (QDHase) or shikimic acid dehydrogenase (SDHase) activity, suggesting that the *qa-3* gene product catalyzed both quinate and shikimate dehydrogenation (Chaleff 1974).

Studies on a *qa-4* mutant isolated by Case showed it to be unable to convert dehydroshikimate to protocatechuic acid, suggesting that the *qa-4* gene product was the enzyme dehydroshikimate dehydrase (DHS-Dase), the enzyme responsible for this conversion (Chaleff 1974).

It was initially believed that a single, positively acting regulatory gene, *qa-1*, regulated the cluster. Two types of mutants were isolated: pleiotropic negative (noninducible) and constitutive mutants. Additionally, two types of noninducible mutants were described based on their ability to complement *qa-2* mutants. One group showed slow (weak) complementation and was designated *qa-1S*; the other showed fast (strong) complementation and was designated *qa-1F*. Case and Giles (1975) performed mapping studies and determined that *qa-1S* and *qa-1F* mutants map in discrete, non-overlapping regions at opposite ends of the *qa-1* locus. This discovery led to the hypothesis that the *qa-1* protein was a multimeric protein with separate domains for DNA and inducer binding (Case and Giles 1975). Later studies would reveal the existence of two separate regulatory genes (Huiet 1984).

V. Cloning of the Quinic Acid Gene Cluster of *Neurospora crassa*

Advances in recombinant DNA technology allowed for major advances in the understanding of the regulation of the *qa* gene cluster, beginning with the cloning and expression of *qa-2* in *E. coli*. This was possible by using *E. coli* with a defect in the *aroD* gene, thus lacking biosynthetic DHQase (B-DHQase) activity, which was complemented by the *N. crassa qa-2* gene (Vapnek, et al 1977, Alton, et al 1978).

Unfortunately, this approach was not possible for any other *qa* genes. Not until the development of a new technique for *N. crassa* transformation were the other genes able to be cloned.

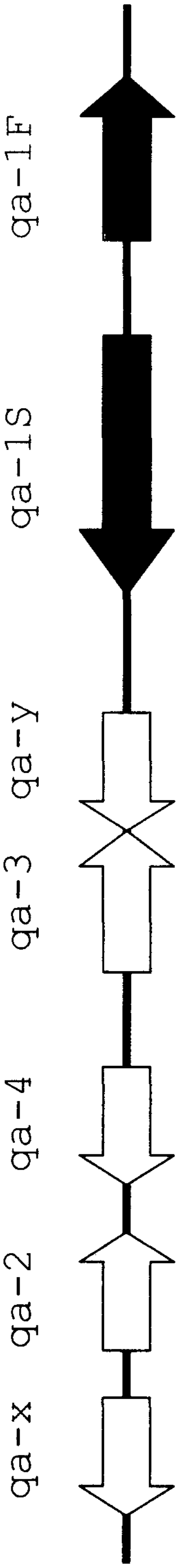
With these new techniques, it was discovered that what was believed to be the *qa-1* gene was actually two separate genes, designated *qa-1S* and *qa-1F*. The genes encode two separate mRNA species that are transcribed in opposite directions. It was also discovered that the *qa-1S* gene was transcribed at low basal levels and was also induced by quinic acid. This induction requires the presence of both the *qa-1S* gene product, believed to be a repressor protein, and *qa-1F* gene product, believed to be an activator protein (Huiet 1984).

Cloning the entire cluster allowed the identification of the mRNAs of all the structural genes of the *qa* cluster. In addition to the three known genes, two additional structural genes were identified by DNA-RNA hybridization studies and named *qa-x* and *qa-y* (Patel, et al 1981). Originally identified merely as a quinic acid inducible transcript with unknown function, *qa-y* is now believed to encode a quinic acid permease. This belief is based on two lines of evidence. First, the *qa-y* gene product has been shown to be quite similar to other known quinate permeases, such as the *qutD* gene of *Aspergillus nidulans* (Geever, et al 1989, Whittington, et al 1987), as well as other types of permeases, including the glucose transporter of human hepatoma cells (Mueckler, et al 1985) and bacterial transporters for arabinose (*AraE*), xylose (*XylE*), and citrate (*Cit*$^+$) (Maiden, et al 1987). Also, deletion experiments result in strains in which *qa-2* and *qa-3* are expressed at only 5-10% of the levels in induced wild-type, as would be expected if quinic acid, the inducer, were not being actively transported into the cell (Case, et al 1992).

The function of *qa-x* remains unclear. Deletion mutants retain the ability to grow on quinic acid as a sole carbon source (Case, et al 1992). The only observable effect of *qa-x* deletion was the accumulation of a brown pigment in the cells (Giles, et al 1991). A theory that the *qa-x* gene product encoded an enzyme responsible for utilizing chlorogenic acid as a carbon source (Giles, et al 1985) was disproved by demonstrating the ability of *qa-x* deletion mutants to grow on chlorogenic acid as a sole carbon source (Case, et al 1992). Recent evidence points to a possible role in catabolite repression—transcription of *qa-x* mRNA increases 20-fold when a culture is shifted to a carbon limiting food source, suggesting that the preferred carbon source directly represses *qa-x* expression—but the true function of the *qa-x* gene product remains uncertain (Giles, et al 1985, Giles, et al 1991).

VI. Organization of the Quinic Acid Gene Cluster

The organization of the *qa* gene cluster has been determined from transformation experiments, Northern blot analyses, S1 mapping, and RNA sequencing. Located on linkage group VII, this 17.3 kb cluster is composed of seven genes: five structural genes *qa-2, qa-3, qa-4, qa-x,* and *qa-y*; and two regulatory genes *qa-1S* and *qa-1F* (Geever, et al 1989). The two regulatory genes are located at one end, the five structural genes at the other (Figure 1). The cluster is composed of three pairs of divergently transcribed genes (*qa-x/qa-2*, *qa-4/qa-3*, and *qa-1S/qa-1F*).

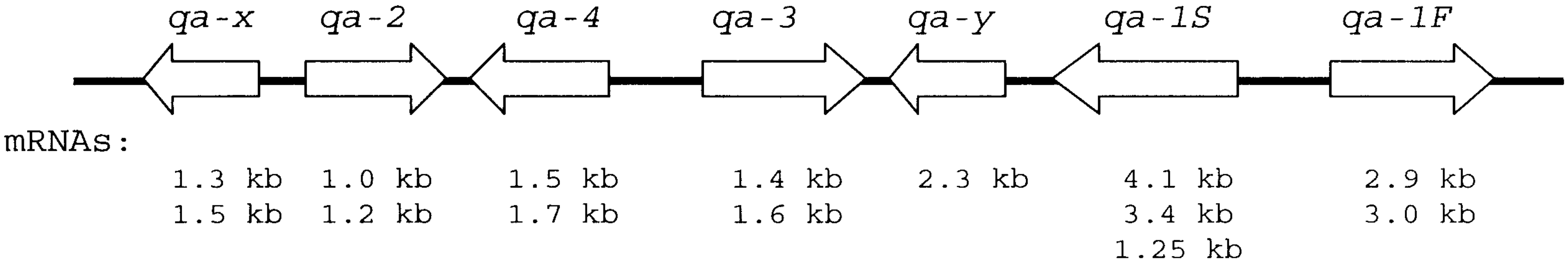

qa-x
qa-2
qa-4
qa-3
qa-y
qa-1S
qa-1F

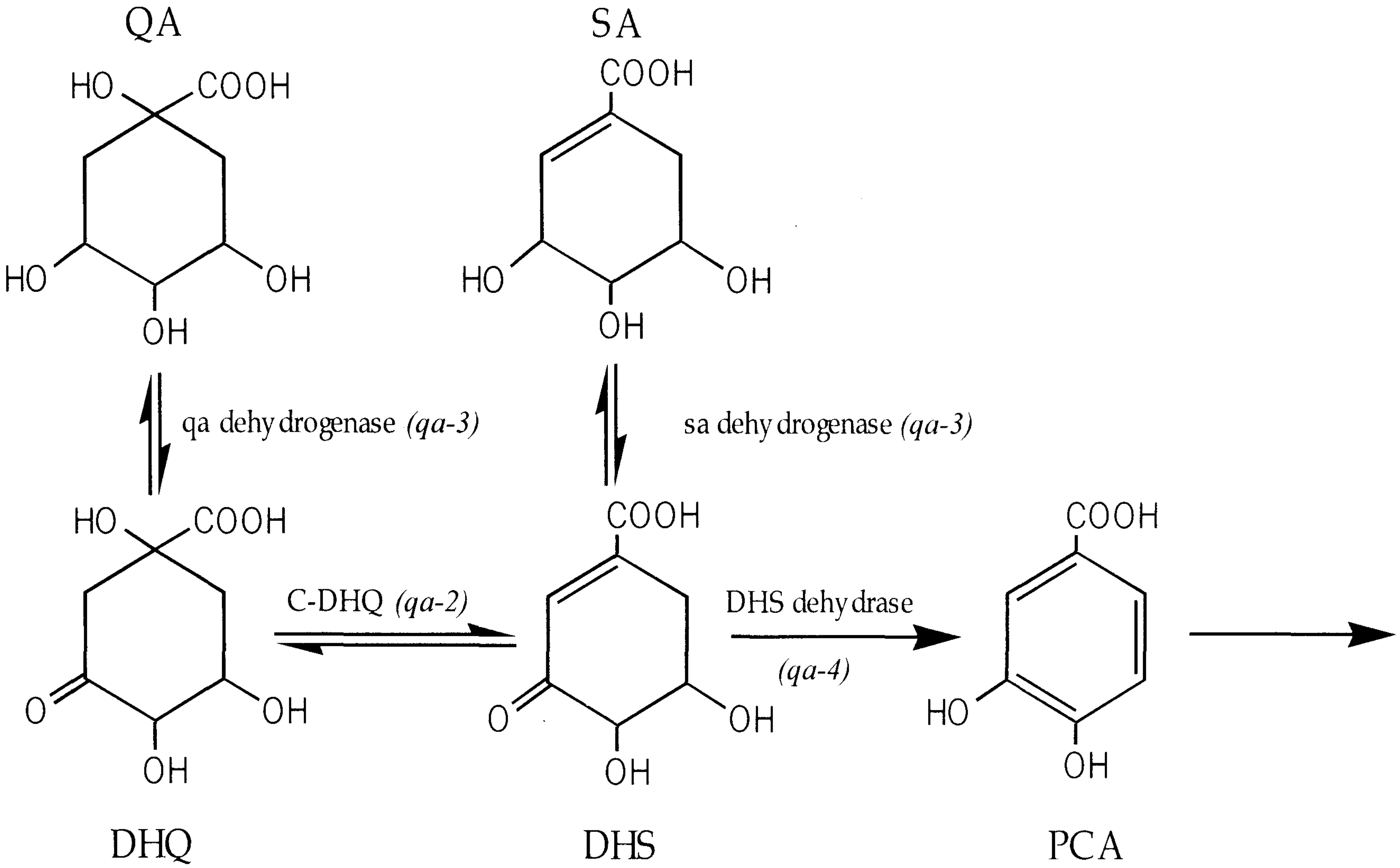
The single unpaired gene, *qa-y*, separates the regulatory genes from the structural genes (Giles, et al 1985).

Cloning of the *qa* gene cluster also permitted the identification of all of the mRNAs of the *qa* genes (Figure 2). Only *qa-y* is represented by a single transcript—the remaining genes all have multiple mRNA species. Following the identification of the various mRNA, studies were performed to determine the degree of induction of each of the mRNAs. The levels of mRNAs of *qa-2, qa-3, qa-4,* and *qa-y* were all found to be increased 300-fold when shifted to quinic acid as the sole carbon source (Giles, et al 1985).

VII. Quinic Acid Metabolism

The gene products of three of the structural genes—*qa-2*, *qa-3*, and *qa-4*—are the enzymes responsible for quinic acid metabolism (Figure 3). *Qa-3* encodes quinic acid/shikimic acid dehydroquinase, which converts quinic and shikimic acid to dehydroquinic acid (DHQ) and dehydroshikimic acid (DHS), respectively. Dehydroquinic acid is then converted to dehydroshikimic acid by catabolic dehydroquinase (C-DHQ), the product of the *qa-2* gene. The product of the *qa-4* gene, DHS dehydrase, converts the dehydroshikimic acid to protocatechuic acid (PCA), which can then be further broken down in other metabolic pathways and thus utilized as a carbon source (Geever, et al 1987).


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


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

VIII. Regulation of the Quinic Acid Cluster

The *qa* gene cluster is regulated by two main mechanisms, both of which occur at the level of transcription (Patel, et al 1981). The primary mechanism involves an inducer, quinic acid, and two regulatory proteins, *qa-1S* and *qa-1F*. Catabolite repression provides secondary level of control, whereby *qa* gene expression is repressed in the presence of a preferred carbon source, especially glucose.

The primary control mechanism of the *qa* gene cluster requires the coordinated actions of the two quinic acid regulatory proteins, *qa-1F* and *qa-1S*, and the inducer quinic acid. This conclusion is supported by mutant studies. Only low levels of *qa* mRNAs are found in noninducible *qa-1S*$^-$and *qa-1F*$^-$ mutants grown with or without quinic acid, while noninduced constitutive *qa-1S*^{C} mutants contain high levels of *qa* mRNAs (Giles, et al 1991).

The *qa-1S* gene encodes a 918 amino acid, 101 kD, protein (Giles, et al 1985, Huiet and Giles 1986, Geever, et al 1989). Deletion experiments have shown the protein to be a presumptive repressor: once deleted, *qa* genes are constitutively transcribed at high levels (Case, et al 1992). The repressor protein is believed to have at least two functional domains: one that interacts with the inducer, quinic acid, and one that interacts with a target. Evidence for the locations of the functional domains has come from studies of both classes of *qa-1S* pleiotropic mutants (noninducible and constitutive). The mutants in the semi-dominant noninducible (*qa-1S*) class each contain a missense mutation causing them to encode functional proteins that act as superrepressors. These mutations are located between codons 627 and 743 suggest that this region is the domain of the repressor that interacts with the inducer quinic acid. The inability of these superrepressors to bind the inducer would cause the repressor protein to remain bound to its target (presumably the activator protein), blocking induction of the *qa* genes (Huiet and Giles 1986). An alternative interpretation also cannot be ruled out—the *qa-1S* mutations may affect the affinity of the repressor for the activator as seems to be the case in comparable GAL 80^{S} mutations in yeast (Salmeron, et al 1990).

The constitutive class ($qa\text{-}1S^{C}$) of mutations is composed mainly of frameshift or nonsense mutations, suggesting that these mutants encode inactive repressors. The locations of the mutations seem to imply that the carboxy terminus is the region of the repressor that interacts with the target. Attempts to show repressor protein-DNA binding using repressor protein overexpressed in a baculovirus system failed to show any protein-DNA binding. Because no evidence has been found to indicate DNA binding by the repressor, its target is believed to be the activator protein (Giles, et al 1985, 1987, 1991).

The *qa-1F* gene product, the activator protein is an 816 amino acid, 89 kD protein (Giles, et al 1985). Two lines of evidence point to an activating role for this protein. When this gene is experimentally disrupted, the *qa* genes are no longer inducible and are only transcribed at basal levels. Second, other studies have shown that the activator protein increases transcription of all of the *qa* genes, including itself (Patel and Giles 1985).

Comparison of the *qa-1F* activator protein to the *qutA* activator in *A. nidulans* reveals three regions of homology (Beri, et al 1987). "Domain swapping" experiments between the two activator genes point to a possible fourth domain (Avalos, Geever, and Case, unpublished). Based on this evidence, it was determined that the *qa-1F* activator protein contains at least four domains: a DNA-binding domain, a transcriptional activating domain, a dimerization domain, and a domain for interacting with the *qa-1S* repressor protein (Geever, et al 1987, 1989, Salmeron and Johnston 1986, Giles, et al 1991).

DNaseI footprinting experiments using activator protein isolated following overexpression of *qa-1F* protein in a baculovirus system (Miller, et al 1986) located the DNA binding domain to the first 183 amino acids (Baum, et al 1987). A 28 amino acid sequence in this region shows conservation with several lower eukaryotic activator proteins (Baum, et al 1987, Pfeifer, et al 1989).

The second region of homology with *qutA* occurs over a broad central segment. Studies of *qa-1F* mutants with changes in this region show alterations in DNA binding. It is believed that the region between codons 296 and 562 may be important to protein dimerization, although it remains to be proven that a dimer is in fact the active binding form of the *qa-1F* protein (Geever, et al 1989, Giles, et al 1991).

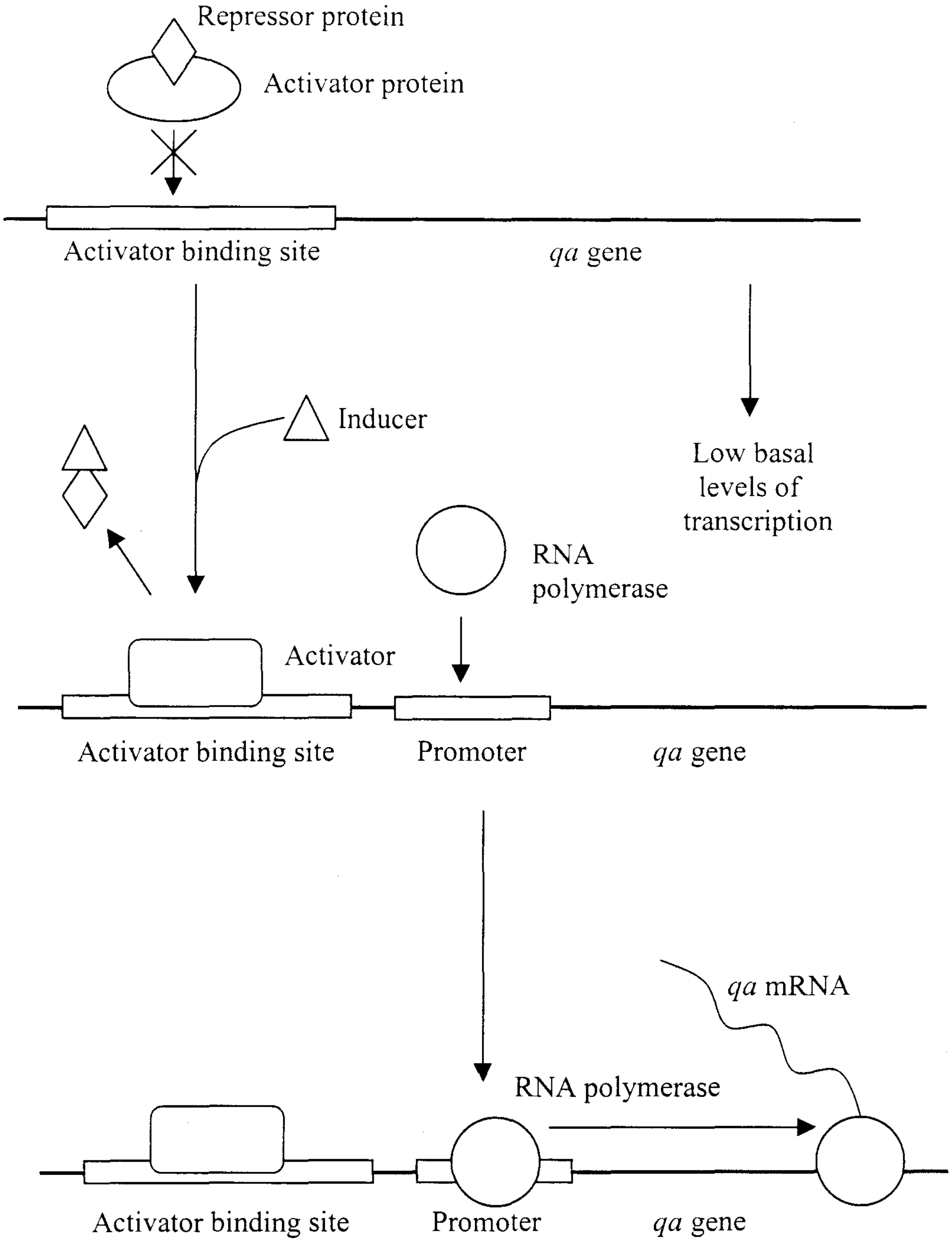
Located at the carboxy terminus of the activator protein, the third domain is comprised mainly of acidic residues. Based on comparisons with a similar region in the GAL4 activator protein of *S. cerevisiae*, this region may be involved in interactions with transcriptional factors (Ma and Ptashne 1987).

The final domain, also located in the carboxy terminus, overlaps with the region of the activator. It is this domain that is believed to interact with the *qa-1S* repressor protein. When this region of the activator is deleted, the resulting mutant displays low constitutive levels of *qa* enzyme activity. This activity was not inducible, suggesting that this region is responsible for interacting with the repressor protein (Giles, et al 1991).

Currently, it is believed that the repressor binds to the activator (figure 4). This binding blocks the positive effect of the activator protein on transcription of the *qa* genes. As a result, transcription occurs only at low basal levels in the absence of quinic acid. The presence of the inducer, quinic acid, allows release of the activator by binding the repressor. The activator is then able to initiate transcription of all of the *qa* genes, including *qa-1S* and *qa-1F*. The increased transcription of the *qa-1S* gene probably maintains repressor protein at a high enough level to allow rapid shutoff when inducer is depleted (Giles, et al 1991).

IX. Catabolite Repression

Catabolite repression provides the second level of regulatory control. Under catabolite repression, the *qa* genes are expressed at a level approximately equal to 10% of gene expression when quinic acid is the carbon source and a preferred source, such as glucose, is absent. The exact mechanism of catabolite repression is not clear. As mentioned above, some evidence points to a possible role for *qa-x* in this process because its transcription appears to be more strongly repressed by glucose than is


Repressor protein
Activator protein
Activator binding site
qa gene
Inducer
Low basal levels of transcription
RNA polymerase
Activator
Activator binding site
Promoter
qa gene
qa mRNA
RNA polymerase
Activator binding site
Promoter
qa gene

transcription of the other qa genes, suggesting catabolite repression has a direct effect on *qa-x* transcription (Giles, et al 1987, 1991). It has also been suggested that the *qa-x* gene product is itself responsible for causing catabolite repression. The *qa-x* protein exhibits a 31% amino acid identity with *GAM1*, a recently identified gene in the GAL system of *S. cerevisiae* believed to play a role in affecting catabolite repression by modifying the GAL4 activator protein (Giles, et al 1991).

The GAL system of *S. cerevisiae* provides three possible reasons for catabolite repression. Protein modification or proteolysis may prevent the *qa-1F* activator protein from binding to its activator sites in the presence of a preferred carbon source. Second, carbon repressors may interact with sequences 5' to the *qa* genes in the presence of a preferred carbon source, blocking transcription. These sequences have not yet been identified, however. A final possibility is direct repression of the *qa-1F* gene, leading to repression of the other *qa* genes due to a lack of activator protein (Flick and Johnston 1990, Johnston, et al 1994).

X. Activator Binding Sites

Genetic analysis of mRNA transcription and studies of the chromatin structure of the *qa* gene cluster show that the activator protein has specific binding sites located in the 5' flanking regions of the *qa* genes (Baum and Giles 1985). These intergenic sites have been termed the activator binding sites. These presumptive binding sites are composed of a 16 bp conserved sequence element. At present, 14 of these sites have been identified by DNA binding and DNaseI footprinting studies and found to have the consensus sequence GGRTAARYRYTTAYCC (Baum, et al 1987).

Prior to the discovery of the *qa* gene cluster, work focused on an apparent gene cluster, the *arom* cluster, encoding five enzymes in the biosynthetic pathway. Researchers were able to isolate mutants lacking one of these enzyme activities for all but one activity—biosynthetic dehydroquinase (B-DHQase), the enzyme responsible for the conversion of dehydroquinate (DHQ) to dehydroshikimate (DHS). It was theorized that *N. crassa* encodes two distinct DHQases: the constitutive biosynthetic form, and an inducible form. Further examination of *arom* mutants led to the discovery of a single mutant lacking in DHQase activity. This double mutant contained a second mutation in a gene unlinked to the *arom* cluster, and was designated a *qa* mutant because it was unable to grow on quinic acid as a sole carbon source. In addition to fortuitously leading to the discovery of the *qa* gene cluster, this finding allowed researchers to isolate mutants (*arom-9*) lacking B-DHQase activity. The knowledge gained studying *arom-9* mutants would prove instrumental in understanding the *qa-1F* activator binding sites.

The *qa-1F* activator protein binding sites were discovered during studies of several *qa-2*^{ai} (activator-independent) mutants. Such mutants are able to grow on minimal media even in the absence of *arom-9* and of *qa-1F*, which is normally required for expression of *qa-2*. These mutants were created by exposing *qa-2*$^{+}$ *qa-1F*$^{-}$ *arom-9*$^{-}$ double mutants to ultraviolet radiation and isolating revertants able to grow on quinic acid as a sole carbon source. The initial double mutant was unable to grow on quinic acid because it lacked both B-DHQase and C-DHQase activities. The newly created mutations permitted constitutive expression of C-DHQase at levels equal to 1-45% of induced wild-type, allowing growth on quinic acid. The mutants were sequenced and found to contain rearrangements, point mutations, and small duplications in the region 5' to the *qa-2* gene (Geever, et al 1983).

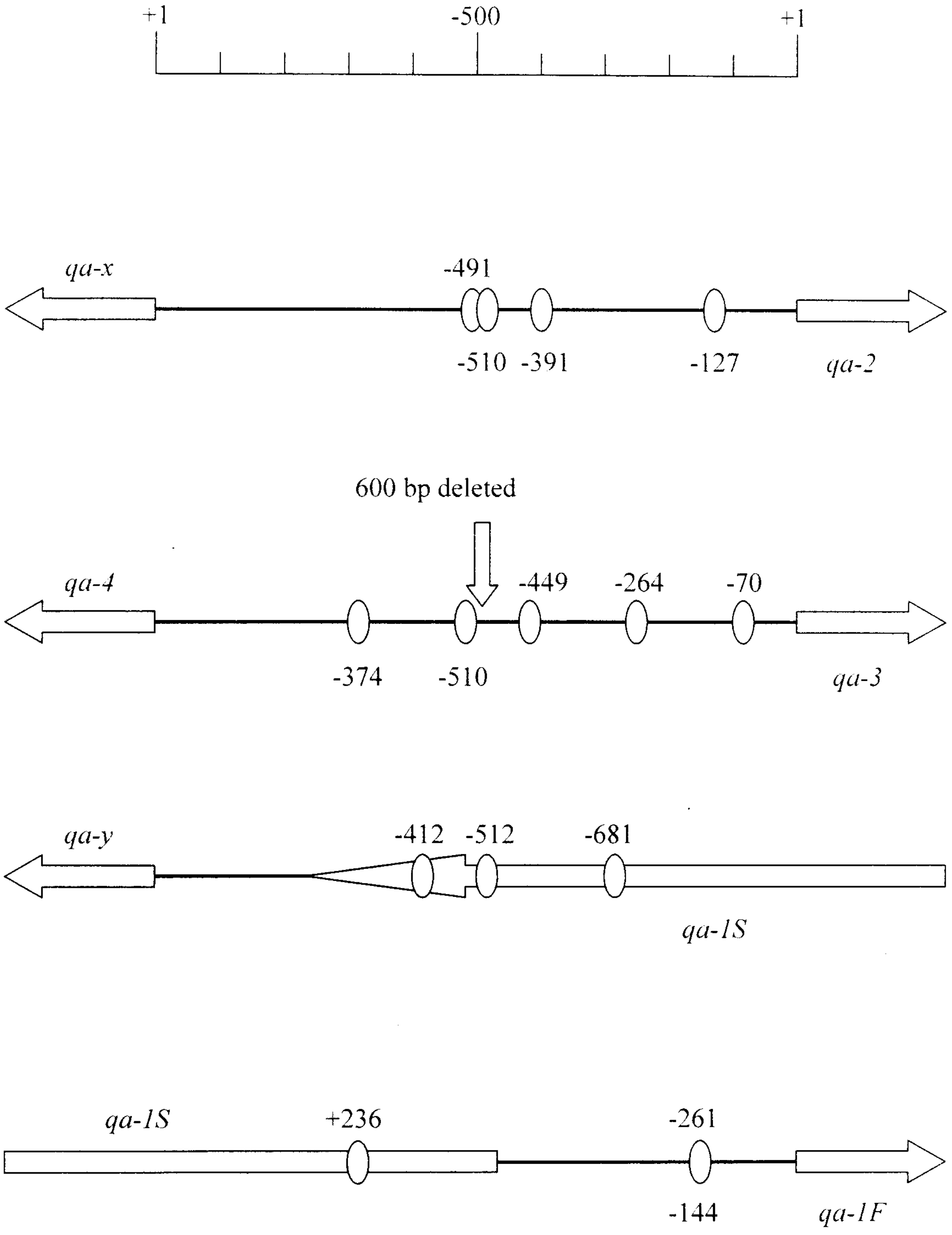
To further understand activator protein action, the transcription initiation sites of the *qa-2*^{ai} mutants were mapped and compared to that of temperature-sensitive (*qa-1F*^{ts}—inducible at 25°C but noninducible at 35°C) mutants and wild-type *N. crassa*. In the induced wild-type, transcription is initiated from two to four *qa-1F* dependent promoters. Transcription from these promoters required the *qa-1F* activator protein. The promoters are divided into two types: type I and type II promoters, based on their responses to certain mutations in the regulatory genes. Unlike type I promoters, type II promoters can be activated by *qa-2*^{ai} mutations, suggesting that these mutants contain enhancer-like elements (Tyler, et al 1984). Gene conversion experiments, in which the *qa-1F*$^{-}$ gene of a *qa-2*^{ai} mutant is replaced by *qa-1F*$^{+}$, demonstrate that the *qa-2* transcripts initiate from the type II (position -45) promoter in the absence of inducer. Upon the addition of inducer, however, transcription initiates instead from the type I (position +1) promoter, suggesting that the two promoter types have different requirements for activation (Giles, et al 1985). In a further study, seven of the *qa-2*^{ai} mutants containing 5' rearrangements were examined. The *qa-1F*$^{+}$ activity was restored to these strains, accompanied by reinitiation at position +1. The experiments demonstrated that the region located between position –190 and –86 is required for induction of *qa-2* expression by *qa-1F*. A symmetrical 16 base pair sequence was identified as a possible site for activator protein binding (Geever, et al 1986, Giles, et al 1987).

A computer search was performed to try to identify any conserved sequences as possible binding sites. The results were correlated with studies of DNase-I hypersensitive sites (HSSs). The 105 bp region 5' to the *qa-2* gene was found to contain two DNaseI HSSs: one of these sites is detectable in inducible (*qa-1F*$^{+}$) strains but not in noninducible (*qa-1F*$^{-}$) strains, suggesting activator protein binding (Baum and Giles 1985). Further studies identified thirteen related sequences that fell within DNase-I protected regions near DNase-I HSS (Baum and Giles 1986). DNase-I footprinting experiments using partially purified *qa-1F* activator protein demonstrated binding of the activator to these sequences. The protected regions, approximately 20-25 bp in length, all contain a partially symmetrical 16 bp sequence (Figure 5) (Baum, et al 1987). Four of these binding sites are located in the *qa-2-qa-x* intergenic region, three of which appear to affect *qa-2* transcription (Figure 6). The fourth site presumably affects *qa-x* transcription (Geever, et al 1989). Interestingly, only one such sequence is found in the *qa-1S-qa-1F* intergenic region, implying bidirectional control of these genes. This is consistent with the theories of activator autoregulation and of activator control of repressor transcription (Giles, et al 1985).

Baum et al (1987) also performed a DNA binding assay to determine the relative *in vitro* binding strengths of each activator binding site (Figure 7). The –127 site, located in the *qa-2-qa-x* intergenic region, showed the highest affinity of any binding site in the cluster. Mutants that lose the other two activator binding sites for the *qa-2* gene but that retain the –127 site are able to express *qa-2* at levels equal to

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

Consensus Sequence: $G^{93}G^{100}R^{57}T^{76}A^{76}A^{93}\ \ R^{64}Y^{71}R^{71}Y^{86}\ \ T^{100}T^{71}A^{86}T^{64}C^{76}C^{76}$


+1
-500
+1
qa-x
-491
-510
-391
-127
qa-2
600 bp deleted
qa-4
-449
-264
-70
-374
-510
qa-3
qa-y
-412
-512
-681
qa-1S
qa-1S
+236
-261
-144
qa-1F

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

15-30% of induced wild-type levels. This would seem to imply that at least one of the other two binding sites, located at positions –391 and –510, is needed for complete induction (Baum, et al 1987).

While it is known that the 16 bp consensus sequence is sufficient to allow activator binding (Baum, et al 1987), the importance of the individual bases in said sequence is not yet known. We propose to isolate a fragment of the *qa-2-qa-x* intergenic region containing the –127 binding site. We will then mutate each highly conserved base to prepare a series of mutants that can be transformed back into *N. crassa*. The effect of the mutations will then be determined by measuring *in vivo* levels of *qa-2* expression.

MATERIALS AND METHODS

I. Materials

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*, *KpnI*, *HindIII*, and *PstI*), RNaseA, single-stranded binding protein, T4 DNA ligase, T7 DNA polymerase, polynucleotide kinase, pBR322, and M13mp18 were purchased from Boehringer Mannheim, Indianapolis, 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, sodium citrate, and acetic acid were purchased from Fisher Scientific, Fairlawn, NJ; QIAGEN columns were purchased from QIAGEN, Inc., Chatsworth, CA; Elutip columns were purchased from Scleicher & Schuell, Keene, NH; pBluescript was purchased from Stratagene, LaJolla, CA; Polaroid film yeast extract, sodium chloride, ampicillin, isopropyl-β-D-thiogalactoside (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-propanesulfonic acid (MOPS), octyl phenoxy polyethoxyethanol (Triton X-100), phenol, cresol, chloroform, sodium acetate, magnesium chloride, glycerol, polytheylene glycol (PEG), and adenosine triphosphate (ATP) were purchased from Sigma Chemical

Company, St. Louis, MO; dNTPs 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.

II. Strains and Media

Two strains of *Escherichia coli* were used, both of which were obtained from the *E. coli* Genetic Stock Center (CGSC) at Yale University. Strain JM101 was used for all experiments involving pBluescript-based recombinant plasmids. Cells were cultured in Luria Broth [LB] (1% bacto-tryptone, 0.5% yeast extract, 1% NaCl). Transformants were selected on Luria Agar with ampicillin [LA_{100}] (1% bacto-tryptone, 0.5% yeast extract, 1% NaCl, 0.01% ampicillin) using 100 μl of 200 mM IPTG and 50 μl of 2% X-Gal in formamide.

Site-directed mutagenesis was performed using *E. coli* strain CJ236 (CGSC# 6793). Deficient in activities of the enzymes *dut* (dUTPase), the enzyme responsible for reducing the intracellular level of uracil and *ung* (uracil *N*-glycosylase), which removes any uracil that become mistakenly incorporated into the DNA, plasmid DNA replicated in CJ236 will contain uracil in place of several thymine residues. *E. coli* strain CJ236 was cultured in 2xYT (1.6% bacto-tryptone, 0.5% yeast extract, 0.5% NaCl). M13 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

pBR322, a 4.36 kb cloning vector, is among the most commonly used vectors (Figure 8). In addition to a multiple cloning site (MCS), it contains both ampicillin and tetracycline resistance genes to be used for selection. Both of these genes contain unique restriction sites allowing for reverse selection.

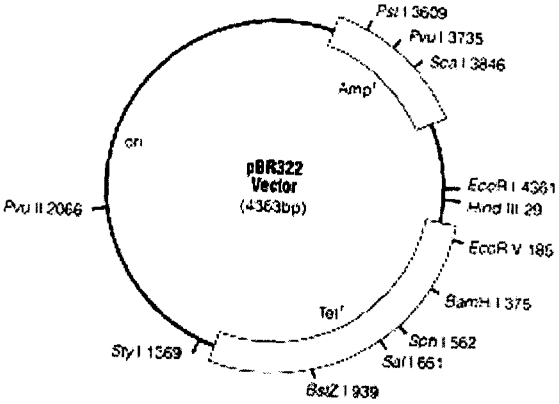
IV. pBluescript II KS (+/-) Phagemid

A pUC19 derivative (Figure 9), this 2,961 bp phagemid contains an ampicillin resistance gene allowing for antibiotic selection of recombinants. It also contains a portion of the *lac Z* gene, allowing for blue-white color selection. Cloning into the multiple cloning site (MCS) disrupts the *lac Z* 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 with a portion of the β-galactosidase gene (Figure 10). Cloning into the MCS disrupts the β-galactosidase gene, allowing for blue-white screening for recombinants.

M13 exists in two different forms—a double-stranded, intracellular replicative form and a single-stranded bacteriophage. The replicative form can be manipulated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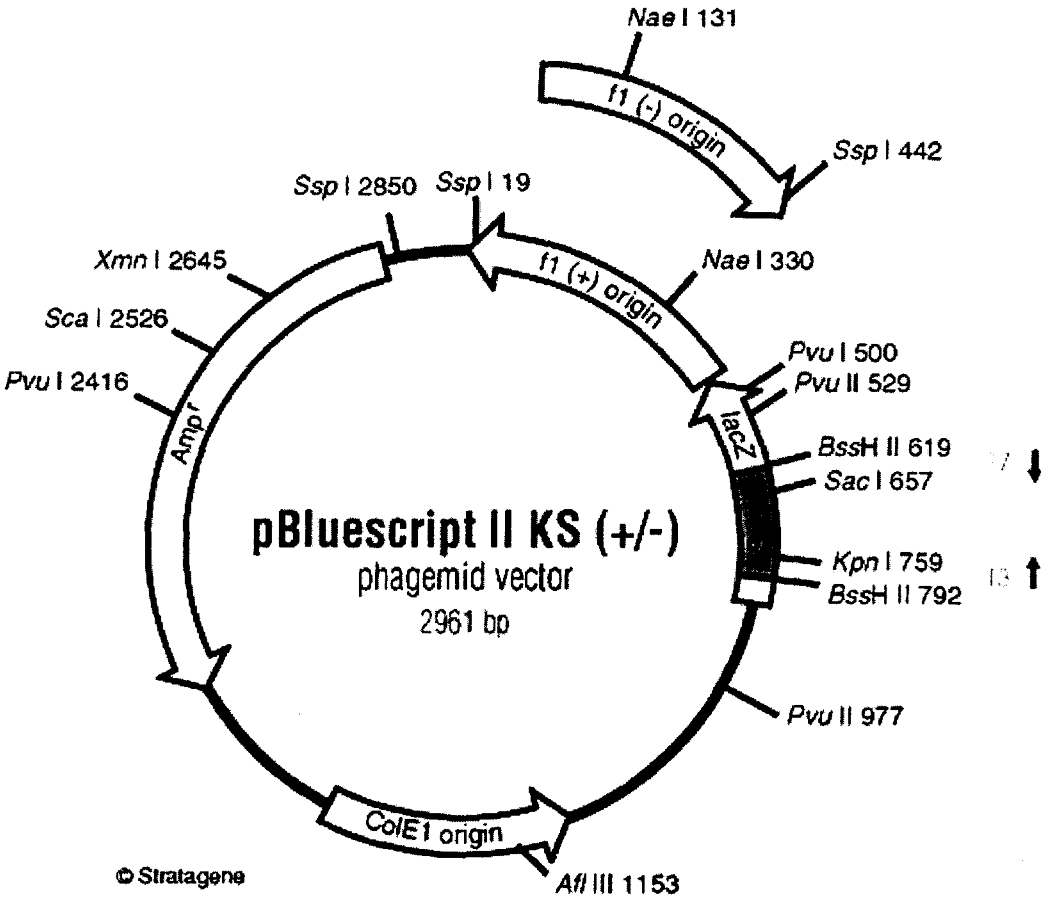
Pvu I 3735
Sca I 3846
Amp^r
ori
pBR322
Vector
(4363bp)
EcoR I 4361
Hind III 29
EcoR V 185
BamH I 375
Tet^r
Sph I 562
Sal I 651
BstZ I 939
Sty I 1369
Pvu II 2066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f1 (-) origin
Ssp I 442
Ssp I 2850
Ssp I 19
f1 (+) origin
Nae I 330
Xmn I 2645
Sca I 2526
Pvu I 2416
Pvu I 500
Pvu II 529
lacZ
BssH II 619
Sac I 657
T7 ↓
Amp r
pBluescript II KS (+/-)
phagemid vector
2961 bp
Kpn I 759
BssH II 792
T3 ↑
Pvu II 977
ColE1 origin
Afl III 1153

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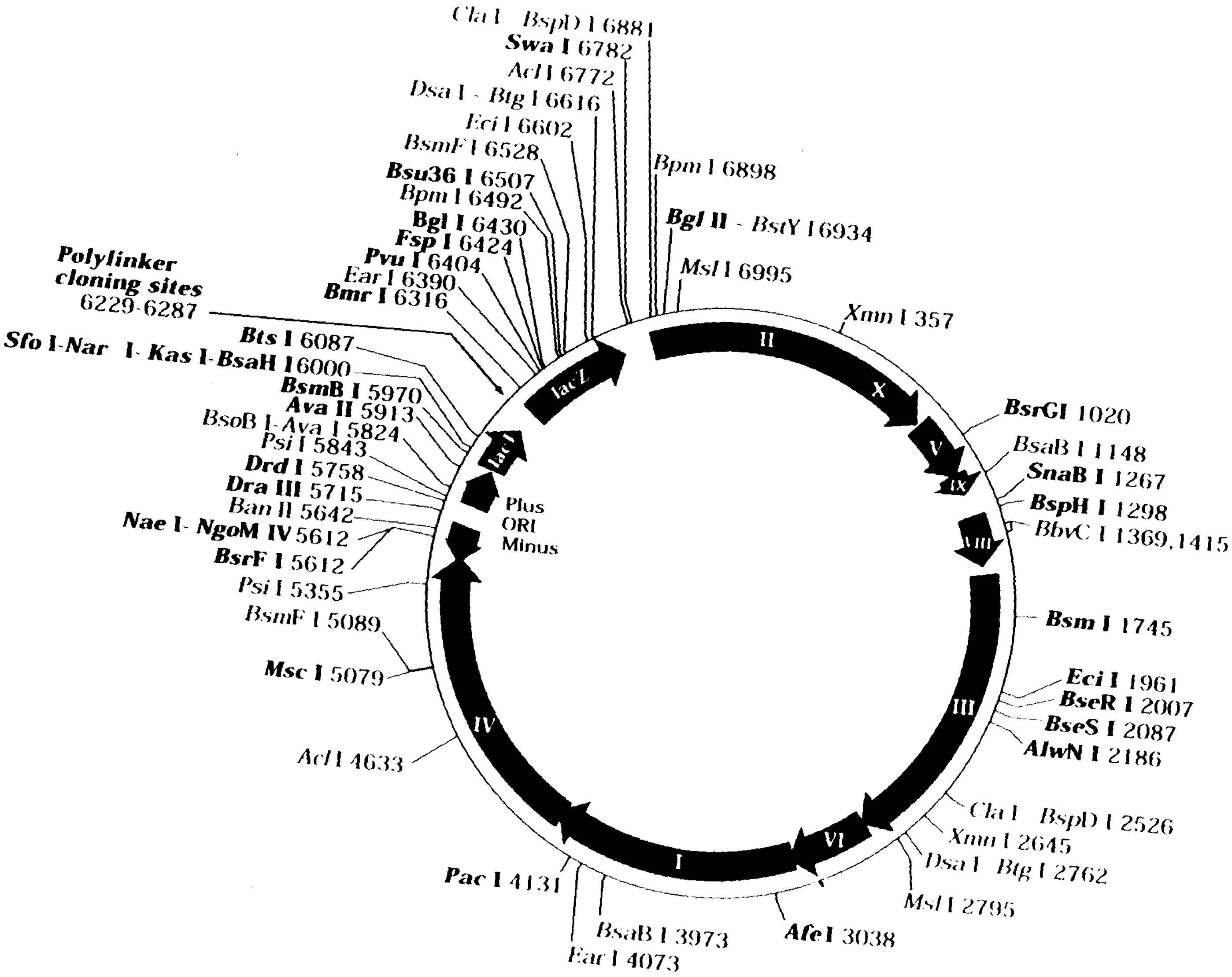
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Swa I 6782
Acl I 6772
Dsa I - Btg I 6616
Eci I 6602
BsmF I 6528
Bsu36 I 6507
Bpm I 6492
Bgl I 6430
Fsp I 6424
Pvu I 6404
Ear I 6390
Bmr I 6316
Polylinker cloning sites 6229-6287
Bts I 6087
Sfo I-Nar I- Kas I-BsaH I 6000
BsmB I 5970
Ava II 5913
BsoB 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
II
X
V
IX
VIII
III
VI
I
IV
lacZ
lacI
Plus
ORI
Minus

as a plasmid. The phage coat can be removed from the single-stranded phage to isolate the single-stranded DNA.

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 $CaCl_2$ and placed in 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 $CaCl_2$. 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 milliliter of LB was then added to each tube and the tubes were incubated at 37°C for one hour. Finally, 100 µl from each tube was spread onto LA_{100} plates, which were incubated overnight at 37°C.

VII. Alkaline Plasmid Screen

Colonies were selected from LA_{100} plates and picked to 2 ml of LA_{100} 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 microcentrifuge 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 microcentrifuge 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 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% H_3PO_4 (1.679 mg/ml)]. Gels were stained with ethidium bromide (EtBr) [50 mg/ml] and the DNA visualized on a transilluminator.

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

A colony was picked from an LA_{100} plate to 50 ml of LA_{100} 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 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 ice-cold neutralizing solution was added. The tube was immediately mixed and placed in an ice bath 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 15,000x g for 30 minutes. A Qiagen column was equilibrated by applying 7 ml buffer QBT (0.75 M NaCl, 0.05 M MOPS, 15% EtOH, 0.15% Triton X-100; pH 7.0) and allowing it to empty by 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 buffer QC (1M 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 addition of 7 ml of isopropanol followed by centrifugation at 15,000x g for 30 minutes at 4°C. The pellet was washed two times 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 which 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. Restriction Digest of Vector and Vector Isolation

Approximately 3 μg of vector 449 was digested with restriction enzymes. Into an eppendorf tube was placed 50 μl (1μg/20μl) of plasmid 449, 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 verified 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 phenol-*m*-cresol and once with an equal volume of chloroform. Two volumes 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 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 stored at -20°C.

XI. Restriction Digest of Fragment and Fragment Isolation

Approximately 1 μg of target DNA was digested. The reaction was incubated overnight at 37°C. The entire reaction mixture was run 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 Na_2EDTA, pH 7.9) and placed in an electrophoresis tank filled with 0.5x TAE. After 45 minutes of electrophoresis, an Elutip column was primed by passing first 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 buffer containing the DNA was then drawn from the bag and passed over the Elutip column. The DNA was then eluted from the column with 400 µl of high salt buffer and collected in an eppendorf tube. The mixture was then extracted, precipitated, and washed as described above. The pellet was 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 desire fragment. The remainder of the sample was stored at -20°C.

XII. Ligation, Transformation and Isolation of Recombinant Plasmids

The isolated fragments were ligated into the digested vectors 449 by combining the following in an eppendorf tube: 3 µl of vector, 3 µl of fragment, 3 µl of 10x ligase buffer, 20 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 was used in the transformation. In addition to ampicillin for selection, IPTG and X-Gal were used to allow blue-white selection of recombinants. White colonies were selected for alkaline plasmid screening.

XIII. Transformation of Frozen Competent *E. coli* JM101 with 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 $CaCl_2$. 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 0.1M $CaCl_2$ and frozen at -70°C in 0.5 ml aliquots.

For the transformation, 2 ml of LB was inoculated with JM101 and incubated overnight at 37°C. The next day, 50 ml of LB was inoculated with 500 μl of the overnight growth and incubated at 37°C for 2 hours. These cells would serve as lawn cells for the bacteriophage. A tube of frozen competent JM101 was thawed and 100 μl was dispensed into each of two eppendorf tubes. To the experimental tube 10 μl of the ligation mix was added, and no DNA was added to the control tube. The cells were placed on ice for 30 minutes, followed by a 5 minute heat shock at 37°C. The cells were plated by combining the following: 3 ml 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.

XIV. Direct Electrophoresis of M13 DNA

The following 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 microcentrifuge. The supernatants containing phage were transferred to eppendorf tubes and stored at 4°C. Direct electrophoresis was performed by combining 12 μl of supernatant with 2 μl of 2% SDS and running the resulting mixture on a 0.8% agarose gel.

XV. Isolation of Uracil-Containing Single-Stranded M13

First, 8 ml of 2xYT was inoculated with CJ236 and incubated overnight at 37°C. The following morning, 2 liters of 2xYT was inoculated with the entire overnight growth. The cells were incubated at 37°C for 2 hours. Next, 400 μl of phage lysate was added and was grown at 37°C for 6 hours. The cells were spun at 5,000 x *g* for 30 minutes and the supernatants removed and stored at 4°C overnight. Approximately 1 ml of lysate was set aside for a plaque assay. The pellets were discarded.

The next morning, ¼ volume of 15% PEG, 2.5 M NaCl was added to the supernatant. The mixture was placed on ice for 1 hour, and then spun for 20 minutes at 5,000 x *g*. The supernatants were drained well and the pellets resuspended in 5 ml 1xTE. The centrifuge bottles were then rinsed with an additional 2 ml of 1xTE, which was combined with the original 5 ml. The mixtures were vortexed briefly and placed on ice for 1 hour before spinning for 20 minutes at 5,000 x *g*. The supernatants were transferred to clean centrifuge tubes and extracted twice with alkaline-buffered phenol-*m*-cresol and once with chloroform. The DNA was precipitated by addition of 1/10 volume of 3 M NaOAc, pH 5.2 and 2 volumes of ice-cold ethanol, followed by incubation on ice for 30 minutes and centrifugation for 20 minutes at 5,000x g at 4°C. The pellet was air dried and resuspended in 500 µl of 1xTE, which was then transferred to an eppendorf tube. One milliliter of isopropanol was added, and the DNA was re-precipitated by spinning in a microcentrifuge at 15,000 x *g* for 15 minutes. The pellet was dried in a speed vac and resuspended in 25 µl of 1x TE. The concentration of DNA was determined both by electrophoresis on a 0.8% agarose gel and by spectrophotometry.

XVI. Plaque Assay

A serial dilution was performed on the lysate to obtain phage concentrations of 10^{-3} through 10^{-6}. Both CJ236 and JM101 were grown to an OD of 0.2 to serve as lawn cells for the phage. Phage dilutions were plated in both JM101 and CJ236 by combining 3 ml of soft YT, 200 µl of uninfected lawn cells, and 30 µl of phage dilution and pouring the resultant mixture over YT plates. The plates were incubated at 37°C overnight. The plaques were counted the following day and growth on JM101 was compared to growth on CJ236.

XVII. Mutagenesis

First, the mutagenic oligonucleotide was phosphorylated. In an eppendorf tube was added: 2 µl 10x T4 polynucleotide kinase (PNK) buffer, 2 µl 10 mM ATP, 2 µl primer qa-2-1, 14 µl water, and 1 µl T4 PNK. The mixture was incubated 1 hour at 37°C then the reaction was quenched by addition of 3 µl of 100 mM EDTA. The tube was then heated to 70°C for 5 minutes to denature the enzyme and allowed to cool to room temperature.

Next, 1 µg of single-stranded uracil-containing DNA template was added, along with 1.25 µl of 20x SSC (3 M NaCl; 0.3 M Sodium citrate; pH 7.0). The mixture was vortexed briefly, and then spun for 5 seconds in a microcentrifuge. The reaction was incubated in a heat block at 37°C for 5 minutes, after which the heat block was shut off and the tube was allowed to cool to room temperature in the heat block. The tube was spun in a microcentrifuge for 5 seconds to collect any moisture from the sides of the tube. The remainder of the reactants were then added: 10 µl 10 mM ATP, 5 µl each 10 mM dNTPs, 2 µl single-stranded binding protein (ssbp), 2.5 U T7 DNA polymerase, 2 U T4 DNA ligase, and sterile water to a 100 µl total volume. The mixture was mixed well, then incubated for 5 minutes at 0°C, followed by 5 minutes at room temperature, and then 2 hours at 37°C. The reaction was quenched by the addition of 3 µl of 500 mM EDTA and stored at -20°C.

XVIII. Isolation of Replicative-Form M13 for Sequencing

The mutagenesis mixture was transformed into JM101 as described in section XII. Plaques were picked to JM101 and grown overnight at 37°C as described in section XIII and were harvested the following morning. Fifty milliliters of LB were inoculated with and overnight growth of JM101 and incubated at 37°C for 1 hour.

Thirty microliters of phage lysate was added and grown for 3 hours at 37°C. Replicative-form M13 was isolated using the QIAGEN protocol.

XIX. Sequencing

Sequencing was performed on the Beckman CEQ 2000, a dye terminator sequencer using the protocol developed by Beckman-Coulter. The dye terminator reaction was prepared by combining 25-50 fmol of ssDNA (50-100 fmol of dsDNA) with the four dye terminators (ddATP, ddCTP, ddGTP, and ddUTP), 10x reaction buffer, dNTP mix, polymerase enzyme, a primer, and sterile water to a 20 μl total volume. The primers used were the standard Beckman –47 pUC primer, M13 forward primer, and M13 reverse primer. The mixture was placed in a thermal cycler on the following program: 96°C for 20 seconds, 50°C for 20 seconds, 60°C for 4 minutes for 45 cycles followed by holding at 4°C.

Following thermal cycling, the reaction was stopped by the addition of stop solution (1.5 M NaOAc, 50 mM EDTA) and glycogen. The DNA was precipitated with 95% ethanol and washed two times with 70% ethanol. The pellets were vacuum dried for 40 minutes and resuspended in deionized formamide. The samples were then loaded into the CEQ 2000 and run with the desired method.

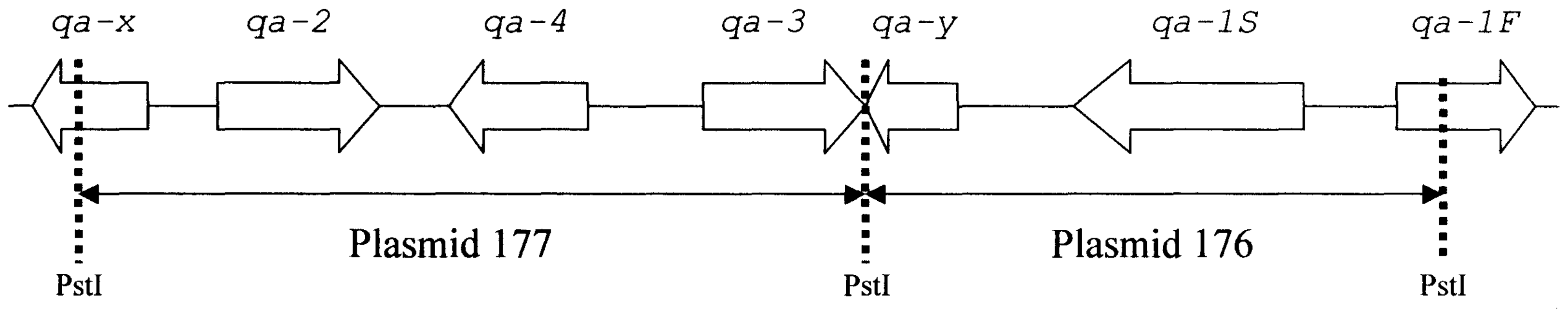
RESULTS

I. Construction of Plasmids 176 and 177

A clone known to contain the entire *qa* gene cluster was previously digested with the restriction endonuclease *PstI* and two fragments were isolated, which collectively represent bases 718 to 15416 of the cluster (Figure 11). These fragments were cloned into pBR322 to form two plasmids. The resulting constructs were labeled #176 & #177. Plasmid 176 contains the *qa-y* and *qa-1S* genes as well as a fragment of *qa-1F*, while plasmid 177 contains *qa-2*, *qa-3*, and *qa-4*, plus part of *qa-x*. Importantly, plasmid 177 contains the entire *qa-x-qa-2* intergenic region (Asch, unpublished data).

II. Construction of Plasmid pEP (*Eco-Pst* Clone)

Plasmid 177, containing the fragment comprised of bases 718 to 7953, was digested further with *EcoRI* and *PstI*, yielding a fragment approximately 2.5 kb in length (Figure 12). This smaller fragment was cloned into pBluescript and the resultant construct named pEP (Asch, unpublished data).


qa-x
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qa-3
qa-y
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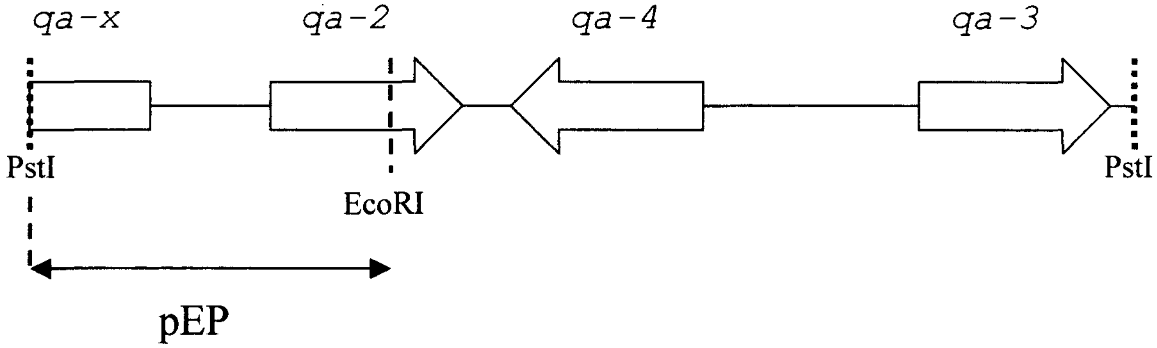
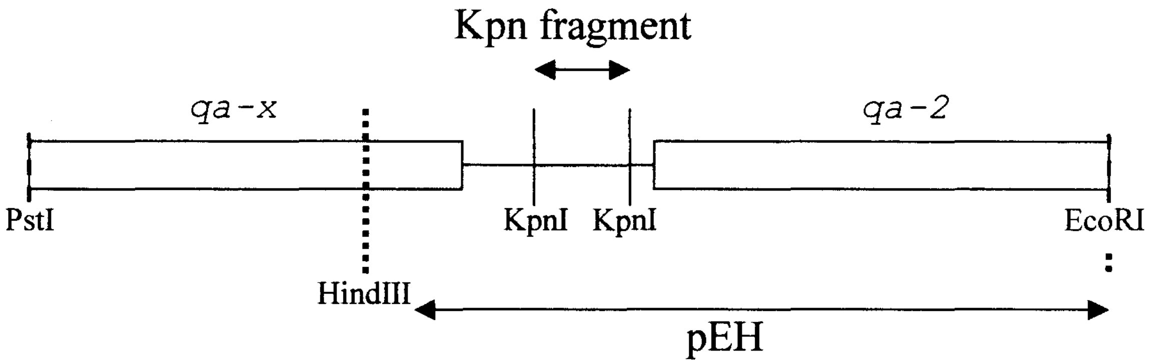
Plasmid 177
Plasmid 176
PstI
PstI
PstI


qa-x
qa-2
qa-4
qa-3
PstI
EcoRI
PstI
pEP

III. Construction of Plasmid pEH (*Eco-Hind* Clone)

Plasmid pEP was transformed into JM101. Based on the results of an alkaline plasmid screen, a promising clone was selected and a large amount of pEP was isolated and purified by QIAGEN purification. Both vector (449) and plasmid pEP were digested with the restriction endonucleases *EcoRI* and *HindIII.* The vector digestion yielded a single fragment, which was purified by phenol and chloroform extractions. pEP digestion resulted in two fragments: a 1.8 kb fragment, the *Eco-Hind* fragment; and an approximately 3.7 kb fragment containing the vector pBluescript. The *Eco-Hind* fragment was gel purified and isolated by electroelution. The fragment was further purified by passage over an Elutip column and extraction with phenol and chloroform. The fragment was then ligated into the cut pBluescript vector to form the pEH plasmid (Figure 13). Following transformation into JM101, pEH was grown up on a large scale and purified by the QIAGEN method.

A test restriction digest (Figure 14) of this plasmid with both *EcoRI* and *HindIII* resulted in two fragments, one approximately 3.7 kb in length corresponding to the vector, the second 1.8 kb in size, representing the fragment. Other restriction endonucleases used included *PstI*, *HindIII*, *BamHI*, and *EcoRI*, each of which was predicted to cut the clone only once—at a unique site in the pBluescript MCS. As expected, each digest produced a single band of approximately 5 kb. pEH was also digested with *KpnI*, which was predicted to cut in three places—once in the pBluescript MCS and twice within the fragment. The digest yielded three fragments: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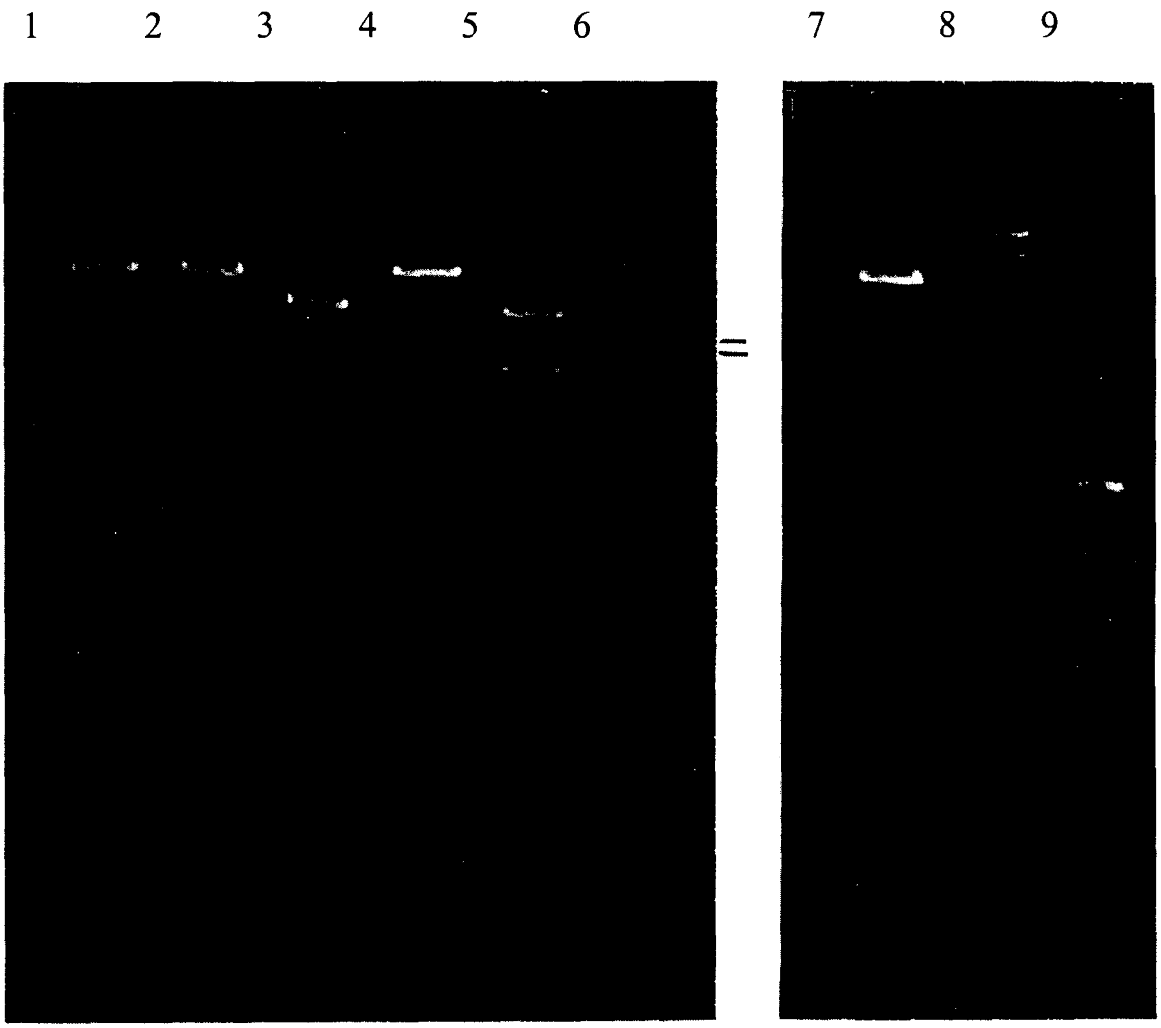
qa-2
PstI
KpnI
KpnI
EcoRI
HindIII
pEH


1
2
3
4
5
6
7
8
9

the 500 bp fragment containing the activator binding site, an 800 bp fragment, and an approximately 4 kb fragment.

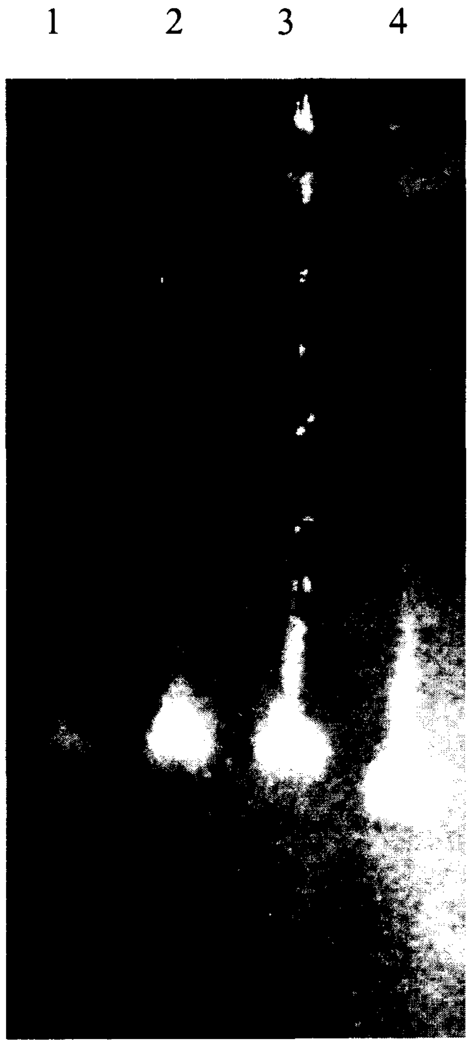
IV. Construction of the M13 Clone (*KpnI* Clone)

Plasmid pEH was further digested with *KpnI* to yield a 500 bp fragment (figures 13 and 14) that was cloned into M13mp18. Bacteriophage was harvested and the M13 DNA was directly electrophoresed on an agarose gel after treatment with SDS to remove the phage coats (figure 15). The shift in size observed in the lanes containing the M13 clones (lanes 1-3) when compared to the control (M13mp18 with no insert) in lane 4 shows that the insert was successfully ligated.

V. Isolation of Uracil-Containing Single-Stranded DNA

Prior to performing site-directed mutagenesis, a protocol had to be developed that would allow the isolation of uracil-containing single-stranded (ss) M13 in concentrations of at least 1 μg/μl. The protocol used is based on the method described by Kunkel et al (1991). Large volumes (at least two liters) of cells are required to yield sufficient concentrations of DNA.

Bacteriophage was grown in CJ236 for six hours—longer growing times had to be avoided due to the production of large numbers of deletion mutants. The cells were pelleted and discarded; the phage-containing lysate was saved.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A plaque assay was performed on the lysate. The phage titer was determined in two different strains of *E. coli*: JM101 and CJ236. Because of the uracil in the DNA, it would be predicted that the phage would tend to be broken down in the JM101. As a result, the titer on CJ236 should be greater than that on JM101 by four to five orders of magnitude. As expected, the titer on CJ236 was approximately 100,000 times greater than the titer on JM101.

The ssDNA was isolated by first removing the phage coats with a PEG solution. The exposed DNA was then phenol and chloroform extracted, precipitated, and washed. The final concentration of DNA was determined spectrophotometrically by measuring the absorbance at 260 nm and was found to be 1.040 ng/μl.

The sequence of the template was confirmed by sequencing on the Beckman-Coulter CEQ 2000 automated sequencer, followed by comparison with known sequences in the FASTA database (http://www2.ebi.ac.uk/fasta3/). The template was indeed found to contain the KpnI insert (figures 16 and 17). The template was resequenced several times with different primers to confirm this finding (data not shown).

VI. Site-directed Mutagenesis

The first step in the mutagenesis was the choice of a mutagenic oligonucleotide to serve as primer. As mentioned above, the sequence of the –127 activator binding site is GGGTAATCGCTTATCC. The first set of primers used substituted an A for the initial G, with the sequence

GCCAGTGAATTCGAGCTC**GGTACC**TCTCGGTTGGGTGCGATGGGCTTGTG
GGAGATGGGCGCAACAGGGAATGCGTCCGAAAGATGCCGCCGCACTATA
CCCAACAACTCACAGGCCAGAAGGTTGAATTGAGATGTGTTTATGTTTTG
TTGTTTAATCACGGGTTCCCTCATCATTCCCATCTCTAATACACGGACAAG
TTGTCTGCACGACGAGC***GGATAAGCGATTACCC***GTCCCCGCTCCGCTTCCG
TGACATTGCTCGTCACTGCACTGATGACCATTCGAAAGGCCGTCATGGGT
TTGTTTTCTGCTTCTGCCATTGCACCTTGGATTAATGGGCGAGACCCCCCG
TCTTCGATTTGCACGATTGGAACGTACGGAGTGGTGAAGAGGCTGTATTG
GTTTAGTGTTCTCTGACATGATACCTCCGGTTGAGTGGTTGAGGTCGTGCA
AGTCGTTAACCCTTCAGCCACCAAGAAAGGGTTGAGTGTTGTTCCAGTTT
GGAACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAGGGGCG
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FASTA searches a protein or DNA sequence data bank
 version 3.2t09 December 7, 1999
Please cite:
 W.R. Pearson & D.J. Lipman PNAS (1988) 85:2444-2448

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 >EMBOSS_001
 vs  EMBL Fungi library
searching /ebi/services/idata/fastadb/em_fun library

72346332 residues in 39343 sequences
  Expectation_n fit: rho(ln(x))= 5.4132+/-0.000528; mu= 22.1707+/- 0.036;
 mean_var=91.8229+/-18.430, 0's: 6 Z-trim: 16  B-trim: 1288 in 2/81

The best scores are:                                      initn init1 opt z-sc E(39331)

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       530       520       510       500       490       480
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                                      ::::  ::  :: :::::::::::::::::
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       470       460       450       440       430       420
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        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
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       410       400       390       380       370       360
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        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
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       350       340       330       320       310       300
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        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
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       290       280       270       260       250       240
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       230       220       210       200       190       180
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        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_FUN  AATCGCTTATCCGCTCGTCGTGCAGACAACTTGTCCGTGTATTAGAGATGGGAATGATGA
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       170       160       150       140       130       120
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        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_FUN  GGGAACCCGTGATTAAACAACAAAACATAAACACATCTCAATTCAACCTTCTGGCCTGTG
          2710      2720      2730      2740      2750      2760

       110       100        90        80        70        60
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        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
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        50        40        30        20        10
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        ::::::::::::::::::::: ::::::::
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          2830      2840       2850      2860      2870      2880
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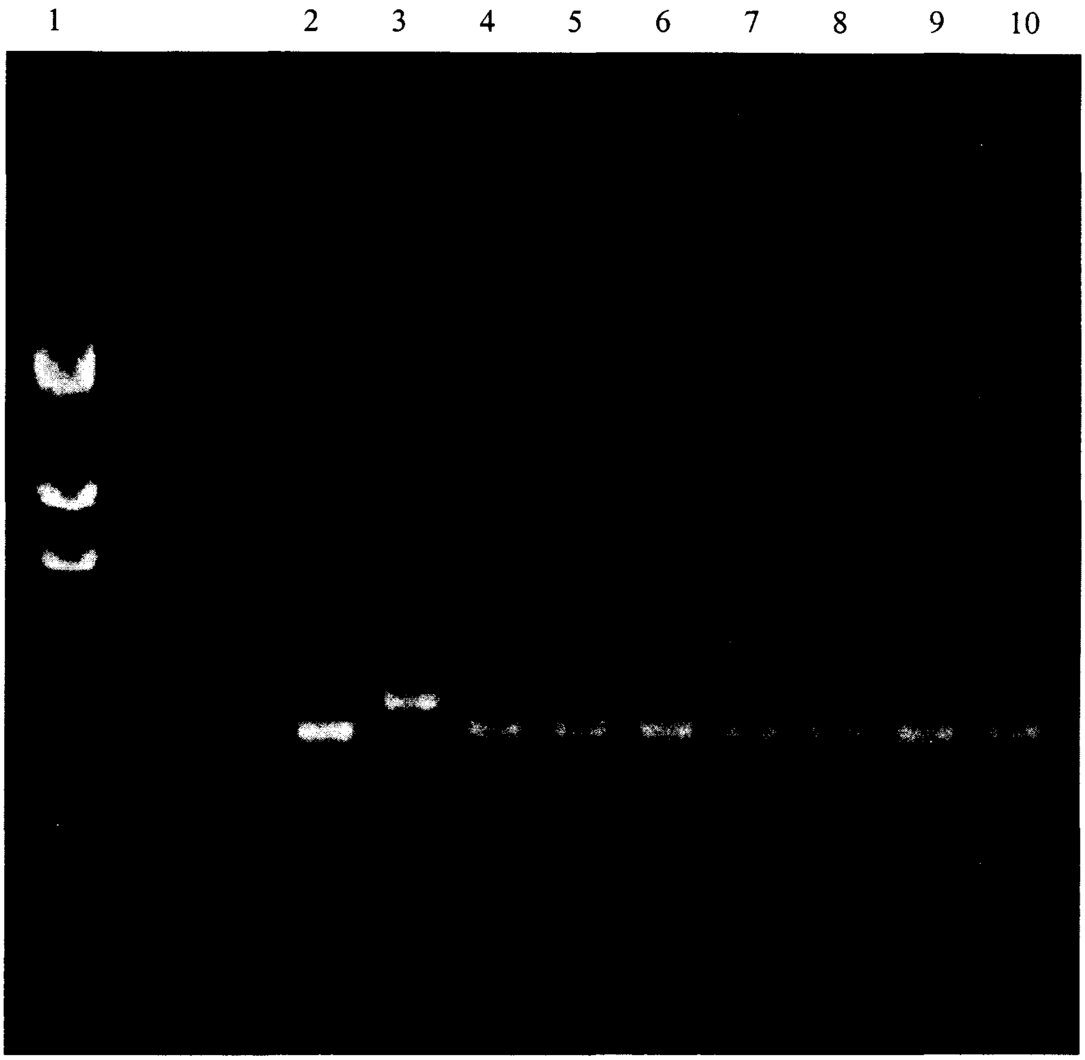
GGAGCGGGGACAGGTAATCGCTTATCCG. The primers had to be phosphorylated with polynucleotide kinase prior to their use in the mutagenesis reaction.

The single-stranded, uracil-containing DNA template was combined with the phosphorylated mutagenic oligonucleotide, single-stranded binding protein (ssbp), dNTPs, ATP, T4 DNA ligase, and T7 DNA polymerase to allow *in vivo* synthesis of a second DNA strand that would bear the desired mutation.

The newly double-stranded M13 was then transformed into JM101. The cells destroyed the uracil-containing strands and new thymine-containing strands were synthesized *in vivo*. The new phage was harvested from the resulting plaques and analyzed by direct electrophoresis (figure 18). RF M13 was grown and purified by the QIAGEN protocol (figure 19). The clone chosen for analysis was sequenced on the CEQ 2000 using various primers and found to contain the insert but not the desired mutation (figures 20, 21, and 22).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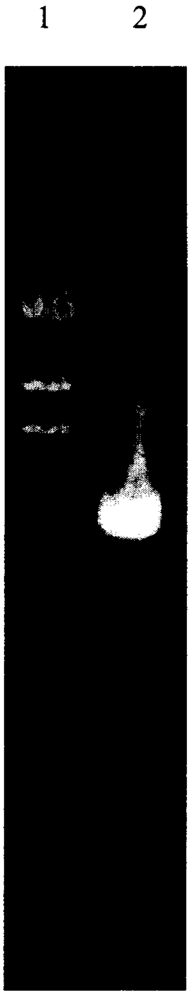
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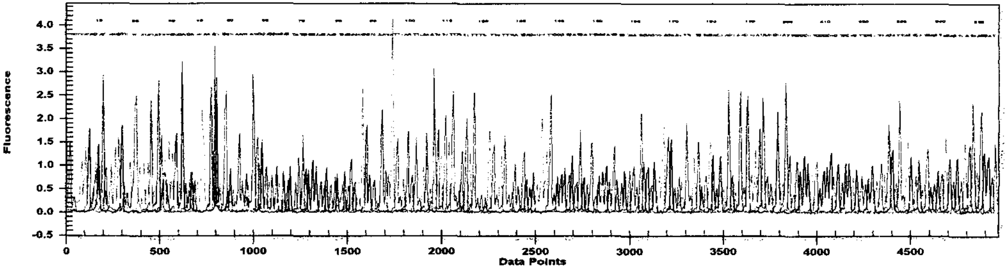

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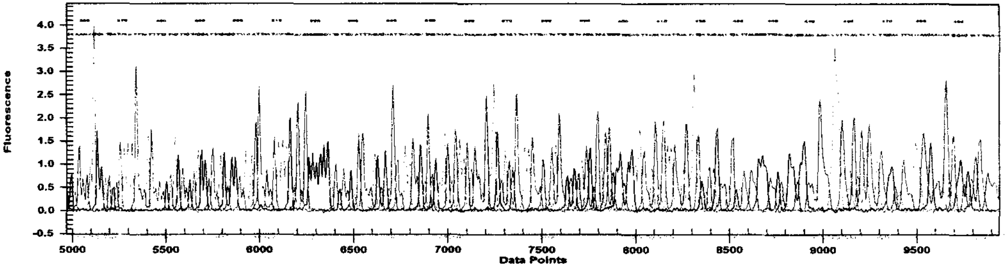

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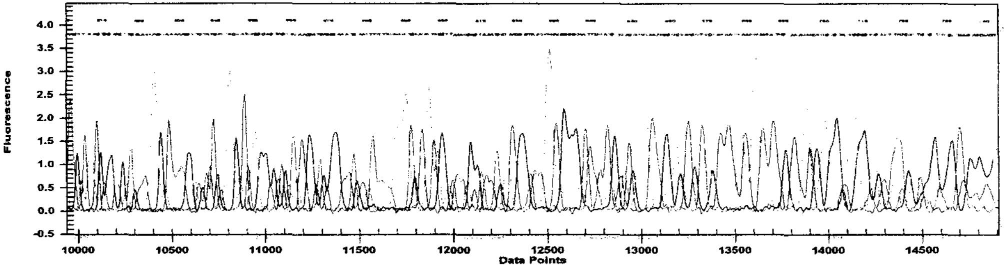

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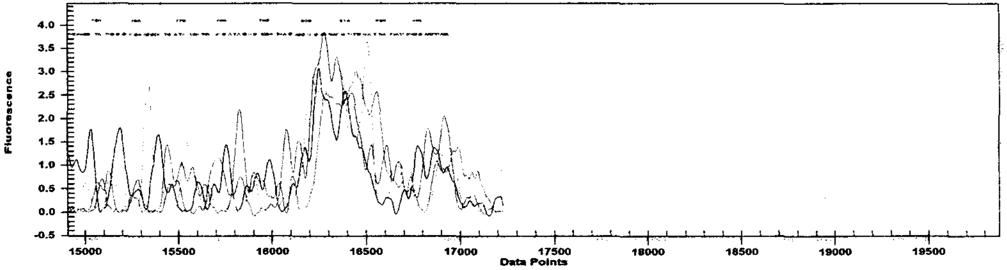

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GATGTGTTTATGTTTTGTTGTTTAATCACGGGTTCCCTCATCATTCCCATCTCT
AATACACGGACAAGTTGTCTGCACGACGAGC***GGATAAGCGATTACCC***GTCCC
CGCTCCGCTTCCGTGACATTGCTCGTCACTGCACTGATGACCATTCGAAAGGC
CGTCATGGGTTTGTTTTCTGCTTCTGCCATTGCACCTTGGATTAATGGGCGAG
ACCCCCCGTCTTCGATTTGCACGATTGGAACGTACGGAGTGGTGAAGAGGCT
GTATTGGTTTAGTGTTCTCTGACATGATACCTCCGGTTGAGTGGTTGAGGTCG
TGCAAGTCGTTAACCCCTTCAGCCACCAAGAAAGGGTTGAGTGTTGTTCCAGT
TTGGGAACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCG
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AGTTTTTGGGGTCGAGGTGCCGTAAAGCACTAAATCGGAACCCTAAGGGGA
GCCCCGAATTTAGAGCTGACGGGGAAAGCCGCGAACGTGGCGAGAAGGAAG
GGAAGATGCGAAAGAACGGCGCTAAGCCCCGCAGTTTATCGGTCGCTGGCGT
AACACCAACCGCGGCCTATGCCGCTACAGCCCGTCATGTGCGTGAAGCATAT
CGCTCCGTGATCACCGCATCTGATCTGTTCGATAGATGATGTGACCAACTGAC
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FASTA searches a protein or DNA sequence data bank
 version 3.2t09 December 7, 1999
Please cite:
 W.R. Pearson & D.J. Lipman PNAS (1988) 85:2444-2448

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EM_FUN:NCQA2MUD K00981 N.CRASSA QA-2  ( 505) [r] 2092 1987 2202 2128.9 1.3e-111
EM_FUN:NCQA2MUE K00982 N.CRASSA QA-2  ( 505) [r] 2101 1996 2153 2081.4 5.7e-109
EM_FUN:NCQA2MUC K00980 N.CRASSA QA-2  ( 503) [r] 2036 1341 2136 2065.0 4.7e-108
EM_FUN:NCQA2MUA K00978 N.CRASSA QA-2  ( 589) [r] 1406 1301 1398 1349.2   3e-68
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7.1e-113
 97.665% identity in 471 nt overlap (471-1:2377-2844)
       500       490       480       470       460       450
EMBOS-  TTCTTTAATAGTGGACTCTTGTTCCCAAACTGGAACAACACTCAACCCTTTCTTGGTGGC
                                      ::::  ::  :: :::::::::::::::::
EM_FUN  TAGGTACCAAATTTTTCTATGCCTTTTTCTTGGATAAATCCT-AACCCTTTCTTGGTGGC
        2350      2360      2370      2380      2390      2400

       440       430       420       410       400       390
EMBOS-  TGAAGGGGTTAACGACTTGCACGACCTCAACCACTCAACCGGAGGTATCATGTCAGAGAA
        :::: :::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_FUN  TGAA-GGGTTAACGACTTGCACGACCTCAACCACTCAACCGGAGGTATCATGTCAGAGAA
          2410      2420      2430      2440      2450      2460

       380       370       360       350       340       330
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        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_FUN  CACTAAACCAATACAGCCTCTTCACCACTCCGTACGTTCCAATCGTGCAAATCGAAGACG
          2470      2480      2490      2500      2510      2520

       320       310       300       290       280       270
EMBOS-  GGGGGTCTCGCCCATTAATCCAAGGTGCAATGGCAGAAGCAGAAAACAAACCCATGACGG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_FUN  GGGGGTCTCGCCCATTAATCCAAGGTGCAATGGCAGAAGCAGAAAACAAACCCATGACGG
          2530      2540      2550      2560      2570      2580

       260       250       240       230       220       210
EMBOS-  CCTTTCGAATGGTCATCAGTGCAGTGACGAGCAATGTCACGGAAGCGGAGCGGGGACGGG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_FUN  CCTTTCGAATGGTCATCAGTGCAGTGACGAGCAATGTCACGGAAGCGGAGCGGGGACGGG
          2590      2600      2610      2620      2630      2640

       200       190       180       170       160       150
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       140       130       120       110       100        90
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        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
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        80        70        60        50        40        30
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        ::::::::::::::::::::::::::::::::::::::::::::: ::::::::::::::
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        20        10
EMBOS-  CCACAAGCCCATGCTACCCCA
        ::::::::::::   :::: :
EM_FUNCCACAAGCCCATCGCACCCAACCAGAGGTACCAAACACAATGGCGTCCCCCCGTCACATT
2830      2840      2850      2860      2870      2880
```

DISCUSSION

Transcription of the *qa* genes at levels sufficient to allow growth on quinic acid as the sole carbon source depends on the interaction of the *qa-1S* repressor, the *qa-1F* activator, and the inducer, quinic acid. In the absence of inducer, the repressor binds the activator protein, only allowing transcription of the *qa* genes at low basal levels. The addition of quinic acid in the absence of a preferred carbon source releases the activator protein, which is then able to bind to activator binding sites, enhancing transcription of the *qa* genes.

These activator binding sites have been found to consist of a 16 base pair conserved sequence. Currently, fourteen of these sites have been discovered; four of these sites are located in the *qa-2-qa-x* intergenic region, three of which affect *qa-2* transcription. Of these three sites the –127 activator binding site has been demonstrated to have the highest binding affinity; in fact, this site has the highest affinity of the entire cluster.

The sixteen bases of the consensus sequence are highly conserved, but the relative importance of each base is still unknown. In order to determine this, the section of DNA containing the binding site had to first be isolated. A clone known to contain most of the *qa* gene cluster was digested with the restriction endonuclease *PstI.* The fragment containing the *qa-2-qa-x* intergenic region was isolated and cloned into pBR322 to form plasmid 177. This was further digested with the restriction enzymes *PstI* and *EcoRI.* The resulting 2.5 kb fragment was cloned into pBluescript to form the construct pEP. By cutting with the enzymes *EcoRI* and

HindIII it was possible to further reduce the size of the insert to 1.8 kb. This fragment was ligated into pBluescript to form the clone pEH. It was then possible to digest the fragment with the restriction enzyme *KpnI* to yield a 500 bp fragment, which was cloned into M13mp18 to yield the M13 clone. This construct was sequenced from both the standard pUC sequencing primer and the M13 forward primer. Sequencing demonstrated the presence of the desired insert DNA.

The M13 clone was grown in the *dut*$^-$ *ung*$^-$ *E. coli* strain CJ236. As a result, the DNA formed contained uracil, rendering it unstable in a wild-type strain. Single-stranded M13 was isolated and combined with a mutagenic primer, dNTPs, polymerase, and ligase to allow *in vitro* synthesis of a second strand of DNA. Because no dUTP was provided, the new strand did not contain any uracil. The resulting double-stranded plasmid was transformed into JM101, wherein the uracil-containing strand was destroyed and the newly synthesized, mutagenized strand was preserved. This strand was used as a template for *in vivo* synthesis of a new second strand. This procedure was chosen to increase the amount of mutagenized product formed, making selection of a mutant clone easier.

The resulting bacteriophages were isolated and double-stranded DNA was recovered. We initially attempted to sequence the DNA following a simple alkaline plasmid screen with no success. We also attempted to isolate single-stranded DNA to sequence but were still unable to obtain any sequence data. Only following QIAGEN purification of the double-stranded replicative form were we able to obtain any sequence data. Unfortunately, the clone we were able to sequence did not contain the desired mutation. Possible reasons for this include leakage and escape. With leakage, not all the DNA isolated from CJ236 contain uracil. If a few strands "leak" through, they will be replicated using the mutagenic primer, but the original strand will not be destroyed when transformed into wild-type, providing a template for replication of non-mutated DNA. The uracil-containing strands can also escape destruction in the wild-type cells long enough to be replicated, also providing a reservoir of non-mutant DNA.

While we were unable to isolate the desired mutant, we did, however, isolate and clone the desired fragment. We also developed protocols that will allow the work to be completed by future students.

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Geever, R.F., Huiet, L., Baum, J.A., Tyler, B.M., Patel, V.B., Rutledge, B.J., Case, M.E., and N.H. Giles. (1989). DNA sequence, organization and regulation of the *qa* gene cluster of *Neurospora crassa. J. Mol. Biol.* **207**, 15-34.

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