The Localization of the *qa-1S-qa-1F* Intergenic Region of *Neurospora africana*

by

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Scott Michael Raidel

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ABSTRACT

Many microrganisms, in the presence of a preferred carbon source, repress genes which are used to metabolize other carbon sources, a process call carbon catabolite repression. Carbon catabolite repression has been shown to operate within the quinic acid utilization pathway (*qa*) of *Neurospora crassa.* The mechanisms acting to cause this repression remain unknown. However, a strain of *N. crassa* containing a deletion of the *qa-1S* repressor gene showed that some *qa* genes remained slightly repressed while others remained highly repressed, in the presence of a preferred carbon source. One of these directly repressed genes is the gene coding for the *qa-1F* activator protein.

To investigate this phenomenon the *qa-1S-qa-1F* intergenic region of *Neurospora africana* was chosen for study. *N. africana* was chosen because the entire *qa* gene sequence of *N. crassa* is known and provided a comparison between the two species. First, subclones of plasmid pR1, which contained a 3.8 kb insert known to contain the *qa-1S-qa-1F* intergenic region of *N. africana*, were constructed. Southern blot analysis of the subclones plasmid pRX1 and pRX2 revealed that the entire *qa-1S-qa-1F* intergenic region was contained within plasmid pRX2. Further sequence analysis revealed the existence of portions of the *qa-1F* and *qa-1S* genes and the location the intergenic region within the original 3.8 kb insert.

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LIST OF ABBREVIATIONS

ABBREVIATION	MEANING
GAL	Galactose System
UAS	Upstream Activating Sequences
URS	Upstream Repression Sequences
qa	Quinic Acid System
WT	Wild-Type
kb	kilobases
bp	basepairs
MCS	Multiple Cloning Site
Amp^r	Ampicillin Resistance Gene

INTRODUCTION

I. Kingdom Fungi

The kingdom fungi, which is divided into three divisions (Zygomycota, Ascomycota, and Basidomycota), represents a large, diverse group of eukaryotic organisms, which number more then 100,000 known species. Fungi are decomposers, whose metabolism releases carbon dioxide and nitrogenous materials into the environment. Some fungi are unicellular but most are filamentous and may be organized into highly structured shapes. All fungi are heterotrophic (can not make own food from inorganic materials), and obtain their food as saprobes (live on nonliving organic matter) or as parasites (feed on living organic matter). Fungi do not ingest their food, but use enzymes to break it down and then absorb it. Finally, all fungi have cell walls and most produce some type of spores.

II. Division Ascomycota

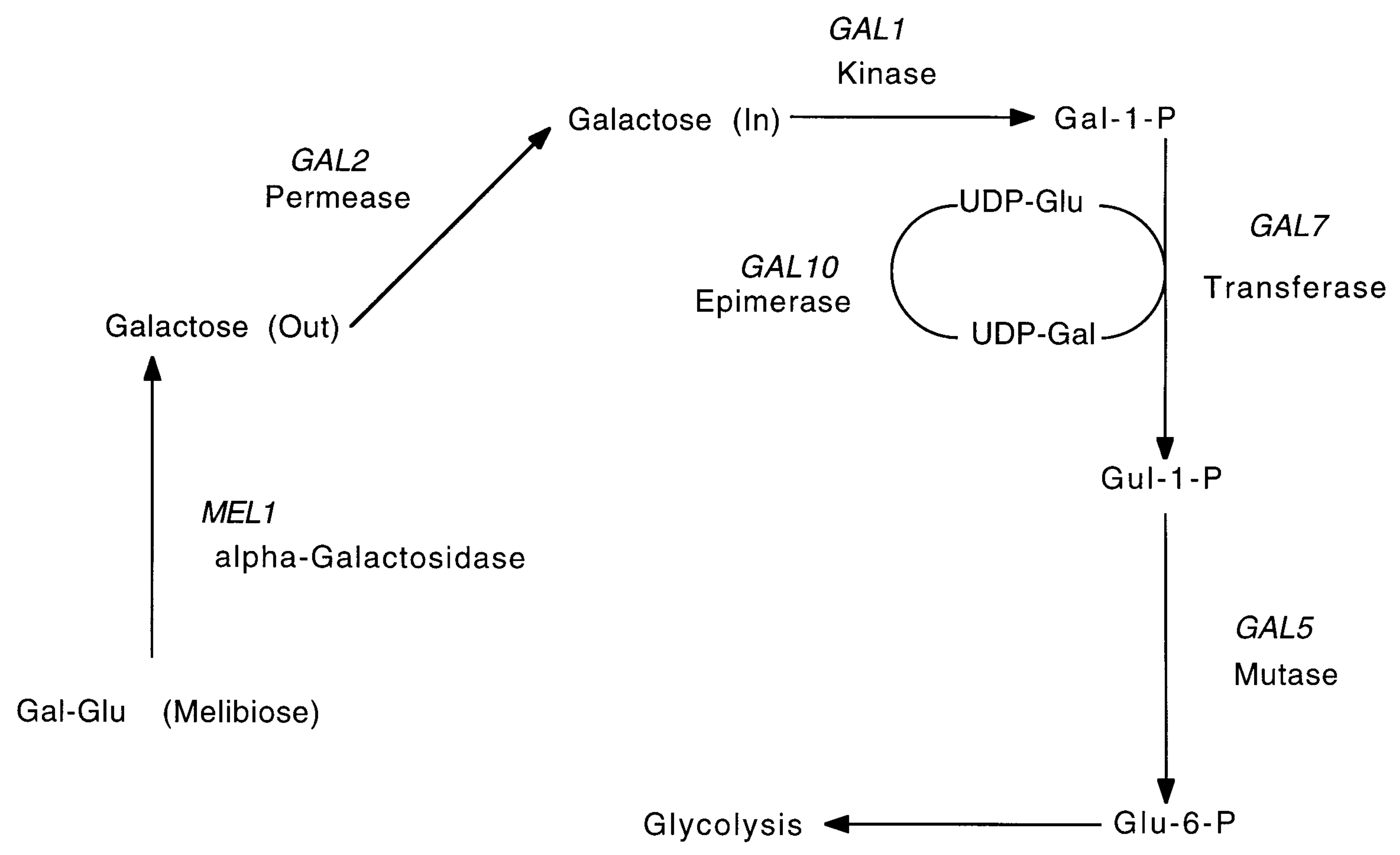
This is the largest division of the kingdom fungi, representing about 30,000 species. Included in this division are yeast, powdery mildews, molds, morels, and truffles. Two groups, the yeasts of *Saccharomycetales* and the filamentous ascomycetes, are of particular interest.

III. The *Saccharomycetales*

The *Saccharomycetales* are unicellular, eukaryotic organisms which reproduce either sexually or asexually, and are characterized by the absence of an ascocarp. One species within this group, *Saccharomyces cerevisiae*, has many features which make it an ideal model for biological research. Some of these are, that it has an ancient history in baking and brewing, and from this it is regarded as a safe organism. Furthermore, its growth cycle allows for a homogeneous population to be produced in a short time period and at relatively inexpensive costs. These features have made it an attractive host system to study eukaryotic gene regulation.

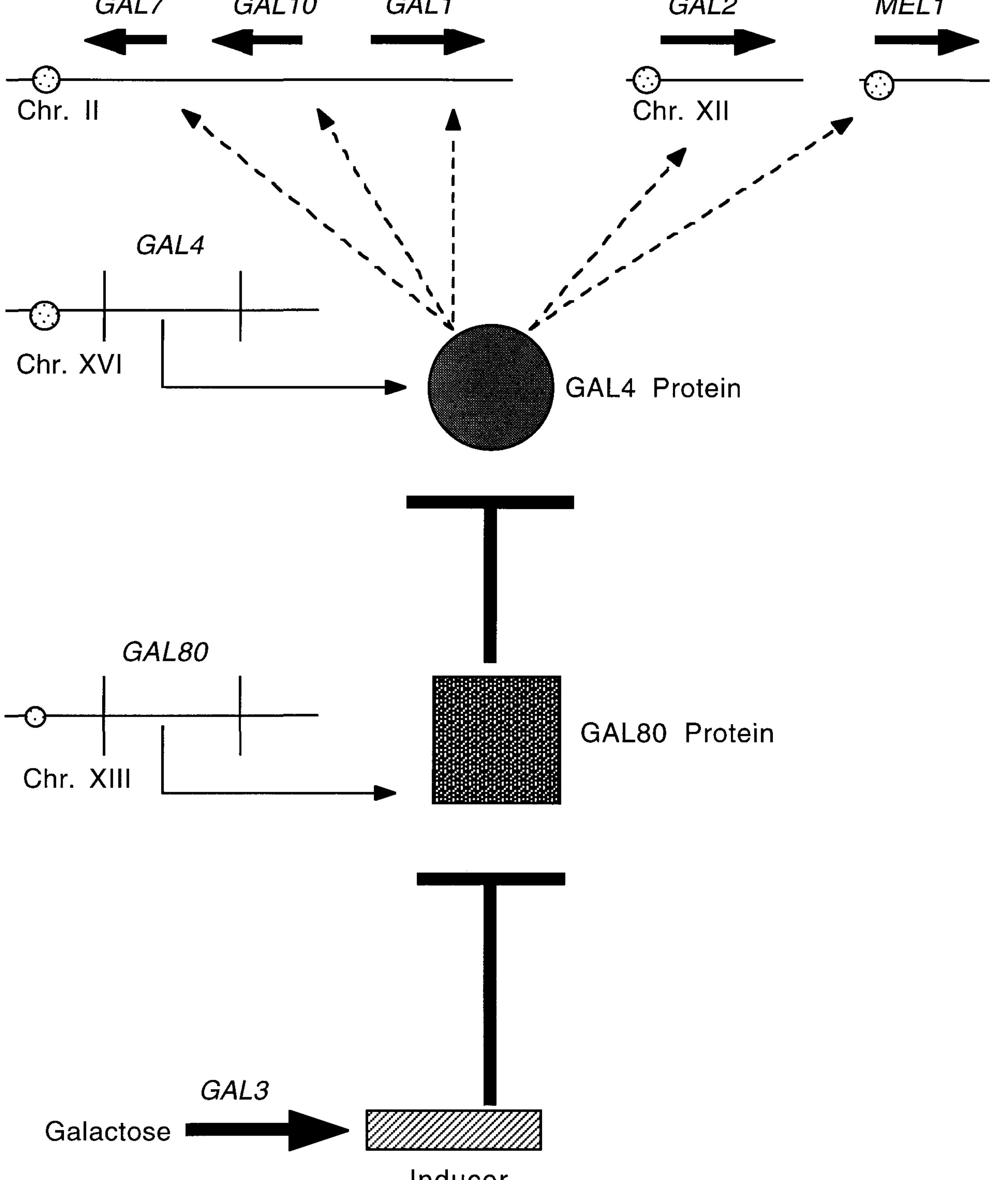
IV. The Galactose (*GAL*) System of *Saccharomyces cerevisiae*

The galactose (*GAL*) system, which encodes enzymes for galactose utilization, is one of the most intensively studied and best understood genetic regulatory circuits in yeast. *S. cerevisiae* utilizes galactose by the enzymes of the Leloir pathway (Kosterlitz, 1943; Leloir, 1951). These enzymes are encoded by the structural genes *Gal1* (galactokinase), *Gal7* (galactose-1-phosphate uridylyltransferase), *Gal10* (uridine diphosphoglucose-4-epimerase), and *Gal5* (phosphoglucomutase) (Douglas and Hawthorne, 1964, 1972) (Figure 1). Expression of these genes, except *Gal5*, is tightly regulated and their expression is induced by galactose and repressed by


GAL1
Kinase
Galactose (In)
Gal-1-P
GAL2
Permease
UDP-Glu
GAL7
GAL10
Epimerase
Transferase
Galactose (Out)
UDP-Gal
Gul-1-P
MEL1
alpha-Galactosidase
GAL5
Mutase
Gal-Glu (Melibiose)
Glycolysis
Glu-6-P

glucose. The *Gal5* gene is unregulated and expressed under all conditions (Bevan and Douglas, 1969). Two other genes (*Gal2* and *MEL1*) are also known to participate in this galactose pathway. The gene *MEL1* allows the cell to use the sugar melibiose in the galactose pathway. It does this by encoding an alpha galactosidase, which cleaves the disaccharide (melibiose) into its component sugars galactose and glucose (Lazo et al., 1978) (Figure 1). The gene *GAL2* encodes a specific permease which allows galactose to enter the cell (Figure 1). Finally, three other genes (*GAL4, GAL80,* and *GAL3*) act as regulators of the pathway. The *GAL3* gene appears to encode an enzyme that catalyzes synthesis of the inducer from galactose. While the genes *GAL4* and *GAL80* act to regulate transcription of these *GAL* genes. The *GAL4* gene acts to activate transcription, while *GAL80* prevents transcription.

The essential elements of the *GAL* regulatory circuit (*GAL1-7* and *-10*) are clustered near the centromere of chromosome II (St. John and Davis, 1981) (Figure 2). These genes have been isolated (St. John and Davis, 1979), sequenced (Citron and Donelson, 1984), their transcripts identified and mapped (St. John and Davis, 1981; St. John et al., 1981), and their sites of transcription have been located (Citron and Donelson, 1984). The *GAL2* gene and *MEL1* lie on a separate chromosome, chromosome XII (Figure 2). While the regulatory genes also lie on separate chromosomes. *GAL4* lies on chromosome XVI and *GAL80* is found on chromosome XIII (Figure 2).



GAL7
GAL10
GAL1
GAL2
MEL1
Chr. II
Chr. XII
GAL4
Chr. XVI
GAL4 Protein
GAL80
Chr. XIII
GAL80 Protein
GAL3
Galactose
Inducer

The *GAL4* encodes a protein that activates transcription of these genes, while *GAL80* encodes a protein that binds to the GAL4 protein preventing transcription of the other genes (Figure 2). The inducer (produced by *GAL3*) prevents the GAL80 protein from inhibiting GAL4 function, by binding to GAL80. How the inducer accomplishes this is uncertain, but it may be due to the inducer causing a transformation of the GAL80•GAL4 complex that exposes the GAL4 activation domain (Leuther and Johnston, 1992). A second regulatory circuit, catabolite repression, also acts to prevent *GAL* gene expression during growth on a preferred carbon source, such as glucose (for review, see Johnston, 1987). However little is known about this system.

V. Mechanisms of the GAL4 Activator Protein

The *GAL4* gene encodes a protein of 881 amino acids, which activates transcription of the genes required for galactose catabolism (Oshima, 1982; Johnston and Hopper, 1982). Analysis of the GAL4 protein has revealed some of the regions responsible for its function and include: (1) DNA-binding, (2) transcription activation, (3) ability to enter the nucleus, (4) interaction with the GAL80 protein, (5) possible involvement in catabolite repression, and (6) multimer function.

The GAL4 protein is made in the cytoplasm but acts in the nucleus. Therefore, a specific transport system is needed

and believed to be found in the N-terminal region, near the DNA-binding domain, of the GAL4 protein (Silver et al., 1984). The most important domain is the DNA-binding domain. This domain resides in the N-terminal 65 amino acids (Marmorstein et al., 1992). This region is homologous to other eukaryotic DNA binding proteins (Johnston and Dover, 1987) and contains six cysteine residues which form a structure called the "cysteine-zinc DNA binding finger". Evidence for this zinc finger has come from *gal4* mutants that alter this structure abolishing DNA binding of the GAL4 protein (Johnston and Dover, 1987). Experiments have identified 11 known GAL4 DNA binding sites bearing the consensus 17 base-pair (bp) palindrome CGGAGGACTGTCCTCCG (Giniger et al., 1985) existing in the upstream activating sequences (UAS_{GAL}) for the various *GAL* genes. The structure of this binding site suggests that the GAL4 protein binds to the DNA as a multimer, probably a dimer or tetramer, hence the GAL4 protein bears a multimer domain (Giniger et al, 1985; Marmorstein et al., 1992; Kang et al., 1993). The residues of the central region (238-767) of the protein are believed to work in catabolite repression. It has been found that this region is required for inhibition of the activator by glucose as well as for the activation of GAL4 in the absence of glucose (Kang et al., 1993). The GAL80 repressor protein is believed to bind to the C-terminal 30 residues (851-881) of GAL4 and is responsible for repression of the GAL4 protein activity (Marmorstein et al., 1992). The transcription activation domain activates transcription through contacts with

other proteins more directly responsible for transcription. Two regions of the GAL4 protein are known to contribute to transcriptional activation, these are residues 148-196 and 768-881 (Ma and Ptashne, 1987a; Lin et al., 1988).

The conditions under which the GAL4 protein binds to DNA were found to be both in the presence and absence of galactose (Giniger et al, 1985; Lohr and Hopper, 1985; Selleck and Majors, 1987). Thus, expression of the *GAL* genes must involve modifications of the GAL4 protein, but not the DNA-binding domain. It is thought that the GAL80 protein, in the absence of inducer, interacts with GAL4, which is bound to the DNA, and prevents transcription without changing GAL4 DNA binding properties. In contrast, these same experiments have also shown the GAL4 protein does not bind DNA while grown on glucose. This condition is believed to be the cause of catabolite repression of *GAL* gene expression. Thus, it appears that one mechanism of glucose repression is the prevention of DNA binding by the GAL4 protein (for review, see Johnston, 1987).

VI. Mechanisms of the GAL80 Repressor Protein

The GAL80 protein encodes a protein of 435 amino acids, which inhibits the GAL4 activator protein (Lue et al., 1987; Ma and Ptashne, 1987b). Analysis of the GAL80 protein has revealed at least three functional domains. These domains are

involved in: (1) interaction with the GAL4 protein, (2) interaction with the inducer, and (3) targeting to the nucleus.

The GAL80 protein is made in the cytoplasm but acts in the nucleus. Therefore, a specific transport system is needed and believed to be found in residues 1-109 and 341-405 of the GAL80 protein. The GAL80 protein is thought to interact with the inducer and this has been identified to occur at residues 322-340 (Yun et al., 1991). Evidence that the inducer acts on the GAL80 protein came from *gal80* mutants. These mutants were constitutive for *GAL* gene expression showing that the inducer is not needed for expression of the *GAL* genes in the absence of GAL80 protein (for review, see Johnston, 1987). Also, mutations which lie within the inducer binding domain resulted in GAL80 protein unable to recognize the inducer and allow transcription to occur (Douglas and Hawthorne, 1964; Nogi and Fukasawa, 1984). These results together suggested that the inducer binds directly to the GAL80 protein. The final domain consists of two distinct regions, residues 1-321 and 341-423, which are proposed to bind to the GAL4 protein and inhibit its function. The conclusion that GAL80 indirectly repress *GAL* gene expression by inhibiting GAL4 is based on the findings that *gal4* mutants are epistatic to *gal80* mutants (Douglas and Hawthorn, 1964; Torchia et al., 1984). This means that *gal4-gal80* double mutants have the same phenotype (GAL$^-$) as *gal4* mutants, suggesting the GAL4 functions after GAL80.

Transcription of the *GAL80* gene is regulated by the GAL4 protein. This is seen by an approximate 5-fold increase in its basal level expression when grown on galactose. This is caused by a binding site for the GAL4 protein within the *GAL80* promoter (Buam et al., 1986). This expression acts to regulate *GAL* gene expression because higher concentrations of *GAL* genes requires higher galactose concentrations, which may not be available. These results also show why inducer levels, while on galactose, are insufficient to saturate all GAL80 protein. This antagonistic effect of increasing GAL80 while reducing their activity is a way yeast maintain homeostasis of the *GAL* genes.

VII. Activation of the Galactose (*GAL*) System

In the absence of galactose, the inducer, the GAL4 protein is bound to the UAS_{GAL} sites but is prevented, by its interactions with the GAL80 protein, from activating transcription of the *GAL* genes. The GAL80 protein accomplishes this by covering the C-terminal transcriptional activation domain of the GAL4 protein. However, in the presence of galactose, the inducer causes a transformation of the GAL80•GAL4 protein complex by binding to the GAL80 protein (Leuther and Johnston, 1992). This change allows the exposure of the GAL4 protein activation domain, and hence activates transcription of the *GAL* genes. This is accomplished by the GAL4 protein, while bound to the UAR_{GAL} by its DNA-binding

domain, interacts with other proteins essential for transcription, such as a TATA box binding factor (Selleck and Majors, 1987).

Expression of the *GAL1,-2,-7,* and *-10* genes requires the GAL4 protein and are completely inhibited by the GAL80 protein (St. John and Davis, 1981). Indeed, experiments have shown that the GAL4 protein induces a 1,000-fold increase in transcription of these genes. However, other genes involved in the utilization of galactose do not behave this way. The *MEL1* gene is not completely inhibited by GAL80, but does require GAL4 (Post-Beittenmiller et al., 1984). Also, basal levels of GAL80 do not depend on GAL4 but are found to increase 2-100-fold in its presence (Shimada and Fukasawa, 1985).

Other genes have also been implicated in the activation of GAL4 activity. One of these is the *GAL3* gene. This gene is thought to encode an enzyme that catalyzes synthesis of inducer from galactose, although its precise role in *GAL* gene regulation is unknown. Experiments using *gal3* mutants showed an induction lag of 2-5 days in response to galactose, while wild-type showed an induction lag of only a few minutes (Spiegelman et al., 1950; Kew and Douglas, 1976). Another gene, *GAL11*, has also been shown to be required for full induction of *GAL* gene expression (Himmelfarb et al., 1990). Experiments using *gal11* mutants displayed a 5-fold reduction in *GAL* gene expression (Suzuki et al., 1988). These results suggested that the GAL11 protein complexes with GAL4 to

increase its activity (Nishizawa et al., 1990) but its precise function still remains unknown.

VIII. Carbon Catabolite Repression of the Galactose (*GAL*) System

Glucose enters the glycolytic pathway directly and cells prefer to use it instead of other sugars which require conversion to be utilized, such as galactose. This effect is seen in *S. cerevisiae* when *GAL* gene expression is repressed when grown on glucose (for review, see Johnston, 1987). This regulatory circuit is superimposed upon the circuit which induces *GAL* gene expression and is termed catabolite repression. Catabolite repression appears to act on at least three separate levels in the *GAL* gene regulatory circuit. These include: (1) directly on the promoters of the *GAL* genes, (2) directly on the GAL4 protein, and (3) on the inducer levels. Several genes have been identified, using mutational studies that express *GAL* genes while on glucose, which may be responsible for catabolite repression (for review, see Johnston, 1987).

The mechanisms of catabolite repression proposed to act by reducing inducer levels are that glucose could cause repression of *GAL* gene expression by preventing the inducer from inactivating GAL80 protein activity. Also, since *GAL3* is regulated by the GAL4 and GAL80 proteins (Torchia and Hopper, 1986), glucose also appears to inhibit synthesis of the

inducer by repressing transcription of *GAL3*. Furthermore, the transport of galactose is inhibited by glucose at two levels. The first is that synthesis of the permease is repressed by glucose because *GAL2* expression is subject to catabolite repression (Tschopp et al., 1986), and by a process called catabolite inactivation. Here, glucose inactivates preexisting permease molecules (Ma and Ptashne, 1987c).

Catabolite repression may cause inhibition of the GAL4 protein DNA binding ability. This mechanism of glucose repression acts to prevent the binding of the GAL4 protein to the DNA, which might be due to the action of GAL80 or other unidentified gene products. Catabolite repression may also act by directly repressing only the *GAL4* gene. This repression would limit the amount of GAL4 protein, and therefore directly repress the expression of the *GAL* genes (Johnston et al., 1994).

Finally, catabolite repression may act directly on the *GAL* promoters. This conclusion is based on findings that *GAL* gene regulatory sequences found between the UAS_{GAL} and transcriptional initiation sites (TATA box) seem to be sufficient to provide catabolite repression (Flick and Johnston, 1991; 1992). These sequences are termed upstream repression sequences (URS_{GAL}). It is thought that the repression that operates through these URS_{GAL} could be due to unidentified repressor proteins (Erickson and Johnston, 1993). One such protein is encoded by *MIG1*. This protein was found to bind to *GAL* promoters in the presence of glucose and may possibly play a role in repression. Other experiments suggest that

several genes (*MIG1*, *SSN6*, and *TUP1*) may form a complex required for repression of *GAL* genes in the presence of glucose (Kelcher et al., 1992). Still the exact mechanisms of catabolite repression are unknown, but it is believed that these methods evolved to control other genes and adapted to cause repression of the *GAL* genes.

IX. Filamentous Ascomycetes

The filamentous ascomycetes are distinguished from the *Saccharomycetales* by being more complex morphologically and by the formation of an ascocarp. Like the *Saccharomycetales*, filamentous ascomycetes are important economically and are an ideal model for biological research. With the discovery of *Neurospora*, (Shear and Dodge, 1927) and a particular species within this genus, *Neurospora crassa*, which gained noteworthy status by being used to discover the "one gene, one enzyme" theory, which lead to a Nobel Prize in 1958, (Beadle and Tatum, 1941), all of the features which make this genus an ideal model system for study were quickly seen. Some of these are that they have a short growth time allowing for consistent and ongoing experimentation. They are capable of growing on simple media and are therefore relatively inexpensive to maintain. Finally, and most important, they contain haploid genomes allowing mutations to be identified quickly by ruling out dominant alleles which may hide a mutation. These factors have made *Neurospora* a great host system to study eukaryotic

gene regulation, such as that seen with the quinic acid (*qa*) gene cluster.

X. Discovering the Quinic Acid (*qa*) Gene Cluster of *Neurospora crassa*

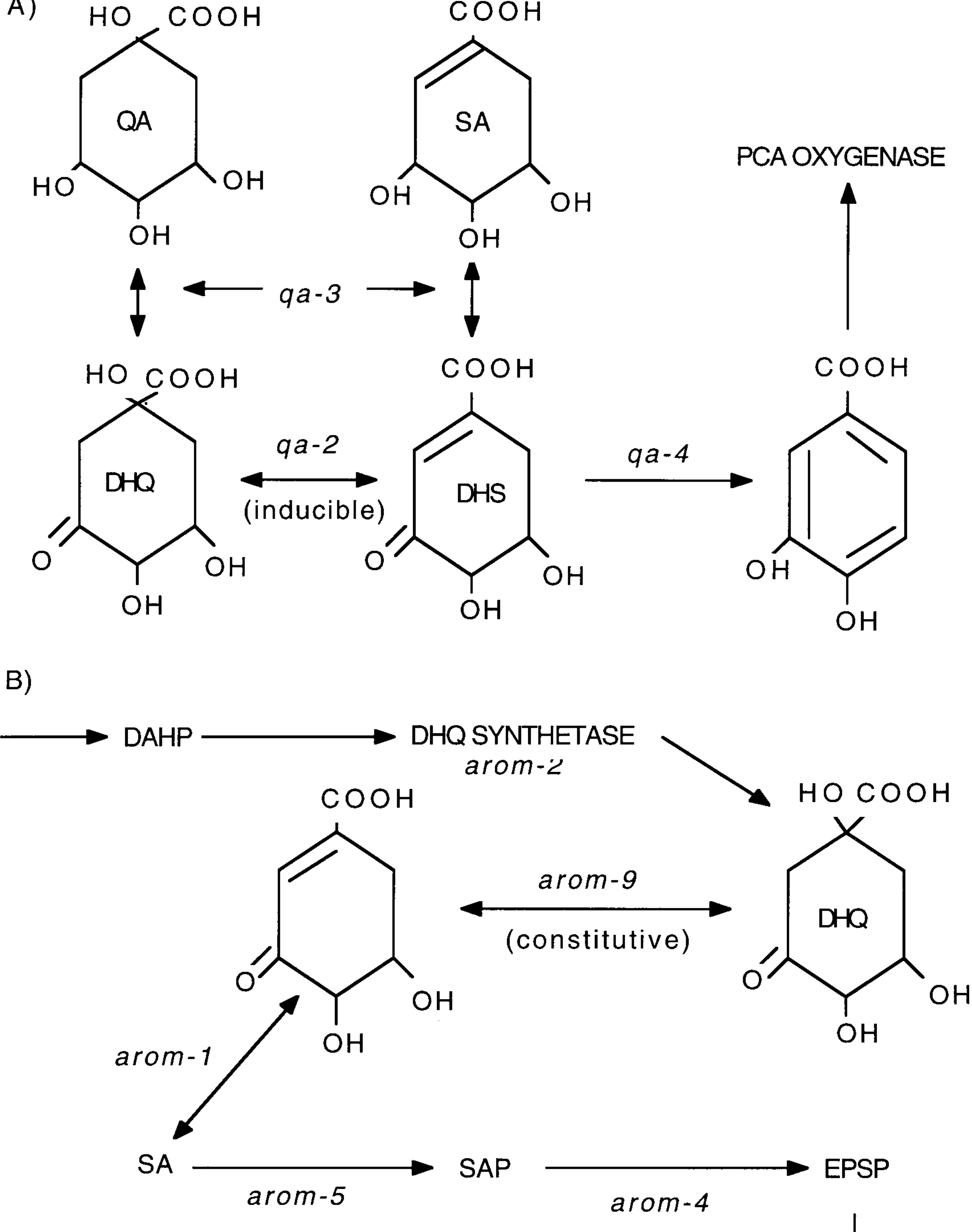
Clustering of genes involved in a metabolic pathway is an organizational feature used in many eukaryotic genomes. Two different forms of clustering have been identified in fungi (Giles, 1978). One of these is the gene cluster. A gene cluster is a section of the genome consisting of separate genes in a contiguous array. The quinic acid (*qa*) gene cluster of *N. crassa* is a well characterized example of this type of gene organization, and provides an excellent system to study genetic regulation in a simple, multicellular eukaryotic organism.

Early studies of the *qa* gene cluster established the existence of four genes in the cluster. Three of these genes (*qa-2, qa-3, qa-4*) encoded inducible enzymes that catalyze the catabolism of quinic acid to protocatechuic acid, and are termed the structural genes (Giles et al., 1973; Chaleff, 1974) (Figure 3A). The other gene, *qa-1*, was found to encode a regulatory protein. This protein when combined with the inducer, quinic acid, acted as a positive regulator and controlled transcription of the three structural genes (Valone et al., 1971; Case and Giles, 1975; Patel et al., 1981).

Experiments involving *qa-2* mutants showed that these mutants did not display catabolic dehydroquinase (C-DHQase)

activity (Giles et al., 1985). This provided evidence that the gene *qa-2* encoded a C-DHQase that catalyzes the breakdown of 5-dehydroquinate to 5-dehydroshikimate (Figure 3A). Other experiments involving a *qa-2* mutant strain of *N. crassa* exposed another type of mutant strain called *arom-9* [no biosynthetic dehydroquinase (B-DHQase) activity]. This mutant strain could not convert 5-dehydroquinate to 5-dehydroshikimate. This discovery led to the mapping of the *arom* gene cluster (Rines et al., 1969). Studies using a pleiotropic *arom* mutant strain of *N. crassa* [lacks all enzymes needed in the aromatic biosynthetic pathway (Figure 3B)], which also contained a mutation in the *qa-1* gene, identified a strain which could not synthesize dehydroquinase (DHQase) or grow on quinic acid as a sole carbon source. This strain showed the *qa-1* regulatory gene was unlinked to the *arom* gene cluster (Giles et al., 1985).

Studies using a *N. crassa* strain with a mutation in the *qa-3* gene had no quinic acid dehydrogenase (QDHase) activity, nor shikimic acid dehydrogenase (SDHase) activity (Chaleff, 1974). In these studies, one mutant strain was found that reverted and obtained both QDHase and SDHase activity. This suggested the *qa-3* gene encoded a bifunctional enzyme, which catalyzed quinate and shikimate dehydrogenation (Figure 3A). Studies using a strain of *N. crassa* containing a mutation in the *qa-4* gene (originally obtained by Case) could not convert dehydroshikimate to protocatechuic acid (Chaleff, 1974)


A)
HO COOH
QA
HO
OH
OH
COOH
SA
OH
OH
OH
PCA OXYGENASE
qa-3
HO COOH
DHQ
O
OH
OH
qa-2
(inducible)
COOH
DHS
O
OH
OH
qa-4
COOH
OH
OH
B)
DAHP
DHQ SYNTHETASE
arom-2
HO COOH
COOH
O
OH
OH
arom-9
(constitutive)
DHQ
O
OH
OH
arom-1
SA
arom-5
SAP
arom-4
EPSP

(Figure 3A). This suggested that the *qa-4* gene encoded the enzyme dehydroshikimate dehydrotase (DHS-Dase), whose activity was needed for this conversion to occur.

Finally with the *qa-1* regulatory gene, two types of mutations were isolated. These were pleiotropic negative (noninducible) and constitutive mutants. Each affected the synthesis of the three structural genes in different manners. Two types of noninducible *qa-1* mutants were described according to their ability to complement *qa-2* mutants. These were, semidominant $qa\text{-}1^S$ mutants, which showed slow (weak) complementation, and recessive $qa\text{-}1^F$ mutants, which showed fast (strong) complementation (Rines, 1969). Mapping experiments of these ($qa\text{-}1^S$, and $qa\text{-}1^F$) mutants found two distinct nonoverlapping regions at opposite ends of the *qa-1* gene (Case and Giles, 1975). Constitutive $qa\text{-}1^C$ mutants were found to be obtained directly from wild-type *N. crassa* strains (Partridge et al., 1972), as well as from $qa\text{-}1^S$ mutants. However $qa\text{-}1^F$ mutants did not give rise to constitutives (Valone et al., 1971). The results from these two types of mutations apparently suggested the existence of two domains within the *qa-1* regulatory protein, one for inducer binding and the other for DNA binding. Subsequent studies revealed the presence of two separate regulatory genes.

XI. Cloning of the Entire Quinic Acid (*qa*) Gene Cluster of *Neurospora crassa*

Recombinant DNA technology provided a major breakthrough in studying the *qa* gene cluster. Early experiments found that the structural gene *qa-2* could be expressed in *Escherichia coli* (Vapnek et al., 1977; Alton et al., 1978). This was possible because the *qa-2* gene, when expressed in a strain of *E. coli* with a mutation in the *aroD* gene (lacks biosynthetic B-DHQase activity) would complement this mutation. However, it was found that none of the other *qa* genes (*qa-3, qa-4,* and *qa-1*) could be expressed in *E. coli.* However, with the subsequent development of a new *N. crassa* transformation technique (Case et al., 1979) it became possible to clone the *qa* genes by complementation of *Neurospora qa* mutants.

Using these techniques the *qa-1* regulatory gene was localized to a 5.8 kilobase (kb) region at the centromere-proximal end of the cluster (Schweizer et al., 1981b). To characterize the *qa-1* regulatory gene, transformation experiments were done on both *qa-1*^{S} and *qa-1*^{F} mutants (Huiet, 1984). These DNA subclones were also hybridized to poly(A)$^+$ RNA to identify the messenger ribonucleic acids (mRNAs) associated with the *qa-1* gene. These experiments indicated that the *qa-1*^{S} and *qa-1*^{F} regions constituted two different genes that encoded distinct mRNAs, which were transcribed in opposite directions (Huiet, 1984). These two regulatory genes were then termed *qa-1S* and *qa-1F*

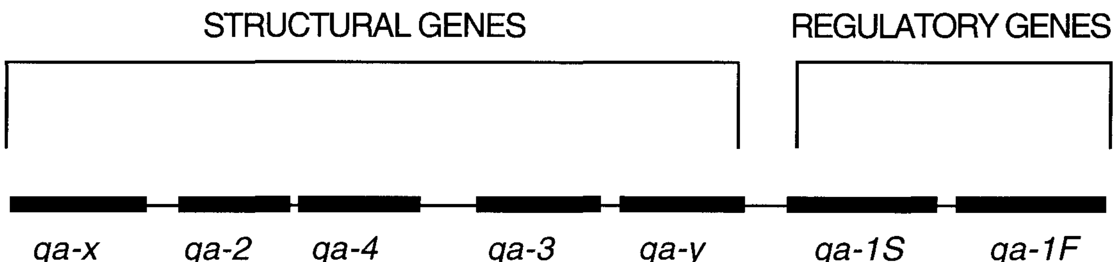
respectively. Based on these results it was now necessary to revise the original hypothesis that the *qa-1* gene encoded a single regulatory protein with separate functional domains (Case and Giles, 1975). The new hypothesis proposed that the two *qa* genes (*qa-1S* and *qa-1F*) encoded a repressor protein (*qa-1S*) and an activator protein (*qa-1F*), whose interactions controlled *qa* gene expression (Huiet, 1984).

Using subclones of a 42 kb region of cloned *N. crassa* DNA, which was centered around the gene *qa-2*, it was possible to localize and determine the order of the three structural genes as *qa-2* -->*qa-4* -->*qa-3* (Schweizer et al., 1981a; 1981b) (Figure 4). It was also determined using transformation experiments involving stable *qa-1S* and *qa-1F* mutants as recipients that the regulatory genes were located to the right of the gene *qa-3*. These experiments, along with genetic mapping, also determined the order of these regulatory genes (Figure 4) (Schweizer et al., 1981a; 1981b). This cloning of genes allowed for the identification of all the structural genes mRNAs. DNA-RNA hybridization studies revealed the existence of five, rather then three structural genes. Each structural gene was transcribed as a separate mRNA (Giles et al., 1985). The two additional structural genes which were identified were termed *qa-x* and *qa-y*.

The *qa-y* gene was originally identified as a quinic acid-inducible transcript of unknown function (Patel et al., 1981). The *qa-y* gene was found to be located between *qa-3* and *qa-1S* (Giles et al., 1985) (Figure 4). The *qa-y* gene showed 61%

amino acid identity with the *qutD* gene of *Aspergillus nidulans*, which was predicted to encode a quinate permease (Whittington et al., 1987). Experiments also showed amino acid structural similarities to a family of human hepatoma cells (Mueckler et al., 1985) and bacterial transporters for arabinose (*AraE*), xylose (*XylE*) and citrate (cit^+) (Maiden et al., 1987; Geever et al., 1989). In addition to these results, it was found that when the *qa-y* gene of *N. crassa* was mutated these strains had a reduced ability to absorb quinic acid and grow on quinic acid as a sole carbon source. These strains also had very low levels of quinate pathway enzymes (*qa-2, qa-3,* and *qa-4*) when compared to wild-type strains (Case et al., 1992). With this comparative and experimental evidence it has been established that the *qa-y* gene encodes a quinic acid permease.

The *qa-x* gene was also originally identified as a quinic acid inducible transcript of unknown function (Patel et al., 1981). It was found that the *qa-x* gene was located to the left of *qa-2* (Giles et al., 1985) (Figure 4). Using null mutations of the *qa-x* gene it was found that these strains could still grow on quinic acid as a sole carbon source, and over time they accumulated a brown pigment (Asch, unpublished data). It was hypothesized that the *qa-x* gene encoded an enzyme capable of hydrolyzing chlorogenic acid (Giles et al., 1985). However, these mutants still grew on chlorogenic acid seemingly to disprove this hypothesis. A possible role for the *qa-x* gene was seen by evidence obtained with the galactose (*GAL*) system in *S. cerevisiae*. Both the *GAL* and *qa* systems


STRUCTURAL GENES
REGULATORY GENES
qa-x
qa-2
qa-4
qa-3
qa-y
qa-1S
qa-1F

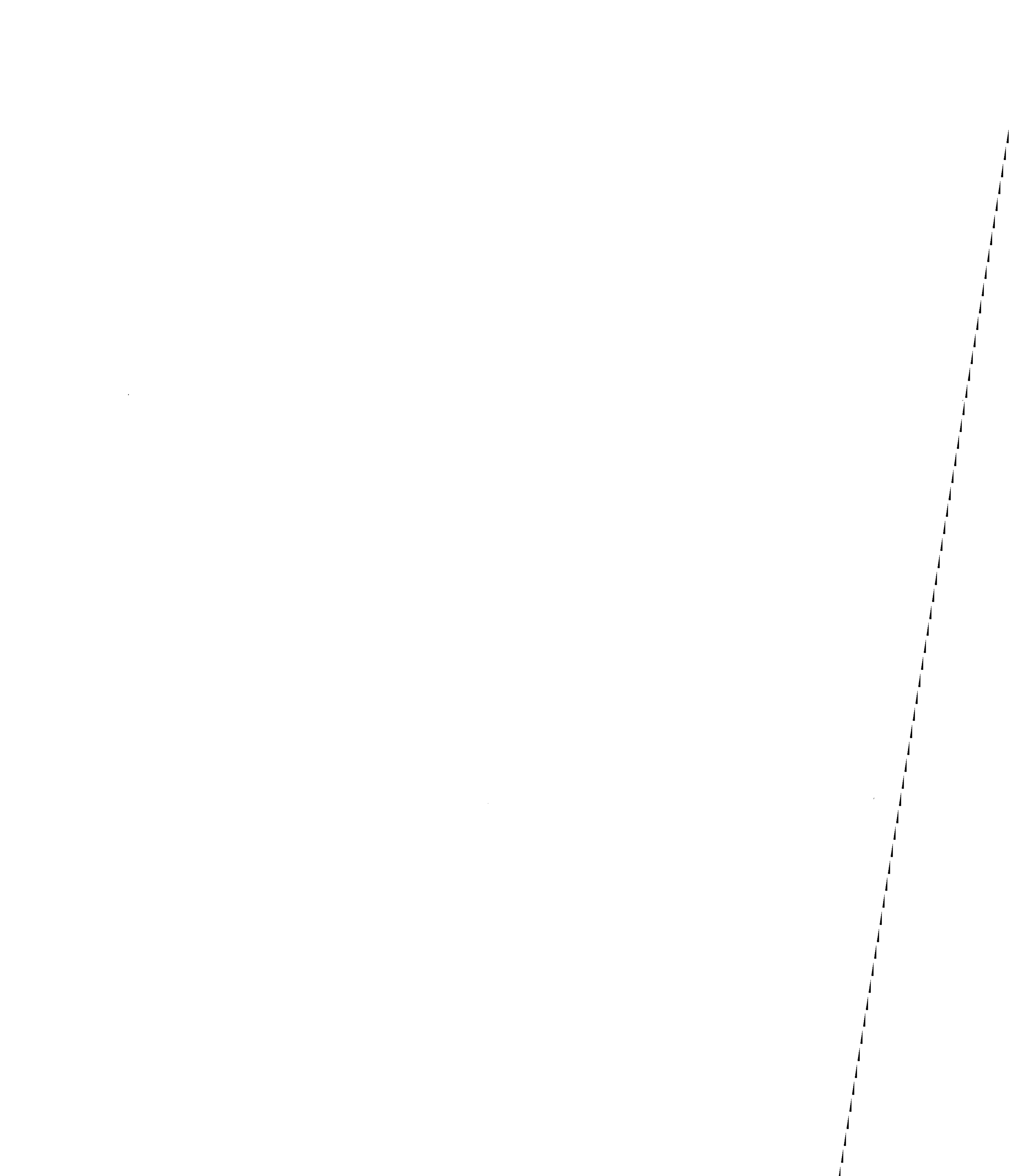
are subject to carbon catabolite repression. It was found that expression of the *qa-x* gene was strongly affected by catabolite regression, more so than the other *qa* genes. This was shown by a 20-fold increase in *qa-x* mRNA when a culture was shifted to a carbon limiting growth condition (Tyler and Geever, unpublished data). This suggested that a preferred carbon source may directly effect repression of *qa-x* transcription (Giles et al., 1991). However, it was also suggested that the product of *qa-x* is itself involved directly in affecting catabolite repression. This is supported by the comparison of *qa-x* to a gene (GAM1) implicated in carbon-regulated dephosphorylation of the *GAL4* activator. The gene *qa-x* was found to have 31% amino acid identity to the GAM1 gene, suggesting homology (Giles et al., 1991). If *qa-x* plays a similar role to GAM1 it has yet to be determined. Therefore, the function of the gene *qa-x* still remains unknown.

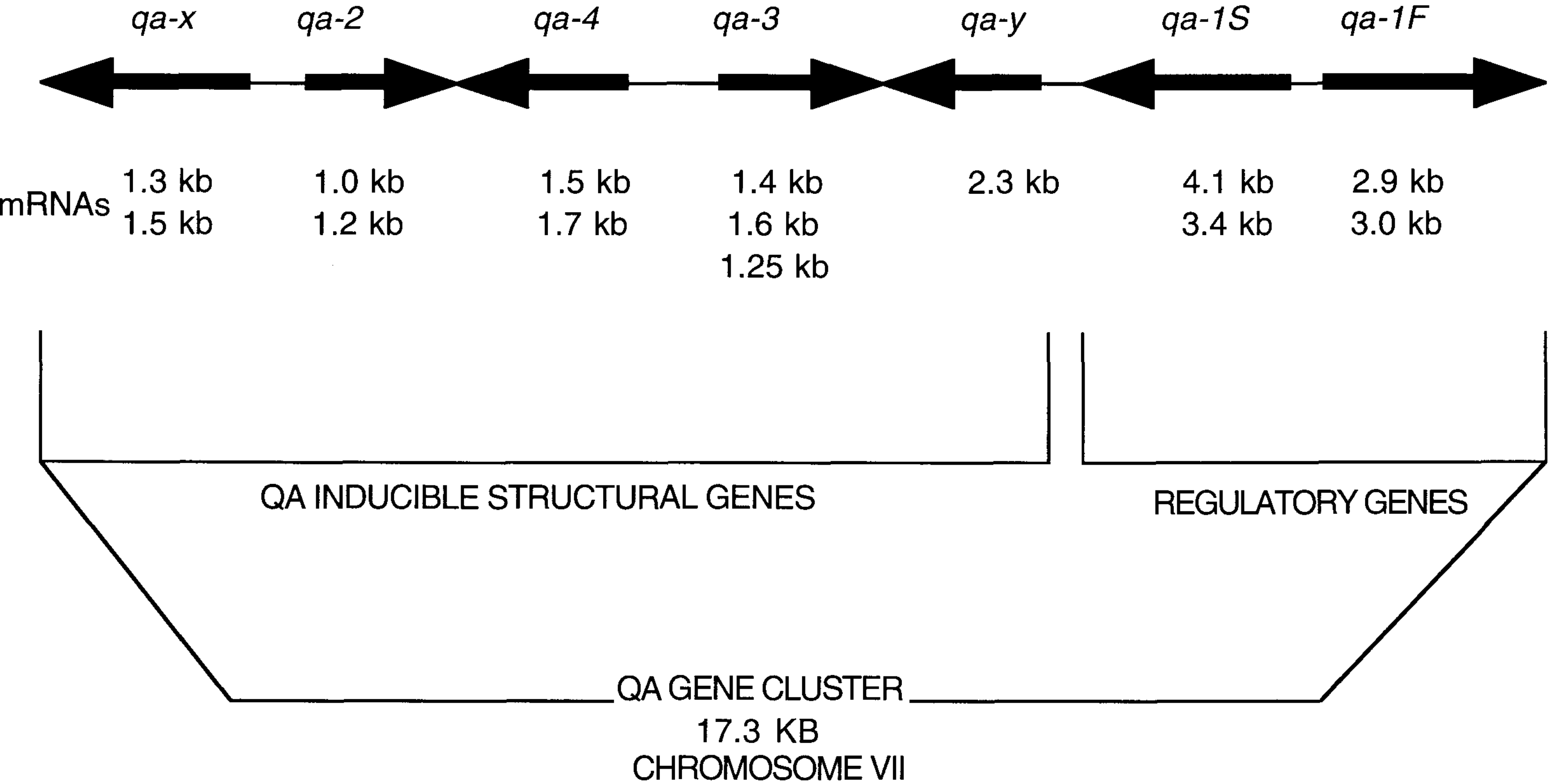
Finally, by using transformation experiments, Northern blot analysis, SI nuclease mapping and nucleotide sequencing the structure of the *qa* gene cluster has been determined (Giles et al., 1985; Geever et al., 1989). The seven *qa* genes were found to cover approximately 17.3 kb of DNA on chromosome VII (Figure 5). The locations of each gene and lengths of their mRNAs was also established (Geever et al., 1989) (Figure 5). The direction of transcription for each gene has also been established and the genes were found to be divergently transcribed in pairs (*qa-x/qa-2*, *qa-4/qa-3*, and *qa-1S/qa-1F*)

with the unpaired *qa-y* gene separating the structural pairs from the regulatory pair (Figure 5).

XII. Mechanisms of the *qa-1S* Repressor Protein

It was found using DNA sequence analysis that the *qa-1S* gene encodes a protein of 918 amino acids (Huiet, 1983; Huiet and Giles, 1986; Geever et al., 1989). Evidence to support the thought that the *qa-1S* gene encodes a repressor protein was seen when a deletion of the gene caused constitutive transcription of all the *qa* genes at high levels (Case et al., 1992). Studies using the two classes of *qa-1S* pleiotropic mutations (noninducible and constitutive) showed the possible location of two functional domains within the repressor protein (Huiet and Giles, 1986). The semidominant noninducible (*qa-1S*$^{-}$) class of mutants all contained a missense mutation which caused these mutants to encode a functional repressor that acted as a superepressor. The location of these mutations (between codons 627 and 743) showed that this domain of the repressor protein interacted with the inducer quinic acid (Huiet and Giles, 1986). Here these mutant superepressor proteins failed to bind the inducer quinic acid and caused them to remain bound to its target. However, an alternative thought believes that these *qa-1S*$^{-}$ mutations affect the affinity of the repressor for the activator protein, as in the comparable GAL80^{S} mutations in yeast (Salmeron et al., 1990).





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
1.25 kb
2.3 kb
4.1 kb
3.4 kb
2.9 kb
3.0 kb
QA INDUCIBLE STRUCTURAL GENES
REGULATORY GENES
QA GENE CLUSTER
17.3 KB
CHROMOSOME VII

The constitutive (*qa-1S^c*) mutants were the result of a frameshift or nonsense mutation, which caused these mutants to encode inactive repressors. These mutations appeared to show that the carboxy terminus of the repressor protein interacts with the target. Experiments using overexpressed repressor protein produced in baculovirus showed that the *qa-1S^c* repressor protein failed to bind to the DNA itself (Geever and Baum, unpublished data). This along with other evidence (Giles et al., 1985; 1987) suggested that the target for the repressor protein is the activator protein.

XIII. Mechanisms of the *qa-1F* Activator Protein

It was found using DNA sequence analysis that the *qa-1F* gene encodes a protein of 816 amino acids (Huiet, 1983; Geever et al., 1989). Experiments have shown that *qa-1F* mRNAs of *Neurospora* wild-types are produced at basal levels in the absence of quinic acid. However, a 50-fold increase was observed upon quinic acid induction (Giles et al., 1985). Additional experiments using noninducible *qa-1F*$^-$ mutations resulted in noninduced transcription of all the *qa* genes at low basal levels, like uninduced wild-type (Avalos, Geever, and Case, unpublished data). Genetic and molecular studies (Patel and Giles, 1985) have also shown that the *qa-1F* activator protein plays a positive role in transcription of all the *qa* genes, including itself (autoregulation).

On the basis of certain studies (Geever et al., 1987; 1989; Beri et al., 1987; Salmeron and Johnston, 1986; Avalos, Geever, and Case, unpublished data) at least four functional domains within the activator protein have been identified, when compared to the *A. nidulans* activator. These are: a DNA-binding domain, a dimerization domain, a transcriptional activation domain, and a domain for interaction with the *qa* repressor.

The DNA-binding domain was localized to the first 183 amino acids (Baum et al., 1987). Within this region a 28 amino acid sequence containing a six cysteine motif shows conservation with several lower eukaryotic activator proteins (Baum et al., 1987; Pfeifer et al., 1989). Direct evidence was obtained which implicated this conserved segment in DNA binding (Geever et al., 1987; 1989, and unpublished data).

The second domain occurs over a broad central segment. Studies using *qa-1F* noninducible and inducible mutants indicated that alterations in this region affected DNA binding. However, within this domain is a yet identified segment between codons 296 and 562 which did not bind to DNA. This segment is believed to contain residues needed for protein dimerization (Giles et al., 1991).

The third domain is located at the carboxy terminus of the activator and contains mainly acidic residues. This region is believed to be implicated in interactions with transcriptional factors by comparison to a similar region in the *GAL4* activator (Ma and Ptashine, 1987a).

The final fourth domain is a region that overlaps with the acidic region in the carboxy terminus. It is proposed that this region interacts with the *qa* repressor protein. Experiments which exchanged the C-terminus of the *N. crassa* activator with that of *A. nidulans* found that when this chimeric activator was transformed into *N. crassa* modest levels of transcription was seen. These transformants grew slowly on quinic acid, which said that the chimeric was capable of activating transcription. However, transcription was found to be constitutive and not inducible (Avalos, Geever, and Case, unpublished data). These results suggested that the carboxy terminus of the activator was involved in interactions with the repressor protein. This was supported by the finding that a deletion of this carboxy terminus produced a mutant with constitutive activity greater than (20%) that of an induced wild-type (Giles et al., 1991). Evidence supporting the mechanisms of the activators function came from studies using genetic analysis of mRNA transcription and chromatin structure in the *qa* gene cluster. These studies provided evidence that the activator protein interacts at specific sites in the 5' flanking region of the *qa* genes (Baum and Giles, 1985; 1986; Geever et al., 1983; 1986). From these studies, a 16 base-pair (bp) sequence element, found one or more times 5' to each of the *qa* genes, was identified as a potential binding site for the activator protein. Evidence for the activator binding DNA was found in overexpressing the *qa-1F* gene in a baculovirus expression vector (Miller et al., 1986). This overexpressed *qa-1F* activator protein was used in DNA

binding and DNase I footprinting experiments. These identified the precise location of 14 sites in the cluster, each characterized by a conserved, symmetrical 16 bp sequence (GGRTAARYRYTTAYCC) to which the activator bound (Buam et al., 1987; Geever et al., 1989). Of particular interest was the finding of a single binding site in the common 5' region of the two regulatory genes. This suggested bidirectional control and supported the findings for activator autoregulation and transcriptional control of repressor gene expression by the activator.

XIV. Comparisons Between the Quinic Acid (*qa*) Gene Cluster of *Neurospora crassa* and the Quinic Acid Utilization (*qut*) Gene Cluster of *Aspergillus nidulans*

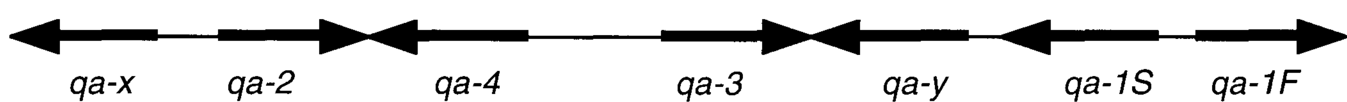
Comparative studies of the quinic acid pathways in *A. nidulans* and *N. crassa* revealed many similarities and differences between the two. First, the regulation of the pathway in *A. nidulans*, which is controlled by the *qutA* activator protein and the *qutR* repressor protein, seem to be analogous to the regulation caused by the *qa-1F* activator protein and the *qa-1S* repressor protein in the *N. crassa* pathway. However, when the sequence of the *qut* regulatory proteins (Beri et al., 1987; Geever, unpublished data) was compared to the *qa* regulatory proteins they were found to diverge substantially. The amino acid identity of the two activators was only 25%, and 50% between the two repressors.

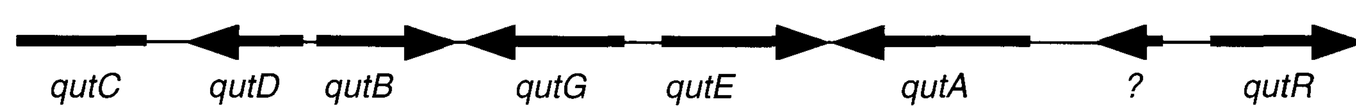
Despite this divergence, the functional domains in both regulatory pairs appear to be conserved. Next, when the *qutD* gene product of *A. nidulans* was compared to the *qa-y* gene product of *N. crassa* 61% amino acid identity was seen, which suggested that *qutD* also encoded a quinic acid permease. Also, the *qutG* gene product of *A. nidulans* showed 68.5% amino acid identity to the *qa-x* gene product of *N. crassa*, and both were found to encode quinate-inducible messages of unknown function. Two organizational features of both clusters stand out: (1) their genes are arranged as divergently transcribed pairs; and (2) structural and regulatory genes occupy separate regions of the cluster. Eventhough the genes of both remained clustered, their gene orders are different (Grant et al., 1988; Hawkins et al., 1988) (Figure 6). Not all the same pairs of genes are divergently transcribed in the two, and the order of the regulatory genes in *A. nidulans* is inverted (Figure 4). Finally, the gene of unknown function between the two *qut* regulatory genes (Figure 6) appears not to be under quinic acid regulation and *N. crassa* does not posses this gene.

XV. Comparisons Between the Quinic Acid (*qa*) Gene Clusters of *Neurospora* Species

With the detection of *qa* catabolic enzymes in other fungi (Ahmed and Giles, 1969; Berlyn and Giles, 1972) comparative studies of the *qa* gene cluster were initiated. Now with all of the information gathered on the *qa* gene cluster of *N. crassa*

Neurospora
QA cluster





Aspergillus
QUT cluster

more detailed comparative studies could be done on different *Neurospora* species. One such study, (Asch et al., 1991) compared the *qa* genes of various heterothallic (different nuclei) and homothallic (same nuclei) *Neurospora* species. It was found that the *qa* gene cluster of *N. crassa* (heterothallic) was highly conserved in various species of *Neurospora*. However, there were many restriction fragment length polymorphisms that distinguished *N. crassa* from the homothallic species. Despite this difference, the gene organization of the cluster remained highly conserved (*qa-x, qa-2, qa-4, qa-3, qa-y, qa-1S,* and *qa-1F*). With the amount of conservation observed in the various species focused then turned to examine if the mechanisms controlling expression of the *qa* genes in both heterothallic and homothallic species were similar.

To determine if the same control circuits operate in homothallic and heterthallic species, Asch et al. (1991) measured *qa-2* gene expression in *N. africana* (homothallic) under non-inducing conditions (no carbon source), inducing conditions (quinic acid alone), and catabolite repression conditions (quinic acid and dextrose). Results showed at least a 1,500-fold induction of the *qa-2* gene in the presence of quinic acid over basal levels compared to a 2,000-fold in *N. crassa*. Under catabolite repression, *N. africana* showed a 3-fold reduction in expression of *qa-2* verse a 65-fold reduction in *N. crassa*. It was thought that this difference might be due to specific sequence differences between the homothallic species and *N. crassa*.

To examine this the intergenic region between *qa-x* and *qa-2* of *N. africana* (homothallic) was sequenced and compared to its counterpart in *N. crassa* (Asch et al., 1991). An earlier study (Asch and Case, unpublished data), found that the *N. africana qa-1F* binding domain was identical to the *N. crassa* domain (GGRTAARYRYTTATCC). This seemed to state that *N. africana* employs the same binding sites as *N. crassa* for activation. Indeed, it was found that all four binding sites for the activator protein located in the *qa-x-qa-2* intergenic region of *N. crassa* could be aligned with those of *N. africana*, eventhough the *N. africana* sequence (1088 bp) was smaller than the *N. crassa* region (1194 bp) (Asch et al., 1991).

Since sequence analysis provided no conclusive evidence to the differences of the *qa* expression between species under carbon catabolite repressing conditions, Asch et al. (1991) examined whether the control circuits expressing the *qa* genes in *N. crassa* would operate in the presence of *N. africana* sequences. To do this the *qa-x-qa-2* intergenic region of *N. crassa* was replaced with the *qa-x-qa-2* intergenic region of *N. africana*. Using this transformant, *qa-2* gene expression was measured under non-inducing conditions (no carbon source), inducing conditions (quinic acid alone), and catabolite repression conditions (quinic acid and dextrose). Results showed increased expression of the *qa-2* gene, which were approximately 70% of those seen in wild-type *N. crassa*, under inducing conditions. Furthermore, under catabolite repression conditions, the transformed strains showed over 100-fold

repression of the *qa-2* gene, which were highly comparable to wild-type *N. crassa*. The latter result suggested that any sequence differences between the *N. crassa* and *N. africana qa-x-qa-2* intergenic region had no impact on catabolite repression of the *qa* genes (Asch et al., 1991).

XVI. Two Regulatory Circuits Regulate the Quinic Acid (*qa*) Gene Cluster of *Neurospora crassa*

The expression of the *qa* genes appear to be controlled by two levels of genetic regulation. The first regulatory circuit controlling transcription of the *qa* genes is mediated by the interactions of the *qa-1S* and *qa-1F* proteins in response to quinic acid. This is supported by the findings that uninduced wild-type and mutants (*qa-1S*$^-$ and *qa-1F*$^-$) which were grown in the presence or absence of quinic acid both contained only small amounts of *qa* mRNAs. However, constitutive *qa-1S*^{C} mutants grown in the absence of quinic acid contained elevated levels of *qa* mRNAs (Giles et al., 1985). These findings together, showed that *qa* gene expression is controlled at the transcriptional level by the *qa-1S* and *qa-1F* gene products, and is regulated by the presence or absence of the inducer, quinic acid (Patel et al., 1981; Huiet, 1984). It has been shown that in the presence of the inducer, quinic acid, transcription of all the *qa* genes is increased 50- to 1,000-fold by the action of the activator. This is also seen in the galactose (*GAL*) system in yeast. Here the inducer, galactose, induces transcription of the

GAL4 activator gene, which in turn induces transcription of the *GAL* genes (*GAL1,-7,* and-*10*).

In the absence of the inducer all of the *qa* genes are transcribed at low basal levels. This is attributed to the interaction of the *qa-1S* repressor protein with the *qa-1F* activator protein, which in turn inhibits activator function (Geever et al., 1989; Giles et al., 1991). Again, this is also seen in the *GAL* system. Here in the absence of the inducer, the GAL80 repressor product interacts with the GAL4 activator product to inhibit activator function.

The second regulatory circuit, which is superimposed on the first, acts to repress *qa* gene transcription in the presence of a preferred carbon source, such as glucose or dextrose. It has been shown that wild-type *N. crassa* when grown in the presence of quinic acid and a preferred carbon source, such as glucose, have a reduced level of *qa* gene transcription compared to wild-type *N. crassa* grown on quinic acid alone. The mechanism by which this apparent catabolite repression is acting to repress *qa* transcription is unknown. However, evidence from the *GAL* system of *S. cerevisiae* (Flick and Johnston, 1990; Johnston et al., 1994) provides three possible explanations to this repression. The first is that the *qa-1F* activator protein may not be able to bind to the activator sites when in the presence of a preferred carbon source, which represses transcription of the *qa* genes. This is believed to be due to protein modification, proteolysis, or direct repression of the *qa-1F* gene expression. The second is the possible

interactions of carbon repressors with sequences 5' to the various *qa* genes which act to block transcription while in the presence of a preferred carbon source. However, the presence of such sequences within the *qa* gene cluster have not yet been identified. The third possible mechanism might be the direct repression of the *qa-1F* gene. This repression would in turn repress the other *qa* genes by limiting the amount of the *qa-1F* activator protein.

In order to isolate the mechanism of carbon repression, *N. crassa* strains carrying a complete deletion of the *qa-1S* gene were examined. These strains displayed increased levels of *qa* expression while in the absence of quinic acid (Case et al., 1992). These mutants also showed slightly repressed *qa-x, qa-2* and *qa-4* gene expression and highly repressed *qa-3, qa-y,* and *qa-1F* gene expression while in the presence of glucose (Asch and Case, unpublished data). This suggests that each gene of the *qa* gene cluster may be regulated by different mechanisms, or that carbon catabolite repression acts on the *qa-1F* gene. Evidence for this type of repression was not seen when the *qa-x-qa-2* intergenic region of *N. africana* was compared to its counterpart in *N. crassa* (Asch et al., 1991). However, *qa-2* was shown to not be dramatically affected by carbon repression directly. Therefore, since *qa-1F* seems to be directly affected by a preferred carbon source, the *qa-1S-qa-1F* intergenic region of *N. africana* will be examined and compared to its counterpart in *N. crassa* to see if it contains sequences which may play a role in carbon catabolite repression of the *qa*

genes, in the presence of a preferred carbon source.

MATERIALS AND METHODS

Materials

I. Ethanol was purchased from Aaper Alcohol and Chemical Company, Shelbyville, KY; isopropanol was purchased from Baxter Healthcare Corporation, McGraw Park, IL; restriction endonucleases [*Eco*R1 (10 U/ul), *Bam*H1 (10 U/ul), *Kpn*I (10 U/ul), *Sac*I (10 U/ul), *Sma*I (10 U/ul)], T4 DNA ligase (1 U/ul), DIG *Taq* DNA Sequencing Kit for Standard and Cycle Sequencing, DIG DNA Labeling and Detection Kit, Blocking reagent, disodium 3-(4-methoxyspiro {1,2-dioxetane 3,2'(5'chloro)tricyclo[3.3.1.1.^{3,7}]decan}-4-yl) phenyl phosphate [CSPD], anti-digoxigenin-AP fab fragments, acrylamide, bis-acrylamide, and positively charged nylon membranes were purchased from Boehringer Mannheim, Indpls, IN; bacto-trypton, bacto-agar and yeast extract were purchased from Difco Laboratories, Detroit MI; Polaroid film, developer and replenisher, fixer and replenisher and maleic acid were purchased from Eastman Kodak Co., Rochester, NY; agarose was purchased from EM Science, Cherry Hill, NJ; ethidium bromide [EtOH], 85% phosphoric acid [H_3PO_4], sodium citrate and acetic acid [HOAc] were purchased from Fisher Scientific, Fair Lawn, NJ; *PERFECTprep* Plasmid DNA Kit, and PCR SELECT-II Spin Columns were purchased from 5 Prime———> 3 Prime, Inc., Boulder, Co; restriction endonucleases [*Pst*I (15 U/ul), *Hind*III (15 U/ul), *Xho*I (15 U/ul)], isopropyl-ß-D-thiogalactoside [IPTG],

5'bromo-4-chloro-3-indoyl-ß-D-galactopyranoside [X-gal], ethylenediaminetetraacetic acid-disodium salt [EDTA], sodium dodecyl sulfate [SDS] and ammonium persulfate [AMPS] were purchased from International Biotechnologies, Inc., New Haven, CT; chloroform and phenol were purchased from J.T. Baker Chemical Co., Phillipsburg, NJ; calcium chloride [$CaCl_2$] and magnesium chloride [$MgCl_2$] were purchased from Mallinckrodt, Inc., Paris, KY; QIAGEN 100 Tips were purchased from QIAGEN Inc., Chatsworth, CA; ELUTIP-D columns were purchased from Schleicher & Schuell, Keene, NH; ampicillin, sodium chloride [NaCl], potassium acetate [KOAc], 3-N-morpholino-propanesulfonic acid [MOPS], Trizma base, RNase A, octyl phenoxy polyethoxyethanol [Triton X-100], urea, polyoxythlene-sorbitan monolaurate [Tween 20], N,N,N',N'-Tetramethylethylenediamine [TEMED], sigmacote, lithium chloride [LiCl], and N-lauroylsarcosine were purchased from Sigma Chemical Co., St. Louis, MO;sodium hydroxide [NaOH] was purchased from VMR, Media, PA.

Methods

II. Strains and Media

Recombinant plasmids were transformed into *Escherichia coli* strain JM101. *E. coli* JM101 was cultured in Luria Broth [LB] (1% bacto-tryptone; 0.5% yeast extract; 1% NaCl). Transformants were selected on Luria Agar [LA_{100}] (1% bacto-

tryptone; 0.5% yeast extract; 1% NaCl; 1.5% bacto-agar) using ampicillin (100 ug/mL), 100 uL IPTG (200 mM) and 50 uL X-gal (2%). Transformants were picked to LB_{100} [LB; ampicillin (100 ug/mL)].

Single stranded phages were infected into *E. coli* strain JM101. *E. coli* JM101 was cultured in LB. Transformants were selected on YT plates (0.8% bacto-tryptone; 0.5% yeast extract; 0.5% NaCl; 1.5% bacto-agar) using 100 uL IPTG (200 mM) and 50 uL X-gal (2%). Transformants were picked to 2xYT (1.6% bacto-tryptone; 1% yeast extract; 0.5% NaCl) containing 200 uL of *E. coli* JM101 cells.

III. pBluescript II KS (+/-) Phagemid

This 2,961 basepair (bp) phagemid, which was derived from pUC19, was purchased from Stratagene, La Jolla, CA. Located within this phagemid is a portion of the *lacZ* gene, which confers blue/white color selection of recombinants in the presence of IPTG and X-gal. It also contained a multiple cloning site (MCS) which was oriented in such a way that cloning into this region resulted in the disruption of *lacZ* translation producing white recombinants. Finally it contained an ampicillin resistance gene which was utilized in antibiotic selection of recombinants.

IV. Single-Stranded M13mp18

This phage is described by Messing and Vieira (1982).

V. Restriction Digest of the Vector

Approximately five micrograms of either vector was placed in the presence of sterile water, enzyme of choice, and that enzymes 10X reaction buffer and incubated at 37°C overnight. Next a small sample was run on a 1% agarose gel. If the DNA sample was properly digested, then 200 uL of neutralized phenol was added to the eppendorf tube. The sample was then centrifuged at 12,000-16,000 xg for 10 minutes. The top layer was then removed and placed in a clean eppendorf tube. Next, 200 uL of chloroform was added and then this was also centrifuged at 12,000-16,000 xg for 5 minutes. The top layer was removed again and placed into a clean eppendorf tube and 200 uL of isopropanol was added. This was then centrifuged at 12,000-16,000 xg for 15 minutes. After this centrifugation the liquid was decanted, the DNA pellet was washed two times in 80% ethanol and then allowed to dry for 10-20 minutes. The pellet was then resuspended in 20 uL of 1X TE buffer (0.001 M Trizma base; 0.001 M EDTA, pH 8.0) and stored at -20°C for later use.

VI. Agarose Gel Electrophoresis

The condition of the DNA used in all of the experiments was analyzed by electrophoresis. Here, DNA fragments were resolved by a 1% agarose gel electrophoresis in 1X tris-phosphate (TPE) buffer [0.08 M Trizma base; 0.005 M EDTA; 85% H_3PO_4 (1.679 mg/mL)]. The gel was stained with EtBr (50 mg/mL) and the DNA was visualized on a transilluminator

VII. Preparation of Fragments

Fragments were prepared by digesting approximately 10 ug of the plasmid pR1 (supplied by Kim Rutledge) with the restriction enzyme(s) of choice. The fragments were then resolved by a 1% agarose gel electrophoresis. The fragment of interest was then cut from the gel and placed into a dialysis bag filled with 0.5X tris-acetate [TAE] buffer (0.04 M Trizma base; 0.2 M NaOAc; 0.002 M Na_2EDTA, pH 7.9). The bag containing the fragment was then placed into an electrophoresis chamber and electrophoresised for 45 minutes. After this time period the polarity was switched and the sample was electrophoresied for 1 minute to release any DNA bound to the inside of the bag. The liquid, containing the DNA, was then drawn out of the bag and placed into an eppendorf tube. Next, an Elutip column was primed by passing 3 mL of high salt buffer (1 M NaCL; 0.02 M Trizma base; 0.001 M EDTA; pH 7.4) and then 3 mL of low salt buffer (0.02 M NaCL; 0.02 M

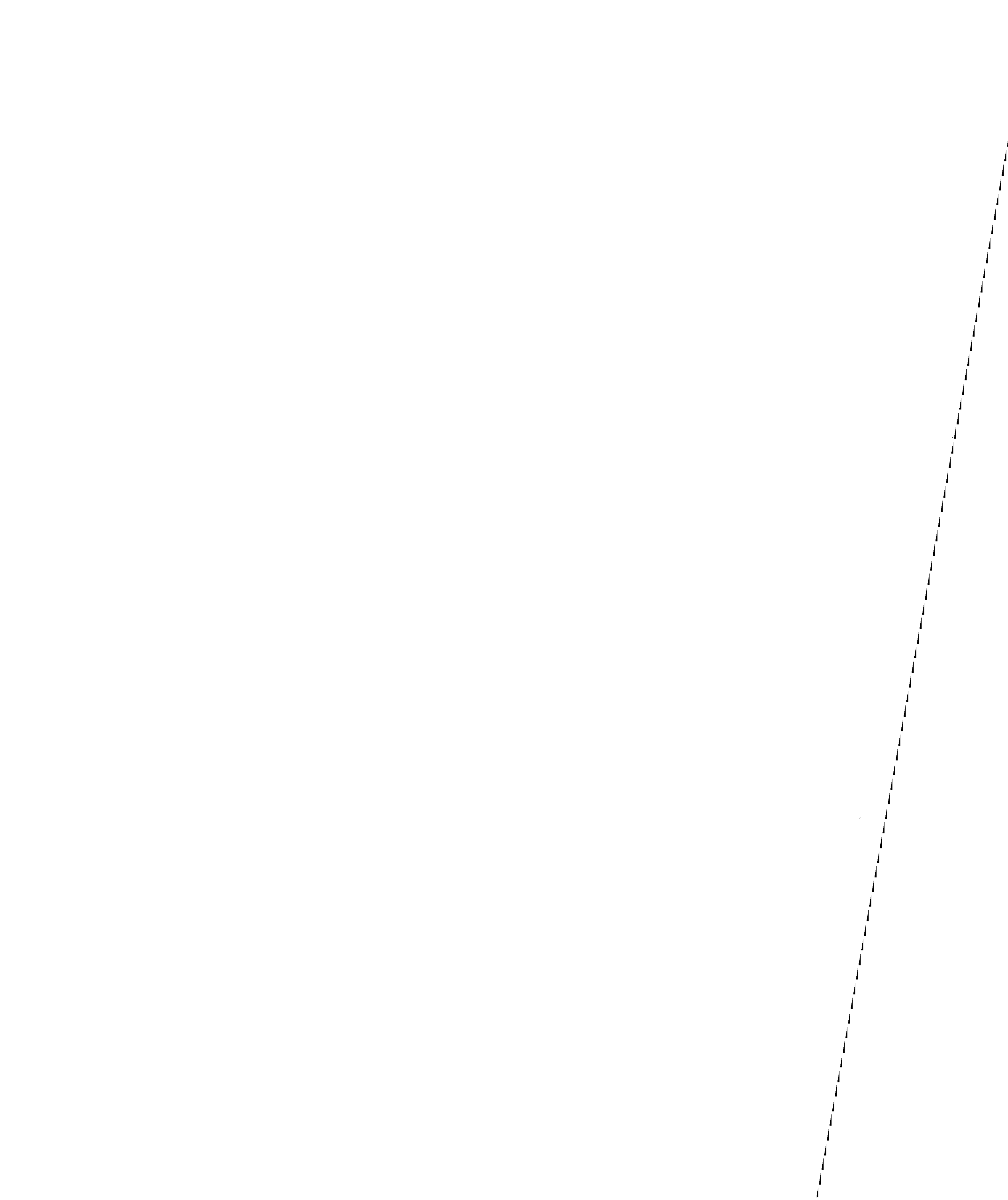
Trizma base; 0.001 M EDTA; pH 7.4) through the column. Next, the DNA collected from the dialysis bag was then passed over the primed column as described by the manufacturer. Finally, the DNA was eluted from the column with 400 uL of high salt buffer and collected (Figure 7). The DNA was then washed and extracted as above (see section V).

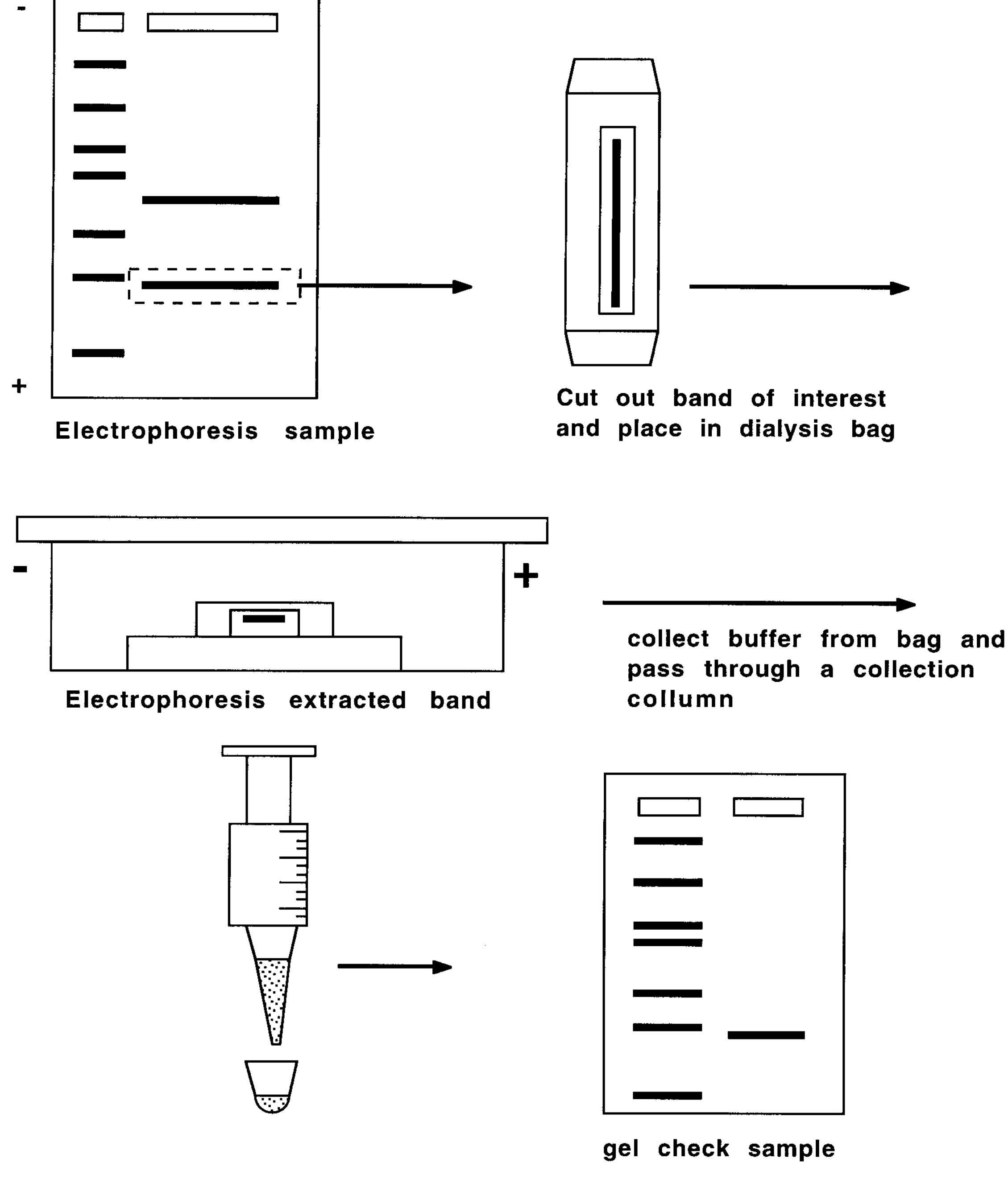
VIII. Construction of Recombinant Plasmids and Phages

Recombinant DNA was constructed by ligating an isolated fragment from the plasmid pR1 with the vector (pBLUESCRIPT; M13) which was cut with the same enzyme(s). This was done by placing 5 uL of the fragment, 5 uL of the vector, 3 uL of 10X ligase buffer, 16 uL of sterile water, and 1 uL of T4 DNA ligase into a sterile eppendorf tube. This mixture was then incubated at 15°C overnight.

IX. Transformation of *E. coli* JM101 with pBluescript DNA

First 2 mL of LB was inoculated with JM101 and incubated at 37°C overnight. The following day, 50 mL of LB was inoculated with 0.5 mL of the overnight growth and incubated at 37°C for 3 hours. These cells were then placed on ice for 10 minutes and then centrifuged (10K; 4°C) for 10 minutes. The resulting pellet was resuspended in 15 mL of 0.1

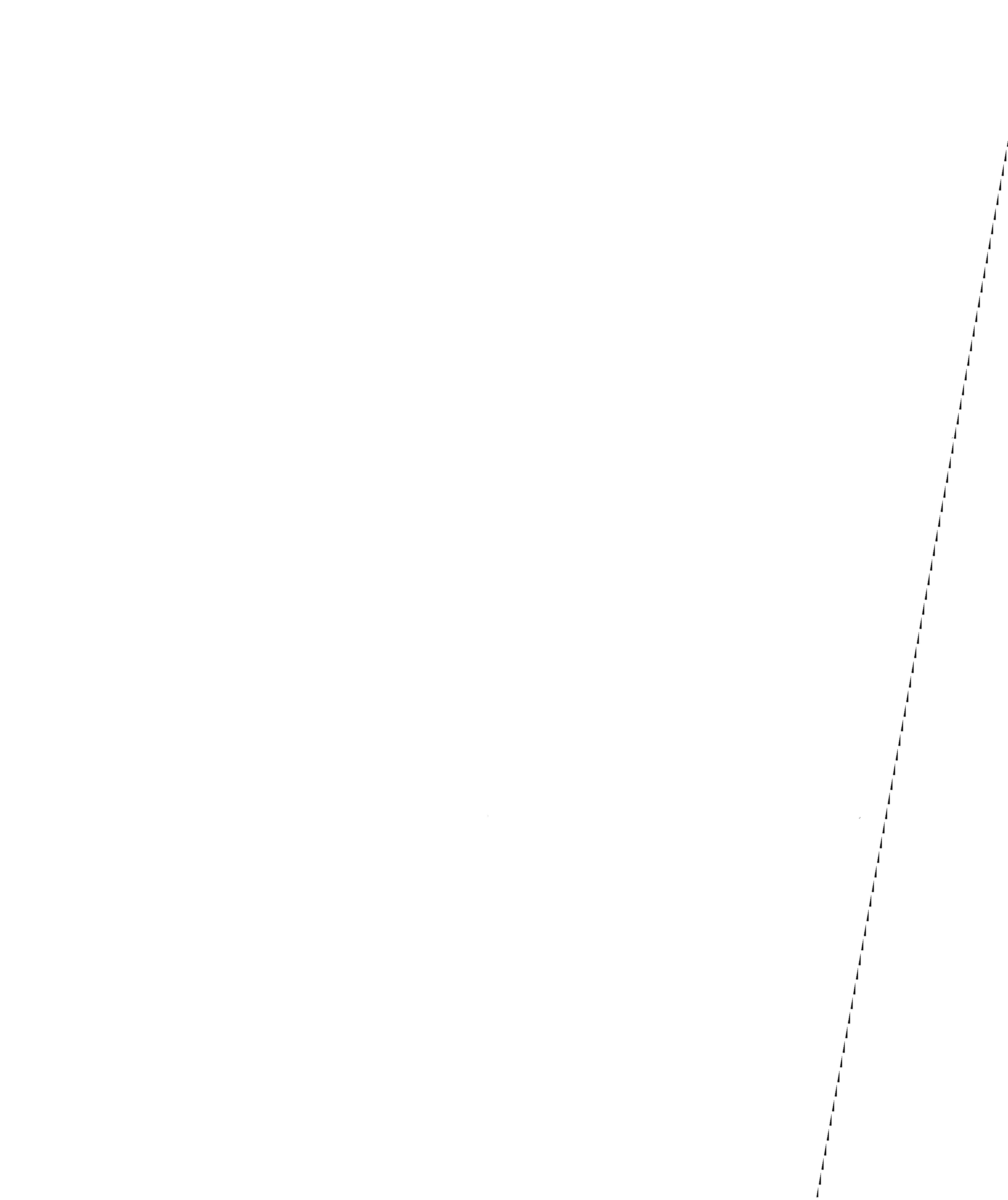


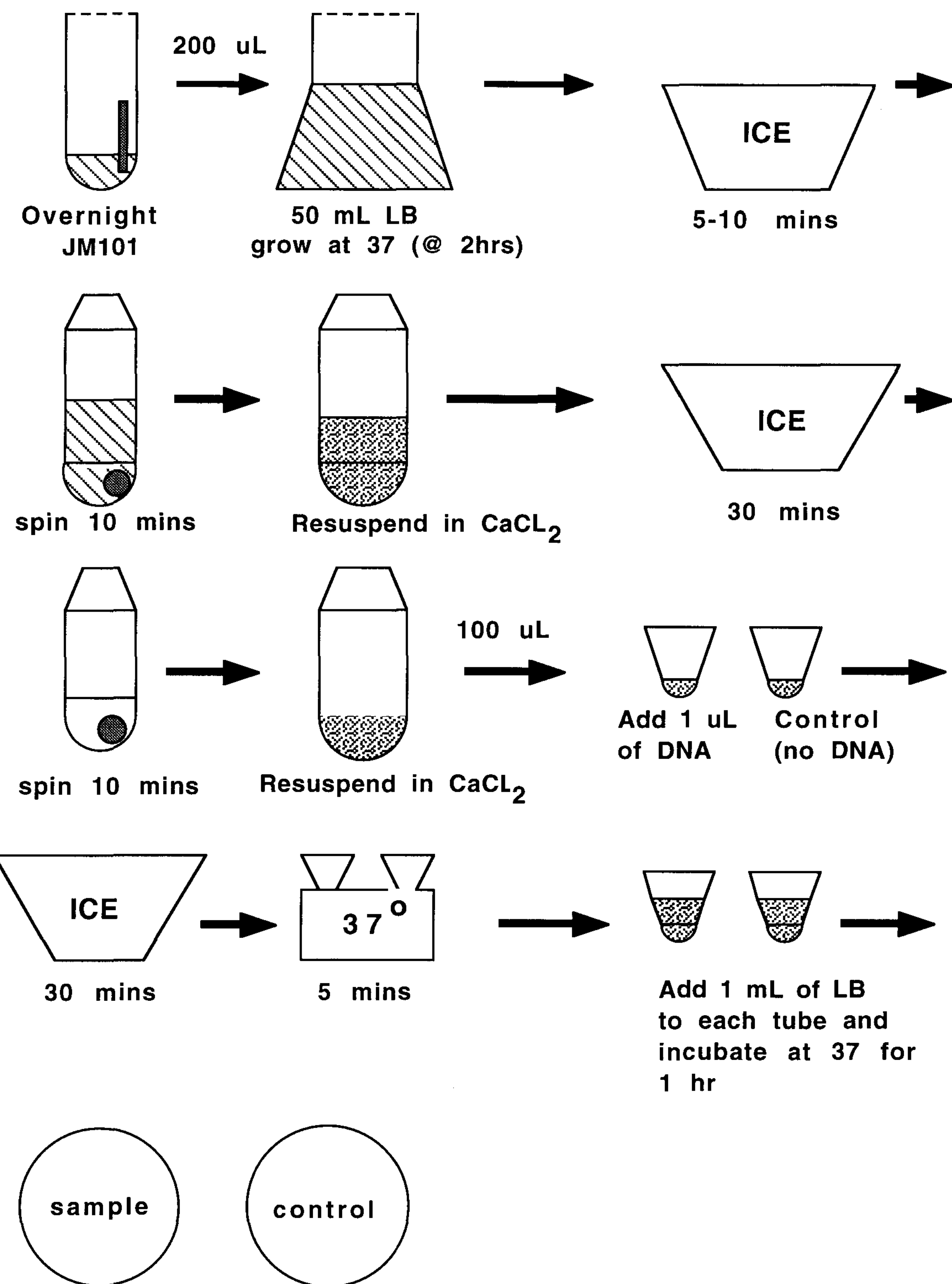

-
+
Electrophoresis sample
Cut out band of interest
and place in dialysis bag
-
+
Electrophoresis extracted band
collect buffer from bag and
pass through a collection
collumn
gel check sample

M $CaCl_2$ and placed on ice for 30 minutes. Next, the cells were centrifuged (10K; 4°C) for 10 minutes and the pellet was resuspended in 0.5 mL of $CaCl_2$. Then, 100 uL of these competent cells were then dispensed into two eppendorf tubes, one being the control and the other the experimental. To the experimental tube 10 uL of the ligation mix was added and no DNA was added to the control tube. These tubes were then incubated on ice for 30 minutes and transferred to a 37°C heat block for 5 minutes. Next, 1 mL of LB was added to each tube and they were incubated at 37°C for 60 minutes. After this incubation 100 uL of the transformation mixes were spread onto selective media (LA_{100}; ampicillin; IPTG; X-gal). These plates were then incubated at 37°C overnight (Figure 8).

X. Transformation of *E. coli* JM101 with M13 DNA

First, 2 mL of LB was inoculated with JM101 and incubated at 37°C overnight. The following day, 50 mL of LB was inoculated with 0.5 mL of the overnight growth and incubated at 37°C for 3 hours. Next, 5 mL of these cells were then collected and placed back at 37°C while the rest of the cells were placed on ice for 10 minutes. These cells were then centrifuged (10K; 4°C) for 10 minutes. The resulting pellet was then resuspended in 15 mL of $CaCl_2$ and placed on ice for 30 minutes. After the incubation, the cells were centrifuged (10K;




200 uL
Overnight
JM101
50 mL LB
grow at 37 (@ 2hrs)
ICE
5-10 mins
spin 10 mins
Resuspend in $CaCL_2$
ICE
30 mins
spin 10 mins
Resuspend in $CaCL_2$
100 uL
Add 1 uL
of DNA
Control
(no DNA)
ICE
30 mins
37°
5 mins
Add 1 mL of LB
to each tube and
incubate at 37 for
1 hr
sample
control
Plate 200 uL of each and grow at 37° overnight

4°C) for 10 minutes and the pellet was resuspended in 0.5 mL of $CaCl_2$. Then, 100 uL of these competent cells were then dispensed into two sterile eppendorf tubes, one being the control and the other the experimental. To the experimental tube 10 uL of the ligation mix was added and no DNA was added to the control tube. These tubes were then incubated on ice for 30 minutes and then transferred to a 37°C heat block for 5 minutes. While the samples were on ice, YT soft agar was melted and into two falcon tubes 100 ul IPTG and 50 uL of X-gal was dispensed, and labeled control and experimental. After the heat shock, 3 mL of YT soft agar, 35 uL of the ligation mix, and 200 uL of JM101 lawn cells was added to the experimental tube. While to the control tube only 3 mL of YT soft agar, and 200 uL of lawn cells were added. Each of these mixtures were then spread out on YT plates and incubated at 37°C overnight.

XI. Direct Electrophoresis of M13 DNA

White transformants were picked to 2 mL of 2XYT and incubated at 37°C overnight. While, one blue plaque was also picked to 2 mL of 2XYT broth and incubated at 37°C overnight. Next, 1.5 mL of the overnight cultures were placed into sterile eppendorf tubes and placed into a microcentrifuge (12,000-16,000 xg) for 5 minutes. The supernatants were then drawn off and placed into sterile eppendorf tubes while the pellets were discarded. Next, 50 uL of each supernatant were then placed into sterile eppendorf tubes along with 5 uL of 2% SDS

and 5 uL of tracking dye. Each sample was then loaded in a 1% agarose gel and electrophoresied to view for shifts in the samples.

XII. Isolation of Single-Stranded M13 Phages

E. coli JM101 was inoculated in 2 mL of 2XYT and grown at 37°C for 3 hours. One milliliter of this culture was then used to inoculate 50 mL of 2XYT along with 200 uL of the supernatant from section XI, that contained the shift, and incubated at 37°C overnight. This culture was then centrifuged (10K; 4°C) for 10 minutes and the supernatant was collected. The supernatant was then checked that it contained the phage (as in section XI.).

If the phage was present 2 mL of LB was inoculated with *E. coli* JM101 and grown at 37°C overnight. The next day 200 uL of the overnight and 50 uL of the phage suspension was used to inoculate 2 mL of YT and grown overnight at 37°C. Next, 1.5 mL of this culture was transferred into a sterile eppendorf tube and placed into a micro-centrifuge (12,000-16,000 xg) for 5 minutes. Next, 1 mL of the supernatant was transferred into a sterile eppendorf tube along with 200 uL of 27% PEG 8000 and 200 uL of 3.3M NaCl and mixed. The phages were allowed to precipitate for a minimal 15 minutes at room temperature [RT]. This mixture was then placed into a microcentrifuge (12,000-16,000 xg) for 5 minutes. The supernatant was decanted and the sides of the eppendorf tube

were wiped down with a Kimwipe to remove residual liquid. The pellet was then resuspended in 90 uL TE, 10 uL 10X buffer (0.2% Sarkosyl; 0.1 M Trizma base, pH 7.8; 0.01 M EDTA), and 1 uL 5 mg/mL Proteinase K (50 ug/mL Proteinase K; 500 uL glycerol; 500 uL 1X TE). This was incubated at 55°C for 20 minutes, allowed to cool to RT., and 8 uL of 5M NaCl was added. This sample was the extracted one time with phenol and two times with chloroform (as in V.) to remove the PEG. The phages were then precipitated with 2 volumes of isopropanol, washed one time with 80 % EtOH, and allowed to dry. The pellet was then resuspended in 20 uL of 1X TE. Next, 5 uL of this sample was gel checked in a 1% agarose gel and the remainder of the sample was stored at 4°C until needed for sequencing

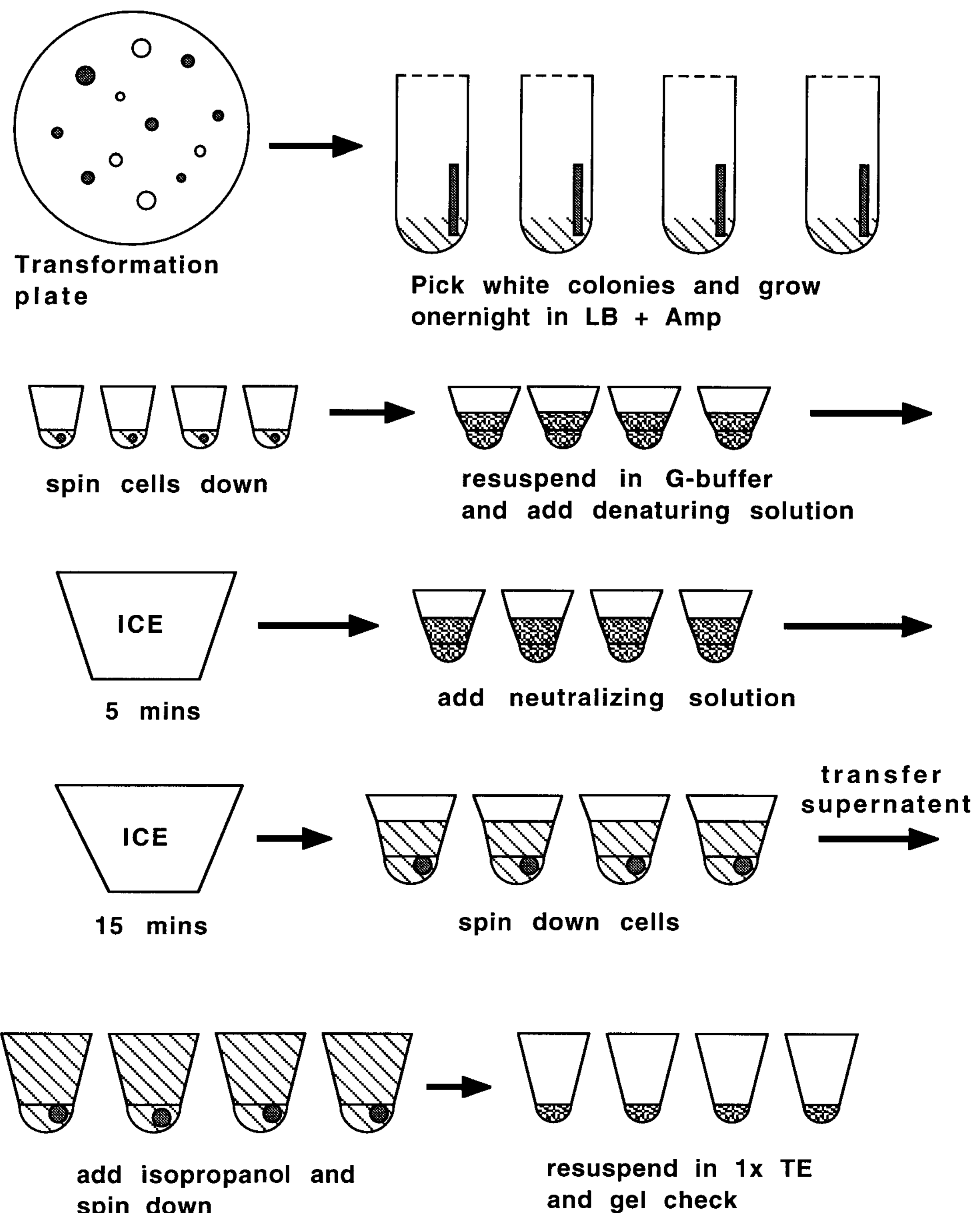
XIII. Isolation of Recombinant Plasmid DNA (Alkaline Plasmid Screen)

White transformants were picked to 2 mL of LA_{100} broth and incubated at 37°C overnight. Then, 1.5 mL of the overnight culture was placed into a sterile eppendorf tube and placed in a microcentrifuge (12,000-16,000 xg) for 15 seconds. The supernatant was decanted and the pellet was resuspended in 200 uL of G buffer (0,05M dextose; 0.025M Trizma base, pH 8.0; 0.01M EDTA, pH 8.0). Next, 400 uL of Denaturing solution (0.2N NaOH; 1% SDS) was added and the tube was inverted several times to mix the solution. The mixture was then placed

on ice for 5 minutes. Next, 300 uL of prechilled Neutralizing solution (3M KOAc; 2M HOAc) was added and the tube was again inverted several times to mix the solution. The mixture was then placed on ice for 15 minutes and then centrifuged (12,000-16,000 xg) for 5 minutes. The supernatant was then transferred to a sterile eppendorf tube, 540 uL of isopropanol was added, and the mixture was mixed well. The tube was then placed into a micro-centrifuge (12,000-16,000 xg) for 5 minutes. The pellet was then washed two times in 80% EtOH and allowed to dry. The pellet was then resuspended in 50 uL of 1X TE. Next, 20 ul of this sample was then gel checked in a 1% agarose gel to observe shifts, while the remainder was stored at 4°C (Figure 9).

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

Transformants (from section IX.) were picked to 2 mL of LA_{100} broth and incubated at 37°C overnight. The following day, 50 mL of LA_{100} broth was inoculated with 500 uL of the overnight culture and incubated at 37°C overnight. The cells were then transferred to a sterile centrifuge tube and placed into an ultracentrifuge (10K; 4°C) for 15 minutes. The pellet was resuspended in 7.5 mL of buffer P1 (100 ug/mL RNase A; 0.05M Trizma base; 0.01M EDTA, pH 8.0). Next 7.5 mL of buffer P2 (0.2M NaOH; 1% SDS) was added and the tube was


Transformation
plate
Pick white colonies and grow
onernight in LB + Amp
spin cells down
resuspend in G-buffer
and add denaturing solution
ICE
5 mins
add neutralizing solution
ICE
15 mins
spin down cells
transfer
supernatent
add isopropanol and
spin down
resuspend in 1x TE
and gel check

inverted gently and incubated on ice for 5 minutes. Finally, 7.5 mL of buffer P3 (3M KOAc, pH 5.5) was added and mixed gently. The sample was then placed on ice for 20 minutes and centrifuged (15K; 4°C) for 30 minutes. The supernatant was then transferred to a sterile centrifuge tube and recentrifuged (15K; 4°C) for 30 minutes. During this centrifugation, a Qiagen tip was equilibrated with 4 mL of buffer QBT (0.75M NaCl; 0.05M MOPS; 15% EtOH, pH 7.0; 0.15% Triton X-100) and allowing it to empty by gravity flow. The supernatant, containing the recombinant plasmid, was then applied to the tip allowing the plasmid DNA to bind to the primed resin. Next, the plasmid DNA was washed 2 times using 10 mL of buffer QC (1M NaCl; 0.05M MOPS; 15% EtOH, pH 7.0) each time. The DNA was then eluted with 5 mL of buffer QF (1.25M NaCl; 0.05M Trizma base; 15% EtOH, pH 8.5) and precipitated with 0.7 volumes of isopropanol and centrifuged (13K; 4°C) for 30 minutes. The pellet was then washed in ice cold 70% EtOH and allowed to dry. The pellet was then resuspended in 3 mL of 1x TE and stored at 4°C.

XV. Isolation of Plasmid DNA (*PERFECT* prep Preparation)

Here, isolation was done as described by the manufacturer (*PERFECT* prep Plasmid DNA Kit). The protocol was as follows. First, 2 mL of LB was inoculated with JM101 containing the plasmid of interest and incubated at 37° overnight. Next, 1.5 mL of this bacterial culture was

transferred into a sterile eppendorf tube and centrifuged (12,000-16,000 xg) for 20 seconds. The supernatant was then removed and the pellet was resuspended in 100 uL of Solution I (0.05 M Tris-HCL, pH 7.6; 0.01 M EDTA, pH 8.0; 100 ug/mL RNase A). The cells were then lysed by adding 100 uL of Solution II (0.2 N NaOH; 1.0% SDS) and inverting the tube several times. The mixture was then neutralized by adding 100 uL of Solution III (1.32 M Potassium Acetate, pH 5.2) and inverting the tube vigorously. The mixture was then centrifuged (12,000-16,000 xg) for 30 seconds and the supernatant was then transferred to a *PERFECT* prep spin column in a collection tube. To this solution, 450 uL of *PERFECT* prep DNA Binding Matrix (*PERFECT* prep DNA Binding Matrix Suspension in Guanidine-HCL) was added and mixed by pipetting. The plasmid was then bound by centrifuging the *PERFECT* prep spin column/collection tube assembly (12,000-16,000 xg) for 30 seconds. Next, the filtrate was decanted, and 400 uL of diluted Purification Solution [Purification Solution Concentrate (Tris-CL; NaCL; EDTA) diluted 1:1 in 95% ethanol] was added to the same *PERFECT* prep spin column. This was then centrifuged (12,000-16,000 xg) for 60 seconds. The *PERFECT* prep spin column was then transferred to a new collection tube and centrifuged (12,000-16,000 xg) again for 60 seconds to remove residual Purification Solution. The same *PERFECT* prep spin column was then transferred to another clean collection tube, and the purified plasmid DNA was eluted by adding 50 uL of TE and centrifuging (12,000-16,000 xg) for

60 seconds. Finally, 10 uL of this sample was gel checked to make sure the plasmid was isolated and, the remainder for the sample was stored at 4°.

XVI. Restriction Digest of Recombinant DNA

Recombinant DNA was digested with various enzymes (*Eco*R1; *Bam*H1; *Kpn*I; *Sac*I; *Pst*I; *Hind*III; *Xho*I; and *Sma*I) as described by the manufacturer. Here, 10 uL of recombinant DNA was incubated at 37°C for 90 minutes in the presence of 16 uL of sterile water, 3 uL of the appropriate 10X reaction buffer, and 1 uL of enzyme.

XVII. Sequencing Reactions for Single-Stranded DNA

First, a primer annealing mixture was prepared as described by the manufacturer (DIG Taq DNA Sequencing Kit for Standard and Cycle Sequencing). This was done by adding 5-10 uL of single-stranded M13 (from section XII.), 2 uL of 10x reaction buffer, 2 uL of DIG-labeled M13/pUC19 forward sequencing primer, 10 uL of sterile water, and 1 uL of *Taq* DNA polymerase (3 U/uL) into a sterile eppendorf tube. Four 300 uL eppendorf tubes, labeled G, A, T, and C, were filled with 2 uL of the appropriate extension/termination mixture. Next, 4 uL of the primer annealing mixture was added to each PCR tube and overlaid with 10 uL of mineral oil. Each tube was then placed in the thermocycler. Here first, the mixture was

denatured by heating at 95°C for 5 minutes. Following this step, the PCR reaction used for the forward primer was as follows, one cycle included; 95°C for 30 seconds, 60°C for 30 seconds, and 70°C for 60 seconds. The reaction cycled 29 times and after amplification the samples were stored at 4°C. To end the reaction 2 uL of formamide buffer was added to each tube.

XVIII. Sequencing Reactions for Double-Stranded DNA

First, a primer annealing mixture was prepared as described by the manufacturer (DIG Taq DNA Sequencing Kit for Standard and Cycle Sequencing). This was done by adding 5-10 uL of double stranded plasmid DNA (from section XV.), 2 uL of 10X reaction buffer, 2 uL of DIG-labeled M13/pUC19 forward or reverse sequencing primer, 10 uL of sterile water, and 1 uL of *Taq* DNA polymerase (3 U/uL) into a sterile eppendorf tube. Next, four 300 uL eppendorf tubes, labeled G, A, T, and C were filled with 2 uL of the appropriate extension/termination mixture. To these, 4 uL of the primer annealing mixture was added and, each was overlaid with 10 uL of mineral oil. Each tube was then placed into the thermocycler. Here first, the mixtures were denatured by heating at 95° for 5 minutes. Following this step, the PCR reaction varied depending on the primer used. For the forward primer, one cycle included; 95°C for 30 seconds, 60°C for 30 seconds, and 70°C for 60 seconds. The reverse primers cycle was, 95°C for 60 seconds, 56°C for 60 seconds, and 70°C for 60

seconds. Both reaction cycled 29 times and after amplification the samples were stored at 4°C. To end the reaction 2 uL of formamide buffer was added to each tube.

XIX. Sequencing Gel Electrophoresis and Contact Blot

An 8% polyacrylamide gel was cast in a mold between two sequencing plates, one of which was treated with siliconizing solution. The PCR products (from sections XVII. or XVIII.) were denatured at 95°C for 5 minutes and transferred to ice. Next, 3 uL of each of the four PCR reactions (G, A, T, and C) were loaded into the wells of the sequencing gel. After electrophoresis (2000V, 29 mAmps, 60 Watts; short run/about 4 hours; long run/about 8 hours) the plate treated with the siliconizing solution was removed. A positively charged nylon membrane, cut to match the size of the run, was then placed on the gel. The plate that was removed was then placed back on the gel along with approximately 20 kilograms of weight. After 20 minutes, the sandwich was disassembled. The DNA, now attached to the membrane was crossliked in a UV crosslinker under optimal conditions set by the manufacturer. The membrane then proceeded to the detection process.

XX. Detection

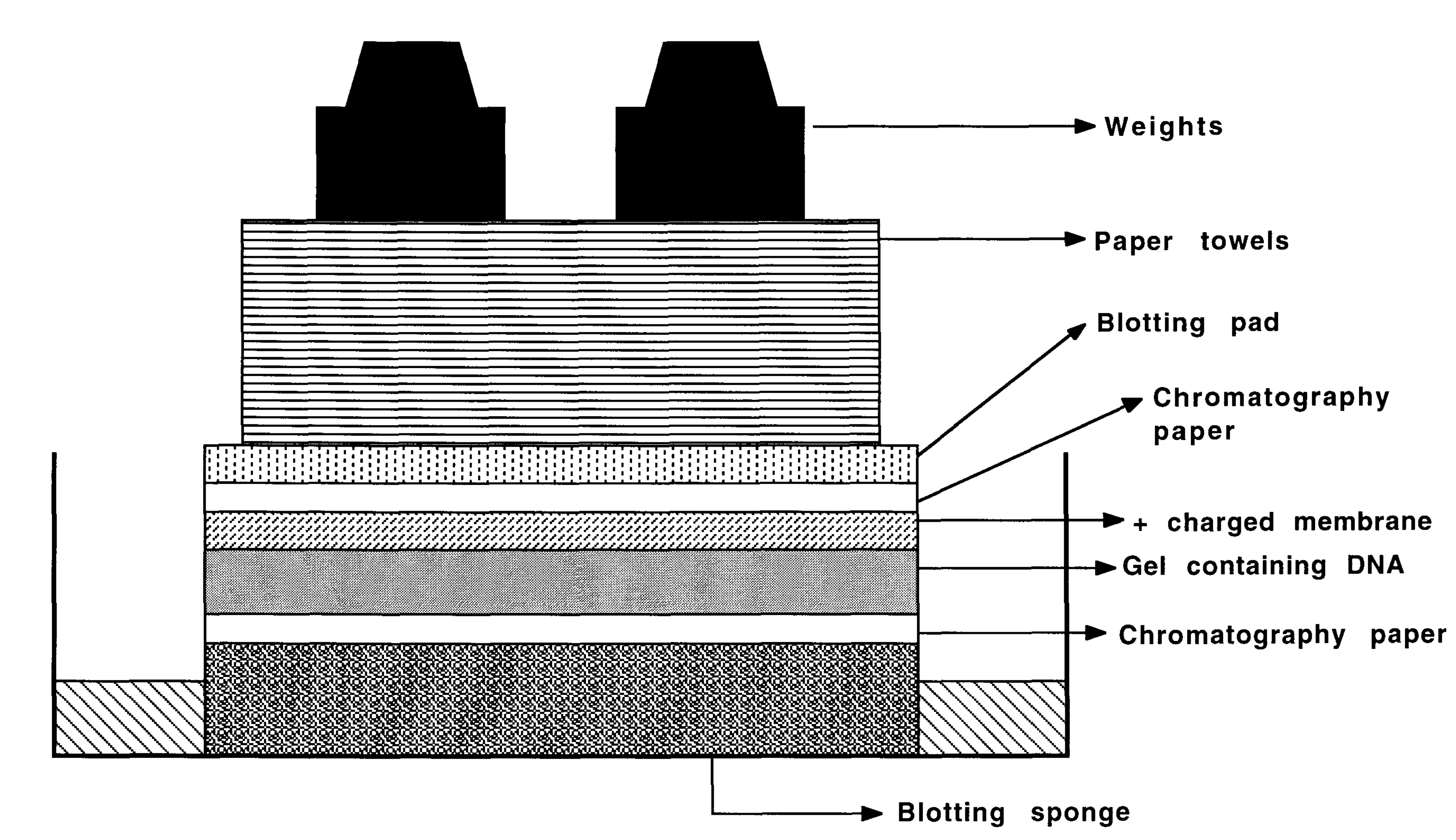
All of the incubations here were performed at RT. First, the membrane was rinsed for 1 minute in 50 mL of washing buffer (Buffer 1: 0.1M maleic acid; 0.15M NaCl, pH 7.5 plus 0.3% Tween 20). The washing buffer was removed and the membrane was incubated for 30 minutes in 50 mL of Buffer 2 (10% Blocking Stock Solution diluted 1:10 in Buffer 1). The Blocking Solution was decanted and 50 mL of antibody solution (anti-DIG-AP conjugate diluted 1:5000 in Buffer 2) was added and incubated for 30 minutes. Next, the antibody solution was removed and the membrane was washed 2 times, 15 minutes per wash, in 50 mL of Washing solution. The membrane was then equilibrated in 20 mL of Detection buffer, or Buffer 3 (0.1M Trizma base; 0.1M NaCl; 0.05M $MgCl_2$, pH 9.5). The membrane was then placed on plastic wrap and 2 mL of a CSPD solution (diluted 1:100 in Buffer 3) was placed on the membrane. The membrane was incubated for 5 minutes at RT. and then the CSPD solution was removed. The membrane was then sealed in a hybridization bag and incubated at 37°C for 15 minutes. The membrane was then exposed to X-ray film for about 3 hours. This film was then exposed to visualize the sequencing reactions.

XXI. Southern Transfer

Recombinant DNA was digested with selected restriction enzymes, and run on a 1% agarose gel (17V for 8 hours) and stained in EtBr (50 mg/mL) to visualize the cut DNA. If the DNA was cut, the gel was incubated at RT. for 10 minutes in 0.5N HCl. The gel was then washed briefly in water, and incubated at RT. for 60 minutes in Denaturing solution (0.5N NaOH; 1.5M NaCl) with gentle shaking. The gel was then placed in Neutralization solution (0.5M Trizma base; 3M NaCl, pH 7.5) for 60 minutes at RT. with gentle shaking. The gel was then blotted overnight to remove the DNA by capillary transfer, and bind it to a positively charged nylon membrane using 20X SSC buffer (3M NaCl; 0.3M Sodium citrate, pH 7.0). See figure 10 for the setup of the transfer.

XXII. DNA Fixation

After the Southern Transfer the membrane was rinsed in 5X SSC (1:4 dilution of 20X SSC buffer) buffer at RT. for 60 seconds. The membrane was then placed on Whatman paper and baked for 60 minutes at 80°C. The membrane was place into a hybridization bag and now ready for hybridization of the probe (see section XXVI.)


Weights
Paper towels
Blotting pad
Chromatography paper
+ charged membrane
Gel containing DNA
Chromatography paper
Blotting sponge

XXIII. Probe Preparation

First, 10 uL of DNA template (supplied by John Troutman) was placed into a PCR tube along with 2 uL of the primer C1, 2 uL of the primer C2, # uL of 10x dNTP's, 3 uL of 10x reaction buffer, and 9 uL of sterile water. This mixture was heated at 95°C for 5 minutes, then 1 uL of *Taq* DNA polymerase was added, and the mixture was overlaid with 10 uL of mineral oil. The tube was then placed into the thermocycler and run under the program PCREX45 (92.5°C for 5 minutes, 45°C for 2 minutes, 72°C for 2 minutes, 92.5°C for 30 seconds; it then cycled 42 times at 45°C for 30 seconds, 72°C for 2 minutes, and 92.5°C for 30 seconds; the reaction was finalized by 45°C for 30 seconds, 72°C for 5 minutes, and 4°C for 1 minute; the mixture was then held at 4°C until needed). The following day, the product was collected and 5 uL was gel checked in a 1% agarose gel to see if the reaction occurred. If the reaction was successful the product was placed through a *PCR* SELECT-II spin column according to the manufactures protocol in order to purify the PCR product.

XXIV. Labeling of the Probe

After the PCR sample was cleaned the DNA was labeled using the DIG DNA Labeling and Detection Kit as describe by the manufacturer. Here, 15 uL of the sample was place into a sterile eppendorf tube and denatured at 95°C for 5 minutes

and then transferred to ice. Next, 2 uL of 10X hexanucleotide mixture, 2 uL of 10X dNTP labeling mixture, and 1 uL of Klenow enzyme were added and the tube was incubated at 37°C overnight. The following day, 2 uL of 0.2M EDTA was added and the DIG-labeled nucleic acid was precipitated with 0.1 volume of 4M LiCl and 2.5-3.0 volumes of cold 70% EtOH. The tube was incubated at -70°C for 30 minutes and then centrifuged (12,000-16,000 xg) for 15 minutes. The liquid was then decanted and the pellet was allowed to dry. The pellet was then resuspended in 50 uL of 1X TE.

XXV. Quantitation of the Probe

Serial 10-fold dilution's of the DIG-labeled control DNA and the generated DIG-labeled experimental probe DNA were prepared as described by the manufacturer (DIG DNA Labeling and Detection Kit). Next, 1 uL of each dilution were spotted onto a positively charged nylon membrane and each corresponding dilution was marked near the appropriated spot. This membrane was then baked at 80°C for 30 minutes to fix the DNA for detection (all solutions used were the same as in section XX. unless noted).

After baking, the membrane was washed for 1 minute in Washing buffer. The membrane was then incubated in Blocking solution for 5 minutes. The Blocking solution was then removed and the membrane was placed in antibody solution and incubated for 10 minutes. After the Blocking solution was

removed, the membrane was then washed two times in Washing buffer, 5 minutes per wash. After then final wash the membrane was placed in Detection buffer and incubated for 2 minutes. The membrane was then removed from the Detection buffer and a Color Substrate Solution (45 uL of NBT; 35 uL of X-phosphate solution in 10 mL of Detection buffer) was added. Color development was then allowed to occur in the dark for 45 minutes. This reaction was then terminated by washing the membrane in sterile water for 5 minutes. Finally, spot intensities of the control and experimental dilution's were compared to estimate the concentration of the experimental probe.

XXVI. Prehybridization and Hybridization

First, 50 mL of Prehybridization solution (5X SSC; 0.1% N-lauroylsarcosine; 0.02% SDS; 1% Blocking Reagent) was added to the bag containing the membrane (from section XXII). The membrane was then incubated at 65°C for 2-3 hours. After the incubation, the Prehybridization solution was collected and 20 mL of the Hybridization solution (contains the DIG-labeled probe from section XXIV.) was added to the bag. The probe was allowed to hybridize overnight at 65°C. The next day, the Hybridization solution was collected and the membrane was removed from the bag. The membrane was then wash two times, 5 minutes each wash, in 2X Wash solution (2X SSC; 0.1% SDS) at RT.. The membrane was then washed two times, 15

minutes each wash, in 0.5X Wash solution (0.5X SSC; 0.1% SDS) at 65°C. The membrane was now prepared for detection as in section XX.

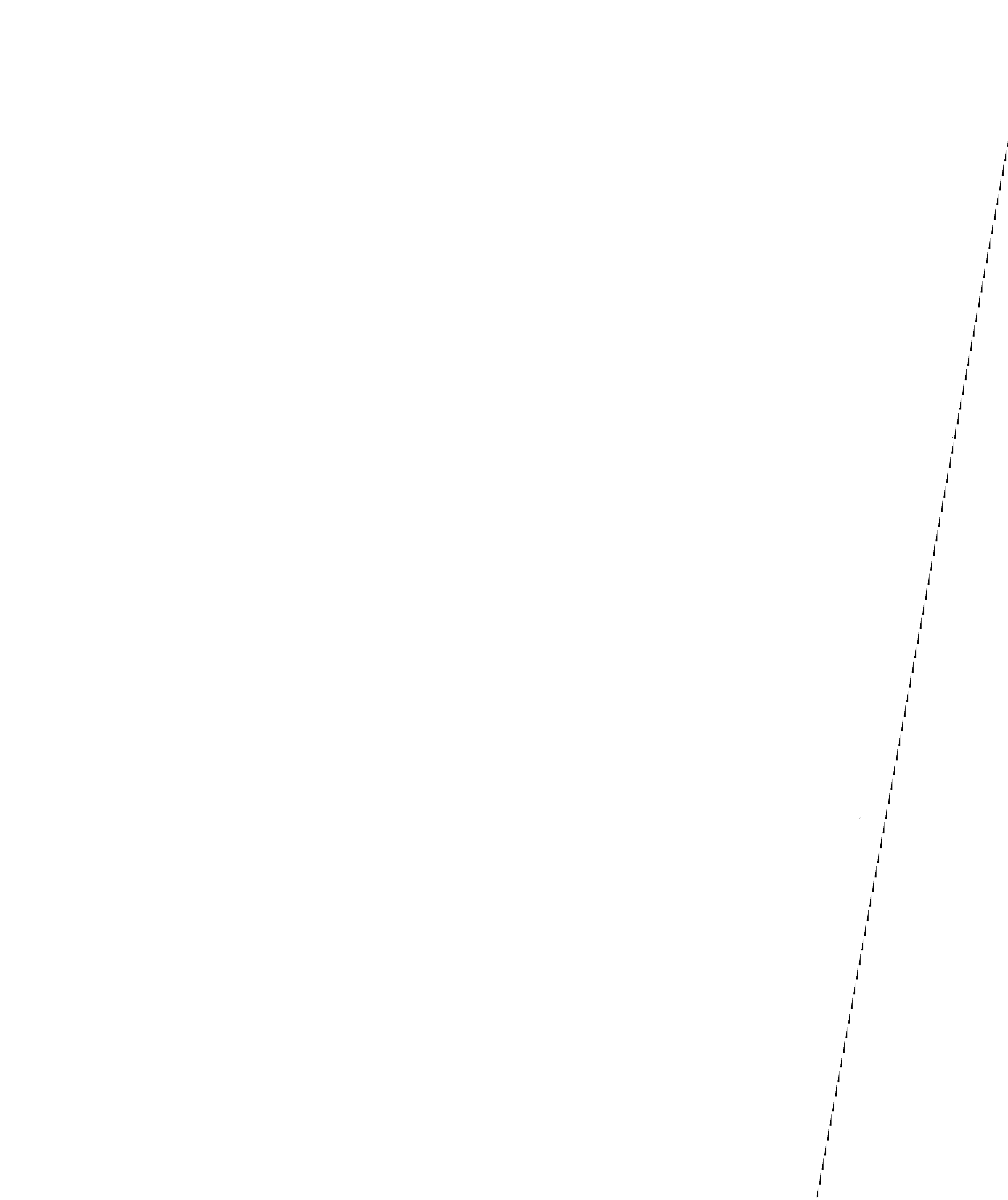
RESULTS

I. Construction of Plasmid pR1

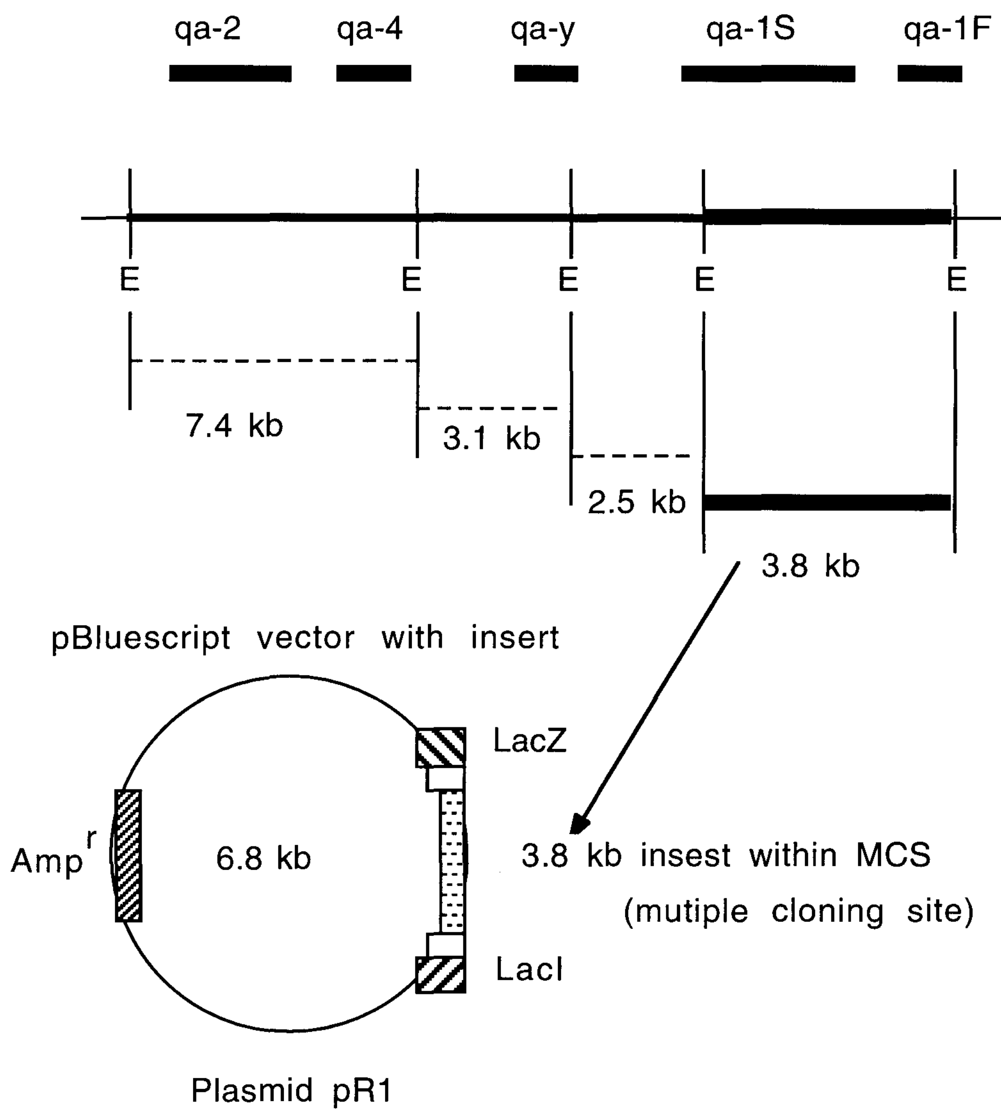
The *qa-1S-qa-1F* intergenic region of *N. africana* was chosen for study based on the role it may play in carbon catabolite repression of the *qa* genes. To start the characterization of the *qa-1S-qa-1F* intergenic region of *N. africana* the lambda clone NA3 was examined. This particular clone was known to contain most of the *qa* gene cluster (Asch, unpublished data) (Figure 11). It was found that when this clone was digested with the restriction endonuclease *Eco*R1, six fragments of various sizes were produced, four of which contained parts of the *qa* gene cluster (Figure 11) (Rutledge, unpublished data). In another study, it was found that the *qa-1S-qa-1F* intergenic region was contained within the 3.8 kb fragment of the NA3 clone, after digestion with *Eco*R1 (Roys, unpublished data). Thus, this particular fragment was isolated, ligated into an *Eco*R1 digested pBluescript vector, and transformed into *E. coli* JM101. The resulting subclone was designated plasmid pR1 (Rutledge, unpublished data) (Figure 11).

II. Characterization of Plasmid pR1

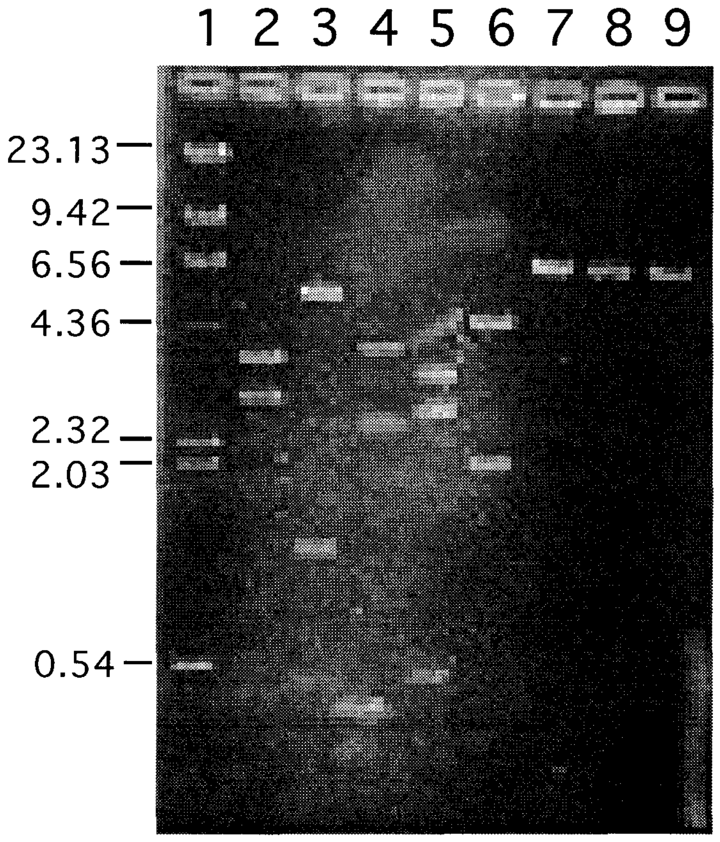
To further characterize the *qa-1S-qa-1F* intergenic region of *N. africana*, the subclone pR1 was examined. Here, a series



NA3 Clone



of different restriction digests were performed to produce a restriction map of the plasmid pR1. The restriction enzymes used all contained a unique (or one) restriction site within the pBluescript vector. Therefore, if a restriction site for a particular enzyme also existed within the 3.8 kb insert, multiple fragments would be seen after digestion. The sizes of these fragments could then be estimated by comparison to a size standard (lambda DNA cleaved with *Hind*III) (Figure 12, lane 1). The restriction enzyme *Eco*R1 produced two fragments of approximately 3.8 kb and 2.9 kb in size (Figure 12, lane 2). This result confirmed the presence of both the insert (3.8 kb) and the vector (2.9 kb) whose sizes were already known. The enzyme *Xho*1 generated two fragments, one of 5.370 kb and the other 1.330 kb in length (Figure 12, lane 3). These two fragments suggested that a *Xho*1 restriction site existed within the insert. The enzyme *Pst*1 produced three fragments of 3.490 kb, 2.710 kb and 0.500 kb in size (Figure 12, lane 4). With the production of three fragments this suggested that the insert contained two *Pst*1 restriction sites. Next, the enzyme *Sac*1, also generated three fragments of 3.360 kb, 2.800 kb and 0.540 kb in size (Figure 12, lane 5). This also suggested that the insert contained two restriction sites for the enzyme *Sac*1. The enzyme *Bam*H1 generated two fragments of the lengths, 4.570 kb and 2.130 kb (Figure 12, lane 6). This result suggested, like *Xho*1, that only one restriction site for *Bam*H1 could be found within the insert. The restriction enzymes *Kpn*1 (Figure 12, lane 7), *Hind*III (Figure 12, lane 8), and *Sma*1

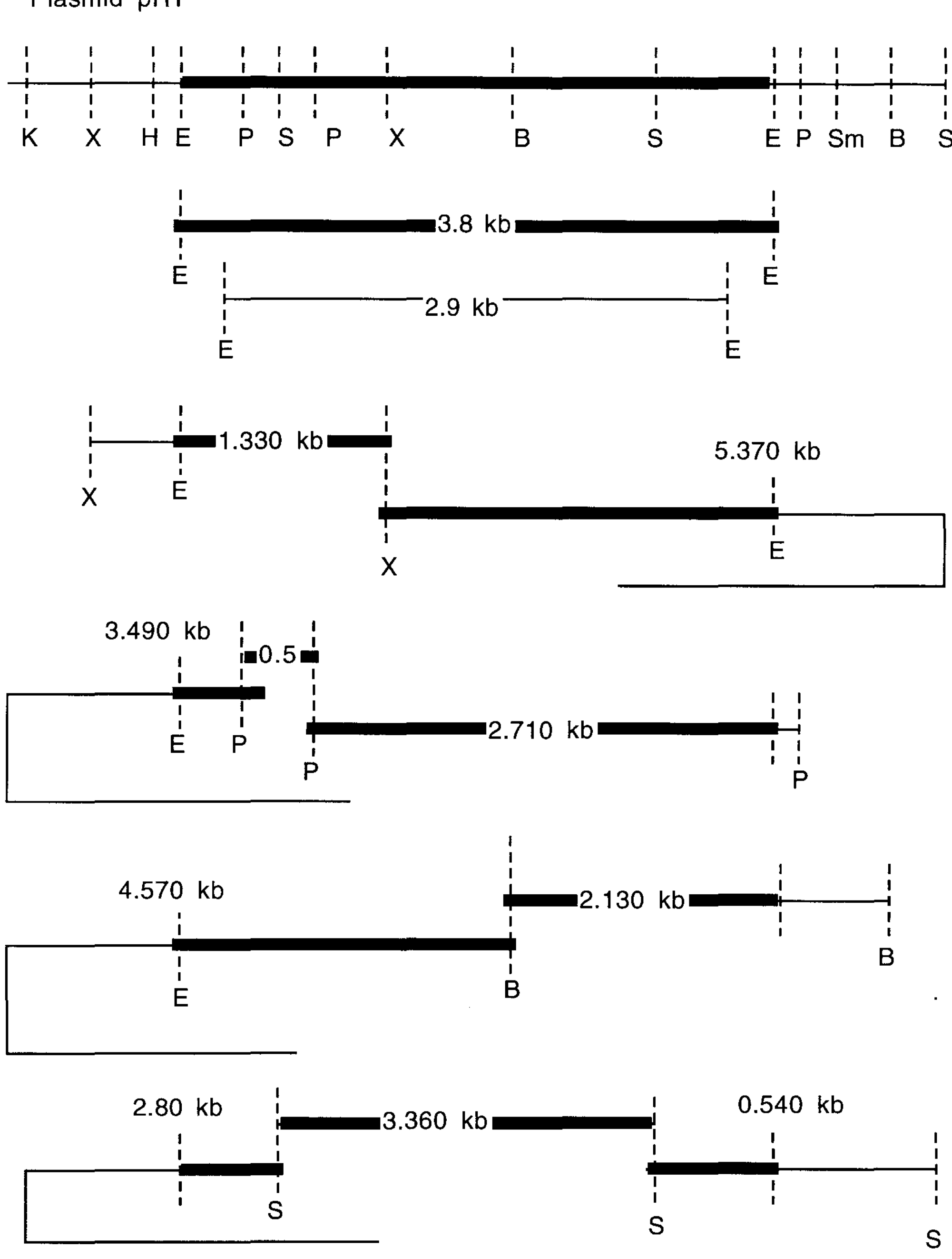

1 2 3 4 5 6 7 8 9
23.13
9.42
6.56
4.36
2.32
2.03
0.54

(Figure 12, lane 9) each only generated one fragment. These results suggested that no restriction site for these enzymes existed within the insert. Now based on this information a preliminary restriction map was deduced (Figure 13).

Localization of the *qa-1S-qa-1F* Intergenic Region

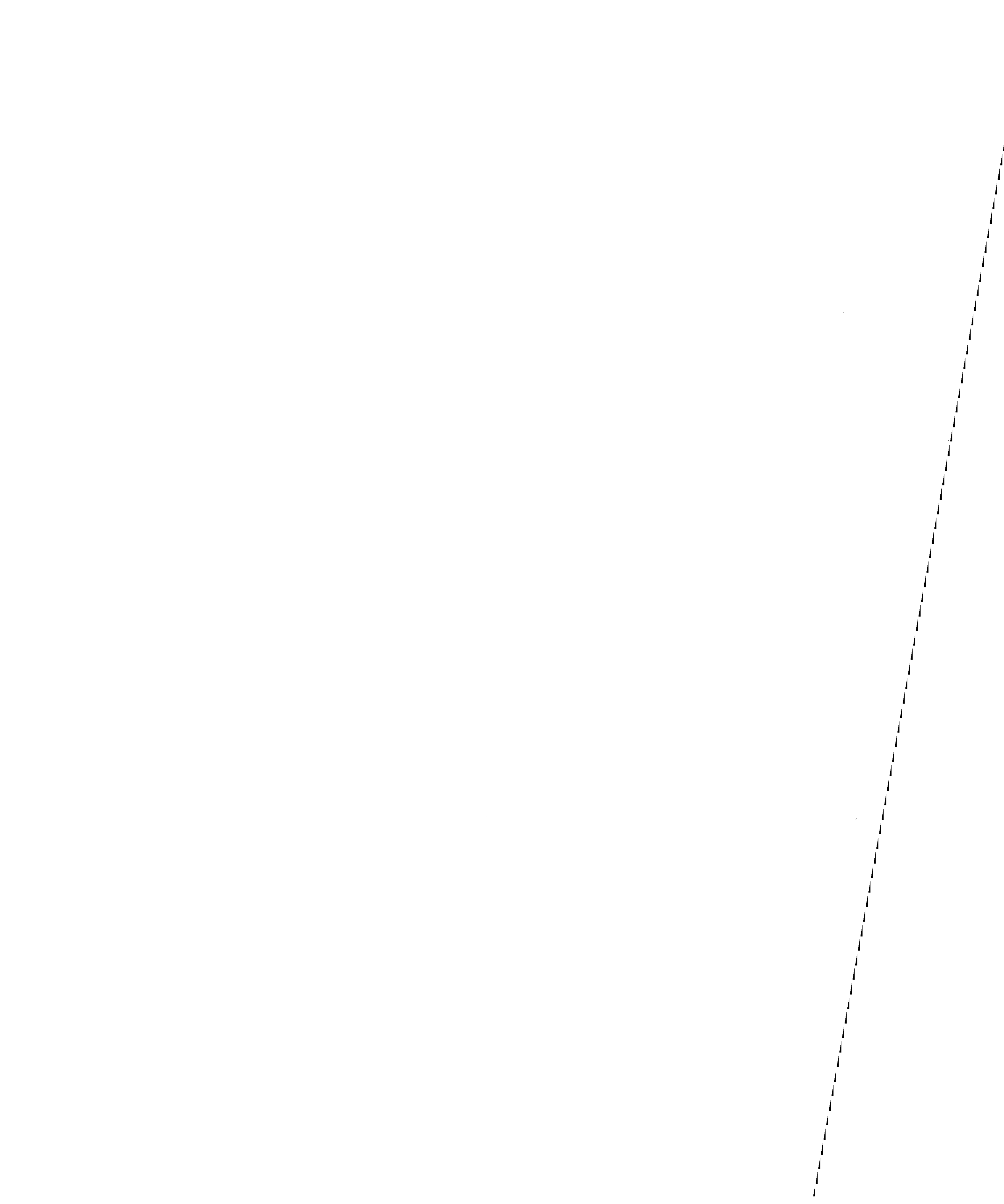
III. Southern Blot Analysis of Plasmid pR1

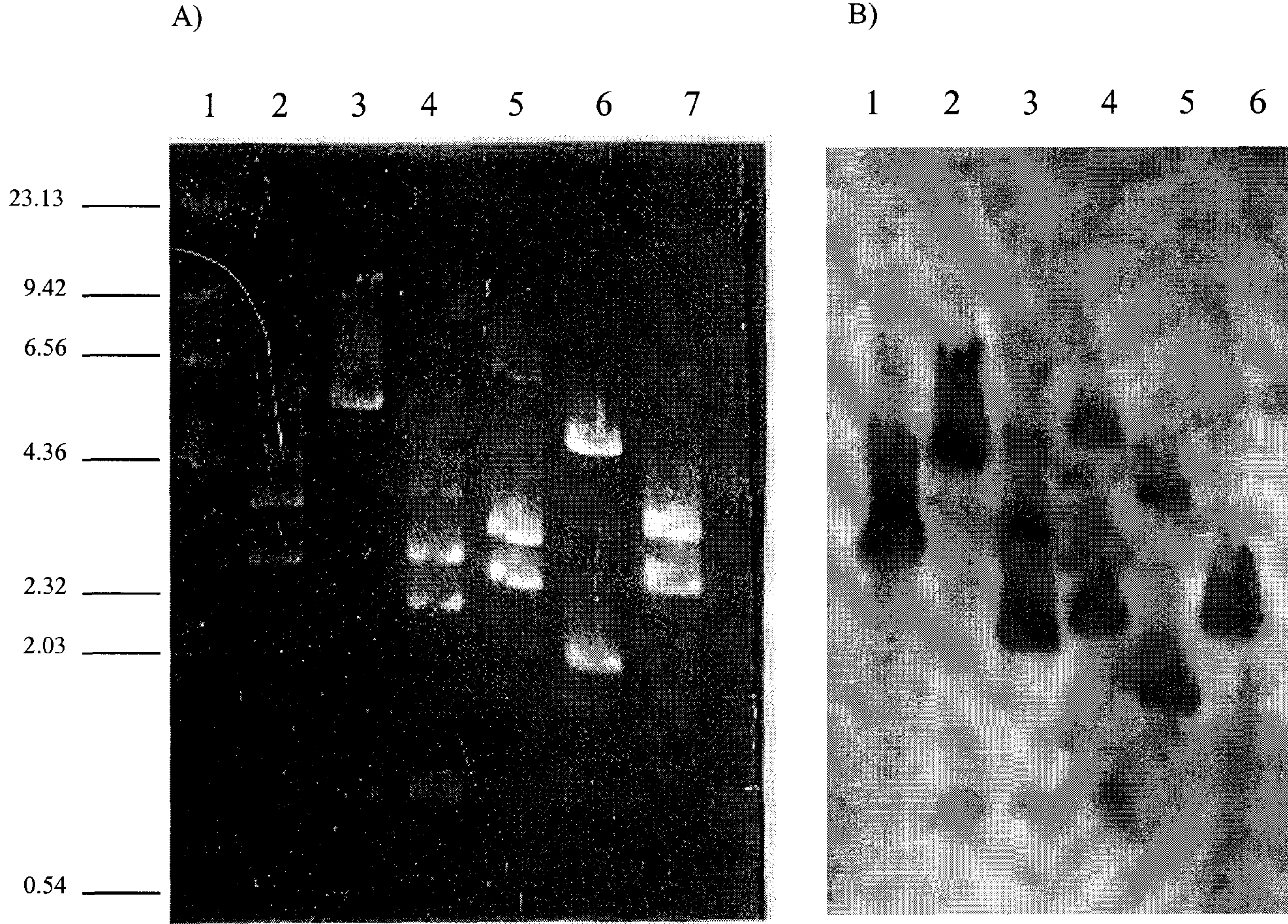
To localize the *qa-1S-qa-1F* intergenic region contained within the 3.8 kb insert, a Southern blot analysis was performed on the plasmid pR1. Here the plasmid pR1 was subjected to a series of restriction digests. The restriction endonucleases chosen (*Eco*R1, *Xho*1, *Pst*1, *Bam*H1, and *Sac*1) as the insert was known to contain these restriction sites (Figure 13). Next, an 800 bp DIG-labeled probe, which was a PCR product, spanning a portion of the *qa-1S-qa-1F* intergenic region of *N. crassa* was generated from *N. crassa* genomic DNA (Roys, unpublished data). Based on an earlier study (Asch et al., 1991), it was thought that this *N. crassa* probe would hybridize to complementary *N. africana qa-1S-qa-1F* intergenic sequences, and was therefore used to confirm which fragments contained *qa-1S-qa-1F* intergenic sequences complementary to the probe. This probe covered from 14,300 to 15,000 on the *qa* gene sequence (Geever et al., 1989). and contained only sequences derived from the *qa-1S-qa-1F* intergenic region of *N. crassa* (Roys, unpublished data). As the blot shows, for the



Plasmid pR1
K X H E P S P X B S E P Sm B S
E 3.8 kb E
E 2.9 kb E
X E 1.330 kb X
5.370 kb
E
3.490 kb
0.5
E P P
2.710 kb
P
4.570 kb
E
B 2.130 kb B
B
2.80 kb
S 3.360 kb S
0.540 kb
S
S

*Eco*R1 digest (Figure 14A, lane 1) only the 3.8 kb fragment hybridized the probe (Figure 14B, lane 1). This result confirmed the previous data indicating that this fragment contained the *qa-1S-qa-1F* intergenic region of *N. africana* (Roys, unpublished data). The two fragments produced by the *Xho*1 digest (Figure 14A, lane 2) both hybridized the probe (Figure 14B, lane 2). However the 5.370 kb fragment produced a greater intensity than the 1.330 kb fragment. This result suggested that only a small portion of the *qa-1S-qa-1F* intergenic region existed within the 1.330 kb fragment. The double digest of *Eco*R1 and *Xho*1 generated three fragments of 2.870 kb, 2.500 kb, and 1.300 kb (Figure 14A, lane 3). Of these three fragments only the 2.500 kb and the 1.300 kb hybridized the probe (Figure 14B, lane 3). However like the *Xho*1 digest, the 2.500 kb fragment produced a greater intensity than the 1.300 kb fragment. This again suggested that the 1.300 kb fragment contained only a small portion of the *qa-1S-qa-1F* intergenic region. Of the three generated *Pst*1 fragments (Figure 14A, lane 4), only the 2.710 kb fragment hybridized the probe (Figure 14B, lane 4). This result showed that this particular fragment contained the entire *qa-1S-qa-1F* intergenic region. Both of the *Bam*H1 generated fragments (Figure 14A, lane 5) hybridized the probe (Figure 14B, lane 5). This provided evidence that the *Bam*H1 restriction site existed within the *qa-1S-qa-1F* intergenic region. Finally, of the three fragments produced by *Sac*1 (Figure 14A, lane 6), only the 2.800 kb fragment hybridized the probe (Figure 14B, lane 6).

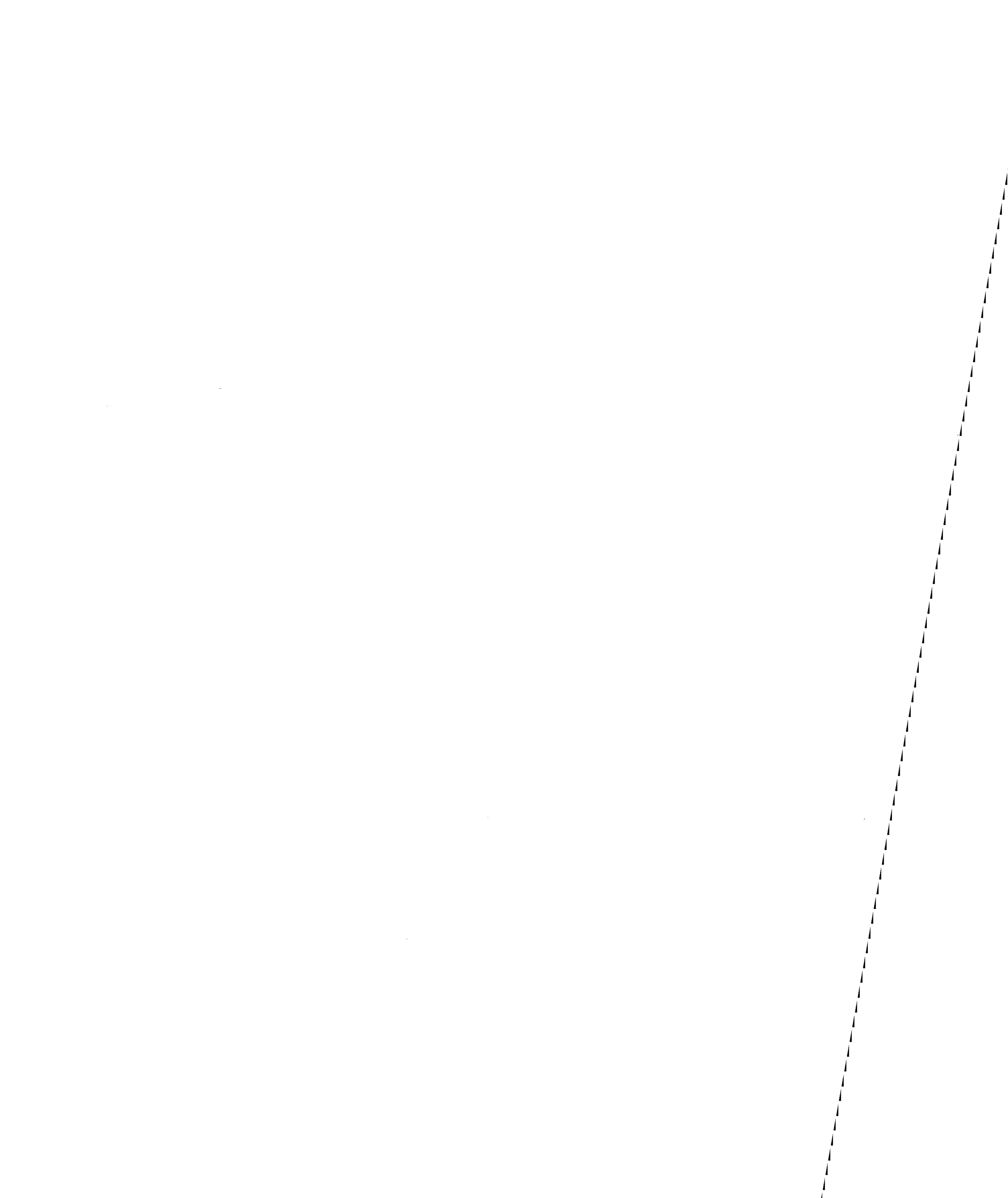


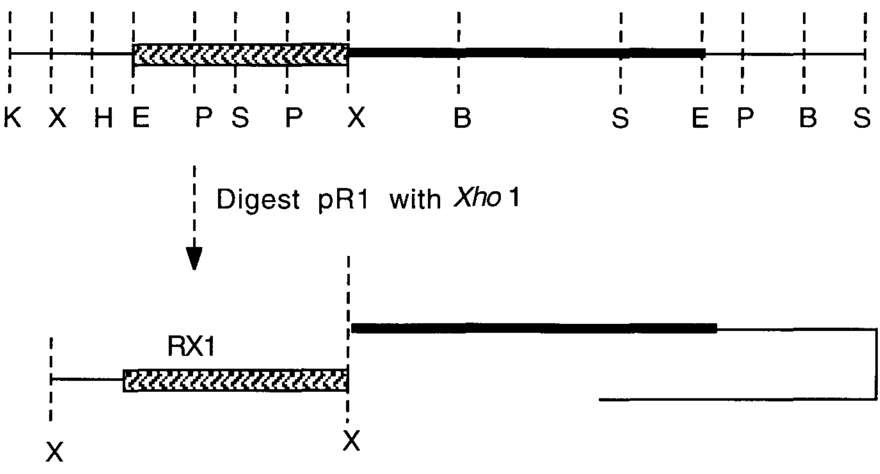

A)
1 2 3 4 5 6 7
23.13
9.42
6.56
4.36
2.32
2.03
0.54
B)
1 2 3 4 5 6

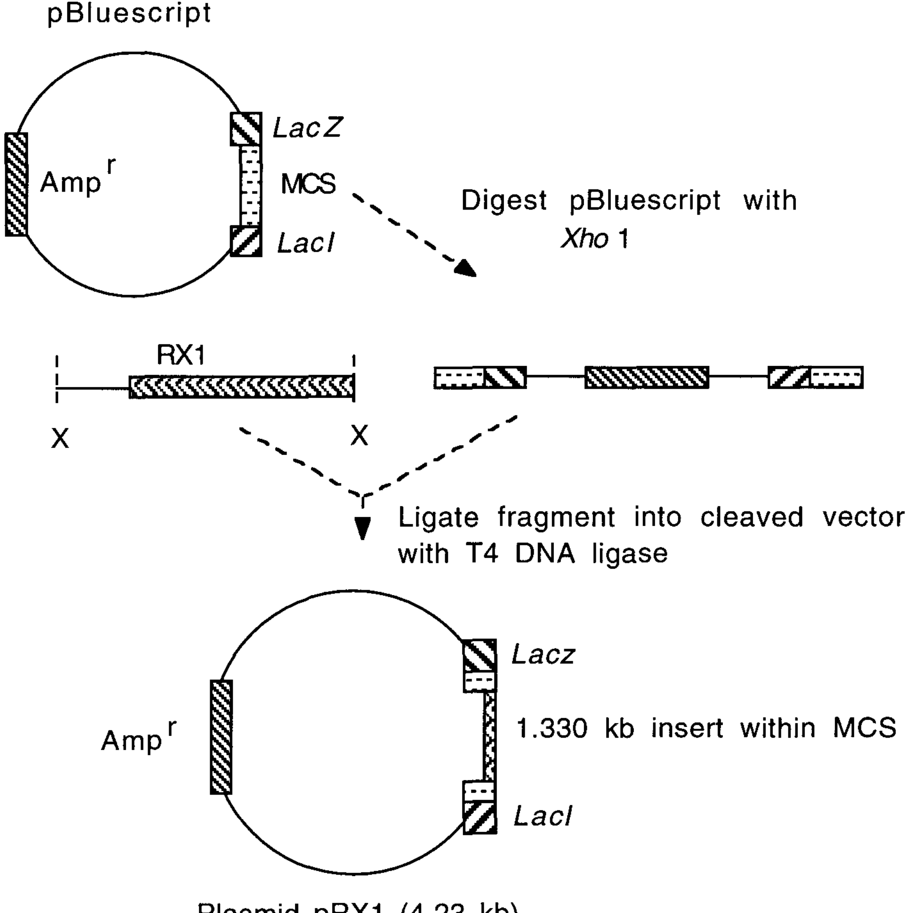
This suggested that this fragment also contained the entire *qa-1S-qa-1F* intergenic region. This information was then used to construct subclones of plasmid pR1 in a hope to further localize the *qa-1S-qa-1F* intergenic region, and initiate DNA sequencing of this region.

IV. Construction and Characterization of the Subclone Plasmid pRX1

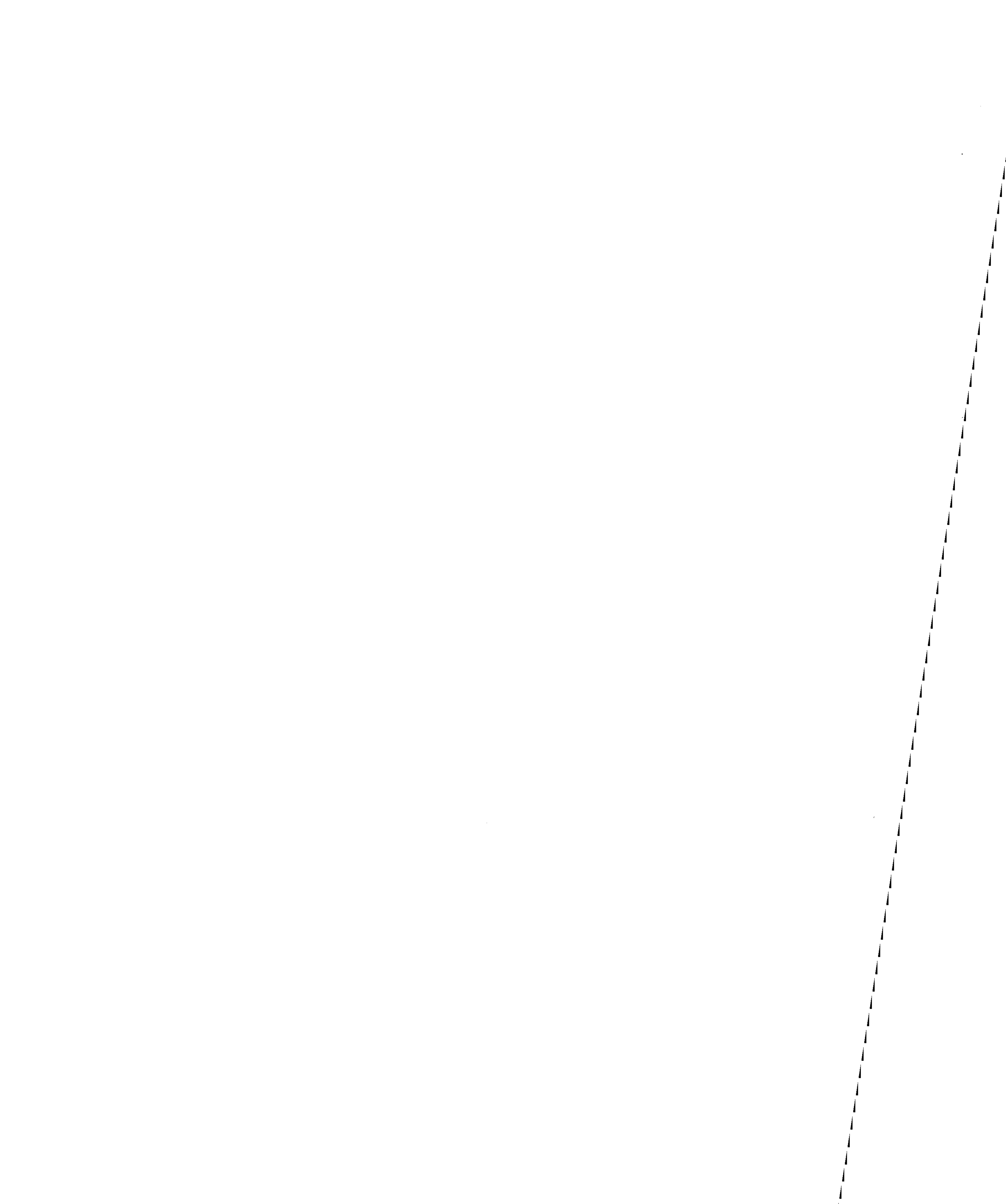
To further localize the *qa-1S-qa-1F* intergenic region, the 1.330 kb fragment produced by a *Xho*1 digest of plasmid pR1 was isolated and ligated into a *Xho*1 cleaved pBluescript vector (Figure 15). This particular fragment was chosen because the location of the *Xho*1 restriction site essentially split the 3.8 kb insert into two halves and based on the Southern blot analysis of the plasmid pR1 (Figure 14B, lane 2) where this fragment showed apparent complementarity to the DIG-labeled probe. The resulting subclone, plasmid pRX1, was then subjected to a series of restriction enzymes to further identify the locations of there restriction sites within the insert. Again, as in section II, the sizes of the fragments produced by the restriction digests were compared to a size standard (Figure 16, lane 1). The restriction enzyme *Xho*1 generated two fragments of 2.900 kb and 1.330 kb (Figure 16, lane 2). This result confirmed the presence of both the vector (2.900 kb) and the *Xho*1 generated fragment (1.330 kb). The enzyme *Sac*1 produced two

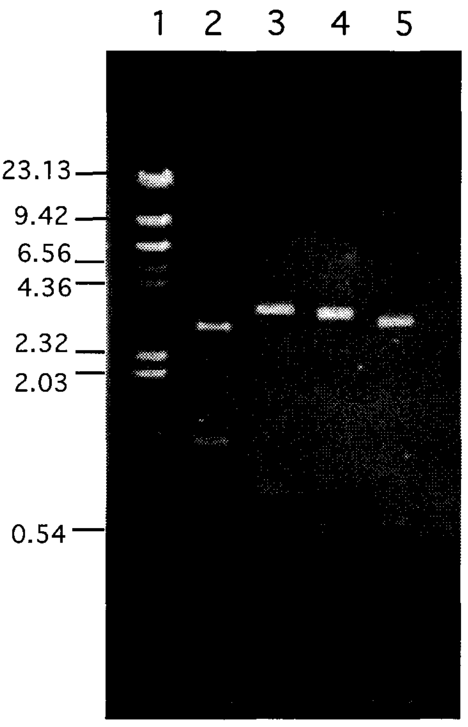






Plasmid pRX1 (4.23 kb)





1
2
3
4
5
23.13
9.42
6.56
4.36
2.32
2.03
0.54

fragments of approximately 3.430 kb and 0.800 kb (Figure 16, lane 3). The enzyme *Pst*1 generated three fragments. There sizes were approximately 3.360 kb, 0.500 kb, and 0.370 kb (Figure 16, lane 4). Finally, the double digest of *Eco*R1 and *Pst*1 generated four fragments. The approximate sizes of these fragments were 2.880 kb, 0.500 kb, 0.500 kb, and 0.350 kb (Figure 16, lane 5). However, only three of these fragments can be seen, because two of the four were approximately the same size (0.500 kb). The information from this gel was then used to draw a restriction map of the subclone plasmid pRX1 (Figure 17).

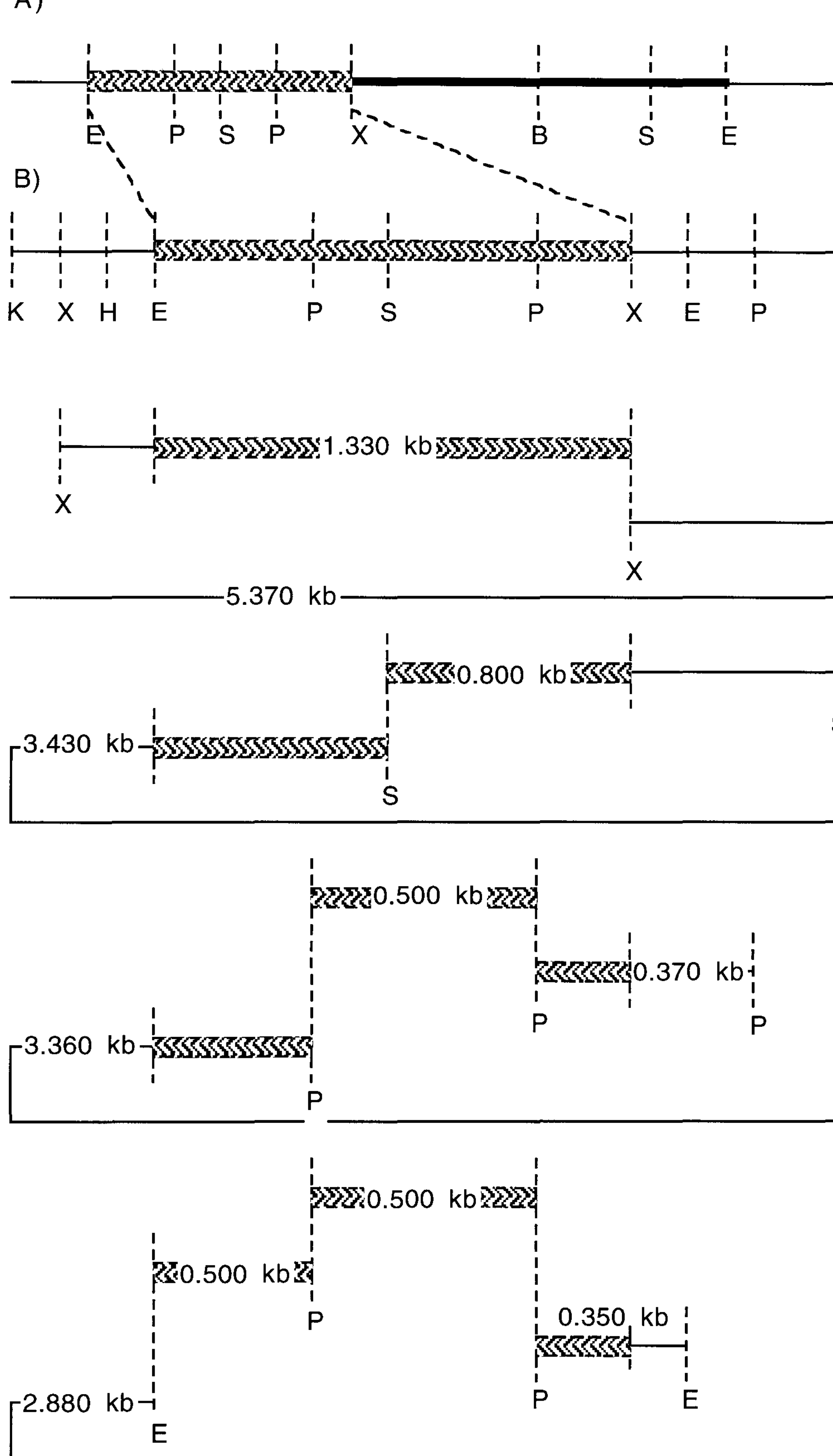
V. Southern Blot Analysis of the Subclone Plasmid pRX1

To further isolate portions of the insert, possibly containing *qa-1S-qa-1F* intergenic regions, a Southern blot analysis was performed on the subclone plasmid pRX1. Here, the subclone plasmid pRX1 was subjected to a series of restriction digests as in section IV (*Xho*1, *Sac*1, *Pst*1, and *Eco*R1). Next, the same 800 bp DIG-labeled probe used in section III was used to confirm the presence of *qa-1S-qa-1F* intergenic sequences within the fragments. As a positive control the plasmid pR1 was digested with *Eco*R1 to produce the entire 3.8 kb original insert, which is known to contain the *qa-1S-qa-1F* intergenic region. As the blot shows the positive control did hybridize the probe (Figure 18, lane 1). However, neither the *Xho*1 (Figure 18, lane 2), *Sac*1 (Figure 18, lane 3),

Figure 17. Restriction map of the subclone plasmid pRX1

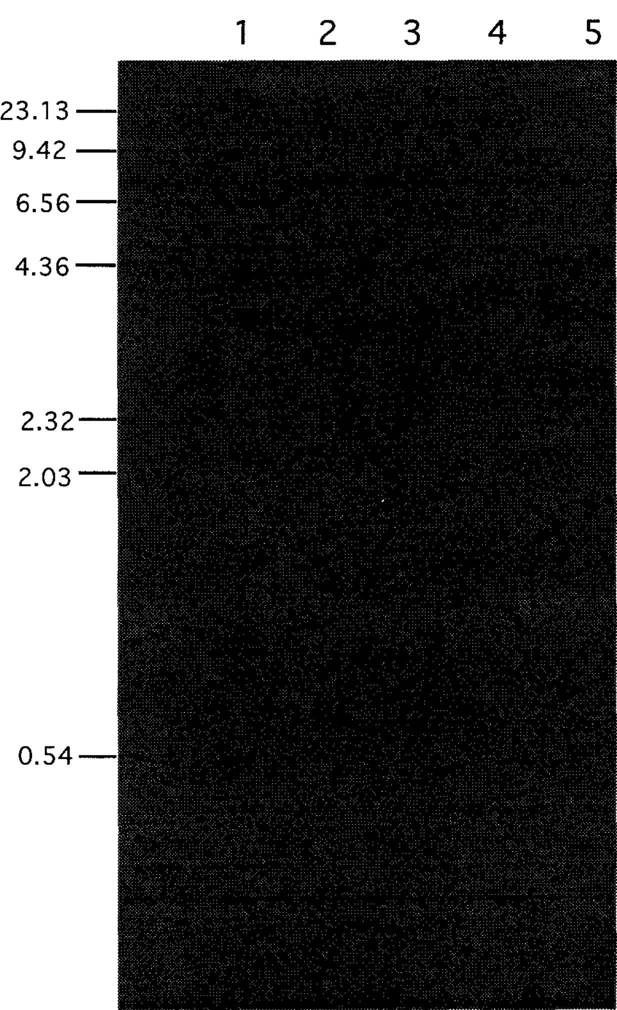
A) Shows the original plasmid pR1, with its restriction sites. Shaded area represents the portion subcloned.

B) Shows the restriction map of the subclone plasmid pRX1. The restriction enzymes are represented by the following letters: K=*Kpn*1, X=*Xho*1, H=*Hind*III, S=*Sac*1, P=*Pst*1, and E=*Eco*R1. The thin line represents the pBluescript vector, while the thick line is the portion of the 3.8 kb insert subcloned. The numbers represent the sizes of the fragments generated by each enzyme (Figure 16).



A)
E
P
S
P
X
B
S
E
B)
K
X
H
E
P
S
P
X
E
P
S
1.330 kb
X
X
5.370 kb
0.800 kb
S
3.430 kb
S
0.500 kb
0.370 kb
P
P
3.360 kb
P
0.500 kb
0.500 kb
P
0.350 kb
P
E
2.880 kb
E

Figure 18. Southern blot analysis of the subclone plasmid pRX1. Lane 1 is the positive control (pR1 cleaved with *Eco*R1), showing the 3.800 kb fragment. Lane 2 is the *Xho*1 digest. Lane 3 is the *Sac*1 digest. Lane 4 is the *Pst*1 digest. Lane 5 is the double digest of *Eco*R1 and *Pst*1. Lanes 2-5 each show no activity, suggesting no complementarity to the probe.


1
2
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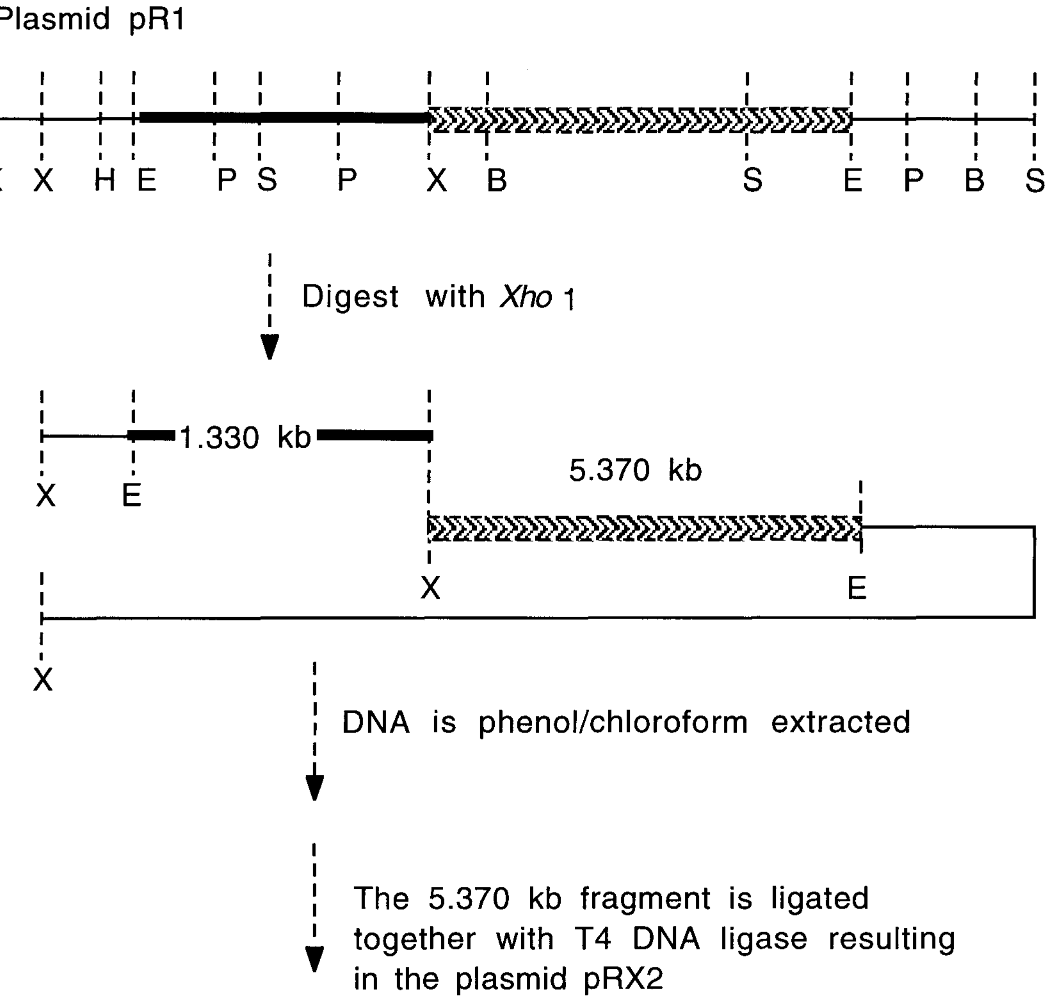
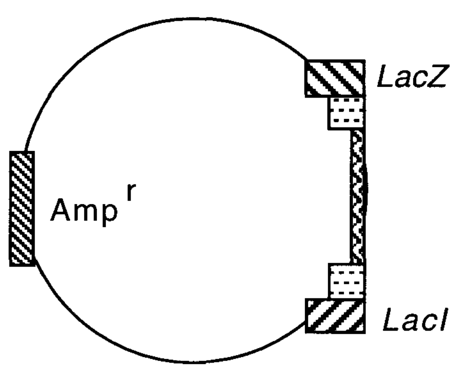
4
5
23.13
9.42
6.56
4.36
2.32
2.03
0.54

*Pst*1 (Figure 18, lane 4), or the double digest of *Eco*R1 and *Pst*1 (Figure 18, lane 5) generated fragments hybridized the probe. This suggested that no *qa-1S-qa-1F* intergenic sequences could be found in these portions of the original 3.8 kb insert. It is believed that this particular portion of the insert contains most of the *qa-1F* gene of *N. africana*. This result contradicts the Southern blot analysis of the plasmid pR1 (Figure 14B, lane 2), which showed this portion of the insert hybridizing the DIG-labeled probe. However, it is believed that this hybridization was a false positive and is hoped to be confirmed with DNA sequencing of this region of the insert.

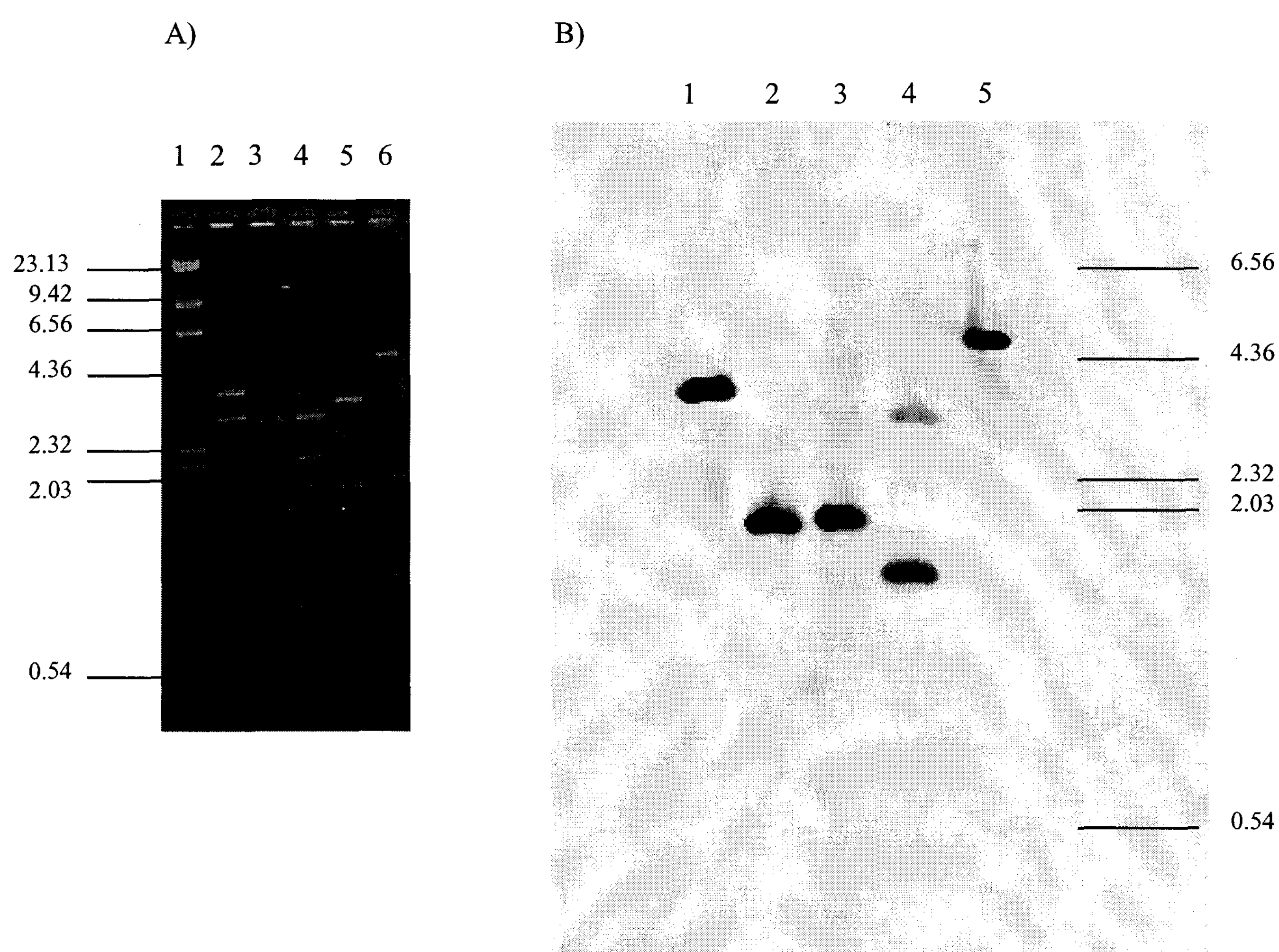
VI. Construction and Characterization of the Subclone Plasmid pRX2

To further localize the *qa-1S-qa-1F* intergenic region, the subclone plasmid pRX2 was generated (Figure 19). This plasmid was generated based on the location of the *Xho*1 restriction site, which split the 3.8 kb insert into two halves and the Southern blot analysis of the plasmid pR1 (Figure 14B, lane 2) which showed this fragment hybridized the DIG-labeled probe. This plasmid was then also subjected to a series of restriction enzymes to further reinforce there locations within the original 3.8 kb insert. The sizes of the fragments generated by these digests were then compared to a size standard to estimate their lengths (Figure 20A, lane 1). The double digest of *Sac*1 and *Xho*1 generated three fragments of 2.830 kb, 2.000





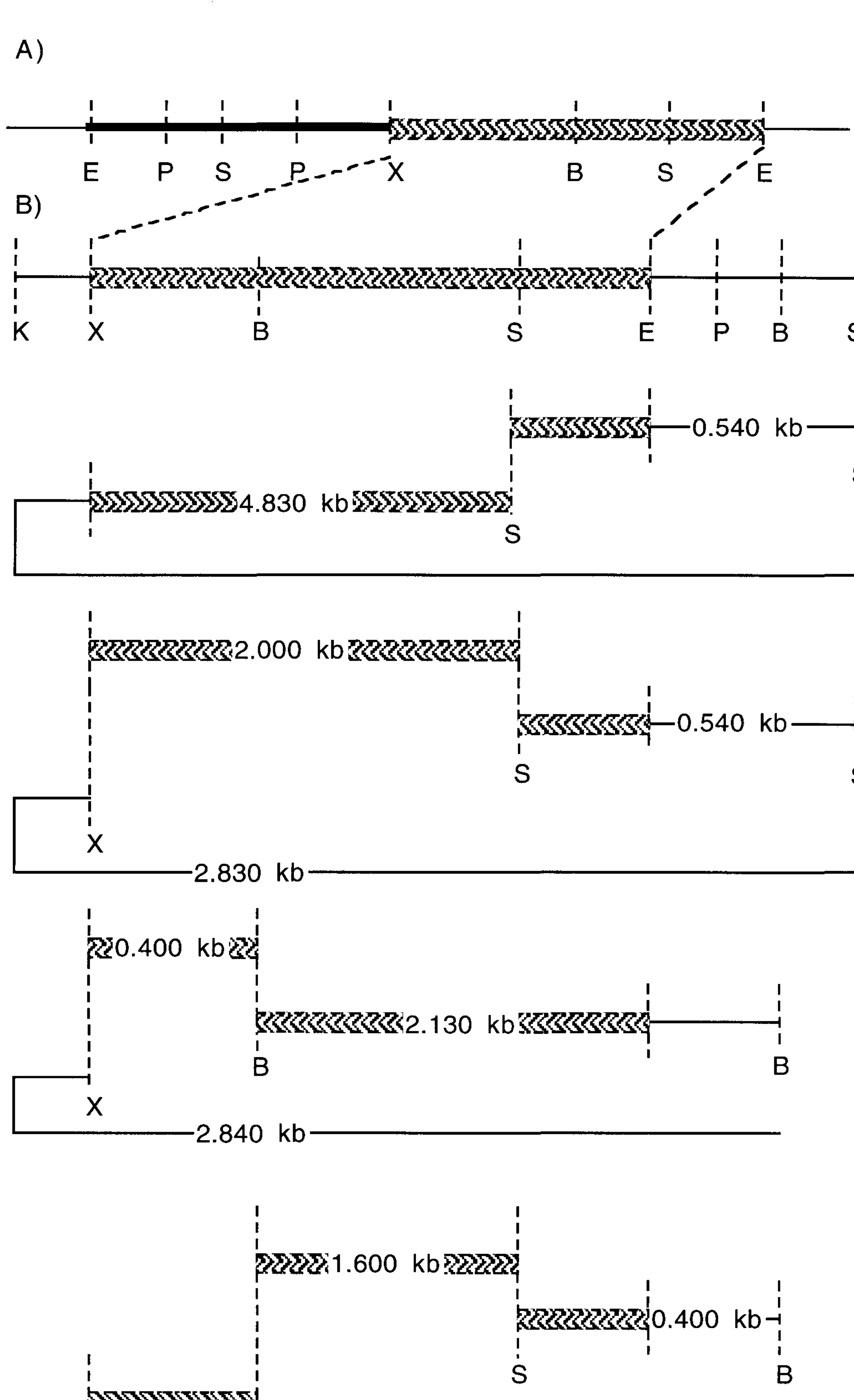
Plasmid pRX2 (5.370 kb)


A)
1 2 3 4 5 6
23.13
9.42
6.56
4.36
2.32
2.03
0.54
B)
1 2 3 4 5
6.56
4.36
2.32
2.03
0.54

kb, and 0.540 kb (Figure 20A, lane 3). The double digest of *Bam*H1 and *Xho*1 produced three fragments of 2.840 kb, 2.130 kb, and 0.400 kb (Figure 20A, lane 4). Next, the double digest of *Bam*H1 and *Sac*1 also generated three fragments of 3.230 kb, 1.600 kb, and 0.540 kb (Figure 20A, lane 5). Finally the enzyme *Sac*1, when used alone generated two fragments of 4.830 kb and 0.54 kb (Figure 20A, lane 6). All of this information was then used to generate a restriction map the of the subclone plasmid pRX2 (Figure 21).

VII. Southern Blot Analysis of the Subclone Plasmid pRX2

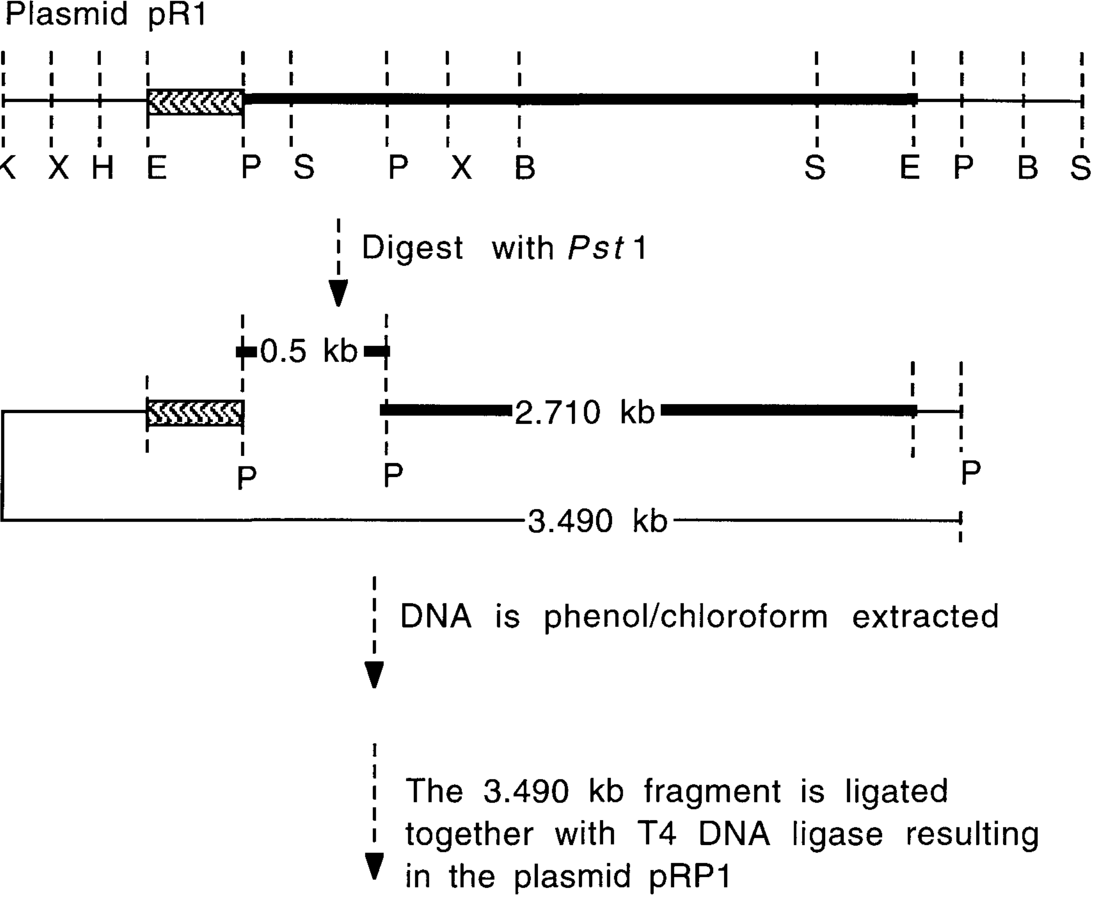
To isolate portions of the subclone plasmid pRX2 possibly containing *qa-1S-qa-1F* regions, a Southern blot analysis was performed on the subclone plasmid pRX2. Here, the subclone plasmid pRX2 was subjected to a series of restriction digests (Figure 20A, lanes 3-6). Next, the same DIG-labeled 800 bp probe used in sections III and V was used to confirm the presence of *qa-1S-qa-1F* intergenic sequences within these fragments. Here, as a positive control the plasmid pR1 was digested with *Eco*R1 to produce the entire 3.8 kb original insert (Figure 20A, lane 2). As the blot shows, the positive control hybridized to the probe (Figure 20B, lane 1). Of the three fragments generated by the double digest of *Sac*1 and *Xho*1, only the 2.000 kb fragment hybridized the probe (Figure 20B, lane 2). With the three fragments generated by the double

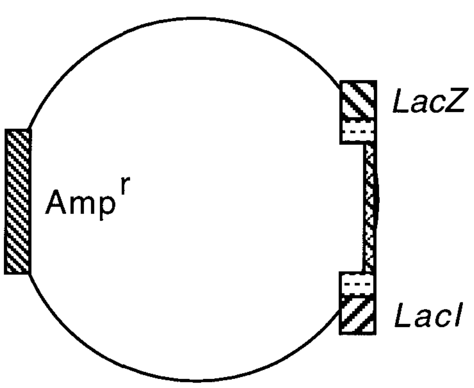

A)
E
P
S
P
X
B
S
E
B)
K
X
B
S
E
P
B
S
0.540 kb
S
4.830 kb
S
2.000 kb
0.540 kb
S
S
X
2.830 kb
0.400 kb
2.130 kb
B
B
X
2.840 kb
1.600 kb
0.400 kb
S
B
B
3.230 kb

digest of *Bam*H1 and *Xho*1, only the 2.130 kb and the 0.400 kb hybridized the probe (Figure 20B, lane 3). Here, the 0.400 kb fragment generated by this digest hybridized the probe very weakly suggesting only slight complementarity to the probe. The double digest of *Bam*H1 and *Sac*1 generated three fragments, of which the 3.230 kb and the 1.600 kb hybridized the probe (Figure 20B, lane 4). Finally, of the two fragments generated by the enzyme *Sac*1, only the 4.830 kb fragment hybridized the probe (Figure 20B, lane 5). The 0.540 kb fragment generated by the *Sac*1 and *Xho*1 double digest and the *Bam*H1 and *Sac*1 double digest was believed to contain a portion of the *qa-1S* gene of *N. africana*. With this information, along with the Southern blot analysis of subclone plasmid pRX1 (section V) the *qa-1S-qa-1F* intergenic region of *N. africana* was believed to be narrowed down to a select region in the original 3.8 kb insert.

VIII. Construction and Characterization of the Subclones Plasmid pRP1 and Plasmid pRB1

With the thought that the *qa-1S-qa-1F* intergenic region had been narrowed down to a select region within the original 3.8 kb insert, subclones of plasmid pR1 could now be constructed to initiate DNA sequencing. First, the plasmid pRP1 was generated. This was done by digesting the plasmid pR1 with the restriction enzyme *Pst*1, and then ligating the 3.490 kb fragment together (Figure 22). This particular plasmid was

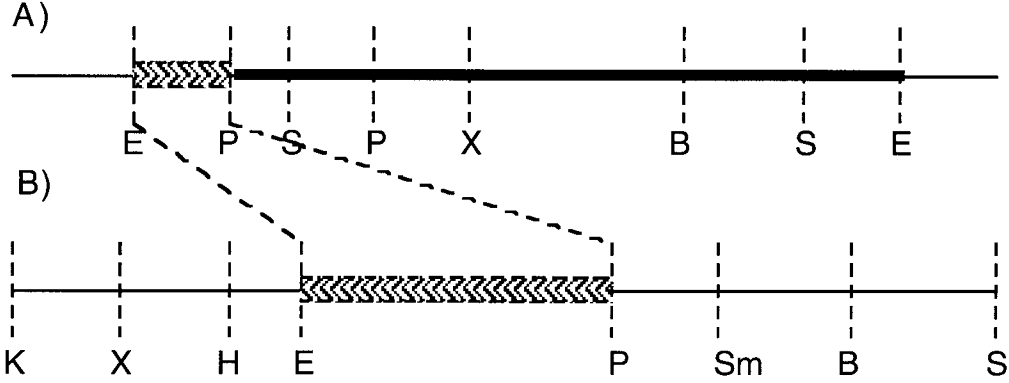


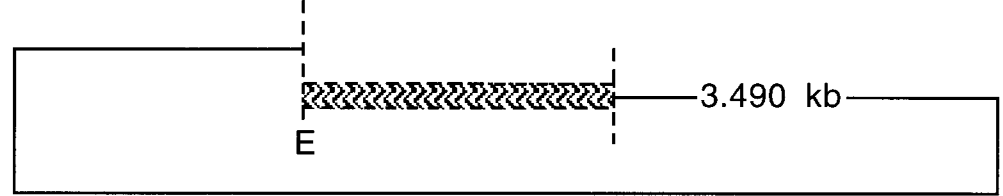


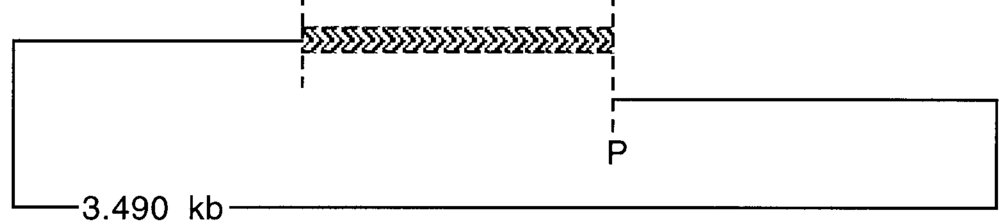
Plasmid pRP1 (3.490 kb)

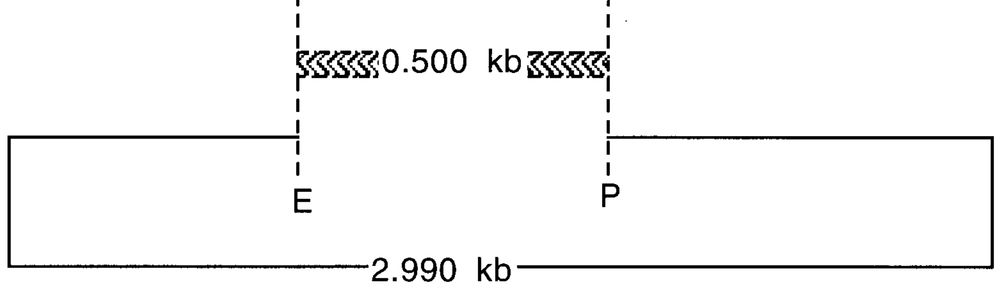
made with the hope that DNA sequencing would reveal that indeed this portion of the 3.8 kb insert contained a section of the *qa-1F* gene. This plasmid, like the others was also subjected to a series of restriction digests to generate a restriction map of the subclone (Figure 23).

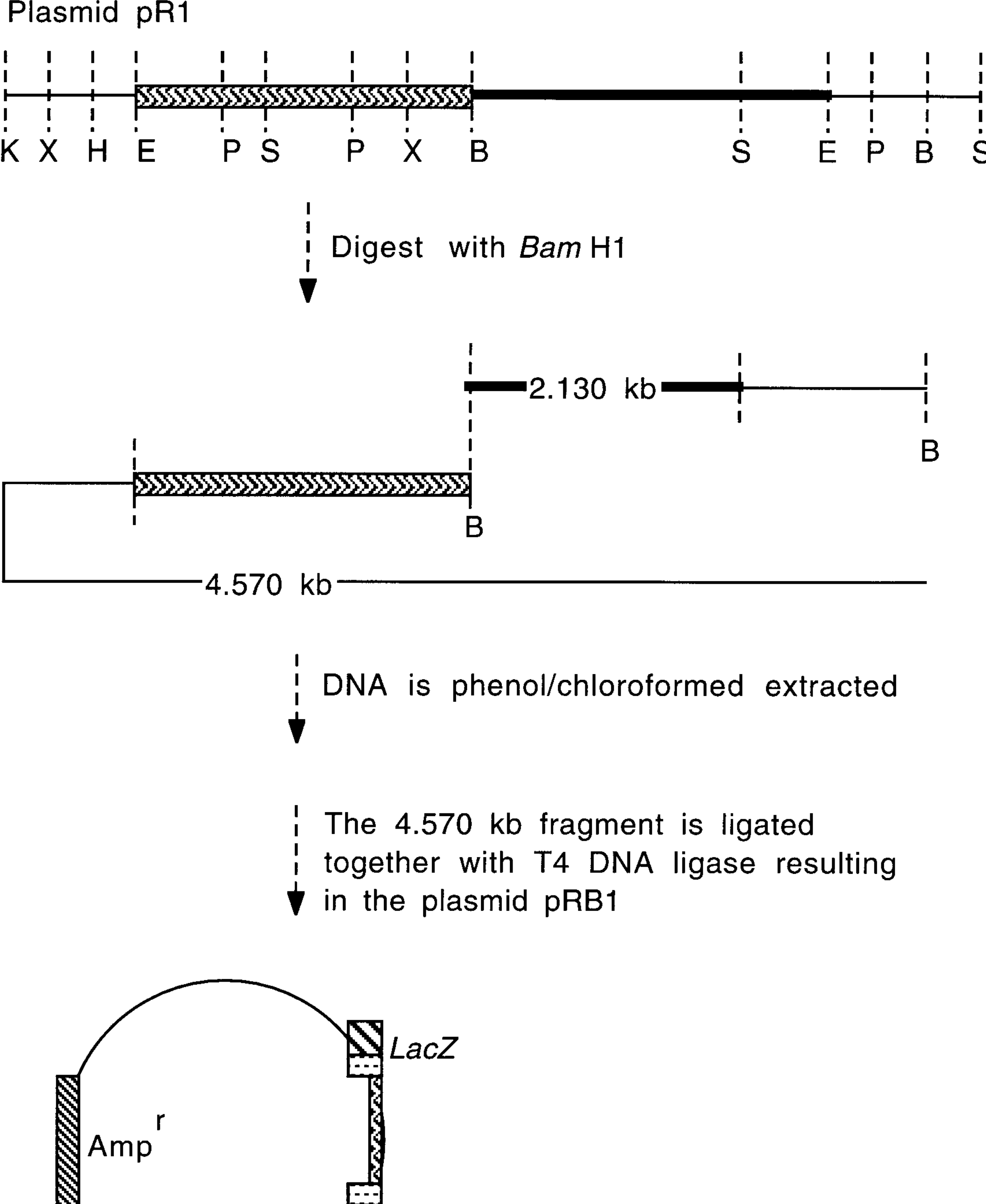
Next, the plasmid pRB1 was generated. This was done by digesting the plasmid pR1 with the restriction enzyme *Bam*H1, and then ligating the 4.570 kb fragment together (Figure 24). Again, a restriction map was deduced for this subclone, to reinforce the restriction sites contained within the original 3.8 kb insert (Figure 25). This plasmid was made based on the Southern blot analysis of plasmid pR1 (Figure 14B) and the subclone plasmid pRX2 (Figure 20B). Since both the fragments produced by the *Bam*H1 digest (Figure 14A, lane 6) hybridized the DIG-labeled probe (Figure 14B, lane 5), the Southern blot of pR1 showed that the *Bam*H1 site was located in the *qa-1S-qa-1F* intergenic region. While the double digest of *Bam*H1 and *Xho*1 performed on the subclone plasmid pRX2 generated three fragments (Figure 20A, lane 4). Two of these (2.130 kb and 0.400 kb) three hybridized the probe (Figure 20B, lane 3), but the 0.400 kb fragment hybridized the probe very weakly. This suggested that it contained only a small portion of the *qa-1S-qa-1F* intergenic region. It is hoped that DNA sequencing will reveal this section of the *qa-1S-qa-1F* intergenic region contained within the subclone plasmid pRB1.


A)
E
P
S
P
X
B
S
E
B)
K
X
H
E
P
Sm
B
S


3.490 kb
E


P
3.490 kb


0.500 kb
E
P
2.990 kb


Plasmid pR1
K X H E P S P X B S E P B S
Digest with *Bam* H1
2.130 kb
B
B
4.570 kb
DNA is phenol/chloroformed extracted
The 4.570 kb fragment is ligated together with T4 DNA ligase resulting in the plasmid pRB1
LacZ
Amp^r
LacI
Plasmid pRB1 (4.570 kb)

IX. Sequencing the Subclone Plasmid pRP1

To establish that a portion of the *qa-1F* gene was located to the left of the *Xho*1 restriction site within the original 3.8 kb insert, DNA sequence analysis was performed on the subclone plasmid pRP1. This subclone was sequenced using an M13/pUC forward sequencing primer. This primer recognizes a sequence in the 3' end of the multiple cloning site of the pBluescript vector and allows DNA sequencing to proceed towards the 5' end of the multiple cloning site. Therefore, sequencing of the insert within the subclone plasmid pRP1 started at the *Pst*1 site and moved towards the *Eco*R1 site (Figure 23). The DNA sequence which was generated by this reaction was then analyzed on DNA Strider 1.0. This software locates any restriction site which is located within the sequence entered and produces a restriction map of that sequence. Figure 26 shows the sequence generated with the subclone plasmid pRP1 and its restriction sites.

Next, it had to be determined if any sequence homology existed between the portions of the 3.8 kb insert of *N. africana*, contained within this subclone, and the *qa* gene cluster of *N. crassa*. To do this, the sequence generated by this subclone was entered into a database on the World Wide Web. The resource (URL) used was Bioscan Online (http://genome.cs.unc.edu/bin/nuc1-match). This web site allows the user to enter sequences and it will compare and match the users sequence to know sequences contained within

```
DNA  sequence    69 b.p.   GACTAGTTGCCT ... GTCTATGGAACA   linear

                          Msp I
  Mae I                   Hpa II            Hga I          Hga I
 Spe I   Mnl I           Cfr10 I         Bsm I            BstU I
 ||       |               ||               |    |           ||
GACTAGTTGCCTCTTGATGATGACCGGCTACGACAAGAATGCGTCGCTACCTGTCGCGTCTATGGAACA  69
CTGATCAACGGAGAACTACTACTGGCCGATGCTGTTCTTACGCAGCGATGGACAGCGCAGATACCTTGT
 ||       |         .     ||      .        |   .|        .  ||   .
 2        10              23               37                55
  3                        24                   41            56
                           24
```

several databases. The sequence data from the subclone plasmid pRP1 provided evidence that this portion of the insert contained part of the *qa-1F* gene of *N. africana*. This was seen with nucleotides 3 to 39 of the *N. africana* generated sequence showing homology with nucleotides 16547 to 16583 of the *N. crassa qa* gene cluster, which is a conserved *qa-1F* coding region (Figure 27). Based on this information, Southern blot analysis of the subclone pRX1 (Figure 18), and physical analysis of the location of these complementary sequences with the *qa* gene cluster of *N. crassa* (Geever et al., 1989) a conclusion can be drawn. It can be stated, with some certainty that the portion of the 3.8 kb insert located to the left of the *Xho*1 restriction site contains no *qa-1S-qa-1F* intergenic sequences, but a large portion of the *qa-1F* gene of *N. africana*.

X. Sequencing the Subclone Plasmid pRB1

DNA sequencing analysis of the subclone plasmid pRB1 was performed based on the Southern blot analysis of the subclone plasmid pRX2. Here, the 400 bp fragment, produced by the *Bam*H1/*Xho*1 digest, hybridized the DIG-labeled probe very weakly (Figure 20B, lane 3) suggesting that it contained only a small portion of the *qa-1S-qa-1F* intergenic region. Therefore, DNA sequencing was performed on this fragment, using the subclone plasmid pRB1, in a hope to identify if *qa-1S-qa-1F* intergenic sequences existed within it. Sequencing of the

Best Sum Statistic for Each Similar Database Sequence

Db|Acc|Name	Description	P(n)	n
	Neurospora crassa qa gene cluster.	6.36e-03	1

Alignments

gb|X14603|NCQA

Best Sum Statistic P(1) = 6.9e-03 Length: 18120 Date: 30-MAY-1996
Neurospora crassa qa gene cluster.
Score = 169 Length = 37 Expect = 6.9e-03 P = 6.9e-03

```
Query:      3 CTAGTTGCCTCTTGATGATGACCGGCTACGACAAGAA 39
              C AGTTGCCTC TGATGATGACCGGC ACGAC A  A
Entry: 16547 ccagttgcctcctgatgatgaccggccacgaccaaga 16583
```

subclone plasmid pRB1 was done using the same M13/pUC forward primer used in section IX. Hence, sequencing of the insert within the subclone plasmid pRB1 started at the *Bam*H1 site and moved towards the *Xho*1 site (Figure 25). The sequence generated by this reaction was, as before, analyzed on DNA Strider 1.0. Figure 28 shows the sequence generated with the subclone plasmid pRB1 and its restriction sites.

Next, as in section IX, Bioscan Online was used to determine if any sequence homology existed between this portion of the 3.8 kb insert of *N. africana* and the *qa* gene cluster of *N. crassa*. The sequence generated by the subclone plasmid pRB1 (Figure 29) regretfully did not provide any homology to the *qa* gene cluster of *N. crassa* . However, this particular sequence did show complementary sequences to *E. coli* DNA, raising numerous questions. It was thought that since *E. coli* was the host used to propagate the subclone plasmid pRB1, perhaps a section of *E. coli* DNA was inserted into the plasmid. To examine this, the plasmid pR1 and the subclone plasmid pRB1 were both digested with *Bam*H1 and *Sac*1, used in concert (Figure 30). Therefore, if the subclone plasmid pRB1 contained only its portion of the original 3.8 kb insert, it would be seen by producing two fragments the same size as two of the four produced by the digest of plasmid pR1. Indeed, this was seen in figure 30, where the digested subclone plasmid pRB1 (Figure 30, lane 2) showed the two expected fragments, of the same size, as two of the four produced by plasmid pR1 (Figure 30, lane 3). Hence, more examination of this subclone

```
DNA  sequence    324 b.p.   AGCAGCACGCCG ... CATATCGTGCGT   linear

                                                HinP I
                                        ScrF I  Fnu4H I
 Fnu4H I                                EcoR II    Hha I    Mnl I
 Bbv I                                  BstN I  Bbv I    Hinf I
 |                                      |       |  |     |   |
AGCAGCACGCCGTTGCCGTCAGAAATCCTACCTGGCACGCTGCGCTGAATCCTCTGGTGTGAGCAGTTATCCACTTGTTG   80
TCGTCGTGCGGCAACGGCAGTCTTTAGGATGGACCGTGCGACGCGACTTAGGAGACCACACTCGTCAATAGGTGAACAAC
 |        .         .         .|        |.  |     |  .|        .         .         .
 2                             31       39       47
 2                             31          42       51
                               31       39
                                           42
                                                                NspB II
                                                             Sau3A I
        HinP I                      Mnl I                    Mbo I
        Hha I                       Sec I                    Dpn I
     BspM I                    Mae III    Mse I             Taq I    Xmn I              BspM I
     |   |                     |   | |    |                 | |  |    |                  |
CTTTTGTCCACCTGCGCCACCAGTTTGTAACCGAGGCTTAAACTATAGGCTCGATCAGCGGAATGGTTTCCCAACTGACC  160
GAAAACAGGTGGACGCGGTGGTCAAACATTGGCTCCGAATTTGATATCCGAGCTAGTCGCCTTACCAAAGGGTTGACTGG
     |   |  .              |  .| |    | .             .| |  |    .|         .         | .
     90                   107       118                131       141                158
         94                    111                       133
         94                      113                     133
                                                         133
                                                            136
 Fnu4H I                                Mnl I              Mnl I
 Bbv I                                Taq I        Mbo II        Fnu4H I    Tth111 II
 |                                    | |          |       |     |          |
TGCTGCTCGCTGGTTTCGCTGATGGTGCTGGTCAGGTTTCGAGGCTTTTCTCTTCCGCCTCCGCCGCCAGCGTGTTTGCT  240
ACGACGAGCGACCAAAGCGACTACCACGACCAGTCCAAAGCTCCGAAAAGAGAAGGCGGAGGCGGCGGTCGCACAAACGA
 |        .         .         .       |.|         .|       | .   |         .|        .
 162                                  199          211           223        233
 162                                     201              218
                                                     Sau3A I
                                                     Mbo I    Rsa I
                                                     Dpn I   Spl I
                                                    Pvu I  Mae II
                                               Ple I     Rsa I    Fnu4H I
      HinP I                          Tth111 II    Taq I  SnaB I
Fok I     Hha I                   Mae II       Hinf I  Spl I      Bbv I
|         |                       |     |      |   |||  ||||||    |
CATCCGTCCAGCGCATAAAACTCAAAGCCAGCACGTTCAAGCAACCGAGTCGATCGTACGTACGCAGCACGACATATCGT  320
GTAGGCAGGTCGCGTATTTTGAGTTTCGGTCGTGCAAGTTCGTTGGCTCAGCTAGCATGCATGCGTCGTGCTGTATAGCA
|        .|         .         .   |     | .    |   |||  ||||||    |         .         .
241       251                     273          287    295         304
          251                           278       290    297
                                               287    296         304
                                                   291    298
                                                    292    299
                                                    292     300
                                                    292

GCGT  324
CGCA
```

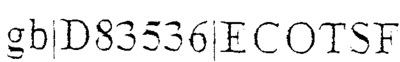
Best Sum Statistic for Each Similar Database Sequence

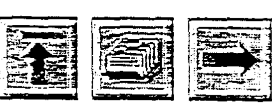
```
Db:Acc:Name              Description                                 P(N)        N

gb|D38582|ECODINJ        Escherichia coli genes for  YafH, YafI...   3.3e-29     3
gb|D83536|ECOTSF         Escherichia coli DNA.                       1.8e-27     3
```

Alignments

gb|D38582|ECODINJ

Best Sum Statistic P(3) = 3.3e-29 Length: 11295 Date: 19-DEC-1995
Escherichia coli genes for YafH, YafI, YafJ, YafK, YafQ, DinJ, YafL, YafM, FhiA, MbhA, DinP, YafN YafO and YafP.





Best Sum Statistic P(3) = 1.8e-27 Length: 91430 Date: 10-APR-1996
Escherichia coli DNA.
Score = 402 Length = 92 Expect = 8.4e-19 P = 8.4e-19

```
Query:    124 ATAGGCTCGATCAGCGGAATGGTTTCCCAACTGACCTGCTGCTCGCTGGTTTCGCTGATG 183
              ATAGGCTCGATCAGCGGAATGGTTTCCCAACTGACCTGCTGCTCGCTGGTTTCG TGATG
Entry: 58908 ataggctcgatcagcggaatggtttcccaactgacctgctgctcgctggtttcggtgatg 5896

Query:    184 GTGCTGGTCAGGTTTCGAGGCTTTTCTCTTCC 215
              GTGC GGTCAG  TT    G  TTT    T C
Entry: 58968 gtgcgggtcagcgtttcgaggcttttctctcc 58999
```

Score = 222 Length = 37 Expect = 4.6e-06 P = 4.6e-06

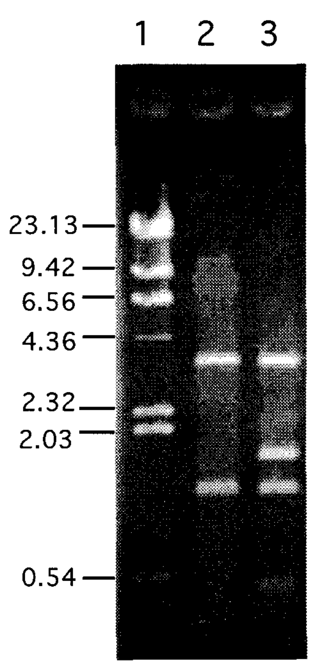
```
Query:    195 GTTTCGAGGCTTTTCTCTTCCGCCTCCGCCGCCAGCG 231
              GTTTCGAGGCTTTTCTCTTCCGCCTCCGCCGCCAGCG
Entry: 58980 gtttcgaggcttttctcttccgcctccgccgccagcg 59016
```

Score = 180 Length = 44 Expect = 4.3e-03 Sum-Stat P(2) = 2.7e-23

```
Query:      1 AGCAGCACGCCGTTGCCGTCAGAAATCCTACCTGGCACGCTGCG 44
              AGCAGCACGCCGTTGCCGTCAGAAATC       GCACGC GCG
Entry: 58791 agcagcacgccgttgccgtcagaaatcacctgccgcacgccgcg 58834
```

Score = 171 Length = 39 Expect = 1.9e-02 Sum-Stat P(3) = 1.8e-27

```
Query:     83 TTTGTCCACCTGCGCCACCAGTTTGTAACCAGGCTTAAA 121
              TTTGTCCACC GCGCCACCAGTTTGTAACC  G  T AA
Entry: 58865 tttgtccaccagcgccaccagtttgtaaccgaggcttaa 58903
```

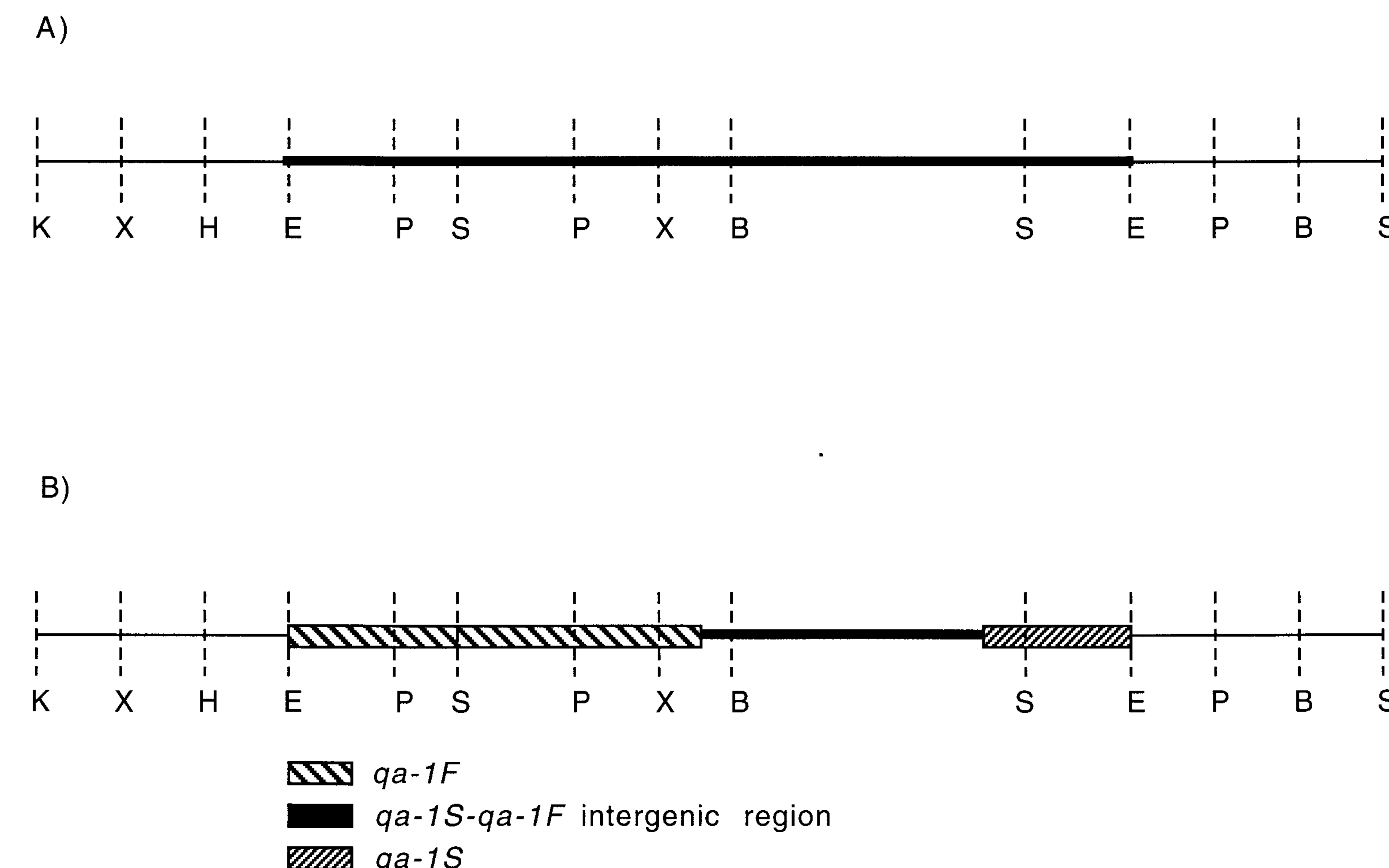

1
2
3
23.13
9.42
6.56
4.36
2.32
2.03
0.54

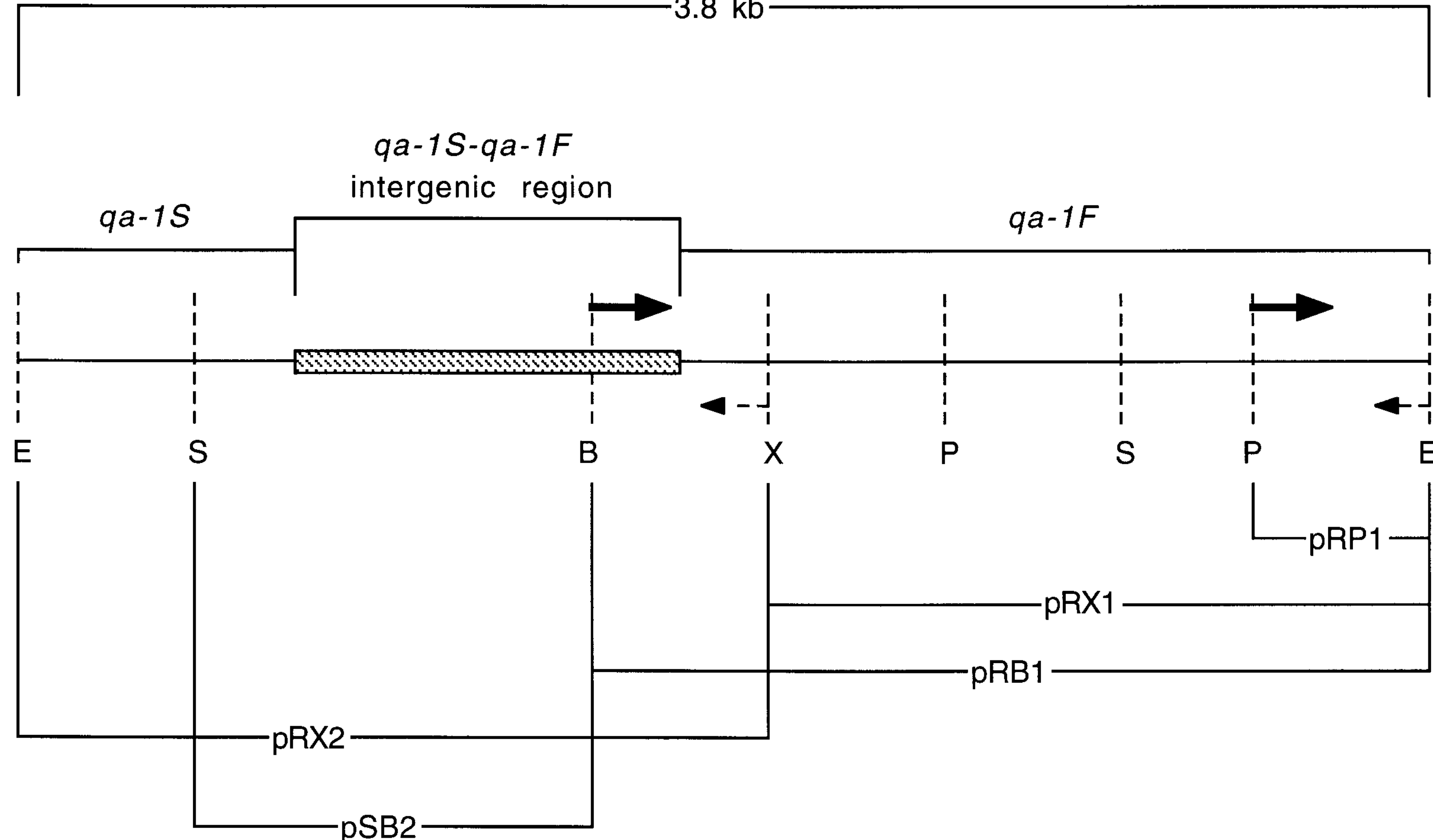
needs to be accomplished before a definitive explanation for this sequence can be reported.

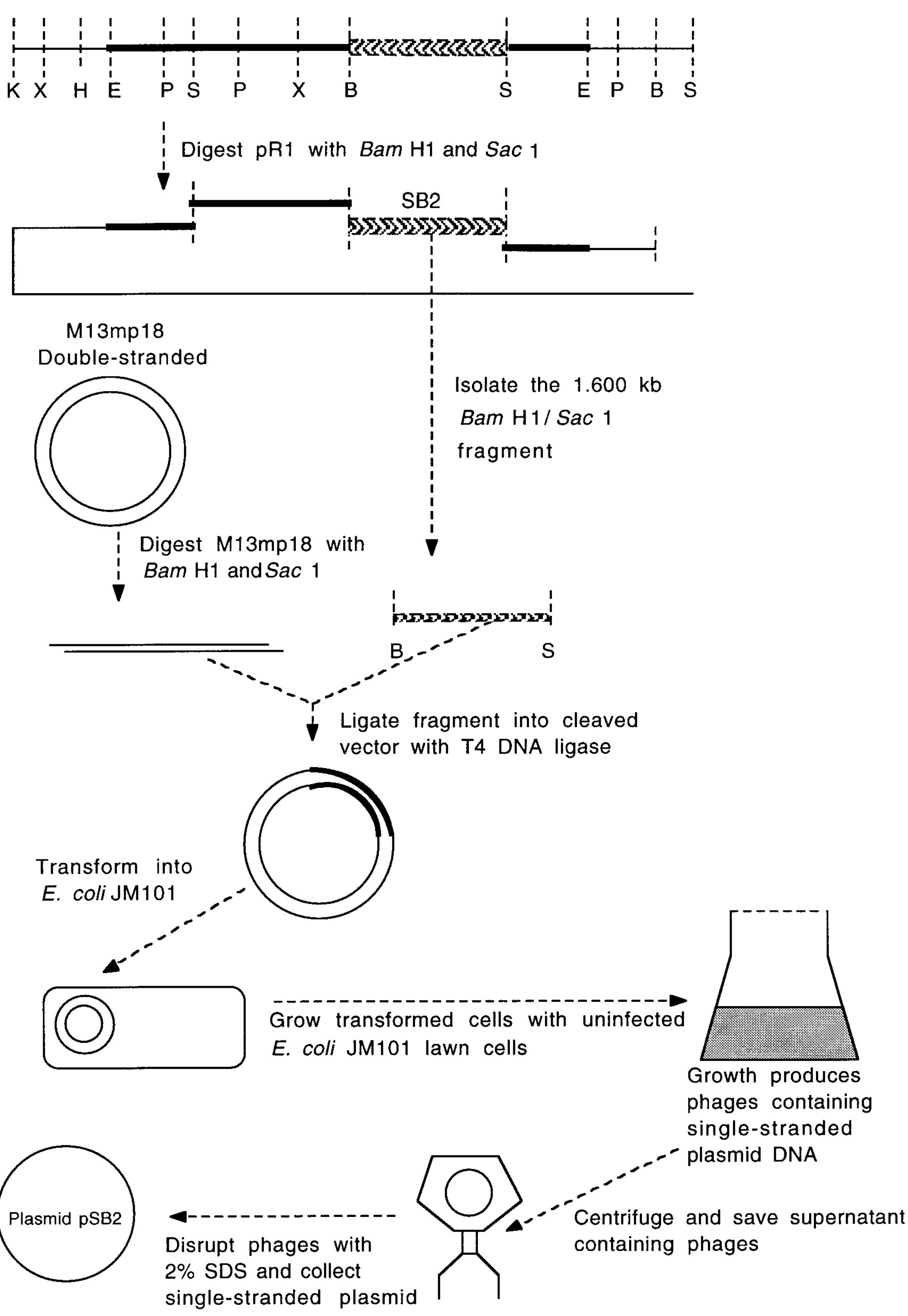
Finally, as an overview figure 31 shows the location of the *qa-1S-qa-1F* intergenic region of *N. africana*, while figure 32 shows all of the start sites of sequencing performed within the original 3.8 kb insert contained within the plasmid pR1.

XI. Construction of the M13mp18 Subclone Plasmid pSB2

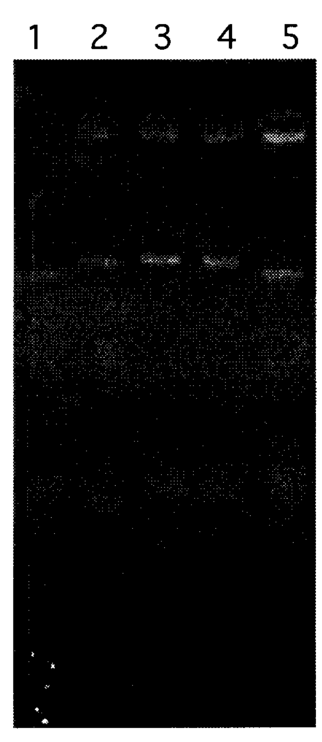
The Southern blot analysis of plasmid pR1 (Figure 14) and the subclone plasmid pRX1 (Figure 18) and plasmid pRX2 (Figure 20), along with the sequence analysis of the subclone plasmid pRP1 (Figure 27) provided evidence to the location of the *qa-1S-qa-1F* intergenic region of *N. africana* (Figure 31). The entire *qa-1S-qa-1F* intergenic region is believed to be contained within the 2.000 kb *Xho*1/*Sac*1 fragment (Figure 20B, lane 2), and most within the 1.600 kb *Bam*H1/*Sac*1 fragment (Figure 20B, lane 4) produced by the subclone plasmid pRX2. With this information, the 1.600 kb *Bam*H1/*Sac*1 fragment was isolated and ligated into an M13mp18 vector (Figure 33). The resulting subcloned plasmid pSB2 was then transformed into *E. coli* JM101, and directly electrophoresised (Figure 34, lanes 2, 3, and 4) and compared to a control (Figure 34, lanes 1 and 5) to ensure that the 1.600 kb insert was successfully ligated into the vector. This was observed as the shift in size seen in figure 34.


A)
K X H E P S P X B S E P B S
B)
K X H E P S P X B S E P B S
qa-1F
qa-1S-qa-1F intergenic region
qa-1S


3.8 kb
qa-1S-qa-1F
intergenic region
qa-1S
qa-1F
E
S
B
X
P
S
P
E
pRP1
pRX1
pRB1
pRX2
pSB2



K X H E P S P X B S E P B S
Digest pR1 with Bam H1 and Sac 1
SB2
M13mp18
Double-stranded
Isolate the 1.600 kb
Bam H1/ Sac 1
fragment
Digest M13mp18 with
Bam H1 and Sac 1
B
S
Ligate fragment into cleaved
vector with T4 DNA ligase
Transform into
E. coli JM101
Grow transformed cells with uninfected
E. coli JM101 lawn cells
Growth produces
phages containing
single-stranded
plasmid DNA
Centrifuge and save supernatant
containing phages
Plasmid pSB2
Disrupt phages with
2% SDS and collect
single-stranded plasmid


1 2 3 4 5

This M13mp18 vector was chosen over the standard pBluescript vector for sequencing purposes. DNA sequencing requires a single-stranded template to work correctly. Since pBluescript is double-stranded it requires denaturing to allow sequencing. However, M13mp18 exists in a single-stranded state and eliminates this variable from the sequencing reaction, allowing for simpler sequencing. It is hoped that sequencing of this subclone will reveal *qa-1S-qa-1F* intergenic sequences. However, this has yet to be accomplished.

DISCUSSION

Carbon catabolite repression acts to regulate gene expression in many microorganisms. Two examples of this are the regulation of the galactose (*GAL*) system of *Saccharomyces cerevisiae* and the quinic acid (*qa*) system of *Neurospora crassa* in the presence of a preferred carbon source. Wild-type *N. crassa*, grown in the presence of quinic acid and a preferred carbon source, displays a greatly reduced level of *qa* gene expression compared to wild-type *N. crassa* grown on quinic acid alone. The mechanisms which are acting to cause this repression remain unknown. However, the *GAL* system of *S. cerevisiae* may offer some explanations. Carbon catabolite repression of the *GAL* regulatory circuit appears to act on at least three separate levels. These include: (1) directly on the level of GAL4 activator protein, (2) on inducer levels, and (3) directly on the *GAL* gene promoters.

The catabolite repression seen in *S. cerevisiae* may be caused by the direct inhibition of the GAL4 activator protein. This inhibition may be an effect of the preferred carbon source: 1) directly repressing the expression of the GAL4 activator protein, 2) acting on the GAL80 repressor protein, 3) or recruiting unidentified gene products to prevent the GAL4 activator from binding to its activation sites. This same effect may be occurring with *qa* gene expression in *Neurospora*. Here, the *qa-1F* activator protein, in the presence of a preferred carbon source, may not be able to bind to its activation sites.

This may be due to the direct repression of the *qa-1F* activator gene or protein modifications and proteolysis of the activator by unidentified gene products.

Both the *GAL* system and *qa* system encode a specific premease (*GAL2* and *qa-y*) for their respective sugars. Within the *GAL* system the transport of galactose appears to be inhibited by a preferred carbon source at two levels. The first being that the *GAL2* gene, which encodes the premease, is subject to catabolite repression (Tschopp et al., 1986) and the second is that a preferred carbon source may interact with preexisting premeases inactivating them, a process called catabolite inactivation (Ma and Ptashne, 1987c). These same effects may occur within the *qa* gene cluster. Indeed this was seen, when a *N. crassa* strain containing a deletion of the *qa-1S* gene was created. This particular strain should have displayed constitutive expression of the *qa* genes. However, when grown in the presence of glucose alone the *qa-3, qa-y,* and *qa-1F* genes remained highly repressed (Asch and Case, unpublished data). This result suggested two things. First, that like *GAL2*, the quinic acid premease *qa-y* gene is affected by catabolite repression. Second, it seemingly disproved any thought that the *qa-1S* repressor protein acts on the *qa-1F* activator protein during carbon catabolite repressing conditions. These results when taken together suggest the possible role of yet identified gene products acting to cause repression.

Finally, catabolite repression may act directly on the promoters of each system. This is the most compelling scheme

for catabolite repression. In the *GAL* system sequences termed upstream repression sequences (URS_{GAL}) were found to exist between the upstream activating sequences (UAS_{GAL}) and the transcriptional initiation sites. These URS_{GAL} sites are thought to act under catabolite repression conditions by binding unidentified repressor proteins (Erickson and Johnston, 1993). Recent experiments to find these unidentified proteins has yielded the MIG1, SSN6, and TUP1 proteins. MIG1 was found to bind to *GAL* promoters in the presence of glucose and may play a role in repression alone, or it may complex with SSN6 and TUP1 (Kelcher et al., 1992). These possible interactions of carbon repressor proteins, with sequences 5' to the various *GAL* genes, which act to block transcription while in the presence of a preferred carbon source, may also act within the *qa* system of *Neurospora*. If similar sequences do exist within the *qa* gene cluster of *Neurospora*, they would most likely be found before the *qa-3, qa-y,* and the *qa-1F* genes. The reason for this, is that an *N. crassa* strain carrying a complete deletion of the *qa-1S* gene displayed highly repressed *qa-3, qa-y,* and *qa-1F* gene expression and slightly repressed *qa-x, qa-2,* and *qa-1S* gene expression when grown in the presence of a preferred carbon source. However, the existence of such sequences before these genes (*qa-3, qa-y,* and *qa-1F*) has yet to be determined.

In an attempt to see if such sequences exist within the *Neurospora qa* gene cluster the *qa-1S-qa-1F* intergenic region of *N. africana* was chosen for study. This region was chosen

again based on the results that a *N. crassa* strain carrying a deletion of the *qa-1S* gene displayed slightly repressed *qa-x, qa-2,* and *qa-4* gene expression and highly repressed *qa-3, qa-y,* and *qa-1F* gene expression, when grown in the presence of glucose. Therefore, if sequences like the URS_{GAL} existed within the cluster, they would most likely be found 5' to the genes which remained highly repressed when grown on glucose. Since the *qa-1F* gene remains highly repressed and since the sequence of this *qa-1S-qa-1F* intergenic region is known in *N. crassa* (Geever et al., 1989) it allows comparisons to be made between the two species (*N. crassa*/heterothallic and *N. africana*/ homothallic). To enable the isolation and characterization of the *N. africana qa-1S-qa-1F* intergenic region, a 3.8 kb fragment from the lambda clone NA3, known to contain the *qa-1S-qa-1F* intergenic region was isolated and ligated into a pBluescript vector, and termed pR1 (Rutledge, unpublished data) (Figure 11).

Plasmid pR1 was then subjected to a series of restriction enzymes to establish a preliminary restriction map of the 3.8 kb insert (Figure 13). Next, a Southern blot analysis was performed on the plasmid pR1 to localize those fragments which contained *qa-1S-qa-1F* intergenic sequences (Figure 14). The most interesting portion of this blot, for two reasons, was the two fragments generated by the restriction enzyme *Xho*1 (Figure 14A, lane 3). First, the location of this restriction site essentially split the insert into two halves, and second, that both fragments hybridized the DIG-labeled probe (Figure 14B,

lane 2). However, the 1.330 kb fragment produced a weaker intensity than the 5.370 kb fragment (Figure 14B, lane 2). This result suggested that only a small portion of the *qa-1S-qa-1F* intergenic region existed within the 1.330 kb fragment. Based on these results, the subclones plasmid pRX1 and plasmid pRX2 were produced (Figures 15 and 19, respectively). Like plasmid pR1, both subclones were then subjected to a series of restriction enzymes to generate restriction maps of their portions of the original 3.8 kb insert (Figures 17 and 21). Southern blot analysis was then performed on both subclones to establish if they both indeed contained portions of the *qa-1S-qa-1F* intergenic region of *N. africana.*

The Southern blot analysis of the subclone plasmid pRX1 revealed that none of the restriction fragments generated hybridized the DIG-labeled probe (Figure 18). This result contradicted that of the plasmid pR1 (Figure 14B, lane 2) and suggested that none of the *qa-1S-qa-1F* intergenic region existed to the left of the *Xho*1 site within the 3.8 kb insert. The construction (Figure 22) and subsequent sequencing (Figure 26) of the subclone plasmid pRP1 provided evidence, based upon its location within the *qa* gene cluster of *N. crassa* (Figure 29), that this portion of the 3.8 kb insert contained a section of the *qa-1F* gene of *N. africana* (Figure 31). Thus the entire *qa-1S-qa-1F* intergenic region had to be located in the subclone plasmid pRX2 (Figure 32).

The Southern blot analysis of the subclone plasmid pRX2 (Figure 20) indeed provided evidence to support the conclusion

that it contained the entire *qa-1S-qa-1F* intergenic region. This was seen with the double digest of the plasmid with *Xho*1 and *Sac*1. Here, the 2.000 kb fragment hybridized the DIG-labeled probe, while the 0.540 kb fragment did not (Figure 20B, lane 2). This suggested that this 2.00 kb fragment contained the entire *qa- 1S-qa-1F* intergenic region of *N. africana*. When this 2.000 kb *Xho*1/*Sac*1 fragment was digested with the enzymes *Bam*H1 and *Sac*1, it produced a 0.400 kb fragment and a 1.600 kb fragment (Figure 21), both of which hybridized the DIG-labeled probe. However, the 0.400 kb *Xho*1/*Bam*H1 fragment produced a weaker intensity than that of the 1.600 kb *Bam*H1/*Sac*1 fragment (Figure 20B, lanes 4 and 5, respectively). This result suggested that the 0.400 kb fragment contained only a small portion of the *qa-1S-qa-1F* intergenic region. While, the 1.600 kb fragment contained most of the *qa-1S-qa-1F* intergenic region. Based on this, the subclone plasmid pRB1 was constructed (Figure 24). Next, sequencing of the subclone plasmid pRB1 was conducted to try and identify the *qa-1S-qa-1F* intergenic sequences contained within this 0.400 kb *Xho*1/*Bam*H1 fragment. The sequence generated by this subclone (Figures 28) did not identify any homology to the *qa* gene cluster of *N. crassa*, in particular to the *qa-1S-qa-1F* intergenic region of *N. crassa* (Figure 29). However, a more detailed analysis of this sequence is needed before it is dismissed as not containing *qa-1S-qa-1F* intergenic sequences of *N. africana*.

The Southern blot analysis of the subclone plasmid pRX2 also showed that the 0.540 kb fragment produced by the *Sac*1/*Xho*1, *Bam*H1/*Sac*1, and *Sac*1 digests (Figure 20A, lanes 3,5, and 6, respectively) did not hybridize the DIG-labeled probe (Figure 20B, lanes 2, 4, and 5). Since, the Southern blot of the subclone plasmid pRX1 (Figure 18), and the sequencing of the subclone plasmid pRP1 (Figure 27) showed that the portion of the 3.8 kb fragment to the left of the *Xho*1 site contained a section of the *qa-1F* gene of *N. africana* (Figure 31), it was thought that this 0.540 kb fragment contained a section of the *qa-1S* gene of *N. africana.* To verify this, the subclone plasmid pRX2 can be used to sequence this region of the insert. However, this has not been accomplished yet. Therefore, more analysis is needed before it can be definitively stated that this 0.540 kb fragment contains a section of the *qa-1S* gene of *N. africana*.

The lack of sequence homology which was encountered with the sequencing performed was attributed to the use of the double-stranded pBluescript vector. Since, DNA sequencing requires a single-stranded template to work correctly, the double-stranded pBluescript vector needs to be denatured to allow sequencing. In an attempt to eliminate this variable from the sequencing reaction, the single-stranded M13mp18 vector was chosen for the construction of any new subclones which were intended for DNA sequencing purposes. The first subclone to be constructed using this procedure was the subclone plasmid pSB2 (Figure 33). This plasmid was

generated based on the Southern blot analysis of the subclone plasmid pRX2. As previously mentioned, the 1.600 kb *Bam*H1/*Sac*1 fragment (Figure 21), which hybridized the DIG-labeled probe (Figure 20B, lane 5), is believed to contain most of the *qa-1S-qa-1F* intergenic region of *N. africana*. Therefore, it is thought that the sequencing of this subclone will reveal these *qa-1S-qa-1F* intergenic sequences. However, the sequencing of this subclone plasmid pRB2 has yet to be accomplished.

In conclusion, the Southern blot analysis of plasmid pR1 (Figure 14), subclone plasmid pRX1 (Figure 18), and subclone plasmid pRX2 (Figure 20), along with the DNA sequencing performed on the original 3.8 kb insert, it is believed that the *qa-1S-qa-1F* intergenic region of *N. africana* has been isolated (Figure 32). In the future, the subclones plasmid pRB1, plasmid pRX2, and plasmid pSB2 can be used to sequence the entire *qa-1S-qa-1F* intergenic region. Once this has been accomplished, the *qa-1S-qa-1F* intergenic region of *N. africana* can be compared to its *N. crassa* counterpart and examined for the existence of sequences 5' to the *qa-1F* gene acting under carbon catabolite repressing conditions. Ultimately, the *qa-1S-qa-1F* intergenic region of *N. africana* will be used to replace its *N. crassa* counterpart to determine if the *qa-1S-qa-1F* intergenic sequences of *N. africana* can operate to cause carbon catabolite repression of the *qa* genes of *N. crassa*.

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