Studies on the quinic acid (*qa*) gene cluster of *Neurospora africana*

by

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Neurospora africana

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ABSTRACT

The *qa-1S-qa-1F* intergenic sequence of *N. africana* was chosen for study because this region may play a role in the carbon catabolite repression of the genes within the quinic acid (*qa*) gene cluster. Evidence has indicated that expression of the *GAL* genes, within the galactose pathway of *S. cerevisiae* , is reduced in the presence of a preferred carbon source such as glucose. This process results from the inability of the GAL4 activator protein to bind to its GAL4 binding sites within the *GAL* promoters. However, the exact mechanism of GAL4 inhibition is unknown. A similar mechanism may occur within the *qa* gene cluster of *N. crassa.* In the presence of a preferred carbon source, *qa* gene expression is repressed. Asch and Case (1992) provided evidence that some *qa* genes were slightly repressed in the presence of glucose, while other *qa* genes were highly repressed. The *qa-1F* activator gene was amongst the highly repressed genes. The exact mechanism of catabolite repression is unclear.

We attempted to study the *qa-1S-qa-1F* intergenic sequence of *N. africana* because this region may influence the *qa* gene repression in the presence of glucose. Two plasmid clones, pR1 and pRX2, were established. Plasmid pR1 contains a 3.8-kb insert spanning the entire *qa-1S-qa-1F* region of *N. africana.* Whereas, plasmid pRX2 contains a 2.7-kb fragment spanning a portion of the *qa-1S-qa-1F* intergenic region. Southern blot analysis of both plasmids pR1 and pRX2 confirmed that these clones contain the *qa-1S-qa-1F* intergenic sequence. Sequence analysis of the plasmid pR1, using the M13/pUC reverse primer, determined the *qa-1F* end of the 3.8-kb insert. Although sequencing of both plasmids was limited, the established clones were characterized and can be used in future experiments.

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LITERATURE REVIEW

I. Fungi

Fungi are an enormous and diverse group of organisms comprising well over 60,000 species. The Ascomycetes, containing some 15,000 species, make up the largest class of fungi. The primary morphological feature that distinguishes this class from other classes of fungi is the ascus, a specialized, elongated sporangium enclosing spores. Two groups within the ascomycetes, the yeasts of *Saccharomycetales* and the filamentous ascomycetes, serve as ideal models in the biological research of eukaryotic organisms (Taylor *et al.*, 1993).

II. Yeast

The ascomycetous yeasts, *Saccharomycetales,* are characterized by the absence of ascogenous hyphae and ascocarps. These unicellular, eukaryotic organisms reproduce either asexually (budding or fission) or sexually. Although the economic significance of yeast can hardly be overemphasized, their role in scientific research has been profound. One species, the common yeast *Saccharomyces cerevisiae*, has several features which make it attractive as a host system. First, yeast has an ancient history in the brewing and baking industries and has been accepted as a GRAS (generally regarded as safe) organism. Second, *S. cerevisiae* may be grown inexpensively in vast quantities over a short time. Third, these organisms can be grown in a synchronous manner as they progress through the cell cycle. This permits the generation of a homogenous population of cells.

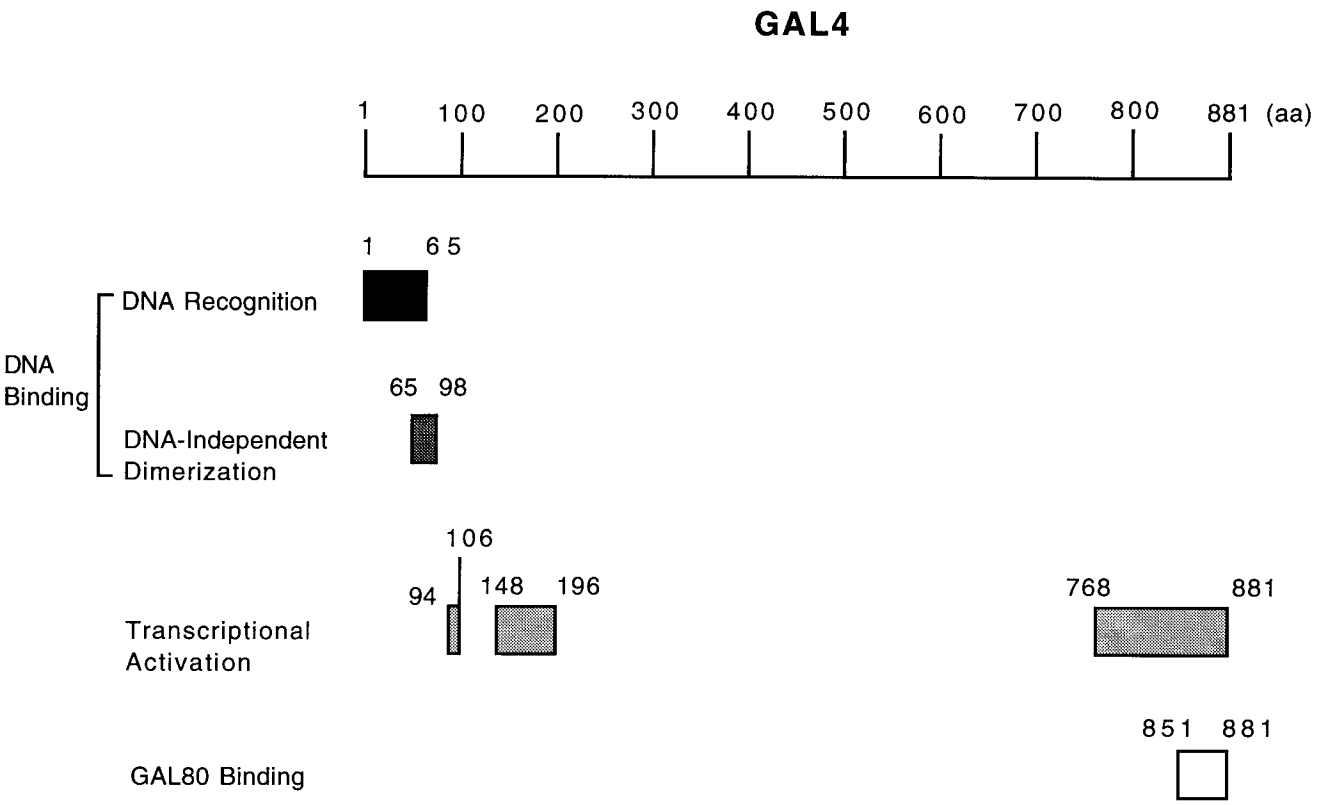
III. Galactose (*GAL*) System of *Saccharomyces cerevisiae*

S. cerevisiae has served as a model system for many genetic and biochemical studies. One of the best examples of eukaryotic gene regulation involves the *GAL* genes of yeast.

The ability of the simple eukaryote, *S. cerevisiae,* to utilize galactose as a source of carbon is conferred by the *GAL1*, *GAL7*, and *GAL10* genes which encode galactokinase, alpha-D-galactose-1-phosphate uridyl transferase, and uridine diphosphogalactose-4-epimerase (for review, see Johnston, 1987). These inducible enzymes catalyze the conversion of galactose to glucose 1-phosphate which is then converted to glucose 6-phosphate by a phosphoglucomutase for use in glycolysis (Kang *et al.*, 1993). A fourth structural gene, *GAL2*, encodes a galactose permease which catalyzes the transport of galactose into the cell. Mutations defining the structural genes *GAL1*, *-7,* and *-10* map near the centromere of chromosome II (Douglas and Hawthorne, 1969). The *GAL1* gene is transcribed divergently from the *GAL7* and *GAL10* genes (St. John and Davis, 1981). Mutations occurring at the *GAL2* locus map to a different linkage group (chromosome XII) (Douglas and Hawthorne, 1969). Two additional genes, *GAL4* and *GAL80*, are involved in regulating the transcription of the *GAL* genes. Douglas and Hawthorne (1964) demonstrated that *gal80* mutants constitutively induced *GAL1*, *GAL7*, and *GAL10* gene expression, while *gal4* mutants were noninducible. The results suggest that the *GAL80* product acts as a repressor and that the *GAL4* product acts as an inducer of *GAL1*, *-7*, and *-10* gene expression. The two regulatory genes *GAL4* and *GAL80* are not linked to each other and are not linked with the other genes of the galactose pathway (Hopper *et al*, 1978).

IV. GAL4 Activator Protein

The *GAL4* gene product is an 881-amino acid protein that activates the transcription of the genes required for the utilization of galactose in *S. cerevisiae* (St. John and Davis, 1979; Post-Beittenmiller, 1984; Giniger *et al.*, 1985). Molecular analysis of the GAL4 protein indicates the existence of several functional domains (Figure 1). The specific binding of the activator protein to the GAL4 binding site is facilitated by a DNA binding-domain contained within the N-terminal 65 amino acid residues of the activator protein


GAL4
1
100
200
300
400
500
600
700
800
881
(aa)
1
65
DNA Recognition
DNA
Binding
65
98
DNA-Independent
Dimerization
106
94
148
196
768
881
Transcriptional
Activation
851
881
GAL80 Binding

(Marmorstein *et al.*, 1992). This domain contains six cysteine residues and a zinc-finger region. Residues 65-94 of the GAL4 protein mediate dimer formation of the GAL4 protein (Marmorstein *et al.*, 1992). Two activating regions, GAL4 (148-196) and GAL4 (768-881), contribute to transcriptional activation (Ma and Ptashne, 1987a; Lin *et al.*, 1988) when fused independently to the DNA-binding domain (Figure 1). *In vitro* studies have demonstrated that sequences contained within residues 94-106 of the GAL4 protein play a role in transcriptional activation as well (Marmorstein *et al.*, 1992). The negative regulator protein, GAL80, has been shown to bind directly to the C-terminal 30 residues (851-881) of the GAL4 activator protein (Marmorstein *et al.*, 1992). This sequence is required for repression of GAL4 protein activity by the GAL80 protein (Ma and Ptashne, 1987b). The central region (residues 238-767) of the GAL4 protein is required for the inhibition of the activator by glucose as well as for the activation of GAL4 in the absence of glucose (Kang *et al.*, 1993).

Most of the *GAL* genes contain multiple GAL4 protein binding sites in their upstream activating sequences (UAS_{GAL}) for galactose. The GAL4 protein specifically binds to these sites causing transcription of the affected *GAL* genes. These binding sites consist of a 17-bp palindrome (CGGAGGACTGTCCTCCG) that is a consensus sequence for the 11 known GAL4 protein binding sites (Giniger *et al.*, 1985). Four elements similar to this consensus sequence are located in the UAS_{GAL} of the divergent *GAL-1-10* promoter, while the *GAL7* and *GAL2* promoters each contain two such binding sites (Johnston, 1987). The GAL4 protein binds as a dimer to the 17-bp sequence and induces the activity of the various *GAL* genes (Marmorstein *et al.*, 1988; Kang *et al.*, 1993).

V. GAL80 Repressor Protein

The repressor protein GAL80, consisting of 435 amino acids, appears to be responsible for inhibiting the activation function of the GAL4 activator protein (Lue *et al.*,

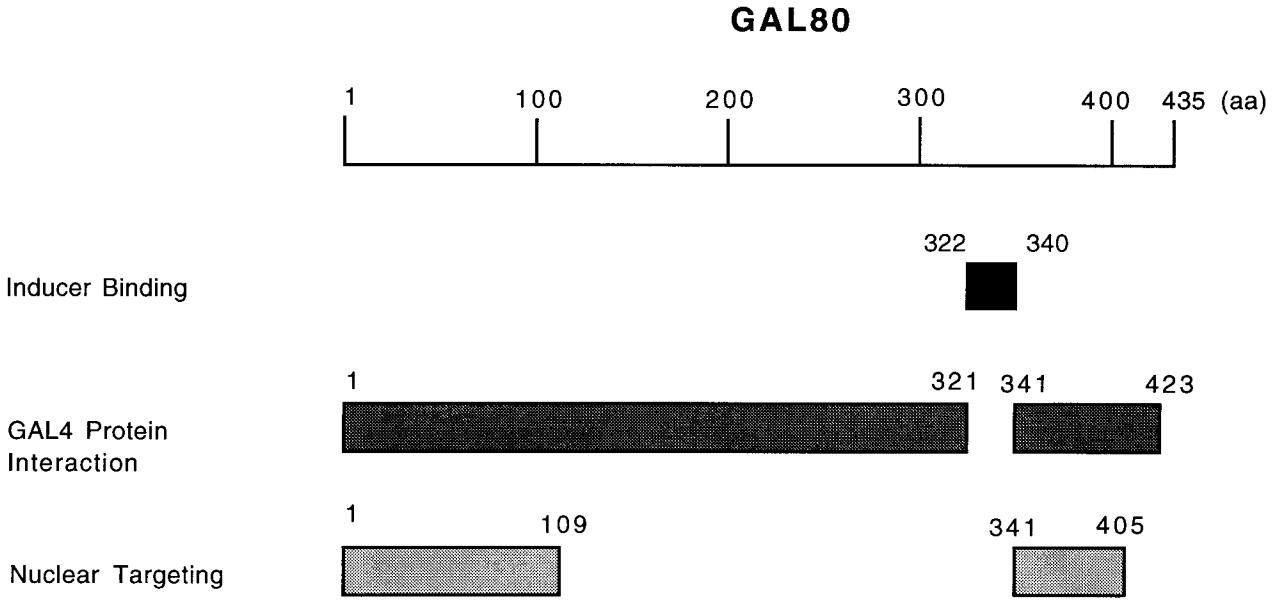
1987; Ma and Ptashne, 1987b). Molecular genetic analysis of GAL80 indicates that the repressor contains at least three functional domains (Figure 2). Two separate regions (amino acid residues 1-321 and 341-423) of the GAL80 protein are proposed to have the GAL4-repressing function. Experiments involving mutant GAL80 proteins carrying missense mutations in this domain indicated that the mutant proteins failed to bind GAL4 (Yun *et al.*, 1991). This GAL4 interaction domain appears to overlap those regions GAL80 (1-109) and GAL80 (341-405) presumably involved in targeting the GAL80 protein to the nucleus (Figure 2). Residues 322-340 of the GAL80 protein are involved in the interaction with the inducer (galactose) (Yun *et al.*, 1991).

According to the current model, the GAL80 protein binds to the GAL4 protein and inhibits its activator function in the absence of the inducer (galactose) (Ma and Ptashne, 1987b). Experiments have demonstrated that the GAL80 protein recognizes the carboxy-terminal 30 amino acids of the GAL4 protein and forms a GAL80•GAL4 protein complex. As a result, the GAL4 protein cannot activate transcription (Ma and Ptashne, 1987b). *In vitro* experiments performed by Lue *et al.* (1987) demonstrated that the GAL80 repressor protein does bind to the GAL4 protein. In addition, Yun *et al.* (1990) provided evidence that purified GAL80 protein was capable of binding to GAL4 protein. However, the exact mechanism by which this GAL80•GAL4 interaction interferes with GAL4 activity remains unclear.

VI. Regulation of the Galactose (*GAL*) system

A. Activation of the *GAL* System

The presence of galactose induces the transcription of the *GAL* genes by preventing the direct interaction of GAL4 activator protein with the repressor protein produced by *GAL80* (Lue *et al.*, 1987; Ma and Ptashne, 1987b). The GAL4 protein can then bind specific 17-bp sites within the UAS_{GAL}, found in regions upstream of *GAL1*, *-7*, and *-10*,


GAL80
1
100
200
300
400
435 (aa)
322
340
Inducer Binding
1
321
341
423
GAL4 Protein
Interaction
1
109
341
405
Nuclear Targeting

resulting in a significant induction in gene expression (Giniger *et al.*, 1985). The inducer (galactose) disassociates the GAL80•GAL4 protein complex and allows the GAL4 protein to function, resulting in the expression of the *GAL1*, *-7*, and *-10* genes (Ma and Ptashne, 1987b). Experiments have demonstrated that the GAL4 protein induces a 1000-fold increase in the transcription of *GAL1*, *-7*, *-10*, and *-2* genes, while inducing a 2-100-fold increase in the transcription of *GAL80* (Johnston, 1987) in the presence of the inducer (galactose). Studies involving constitutive GAL4^{c} mutants (always expressing *GAL* genes) indicate that changes in the GAL4 protein can presumably prevent interaction with the GAL80 protein (Ma and Ptashne, 1987b). An additional regulatory gene *GAL3*, whose function remains unknown, is also required for the efficient induction of *GAL4* activity in the presence of the inducer. In experiments involving *gal3* mutants (Spiegelman et al., 1950; Kew and Douglas, 1976), investigators noted an induction lag of 2-5 days in response to galactose; however, wild-type cells displayed an induction lag of only several minutes. Another gene , *GAL11*, is required for the full induction of *GAL* gene expression (Himmelfarb *et al.*, 1990). Investigators have demonstrated that the activity of the GAL4 protein on the *GAL* genes is five-fold less in strains containing a *GAL11* deletion as compared to wild-type cells (Suzuki *et al.*, 1988). The function of this gene remains unknown. However, Nishizawa *et al.* (1990) have suggested that *GAL11* product forms a complex with the GAL4 protein and somehow increases the GAL4 protein activity.

B. Carbon Repression of the *GAL* System

The expression of *GAL* genes is repressed in yeast growing on glucose. This mechanism of glucose repression results in the preferential utilization of glucose by cells in the presence of other carbon sources. Glucose repression of the *GAL* genes occurs by at least two mechanisms. First, glucose represses *GAL2* and *GAL3* gene expression. The reduced expression of the *GAL2* (encodes a galactose permease) and *GAL3* (required for the synthesis of the GAL4 protein) genes leads to a reduction in the level and function of

the GAL4 activator protein. As a result, the activity of the GAL80 protein increases and there is a decrease in *GAL* gene expression. Experiments have demonstrated that *GAL4* gene expression is transcriptionally repressed about five-fold in cells growing on glucose this, in turn, results in a 50-fold reduction in *GAL1* gene expression (Griggs and Johnston, 1991). Giniger and Ptashne, in 1988, established that the GAL4 protein binds cooperatively to its four UAS_{GAL} in the *GAL1* promoter. As stated earlier, the promoters of *GAL7*, *GAL10*, and *GAL2* also contain multiple sites for GAL4 protein binding (Johnston, 1987); therefore, the repression of *GAL4* may affect the expression of these genes in a similar fashion. *GAL80*, on the other hand, contains a single GAL4 binding site and is not repressed significantly in the presence of glucose (Griggs and Johnston, 1991). These findings suggest that *GAL4* functions synergistically with galactose to activate *GAL1* gene expression. Second, glucose repression can operate through UAS_{GAL} and upstream repression sequences (URS_{GAL}) in the *GAL* promoters. UAS_{GAL}- mediated glucose repression results in the failure of the GAL4 protein to bind UAS_{GAL} (Giniger et al., 1985). This could be due to the repression of *GAL4* gene expression, to the modification of the GAL4 protein, or to GAL4 proteolysis in the presence of glucose (Griggs and Johnston, 1991). Repression that operates through URS_{GAL}, located between UAS_{GAL} and the TATA box (Flick and Johnston, 1991; 1992), is possibly due to unidentified repressor proteins (Erickson and Johnston, 1993). One example is the MIG1protein which is produced by the *MIG1* gene. In the presence of glucose, this repressor binds to *GAL1* and *GAL4* promoters and plays a key role in the mechanism of repression operating through promoters. In addition to *MIG1*, several other genes are required for the repression of *GAL* genes in the presence of glucose. The actual repressor complex is formed through the probable association of MIG1 with the products of two genes, *SSN6* and *TUP1* (Keleher *et al.*, 1992). Three additional genes (*HXK2*-encodes a hexokinase; *REG1*-function unknown; *GRR1*-function unknown) have been associated

with glucose repression of gene expression, but their actual roles remain unclear.

VII. Filamentous Fungi

The filamentous ascomycetes are morphologically more complex than the yeasts of *Saccharomycetales*. The distinguishing features of the members within this group are the presence of ascogenous hyphae containing many asci and the formation of an ascocarp. In addition, these multicellular, eukaryotes are known for their conidium elaboration. A variety of different filamentous fungi have become important experimentally for the studies of biological processes. Aside from their economic importance, these organisms have a number of properties which make them important scientifically. First, these fungi can be grown on simple media and require less expensive laboratory equipment than most plants and animals. Second, They contain haploid genomes during most of their life cycle. This permits rapid identification of mutations and rules out the masking of a mutation by a dominant allele. Third, their sexually-derived spores can be harvested and cultured on media. Fourth, the rapid cycles and short generation times of these organisms enable the consistent and ongoing continuation of experiments. Finally, these filamentous fungi have been well-characterized genetically and therefore serve as attractive model systems.

In 1927, *Neurospora*, was discovered by Shear and Dodge, who pointed out the properties that make this filamentous fungus ideal for the study of genetics (Shear and Dodge, 1927). One species within this genus, *Neurospora crassa,* gained respect in 1958 as the experimental tool in the Nobel Prize-winning research which ultimately led to the theory "one gene, one enzyme." (Beadle and Tatum, 1941).

VIII. The Quinic Acid (*qa*) Gene Cluster of *Neurospora crassa*

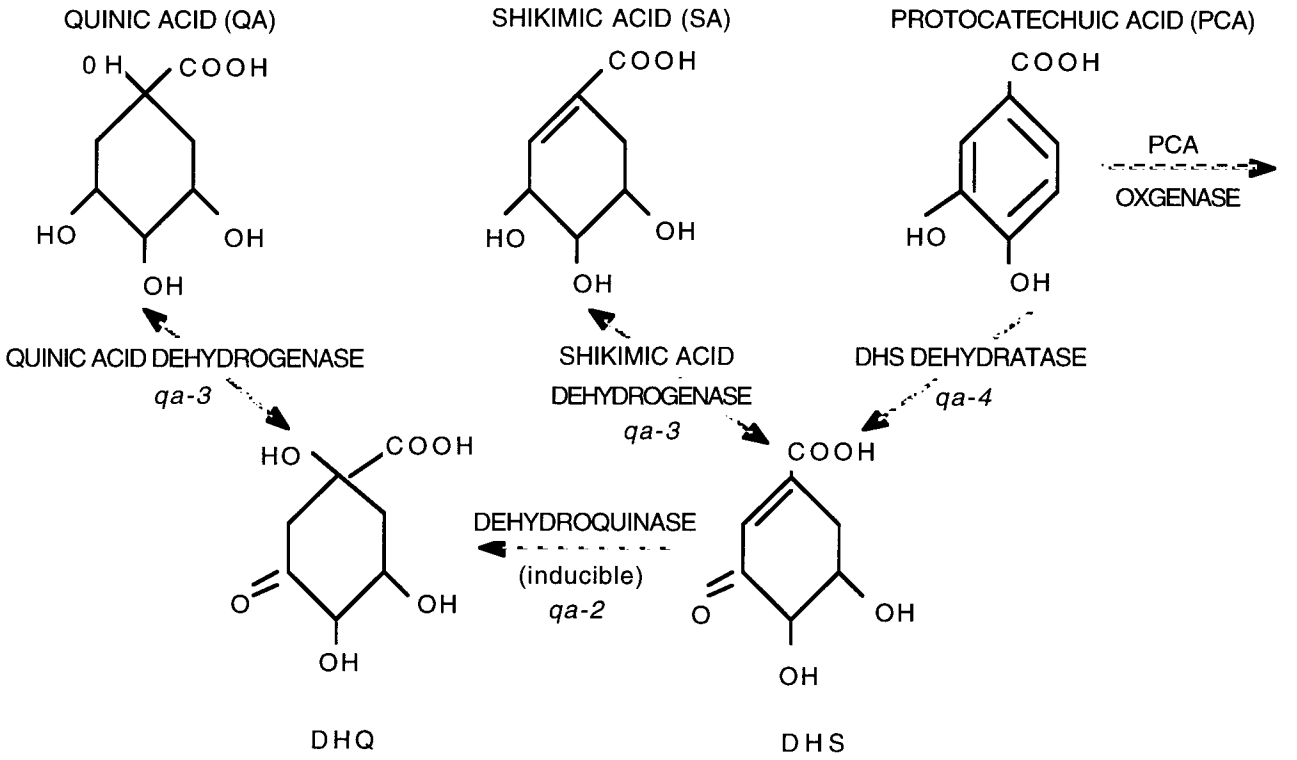
The quinic acid (*qa*) gene cluster of *N. crassa* provides a well-characterized system for the study of eukaryotic gene expression. Almost 30 years ago, early studies using *N.*

crassa mutants unable to use quinic acid, along with additional experiments conducted during the early 1970s, identified the existence of three distinct structural genes encoding three inducible enzymes which catalyze quinic acid catabolism (Figure 3). These initial studies also indicated the existence of a regulatory gene that controlled the synthesis of the three enzymes (Giles *et al.*, 1985).

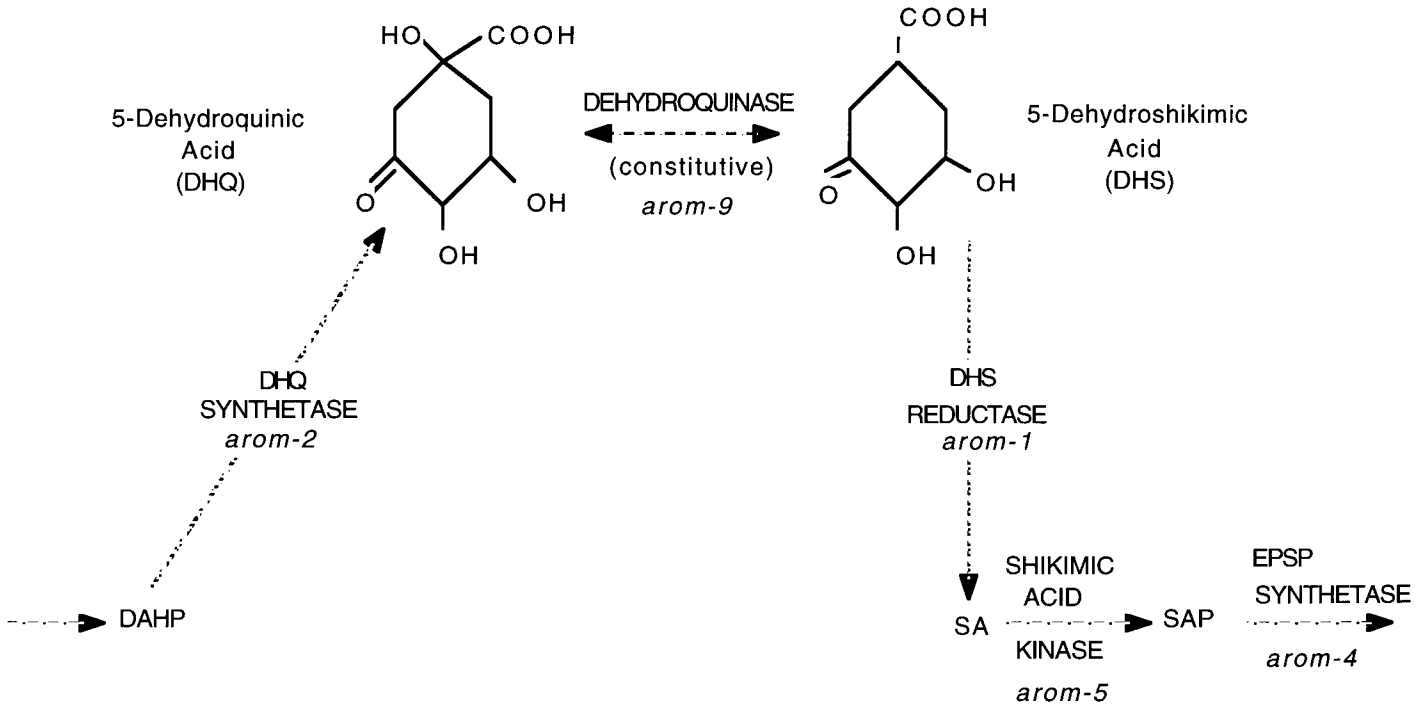
Experiments conducted by Rines (Ph.D. thesis, reviewed by Giles *et al.*, 1985), involving *qa-2* mutants, showed that these mutants did not display catabolic dehydroquinase (C-DHQase) activity. These findings provided evidence that the *qa-2* gene encoded a C-DHQase that catalyzes the conversion of 5-dehydroquinate to 5-dehydroshikimate (Figure 3). Experiments involving *N. crassa* strains which contained a *qa-2* mutation [lacking catabolic dehydroquinase (C-DHQase) activity] revealed a class of mutants called *arom-9 (*lacking biosynthetic dehydroquinase (B-DHQase) activity (cannot convert 5-dehydroquinate to 5-dehydroshikimate in the biosynthetic pathway) which mapped to the *arom* gene cluster (Rines *et al.*, 1969). Studies with pleiotropic *arom* mutants (lacking the five biosynthetic enzymes in the aromatic biosynthetic pathway) led to the isolation of a *N. crassa* double mutant strain which could not synthesize dehydroquinase (DHQase) or grow on quinic acid as a sole source of carbon. This strain contained a second mutation within a gene, designated *qa-1*, which was unlinked to the *arom* gene cluster (Giles *et al.*, 1985). Studies of *arom-9*$^{-}$-*qa-2*$^{+}$ mutants (lacking B-DHQase activity) demonstrated that C-DHQase activity (*qa-2*) can substitute for the absent B-DHQase activity. The reverse was also observed, *qa-2*$^{-}$-*arom-9*$^{+}$ mutants can grow at a reduced rate on quinic acid as the sole carbon source (Giles and Case, 1975). These results suggest that the *qa-2* product (C-DHQase) is complemented by the B-DHQase of *arom-9*.

Strains which contained a mutation in the *qa-3* gene showed no quinic acid dehydrogenase (QDHase) activity nor shikimic acid dehydrogenase (SDHase) activity (Chaleff, 1974). However, one of the *qa-3* mutants reverted and displayed both QDHase

A


QUINIC ACID (QA)
SHIKIMIC ACID (SA)
PROTOCATECHUIC ACID (PCA)
PCA
OXGENASE
QUINIC ACID DEHYDROGENASE
qa-3
SHIKIMIC ACID
DEHYDROGENASE
qa-3
DHS DEHYDRATASE
qa-4
DEHYDROQUINASE
(inducible)
qa-2
DHQ
DHS

B


5-Dehydroquinic
Acid
(DHQ)
DEHYDROQUINASE
(constitutive)
arom-9
5-Dehydroshikimic
Acid
(DHS)
DHQ
SYNTHETASE
arom-2
DHS
REDUCTASE
arom-1
DAHP
SHIKIMIC
ACID
KINASE
arom-5
SA
SAP
EPSP
SYNTHETASE
arom-4

and SDHase activity. This suggested that *qa-3* gene encoded a bifunctional enzyme which catalyzes quinate and shikimate dehydrogenation (Figure 3). Chaleff (1974) also determined that one mutant (originally obtained by Case) lacked dehydroshikimate dehydrotase (DHS-Dase) activity which catalyzes the conversion of dehydroshikimate to protocatechuic acid. This mutation was identified as *qa-4* (Figure 3).

Experiments involving heterokaryons (fungal cells containing two different nuclei - a *qa-1* mutation in one nucleus and in the other nucleus a mutation in another gene) revealed the presence of two classes of *qa-1*mutations. One class, designated as fast-complementing *qa-1F*$^-$ mutants, are recessive to *qa-1F*$^+$ in heterokaryons.(Giles *et al.*, 1985) when grown on quinic acid as the sole carbon source. The other class of mutants, designated as slow-complementing *qa-1S*$^-$, were selected after mutagenesis of an *arom-9*$^-$ mutant. These mutants were distinguished from *qa-1F*$^-$ mutants in being only partially or almost completely dominant to *qa-1S*$^+$ in heterokaryons (Giles *et al.*, 1985). In heterokaryons, these *qa-1S*$^-$ mutants are slow-complementing when cultured on minimal media, but are fast-complementing with mutations in unrelated genes.

IX. Cloning of the Entire Quinic Acid (*qa*) Gene Cluster of *N. crassa*

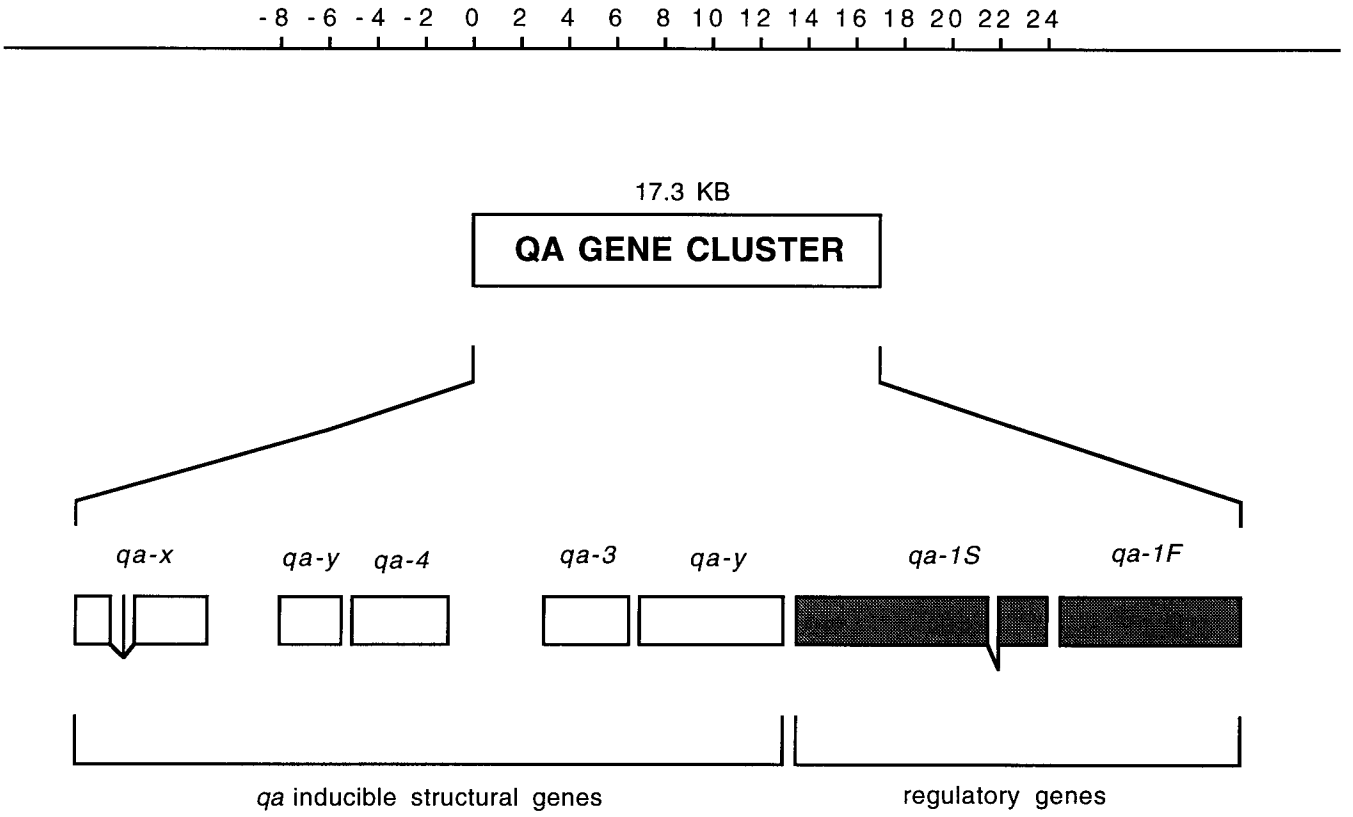
The emergence of recombinant deoxyribonucleic acid (DNA) technology made possible the cloning of the entire *qa* gene cluster. First, one of the structural genes, *qa-2*, was successfully cloned into *Escherichia coli* (Vapnek et al., 1977) by complementation. This was accomplished because the *qa-2* gene, if expressed in *E. coli*, could complement the *E. coli aroD* mutant (lacks biosynthetic (B-DHQase) activity). Attempts to clone the other genes, *qa-3* and *qa-4*, in *E. coli* were unsuccessful and a test for *qa-1F* had not been not developed at the time (Schweizer *et al.*, 1981b). Soon however, the development of a new *N. crassa* transformation technique (Case *et al.*, 1979) permitted the cloning of the additional *qa* genes by complementation of *Neurospora qa* mutants. Subclones from a 42-

kilobase (kb) region of cloned *N. crassa* DNA (centered around *qa-2*) (Schweizer *et al.*, 1981a, 1981b) were successful in localizing and determining the order of the three structural genes (*qa-2* --> *qa-4* --> *qa-3*) (Figure 4). Transformation experiments (Schweizer *et al.*, 1981a, 1981b) using stable, *qa-1F* mutants as recipients determined that the *qa-1* region was located to the right of *qa-3* on the genetic map. Additional experiments used *qa-1S* mutants as recipients. Subclones in the *qa* region transformed *qa-1F* mutants but not *qa-1S* mutants, while others transformed *qa-1S* and not *qa-1F* mutants. These results, along with genetic mapping, established the order of the two regulatory genes (*qa-3* --> *qa-1S* --> *qa-1F*) (Figure 4).

The cloned genes permitted the identification of *qa-2*, *qa-3*, and *qa-4* messenger ribonucleic acids (RNAs). DNA-RNA hybridization studies have revealed the existence of five, rather than three structural *qa* genes, transcribed as separate mRNAs (Giles *et al.*, 1985). In addition to the known structural genes *qa-2*, *qa-3*, and *qa-4*, two other genes (*qa-x* and *qa-y*) were identified as part of the *qa* gene cluster.

The *qa-x* gene is located to the left of *qa-2* (Figure 4) (Giles *et al.*, 1985). The function of *qa-x* is still unknown. In the presence of quinic acid, *qa-x*$^-$ mutants grow, and after a while, begin to produce a brown pigment (Asch, unpublished). The fact that these mutants also grow on chlorogenic acid ruled out the hypothesis that *qa-x* may encode an enzyme that hydrolyzes chlorogenic acid (Giles *et al.*, 1985). Studies of the *qa-x* gene indicated a 31% homology with a gene (*GAL12*) that controls carbon-regulated GAL4 protein dephosphorylation (Hopper and Mattes, personal communication, reviewed by Case *et al.*, 1992). However, Case *et al* (1992) demonstrated that deletion of the *qa-x* gene in *N. crassa* (Δ*qa-x* mutants) did not affect carbon-mediated *qa-2* gene repression.

The structural gene *qa-y* is located between *qa-3* and *qa-1S* (Figure 4) (Giles *et al.*, 1985). Indirect evidence indicates that the *qa-y* gene encodes a quinic acid permease. In 1989, Geever *et al.* conducted a Genbank data base search and found that the *qa-y* gene


-8 -6 -4 -2 0 2 4 6 8 10 12 14 16 18 20 22 24
17.3 KB
QA GENE CLUSTER
qa-x
qa-y
qa-4
qa-3
qa-y
qa-1S
qa-1F
qa inducible structural genes
regulatory genes

shared probable homology with a glucose transporter of human hepatoma cells. In addition, Case *et al.* (1992) found that *Neurospora* mutants Δ*qa-y* (deletion of *qa-y*) could not grow on quinic acid as a sole carbon source and had low levels of *qa-2* and *qa-3* enzyme activities. This suggests that *qa-y* encodes a quinate permease whose absence in Δ*qa-y* mutant strains leads to reduced quinic acid uptake.

The availability of cloned DNAs for transformation and DNA-RNA hybridization indicated that the fast- and slow-complementing regions of the *qa-1* gene make up two distinct genes, *qa-1F* and *qa-1S* respectively (Huiet, 1984). These two genes, which encode separate mRNAs, are transcribed divergently (Figure 4).

Transformation experiments, Northern blot analysis, SI nuclease mapping, and nucleotide sequence have illustrated the structure of the *qa* gene cluster (Giles *et al.*, 1985; Geever *et al.*, 1989). The seven *qa* genes occupy approximately 17.3-kb of DNA in chromosome VII (Figure 4). The organization of the *qa* genes and the lengths of their mRNAs is illustrated in Figure 4. The *qa* regulatory genes, *qa-1F* and *qa-1S*, are located at one end of the cluster, while the structural genes, *qa-2*, *qa-3*, *qa-4*, *qa-x*, and *qa-y*, are grouped together. The genes are divergently transcribed in the pairs *qa-x*/*qa-2*, *qa-4*/*qa-3*, and *qa-1S*/*qa-1F*. Only the *qa-y* gene is unpaired.

X. The *qa-1F* Activator Protein

The *qa-1F* gene encodes an activator protein of 816 amino acids (Huiet, 1983; Geever *et al.*, 1989). At least four functional domains have been identified within the *qa-1F* protein (Salmeron and Johnston, 1986; Beri *et al*, 1987; Geever *et al.*, 1987, 1989). DNase I footprinting experiments implicated the first 183 amino acids of the *qa-1F* protein as being responsible for specific DNA binding (Baum *et al.*, 1987). This region contains a six cysteine motif which shows conservation with lower eukaryotic activators such as the GAL4 protein of *S. cerevisiae* (Baum *et al.*, 1987; Pfeifer *et al.*, 1989). A second domain,

expanding a broad central segment of the *qa-1F* protein, may contain residues essential for dimerization. Ma and Ptashne (1987) have suggested that dimer formation may constitute the *qa-1F* active binding form, but no data exists to support this hypothesis. The third region, located at the carboxy terminus of the activator, involves mainly acidic residues. This region has been identified in the GAL4 protein and is responsible for transcriptional activation (Ma and Ptashne, 1987). By analogy, this region is likely to play a similar role of transcriptional activation in *qa-1F*. Experiments involving frameshift and nonsense mutations (located proximal to the carboxy terminus of the *qa-1F* protein) resulted in the production of noninducible *qa-1F*$^-$ mutants lacking this domain (Geever *et al.*, 1987, 1989). A fourth domain presumably interacts with the *qa-1S* repressor protein.

Patel and Giles (1985) provided evidence that the *qa-1F* protein plays a role in transcription of all the *qa* genes, including itself. Studies have demonstrated that *qa-1F* mRNAs of *Neurospora* wild-types are produced at basal levels in the absence of quinic acid, but a 50-fold elevation is observed upon quinic acid induction (Giles *et al.*, 1985). Also, the same basal levels of *qa-1F* mRNA found in the wild-type were observed in noninducible *qa-1F* mutants (Giles et al., 1985). Additional experiments involving noninducible *qa-1F*$^-$mutants indicate that transcription of all the *qa* genes occurred at the same low basal levels found in the uninduced wild-type (Avalos, Geever, and Giles, unpublished data). In uninduced *Neurospora* wild-type cultures low levels of *qa-1S* mRNAs are present. However, upon quinic acid induction, this level increases approximately 10-fold. These results suggest that the *qa-1F* activator protein plays a positive role in *qa* gene transcription and is autoregulated.

In vivo studies have provided evidence that the *qa-1F* activator protein binds to a conserved, symmetrical 16-bp sequence (GGRTAARYRYTTAYCC) in the 5' flanking regions of the *qa* genes (Baum *et al.*, 1987; Geever *et al.*, 1989) and initiates transcription of itself and all of the *qa* genes. A comparable binding sequence has been identified as the

GAL4 binding site in yeast (Giniger *et al.*, 1985). DNA binding and DNase I footprinting experiments have identified the locations of 14 of these *qa-1F* protein binding sites throughout the *qa* gene cluster of *N. crassa* (Baum *et al.*, 1987; Geever *et al.*, 1989).

XI. The *qa-1S* Repressor Protein

Giles and Case (1975) distinguished two classes of *qa-1S* mutants. First, noninducible, dominant *qa-1S*$^{-}$ mutants which are partially to completely dominant to *qa-1S*. These proteins seem to act as super-repressors. A second class, constitutive, recessive *qa-1S*^{c} mutants, produce all three enzymes in the absence of the inducer. These proteins seem to act as inactive repressors. The *qa-1S*^{c} mutants are derived either from wild-type or as "revertants" of the non-inducible *qa-1S*$^{-}$ mutants. These two classes of mutants defined the *qa-1S* gene (Giles *et al.*, 1985) which codes for a 918 amino acid repressor protein (Geever *et al.*, 1987).

Indirect evidence suggests that the *qa-1S* protein interacts with the *qa-1F* activator protein (Giles *et al*, 1987; Avalos *et al.*, unpublished) resulting in the formation of a *qa-1S•qa-1F* protein complex. This complex inhibits *qa-1F* activity, thus preventing *qa* gene induction. This same phenomenon is observed between the GAL4 activator protein and the GAL80 repressor protein within the *GAL* system of *S. cerevisiae*.

XII. Regulation of the Quinic Acid (*qa*) Gene Cluster of *N. crassa*

A. First Regulatory Circuit

The expression of the *qa* genes appears to be controlled by two levels of genetic regulation. In the first regulatory level, interaction of the *qa-1F* and *qa-1S* proteins in response to quinic acid levels in the cell, control transcription of the *qa* genes. Uninduced wild-type cultures (grown in the absence of quinic acid) and uninduced mutants (*qa-1F*$^{-}$ and *qa-1S*$^{-}$) grown in the absence or presence of quinic acid contained only trace amounts

of *qa* mRNAs. However, constitutive (*qa-1S*^{c}) mutants grown in the absence of quinic acid contained elevated amounts *qa* mRNAs (Giles et al., 1985). The results indicate that *qa* gene expression is regulated at the transcriptional level by both the *qa-1F* gene and the *qa-1S* gene. And this regulation is dependent on the presence of quinic acid (Patel *et al.*, 1981; Huiet, 1984). In the presence of the inducer, transcription of all of the genes in the *qa* cluster is induced 50- to 1,000-fold by the action of the activator. Studies (Baum et al., 1987) have shown that this results from binding of the activator to conserved 16-bp sequences (GGRTAARYRYTTATCC) as determined by DNA I protection experiments resulting in the transcription of the *qa* genes. A similar example involves the galactose (*GAL*) system in yeast. The presence of galactose (inducer) switches on the *GAL4* gene whose product induces *GAL1*, *-7*, *-10* gene transcription.

In absence of quinic acid, all *qa* genes are transcribed at low basal levels. This is probably due to the direct interaction of the *qa-1S* repressor product (Geever *et al.*, 1989) with the *qa-1F* activator protein which inhibits activator function (Giles *et al.*, 1991). The same can be observed in the *GAL* system of *S. cerevisiae*. In the absence of the inducer galactose, GAL80 (repressor) forms a complex with GAL4 (activator) inhibiting the activity of the activator.

B. Second Regulatory Circuit

A second regulatory circuit represses *qa* gene transcription in response to a preferred carbon source such as glucose or sucrose. In the presence of quinic acid and a preferred carbon source, wild-type *N. crassa* strains show a repressed level of *qa* gene induction as when observed with quinic acid alone. The mechanisms by which catabolite repression affects *qa* gene expression is unknown. The *GAL* system of *S.cerevisiae* , which has regulatory mechanisms similar to the *qa* gene cluster, offers two possible explanations. First, the *qa-1F* activator protein may not be able to bind to the activator binding sites in the presence of glucose. As a result, *qa* gene expression is reduced. This may be due to

protein modification, proteolysis, or repression of *qa-1F* gene expression. Second, interaction of carbon repressors, in the presence of a preferred carbon source such as glucose, with sequences 5' to *qa* genes to prevent transcription may be another possible mechanism of repressor action. However, the presence of such sequences have not been identified in the *qa* system.

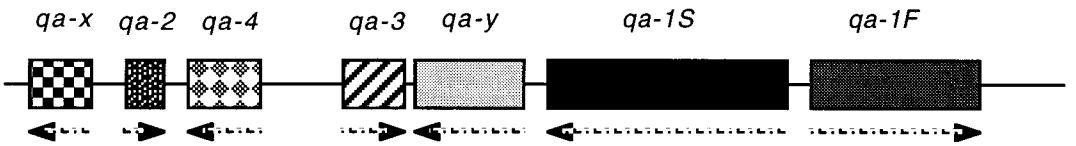
VIII. Similarities between the Quinic Acid (*qa*) System of *N. crassa* and the Galactose (*GAL*) System of *S. cerevisiae*

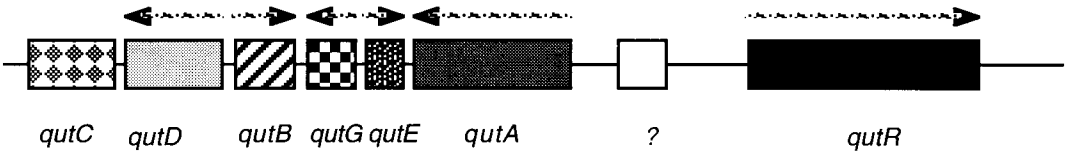
Similarities of gene regulation are observed in both the quinic acid (*qa*) system of *N. crassa* and the galactose (*GAL*) system of *S. cerevisiae*. First, in the presence of the inducer (quinic acid; galactose), both activator proteins (*qa-1F*; GAL4) induce the transcription of various genes. However, in the absence of an inducer, a repressor protein (*qa-1S*; GAL80) inhibits activator function by forming a complex with the activator protein. Second, gene expression of both systems is repressed in the presence of a preferred carbon source (glucose), even in the presence of an inducer.

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

Regulation of the *qut* gene cluster of the related species *Aspergillus nidulans* appears to be analogous to the *N. crassa qa* cluster (Figure 5). First, the regulatory genes, *qutA* (activator) and *qutR* (repressor), of the quinic acid utilization (*qut*) cluster seem to be homologous to the *qa-1F* and *qa-1S* genes. Second, amino acid comparisons show a 68.5% identity between *qa-x* and the gene *qutG* (within the (*qut*) cluster of *A. nidulans*). The *qutG* gene has a quinate-inducible message, but its function is also unknown. Third, the corresponding gene *qutD* of *A. nidulans* was predicted to encode a quinic acid permease (transports quinic acid into the cell) based on a 61% amino acid similarity with the *qa-y*

QA GENE CLUSTER





QUT GENE CLUSTER

gene (Whittington *et al.*, 1987). Fourth, both clusters are arranged in pairs of divergently transcribed genes. Fifth, structural and regulatory genes are found in different regions of the cluster.

Differences have also been observed. The order of the regulatory genes in *A. nidulans* (*qutA*-activator; *qutR*-repressor) is inverted as compared to the regulatory genes of *N. crassa* (*qa-1S*-repressor; *qa-1F*-activator) (Figure 5). Also an unknown gene is located between the two regulatory genes of *A. nidulans*. And not all of the same gene pairs are divergently transcribed in the two related species.

XV. Comparative Studies Between the Quinic Acid (*qa*) Gene Clusters of *Neurosprora* Species

Comparative studies of the *qa* gene cluster have been initiated in various fungi as a result of the prior detection of *qa* catabolic enzymes in other fungi (Ahmed and Giles, 1969; Berlyn and Giles, 1972). In an attempt to elucidate the mechanisms that control *qa* gene expression, Asch et al. (1991) decided to study and compare the *qa* genes of various species of *Neurospora*. A high degree of conservation was found within the *qa* clusters of both heterothalic (different nuclei) and homothalic (same nuclei) *Neurospora* species. Southern blot analysis also revealed many restriction fragment length polymorphisms in homothalic species as compared to their heterothallic *N. crassa* counterpart. However, the *qa* gene organization of *N. crassa* seemed to be conserved (*qa-x*, *qa-2*, *qa-4*, *qa-3*, *qa-y*, *qa-1S*, *qa-1F*) in the homothalic species. Although sequencing results indicated that the *qa-x-qa-2* intergenic region of the homothalic species *Neurospora africana* (1088 bp) is smaller than that of the heterothallic *N.crassa* (1194), all four *N. africana qa-1F* activator binding sites could be aligned with the activator binding sites of *N. crassa*. Case and Asch (1992) provided evidence that the *N. africana qa-1F* binding domain is identical to the *N. crassa* domain, suggesting that the *qa-1F* protein probably induces the expression of the *N.*

africana qa-x and *qa-2* genes by the same mechanisms occurring in *N. crassa.*

In the presence of quinic acid as a sole carbon source, catabolic dehydrogenase assays indicated a similar induction of *qa-2* gene expression (over basal levels) in *N. africana* as observed in *N. crassa.* However, a 3-fold reduction (as compared with a 65-fold reduction in *N. crassa*) was observed in *N. africana qa-2* gene expression in the presence of quinic acid and a preferred carbon source such as glucose. These results indicated that both circuits were operating in *N. africana*, but repression of *qa-2* gene expression did not occur to the same extent in *N. africana* as in *N. crassa.*

When the *qa-x-qa-2* intergenic region of *N. crassa* was replaced with the *qa-x-qa-2* intergenic region of *N. africana*, transformants showed about a 70% induction in *qa-2* gene expression as compared to wild-type *N. crassa* in the presence of quinic acid. Whereas, in the presence of dextrose, the level of repressed catabolic dehydrogenase activity was similar between the both wild-type *N. crassa* and the transformed *N. africana* strain. The results indicate that sequence differences between the *Neurospora* species did not affect catabolite repression.

Studies by Case *et al.* (1992) revealed that *N. crassa* strains containing a Δ*qa-1S* deletion displayed elevated *qa-2* gene expression in the absence of quinic acid and even in the presence of glucose. Asch and Case (1992) further demonstrated that Δ*qa-1S* mutants showed slightly repressed *qa-x*, *qa-2*, and *qa-4* gene expression the presence of glucose. While the *qa-3*, *qa-y*, and *qa-1F* genes were more highly repressed. The results suggest that some of the *qa* genes may be regulated by different mechanisms and that the *qa-1S* protein may play a role in carbon catabolite repression. The *qa-1S-qa-1F* intergenic region of *N. africana* will be analyzed to further understand the mechanisms regulating the *qa* gene cluster of *N. crassa.* The *qa-1S-qa-1F* intergenic region of *N. africana* was chosen for study because it may play a role in the carbon catabolite repression of the *qa* genes in the presence of a preferred carbon source (glucose).

MATERIALS AND METHODS

I. Materials

T4 DNA ligase (1 U/µl), restriction endonucleases [*Eco*RI (10 U/µl), *Bam*HI (10 U/µl), *Kpn*I (10 U/µl), *Sac*I (10 U/µl), *Sma*I (10 U/µl)], DIG *Taq* DNA Sequencing Kit for Standard and Cycle Sequencing, DIG DNA Labeling and Detection Kit, blocking reagent, disodium 3-(4-methoxyspiro {1,2-dioxetane3,2'(5'chloro)tricyclo[3.3.1.1.^{3,7}] decan}-4-yl) phenyl phosphate [CSPD], anti-digoxigenin-AP fab fragments, bis-acrylamide, acrylamide, and positively charged nylon membranes were purchased from Boehringer Mannheim, GmbH, Germany. Restriction endonucleases [*Pst*I (15 U/µl), *Hin*dIII (15 U/µl), *Xho*I (15 U/µl)], ethylenediaminetetraacetic acid • disodium salt [EDTA], isopropyl-ß-D-thiogalactoside [IPTG], 5'bromo-4-chloro-3-indoyl-ß-D-galactopyranoside [Xgal], sodium dodecyl sulfate [SDS] and ammonium persulfate [AMPS] were purchased from International Biotechnologies, Inc., New Haven, CT. Ampicillin, sodium chloride [NaCl], potassium acetate [KOAc], sodum acetate [NaOAc], 3-N-morpholino-propanesulfonic acid [MOPS], (tris[Hydroxymethyl]aminomethane) [Trizma Base], RNase A, octyl phenoxy polyethoxyethanol [Triton X-100], urea, polyoxyethylene-sorbitan monolaurate [Tween 20], N,N,N',N'-tetramethylethylene-diamine [TEMED], sigmacote, developer & replenisher, fixer & replenisher, lithium chloride [LiCl], and N-lauroyl-sarcosine were purchased from Sigma Chemical Co., St. Louis, MO. *PERFECTprep* Plasmid DNA Kit was purchased from 5 Prime —› 3 Prime, Inc., Boulder, CO. Phosphoric acid [H_3PO_4] (85%), ethidium bromide [EtOH], chloroform, and sodium citrate were purchased from FisherScientific, Fair Lawn, NJ. Bacto-trypton, bacto-agar and bacto-yeast extract were purchased from Difco Laboratories, Detroit, MI. Hydrochloric acid [HCl], magnesium chloride [$MgCl_2$], and calcium chloride [$CaCl_2$] were made by Mallinckrodt, Inc., Paris, KY. Agarose was made by EM Science,

Gibbstown, NJ. Maleic acid and polaroid film were purchased from Eastman Kodak Co., Rochester, NY. Dextrose, phenol, and acetic acid [HOAc] were made by J.T. Baker Chemical Co., Phillipsburg, NJ. Sodium hydroxide [NaOH] was purchased from VWR, Media, PA. Isopropanol was made by Baxter Healthcare Corp., McGraw Park, IL. Ethanol [EtOH] was purchased from Aaper Alcohol and Chemical Co., Shelbyville, KY.

II. Strains and Media

Recombinant plasmids were propagated on *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 + ampicillin [LA_{100}] (LB; 1.5% bacto-agar; ampicillin (100 µg/ml), also containing 100 µl IPTG (200 mM) and 50 µl Xgal (2%). Transformants were picked to LB containing ampicillin (100 µg/ml) [LA_{100} broth].

III. pBluescript II KS (+/-) Phagemid

The 2,961 basepair [bp] phagemid, derived from pUC19, was purchased from Stratagene, La Jolla, CA. A portion of the *lacZ* gene (encoding ß-galactosidase), located within the phagemid, confers blue/white color selection of recombinants in the presence of IPTG and Xgal. The multiple cloning site [MCS] is oriented such that cloning into the region results in the disruption of *lacZ* translation. Antibiotic selection utilized the ampicillin resistance gene located within the vector.

IV. Agarose Gel Electrophoresis

The condition of the DNA used in all of the experiments was analyzed by agarose gel electrophoresis. Briefly, DNA was loaded into wells and run on a 1% agarose gel in 1X Tris-Phosphate [TPE] buffer (0.08 M Trizma base; 0.5 M EDTA; 85% H_3PO_4 [1.679 mg/ml]). The gel was stained with EtBr (50 mg/ml) and visualized on a transilluminator.

V. Restriction Digest of Vector

Five micrograms of the pBluescript vector was incubated at 37°C overnight in the presence of sterile H_2O, a restriction enzyme, and 10X buffer as described by the manufacturer. A small sample was run on a 1% agarose gel. If the DNA was digested, 400 µl of neutralized phenol was added to the eppendorf tube. The sample was then centrifuged (12,000-16,000 xg) at room temperature [RT] for 10 minutes. The top layer was removed and placed into a sterile eppendorf tube. Four hundred microliters of chloroform was added to the tube and it was placed into a microcentrifuge (12,000-16,000 xg) for 5 minutes at RT. After centrifugation, the top layer was transferred to another sterile tube and 400 µl of isopropanol was then added. The sample was centrifuged (12,000-16,000 xg) at RT for 5 minutes. The liquid was decanted and the DNA pellet dried for 20 minutes. The pellet was resuspended in 20 µl of 1X TE buffer (0.01 M Trizma base, pH 8.0; 0.001 M EDTA, pH 8.0).

VI. Preparation of Fragments

Fragments were prepared by digesting 10 µg of the insert (supplied by Dr. Asch) with a restriction endonuclease. The fragments were resolved by agarose gel (1%) electrophoresis in 1X TPE buffer, cut from the gel and placed into a dialysis bag filled with 0.5X Tris-Acetate [TAE] buffer (0.04 M Trizma base; 0.02 M NaOAc; 0.002 M EDTA, adjust to pH 7.9 with 121 ml glacial acetic acid). The bag was clamped at both ends and placed into a gel tank to undergo electrophoresis for 45 minutes. Meanwhile, an Elutip column was primed by passing 3 ml of high salt buffer, pH 7.4 (1 M NaCl; 0.02 M Trizma base; 0.001 M EDTA) and then passing 3 ml of low salt buffer, pH 7.4 (0.2 M NaCl; 0.02 M Trizma base; 0.001 M EDTA) through the column. After electrophoresis, the liquid was drawn out of the bag and passed over the primed Elutip column as described by the manufacturer. The DNA was then eluted from the column with 400 µl of high salt buffer,

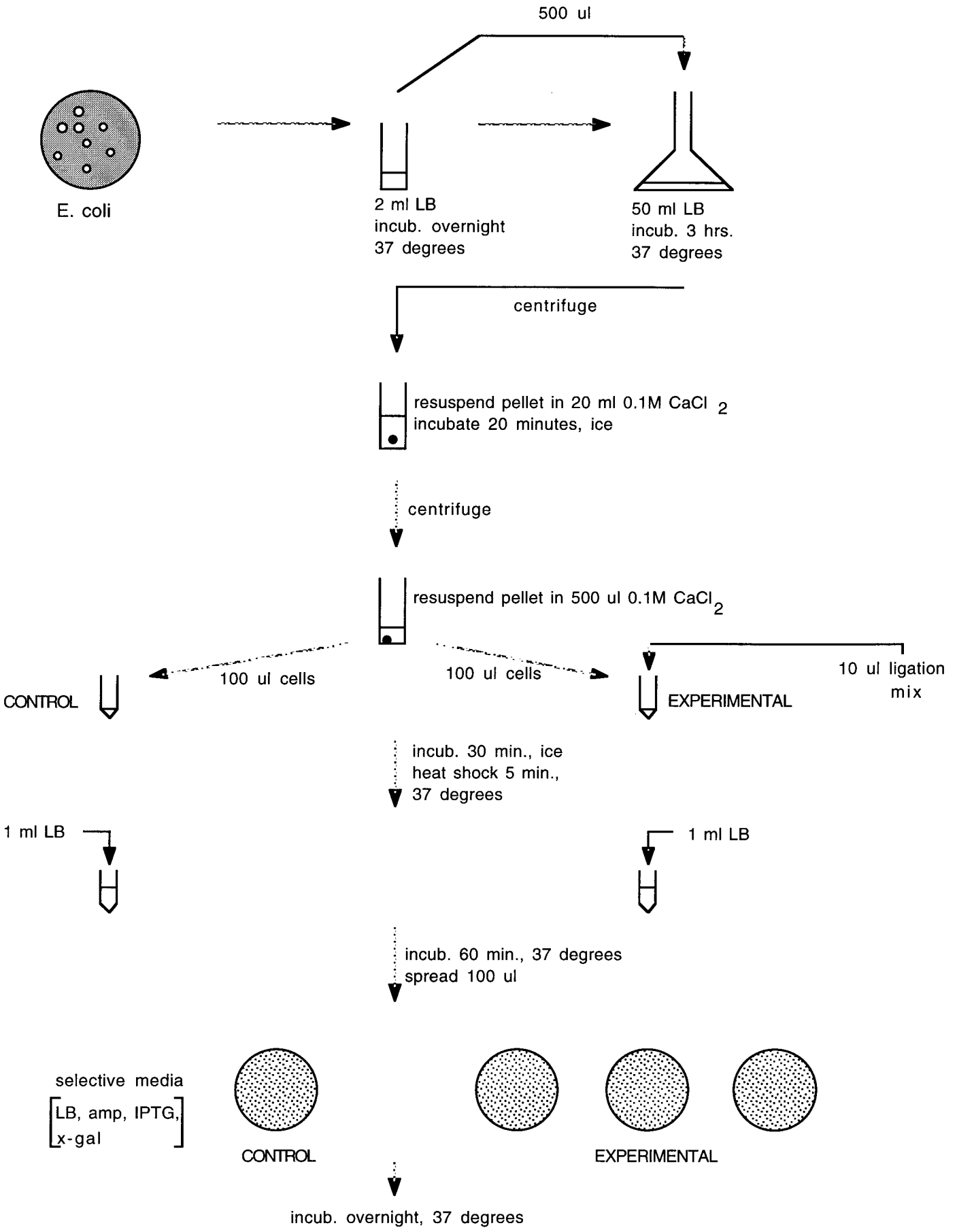
extracted and precipitated as described above (see section V: Restriction Digest of Vector). The supernatant was decanted. After the DNA pellet dried for 20 minutes, it was redissolved in 20 µl of 1X TE buffer.

VII. Construction of Recombinant Plasmid

Recombinant DNA was constructed by ligating an isolated fragment to a cleaved pBluescript vector. T4 DNA ligase was used in accordance with the manufacturer's instructions. Briefly, 5 µl of the insert, 3 µl of the vector, 3 µl of 10X ligase buffer,18 µl of sterile H_20, and 1 µl of T4 DNA ligase were added to a sterile eppendorf tube and incubated at 15°C overnight.

VIII. Transformation of *Escherichia coli* JM101

E. coli JM101 was transformed with recombinant plasmid DNA using the $CaCl_2$ procedure (Figure 6). Two milliliters 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 overnight growth and incubated at 37°C for 3 hours. The cells were chilled on ice for 10 minutes and then centrifuged (12,000 xg; 4°C; 10 minutes). The pellet was resuspended in 20 ml of cold 0.1 M $CaCl_2$ and placed on ice for 30 minutes. After the incubation, the cells were centrifuged (12,000 xg; 4°C; 10 minutes) and the pellet was resuspended in 0.5 ml of cold 0.1 M of $CaCl_2$. One hundred microliters of competent cells were dispensed into two sterile eppendorf tubes labeled "control" and "experimental" respectively. Ten microliters of ligation mix was added to the "experimental" tube only, and both tubes were placed on ice for 30 minutes. The tubes were then transferred to a 37°C heat block for 5 minutes. One milliliter of LB was then added to both tubes and they were placed into a 37°C incubator for 60 minutes. Afterwards, 100 µl of the transformation mix was spread onto selective medium (LA_{100}; IPTG; Xgal) and the plates were incubated at 37°C overnight.


500 ul
E. coli
2 ml LB
incub. overnight
37 degrees
50 ml LB
incub. 3 hrs.
37 degrees
centrifuge
resuspend pellet in 20 ml 0.1M CaCl 2
incubate 20 minutes, ice
centrifuge
resuspend pellet in 500 ul 0.1M CaCl2
100 ul cells
100 ul cells
CONTROL
EXPERIMENTAL
10 ul ligation mix
incub. 30 min., ice
heat shock 5 min.,
37 degrees
1 ml LB
1 ml LB
incub. 60 min., 37 degrees
spread 100 ul
selective media
[LB, amp, IPTG, x-gal]
CONTROL
EXPERIMENTAL
incub. overnight, 37 degrees

IX. Isolation of Recombinant DNA (Alkaline Plasmid Screen)

Transformants were picked to 2 ml of LA_{100} broth and incubated at 37°C overnight. One and one-half milliliters of overnight culture was harvested into a sterile eppendorf tube. The tube was placed into a microcentrifuge (12,000-16,000 xg) for 15 seconds at RT. The pellet was drained and resuspended in 0.2 ml of G buffer (0.05 M dextrose; 0.025 M Trizma base, pH 8.0; 0.01 M EDTA, pH 8.0). Four hundred microliters of Denaturing solution (0.2 N NaOH; 1% SDS) was then added and the tube was inverted several times. The cells were placed on ice for 5 minutes. Three hundred microliters of chilled Neutralizing solution (3 M KOAc; 2 M HOAc) was added to the eppendorf tube and it was inverted several times. The cells were incubated on ice for 15 minutes. Afterwards, the cells were centrifuged (12,000-16,000 xg) at RT for 5 minutes. The supernatant was transferred to a sterile eppendorf tube and mixed well with 0.54 ml of isopropanol. After a 5 minute centrifugation (12,000-16,000 xg) at RT, the isopropanol was decanted and the pellet was washed twice at RT in 80% EtOH. After drying, the DNA pellet was resuspended in 50 µl of 1X TE buffer and stored at 4°C.

X. Restriction Digest of recombinant DNA

Recombinant DNA was digested with various restriction endonucleases as described by the manufacturer. Briefly, 10 µl of recombinant DNA was incubated at 37°C for 3 hours in the presence of 15-16 µl of sterile H_2O, 1 µl of enzyme and 3 µl of 10X enzyme buffer.

XI. Large Scale Isolation of Plasmid DNA (Qiagen Preparation)

Transformants were picked to 2 ml of LA_{100} broth and incubated at 37°C overnight. A flask, containing 50 ml of LA_{100} broth, was inoculated with 0.5 ml of the overnight growth and incubated at 37°C overnight. The cells were harvested into a sterile centrifuge

tube and placed inside an ultracentrifuge (12,000 xg; 4°C; 15 minutes). The pellet was resuspended in 7.5 ml of buffer P1 (100 µg/ml RNase A; 0.05 M Trizma base; 0.01 M EDTA, pH 8.0). Seven and one-half milliliters of buffer P2 (0.2 M NaOH; 1% SDS) was then added to the tube. The sample was inverted gently and the cells were incubated at RT for five minutes. Seven and one-half milliliters of buffer P3 (3 M KOAc, pH 5.5) was added to the tube and it was mixed gently. The sample was placed on ice for 20 minutes and centrifuged (17,000 xg; 4°C) for 30 minutes. The supernatant was transferred to another sterile centrifuge tube. A sample of the supernatant was prepared for agarose gel electrophoresis. Two hundred and fifty microliters of the supernatant along with 250 µl of isopropanol were placed into a sterile eppendorf tube. The tube was centrifuged (12,000-16,000 xg) at RT for 15 minutes and the supernatant was decanted. After the DNA pellet dried for 10 minutes, it was redissolved in 30 µl of 1X TE buffer. The entire sample was resolved on a 1% agarose gel. If DNA was present, a Qiagen tip was equilibrated by applying 4 ml of buffer QBT (0.75 M NaCl; 0.05 M MOPS; 15 % EtOH, pH 7.0; 0.15% Triton X-100) to the column and allowing it to empty by gravity flow. Afterwards, the supernatant was applied to the tip and allowed to enter the resin via gravity flow. The tip was washed twice with 10 ml of buffer QC (1 M NaCl; 0.05 M MOPS; 15% EtOH, pH 7.0). The DNA was eluted with 5 ml of buffer QF (1.25 M NaCl; 0.05 M Trizma base; 15% EtOH, pH 8.5), precipitated with 0.7 volumes of isopropanol and centrifuged (15,000 xg; 4°C) for 30 minutes. Chilled 70% EtOH was used to wash the DNA pellet. After centrifugation (17,000 xg; 4°C; 10 minutes), the pellet was allowed to dry for 5 minutes. The DNA pellet was then redissolved in 3 ml of 1X TE buffer and stored at -20°C.

XII. Preparation of Double-Stranded Template For Sequencing

Transformants were picked to 2 ml of LA_{100} broth and incubated at 37°C overnight.

One and one-half milliliters of the overnight growth was transferred to a sterile eppendorf tube and it was placed into a microcentrifuge (12,000-16,000 xg) at RT for 20 seconds. After centrifugation, the supernatant was removed and the pellet was resuspended in 100 µl of Solution I (0.05 M Tris-Cl, pH 7.6; 0.01 M EDTA, pH 8.0; 100 µg/ml RNase A). One hundred microliters of Solution II (0.2 N NaOH; 1% SDS) was added and the tube was gently inverted. One hundred microliters of Solution III (1.32 M KOAc, pH 5.2) was then added to the tube and it was mixed by vigorous inversion. The lysate was centrifuged (12,000-16,000 xg) for 30 seconds at RT and the supernatant was transferred to a sterile *PERFECTprep* spin column. Four hundred and fifty microliters of *PERFECTprep* DNA binding matrix was added to the supernatant. The sample was mixed well and centrifuged (12,000-16,000 xg) at RT for 30 seconds. The filtrate was decanted and 400 µl of diluted Purification Solution (Purification Solution Concentrate was diluted with an equal volume of 95% EtOH) was added to the same spin column. The collection tube was capped, inverted and centrifuged (12,000-16,000 xg) at RT for 60 seconds. The spin column was transferred to a fresh collection tube and placed into a microcentrifuge (12,000-16,000 xg) at RT for 60 seconds. After centrifugation, the spin column was transferred to another fresh collection tube. Fifty microliters of 1X TE buffer was added to the spin column. It was vortexed briefly and centrifuged at RT for 60 seconds as above. the spin column was then discarded and the purified DNA was stored at 4°C.

XIII. Sequencing Reactions

A primer annealing mixture was prepared by adding 5 µl of recombinant DNA (prepared by *PERFECTprep* method), 2 µl of 10X reaction buffer, 2 µl of DIG-labeled M13/pUC19 forward or reverse sequencing primer, 10 µl of sterile H_2O and 1 µl of *Taq* DNA polymerase (3 U/µl) in accordance with the manufacturer's instructions. Four vials, labeled G, A, T and C respectively, were filled with 2 µl of the appropriate extension/

termination mixture. Four microliters of primer annealing mixture was then added to each vial. The vials were centrifuged (12,000-16,000 xg) at RT briefly. After centrifugation, a drop of sterile mineral oil was placed into each vial and they were placed inside a thermocycler. The mixture was denatured by heating at +95°C for 5 minutes. For the forward sequencing primer, one cycle included 95°C for 30 seconds, 60°C for 30 seconds and 70°C for 1 minute. The reverse sequencing primer cycle included 95°C for 1 minute, 56°C for 1 minute and 70°C for 1 minute respectively. Both cycles repeated 29 times. After amplification, the samples were stored at 4°C. Two microliters of formamide buffer was added to each of the vials to stop the reaction.

XIV. Sequencing Gel Electrophoresis

An 8% polyacrylamide gel (1X TBE: 0.135 M Trizma base, 0.045 M Boric acid, 0.0025 M EDTA; 8 M Urea) was produced in a mold. The reaction products (from the extension/termination reaction), contained within the four vials, were denatured at 95°C for 3 minutes. The vials were transferred to ice and centrifuged (RT; 12,000-16,000 xg) briefly. Three microliters of each of the four extension/termination reactions (G, A, T, C) was dispensed into corresponding wells of the sequencing gel. The gel ran at 60 watts for approximately 8 hours in 1X TBE buffer.

XV. Detection

Following electrophoresis, the siliconized glass plate was removed. A nylon membrane, cut to match the size of the gel, was carefully place onto the sequencing gel. The membrane was covered with a sheet of 3 mm Whatman chromatography paper and the glass plate was placed on top to complete the sandwich. A weight of approximately 2 kilograms was applied to the sandwich. After 20 minutes, the sandwich was disassembled. The nylon membrane was pulled off and exposed to UV-light for 3

minutes. The membrane was sealed in a plastic bag to await detection. All of the incubations were performed at RT in sealed hybridization bags. The membrane was rinsed for 1 minute in 50 ml of Washing buffer (Buffer 1: 0.1 M Maleic acid, 0.15 M NaCl, adjusted to pH 7.5 with solid NaOH; 0.3% Tween 20). The Washing buffer was decanted and the membrane was incubated for 30 minutes in 50 ml of Buffer 2 (10% Blocking stock solution diluted 1:10 in Buffer 1). After the incubation, the Blocking solution was discarded and 50 ml of antibody solution (anti-DIG-AP conjugate diluted 1:10,000 in Buffer 2) was added to the bag. After a 30 minute incubation, the antibody solution was poured off. The membrane was washed twice, 15 minutes per wash, in 50 ml of Washing buffer. The Washing buffer was discarded and the membrane was equilibrated in 20 ml of Detection buffer (0.1 M Trizma base, 0.1 M NaCl, 0.05 M $MgCl_2$, pH 9.5). The damp membrane was transferred to another hybridization bag and 1-2 ml of diluted CSPD (diluted 1:100 in Buffer 3) was added. The bag was sealed and placed into a 37°C incubator for 15 minutes. After the incubation, the membrane was exposed to x-ray film for 3 hours.

XVI. Southern Transfer

DNA was digested as previously described, run on a 1% agarose gel and stained with EtBr. If the DNA was digested, the gel was incubated at RT for 30 minutes in Denaturing solution (0.5 N NaOH; 1.5 M NaCl) with gentle shaking. The Denaturing solution was poured off and the gel was then submerged in Neutralization solution (0.5 M Trizma base, pH 7.5; 3 M NaCl) for 30 minutes at RT. The Neutralization solution was discarded. The DNA was blotted overnight from the gel onto the nylon membrane using 10X SSC buffer (1:2 dilution of 20X SSC buffer: 3 M NaCl, 0.3 M Sodium Citrate, pH 7.0) by capillary transfer. The membrane was rinsed in 5X SSC (1:4 dilution of 20X SSC buffer) buffer at RT for 1 minute. The damp membrane was placed on a piece of 3 mm Whatman

chromatography paper and baked for 1 hour at +80°C. Afterwards, the membrane was sealed in a plastic bag.

XVII. Probe Preparation

Fifteen microliters of DNA template was denatured at +95° for 10 minutes and immediately transferred to ice. Two microliters of 10X hexanucleotide mixture and 2 µl of 10X dNTP labeling mixture were added to the reaction tube. One microliter of Klenow enzyme was then added and the tube was place into a 37°C heat block overnight. The following day, 2 µl of 0.2 M EDTA was added to stop the reaction. The DIG-labeled nucleic acid was precipitated by adding 0.1 volume of 4 M LiCl and 2.5-3.0 volumes of cold 70% EtOH, and incubating 30 minutes (at -70°C). After the incubation, the tube was centrifuged (13,000 xg) at RT for 15 minutes. The supernatant was decanted. After the pellet dried, it was redissolved in 50 µl of 1X TE buffer.

XVIII. Quantitation of Probe

Serial 10-fold dilutions of DIG-labeled control DNA were made using the supplied DNA dilution buffer. One microliter of each dilution was spotted onto a positively charged nylon membrane. The corresponding dilution was marked next to the appropriate spot. Serial 10-fold dilutions of the DIG-labeled experimental probe were made and 1 µl of each dilution was spotted onto the membrane. Each dilution was lightly marked on the membrane as before. The membrane was then baked at +80°C for 30 minutes. After baking, a small amount of Washing buffer was used to wet the membrane. The washing buffer was decanted and the membrane was incubated in Blocking solution at RT for 5 minutes. The Blocking solution was discarded and diluted antibody (1: 5,000 dilution in Blocking solution) was added. The membrane incubated at RT for 10 minutes. After the incubation, the antibody solution was poured out and the membrane was washed twice

(5 minutes at RT) in Washing buffer. Detection buffer was added and the membrane incubated at RT for 2 minutes. The Detection buffer was decanted and a Color Substrate Solution (45 µl of NBT + 35 µl of X-phosphate solution in 10 ml of Detection buffer) was added. Color development occurred in the dark for 30-60 minutes. The reaction was terminated by washing the membrane in 50 µl of sterile H_2O for 5 minutes at RT. Spot intensities of the control and experimental dilutions were compared to estimate the concentration of experimental probe.

XIX. Prehybridization and Hybridization

Twenty milliliters of Prehybridization solution (5X SSC; 0.1% N-lauroylsarcosine; 0.02% SDS; 1% Blocking Reagent) was added to the bag. The membrane was incubated at 65°C for 2 hours. After the incubation, the prehybridization solution was discarded and 20 ml of Hybridization solution (Prehybridization solution containing the DIG-labeled probe) was added to the bag. The probe was allowed to hybridize at 65°C overnight. The following day, the Hybridization solution was decanted into a tube and stored at -20°C. The membrane was washed twice, 5 minutes per wash , in 2X Wash solution (2X SSC; 0.1% SDS) at RT. The membrane was then washed twice, 15 minutes per wash, in 0.5X Wash solution (0.5X SSC; 0.1% SDS) at 65°C and detected as described previously in section XV.

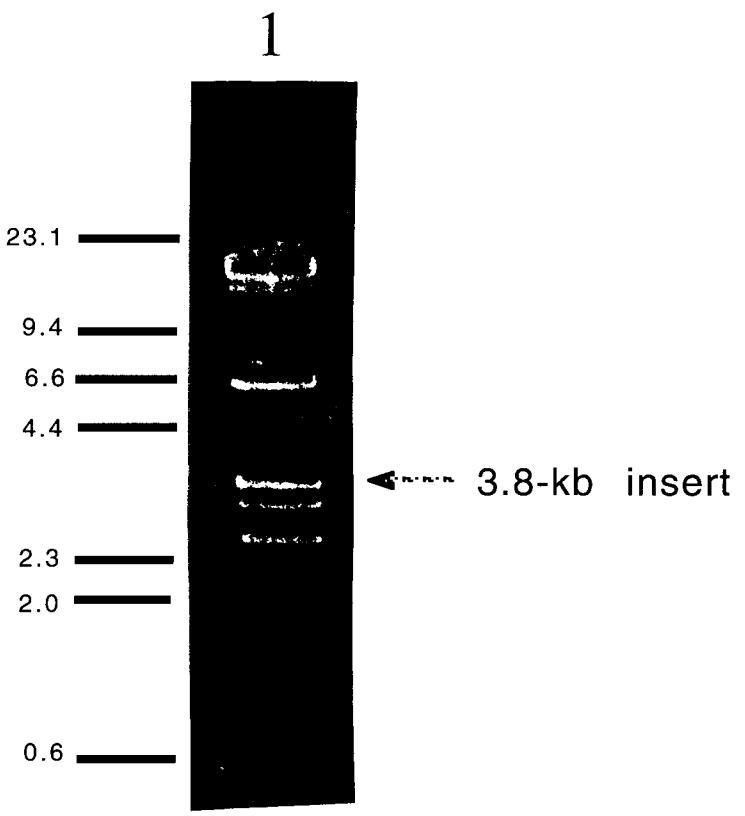
RESULTS

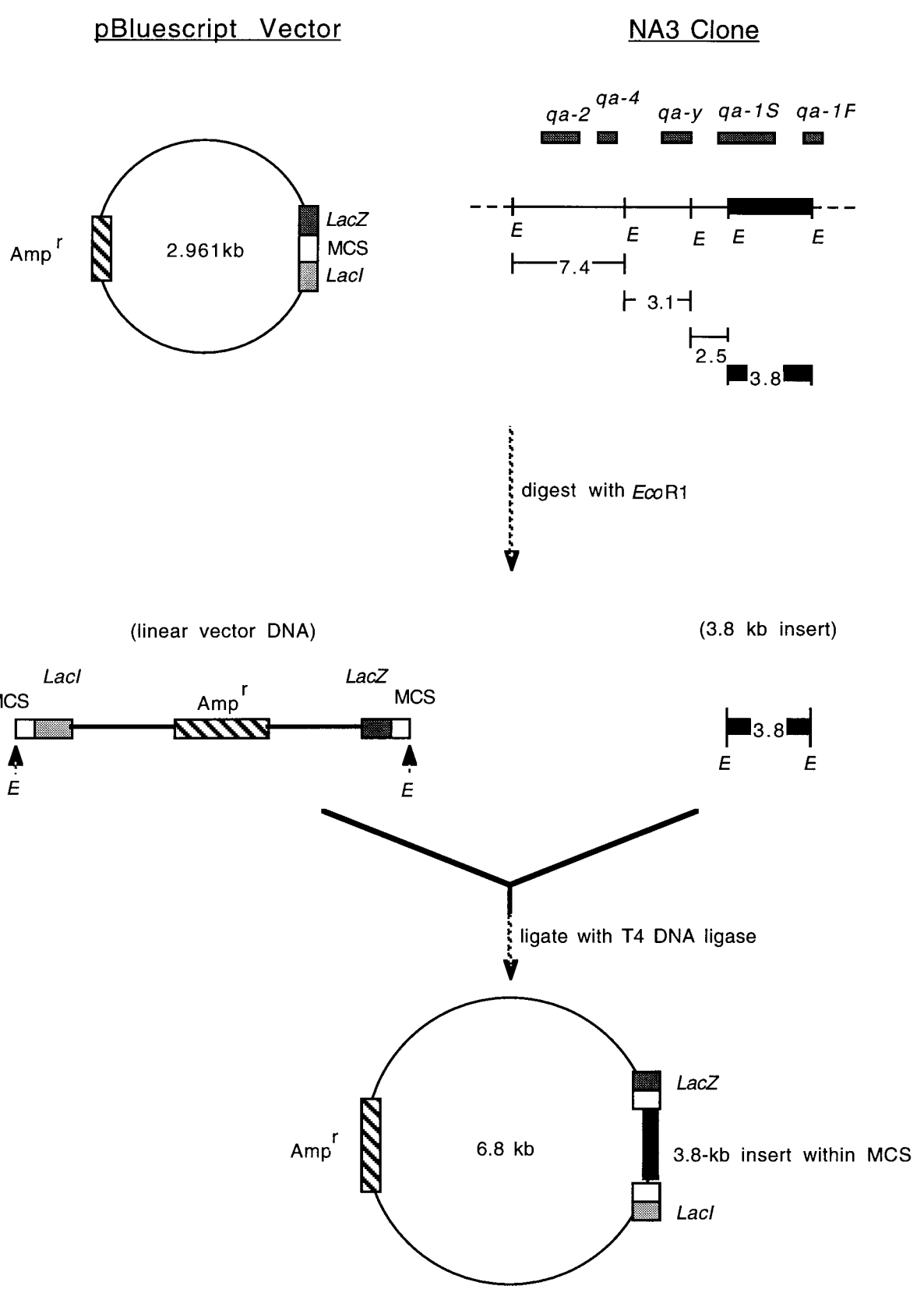
I. Construction of Plasmid pR1

In order to further characterize the *qa-1S-qa-1F* intergenic region of *N. africana*, we began to examine the lambda clone NA3. This clone contains most of the *qa* gene cluster of *N. africana* (Asch, unpublished data). When digested with the restriction endonuclease *Eco*R1, this clone produces six fragments (Figure 7). Four of the six fragments contain part of the *qa* gene cluster (Figure 8). Previous studies had indicated that the 3.8 kb fragment of the NA3 clone contains the *qa-1S-qa-1F* intergenic region of interest (Roys, unpublished data). Therefore, this fragment was isolated, ligated to an *Eco*R1-digested pBluescript vector, and transformed into *E. coli* JM101. The resulting subclone, containing the 3.8-kb *Eco*R1 fragment spanning the entire *qa-1S-qa-1F* intergenic region, was designated plasmid pR1 (Figure 8).

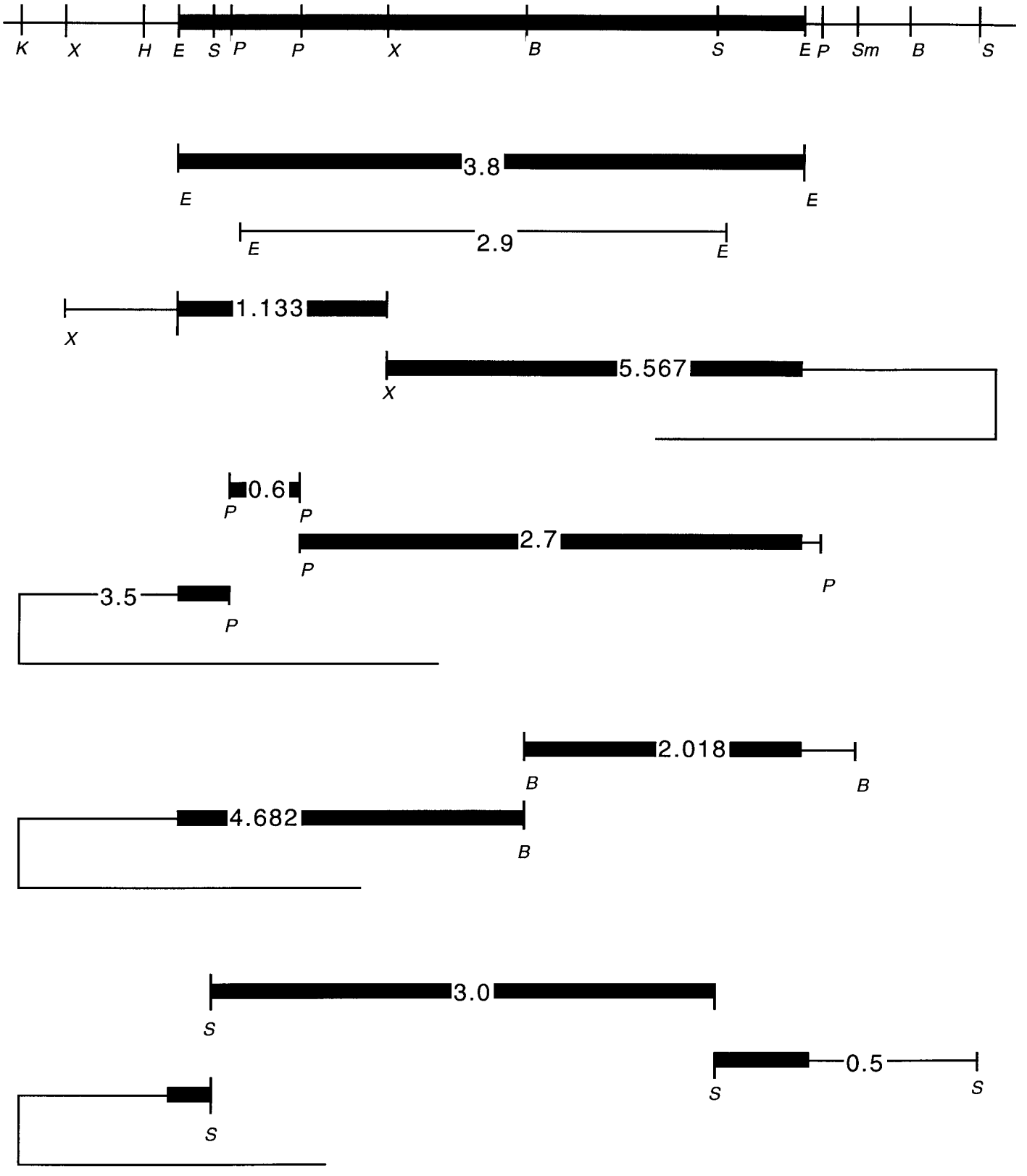
II. Characterization of Plasmid pR1

A series of restriction digests were performed in order to deduce a preliminary restriction map of the plasmid pR1. All of the restriction enzymes used in the experiments contained a unique recognition site within the pBluescript vector. The sizes of the fragments generated from the digests were estimated by comparison with a size standard (lambda DNA cleaved with *Hin*dIII). The restriction endonuclease *Eco*R1 generated two fragments approximately 2.9 kb and 3.8 kb in size (Figure 10A, lane 1). This confirmed the existence of both the vector (2.9 kb) and the insert (3.8 kb) whose sizes were previously known. The fragment sizes generated by the *Xho*1 digest were about 1.133 kb and 5.567 kb (Figure 10A, lane 2). It appeared that most of the 3.8 kb insert was contained within the larger *Xho*1 fragment (Figure 9). The *Eco*R1-*Xho*1 double-digest of plasmid pR1 generated two fragments (1.1 kb and 2.7 kb) which contained most of the


1
23.1
9.4
6.6
4.4
2.3
2.0
0.6
3.8-kb insert


pBluescript Vector
NA3 Clone
qa-2
qa-4
qa-y
qa-1S
qa-1F
Amp r
2.961kb
LacZ
MCS
LacI
E
E
E
E
E
7.4
3.1
2.5
3.8
digest with EcoR1
(linear vector DNA)
(3.8 kb insert)
LacI
MCS
Amp r
LacZ
MCS
E
E
3.8
E
E
ligate with T4 DNA ligase
Amp r
6.8 kb
LacZ
3.8-kb insert within MCS
LacI

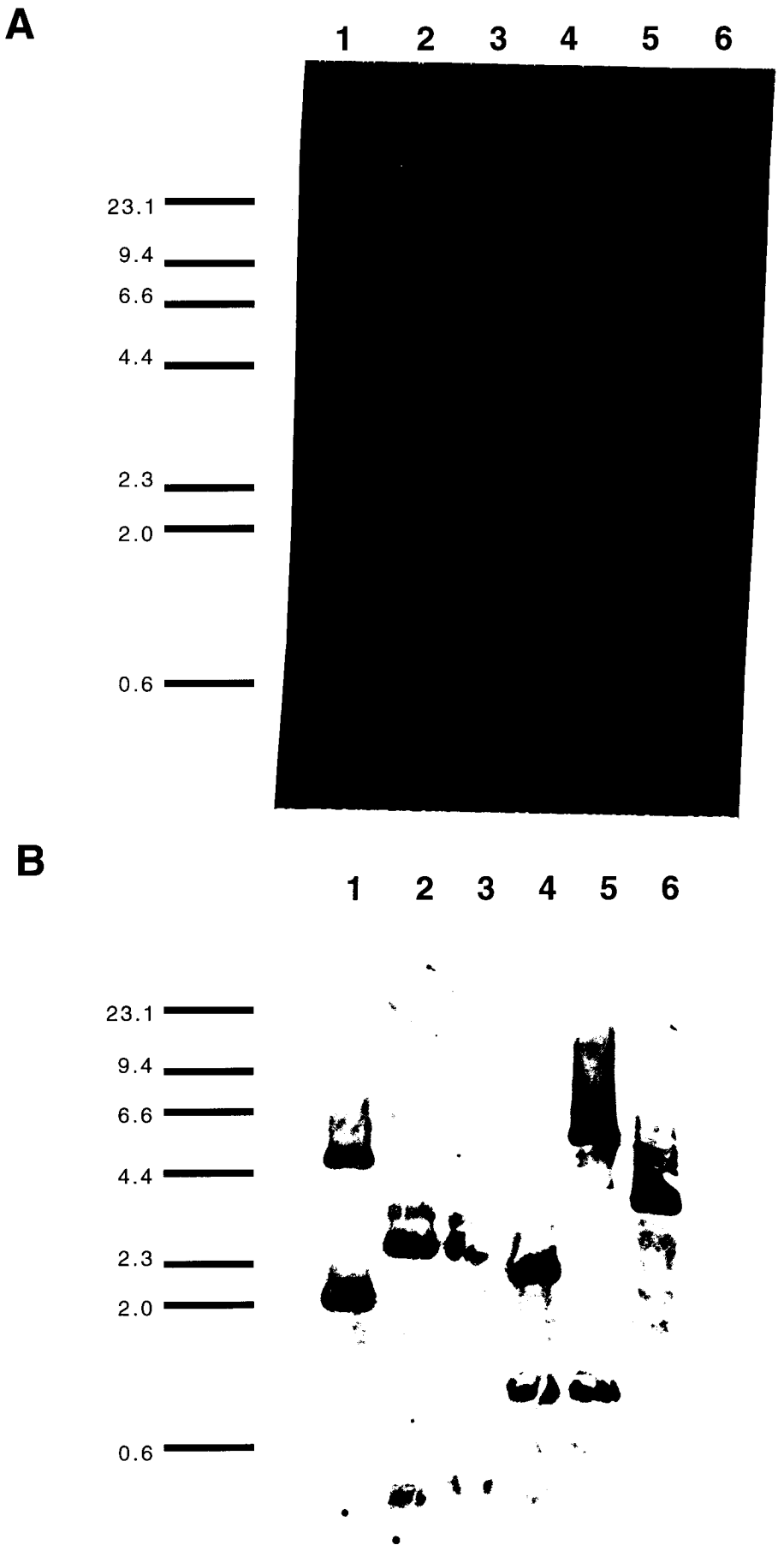
pR1


K X H E S P P X B S E P Sm B S
3.8
E E
E 2.9 E
X 1.133
X 5.567
P 0.6 P
P 2.7 P
3.5 P
B 2.018 B
4.682 B
S 3.0
S 0.5 S
S

insert (Figure 10A, lane 3). The estimated sizes of the *PstI* fragments were 500 bp, 2.8 kb, and 3.4 kb respectively (Figure 10A, lane 4). The two smaller fragments contained most of the 3.8-kb insert (Figure 9). The presence of the 500-bp *Pst*1 fragment suggests that both *Pst*1 sites, located within the plasmid pR1 insert, are probably in close proximity to one another. Of the three *Sac*I-generated fragments (Figure 10A, lane 5), the 3.0-kb fragment contained most of the insert (Figure 9). Cleavage by *Bam*HI resulted in two fragments (Figure 10A, lane 6) approximately 2.018 kb and 4.682 kb (which contained most of the insert) in size. Agarose gel electrophoresis of plasmid pR1 digested with the restriction endonuclease *Xho*I or *Bam*HI each revealed two fragments. This suggests that one recognition site for each enzyme (*Xho*1 and *Bam*H1) exists somewhere within the 3.8-kb insert. Cleavage with the restriction enzyme *Pst*I or *Sac*I revealed three fragments, indicating that two recognition sites for both *Pst*I and *Sac*I are located somewhere within the 3.8-kb insert (Figure 9). Since *Hin*dIII and *Sma*I linearized the pR1 recombinant construct, cutting only at the vector, these enzymes do not have a restriction site within the insert.

III. Southern Blot Analysis of Plasmid pR1

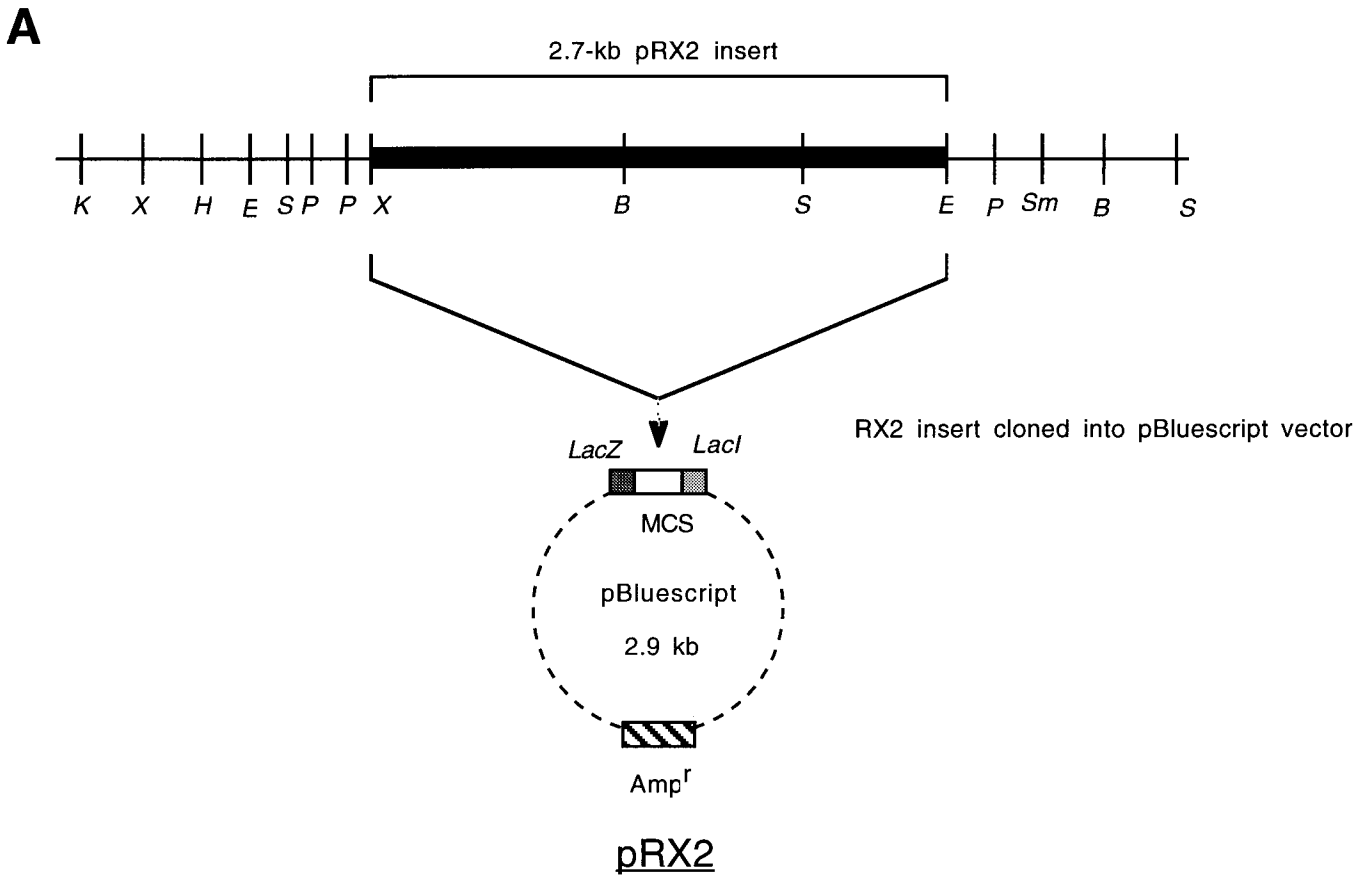
Southern blot analysis of plasmid pR1 was used to localize the *qa-1S-qa-1F* intergenic sequences of *N. africana* on our restriction map. The pR1 recombinant plasmid was digested with a series of enzymes (*Eco*R1, *Xho*1, *Pst*1, *Bam*H1, and *Sac*I) known to contain recognition sites within the insert. An 800 bp DIG-labeled probe, which is a PCR (polymerase chain reaction) product spanning the entire *qa-1S-qa-1F* intergenic region of *N. crassa* (Roys, unpublished data), was used to confirm the existence of those fragments which contain the *qa-1S-qa-1F* intergenic sequences. This probe extends from 14,300 to 15,000 on the *qa* gene sequence (Geever *et al.*, 1989) and contains only the sequences derived from the *qa-1S-qa-1F* intergenic region. Of the two *Eco*R1-generated fragments,

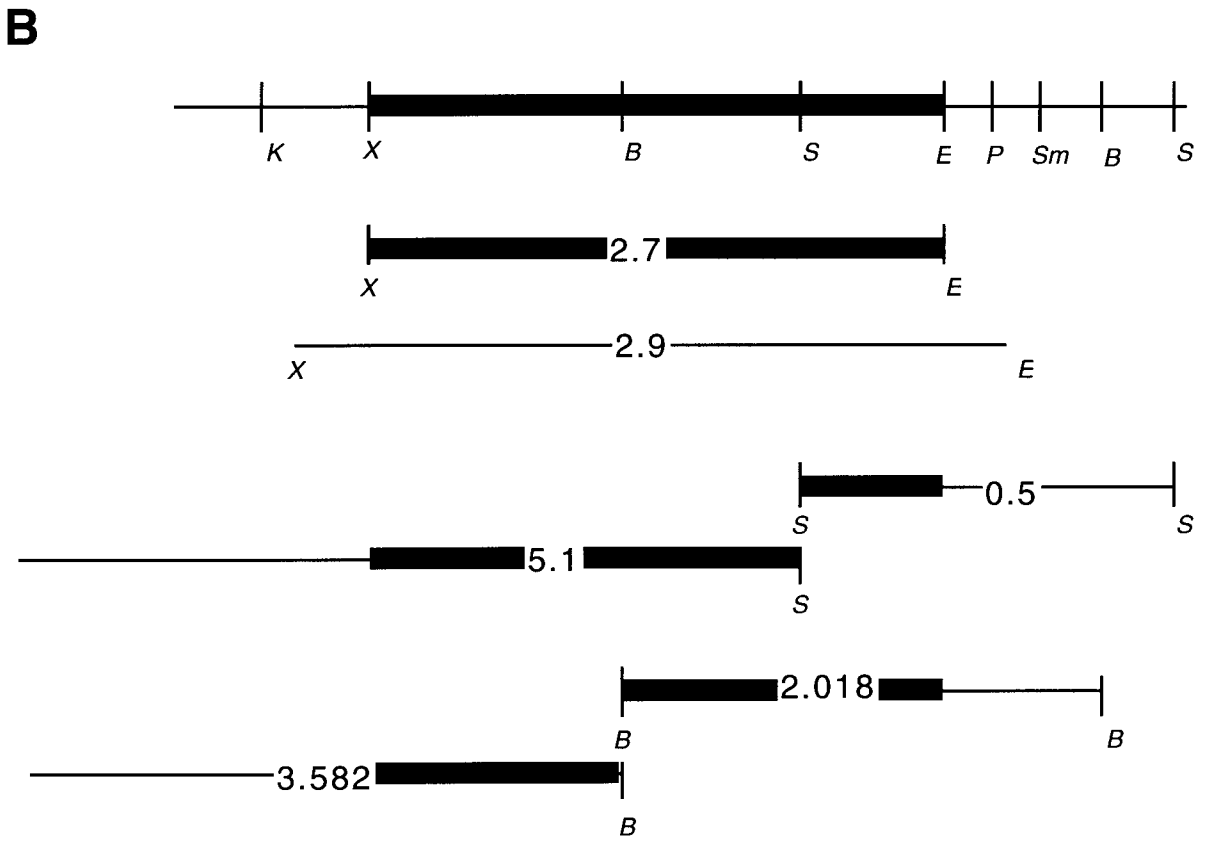

A
1 2 3 4 5 6
23.1
9.4
6.6
4.4
2.3
2.0
0.6
B
1 2 3 4 5 6
23.1
9.4
6.6
4.4
2.3
2.0
0.6

only the 3.8-kb fragment hybridized to the probe (Figure 10B, lane 6). This finding was consistent with previous data indicating that the 3.8-kb fragment contained the region between *qa-1S* and *qa-1F* (Roys, unpublished). Both of the *Xho*1 fragments (1.133 kb, 5.567 kb) hybridized to the probe (Figure 10B, lane 4). The 1.1-kb and 2.7-kb fragments generated from a *Eco*R1-*Xho*1 double digest of plasmid pR1 hybridized to the DIG-labeled probe. However, the larger *Eco*R1-*Xho*1-generated fragment (2.9 kb) did not hybridize to the probe (Figure 10B, lane 4). These data provide evidence for the existence of a *Xho*1 recognition site within the *qa-1S-qa-1F* intergenic region. Hybridization was observed between all three *Pst*1-generated fragments (500 bp, 2.8 kb, 4.3 kb) and the probe, suggesting that both *Pst*1 sites were located somewhere within the *qa-1S-qa-1F* intergenic region (Figure 10B, lane 3). All of the fragments generated from *Sac*1 or *Bam*H1 digests hybridized to the probe, proving that recognition sites for both restriction endonucleases were located somewhere within the *qa-1S-qa-1F* intergenic region (Figure 10B, lanes 2,1).

IV. Construction and Characterization of Subclone Plasmid pRX2

In order to further localize the *qa-1S-qa-1F* intergenic region and to initiate DNA sequencing, a 2.7-kb fragment generated from an *Eco*R1-*Xho*1 digest of plasmid pR1 was isolated and ligated into an *Eco*R1-*Xho*1 cleaved pBluescript vector (Figure 11A). The resulting plasmid pRX2 construct was then cleaved with six different enzymes (*Eco*R1, *Xho*1, *Pst*1, *Sac*1, *Bam*H1, and *Hin*dIII) to further reinforce the locations of the recognition sites within the *qa-1S-qa-1F* intergenic region. Single digests using either *Eco*R1 or *Xho*1 simply linearized the recombinant construct as expected. The restriction enzyme *Hin*dIII did not cleave plasmid pRX2 either. This was expected because the *Hin*dIII site was deleted with the *Eco*R1-*Xho*1 digest of plasmid pR1. The double digest of plasmid pRX2 with *Eco*R1 and *Xho*1 resulted in two fragments (2.7-kb and 2.9-kb). This data reinforces the existence of the vector (2.9 kb) and the insert (2.7 kb)

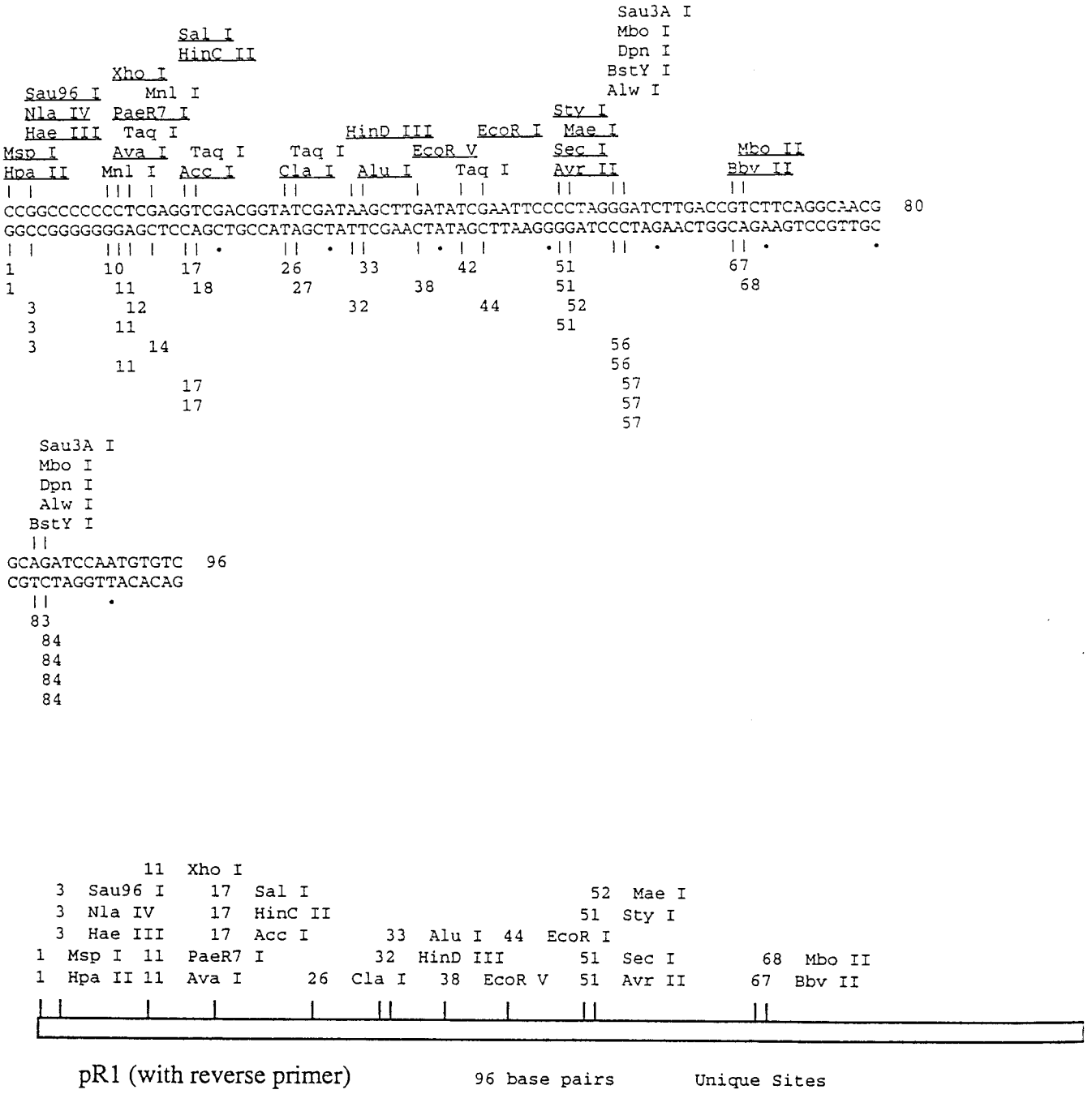

A
2.7-kb pRX2 insert
K X H E S P P X B S E P Sm B S
RX2 insert cloned into pBluescript vector
LacZ
LacI
MCS
pBluescript
2.9 kb
Amp^r
pRX2


B
K X B S E P Sm B S
2.7
X E
2.9
X E
0.5
S S
5.1
S
2.018
B B
3.582
B

(Figure 11B). The *Pst*1 restriction endonuclease linearized the plasmid, providing evidence that the two *Pst*1 recognition sites are located outside the plasmid pRX2 insert. Digestion of plasmid pRX2 with *Sac*1 generated only two fragments, one approximately 500 bp and the other approximately 6.2 kb (Figure 11B). However, the *Sac*1 digest of plasmid pR1 resulted in three fragments (Figure 9; Figure 10A, lane 5). These results indicate that only one of the *Sac*1 sites exists somewhere within the plasmid pRX2 insert, while the other is located somewhere outside the plasmid pRX2 insert. The *Bam*H1 restriction digest of plasmid pRX2 (Figure 9) generated two fragments (2.018 kb and 4.682 kb) identical in size as the *Bam*H1-generated fragments of plasmid pR1(Figure 10A, lane 6; Figure 11B). This suggests that a *Bam*H1 site is located somewhere within the pRX2 insert only.

V. Sequencing Plasmid pR1 and Subclone Plasmid pRX2

To further analyze the *qa-1S-qa-1F* intergenic region of *N. africana*, sequence analysis was performed on both plasmids pR1 and pRX2. The DNA sequences of plasmid pR1 and plasmid pRX2 were analyzed using DNA Strider 1.0 which automatically locates any enzyme recognition sites the sequences present. A restriction map displaying the enzyme recognition sites was also generated from the available DNA sequences using the same computer program. Figure 12 illustrates the results of the reverse primer sequencing of plasmid pR1. The M13/pUC reverse sequencing primer, which is labeled with dioxygenin at the 5' end, is homologous to 17 nucleotides 5' to the polylinker. This primer will anneal to the 5' portion of the polylinker and DNA synthesis will proceed toward the insert located within the polylinker. Some of the unique recognition sites for the enzymes such as *Eco*R1, *Xho*1, and *Hin*dIII, within the polylinker were observed with the plasmid pR1 DNA sequence. Sequencing beyond the *Eco*R1 site of the polylinker was limited for which a small amount of sequence data was generated for plasmid pR1 (Figure 12). The

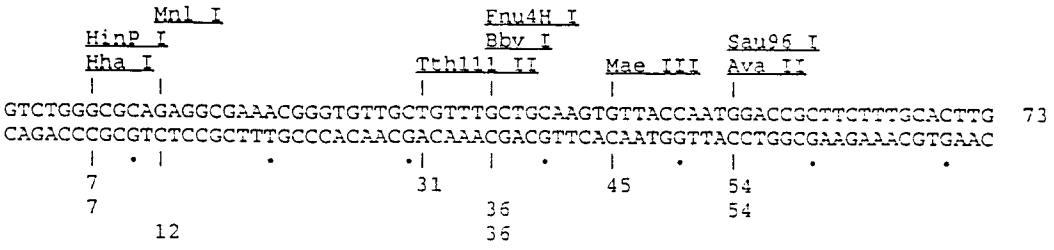


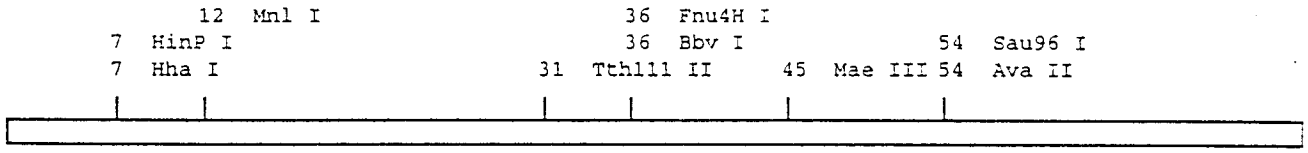
pR1 (with reverse primer) 96 base pairs Unique Sites

same reverse sequencing primer was used to analyze plasmid pRX2. Recognition sites were observed, but they differed from those recognition sites seen in the plasmid pR1 sequence. Although enzyme recognition sites were located within the plasmid pRX2 sequence, there is no indication of the presence of any polylinker (Figure 13).

VI. Sequence Comparisons of Plasmid pR1 With the Quinic Acid (*qa*) Gene Cluster of *N. crassa.*

To determine the existence of any sequence homology between the pR-1 insert of *N. africana* and the *qa* gene cluster of *N. crassa*, the plasmid pR1 (figure 12) and plasmid pRX2 sequences were entered onto databases on the World Wide Web. The URLs are as follows: 1) Bioscan Online (URL http://genome.cs.unc.edu/bin/ mac1-match); 2) Pedro's Molecular Biological Tools (URL http://bchm 20.aclcb.purdue.edu/rt 95.06.16 research-tools htm). Sequence homology was observed between the *N. africana* sequence of plasmid pR1 and a sequence located within the *N. crassa qa* gene cluster using the M13/ pUC reverse sequence primer (Figure 14). However, the *N. africana* sequence was found to be located within a conserved *N. crassa qa-1F* coding region (16668-16715) and not within the *qa-1S-qa-1F* intergenic region. This 41 bp *N. africana* sequence is found to be located within the *qa-1F* gene of *N. crassa*. The plasmid pRX2 sequence (Figure 13), which was generated using the same M13/pUC reverse sequence primer, did not display any sequence homology with the *N. crassa* sequence.





pRX2 (with reverse primer) 73 base pairs Unique Sites

gb|X14603|NCQA

Best Sum Statistic P(1) = **6.0e-05** Length: **18120** Date: **04-OCT-1993**
Neurospora crassa qa gene cluster.

MINUS STRAND SEGMENT PAIRS

Score = **196** Length = **48** Expect = **6.0e-05** P = **6.0e-05**

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Query:      6 CCCCTAGGGATCTTGACCGTCTTCAGGCAACGGCAGATCCAATGTGTC 53
              C CC A  GATCTTGACCGTCTTCAGG AACGGCAGATCC    TGTC
Entry: 16715 ctcccattgatcttgaccgtcttcaggtaacggcagatccgcaatgtc 16668
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DISCUSSION

To elucidate the mechanisms involved in the regulation of the *qa* gene cluster in *N. crassa*, the *qa-1S-qa-1F* intergenic region of *N. africana* was isolated and analyzed. We believe that this region is important because it may play a role in repressing *qa* gene expression in the presence of glucose. Although the mechanism(s) of carbon catabolite repression in *Neurospora* remain(s) unclear, the galactose (*GAL*) system of *S. cerevisiae* may offer some possible explanations.

In the presence of glucose, expression of the *GAL* genes is reduced. This results from the inability of the GAL4 activator protein to bind to the GAL4 binding sites within the UAS_{GAL} of the *GAL* promoters. Since the *GAL* genes vary in their number of GAL4 binding sites, and the GAL4 protein binds cooperatively to these sites, some *GAL* genes may be highly repressed (*GAL1-10* promoter - four GAL4 binding sites) while others may be only slightly repressed (*GAL80* - 1 GAL4 binding site) in the presence of a preferred carbon source . Griggs and Johnston (1991) observed a 50-fold reduction in the expression of *GAL1* due to a five-fold reduction of *GAL4* expression in the presence of glucose. The same phenomenon may occur within the *qa* gene cluster of *N. crassa*. The *qa-1F* activator protein may undergo reduced repression, modification, proteolysis, or may simply interact with the *qa-1S* repressor in the presence of a preferred carbon source. As a result, the *qa-1F* activator protein may not be able to bind effectively, if at all, to the *qa-1F* binding sites within the *qa* gene cluster. As in the *GAL* system, the number of *qa-1F* binding sites varies between the *qa* genes. This factor may play a role in determining to what extent a gene will be repressed. Asch *et al.* (1992) demonstrated that *Neurospora* mutants (Δ*qa-1S*) containing a deletion in *qa-1S* displayed slightly repressed *qa-x*, *qa-2*, and *qa-4* expression. However, *qa-3*, *qa-y*, and *qa-1F* gene expression was highly repressed.

Another possible mechanism of carbon repression may operate through direct repression. In the presence of glucose, repressor proteins may bind to *qa-1F* promoters to prevent *qa-1F* transcription which would result in reduced expression of all of the *qa* genes. In the *GAL* system, the MIG1 repressor protein forms a complex with the products of two genes, *SSN6* and *TUP1* and binds to *GAL1* and *GAL4* promoters. Unidentified repressor proteins may also play a role in repressing *qa* gene expression in the presence of glucose. Carbon repressor proteins may specifically bind to certain *qa* gene promoters (*qa-y, qa-1F, qa-4*) to reduce transcription, resulting in a reduction of *qa* gene expression.

In an attempt to understand the mechanisms involved in the catabolite repression of the *qa* genes, the *qa-1S-qa-1F* intergenic sequence of *N. africana* was cloned into a vector. This enabled the isolation and characterization of the *qa-1S-qa-1F* intergenic region.

The restriction digests of the pR1 recombinant plasmid localized various enzyme recognition sites within the plasmid pR1 insert. The existence of the plasmid pR1 was confirmed through an *Eco*R1 digest. The restriction enzymes *Xho*1 and *Bam*H1 each revealed only a single recognition site within the plasmid pR1 insert. Whereas, *Pst*1 and *Sac*1 contained more than one recognition site within the insert. Two additional restriction endonucleases, *Kpn*1 and *Sma*1, simply linearized the pR1 plasmid indicating that these two enzymes contain a single recognition site in the vector but not in the insert. The data generated from the restriction digest was used to construct a restriction map (Figure 9). However, the exact location of all the restriction sites have yet to be determined.

Southern blot analysis revealed only the fragments that contained the *qa-1S-qa-1F* intergenic region of *N. africana*. All of the fragments generated from *Xho*1, *Pst*1, *Sac*1, and *Bam*H1 digests hybridized to the DIG-labeled probe. This suggests that all three enzyme recognition sites are located within the *qa-1S-qa-1F* intergenic region of *N. africana*. The 3.8-kb fragment, which was generated from an *Eco*R1 digest, hybridized to

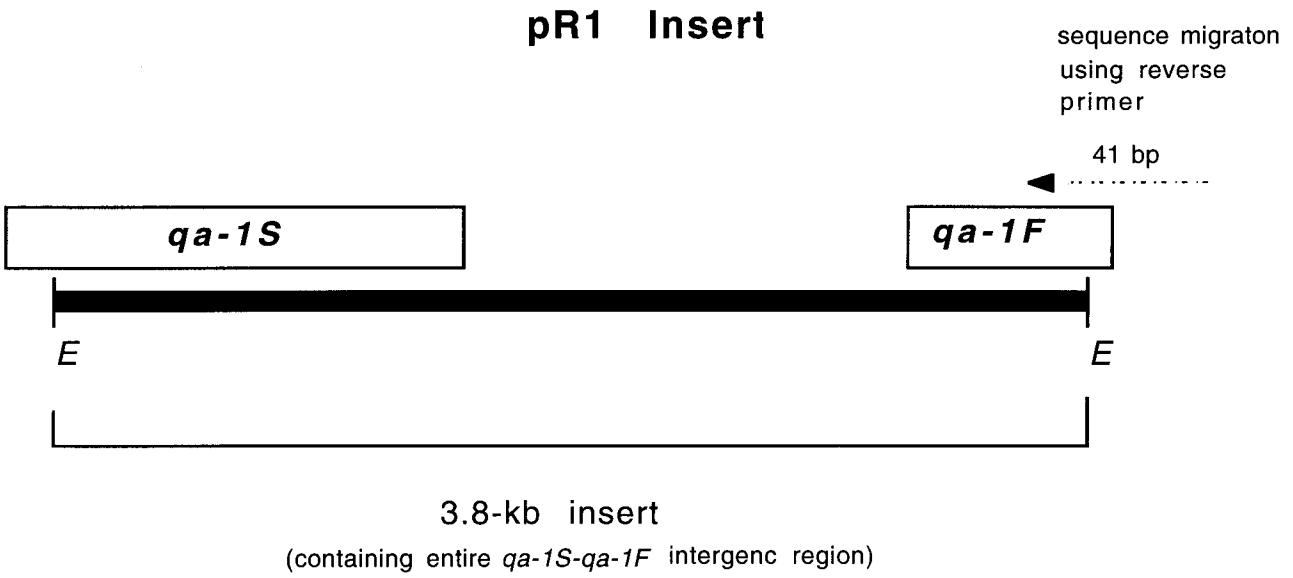
the probe. This finding was consistent with previous data indicating that the 3.8-kb insert contains the *qa-1S-qa-1F* intergenic region. The 1.1- and 2.7-kb *Eco*R1-*Xho*1 fragments also hybridized to the probe.

To further characterize the *qa-1S-qa-1F* intergenic region, a 2.7-kb fragment generated from an *Eco*R1-*Xho*1 digest was subcloned. The pRX2 plasmid construct was characterized by a series of restriction digests. Linearization of the pRX2 plasmid was expected with *Eco*R1 and *Xho*1 single digests since both recognition sites where excised from the pBluescript phagemid during ligation of the 2.7-kb fragment to the vector. The *Hin*dIII digest did not affect the plasmid pRX2. This observation confirmed the existence of the plasmid pRX2 insert within the vector because the *Hin*dIII site was removed with the construction of the pRX2 plasmid. The restriction endonucleases *Bam*H1and *Sac1* generated two pRX2 fragments which suggests that a single recognition site for both enzymes exists within the *qa-1S-qa-1F* intergenic region of *N. africana.* However, an additional *Sac*1 site is located within the plasmid pR1 insert (Figure 9). A single *Pst*1 site is located within the pBluescript vector because the plasmid pRX2 was linearized by the *Pst*1 digestion.

Both the pR1 and pRX2 plasmids were sequenced in order to analyze the *qa-1S-qa-1F* intergenic region of *N. africana.* The location of various enzyme recognition sites within the pR1 and pRX2 inserts were generated through the computer program Strider 1.0. A polylinker was not observed within the pRX2 sequence. Therefore, the location of the pRX2 sequence relative to the *N. crassa* sequence could not be determined. Although the plasmid pR1 sequence revealed several unique recognition sites within the pBluescript polylinker, sequence beyond the polylinker was limited. The *N. africana* sequence (Figure 12) generated from plasmid pR1 was located within a conserved 47-bp *qa-1F* coding region of *N. crassa* . This data indicated that the sequence was not located within the *qa-1S-qa-1F* intergenic region of *N. africana*, but was within the *qa-1F* sequence.

Although the sequencing of plasmids pR1 and pRX2 was limited, one end of the 3.8-kb insert was determined using the reverse sequencing primer. The sequence generated from plasmid pR1, using reverse primer, identified the *qa-1F* end of the insert (Figure 15). In addition, both plasmids pR1 and pRX2 are established and can be used in future experiments. The plasmid pR1 contains sequences spanning the entire *qa-1S-qa-1F* intergenic region.

In the future, the plasmid clones pR1 and pRX2 can be used to help locate, isolate, and sequence the *qa-1S-qa-1F* intergenic region of *N. africana.* The well-characterized *N. africana qa-1S-qa-1F* sequence can then be used to replace its *N. crassa* counterpart. This will enable one to determine if the *qa-1S-qa-1F* intergenic sequence of *N. africana* plays a role in carbon catabolite repression of the *qa* gene cluster of *N. crassa.*


pR1 Insert
sequence migraton
using reverse
primer
41 bp
qa-1S
qa-1F
E
E
3.8-kb insert
(containing entire qa-1S-qa-1F intergenc region)

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