IDENTIFICATION AND LOCATION OF THE *QUT* GENES IN *ASPERGILLUS NIDULANS* USING DNA - MEDIATED TRANSFORMATION

HAYLEY ANN WHITTINGTON

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

To my family for their constant

encouragement, love and support

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CHAPTER 1

INTRODUCTION

This introductory chapter has been divided into two sections. Section One aims to show why *Aspergillus nidulans* is a good experimental system for studying gene expression and regulation in eukaryotic micro-organisms and outlines what is known about gene organisation and regulation in filamentous fungi. Section Two describes the state of the art in relation to the quinic acid gene cluster of *A.nidulans* at the commencement of the experimental work that forms the basis of this thesis.

SECTION ONE: *ASPERGILLUS NIDULANS*: A SYSTEM FOR THE STUDY OF GENE EXPRESSION AND REGULATION IN EUKARYOTIC MICRO-ORGANISMS

The ability to perform sophisticated genetic manipulations has been of fundamental importance in establishing models for the regulation of gene expression. Among the lower eukaryotes only the regulatory systems in *Aspergillus nidulans*, *Neurospora crassa* and *Saccharomyces cerevisiae* have been submitted to a genetic analysis comparable in detail to that possible in some prokaryotic systems.

The use of *Aspergillus nidulans* as a genetic system was established by Pontecorvo and his associates in 1953 (Pontecorvo *et al*, 1953) and considerable progress has been

made recently in its development as an organism for molecular studies. Following the biochemical and genetical studies of the sixties, knowledge of the physiology of this organism has been accumulating such that it now represents one of the best systems for the study of gene expression and regulation in eukaryotic micro-organisms.

A.nidulans has two main attractions as an organism for genetical studies. Firstly, it forms discrete colonies but is a differentiating and multicellular micro-organism which can grow on simple defined media and is able to utilize a wide range of nutrients. Thus a large number of biosynthetic and degradative pathways have been amenable to study. Extensive nutritional screening in growth tests has allowed the isolation and characterisation of a large number of mutant strains, with over 350 mutant gene loci now being available on the A.nidulans genetic map (Clutterbuck, 1982). Secondly, it has an advantage over other filamentous fungi in the availability of a powerful system of genetic analysis in the sexual and parasexual processes. This has been exploited in the investigation of regulatory systems controlling a wide variety of metabolic pathways, particularly in nitrogen (Kinghorn and Pateman, 1977; Cove, 1979) and carbon (McCollough et al, 1977; Arst, 1981) assimilation.

As A.nidulans is a homothallic ascomycete, the absence of mating types means that the out-crossing of strains and

hence genetic heterogeneity can be avoided. The large numbers of progeny obtained from a cross and the wide variety of selective techniques allow a gene to be positioned within a particular linkage group and the production of detailed fine structure maps. In the parasexual cycle, A.nidulans heterokaryons may produce sectors of diploid mycelium which are easily identifiable if the heterokaryon is between two complementary conidial colour mutants. Haploidisation of such diploids can be enhanced by the use of benlate (Hastie, 1970) and this technique is used to map new mutations to chromosomal linkage groups (McCully and Forbes, 1965). Diploids are commonly used in complementation experiments, as there are two disadvantages associated with the use of heterokaryons for such tests. Firstly, no satisfactory method has been devised for growing heterokaryons in liquid culture to obtain mycelia for enzyme studies but diploids may be cultured under the same conditions as haploids providing that selection is maintained. Secondly, heterokaryons exhibit variation in their nuclear ratios and are therefore unsuitable if the product under study is nucleus limited (Pontecorvo, 1963) or if its production is limited such that the nuclear ratios prevent its normal effect (Casselton and Lewis, 1967). This is often the case for regulatory gene products and has been observed in studies on the regulatory genes for nitrate assimilation (Cove, 1969), purine degradation (Scazzocchio and Darlington, 1967; Scazzocchio, 1973) and quinic acid utilization (Giles et al, 1985)

pathways. The application of A.nidulans heterokaryons in complementation tests has been of limited value because of these problems.

Although Neurospora crassa, a related ascomycete, produces segregationally stable heterokaryons, it does not produce heterozygous diploids and hence A.nidulans is the preferred organism for complementation studies because of its stable diploids.

The advent of techniques for gene isolation and analysis, first in bacteria and then in yeast, has resulted in rapid progress in the understanding of gene structure and expression. In A.nidulans a firm base had been established by genetical and biochemical studies which could be advanced by the development and application of these new techniques in order to obtain a similar understanding of gene expression in filamentous fungi. Recently an advance of central importance has been made by the development of a DNA-mediated transformation system for A.nidulans (Ballance et al, 1983; Tilburn et al, 1983). This provides a method for the isolation of specific genes from gene libraries constructed in plasmid or cosmid vectors through complementation of the mutant allele by the wild-type gene. Genes that have been cloned may then be reintroduced into the appropriate mutant strain in order to verify the presence and integrity of the particular gene in its entirety and to identify its biological function. This

technique also allows genes manipulated *in vitro* to be reintroduced into the organism so that DNA sequences important for gene regulation and expression may be identified.

Two transformation systems were developed independently for *A.nidulans* by Ballance *et al* (1983) and Tilburn *et al* (1983) using different selectable markers. Both methods are based on those developed for *S.cerevisiae* (Hinnen *et al*, 1978; Beggs, 1978) and *N.crassa* (Case *et al*, 1979) where protoplasts prepared from mycelium are made competent for DNA uptake by the addition of polyethylene glycol (PEG) and calcium chloride.

Transformation requires the presence of a marker on a transforming plasmid that allows selective growth of only transformed colonies. Selection may be by complementation of mutant alleles with cloned genes or by the use of dominant resistance markers. As *A.nidulans* has many defined mutations in known genes the former approach was used in preliminary transformation experiments. Ballance *et al* (1983) used the *pyr4* gene of *N.crassa* to transform an *A.nidulans* strain carrying the corresponding mutant *pyrG* gene while Tilburn *et al* (1983) used the *amdS* gene of *A.nidulans*, encoding acetamidase. Many other selection systems based on complementation have since been developed for *A.nidulans* (Turner and Ballance, 1985).

Figure 1.1

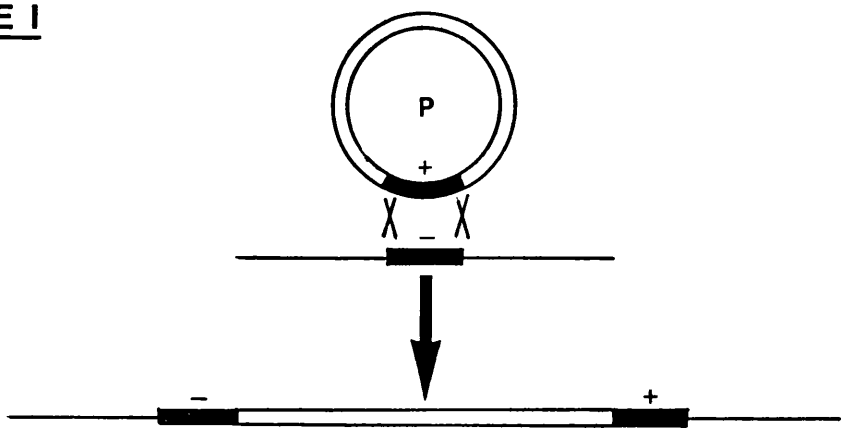
A schematic representation of the integration events proposed for Type I, II and III transformants

a. Type I : The plasmid (P) integrates into the genomic DNA at the host gene locus, which is homologous to the cloned sequences present in the plasmid, resulting in the bacterial vector sequences being flanked by the wild-type and mutant gene loci.

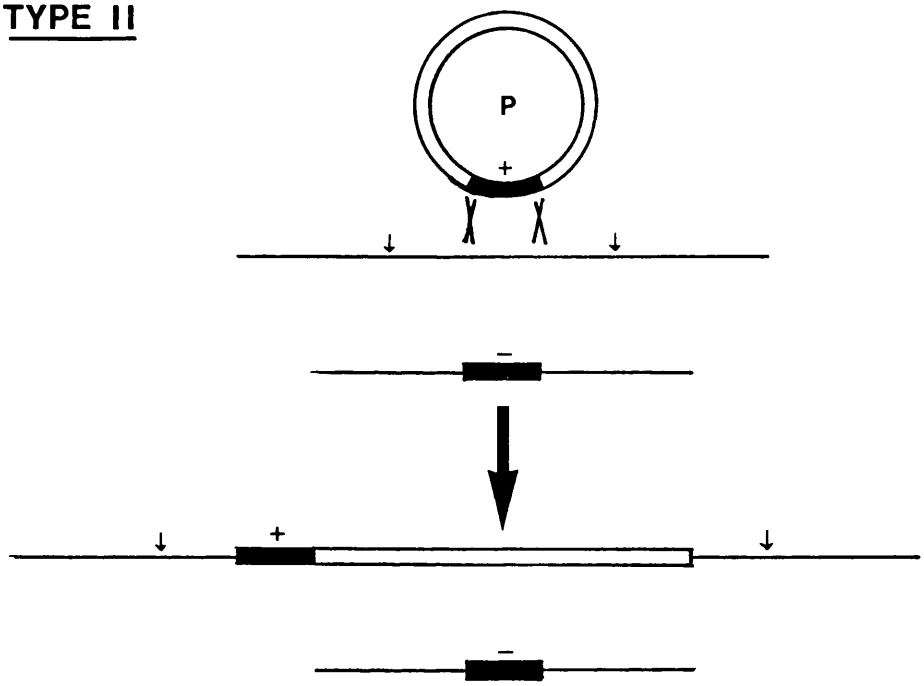
b. Type II : The plasmid (P) integrates into the genomic DNA at a sequence which is not homologous to the cloned sequences present in the plasmid.

c. Type III : Integration of the cloned DNA sequences on the plasmid (P) into the genomic DNA by a 'gene conversion' event at the host gene locus resulting in repair of the resident mutant gene.

TYPE I



TYPE II



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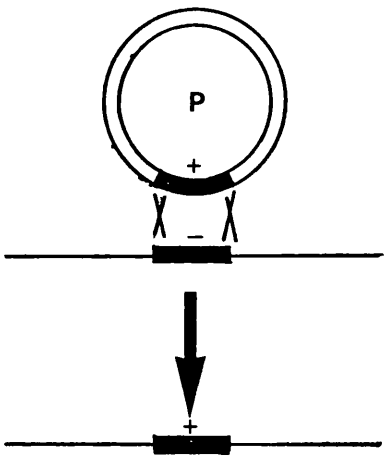


A.nidulans has not, to date, been shown to contain any native plasmids and as yet none of the vectors constructed for use in transformation experiments are able to replicate autonomously. Stable transformants are obtained by integration of the transforming DNA into the genome. The position of integration can be determined by analysis of the genomic DNA which allows transformants to be classified according to the type of integration event. When a high degree of homology exists between the cloned sequences in the vector and the A.nidulans genome, integration frequently occurs at the host gene locus. This results in the bacterial vector sequences being flanked by the wild-type and mutant genes (Figure 1.1a). This is referred to as a Type I transformation event by analogy to the system of classification used in yeast (Hinnen et al, 1978). Homologous transformation may repair the resident mutant gene by a 'gene conversion' type event, which does not result in integration of the bacterial vector sequences, that is referred to as a Type III transformation (Figure 1.1c). Integration of the vector may also occur at a non-homologous site, which is a Type II transformation, and this is the type of integration event predominantly observed when the 'foreign' N.crassa pyr4 gene is used to transform A.nidulans probably because of the low degree of homology between the pyr4 and resident pyrG gene (Turner and Ballance, 1985; Figure 1.1b). The relative frequencies of the three types of transformants varies with the selection system used, the nature of the mutation and possibly with

the genetic background of the transformed strain, as multiple integration of plasmid DNA in Type I and II transformants has been reported to occur in a strain-dependent way (Wernars et al, 1985). The effects of linearisation on circular vectors has been studied, as in S.cerevisiae it is known to increase the transformation frequency for integrating vectors and to direct integration by the production of recombinogenic ends (Orr-Weaver et al, 1983). In A.nidulans linearization of the transforming vector within the trpC gene increases the proportion of Type I and III transformants but does not seem to increase the transformation frequency (Yelton et al, 1984).

The stability of the integrated transforming DNA at different stages of the A.nidulans life cycle varies between individual transformants. The phenotypic stability of transformants during vegetative growth, conidiation and germination under non-selective conditions varies between 97 to >99.9% depending upon the transformation system used. However, there may be some genetic instability as transformants exhibit a wide variety of morphological phenotypes. Transforming markers also vary in their stability through the sexual cycle and it has been observed that the progeny from selfed cleiostothecia frequently lose the integrated transforming DNA (Tilburn et al, 1983; Yelton et al, 1984). The limited instability of the integrated transforming DNA has been utilised in the reisolation of the vector containing either the selectable marker or the

resident mutant gene by transformation of E.coli with total genomic DNA (Johnstone et al, 1985).

Low frequency integrative transformation is sufficient for studying gene expression; however improvements have been made to the transformation systems to allow the identification and cloning of genes from gene banks by the transformation of mutant strains. Previously, gene cloning had relied upon gene expression in a heterologous host or hybridization of gene banks to heterologous gene probes. Yelton et al (1985) have constructed a cosmid vector, suitable for isolating genes by transformation, which contains the A.nidulans trpC gene for selection, a lambda cos site and the ampicillin resistance gene to allow propagation in E.coli. Although this vector transforms A.nidulans at a low frequency, inserts of 35-40 kb within the cosmid effectively reduces the number of transformants required to screen a gene bank. The transforming sequences can be reisolated by subjecting total A.nidulans genomic DNA to in vitro lambda packaging followed by transduction into E.coli. This method was successfully used to clone the yA locus of A.nidulans by visual selection.

Attempts to improve the transformation efficiency of existing vectors has centred around the search for autonomously replicating sequences, however no such sequences have yet been isolated for filamentous fungi. Recently a sequence of A.nidulans DNA has been isolated,

designated ans1, which when inserted in a vector containing the pyr4 gene, increases the transformation frequency 50-100 fold to yield around 5000 transformants per μg of DNA (Ballance and Turner, 1985). Transformation does not occur by autonomous replication and it is considered that the ans1 sequence improves the transformation frequency by enhancing integration. This sequence is very AT rich and is reiterated many times throughout the genome, however its biological function is not known. Transforming sequences can be reisolated from total genomic DNA by transformation of E.coli to ampicillin resistance, which occurs at a low frequency. This suggests that a low copy number of free plasmids is present in the transformant and these are believed to be due to excision of the integrated DNA. The vector containing ans1 and the pyr4 gene has been used to self clone the acuD gene encoding isocitrate lyase, from a gene bank (Ballance and Turner, 1986).

A vector containing the argB gene in pUC8 also exhibits an increased transformation frequency, yielding approximately 500 transformants per μg DNA. Transformation occurs by integration and the reason for the improved transformation frequency is not known. As in the pyr4 system, transforming sequences can be reisolated in E.coli and this vector has been used to clone the A.nidulans brlA gene (Johnstone et al, 1985). A temperature sensitive brlA allele has also been isolated from a wild-type transformant supporting the theory that the transforming DNA is excised from the genome

and may contain the mutant allele instead of the wild-type gene.

The development of vectors for the selection of genes by complementation of auxotrophic mutations in A.nidulans, in conjunction with methods for the isolation of genes using heterologous gene probes, means that an increasing number of genes are becoming available for detailed study. Sequence analysis and comparative studies of the 5' and 3' non-coding regions of these cloned genes may enable sequences important in gene expression and regulation to be identified.

The production of a transformation system for A.nidulans has allowed techniques developed for S.cerevisiae and E.coli to be applied to this organism. A method is now available for the precise replacement of A.nidulans genes with mutant alleles, made in vitro, using either a one- or two-step procedure (Miller et al, 1985) adapted from one-step (Rothstein, 1983) and two-step (Scherer and Davies, 1979) methods developed for S.cerevisiae. This allows the biochemical and biological consequences of introducing a specific mutation into the genome of an otherwise unaltered cell to be studied. The E.coli lacZ gene has been used as an aid in the analysis of expression signals in several organisms by constructing transcriptional or translational fusions of particular genes with the lacZ gene sequences and this method of analysis has now been applied to A.nidulans (Van Gorcom et al, 1986).

The sound scientific base established by genetical studies in A.nidulans and the application of recombinant DNA techniques for molecular analyses will allow detailed studies of gene structure and function in a variety of metabolic pathways and the identification of specific regions and DNA sequences involved in gene expression and regulation.

1-1 GENE ORGANISATION IN FILAMENTOUS FUNGI

The functional significance and occurrence of gene clusters has been well documented for prokaryotes, such as Salmonella typhimurium and Escherichia coli, since the classical studies of Demerec (1964) and Jacob and Monod (1961). It has been demonstrated that many clusters of functionally related bacterial genes constitute operons, a unit of genetic regulation generally under negative control which is transcribed as a polycistronic mRNA. The subsequent translation of this mRNA ensures the co-ordinate synthesis of proteins encoded in a particular operon.

In contrast to bacteria, there is no evidence in eukaryotes for polycistronic transcripts. In fungi related genes tend to be located within separate linkage groups and their expression is generally subject to positive control by, one or more, unlinked regulatory genes. However, a number of metabolic pathways have been identified that do exhibit gene

clustering but to varying degrees. These gene clusters are present within a single linkage group but may be either loosely linked, that is freely recombining, or closely linked.

There are four well documented examples in fungi of metabolic pathways whose genes are tightly clustered; the proline utilization and nitrate utilization pathways of A.nidulans, the quinic acid degradation pathway of N.crassa and the galactose metabolic pathway of S.cerevisiae. These are 'true gene clusters' of contiguous but separate genes and will be described further below.

A second type of gene clustering has been described. Many gene clusters identified have been shown to be "cluster-genes" which are single genes encoding multifunctional polypeptide chains typically associated as homopolymeric aggregates (Giles, 1978). Examples of 'cluster-genes' in fungi are the tryptophan synthetase gene of N.crassa and A.nidulans; the HIS4 gene of S.cerevisiae; the genes for fatty acid synthetase, FAS1 and FAS2, of S.cerevisiae; the AROM genes of N.crassa and A.nidulans encoding enzymes for the polyaromatic biosynthetic pathway, which will be described later.

1-1.1 TRUE GENE CLUSTERS

"True gene clusters" may be distinguished from "cluster-genes" on the basis of three criteria. Firstly, the products of individual genes within a gene cluster are different polypeptide chains. Secondly, no mutations identified to date exert a pleiotropic effect on a different gene within the cluster. Pleiotropic mutations eliminating some or all of the enzyme activities encoded by the gene cluster have been observed but these have been shown to be deletions or mutations within a trans-acting regulatory gene either linked to or distant from the gene cluster. Thirdly, cloning of the gene cluster and subsequent sequence analysis allows the presence of separate transcripts to be demonstrated (Fincham, 1985). These criteria have been satisfied by the proline catabolism gene cluster of A.nidulans and the quinic acid degradation gene cluster of N.crassa.

The proline catabolism gene cluster in linkage group VII of A.nidulans contains four genes whose products are sufficient for the conversion of exogenous L-proline to internal L-glutamate. The prnB locus encodes the major proline permease, prnD and prnC are the structural genes for proline oxidase and Δ' - pyrroline-5-carboxylase (P5C) hydrogenase respectively and prnA is a pathway specific positively acting regulatory gene for proline induction (Arst et al, 1980; Jones et al, 1981; Sharma and Arst, 1985). Some

cis-acting regulatory mutations, designated *prn*^{c}, have been identified which map to the central region of the *prn* cluster and control expression of *prnB*; however there is reduced expression of the *prnC* gene when the cis-acting region is deleted suggesting that this region contains an enhancer type element for *prnC* expression (Arst and Scazzocchio, 1985). The entire proline catabolism gene cluster has been cloned and RNA transcripts have been identified for all the genes described above with the addition of another proline-inducible transcript mapping between the *prnA* and *prnD* genes (Arst and Scazzocchio, 1985). Transformation experiments have also been carried out with the cloned *prn* cluster in order to confirm the integrity and biological functions of the cloned genes.

The most extensively researched and well documented gene cluster in filamentous fungi is the quinic acid (*qa*) gene cluster of *Neurospora crassa*. Genetical and biochemical studies of mutants of *N.crassa* unable to utilize quinic acid as a carbon source identified four distinct genes involved in the catabolism of quinic acid to protocatechuic acid and determined that these genes occurred as a tightly linked cluster, the *qa* gene cluster (Rines, 1969; Chaleff, 1974; Case and Giles, 1976). Three of these genes, *qa-2*, *qa-3* and *qa-4* are structural genes encoding the enzymes catabolic dehydroquinase, quinate dehydrogenase and dehydroshikimate dehydratase respectively. Mutations in these result in loss of a single enzyme activity (Rines,

1969; Chaleff, 1974). The enzyme activities are co-ordinately induced in the presence of the inducer quinic acid and the enzyme activities are physically separable by sucrose density centrifugation. Mutations in the fourth gene, qa-1, are pleiotropically non-inducible for the above three enzyme activities. Two types of qa-1 mutations were identified, fast (qa-1F) and slow (qa-1S), based upon their rapidity in complementing qa structural gene mutations in heterokaryons, which map to two different regions of the qa1 locus (Rines, 1969). Constitutive qa-1 mutants were obtained as revertants of qa-1S but not qa-1F mutants and some of these were semi-dominant to wild-type in heterokaryons (Valone et al, 1971). Based on these and related observations it was proposed that the regulation of the qa gene cluster involved the action of a single multimeric activator protein (qa-1) containing two distinct functional domains. The activator domain, defined by qa-1S mutations, interacted with the inducer while the domain defined by qa-1F mutations interacted with the 5' regions adjacent to each of the three structural genes to facilitate transcription. Constitutive mutants were considered to produce activator proteins capable of initiating transcription in the absence of inducer because the usual allosteric transitions assumed to be produced by activator binding were no longer required (Giles et al, 1973; Case and Giles, 1975).

Major advances in the analysis of the qa gene cluster became possible with the cloning of the qa-2 gene. Vapnek et al (1977) demonstrated that clones containing the qa-2 gene of N.crassa could be selected by complementation of E.coli aroD mutants which lack the biosynthetic dehydroquinase of the polyaromatic amino acid pathway. Using this procedure a 36.6 kb fragment in a cosmid containing the complete qa gene cluster was isolated (Schweizer et al, 1981). The newly developed transformation system for N.crassa was used to locate and confirm the functional integrity of the remaining structural genes, qa-3 and qa-4, and the regulatory gene qa-1 (Schweizer et al, 1981). The gene order qa-2 - qa-4 - qa-3 - qa-1S - qa-1F was as had been previously determined by genetic analysis (Case and Giles, 1976). The transformation experiments provided the first evidence for two regulatory genes controlling the qa cluster corresponding to the two presumptive regions of the qa-1 gene, qa-1S and qa-1F, which was confirmed by DNA-RNA hybridization studies (Huiet, 1984). Separate quinate inducible mRNAs were demonstrated for the qa structural genes and two additional quinate inducible transcripts of unknown function, qa-x and qa-y, were also identified (Patel et al, 1981). Possible functions for the qa-x and qa-y genes have been suggested. First, that one might encode a permease as there is some indirect evidence for the presence of a permease for quinic acid uptake in N.crassa (Rines, 1973) and second, that the other gene might encode an enzyme for the conversion of chlorogenic acid to quinic acid and

caffeic acid as in higher plants, on which N.crassa grows as a saprophyte, quinic acid exist primarily as the caffeic acid ester chlorogenic acid (Giles et al, 1985).

The qa structural genes are regulated by the inducer and the products of the qa-1S and qa-1F genes at the level of transcription. The qa mRNAs are present in only trace amounts in uninduced wild-type cultures and in cultures of non-inducible qa-1F and qa-1S mutants but in constitutive ($qa\text{-}1^c$) strains mRNA induction does not require the presence of the inducer, quinic acid. Catabolite repression appears to have little effect on the qa genes except qa-x which may be regulated differently. A qa-1F mutation reduces transcription only 2 to 3 fold but a $qa\text{-}1S^-$ mutation blocks transcription of the qa-x gene. Therefore the qa-x gene may be controlled primarily by qa-1S and catabolite repression. The transcripts of the two regulatory genes, qa-1S and qa-1F, are also inducible, with transcript levels being increased 50 fold in the presence of quinic acid, and their induction is dependent on the presence of wild-type qa-1S and qa-1F products suggesting that these genes are autogenously regulating (Huiet, 1984; Patel and Giles, 1985).

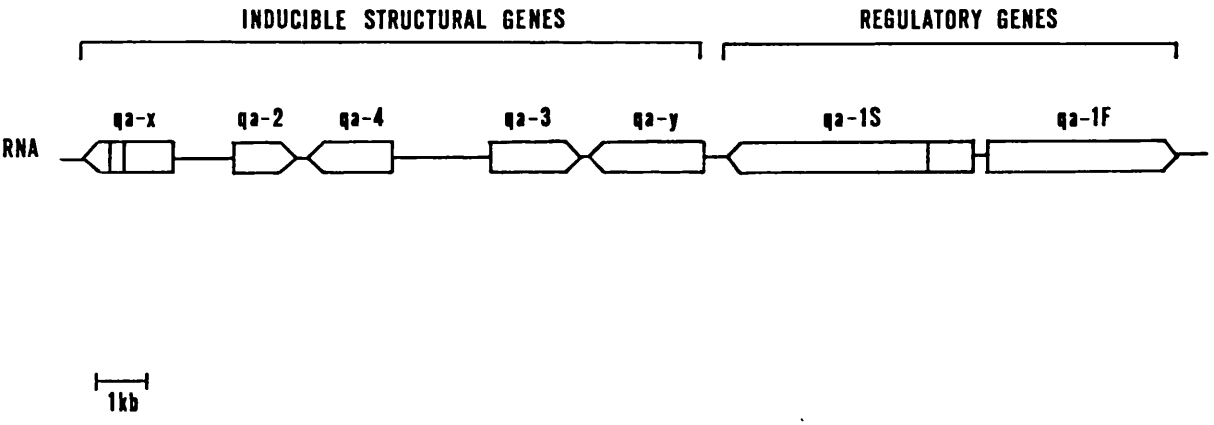
The two qa regulatory genes, located at one end of the gene cluster, are divergently transcribed from a short, common 5' region for each gene. The five qa structural genes are all transcribed from distinct promoter regions. Pairs of genes

Figure 1.2

A transcriptional map of the N.crassa qa gene cluster

The diagram indicates the size and transcriptional direction of the major qa mRNAs of the inducible structural genes and the regulatory genes. Vertical lines in the qa-x and qa-1S transcripts show the position of small intervening sequences.

FIGURE 1.2


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

qa-x qa-2 and qa-3 qa-4 are divergently transcribed in different directions from common regions 5' to each pair of genes. The qa-y gene has a single flanking 5' region (Giles et al, 1985; Figure 1.2).

The model for the regulation of the qa gene cluster has been modified in the light of the available molecular data. There are two regulatory products acting upon the qa cluster, a negatively acting repressor protein produced by the qa-1S gene and a positively acting activator protein produced by the qa-1F gene. These two molecules in association with the inducer, quinic acid, regulate the utilization of quinic acid in N.crassa. It has been postulated that the qa-1S repressor product controls the expression of the activator-encoding qa-1F gene. In a wild-type strain, in the absence of quinic acid, the qa genes products are produced at low basal levels. The addition of quinic acid releases the inhibition of qa-1F expression by the repressor and the activator protein then initiates its own synthesis and that of the other qa transcripts. The activator also stimulates production of the repressor protein and this may be to ensure that there is enough repressor protein to turn off the system when levels of quinic acid fall. However a positive role for the qa-1S gene product has not been excluded as a possibility (Giles et al, 1985). Evidence for a positive and negative regulatory role for the qa-1F and qa-1S gene products respectively is provided by the types of mutations observed in these two genes. Two types of qa-1S

mutants have been observed, recessive constitutive mutants (qa-1S^{c}) and semi-dominant non-inducible (qa-1S^{-}) mutants. The qa-1S^{c} mutants are considered to produce 'inactive' repressor protein while the qa-1S^{-} mutants may produce a 'super-repressor', insensitive to inducer. The single class of recessive non-inducible qa-1F mutants (qa-1F^{-}) may be due to loss of activator function.

The DNA sequence of the entire qa cluster has been obtained. The seven genes are contained within 17.3 kb. Approximately 60% of this DNA is coding sequence and 80% of the total sequence is present in the mRNAs. None of the transcripts appear to overlap and no regions of repetitive DNA sequence were detected (Giles et al, 1985).

1-1.2 "CLUSTER GENES"

A "cluster gene" is a single gene encoding a multifunctional protein. Examples of 'cluster genes' in filamentous fungi are pyr3 (Mackoff et al, 1978), his3 (Minson and Creaser, 1969) and trp1 (Schlechtman and Yanofsky, 1983) of N.crassa and trpC (Kafer, 1978) of A.nidulans.

Early studies of "cluster gene" mutants in fungi, for example the his4 mutants of S.cerevisiae (Fink, 1965), by combined genetic, biochemical and complementation analyses revealed that they had many of the properties of bacterial operons: biochemical heterogeneity, polarized

complementation, asymmetric localization of entirely non-complementing mutants and suppressibility of polar mutations. However, the products of fungal "cluster genes" tended to remain associated after extensive purification, although separation of these polypeptides had been reported in some cases. Because of these contradictory findings it was unclear whether the fungal "cluster genes" were monocistronic or polycistronic and the presence of polycistronic messenger RNAs, characteristic of bacterial operons, in eukaryotes remained a possibility. This problem was finally resolved when Bigelis *et al* (1977) demonstrated that the *his4* gene of *S.cerevisiae* was monocistronic, specifying a single polypeptide of 95,000 molecular weight, which is multifunctional and catalyzes the 2nd, 3rd and tenth steps in the histidine biosynthetic pathway. Proteins smaller than 95 K, capable of carrying out one or other of the reactions, were the result of proteolysis or premature termination by nonsense mutations.

A well characterized system, both genetically and biochemically is the *arom* "cluster gene" of *N.crassa* (Giles *et al*, 1967). This "cluster gene" in linkage group II encodes five enzymes catalyzing reactions two to six in the polyaromatic biosynthetic pathway. Mutants lacking single enzyme activities map to discrete non-overlaping segments of the *arom* region corresponding to the five *arom* enzymes and pleiotropic mutants, lacking all five enzyme activities, map exclusively within the *arom-2* locus at one end of the

genetic map and are suppressed by nonsense suppressors (Case and Giles, 1968; 1974). Biochemical analyses have demonstrated that the *arom* enzymes co-sediment and co-purify (Ahmed and Giles, 1969; Burgoyne *et al*, 1969) and that the purified enzyme complex is a dimer of a pentafunctional polypeptide (Gaertner and Cole, 1976; Lumsden and Coggins, 1977). These observations led to the conclusion that the *AROM* region was monocistronic and hence a 'cluster gene'. A similar genetical organisation has been shown for the *AROM* genes of *A.nidulans* (Roberts, 1969), *S.cerevisiae* (Leeuw, 1968), *Ustilago maydis* and *U.violecea* (Berlyn and Giles, 1972) and *Schizosaccharomyces pombe* (Strauss, 1979). In contrast, the prokaryotic polyaromatic biosynthetic enzymes are clearly separable (Berlyn and Giles, 1969; 1973) and the *aro* genes are not contiguous (Pittard and Wallace, 1966).

The entire *aro* loci from *S.cerevisiae* (Larimer *et al*, 1983) and *S.pombe* (Nakanishi and Yamamoto, 1984), the *A.nidulans* *AROM* locus (Kinghorn and Hawkins, 1982; Charles *et al*, 1986) and part of the *N.crassa* *arom* locus (Catcheside *et al*, 1985) have been cloned. DNA sequence analysis of the *A.nidulans* *AROM* gene revealed a single open reading frame of 4812 bp and the inferred molecular weight of the *AROM* polypeptide is 175.101 (Charles *et al*, 1986), similar to that of 165,000 for the *arom* polypeptide of *N.crassa* as determined by SDS polyacrylamide gel electrophoresis (Smith and Coggins, 1983).

1-1.3 THE EVOLUTION OF "CLUSTER GENES" AND GENE CLUSTERS

The evolution of "cluster genes" has been examined for the biosynthetic aromatic amino acid pathway (Giles, 1978). Comparative biochemical studies have revealed that for all fungi examined the enzymes of this pathway are physically associated while in prokaryotes they are physically separable on sucrose density gradients. For other eukaryotes examined (Chlamyodomonas, a moss and tobacco) two of the five activities are physically associated (Berlyn et al, 1970) while the other three activities are separable. Hence the gene-enzyme relationships in this pathway for bacteria and fungi are in contrast to that usually observed in these two groups, as the genes are scattered in bacteria but clustered or even fused in fungi. Information on the molecular weights of the five aro enzymes in bacteria is consistent with the hypothesis that the five functional segments of the pentafunctional arom polypeptide are each homologous to the corresponding bacterial enzymes (Berlyn and Giles, 1969). There is distinct homology between the DNA sequences of the E.coli aroA gene and 1356 bp of the A.nidulans AROM gene and there is 36% homology between their amino acid sequences. The region of AROM DNA encoding the aroA function will complement an aroA mutation in E.coli (Charles et al, 1986). These observations suggest that the A.nidulans AROM and E.coli aro genes have a common ancestor.

In A.nidulans quinate catabolism and aromatic amino acid biosynthesis share two intermediates, dehydroquinate and dehydroshikimate, and have two distinct dehydroquinase iso-enzymes (Kinghorn and Hawkins, 1982; Hawkins et al, 1982). The DNA sequences encoding these two iso-enzymes exhibit no sequence homology suggesting that they have evolved by convergent evolutionary processes (Charles et al, 1985). The enzymes encoded by the AROM locus may channel the intermediates of aromatic biosynthesis so that levels of these common intermediates are low enough to prevent induction of the catabolic pathway (Giles, 1978) and hence the evolution of the AROM "cluster gene" and the quinic acid gene cluster may be interrelated.

Fincham (1985) has addressed the significance of the clustering of functionally related but separately transcribed genes. First, that clustering reflects a common mode of regulation and second, that clustering of complementary genes performing a specialized function reflects on their evolution as linkage may be selected for by the stabilization of association of mutually adapted alleles at different loci.

1-2 GENE REGULATION IN FILAMENTOUS FUNGI

Three components of regulatory systems have been recognized by the identification and analysis of mutants affected in regulation. Firstly, there are the genes subject to

regulation whose protein products are required and produced when the fungus needs to utilize a special source of carbon, nitrogen, phosphate or sulphate or are required for development and differentiation during the growth cycle. Functionally related genes are usually scattered throughout the genome but a number of metabolic pathways have been identified that exhibit varying degrees of gene clustering. Secondly, there are the regulatory genes which act in trans on the regulated genes. The regulatory genes products may be negatively acting repressors or, as is more commonly observed in fungi, positively acting activators of transcription. Mutations result in either an enzyme-negative or enzyme-constitutive phenotype which in a positively acting regulatory gene are generally recessive and dominant respectively in heterokaryon or diploid strains. Thirdly, there are sites in the DNA sequence adjacent to the protein coding sequences of the regulated genes that have cis-limited effects on gene expression in relation to their response to trans-acting regulatory genes.

Regulatory genes may be divided into two categories, pathway specific regulatory genes and wide domain regulatory genes and examples of these two forms of regulatory control are described below.

1-2.1 PATHWAY SPECIFIC GENE REGULATION

Pathway specific regulatory genes mediate induction or

repression of the synthesis of a number of enzymes and permeases of a single metabolic pathway. A number of these regulatory genes have been identified, for example, *amdA*, *facB*, *alcR*, *aplA*, *prnA*, *nirA*, *uaY*, *galA* and *arcA* in *A.nidulans*; *GAL4* and *GAL80* in *S.cerevisiae* and *qa-1F* and *qa-1S* in *N.crassa*.

Studies to date in filamentous fungi suggest that positively acting regulatory genes predominate in pathway specific gene regulation. An example of a positively acting regulatory gene is the *nirA* gene which mediates induction of the nitrate and nitrite reductases by nitrate and nitrite in the nitrate assimilation pathway of *A.nidulans*. Loss of function mutations, *nirA*$^{-}$, lead to non-inducibility and the inability to utilize nitrate and nitrite (Cove, 1979) and rarer constitutive mutations, *nirA*^{c}, remove the requirement for a co-inducer (Pateman and Cove, 1967; Rand and Arst, 1978). Another gain-of-function mutation, *nirA*^{d}, removes the need for the *areA* gene product resulting in nitrogen metabolite derepressed expression of activities under *nirA* control (Rand and Arst, 1978; Tollervey and Arst, 1981). Intragenic recombination between the *nirA*^{c} and *nirA*^{d} mutations suggests that the *nirA* gene product has two domains, a region affected by co-inducer binding defined by *nirA*^{c} mutations and a region interacting with the *areA* gene product or initiator sites adjacent to the structural genes under *nirA* and *areA* control, defined by *nirA*^{d} mutations.

In the ethanol utilization pathway of A.nidulans, the alcA and aldA genes encode the enzymes alcohol dehydrogenase I and aldehyde dehydrogenase respectively. Mutations in a third locus, alcR, have been shown to result in pleiotropic loss of the above enzyme activities which suggests that the alcR gene product is a positive activator mediating production of these enzymes in the presence of co-inducer (Pateman et al, 1983; Sealy-Lewis and Lockington, 1984).

In the proline catabolism gene cluster, the prnA gene has been shown to encode a protein which is positively acting as some prnA$^-$ mutations are suppressible by translational suppressors (Sharma and Arst, 1985). Translational suppressors have also been used to demonstrate that the products of the A.nidulans regulatory genes, alcR (Roberts et al, 1979) and areA (Al Taho et al, 1984) are proteins. A protein considered to be the product of the uaY regulatory gene has been identified by chromatography as binding to the inducer, uric acid. Uric acid is the inducer of the synthesis of a number of enzymes and permeases involved in purine degradation (Scazzocchio and Darlington, 1968) and mutations in the uaY gene result in non-inducibility by uric acid (Scazzocchio et al, 1982). The protein identified is missing in strains carrying null non-reversible mutations in uaY and has a modified elution pattern in strains carrying 'leaky' uaY mutations where the levels of one enzyme, urate oxidase, are reduced in relation to the other enzymes under the control of the uaY gene (Philippides and Scazzocchio,

1981).

1-2.2 WIDE DOMAIN GENE REGULATION

Two forms of wide domain regulatory control are well documented, nitrogen metabolite repression and carbon catabolite repression. Wide domain regulatory genes control the expression of structural genes from a number of different pathways, mediating responses to environmental factors that have wider ranging metabolic consequences.

In nitrogen metabolite repression, a positively acting regulatory gene, *areA*, mediates repression of the synthesis of a large number of enzymes and permeases involved in nitrogen metabolism by preferred nitrogen sources, particularly ammonium and L-glutamine (Arst and Cove, 1973). Loss of function mutations, *areA*^{r}, result in low, repressed levels of the enzymes and permeases involved in nitrogen source utilization and an inability to utilize nitrogen sources other than ammonium (Arst and Scazzocchio, 1985). These mutations have been classed as loss of function mutations on the basis of their frequency and recessivity (Arst and Cove, 1973) and from studies of *areA*^{r} alleles (Rand and Arst, 1977; Arst, 1981). Rarer mutant alleles, *areA*^{d}, lead to metabolite derepressed expression of one or more activities under *areA* control and have been shown to enhance expression of some enzyme activities while reducing or not affecting others (Arst and Scazzocchio, 1985). It is

considered that these products probably alter the structure of the areA gene product. It has been established that the product of the areA gene is a protein (Al Taho et al, 1984) and evidence indicates that the areA product is directly involved in regulation of gene expression and that receptor sites for the areA product differ in structure (Arst and Cove, 1973; Arst and Bailey, 1977). The product of the equivalent gene in N.crassa, nit-2, has been isolated as a DNA binding protein located in the nucleus (Grove and Marzluf, 1981). Evidence suggests that L-glutamine is the effector for the areA product as in N.crassa. The presence of glutamine prevents the equivalent nit-2 product from activating expression of the structural genes under its control (Marzluf, 1981).

In the pathway for carbon source utilization many structural genes are subject to carbon catabolite repression. This is mediated by the creA regulatory gene which is probably negatively acting. Loss of function mutations, $creA^{d}$, lead to carbon catabolite derepression (Arst and Scazzocchio, 1985). Evidence suggests that the creA gene product is directly involved in carbon catabolite repression (Arst and Bailey, 1977). The identity of the effector(s) has not been established but it is likely that some metabolism of repressing carbon sources must occur for carbon catabolite repression to be affected (Arst and Bailey, 1977).

Some genes are subject to a number of controls, for example

the utilization of a compound such as acetamide, which may act as a source of both nitrogen and carbon, is subject to both nitrogen and carbon catabolite repression.

Other forms of wide domain regulatory control have been studied. These are phosphorus repression mediated by the palcA gene product, sulphur repression mediated by the suAmeth gene product and pH regulation, where the secretion of enzymes and synthesis of permeases are dependent upon the pH of the growth medium (Arst and Scazzocchio, 1985).

1-2.3 REGULATORY PROTEIN RECEPTOR SITES

The third component of regulatory systems is, as previously described, DNA sequences adjacent to the protein coding sequences of the regulated genes that have cis-limited effects on gene expression particularly in their response to trans-acting regulatory genes. The isolation of cis-acting regulatory mutations has identified potential receptor sites for regulatory gene products and yielded information concerning the phenotypes of other regulatory mutations. A number of such mutations have been isolated and characterised during studies on the amdS gene of A.nidulans, which encodes acetamidase and is subject to a multiplicity of regulatory controls (Hynes, 1975; 1977; 1978a; 1978b; 1980; 1982; Hynes et al, 1983). In the proline utilization gene cluster, prn^d mutations, which were selected as suppressing are^r mutations for L-proline

utilization, map adjacent to prnB, the gene for L-proline permease, in the central cis-acting regulatory region of the prn gene cluster (Arst and MacDonald, 1975; Arst et al, 1980; 1981). The prn^d mutations have cis-acting effects on prnB expression but not on the other prn genes and from their effects on permease levels it is suggested that they lead mainly to derepression but it is not clear whether they relieve nitrogen metabolite repression, carbon catabolite repression or both.

The molecular characterisation of such cis-acting sites of co-ordinately regulated genes would yield information at the DNA sequence level of sites involved in regulatory protein binding, whether they are pathway specific regulatory genes or wide domain regulatory genes.

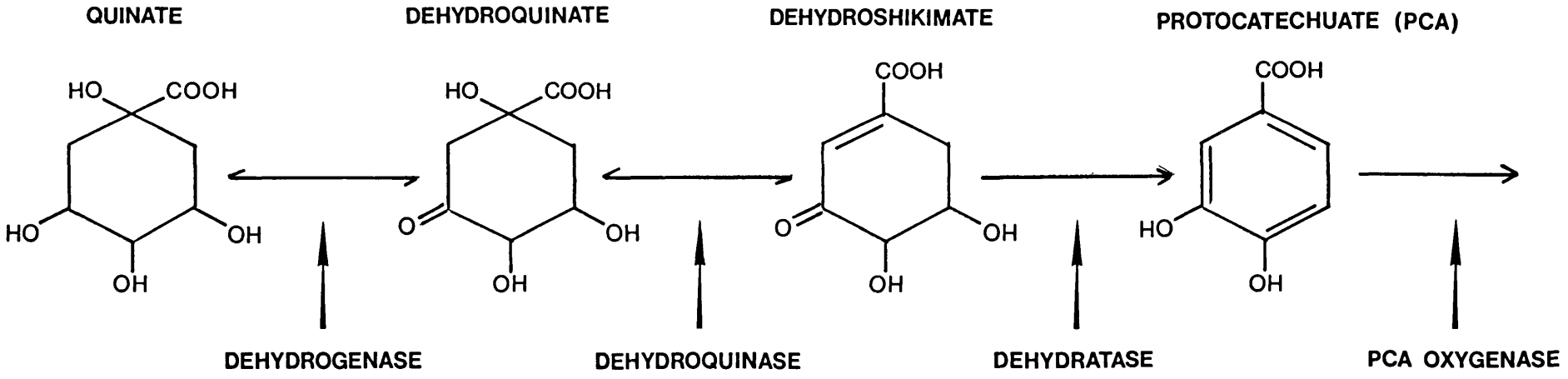
SECTION TWO: THE QUINIC ACID UTILIZATION GENE CLUSTER OF ASPERGILLUS NIDULANS

Aspergillus nidulans is able to utilize quinate, a possible product of lignin catabolism in decaying leaves, as a sole carbon source. Three enzyme activities required for the catabolism of quinate to protocatechuic acid are induced and can be assayed in vitro (1) a dehydrogenase activity converting quinate to dehydroquinate (2) a heat stable dehydroquinase converting 3-dehydroquinate to 3-dehydroshikimate and (3) a dehydratase converting

Figure 1.3

The enzyme pathway for the catabolism of quinate to protocatechuic acid (PCA)

FIGURE 1.3


QUINATE
DEHYDROQUINATE
DEHYDROSHIKIMATE
PROTOCATECHUATE (PCA)
HO
COOH
HO
OH
OH
O
COOH
OH
OH
HO
COOH
OH
OH
DEHYDROGENASE
DEHYDROQUINASE
DEHYDRATASE
PCA OXYGENASE

3-dehydroshikimate to protocatechuate (Figure 1.3). These enzyme activities are absent in cultures grown on glucose as a carbon source and enzyme induction by quinic acid is subject to carbon catabolite repression when glucose is also present (Hawkins *et al*, 1982; Hawkins *et al*, 1984).

Mutants designated *qut* (quinate utilization) were isolated on their inability to grow on quinate, but with wild-type growth on protocatechuate, as sole carbon sources. Assays for the quinate utilization enzymes identified four distinct groups of mutants; *qutA* are pleiotropically non-inducible and lack all three enzyme activities, *qutB* lack quinate dehydrogenase activity, *qutC* lack dehydroshikimate dehydratase activity and *qutE* lack catabolic dehydroquinase activity (Hawkins *et al*, 1982; DaSilva, 1985). The recombination frequency is low between the various *qut* alleles indicating a gene cluster, as in the analogous system in *N.crassa*, and the *qutC* allele has been located in linkage group VIII by the haploidization of diploids (Hawkins *et al*, 1982). Mutants in the *qutA* locus that have lost all three enzyme activities were considered to delineate a control gene, analogous to the *qa-1* gene of *N.crassa* (Case and Giles, 1975), whose product regulated the synthesis of the three enzymes with a positive mode of action (Hawkins *et al*, 1982).

The two pleiotropic non-inducible mutants, *qutA4* and *qutA8*, and the *qutB* structural gene mutant were further analysed in

heterokaryons and diploid strains (Hawkins et al, 1984). Results from growth tests on quinic acid with diploid strains were more consistent than those obtained from heterokaryons. This was probably because A.nidulans heterokaryons are unstable and the ratios of component nuclei fluctuate widely. Therefore the analysis of these mutations were based upon data obtained from growth tests and enzyme assays of diploid strains grown on quinic acid.

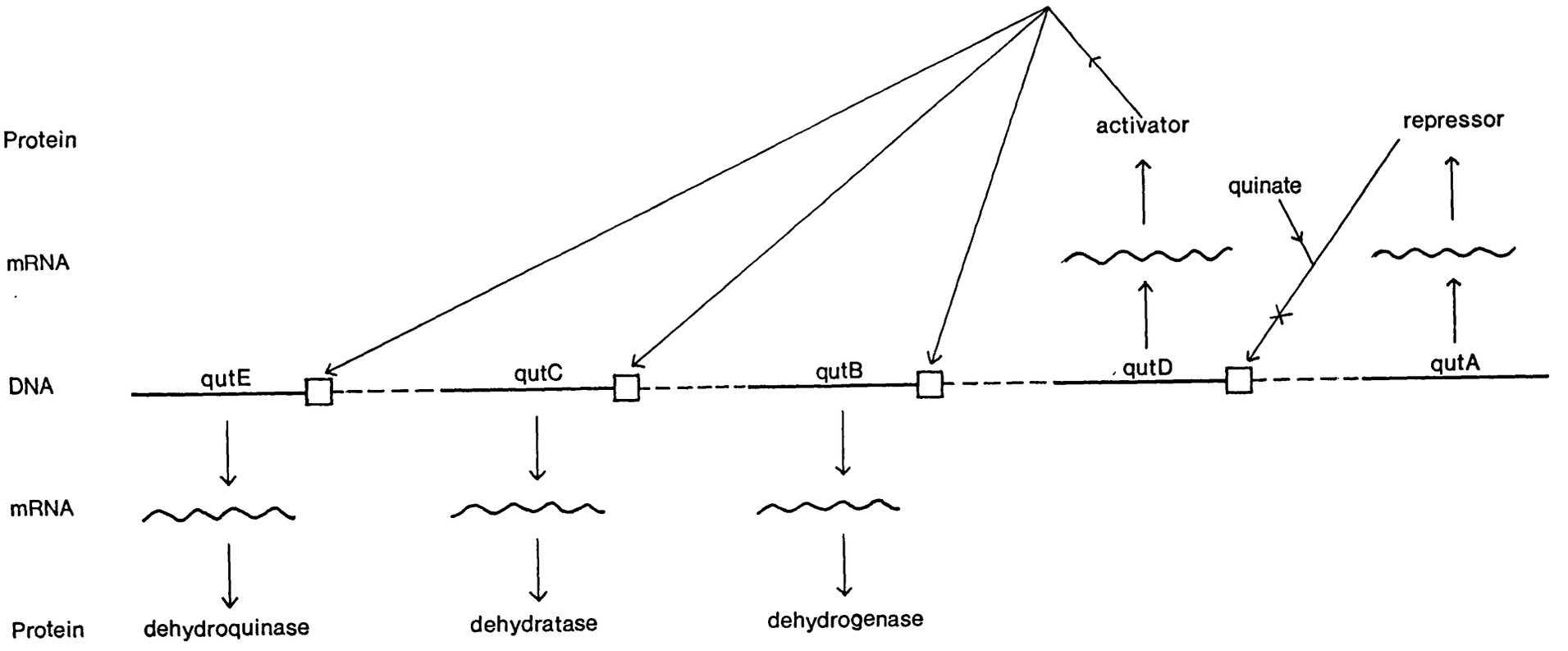
The qutB42 mutant, which lacks dehydrogenase activity, is recessive to wild-type in growth of heterozygous diploid strains on quinic acid and exhibits gene dosage with respect to enzyme activity, producing 60-70% of the wild-type dehydrogenase activity. The non-inducible qutA8 mutant, which lacks all three enzyme activities is fully recessive with respect to growth on quinic acid and enzyme induction in heterozygous diploid strains, for wild-type levels of the enzymes are produced. It was interpreted that the qutA8 allele was a recessive mutation in a regulatory gene, whose product was actively required for expression of the three structural genes. In contrast, the qutA4 non-inducible mutant, which was semi-dominant to wild-type with respect to growth of heterozygous diploid strains on quinic acid, shows an intermediate phenotype for induction of the three enzymes to 60-80% of wild-type levels. Similar levels are observed when the qutA4 mutant is in trans to the qutB42 mutant in heterozygous diploids. From these observations it was suggested that the qutA4 mutation identifies a regulatory

Figure 1.4

A basic model for the regulation of the A.nidulans gut gene cluster

The central solid line represents the gut gene cluster DNA, the broken lines joining the seperate genes indicating genetic linkage. The open boxes at the start of each gene define 5' regulatory sites that interact with RNA polymerase and / or the inducer and repressor proteins. The wavy lines represent the mRNA transcripts (Hawkins et al, 1984)

FIGURE 1.4


Protein
mRNA
DNA
mRNA
Protein
activator
repressor
quinate
qutE
qutC
qutB
qutD
qutA
dehydroquinase
dehydratase
dehydrogenase

gene repressing expression of the structural genes, with this mutation conferring a non-inducible phenotype. The hypothesis that the qutA4 and qutA8 mutations define two separate genes was supported by evidence that the qutA4 and qutA8 mutant genomes complement in trans in heterozygous diploid strains with respect to growth on quinic acid and enzyme induction. The qutA8 allele was designated qutD8, defining a second regulatory gene, qutD. From these observations it was suggested that the two classes of qa-1 mutation, qa-1S and qa-1F, might define two regulatory genes equivalent to qutA and qutD respectively (Case and Giles, 1975; Hawkins et al, 1984) which was confirmed by Huiet (1984) from data obtained in transformation experiments with N.crassa.

From the above experiments a basic model was constructed to explain how the two regulatory genes might interact to control expression of the structural genes (Figure 1.4) (Hawkins et al, 1984). It was proposed that the negatively acting repressor gene qutA is constitutively transcribed and inhibits transcription of the positive activator gene qutD by binding of the repressor protein at the 5' control region of qutD. In the presence of quinic acid, either the repressor protein is inactivated allowing transcription of qutD or binding quinic acid may convert the repressor protein into a positive signal for qutD transcription. Transcription of the qutD gene would result in synthesis of an activator protein which binds to the 5' regions of each

of the structural genes, stimulating their transcription. Alternative models for the interaction of the repressor protein and quinic acid may also be proposed (Hawkins *et al*, 1984). Interaction could occur at two levels, firstly to release transcription of the activator gene by altering the binding of the repressor protein to the *qutD* 5' control region and secondly, an interaction between the repressor protein - quinic acid complex and the *qutD* protein product to produce a structural gene - activator protein complex. Simple genetic models, similar to that proposed above, involving cascades of negative and positive-acting regulatory genes have been proposed for the galactose utilization system of yeast (Oshima, 1982) and the phosphatases in *N.crassa* (Littlewood *et al*, 1975).

From the model of control of the *QUT* gene cluster in *A.nidulans* a number of predictions were made that would be amenable to further investigation (Hawkins *et al*, 1984). At the genetic level two further classes of regulatory mutation should occur, recessive constitutive mutants in the repressor gene (*qutA*) defective in repressor protein or its binding to the activator gene control region and cis-acting dominant constitutive mutations in this control region which will not bind repressor protein. In the analysis of the mRNA encoded by the *qut* genes, the model predicts a constitutively expressed mRNA transcript from the repressor (*qutA*) gene and four further transcripts corresponding to the activator (*qutD*) gene and the three structural genes

induced by quinic acid.

The catabolic dehydroquinase enzyme has been purified from A.nidulans by ammonium sulphate precipitation and its molecular weight has been determined by gel electrophoresis in non-denaturing and SDS denaturing gels (Hawkins et al, 1982). Under non-denaturing conditions the enzyme has a molecular weight greater than 200,000 D and under denaturing conditions a series of bands are observed at molecular weight 10,000, 20,000, 30,000 and 40,000, corresponding to putative monomer, dimer and trimer and tetramer subunits. After extensive heating at 95°C the 10,000 D band increases relative to the other bands, which become diminished, suggesting that the protein has a basic monomer molecular weight of approximately 10,000 D. Early studies in N.crassa suggested that the catabolic dehydroquinase enzyme was composed of 22 identical subunits of approximately 10,000 D (Hautala et al, 1975) which indicates that the A.nidulans and N.crassa enzymes are closely related. Further data has since shown that the N.crassa enzyme subunits are polypeptides of 173 amino acids with a molecular weight of 18,270 D and twelve of these subunits are present in the native enzyme (Vapnek et al, 1977; Alton et al, 1982; Hawkins et al, 1982). The lower molecular weight obtained in earlier experiments was due to degradation of the polypeptide in a proline rich region during heat treatment to solubilize and denature the enzyme in SDS. A similar situation might also apply to A.nidulans. Further evidence

that the A.nidulans and N.crassa enzymes are closely related and may have amino acid sequence homology was obtained from the observation that the A.nidulans enzyme immunologically cross-reacts with rabbit antiserum raised to the N.crassa purified enzyme (Hawkins et al, 1982). Therefore there is evidence of amino acid homology between the A.nidulans and N.crassa enzymes and hence potential DNA sequence homology between the corresponding QUTE and qa-2 genes.

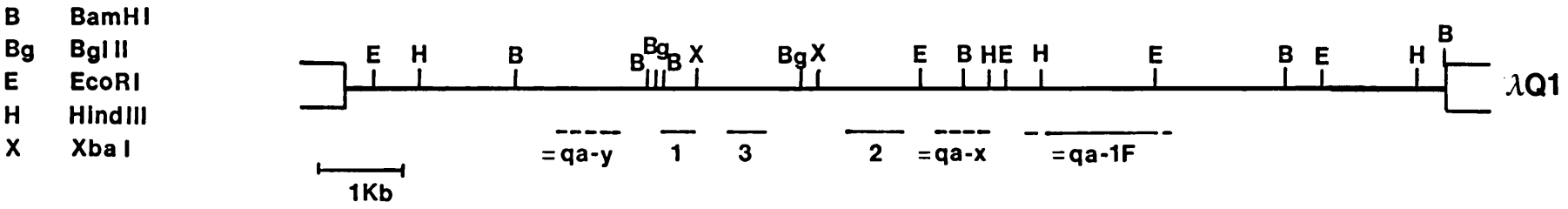
The A.nidulans QUTE gene, encoding catabolic dehydroquinase, has been cloned from an A.nidulans genomic DNA library by cross hybridization to the N.crassa qa-2 gene (Hawkins et al, 1985; DaSilva, 1985). The A.nidulans genomic DNA library was constructed by ligating size selected Sau3A partially restriction digested DNA in the BamHI sites of the replacement vector λDB286 (Brammer, 1982). This gene library was probed with a N.crassa 3.1 kb DNA sequence from the plasmid pVK57 (Kushner et al, 1977) containing the entire qa-2 gene and part of the qa-4 dehydratase-encoding gene from the qa gene cluster (Schweizer et al, 1981). A single recombinant phage, λQ1, was isolated, which contains a 13.2 kb insert of A.nidulans DNA. A second recombinant phage, λQ2, was isolated using a 3.3 kb fragment from λQ1, which cross-hybridizes to the 3.1 kb fragment from pVK57. This phage contains an 8.9 kb fragment of A.nidulans genomic DNA which maps wholly within the genomic DNA contained in λQ1.

Figure 1.5

Restriction map of the recombinant phage λQ1

A restriction map of the A.nidulans genomic DNA present within the recombinant phage λQ1 indicating the location of DNA sequences homologous to the qa-3 (1), qa-4 (3) and qa-2 (2) genes of N.crassa. The qa-2, qa-3 and qa-4 genes are equivalent to the A.nidulans QUTE, QUTB and QUTC genes respectively. The region homologous to the N.crassa activator- encoding qa-1F gene is considered to contain the A.nidulans QUTD gene. The location of DNA sequences homologous to the N.crassa quinate inducible qa-x and qa-y genes is also shown (Hawkins et al, 1985)

FIGURE 1.5


B BamHI
Bg BglII
E EcoRI
H HindIII
X Xba I
E H B B Bg B X Bg X E B HE H E B E H B
λQ1
=qa-y
1
3
2
=qa-x
=qa-1F
1Kb

The recombinant λ phage has been mapped with restriction enzymes and the positions of the QUTB, QUTC and QUTE genes determined using three N.crassa DNA fragments as probes in hybridization experiments, each of which is largely contained within the three enzyme structural genes cloned in pVK88 (Figure 1.5). The relative order of enzyme structural genes is the same in A.nidulans as in N.crassa (Schweizer et al, 1981) although they are contained within a smaller DNA sequence of 3.4 kb in A.nidulans compared to 5.4 kb in N.crassa (Hawkins et al, 1985).

The QUTD gene of A.nidulans was considered to be equivalent to the qa-1F gene, which encodes the activator protein in N.crassa. DNA sequences homologous to the qa-1F gene have been located in the recombinant phage λQ1 (DaSilva, 1985). A 2.1 kb restriction fragment from pMSK375 (Schweizer et al, 1981), wholly within the coding sequence of the qa-1F gene hybridized strongly to λQ1 DNA and located the equivalent gene (QUTD) in A.nidulans to a 1.7 kb EcoRI restriction fragment (Figure 1.5). The qa-1S gene, which encodes the repressor protein in N.crassa, did not exhibit any homology to the λQ1 DNA when used as a probe in hybridization experiments indicating that the equivalent gene (QUTA) in A.nidulans is not present on this clone (Da Silva, 1985; Hawkins et al, 1985).

The ability of the A.nidulans QUTE gene to complement an aroD6 biosynthetic dehydroquinase mutation in E.coli was

examined (Hawkins *et al*, 1985). Previous studies had shown that the *qa-2* gene, encoding catabolic dehydroquinase in *N.crassa*, and the gene for the biosynthetic isoenzyme in *A.nidulans* complement the *aroD6* mutation in *E.coli* (Kushner *et al*, 1977; Hawkins *et al*, 1982; Kinghorn and Hawkins, 1982). A 3.3 kb fragment containing the entire *QUTE* gene, as determined by hybridization studies, was sub-cloned into pBR322 and transformed into an *aroD6* mutant strain SK3430 of *E.coli*. Slow growth representing weak complementation was observed on plates at 30°C after 4-6 days incubation but not at 37°C. This observation explained why attempts to clone the *QUTE* gene by complementation in the *E.coli* *aroD6* mutant were unsuccessful as all incubations were at 37°C (Hawkins, Kinghorn and Giles, unpublished work). A sequence allowing expression of the *N.crassa* *qa-2* gene in *E.coli* is probably absent in the 5' region of the *A.nidulans* *QUTE* gene, which is consequently expressed with low efficiency.

THE AIM OF THE RESEARCH PROJECT

The aim of the research project, that forms the basis of this thesis, was to identify the function and confirm the integrity of *A.nidulans* genomic DNA sequences cloned within the recombinant phage λQ1 by transformation of *qut*$^-$ mutant strains of *A.nidulans*. Attention has been focused on the identification of the *QUTE* gene, encoding catabolic dehydroquinase and the *QUTA* and *QUTD* genes that are

considered to encode two regulatory proteins.

DNA sequences homologous to the N.crassa qa-2 gene, encoding catabolic dehydroquinase, have been isolated within the recombinant phage λQ1 from an A.nidulans gene library and a qutE⁻ mutant strain of A.nidulans has been identified. The qa-2 gene has been used as a selectable marker to develop an efficient transformation system for N.crassa (Case et al, 1979). The initial aim was to transform an A.nidulans qutE mutant strain with DNA sequences from λQ1 homologous to the qa-2 gene in order to confirm the location and integrity of the QUTE gene and to test the application of the newly developed A.nidulans transformation system for the selection of transformants able to utilize quinate as a sole carbon source.

The location of the QUTA and QUTD genes was of particular interest. These genes were considered to encode regulatory proteins as qutA and qutD mutant strains are pleiotropically non-inducible with respect to the production of the quinate utilization enzymes. The location of these genes may be determined by the transformation of appropriate mutant strains with DNA sequences isolated from phage λQ1.

A number of transformants have been analysed using molecular biological and biochemical techniques to confirm their integrity and potentially to learn more about the regulation of the QUT gene cluster.

CHAPTER 2

MATERIALS AND METHODS

2-1 GENETICS AND BIOCHEMISTRY OF *ASPERGILLUS NIDULANS*

2-1.1 STRAINS

The strains of *A.nidulans* (Eidam Winter) used were all derived from the Glasgow stock (Pontecorvo *et al*, 1953).

The following strains were utilized for the meiotic mapping of the *qut* gene cluster.

G191	*pyrG89 pabaA1; fwA; uaY9*
R153 *qutA4*	*wA3; pyroA4; qutA4*
R153 *qutB42*	*wA3; pyroA4; qutB42*
R153 *qutC113*	*wA3; pyroA4; qutC113*
R153 *qutD8*	*wA3; pyroA4; qutD8*
R153 *qutE208*	*wA3; pyroA4; qutE208*

Quinic acid non-utilizing mutants of *A.nidulans* were the recipient strains in transformation experiments, as follows:

WA53	*pyrG89; pyroA4; qutE208*
GD22	*pyrG89 pabaA1; wA3; pyroA4; qutD8*
R153 *qutA361*	*wA3; pyroA4; qutA361*

The strain R153 (wA3; pyroA4) constructed from a translocation free strain of Kafer (1965) was used as a standard 'wild-type' strain for biochemical studies and molecular analyses.

Strains were cultured on complete medium slants for medium term storage and held on silica gel for long term storage. Stocks were stored at 4°C.

2-1.2 CULTURE MEDIA

(i) Minimal Medium

The defined minimal medium used throughout this work is basically that of Pontecorvo et al (1953) modified by Roberts (1963). This is prepared as a 10 x solution of stock salts (see below) to which a final concentration of 10 mM $MgSO_4$ is added.

10 x Stock Salts Solution

Substance	Amount per litre
$NaNO_3$	6.0g
KCl	0.52g
KH_2PO_4	1.52g
Trace elements solution	1.0 ml

Trace Elements Solution

Substance	Amount per litre
$FeSO_4.7H_2O$	1.00 g
$ZnSO_4.7H_2O$	8.80 g
$CuSO_4.5H_2O$	0.40 g
$MnSO_4.4H_2O$	0.15 g
$Na_2B_4O_7.10H_2O$	0.10 g
$(NH_4)_6Mo_7O_{24}$	0.05 g

The trace elements were added to distilled water and the undissolved crystals brought into solution by the addition of concentrated HCl (2ml) directly onto the crystals. The solution was adjusted to the final volume by the addition of distilled water.

Minimal medium plus $MgSO_4$ for plates was solidified with 1.5% (w/v) agar. Minimal medium for liquid culture contained the wetting agent Tween 80, diluted 10^{-5} (v/v), and sterile $MgSO_4$ was added to a final concentration of 10 mM after autoclaving the medium.

(ii) Complete Medium

Malt extract agar is a complex medium and was used when fast growth and good conidiation of strains was required.

Substance	Amount per litre
Malt extract	20 g
Bacto-peptone	1 g
Agar	15 g

All media were sterilized by autoclaving at 15 lb pressure for 15 minutes.

(iii) Carbon Sources

Carbon sources were added aseptically to sterile media as follows:

Carbon Source	Final Concentration
Glucose (solid medium)	40 mM
Glucose (liquid medium)	20 mM
Quinic acid (growth medium)	1% (w/v)
Quinic acid (induction medium)	0.1% (w/v)
Glycerol	20mM

Quinic acid was prepared as a 10% stock solution in sterile distilled water, brought to pH 6.5 with NaOH and sterilized by filtration through a 0.45 μm membrane filter (Whatman).

1 M glucose and 2 M glycerol stock solutions were sterilized by autoclaving at 10 lb pressure for 10 minutes.

(iv) Nutritional Supplements

The nutritional supplements for auxotrophic strains were added as required prior to autoclaving the medium.

Supplement	Stock solution (mg ml^{-1})	Amount per litre
p-Aminobenzoic acid (paba)	1.0	1.0 ml
pyridoxin.HCl (pyro)	0.5	1.0 ml
uracil	-	1.1 g
uridine	-	2.4 g

(v) Incubation Temperature

All cultures were grown at 37°C unless otherwise stated.

2-1.3 PREPARATION OF SUSPENSIONS OF CONIDIOSPORES

Dilute suspensions of conidiospores were spread onto the surface of malt extract agar plates at such a concentration to produce confluent growth and good conidiation. Each plate was then flooded with approximately 10 ml of sterile 'Tween-saline', a solution of 0.08% (w/v) NaCl plus

0.025% (v/v) of the wetting agent Tween 80.

The conidia were harvested by repeatedly drawing a sterile wire across the surface of the plate. The suspensions were collected in sterile 25 ml disposable universal bottles and vigorously shaken on a 'Whirlimixer' to disrupt the conidial chains. The suspensions were washed by repeated centrifugation in neutral phosphate buffer (20 mM KH_2PO_4; 50 mM Na_2HPO_4, 50 mM NaCl, 0.4 mM $MgSO_4.7H_2O$) and finally resuspended in 10 ml of the same buffer. The conidiospores were stored at 4°C and retained good viability for at least four weeks.

2-1.4 STANDARD TECHNIQUES FOR GENETIC ANALYSIS

The techniques used were basically those described by Pontecorvo et al (1953).

Sexual crosses were set up between haploid strains carrying complementary nutritional markers. Dense suspensions of conidiospores of the two strains were mixed on the surface of thick minimal medium plates and 'dug' into the medium with a sterile wire loop. The plates were then sealed with adhesive tape and incubated at 30°C for about three weeks.

Fruiting bodies (cleiostothecia) were picked from mature crosses and rolled across the surface of 4% (w/v) hard agar plates to remove contaminating mycelia and conidiospores.

Individual cleiostothecia were then crushed in 1 ml of neutral phosphate buffer and a sample of the resultant ascospore suspension streaked on complete medium to identify hybrid cleiostothecia by the presence of recombinant conidial colours. Suitable dilutions of ascospore suspensions from hybrid cleiostothecia were plated to produce separate colonies and the progeny tested on appropriate media for the segregation of markers.

2-1.5 A QUALITATIVE "SPOT TEST" FOR THE PRODUCTION OF PROTOCATECHUIC ACID

The method used is adapted from that described by Partridge et al (1972) for the visual identification of constitutive quinic acid mutants in N.crassa. Salamon and Davies (1953) reported that $FeCl_3$ produced a strong visible colour with protocatechuic acid (PCA) which should be accumulated by any strain of N.crassa which has activity for the enzymes leading from quinic acid to protocatechuic acid providing that quinic acid is supplied and protocatechuic acid oxygenase is essentially absent or inactive. These requirements would be met by a specific constitutivity of the pre-protocatechuic acid enzymes together with an absence of protocatechuic acid oxygenase by, for example, exclusion of oxygen from the system. The rapid qualitative assay for protocatechuic acid was derived by Partridge et al (1972) on the basis of the above report.

The method, adapted for use with A.nidulans by Grant et al (1988), is as follows. Conidiospores from single colonies were used to "patch" a thick inoculum onto minimal medium containing either quinate or glycerol as a carbon source and incubated for 24 hours at 37°C. Approximately equal sized blocks of agar containing mycelium were excised from the plates and placed in individual test tubes containing 0.75 ml of the following mixture: 200 ml quinic acid pH6.5, 100 ml 0.4 M potassium glycinate buffer pH9.25, 26 ml 1% (w/v) $FeCl_3$, 2 ml 60 mM NAD, 100 ml dimethylsulphoxide and distilled water to a final volume of 486 ml. To prevent induction of the quinic acid utilization enzymes, cycloheximide was included at 30 $\mu g\ ml^{-1}$. The surface of the mixture was covered with a small volume of paraffin oil to exclude oxygen. The tubes were placed in a 30°C incubator during observation. Mycelia producing protocatechuic acid developed a strong purple colouration during a period of time varying from almost immediately to up to one hour. All tubes were observed for a period of 24 hours.

2-1.6 PREPARATION OF CELL-FREE EXTRACTS FOR ENZYME ASSAYS

Mycelium was grown in liquid culture using 2 litre Erlenmeyer flasks with vertical baffles, formed by indenting the walls of the flask, and treated with water repellent silicone film (Armitt et al, 1976). Each flask contained 250 ml liquid minimal medium supplemented with 10 mM uridine and containing 20 mM glucose as a carbon source. Each flask

was inoculated with a suspension of conidiospores to yield 10^6 spores ml^{-1} and the cultures were incubated for 18 hours at 30°C on a gyratory shaker. The mycelium was harvested aseptically by filtration through Whatman No 1 filters, washed with 1 x minimal salts solution and divided equally between three different types of uridine-supplemented liquid minimal medium for a further four hours incubation at 30°C. The three media were as follows -

(1) minimal medium containing 20 mM glucose

(2) minimal medium containing 20 mM glucose and 0.1% (w/v) quinate (pH6.5)

(3) minimal medium containing 0.1% (w/v) quinate (pH6.5)

Following incubation the mycelium was harvested by filtration, washed thoroughly in situ with buffer (0.1 M potassium phosphate, pH7.2; 10 mM EDTA; 1 mM DTT) and stored at -18°C wrapped in aluminium foil. A thin pad of frozen mycelium (approximately 1 g) was immersed in liquid nitrogen in a pre-cooled mortar and ground to a fine powder with a cold pestle. The frozen powdered mycelium was resuspended in 1 ml extraction buffer (0.1 M potassium phosphate, pH7.2; 10 mM EDTA; 1 mM DTT; 1 mM PMSF (phenylmethyl-sulphonylfluoride)) and, after thawing, the mycelium was extracted by gently shaking the slurry in a polypropylene tube on ice for one hour. The cell debris was then removed by centrifugation for 15 minutes in an Eppendorf microfuge at 4°C. The supernatant was decanted, stored on ice and the

enzymes assayed within six hours of extraction. A sample of the supernatent was heated at 70°C for 10 minutes to denature the biosynthetic dehydroquinase (Kinghorn and Hawkins, 1982). The heated sample was cooled on ice and recentrifuged to pellet denatured proteins.

2-1.7 ENZYME ASSAYS

Each of the three enzymes was assayed using a Unicam (Cambridge) SP1800 recording spectrophotometer at 37°C in 1 ml reaction mixtures containing 5 to 20 µl samples of the cell-free extracts.

(i) Quinate dehydrogenase (enzyme 1) was assayed by following the reduction of NAD at 340 nm in a reaction mixture containing 10 mM Tris.HCl (pH8.6); 1 mM quinic acid; 2.5 mM NAD.

(ii) Catabolic dehydroquinase (enzyme 2) was assayed by monitoring the increase in absorbance at 240 nm in a reaction mixture containing 10 mM Tris.HCl (pH7.2); 1 mM EDTA; 20 µl dehydroquinate solution (see source of materials). The volume of dehydroquinate solution added gives an A_{240} of 0.2 prior to addition of the cell-free extract. Thermolabile biosynthetic dehydroquinase had been inactivated by heating (see above).

(iii) 3-dehydroshikimate dehydratase (enzyme 3) was assayed

by measuring the increase in absorbance at 290 nm in a reaction mixture containing 0.1 M Tris.HCl (pH7.5); 2.5 mM $MgCl_2$ and 30 µl dehydroshikimate solution. The latter was freshly prepared enzymatically from dehydroquinate (20 µl, A_{240} 0.2) using purified A.nidulans catabolic dehydroquinase (see source of materials).

The assay for each enzyme activity was repeated once and the average value taken to calculate the specific enzyme activities.

2-1.8 PROTEIN ESTIMATIONS

The concentration of soluble protein was assayed in each cell-free extract by the method of Lowry et al (1951). Calibration curves were constructed using bovine serum albumin standards each time protein estimations were performed.

2-2 RECOMBINANT DNA TECHNIQUES FOR ASPERGILLUS NIDULANS

2-2.1 BACTERIAL STRAINS, PLASMIDS AND BACTERIOPHAGE

(i) Strains of Escherichia coli

JA221 hsdR, mk$^+$, trpESD, leuB6, recA$^-$, lacY
(Clarke and Carbon, 1978)

DH5α F$^-$, endA1, hsdR17 ($r_k^- m_k^+$), supE44, thi-1, λ$^-$, recA1, gyrA96, relA1, Δ (lacZYA - argF), U169, φ80 d lacZ ΔM15.

The above bacterial strains were used for harbouring recombinant DNA plasmids. Bacterial strains for new cultures were reisolated by inoculation from single isolated colonies. Cultures were incubated at 37°C.

(ii) Plasmids

Plasmids pBR322 (Bolivar et al, 1977, a,b), pBR325 (Bolivar, 1978) and pUC19 (Yanisch-Perron et al, 1985) were used as controls in transformation experiments and in the subcloning of DNA fragments from recombinant λ DNA. Plasmid pVK57 (Kushner et al, 1977) containing a DNA fragment from the N.crassa qa gene cluster was generously supplied by

Professor N.H. Giles and co-workers. The *N.crassa* *pyr4* plasmids pDJB1 and pDJB2 (Ballance and Turner, 1985) for use in the transformation of *A.nidulans* were kindly supplied by Dr G. Turner. The plasmid pEH1 containing a fragment from the *A.nidulans* *QUT* gene cluster was constructed in our laboratory by Dr A.R. Hawkins.

(iii) Bacteriophages

The recombinant λ clone λQ1 (Hawkins *et al*, 1985) was isolated in our laboratory by Dr A.R. Hawkins and Dr A.J.F. Da Silva.

2-2.2 MEDIA

(i) Luria Broth

Substance	Amount per litre
Difco Bacto Tryptone	10g
Difco Bacto Yeast Extract	5g
NaCl	5g

Luria agar was prepared by solidifying liquid medium with 15 g Difco Bacto agar per litre. Luria broth and agar were used for bacterial cultures.

Antibiotics were added to media for the selection of plasmids as follows:

Antibiotic	Stock solution (mg/ml)	Final concentration (μg/ml)
Ampicillin (sodium salt)	25	50
Chloramphenicol	34	30
Tetracycline HCl	12.5	15

Ampicillin was prepared in water, chloramphenical in ethanol and tetracycline in 50% (v/v) ethanol : water. The ampicillin solution was sterilized by filtration (0.45 μm Whatman). The antibiotics were added to molten agar media cooled to 55°C.

2-2.3 EXTRACTION WITH PHENOL/CHLOROFORM AND ETHANOL PRECIPITATION OF DNA

Proteins were removed from nucleic acid solutions by extraction with an equal volume of phenol : chloroform reagent (phenol, 100g; chloroform, 100 ml; isoamyl alcohol, 4 ml; 8-hydroxyquinoline, 0.1 g) saturated with 10 mM Tris HCl pH7.5.

DNA solutions were concentrated by precipitating the DNA with two volumes of ethanol after the addition of 0.1 volume of 3M NaAc, pH5.2. The solution was mixed and chilled in a methanol/dry ice bath for 10 minutes or at -18°C overnight. DNA precipitates were pelleted by centrifugation at

13,500 x g for 10 minutes, rinsed in cold 70% (v/v) ethanol, dried under vacuum and resuspended in appropriate volumes of sterile distilled water or TE buffer (10 mM Tris.HCl, pH7.5; 1mM EDTA).

2-2.4 PLASMID DNA PREPARATIONS

(i) Small scale plasmid DNA preparation

This preparation is a modification of the method of Birnboim and Doly (1979).

Cultures of the E.coli strain carrying the plasmid were grown overnight at 37°C in Luria broth containing the appropriate antibiotic. A 1.5 ml sample of the culture in an Eppendorf tube was centrifuged for 1 minute and the medium removed by aspiration. The bacterial pellet was resuspended in 100 µl of a lysis solution (50 mM glucose; 10 mM EDTA; 25 mM Tris HCl pH8.0; 4mg/ml of freshly added lysozyme) and left at room temperature for 5 minutes, when 200 µl of a freshly prepared, ice cold solution of 0.2N NaOH, 1% (w/v) SDS was added. The solutions were mixed by inverting the tube several times and held on ice for 5 minutes; then 150 µl of 3M potassium acetate (pH4.8 with glacial acetic acid) was added and again the mixed solution left for 10 minutes on ice, when it was centrifuged for 5 minutes in a precooled microfuge in a 4°C cold room. The supernatent was transferred to a fresh Eppendorf tube,

phenol : chloroform extracted and the DNA precipitated with ethanol for two minutes at room temperature. The plasmid DNA was resuspended in 50 µl TE buffer containing 20 µg/ml DNase-free RNase.

(ii) Large scale plasmid DNA preparation

This method is based on that of Ish-Horowicz and Burke (1981).

Two 400 ml volumes of Luria broth, containing appropriate antibiotic, were inoculated with 8 ml of an overnight culture of the E.coli strain carrying the required plasmid and incubated at 37°C overnight with shaking. The bacteria were harvested by centrifugation at 4000 g for 10 minutes at room temperature. The pellets of bacteria were resuspended in 12 ml of solution I (50 mM glucose, 25 mM Tris.HCl pH8.0, 10mM EDTA) and left to stand at room temperature for 10 minutes, when 24 ml of solution II (freshly prepared 0.2N NaOH, 1% (w/v) SDS) was added, mixed gently and placed on ice for 5 minutes. A 12 ml volume of 3M potassium acetate pH4.8 was then added, mixed gently and returned to ice for a 15 minute period. The precipitate of chromosomal DNA and proteins was removed by centrifugation at 6000 g for 10 minutes at 4°C and the supernatent was passed through polyallomer wool to separate the solution from remaining cell debris and precipitate. The nucleic acids were precipitated by the addition of 0.6 volumes of propan-2-ol

and recovered by centrifugation at 6000 g for 10 minutes at 4°C. The pellets of nucleic acid were rinsed in 70% (v/v) ethanol, dried under vacuum and each pellet was resuspended in 3 ml TE buffer (10 mM Tris Cl, 1 mM EDTA pH8). The nucleic acids solutions were pooled in a 15 ml Corex tube to which 1 g of analar grade caesium chloride and 0.1 ml ethidium bromide (10 mg/ml) was added for each ml of nucleic acid solution. The density of the solution was measured to determine that it was 1.57 g ml^{-1} and adjusted to this density if necessary. The solution was loaded into Beckman polyallomer 'Quick-Seal' (5/8" x 3") tubes and the volume adjusted with a 1.57 g ml^{-1} solution of CsCl. The tubes were centrifuged overnight at 270,000 g in a 75Ti rotor at 15°C. After centrifugation, the band of plasmid DNA was recovered from the gradient using a syringe and 18 g needle and the ethidium bromide removed by repeated extractions with an equal volume of CsCl saturated propan-2-ol. The plasmid DNA solution was extensively dialysed aganst TE buffer to remove the CsCl and the plasmid DNA precipitated with ethanol and re-dissolved in an appropriate volume of sterile distilled water or TE buffer.

The concentration of the plasmid solution was determined by optical density at 260 nm, where an OD of 1 corresponds to approximately 50 μg ml^{-1} of DNA. The purity of the DNA preparation was estimated from measurements of the OD_{260}/OD_{280} ratio. If the preparation is pure, the ratio should be approximately 1.8 (Maniatis et al, 1982).

2-2.5 DIGESTION OF DNA WITH RESTRICTION ENDONUCLEASES

The DNA solution, typically containing between 0.2 - 1 μg of DNA, was mixed with sterile distilled water to a total volume of 18 μl. A 2 μl volume of the appropriate digestion buffer was then added and the solution gently mixed. The 10 x digestion buffers used were as described by the enzymes' manufacturer. This solution was then incubated with a 2 to 5 fold excess of restriction enzyme (manufacturers definition) at 37°C for 1 hour. Samples of genomic DNA were incubated at 37°C for 3 hours.

When further manipulation of the DNA was necessary, one phenol/chloroform extraction and ethanol precipitation was performed.

2-2.6 GEL ELECTROPHORESIS

Gel electrophoresis was used to separate, identify and purify DNA fragments.

Horizontal slab agarose gels (0.8% - 1.5% (w/v) agarose) were prepared and electrophoresed in Tris-acetate buffer (40 mM Tris-HCl, 2 mM EDTA; pH8.0 with glacial acetic acid) containing 0.5 μg ml^{-1} ethidium bromide (Maniatis et al, 1982).

Molecular weight markers were provided by HindIII digestion of λ DNA or HaeIII digestion of pBR322. DNA samples were mixed with 0.1 x volume of electrophoresis loading buffer (0.25% (w/v) bromophenol blue, 15% (w/v) Ficoll 400) and electrophoresed through the agarose gel until the bromophenol blue dye had travelled to within the last 1/3 of the gel.

DNA binding ethidium bromide in agarose gels was visualized using a short wavelength ultra-violet transilluminator (Ultra-violet Products Inc, California, USA) and photographed using a Polaroid MP-3 land camera and Polaroid 4 x 5 Type-55 or Type-57 film.

2-2.7 RECOVERY OF DNA FRAGMENTS FROM AGAROSE GELS

(i) Electroelution onto Whatman DE81 DEAE-Cellulose Paper

DNA was electroeluted onto Whatman DE81 DEAE-cellulose paper and recovered by a modification of the method of Dretzen et al (1981).

DE81 paper was presoaked in 2.5 M NaCl for 15 minutes and rinsed three times in distilled water. After separating DNA molecules by gel electrophoresis (using Seakem HGT agarose), DNA bands were visualised under a long wavelength UV source and a slice of gel, containing the band to be purified, excised. This slice was then wrapped in DE81 paper and

inserted into a slot of equivalent size in an unused part of the gel. Electrophoresis was resumed for 20 to 30 minutes at 100 V until the DNA was bound to the paper. The DE81 paper was removed, rinsed in distilled water and blotted dry on Whatman 3 MM paper. Excess paper was removed and the strip containing the bound DNA placed in an Eppendorf tube. After addition of 450 μl of a high salt buffer (1M NaCl, 50 mM Tris.HCl pH7.5, 1mM EDTA pH7.5), the paper was shredded by dragging the tube over an Eppendorf tube rack and incubated at 37°C for 1 hour.

The DNA solution was recovered by inverting the tube, making a small hole in the bottom with a red hot needle, and centrifuging the solution for 1 minute through the hole into a second Eppendorf tube. The DNA solution was then centrifuged through a 1 ml plastic pipette tip plugged with polyallomer wool into a third Eppendorf tube to remove any traces of DE81 paper. The DNA solution was then phenol/chloroform extracted, ethanol precipitated and the DNA then resuspended in an appropriate volume of TE buffer. The concentration of DNA was estimated by electrophoresing an aliquot of this solution through an agarose gel against DNA molecular weight markers of a known concentration.

(ii) Recovery of DNA from Low-gelling-temperature Agarose

A horizontal 0.6% (w/v) agarose gel was prepare by dissolving the low-gelling-temperature agarose (FMC Bioproducts) in Tris-Acetate electrophoresis buffer at 70°C. After cooling to 37°C, ethidium bromide was added to a final concentration of 0.5 $\mu g\ ml^{-1}$ and the gel was poured at 4°C. After electrophoresis at a low voltage to prevent heating, the DNA bands were visualized using a long wavelength ultra-violet source. The required DNA fragments were excised from the gel in the minimum amount of agarose and the volume of the gel slices was estimated by weighing on a fine balance assuming that 1 ml of agarose gel weighs 1 g. The volume of the gel slice was increased six-fold with 10 mM Tris HCl pH7.4 such that the low-gelling-temperature agarose represents 0.1% (w/v) of the total volume. The solution containing the gel slice was heated for 5 minutes at 65°C to melt the gel and then equilibrated at 37°C.

This DNA solution containing 0.1% (w/v) agarose may be used as a substrate for DNA ligase in high volume ligation mixtures (Crouse et al, 1983).

This method was used to isolate the 6.1 kb and 7 kb BamHI fragments from partially digested λQ1 DNA.

2-2.8 CONSTRUCTION OF HYBRID PLASMIDS

Fragments of A.nidulans DNA were amplified using plasmid vectors in E.coli strains.

(i) Ligation of DNA into Plasmid Vectors

1 μg of vector DNA was digested to completion with the appropriate restriction endonuclease(s). Protruding 5' termini were dephosphorylated by the addition of 1 unit (manufacturers definition) of calf intestinal alkaline phosphatase (CIP) and incubation at 37°C for 15 minutes. Vector DNA with blunt ends or recessed 5' termini was dephosphorylated by incubation with 1 unit of CIP for 15 minutes at 37°C, followed by 15 minutes at 56°C. Following one phenol/chloroform extraction and ethanol precipitation, the DNA was resuspended in 100 μl of sterile distilled water (final concentration 10 ng/ μl).

DNA ligations were carried out using a 10 x ligase buffer (final concentration of 50 mM Tris.HCl pH7.5; 10mM $MgCl_2$; 1mM ATP), 20 ng of cut vector DNA, samples of purified A.nidulans DNA fragment that are in 0.5, 1 or 2 molar excess of vector DNA and 1 unit (manufacturers definition) of T4 DNA ligase, in a final volume of 10 μl. Vector DNA alone is also religated to monitor the effectiveness of dephosphorylation. Ligation reactions were incubated at 15°C for 12 hours.

(ii) Transformation of E.coli Strains

E.coli strains JA221 and DH5α were transformed with plasmid DNA using a modified version of the method of Kushner (1978).

The host bacterial strain was grown overnight in Luria broth, diluted 1/100 in the same medium and incubated with vigorous aeration until reaching an OD_{550} of 0.2 in logarithmic growth.

1.5 ml samples of cells in Eppendorf tubes were pelleted by centrifugation for 10 seconds in an Eppendorf centrifuge. The cells were resuspended in 500 µl 0.1M MOPS (3-[N-Morpholino] propanesulphonic acid) solution, pH7.0 containing 10 mM RbCl, pelleted by centrifugation for 10 seconds, resuspended in 500 µl 0.1M MOPS solution pH6.5, 10 mM RbCl, 50 mM $CaCl_2$ and held on ice for 90 minutes. The cells were again pelleted and resuspended in 150 µl of the same buffer solution, to which the DNA ligation mixture (see above) and 3 µl of DMSO (Dimethyl Sulphoxide) were added. After a minimum of 30 minutes incubation on ice, the cells were heat shocked for 30 seconds at 55°C and then transferred immediately to an ice-water bath for 2 minutes.

Chilled cells containing hybrid pBR322 or pBR325 vectors were plated directly by spreading on Luria agar plates

containing 50 μg ml^{-1} ampicillin to select transformed cells. Cells containing hybrid pUC19 vectors were spread onto Luria agar plates containing 40 μg ml^{-1} X-gal (20mg ml^{-1} X-gal stock in dimethylformamide). Plates were incubated overnight at 37°C to yield transformed colonies.

This method usually yields approximately 10^6 transformed bacteria per μg supercoiled plasmid DNA.

2-2.9 TRANSFORMATION OF ASPERGILLUS NIDULANS

Sterile cellophane discs, the size of a petri dish base, were placed on the surface of 20 appropriately supplemented complete medium plates and any excess moisture removed from the surface of the cellophane by drying for 30 minutes in a 37°C incubator.

A conidiospore suspension (1-5 x 10^8 conidiospores ml^{-1}) of the appropriate A.nidulans strain was inoculated onto the surface of the cellophane discs as multiple, closely spaced spots using a cotton wool swab and the cultures were incubated for 12 to 14 hours at 37°C.

After incubation the cellophane discs containing young mycelium were divided equally between 4 clean petri dishes each containing a 15 ml solution of the cell wall enzyme Novozym 234 (5 mg ml^{-1}) in an osmotic stabilizer, 0.6 M KCl, and placed at 30°C for one and half hours. The cellophane

discs were then removed from the resulting protoplast suspension and rinsed in 20 ml of 0.6 M KCl in a sterile beaker to remove any adhering protoplasts. The solutions containing protoplasts were filtered through a nylon filter (Gallenkamp GMX-500-V) and a sintered glass filter (porosity 1) to remove cell debris. The protoplasts were pelleted by centrifugation at 1500 x g for 5 minutes and washed three times, twice with 0.6M KCl and once with 0.6M KCl, 50 mM $CaCl_2$. The protoplasts were resuspended in 0.6M KCl, 50 mM $CaCl_2$ at a concentration of 1 - 5 x 10^7 ml^{-1}. Up to 20 μg of DNA in 20 μl TE buffer pH8 was added to 200 μl aliquots of the protoplast suspension in 20 ml screw capped tubes (Sterilin), followed by the addition of 50 μl of 25% PEG (polyethylene gycol) 6000, 50 mM $CaCl_2$, 10 mM Tris.HCl pH7.5. After 20 minutes on ice a further 2 ml of the PEG solution was added and the mixture left at room temperature for 5 minutes. Following the addition of 4 ml 0.6M KCl, 50 mM $CaCl_2$, the total transformation mixture was added to 100 ml of appropriately supplemented molten minimal medium containing 0.6 M KCl and 2% (w/v) agar. This molten agar was poured as a top layer on to ten similarly supplemented minimal medium plates. Aliquots of the transformation mixture may be diluted in 0.6 M KCl, 50 mM $CaCl_2$ prior to addition to the molten agar if a high number of transformants are anticipated.

The efficiency of protoplast regeneration was assessed by

plating 50 µl and 100 µl aliquots of a 10^{-2} and 10^{-3} dilution of the final transformation mixture in complete medium containing 0.6M KCl. The figure obtained was compared to a visual enumeration of the initial protoplast suspension. The number of osmotically resistant particles within the protoplast suspension was estimated by the addition of an aliquot of protoplasts to distilled water which, after 10 minutes at room temperature, were added to complete medium within a molten agar top layer.

All plates were incubated at 37°C for 48 to 72 hours.

2-2.10 PREPARATION OF A.NIDULANS GENOMIC DNA

Genomic DNA was prepared by a modification of the method described by Hawkins et al (1985).

Mycelium of the appropriate A.nidulans strain was grown in liquid culture inoculated with 10^6 conidiospores/ml medium. The mycelium was harvested by filtration, washed in distilled water, rapidly frozen in liquid nitrogen and then powdered in a pestle and mortar under liquid N_2. The powdered mycelium was carefully resuspended in an extraction buffer (10 mM Tris.Cl pH8.0, 1 mM EDTA pH8.0, 4% (w/v) sodium dodecyl sulphate (SDS), 25% (w/v) sucrose) at 20 ml g^{-1} and gently agitated at room temperature for 15 minutes. The solution was extracted with phenol and chloroform, the solvents being separated by centrifugation at 20,000 x g for

10 minutes. The aqueous phase is treated with proteinase K (0.5 mg ml^{-1}) and DNase-free RNase (10 μg ml^{-1}) and then further extracted with phenol and chloroform. After centrifugation at 60,000 x g in an ultracentrifuge to pellet contaminating carbohydrates, the solution was dialysed against TE buffer (4 x 500 ml in 2 hours) and then ethanol precipitated to increase the final concentration of DNA. This method yields high molecular weight genomic DNA (50 kb) which digests efficiently with restriction endonucleases.

2-2.11 ^{32}P - LABELLING OF DNA FRAGMENTS

(i) Nick Translation

The method used was essentially that of Jefferys *et al* (1980).

A sample of 50 to 100 ng of DNA was added to the following reaction mixture:

2.5 μl 10 x nick-translation buffer (500 mM Tris.Cl pH7.5, 50 mM $MgCl_2$, 100 mM 2-mercaptoethanol

2 μl each of 50 μM dGTP, dATP and dTTP

1 μl 8 mg ml^{-1} DNase I (freshly diluted from a 1 mg ml^{-1} stock in 10mM Tris.Cl pH7.5)

1.5 μl $\alpha^{32}P$ - dCTP (10 μCi/ μl 3000 Ci/mmol)

1.5 μl *E.coli* DNA polymerase I (5 units)

H_2O to 25 μl

After incubation for 60 minutes at 15°C, the reaction was stopped by the addition of 25 µl of 0.5% (w/v) SDS, 12.5 mM EDTA, 10 mM Tris.Cl pH7.5. The solution was phenol extracted and the phenol then re-extracted by the addition of 50 µl of 10 mM Tris.Cl pH7.5 and the aqueous phases combined. A 100 µg of high molecular weight salmon sperm DNA was added as a carrier and the DNA precipitated with ethanol, then rinsed in 70% (v/v) ethanol. No chilling or centrifugation was required due to the high concentration of DNA. The precipitated DNA was redissolved in 200 µl of Tris.Cl pH7.5 and a second ethanol precipitation repeated as above. The final DNA precipitate was redissolved in 500 µl of 10 mM Tris.Cl pH7.5.

High molecular weight salmon sperm DNA was prepared as follows: 200 mg of salmon sperm DNA was dissolved in 200 ml of 10 mM Tris.Cl pH7.5 overnight at 4°C. The DNA solution was then phenol/chloroform extracted, ethanol precipitated, dried under vacuum and redissolved in 70 ml of 10 mM Tris.Cl pH7.5. The DNA concentration was calculated from the optical density at 260nm.

The DNA probes used to analyze the genomic DNA of <u>QUTE</u> transformed strains was labelled with $\alpha^{32}P$ - dCTP by this method.

(ii) Oligo-labelling of DNA fragments by hexadeoxynucleotide primers

The labelling reaction was carried out at room temperature by the addition of the following reagents in the stated order:

X µl H_2O to a total volume of 25 µl
5 µl OLB Buffer
1 µl BSA (10 mg/ml)
X µl DNA fragment (25 mg) up to 16.25 µl
2.5 µl $\alpha^{32}P$ - dCTP (10 µCi/ µl)
0.5 µl Klenow (large fragment) Polymerase I (2 units)

The reaction mixture was incubated at room temperature for at least five hours. The reaction was then stopped by the addition of 100 µl stop buffer (Feinberg and Vogelstein, 1984).

Solutions

OLB Buffer

The Solutions A:B:C were mixed in the ratio 10:25:15 to give a total volume of 50 µl, which is sufficient for 10 labelling reactions.

Solution A

100 µl 1.25 M Tris Cl, 0.125 M $MgCl_2$, pH8

18 µl 2-mercaptoethanol

5 µl each of 0.1 M dATP,0.1 M dGTP,0.1 M dCTP

Solution B

2M HEPES pH6.6

Solution C

Hexadeoxynucleotides 90 OD units ml^{-1} in 3 mM Tris.Cl, 0.2 mM EDTA, pH7.0 (Pharmacia P-L)

Stop Buffer

20 mM NaCl, 20 mM Tris.Cl pH7.5, 2 mM EDTA, 0.25% (w/v) SDS, 1 µM dCTP

Measurement of $\alpha^{32}P$ - dCTP incorporation

The efficiency of $\alpha^{32}P$ - dCTP incorporation was determined by TCA (Trichloroacetic acid) precipitation. A 1.25 µl aliquot of the reaction mixture was mixed with 500 µl salmon sperm DNA (50 µg ml^{-1}) followed by the addition of 150 µl 50% TCA. The precipitate was collected by filtration of the solution through a 2.5 cm GF/C glass-fibre disc. The disc was washed with 50 ml of ice-cold 5% TCA and 10 ml of

ethanol, then dried at 60°C for 10 minutes. A second disc was prepared by pipetting a 1.25 µl aliquot of the reaction mixture onto the disc. Both discs were then placed in scintillation vials with 5 ml of the toluene-based scintillation fluid Fisofluor. The number of radioactive counts per minute were measured in a scintillation counter.

The first disc indicates the number of counts incorporated into the nucleic acid and the second disc, the total number of counts in the reaction mixture.

Removal of unincorporated nucleotides

The tip of a siliconized Pasteur pipette was plugged with polyallomer wool and carefully packed with Sephadex G50 suspended in TE buffer to within 5-10 mm of the top. After washing through with 2 ml TE buffer, the column was allowed to drain. The reaction mixture was loaded onto the column. A 100 µl of TE buffer was then added and a 100 µl fraction from the column collected in an Eppendorf tube. This was repeated up to 16 times. A µl aliquot of each 100 µl fraction was pipetted onto GF/C glass fibre discs and allowed to dry. Each glass fibre disc was placed in scintillation fluid in a scintillation vial and counted in a scintillation counter to determine which fraction(s) contained the labelled DNA.

2-2.12 SOUTHERN BLOT FILTER HYBRIDIZATION

(i) The Transfer of DNA onto Nitrocellulose or Hybond Filters

DNA was transferred to 0.45 μ nitrocellulose or Hybond-N nylon filters as described by Maniatis *et al*, (1982). Genomic DNA samples of the *QUTE* transformants were transferred to 0.45 μ nitrocellulose while those of the *QUTD* and *QUTA* transformants were transferred to Hybond-N nylon filters (Amersham).

The DNA samples were separated by electrophoresis and unused areas of the gel were removed. The DNA was denatured by immersing the gel in 1.5 M NaCl, 0.5 M NaOH for 1 hour at room temperature with gentle shaking. This step was then repeated with an equal volume of 1M Tris.Cl pH8.0, 1.5M NaCl, to neutralize the gel. The gel was inverted and laid on top of a glass plate, which was covered in 3MM paper soaked with 10 x SSC, taking care to avoid trapping air bubbles between the gel and the paper. The plate was suspended 2-3 cm above a tray filled with 10 x SSC, such that only the ends of the 3 MM paper were immersed in the liquid. The 0.45 μ nitrocellulose or Hybond-N nylon filter and two pieces of 3 MM paper, cut to a size slightly greater than that of the gel, were soaked in 2 x SSC. The filter followed by the two sheets of 3 MM paper were laid on top of the gel and any air bubbles removed by rolling a glass rod

over the surface. A stack of paper towels was placed over the 3 MM paper, covered with a glass plate and compressed with a 500 g weight. DNA transfer was allowed to proceed for 16 to 18 hours, with the paper towels being replaced at intervals. After this period the towels and 3 MM paper were removed. The dehydrated gel and filter were turned over and placed, gel side up, on a dry sheet of 3 MM paper. The positions of the gel slots were marked on the filter with a ball-point pen. The gel was discarded and the filter soaked in 6 x SSC at room temperature for 5 minutes. The filter was then dried at room temperature on 3 MM paper. The DNA was bound to the nitrocellulose filter by baking at 80°C for four hours. Hybond-N nylon filters were wrapped in Saran cling film and placed on a short wavelength ultra-violet transilluminator (Ultra-violet Products Inc, California, USA) for five minutes to bind the DNA to the filter.

(ii) Preparation of Genomic DNA "Dot Blots"

An equal amount of genomic DNA in distilled water from each of the A.nidulans transformants and control strains was added to an equivalent amount of 2 x Denaturation solution (2M NaCl, 0.2M NaOH, 20 mM EDTA) in an Eppendorf tube and heated for 5 minutes in a boiling water bath. The DNA solutions were rapidly cooled in an ice-water bath and centrifuged briefly in a microfuge, before being two-fold serially diluted in 1 x Denaturation solution. A piece of 0.45 μ nitrocellulose or HybondN nylon filter was placed on

impervious paper and an aliquot of each dilution was pipetted onto the filter to form "dots" of approximately equal size. After the "dots" had dried at room temperature, the filter was rinsed in 6 x SSC, dried and the DNA bound to the filter as described in the previous section.

(iii) Hybridization with $\alpha^{32}P$ - labelled DNA Probe

a) Analysis of the A.nidulans QUTE transformed strains

The conditions used were those commonly practised in our department at this time (Jeffreys et al, 1980).

The filters were cut into convenient strips and pre-hybridized with gentle agitation at 65°C in hybridization boxes made of perspex, as follows:

- one wash in 3 x SSC for 30 minutes
- one wash in 1 x Denhardts for 30 minutes
- one wash in filter hybridization mixture (FHM) for one hour
- one wash in FHM plus 6% (w/v) PEG 8000 (Polyethylene Glycol - Sigma, grade 8000)

The final hybridization solution contained the ^{32}P - labelled DNA which had been denatured by heating at 100°C for five minutes prior to addition to the hybridization solution. Hybridization was allowed to proceed overnight.

Unbound ^{32}P - labelled DNA was removed from the filters by repeated washing in filter hybridization solution (15 minutes at 65°C each time) until no detectable ^{32}P could be measured in the wash solution. The stringency of the hybridization and wash solutions was always 1 x SSC, 0.1 % SDS. The filters were rinsed in 3 x SSC and dried at room temperature. The strips of filter were then reassembled.

Solutions

All solutions were de-gased under vacuum before use.

10 x Denhardts Solution

10 g Bovine serum albumin (Sigma Fraction V)

10 g Polyvinylpyrrolidone

10 g Ficoll 400

Made up to 500 ml with H_2O

1 x Filter Hybridization Mixture

1 x Denhardts

50 µg/ml alkali denatured salmon sperm DNA

0.1% SDS

1 x SSC

b) Analysis of the A.nidulans QUTD and QUTA transformed strains

The following method utilizes polyethylene glycol in the hybridization solution (Amasino, 1986) The strips of Hybon-N nylon filter in hybridization boxes were pre-hybridized at 65°C for at least one hour in the following solution:

3 x SSC

5 x Denhardts solution

200 µg/ml alkali denatured salmon sperm DNA

0.1% SDS

6% PEG 8000

Hybridization was allowed to proceed overnight at 65°C in the following solution:

3 x SSC

2 x Denhardts solution

200 µg/ml alkali denatured salmon sperm DNA

0.1% SDS

6% PEG 8000

α ^{32}P - dCTP labelled DNA

The filters were then washed at 65°C as follows:

- 4 brief rinses in 3 x SSC, 0.1% SDS
- 4 x 30 minute washes in 3 x SSC, 0.1% SDS
- 2 x 30 minute washes in 0.5 x SSC, 0.1% SDS

Excess liquid was removed from the filters by blotting with 3 MM paper and the damp Hybon-N filters were reassembled and wrapped in cling film to prevent them drying out completely.

The ^{32}P - labelled DNA may be stripped off the Hybon-N filter to allow re-use by heating at 45°C for 30 minutes in 0.4M NaOH, followed by a further 30 minutes at 45°C in 0.1 x SSC, 0.1% SDS, 0.2M Tris.Cl pH7.5. The filters can then be stored at 4°C until required.

Solutions

10 x Denhardts Solution

As above

20 x SSC

175.3 g of NaCl and 88.2 g sodium citrate in 1 litre of distilled water, adjusted to pH7.0 with a few drops of 10 N NaOH.

Alkali denatured salmon sperm DNA (10 mg/ml)

1 g of salmon sperm DNA (Sigma Type III) was added to 500 ml of 20 mM EDTA pH8.0. The DNA was dissolved by heating the solution to 100°C, followed by the addition of 15 ml of 10 M NaOH and incubation for a further 20 minutes to denature the DNA. The solution was cooled on ice, then 20 ml of 1 M Tris.Cl pH7.5 was added and the pH adjusted to 7-8 with concentrated HCl, added in small volumes with rapid mixing. The denatured DNA solution was phenol/chloroform extracted, precipitated with IMS (Industrial Methylated Spirit) and rinsed with 70% (v/v) ethanol. The DNA was initially dissolved in 50 ml water, the concentration was then determined from the optical density at 260 nm and the volume adjusted to a final DNA concentration of 10 mg/ml.

(iv) Autoradiography

The nitrocellulose or Hybon-N filter strips, which had been reassembled in their original order, were covered with "cling" film and placed in a Kodak cassette against Fuji RX X-ray film. If autoradiography required prolonged periods of exposure, the filter and film were placed between intensifying screens within the cassette and left at -70°C. Exposure times varied from 3 hours to 7 days.

2-2.13 SOURCE OF MATERIALS

All reagents and chemicals were of analar or greater purity and supplied by Fisons plc, BDH Ltd and Sigma Chemical Co Ltd, UK.

Chemicals obtained from other sources are listed below:

GIBCO BRL LIMITED, SCOTLAND

- Restriction endonucleases
- T4 DNA ligase
- DNA polymerase I
- DNase I
- Deoxyribonucleoside triphosphates

AMERSHAM INTERNATIONAL PLC, ENGLAND

- Radionucleotides

ANGLIAN BIOTECHNOLOGY

- 5-bromo-4-chloro-3-indolyl- -D-galactopyranoside (X-gal)

BOEHRINGER

- Calf intestinal phosphatase

PHARMACIA LIMITED, ENGLAND

- DNA polymerase I (large fragment, Klenow enzyme)

FMC BIOPRODUCTS, USA

Agarose

NOVO BIOLAB, DENMARK

Novozym 234

CHAPTER 3

THE CONSTRUCTION OF RECIPIENT STRAINS FOR TRANSFORMATION EXPERIMENTS

INTRODUCTION

The aim of the experimental work described in the following chapters has been to confirm the presence and locate the positions of the QUTE, QUTD and QUTA genes of Aspergillus nidulans on the recombinant bacteriophage λQ1 DNA by transformation of the appropriate qut mutant strains.

In order to monitor the efficiency of the transformation system it was considered desirable to use a known genetic marker as a positive control. This would show that negative results obtained in the transformation of qut mutant strains were not due to technical problems in the protocol for transformation. For historical reasons the Neurospora crassa pyr4 gene, which encodes orotidine 5' phosphate decarboxylase, was selected (Buxton and Radford, 1983). The plasmid pDJB1, which contains the pyr4 gene in pBR325, will transform A.nidulans pyrG^{-} strains at a frequency of 50-150 transformed colonies / μg plasmid DNA (Ballance and Turner, 1985).

In order to use the N.crassa gene as a marker in

Table 3.1

Characteristics of certain quinate non-utilising (qut) mutant strains of A.nidulans

MUTANT ALLELE	PHENOTYPE	DOMINANCE TO WILD TYPE ALLELE IN DIPLOID STRAIN	PROPOSED GENE PRODUCT
qutB42	loss of quinate dehydrogenase activity (1)	recessive	quinate dehydrogenase
qutC113	loss of 3-dehydroshikimate activity (3)	recessive	3-dehydroshikimate dehydrogenase
qutE208	loss of catabolic dehydroquinase activity (2)	recessive	catabolic dehydroquinase
qutD8	pleiotropic non-inducible	recessive	activator protein *
qutA4	pleiotropic non-inducible	semi-dominant	repressor protein *

* Hypothesis at the start of this Thesis work

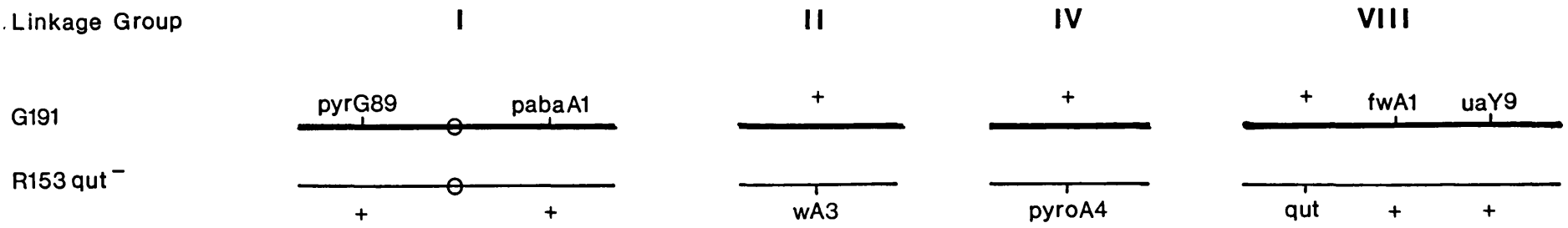
Figure 3.1

A. A chromosomal map of the *A.nidulans* *pyrG*$^-$ strain, G191, and the R153 *gut*$^-$ strains to show the location of the markers used in the crosses to obtain *pyrG*$^-$ *gut*$^-$ double mutants as *A.nidulans* recipient strains for transformation.

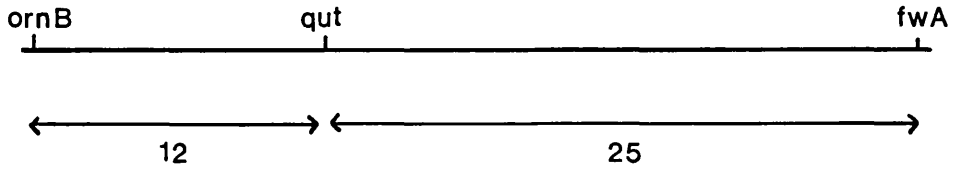
B. A linkage map showing the location of the *gut* gene cluster within linkage group VIII. The *fwA* and *gut* gene loci are linked within chromosome VIII, having an average recombination frequency of 25%. This observation combined with data on the linkage of the *gutE208* and *ornB7* alleles (Da Silva , 1985) suggests that the *gut* genes are located between the *fwA* and *ornB* gene loci on chromosome VIII.

FIGURE 3.1

A


Linkage Group
I
II
IV
VIII
G191
pyrG89
pabaA1
+
+
+
fwA1
uaY9
R153 qut⁻
+
+
wA3
pyroA4
qut
+
+

B


ornB
qut
fwA
12
25

transformation experiments it was necessary to construct some *pyr*,*qut* double mutant strains of *A.nidulans* which require uridine and are unable to utilize quinic acid as a carbon source for growth. Crosses were carried out between the *pyrG*$^{-}$ strain G191 and five R153 strains (*wA3*; *pyroA4*) each of which carry one of the mutations, *qutB42*, *qutC113*, *qutE208*, *qutD8* and *qutA4*, whose characteristics are shown in Table 3.1, and genotypes in Figure 3.1A.

The technique of mitotic haploidisation has been used to locate the *qutC* gene to linkage group VIII. Analysis of crosses between various *qut* strains has shown that the *qut* genes are closely linked with a recombination frequency of no greater than 1% suggesting that these genes are clustered on chromosome VIII (Hawkins *et al*, 1982). The further analysis of crosses has demonstrated that the *qutE* gene is linked to *ornB* within linkage group VIII and the recombination frequency between these two gene loci is approximately 12.5% (Da Silva, 1985). The *fwA* allele, present in the *pyrG*$^{-}$ strain G191, is 25 units from *ornB* on linkage group VIII in the standard map (Clutterbuck, 1982), therefore it is expected that measurement of the recombination frequency between *fwA* and the *qut* gene loci will map the *qut* gene cluster with respect to *fwA* and *ornB*.

Crosses between strain G191 (*pyrG89* *pabaA1*; *fwA1*) and representative *qut* mutant strains in the R153 genotype (shown in Table 3.1) were analysed. The phenotypes of the

TABLE 3.2: ASSORTMENT OF THE fwA AND qut MARKERS

GENOTYPES		CROSSES					TOTALS
		G191 x qutB42	G191 x qutE208	G191 x qutA4	G191 x qutC113	G191 x qutD8	
P	fw +	29	58	15	23	24	62
P	+ qut	13	21	17	18	21	56
R	+ +	8	4	7	7	8	22
R	fw qut	3	5	9	4	4	17
TOTALS		53	88	48	52	57	157
RECOMBINATION FREQUENCY (%)		21	10.2	33	21	21	24.8
SEGREGATION							
fw/+		32/21	63/25	24/24	27/25	28/29	79/78
qut/+ (1)		16/37	26/62	26/22	22/30	25/32	73/84
qut/+ (2)		32/68	54/90	57/43	52/48	48/52	157/143

(1) fw progeny
(2) all progeny

progeny obtained in each cross were determined by growth on the appropriate test media. No tests were done for the presence of the uaY9 marker since this is difficult to score.

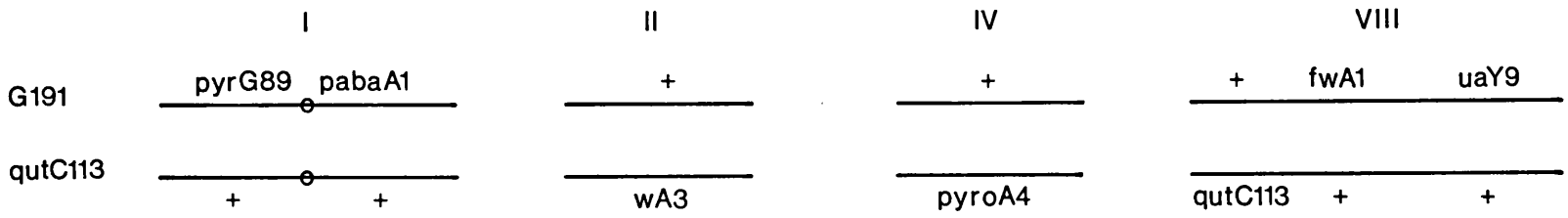
3-1 LINKAGE BETWEEN THE fwA AND gut GENES

Progeny from each cross were classified for assortment of the fwA and gut markers and the degree of linkage between these gene loci calculated. As it is not possible to determine which fwA allele is present in wA3 (white) progeny, only those which produce fawn or green conidiospores were scored. The results are shown in Table 3.2.

In two of the crosses, G191 x gutB42 and G191 x gutE208 the fwA and gut markers did not segregate equally in the progeny scored. This may be due to a bias in the type of progeny tested and inexperience in scoring the gut phenotype, particularly for gutB42 mutants which have a leaky phenotype. Estimates of the recombination frequency between the fwA and gut genes are therefore discounted for these two crosses.

For the remaining three crosses the segregation of the fwA and gut markers is, in each case, 1:1 as expected. The average recombination frequency between these genes is 25% and thus the fwA and gut gene loci are linked within

TABLE 3.3 : THE ASSORTMENT OF MARKERS IN THE CROSS G191 x qutC113



GENOTYPE	NUMBER OF PROGENY IN THE CROSS G191 x qutC113 HAVING THE FOLLOWING COMBINATION OF MARKERS													
	pyrG / pabaA	pyrG / wA	pyrG / pyroA	pyrG / qutC	pyrG / fwA	pabaA / wA	pabaA / pyroA	pabaA / qutC	pabaA / fwA	wA / pyroA	wA / qutC	pyroA / qutC	pyroA / fwA	qutC / fwA
+ / -	19	24	30	32	18	26	29	28	13	24	23	20	14	23
- / +	17	18	19	21	9	22	20	19	8	19	18	20	11	18
+ / +	39	34	28	26	16	30	27	28	17	28	29	27	14	7
- / -	25	24	23	21	9	22	24	25	14	29	30	33	13	4
RECOMBINATION FREQUENCY (%)	36	58	51	47	52	52	51	53	40	43	41	40	52	21
ρ (χ^2 TEST)	0.01	0.1	0.5	0.5	0.1	0.5	0.5	0.5	0.5	0.5	0.5	0.1	0.9	<0.005

chromosome VIII. This observation combined with the data on the linkage of the qutE208 and ornB7 alleles (Da Silva, 1985) suggests that the qut genes are located between the fwA and ornB gene loci on chromosome VIII (Figure 3.1B).

3-2 THE ANALYSIS OF ONE CROSS TO TEST THE INDEPENDENT ASSORTMENT OF GENE LOCI

The progeny of one cross, G191 x R153 qutC113, were classified for assortment of all available markers to test for the independent assortment of gene loci. The results are shown in Table 3.3.

Gene loci exhibiting independent assortment have a recombination frequency of 50%. All the markers located on different linkage groups show, as expected, independent assortment and their recombination frequencies are approximately 50% (within the limits of the X^2 test). The pyrG89 and pabaA1 markers in linkage group I have a recombination frequency of 36% ($p = 0.01$) demonstrating weak linkage on chromosome I. As previously shown, the qutC113 and fwA1 markers are linked on chromosome VIII.

SAMPLE CALCULATIONS OF X^2

X^2 = total of $\frac{(O - E)^2}{E}$ over all classes.

1. pyrG89/pabaA1

O = observed number in any class
E = expected number in any class

CLASS	O	E	$(O - E)^2$	$\frac{(O - E)^2}{E}$
$pyrG^+/pabaA^+$	39	25	196	7.84
$pyrG^-/pabaA^-$	25	25	0	0
$pyrG^+/pabaA^-$	19	25	36	1.44
$pyrG^-/pabaA^+$	17	25	64	2.56
	100	100		X^2 = 11.84
				p = 0.01

The pyrG89 and pabaA1 markers are weakly linked on chromosome I.

2. pyrG89/wA3

CLASS	O	E	$(O - E)^2$	$\frac{(O - E)^2}{E}$
$pyrG^+/wA^-$	24	25	1	0.04
$pyrG^-/wA^+$	18	25	49	1.96
$pyrG^+/wA^+$	34	25	81	3.24
$pyrG^-/wA^-$	24	25	1	0.04
	100	100		X^2 = 5.28
				p = 0.10

The pyrG89 and wA3 markers are, as expected, unlinked.

3. qutC113/fwA1

CLASS	O	E	$(O - E)^2$	$\frac{(O - E)^2}{E}$
$qutC^+/fwA^-$	23	13	100	7.69
$qutC^-/fwA^+$	18	13	25	1.92
$qutC^+/fwA^+$	7	13	36	2.77
$qutC^-/fwA^-$	4	13	81	6.23
	52	52		X^2 = 18.61
				p = <0.005

The qutC113 and fwA1 markers are linked with chromosome VIII.

Table 3.4

The A. nidulans pyrG⁻ qut⁻ strains selected for use in transformation experiments

CROSS	STRAIN NUMBER	GENOTYPE
G191	GA 28	pyrG89 pabaA1; wA3; qutA4
X	80	pyrG89 pabaA1 wA3; pyroA4; qutA4
R153 qutA4	87	pyrG89; wA3; pyroA4; qutA4
G191	GB 21	pyrG89 pabaA1; qutB42
X	81	pyrG89 pabaA1; qutB42
R153qutB42	96	pyrG89 pabaA1; pyroA4; qutB42
G191	GC 6	pyrG89 pabaA1; qutC113
X	50	pyrG89 pabaA1; pyroA4; qutC113
R513 qutC113	83	pyrG89 pabaA1; pyroA4; qutC113
G191	GC 22	pyrG89 pabA1; wA3; pyroA4; qutD8
X	96	pyrG89 pabaA1; wA3; qutD8
R153 qutD8	98	pyrG89 pabaA1; wA3; qutD8
G191	WA 53	pyrG89 pyroA4; qutE208
X		
R153 qutE208		

3-3 THE ISOLATION OF PARTICULAR *pyrG*$^-$ *qut*$^-$ RECOMBINANTS AS RECIPIENT STRAINS FOR TRANSFORMATION

Particular factors were taken into consideration when strains obtained as recombinants from these crosses were chosen for use in transformation experiments with the cloned *QUT* genes.

Since the plasmid pDJB1, which carries the *N.crassa* *pyr4* gene (Ballance and Turner, 1985), was to be used as a positive control for transformation efficiency, all strains are required to be *pyrG*$^-$ *qut*$^-$ double mutants. It was also desirable to pick strains with different combinations of the two nutritional markers, *pabaA* and *pyroA*, to other *A.nidulans* strains already being used within our laboratory. The preferred conidiospore colour was white or green as the *pyrG*, *fwA*, *qut*$^+$ strain G191 was already in use.

The *pyrG*$^-$ *qut*$^-$ recombinants isolated for use are shown in Table 3.4. It was noted that the *pyrG* mutation produces a slower rate of growth than in the equivalent wild-type (*pyrG*$^+$) strains.

DISCUSSION

A number of *pyrG*$^-$ *qut*$^-$ double mutant strains have been constructed for use in transformation experiments and

linkage between the *fwA* and *qut* gene loci on chromosome VIII has been determined.

The *ornB* and *fwA* markers are shown to be 25 units apart in standard linkage maps of *A.nidulans* (Clutterbuck, 1982) and the recombination frequency between *ornB7* and *qutE208* markers is 12% (Da Silva, 1985). As the average recombination frequency between the *fwA* and *qut* gene loci is 25% it is probable that the *qut* gene cluster is located between the *ornB* and *fwA* gene loci on chromosome VIII (Figure 3.1B).

It is recognised that the *qutA4* mutant strain may not be suitable for use in transformation experiments because this allele is semi-dominant to wild-type in heterozygous diploid strains (Hawkins *et al*, 1982); however at the time that these crosses were undertaken no other mutant alleles were available at the *qutA* locus. At a later date further alleles were isolated that were shown to be fully recessive in heterozygous diploids (Grant *et al*, 1988). One of these, *qutA361*, was selected for use in transformation experiments, but there was insufficient time to cross this mutant strain with the *pyrG*$^{-}$ strain and hence in these experiments the *N.crassa pyr4* gene could not be used as a positive control for transformation efficiency.

The following mutant strains were used in transformation experiments:

WA53	(pyrG89; pyroA4; qutE208)
GD22	(pyrG89 pabaA1; wA3; pyroA4; qutD8)
R153 qutA361	(wA3; pyroA4; qutA361)

The strains GB and GC (Table 3.4) were supplied to Mrs H Lamb at the University of Newcastle upon Tyne for use in transformation experiments to identify and locate the QUTB and QUTC genes on λQ1 DNA.

CHAPTER 4

IDENTIFICATION OF THE CLONED *QUTE* GENE BY TRANSFORMATION OF A *qutE* MUTANT STRAIN OF *A.NIDULANS*

INTRODUCTION

The position of the enzyme structural genes of the *A.nidulans* *QUT* gene cluster within the recombinant bacteriophage λQ1 was determined by using the corresponding *qa* genes of *Neurospora crassa* as heterologous DNA probes (Hawkins *et al*, 1985). The genes are apparently in the same relative order in *A.nidulans* as in *N.crassa* although they are contained within a smaller length of DNA sequence in *Aspergillus* compared to *Neurospora* (Figure 1.5).

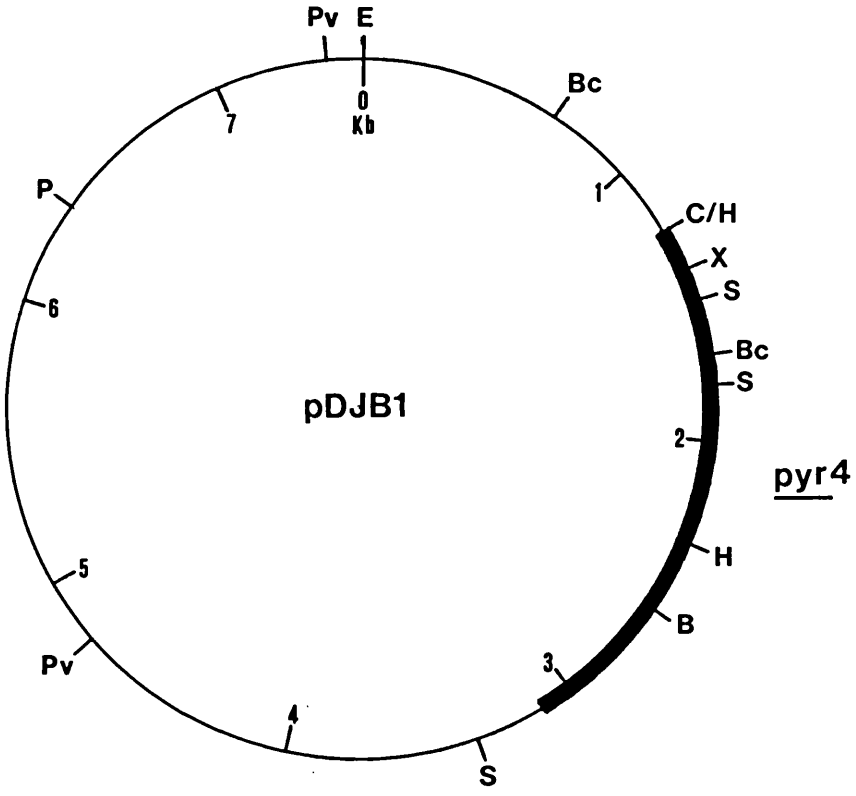
At the start of my work only one of the cloned enzyme structural genes had been tested for its functional identity and integrity. This was the *QUTE* gene, encoding the enzyme catabolic dehydroquinase. A 3.3kb fragment from λQ1, containing DNA sequence homologous to the *qa-2* gene, was introduced into the *Bam*HI site of the vector pBR322. This plasmid was shown to weakly complement an auxotrophic mutant of *aroD6* in *Escherichia coli*, which encodes biosynthetic dehydroquinase, when tested for growth at 30°C, though the *N.crassa* *qa-2* gene complements well at this temperature (Hawkins *et al*, 1985). It has been found

Figure 4.1

A restriction map of the plasmid pDJB1

A restriction map of the plasmid pDJB1 which contains the *N.crassa* *pyr4* gene, encoding orotidine 5' phosphate decarboxylase, subcloned into the bacterial vector pBR325.

FIGURE 4.1



———	pBR325
▬▬	N.crassa DNA
B	BamH I
Bc	Bcl I
C	Cla I
E	EcoR I
H	Hind III
P	Pst I
Pv	Pvu II
S	Sal I
X	Xho I

that the 5' non-coding region of the N.crassa qa-2 gene contains a sequence, 11 bp upstream of the translation initiation codon, which has homology to the canonical sequence for the E.coli ribosome binding site (Shine and Dalgarno, 1975; Alton et al, 1982). This sequence may facilitate expression of the N.crassa qa-2 gene in E.coli and its absence from the 5' non-coding region of the A.nidulans QUTE gene may be responsible for the genes' low efficiency of expression (Hawkins et al, 1985).

The location and functional integrity of the QUTE gene has been determined by transformation of a quinic acid non-utilising qutE mutant of A.nidulans and is reported in this chapter. Subsequent DNA sequence analysis has unambiguously confirmed this result (Da Silva et al, 1986). The expression of multiple copies of the QUTE gene in transformants has also been studied.

4-1 TRANSFORMATION OF A qutE MUTANT OF A.NIDULANS WITH THE QUTE GENE

The strain WA53 (pyroA4; pyrG89; qutE208) was used as a recipient in transformation experiments. This recipient strain allowed the plasmid pDJB1, which carries the pyr4 gene of N.crassa in pBR325, to be used as a positive control for the efficiency of transformation (Ballance and Turner, 1985; Figure 4.1). This plasmid will transform a uridine auxotrophic strain of A.nidulans, G191, at a

frequency of 50-150 transformants / μg plasmid DNA, but "abortive" transformants occur at 100 times the frequency of stable transformants (Ballance and Turner, 1985).

The method for the preparation of A.nidulans protoplasts and their subsequent transformation used for the experiments described within the results chapters was that devised by Ballance and Turner (1985). The standard procedure is outlined here and described in detail in the Materials and Methods.

Mycelium from the appropriate A.nidulans strain was grown from a standard spore inoculum on discs of cellophane placed on the surface of complete culture medium at 37°C for 12-14 hours. Protoplasts were prepared from this mycelium by incubation with the cell wall enzyme Novozym 234 (5mg/ml) for 2 hours at 30°C in the presence of the osmotic stabilizer 0.6M KCl. After filtration through a nylon filter (Gallenkamp GMX-500-V) and sintered glass filter (porosity 1) to remove mycelial debris, the protoplasts were centrifuged at 1500 xg for 5 minutes and washed three times, twice with 0.6M KCl and once with 0.6M KCl, 50mM $CaCl_2$. The protoplasts are resuspended in 0.6M KCl, 50mM $CaCl_2$ at a concentration of 1-5 x 10^7/ml. Up to 20 μg of DNA in 20 μl of TE pH8.0 was added to 200 μl aliquots of the protoplasts in 20ml screw scrapped tubes, followed by the addition of 50 μl of 25% PEG 6000, 50mM $CaCl_2$, 10mM Tns.Cl pH 7.5. After 20 minutes on ice a further 2ml of the PEG solution was

added and the mixture left at room temperature for 5 minutes. After the addition of 4ml of 0.6M KCl, 50mM $CaCl_2$, the total transformation mixture was added to 100ml of appropriately supplemented molten minimal medium containing 0.6M KCl and 2% (w/v) agar. This molten agar was poured as a top layer onto ten similarly supplemented minimal medium plates.

The efficiency of protoplast regeneration was assessed by plating 50 μl and 100 μl aliquots of a 10^{-2} and 10^{-3} dilution of the final transformation mixture in complete medium containing KCl. The figure obtained was compared to a visual enumeration of the initial protoplast suspension. The number of osmotically resistant particles within the protoplast suspension was estimated by the addition of an aliquot of protoplasts to distilled water which, after 10 minutes at room temperature, were added to plates of complete medium within a molten top agar layer. The protoplast regeneration frequency varied between 10-40% within which 1-2% of colonies obtained were derived from osmotically resistant material. All plates were incubated at 37°C for 48-72 hours. Any departure from this standard method will be noted when appropriate.

In order to be assured that colonies from a transformation experiment were unlikely to be the result of reversion of the original mutation, in all experiments protoplasts were either exposed to an equivalent amount of vector DNA alone

(pBR325 or pBR322) or alternatively no DNA was added to an aliquot of the protoplast suspension.

Colonies obtained upon selective medium were picked and purified by growth of a high dilution of conidiospores yielding single colonies on supplemented minimal medium plates containing quinate (pH 6.5) as a carbon source. Only those colonies that were conidiating and grew vigorously upon transfer to new medium were scored to measure the transformation frequency. Most *qut*$^-$ *A.nidulans* strains exhibit slight "leaky" growth visible as a low level growth of mycelial filaments. This may be due to the metabolism of small amounts of alternative carbon sources present in agar plates (Payton *et al*, 1976). Colonies producing conidiospores are clearly visible against this low background growth though any "abortive" transformants that might occur may be masked by the low level growth of mycelium.

The plasmid pEH1 was used in the initial transformation of strain WA53 (Da Silva, 1985; Figure 4.2).

An aliquot containing 10^4 viable protoplasts/ml was transformed with 15 μg of pDJB1 as a positive control for the efficiency of transformation and plated onto minimal medium lacking uracil. Independently, further aliquots were transformed with 15 μg of pEH1 and 15 μg of pBR322, which is a negative control for reversion of the *qutE* mutation, and

Table 4.1

Transformation of the qutE mutant strain with pEH1

EXPERIMENT NUMBER	PLASMID USED	AMOUNT OF DNA(μg)	TOTAL NUMBER OF COLONIES	TRANSFORMATION FREQUENCY
1	pEH1	15	8 [a]	0.5
	pDJB1	15	66 [b]	4.5
	pBR322	15	0 [a]	0
2	pEH1	15	31 [a]	2
	pDJB1	15	87 [b]	6
	pBR322	15	0 [a]	0
3	pEH1	15	92 [a]	6
	pDJB1	15	123 [b]	8
	pBR322	15	0 [a]	0

(a) *qut*$^+$ selection (b) *pyr*$^+$ selection

The transformation frequency is expressed as the number of colonies/μg transforming DNA. The number of protoplasts/ml of the protoplast suspension and their percentage regeneration frequency in the above experiments was as follows - Experiment 1 : 2.3 x 10^7, 31%, Experiment 2 : 2.6 x 10^7/ml, 27%; Experiment 3 : 1.8 x 10^7/ml, 27%

then plated onto minimal medium containing quinate (pH6.5) as a sole carbon source and supplemented with 20mM uracil. Plates were incubated at 37°C for 48-92 hours. This experiment was repeated three times and the resulting frequencies of transformation and protoplast regeneration are listed in Table 4.1.

Transformants, recovered as strongly growing colonies, were obtained with plasmids pEH1 and pDJB1. The transformation frequency of WA53 by pDJB1 was 8 transformants/ μg plasmid DNA. The colonies observed had a "ragged" appearance but only those that were conidiating and showed continued growth upon transfer were counted. Plasmid pEH1 transformed strain WA53 at a frequency of 6 transformants/ μg plasmid DNA and the colonies obtained were clearly visible against the low background growth of mycelium. Protoplasts that were exposed to pBR322 DNA in the transformation experiments did not produce any colonies on the selective medium. The background growth of strain WA53 was clearly visible, indicating that the colonies obtained with plasmid pEH1 were unlikely to be the result of reversion of the qutE mutation. These results show that the entire functional QUTE gene is present in the DNA sequences contained within the 6.5 kb HindIII fragment of pEH1.

In order to further define the location of the QUTE gene, a smaller 3.3 kb BamHI restriction fragment was isolated from the plasmid pEH1 by restriction endonuclease digestion

Figure 4.2

Restriction maps of plasmids pEH1, pAL3.3A and pAL3.3B

The plasmid pEH1 contains 6.5 kb of A.nidulans genomic DNA isolated as a HindIII restriction fragment from the recombinant clone λQ1 and subcloned into the HindIII restriction endonuclease site of bacterial vector pBR322 (Da Silva, 1985) The 6.5 kb of A.nidulans DNA is considered to contain the QUTB, QUTC and QUTE genes which are homologous to the qa-3, qa-4 and qa-2 genes of N.crassa respectively.

The plasmids pAL3.3A and pAL3.3B contain a 3.3 kb BamHI fragment of A.nidulans DNA, isolated from the plasmid pEH1, subcloned into the BamHI site of the bacterial vector pBR325. Plasmids pAL3.3A and pAL3.3B differ by the orientation of the 3.3 kb BamHI fragment relative to the pBR325 vector sequences. The 3.3 kb fragment of A.nidulans DNA is considered to contain the QUTC and QUTE genes but only part of the QUTB gene. The QUTC and QUTE genes are homologous to the N.crassa qa-4 and qa-2 genes respectively.

FIGURE 4.2

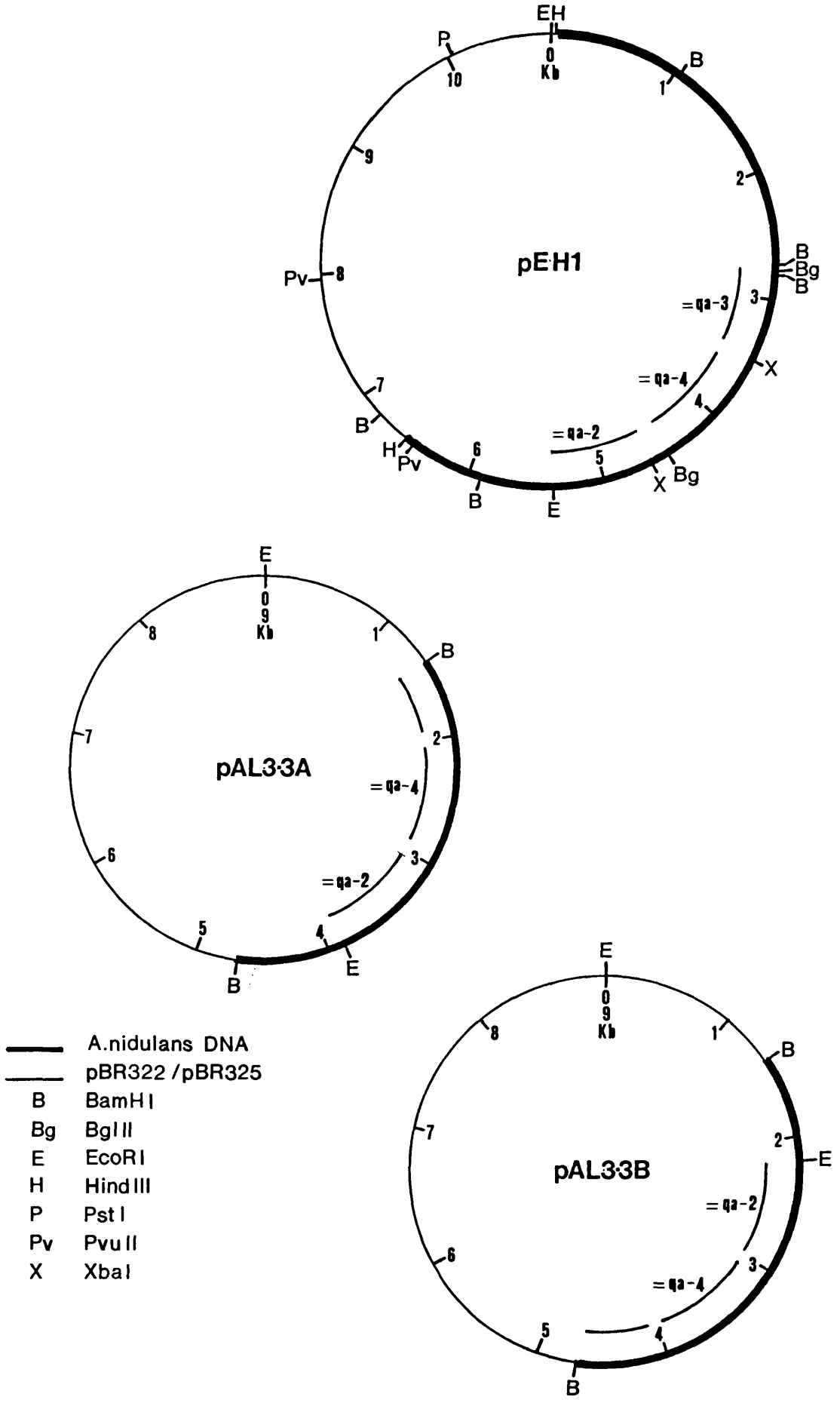

EH
0
Kb
P
10
B
1
2
9
B
Bg
B
Pv
8
pE·H1
=qa-3
3
X
=qa-4
4
7
B
=qa-2
H
Pv
6
5
X
Bg
B
E
E
0
9
Kb
8
1
B
2
7
pAL3·3A
=qa-4
6
3
=qa-2
5
4
E
B
E
0
9
Kb
8
1
B
2
E
7
pAL3·3B
=qa-2
6
3
=qa-4
5
4
B
A.nidulans DNA
pBR322 /pBR325
B BamH I
Bg BglII
E EcoR I
H HindIII
P Pst I
Pv PvuII
X Xba I

Table 4.2

Transformation of the qutE mutant strain with the pAL3.3 plasmids

PLASMID USED	AMOUNT OF DNA(μg)	TOTAL NUMBER OF COLONIES	TRANSFORMATION FREQUENCY
pDJB1	10	37 [b]	4
pAL3.3A	10	45 [a]	4.5
pAL3.3B	10	40 [a]	4
No DNA	-	0 [a]	0

(a) *qut*$^+$ selection (b) *pyr*$^+$ selection

The transformation frequency is expressed as the number of colonies/μg transforming DNA. There were 1.8×10^7 protoplasts/ml of the protoplast suspension of which 47% were regenerable.

of pEH1 with BamHI, electrophoresis through a 0.8% agarose gel and electroelution of the appropriate DNA fragment onto Whatman DE81 DEAE-cellulose paper. The fragment was then recovered by a modification of the method by Dretzen et al (1981) and ligated into the BamHI restriction site of pBR325 to create plasmid pAL3.3. This plasmid was recovered in two forms, differing by the orientation of the 3.3 kb BamHI fragment relative to pBR325, designated pAL3.3A and pAL3.3B. The 3.3 kb fragment present in these plasmids spans the putatative QUTC and QUTE genes, but contains only part of the QUTB gene (Figure 4.2). Aliquots of protoplasts from strain WA53 were transformed with 10 µg each of pAL3.3A, pAL3.3B and pDJB1. Protoplasts that were not exposed to DNA were used as a negative control. The results are shown in Table 4.2. The frequency of transformation of WA53 with pAL3.3A and pAL3.3B is 4.5 and 4.0 transformants/ µg plasmid DNA respectively. These results show that the functional QUTE gene is present within the 3.3 kb fragment in pAL3.3 and that the frequency of transformation is not affected by the orientation of this fragment relative to the vector pBR325.

4-2 THE EFFECT OF THE ans1 SEQUENCE ON THE TRANSFORMATION FREQUENCY OF AN A.NIDULANS qutE MUTANT WITH THE QUTE GENE

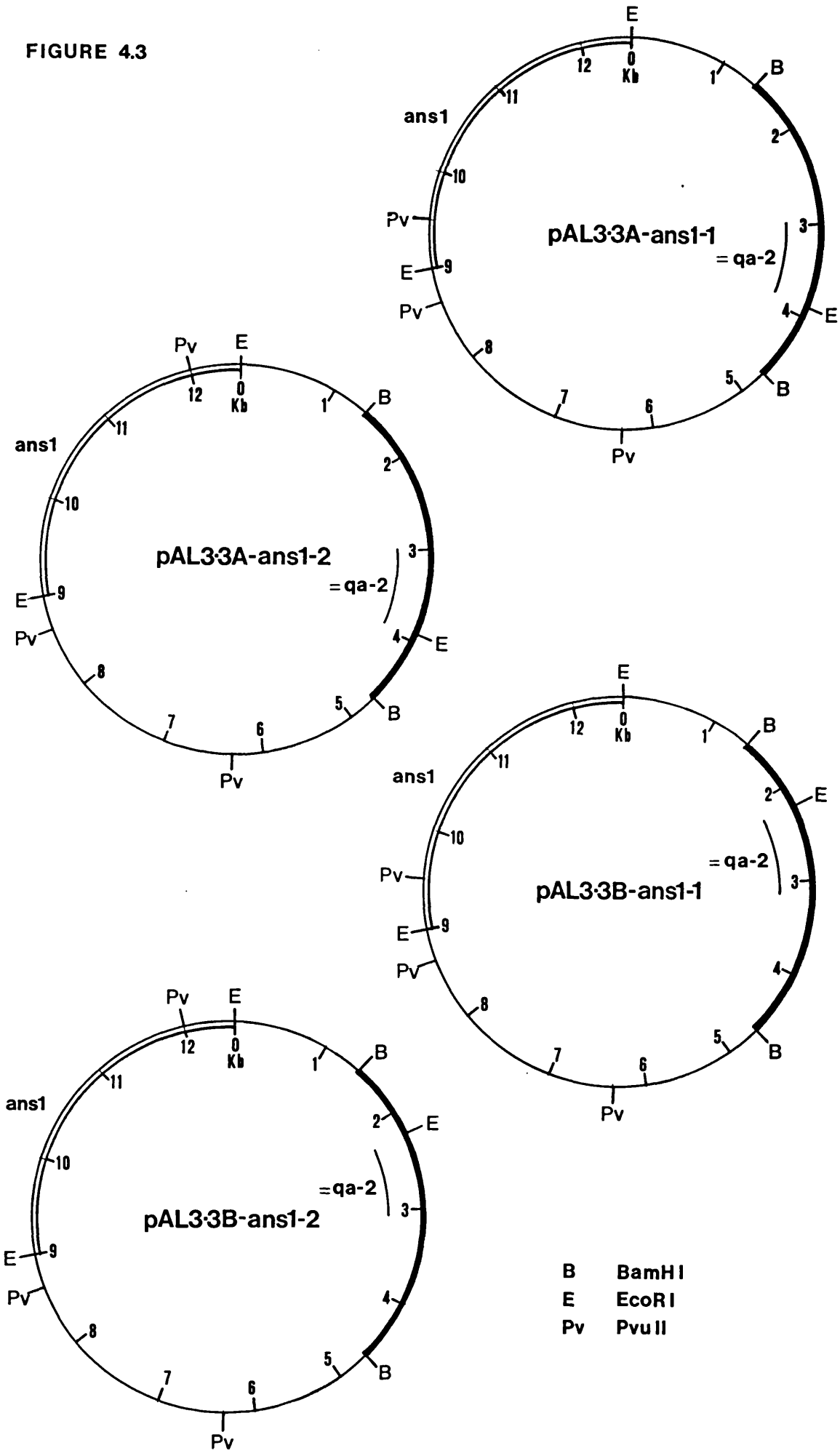
The ans1 sequence was isolated from A.nidulans by Ballance and Turner (1985). This genomic sequence was isolated on the

Figure 4.3

Restriction maps of the pAL3.3-ansi plasmids

The pAL3.3-ansi plasmids each contain the ansi sequence present on a 3.5 kb EcoRI fragment of A.nidulans DNA, isolated from the plasmid pDJB2 (Ballance and Turner, 1985). The 3.5 kb EcoRI fragment was ligated in both orientations into the plasmids pAL3.3A and pAL3.3B, which were partially restriction endonuclease digested with EcoRI. This yielded four plasmids, pAL3.3A-ansi-1, pAL3.3A-ansi-2, pAL3.3B-ansi-1 and pAL3.3B-ansi-2 which exhibit all four possible orientations of the ansi sequence relative to the QUTE gene. The location of the QUTE gene, which is equivalent to the N.crassa qa-2 gene, within the 3.3 kb BamHI fragment of A.nidulans DNA is shown on the diagrams.

FIGURE 4.3


pAL3-3A-ans1-1
pAL3-3A-ans1-2
pAL3-3B-ans1-1
pAL3-3B-ans1-2
ans1
=qa-2
E
B
Pv
0 Kb
B BamH I
E EcoR I
Pv Pvu II

basis of its ability to confer on a pyr4 plasmid the capability of replicating in yeast. When used to transform an A.nidulans pyrG strain the ans1 sequence was found to increase the transformation frequency by two orders of magnitude to 3-5 x 10^3 transformants/ µg plasmid DNA. In contrast to yeast, transformation was shown to occur by integration rather than by autonomous replication of the plasmid (Ballance and Turner, 1985).

The ans1 sequence has been isolated and inserted into the plasmid pAL3.3, which is able to transform on A.nidulans qutE mutant, in order to answer two questions. Firstly, does the ans1 sequence increase the frequency at which the QUTE gene can transform a qutE mutant and secondly, does the relative orientation of the ans1 sequence and the QUTE gene significantly affect this transformation frequency.

The ans1 sequence was isolated on a 3.5 kb EcoRI restriction fragment from a pyr4 plasmid pDJB2 and ligated into the EcoRI restriction site of plasmids pAL3.3A and pAL3.3B to create all four possible orientations of ans1 relative to the QUTE gene (Figure 4.3). In the subsequent transformation experiment 100 µl aliquots of protoplasts from strain WA53 were transformed with 1µg each of pDJB1, pDJB2, pAL3.3A, pAL3.3B and the four pAL3.3 - ans1 plasmids. The protoplast suspension transformed with the plasmid pDJB2 was diluted 10-fold prior to plating because of the high number of transformants expected, the other protoplast

Table 4.3

Transformation of the gutE mutant strain with the pAL3.3-ansl plasmids

PLASMID USED	AMOUNT OF DNA(μg)	TOTAL NUMBER OF COLONIES	TRANSFORMATION FREQUENCY
pDJB1	1	26 [b]	26
pDJB2	1	2000 [b]	2000
pAL3.3A	1	5 [a]	5
pAL3.3B	1	4 [a]	4
pAL3.3A ansl.1	1	300 [a]	300
pAL3.3A ansl.1	1	264 [a]	264
pAL3.3A ansl.2	1	605 [a]	605
pAL3.3B ansl.2	1	220 [a]	220

(a) gut$^+$ selection (b) pyr$^+$ selection

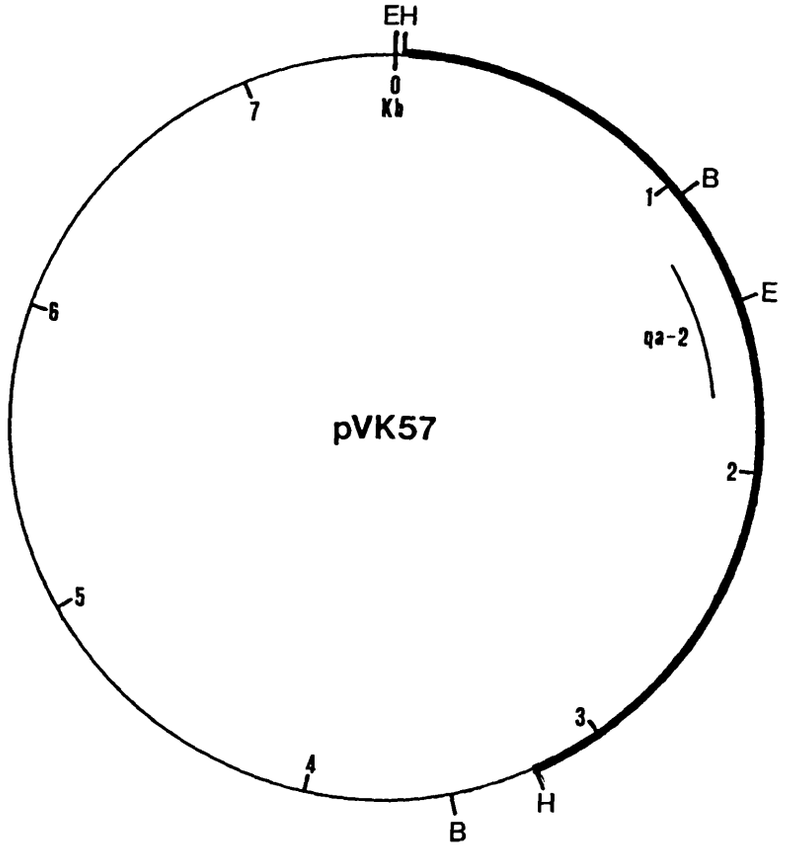
The transformation frequency is expressed as the number of transformants /μg transforming DNA. There were 1.4 x 10^7 protoplasts /ml of the protoplast suspension of which 32% were regenerable.

Figure 4.4

Restriction map of the plasmid pVK57

The plasmid pVK57 contains a 3.2 kb HindIII fragment of N.crassa DNA in the bacterial vector pBR322. The 3.2 kb HindIII fragment contains the N.crassa qa-2 gene, encoding catabolic dehydroquinase, and the location of this gene within the N.crassa DNA is shown on the diagram.

FIGURE 4.4



pBR322

N.crassa DNA

B BamH I

E EcoR I

H Hind III

suspensions were plated undiluted. The results are shown in Table 4.3.

The ans1 sequence increased the transformation frequency of the pyr4 gene by 100-fold which is consistent with that observed by Ballance and Turner (1985). For the QUTE gene, the transformation frequency was increased by 50-100 fold, the same order of magnitude as that observed for the pyr4 gene. It appears that the relative orientation of the ans1 sequence to the QUTE gene has no significant effect on the frequency of transformation as shown by the pAL3.3 and pAL3.3 - ans1 plasmids.

4-3 AN ATTEMPT TO TRANSFORM AN A.NIDULANS qutE MUTANT STRAIN WITH THE EQUIVALENT qa-2 GENE OF N.CRASSA

The qa-2 gene of N.crassa, which encodes the enzyme catabolic dehydroquinase, was used as a heterologous DNA probe in the cloning of the A.nidulans QUTE gene. An attempt has been made to express the qa-2 gene in A.nidulans through the introduction of this gene into the organism by transformation. The qa-2 gene is present in the plasmid pVK57, which contains a 3.2 kb HindIII fragment in vector pBR322 that spans the qa-2 gene of N.crassa (Kushner et al, 1977; Schweizer et al, 1981) (Figure 4.4). The 3.2 kb HindIII fragment has been shown to complement an aroD6 mutant of E.coli (Kushner et al, 1977; Vapnek et al, 1977) and is able to transform a N.crassa qa-2 mutant (Schweizer

Table 4.4

Transformation of the qutE mutant strain with the N. crassa qa-2 gene on plasmid pVK57

PLASMID USED	AMOUNT OF DNA(μg)	TOTAL NUMBER OF COLONIES	TRANSFORMATION FREQUENCY
pDJB1	15	61 [b]	4
pEH1	15	46 [a]	3
pVK57	15	0 [a]	0
pBR322	15	0 [a]	0

(a) *qut*$^+$ selection (b) *pyr*$^+$ selection

The transformation frequency is expressed as the number of colonies/μg transforming DNA. There were 4.9 x 10^7 protoplasts /ml of protoplast suspension of which 36% were regenerable.

et al, 1981).

Aliquots of protoplasts from strain WA53 were transformed with 15 μg each of plasmids pDJB1, pEH1, pVK57 and pBR322 and plated onto selective medium. The results are shown in Table 4.4. No colonies were observed on plates where protoplasts had been exposed to the plasmid pVK57 containing the N.crassa qa-2 gene and so it can be concluded that the qa-2 gene was unable to complement a qutE mutation in A.nidulans.

4-4 THE MOLECULAR ANALYSIS OF QUTE TRANSFORMANTS

A number of QUTE transformants were analysed by Southern hybridization using a homologous DNA probe to establish the presence and position of the transforming DNA within the A.nidulans genome.

This technique, which was used later to analyse transformants from other transformation experiments, will be outlined briefly. Independently isolated transformants from selective medium were picked and purified to obtain single colonies that were used to prepare conidial suspensions. Genomic DNA was prepared by a modification of the method described by Hawkins et al (1985). Mycelium was grown in liquid culture under selective conditions from an inoculum of 10^6 conidiospores ml^{-1} medium. The mycelium was harvested by filtration, washed in distilled water, rapidly

frozen in liquid nitrogen and then ground in a pestle and mortar under liquid nitrogen. The powdered mycelium was carefully resuspended in an extraction buffer (TE, 4% (w/v) sodium dodecyl sulphate (SDS), 25% (w/v) sucrose) at 20ml g^{-1} and gently agitated at room temperature for 15 minutes. After phenol and chloroform extractions, the aqueous phase was treated with proteinase K (0.5 mg ml^{-1}) and DNAse-free RNAse (10μg ml^{-1}) and then further extracted with phenol and chloroform. After high speed centrifugation (60,000 xg) in an ultracentrifuge to pellet contaminating carbohydrates, the solution was extensively dialysed againsed TE buffer and ethanol precipitated to increase the final concentration of DNA. This method yields high molecular weight DNA (50 kb) which digests efficiently with restriction endonucleases.

In order to analyse the genomic DNA by Southern hybridization (Southern, 1975), the genomic DNA (3 μg) was digested with the appropriate restriction endonuclease and the products separated by electrophoresis through a 0.8% agarose gel. The DNA was then transferred to a nitrocellulose filter and immobilised. The filters were then probed with an appropriate $\alpha^{32}P$ - dCTP labelled DNA probe in hybridization solution (Rigby et al, 1977; Jeffreys et al, 1980). The filters were placed in a cassette with intensifying screens and exposed to X-ray film for varying periods of time. In all experiments genomic DNA from a wild type strain (R153) and the strain used as a recipient in the

Figure 4.5

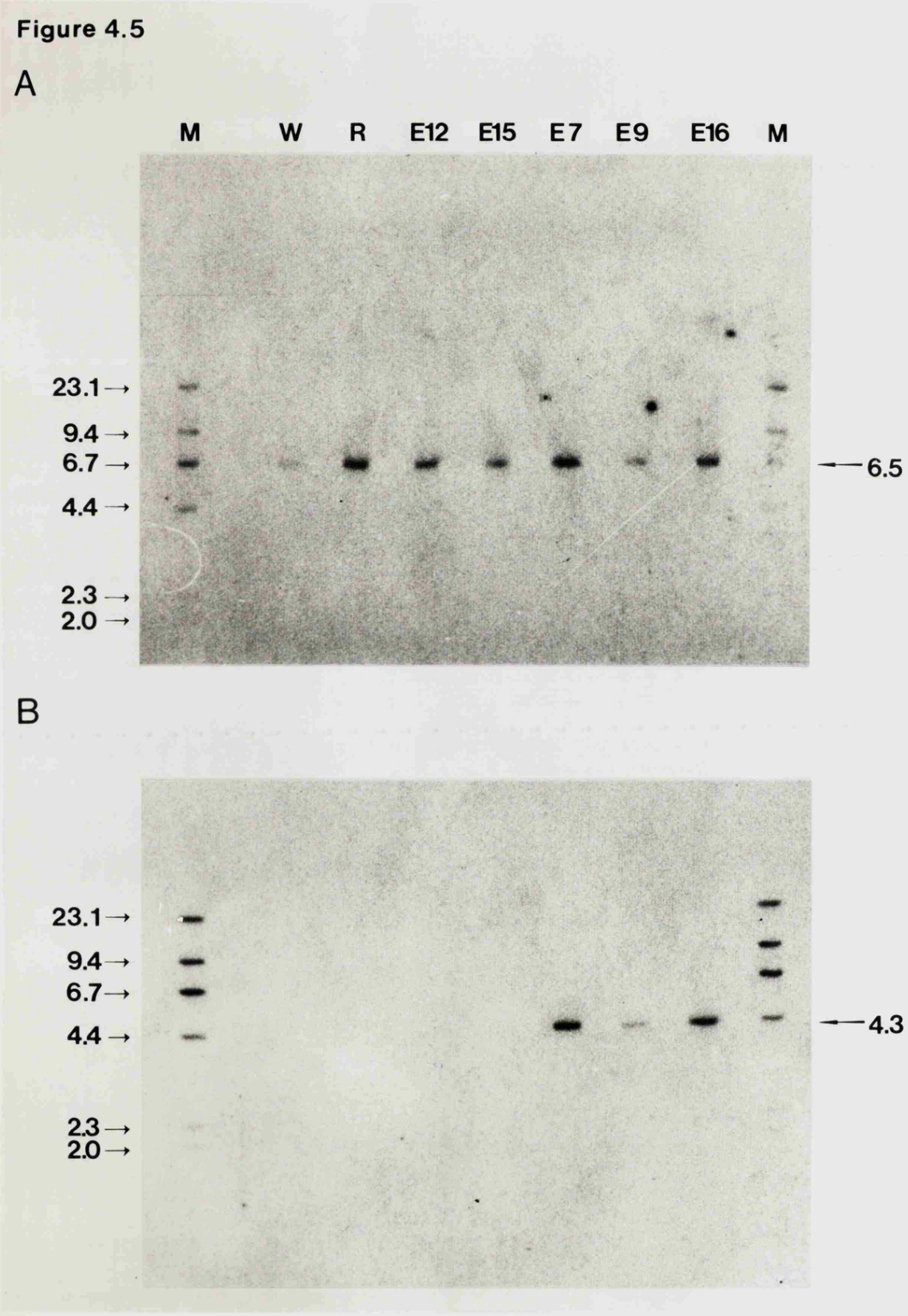
The analysis of QUTE transformants by DNA hybridization

Twelve transformants have been physically analysed by Southern hybridization. The data exhibited shows the results for a representative number of QUTE transformants. For the remaining seven transformants, two exhibit the same pattern of bands upon hybridization as QUTE transformants, E12 and E15, and one exhibits the same pattern of bands as QUTE transformants, E7, E9 and E16. The molecular weight markers (M) are HindIII digested λ DNA.

A. Genomic DNA from five QUTE transformants (E12, E15, E7, E9 and E16) and two control strains, R153 (W) and WA53 (R), was hybridized to 100 ng of the 6.5 kb HindIII fragment of A.nidulans DNA, isolated from pEH1, that was labelled with α^{32}P-dCTP by nick translation. The probe hybridized to a 6.5 kb HindIII fragment of genomic DNA for each of the five QUTE transformants and two control strains.

B. A replicate filter to that used in (A) was hybridized to 100 ng of pBR322 labelled with α^{32}P-dCTP by nick translation. The probe did not hybrize to the two control strains, R153 (W) and WA53 (R), and two of the QUTE transformants, E12 and E15. The probe hybridized to a 4.3 kb HindIII fragment in three of the QUTE transformants, E7, E9 and E16.

Figure 4.5



transformation experiment was analysed with that of the transformants as a control. The molecular weight DNA markers used in all hybridization experiments were λ DNA digested with restriction endonuclease HindIII, unless otherwise stated.

Genomic DNA from twelve QUTE transformants and two control strains, wild-type (R153) and the pyrG qutE recipient strain (WA53), digested with restriction endonuclease HindIII was processed as described above and replicate filters were probed with either α^{32}P - dCTP labelled 6.5 kb HindIII fragment of A.nidulans DNA containing the QUTE gene, which was isolated from plasmid pEH1, or labelled pBR322 DNA. This would establish which of the transformants contained pBR322 DNA sequences integrated into their genome.

The autoradiographs (Figure 4.5) show that for all the transformants and control strains analysed the 6.5 kb fragment isolated from pEH1 has hybridized to a 6.5 kb HindIII fragment in the genomic DNA. No bands of any other size were observed for the transformants on the autoradiograph. The pBR322 DNA probe hybridized to a 4.3 kb HindIII fragment in the genomic DNA of four of the twelve transformants, indicating that the plasmid DNA had integrated into the genomes of these transformants, but failed to hybridize to any DNA sequences in the remaining eight transformants or the two control strains showing that pBR322 sequences are not normally present in wild-type

A.nidulans or the recipient gutE mutant strain (WA53).

The eight transformants that fail to hybridize to labelled pBR322 DNA are classified as Type III transformants, as described by Hinnen et al (1978) (Figure 1.1). In this class of transformant repair of the mutation occurs by "gene conversion" between the transforming DNA and the genomic DNA at the site of the resident gene. This produces a pattern of bands upon hybridization and autoradiography that are indistinguishable to that of the wild-type A.nidulans.

The four transformants that contain pBR322 DNA sequences may be classified as either Type I or Type II transformants (Hinnen et al, 1978), depending on whether integration of the transforming DNA occurred at either the resident gene or at a distant unlinked site, respectively (Figure 1.1). In order to distinguish between the Type I and Type II events, genomic DNA of these four transformants, together with that of two Type III transformants (E12 and E15) and the two control strains, was digested singly with restriction endonucleases PstI and PvuII then transferred to and immobilised on nitrocellulose by the processes described previously. The genomic DNA was then probed with α^{32}P - dCTP labelled pEH1 plasmid.

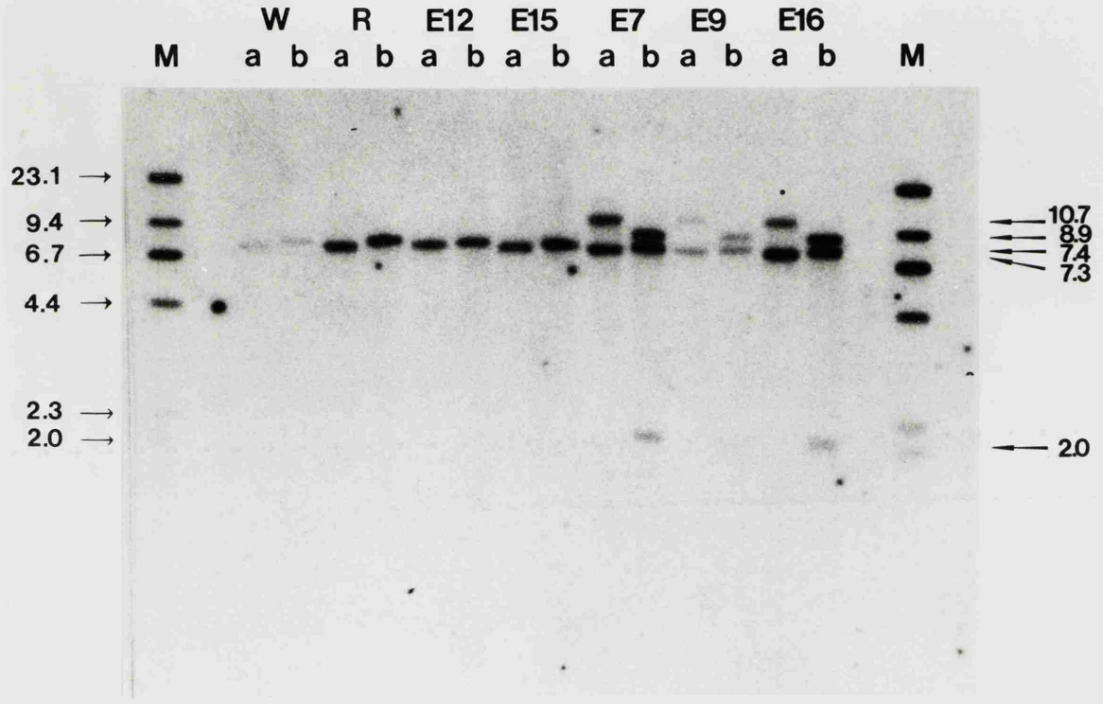
Restriction endonuclease PstI has only one site within the plasmid pEH1, in the pBR322 sequences, while PvuII has two

Figure 4.6

The analysis of QUTE transformants to determine the location of the integrated transforming DNA

An autoradiograph of genomic DNA from the two control strains, R153 (W) and WA53 (R), and five QUTE transformants E7, E9, E12, E15 and E16 digested with (a) PstI and (b) PvuII and hybridized to pEH1 plasmid labelled with α^{32}P-dCTP by nick translation (0.1 μg, 10^8 cpm μg^{-1}). The pEH1 plasmid hybridized to 7.3 kb PstI and 7.4 kb PvuII fragments in the genomic DNA of the two control strains and the two Type III transformants, E12 and E15. Plasmid pEH1 hybridized to PstI fragments of 10.7 and 7.3 kb and to PvuII fragments of 8.9, 7.4 and 2.0 kb in the genomic DNA of the three QUTE transformants E7, E9 and E16. The QUTE transformant E2 exhibited the same pattern of bands as E7, E9 and E16 (data not shown). The molecular weight markers (M) are HindIII digested λ DNA, as described previously, and the sizes of the bands are shown at the left of the figure.

Figure 4.6


W R E12 E15 E7 E9 E16
M a b a b a b a b a b a b a b M
23.1
9.4
6.7
4.4
2.3
2.0
10.7
8.9
7.4
7.3
2.0

sites, one in the pBR322 sequences and the other in the 6.5 kb fragment of A.nidulans DNA very close to the junction with the pBR322 sequences (Figure 4.2). The autoradiograph (Figure 4.6) showed that the two type III transformants (E12 and E15) exhibit the same pattern of bands as the two control strains WA53 and R153. The hybridization produced bands of 7.1 and 7.4 kb when the genomic DNA was restriction digested with PstI and PvuII respectively. Only one band is observed on digestion with PvuII because the site of this enzyme is so close to the end of the 6.5 kb HindIII fragment within pEH1 that no significant hybridization of the DNA probe occurs to such a small region of homology. All four transformants that contain pBR322 sequences exhibit the same pattern of bands on the autoradiograph, which is different from that of the Type III transformants and the control strains. When cut with restriction endonuclease PstI two bands occur on the autoradiograph of 7.3 and 10.7 kb, while PvuII digestion produces three bands of 8.9, 7.4 and 2.0 kb (Figure 4.6). This pattern of bands is consistent with those expected for a Type I transformant where integration of the transforming plasmid occurs at the site of the resident gene with vector sequences also being integrated.

These Type I transformants may contain multiple copies of the transforming DNA tandomly integrated into the genome and hence contain multiple copies of the QUTE gene and possibly the QUTB and QUTC genes.

4-5 THE DETERMINATION OF THE COPY NUMBER OF THE QUTE GENE WITHIN THREE TYPE I TRANSFORMANTS

Genomic DNA "dot-blots" have been carried out to determine how many copies of the transforming DNA and hence, copies of the QUTE gene have integrated into the genomic DNA of three Type I transformants.

In a DNA "dot-blot" equal amounts of genomic DNA from strains having an indeterminate number of copies of a particular gene are compared to that of a strain known to carry only a single copy of the gene. Each DNA sample is denatured, serially diluted and immobilised on a nitrocellulose filter in the form of a spot. After hybridization to the appropriately labelled DNA probe and autoradiography, the intensity of the resulting dots on the autoradiograph are compared to those of the single copy number control to obtain the relative gene copy number. This comparison may be done qualitatively by eye or, if necessary, quantitatively either using a densitometer to measure the intensity of each dot or a liquid scintillation counter to determine the level of radiation emitted by each dot.

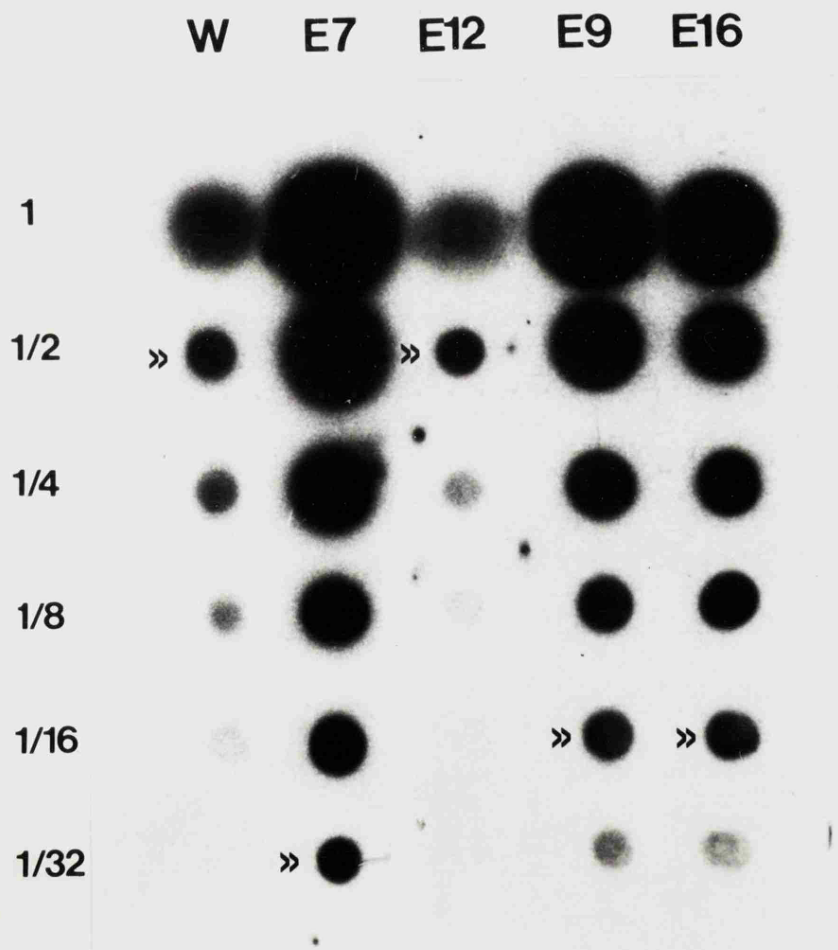
For this experiment the wild-type strain R153 and a Type III transformant are assumed to have a single copy of the QUT structural genes and hence a single copy of the QUTE gene and were used as a reference. Denatured genomic DNA (1μg)

Figure 4.7

Genomic DNA "dot blots" to determine the copy number of the QUTE gene in three Type I transformants

Denatured genomic DNA (1 µg) of three Type I transformants (E7, E9 and E16), one Type III transformant (E12) and the wild-type strain R153 (W) was serially two-fold diluted to 1/32 of its original concentration and immobilized on nitrocellulose in the form of spots. The DNA was hybridized to the 6.5 kb HindIII fragment, isolated from pEH1, labelled with α^{32}P-dCTP by nick translation (0.1µg, 10^8 cpm µg^{-1}). The control strain R153 (W) and the Type III transformant, E12, are considered to contain a single copy of the QUTE gene and are used as a reference. Dots of equal intensity are indicated on the autoradiograph.

Figure 4.7



of the three Type I transformants, one Type III transformant and the wild-type strain R153 was serially two-fold diluted to 1/32 of its original concentration and immobilised on nitrocellulose in the form of spots. The DNA was then hybridized to $\alpha^{32}P$ - dCTP labelled 6.5 kb HindIII fragment from plasmid pEH1 (Rigby et al, 1977; Jefferys et al, 1980)

A qualitative comparison of the density of the dots on the autoradiograph (Figure 4.7) shows that, as expected, the wild type strain R153 and the Type III transformant E12 have dots of equal intensity and hence the same copy number of the QUTE gene, that being a single copy. By reference to these two strains the three Type I transformants,E7,E9 and E16, have copy numbers of the QUTE gene of 16, 8 and 8 respectively.

Hence these Type I transformants contain multiple copies of the 6.5 kb HindIII fragment present on pEH1 integrated in tandem in their genomes and therefore multiple copies of the QUTE gene.

The presence of a functional QUTB gene, encoding quinate dehydrogenase, within the 6.5 kb HindIII fragment of pEH1 has been demonstrated by transformation of an A.nidulans qutB mutant (H Lamb, personal communication) but the presence of a functional QUTC gene has yet to be proven, though sequences homologous to the equivalent qa-3 gene of N.crassa, as determined by hybridization, are

apparently present on this fragment. If the functional QUTC gene is present then the Type I transformants will contain multiple copies of the three QUT structural genes but only a single copy of each of the regulatory genes.

4-6 THE EXPRESSION OF THE QUT ENZYME STRUCTURAL GENES IN SELECTED QUTE TRANSFORMANTS

A study of the expression of the QUT enzyme structural genes in certain QUTE transformants was undertaken to establish two points: first, are multi-copy number transformants subject to the same regulatory controls. Second, if subject to the same regulatory controls, does the induction of the multi-copy number transformant by quinate lead to higher than wild-type levels of the QUTE, QUTB and QUTC gene products.

To resolve these two questions a Type I transformant (E7), which has 16 copies of the transforming DNA integrated into the genome, was compared to a Type III transformant (E15), the recipient qutE,pyrG mutant strain (WA53) and a pyrG wild-type strain (G191), which are considered to contain a single copy of each of the QUT enzyme structural genes. These strains were grown in liquid minimal medium supplemented with 10mM uridine and 20mM glucose for 18 hours at 30°C. The mycelium was then harvested, washed and divided equally between three different types of uridine-supplemented liquid minimal medium and incubated for

a further 4 hours. These three media were as follows:-

(1) minimal medium containing 20mM glucose

(2) minimal medium containing 20mM glucose and 0.1% (w/v) quinate (pH6.5)

(3) minimal medium containing 0.1% (w/v) quinate (pH6.5)

The mycelium was harvested from these cultures, washed and stored at -20°C. Cell free extracts were prepared from equal amounts of each mycelium (1g wet weight). The specific activities of the three enzymes (1) quinate dehydrogenase (2) catabolic dehydroquinase and (3) dehydroshikimate dehydratase were assayed for all the cell free extracts in a Unicam SP1800 recording spectrophotometer at 37°C, as described in the Materials and Methods.

The results are shown in Table 4.5. The glucose grown cultures all showed no significant measurable activity of the quinate inducible enzymes showing that under non-inducing growth conditions none of the QUT enzymes are produced. Hence the QUT structural genes are not constitutively expressed but normally regulated even in a high copy number transformant.

Expression of the QUT genes is subject to carbon catabolite repression, that is the enzymes are poorly induced by quinate when glucose is also present as a carbon source in

TABLE 4.5 : LEVELS OF THE QUINATE INDUCIBLE ENZYMES IN PARTICULAR QUTE TRANSFORMANTS

	INDUCTION CONDITIONS	GLUCOSE (20 mM)			GLUCOSE / QUINATE (20 mM/0.1%)			QUINATE (0.1%)		
	ENZYME ASSAYED	1	2	3	1	2	3	1	2	3
STRAINS ASSAYED	WT	0.010	0.100	ND	0.016	0.120	ND	0.090	2.570	0.380
	RECIP.	0.011	0.080	ND	0.025	0.070	ND	0.090	ND	0.470
	TYPE III	0.003	0.060	ND	0.045	0.200	ND	0.140	1.960	0.270
	TYPE I	0.012	0.090	ND	0.023	0.140	ND	0.120	3.150	0.340

1 - QUINATE DEHYDROGENASE (uM/min/mg protein)

2 - CATABOLIC DEHYDROQUINASE (OD/min/mg protein)

3 - DEHYDROSHIKIMATE DEHYDRATASE (uM/min/mg protein)

ND - NOT DETECTABLE

the medium. It has been shown that 20mM glucose in growth medium containing 0.1% (w/v) quinate represses induction of the three enzymes. The results show that under these growth conditions no enzyme induction occurred in the multi-copy number Type I transformant E7 nor in the other strains tested indicating normal carbon catabolic repression.

The enzyme activities are induced in all of the strains grown on quinate and, as expected, the recipient strain WA53, which is a qutE mutant, does not exhibit any catabolic dehydroquinase activity. The enzyme activities in the multicopy Type I transformant E7 are not higher than that of the wild-type pyrG strain G191 or the Type III transformant E15 which indicates that increasing the copy number of the QUT enzyme structural genes alone does not lead to increased levels of enzyme activity.

This observation was confirmed when the levels of catabolic dehydroquinase activity were measured for three Type III transformants (E8, E12 and E15) and three multi-copy Type I transformants (E7, E9 and E16). In this case the strains were grown in liquid minimal medium containing glucose for 18 hours at 30°C and the mycelium was then transferred to fresh minimal medium containing 0.1% (w/v) quinate for a further 4 hours incubation at 30°C to allow induction of the quinate specific enzymes. It can be seen (Table 4.6) that there is no significant increase in the level of catabolic dehydroquinase activity in the multi-copy Type I

Table 4.6

Dehydroquinase activity in Type I and Type III transformants

A.NIDULANS STRAIN	QUTE GENE COPY NUMBER	OD ^{240} $min^{-1}ml^{-1}$	TOTAL PROTEIN mg ml^{-1}	DEHYDROQUINASE ACTIVITY OD ^{240} mg^{-1} min^{-1}
G191	1	3.8	4.05	0.94
WA53	1*	0.0	4.55	0.00
E7	16	6.4	4.50	1.42
E8	1	10.6	4.65	2.28
E9	8	7.2	4.25	2.40
E12	1	10.0	5.25	1.90
E15	1	5.8	3.20	1.81
E16	8	5.0	3.65	1.37

*The strain WA53 is a qutE mutant and therefore lacks catabolic dehydroquinase.

Aliquots of the protein extracts from the above strains were assayed for dehydroquinose activity in triplicate and the average OD^{240} $min^{-1}ml^{-1}$ used to calculate the dehyroquinose activity.

transformants (E7, E9 and E16) over that of the Type III transformants (E8, E12 and E15) and the control G191.

Hence it can be concluded that increasing the copy number of the QUT enzyme structural genes alone does not lead to increased enzyme activity under inducing conditions.

DISCUSSION

During the course of these experiments it has been shown that fragments of A.nidulans genomic DNA which hybridize to the qa-2 gene of N.crassa, encoding catabolic dehydroquinase, are able to transform a qutE mutant strain of A.nidulans, thus enabling it to utilize quinate as a carbon source. This indicates that these DNA fragments, the smallest of which is 3.3 kb, contain the QUTE gene of A.nidulans encoding the enzyme catabolic dehydroquinase. The plasmid pEH1 also contains the QUTB gene as demonstrated by its ability to transform a qutB mutant strain of A.nidulans (H Lamb, personal communication) however transformation of a qutC mutant strain indicating the presence of the QUTC gene has yet to be achieved with this plasmid.

A study of particular transformants has shown that increasing the copy number of the genomic DNA contained within pEH1 does not lead to an increase in the levels of enzyme activity encoded by the QUT structural genes. This

suggests that there is a rate limiting step in the induction of these three enzymes, the most obvious candidate being the positively acting regulatory protein encoded by one of the regulatory genes. Alternatively there may be sequences absent on the plasmid pEH1 that are essential for gene expression.

Similar studies have been undertaken on the expression of the alcA gene of A.nidulans in multicopy number transformants (D Gwynne, personal communication). The alcR gene product positively regulates the alcA gene. When single copies of both genes are present there are normal (wild-type) levels of mRNA induced and alcohol dehydrogenase activity. The alcA promoter region has been fused to the human interferon α2 coding region and in subsequent transformation experiments it has been shown that introducing multiple copies of this fusion gene into a strain having single copies of alcA and alcR results in decreased alcA gene expression, characterised by lower than wild-type levels of mRNA and alcohol dehydrogenase activity. This suggested that the multiple alcA promoter regions were removing the pool of alcR gene product hence limiting the expression of the alcA gene. This situation was relieved by increasing the copy number of the alcR gene. It was also shown that multiple copies of alcA and alcR lead to proportionately higher than wild-type levels of alcA expression. This demonstrates that the alcR gene product was the rate limiting factor in multicopy alcA gene expression.

There may be similarities between that observed for alcA and alcR and that of the multi-copy QUTE transformants. If multiple copies of both the QUT structural genes (QUTB, QUTC and QUTE) and the activator gene are introduced into a strain containing a single copy of the repressor encoding gene, not only may there be increased expression of the QUT structural genes under inducing conditions but dilution of the repressor gene product relative to the activator gene, on which it may have a negative regulatory effect, could lead to constitutive expression of the QUT structural genes under non-inducing conditions.

An attempt to transform a qutE mutant of A.nidulans with the qa-2 gene of N.crassa has not been successful. It has already been shown that homology exists between the qa-2 and QUTE genes, in that they are able to cross hybridize (Hawkins et al, 1985) and that rabbit antibodies raised against the qa-2 gene product cross react with the QUTE gene product (Hawkins et al, 1982). It is possible that the A.nidulans QUT activator protein is unable to recognise cis-acting regulatory sites in the 5' region of the N.crassa qa-2 gene so preventing induction of the qa-2 product, catabolic dehydroquinase.

CHAPTER 5

THE IDENTIFICATION AND FUNCTION OF THE QUTD GENE IN A.NIDULANS

INTRODUCTION

One class of A.nidulans mutant strains unable to utilize quinic acid as a carbon source for growth are non-inducible for the three quinate specific enzyme activities (Hawkins et al, 1982). The analysis of a small number of these non-inducible mutants revealed two distinct gene loci, QUTA and QUTD that map within the QUT gene cluster (Hawkins et al, 1984). The mutant alleles qutA4 and qutD8 were shown to be partially dominant and fully recessive to the wild-type respectively and the mutant genomes were able to complement when combined in heterozygous diploid strains. It was suggested that these two genes encoded functions required for the regulation of the QUT enzyme structural genes. The QUTA gene was thought to encode a repressor protein, the qutA4 allele producing a repressor protein insensitive to the presumed inducer quinic acid, and the QUTD gene was considered to encode an activator protein which may be absent in the qutD8 mutant (Hawkins et al, 1984).

Mutations in the QUTA and QUTD genes exhibited characteristics in common with those in the qa-1S and qa-1F

genes of N.crassa suggesting that these genes had common modes of action and hence were equivalent in the two organisms. As it was possible that there was sequence homology between these genes, the qa-1F gene which encodes the activator protein in N.crassa was used as a DNA probe in hybridization experiments to locate the position of the activator encoding gene (QUTD) of A.nidulans on the recombinant phage λQ1 DNA (Da Silva, 1985). A 2.1 kb BamHI-XbaI restriction fragment from the plasmid pMSK375 (Schweizer et al, 1981), wholly within the coding sequence of the qa-1F gene, hybridized strongly to the λQ1 DNA and located the equivalent gene in A.nidulans (QUTD) to a 1.7 kb EcoRI restriction fragment (Figure 1.5). The repressor encoding gene, qa-1S, of N.crassa did not exhibit any homology to the λQ1 DNA when used as a DNA probe in hybridization experiments indicating that the equivalent gene (QUTA) in A.nidulans is not present on this clone.

Further observations made by the research group during 1984 has led to a revision of the original hypothesis that the QUTA and QUTD genes of A.nidulans are equivalent to the qa-1S and qa-1F genes of N.crassa. My own contribution to this advance has been in the transformation of qutD (Chapter 5) and qutA (Chapter 6) mutant A.nidulans strains. This will be described in the results section of these chapters and related to the work of the group in the Discussion sections.

The aim of the work described in this Chapter is to locate the position of the QUTD gene on the λQ1 DNA and to assess the function of this gene in relation to the QUT gene cluster in A.nidulans.

5-1 TRANSFORMATION OF A qutD MUTANT STRAIN WITH FRAGMENTS OF λQ1 DNA THAT EXHIBIT HOMOLOGY TO THE qa-1F GENE OF N.CRASSA

A qutD mutant strain of A.nidulans was transformed with fragments of DNA isolated from λQ1 that hybridize to the activator encoding gene, qa-1F, of N.crassa, to confirm that QUTD is the equivalent gene in A.nidulans.

A qutD,pyrG double mutant (GD22) was used in the transformation experiments. This strain, whose construction is described in Chapter 3, is both a non-inducible quinic acid non-utilising mutant and a uridine auxotroph. The qutD8 allele used is fully recessive to wild-type in heterozygous diploid strains and the pyrG mutation allows the plasmid pDJB1 (Ballance and Turner, 1985), containing the pyr4 gene of N.crassa, to be used as a positive control for the efficiency of transformation.

The method used for the preparation of A.nidulans protoplasts and their subsequent transformation was that described in Chapter 4. As before, the addition to

Figure 5.1

Restriction maps of the plasmids pAL3.7 and pAL4.4

The plasmid pAL3.7 contains a 3.7 kb BamHI fragment of A.nidulans DNA excised from λQ1 and subcloned into the BamHI restriction site of pBR322.

The plasmid pAL4.4 contains a 4.4 kb HindIII fragment of A.nidulans DNA excised from λQ1 and subcloned into the HindIII restriction site of pBR325.

Both restriction fragments span the region of A.nidulans DNA in λQ1 that is homologous to the N.crassa qa-1F gene.

FIGURE 5.1

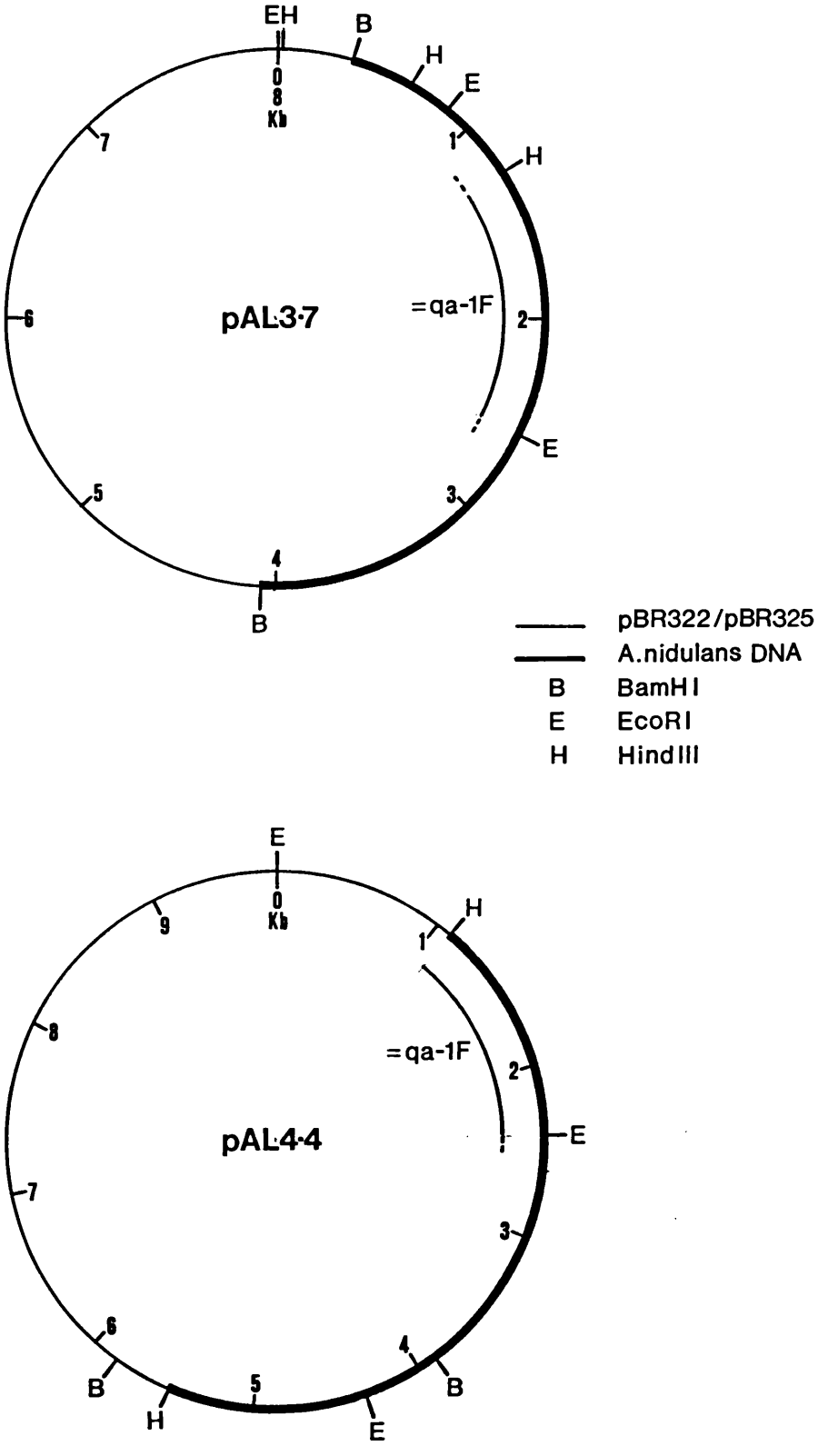

EH
B
H
E
H
E
B
0
8
Kb
1
2
3
4
5
6
7
pAL3·7
=qa-1F
pBR322/pBR325
A.nidulans DNA
B BamHI
E EcoRI
H HindIII
E
H
E
E
B
H
B
0
Kb
1
2
3
4
5
6
7
8
9
pAL4·4
=qa-1F

Table 5.1

Transformation of the qutD pyrG mutant strain, GD22, with the plasmids pAL3.7 and pAL4.4

PLASMID USED	AMOUNT OF PLASMID (μg)	TOTAL NUMBER OF COLONIES	TRANSFORMATION FREQUENCY
pDJB1	10	44 [b]	4.4
pAL3.7	10	0 [a]	0
pAL3.7	20	0 [a]	0
pAL4.4	10	0 [a]	0
pAL4.4	20	0 [a]	0
No DNA	-	0 [a]	0

(a) *qut*$^+$ selection (b) *pyr*$^+$ selection

The transformation frequency is expressed as the number of colonies/μg plasmid DNA. There were 1.7×10^7 protoplasts/ml of the protoplast suspension of which 23% were regenerable.

protoplasts of plasmid pBR325 or no DNA was used as a negative control for reversion of the original qutD mutation.

Two plasmids were constructed containing restriction fragments of A.nidulans genomic DNA that span the region homologous to the qa-1F gene of N.crassa to the right of the QUT enzyme structural genes on λQ1. Plasmid pAL3.7 contains a 3.7 kb BamHI fragment isolated from λQ1 subcloned into the BamHI site of pBR322 while pAL4.4 contains a 4.4kb HindIII fragment isolated from λQ1 subcloned into the HindIII site of pBR325 (Figures 5.1 and 5.3).

Suitable amounts of the two plasmids were added to 200 µl aliquots of protoplasts and then plated onto appropriately supplemented minimal medium for incubation. The results are shown in Table 5.1. Protoplasts transformed with plasmid pDJB1 (pyr4) DNA yielded uridine independent transformants at a frequency of 4.4/ µg plasmid DNA after 48 hours incubation whereas protoplasts exposed to the pAL3.7 and pAL4.4 plasmids produced no colonies, even after extended incubation. No colonies appeared on plates of protoplasts that were not exposed to DNA indicating that there was no reversion of the qutD8 mutation.

It can be concluded that the plasmids pAL3.7 and pAL4.4 are unable to transform a qutD mutant strain. In N.crassa the

qa-1F gene produces two quinate inducible mRNA species of 3.0 and 2.9 kb respectively which differ in size at their 5' end only (Patel and Giles, 1985). If the QUTD gene is similar in size to the qa-1F gene and considering the position of the DNA sequences homologous to the qa-1F gene within the restriction fragments present in pAL3.7 and pAL4.4, it is possible that the entire QUTD gene may not be present within either of these two plasmids which may account for their inability to transform a qutD strain.

5-2 TRANSFORMATION OF THE qutD MUTANT STRAIN WITH THE RECOMBINANT BACTERIOPHAGE λQ1 DNA

The qutD pyrG strain was transformed with λQ1 DNA to demonstrate that sequences capable of transforming a qutD mutant are present within the 13.6 kb of A.nidulans genomic DNA on this clone. Phage λQ1 DNA has two sites for the restriction endonuclease XbaI close together within the region of the QUT enzyme structural genes (Figure 5.3). If the cohesive ends of the DNA are annealed, restriction digestion of λQ1 DNA with this enzyme yields two fragments. The larger fragment spans the regions of DNA to the left and right of the QUT enzyme structural genes and has DNA ends homologous to the A.nidulans genome. It has been demonstrated for Saccharomyces that cleavage of a chaemeric plasmid within the yeast DNA sequences enhances the efficiency of integration during yeast transformation

Table 5.2

Transformation of the qutD pyrG mutant strain, GD22, with uncut and XbaI digested λQ1 DNA.

DNA USED	AMOUNT OF DNA (μg)	TOTAL NUMBER OF COLONIES	TRANSFORMATION FREQUENCY
pDJB1	10	64 [b]	6.4
λQ1	10	36 [a]	3.6
XbaI digested λQ1	10	228 [a]	22.8
No DNA	-	0 [a]	0

(a) *qut*$^+$ selection (b) *pyr* selection

The transformation frequency is expressed as the number of colonies/μg transforming DNA. There were 1.9 x 10^7 protoplasts/ml of the protoplast suspension of which 39% were regenerable

(Struhl, 1983). Similar studies in N.crassa on the effect of plasmid linearisation in qa-2 transformation (Dhawale and Marzluf, 1985) have also demonstrated a 3-4 fold increase in the frequency of transformation of a plasmid linearised within the inserted N.crassa DNA in comparison to that linearised within plasmid sequences or to circular plasmid. Linearised plasmid having only one end homologous to the N.crassa genome exhibits an intermediate level of transformation. It was hoped that cleaving the λQ1 DNA to produce free homologous DNA ends would enhance the transformation frequency of what is a relatively large DNA molecule (50 kb).

Equal amounts (10 μg) of digested or undigested λQ1 DNA and plasmid pDJB1 was added to protoplasts from the qutD pyrG strain and plated onto the appropriate selective medium. The results are shown in Table 5.2. Protoplasts transformed with the plasmid pDJB1 yielded uridine independent transformants at a frequency of 6.4/ μg plasmid DNA. Protoplasts transformed with λQ1 DNA produced conidiating colonies clearly visible after 48 hours incubation at a frequency of 3.6 transformants / μg DNA. Digestion of the λQ1 DNA with Xbal increased the frequency of transformation six fold to 23 transformants/ μg DNA which probably represents improved integration of the transforming DNA into the genome due to the recombinogenic homologous ends of A.nidulans DNA. No colonies were observed on plates where protoplasts had not been exposed to DNA.

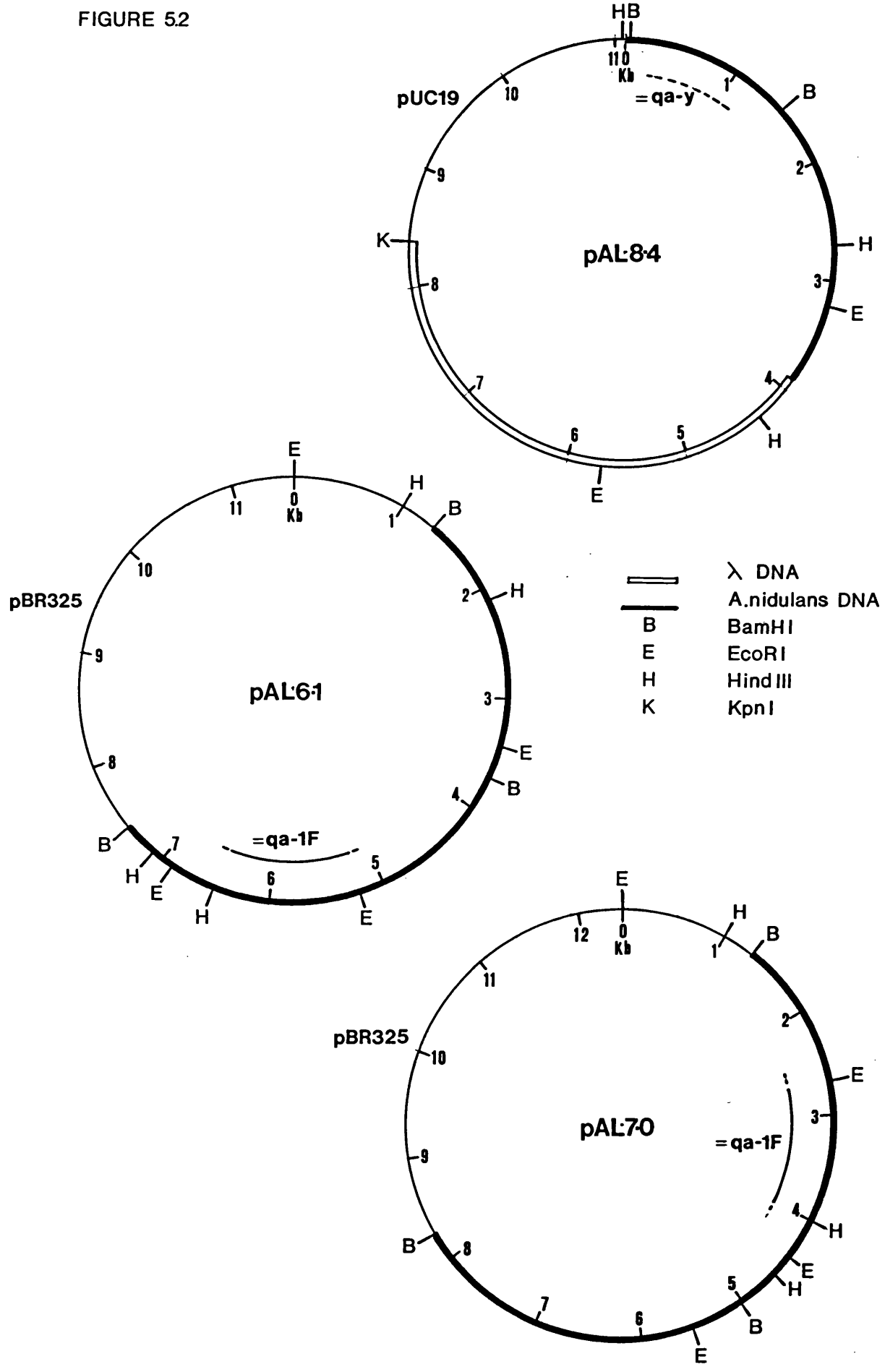
Figure 5.2

Restriction maps of the plasmids pAL8.4, pAL6.1 and pAL7.0

Plasmid pAL8.4 contains an 8.4 kb fragment which spans the entire region of A.nidulans DNA to the left of the QUT enzyme structural genes in λQ1 as well as 4.5 kb of λDB286 DNA and contains DNA sequences homologous to the N.crassa qa-y gene. The 8.4 kb fragment was excised from λQ1 DNA digested with KpnI and BglII and ligated into pUC19 which had been digested with KpnI and BamHI in the 54 bp polylinker sequence.

Two fragments of 6.1 and 7.0 kb were isolated from λQ1 DNA which had been partially digested with BamHI. These fragments overlap by the 3.7 kb BamHI fragment present in pAL3.7 and together span the entire region of A.nidulans DNA to the right of the QUT structural genes in λQ1 that contains DNA sequences homologous to the N.crassa qa-1F gene. Plasmids pAL6.1 and pAL7.0 contain the 6.1 and 7.0 kb fragments respectively subcloned into the BamHI site of pBR325.

FIGURE 5.2


pAL:8-4
pUC19
=qa-y
HB
11 0 Kb
1
2
3
4
5
6
7
8
9
10
B
H
E
K
pAL:6-1
pBR325
=qa-1F
0 Kb
11
12
λ DNA
A.nidulans DNA
BamHI
EcoRI
HindIII
KpnI

Twelve colonies from protoplasts transformed with λQ1 DNA and twenty eight from those transformed with digested λQ1 DNA were picked and purified by single colony isolation. The phenotypes of these strains were tested by growth on the appropriately supplemented minimal medium. All were found to be able to utilize quinate as a carbon source (*QUT*$^+$) but as expected were still uridine auxotrophs (*pyrG*$^-$). The other genetic markers were also present.

This result confirmed that the entire *QUTD* gene is present within the 13.6 kb insert of *A.nidulans* DNA in λQ1.

5-3 THE LOCATION OF THE *QUTD* GENE IN THE λQ1 DNA

In order to locate the position of the *QUTD* gene on the λQ1 DNA, further DNA fragments to the left or right of the *QUT* enzyme structural genes in λQ1 were subcloned into plasmid DNA vectors.

To span the total region of *A.nidulans* DNA to the right of the *QUT* enzyme structural genes in λQ1, two fragments of 6.1 and 7.0 kb were isolated. These were prepared by a partial *Bam*HI digestion of λQ1 DNA and isolated from a 0.6% (w/v) low-gelling point agarose gel (Crouse *et al*, 1983). The fragments were ligated into the *Bam*HI site of pBR325 to create plasmids pAL6.1 and pAL7.0 respectively (Figure 5.2). These two restriction fragments overlap by the 3.7 kb

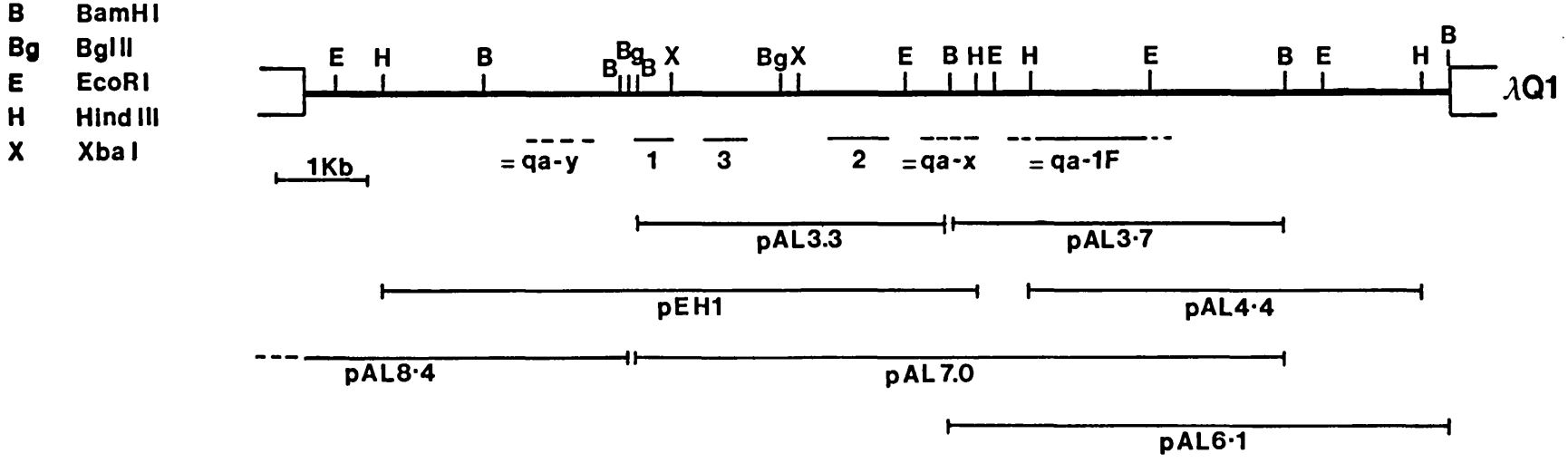
Figure 5.3

The location of A.nidulans DNA restriction fragments within λQ1 that have been subcloned into plasmid vectors

A restriction map of the A.nidulans DNA in λQ1 showing the position of restriction fragments that have been subcloned into plasmid vectors for the transformation of A.nidulans gut$^-$ strains. The bars indicate the location of restriction fragments and the name of the plasmid containing each fragment is situated below.

The position of the QUTE gene (2) and DNA sequences homologous to the qa-3 (1), qa-4 (3), qa-x, qa-y and qa-1F genes of N.crassa is indicated.

FIGURE 5.3


B BamHI
Bg BglII
E EcoRI
H HindIII
X XbaI
E H B Bg B X BgX E B HE H E B E H B
λQ1
1Kb
= qa-y
1
3
2
= qa-x
= qa-1F
pAL3.3
pAL3·7
pEH1
pAL4·4
pAL8·4
pAL7.0
pAL6·1

fragment present in pAL3.7 (Figure 5.3).

Attempts were made to subclone a 9.6 kb BglII restriction fragment from the right hand end of the A.nidulans DNA in λQ1, which spans the QUTE gene, the DNA sequences that hybridize to the qa-1F gene and 1.2 kb of λDB286 DNA. However these 1.2 kb of DNA contain the P_L promoter but not the cI repressor gene and it was found that when the 9.6 kb fragment was subcloned into the BamHI site of pBR325 or pUC19 and subsequently used to transform E.coli, deletions and rearrangements occur within the DNA resulting in altered restriction patterns.

An 8.4 kb fragment was isolated which spans the entire region of A.nidulans DNA to the left of the QUT enzyme structural genes in λQ1 as well as 4.5 kb of λDB286 DNA (Figure 5.3). This fragment was prepared by digestion of λQ1 DNA with BglII and KpnI, separated by electrophoresis in a 0.8% (w/v) agarose gel, electroeluted onto Whatman DE81 DEAE-cellulose paper and recovered following the the method of Dretzen et al (1981). The fragment was ligated into pUC19, digested by BamHI and KpnI in the 54 bp polylinker sequences, to create plasmid pAL8.4 (Figure 5.2).

Appropriate amounts of the plasmids pAL6.1, pAL7.0, pEH1 and pAL8.4 were added to protoplasts of the qutD pyrG strain which were then plated onto selective medium for incubation. Protoplasts exposed to λQ1 DNA or vector pBR325 DNA were

Table 5.3

Transformation of the qutD pyrG mutant strain, GD22, to determine the location of the QUTD gene within λQ1

	DNA USED	AMOUNT OF DNA (μg)	TOTAL NUMBER OF COLONIES	TRANSFORMATION FREQUENCY
(A)	λQ1	20	22 [a]	1.1
	pAL6.1	10	0 [a]	0
	pAL7.0	10	0 [a]	0
	pAL8.4	10	44 [a]	4.4
	pEH1	10	38 [a]	3.8
	pBR325	10	0 [a]	0
(B)	pDJB1	5	12 [b]	2.4
	λQ1	10	16 [a]	1.6
	pAL8.4	5	18 [a]	3.6
	pEH1	5	17 [a]	3.4
	pBR325	5	0 [a]	0

(a) qut$^+$ selection (b) pyr$^+$ selection

The number of protoplasts/ml of the protoplast suspension and the percentage that were capable of regeneration on supplemented medium in the above experiments was as follows - (A) 1.1 x 10^7/ml, 21%; (B) 1.4 x 10^7/ml, 23%.

Figure 5.4

The analysis of ten QUTD transformants by DNA hybridization

Samples of BamHI digested genomic DNA from ten QUTD transformants (1-10), the wild-type R153 (W) and the recipient GD22 (R) hybridized to either BamHI digested pUC19 or the 8.4 kb BglII - KpnI fragment labelled with α^{32}P-dCTP. The molecular weight markers (M) are HindIII digested λ DNA

A. An autoradiograph of the genomic DNA samples hybridized to the 8.4 kb BglII - KpnI fragment. The 8.4 kb fragment hybridized to BamHI fragments of 2.8 and 1.6 kb in the genomic DNA of the two control strains (W & R) and all ten transformants. A third BamHI fragment of 9.1 kb is identified in transformants number 7 and 8. Transformant number 9 exhibits a more complex pattern of bands, with six BamHI fragments of genomic DNA being identified including the 2.8 and 1.6 kb fragments.

B. An autoradiograph of the genomic DNA samples hybridized to BamHI digested pUC19.No sequences homologous to pUC19 are present in the genomic DNA of the two control strains (W & R) and six transformants (1-6). pUC19 hybridized to a 9.1 kb BamHI fragment in transformants number 7 and 8 and a 4.0 kb fragment in transformant number 10. Transformant number 9 has two fragments of 11.5 and 7.0 kb identified by pUC19.

Figure 5.4

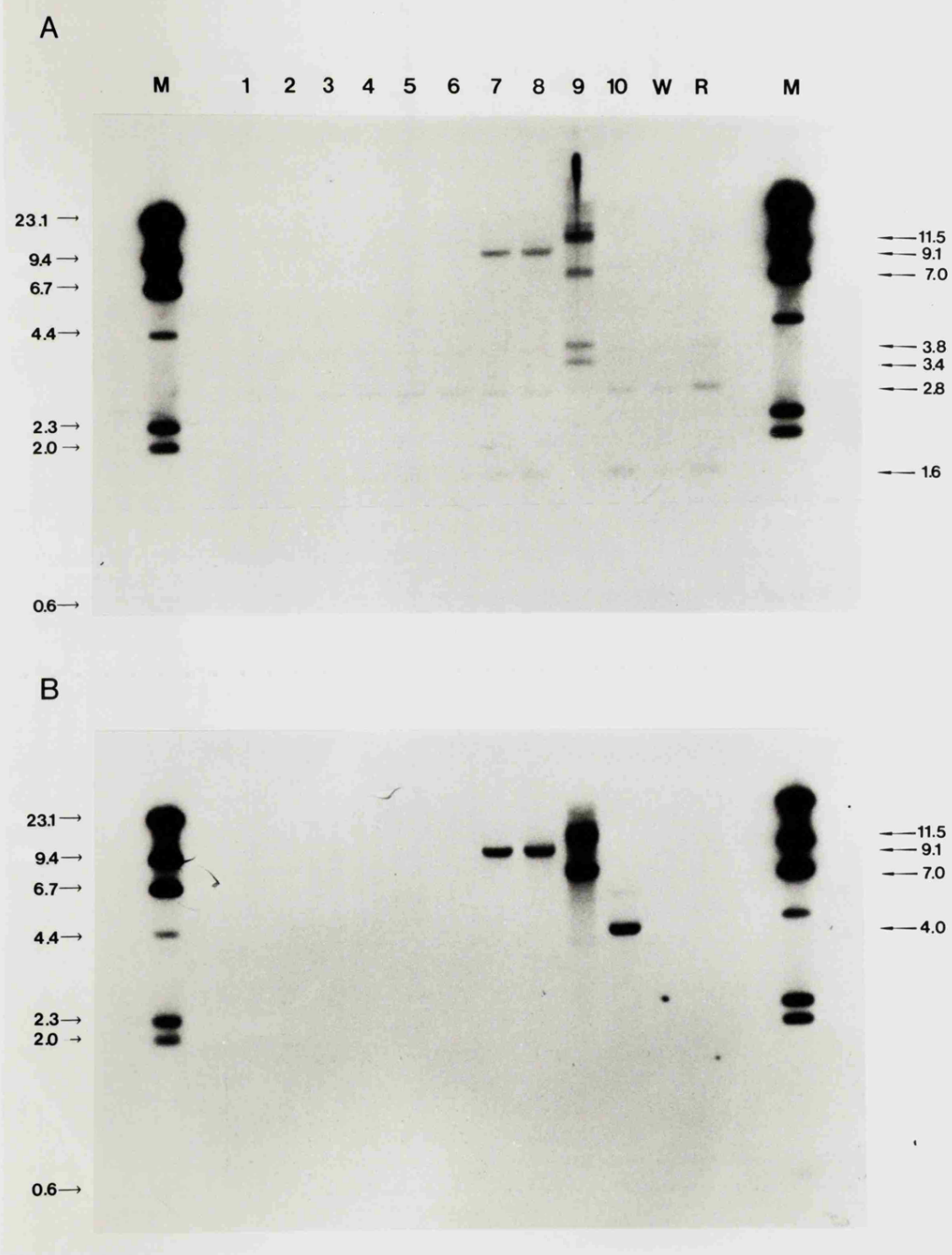

A
M
1
2
3
4
5
6
7
8
9
10
W
R
M
23.1
9.4
6.7
4.4
2.3
2.0
0.6
11.5
9.1
7.0
3.8
3.4
2.8
1.6
B
23.1
9.4
6.7
4.4
2.3
2.0
0.6
11.5
9.1
7.0
4.0

Figure 5.5

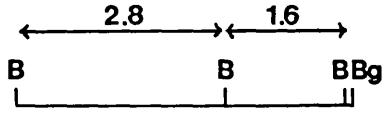
The restriction fragments expected in Type I and Type III transformants obtained by transformation with pAL8.4

A. The 8.4 kb BglII - KpnI fragment hybridized to BamHI fragments of 2.8 and 1.6 kb in the genomic DNA of the two control strains (W and R) and six transformants (1-6). No DNA sequences homologous to pUC19 were present in the genomic DNA. Therefore the control strains and the six transformants exhibit the same pattern upon hybridization indicating that these six transformed strains may be classified as Type III transformants.

B. The genomic DNA of two transformants (7 and 8) has BamHI fragments of 2.8 and 1.6 kb that hybridize to the 8.4 kb fragment and a BamHI fragment of 9.1 kb which hybridizes to both the 8.4 kb fragment and pUC19. The restriction fragments identified in these two strains suggests that they may be classified as Type I transformants.

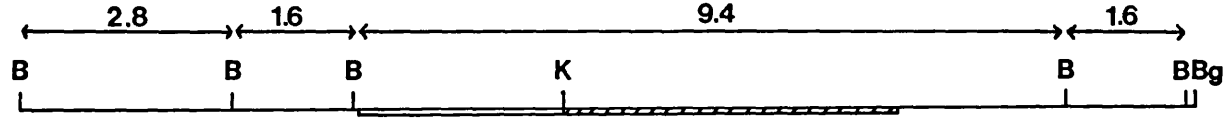
FIGURE 5.5

A



B BamH I
Bg BglII
K KpnI
A.nidulans DNA
pUC19
λ DNA

B



1kb

Figure 5.6

The analysis of ten QUTD transformants by DNA hybridization

Genomic DNA, digested with KpnI, from ten QUTD transformants (1-10) and two control strains, R153 (W) and GD22 (R), was hybridized to either the 8.4 kb BglII - KpnI fragment or pUC19 which were labelled with α^{32}P-dCTP. The molecular weight markers (M) are HindIII digested λ DNA.

A. An autoradiograph of the KpnI digested genomic DNA samples hybridized to the 8.4 kb BglII - KpnI fragment. The 8.4 kb fragment hybridizes to a 14.5 kb KpnI fragment in the genomic DNA of the two control strains (W & R) and seven transformants (1-6 & 10). Fragments of 14.5 and 11.9 kb are identified in the genomic DNA of two transformants, number 7 and 8, and transformant number 9 exhibits a complex pattern upon hybridization.

B. An autoradiograph of the KpnI digested genomic DNA samples hybridized to BamHI digested pUC19. The genomic DNA of the two control strains (W & R) and six transformants (1-6) does not contain sequences homologous to pUC19. A KpnI fragment of 14.0 kb is identified in the genomic DNA of two transformants, number 7 and 8, and DNA sequences homologous to pUC19 are also present in the genomic DNA of transformant number 9 and 10.

Figure 5.6

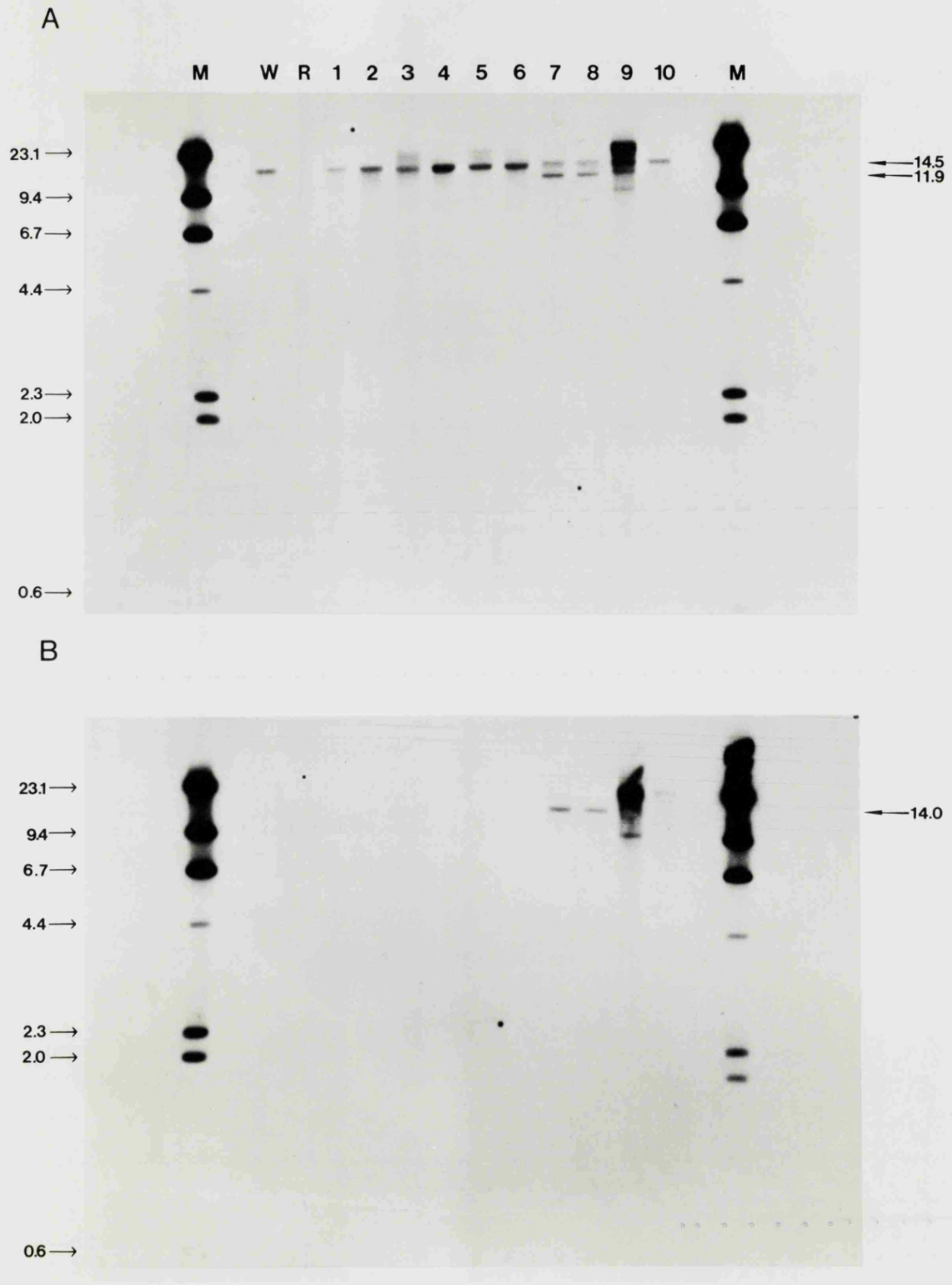

A
M
W
R
1
2
3
4
5
6
7
8
9
10
M
23.1
9.4
6.7
4.4
2.3
2.0
0.6
14.5
11.9
B
23.1
9.4
6.7
4.4
2.3
2.0
0.6
14.0

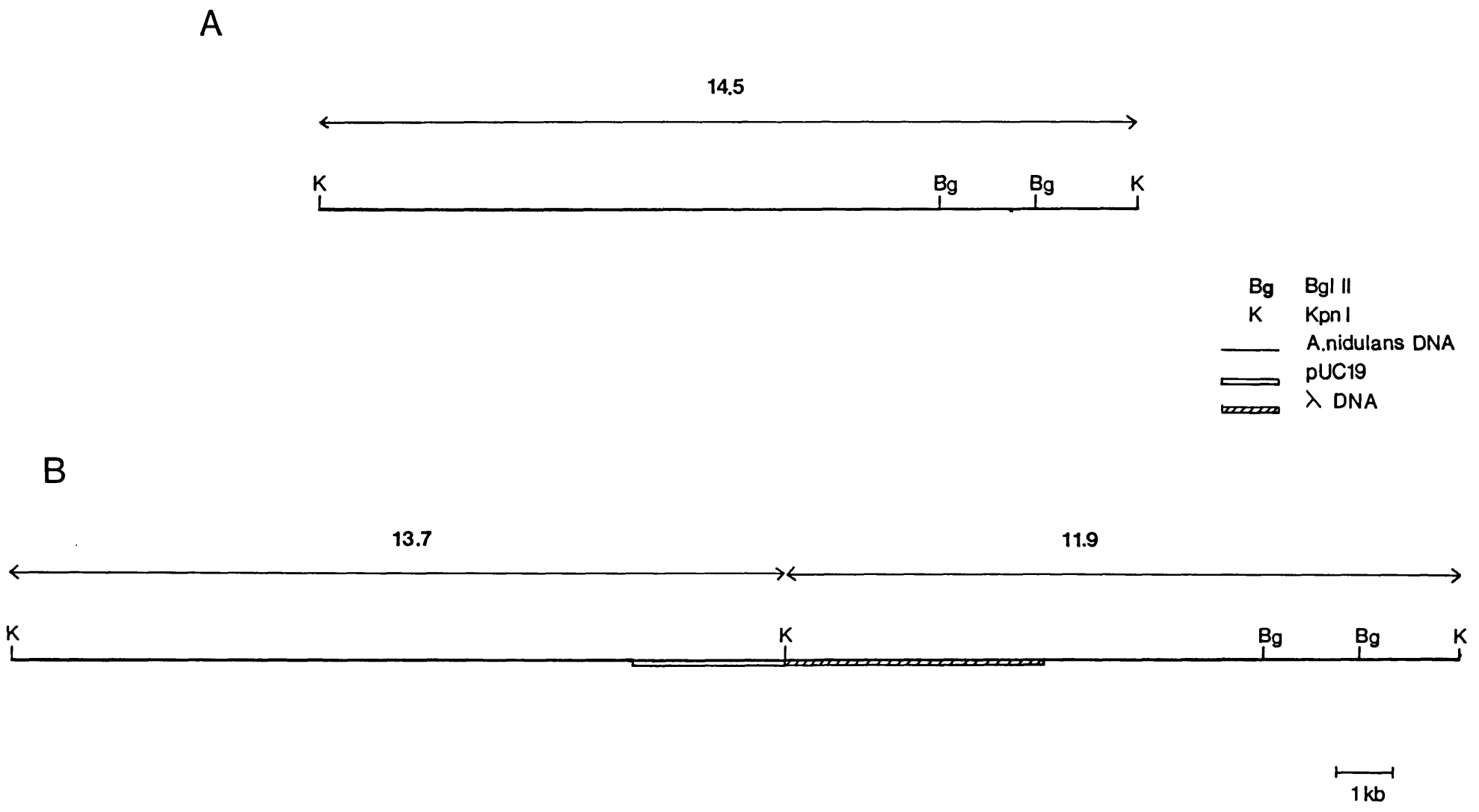
Figure 5.7

The restriction fragments expected in Type I and Type III transformants obtained by transformation with pAL8.4

A. The genomic DNA of the two control strains, R153 (W) and GD22 (R), and six QUTD transformants (1-6) contains a 14.5 kb KpnI fragment that hybridizes to the 8.4 kb BglII - KpnI fragment and no sequences homologous to pUC19. The hybridization pattern is consistent with the six QUTD transformants being classified as Type III transformants.

B. The genomic DNA of two QUTD transformants, number 7 and 8, contains two KpnI fragments of 14.5 and 11.9 kb that hybridize to the 8.4 kb BglII - KpnI fragment and a 14.0 kb KpnI fragment that hybridizes to pUC19. The hybridization pattern is consistent with these two strains being Type I transformants.

FIGURE 5.7


A
14.5
K
Bg
Bg
K
Bg Bgl II
K Kpn I
A.nidulans DNA
pUC19
λ DNA
B
13.7
11.9
K
K
Bg
Bg
K
1 kb

used as positive and negative controls respectively. The results are shown in Table 5.3A. Protoplasts exposed to pAL6.1, pAL7.0 or pBR325 DNA yielded no colonies upon plating even after extended incubation. Colonies were obtained from protoplasts transformed with λQ1, pAL8.4 or pEH1 DNA after 48 hours incubation and were forming conidiospores by 72 hours of incubation. The transformation frequencies obtained with these vectors are shown in Table 5.3A. All the colonies obtained by transformation with pAL8.4 and pEH1 were picked and purified by single colony isolation on supplemented minimal medium. When tested all the transformed strains isolated were able to utilise quinate as a carbon source (QUT+) and were uridine auxotrophs.

The ability of the plasmids pAL8.4 and pEH1 to transform a qutD strain was confirmed by the transformation of protoplasts with appropriate amounts of these plasmids. Protoplasts exposed to λQ1 DNA, pDJB1 and pBR325 DNA were used as controls (Table 5.3B). Colonies were observed on plates of protoplasts transformed with pAL8.4, pEH1 and the controls λQ1 and pDJB1 DNA. No colonies were obtained from plates of protoplasts exposed to pBR325 DNA showing that any colonies obtained above were not a result of reversion of the qutD mutation.

It is apparent that the QUTD gene is not equivalent to the activator encoding qa-1F gene of N.crassa as the DNA

fragments to the right of the QUT enzyme structural genes on λQ1, that contain sequences homologous to the qa-1F gene, are unable to transform a qutD mutant strain. Instead, the transformation experiments suggest that the QUTD gene is located to the left of the QUT enzyme structural genes in λQ1 within a 3 kb HindIII-BglII fragment of A.nidulans DNA that is common to both the pAL8.4 and pEH1 plasmids (Figure 5.3) and which has no homology detectable by hybridization to the qa-1F gene (Da Silva, 1985).

5-4 ANALYSIS OF THE TRANSFORMED qutD STRAINS

A number (10) of qutD strains transformed with pAL8.4 were analysed by DNA hybridization (Southern, 1975) using homologous DNA probes to establish the presence and position of the transforming DNA within the genome.

The strains were picked and purified by single colony isolation on supplemented minimal medium. High molecular weight genomic DNA was prepared from mycelium grown in liquid culture as described previously (Chapter 2). The concentration of the DNA in the preparations was measured spectrophotometrically and its quality and size estimated by gel electrophoresis against appropriate DNA size markers. Genomic DNA prepared from a wild-type (R153) and qutD,pyrG strains were used as controls.

Samples of genomic DNA (3 μg), from each of the above

strains, digested with either BamHI or KpnI were electrophoresed through a 0.8% (w/v) agarose gel. The DNA was transferred to a nylon filter, immobilised and then hybridized to either BamHI digested pUC19 or to the 8.4 kb DNA fragment labelled with $\alpha^{32}P$ - dCTP (Amasino, 1986; Feinberg and Vogelstein, 1984). The 8.4 kb fragment was that used previously in the construction of pAL8.4 (Section 5.3). The resulting autoradiographs are shown in Figure 5.4 and 5.6.

In the wild-type (R153) and qutD pyrG control strains the 8.4 kb fragment DNA probe identifies two restriction fragments of 2.8 and 1.6 kb in the BamHI digested genomic DNA (Figure 5.4A, 5.5) and a single restriction fragment of 14.5 kb in the KpnI digested genomic DNA (Figure 5.6A, 5.7). As expected no restriction fragments were identified by the vector pUC19 DNA probe (Figure 5.4B, 5.6B).

Six of the transformed strains also contained no vector pUC19 DNA and the same restriction fragments were identified by the 8.4 kb fragment DNA probe as in the control strains (Figure 5.4, 5.5, 5.6 and 5.7). These six transformed strains may be classified as Type III transformants as defined by Hinnen et al (1978).

In the remaining four transformed strains, novel restriction fragments are identified by the vector pUC19 DNA probe within the genomic DNA showing that they contain vector DNA

sequences. Two of these strains (nos. 7 and 8) contain a 9.1 kb fragment identified by both the pUC19 and the 8.4 kb fragment DNA probes in BamHI digested genomic DNA (Figure 5.4, 5.6). The size of this restriction fragment is consistent with that expected from tandem integration of the transforming pAL8.4 DNA into the genome at homologous DNA sequences within the QUT gene cluster. This is confirmed by the sizes of the restriction fragments identified in the KpnI digested genomic DNA, where an 11.9 kb restriction fragment is identified by the 8.4 kb fragment DNA probe and a 14.0 kb fragment is identified by both DNA probes (Figure 5.7). These two transformed strains (nos. 7 and 8) may therefore be classified as Type I transformants (Hinnen et al, 1978).

The remaining two transformed strains (nos. 9 and 10) that hybridize to vector DNA exhibit a more complex pattern of bands on the autoradiographs (Figure 5.4B, 5.6B) due to novel restriction fragments created by integration of the transforming DNA into the genome. The position at which the transforming DNA has integrated cannot be determined.

Thus four of the ten transformed qutD strains analysed contain pUC19 DNA sequences from pAL8.4 within their genome, the remaining six strains being indistinguishable from the control strains after "repair" of the qutD mutation by the transforming DNA.

5-5 THE REGULATION OF THE QUT ENZYME STRUCTURAL GENES IN TRANSFORMED qutD STRAINS

A number of transformed qutD strains were examined to determine whether they exhibit normal regulation of the QUT enzyme structural genes. Of the strains tested for production of the quinate specific enzymes under inducing and non-inducing growth conditions, 20 were transformed with λQ1 DNA, 80 with XbaI digested λQ1 DNA and 50 with the plasmid pAL8.4. The wild-type (R153) and qutD pyrG mutant strain were used as controls. All the transformed qutD strains were picked and purified by single colony isolation on minimal medium containing quinate as a carbon source. Conidiospores from these colonies were used to "patch" a thick inoculum onto minimal medium containing either quinate or glycerol as a carbon source and incubated for 24 hours at 37°C. Approximately equal sized blocks of agar containing mycelium were used in the qualitative "spot test".

This test was adapted for use in A.nidulans from a method described by Partridge et al (1972) for the visual identification of constitutive quinic acid mutants in N.crassa. Salamon and Davies (1953) reported that $FeCl_3$ produced a strong colour with protocatechuic acid, which should be accumulated by any strain of N.crassa which has activity for the enzymes leading from quinic acid to protocatechuic acid provided that quinic acid is supplied

and protocatechuic acid oxygenase is essentially absent or inactive. These requirements would be met by a specific constitutivity of the pre-protocatechuic acid enzymes together with an absence of protocatechuic acid oxygenase by, for example, exclusion of oxygen from the system. Mycelium grown on glycerol as a carbon source will only produce protocatechuic acid and hence a colour reaction with $FeCl_3$ if the quinate specific enzymes are produced constitutively. This method is described in detail in Chapter 2, Section 2-1.5.

All (150) of the transformed qutD strains exhibited normal regulation of the QUT enzyme structural genes, that is when grown under inducing conditions, in the presence of quinate, the three quinate specific enzymes were produced, however under non-inducing growth conditions, on medium containing glycerol, these enzymes were not produced.

DISCUSSION

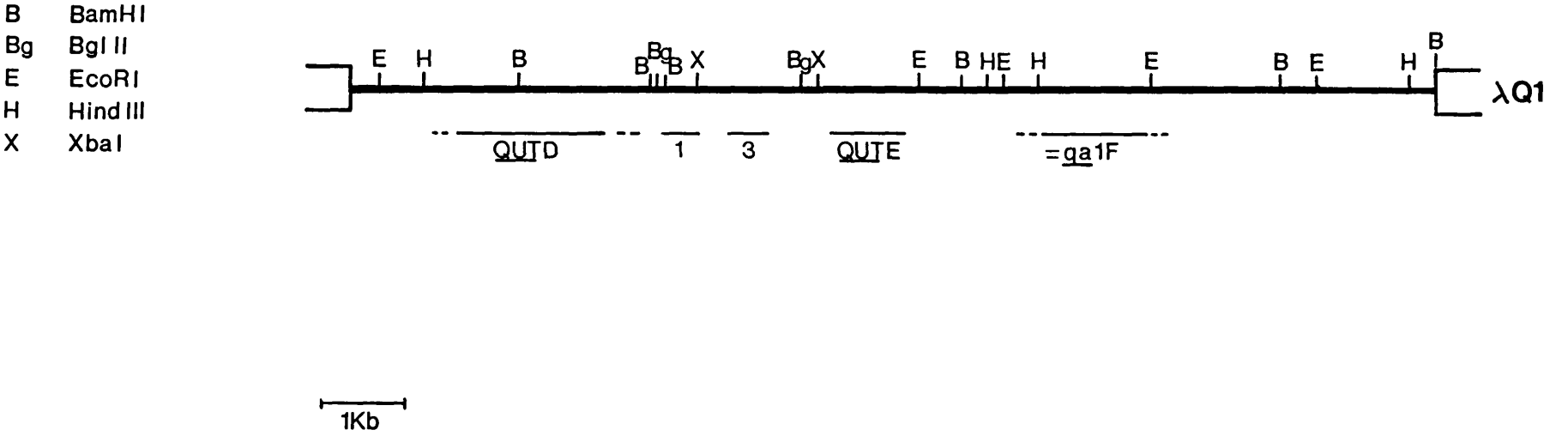
Earlier studies of the pleiotrophic non-inducible qutD8 mutant in heterozygous diploid strains had shown that the qutD8 allele was fully recessive to wild-type with respect to growth on quinic acid and that all three quinate specific enzyme activities were induced to the same level as in the wild-type. In view of thèsedata it was initially suggested that the qutD8 allele was a recessive mutation in a regulatory gene whose product was actively required for the

Figure 5.8

A restriction map of λQ1 showing the location of the QUTD gene

A restriction map of the A.nidulans DNA within λQ1 showing the position of the QUTD gene in a 3 kb BglII - HindIII fragment. The location of the QUTE gene and DNA sequences homologous to the N.crassa qa-3 (1), qa-4 (3) and qa-1F genes is also indicated.

FIGURE 5.8


B BamHI
Bg BglII
E EcoRI
H HindIII
X XbaI
E H B Bg B B X Bg X E B HE H E B E H B
λQ1
QUTD
1
3
QUTE
=ga1F
1Kb

expression of the QUT enzyme structural genes, that is it encoded an activator protein (Hawkins et al, 1984). Similar genetic studies in N.crassa indicated that the qa-1F gene encoded a positive acting gene product and this was confirmed by a detailed molecular analysis of the qa gene cluster (reviewed in Giles et al, 1985).

The transformation of a qutD mutant strain and the analysis of further newly isolated qutD mutant alleles has led to a revision of our original hypothesis. Transformation of a qutD strain with various fragments from the recombinant phage λQ1 DNA has revealed that the QUTD gene is located within a 3 kb BglII-HindIII fragment to the left of the QUT enzyme structural genes on λQ1 (Figure 5.8). This DNA fragment exhibits no homology detectable by hybridization to the qa-1F gene of N.crassa which encodes the activator protein and is known to have homology to a 1.7 kb EcoRI fragment to the right of the QUT enzyme structural genes on λQ1 DNA (Da Silva, 1985). As the QUTD gene is not located within DNA sequences homologous to the qa-1F gene it is unlikely that it encodes an activator protein in A.nidulans.

Hybridization studies on λQ1 DNA have revealed that this 3 kb BglII-HindIII fragment contains sequences with homology to the qa-y gene of N.crassa (Hawkins, personal communication) (Figure 1.5). The qa-y gene is one of two, the other being qa-x, additional presumptive qa structural genes that were initially identified by DNA-RNA

hybridization studies using cloned qa DNA (Patel et al, 1981). No mutations have yet been identified within these two genes but they encode quinate inducible mRNAs and their DNA has been sequenced. The polypeptides produced by the qa-x and qa-y genes are 340 and 537 amino acids long respectively (Geever and Huiet; unpublished data). These cloned DNAs are unable to transform any of the existing qa mutants (Giles et al, 1985).

It has been postulated that one of these two genes, qa-y, might encode a permease for the uptake of quinic acid in N.crassa. Indirect evidence for the presence of a permease in this organism has been obtained during studies on the uptake of radioactive quinate or shikimate in strains having different combinations of mutations in the repressor (qa-1S) and activator (qa-1F) encoding genes (Rines, 1973). It was found that qa-1S^{c},qa-1F^{+} mutants, which express the qa genes constitutively under non-inducing conditions due to loss of a functional repressor, were able to take up the inducing quinate molecules much more rapidly than a wild-type strain, which takes up quinate at a comparable rate only after a considerable time lag. For both the mutant and wild-type strains uptake of quinate was inhibited by the presence of glucose. A qa-1S^{-},qa-1F^{+} strain, which is unable to induce the qa genes on quinate possibly due to the production of a super-repressor protein, and a qa-1S^{+},qa-1F^{-} strain, which also cannot induce the qa genes due to the loss of a functional activator protein, fail to take up significant

amounts of the inducer quinate either with or without glucose present. It was considered that these results implied that there was a permease involved in the uptake of quinate in N.crassa whose synthesis is regulated by the qa-1S and qa-1F genes.

My own efforts have been directed towards the transformation of qutA mutant strains (Chapter 6) but parallel work in the laboratory has provided evidence that the QUTD gene might encode a permease for the uptake of quinate in A.nidulans (Whittington et al, 1987).

In growth tests using quinate (1% w/v) at pH3.5 or 6.5 in minimal medium plates, it was observed that the qutD8 strain grew strongly at the low pH but exhibited the typical qut$^-$ phenotype of no growth at pH6.5, the normal pH for growth of A.nidulans. The wild-type control grew strongly at both pHs. In contrast, the non-inducible qutA4 strain or other mutants deficient for a single quinate specific enzyme activity retained their mutant phenotype on quinate at low pH. Nine new recessive qutD mutants identified by their inability to complement the qutD8 mutation in heterozygous diploid strains and tightly linked (<0.7% units) to the qutD8 allele, have the same phenotypes as the qutD8 allele at pH3.5 and 6.5, that is growing strongly at pH3.5 but not at pH6.5. This phenotype is therefore locus specific and not allele specific within the QUTD gene.

TABLE 5.4

Table 1. The effect of pH upon enzyme induction in *qut*D312. Condispores of a wild-type strain (R153) and the mutant strain *qut*D312 were grown for 15 h in glucose minimal medium and the resulting mycelium harvested aseptically by filtration. For each strain the pad was washed briefly with pre-warmed (37 °C) minimal medium without carbon source and divided to four equal parts which were separately transferred to fresh growth media with 0.5% (w/v) quinic acid at the final pH values shown. Following incubation for a further 5 h at 37 °C the mycelium was harvested by filtration, washed and stored at −18 °C. The pH of the media following enzyme induction were found to be for the wild-type strain, pH 6.95, 6.08, 5.40 and 3.54 and for *qut*D312 pH 6.90, 5.53, 4.38 and 3.55

pH of inducing medium	Enyzme specific activity					
	Dehydrogenase (1)		Dehydroquinase (2)		Dehydratase (3)	
	R153 (*qut*$^+$)	*qut*D312	R153 (*qut*$^+$)	*qut*D312	R153 (*qut*$^+$)	*qut*D312
6.5	0.44	0.07	53.9	0.3	0.26	nd
5.5	0.36	0.08	44.2	1.4	0.26	0.04
4.5	0.42	0.08	56.5	6.2	0.19	0.08
3.5	0.39	0.12	42.6	7.6	0.38	0.10

Cell free extracts were prepared and the enzyme specific activities assayed following procedures given under Methods. The activities of catabolic dehydroquinase were assayed in aliquots of the cell free extracts heated at 70 °C for 10 min to inactive biosynthetic dehydroquinase (Charles et al. 1986).

Specific activities for dehydrogenase (*1*) and dehydratase (*3*) are μmol product min^{-1} mg^{-1} protein; for dehydroquinase (*2*), A240 min^{-1} mg^{-1} protein (1 unit is OD240 of 0.1 min^{-1}); *nd* not detected

TABLE 5.5

Table 2. Enzyme formation at pH 3.5 in *qut*D312. The two strains were treated as described in Table 1 but the mycelium grown in glucose minimal medium (pH 6.5) was resuspended in fresh growth media contianing 0.05 M citric acid – 0.1 M Na_2HPO_4 buffer at pH 3.5 with either glucose or quinic acid or no carbon source and incubation continued for a further 5 h at 37 °C. The preparation of cell free extracts, enzyme assay procedures and enzyme specific activities were the same as in Table 1. The pH of the media at the end of the second incubation was unaltered. The two strains were grown with quinic acid at pH 3.5 in minimal medium for 21 h, when harvested for enzyme assay and the media had pH 3.6

Treatment: Carbon source	Enyzme specific activities					
	Dehydrogenase (1)		Dehydroquinase (2)		Dehydratase (3)	
Glucose grown mycelium (pH 6.5) transferred to:	R153 (*qut*$^+$)	*qut*D312	R153 (*qut*$^+$)	*qut*D312	R153 (*qut*$^+$)	*qut*D312
Citrate buffer	0.08	0.10	nd	nd	nd	nd
Citrate + Glucose	nd	nd	nd	nd	nd	nd
Citrate + Quinic acid	0.21	0.15	9.6	8.1	0.08	0.06
Mycelium grown Quinic acid[1]	0.30	0.18	24.5	31.0	0.19	0.23

Whittington et al (1987)

When a *qutD312* mutant strain cultured in liquid glucose medium was transferred to quinic acid growth medium at pH3.5 significant induction of the quinate specific enzymes was observed consistent with the observed effect of pH on growth, but no induction of these enzymes was observed at pH6.5 (Table 5.4). Growth of the wild-type strain (R153) or the *qutD312* mutant in glucose medium at pH3.5 did not induce the formation of these enzymes (Table 5.5).

Among revertants of *qutD* mutants, selected for growth on quinate (pH6.5), a high proportion were shown to form the quinate specific enzymes constitutively as the result of a recessive mutation at a closely linked gene, designated *qutR* (quinate utilization regulator). This mutation also serves as genetic suppressor of *qutD* mutations but not of non-inducible *qutA* mutations (Grant *et al*, 1988). Growth of a *qutD qutR* mutant strain was poor, qualitatively intermediate between that of the *qutD* mutant and the wild-type strain, although the enzyme levels present were characteristic of fully induced wild-type strains. This suggests that the growth of the suppressed *qutD* strain is limited by the uptake of quinate rather than by a deficiency in enzyme induction.

All the above results strongly suggest that the *QUTD* gene encodes an essential component of a permease system required for the transport of quinate into the mycelium. The effect of low pH is to suppress the ionisation of quinic acid,

which has an apparent pKa of 3.6 (C.F Roberts, personal communication), enabling sufficient non-dissociated quinic acid to enter the mycelium by diffusion, inducing formation of the quinate specific enzymes and allowing growth. At pH values above 4.5 the major proportion of the compound is present in solution as the conjugate base quinate ion which can only enter the mycelium with the aid of a permease. Direct evidence for a permease might have been obtained through studies on the uptake of radioactively labelled quinate in wild-type and qutD mutant strains, similar to the studies in N.crassa by H. Rines (1973), however the cost of such an experiment was prohibitive in our laboratories.

The 3 kb BglII-HindIII fragment of λQ1 DNA, that exhibits homology by hybridization to the qa-y gene of N.crassa and which is considered to contain the QUTD gene, has been sequenced by Dr A.R Hawkins at the University of Newcastle upon Tyne. Results from the analysis of the DNA sequence are discussed in Chapter 7.

CHAPTER 6

IDENTIFICATION OF THE A.NIDULANS QUTA GENE ON λQ1 PHAGE DNA

INTRODUCTION

In earlier studies of quinic acid non-utilising mutants in A.nidulans, the qutD8 mutant allele, which is non-inducible for all three quinate specific enzyme activities (Hawkins et al, 1982) was shown to be fully recessive to wild-type in heterozygous diploid strains (Hawkins et al, 1984). At this time it was considered that the QUTD gene encoded an activator protein which was absent in the qutD8 mutant. The transformation of a qutD strain (Chapter 5) together with the isolation and analysis of further qutD mutant alleles (Whittington et al, 1987) has led to a revision of the original hypothesis. Newly obtained evidence strongly suggests that the QUTD gene encodes an essential component of a permease system required for the transport of quinate into the mycelium (Whittington et al, 1987). In view of this conclusion, the question of the role of the QUTA gene in the regulation of the QUT gene cluster must be re-examined.

The qutA4 allele was shown to be semi-dominant for growth on quinic acid in heterozygous diploid strains and to exhibit an intermediate phenotype in the induction of all three

quinate specific enzymes to 60-80% of wild type levels. Similar levels were observed when the qutA4 allele was in trans with the qutB42 allele, which lacks quinate dehydrogenase activity, in heterozygous diploid strains. It was concluded from these observations that the qutA4 mutation defines a regulatory gene repressing the expression of all three QUT structural genes and hence confers a non-inducible phenotype (Hawkins et al, 1984). Therefore at this time it was considered that the QUTA gene encoded a repressor protein.

In N.crassa the qa-1S gene has been identified as encoding a repressor protein. One class of mutations at this locus, qa-1S^{-}, are non-inducible for the three quinate specific enzyme activities and are partially dominant to the wild-type in heterokaryons (Rines, 1968). Constitutive mutants (qa-1S^{c}), which produce high levels of the three quinate specific enzymes in the absence of the inducer, have been isolated as quinic acid utilising revertants of qa-1S^{-} mutant strains (Valone et al, 1971) and also directly by ultra-violet irradiation of a wild-type strain (Partridge et al, 1972). The constitutive mutations map approximately to the qa-1 locus and are recessive to wild-type in heterokaryons (Partridge et al, 1972). It has been postulated that these recessive constitutive mutations (qa-1S^{c}) encode an inactive repressor molecule and the dominant non-inducible mutations (qa-1S^{-}) encode super-repressing molecules. In contrast, no constitutive

mutants have been isolated as revertants of strains containing mutations (qa-1F$^-$) within the gene encoding the activator protein (Valone et al, 1971).

Recently 62 new non-inducible qut mutants have been isolated of which 53 failed to complement the qutA4 mutation in heterozygous diploid strains. The majority were fully recessive to wild-type but a few were semi-dominant or dominant. Dominant mutation (qut214), which could potentially be a qutA allele, was isolated however it cannot be assigned to the QUTA or QUTD loci on present data. In seeking to isolate constitutive mutant strains, reversion studies were undertaken using various qutA$^-$ mutants, including qutA4, however no closely linked constitutive mutants were obtained amongst some 300 revertants tested (Grant et al, 1988). The inability to isolate constitutive mutations within the QUTA gene, which is considered to encode the repressor protein in A.nidulans, is in direct contrast to the ease with which such mutations occur within the qa-1S gene of N.crassa. The average apparent rate of induction of constitutive mutants from qa-1S$^-$ mutants was 7.3×10^{-5} per viable conidium and they were probably induced in wild-type strains at a 10-fold higher frequency than qa-1S$^-$ mutants (Partridge et al, 1972). It has also been shown that all qa-1S$^-$ mutants can revert to yield the constitutive phenotype after ultra-violet irradiation.

These fundamental differences between the mutations in the QUTA and qa-1S genes suggests that the QUTA gene may not encode a repressor protein but may instead encode the activator protein and hence be equivalent to the qa-1F gene of N.crassa. This revision of our original theory leaves open the question of which gene encodes the repressor protein. Constitutive mutant strains, similar to the qa-1S^{c} mutant strains of N.crassa, have been obtained amongst revertants of qutD mutants (Whittington et al, 1987). These constitutive mutations are recessive and are not within the QUTA locus but identify a closely linked (3% recombination frequency) gene locus, designated qutR. None of the 65 non-inducible strains analysed to date have a mutation at this locus. Genetic mapping has shown the gene order to be QUTD - QUTA - QUTR (Grant et al, 1988). It is possible that the qutR gene locus may encode a repressor protein.

A region of DNA sequence homology within the 1.7 kb EcoRI fragment of A.nidulans DNA on λQ1 has been identified using a 2.1 kb BamHI-XbaI restriction fragment, wholly within the coding sequence of the qa-1F gene of N.crassa, as a DNA probe in hybridizations (Da Silva, 1985; Figure 1.5). In order to establish the biological function of this DNA, λQ1 DNA and plasmid vectors containing fragments of A.nidulans DNA, which span this region of homology, have been used in the transformation of a qutA mutant strain of A.nidulans. The role of the QUTA gene in the regulation of

the QUT gene cluster has also been studied in certain transformed qutA strains.

6-1 TRANSFORMATION OF A qutA MUTANT STRAIN WITH λQ1 DNA AND PLASMID pAL3.7

A qutA4 pyrG double mutant strain was constructed for use in transformation experiments to identify and locate the QUTA gene (Chapter 3). However the qutA4 mutant allele is semi-dominant in diploid strains and it was considered that integration of the wild-type gene together with the qutA4 allele might yield transformants not readily recovered by growth on quinic acid. Hence a strain containing a fully recessive qutA mutant allele, qutA361, was used. Since a qutA361 pyrG double mutant was not available, the plasmid pDJB1, which contains the N.crassa pyr4 gene, could not be used as a positive control for the efficiency of transformation (Ballance and Turner, 1985).

The plasmid pAL3.7 contains a 3.7 kb BamHI fragment isolated from phage λQ1 subcloned into the plasmid pBR322 (Da Silva, 1985). The 1.7 kb of λQ1 DNA containing DNA sequences homologous to the qa-1F gene of N.crassa is present within this 3.7 kb BamHI fragment and the plasmid pAL3.7 was previously used in transformation experiments with a qutD mutant strain of A.nidulans (Chapter 5; Figure 5.1).

The method used for the preparation of qutA361 protoplasts

Table 6.1

Transformation of the qutA mutant strain, R153 qut A361, with λQ1 DNA and the plasmid pAL3.7

EXPERIMENT NUMBER	DNA USED	AMOUNT OF DNA (μg)	TOTAL NUMBER OF COLONIES	TRANSFORMATION FREQUENCY
	λQ1	15	10 [a]	0.7
1	pAL3.7	15	0 [a]	0
	No DNA	-	0 [a]	0
	λQ1	15	14 [a]	1.0
2	pAL3.7	15	0 [a]	0
	No DNA	-	0 [a]	0

(a) *qut*$^+$ selection

The transformation frequency is expressed as the number of colonies /μg transforming DNA. The number of protoplasts/ml of the protoplast suspension and their percentage regeneration on supplemented medium in the above experiments is as follows - Experiment 1 : 1.1 x 10^7/ml, 15%; Experiment 2 : 1.3 x 10^7/ml, 14%.

and their subsequent transformation was that described in Chapter 4. Suitable amounts of phage or plasmid DNA were added to 200 µl aliquots of protoplasts and these were subsequently plated onto the appropriately supplemented minimal medium containing quinate (pH6.5) as a carbon source and incubated at 37°C for up to 96 hours. Protoplasts to which no DNA was added were used as a control to monitor reversion of the qutA361 mutant strain.

The results of two such experiments are shown in Table 6.1. Protoplasts transformed with λQ1 DNA yield qut$^+$ transformants at a frequency of around 1/ µg DNA. In neither experiment were colonies observed on plates of protoplasts exposed to plasmid pAL3.7, even after prolonged incubation.

It can be concluded that the QUTA gene is present on the recombinant phage λQ1 DNA by the ability of this DNA to transform a qutA mutant strain. The plasmid pAL3.7, which contains DNA sequences homologous to the qa-1F gene of N.crassa is unable to transform a qutA mutant strain. The qa-1F gene produces quinate inducible mRNA in N.crassa of around 3 kb (Patel and Giles, 1985) and thus if the QUTA gene is of a similar size, the entire gene may not be contained within the 3.7 kb BamHI fragment in plasmid pAL3.7.

6-2 TRANSFORMATION OF THE *qutA* MUTANT STRAIN WITH PLASMIDS pAL6.1, pAL7.0 AND pAL3.7

As the entire *QUTA* gene might not be contained within plasmid pAL3.7, two larger restriction fragments isolated from λQ1 DNA and containing DNA sequences homologous to the *qa-1F* gene of *N.crassa* were used in the transformation of the *qutA361* mutant strain. These are contained within plasmids pAL6.1 and pAL7.0, whose construction is described in Chapter 5 (Section 5-3). Plasmids pAL6.1 and pAL7.0 contain *Bam*HI restriction fragments of 6.1 and 7.0 kb respectively subcloned into pBR325 (Figure 5.2) and overlap by the 3.7 kb *Bam*HI fragment of pAL3.7 (Figure 5.3).

Protoplasts from strain *qutA361* were transformed with λQ1 DNA and plasmids pAL3.7, pAL6.1, and pAL7.0. The results are shown in Table 6.2A. Protoplasts transformed with either λQ1 DNA, pAL6.1 or pAL7.0 yielded *qut*$^+$ transformants. No colonies were obtained from protoplasts exposed to pAL3.7 or no DNA.

These results suggest that the *QUTA* gene is located within 9.4 kb of *A.nidulans* DNA on λQ1 spanned by the DNA restriction fragments within plasmids pAL6.1 and pAL7.0. As 3.0 kb of the 9.4 kb of *A.nidulans* DNA contains the *QUT* enzyme structural genes, the *QUTA* gene is likely to be present in the 6.4 kb of DNA to the right of these genes on λQ1. However, an anomaly is that the 3.7 kb *Bam*HI fragment,

Table 6.2

Transformation of (A) R153 qutA361 and (B) R153 qutA444 to determine the location of the QUTA gene in λQ1

	DNA USED	AMOUNT OF DNA (μg)	TOTAL NUMBER OF COLONIES	TRANSFORMATION FREQUENCY
(A)	λQ1	20	8 [a]	0.4
	pAL37	10	0 [a]	0
	pAL6.1	10	10 [a]	1.0
	pAL7.0	10	8 [a]	0.8
	No DNA	-	0 [a]	0
(B)	λQ1	20	13 [a]	0.7
	pAL37	10	0 [a]	0
	pAL61	10	13 [a]	1.3
	pAL70	10	26 [a]	2.6
	pAL8.4	10	0 [a]	0
	No DNA	-	0 [a]	0

(a) qut$^+$ selection

In Experiment (A) there were 1.9 x 10^7 R153 qutA361 protoplasts/ml of the protoplast suspension of which 33% were capable of regeneration on supplemented medium. In experiment (B) there were 1.5 x 10^7 R153 qutA444 protoplasts/ml of the protoplast suspension of which 25% were capable of regeneration on supplemented medium

which is common to pAL6.1 and pAL7.0, alone will not transform the *qutA361* mutant strain.

In order to test whether this anomalous result is specific to the particular *qutA* allele used, a strain containing a different fully recessive *qutA* allele, *qutA444*, was used as a recipient for transformation. The transformation experiment was repeated except that the plasmid pAL8.4 was included. Plasmid pAL8.4, whose construction was described in Chapter 5 (Section 5-3), contains an 8.4 kb *Bgl*II-*Kpn*I fragment of Q1 DNA subcloned into pUC19 (Figure 5.2). The results of the transformation are shown in Table 6.2B. Protoplasts transformed with either pAL6.1 or pAL7.0 again yielded *qut*$^+$ transformants suggesting that the *QUTA* gene is located to the right of the *QUT* enzyme structural genes on λQ1. This is confirmed by the inability of plasmid pAL8.4, which contains the entire region of *A.nidulans* DNA to the left of the *QUT* enzyme structural genes on λQ1, to transform the *qutA* mutant strain. The plasmid pAL3.7 is unable to transform the *qutA444* strain showing that this result is not specific to a particular *qutA* mutant allele. As yet this anomaly remains unresolved and possible reasons for the observation will be discused below.

6-3 THE REGULATION OF THE *QUT* GENE CLUSTER IN TRANSFORMED *qutA* STRAINS

A number of transformants from the *qutA* mutant strains,

Table 6.3

The qualitative analysis of QUTA transformants to determine the number exhibiting constitutive expression of the QUT genes.

TRANSFORMING DNA	NUMBER OF QUTA TRANSFORMANTS TESTED		TOTAL NUMBER OF QUTA TRANSFORMANTS	NUMBER HAVING NORMAL REGULATION	NUMBER HAVING CONSTITUTIVE EXPRESSION
	R153qutA361	R153qutA444			
λQ1	20	13	33	30	3
pAL6.1	10	13	23	23	0
pAL7.0	8	26	34	34	0

qutA361 and qutA444, were examined to discover if they exhibited normal regulation of the QUT enzyme structural genes.

Colonies from selective plates of strains qutA361 and qutA444 transformed with either λQ1 DNA, plasmids pAL6.1 or pAL7.0 were purified by single colony isolation on minimal medium containing quinate. Conidiospores from these colonies were used to "patch" a thick inoculum onto minimal medium containing either quinate or glycerol as a carbon source and the plates incubated for 24 hours at 37°C. Approximately equal sized blocks of agar containing mycelium were used in the qualitative "spot test" as described in Chapter 5 (Section 5-5). The results of these tests are shown in Table 6.3.

All (57) colonies isolated from the qutA strains transformed with plasmids pAL6.1 and pAL7.0 exhibit normal regulation of the QUT enzyme structural genes. That is, when grown under inducing conditions, in the presence of quinate, the three quinate specific enzymes are present and quinate is converted to protocatechuic acid giving a positive result in the "spot test". However when grown in the absence of quinate, on medium containing glycerol, the quinate specific enzymes are not induced producing a negative result in the "spot test". Therefore all strains transformed by the two plasmids pAL6.1 and pAL7.0 exhibit normal regulation.

In contrast, 3 of the 33 colonies from *qutA* strains transformed with λQ1 DNA produce the quinate specific enzymes under non-inducing conditions of growth and therefore exhibit constitutive expression of the *QUT* enzyme structural genes. The wild-type (R153) and *qutA* mutant (*qutA361* and *qutA444*) control strains, as expected, exhibit normal regulation of these genes.

The constitutive expression of the *QUT* enzyme structural genes in *qutA* strains transformed with λQ1 DNA will be discussed below.

6-4 ANALYSIS OF *qutA* STRAINS TRANSFORMED WITH λQ1 DNA EXHIBITING NORMAL OR CONSTITUTIVE EXPRESSION OF THE *QUT* ENZYME STRUCTURAL GENES

A number of transformed *qutA* strains, which exhibit either normal or constitutive expression of the *QUT* enzyme structural genes, were analysed to confirm the presence of the transforming DNA, λQ1, within their genome. It was also hoped that such studies might reveal differences between the strains that express the *QUT* genes constitutively in relation to those in which these genes are regulated normally.

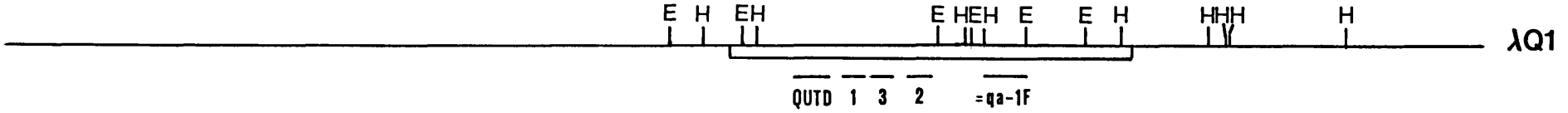
A total of 12 transformed *qutA* strains were examined. Eleven originated from the *qutA361* mutant strain of which two (nos. 3 and 11) exhibited constitutive expression of the

Figure 6.1

The position of HindIII and EcoRI restriction sites in λQ1

A restriction map of the A.nidulans and λDB286 DNA in the recombinant phage λQ1 showing the positions of the HindIII and EcoRI restriction endonuclease sites. The location of the QUTD and QUTE (2) genes and DNA sequences homologous to the N.crassa qa-3 (1), qa-4 (3) and qa-1F genes are also shown.

FIGURE 6.1



λDB286
A.nidulans DNA
E EcoRI
H Hind III

QUT genes. The twelfth was from the qutA444 strain and also constitutively expresses these genes. The remaining nine transformants are regulated normally. A wild-type strain (R153) and the qutA361 mutant strain were used as controls.

High molecular weight genomic DNA was prepared from mycelium grown in liquid culture (Chapter 2). The concentration of the DNA in the preparations was measured spectrophotometrically and its quality and size estimated by gel electrophoresis against appropriate DNA size markers. In each case the DNA was found to be greater than 30 kb in size.

The genomic DNA was digested with HindIII or EcoRI restriction endonucleases for analysis by DNA hybridization (Southern, 1975). These two enzymes have sites within both the A.nidulans and DNA in phage λQ1 (Figure 6.1). Each sample was then divided in two and separated by electrophoresis through a 0.8% agarose gel. The DNA samples were transferred to nylon filters (Hybond), immobilised and then hybridized to either λDB286 DNA or the 7 kb A.nidulans DNA fragment labelled with $\alpha^{32}P$ - dCTP (Amasino, 1986; Feinberg and Vogelstein, 1984). The phage λDB286 DNA was the vector used in the isolation of λQ1 (Hawkins et al, 1985). The 7 kb A.nidulans DNA fragment was prepared by partial digestion of plasmid pAL7.0 with BamHI and isolated from a 0.6% (w/v) low-gelling point agarose gel (Crouse et al, 1983).

The autoradiographs of HindIII digested genomic DNA hybridized to λDB286 DNA and the 7 kb A.nidulans DNA fragment are shown in Figure 6.2A, B. The sizes of the bands obtained for each transformed qutA strain and the control strains were estimated by comparison to the HindIII digested λ DNA size markers. The origin of each band is shown in Figure 6.3.

As expected the two control strains contain no λ DNA within their genome (Figure 6.2A) and when hybridized to the 7 kb A.nidulans DNA probe restriction fragments of 6.4, 4.4 and 0.6 kb are identified (Figure 6.2B). The 4.4 kb fragment contains sequences that exhibit homology to the qa-1F gene of N.crassa (Da Silva, 1985).

Eleven of the twelve transformed qutA strains, including the three (nos. 3, 11 and 12) that express the QUT genes constitutively, produce the same pattern of bands on the autoradiographs when hybridized to the two DNA probes (Figure 6.2 and 6.3). This is also the case for the EcoRI digested genomic DNA of these strains (Figure 6.4 and 6.5). All the restriction fragments generated by these two enzymes, HindIII and EcoRI, in λQ1 are present.

The absence of novel restriction fragments for these eleven transformed strains suggests that the λQ1 DNA has integrated into the genome at the site of the resident QUT

Figure 6.2

Analysis of twelve QUTA transformants by DNA hybridization

The genomic DNA of twelve QUTA transformants and two control strains, R153 (W) and the qutA361 mutant (R), was digested with HindIII and hybridized to either the 7.0 kb A.nidulans fragment from pAL7.0 or to λDB286 DNA which were labelled with α^{32}P-dCTP. The molecular weight markers (M) are HindIII digested λ DNA.

A. An autoradiograph of the HindIII digested genomic DNA samples hybridized to λDB286 DNA. The λDB286 DNA hybridizes to HindIII fragments of >23, 3.7, 2.8, 1.6 and 0.6 kb in the genomic DNA of eleven of the twelve QUTA transformants (1-7,9-12). The twelfth QUTA transformant (8) contains the 2.8 kb HindIII fragment and two novel HindIII restriction fragments. The two control strains (W & R), as expected, do not contain DNA sequences homologous to the λDB286 DNA.

B. An autoradiograph of the HindIII digested genomic DNA samples hybridized to the 7.0 kb A.nidulans DNA fragment from pAL7.0. The 7.0 kb fragment hybridizes to HindIII fragments of 6.4,4.4 and 0.6 kb in the genomic DNA of the two control strains and all twelve transformants. Transformant number 8 also exhibits a novel HindIII restriction fragment.

Figure 6.2

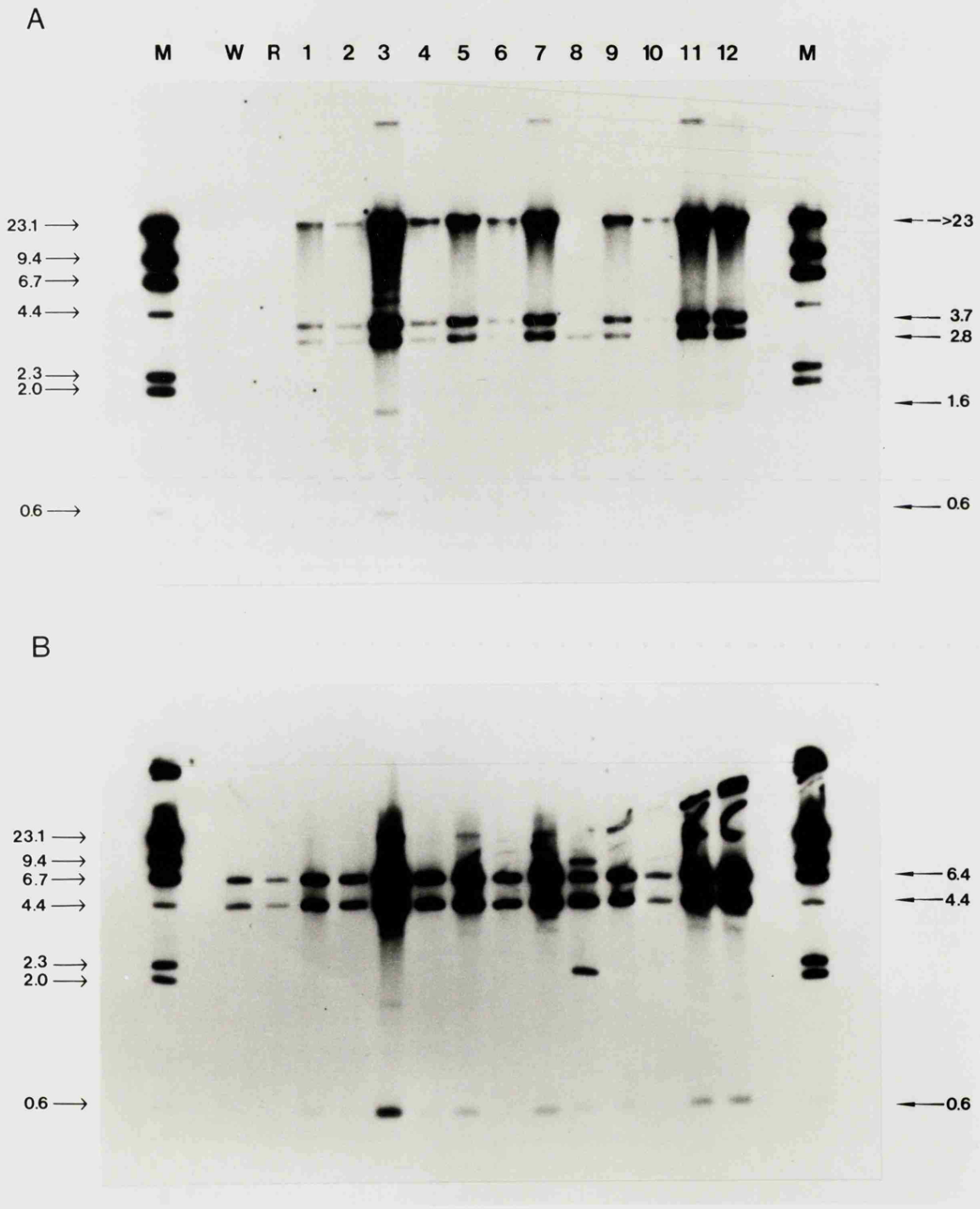

A
M W R 1 2 3 4 5 6 7 8 9 10 11 12 M
23.1
9.4
6.7
4.4
2.3
2.0
0.6
>23
3.7
2.8
1.6
0.6
B
23.1
9.4
6.7
4.4
2.3
2.0
0.6
6.4
4.4
0.6

Figure 6.3

The restriction fragments expected in (A) a wild-type strain and (B) a Type I transformant containing λQ1 DNA

A. The HindIII restriction fragments of A.nidulans DNA that hybridize to the 7.0 kb fragment in the genomic DNA of a wild-type strain. Restriction fragments of this size were observed in the genomic DNA of the two control strains, R153 and the qutA361 mutant.

B. The restriction fragments expected in the genomic DNA of a qutA strain transformed with λQ1 that has been classified as a Type I transformant. The 7.0 kb fragment would hybridize to HindIII restriction fragments of 6.5, 4.4 and 0.6 kb and λDB286 DNA would hybridize to fragments of 27, 3.7, 3.2, 1.6 and 0.6 kb. This pattern of hybridization was observed in eleven of the twelve QUTA transformants.

FIGURE 6.3

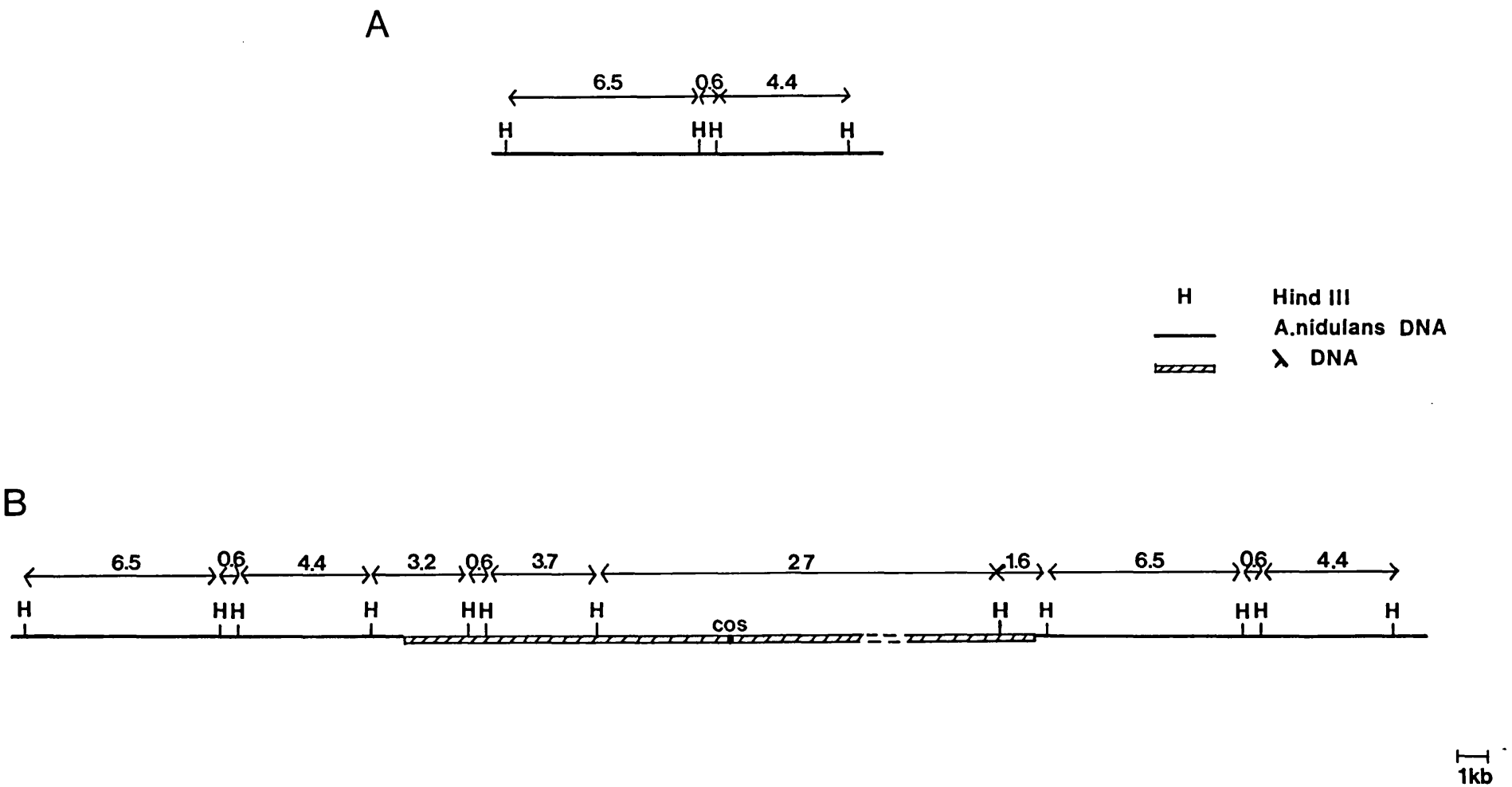

A
6.5
0.6
4.4
H
H H
H
H
Hind III
A.nidulans DNA
DNA
B
6.5
0.6
4.4
3.2
0.6
3.7
2.7
1.6
6.5
0.6
4.4
H
H H
H
H H
H
cos
H
H
H H
H
1kb

Figure 6.4

The analysis of twelve QUTA transformants by DNA hybridization

The genomic DNA of twelve QUTA transformants and two control strains, R153 (W) and the qutA361 mutant (R), was digested with EcoRI and hybridized to either the 7.0 kb A.nidulans fragment from pAL7.0 or to DB286 DNA, which were labelled with α^{32}P-dCTP.

A. An autoradiograph of the EcoRI digested genomic DNA samples hybridized to λDB286 DNA. The λDB286 DNA hybridized to EcoRI fragments of >23 and 2.6 kb in the genomic DNA of eleven of the twelve QUTA transformants (1-7,9-12). The twelfth QUTA transformant (8) contains the 2.6 kb fragment and a novel HindIII restriction fragment that hybridizes to λDB286 DNA. The two control strains (W & R), as expected, do not contain sequences homologous to the λDB286 DNA.

B. An autoradiograph of the EcoRI digested genomic DNA samples hybridized to the 7.0 kb A.nidulans DNA fragment. The 7.0 kb fragment hybridizes to EcoRI fragments of 6.0, 1.7, 1.5 and 0.9 kb in the genomic DNA of the two control strains and all twelve QUTA transformants.

The molecular weight markers (M) are HindIII digested λ DNA.

Figure 6.4

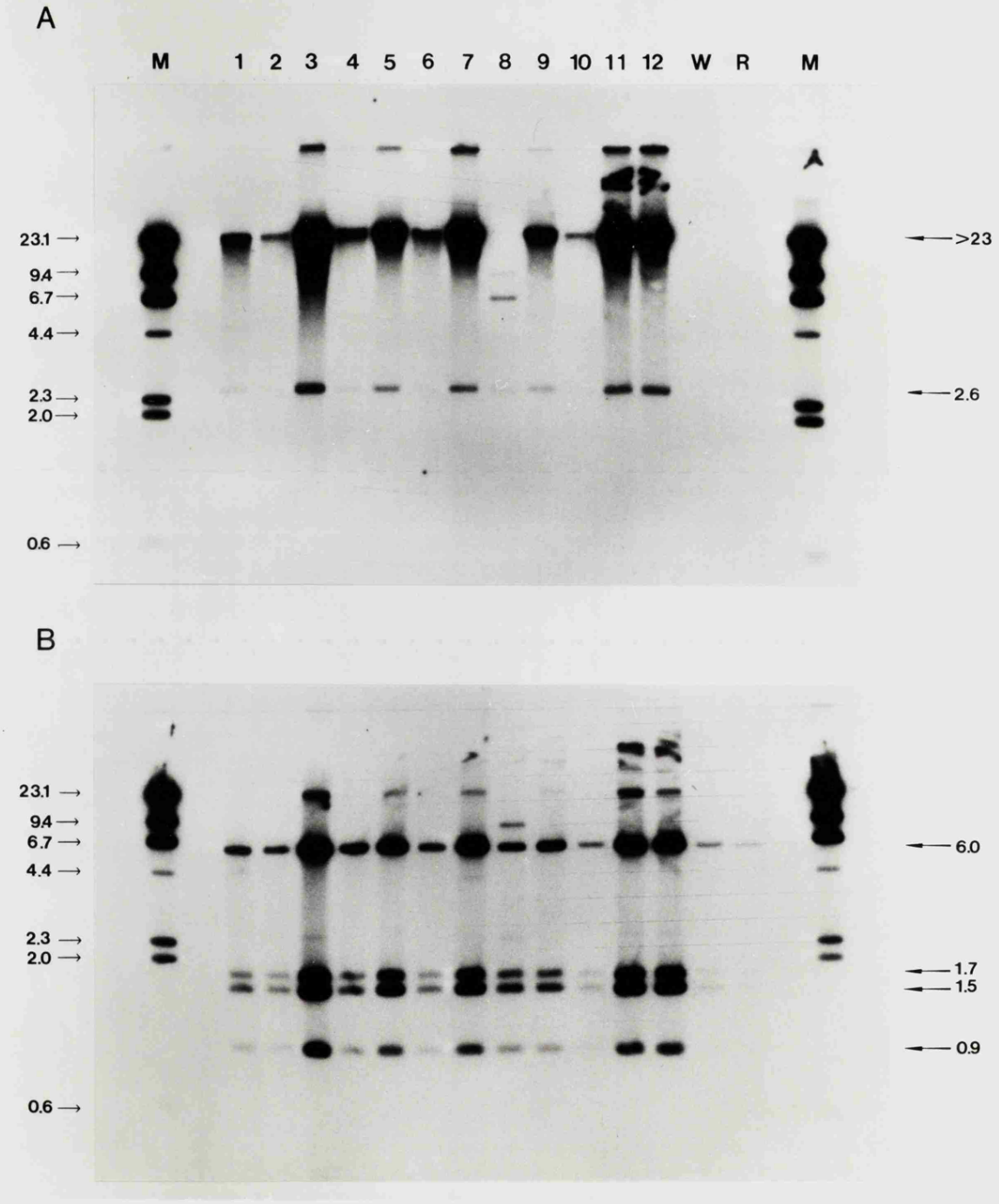

A
M 1 2 3 4 5 6 7 8 9 10 11 12 W R M
23.1
9.4
6.7
4.4
2.3
2.0
0.6
>23
2.6
B
23.1
9.4
6.7
4.4
2.3
2.0
0.6
6.0
1.7
1.5
0.9

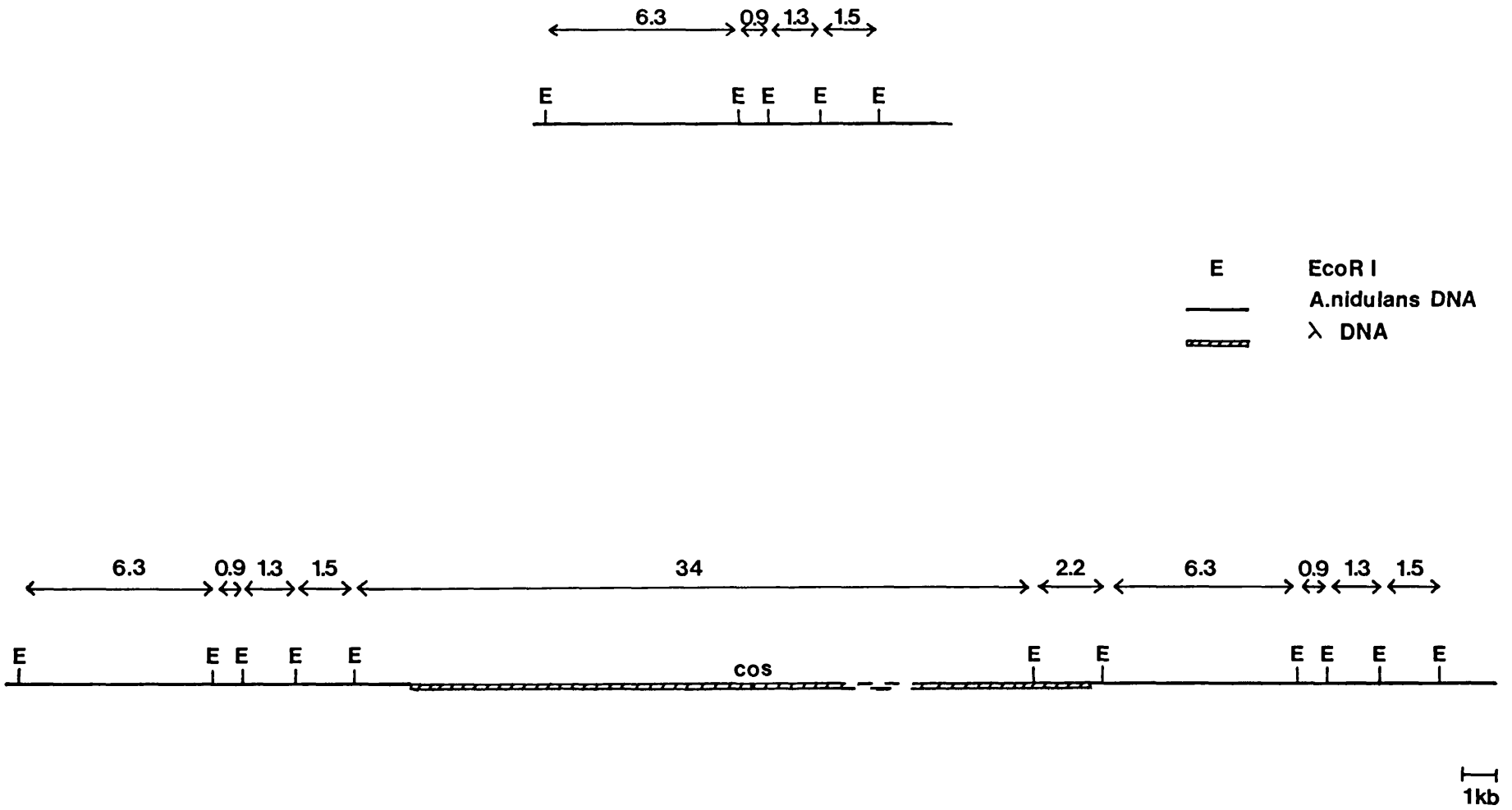
Figure 6.5

The restriction fragments expected in the genomic DNA of (A) a wild-type strain and (B) a Type I transformant containing λQ1 DNA

A. The EcoRI restriction fragments of A.nidulans DNA that hybridize to the 7.0 kb fragment in the genomic DNA of a wild-type strain. Restriction fragments of this size were observed in the genomic DNA of the two control strains, R153 and the qutA361 mutant.

B. The restriction fragments expected in the genomic DNA of a qutA strain transformed with λQ1 that has been classified as Type I transformant. The 7.0 kb fragment would hybridize to EcoRI restriction fragments of 6.3, 1.5, 1.3 and 0.6 kb and λDB286 DNA would hybridize to fragments of 34 and 2.2 kb. The restriction fragments identified in the genomic DNA of eleven of the twelve QUTA transformants suggests that they are Type I transformants.

FIGURE 6.5


6.3
0.9
1.3
1.5
E
E E E E
E
EcoR I
A.nidulans DNA
λ DNA
6.3
0.9
1.3
1.5
34
2.2
6.3
0.9
1.3
1.5
E
E E E E
cos
E E
E E E E
1kb

gene cluster (Figure 6.3 and 6.5). It is significant that this is the case for both strains that express the QUT genes constitutively and also those with normal expression. This observation suggests that the altered regulation of the QUT genes is not related to the position at which the transforming DNA is integrated into the genome.

It is possible that these transformed strains may contain multiple copies of the transforming DNA, λQ1, tandemly integrated into the genome. The constitutive expression of the QUT genes could be due to an increase in the number of copies of the activator gene present on the transforming DNA relative to the single copy of the repressor encoding gene present in the genomic DNA. These multiple copies of the activator gene may "titrate" the level of repressor protein resulting in derepression of the QUT genes.

Examination of the autoradiographs of genomic DNA from these strains (Figures 6.2B and 6.4B) suggests that the copy number of the restriction fragments identified by the 7 kb A.nidulans DNA probe is indeed higher in strains that express the QUT genes constitutively than in the wild-type control strain but does not, in some cases, differ significantly from those transformed strains that express these genes normally, for example numbers 5 and 7 in Figure 6.2B.

Only one transformed stain (Number 8) exhibited an altered

pattern of bands on the autoradiograph produced by novel restriction fragments (Figures 6.2 and 6.4). This may be due to integration of only part of the λQ1 DNA into the genome, but the position of integration cannot be determined from the information available. This strain expresses the QUT genes normally.

6-5 ESTIMATION OF THE QUTA GENE COPY NUMBER IN TRANSFORMED qutA STRAINS

A number of qutA transformed strains were chosen for further study. The QUTA gene copy number of transformed strains that exhibit constitutive expression of the QUT genes (number 3, 11 and 12) was compared to that of transformed strains that exhibit normal regulation (number 1, 7 and 8) by the use of "DNA dot blots", as described previously (Chapter 4, section 4-5). Two strains, a qutA$^+$ wild-type (R153) and the mutant qutA361, that are considered to contain only a single copy of the QUT gene cluster, were used as a reference for comparison to the transformed qutA strains.

Samples of genomic DNA (8 μg in 80 μl Denaturation solution) from each of these strains was two-fold serially diluted in buffer to 1/64 of its original concentration and denatured by heating at 100°C for 5 minutes. Aliquots (2.5 μl) of each dilution were bound to a nylon filter and the DNA hybridized to the 7 kb A.nidulans DNA fragment

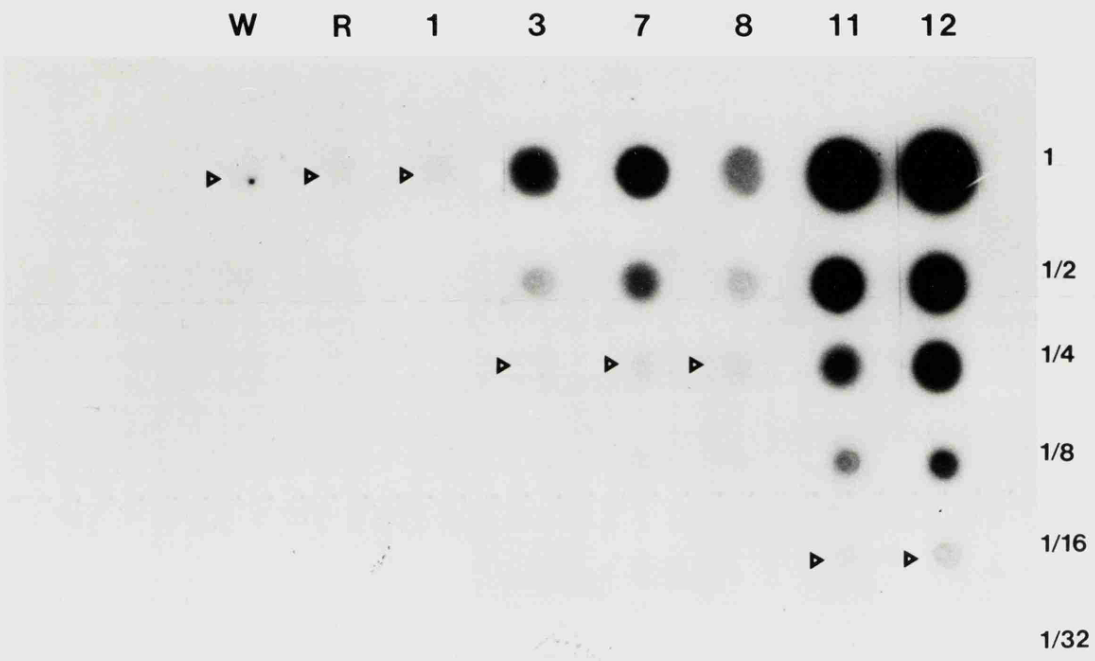
Figure 6.6

Estimation of the QUTA gene copy number in QUTA transformants

The copy number of the QUTA gene in transformed strains that exhibit constitutive expression of the QUT structural genes (number 3,11 and 12) was compared to that in transformed strains exhibiting normal regulation (number 1,7, and 8). Two strains, a gut^{+} wild-type R153 (W) and the mutant qutA361 (R), that are considered to contain a single copy of the QUT gene cluster, were used as a reference for comparison to the QUTA transformants.

Denatured genomic DNA from the above strains was immobilized in the form of spots on nitrocellulose and hybridized to the 7.0 kb fragment, from pAL7.0, labelled with α^{32}P-dCTP. This 7.0 kb fragment spans DNA sequences homologous to the N.crassa qa-1F gene which are considered to contain the QUTA gene. Dots of approximately equal intensity are indicated on the figure.

Figure 6.6



labelled with $\alpha^{32}P$ - dCTP (Feinberg and Vogelstein, 1984; Amasino, 1986). This 7 kb fragment of A.nidulans DNA (derived from pAL7.0) spans DNA sequences of the QUT enzyme structural genes and the region homologous to the N.crassa qa-1F gene.

The autoradiograph obtained is shown in Figure 6.6. A qualitative comparison of the density of the dots of the autoradiograph shows that the three transformed strains that express the QUT genes constitutively (number 3, 11 and 12) have a higher number of copies of the A.nidulans DNA spanned by the 7 kb DNA probe than the reference strains, R153 and qutA361. Transformed strains numbers 3, 11 and 12 have approximately 4, 16 and 16 copies of this DNA respectively. However transformed strain number 7 that exhibits normal regulation of QUT genes has four copies of this DNA which is the same as strain number 3 which expresses these genes constitutively.

Thus evidence does not support the suggestion that the constitutive expression of the QUT genes is related simply to an increase in the copy number of the activator encoding gene, present on the λQ1 transforming DNA in the genome, as the presence of multiple copies of this DNA is a feature of strains that express the QUT genes constitutively and those in which the regulation of these genes is normal.

6-6 THE STABILITY OF THE λQ1 TRANSFORMED *qutA* STRAINS

The three λQ1 transformed *qutA* strains that express the *QUT* enzyme structural genes constitutively were tested after vegetative growth on non-selective medium to examine the stability of their phenotype with respect to growth on quinic acid (*QUT*$^+$) and constitutive formation of the quinate specific enzymes.

These three strains were isolated originally from selective platings of protoplasts from the *qutA361* and *qutA444* strains transformed with λQ1 DNA. The colonies were replated at high dilution to isolate a single colony on minimal medium containing quinate as a carbon source. Conidiospores, taken from an isolated single colony of each strain, were used to grow mycelium that was examined for formation of the quinate specific enzymes using the "spot test" and also to provide confluent growth of conidiating mycelium on minimal medium containing quinate from which conidiospore suspensions were prepared. These were used to grow mycelium in liquid culture from which genomic DNA was isolated. No variation was noted in the appearance of the colonies of the transformed strains during the operations described above. However when suspensions of conidiospores from the original isolated single colony were later replated on complete medium containing glucose as a carbon source, two types of colony morphology was observed after 48 hours incubation at 37°C. The two types of colony morphology were produced by

Table 6.4

Stability of the quinate utilization and constitutive expression phenotypes in a QUTA transformant.

GROWTH ON QUINATE	EXPRESSION OF THE QUINATE INDUCIBLE ENZYMES	COLONIES WITHOUT CLEISTOTHECIA	COLONIES WITH CLEISTOTHECIA
NORMAL	INDUCIBLE	2	3
	CONSTITUTIVE	23	20
NO GROWTH	NON-INDUCIBLE	0	2
	TOTAL NO OF COLONIES	25	25

all three of the above strains. In one type, the colony (white) produced normal white conidiospores while in the other type many immature cleiostothecia were produced giving the colonies a 'buff' colouration. Colonies producing sectors of both phenotypes were also observed.

A number of colonies (25) of each type from one of these strains (number 3) were examined using the "spot test" to determine whether they have the same phenotype as the original isolated single colony, that is constitutive formation of the quinate specific enzymes. Their phenotype with respect to growth on quinic acid was also tested. The results are shown in Table 6.4.

It can be observed that the colony morphology does not appear to be related to the properties of the strain with respect to growth on quinic acid and constitutive formation of the quinate specific enzymes. Both the QUT$^+$ phenotype and the phenotype with respect to constitutive formation of the quinate specific enzymes are unstable, being lost by 4% and 14% of the colonies tested respectively. It has been noted that "buff" colonies, upon replating, produce colonies of both morphological types, while white colonies only produce colonies of the same morphological type.

The loss of ability to utilise quinate as a carbon source or to form the quinate specific enzymes constitutively in some colonies of this strain suggests that the transforming λQ1

DNA is not stably integrated into the genome in these cases. Rearrangement or excision of the λQ1 DNA has probably resulted in the loss of these characteristics. The production of sexual structures by the "buff" colonies could be related to the size of the integrated λQ1 DNA (approximately 50 kb) or to the number of copies of this DNA integrated into the genome.

The above result suggests that the conidiospore suspension, used to grow mycelium from which genomic DNA was isolated, might be heterogenous with respect to these phenotypes. This was investigated by plating dilutions of this suspension on non-selective medium and testing the colonies obtained with respect to growth of quinate and constitutive formation of the quinate specific enzymes, as previously described. Again the two types of colony morphology were observed however all the colonies (50) tested had the same characteristics as the original isolated single colony.

A brief examination of the purified colonies from the remaining two transformed strains (number 11 and 12) that form the quinate specific enzymes constitutively has also revealed them to be unstable with respect to this phenotype.

Although the reason for the stability of the phenotypes in colonies from the conidiospore suspension is unresolved, it is apparent that the phenotype with respect to constitutive formation of the quinate specific enzymes is unstable in

many colonies isolated from transformed strain number 3. Therefore further studies on the levels of the quinate specific enzymes produced in the mycelium of these strains would be of no value as such mycelium is likely to be heterokaryotic, that is containing nuclei of different genetic constitution due to the instability of the integrated transforming DNA.

6-7 THE ANALYSIS OF *qutA* MUTANT STRAINS TRANSFORMED WITH THE PLASMID pAL6.1

A number (10) of quinate utilizing strains from the mutant *qutA361* transformed with pAL6.1 were analysed by DNA hybridization (Southern, 1975) to confirm the presence of the transforming vector DNA within the genome.

The strains were purified by single colony isolation and high molecular weight genomic DNA was isolated from mycelium grown in liquid culture (Chapter 2). Genomic DNA prepared from a *QUTA*$^+$ wild type (R153) and *qutA361* strains were used as controls. The quality and quantity of the genomic DNA was measured spectrophotometrically and by agarose gel electrophoresis. In all cases the genomic DNA was approximately 50 kb in size and there was no degradation.

Samples of genomic DNA (3 μg) from these strains, digested with either *Bam*HI or *Hind*III, were separated by electrophoresis through a 0.8% agarose gel. The DNA was

Figure 6.7

The analysis of genomic DNA from gutA strains transformed with pAL6.1 by DNA hybridization

Genomic DNA, digested with BamHI, from ten gutA strains transformed with pAL6.1 and two control strains, R153 (W) and the gutA361 mutant (R), was hybridized to either the 6.1 kb fragment from pAL6.1 or pBR325 DNA, which were laballed with α^{32}P-dCTP.

A. The BamHI digested genomic DNA samples hybridized to the 6.1 kb fragment. The genomic DNA of the two control strains has BamHI fragments of 10.5 and 3.8 kb that hybridize to the 6.1 kb fragment. The same pattern of hybridization is observed in three of the transformed strains (number 1,7 and 10), suggesting they are Type III transformants. Restriction fragments of 10.5, 3.8 and 2.4 kb are identified in two transformed strains (number 8 and 9) which is the pattern expected in Type I transformants.

B. The BamHI digested genomic DNA samples hybridized to pBR325 DNA. The two control strains (W & R) and five of the transformed strains (number 1,3,5,7 and 10) do not contain DNA sequences homologous to pBR325. The genomic DNA of two tansformed strains (number 8 and 9) contains a 6.0 kb BamHI fragment which hybridizes to pBR325.

Figure 6.7

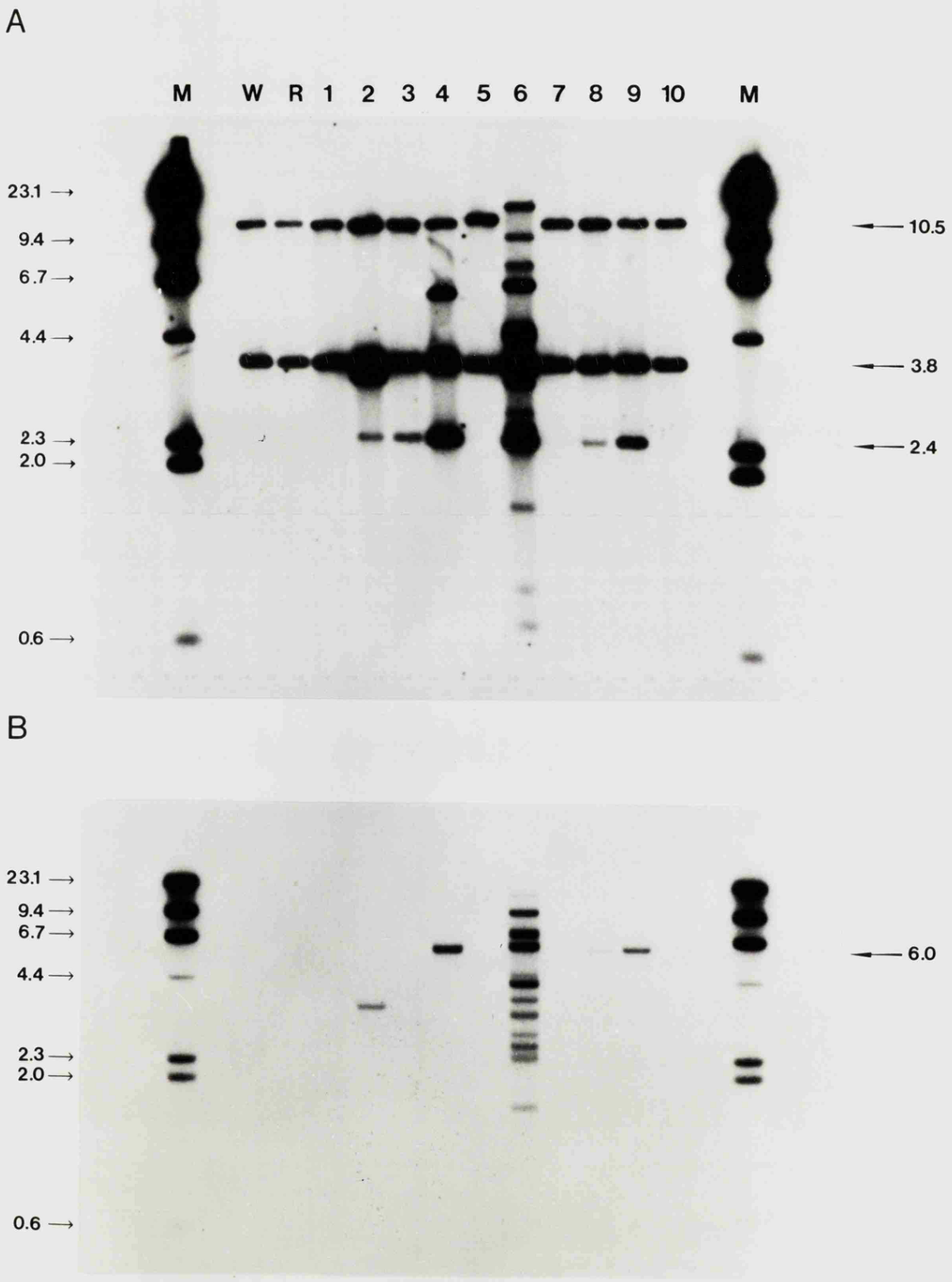

A
M W R 1 2 3 4 5 6 7 8 9 10 M
23.1
9.4
6.7
4.4
2.3
2.0
0.6
10.5
3.8
2.4
B
23.1
9.4
6.7
4.4
2.3
2.0
0.6
6.0

Figure 6.8

The analysis of genomic DNA from gutA strains transformed with pAL6.1, by DNA hybridization

Genomic DNA, digested with HindIII, from ten gutA strains transformed with pAL6.1 and two control strains, R153 (W) and the gutA361 mutant (R), was hybridized to either the 6.1 kb fragment from pAL6.1 or to pBR325 DNA, labelled with α^{32}P-dCTP.

A. The HindIII digested genomic DNA samples hybridized to the 6.1 kb fragment from pAL6.1. The genomic DNA from the two control strains (R & W) has HindIII fragments of 6.7, 4.4, 2.0 and 0.6 kb that hybridize to the 6.1 kb fragment. The same hybridization pattern is observed in three of the transformed strains (number 1, 7 and 10) suggesting that they are Type III transformants. Restriction fragments of 6.7, 4.4, 2.0, 1.1 and 0.6 kb are identified in two transformed strains (number 8 and 9) which is the pattern expected in a Type I transformant.

B. The HindIII digested genomic DNA samples hybridized to pBR325. The two control strains (W & R) and five of the transformed strains (number 1, 3, 5, 7 and 10) do not contain DNA sequences homologous to pBR325. The genomic DNA of two transformed strains (number 8 and 9) contains 6.0 and 1.1 kb HindIII fragments which hybridize to pBR325.

Figure 6.8

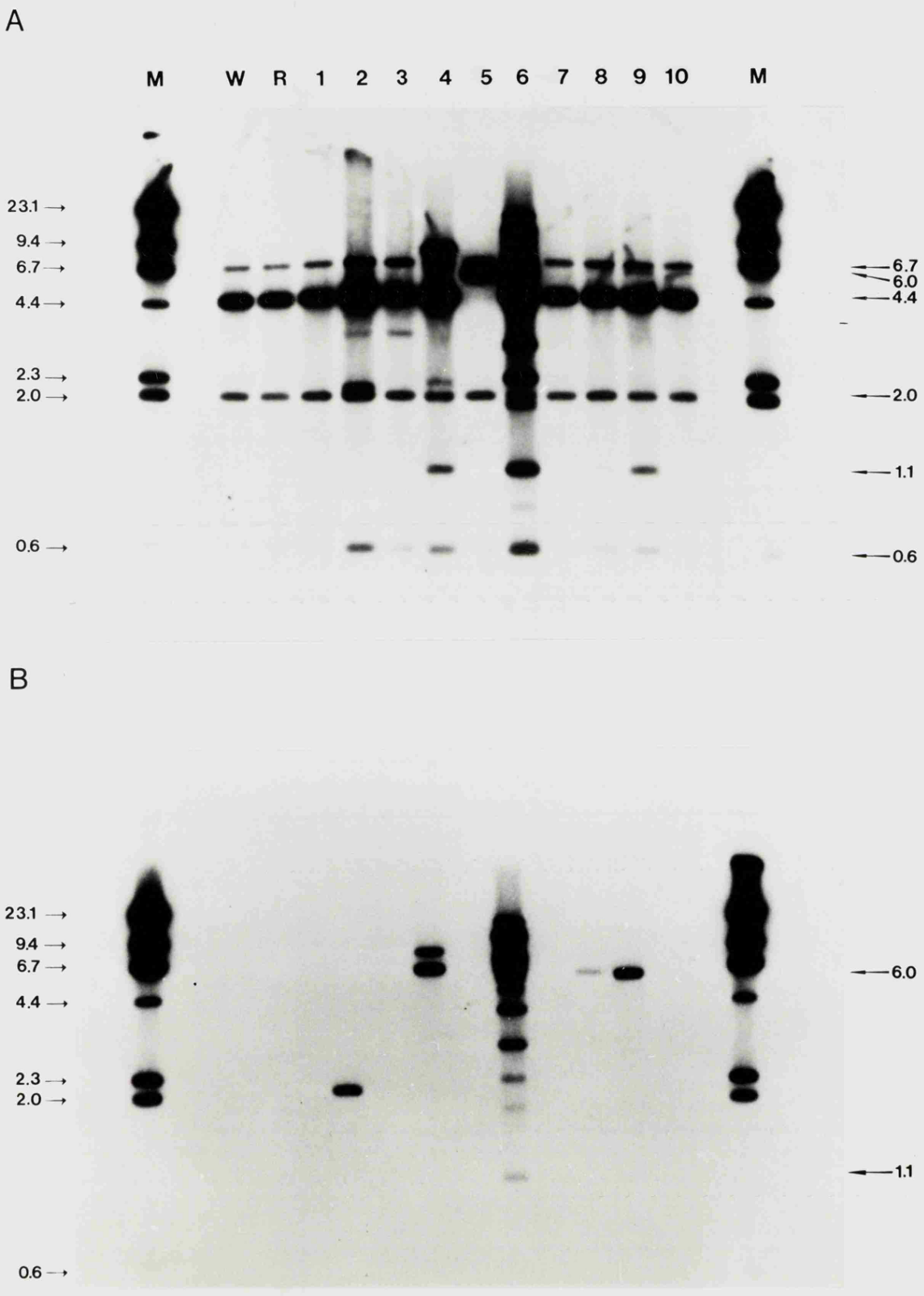

A
M
W
R
1
2
3
4
5
6
7
8
9
10
M
23.1
9.4
6.7
4.4
2.3
2.0
0.6
6.7
6.0
4.4
2.0
1.1
0.6
B
23.1
9.4
6.7
4.4
2.3
2.0
0.6
6.0
1.1

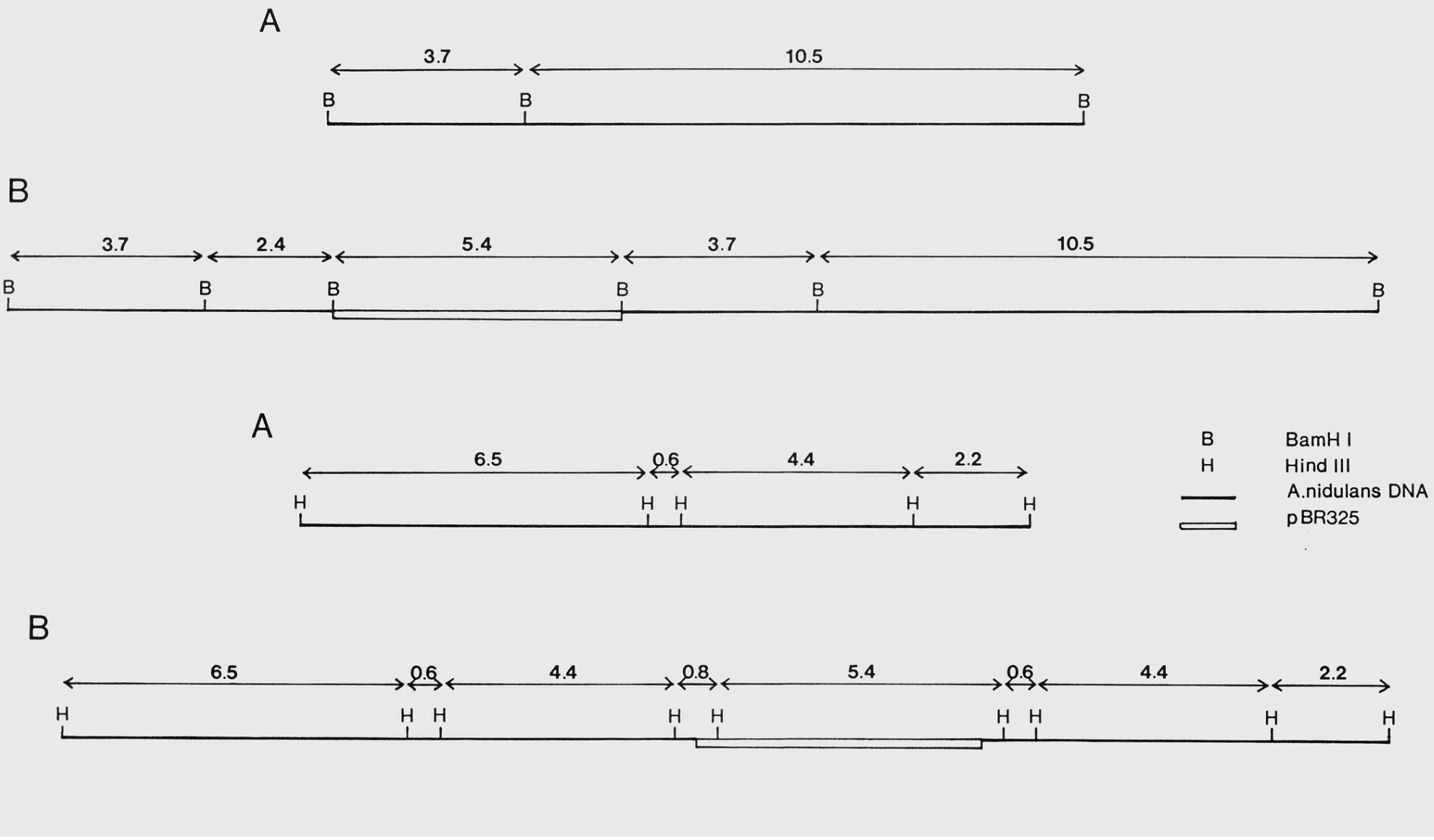
Figure 6.9

The BamHI and HindIII restriction fragments expected in (A) a wild-type strain and (B) a Type I transformant

A. The two figures marked (A) show the sizes of the BamHI and HindIII fragments within the genomic DNA of a wild-type strain that would hybridize to the 6.1 kb fragment from pAL6.1. A Type III transformant would exhibit the same pattern of restriction fragments.

B. The two figures marked (B) show the sizes of the BamHI and HindIII restriction within the genomic DNA of a Type I transformant that would hybridize to the 6.1 kb fragment from pAL6.1 and to pBR325 DNA. Two transformed strains (number 8 and 9) exhibited this pattern of restriction fragments in their genomic DNA when hybridized to the above DNA probes.

FIGURE 6.9


A
3.7
10.5
B
B
B
B
3.7
2.4
5.4
3.7
10.5
B
B
B
B
B
B
A
6.5
0.6
4.4
2.2
H
H
H
H
H
H
B
BamH I
H
Hind III
A.nidulans DNA
pBR325
B
6.5
0.6
4.4
0.8
5.4
0.6
4.4
2.2
H
H
H
H
H
H
H
H
H
H

transferred to a nylon filter, immobilised and hybridized to either pBR325 or the 6.1 kb A.nidulans DNA fragment labelled with α^{32}P - dCTP (Feinberg and Vogelstein, 1984; Amasino, 1986). The 6.1 kb A.nidulans DNA fragment was prepared by partial digestion of pAL6.1 with BamHI and isolated from a 0.6% (w/v) low-gelling point agarose gel (Crouse et al, 1983).

The autoradiographs obtained are shown in Figure 6.7 and 6.8. The sizes of the DNA restriction fragments identified by the two DNA probes and how they are derived within the genomic DNA is shown in Figure 6.9.

The QUT$^+$ wild-type (R153) and qutA361 mutant control stains have restriction fragments of 10.5 and 3.8 kb (Figure 6.7) identified by the 6.1 kb A.nidulans fragment in BamHI digested genomic DNA. No restriction fragments are identified by the vector pBR325 DNA. Five of the ten transformed strains have no DNA sequences identified by the vector pBR325 DNA and, of these, three strains (number 1, 7 and 10) have restriction fragments identified by the 6.1 kb A.nidulans fragment identical to those of the control strains, showing that they are Type III transformants (Hinnen et al, 1978). The remaining five strains all contain vector pBR325 DNA sequences within their genomic DNA and the restriction fragments identified by the DNA probes for two of these strains is that expected from integration of the transforming pAL6.1 DNA at homologous DNA sequences

with the resident QUT gene cluster (Figure 6.9). It is not possible to determine the position of integration of the transforming DNA in the remaining transformed strains as they exhibit a more complex pattern of restriction fragments identified by the DNA probes.

The presence of plasmid pAL6.1 DNA within the genomic DNA of transformed qutA strains suggests that the QUTA gene is present within the 6.1 kb of A.nidulans DNA in plasmid pAL6.1.

DISCUSSION

The aim of the work described in this chapter has been to identify the position of the QUTA gene on the recombinant phage λQ1 DNA by transformation of an A.nidulans qutA mutant strain. The opportunity has also arisen to examine the expression of the QUT enzyme structural genes in the transformed qutA strains.

The genetic analysis of newly isolated qutA mutants (Grant et al, 1988) led to the revision of our original hypothesis that the QUTA gene encoded a repressor protein (Hawkins et al, 1984). The current hypothesis is that the gene may encode an activator protein and thus be equivalent to the qa-1F gene of N.crassa. This hypothesis is supported by the transformation of a qutA mutant strain with DNA fragments isolated from λQ1 DNA. Transformation has been obtained with

two overlapping restriction fragments, of 6.1 and 7.0 kb respectively, spanning a 6.4 kb region of A.nidulans DNA to the right of the QUT enzyme structural genes within which are DNA sequences homologous to the qa-1F gene of N.crassa (Da Silva, 1985). This suggests that the QUTA gene is located within these 6.4 kb of A.nidulans DNA.

One anomaly is that a 3.7 kb fragment, common to both the 6.1 kb and 7.0 kb fragments described above, alone cannot transform a qutA mutant strain. It is possible that this 3.7 kb fragment may not contain the entire QUTA gene and hence could only transform a qutA strain by integration of this DNA at homologous DNA sequences via a "gene conversion" event. The frequency at which this event may occur could be too low for detection within these particular experiments.

The expression of the QUT enzyme structural genes in particular transformed qutE strains (Chapter 4) has shown that increasing the copy number of DNA spanning the QUT enzyme structural genes does not lead to an increase in the levels of the quinate specific enzymes. It has been suggested that there is a rate limiting element in the induction of these genes, the most obvious candidates being the activator and repressor proteins. In these transformed strains it is possible that the single QUTA gene produces insufficient activator protein to saturate the promoters of the multiple copy QUT structural genes. Hence, introducing multiple copies of the QUTA gene might relieve the situation

and allow an increase in the level of expression of these QUT genes. Constitutive expression of the QUT genes could also occur as there might no longer be enough repressor protein being formed by the single copy of the repressor encoding gene, to saturate the 5' regions of the multiple QUTA genes and effectively repress their expression.

Constitutive formation of the quinate specific enzymes has been shown to occur in three qutA strains transformed with λQ1 DNA, which contains the activator encoding QUTA gene. It was therefore suggested that the situation described above was occurring and that these strains might contain multiple tandemly integrated copies of λQ1 DNA within their genome. However analysis of the genomic DNA revealed that one of the strains (number 3) that exhibits constitutive formation of the quinate specific enzymes did not have a significantly higher number of copies of the λQ1 DNA than other transformed strains in which the regulation of the QUT enzyme structural genes was normal. Further studies were not carried out on these transformed strains because of their instability. If the above hypothesis was correct, constitutive formation of the quinate specific enzymes should have been observed among strains transformed with the plasmids pAL7.0 and pAL6.1, that may also contain the QUTA gene, but none of the 57 strains tested exhibited this phenotype. However the copy number of the plasmid DNA within the genomes of these strains was not determined.

As the above evidence has suggested that constitutive expression of the QUT enzyme structural genes is not related to the copy number of the QUTA gene, other possible explanations must be examined. An alternative hypothesis is related to the position at which the transforming DNA integrates into the genome. Mutations in the closely linked qutR gene, which is thought to encode the repressor protein, results in constitutive formation of the quinate specific enzymes (Whittington et al, 1987). This phenotype would be observed in transformed strains if the integrated DNA disrupted the qutR gene locus. The data available for the strains transformed with λQ1 DNA does not support this hypothesis as the λQ1 DNA appears to have integrated into the genome at homologous DNA sequences within the QUT gene cluster and there is no obvious difference between strains that exhibit constitutive formation of the quinate specific enzymes and those in which the regulation of the QUT genes is normal. Hence it is unlikely that, in this case, the constitutive formation of the quinate specific enzymes is related to the position of integration of the transforming DNA.

It should be noted that none of the 36 qutD strains transformed with λQ1 DNA that were tested exhibit this phenotype indicating that it may be specific to these particular qutA transformed strains. In view, however, of the instability of this phenotype no further studies have been carried out.

In summary, constitutive formation of the quinate specific enzymes in gutA strains transformed with λQ1 DNA does not appear to be directly related to the copy number of this DNA or to the position at which this DNA has integrated into the genome.

CHAPTER 7

DISCUSSION

Early genetic studies of A.nidulans quinate non-utilizing mutants identified five genes mapping within the QUT gene cluster; the three enzyme structural genes (QUTB, QUTC and QUTE) and two genes, QUTA and QUTD, which were considered to have a regulatory function. The QUTA gene was interpreted as encoding a repressor protein and the QUTD gene as encoding an activator protein (Hawkins et al, 1984). Heterologous DNA probes from the N.crassa qa gene cluster were used to isolate the corresponding A.nidulans QUT gene cluster in a recombinant clone, λQ1. Within this clone, regions of DNA homologous to the three qa enzyme structural genes (qa-2, qa-3 and qa-4), the activator encoding qa-1F gene and the qa-x and qa-y genes were considered to contain the equivalent QUT genes of A.nidulans (Hawkins et al, 1985; Da Silva, 1985). The hybridization data suggests that the qa and QUT gene clusters are similarly organized as the structural genes are in the same relative order but are inverted relative to the putative activator encoding gene in A.nidulans.

The aim of the work described in this thesis was to confirm the identity and integrity of the QUT gene cluster within the recombinant clone,λQ1 particularly with respect to the

QUTE, QUTD and QUTA genes, using the newly developed transformation system for A.nidulans (Ballance et al, 1983). The QUTB and QUTC genes have been similarly analysed by H. Lamb (Hawkins et al, 1988). The qutE, qutD and qutA mutant A.nidulans strains have been successfully transformed with genomic DNA, isolated from λQ1, in plasmid vectors and quinate utilizing transformants selected. From the results of the transformation experiments the following conclusions can be made with respect to the QUTE, QUTD and QUTA genes.

First, DNA homologous to the N.crassa qa-2 gene, encoding catabolic dehydroquinase, is able to transform an A.nidulans qutE mutant strain. Hence, the entire functional QUTE gene is located in this region. This has been confirmed by DNA sequence analysis of the QUTE gene which shows that the QUTE and qa-2 genes have regions of extensive DNA homology (Da Silva et al, 1986).

Second, biochemical and genetical studies of a number of qutD mutants strongly suggest that such strains are defective in an essential element of a quinate permease system (Whittington et al, 1987). This evidence was supported by the observation that DNA homologous to the qa-y gene was able to transform a qutD mutant strain. The function of the qa-y gene in N.crassa has not been determined, however indirect evidence has been obtained for a permease in N.crassa involved in the uptake of quinate and it has been suggested that such a function might be

encoded by either the qa-x or qa-y genes (Rines, 1973; Giles et al, 1985). Hence it was concluded that the QUTD gene, which was originally considered to encode an activator protein, encodes an essential component of a permease system for quinate uptake (Whittington et al, 1987). DNA sequence analysis has confirmed that the QUTD gene is homologous to the N.crassa qa-y gene and that the inferred molecular weight of the QUTD gene product is in close agreement with the molecular weight of the qa-y gene product. Visual inspection of the deduced protein sequence of the QUTD gene product reveals the presence of 8 highly hydrophobic sequence motifs bounded by charged or hydrophilic regions which are consistent with the conclusion that the QUTD gene encodes a permease (Hawkins et al, 1988).

Third, the QUTA gene of A.nidulans was initially interpreted as encoding a repressor protein (Hawkins et al, 1984) however transformation of a qutA A.nidulans mutant strain with DNA homologous to the qa-1F gene of N.crassa suggests that the QUTA gene encodes an activator protein which may act alone or in concert with other proteins to positively regulate the QUT gene cluster. This conclusion is supported by evidence from the genetic analysis of qutA mutants. In N.crassa the qa-1S gene encodes a repressor. A high proportion of prototrophic revertants of non-inducible qa-1S mutant strains selected for growth on quinic acid are constitutive mutants however no such mutants are found amongst qa-1F revertants (Giles et al, 1985). In

A.nidulans, a total of 500 revertants of semi-dominant and recessive qutA mutants were screened and none were found to be constitutive mutants mapping to the qutA locus. Hence this observation is in contrast to that observed for N.crassa qa-1S mutants (Grant et al, 1988). The region containing the QUTA gene has been subject to DNA sequence analysis and shares significant homologies with the N.crassa qa-1F gene and to other positively acting eukaryotic DNA regulatory proteins (Beri et al, 1987). The above data concurs strongly with the view that the QUTA gene is a positively acting DNA regulatory gene.

Hence data obtained from the transformation of A.nidulans qut mutant strains has identified the location and confirmed the functional integrity of the QUTE, QUTD and QUTA genes within the recombinant clone, λQ1. The functions of the QUTA and QUTD genes differ from that suggested by early genetic studies (Hawkins et al, 1984) however the conclusions drawn from the transformation experiments are strongly supported by more detailed genetic and DNA sequence analysis (Da Silva, 1985; Beri et al, 1987; Whittington et al, 1987; Grant et al, 1988; Hawkins et al, 1988).

The above conclusions raised the question of the identity and location of the repressor encoding gene within the QUT gene cluster. A high proportion of constitutive strains were recovered among revertants of a number of qutD mutants. The constitutive strains all had similar phenotypes, growing

poorly on quinate although producing substantial levels of the three enzymes for conversion of quinate to protocatechuate when grown on glycerol. It was this observation that strongly suggested that the QUTD gene encoded a permease. The analysis of one constitutive revertant strain to establish the genetic basis of constitutive enzyme formation has shown that the revertant was a double mutant with the second mutation identifying a new gene locus, qutR, controlling the induction of the quinate specific enzymes and closely linked to qutD. The constitutive mutation was fully recessive to wild-type in heterozygous diploids exhibiting no enzyme activity when grown in the absence of quinic acid. It has also been demonstrated that each of the constitutive revertant strains contained a new mutation functionally allelic to the qutR mutation. Since the qutR mutations result in loss of repression of the quinate specific enzymes the qutR gene is interpreted as encoding a repressor protein (Grant et al, 1988).

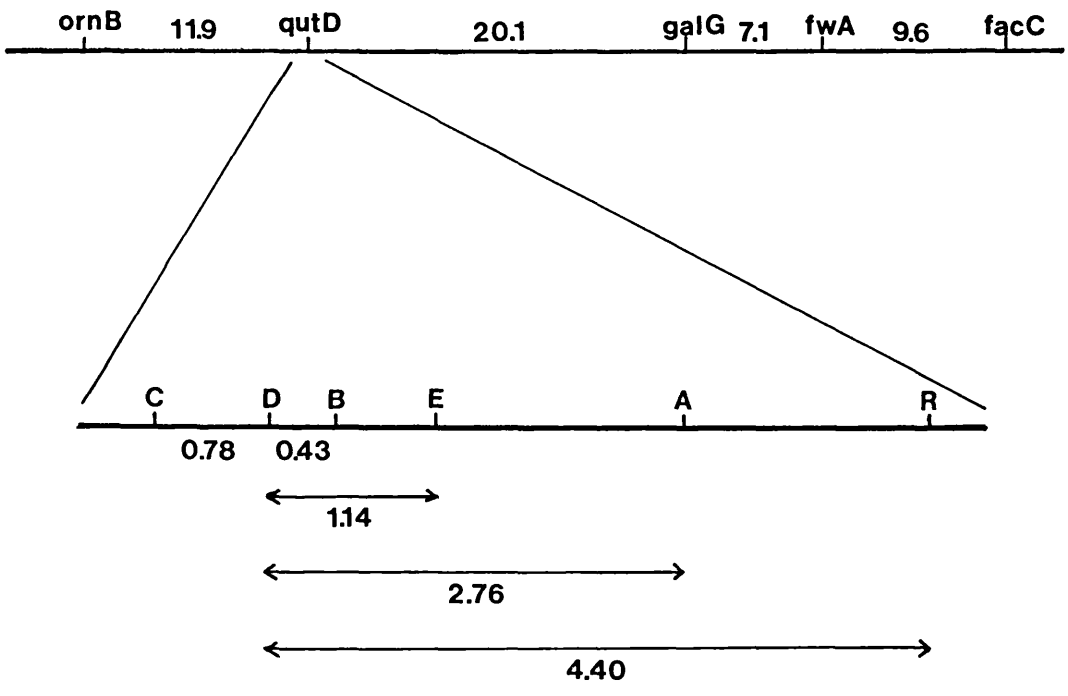
The above conclusion was supported by the observation that 95% of revertants from the dominant non-inducible strain qut365, which maps to the qutR locus, were constitutive for formation of the quinate specific enzymes when grown on glycerol. The strain reverts spontaneously at a high rate which is increased by exposure to a low dose of UV irradiation (Grant et al, 1988). The properties of the dominant mutant are the same as that observed for N.crassa

Figure 7.1

A fine-structure map of the QUT gene cluster in A.nidulans

A map of the QUT gene cluster in chromosome VIII of A.nidulans showing the map distances of the qutC, qutB, qutE, qutA and qutR loci relative to the qutD locus expressed as the percentage recombination frequency. The positions of the flanking markers used to orientate the gene cluster are also indicated (Grant et al, 1988).

FIGURE 7.1


ornB
11.9
qutD
20.1
galG
7.1
fwA
9.6
facC
C
D
B
E
A
R
0.78
0.43
1.14
2.76
4.40

strains carrying a mutation in the qa-1S repressor encoding gene (Giles et al, 1985). Hybridization studies have shown that DNA homologous to the qa-1S gene is not present within the recombinant clone, λQ1 (Da Silva, 1985).

The positions of the genes with the QUT gene cluster have been mapped by three-point crosses using a qutR mutation, in a qutD qutR double mutant, as a flanking marker. The recombination frequency values order the genes in the sequence qutD - qutB - qutE - qutA - qutR. The qutC gene has been mapped distal to qutD with respect to qutR (Figure 7.1, Grant et al, 1988).

The location of the QUTB gene is as determined by hybridization, transformation and DNA sequence analysis (Hawkins et al, 1985; Hawkins et al, 1988) however the location of the qutC gene is not consistent with the position determined by hybridization studies with the equivalent N.crassa qa-3 gene (Hawkins et al, 1985). The region of DNA homologous to the qa-3 gene in λQ1 is unable to transform a qutC mutant strain and DNA sequence analysis of this DNA has shown that it is homologous to the N.crassa qa-x gene. This DNA therefore contains the previously unidentified QUTG gene. The plasmid pEH1 and λQ1 DNA are also unable to transform a qutC mutant indicating that the QUTC gene is not present within λQ1 (Hawkins et al, 1988). Recently DNA homologous to the qa-3 gene has been isolated within a new recombinant λ clone and it is considered that

this region of DNA contains the QUTC gene (A.R Hawkins, personal communication)

The detailed genetical and molecular analysis of the organization of the quinic acid utilization gene clusters in the closely related Ascomycetes N.crassa and A.nidulans allows a comparison to be made between these two systems. Both systems contain five quinate inducible genes and an activator and repressor regulatory gene. DNA sequence obtained for five of the QUT genes exhibits extensive homology to the equivalent qa genes suggesting that they are closely related and probably diverged from a common ancestor (Da Silva et al, 1986; Beri et al, 1987; Hawkins et al, 1988). There are also strong similarities in the phenotypes of the different classes of mutants isolated within the QUT and qa gene clusters. However, there are major differences in the organization of these two systems. In A.nidulans, the QUTC gene, encoding dehydroshikimate dehydratase, and the QUTR gene, encoding the putative repressor protein, are displaced. This is particularly significant for the QUTR gene, as in N.crassa the activator-encoding qa-1F and repressor-encoding qa-1S genes are divergently transcribed from a common promoter region while in A.nidulans the equivalent genes are transcribed from separate individual promoters. Whether this has implications for the regulatory control of these gene clusters remains to be discovered.

A basic model proposed for the regulation of the QUT gene cluster considered that regulation was at the level of transcription (Hawkins et al, 1984) and this has been confirmed by studies on the 3-dehydroquinase-encoding QUTE gene (Da Silva et al, 1986). Studies of the qa gene cluster have shown that the qa structural genes are regulated primarily at the level of transcription and are dependent on the presence of the inducer, quinate, and the product of the qa-1F gene. There is also evidence that the qa-1S gene is involved in their expression (Huiet, 1984; Patel et al, 1981). Studies have also shown that the qa-1F gene is subject to autogenous regulation as well as control by the negative regulatory gene qa-1S and the inducer quinate (Patel and Giles, 1985).

An attempt has been made to learn more about the regulation of the QUT gene cluster by molecular and biochemical studies of particular multi-copy QUT transformants. It has been observed in QUT transformants that multiple copies of the QUT structural genes do not result in higher than wild-type levels of induced enzyme activity and it has been suggested that this may be due to "titration" of the activator protein by the cis-acting regulatory sites in the 5' regions of the multi-copy number QUT structural genes. The "titration" of specific positively acting regulatory proteins has been observed in multi-copy number transformants during studies on the amdS (Kelly and Hynes, 1987) and alcA (D Gwynne; personal communication) genes of A.nidulans.

From the above observations it was proposed that increasing the copy number of the positively-acting QUTA regulatory gene and the QUT structural genes would lead to increased gene expression and possibly constitutive expression of the QUT structural genes due to "titration" of the repressor protein by multiple copies of the activator-encoding QUTA gene on which it may have a negative regulatory effect. Among strains transformed with the QUTA gene and QUT structural genes contained in the recombinant clone λQ1, a significant number were obtained that produce the quinate specific enzymes constitutively, however this phenotype could not be correlated directly to an increase in copy-number and was unstable yielding non-constitutive quinic acid utilizing segregants. The instability may result from rearrangement or excision of the integrated DNA.

Recently a transformant has been obtained carrying approximately 20 copies each of the QUTA and QUTE genes. When induced by quinate the transformant produces wild-type levels of quinate dehydrogenase and dehydroshikimate dehydratase but the level of dehydroquinase is increased approximately 20-fold. The QUTE gene is still subject to normal regulatory control. A second transformant containing approximately 20 copies of the QUTA gene alone produces wild type levels of the three quinate specific enzymes under inducing conditions (C. F. Roberts, personal communication). These observations suggest that 20 copies of the QUTA gene

are insufficient to cause "titration" of the repressor protein.

During studies of the cis-acting regulatory regions of the amdS gene, it has been observed that a transformant containing more than 100 copies of the entire 5' region of the amdS gene exhibits "titration" of the product of the positively-acting amdR regulatory gene, resulting in reduced expression of the lamA, gatA and/or gabA genes which are co-regulated with amdS by the amdR product. This effect can be reversed by the introduction of multiple copies of amdR and is referred to as "anti-titration" (Andrianopoulos and Hynes, 1988). This observation supports the suggestion that the wild-type levels of the quinate specific enzymes in a QUT transformant having a single copy of the QUTA gene and multiple copies of the QUT structural genes (QUTB, QUTD, QUTE and QUTG) is the result of "titration" of the QUTA product. The 20-fold increase in catabolic dehydroquinase in a QUT transformant having approximately 20 copies each of the QUTA and QUTE genes may be either the result of "anti-titration" by the QUTA gene product or insufficient titration of the QUTA gene product by 20 copies of the QUTE gene alone.

Studies of the mechanisms involved in the regulation of the quinic acid gene cluster are currently at a more advanced stage in N.crassa. These have been focused on the cis-acting regulatory sites in the 5' regions of the qa

genes and the regions of the two regulatory proteins involved in binding the inducer, quinate, or binding to their target site of action.

A 105 base pair region was identified 5' to the qa-2 gene that is required for qa-1F-mediated induction of qa-2 transcription (Geever et al, 1986). This region is flanked by two DNase I hypersensitive sites (HSSs), one of which is qa-1F inducible and hence it was considered that this HSS might have resulted from activator protein binding (Baum and Giles, 1985). Examination of the qa gene cluster has revealed that HSSs are present in the 5' regions of all the qa genes. Two pairs of the structural genes are divergently transcribed and the chromatin organisation of these qa-x-qa-2 and qa-4-qa-3 intergenic regions appears to be similar with respect to the positions of the DNase I HSSs in induced and uninduced wild-type chromatin. The majority of the inducible DNase I HSSs are qa-1F - dependent however in the qa-x gene there is evidence for differential regulation of the DNase I HSSs suggesting that qa-x transcription may be directly controlled by more than one regulatory factor. This is supported by evidence from transcriptional studies which indicate that qa-x transcription may be partially regulated by carbon catabolite repression (Geever et al, 1986; Tyler et al, 1984). The hypersensitive regions often extend for 100-500 base pairs and are interrupted by short regions protected from DNase I digestion, that are considered to be sites of specific DNA - protein interaction

(Geever et al, 1986; Baum and Giles, 1986). A 75 base pair sequence, including the DNase I protected region 5′ to the qa-2 gene, was used in a homology search of the qa gene cluster sequence from which was derived a consensus 16 base pair sequence of imperfect dyad symmetry. Thirteen related sequences are located in the qa gene cluster and are closely associated with the inducible HSSs (Baum and Giles, 1986).

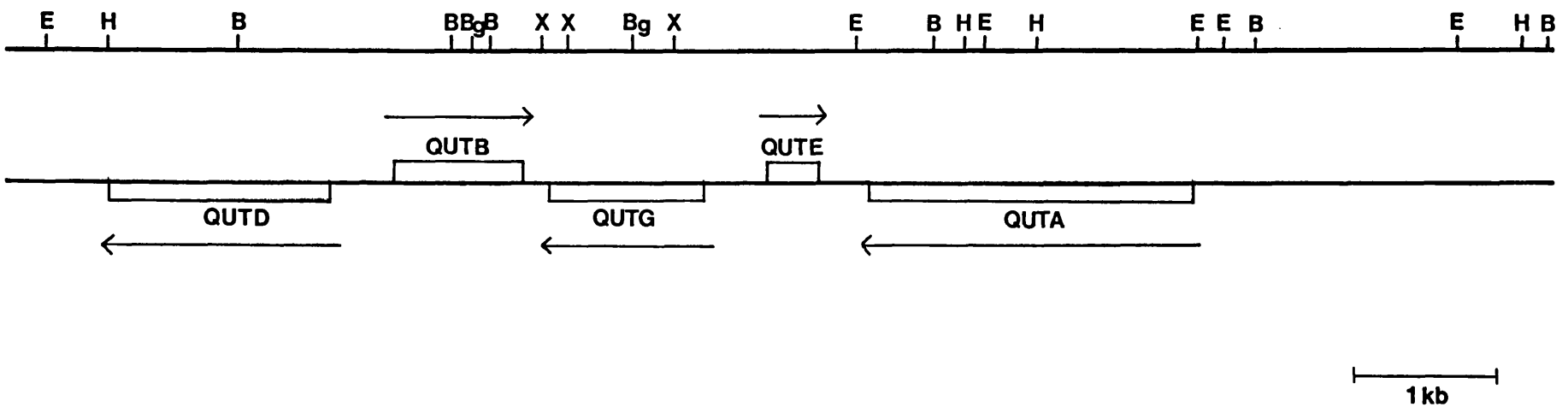
The qa-1F activator protein has been over-expressed in insect cell culture using a baculovirus expression vector and, using a DNA binding assay and DNase I footprinting analysis, it has been shown to bind to thirteen regions in the qa gene cluster. These are usually 21 to 22 base pairs in length and each contains a 16 base pair sequence related to the previously identified consensus sequence. Several 16 base pair synthetic oligonucleotides which are variants of the consensus sequence bind activator protein indicating that the 16 base pair sequence is sufficient for activator binding (Baum et al, 1987). The qa regulatory genes, which are induced 40 to 50-fold by quinate, contain only one detectable activator binding site in their 370 base pair common 5′ flanking region (Huiet, 1984; Patel and Giles, 1985). The qa structural genes, which are induced 300 to 1000-fold by quinate (Tyler et al, 1984),contain multiple activator binding sites in their flanking 5′ regions. The spacing of the binding sites and their positions relative to the transcriptional start sites are variable, with no apparent correlation to inducibility. The single activator

Figure 7.2

A restriction map of the A.nidulans DNA in λQ1 showing the positions and extent of the QUT genes

The boxes indicate the size of the individual genes and the arrows show the direction of transcription (Hawkins et al, 1988)

FIGURE 7.2



B BamH I
Bg Bgl II
E EcoR I
H Hind III
X Xba I

binding site between the divergently transcribed qa-1F and qa-1S genes suggests that this site functions bidirectionally and corroborates prior evidence that the qa-1F gene is autoregulated and that the activator protein also controls expression of the qa-1S repressor. This may be to ensure that enough repressor is present to switch off the qa system when inducer levels fall (Patel and Giles, 1985; Baum et al, 1987). It has been suggested that the multiple binding sites in the 5'-flanking regions of the qa structural genes may facilitate greater transcriptional control by the qa-1F activator protein (Baum et al, 1987).

In A.nidulans DNA sequence has been obtained for five genes, QUTA, QUTB, QUTD, QUTE and QUTG in the QUT gene cluster (Da Silva et al, 1986; Beri et al, 1987; Hawkins et al, 1988). Two pairs of genes, QUTD QUTB and QUTG QUTE , are divergently transcribed and the distance between their translational start codons are 444 and 461 nucleotides respectively (Figure 7.2, Hawkins et al, 1988). A computer aided homology search of the above intergenic regions and the 5' non-translated region of the QUTA gene has revealed a number of conserved sequence elements. The longest conserved motif is 22 nucleotides long, present once in each of the two intergenic regions and has the consensus CANCCTTGATCTAATGCAGTTT. A truncated 16 nucleotide variation of this sequence is present in the 5' non-translated region of the QUTA gene. It is suggested that this sequence motif may be the primary target for the QUTA gene product (Hawkins

et al, 1988). The two intergenic regions have four variations of a consensus 16 nucleotide sequence (GCCAGANCGTTCTNCC) and each of the four genes has one of these sequences downstream of the 22 nucleotide motif. These sequences have an imperfect inverted repeat structure and their consensus sequence exhibits 50% homology to the UAS_{GAL} motif found in the GAL system of S.cerevisiae (Giniger et al, 1985) and 43% homology to the 16 nucleotide motif found in the qa gene cluster of N.crassa (Giles et al, 1985). Seven variations of a second 16 nucleotide sequence have been identified that show 66% homology to the 16 nucleotide consensus sequence (GGATAANNNNTTATCC) present in the qa gene cluster. A 16 nucleotide sequence exhibiting 67% homology to this consensus is present in the 5' non-translated region of the QUTA gene (Beri et al, 1987). Five of these 16 nucleotide motifs are present in the QUTD/QUTB intergenic region and two in the QUTG/QUTE intergenic region. The position of these 16 nucleotide motifs is variable with respect to the 22 nucleotide motifs. In N.crassa the 16 nucleotide sequence has been shown to bind the qa-1F activator protein (Baum et al, 1987) and it is considered that these sequences may similarly bind the QUTA gene product in A.nidulans (Hawkins et al, 1988). Each of the five genes contains a variation of a 9 nucleotide motif which bears striking homology to the consensus CAAT sequence of mammalian genes (Benoist et al, 1980) that is considered to be involved in binding of RNA polymerase II (Beri et al, 1987; Hawkins et al, 1988). For the two

intergenic regions, the arrangement in the 5' region of each gene is a 22 nucleotide motif flanked by a 9 nucleotide motif and one 16 nucleotide motif (GCCAGANCGNTCTNCC) (Hawkins et al, 1988). The homology between the 16 nucleotide motifs of the qa and QUT gene cluster suggests that these systems may share a common mode of regulation and re-emphasizes their potential common ancestry.

In N.crassa the DNA binding domain of the qa-1F activator protein has been located in the first 183 amino acids and contains an arginine-lysine rich domain and six cysteines (Baum et al, 1987). Analysis of the A.nidulans QUTA protein sequence reveals a similar sequence in the amino terminus of the protein (Beri et al, 1987) indicating that this region may be involved in DNA binding. The homology in the amino terminus sequence is also present in other lower eukaryotic activator proteins, for example GAL4 (Keegan et al, 1986), PPR1 (Kammerer et al, 1984), ARGRII (Messenguy et al, 1986) and LAC9 (Salmeron and Johnston, 1986), and is considered to form a "DNA finger", a structure which is proposed to probe and disrupt the structure of chromatin so allowing access to the DNA to facilitate DNA binding. Studies in S.cerevisiae have shown that although the 73 N-terminal amino acids of the GAL4 activator protein are sufficient for DNA binding, such truncated proteins are unable to activate transcription in vivo (Struhl, 1987). Deletion analysis of the GAL4 activator indicates that two short separate regions of 50 to 100 amino acids that contain

a high proportion of acidic residues are each capable of transcriptional activation when fused to the GAL4 DNA binding domain (Ma and Ptashne, 1987). A non-homologous but similarly acidic region is required for transcriptional activation in the yeast GCN4 activator (Struhl, 1987). This suggests that in yeast the transcriptional activation regions are short and not defined by primary sequence but rather by a structural feature (Struhl, 1987). It will be interesting to see whether similar regions are involved in transcriptional activation in the N.crassa qa-1F and A.nidulans QUTA proteins.

In N.crassa, the qa-1S gene is considered to encode a negative regulatory protein, which interacts with quinic acid and, in the absence of inducer, blocks the action of the qa-1F activator protein. Its negative role is based on the phenotypes of the dominant non-inducible qa-1S^{-} and recessive constitutive qa-1S^{c} mutants. The DNA sequence has been obtained for the qa-1S gene from a wild-type strain, two constitutive and two non-inducible mutants (Huiet and Giles, 1986).

The amino acid sequence of the qa-1S repressor has been compared to the amino acid sequences of the S.cerevisiae and A.nidulans AROM proteins and their monofunctional E.coli counterparts (Anton et al, 1987). The N.crassa arom gene has been cloned (Catcheside and Storer, 1984) and, although its DNA sequence is not yet available, the organisation of

the functional domains in the pentafunctional polypeptide appear to be the same as that established for A.nidulans and S.cerevisiae (Duncan et al, 1987; Charles et al, 1986). The result of these comparisons is that the qa-1S protein is homologous to the three C-terminal domains of the AROM proteins and the three corresponding monofunctional E.coli enzymes, shikimate kinase, 3-dehydroquinase and shikimate dehydrogenase. The spacing of the blocks of homology in the qa-1S protein are basically similar to that of the AROM proteins, except that the qa-1S protein has an extra 100 amino acids compared to the S.cerevisiae AROM protein (Anton et al, 1987). The A.nidulans QUTB gene, encoding shikimate dehydrogenase, also exhibits significant homology to the C-terminal region of the qa-1S protein and the shikimate dehydrogenase domain of the A.nidulans AROM protein (Hawkins et al, 1988). The above observations have lead to the conclusion that the qa-1S protein probably evolved by adaptation of a duplicated gene for the partially or fully fused arom multifunctional enzyme (Anton et al, 1987).

The mutations in the two constitutive qa-1S^{c} mutants were located in the C-terminal region of the qa-1S protein which may be involved in either binding the repressor to its target, possibly the qa-1F activator protein, or forming active dimers and tetramers (Huiet and Giles, 1986). This region is homologous to the shikimate dehydrogenase domain of the AROM proteins (Anton et al, 1987). Evidence suggests

that there are quarternary interactions between the shikimate dehydrogenase and dehydroquinate synthase domains of the N.crassa arom protein and that part(s) of the proteins' C-terminal region are involved in dimer interactions (Giles et al, 1967; Coggins et al, 1985). The mutations in the two non-inducible qa-1S$^-$ mutants occur in a region proximal to the constitutive mutations in the qa-1S protein (Huiet and Giles, 1986). This region is homologous to quinate and shikimate dehydrogenases (Anton et al, 1987) and is considered to be involved in binding the inducer (Huiet and Giles, 1986).

A.nidulans genomic DNA homologous to the qa-1S gene has recently been obtained in a recombinant λ clone, which shares no common sequences with λQ1 (A.R Hawkins, personal communication). This homologous DNA is considered to span the qutR gene, though this has not yet been proven. It will be interesting to observe if the QUTR DNA sequence, when it is available, is equally homologous at the protein level to the A.nidulans AROM gene and whether the non-inducible and constitutive mutations identified to date affect the same regions as those in N.crassa.

Studies, to date, of the qa and QUT gene clusters have revealed both similarities and differences between these two systems, however it is hoped that further detailed analysis and subsequent comparisons will enhance our understanding of gene regulation and expression in these two closely related

systems.

THE COMMERCIAL APPLICATION OF A.NIDULANS

Studies of gene expression and regulatory processes in A.nidulans have been applied to the development of heterologous gene expression systems. Filamentous fungi of the genus Aspergillus are major sources of industrial enzymes and are able to secrete large quantities of certain proteins. The secretion process circumvents the intracellular accumulation of insoluble forms, which require subsequent denaturation and renaturation; protects the product from hydrolysis and in eukaryotes allows advantage to be taken of modifications such as glycosylation and specific endoproteolysis. For several species large scale fermentation technology is well established and they represent ideal host systems for the production of a wide range of proteins if they can be induced to secrete heterologous proteins by the application of recombinant DNA technology. Although A.nidulans is not noted for producing large amounts of extracellular protein, it is closely related to several commercially important species, for example A.niger, A.awamori and A.oryzae, which have this capacity. Thus information obtained from the study of heterologous gene expression in A.nidulans may be applicable to the development of similar systems for the industrially important Aspergillus species.

The expression and secretion of heterologous proteins in A.nidulans has recently been reported by a number of research groups (Cullen et al, 1987; Gwynne et al, 1987; Upshall et al, 1987).

Cullen et al (1987) have tested the ability of A.nidulans to synthesize and secrete bovine chymosin, an enzyme used in cheese manufacturing. Chymosin is an aspartyl protease found in the fourth stomach of unweaned calves where it cleaves K-casein in milk resulting in clotting. Chymosin is secreted as prochymosin whose 42 amino acid N-terminus is autocatalytically cleaved at low pH (Foltmann, 1970). Sequence analysis indicates that prochymosin is processed from a larger precursor, preprochymosin, containing a 16 amino acid signal peptide. Four vectors were constructed in which the transcriptional, translational and secretory control regions of the A.niger glucoamylase gene were functionally fused to either prochymosin or preprochymosin cDNA. In all constructions the glucoamylase terminator was fused to the 3' end of the prochymosin coding sequence. After transformation of A.nidulans with these plasmids, secretion of polypeptides enzymatically and immunologically indistinguishable from bovine chymosin was achieved. The production of chymosin was inducible under the control of the glucoamylase gene and greater than 90% of the chymosin was extracellular in immunological assays in comparison to that observed for S.cerevisiae where initially only 1% of

the protein was secreted (Moir et al, 1985), though this has been improved by subsequent developments in the system (Smith et al, 1985). The secretion of chymosin by A.nidulans therefore represents an improvement over other microbial systems such as S.cerevisiae (Moir et al, 1985; Smith et al, 1985) and E.coli (Emtage et al, 1983).

Gwynne et al (1987) have used the regulated promoters of the A.niger glucoamylase gene and the A.nidulans alcA gene in vectors combined with secretion signal sequences fused in frame to heterologous coding regions. They have reported the controlled secretion of a Cellulomonas fimi endogluconase and human interferon α2 from transformed strains of A.nidulans. The C.fimi endogluconase is a bacterial secreted protein and the C-terminal coding region of this gene, utilized in this system, was known to be efficiently secreted into the periplasm of E.coli (Wong et al, 1986) and the extracellular medium by S.cerevisiae (Skipper et al, 1985). It is therefore relatively well secreted by heterologous systems. In contrast, human interferon α2 is inefficiently secreted by heterologous systems including yeast (Hitzeman et al, 1983) and B.subtilis (Schein et al, 1986). The secretion of these two proteins by A.nidulans was comparable to that obtained in these alternative systems and for interferon α2 the levels obtained in shaken culture were equivalent to that obtained in the best available fermenter optimised microbial systems including B.subtilis (Schein et al, 1986).

Upshall et al (1987) have studied the ability of A.nidulans to produce human tissue plasminogen activator (t-PA). t-PA is a complex, 68 kD, secreted serine protease which is important in dissolving blood clots. It has 35 cysteine residues of which 34 are involved in disulphide bonds and there are 3 N-linked glycosylation sites which are utilized in the mammalian system. Active t-PA is generated by proteolytic cleavage of a primary translation product. The signal peptide and pro-sequence are removed to generate a mature protein with an NH_2 terminal glycine residue. Four amino acid residues are removed to produce a protein with a NH_2-terminal serine, by aminopeptidase activity. The single chains have plasminogen activating activity in the presence of fibrinogen but internal processing converts the single chain forms into disulphide bonded active two chain forms that do not require fibrinogen for activity. Attempts have been made to produce t-PA in microbial systems however in E.coli t-PA is cytosolic, non-glycosylated and incorrectly folded to produce a poorly soluble molecule (Pennica et al, 1983) and in S.cerevisiae t-PA is secreted only in the periplasmic space and is generally hyperglycosylated (MacKay, 1987; Lemontt et al, 1985).

A vector containing cDNA of the coding region of t-PA fused to promoter and terminator fragments of the tpiA gene of A.nidulans was transformed into A.nidulans. Analysis of

transformants revealed that A.nidulans was able to secrete active t-PA into the culture medium, the protein being correctly processed at its NH_2 terminus and not hyperglycosylated. The highest level of t-PA secreted into the culture medium was 100 μg per litre however this was increased to 1 mg per litre by fusing t-PA cDNA to the promoter fragments of either the A.nidulans alcC or A.niger adhA genes.

The successful expression of active bovine chymosin (Cullen et al, 1987), α-interferon (Gwynne et al, 1987) and t-PA (Upshall et al, 1987) in A.nidulans clearly demonstrates the potential of this organism for the secretion of mammalian proteins.

Continuing studies to further our understanding of gene expression, regulation and secretion in A.nidulans may allow the application of this organism to other areas of industrial interest.

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IDENTIFICATION AND LOCATION OF THE QUT GENES IN ASPERGILLUS NIDULANS USING DNA - MEDIATED TRANSFORMATION

BY H.A. WHITTINGTON

A cluster of QUT genes within A.nidulans, encoding the enzymes required for the catabolism of quinate to protocatechuic acid, has previously been isolated within the recombinant phage λQ1 by hybridization to certain genes in the equivalent N.crassa qa cluster. The location and functional integrity of the QUTE, QUTD and QUTA genes within the QUT gene cluster has been confirmed by the transformation of appropriate A.nidulans qut mutant strains. A.nidulans DNA homologous to the N.crassa qa-2 gene, encoding catabolic dehydroquinase, is able to transform a qutE mutant strain. Biochemical analysis of QUTE transformants containing multiple copies of the QUTE gene has shown that upon induction by quinate there is no increase in the level of catabolic dehydroquinase over that observed in a wild-type strain and that the transformants are subject to normal regulatory control. A.nidulans DNA homologous to the N.crassa qa-y gene is able to transform a qutD mutant strain. Biochemical studies of a number of qutD mutants suggests that in A.nidulans the QUTD gene encodes an essential component of a permease system required for the uptake of quinate. A.nidulans DNA homologous to the N.crassa qa-1F gene is able to transform a qutA mutant strain showing that the QUTA gene is equivalent to the N.crassa qa-1F gene and encodes a positively-acting regulatory protein. A small number of QUTA transformants exhibited constitutive expression of the QUT genes but these strains were subsequently found to be phenotypically unstable and therefore unsuitable for further analysis. DNA sequence analysis of the genes described above by the research group has confirmed their location within λQ1 and their physical organisation in chromosome VIII of Aspergillus nidulans.