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Sequence analysis and transformation by the catabolic 3-dehydroquinase (*QUT*E) gene from *Aspergillus nidulans*

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The induction of catabolic 3-dehydroquinase by quinic acid in *Aspergillus nidulans* has been shown to involve transcriptional control and yields a single major 0.8 kb mRNA. The nucleotide sequence of the catabolic 3-dehydroquinase *QUT*E gene has been determined and contains a single uninterrupted open reading frame of 462 bases encoding a 16505 Da protein of 153 residues. Comparison with the corresponding *QA*2 gene of *Neurospora crassa* reveals the absence of 75 nucleotides encoding 25 amino acids from the centre of the *QUT*E gene of *A. nidulans* and the presence of 21 additional nucleotides at its 3′ end. There is no nucleotide or amino acid homology between these two elements. A 16 bp inverted repeat (5′ GGCAGAGCGTTCTGCC) shows similarity to such repeats found in other fungal promoters. The functional integrity of the *QUT*E gene was demonstrated by the transformation of a *qut*E mutant strain which regains growth on quinic acid as sole carbon source. Four of the twelve transformed strains examined contained vector sequences integrated at the *qut*E locus, and these strains all exhibited normal regulation of 3-dehydroquinase even when 16 copies of the *QUT*E gene were present.

INTRODUCTION

The *QUT*E gene of *Aspergillus nidulans* is one of three structural genes coding for three enzymes required for growth on quinate as sole carbon source and forms part of the quinic acid utilization (*QUT*) gene cluster located on chromosome VIII (Hawkins *et al.*, 1982*a*). The *QUT*E gene encodes catabolic 3-dehydroquinase, one of two 3-dehydroquinase isoenzymes which catalyse the conversion of 3-dehydroquinate to dehydroshikimate. The second isoenzyme, biosynthetic 3-dehydroquinase, is active as part of a pentafunctional polypeptide encoded by the *AROM* locus of *A. nidulans*, and catalyses the same reaction in the synthesis of chorismate, a common precursor for many aromatic compounds. The genetic region encoding the biosynthetic 3-dehydroquinase isoenzyme has been characterized by expression in *Escherichia coli*, identification of its *in vitro* transcription and translation product, and DNA sequence analysis (Kinghorn & Hawkins, 1982; Charles *et al.*, 1985, 1986).

Synthesis of catabolic 3-dehydroquinase is induced by growth in the presence of quinate and requires the products of two control genes, *QUT*A and *QUT*D, which are thought to interact together with quinate in promoting the expression of the enzyme structural genes, which are also subject to carbon catabolite repression (Hawkins *et al.*, 1984). The production of the catabolic 3-dehydroquinase enzyme is therefore highly regulated in response to a series of metabolic signals, the simplest hypothesis being that this regulation is at the level of transcriptional control. The three enzyme structural genes have been isolated, and *QUT*E shown to express weakly in *aro*D mutant strains of *E. coli* deficient in biosynthetic 3-dehydroquinase (Hawkins *et al.*, 1985). In order to understand how the *QUT*E gene is regulated at the molecular level, we have determined the nucleotide sequence of the gene and its 5′ and 3′ flanking regions, and measured the level of *QUT*E-specified mRNA in wild type mycelium grown with glucose or quinate as carbon source. The functional integrity of the cloned *QUT*E gene has been confirmed by the transformation of *qut*E mutant strains of *A. nidulans*, and the effects of the integration of multiple copies of the *QUT*E gene upon enzyme regulation and the levels of induced catabolic 3-dehydroquinase activities have been investigated.

MATERIALS AND METHODS

Organisms and materials

Aspergillus nidulans strain WA53 (*pyro*A4; *pyr*G189; *qut*E208), was the host in all transformation experiments, and the strain R153 (WA3; *pyro*A4) the source of wild type genomic DNA and total cellular RNA, isolated as previously described (Clements & Roberts, 1985). The strains of bacteria, M13 bacteriophage and the origins of the plasmids have been described previously (Hawkins *et al.*, 1985). The plasmid pDJB-1 (Ballance *et al.*, 1983) was generously provided by Dr. G. Turner of Bristol University.

Materials were obtained as described in Charles *et al.* (1985) and media for the growth of *A. nidulans* or *E. coli* were those previously reported (Armitt *et al.*, 1976; Hawkins *et al.*, 1984; Willetts *et al.*, 1969).

Abbreviations used: kb, kilobases; bp, base pairs; SSC, standard saline citrate (20 × : 3.0 M-NaCl/0.15 M-sodium citrate); *QUT*, a wild type allele at a quinic acid utilization gene locus; *qut*, a mutant allele at a quinic acid utilization gene locus.

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Recombinant DNA techniques

Plasmids and phage M13 RF DNA were purified by a scaled up version of the Birnboim & Doly (1979) procedure with minor modifications (Charles *et al.*, 1985).

Transformation of *A. nidulans*

A. nidulans was transformed by the method of Ballance *et al.* (1983) using strain WA53 (*pyro*A4; *pyr*G189; *qut*E208) which requires uracil and cannot utilize quinate as sole carbon source. Each transformation reaction used a suspension of 10^6 regenerable sphaeroplasts mixed with 15 μg of the appropriate plasmid DNA.

Sphaeroplasts were plated in appropriate selective regeneration medium (lacking uracil or with quinate as sole carbon source) and transformants identified as strongly growing colonies that continued vigorous growth on selective medium after transfer of condiospores. Transformants were replated for single colony isolation and subsequently maintained on selective medium.

Southern and Northern blot analysis

Southern and Northern blot analyses were carried out using standard methods (Jeffreys *et al.*, 1980; Clements & Roberts, 1985; Thomas, 1980) using plasmid pBR322 DNA cut with restriction endonuclease *Sau*3A or λ C_I857 DNA cut with endonuclease *Hin*dIII as M_r markers. DNA probes were labelled with [α-^{32}P]dCTP by nick translation (Rigby *et al.*, 1977) or by 'fill in' reactions of cloned DNA fragments in phage M13 as previously described (Charles *et al.*, 1985).

Determination of gene copy number in transformed strains

To determine the copy number of the *QUT*E gene in transformed strains, chromosomal DNA from wild type strain R153 (copy number 1) and transformed strains (unknown copy number) was serially diluted 2-fold to 1:32 from an original concentration of 1 μg/ml. Samples from each of the dilution series were denatured and immobilized on a nitrocellulose filter ('dot blot') and hybridized with labelled 6.5 kb *Hin*dIII fragment isolated from plasmid pEH1.

Purification of DNA fragments

DNA restriction endonuclease generated fragments were purified as previously described (Dretzen *et al.*, 1981).

Cloning sonicated fragments of the *QUT*E gene

The plasmid pEH1 which contains the *QUT*B, *QUT*C and *QUT*E genes on a 6.5 kb *Hin*dIII fragment was physically sheared by ultrasonic vibration. Following treatment with Klenow polymerase to fill in and make sonicated molecules flush ended, fragments in the range 600–1000 bp were isolated and ligated into *Sma*I-cut M13mp9 RF DNA according to the method of Deninger (1983). Phage containing *QUT*E sequences were identified by hybridization to a labelled 1.1 kb *Eco*RI–*Bgl*II fragment containing the *QUT*E gene.

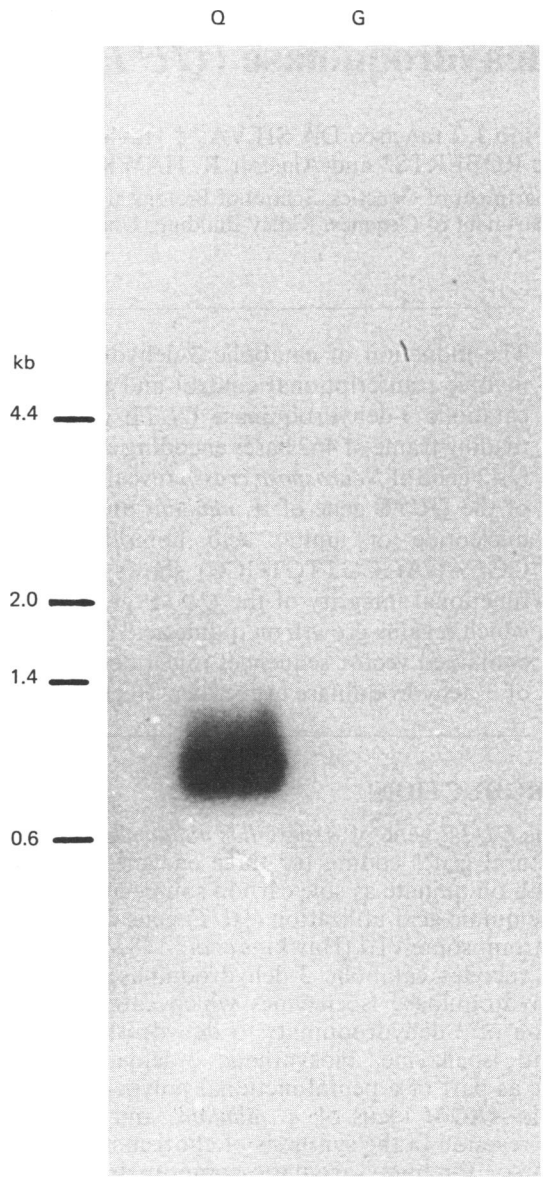

Fig. 1. Northern blot analysis of *QUT*E-specified mRNA

Total cellular RNA (20 μg) isolated from *A. nidulans* mycelium grown with quinate (Q) or glucose (G) as sole carbon sources was electrophoresed through a 1% formaldehyde/agarose gel, immobilized on nitrocellulose filters and probed with an α-^{32}P-labelled 1.1 kb *Eco*RI–*Bgl*II fragment containing the *QUT*E gene. λ phage DNA digested with *Hin*dIII and plasmid pBR322 DNA digested with *Sau*3A were included as M_r markers, and identified by hybridization with appropriately labelled DNA. The positions of relevant markers are indicated.

DNA sequencing

Dideoxy chain terminator sequencing using [^{35}S]dATP and buffer gradient gels was as described previously (Sanger *et al.*, 1977; Biggin *et al.*, 1983).

Oligonucleotide synthesis

Specific oligonucleotides were synthesized by the method of Matthes *et al.* (1984), with modifications to the wash cycle as described by Sproat & Gait (1985).

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                                                                    60
ccgccccatactgcaaccttcatcttatgccgttttgactggGGCAATCTctgttgcagc

                                                                   120
cGGCAGAgcgtTCTGCCaccactttcccttatccgtttccccgcaattttatactccaag

                                                                   180
tccagagacaaggactaaataacaatctacccccccaaaaccaatcggccgtaaaccttca

                                                                   240
ttgctactctgcacaATGGAAAAATCAATCCTCCTCATCAATGGCCCGAACCTCAACCTC
                 MetGluLysSerIleLeuLeuIleAsnGlyProAsnLeuAsnLeu

                                                                   300
TTGGGCACCCGCGAACCGCACATCTACGGCTCAACCACGCTCTCCGACGTCGAAGAATCC
LeuGlyThrAgrGluProHisIleTyrGlySerThrThrLeuSerAspValGluGluSer

                                                                   360
TCCAAAGGTCACGCAGCTTCGCTCGGCGCCTCCCTGCAAACATTTCAGTCAAACCATGAA
SerLysGlyHisAlaAlaSerLeuGlyAlaSerLeuGlnThrPheGlnSerAsnHisGlu

                                                                   420
GGTGCCATTGTTGACCGAATCCATGCCGCTCGCGGGAACACCGACGCGATCATCATCAAC
GlyAlaIleValGluArgIleHisAlaAlaArgGlyAsnThrAspAlaIleIleIleAsn

                                                                   480
CCCGGTGCATATACACACACCTCTGTCGCTATTCGGGATGCCCTGTTAGGAGTTGAGATT
ProGlyAlaTyrThrHisThrSerValAlaIleArgAspAlaLeuLeuGlyValGluIle

                                                                   540
CCCTTCATCGAACTCCATGTGAGCAATGTCCATGCACGAGAGCCGTTCAGACATCACAGT
ProPheIleGluLeuHisValSerAsnValHisAlaArgGluProPheArgHisHisSer

                                                                   600
TACTTTAGCGACAAAGCGTCAGGCATCATTGTGGGACTGGGTGTGTATGGGTACAAGGTC
TyrPheSerAspLysAlaSerGlyIleIleValGlyLeuGlyValTyrGlyTyrLysVal

                                                                   660
GCAGTGGAGCATGTTGCGTTGAACTTCAAGCCTCTGGAGAAAAAGGCTGCTCTTTAAatt
AlaValGluHisValAlaLeuAsnPheLysProLeuGluLysLysAlaAlaLeu***

                                                                   720
cttcttggattatgatactcctgcgacgtatcatgtaatttatgtcaaacaaagtttgct

                                                                   780
ccatataaaatcatctggggttgagtgattctgtcgctatctgcgactcacgcggtagtg

                                                                   840
tattccccggcgacgttaatgtttgttgtgtcggaaggagaaatgaatcagtctggctgg

                                                                   900
tgtaccgatacagctcgggattgcggcttccggaacgaatcccagattctgcatgaattc

                                                                   960
gggctggttgtccatcctagtgctagttagttttataacttgcgtgcagaagagaggttg

                                982
ttacctatctgtcccatcaagc
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Fig. 2. The catabolic 3-dehydroquinase gene *QUT*E of *A. nidulans*

The nucleotide sequence of the *QUT*E gene and its 5′ and 3′ non-translated regions is shown. The single open reading frame which shows homology with the *N. crassa QA*2 gene is shown with its inferred protein sequence. The 'CAAT' sequence (boxed), the regions of the inverted repeat which show perfect homology (arrows) and the protein coding region of the sequence are shown in capital letters.

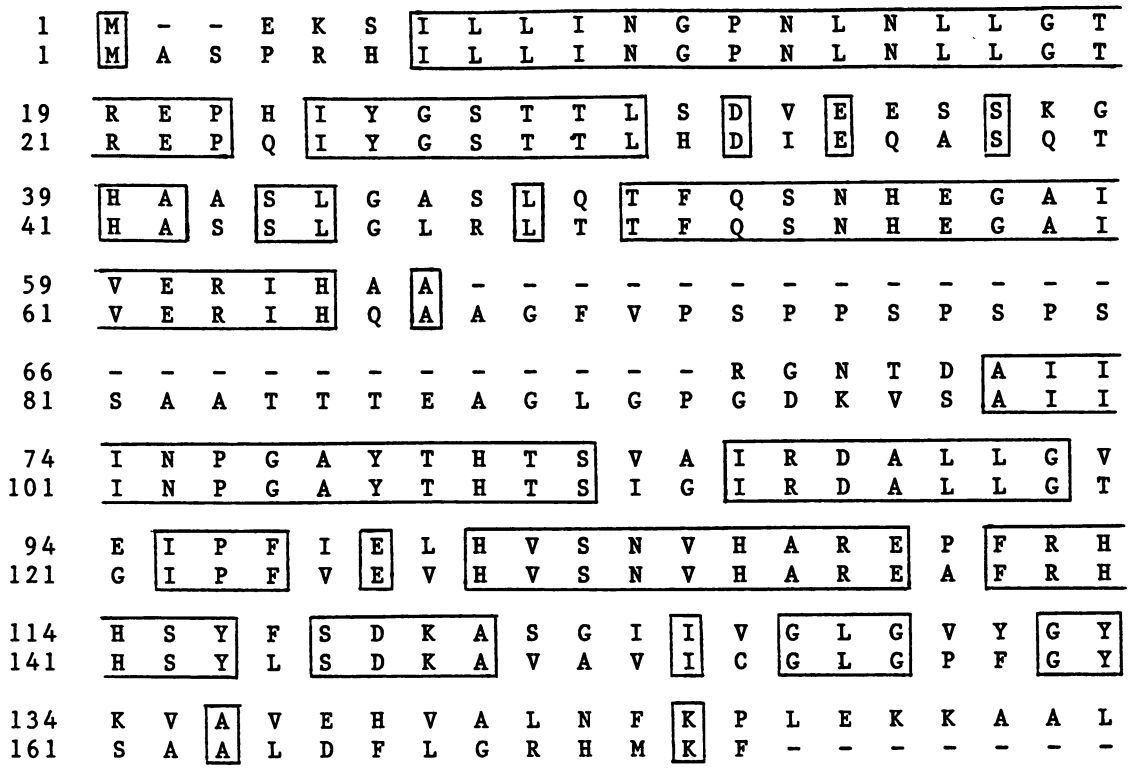

Fig. 3. Comparison of the amino acid sequences specified by the *A. nidulans QUT*E and *N. crassa QA2* genes

The *A. nidulans QUT*E amino acid sequence (upper line) is shown in an alignment with the *N. crassa QA*2 gene (lower line), with gaps introduced to maximize the homology which is indicated by boxes.

Enzyme assays

Growth of mycelium, preparation of cell-free extracts and enzyme assays were as previously described (Hawkins *et al.*, 1984).

RESULTS AND DISCUSSION

Demonstration of the transcriptional control of the *QUT*E gene

The model for the control of the induction of the three quinate-utilization enzymes supposes that regulation is at the level of transcription (Hawkins *et al.*, 1984). In order to test this hypothesis in the case of the catabolic 3-dehydroquinase *QUT*E gene, total cellular RNA from a wild type strain (R153) of *A. nidulans* was isolated from mycelium grown on either quinate or on glucose as sole carbon source.

Each RNA preparation (20 μg) was probed with a labelled 1.1 kb *Eco*RI–*Bgl*II DNA fragment containing the *QUT*E gene, isolated from the recombinant phage λQ1 (Hawkins *et al.*, 1985), using stringency conditions of 0.1 × SSC at 50 °C in the final wash. A photograph of the resulting autoradiograph is shown in Fig. 1 and shows that the probe hybridised to a single RNA species of 0.8 kb, and that this presumed mRNA is only detected in total RNA prepared from quinate-grown mycelium. These data clearly demonstrate that the *QUT*E gene is actively transcribed in mycelium grown with quinate as sole carbon source, but not in mycelium grown on glucose.

Nucleotide sequence of the catabolic 3-dehydroquinase (*QUT*E) gene of *A. nidulans*

M13 templates containing the 1.1 kb *Eco*RI–*Bgl*II fragment or sonicated fragments containing *QUT*E sequences were subjected to DNA squencing reactions using universal primer. Gaps in the sequence were filled by priming further sequencing reactions with specific oligonucleotides to complete the DNA sequence on both strands (results not shown).

The nucleotide sequence of the *QUT*E gene and its 5′ and 3′ flanking regions is shown in Fig. 2. The gene contains a single uninterrupted open reading frame of 462 bases encoding a polypeptide of 16505 Da. This in good agreement with previous estimates of the molecular mass of the 3-dehydroquinase subunit, approx. 18 kDa (Hawkins *et al.*, 1982*a*).

Homology between the catabolic dehydroquinase genes from *Aspergillus nidulans* (*QUT*E) and *Neurospora crassa* (*QA*2)

The hybridization of the *N. crassa QA*2 gene with the *A. nidulans QUT*E gene (Hawkins *et al.*, 1985) and the observation that rabbit antibodies raised against the *N. crassa* catabolic 3-dehydroquinase cross-react with catabolic 3-dehydroquinase enzyme from *A. nidulans* (Hawkins *et al.*, 1982*a*), strongly indicates that regions of homology exist between the two genes. An alignment of the protein sequence of the *QUT*E and *QA*2 genes to obtain maximum homology is shown in Fig. 3.

Inspection of Fig. 3 shows that the two genes have

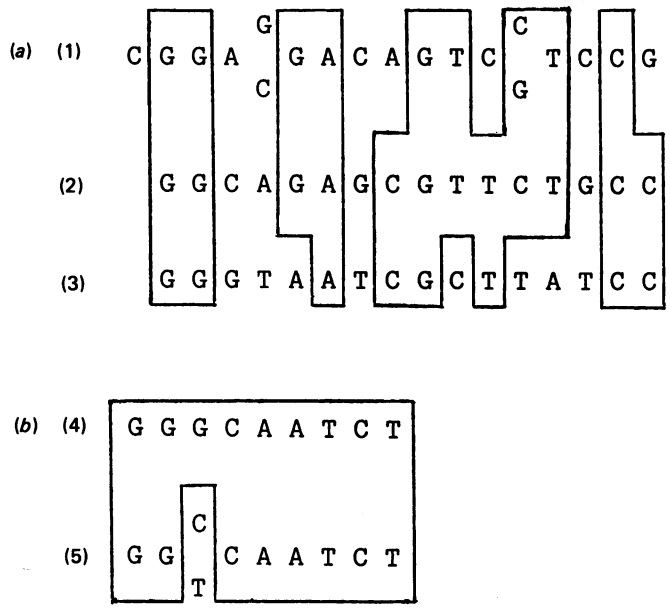

Fig. 4. Homologies between *A. nidulans QUT*E non-coding flanking sequences and other eukaryotic consensus regulatory sequences

(*a*) Homologies between inverted repeats present in the 5′ flanking regions of the *A. nidulans QUT*E (2), *N. crassa QA*2 (3) and *S. cerevisiae Gal* (1) genes. (*b*) Homology between a sequence in the 5′ flanking region of the *A. nidulans QUT*E gene (4) and the mammalian consensus 'CAAT' sequence (5).

regions of extensive homology and reveals the following points: (*a*) the *QUT*E gene lacks two amino acids at its 5′ when compared with the *QA*2 gene; (*b*) the *QUT*E gene lacks 25 amino acids that are present in the middle of the *QA*2 sequence; (*c*) the *QUT*E gene has seven amino acids at the 3′ end of the coding sequence that are not present in the *QA*2 gene; (*d*) there is no discernible sequence homology between these two structural elements in either the nucleotide or the amino acid sequences.

These data demonstrate that 25 amino acids can be deleted from the centre of the enzyme (nucleotide positions 207–282) without disrupting its catalytic activity. It has been shown previously (Hawkins *et al.*, 1982*b*) that the catabolic 3-dehydroquinase enzyme from *N. crassa* is highly susceptible to mechanical shearing or proteolytic degradation or both to produce two co-purifying fragments of approximately equal size. This degradation occurs at amino acid residues 88–92 in the *N. crasa* protein, which follow a proline-rich region, and are located within the 25 amino acids that are absent from the *A. nidulans* enzyme. This finding can be explained if one proposes that the missing segments of polypeptide in the *A. nidulans* enzyme lie on the surface of the protein in *N. crassa* and are not involved in the catalytic site, or that they fall in a non-ordered region of the protein, possibly a peptide link between independently folded domains.

These results clearly show that the *QUT*E gene of *A. nidulans* and the *QA*2 gene of *N. crassa* are closely related and probably have diverged from a common ancestor. In contrast, in *A. nidulans* the DNA sequence of the *QUT*E gene shows no homology with its isoenzyme biosynthetic 3-dehydroquinase encoded within the complex *AROM* locus (Charles *et al.*, 1985, 1986), strongly suggesting separate phylogenetic origins of these two genes and their convergent evolution.

The 5′ and 3′ non coding regions of the *QUT*E gene

Inspection of the DNA sequence flanking the 5′ ends of the *QUT*E gene reveals a 16bp sequence centred around nucleotide 69 that shows a close but imperfect inverted repeat. This sequence shows homology with an imperfect inverted repeat found 5′ to the *QA*2 gene of *N. crassa* and with the consensus sequence derived from the *Gal* genes of *Saccharomyces cerevisiae* (Giniger *et al.*, 1985).

The sequences of these repeats are shown in Fig. 4(*a*) aligned for maximum homology (shown by boxes). In *S. cerevisiae* four such sequences close together in the promoter region have been shown to be involved in regulating levels of transcription of the *Gal* genes (Giniger *et al.*, 1985). We speculate that the *QUT*E inverted repeat may be involved in the regulation of the transcription of this gene.

The 'CAAT' sequence has been recognized as being involved in the binding of RNA polymerase II to the DNA and is usually located 70–80 bp upstream from the start of transcription. We note that a sequence (shown in Fig. 4*b*) in the 5′ untranslated region of *QUT*E shows almost complete identity with the canonical sequence postulated for mammalian genes (Benoist *et al.*, 1980).

Sequences in the 5′ and 3′ untranslated region of *QUT*E that could be involved in the initiation and termination of transcription are present, but the evaluation of their biological significance is the subject of continuing investigation.

It is worth noting, however, that the canonical sequence for the *E. coli* ribosome-binding site that is present in the 5′ non-coding region of the *N. crassa QA*2 gene is absent from the 5′ non-coding region of the *A. nidulans QUT*E gene, as previously predicted (Hawkins *et al.*, 1985). It is highly probable that the presence of this sequence in the *N. crassa* DNA allows efficient expression of the *QA*2 gene in *E. coli* (Alton *et al.*, 1982), and its absence accounts for the very poor expression of the *A. nidulans QUT*E gene in *E. coli* (Hawkins *et al.*, 1985).

Transformation of *QUT*E mutants of *A. nidulans*

The functional identity and integrity of the cloned *QUT*E gene was demonstrated by the transformation of a *qut*E mutant strain of *A. nidulans* to restore the ability to grow on quinic acid as carbon source.

A. nidulans strain WA53 (*pyro*A4; *pyr*G189; *qut*E208) was separately transformed with 15 μg of plasmid pBR322 (negative control) or plasmid pDJB1 containing the *N. crassa pyr*-4 gene (positive control) or plasmid pEH1, containing the *QUT*E gene, (experimental), and sphaeroplasts plated in selective regeneration medium. Sphaeroplasts exposed to pBR322 DNA never gave rise to growing colonies when regenerated in media lacking uracil or containing quinate as carbon source, indicating stability of both the *pyr*G189 and *qut*E208 mutations. Sphaeroplasts exposed to pDJB1 or pEH1 DNA both gave rise to approximately seven transformants per μg of DNA when plated in regeneration medium either lacking uracil or containing quinate as carbon source respectively. A sample of 12 independently isolated colonies

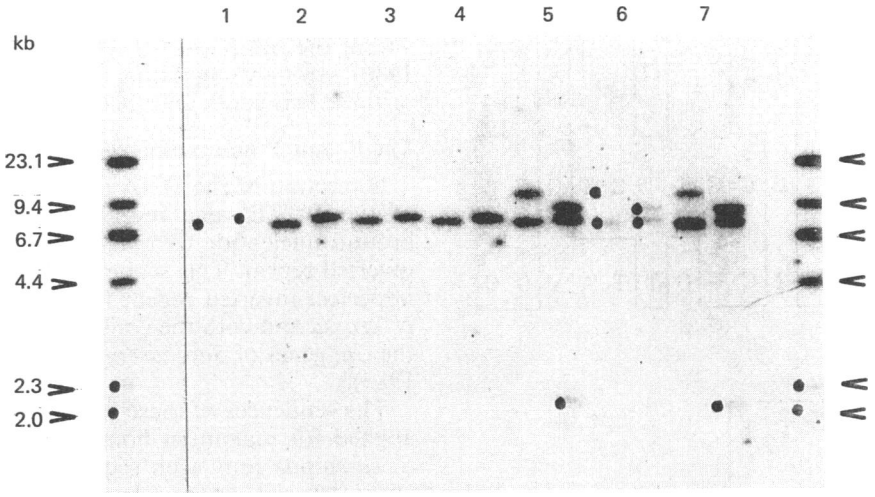

Fig. 5. Physical analysis of selected *A. nidulans* strains transformed with the *QUT*E gene

Genomic DNA (3.5 μg) from strains was digested with endonuclease *Pst*I (left lane of each pair) or *Pvu*II (right lane of each pair), separated by electrophoresis and transferred to a nitrocellulose filter. The DNA samples were hybridized with [α-^{32}P]dCTP-labelled pEH1 plasmid DNA (0.1 μg, 10^8 c.p.m./μg) as previously described (Rigby *et al.*, 1977; Jeffreys *et al.*, 1980). Track 1, wild type strain R153; track 2, recipient strain WA3; tracks 3 and 4, Type III transformed strains E12 and E15 (no vector pBR322 sequences); tracks 5, 6 and 7, Type I transformed strains E2.7, E2.9 and E2.16 (contain vector sequences). The size markers (kb) are provided by phage λ DNA digested with endonuclease *Hin*dIII and hybridized by [α-^{32}P]dCTP-labelled λ DNA. The positions of faint bands are indicated by dots.

produced by transformation with pEH1 DNA were purified as detailed in the Materials and methods section and grown in bulk for DNA extraction. Genomic DNA from these 12 transformants, the wild type (R153) and recipient (WA53) was digested with endonuclease *Hin*dIII and replicate filters subjected to Southern blot analysis by probing with labelled pBR322 DNA or the 6.5 kb *Hin*dIII fragment from pEH1. Analysis of the autoradiograph produced (results not shown) demonstrated that in all cases the 6.5 kB *Hin*dIII fragment hybridized to a 6.5 kb *Hin*dIII fragment in the chromosomal digests, as expected. The labelled pBR322 DNA hybridized to a 4.3 kb *Hin*dIII fragment in the chromosomal DNA of four of the twelve transformants, indicating integration of vector sequences, but failed to hybridize to the wild type control (R153) or recipient (WA53) DNA. The eight transformants lacking vector sequences have most probably arisen by gene conversion or double crossing over within the *qut*E gene, classified as Type III transformation by Hinnen *et al.* (1978). The transformants containing pBR322 vector sequences may have arisen by integration events at the site of the resident *qut*E gene locus or elsewhere in the genome, classified as Type I or Type II transformation by Hinnen *et al.* (1978). In order to distinguish between these two possibilties, DNA from the control strains (R153, WA53) three transformants (E7, E9 and E16) containing vector sequence and two (E12 and E15) lacking them was singly digested with endonucleases *Pst*I or *Pvu*II and subject to Southern blot analysis using labelled pEH1 DNA. Fig. 5 shows a photograph of the resulting autoradiograph. From a knowledge of the restriction map of plasmid pEH1 we can predict that Type I transformants will produce DNA fragments of 7.3 and 10.7 kb in the *Pst*I digest and 8.9, 7.4 and 2.0 kb in the *Pvu*II digest which hybridize to the pEH1 probe. On the other hand, Type III transformants will show hybridization of this probe to 7.1 and 7.4 kb fragments in the *Pst*I and *Pvu*II digests respectively. The data obtained was consistent with these predictions, and Fig. 5 shows that transformants E7, E9 and E16 (Type I) contain vector sequence integrated at the resident gene locus, whereas transformants E12 and E15 (Type III) contain no vector DNA. None of the twelve transformed strains examined resulted from integration of the transforming DNA at sites elsewhere in the genome (Type II).

Copy number of *QUT*E genes in Type I transformants

The copy number of the *QUT*E genes in Type I transformants was determined by using the protocol detailed in the Materials and methods section. A photograph of the autoradiograph produced by a 'dot-blot' filter is shown in Fig. 6, and shows that as expected the Type III transformant (E12) yields the same intensity spot as the wild type (R153), implying a copy number of 1, whereas transformants E7, E9 and E16 display intensities implying that respectively 16, 14 and 4 copies of the *QUT*E gene have been integrated into the chromosome.

Catabolic 3-dehydroquinase activities in transformed strains

A study of the expression of *QUT*E gene in transformed strains was undertaken to answer two questions. First, do the multi-copy number transformants exhibit an altered regulatory control? That is, do the *QUT*E genes still require induction by quinate, or are they constitutively expressed in its absence? Second, if subject to normal regulation, does quinate induction of the multicopy transformants lead to higher levels of catabolic 3-dehydroquinase than found in wild type? These questions arise from the possibility that the presence of such multiple copies of the *QUT*E gene may titrate repressing proteins, particularly the *QUT*A

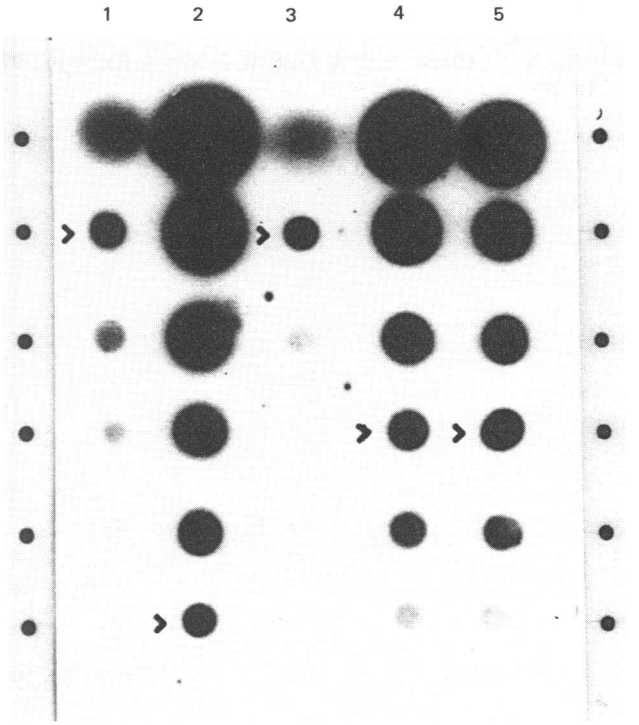

Fig. 6. Determination of the copy number of *QUT*E genes in representative transformants

Genomic DNA was serially diluted 2-fold from an initial concentration of 1.0 μg/ml, denatured and equivalent samples spotted on a nitrocellulose filter at the positions indicated by the dots. Samples showing the same intensities of hybridization are indicated by the arrowheads. The DNA was hybridized to [α-^{32}P]dCTP-labelled 6.5 kb *Hin*dIII fragment (0.3 μg, 10^8 c.p.m./μg) excised from plasmid pEH1. Track 1, wild-type strain (R153) taken as a copy number of 1; tracks 2, 4 and 5, Type I transformed strains E2.7, E2.9 and E2.16 containing respectively 16, 4 and 4 copies of the *QUT*E gene; track 3, a type III transformed strain E2.12.

protein if this has a negative function in the absence of the inducer quinic acid. Three Type I transformants (E7, E9 and E16) containing 16, 4 and 4 copies of the *QUT*E gene respectively were tested together with three Type III transformants (E8, E12 and E15) all with one copy of the *QUT*E gene, the *qut*E mutant recipient strain (WA53) and a *pyr*G$^-$; *QUT*E$^+$ (G191) strain. The specific activity of the catabolic 3-dehydroquinase was assayed in cell-free extracts of all strains from quinate-induced and non-induced cultures as previously described (Hawkins *et al.*, 1984). None of the glucose-grown non-induced strains contained any measurable enzyme activity, showing that the multicopy transformants did not constitutively express the *QUT*E gene. As expected, the recipient *qut*E mutant strain (WA53) showed no catabolic 3-dehydroquinase enzyme activity upon induction. The wild type control (G191) and the transformed strains all showed similar levels of catabolic 3-dehydroquinase activity upon induction, indicating that increasing the copy number of the *QUT*E gene does not lead to increased levels of enzyme activity. This result suggest that the rate limiting step(s) in the expression of these genes is not the number of copies of the enzyme structural genes, but more likely in the concentrations of the protein products of the two control genes *QUT*A and *QUT*D. Analysis of enzyme induction in diploid strains has shown that the *QUT*D gene product is not rate-limiting (Hawkins *et al.*, 1984). We have recently recovered a class of fully recessive non-inducible mutants in the *QUT*A gene which suggest that this gene product is required as a co-activator for enzyme induction (S. Grant, C. F. Roberts, H. Lamb & A. R. Hawkins, unpublished work). The activities of catabolic 3-dehydroquinase found in the transformants discussed above indicate that the concentration of the *QUT*A gene product may be rate-limiting for enzyme induction, and in this context it will be interesting to discover if the integration of multiple copies of either the *QUT*A or *QUT*D genes or both genes alters the levels of gene expression.

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