The QUTA activator and QUTR repressor proteins of *Aspergillus nidulans* interact to regulate transcription of the quinate utilization pathway genes

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Genetic evidence suggests that the activity of the native QUTA transcription activator protein is negated by the action of the QUTR transcription repressor protein. When *Aspergillus nidulans* was transformed with plasmids containing the wild-type *qutA* gene, transformants that constitutively expressed the quinate pathway enzymes were isolated. The constitutive phenotype of these transformants was associated with an increased copy number of the transforming *qutA* gene and elevated *qutA* mRNA levels. Conversely, when *A. nidulans* was transformed with plasmids containing the *qutR* gene under the control of the constitutive *pgk* promoter, transformants with a super-repressed phenotype (unable to utilize quinate as a carbon source) were isolated. The super-repressed phenotype of these transformants was associated with an increased copy number of the transforming *qutR* gene and elevated *qutR* mRNA levels. These copy-number-dependent phenotypes argue that the levels of the QUTA and QUTR proteins were elevated in the high-copy-number transformants. When diploid strains were formed by combining haploid strains that contained high copy numbers of either the *qutA* gene (constitutive phenotype) or the *qutR* gene (super-repressing; non-inducible phenotype), the resulting diploid phenotype was one of quinate-inducible production of the quinate pathway enzymes, in a manner similar to wild-type. The simplest interpretation of these observations is that the QUTR repressor protein mediates its repressing activity through a direct interaction with the QUTA activator protein. Other possible interpretations are discussed in the text. Experiments in which truncated versions of the QUTA protein were produced in the presence of a wild-type QUTA protein indicate that the QUTR repressor protein recognizes and binds to the C-terminal half of the QUTA activator protein.

Keywords: quinate, transcription regulation, protein–protein interaction

INTRODUCTION

Quinic acid is abundant and many prokaryotes and microbial eukaryotes are able to exploit it as a source of carbon (for a review see Hawkins *et al.*, 1993a). In the microbial eukaryotes, as exemplified by *Neurospora crassa* and

Aspergillus nidulans, the quinic acid utilization pathway (*qut*) is completely dispensable and in wild-type strains the enzymes necessary for its catabolism are only produced when quinate is supplied exogenously. This clearly implies that this pathway is regulated, and the mechanism of this regulation has been studied in terms of the control of

Abbreviations and enzyme designations: AROM, a pentadomain protein catalysing the conversion of 3-deoxy-D-arabino-heptulosonic 7-phosphate to 5-enolpyruvyl shikimate 3-phosphate; DHQ, dehydroquininate; DHQ synthase, dehydroquininate synthase, EC 4.6.1.3; dehydroquinase, EC 4.2.1.10; DHS, dehydroshikimate; EPSP synthase, 5-enolpyruvyl shikimate-3-phosphate synthase, EC 2.5.1.19; QUTA, positively acting protein mediating the transcriptional regulation of the quinic acid utilization gene cluster of *A. nidulans*; QUTR, negatively acting protein mediating the transcriptional regulation of the quinic acid utilization gene cluster of *A. nidulans*; shikimate dehydrogenase, EC 1.1.1.25; shikimate kinase, EC 2.7.1.71.

pathway gene expression and the control of metabolic flux (Giles *et al.*, 1985; Grant *et al.*, 1988; Lamb *et al.*, 1991, 1992; Wheeler *et al.*, 1996). Genetic analysis of the ability to utilize quinate as a carbon source identified three classes of mutant corresponding to different complementation groups that pleiotropically affected the production of all three of the *qut* pathway enzymes. One class (*qutD* in *A. nidulans*; *qa-Y* in *N. crassa*) encoded a permease necessary to transport quinate into the mycelium at neutral pH (Whittington *et al.*, 1987; Geever *et al.*, 1989). A second class mainly gave rise to recessive mutations that had a non-inducible phenotype but also rarely to a dominant non-inducible phenotype. These mutations (*qutA* in *A. nidulans*; *qa-1F* in *N. crassa*) were interpreted as identifying a positively acting regulatory protein. The third class was identified in *N. crassa* (designated *qa-1S*) in a colorimetric plate test designed to identify mutants that constitutively produced the quinate pathway enzymes in the absence of quinate (Giles *et al.*, 1985). Such mutants were found to be recessive. In *A. nidulans*, however, the equivalent gene (*qutR*) was identified as a second-site suppressor of *qutD* mutations that when outcrossed conferred a recessive phenotype of constitutive production of the quinate pathway enzymes in the absence of quinate. Dominant mutations conferring the phenotype of quinate non-utilization and apparently mapping at the *qa-1S* and *qutA* loci were identified in *N. crassa* and *A. nidulans* and found to revert at a high frequency to a mutant phenotype of constitutive production of the quinate pathway enzymes in the absence of quinate. This third class of mutant was interpreted as identifying a negatively acting control gene (Giles *et al.*, 1985; Grant *et al.*, 1988).

In both *N. crassa* and *A. nidulans*, the genes involved in the utilization of quinate and its wild-type regulation have been shown to map in a cluster. In both species, the entire gene cluster has been isolated and the genes encoding the quinate pathway enzymes shown to be regulated at the level of transcriptional control (Geever *et al.*, 1989; Lamb *et al.*, 1990; Levesley *et al.*, 1996). The *A. nidulans* QUTA activator protein (encoded by *qutA*) controls the production of its own mRNA and that of the *qutR* repressor gene in an autoregulatory circuit (Levesley *et al.*, 1996), and it contains a putative zinc binuclear cluster motif (which has been shown to bind zinc *in vitro*), which is proposed to facilitate binding to variants of a 16 nt motif found in the promoters of *qut* pathway genes (Beri *et al.*, 1987; Hawkins *et al.*, 1988; Levesley *et al.*, 1996). A similar situation exists in *N. crassa* (Geever *et al.*, 1989).

The repressing effect of the QUTR and QA-1S proteins has been proposed to be negated by the binding of one or more of the *qut* pathway metabolites – quinate, 3-dehydroquinase (DHQ), shikimate or dehydroshikimate (DHS) (Hawkins *et al.*, 1992). The binding of these intermediates is implied to cause some allosteric change in the repressor protein which negates its repressing function. These putative allosteric changes could range from subtle changes in the overall shape of the repressor protein to a complete dissociation of the proposed activator–repressor complex. This model arises from the observation that either the exogenous supply of quinate

or the endogenous supply of DHQ leaking from a mutant form of the shikimate pathway AROM pentafunctional enzyme lacking 3-dehydroquinase activity leads to the production of the quinate utilization pathway enzymes (Case *et al.*, 1972; Giles, 1978). A potential molecular mechanism for this proposed series of complex recognition events has been identified in the proposal that the quinate pathway activator and repressor proteins are homologous with shikimate pathway enzymes that recognize the quinate-pathway-activating metabolites (or closely similar molecules) as substrates (Hawkins *et al.*, 1992, 1993a; Bugg *et al.*, 1991; Hawkins & Lamb, 1995). The activator protein is proposed to be homologous with the two N-terminal domains (DHQ synthase and EPSP synthase), and the QUTR protein is proposed to be homologous with the three C-terminal domains (shikimate kinase, 3-dehydroquinase and shikimate dehydrogenase) of the pentafunctional AROM enzyme active in the shikimate pathways of *A. nidulans* and *N. crassa* (Charles *et al.*, 1986; Hawkins, 1987; Anton *et al.*, 1987; Hawkins *et al.*, 1992, 1993a, b). Biophysical analysis of the proposed homologous AROM 3-dehydroquinase and the QUTR 3-dehydroquinase-like domains has shown that they have similar physical characteristics that derive from a highly conserved tertiary structure (Lamb *et al.*, 1996). Recent analysis of the nucleotide sequence of 13 *qutA* mutations has confirmed the bi-domain structure of the QUTA protein and has located a transcription activation function (TAF) in the C-terminal EPSP-synthase-like domain (Levesley *et al.*, 1996). These two observations lend some credence to the evolutionary hypothesis for the origins of the quinate pathway activator and repressor proteins, and in this communication we address the question of how these two proteins interact to regulate the transcription of the *qut* gene cluster in response to the presence of quinate.

METHODS

Plasmids, recombinant lambda and strains. The origins of the recombinant phage lambda and plasmids, other than those constructed as part of the research described here, have been described previously (Hawkins *et al.*, 1985; Beri *et al.*, 1987, 1990; Moore & Hawkins, 1993; Lamb *et al.*, 1990). The *Escherichia coli* strains used have been described previously, as have methods for their growth and propagation (Kingham & Hawkins, 1982; Grant *et al.*, 1988; Hawkins & Smith, 1991; Lamb *et al.*, 1992). The genotypes of the *A. nidulans* strains used are given in Table 1.

Materials. Chemicals and solvents other than DHQ were of AnalaR or greater purity and were purchased from local suppliers. DHQ was made from quinate and purified by chromatography as described by Grewe & Haendler (1966). Quinate and NAD were from Sigma and dehydroshikimate (DHS) was made enzymically from DHQ using either purified type I 3-dehydroquinase from *Salmonella typhi* or type II 3-dehydroquinase from *Mycobacterium tuberculosis* (Moore *et al.*, 1992). General molecular biology reagents were from Pharmacia or Gibco BRL; *Taq* polymerase was from BCL. Specific 30-mer oligonucleotides were purchased from the University of Newcastle upon Tyne Central Facility for Molecular Biology, and Dynabeads were from Dynal UK.

Table 1. Genotypes of *A. nidulans* strains

Strain	Genotype	Comments	Reference
R153	<i>wA3; pyroA4</i>		Grant <i>et al.</i> (1988)
R21	<i>yA2; pabaA1</i>		Grant <i>et al.</i> (1988)
<i>qutE208</i>	<i>yA2; qutE208; pabaA1</i>		Grant <i>et al.</i> (1988)
G191	<i>fwA1; pyrG189; pabaA1; uaY9</i>		Lamb <i>et al.</i> (1990)
<i>qutA303</i>	<i>wA3; pyroA4; qutA303</i>		Grant <i>et al.</i> (1988)
2035	<i>yA2; pyrG189; qutR^c</i>		Lamb <i>et al.</i> (1992)
2043	<i>fwA1; pyrG189; pabaA1; uaY9</i>	Strain G191 transformed with plasmid pCAP2, containing the <i>N. crassa</i> <i>pyr-4</i> gene	This work

Methods. Restriction endonucleases were used according to the manufacturer's recommendations. PCR amplification of target DNA sequences was as described previously (Van den Hombergh *et al.*, 1992; Moore & Hawkins, 1993). All other routine molecular biology protocols followed individual manufacturer's recommendations or were as described by Maniatis *et al.* (1982). DNA was labelled using a Boehringer Mannheim random-primed DNA-labelling kit, and the labelled probe purified using the NucTrap purification column system from Stratagene. Quinate dehydrogenase, 3-dehydroquinase and dehydroshikimate dehydratase assays were performed as described previously (Grant *et al.*, 1988). Transformation of *A. nidulans* and the preparation of cell-free extracts for enzyme assay were as previously described (Lamb *et al.*, 1991; Grant *et al.*, 1988). An *in vitro* colour test, the 'PCA spot test' was used to detect the presence of quinate pathway enzymes in young mycelium and was carried out as described by Grant *et al.* (1988). This test traps the product of the quinate utilization pathway (protocatechuic acid; PCA) as a highly coloured compound after reaction with iron. This purple-coloured compound is easily visible by eye and can be measured accurately spectrophotometrically (Van den Hombergh *et al.*, 1992) when used in pathway flux experiments. However when used as a test for constitutivity using a single measurement the test is only semi-quantitative, but it easily and accurately identifies constitutive transformants. Purification of total RNA followed the method of Cathala *et al.* (1983) and poly(A⁺) mRNA was prepared from total RNA using Dynabeads Oligo(dT) and following the manufacturer's protocol. Images from Southern and Northern-blot experiments that involved the *qutA*, *qutR* and *actA* genes of *A. nidulans* were recorded by quantitative phosphorimaging, using a Molecular Dynamics phosphorimager model 400b and analysed using Imagequant software version 3.3. Exposures of the probed filters varied between 1 h (for high-copy-number transformants) and 48 h (for low-expressed wild-type regulatory gene mRNA levels). Copy number of the *qutA* gene in Southern blots of transformant DNA was quantified by comparison of duplicate dot-blots probed with the *qutA* gene or the *actA* gene of *A. nidulans* (Fidel *et al.*, 1988). Copy number of the *qutR* gene in Southern blots of transformant DNA was quantified by reference to the intensity of the signal produced by the resident *qutR* gene, as only transformants with integration events away from the resident *qut* gene cluster were utilized in subsequent analysis.

Construction of *A. nidulans* expression vectors and recombinant derivatives expressing the *qa-2*, *qutR* and *qutA* genes. Plasmid pNUFC77 contains the *A. nidulans* *qutE* promoter and terminator sequences separated by a short polylinker region containing *NcoI*, *PstI* and *HindIII* recognition sites, and

additionally has translational stop codons in all three reading frames immediately downstream of the terminator sequence (Moore & Hawkins, 1993). The *qutE* promoter region of pNUFC77 was removed by digestion with *SacI* and *NcoI* and replaced with the promoter region from the *A. nidulans* phosphoglycerate kinase (*pgk*) gene, a promoter that has constitutive activity (Clements & Roberts, 1986). The promoter region from the *pgk* gene extended from the A base preceding the AUG translational codon upstream for a further 372 nt. This promoter region was amplified by PCR using oligonucleotides containing recognition sites for *SacI* (the upstream site) or *NcoI* (the downstream site), and following digestion with *SacI* and *NcoI* was subcloned into appropriately digested pNUFC77 to yield pNUFC100. The pUC18 backbone in plasmids pNUFC77 and 100 contains a unique *NdeI* site located outside of the general-purpose polylinker (which is deleted in these constructs); this site was replaced with a unique *XbaI* site in plasmids pNUFC77 and 100 by site-directed mutagenesis. This was achieved by PCR using 'back-to-back' oligonucleotides, one of which contained the required *XbaI* site, as described by Hemsley *et al.* (1989) and modified by Moore & Hawkins (1993). This mutagenesis produced plasmids pNUFC101 (modified pNUFC100) and pNUFC102 (modified pNUFC77). Plasmid pNUFC200 was constructed by subcloning PCR-amplified DNA containing the *qa-2* coding sequence into plasmid pNUFC101. The amplified sequence was flanked by *NcoI* and *HindIII* sites at the 5' and 3' ends of the gene, respectively, and after suitable digestion was subcloned into the vector pNUFC101. The *N. crassa* *pyr-4* gene was amplified by PCR from plasmid pFB4 and, in addition to the coding sequence, contained the first 219 nucleotides of the upstream 5' non-coding region and the first 165 nucleotides of the 3' non-coding region. The oligonucleotides for PCR of the *pyr-4* gene contained recognition sites for *XbaI* and, following appropriate digestion, the PCR-amplified DNA was subcloned in *XbaI*-digested pUC18 DNA. In all cases, modified plasmids were rescued from ligation mixtures by transforming *E. coli* strain SK3430 and selecting transformants on rich medium containing 50 µg ampicillin ml⁻¹. Individual plasmid constructs were verified by digesting plasmid DNA with appropriate restriction endonucleases. The oligonucleotides used in the various PCR amplifications were: the *pgk* promoter upstream oligonucleotide, 5' CGATGTATAACTGAGCTCAGGAACGGAGCG 3'; the *pgk* promoter downstream oligonucleotide, 5' TGCTGGTGAGAGCCATGGTTGCTATAGCTG 3'; the mutagenic oligonucleotide to produce an *XbaI* site into pNUFC77 and 100, 5' CTGAGAGTGCACCTAGACGGTGTGAAATA 3'; the non-mutagenic oligonucleotide to introduce an *XbaI* site into pNUFC77 and 100, 5' TACA-

ATCTGCTCTGATGCCGCATAGTTAAG 3'; the *pyr-4* gene upstream oligonucleotide, 5' AATTCACGCTGATCTAGA-AAACATTGTGCA 3'; the *pyr-4* downstream oligonucleotide, 5' GCCTTACAAATCTAGATGGTAGTTTCTT 3'; the 5' *qa-2* oligonucleotide, 5' GAGGTACCAAACACCATGGCG-TCCCCCGT 3'; the 3' *qa-2* oligonucleotide, 5' CATCC-CAATGCAAAGCTTCAAACTTCATGT 3'. The template for the *pgk* promoter PCR was plasmid pGK1 (Clements & Roberts, 1985), for the *pyr-4* gene it was plasmid pFB4 (Buxton & Radford, 1983) and for the *qa-2* gene it was pVK88 (Giles *et al.*, 1985).

The purpose behind producing pNUFC101 and 102 was to provide vectors containing a unique *Xba*I site, to facilitate the subcloning of the selectable marker *pyr-4* (Buxton & Radford, 1983; Glazebrook *et al.*, 1987) thereby allowing indirect selection for any other sequence cloned into the plasmids. The *gutA*, *gutR* and *pyr-4* genes do not contain *Xba*I sites; therefore by prior subcloning of *gutA* or *gutR* into these vectors it should be possible to introduce them into *A. nidulans* by indirect selection using the *pyr-4* selectable marker cloned into the *Xba*I site.

Initial experiments showed that the *pyr-4* gene, when amplified by PCR using oligonucleotides incorporating *Xba*I sites, could not be subcloned into the *Xba*I site of pNUFC101 and 102 following suitable digestion. Subsequently, however, the *pyr-4* gene was successfully subcloned into the *Xba*I site in pUC18 to yield pNUFC247 and shown to complement the *pyrG189* mutation. When *pyr-4* was released from pNUFC247 by *Xba*I digestion, and purified by gel electrophoresis, it was found that it could be easily subcloned into the *Xba*I site in pNUFC101 or 102 or recombinant plasmids derived from them. The reason for the difficulty in subcloning fragments of DNA prepared by the direct digestion of the products of a PCR remains unclear. We were able to routinely subclone the products of a PCR provided that the ends were generated by digestion with different restriction endonucleases. If, however, the ends were generated by the same restriction endonuclease we experienced great difficulty and, in some cases, were unable to subclone the fragment and had to employ the alternative strategies described below to construct the plasmids needed.

Experiments with the *gutA* gene made use of the fact that it does not contain a *Bgl*II site and that the *A. nidulans* vector pCAP2 (Turner & Ballance, 1987), has a unique *Bam*HI site in addition to the selectable marker *pyr-4*. Sequences of the whole *gutA* gene or parts thereof were subcloned into pNUFC101 or 102 and subsequently amplified by PCR, using oligonucleotides specific to the promoter and terminator and containing *Bgl*II sites. These PCR products therefore contain the *gutA* sequences fused to the *gutE* or *pgk* promoter and the *gutE* terminator. Following appropriate digestion, the *gutA* sequences could then be successfully subcloned into the *Bam*HI site of pCAP2, thereby allowing transformation into *A. nidulans* using the *pyr-4* selectable marker. This strategy was adopted because at the time of construction it was not possible to utilize the strategy of subcloning in the selectable *pyr-4* marker (see above). *gutA* and *gutR* DNA sequences of the coordinates shown in Table 4 were amplified by PCR using 5' and 3' oligonucleotides that contained *Nco*I/*Bsp*HI or *Hind*III sites, respectively, at the 5' and 3' ends of the target DNA (see below). After suitable digestion, the DNA sequences were subcloned into the vectors pNUFC77 or 101, placing them under the control of the *gutE* or *pgk* promoters and the *gutE* terminator. Oligonucleotides specific to the 5' end of the *pgk* promoter and the 3' end of the *gutE* terminator and containing restriction sites for *Bgl*II were used to PCR-amplify the *gutA* DNA sequences in plasmids pNUFC77 or 101 as a cassette with the promoter and terminator attached. Following

appropriate digestion with restriction endonucleases the PCR-amplified DNA sequences were subcloned in the transformation vector pCAP2 at a unique *Bam*HI site. Plasmid pCAP2 carries the *N. crassa pyr-4* gene, allowing selection for transformants on the basis of uracil independence in strains wild-type for the *gut* gene cluster, but carrying the *pyr-G* mutation.

gutR DNA sequences of the coordinates shown in Table 4 were amplified by the PCR using oligonucleotides containing 5' and 3' *Nco*I sites, and after suitable digestion, subcloned into the vector pNUFC101. Subsequently the recombinant pNUFC101 plasmids were digested with *Xba*I and the *pyr-4* gene from pNUFC247 subcloned on an *Xba*I fragment.

The 5' oligonucleotide for the complete *gutA* gene was 5' TCATTGTATCGATCATGAGTAGCGATA 3'; the 3' oligonucleotides for the complete *gutA* gene was 5' GTGCA-GAAGAGATCATGATTACCTATCTGT 3'; the 5' oligonucleotide to the promoter in pNUFC101 was 5' CGGCC-AGTGCCAAGATCTTTTCATGCAGAAT 3'; the 3' oligonucleotide to the terminator in pNUFC101 was 5' ACGAATTAATTCAGATCTAGGAACGGAGCC 3'; the 5' oligonucleotide for the complete *gutR* gene was 5' CTGATCCACCTGCCATGGTTCATGCGTTCT 3'; the 3' oligonucleotide for the complete *gutR* gene was 5' ACAAGCGTGGCCCCATGGTCACATCGGCTG 3'; the 5' oligonucleotide for the *gutA* sequence in pATR3 was 5' CTGGCAACCATCATGAAAGAGTCGGAGGGG 3'; the 5' oligonucleotide for the *gutA* sequence in pATR4 was 5' CTGGCAACCATCATGAAAGAGTCGGAGGGG 3'; the 3' oligonucleotide for the *gutA* sequence in pATR4 was 5' GTGCTGAGATCATGAAGTCAACCCGCGTTGG 3'.

RESULTS AND DISCUSSION

Construction of *A. nidulans* expression vectors

In order to be able to decouple the expression of the *gutA* and *gutR* genes and fragments thereof from the auto-regulatory circuit mediated by the QUTA protein, *A. nidulans* expression vectors utilizing the constitutive phosphoglycerate kinase (*pgk*) promoter were constructed. These vectors were constructed as part of a larger exercise designed to produce general expression vectors for *A. nidulans* with either inducible or constitutive promoters. Expression vectors utilizing the quinate-inducible *gutE* promoter or the constitutive *pgk* promoter (Clements & Roberts, 1985, 1986) were constructed using pUC18 as a backbone. The construction of pNUFC77 (which contains a quinate-inducible promoter) has been described previously (Moore & Hawkins, 1993), and the construction of plasmids pNUFC100, 101 and 102 is described in Methods. The *gutE* promoter in pNUFC77 has previously been shown to drive effective transcription of heterologous genes in *A. nidulans* (Lamb *et al.*, 1991; Moore *et al.*, 1992; Moore & Hawkins, 1993).

The control of transcription by the *A. nidulans pgk* promoter has been analysed in detail previously, and was shown to be positively influenced by the sequence of the *pgk* chromosomal gene encompassing codons 14–183 and including two introns (Streatfield *et al.*, 1992). In order to assess the ability of the isolated *pgk* promoter – in the absence of the sequences from the *pgk* gene – to drive heterologous gene expression in pNUFC101, the *qa-2* gene of *N. crassa* encoding a type II 3-dehydroquinase was

Table 2. Description and testing of *A. nidulans* expression vectors

Plasmid characteristics are shown in (a). In (b), cell-free extracts of uninduced (U) or induced (I) mycelium prepared from the control wild-type (R153), two transformants of strain *qutE208* transformed with pEH1 (TE1 and 2), and five experimental strains of *qutE208* transformed with pNUFC200 (TQ1–5) grown in minimal medium with glucose as carbon source were assayed for the presence of quinate pathway enzymes. Activities are expressed relative to the value in the R153 control, which has a wild-type *qut* gene cluster. Specific activities are shown in parentheses. ND, Not detectable.

(a) Plasmid characteristics

Plasmid	Vector	Backbone	Promoter	Terminator	Other features
pNUFC77	NA	pUC18	<i>qutE</i>	<i>qutE</i>	
pNUFC100	NA	pUC18	<i>pgk</i>	<i>qutE</i>	
pNUFC101	pNUFC100	pUC18	<i>pgk</i>	<i>qutE</i>	Unique <i>NdeI</i> → <i>XbaI</i>
pNUFC102	pNUFC77	pUC18	<i>qutE</i>	<i>qutE</i>	Unique <i>NdeI</i> → <i>XbaI</i>
pNUFC200	pNUFC101	pUC18	<i>pgk</i>	<i>qutE</i>	Drives transcription of the <i>N. crassa qa-2</i> gene
pNUFC247	NA	pUC18	NA	NA	Contains the <i>N. crassa pyr-4</i> gene

NA, Not applicable.

(b) Ability of the isolated *pgk* promoter to drive constitutive transcription

Strain	Quinate dehydrogenase		3-Dehydroquinase		Dehydroshikimate dehydratase	
	U	I	U	I	U	I
R153	ND	1.00 (0.090)	ND	1.00 (0.285)	ND	1.00 (0.179)
TE1	ND	0.80 (0.073)	ND	0.73 (0.219)	ND	1.00 (0.179)
TE2	ND	1.25 (0.113)	ND	0.20 (0.058)	ND	0.97 (0.174)
TQ1	ND	1.21 (0.110)	2.60 (0.750)	2.60 (0.750)	ND	1.04 (0.187)
TQ2	ND	0.85 (0.077)	0.88 (0.250)	0.88 (0.025)	ND	0.97 (0.174)
TQ3	ND	0.90 (0.080)	3.80 (1.090)	4.50 (1.300)	ND	0.96 (0.172)
TQ4	ND	0.87 (0.079)	5.80 (1.670)	5.70 (1.640)	ND	1.01 (0.182)
TQ5	ND	0.69 (0.063)	0.25 (0.071)	0.33 (0.094)	ND	0.92 (0.167)

amplified by PCR and subcloned into pNUFC101 to yield pNUFC200 (see Table 2a). *A. nidulans* strain *qutE208*, which is a mutant strain unable to grow on quinate as sole carbon source because it lacks the type II 3-dehydroquinase, was transformed with pNUFC200 and transformants were selected by growth on minimal medium with quinate as sole carbon source. As a control, *A. nidulans* strain *qutE208* was transformed with pEH1, which contains the *qutE* gene encoding a type II 3-dehydroquinase (Berl *et al.*, 1990), and with pTR101, which contains the *qa-2* gene under the control of the *E. coli trc* promoter (Hawkins *et al.*, 1993c).

Individual transformants were streaked for single colonies on selective medium, grown in bulk and 3-dehydroquinase assays performed on cell-free extracts as described previously (Grant *et al.*, 1988; Lamb *et al.*, 1991). The results of these assays on control and experimental

transformants (containing the *qa-2* gene under the control of the *pgk* promoter) are shown in Table 2(b); the results show that the *pgk* promoter in pNUFC200 can drive efficient constitutive transcription of heterologous cloned genes with, in this instance, enzyme levels up to 5.8-fold over the wild-type value.

The use of the *qa-2* gene here was merely to confirm that the *pgk* promoter, lacking the sequences of the *pgk* gene that positively affect transcription, drives constitutive transcription when taken out of its usual context and placed in pNUFC101. As direct selection for the function of the *qa-2* gene was used in this experiment, it is possible to argue that these transformants contain complex rearrangements that fuse the *qa-2* genes to unidentified constitutive promoters. This explanation can be precluded for three reasons: firstly, control experiments utilizing pTR101, which contains the *qa-2* gene cloned into the *E.*

Table 3. Constitutive expression of *qut* pathway enzymes in strains transformed with the complete or truncated forms of the *qutA* gene

Cell-free extracts from control and transformed strains were screened for the production of quinate pathway enzymes under conditions of quinate induction (I) or uninduced (U). Each enzyme assay was carried out once, separately and in conjunction with appropriate control PCA spot tests (spot test results: I, inducible; NI, non-inducible; C, constitutive). In all cases enzyme activities are expressed relative to the value in the control, which has a wild-type *qut* gene cluster. Specific activities of the enzymes are shown in parentheses. ND, Not detectable. Strains: (a) wild-type (R153) control, *qutA303* and three transformants (TATRA1, TATRA2, TATRA95) transformed with plasmid pAL7ΔGE; (b) control transformant (2043) transformed with unmodified pCAP2 and four transformants (TATRA41, 43, 44, 45) that were transformed with plasmid pNUFC201 containing the *qutA* gene in the vector pNUFC101 and were positive in the PCA spot test; (c) control transformant (2043) transformed with unmodified pCAP2, one inducible transformant (TATRK189) and three transformants that were constitutive in the PCA spot test transformed with plasmid pATR3.

Strain	PCA spot test	Quinate dehydrogenase		3-Dehydroquinase		Dehydroshikimate dehydratase	
		U	I	U	I	U	I
(a) Expression of the complete <i>qutA</i> gene driven by the native <i>qutA</i> promoter							
R153	I	ND	1.0 (0.142)	ND	1.0 (0.125)	ND	1.0 (0.136)
<i>qutA303</i>	NI	ND	ND	ND	ND	ND	ND
TATRA1	I	ND	1.0 (0.141)	ND	1.4 (0.176)	ND	1.3 (0.170)
TATRA2	C	1.3 (0.190)	1.1 (0.158)	1.7 (0.210)	1.0 (0.130)	1.0 (0.140)	0.8 (0.110)
TATRA95	C	1.1 (0.155)	1.9 (0.270)	1.2 (0.150)	3.0 (0.380)	1.0 (0.136)	2.4 (0.320)
(b) Expression of the complete <i>qutA</i> gene driven by the <i>pgk</i> promoter							
2043	I	ND	1.0 (0.041)	ND	1.00 (0.220)	ND	1.00 (0.051)
TATRK41	C	0.34 (0.014)	2.5 (0.103)	0.32 (0.070)	0.47 (0.104)	0.12 (0.006)	1.18 (0.060)
TATRK43	C	1.20 (0.052)	4.3 (0.178)	0.86 (0.019)	0.41 (0.090)	0.59 (0.030)	1.98 (0.101)
TATRK44	C	1.00 (0.044)	2.5 (0.105)	0.68 (0.150)	0.63 (0.139)	0.94 (0.048)	2.37 (0.121)
TATRK45	C	0.90 (0.040)	1.4 (0.059)	0.42 (0.093)	0.82 (0.180)	0.88 (0.045)	1.18 (0.060)
(c) Expression of the <i>qutA</i> gene lacking the sequence encoding the amino-terminal 32 amino acids, and driven by the <i>pgk</i> promoter							
2043	I	0.03 (0.005)	1.00 (0.183)	0.11 (0.005)	1.00 (0.045)	ND	1.00 (0.194)
TATRK189	I	0.09 (0.017)	0.54 (0.098)	0.06 (0.003)	0.77 (0.035)	0.07 (0.112)	0.97 (0.122)
TATRK195	C	0.22 (0.040)	0.46 (0.085)	0.40 (0.018)	1.24 (0.056)	0.20 (0.038)	0.52 (0.038)
TATRK202	C	0.23 (0.042)	0.40 (0.074)	0.60 (0.027)	1.04 (0.047)	0.17 (0.033)	0.56 (0.033)
TATRK251	C	0.20 (0.037)	0.70 (0.129)	0.55 (0.025)	0.80 (0.036)	0.25 (0.050)	0.51 (0.050)

coli expression plasmid pTrc99a, never gave rise to transformants; secondly, the frequency of transformation ($\sim 50 \mu\text{g}^{-1}$) is far too high to be accounted for by complex gene rearrangements; and thirdly, Southern-blot analysis of six randomly isolated strains transformed with pNUFC200 (and subsequently digested with restriction enzymes designed to release the *qa-2* PCR product) showed that all the transformants had an indistinguishable integration pattern, making it extremely unlikely that complex rearrangements were taking place fusing the *qa-2* gene to unidentified constitutive promoters.

Increasing the copy number of the *qutA* gene in *A. nidulans* leads to a constitutive phenotype for the quinate utilization pathway

Expression from the wild-type *qutA* promoter. *A. nidulans* strain *qutA303* has a mutation in the *qutA* gene which prevents production of the quinate pathway enzymes even in the presence of quinate; this strain is therefore unable to utilize quinate as a carbon source (Grant *et al.*,

1988). The *qutA303* mutation is located within the DHQ-synthase-like domain of the QUTA protein (Levesley *et al.*, 1996).

A. nidulans strain *qutA303* was transformed with plasmid pAL7ΔGE, which contains the wild-type *qutA* gene under its natural promoter (Beri *et al.*, 1990), and transformants were selected by their ability to grow in minimal medium with quinate as carbon source. A total of 104 transformants were screened by the *in vitro* colour test (the PCA spot test; see Methods) for the presence of constitutively produced quinate pathway enzymes. Two transformants, designated TATRA2 and TATRA95, were identified by this screening procedure and the levels of quinate pathway enzymes in these strains and a control transformant (TATRA1) with a wild-type inducible phenotype were determined in cell-free extracts of mycelium grown under inducing (quininate present) or non-inducing (quininate absent) conditions.

The results of these enzyme assays, shown in Table 3(a), demonstrate that transformants TATRA2 and TATRA95

Fig. 1. (a). Northern-blot analysis of wild-type and *qutA* multicopy transformants. Approximately 5 µg poly(A⁺) mRNA isolated from *A. nidulans* strains transformed with plasmids specifying the entire QUTA protein or a deleted form lacking the N-terminal 132 amino acids (and hence lacking the zinc binuclear cluster motif), and driven by the native or *pgk* promoters, was subjected to Northern-blot analysis. Lane 1, TATRA95 multicopy *qutA* transformant (copy number 16; relative message level 5.2) driven by the *qutA* promoter, uninduced. Lane 2, TATRK251 multicopy transformant (copy number 10; relative message level 10) specifying a truncated *qutA* sequence lacking the N-terminal 132 amino acids which include the zinc binuclear cluster motif, and driven by the *pgk* promoter, uninduced. Lane 3, TATRAK41, multicopy *qutA* transformant (copy number 11; relative message level 15) driven by the *pgk* promoter, uninduced. Lane 4, G191, wild-type positive control (copy number 1; relative message level 1), quinate-induced. Lane 5, G191, wild-type negative control (copy number 1; relative message level undetectable), uninduced. The positions of the markers and *qutA* derived mRNA are marked with dots. (b). Northern-blot analysis of host strain and a *qutR* multicopy transformant, R1/27. Approximately 5 µg of poly(A⁺) mRNA isolated from *A. nidulans* strain 2035 transformed with a plasmid specifying the entire *qutR* protein, and driven by the *pgk* promoter, was subjected to Northern-blot analysis. Conidiospores were grown overnight in 0.5% glucose. Resulting mycelial samples were divided in two and grown for 4 h in 0.1% (v/v) glycerol for uninduced samples and 0.1% (v/v) glycerol plus 0.3% (w/v) quinic acid to obtain induced samples. Lane 1, strain 2035, induced (copy number 1; relative message level undetectable); lane 2, strain 2035, uninduced (copy number 1; relative message level undetectable); lane 3, strain R1/27, induced (copy number 4; relative message level 4.5); lane 4, strain R1/27, uninduced (copy number 4; relative message level 0.7). In (a) and (b) the *qutA* or *qutR* message level, where detectable, is expressed relative to the value of the actin control. Even transfer of the various mRNA species was checked by probing for the actin-specific mRNA (the lower row of mRNA species in each case) using a radiolabelled 0.83 kb *NcoI*-*KpnI* fragment from the *actA* gene of *A. nidulans* (Fidel *et al.*, 1988). In both (a) and (b) the unlabelled track contains RNA size markers (1.4, 2.4, 4.4, 7.5 and 9.5 kb, purchased from GIBCO BRL).

constitutively produce all three quinate pathway enzymes. Southern and dot-blot analysis of strains TATRA2 and TATRA95 showed that they were multi-copy transformants (10 and 16 copies respectively; data not shown) and that TATRA1 had only a single extra copy of the *qutA* gene. In each case, however, the *qutR* (repressor-encoding) gene was unaffected by the integration events, precluding the disruption of the gene as a cause of the constitutive phenotype. Northern-blot analysis of transformant TATRA95 demonstrated that a *qutA*-specific mRNA of the appropriate size was being synthesized

constitutively to an elevated concentration compared with the wild-type control (see Fig. 1a).

Biological precedent suggests that the two most likely explanations of these data are that the QUTR repressor protein is being 'titrated out' either by interacting stoichiometrically with the presumed excess QUTA protein being produced by these transformants or by interaction with the multiple copies of the *qutA* promoter. Alternative interpretations of the data are evaluated in the Conclusions.

Table 4. Plasmids containing *qutA* or *qutR* sequences

Plasmid	Vector	Selectable marker for <i>A. nidulans</i>	Promoter	<i>qutA</i> coordinates	<i>qutR</i> coordinates	Encoded domain	Zinc binuclear cluster present?
pAL7ΔGE	pBR325	no	<i>qutA</i>	1–2475	NA	Whole of QUTA	yes
pATR1	pCAP2	<i>pyr-4</i>	<i>pgk</i>	1–2475	NA	Whole of QUTA	yes
pATR3	pCAP2	<i>pyr-4</i>	<i>pgk</i>	396–2475	NA	Whole of QUTA lacking the N-terminal (zinc-binuclear-cluster-containing) 132 amino acids	no
pATR4	pCAP2	<i>pyr-4</i>	<i>pgk</i>	396–1455	NA	DHQ-synthase-like domain lacking the N-terminal (zinc-binuclear-cluster-containing) 132 amino acids	no
pNUFC201	pNUFC101	no	<i>pgk</i>	1–2475	NA	Whole of QUTA	yes
pRTA1	pNUFC101	<i>pyr-4</i>	<i>pgk</i>	NA	1–2790	Whole of QUTR	NA

NA, Not applicable.

Expression from the *pgk* promoter. In order to determine whether the *qutA* promoter in the high-copy-number *qutA* transformants was titrating out the QUTR protein, the *qutA* gene was placed under the control of the *pgk* promoter in pNUFC100 to produce pNUFC201 (see Methods for the construction of pNUFC201).

pNUFC201 was used to transform *A. nidulans* strain *qutA303* and transformants were selected by their ability to utilize quinate as carbon source. Forty transformants were screened by the *in vitro* PCA spot test and 13 were found to constitutively produce the three quinate pathway enzymes. DNA from one transformant (A7/1) which was negative in the PCA spot test for constitutivity and from two transformants (A7/5 and A7/6) which were positive in the test was purified and investigated by Southern-blot analysis (data not shown). Transformant A7/1 had one extra copy of the *qutA* gene, whereas transformant A7/5 had approximately four copies and A7/6 approximately five copies (none of the extra copies had integrated at the *qutA* locus); in each case the analysis showed that the *qutR* genes had not been disrupted.

These results indicate that the QUTR repressor protein does not act solely by binding to the promoter of the *qutA* gene. It is possible, however, that because we were selecting for the growth of transformants on the basis of having a fully functional QUTA protein, the sample is unrepresentative and may have selected for a class of transformants that contain a rare mutation or rearrangement.

In order to explore this possibility, the experiment was repeated using the *qutA* gene under the *pgk* promoter in pATR1. This plasmid includes the selectable marker *pyr-4* (of *N. crassa*) which can complement the *A. nidulans* *pyrG* mutation and allow growth in minimal medium lacking uracil; the selection for transformation is therefore independent of the *qutA* gene in pATR1. *A. nidulans* strain G191 was transformed with plasmids pATR1

(containing the *qutA* gene under control of the *pgk* promoter in the vector pCAP2; see Table 4); 40 transformants were screened by the *in vitro* PCA spot test and 13 were judged to be constitutive (coincidentally, this was an identical number to the experiment described above where the *qutA303* strain was transformed with pNUFC201). The levels of *qut* pathway enzymes in cell-free extracts of four constitutive transformants (TATRK41, 43–45) and one transformant containing unmodified pCAP2 DNA (2043), grown under inducing and non-inducing conditions, are shown in Table 3(b). Southern-blot analysis showed that the *qutR* gene had not been disrupted in these transformants (data not shown). Northern-blot analysis of the transformant TATRK41 demonstrated that a novel *qutA*-specific mRNA of the appropriate size was produced constitutively in this strain, and that the concentration of this mRNA was elevated with respect to the native *qutA* mRNA of the wild-type control (see Fig. 1a).

The combined data in Table 3 show that the effect of the QUTR repressor protein can be negated by transforming in multiple copies of the *qutA* gene under the control of the *qutA* or *pgk* promoters in strains either wild-type or mutant for the host *qutA* gene. The significance of these data lies in the fact that all three quinate pathway enzymes are being produced constitutively in the absence of quinate in some of the transformants and not in the controls, rather than in the values of the specific activities. The apparent enhancement of activity by the addition of quinate in constitutive transformants using the *pgk* promoter to drive transcription of target DNA sequences is probably a reflection of enhanced mRNA production (see Fig. 1a, and below) which then presumably leads to an increase in the concentration of uncomplexed QUTA protein. Furthermore, the effect of transforming in the multiple copies of the *qutA* gene is not an artifact produced by the transformation selection process, and the results confirm that the QUTR protein cannot exert its

effect solely by interacting with the *qutA* promoter. The simplest interpretation of these data is that an increased copy number of the *qutA* gene causes an increase in the levels of the QUTA protein and thereby leads to a constitutive phenotype.

Increasing the copy number of *qutR* leads to a super-repressed phenotype for the quinate utilization pathway

The coding region of the complete *qutR* gene was placed under the control of the *pgk* promoter and the *qutE* terminator in pNUFC 101 to produce pRTA1, which also included the selectable marker *pyr-4* (see Table 4). Expression directed by this plasmid therefore decouples the production of the QUTR protein from the autoregulatory circuit mediated by the *qutA* gene (Levesley *et al.*, 1996), and allows an assessment of the *in vivo* effect of increasing the copy number of the *qutR* gene. Failure to ensure uncoupling would make the overproduction of the QUTR protein unlikely, as it would switch off the transcription of *qutA*, whose product (QUTA) is necessary for induction of the *qutR* gene under the control of its native promoter (Levesley *et al.*, 1996).

In order to confirm that the *qutR* gene in pRTA1 functions normally *in vivo*, *A. nidulans* strain 2035 (which carries a *qutR*^c mutation, thereby leading to constitutive expression of the *qut* pathway enzymes; Lamb *et al.*, 1992) was transformed with pRTA1. Transformants were selected by their ability to grow in minimal medium in the absence of uracil and were subsequently screened in the PCA spot test. More than 95% of the transformants had a quinate-inducible phenotype, demonstrating the presence of a fully functional QUTR protein, whereas the control strain (2035 transformed with pCAP2; Turner & Ballance, 1987) remained constitutive. These wild-type QUTR proteins could be produced either by integration of the transforming plasmid at a site away from the *qutR* locus or by specific integration into the *qutR* gene, thereby giving the possibility of producing a hybrid gene that contained parts of the resident and plasmid-borne *qutR* genes.

Subsequent Southern-blot analyses (data not shown) of selected transformants showed that integration into sites away from the *qutR* locus gave a wild-type phenotype, thereby confirming that the *qutR* gene in pRTA1 was functioning normally *in vivo*. One of these transformants (R1/27, *qutR* copy number 4) was taken at random and poly(A⁺) mRNA prepared from the two halves of a glucose-grown culture, one half of which had been washed free of glucose and incubated in the presence of quinate. The poly(A⁺) mRNA from the two halves of the culture was probed for the presence of *qutR*-specific mRNA by Northern blotting. The results show that *qutR*-specific mRNA, of the appropriate size, was present in the poly(A⁺) mRNA extracted from both cultures (Fig. 1b). This demonstrates that the *qutR* mRNA was being produced even in the absence of quinate and was therefore decoupled from the QUTA-mediated autoregulatory circuit. We also probed for *actA* mRNA, as an internal standard, which indicated that there was sixfold more

Fig. 2. Southern blot analysis of wild-type and *qutR* multi-copy transformants. Approximately 15 µg of DNA isolated from *A. nidulans* strains transformed with plasmids specifying the entire QUTR protein, and driven by the *pgk* promoter, was subjected to Southern blotting. The DNA was digested with *Nco*I prior to electrophoresis, to release the coding region of the *qutR* gene as a 2.8 kb fragment. The probe was generated by using nick-translation to radiolabel a PCR product of the complete *qutA* coding region with [³²P]dCTP. Lane 1, strain 1-4 (*qutR* copy number 7); lane 2, strain R1-80 (*qutR* copy number 3); lane 3, strain R1-64 (*qutR* copy number 5); lane 4, strain R1-60 (*qutR* copy number 5); lane 5, strain R1-36 (*qutR* copy number 23); lane 6, strain R1-19 (*qutR* copy number 22); lane 7, strain G191 wild-type control (*qutR* copy number 1). The unlabelled track contains size markers provided by *Hind*III digestion of lambda DNA. The values for the copy number determination were derived from analysis of a serial twofold dilution series of replicate 'dot-blots', using the *actA* gene of *A. nidulans* (see the legend to Fig. 1) as an internal marker. The samples shown in this figure are a qualitative representation of the data and show that an extra DNA species of the predicted size (2.78 kb) was present in each case following *Nco*I digestion.

qutR-specific mRNA in the half of the culture that had been incubated with quinate (Fig. 1b). This apparent elevation of mRNA levels of heterologous genes fused to the *pgk* promoter in the presence of quinate has been noted previously, although the level of increase seen was not as great as seen here (Streatfield *et al.*, 1992). The mechanism for this increase in *pgk*-promoter-specific mRNA production in the presence of quinate remains unclear. Our data confirm that the increase in copy numbers of the *qutR* gene was associated with an increase in the level of *qutR*-specific mRNA compared with the control strain 2035, which produced levels of *qutR* mRNA that were undetectable under these conditions.

A. nidulans strain G191 (which has a wild-type quinate-inducible *qut* gene cluster) was transformed with pRTA1 and transformants were selected by their ability to grow in minimal medium in the absence of uracil. Six transformants, picked at random, were screened by Southern-blot analysis in an attempt to identify a range that contained different numbers of integrated *qutR* genes (see Fig. 2). Transformants with copy numbers in the range 3 to 23 were identified, and two from the extremes of the

Table 5. Growth tests and enzyme assays on haploid and diploid strains containing multiple copies of the *qutA* and *qutR* genes

(a) Growth tests were carried out on appropriately supplemented minimal medium, using a growth scale of 0–5, where a score of 5 is the extent of wild-type growth and 0.5 is the 'sparse growth' typical of all quinate-non-utilizing mutants (Grant *et al.*, 1988). (b) The *qutE*-encoded type II 3-dehydroquinase levels in the haploid and diploid strains shown were determined in cell-free extracts from mycelium grown with glycerol as carbon source or from mycelium grown with glycerol as carbon source and subsequently induced by the addition of quinate. The extra copies of the *qutA* and *qutR* genes are under the control of the *pgk* promoter. The specific activity for the type II 3-dehydroquinase are shown in parentheses, with the values relative to the appropriate control (given a value of 1) for each of the two sets (haploid and diploid) of transformants. ND, Not detectable.

(a) Growth tests

Strain*	Glycerol	Quinate
R21	5	5
2043	5	5
R1-60	5	3–4
R1-36	5	0.5
<i>qutA</i> 303	5	0.5
<i>qutD</i> 8	5	0.5

* R21, wild type control; 2043, the host strain for transformation; R1-60, a multicopy (5 copies) *qutR* transformant; R1-36, a multicopy (23 copies) *qutR* transformant; *qutA*303, a negative control with a non-functional QUTA protein; *qutD*8, a negative control with a non-functional quinate permease.

(b) 3-Dehydroquinase levels in haploid and diploids strains containing a range of copy numbers for the *qutA* and *qutR* genes

Strain	Ploidy	3-Dehydroquinase activity glycerol grown	3-Dehydroquinase activity quinatc induced	<i>qutA</i> copy number	<i>qutR</i> copy number
2043	N	ND	1.00 (0.890)	1	1
R1-60	N	ND	0.60 (0.530)	1	5
R1-36	N	ND	0.06 (0.054)	1	23
2043/R153	2N	ND	1.00 (0.530)	2	2
TATRA 95/R153	2N	1.00 (0.232)	1.35 (0.730)	17	2
TATRA 95/R1-60	2N	0.53 (0.122)	1.70 (0.920)	17	6
TATRA 95/R1-36	2N	ND	0.42 (0.230)	17	24

range, R1-60 (5 copies of *qutR*) and R1-36 (23 copies of *qutR*), were screened for their ability to produce quinate pathway enzymes and to utilize quinate as carbon source. As shown in Table 5, with an increase in the copy number of the *qutR* gene there was a decreasing ability to utilize quinate as carbon source, and an associated lowering of the *in vivo* levels of the quinate pathway enzymes.

Southern-blot analysis also showed that the *qutA* gene in these multi-copy *qutR* transformants had not been disrupted (data not shown), precluding this as an explanation for the inability of transformant R1-36 to utilize quinate as a carbon source (a super-repressed phenotype). The simplest interpretation of these data is that, in these transformants, an elevated copy number of the *qutR* gene is correlated with an increased concentration of the QUTR protein, thereby producing a super-repressed phenotype.

Combining the super-repressed and constitutive phenotypes in a diploid strain produces an inducible phenotype

If the QUTR protein does mediate its repressing effect on transcriptional regulation by a direct interaction with the QUTA protein, then increasing the concentration of the QUTR protein in a multi-copy *qutA* constitutive transformant should restore the wild-type inducible phenotype. The direct transformation of *qutR*-containing plasmids into a multi-copy *qutA* constitutive transformant could lead to an inducible phenotype by integration into and inactivation of one or more of the *qutA* genes essential to maintain the constitutive phenotype. In order to obviate this possible problem, the facility to produce stable diploid strains in *A. nidulans* was exploited to bring together multi-copy *qutA* (constitutive) and *qutR* (super-

repressed) haploid strains. The rationale was to combine the constitutive transformant strain TATRA95 (containing potentially 15 functional copies of *qutA* under its own promoter, as transformant TATRA95 was generated by transforming a *qutA*⁻ strain), with the multi-copy *qutR* transformant strains R1-60 (5 copies of *qutR*; reduced growth on quinate) and R1-36 (23 copies of *qutR*; unable to utilize quinate; super-repressed). The transcription of these extra *qutR* genes was decoupled from the QUTA-mediated autoregulatory circuit by the substitution of the *pgk* promoter for the wild-type quinate-inducible promoter, as described above.

Multi-copy *qutA* transformant TATRA95 was made diploid with the wild-type strain R153 and with the two multi-copy *qutR* transformants R1-60 and R1-36. The various diploids were screened for the ability of quinate to induce production of the quinate pathway enzymes. The results are shown in Table 5, and demonstrate that an increase in the copy number of the *qutR* gene relative to the copy number of the *qutA* gene in the constitutive strain reduced or, in the case of the diploid with R1-36, eliminated the constitutive phenotype. The effect of the extra copies of the *qutR* gene was also manifest under inducing conditions, as the diploid with R1-36 (24 copies of the *qutR* gene due to the extra *qutR* gene provided by the TATRA95 haploid) was only able to induce the production of the quinate pathway enzymes to approximately half the wild-type level. The biological effects seen in these transformants are therefore correlated with *qutR* gene copy number.

The simplest interpretation of these data is that an elevated *qutR* copy number is associated with elevated QUTR production, and that the QUTR repressor protein mediates its effects by a direct interaction with the QUTA activator protein.

Deletion analysis of the QUTA protein

In order to further test the hypothesis that the QUTR protein interacts directly with the QUTA protein, and to attempt to localize the region of the QUTA protein that mediates this putative interaction, various overlapping subregions of the QUTA protein were produced in *A. nidulans* in the presence of wild-type levels of correctly regulated wild-type intact QUTA protein. The rationale behind these experiments was to produce, *in vivo*, deleted versions of the QUTA protein that lacked the DNA-binding domain; if these could bind to (and effectively 'mop up') the QUTR repressor protein, the endogenous wild-type QUTA protein should be relieved from repression by the endogenous QUTR protein, resulting in a constitutive phenotype.

DNA sequences specifying QUTA protein fragments consisting of (a) the bi-domain QUTA protein lacking the N-terminal (zinc-binuclear-cluster-containing) 132 amino acids (plasmid pATR3); or (b) the N-terminal DHQ-synthase-like domain lacking the N-terminal 132 amino acids, and also lacking the C-terminal EPSP-synthase like domain (plasmid pATR4), were placed under the control

of the *pgk* promoter in the vector pCAP2. These plasmids were transformed (using selection for uracil independence) into *A. nidulans* strain G191 that produces wild-type levels of correctly regulated native QUTA protein. The nucleotide coordinates of the *qutA* gene expressed in *A. nidulans* and driven by the *pgk* promoter are given in Table 4.

When the sequences encoding both of the QUTA protein domains but lacking the N-terminal zinc binuclear motif (pATR3) were expressed in *A. nidulans*, the use of the spot test demonstrated that a constitutive phenotype could be generated (53 transformants out of a total of 258 tested). Three transformants that showed a constitutive phenotype in the spot test (TATRK195, 202 and 251), one that showed an inducible phenotype (TATRK189), and the wild-type control strain 2043 (strain G191 transformed with unmodified pCAP2 DNA) were directly tested for the presence of the quinate pathway enzymes in cell-free extracts. The results of these enzyme assays are shown in Table 3(c); they confirm that the constitutive transformants are indeed producing the quinate pathway enzymes in the absence of quinate. Northern-blot analysis of one randomly selected transformant (TATRK251) demonstrated that the size of the constitutively produced *qutA*-specific mRNA directed by pATR3 was appropriately smaller than the mRNA directed by plasmids specifying the complete QUTA protein (see Fig. 1a). Additionally, the truncated *qutA*-specific mRNA was produced to an elevated concentration compared to the wild-type control (see Fig. 1a). When the sequence encoding the N-terminal DHQ-synthase-like domain of QUTA but lacking the zinc binuclear cluster motif (pATR4) was expressed in *A. nidulans*, the use of the spot test was unable to detect a constitutive phenotype (0 out of 111 transformants tested).

The simplest interpretation of these data is that in the transformants selected for their ability to grow in minimal medium lacking uracil, a range of overproduction of the sub-regions of the QUTA protein was occurring. Furthermore, in the case of pATR3, these subregions are proposed to bind to the QUTR repressor protein and to block the site(s) necessary for a normal stoichiometric interaction with the intact QUTA protein, thereby relieving the endogenous native QUTA protein from repression.

The observation that the truncated QUTA protein encoded by pATR4 does not apparently lead to a constitutive phenotype suggests that the QUTR protein may recognize and bind to the C-terminal EPSP-synthase-like domain of QUTA. This interpretation must be viewed with caution, however, as the truncated protein specified by pATR4 may simply not fold correctly or may be very unstable; either explanation could account for the apparent inability to generate constitutive transformants.

Conclusions

In the experiments described here, we have designed a strategy to elevate the expression *in vivo* of the *qutA* and *qutR* genes, and hence of the QUTA and QUTR proteins,

and we have examined the consequences on the regulation of the quinate utilization pathway. The strategy that we selected was to create multicopy transformant strains that included integrated copies of the *qutA* and *qutR* genes under the control of the *pgk* promoter. The purpose behind the use of the *pgk* promoter was to decouple the transcription of the genes under its control from the action of the QUTA activator protein. This strategy proved successful in that transcription was driven from the *pgk* promoter in the absence of quinate, but this transcription, at least in the case of *qutR*, was shown to be further enhanced by the addition of quinate. The mechanism for this enhancement of transcription from the isolated *pgk* promoter by the presence of quinate remains unclear. That expression driven by the isolated *pgk* promoter drives the production of functional protein products was confirmed by rescue of a *qutE* mutation by the heterologous *qa-2* gene. Transformant strains with multiple copies of the *qutA* gene under the control of the *pgk* promoter, or indeed its own promoter, showed a constitutive phenotype and the elevated expression of the *qutA* mRNA was confirmed. Transformant strains with multiple copies of the *qutR* gene showed a super-repressed phenotype and the elevated expression of the *qutR* mRNA was similarly confirmed. When multicopy *qutA* (constitutive phenotype) and *qutR* (super-repressed phenotype) haploid strains were brought together in diploids, a wild-type inducible phenotype was restored. Multicopy transformant strains expressing a *qutA* gene encoding a truncated QUTA protein also showed a constitutive phenotype, indicating that QUTR-mediated repression is independent of the amino-terminal 132 amino acids of QUTA, which includes the DNA-binding domain.

These data are consistent with three alternative interpretations for how the QUTR protein mediates its repressing effect. Firstly, the elevated levels of the QUTR protein may lead to elevated levels of a second molecule(s), which directly interacts with the QUTA protein and thereby inactivates it. Genetic analysis strongly implies that the *qutR* gene (and the homologous *qa-1S* gene of *N. crassa*) encodes a repressor (Grant *et al.*, 1988; Giles *et al.*, 1985; Geever *et al.*, 1989) and it is possible, therefore, that elevated levels of the QUTR protein could act to repress the transcription of a putative second gene encoding the true repressor protein, thereby leading to a constitutive phenotype. This is unlikely, as the gene encoding this second putative repressor should have been identified in the mutant screens that were originally employed to generate and analyse the *qut* and *qa* mutants of *A. nidulans* and *N. crassa* (Grant *et al.*, 1988; Giles *et al.*, 1985). Furthermore, the QUTR and QA-1S proteins have no recognized motifs capable of DNA binding (Hawkins *et al.*, 1992; Huiet & Giles, 1986) and are therefore unlikely to act by binding to promoter regions to inhibit transcription, or to act as DNA-dependent activators of the transcription of a second repressor gene.

Secondly, the QUTR protein may mediate its repressing effect by potentiating a pool of small organic effector molecules, which subsequently bind to and inactivate the QUTA protein pool. The mechanism for such a putative

effect could be enzymic. For example, the N-terminal sequence of the QUTR and QA-1S proteins are homologous with shikimate kinase and both contain a completely conserved purine nucleotide binding motif; it is possible therefore that this domain could have *in vivo* kinase activity (Anton *et al.*, 1987; Hawkins *et al.*, 1992; Walker *et al.*, 1982). Such an enzymic mechanism is highly unlikely to be dependent on the copy number of the extra *qutR* genes that were introduced into the transformed strains. We note, in addition, that the other two domains of the QUTR protein (dehydroquinase-like and shikimate-dehydrogenase-like) have been screened in an extremely sensitive *in vivo* assay for enzyme activity in *E. coli*, but no such activity was detected (Lamb *et al.*, 1996).

Thirdly, the QUTR protein may mediate its repressing effect on the QUTA protein by a direct protein-protein interaction with the QUTA protein. In this respect, we note that there are significant comparisons to be made with genetic experiments undertaken to understand the control of galactose utilization in *Saccharomyces cerevisiae*. The GAL4 protein is a positively acting transcription-activating protein responsible for switching on the transcription of the genes necessary for the utilization of galactose when this sugar is given as a carbon source. The GAL80 protein is a negatively acting transcription-regulating protein that mediates its action on galactose utilization by a post-translational interaction with the GAL4 protein (for a review see Johnston, 1987). Certain dominant alleles encoding a mutant GAL4 protein that produced the galactose pathway enzymes constitutively in the absence of galactose were shown to complement some dominant mutant alleles encoding a super-repressing GAL80 protein. The resulting diploid strains had the wild-type inducible phenotype and proved to be the first evidence that the GAL4 and GAL80 proteins directly interacted with one another (Nogi *et al.*, 1977).

Taking together the complete package of *in vivo* experiments described here, and viewing the results in the light of previous genetic analysis in both *A. nidulans* and *N. crassa*, and biological precedent, we believe that the most likely interpretation of the data is that the QUTR protein mediates its repressing effect by directly interacting with the QUTA protein.

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