Domain structure and function within the QUTA protein of *Aspergillus nidulans*: implications for the control of transcription

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QUTA is a positively acting regulatory protein that regulates the expression of the eight genes comprising the quinic acid utilization gene (*qut*) gene cluster in *Aspergillus nidulans*. It has been proposed that the QUTA protein is composed of two domains that are related to the N-terminal two domains – dehydroquinase (DHQ) synthase and 5-enolpyruvyl shikimate-3-phosphate (EPSP) synthase – of the pentadomain AROM protein. The AROM protein is an enzyme catalysing five consecutive steps in the shikimate pathway, two of which are common to the *qut* pathway. A genetic and molecular analysis of non-inducible *qutA* mutants showed that all 23 mutations analysed map within the N-terminal half of the encoded QUTA protein. One dominant mutation (*qutA382*) introduces a stop codon at the boundary between the two domains that were identified on the basis of amino acid sequence alignments between the QUTA protein and the N-terminal two domains of the pentadomain AROM protein. The truncated protein encoded by mutant *qutA382* has DNA-binding ability but no transcription activation function. A second dominant mutation (in strain *qutA214*) is missense, changing 457E → K in a region of localized high negative charge and potentially identifies a transcription activation domain in the N-terminus of the EPSP-synthase-like domain of the QUTA protein. A series of qualitative and quantitative Northern blot experiments with mRNA derived from wild-type and mutant *qutA* strains supported the view that the QUTA protein regulates the expression of the *qut* gene cluster, including the *qutA* gene which encodes it. A series of Western blot and zinc-binding experiments demonstrated that a putative zinc binuclear cluster motif located within the N-terminus of the QUTA protein is able to bind zinc *in vitro*.

Keywords: transcription, autoregulation, transcription activation domain, zinc binding

INTRODUCTION

Aspergillus nidulans can utilize quinate as an alternate dispensable carbon source and the production of the three enzymes necessary to convert quinate to protocatechuate is induced by the presence of exogenously supplied

quininate. This induction of enzyme activity is sensitive to carbon catabolite repression, as the presence of glucose inhibits enzyme production (Whittington *et al.*, 1987; Grant *et al.*, 1988). Genetic and biochemical analysis of quinate non-utilizing mutants demonstrated that the *qut* genes map in a cluster and that two genes, *qutA* and *qutR*,

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, dehydroquinase; DHQ synthase, dehydroquinase synthase (EC 4.6.1.3); dehydroquinase, EC 4.2.1.10; EPSP synthase, 5-enolpyruvyl shikimate-3-phosphate synthase (EC 2.5.1.19); GST, glutathione S-transferase; MC, metal-

chelating (domain); quinate dehydrogenase, EC 1.1.1.24; 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*; SSCP, single-strand conformation polymorphism; TAD, transcription activation domain.

are responsible for mediating the regulation of *qut* pathway enzyme production at the level of transcriptional control (Grant *et al.*, 1988; Hawkins *et al.*, 1988; Lamb *et al.*, 1990, 1992). The *qutA* gene was proposed to encode a positively acting regulatory protein, and subsequent sequence analysis of the cloned gene demonstrated (a) the presence of a putative zinc binuclear motif near the N-terminus of the encoded QUTA protein; and (b) a region of high localized negative charge at the extreme C-terminus, which may possibly act as a transcription activation domain (TAD; Beri *et al.*, 1987). Genetic analysis of mutations mapping in the *qutR* gene suggested that the encoded QUTR protein was a negatively acting regulatory protein. Subsequent sequence analysis of the cloned gene demonstrated that the encoded QUTR protein had no known motifs capable of facilitating DNA binding, leading to the proposal that the QUTR protein mediated its negative effect by directly binding to the QUTA protein (Hawkins *et al.*, 1992, 1993a, b).

Comparative sequence analysis of the enzymes and proteins comprising the *qut* and shikimate pathways has revealed a complex web of evolutionary relationships. These relationships have been reviewed (Hawkins *et al.*, 1993a), and in the context of this work the most remarkable proposal is that the QUTA and QUTR proteins are related to metabolic enzymes active in the shikimate pathway. On the basis of amino acid sequence alignments, the QUTA and QUTR proteins are proposed to be homologous with the five domains comprising the AROM protein (Anton *et al.*, 1987; Charles *et al.*, 1986; Hawkins, 1987; Hawkins *et al.*, 1992, 1993b). The AROM protein catalyses the five consecutive steps converting 3-deoxy-D-arabino-heptulosonate 7-phosphate to 5-enolpyruvyl shikimate 3-phosphate in the shikimate pathway, with two of these steps common to the *qut* pathway (for a review see Hawkins *et al.*, 1993a). The QUTA and QUTR proteins have therefore been proposed to have their origins in the splitting of a duplicated copy of the *aromA* gene or its precursor, with the QUTA protein evolving at least in part by acquiring a zinc binuclear cluster motif to facilitate DNA binding (Hawkins *et al.*, 1993b). It has been proposed that the enzymic activities of both proteins (QUTA and QUTR) have been lost, but that the QUTR protein has maintained its ability to recognize its former substrates, which now act as inducers of transcription by binding to the QUTR protein, disrupting its repressing function (Hawkins *et al.*, 1992, 1993b; Bugg *et al.*, 1991; Lamb *et al.*, 1995).

The sequence similarity between the QUTA protein and the N-terminal two domains of the AROM protein (DHQ synthase and EPSP synthase; see Fig. 1) is below 20%, which is in the region where the possibility of fortuitous similarity is significant. The problems of discerning sequence similarity between highly diverged homologous sequences (i.e. those having a common ancestor) that have evolved new functions have been discussed recently (Hawkins *et al.*, 1994; Hawkins & Lamb, 1995; and references therein).

Amongst all quinate non-utilizing (*qut*) mutants analysed, the major proportion (48 of 149) proved non-inducible

for all three enzymes that comprise the metabolic pathway due to mutations in the *qutA* gene. Over half (29) of these mutants proved recessive, many (16) semi-dominant and a few (3) fully dominant in growth tests on quinic acid when combined in heterozygous diploid strains with a wild-type (*qutA*⁺) strain (Grant *et al.*, 1988). The dominance observed was interpreted to indicate that in these mutants, a form of the QUTA protein was produced that competed with the wild-type protein to fully block or diminish its function in promoting gene expression.

In this communication we extend the analysis of domain structure and function within the QUTA protein and report experiments designed to probe the possible involvement of autoregulation of the *qutA* gene.

METHODS

Plasmids, recombinant lambda and strains. The origins of the recombinant phage lambda and plasmids other than those constructed as part of the research reported here have been described previously (Hawkins *et al.*, 1985; Beri *et al.*, 1987, 1990; Moore & Hawkins, 1993). The *Escherichia coli* and *A. nidulans* strains other than those constructed as part of this research have also 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; Studier *et al.*, 1990).

Materials. Chemicals and solvents other than dehydroquininate (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 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, 1993). General molecular biology reagents were from Pharmacia or Gibco BRL. *Taq* polymerase was from BCL, plasmids pGEX-2T, pKK233-2 and pTrc99a were from Pharmacia, and plasmid pRSETB was from Invitrogen. Activated Sepharose 4B and chelating Sepharose FF were from Pharmacia; GSH-Sepharose 4B was produced according to the manufacturer's instructions accompanying the plasmid pGEX-2T. Specific 30-mer oligonucleotides were purchased from the University of Newcastle upon Tyne Central Facility for Molecular Biology. A synthetic QUTA peptide, SNPKRQVRAC, was purchased from Biomac, Glasgow University. ⁶⁵Zn was from Amersham, and Dynabeads were from Dynal UK.

Methods. Restriction endonucleases were used according to the manufacturer's recommendations, and PCR amplification of target DNA sequences was as previously described (Van den Hombergh *et al.*, 1992; Moore & Hawkins, 1993). All other routine molecular biology protocols followed individual manufacturer's recommendations or were as previously described (Maniatis *et al.*, 1982). Quinate dehydrogenase, 3-dehydroquinase and dehydroshikimate dehydratase assays were performed as previously described (Grant *et al.*, 1988). Transformation of *A. nidulans* and the preparation of cell-free extracts from enzyme assay were as previously described (Lamb *et al.*, 1991; Grant *et al.*, 1988). An *in vitro* colour test, 'PCA spot test', was used to detect the presence of quinate pathway enzymes in young mycelium (Grant *et al.*, 1988). Single-strand conformation polymorphism (SSCP) analysis of the *qutA* mutant strains was carried out as described by Orita *et al.* (1989). [α -³²P]dCTP was used to label the DNA produced in the PCR prior

to digestion with selected restriction endonucleases and subsequent separation of single- and double-stranded forms by electrophoresis in a non-denaturing polyacrylamide gel (Mackay *et al.*, 1993). For DNA sequencing, double-stranded PCR products were electrophoresed through low-melting-point agarose and purified by the 'Gene Clean' procedure (TM Company) to ensure removal of unincorporated primers. DNA sequencing was done by standard methods (Sanger *et al.*, 1977; Biggin *et al.*, 1983). *A. nidulans* genomic DNA was isolated as previously described (Charles *et al.*, 1986) and was used in the PCR preceding the SSCP analysis of DNA from wild-type and *qutA* mutant strains.

Protocols for small- and large-scale growth and induction of fusion-protein synthesis in *E. coli* with IPTG were as previously described (Hawkins & Smith, 1991; Moore & Hawkins, 1993; Hawkins *et al.*, 1993c). Purification of fusion proteins containing the GST domain of *Schistosoma japonicum* were as described by Hawkins *et al.* (1993c).

SDS-PAGE was carried out by the method of Laemmli (1970),

and proteins immobilized by electrophoretic transfer to nitrocellulose using standard methods (Burnette, 1981; Samson, 1986). Rabbit antibodies were raised against a synthetic QUTA peptide, and used in Western blot analysis to detect QUTA proteins produced in *E. coli* by the method of Samson (1986). *In vitro* binding of zinc to proteins immobilized on nitrocellulose was determined as described by Barbosa *et al.* (1989). Total RNA was purified by 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.

RESULTS AND DISCUSSION

Mapping and sequence analysis of *qutA* mutations

To gain further insight into the functional domains within the QUTA activator protein we wished to determine the

Table 1. Genetic mapping of the *qutA* activator gene locus in *A. nidulans*

The numbers in the table represent the frequency (per 10⁶ ascospores plated in selective medium) of quinic-acid-utilizing recombinants (*qut*⁺) in crosses between the *qutA* mutants indicated. The arrows show on which side of the sign-post marker particular alleles are located, thereby defining the interval to which the mutation was allocated based upon the distribution of the *qutR16* flanking marker. Numbers in parentheses indicate very close linkage where the mutation could not be ordered with confidence. The phenotypes of heterozygous diploid strains with the *qutA*⁺ wild-type allele in combination with each mutant allele are shown based upon growth on quinic acid plates: diploids with a dominant *qutA* allele (D) produced no growth; recessive *qutA* alleles (R) full normal wild-type growth (identical with that of a *qutA*⁺/*qutA*⁺ homozygote); semi-dominant *qutA* alleles (SD) a marked decrease in growth, both in the density of the mycelium and amount of conidiation, their growth appearing roughly 75% of that compared to wild-type (100%) or the dominant mutants (0%).

R153 <i>qutA</i> strain	Phenotype	R21 <i>qutA</i> 552/633/506/636; <i>qutR16</i> strain			
		R153 <i>qutA</i>	R153 <i>qutA</i>	R153 <i>qutA</i>	R153 <i>qutA</i>
		× 552 (r)	× 633 (r)	× 506 (r)	× 636 (r)
380	R	417←		436←	
404	SD	133←		369←	
402	SD	172←		242←	
422	SD	63←		232←	
423	SD	70←		458←	
400	SD	19←		174←	
394	SD	(3)	88←	245←	106←
341	SD	→6	84←		165←
393	R	→10	136←	193←	214←
386	SD	→17	34←	151←	55←
4	SD	→23	22←	99←	127←
384	SD	→28	21←	83←	117←
399	R	→114	11←	47←	64←
385	R	→104	(0)	41←	10←
379	R	→141	(2)	32←	18←
324	R	→371	→33	(3)	
361	R	→163	→6	)	5←
389	SD			(2)	10←
390	SD	→868	→20	)	7←
303	R	→168	→66		(1)
444	R	→218	→39	→19	
382	D		→132	→23	→17
214	D	→286	→221	→144	→38

Fig. 1. Predicted proteins produced by translation of the *qutA*⁺ gene and *qutA* mutants. DHQS, the region of the QUTA protein homologous with the AROM protein DHQ synthase domain; EPSP, the region of the QUTA protein homologous with the AROM protein EPSP synthase domain; ▨, zinc cluster domain (aa 49–76); ▤, linker region (aa 77–95); ▦, DNA-dependent dimerization domain (aa 95–110); ▧, TAD (aa 448–482); ■, non-QUT amino acids incorporated following a frameshift mutation, or in the case of mutants 214 and 394, following a missense mutation.

nature and locations of the changes within representative classes (recessive, semi-dominant and dominant) of *qutA* non-inducible mutant strains. To attempt to ensure that we determined the changes in mutants distributed throughout the *qutA* gene, the relative positions of 23 *qutA* mutations were determined by genetic analysis, which in turn might guide the choice of mutant strains for DNA sequence analysis.

Close linkage of the *qutR* repressor gene to *qutA* (4% recombination) at the distal end of the *qut* gene cluster suggested the opportunity to extend the method used to map the *qut* gene cluster (Grant *et al.*, 1988) in order to map mutations in the *qutA* locus. In this method the *qut*

genes were ordered by the distribution of a recessive constitutive mutation (*qutR16*) used as a flanking marker in three-point test crosses. This strategy requires the availability of some *qutA* mutations coupled to *qutR16* to serve as 'markers' within the *qutA* locus. For this purpose, a further series of new *qut* mutants were isolated in the strain R21 *qutR16* and classified by complementation analysis in heterozygous diploid strains in combination with known *qut* mutant strains. Four new *qutA* mutants (*qutA506*, 552, 633 and 636) were recovered on the basis of their functional identity with the well-characterized recessive mutant R153 *qutA303*. All four new *qutA* mutations proved to be recessive. Each of the double mutant strains (R21 *qutR16*, *qutA506* or 552 or

Table 2. Frequency of recombination in the *qutA* locus

The frequency of *qut*⁺ recombinants in the crosses between *qutA* mutant strains is shown in relation to the physical separation of the mutations. The value of the ratio ranged from 0.03 to 0.58, the majority of values falling between 0.1 and 0.3 (26 of 31), with an overall mean of 0.21.

Genetic interval	10 ⁶ × Recombination frequency (RF)	Physical distance (bp)	Ratio RF/bp
402–552	172	672	0.26
422–552	67	384	0.17
400–552	19	80	0.24
394–522	3	64	0.05
552–393	10	67	0.15
552–386	17	101	0.17
552–4	23	183	0.13
552–384	28	338	0.08
552–385	104	384	0.27
552–361	163	617	0.26
552–214	286	941	0.30
394–633	88	514	0.17
393–633	136	383	0.36
386–633	34	349	0.10
4–633	22	267	0.08
384–633	21	112	0.19
385–633	0	66	–
633–361	6	167	0.04
633–382	132	432	0.31
633–214	221	488	0.45
402–506	242	1363	0.18
422–506	232	1075	0.22
400–506	174	771	0.23
394–506	245	755	0.32
393–506	193	624	0.31
386–506	151	590	0.26
4–506	99	508	0.19
384–506	83	353	0.24
385–506	41	307	0.13
361–506	2	74	0.03
506–382	23	194	0.12
506–214	144	250	0.58

633 or 636) was crossed with a representative number of R153 *qutA* mutants, and *qutA*⁺ recombinants isolated by plating ascospores pooled from 5–20 hybrid fruiting bodies in selective medium (minimal agar medium with quinic acid as sole carbon source). The mutant strains tested were genetically stable (reversion rates < 10^{−6}), and the recombination frequencies observed fell in the range from not detectable (0) to several hundred *qutA*⁺ progeny in 10⁶ ascospores plated. A number of the *qutA*⁺ recombinants from each cross were classified for their constitutive phenotype using a rapid 'spot-test' for the ability of mycelium grown on a non-inducing carbon source (glycerol) to metabolize quinate to protocatechuate, trapped in a colorimetric reaction (Grant *et al.*, 1988). The resultant matrix (Table 1) reliably mapped

mutations to intervals between the 'sign-post' markers, which fortunately proved reasonably well separated, and resolved the majority of mutants examined (Fig. 1). As expected, recombination frequencies were not additive, but the genetic data proved invaluable in the selection of mutant strains for DNA sequence analysis.

Molecular analysis was initiated by the use of SSCP (Orita *et al.*, 1989; Mackay *et al.*, 1993) to locate mutations to defined restriction fragments in the *qutA* gene. Four mutations (*qutA*422, 400, 552 and 506) were physically mapped by this method and served to align the genetic and physical maps unambiguously. On this basis 14 *qutA* mutations were chosen for DNA sequence analysis (Fig. 1), and in each case the mutation identified by SSCP analysis was confirmed by sequence determination using a newly prepared PCR template. The relationship of recombination frequency and the physical separation of the mutations for each genetically mapped interval is shown in Table 2. There was no obvious bias in recombination across the locus and overall *qut*⁺ recombinants were recovered at a frequency of 1 in 10⁶ for each 56 bp interval.

The positions and nature of the mutant changes are summarized in Fig. 1 and Table 3, inspection of which leads to the following conclusions: (a) the genetic and physical maps are precisely co-linear; (b) none of the mutations are located within the putative zinc binuclear cluster motif; (c) only two of the 14 mutants sequenced were missense mutants (*qutA*394, 214); and perhaps most strikingly (d) all 14 mutations are confined to the N-terminal 55% of the protein, with 13 mutations located within the DHQ-synthase-like domain and only one mutation (*qutA*214) located within the EPSP-synthase-like domain.

The missense dominant mutant *qutA*214 is of particular interest as it specifies a full-length QUTA protein containing the previously identified putative transcription activation domain (TAD) at the extreme C-terminus of the EPSP-synthase-like domain (Berl *et al.*, 1987). This mutant has a dominant phenotype, which strongly implies that the encoded protein is stable and able to fold into a configuration that allows efficient DNA binding, thus out-competing the wild-type QUTA protein, but has no functional TAD. Mutant *qutA*214 is an E → K change in a region of localized high negative charge, 8 acidic residues in a sequence of 29 amino acids, which is 27.5% compared to an average value of 10.7% for the complete QUTA protein. Although it has been shown recently (Van Hoy *et al.*, 1993; Leuther *et al.*, 1993), that TADs do not necessarily have an acidic nature, mutant *qutA*214 may however define a TAD in the QUTA protein. We note that in the same region in the equivalent QA-1F protein of *Neurospora crassa* the E residue is conserved and is also part of a sequence that has 27.5% acidic residues (Geever *et al.*, 1989), supporting the view that this concentration of negative charge is not fortuitous. The E → K change at QUTA amino acid 457 therefore either destabilizes the activity of a spatially distinct TAD, or we believe more likely identifies an *in vivo* functionally active

Table 3. Positions and nature of the *qutA* mutations

DNA from wild-type *A. nidulans* and the *qutA* mutants shown was subjected to SSCP analysis to approximately locate the positions of the mutations. The nucleotide sequence changes were then determined by directly sequencing the PCR-amplified DNA using the dideoxy chain-termination method.

Mutant	Phenotype*	Base alteration†	Base number	AA alteration‡	Stop number§
422	SD	+ A	44	15wt + 23	39
400	SD	- A	348	116wt + 1	117
394	SD	A → G	364	122 I → T	825
552	R	G → A	428	142wt	143
393	R	G → A	495	164wt	165
386	SD	- C	529	177wt + 30	208
4	SD	- A	611	205wt + 2	208
384	SD	+ C	766	255wt + 11	267
385	R	T → G	812	270wt	271
633	R	+ T	878	292wt + 7	300
361	R	+ A	1045	348wt + 48	397
506	R	+ G	1119	373wt + 23	397
382	D	T → A	1313	437wt	438
214	D	G → A	1369	457 E → K	825

* R, SD and D indicate a phenotype of recessive, semi-dominant or dominant for each allele when combined in a diploid with a wild-type *qutA*⁺ allele.

† Arrows between two bases show either transversions or transitions and the designation + A, C, G or T signifies the addition of this base, whereas - A or C indicates the loss of this base.

‡ 'AA alteration' indicates the change induced in the amino acid sequence by each of the mutants studied. 'wt' shows the number of wild-type amino acids produced before either a translational stop is produced or a frameshift occurs. In the latter case the symbol '+' followed by a number indicates the number of adventitious amino acids produced out of frame before a randomly generated stop codon is encountered. In mutants *qutA*394 and 214 the nature of the missense mutation is shown in the single-letter amino acid code.

§ 'Stop number' refers to the number of amino acids encoded by a particular allele before a translational stop is encountered.

TAD in the extreme N-terminus of the EPSP-synthase-like domain in the QUTA protein. We note that the position of this TAD places it immediately adjacent to a proposed linker region between the QUTA DHQ-synthase-like and EPSP-synthase-like domains and that this region may have some flexibility.

All of the recessive *qutA* mutants are nonsense or frameshift mutations producing QUTA proteins containing the putative zinc binuclear cluster motif and the specificity region, but truncated within the limits of the DHQ-synthase-like domain. The probable molecular basis for their recessive phenotype is that the truncated QUTA proteins either are unstable and are proteolytically degraded *in vivo* or simply are unable to produce a folded protein with biological activity.

The existence of the dominant and semi-dominant classes of *qutA* mutant strongly implies that in these mutants a stable form of the QUTA protein is produced that is able to fold and either compete with the wild-type QUTA protein for the binding sites within *qut* gene promoters or interact directly with wild-type QUTA protein to produce inactive heterodimers.

The mutation in *qutA*382 introduces a translational stop codon two amino acids away from the end of the DHQ-synthase-like domain, defined by alignment with the *A. nidulans* pentafunctional AROM protein (Hawkins *et al.*, 1993b). We interpret the dominant phenotype of mutant *qutA*382 to indicate that this strain is producing a stable, soluble but truncated QUTA protein that corresponds only to the DHQ-synthase-like domain. We have recently shown that the DHQ synthase domain of the AROM protein is completely stable and retains efficient enzyme activity when isolated from the rest of the pentafunctional enzyme (Moore *et al.*, 1994). This observation strengthens the following interpretation for the molecular basis of the *qutA*382 mutant phenotype. The DHQ-synthase-like domain of the QUTA protein contains a putative zinc binuclear cluster motif; therefore the simplest interpretation of the dominant non-inducible phenotype is that this domain when isolated in the truncated protein can bind to the target *qut* gene promoters but lacks a transcription activation domain. Binding of this DHQ-synthase-like domain of QUTA will therefore 'mop up' the specific binding sites, and in a heterozygous diploid with the wild-type *qutA* gene will out-compete the wild-type QUTA

protein for these sites, thus producing the dominant phenotype. It is also possible however that the effect of the *qutA382* mutation may be caused by heterodimer formation of the encoded truncated QUTA protein with the endogenous wild-type QUTA protein.

The semi-dominant frameshift mutant *qutA422* is of special interest as it is predicted to produce a truncated QUTA protein 38 amino acids long. The first 15 amino acids are the same as wild-type; however the insertion of an A base at nucleotide 44 alters the reading frame to include a further 23 heterologous amino acids before a stop codon occurs. This region of the QUTA protein contains no recognized functional motifs, and yet the semidominant phenotype displayed by mutant *qutA422* implies that the truncated QUTA protein that it encodes is having some physiological effect. In the absence of any other recognized motif we speculate that the first 15 amino acids of the QUTA protein encode at least part of a nuclear localization signal. It is possible that the extra 23 heterologous amino acids associated with the truncated QUTA protein (caused by translating part of the *qutA* gene out of frame) specified by mutant *qutA422* are responsible for this proposed function, but we feel it is most likely that the semi-dominant phenotype of *qutA422* is a consequence of the first 15 amino acids. The other semi-dominant mutants containing the putative zinc binuclear cluster motif (but lacking the EPSP-synthase-like domain), and the specificity region may have their phenotypic effect either by competitive DNA binding, by heterodimer formation diluting out a pool of transcriptionally active wild-type dimers, or both. Taken together these data provide strong support for the bidomain model for the structure of the QUTA protein and suggest the presence of a novel TAD located adjacent to a hinge region connecting the two domains.

Transcription from *qutA* is autoregulated

The QUTA protein is responsible for directing the transcription of the genes comprising the *qut* gene cluster and has also been shown to direct the transcription of heterologous genes fused to the *qutE* promoter (Grant *et al.*, 1988; Lamb *et al.*, 1991; Moore *et al.*, 1992).

To investigate the pattern and possible regulation of transcription of the *qutA* gene, a series of qualitative and quantitative experiments were performed. Poly(A⁺) mRNA from quinate induced and non-induced cultures of *A. nidulans* strains R153 (wild-type), *qutR16* (constitutive mutant) and *qutA303* (non-inducible mutant) was isolated and subjected to Northern blot analysis with radiolabelled *qutA*, *qutR*, *qutB* and *qutG* DNA. The results are shown in Fig. 2(a).

Northern blot analysis has not been reported for the *qutA*, *qutR*, *qutG* or *qutB* genes; therefore the presence of a quinate-inducible mRNA for each gene was determined. The quinate-inducible mRNA specific to the *qutA*, *qutR* and *qutG* genes is shown in lanes 1–6 of Fig. 2(a); the mRNA for *qutB* was also quinate-inducible (unpublished data). The presence of constitutively produced *qutA*, *qutR*

and *qutB* (as a representative example of one of the genes encoding an enzyme) mRNA in the constitutive mutant *qutR16* is shown in lanes 7, 8 and 11–14 respectively. The absence of *qutA*-specific mRNA in the non inducible mutant *qutA303* is shown in lanes 9 and 10. The *qutA303* mutation is recessive and maps in the sequence encoding the N-terminal DHQ-synthase-like domain of the QUTA protein (see Fig. 1). Taken together with previous genetic experiments (Grant *et al.*, 1988) these observations of the pattern of mRNA expression (Fig. 2a) strongly support the view that the QUTA protein is responsible for controlling transcription of the *qut* gene cluster in response to the presence of quinate, and that the QUTA protein autoregulates the expression of the *qutA* gene which encodes it.

In order to follow the kinetics of induction of *qut*-specific mRNA and enzymes, the levels of *qutA*, *qutR* and *qutB* mRNA and the activity of the *qutB*-encoded quinate dehydrogenase were determined at stages over a 3 h period following the quinate induction of a glucose-grown culture of wild-type R153. The results of this experiment are shown in Fig. 2(b), analysis of which leads to the following observations: (1) the appearance of *qutA*, *qutR* and *qutB* mRNA is concomitant and detectable after 15 min, reaching apparent steady-state levels by 90 min; (2) quinate dehydrogenase activity is detectable 60 min after the addition of quinate and rises approximately linearly for the following 2 h. Taken as a whole, these data lend strong support to the hypothesis that the *qutA* gene of *A. nidulans* is autoregulated.

Truncated N-terminal peptides from the QUTA protein containing the putative zinc binuclear cluster motif are able to bind zinc *in vitro*

The finding that none of the 13 mutations located within the QUTA DHQ-synthase-like domain were located within the putative zinc binuclear cluster motif is surprising and questions if this motif can bind zinc. Specific 30-mer oligonucleotides were used to PCR amplify the DNA sequence of *qutA* from nt 1 to 387, which incorporates the putative zinc binuclear cluster motif. Following restriction endonuclease digestion, the amplified DNA was subcloned into the *E. coli* expression vectors pKK233-2, pTrc99a, pGEX-2T, pETGEXCT and pRSETB. The first two vectors give the potential to produce non-fusion proteins (molecular mass 14.15 kDa). The vectors pGEX-2T and pRSETB give the potential to produce fusion proteins with either the glutathione *S*-transferase (GST) (fusion protein molecular mass 41.15 kDa) protein of *Schistosoma japonicum* or a metal-chelating (MC) domain (fusion protein molecular mass 18.65 kDa) fused to the N-terminal end of the experimental target protein. The plasmid pETGEXCT allows the production of fusion proteins with the GST domain fused to the C-terminus of the experimental target protein (Sharrocks, 1994). Recombinant plasmids were initially identified by colony hybridization using [α -³²P]dCTP-labelled PCR-amplified DNA and their correct construction verified by restriction endonuclease digestion. The

Fig. 2. Transcription studies. (a) Poly(A⁺) mRNA (1.5 µg per track) from quinate-induced (I) and uninduced (U) cultures of strains R153 (wild-type), *qutR16* and *qutA303* was subjected to denaturing agarose gel electrophoresis and transferred to nitrocellulose. The nitrocellulose sheets were then probed with PCR-amplified DNA fragments specifying either the entire coding region of the *qutA* gene, the *qutR* gene, the *qutB* gene or the *qutG* gene and subsequently labelled with [α -³²P]dCTP by nick translation. In all cases an [α -³²P]dCTP-labelled 0.83 kb *NcoI*-*KpnI* fragment from the *A. nidulans actA* gene was used as a probe to check equal loading and even transfer of mRNA species in all 14 lanes; the actin-specific mRNA is shown below the boxed *qut*-specific mRNA. The unlabelled track contains RNA size markers consisting of 0.24, 1.4, 2.4, 4.4, 7.5 and 9.5 kb, and is included as a representative example. Panel (a) is a composite of three separate gels; the data were collected by quantitative phosphorimaging. Probed filters were exposed for between 2 h (enzyme structural genes) and 48 h (regulatory genes). (b-d) Poly(A⁺) mRNA was extracted from replicate cultures of *A. nidulans* strain R153 (wild-type) that had been grown with glucose as carbon source and subsequently induced with 0.1% quinate. Extractions of total and poly(A⁺) mRNA were made from the replicate cultures harvested at intervals over a 3 h period. The poly(A⁺) mRNA was then sequentially probed for the presence of the *qutA*-, *qutR*- and *actA*-specific mRNA and a replicate filter of total RNA probed for the presence of *qutB*-specific mRNA as described in (a). In order to follow the kinetics of induction, phosphorimaging was used to quantify the levels of all four mRNA species at each of the time points and cell-free *in vitro* enzyme assays were used to measure the quinate dehydrogenase activity. Following the subtraction of an average background reading, the ratios of the *qutA*-, *qutR*- and *qutB*-specific mRNA relative to the *actA*-specific mRNA (which was used as an internal constitutive control) were determined. The graphs show the appearance of (b) *qutA*-specific mRNA (c) *qutR*-specific mRNA, and (d) *qutB*-specific mRNA (□) and quinate dehydrogenase (▲). To allow the use of a single scale, in each case the highest value of the ratio with respect to the internal control *actA* mRNA was taken as 100%. The values for the *qutB*-encoded quinate dehydrogenase are shown relative to the value (taken as 100%) found in a replicate culture induced by the presence of quinate for 4 h; it was known from previous data that *qut* pathway enzyme levels reached a maximum value 4–5 h after the addition of quinate. The graphs in (b–d) are therefore directly comparable.

Table 4. Plasmids used for expression studies

Desired target DNA sequences were amplified by PCR using specific 30-mer oligonucleotides incorporating 5' and 3' *Bam*HI and *Eco*RI sites, respectively, for subcloning into the vector pGEX-2T. For subcloning into the vectors pKK233-2, pTrc99a, pETGEXCT and pRSETB, the PCR primers contained 5' and 3' sites for *Bsp*HI and *Hind*III, respectively. For subcloning into pETGEXCT, the PCR product was generated by oligonucleotides introducing 5' and 3' *Bsp*HI and *Hind*III sites respectively and was digested with *Bsp*HI and *Nco*I (there is a naturally occurring *Nco*I site immediately preceding the *Hind*III site artificially introduced on the 3' PCR primer) and subcloned into *Nco*I-cut pETGEXCT. All the *gutA*-derived sequences specified the N-terminal 129 QUTA amino acids. Recombinant plasmids were rescued from ligation mixtures by transforming *E. coli* SK3430 and selection on complete medium containing 50 µg ampicillin ml⁻¹. The correct construction of individual plasmids was directly verified by restriction endonuclease digestion of purified plasmid preparations. NA, Not applicable.

Expression plasmid	Promoter	Recombinant plasmid designation	Potential to produce native (N) or fusion (F) proteins	Fusion domain	Zinc domain production when containing the 5' 387 bp <i>gutA</i> sequence?
pKK233-2	<i>trc</i>	NA	N	NA	No
pTrc99a	<i>trc</i>	NA	N	NA	No
pGEX-2T	<i>trc</i>	pXF32-35	F	GST	Yes, but degraded
pRSETB	T7	pRF2	F	MC	No
pETGEXCT	T7	pCF1	F	GST	Yes, not degraded

plasmids generated are summarized in Table 4. A 12-mer peptide corresponding to QUTA amino acid co-ordinates 38–49 inclusive was used to raise rabbit antibodies to the N-terminal domain of the QUTA protein, and these were subsequently used as a means of identifying heterologous protein production in *E. coli* by Western blotting. The C-terminal Cys residue of this peptide corresponds to the first of the six Cys residues that comprise the putative zinc binuclear cluster (Berl *et al.*, 1987). The antibodies produced were able to recognize and bind to the correct size zinc domain fusion protein and various degradation products of it (see Fig. 3a). The rabbit antibodies were used to screen *E. coli* lysates containing recombinant plasmids derived from pKK233-2, pTrc99a, pGEX-2T and pRSETB after fractionation by SDS-PAGE and Western blotting. Only recombinant plasmids (designated pXF32–35) derived from pGEX-2T were able to direct the production of protein that was specifically recognized by the anti-QUTA antibody; all the other recombinant plasmids based on the commercially available constructs failed to produce *gutA*-specified protein. The fusion protein produced by the pGEX-2T recombinant plasmids was heterogeneous, reproducibly yielding specific degradation products of the expected full-length GST fusion protein (see Fig. 3a). Recombinant plasmids were screened for their ability to specify stable fusions of the expected size by transformation into *recA* (recombination deficient) and *lon* (protease deficient) strains of *E. coli*, but the same pattern of degradation was seen in each case (see Fig. 3a). The purpose of using these strains was to minimize the effects of DNA rearrangements or protease attack, which were considered as plausible explanations for the instability of the GST–QUTA fusion protein.

Recombinant plasmids derived from pETGEXCT, designated pCF, were screened in *E. coli* for their ability to direct the production of a stable QUTA–GST fusion protein. Independently isolated, multiple examples of

these recombinant plasmids were able to direct production of a QUTA–GST fusion protein of approximately the predicted molecular mass (41.15 kDa). The fusion protein migrated slightly faster than expected during SDS-PAGE; however, similar anomalous migration patterns with other proteins have been reported (Samson, 1986). When screened with anti-QUTA rabbit antibody, the fusion protein in strains containing pCF1 cross-reacted strongly, confirming that the protein was the desired QUTA–GST fusion (see Fig. 3d). The C-terminal fusion protein was soluble following sonication; however it was unable to bind to glutathione-substituted Sepharose 4B. To assess whether the QUTA–GST fusion protein could bind zinc *in vitro*, lysates of *E. coli* overproducing the QUTA–GST fusion protein specified by pCF1 (experimental), the GST domain alone (negative control), or a protein consisting of the *gutB*-encoded quinate dehydrogenase (Hawkins *et al.*, 1993c) fused to the MC domain encoded by pRSETB (positive control) were serially diluted, and the proteins were separated by SDS-PAGE and screened with ⁶⁵Zn following Western blotting. The quinate dehydrogenase fusion protein (specified by recombinant plasmid pRF2), acted as a positive zinc-binding control, as the 4.47 kDa MC domain attached to its N-terminus contains six histidine residues that are able to bind zinc atoms, facilitating purification by immobilized metal affinity chromatography. As shown in Fig. 3(b, c), both the experimental and the positive control proteins specifically bound zinc *in vitro*. Zinc binding was still seen in both these protein preparations when they were serially diluted 25-fold lower than the concentration shown in Fig. 3 (data not shown).

These data support the interpretation, but do not unequivocally prove, that it is the putative zinc binuclear cluster motif in the fusion proteins that is specifically binding zinc. However, further analysis of the degraded polypeptides produced in *E. coli* strains containing the

Fig. 3. (a) Detection of the QUTA-GST fusion protein and its breakdown products by *in vitro* antibody binding. Solubilized whole-cell extracts of IPTG-induced and uninduced *E. coli* strains containing recombinant pGEX2T (designated pXF) plasmids specifying the N-terminal 129 amino acids of the QUTA protein were prepared, fractionated by SDS-PAGE, and proteins transferred to nitrocellulose. Equivalent numbers of cells were used in each track. Molecular mass standards [phosphorylase *b* (91 kDa), alcohol dehydrogenase (41 kDa); carbonic anhydrase (29 kDa) and cytochrome *c* (12.8 kDa)] were co-electrophoresed in the un-numbered lane. The nitrocellulose-bound proteins were reacted with anti-QUTA peptide antibody used at a dilution of 1 in 50 (Samson, 1986). (a) Tracks 1-4, *E. coli* BL21 containing plasmids pXF32-35, uninduced; tracks 5-8, *E. coli* BL21 containing plasmids pXF32-35, induced with IPTG; tracks 9-12, *E. coli* SK3430 containing plasmids pXF32-35, uninduced; tracks 13-16, *E. coli* SK3430 containing plasmids pXF32-35, induced with IPTG. *E. coli* BL21 is a protease-deficient *recA*⁺ strain B (Studier *et al.*, 1990), and SK3430 is a *recA*⁺ K12 strain with the normal complement of proteases. GLW55, a *recA* K12 *E. coli* strain (Hawkins & Smith, 1991), gave identical results to those shown in (a) when harbouring the pXF plasmids. (b-d) Identification of the QUTA-GST fusion protein and zinc binding. Equivalent amounts of the GST specified by pGEX-2T (lanes 1 and 2), the QUTA-GST C-terminal fusion protein specified by pCF1 (lanes 3 and 4) and the MC domain-quinolate dehydrogenase N-terminal fusion protein specified by pRF2 (lanes 5 and 6), prepared as total protein extracts from IPTG-induced *E. coli* cells, were subjected to SDS-PAGE in three separate replicate gels. The three proteins of interest are marked by black dots. Following electrophoresis, gels were either stained with Coomassie blue (b) or electroblotted to nitrocellulose in the presence (c) or absence (d) of 0.025% SDS in the transfer buffer. The nitrocellulose filter in (c) was reacted with 50 μ Ci (1.85 MBq) ⁶⁵ZnCl₂ and the filter in (d) was reacted with rabbit anti-QUTA peptide antibody. Molecular mass markers (as in a) were electrophoresed in each gel (shown only in b: un-numbered lane). Following SDS electroblotting the nitrocellulose sheet shown in (c) was incubated in renaturing buffer (100 mM Tris, 50 mM NaCl, 10 mM DTT, pH 6.8) for 1 h with three changes of buffer prior to incubation with 50 μ Ci ⁶⁵ZnCl₂ in 50 ml labelling buffer (100 mM Tris, 50 mM NaCl, pH 6.8). After washing for 1 h with three changes of wash buffer (100 mM Tris, 50 mM NaCl, 1 mM DTT, pH 6.8) the sheet was dried and autoradiographed (Barbosa *et al.*, 1986). The non-SDS electroblotted nitrocellulose sheet (d) was blocked by overnight incubation in 1%

GST-QUTA fusion proteins lends support to this interpretation. The degraded products were purified by affinity chromatography on glutathione-substituted Sepharose 4B, demonstrating that the GST domain was intact and that degradation was taking place at the C-terminal end of the protein within the QUTA sequence. Western blot analysis showed that the higher molecular mass protein bands, that is those least degraded, were both recognized by the antibody to the QUTA peptide and that only these proteins were able to bind zinc (data not shown). Measurement of the molecular masses of the degraded proteins supports the conclusion that the lower molecular mass degraded proteins must have lost all or part of the putative zinc binuclear cluster motif and that this is correlated with the loss of ability to bind zinc.

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