Identification of domains responsible for signal recognition and transduction within the QUTR transcription repressor protein

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QUTR (*qutR*-encoded transcription-repressing protein) is a multi-domain repressor protein active in the signal-transduction pathway that regulates transcription of the quinic acid utilization (*qut*) gene cluster in *Aspergillus nidulans*. In the presence of quinate, production of mRNA from the eight genes of the *qut* pathway is stimulated by the activator protein QUTA (*qutA*-encoded transcription-activating protein). Mutations in the *qutR* gene alter QUTR function such that the transcription of the *qut* gene cluster is permanently on (constitutive phenotype) or is insensitive to the presence of quinate (super-repressed phenotype). These mutant phenotypes imply that the QUTR protein

plays a key role in signal recognition and transduction, and we have used deletion analysis to determine which regions of the QUTR protein are involved in these functions. We show that the QUTR protein recognizes and binds to the QUTA protein *in vitro* and that the N-terminal 88 amino acids of QUTR are sufficient to inactivate QUTA function *in vivo*. Deletion analysis and domain-swap experiments imply that the two C-terminal domains of QUTR are mainly involved in signal recognition.

Key words: AROM enzyme, multi-domain protein, quinic acid utilization.

INTRODUCTION

Biological systems have efficient sensing mechanisms that can detect the presence of both extra- and intracellular signals. Recognition of these signals can lead to cellular responses as diverse as muscle contraction (Ca²⁺ release), post-translational modulation of protein activity (phosphorylation) and transcription regulation. Cells have sophisticated signal-transduction pathways (STPs) that couple the initial recognition event to an appropriate cellular response. The various carriers in a STP involved in transcription regulation may have to recognize and interact with protein and DNA as well as discriminate between related signal molecules. The acquisition within a single protein of signal-transduction and multiple-recognition abilities presents a formidable task in evolutionary terms.

The microbial eukaryotes *Neurospora crassa* and *Aspergillus nidulans* use a three-component STP for the wild-type regulation of quinate utilization as a carbon source. The exogenous supply of quinate or the internal accumulation of dehydroquinate lead to the production of three enzymes necessary to catabolize quinate to protocatechuate [1]. The STP leading to enzyme production consists of a surface receptor and two carriers, and controls the initiation of transcription of the eight genes (in *A. nidulans*) comprising the quinic acid utilization (*qut*) gene cluster. Genetic analysis has shown that (i) the surface signal receptor is a permease necessary to transport quinate into the cell, (ii) the intracellular recognition of quinate and/or a derivative is mediated by a carrier that is a transcription-repressor protein [designated QA-1S in *N. crassa* and QUTR (*qutR*-encoded transcription-repressing protein) in *A. nidulans*], and (iii) transcription initiation is mediated by a carrier that is a transcription

activator containing a zinc bi-nuclear cluster motif. This activator is designated QA-1F in *N. crassa* and QUTA (*qutA*-encoded transcription-activating protein) in *A. nidulans* [1–6]. Experiments *in vivo* have suggested that the QUTR protein mediates its repressing effect by binding to the QUTA protein [7]. The QUTR protein therefore has the complex job of both recognizing and binding to the QUTA protein and relatively small organic signal molecules.

The QA-1F/QUTA activator and the QA-1S/QUTR repressor proteins appear to be related to five individual monofunctional shikimate-pathway enzymes by divergent evolution (see Figure 1; [8,9]). In the microbial eukaryotic lineage these five enzymes are fused to form a single pentafunctional enzyme, denoted the AROM (*aromA*-encoded pentafunctional enzyme) protein, which catalyses five consecutive steps in the shikimate pathway [10–12]. The QA-1F/QUTA proteins are proposed to be homologous to the complete N-terminal-domain dehydroquinate synthase [13] and four-fifths of the second AROM domain enolpyruvyl shikimate 3-phosphate synthase (EPSPS). The QA-1S/QUTR proteins are homologous to the remainder of the AROM protein, the last fifth of the EPSPS domain and the complete shikimate kinase (SK), dehydroquinase (DHQ) and shikimate dehydrogenase (SDH) domains [8,9]. Biophysical evidence has demonstrated that the AROM and QUTR DHQ domains have very similar physical properties [14]. These studies suggest that the recognition of multiple metabolites can be facilitated by fusing modules that have different recognition properties to produce multi-domain proteins. These modules appear to be generated by duplication of genes encoding enzymes that recognize a particular signal molecule as a substrate. In the STP carrier protein these modules have lost their enzymic functions [14] but have retained

Abbreviations used: EMSA, electrophoretic mobility-shift assay; PCA, protocatechuic acid; QUTA, *qutA*-encoded transcription-activating protein; QUTR, *qutR*-encoded transcription-repressing protein; AROM, *aromA*-encoded pentafunctional enzyme; STP, signal-transduction pathway; EPSPS, enolpyruvyl shikimate 3-phosphate synthase; SK, shikimate kinase; DHQ, dehydroquinase; SDH, shikimate dehydrogenase; GST, glutathione S-transferase.

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Figure 1 Proposed evolutionary relationships between the AROM protein and the QUTA and QUTR proteins

the ability to recognize and bind the same or a closely related metabolite, in effect evolution by enzyme recruitment [15].

Enzyme recruitment has played a significant role in the evolution of the galactose STP in *Saccharomyces cerevisiae*. GAL3p functions as the sensor and transducer of the galactose signal in the induction pathway of GAL4p-activated genes. The yeast GAL1-encoded galactokinase enzyme (GAL1p) is able to substitute for the loss of GAL3p activity and shares extensive sequence identity with it, suggesting that GAL3 evolved from a duplicated copy of GAL1 [16–24]. The GAL80p repressor has been reported to be related to a widespread family of oxidoreductases, typified by glucose-fructose oxidoreductase (GFOR) [25].

The QUTR protein provides a tractable paradigm for studying the evolution and molecular mechanisms of signal recognition and transduction and in this paper we address the question of which parts of the QUTR protein are involved in these functions.

MATERIALS AND METHODS

Materials

Chemicals and solvents other than DHQ were of AnalR or greater purity and purchased from local suppliers. DHQ was made from quinate and purified by chromatography as described previously [26]. Specific oligonucleotides were purchased from the University of Newcastle upon Tyne Central Facility for Molecular Biology. Agar, tryptone and yeast extract were from Difco. Reagents for molecular biology were from Gibco, Pharmacia and Boehringer Mannheim.

Molecular biology and biochemistry

The growth and propagation of *A. nidulans* and *Escherichia coli* and preparation of cell-free extracts for 3-DHQ enzyme assay were as described previously [27]. Use of DNA-modifying enzymes followed manufacturers' recommended procedures and standard molecular biology techniques were as described previously [28]. DNA was labelled using a random-primed DNA-labelling kit (Boehringer Mannheim), and the labelled probe

purified using the NucTrap purification column system (Stratagene). PCR amplification was performed according to the following conditions: cycle 1, 94 °C for 2 min, 50 °C for 2 min and 72 °C for 4 min; cycles 2–30, 94 °C for 1 min, 50 °C for 2 min and 72 °C for 4 min using Expand high-fidelity *Taq* polymerase (Boehringer Mannheim). The use of reverse transcriptase (Pharmacia) followed the manufacturer's recommended conditions.

Site-directed mutagenesis of the AROM DHQ domain

Site-directed mutagenesis of the AROM DHQ domain was carried out by the 'back-to-back' method of Hemsley et al. [29] using the forward (mutagenic) oligonucleotide 5'-GGTGAC-ATTATCCGACTTGTGGGTGTT-3' and the reverse (non-mutagenic) oligonucleotide 5'-ATATTCAAGCGCTTTATT-GTA-3'.

Purification of total RNA followed the method of Cathala et al. [30] and poly(A)⁺ mRNA was prepared from total RNA using Dynabeads Oligo (dT) (Dynal UK) and following the manufacturer's protocol. Images from Southern- and Northern-blot, dot-blot, electrophoretic mobility-shift assay (EMSA) and radio-labelled antibody-precipitation experiments were visualized using a Molecular Dynamics PhosphorImager model 400b, and quantified using Imagequant software version 3.3, or with a Fuji PhosphorImager and Tina software. Exposures of the probed filters varied between 1 h (for high-copy-number transformants) to 48 h (for low-expressed wild-type regulatory-gene mRNA levels). Copy number in Southern blots was quantified by using the *actA* gene of *A. nidulans* [31] as an internal marker. With the exception of transformants by plasmid pNUFC311, 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. Copy number in transformants generated by pNUFC311 in which the transforming DNA had integrated into the resident *qutR* gene were quantified relative to an internal standard provided by the actin-encoding *actA* gene. Electro-

Table 1 Recombinant plasmids for expression in *A. nidulans* and *E. coli* or coupled *in vitro* transcription/translation

The amino acid co-ordinates of the QUTA, QUTR, AromA and GST sequences subcloned into the plasmids pNUFC101, pNUFC102, pET9d, pTrc99a and pSPUTK are shown. The domains encoded by these amino acids and, where appropriate, the selectable markers for transformation are also shown. NA, not applicable.

(a)						
Plasmid number	Vector	Amino acid co-ordinates		QUTR domains encoded	Selectable marker	Expression
		QUTR	QUTA			
pRTA1	pNUFC101	1–901	None	Complete QUTR	<i>pyr-4</i>	<i>A. nidulans</i>
pRTA2	pNUFC101	284–901	None	DHQ- and SDH-like	<i>pyr-4</i>	<i>A. nidulans</i>
pRTA3	pNUFC101	1–544	None	EPSPS-, SK- and DHQ-like	<i>pyr-4</i>	<i>A. nidulans</i>
pIVTT1	pSPUTK	1–901	None	Complete QUTR	NA	<i>In vitro</i>
pIVTT2	pSPUTK	None	1–825	None	NA	<i>In vitro</i>
pIVTT3	pSPUTK	None	1–437	None	NA	<i>In vitro</i>
pMUTK10	pET9d	None	1–437	None	<i>Kan^r</i>	<i>E. coli</i>

(b)								
Plasmid number	Vector	QUTR		AromA		GST domain ?	Selectable marker	Expression
		Amino acid co-ordinates	Domains encoded	Amino acid co-ordinates	Domains encoded			
pNUFC286	pNUFC102	1–281	EPSPS- and SK-like	1033–1290	Wild-type DHQ	No	<i>pyr-4</i>	<i>A. nidulans</i>
pNUFC313	pNUFC102	1–281	EPSPS- and SK-like	None	None	Yes	<i>pyr-4</i>	<i>A. nidulans</i>
pNUFC315	pNUFC102	1–281	EPSPS- and SK-like	1033–1290	¹²⁰⁸ K → R mutant DHQ	No	<i>pyr-4</i>	<i>A. nidulans</i>
pNUFC324	pNUFC102	1–88	EPSPS-like	None	None	No	<i>pyr-4</i>	<i>A. nidulans</i>
pNUFC327	pNUFC101	None	None	None	None	Yes	<i>pyr-4</i>	<i>A. nidulans</i>
pTR108	pTrc99a	1–281	EPSPS- and SK-like	1033–1290	Wild-type DHQ	No	<i>bla</i>	<i>E. coli</i>
pTR113	pTrc99a	1–281	EPSPS- and SK-like	1033–1290	¹²⁰⁸ K → R mutant DHQ	No	<i>bla</i>	<i>E. coli</i>

phoresis in the presence of SDS was performed as in [32] with a 5 % polyacrylamide stacking gel and a 7 or 10 % polyacrylamide separating gel. Proteins were made visible by staining with Coomassie Brilliant Blue.

Transformation of *A. nidulans*

At the outset of the project we judged that the biological activity of individual domains of QUTR may be compromised when produced as truncated proteins and that elevated production may be necessary to see a phenotypic effect. We chose to select for uracil-independent transformants using the *pyr-4* gene of *N. crassa*, as this selection system produces transformants with a range of copy number of the transforming plasmid [7]. We chose to use the mutant strain 2035 as the host for transformation as it has a mutation in the *qutR* gene and has a recessive constitutive phenotype. The strategy adopted was to select transformants on the basis of uracil independence and screen for an altered phenotype (inducible or super-repressed) using the protocatechuic acid (PCA) test. The PCA test traps the product of the quinate utilization pathway as a coloured compound after reaction with iron that is easily visible by eye and can be used as a visual screen for the phenotypes of transformants [27]. The phenotype of inducible transformants was confirmed by detecting the presence of type-11 DHQ in cell-free extracts prepared from mycelium grown in the presence of quinate but not when grown in its absence. Transformants selected by this procedure were further screened by Southern-blot analysis to identify those in which the transforming DNA had integrated at a site away from the resident *qutR* gene. This was to ensure that any change of phenotype from constitutive to inducible was due to the production of the desired truncated QUTR proteins and not due to the action of a wild-type QUTR produced by recombination with the resident mutant *qutR* gene. Similarly, transformants

that had a phenotypic change from constitutive to super-repressed were screened by Southern-blot analysis to check that the *qutA* gene had not been disrupted by integration of the transforming plasmid. The super-repressed phenotype was confirmed by demonstrating that transformants were able to utilize PCA, but not quinate, as the sole carbon source, and by the absence of type-11 DHQ in cell-free extracts of mycelium grown in the presence of quinate. Finally, Northern-blot analysis was carried out on representative transformants to check that *qutR*-specific mRNA of an appropriate size was being produced.

Production of recombinant plasmids for expression in *A. nidulans* and *E. coli* or coupled *in vitro* transcription/translation

The amino acid co-ordinates specified by the *qutA* and *qutR* sequences subcloned into the vectors pNUFC101, pNUFC102 [7], pET9d, pTrc99a and pSPUTK are shown in Table 1. The subcloning procedure for plasmids pTR108/113 and pNUFC285/315 is complex and requires explanation. The EPSP/SK-encoding sequence of *qutR* was subcloned into the *A. nidulans* expression vector pNUFC102 (to yield pNUFC284) after PCR amplification using oligonucleotides shown in Table 2. The DHQ-encoding sequence of *arom4* was subcloned into pNUFC284 to yield pNUFC285 after PCR amplification with oligonucleotides shown in Table 2. The sequence of pNUFC285 encoding the *qutR* EPSP/SK fused to the AROM DHQ was released by digestion with *NcoI* and *HindIII* and subcloned into plasmid pRSETb to yield pRF58. This sequence was then released from pRF58 and subcloned into the plasmid pTrc99a to yield pTR108. pNUFC286 was generated by inserting the *pyr-4* gene into pNUFC285. Plasmid pRF58 was used as the substrate for site-directed mutagenesis to convert Lys¹²⁰⁸ into Arg in the AROM DHQ sequence, yielding plasmid pRF64. The *qutR* EPSP/SK fused to the mutant AROM DHQ was released by

Table 2 Oligonucleotides used in the construction of recombinant plasmids for expression in *A. nidulans* and *E. coli* or coupled *in vitro* transcription/translation

All sequences are shown 5' → 3' with the plasmids they were used to construct.

Sense oligonucleotide	Anti-sense oligonucleotide	Plasmid
CGTCGTCTAGCCATGGCAATCCTTGTG	ACCAGTAGCGAGCTGCAGGAAACGAAGAAAATC	pNUFC284
GACTTTAACCGGTTCTCGCAGGTAGCTACC	CGAAGATGGCAAGCTTCTAGGGCTTGATT	pNUFC285
CGTCGTCTAGCCATGGCAATCCTTGTG	ACGATCGATGCCTGCAGGGGAACTTCCTC	pNUFC322
CAGGAAACAGTACTGCGAGTCCCTATACTA	ACGCGCGAGGCAAAAGCTTCAGTCAGTCAC	pNUFC324
CTGATCCACCTGCCATGTTTCATGCGTTCT	ACAAGCGTGGCCCCATGGTCACATCGGCTG	pRTA1
TTCTTCGTTTCGCCATGCTCGCTACTGGTA	ACAAGCGTGGCCCCATGGTCACATCGGCTG	pRTA2
CTGATCCACCTGCCATGTTTCATGCGTTCT	CTTCTGTGCCCATGGTCAGGTTATAGACG	pRTA3
CGTCGTCTAGCCATGGCAATCCTTGTG	ACAAGCGTGGCCCCATGGTCACATCGGCTG	pRF60
TCATTGTATCGATCATGAGTAGCGATAC	ACAAGCGTGGCCGATCCTCACATCGGCTG	pIVT1
TCATTGTATCGATCATGAGTAGCGATAC	GTGCAAGAGATCATGATTACCTATCTGT	pIVT2
TCATTGTATCGATCATGAGTAGCGATAC	GGAAGGCAACAGAATTCATTAGCAGCAAC	pIVT3
CGTCGTCTAGCCATGGCAATCCTTGTG	GTGCAAGAGATCATGATTACCTATCTGT	pMUTK10
GACTTTAACCGGTTCTCGCAGGTAGCTACC (<i>aroA</i>)	ACCAGTAGCGAGCTGCAGGAAACGAAGAAAATC	pNUFC286, pNUFC315
CGTCGTCTAGCCATGGCAATCCTTGTG (<i>qutR</i>)	CGAAGATGGCAAGCTTCTAGGGCTTGATT	pNUFC286, pNUFC315
CAGGAAACAGTACTGCGAGTCCCTATACTA (<i>gst</i>)	ACCAGTAGCGAGCTGCAGGAAACGAAGAAAATC	pNUFC313
CGTCGTCTAGCCATGGCAATCCTTGTG (<i>qutR</i>)	ACGCGCGAGGCAAAAGCTTCAGTCAGTCAC	pNUFC313
CAGGAAACAGTACTGCGAGTCCCTATACTA (<i>gst</i>)	ACGATCGATGCCTGCAGGGGAACTTCCTC	pNUFC324
ACAGGAAACAGATCATGACCCCTATACTAG	ACGCGCGAGGCAAAAGCTTCAGTCAGTCAC	pNUFC324
	GATCGTCAGTCAAAGCTTATGAATCCCATGGATCCACGCGG	pNUFC327

digestion with *Nco*I and *Hind*III and subcloned into plasmid pTrec99a to yield pTR113 and into pNUFC102 to yield pNUFC314. pNUFC315 was generated by inserting the *pyr-4* gene into pNUFC314. Plasmids pTR108 and pTR113 were used to transform the *E. coli aroD*⁻ mutant strain SK3430, which lacks DHQ and consequently has an auxotrophic requirement for the aromatic amino acids. *E. coli* transformants containing the plasmid pTR108 were able to complement the *E. coli aroD*⁻ mutation (confirming *in vivo* DHQ activity), whereas those containing pTR113 could not (confirming lack of DHQ activity), showing that the mosaic proteins had the predicted properties. pNUFC322 was constructed by inserting the *qutR* sequence encoding the N-terminal 88 amino acids into pNUFC102 following PCR amplification using the oligonucleotides shown in Table 2. Following isolation of this plasmid, the sequence encoding the glutathione S-transferase (GST) domain was subcloned out of frame on to the 3' end of the *qutR* sequence following PCR amplification using oligonucleotides shown in Table 2. The recombinant plasmid produced was designated pNUFC324 and encodes a protein that consists of the 88 QUTR amino acids with the additional sequence LQSLY attached to its C-terminus. This sequence of additional amino acids is similar to the sequence NASIV which occurs in the equivalent position of QUTR.

The oligonucleotides used for the remaining subcloning exercises are given in Table 2. These oligonucleotides contained the recognition sequences for *Bam*HI, *Bsp*HI, *Hind*III or *Nco*I. The PCR products were purified from agarose gels as previously described [33] and, after digestion with suitable restriction endonucleases, subcloned into the appropriate vector. Recombinant plasmids were recovered from ligation mixtures by transformation of *E. coli* and selection on suitable drug-containing media.

DNA sequencing

Oligonucleotide primers containing restriction endonuclease sites for *Bam*HI, *Eco*RI or *Hind*III were used to amplify the complete *qutR* gene by PCR from the constitutive mutant strain 2035 as

Table 3 Oligonucleotides used to sequence the *qutR* gene from the wild-type and strain 2035

All sequences are shown 5' → 3' and were used as primers in the di-deoxy sequencing reaction.

TTCAGCCGACTATGGAC	GGAATTAGTTCTACATG	GGAAATTGCGTAATTG
CGTGGCGCTCGCTACTG	AGTATTGACCCCGGTGA	TGTAACACCTTAATAC
CCATGTCTTGAAGTCCC	GGAGGAGTATGACCAGA	TCTGGTCATACTCCTCC
GGGACTTCAAGACATGG	GTATTAAGGTGTTTACA	TCACCGGGGTCAATACT
CATGTAGAATAATTCC	CAGTAGCGAGCGCCACG	CAATTACGCAATTTTCC
GTCCATAGTCGGCTGAA		

three fragments using the cosmid pQ5 [34] as a template. The restriction fragments were subcloned into the sequencing vectors mp18 and mp19 and the sequences of both strands from two independent amplification reactions were determined on an ABI/PE 377 automated DNA sequencer. This procedure was used to eliminate the possibility of PCR artifacts in the sequence, thereby allowing unambiguous determination of the mutations on the mutant *qutR* gene.

The sequences of the oligonucleotides used in the PCR reactions were 5'-GGGATCGCTGAAGCTTGTAGAAGACTCACT-3', 5'-GGGAATATTACCAGGATCCTACGCCACGAAACG-3', 5'-ATTACTGATCCAGAATTCATGTTTCATGCG-3' and 5'-GGATCCATCACAGGATCCCGGCTCGGTAGT-3'.

The primers used to complete the sequence of the mutant *qutR* gene are shown in Table 3. All other DNA sequencing was carried out on double-stranded plasmid DNA using an ABI/PE 377 automated DNA sequencer.

Co-immunoprecipitation of QUTA with QUTR from *in vitro*-synthesized protein mixtures

The plasmid pRF10 [14] was used in conjunction with the *E. coli* strain BL21 DE3 to overproduce the central DHQ-like domain of the QUTR protein. The protein was purified as described

previously [14], with the exception that in the final step of FPLC the buffer used was 20 mM potassium phosphate, pH 7.2/0.05 mM dithiothreitol. The purified protein was then dialysed against PBS (1 % NaCl/0.025 % KCl/0.18 % Na_2HPO_4 /0.03 % KH_2PO_4 , all w/v) and used to initiate the production of rabbit anti-QUTR antibodies. The purified protein (30 mg) was used to make a 10-ml antibody affinity column by covalent linkage to epoxy-activated Sepharose 4B (Pharmacia) as described previously [35]. The QUTR affinity column was used to purify high-avidity QUTR-specific antibodies from rabbit serum as described previously [35].

Protein was synthesized from 2 μg of plasmid using a TNT-SP6-coupled reticulocyte lysate *in vitro* transcription/translation system (Promega), according to the manufacturer's instructions. Proteins were synthesized either using unlabelled amino acids or in the presence of ^{35}S -labelled methionine (Amersham) to allow their visualization by autoradiography. To allow protein–protein interactions to form, aliquots of the QUTA and QUTR proteins were mixed, the volume adjusted to 200 μl with NET buffer (150 mM NaCl/5 mM EDTA/50 mM Tris/HCl, pH 7.5/0.05 %, v/v, Nonidet P-40) and incubated overnight at 4 °C. Complexes were pre-cleared by the addition of 1 mg of pre-immune rabbit serum for every 20 μl of *in vitro*-transcribed/translated product. Following incubation at 22 °C for 1 h, the non-specific antibody–antigen complexes were precipitated by incubation for a further 1 h with Protein A–Sepharose 4B beads (Sigma). Between 2 and 4 μg of the affinity-purified antibody (DHQAB1), directed towards QUTR, was then added and the specific antibody–antigen complexes were precipitated with Protein A–Sepharose beads. The beads were washed once with NET buffer supplemented with NaCl to 0.5 M, once with NET buffer containing 0.1 % SDS and finally with 10 mM Tris/HCl/0.1 % (v/v) Nonidet P-40. Immunoprecipitated proteins were eluted by boiling in SDS/PAGE loading buffer and resolved by SDS/PAGE (7 % gel). The dried gel was dried under vacuum and precipitated proteins visualized by quantitative Phosphor-Imaging.

Partial purification of the N-terminal domain of QUTA

E. coli strain BL21 DE3 was transformed with plasmid pMUTK10 grown at 37 °C to mid-logarithmic phase in drug-supplemented complex medium and then grown for a further 4 h in the presence of 0.2 mg $\cdot$ ml $^{-1}$ isopropyl β -D-thiogalactoside. Cell pastes from 3 ml of isopropyl β -D-thiogalactoside-induced cultures of BL21 DE3 transformed with pMUTK10 or pET9D (control) were sonicated in 500 μl of 100 mM Tris/HCl, pH 8.0/0.5 M NaCl/1 % Triton X-100. After centrifugation at 10000 *g* for 10 min in a bench-top microfuge, the supernatant volume was determined and made to 30 % (w/v) with ammonium sulphate. After incubation on ice for 30 min the suspension was centrifuged at 10000 *g* for 10 min in a bench-top microfuge. The supernatant was removed with a hypodermic needle and, after centrifugation for 1 min at 10000 *g*, remaining traces of supernatant were removed and the pellet dissolved in 500 μl of 100 mM Tris/HCl, pH 8.0/0.5 M NaCl/1 % Triton X-100.

Production of a ^{32}P -labelled DNA probe for EMSA

Oligonucleotides of sequence GGCAATCTCTGTTGCAG and ATAAAATTGCGGGGATCCGGTA were used to amplify the region from nucleotide 4900 to 4959 [4] from the promoter of the *qutE* gene by the PCR. The PCR reaction contained 0.3 μM of each primer, 200 μM dNTPs, 10 ng of DNA from the recombinant lambda λQ1 as the substrate [4], 2.5 units of Promega

Taq polymerase and 10 μCi of [α - ^{32}P]dCTP. The PCR was carried out according to the following regime: 94 °C for 2 min, 35 °C for 2 min and 55 °C for 2 min for 1 cycle; 94 °C for 1 min, 35 °C for 2 min and 55 °C for 2 min for 25 cycles; and 94 °C for 1 min, 35 °C for 2 min and 55 °C for 4 min for 1 cycle. Unincorporated label was removed by spinning through a 1-ml Sephadex G50 column and 2 μl of the spun material was used per EMSA reaction.

EMSA gel mixes

Gels (13.5 $\times$ 16 cm) for EMSA contained, per 100 ml: 13.3 ml of 30 % acrylamide/bisacrylamide (37.5:1), 85.62 ml of water, 1 ml of 10 % (w/v) ammonium persulphate, 10 ml of 5 $\times$ TBE (= 0.45 M Tris/0.32 M boric acid/10 mM EDTA) and 80 μl of *N,N,N',N'*-tetramethyl ethylenediamine (TEMED). This produces a concentration of 0.5 $\times$ TBE in the gel; however, the reservoir buffer contained 1.0 $\times$ TBE.

EMSA reaction mixtures

The standard EMSA reaction mixture was 40 μl and contained 10 mM Hepes buffer, pH 7.9, 4 mM MgCl_2 , 0.1 mM EDTA, 4 mM spermidine, 2 mM dithiothreitol, 1 mg $\cdot$ ml $^{-1}$ BSA, 1 $\mu\text{g} \cdot$ ml $^{-1}$ dI-dC, 50 mM KCl, 15 % (v/v) glycerol and approx. 1 μg of protein. The N-terminal domain of QUTA was recovered from the re-dissolved ammonium sulphate pellet (see above). These reagents were incubated at room temperature for 20 min, and the reaction was initiated by adding 2 μl of labelled probe. Following mixing and incubation at room temperature for 20 min the reaction mixtures were then loaded into individual wells on a gel that had been pre-electrophoresed at 150 V for 15 min and electrophoresis continued until the Bromophenol Blue marker dye had travelled approx. three-quarters of the way down the gel. After electrophoresis the gels were fixed by soaking in 10 % acetic acid for 10 min and then dried using a heated gel dryer attached to a vacuum pump.

Western-blot analysis

Western blotting was carried out as described previously [36].

RESULTS AND DISCUSSION

QUTR binds to the C-terminal domain of QUTA *in vitro*

We have previously shown in *A. nidulans* that over-expression of the *qutA* gene produces a constitutive phenotype and that over-expression of the *qutR* gene produces a super-repressed phenotype in strains that are wild-type for the *qut* gene cluster [7]. When constitutive and super-repressed haploids are combined, the resulting diploid has an inducible phenotype, implying that the QUTA and QUTR proteins interact *in vivo* [7]. To test this implication directly and to determine which of the two domains of the activator QUTR binds to, we carried out co-immunoprecipitation studies *in vitro*. The complete *qutA* and *qutR* genes and sequences encoding the N-terminal 437 residues of *qutA* were subcloned into the vector pSPUTK. The recombinant plasmids (pIVTT1–3, see Table 1a) were shown to direct the production of proteins of the appropriate molecular mass when labelled with [^{35}S]methionine using the rabbit reticulocyte-coupled *in vitro* transcription/translation system (results not shown). The identity of the QUTA protein was confirmed by cross-reaction to an antibody raised against a synthetic peptide derived from the N-terminal domain of QUTA [36]. The nucleotides encoding the N-terminal 437 residues of QUTA were

Figure 2 Immunoprecipitation of QUTA and QUTR by rabbit anti-QUTR antibodies

Lane 1 shows the background levels of QUTA pulled down by Protein A-Sepharose beads when radiolabelled QUTA is mixed with non-labelled QUTR. Lane 2 shows the amount of radiolabelled QUTA pulled down by Protein A-Sepharose beads when anti-QUTR antibody is added to a mixture that also contains non-labelled QUTR. Lane 3 shows the background levels of radiolabelled QUTA pulled down by Protein A-Sepharose beads when anti-QUTR antibody is added to radiolabelled QUTA produced in the absence of QUTR. Lanes 4 and 5 show the background material pulled down by Protein A-Sepharose beads when the radiolabelled N-terminal dehydroquinase synthase-like domain of QUTA is incubated with anti-QUTR antibodies in the presence (lane 4) and absence (lane 5) of non-labelled QUTR.

subcloned into the *E. coli* expression vector pRSET to produce the plasmid pMUTK10. Plasmid pMUTK10 directed the production of low levels of soluble C-terminally truncated QUTA protein, which was recoverable by ammonium sulphate precipitation (see the Materials and methods section). High-avidity antibodies specific to the QUTR DHQ-like domain were purified as described (see the Materials and methods section) and shown to effectively immunoprecipitate radiolabelled QUTR from reticulocyte lysates (results not shown).

Figure 2 shows the results of co-immunoprecipitation experiments between QUTA and QUTR using an anti-QUTR antibody. These data show that the anti-QUTR antibody is only able to immunoprecipitate QUTA over background levels when QUTA has first been incubated with QUTR. The antibody is unable to co-immunoprecipitate the N-terminal 437 residues of QUTA. EMSA experiments (results not shown) demonstrated that the N-terminal 437 residues of QUTA are able to bind to a 60-nucleotide fragment of the *qutE* promoter. This observation makes it unlikely that the failure of this sequence of QUTA to co-immunoprecipitate with QUTR is due to an inability to fold into a native configuration. Additionally, this region of QUTA is proposed to be homologous with the independently folding dehydroquinase synthase domain of the *A. nidulans* AROM protein [13,37]. In these co-immunoprecipitation experiments, the QUTA/QUTR mixtures are subject to a stringent washing regime (that includes SDS) which could disrupt other weak, but potentially biologically significant, interactions.

The N-terminal 88 amino acids of QUTR are sufficient for repression

The hypothesis that the *qutA* and *qutR* genes were generated by splitting a copy of the *arom* gene within the EPSPS-like domain suggests that the QUTR protein may recognize and bind to the QUTA protein through interactions between the fragments of this sequence carried on each protein. In order to determine the smallest part of QUTR that was capable of repression, plasmid pNUFC324 containing the sequence of *qutR* encoding the N-

Figure 3 Northern-blot analysis of transformants expressing the plasmid pNUFC324

Lane 1, an RNA molecular-mass ladder; lane 2, poly(A)⁺ mRNA from the wild-type strain R153; lane 3, poly(A)⁺ mRNA from the constitutive strain 2035; lane 4, poly(A)⁺ mRNA from the constitutive transformant 27B1; lane 5, poly(A)⁺ mRNA from the super-repressed transformant 31C1; lane 6, poly(A)⁺ mRNA from the super-repressed transformant 32A5. The filter was probed initially with [α -³²P]dCTP-labelled *qutR* DNA from co-ordinates 1–264 and then stripped and re-probed with [α -³²P]dCTP-labelled *actA* DNA (shown in the lower box).

terminal 88 amino acids corresponding to the proposed EPSPS-like sequence was used to transform *A. nidulans* strain 2035.

Uracil-independent transformants of strain 2035 were generated and screened in the PCA test where super-repressed strains were identified (see the Materials and methods section); however, no inducible transformants were recovered. In each case the super-repressed phenotype was correlated with elevated copy number (results not shown) and elevated quantities of an appropriately sized mRNA. Figure 3 shows a Northern blot of one constitutive (27B1; lane 4) and two super-repressed (31C1 and 32A5; lanes 5 and 6) transformants. These combined observations show that when the sequence encoding the N-terminal 88 amino acids of QUTR is over-expressed in *A. nidulans*, a repressed phenotype can be recovered, implying that these amino acids can function as a repressor.

The C-terminal SDH-like domain is primarily involved in signal recognition in wild-type QUTR

Genetic and molecular analysis of *N. crassa qa-1S* super-repressed and constitutive mutants has implied that the C-terminal SDH-like domain is involved in signal recognition and signal transduction [1,2]. *A. nidulans* strain 2035 produces the three quinate-pathway enzymes constitutively due to a mutation of the *qutR* gene. The mutation is recessive, implying that the encoded QUTR mutant protein is unable to interact with either wild-type QUTR or QUTA. This mutant was derived from a no-longer-extant dominant non-inducible *qutR* mutant by selection for growth on quinate, which parallels the isolation of the equivalent *qa-1S* constitutive mutants [1,2].

The mutant *qutR* allele in strain 2035 was sequenced and the mutations determined as Gly⁸¹⁰ → Ser and Pro⁸²⁰ → Gln, with both of the mutations located within the C-terminal SDH-like encoding domain. These observations support the view that this domain has both signal-recognition and signal-transduction functions. In order to test this possibility, a *qutR* gene encoding a truncated QUTR protein lacking the C-terminal domain was

Figure 4 Deletion and domain-swap analysis of the QUTR repressor protein

Structures of the QUTR proteins encoded by the plasmids used to transform the *A. nidulans* constitutive strain 2035. The phenotypes indicate whether the transformants were capable of restoring the wild-type inducible regulation, had a mutant repressed (non-inducible) regulation or had no effect, retaining constitutive regulation. E, EPSPS-like sequence; SK, SK-like sequence; DHQ, DHQ-like sequence; SDH, SDH-like sequence.

subcloned to produce plasmid pRTA3 and expressed in *A. nidulans* strain 2035. If the SDH-like domain has an essential direct role in signal transduction then expression of this truncated *qutR* gene in strain 2035 should not lead to a change in phenotype from constitutive to inducible.

As a control to demonstrate that we could restore the inducible phenotype, we transformed the wild-type *qutR* gene (contained within plasmid pRTA1) into strain 2035. A second control, plasmid pRTA2, which contains the *qutR* sequence lacking the N-terminal EPSPS- and SK-like domains, was used to determine if an N-terminally deleted QUTR could mediate signal transduction. If the SDH-like domain of QUTR is directly involved in signal transduction then expression of this truncated gene should produce transformants with an altered inducible phenotype.

Transformants generated by all three plasmids were screened to detect any with an altered phenotype. The results of this analysis are shown in Figure 4, which illustrates the following points. (i) Expression of the wild-type *qutR* gene was able to restore the inducible phenotype. (ii) Expression of the *qutR* gene deleted for the N-terminal EPSPS- and SK-like sequences failed to produce transformants with an altered phenotype. Finally, (iii) expression of a *qutR* gene deleted for the SDH-like domain was able to restore the wild-type inducible phenotype. Figure 5 shows Northern-blot analysis of one constitutive and one inducible pRTA3-derived transformant. These observations imply that in the wild-type QUTR protein the SDH-like domain is involved primarily in signal recognition.

The QUTR DHQ domain is not involved in signal transduction

The observation that expression of a *qutR* gene encoding a protein lacking the SDH-like domain can restore an inducible

Figure 5 Northern-blot analysis of *A. nidulans* transformants expressing the plasmid pRTA3

Lane 1, an RNA molecular-mass ladder; lane 2, poly(A)⁺ mRNA from constitutive transformant R3/11; lane 3, poly(A)⁺ mRNA from inducible transformant R3/25. The filter was probed initially with [α -³²P]dCTP-labelled *qutR* DNA from co-ordinates 1–2706 and then stripped and re-probed with [α -³²P]dCTP-labelled *actA* DNA (shown in the lower box).

phenotype implies that this truncated QUTR protein retains signal-recognition ability. In order to investigate whether the DHQ domain of QUTR could provide the duplicate signal recognition ability we undertook 'domain-swap' experiments. First we swapped the physiologically irrelevant GST protein as it has approximately the same molecular mass as the QUTR DHQ domain. The sequence of the *qutR* gene encoding the EPSPS- and

Figure 6 Northern-blot analysis of transformants expressing the plasmid pNUFC286

Poly(A)⁺ mRNA is shown from (lane 1) the super-repressed transformant 3C1, (lane 2) the super-repressed transformant 1A4, (lane 3) the constitutive transformant 6A5, (lane 4) the constitutive strain 2035 and (lane 5) the wild-type strain R153. Lane 6 contains an RNA molecular-mass ladder. The filter was probed initially with [α -³²P]dCTP-labelled *qutR* DNA from co-ordinates 1–843 and then stripped and re-probed with [α -³²P]dCTP-labelled *actA* DNA (shown in the lower box).

SK-like domains were fused to the GST-encoding sequence to yield plasmid pNUFC313. When *A. nidulans* strain 2035 was transformed with plasmid pNUFC313, phenotypically altered transformants with a super-repressed phenotype were recovered. The super-repressed phenotype was associated with increased copy number and elevated levels of an appropriately sized mRNA (results not shown). No transformants with an inducible phenotype (retaining signal-recognition ability) were recovered.

It could be argued that GST is not physiologically irrelevant because its structure somehow interferes with signal recognition, preventing the recovery of transformants with an inducible phenotype. So, as a control, we swapped the QUTR DHQ domain with the AROM DHQ domain, which is predicted to have a similar overall folding pattern [38]. But the enzymic activity associated with this domain could also interfere with signal recognition, so a mutant enzymically inactive form of the AROM DHQ domain was also used. In the hybrid protein encoded by pNUFC286 the AROM DHQ domain had a wild-type sequence, and in the hybrid protein encoded by pNUFC315 the AROM DHQ has an essential active-site lysine (Lys¹²⁰⁸) mutated to arginine to abolish enzyme activity [39].

When *A. nidulans* strain 2035 was transformed with plasmids pNUFC286 or pNUFC313, phenotypically altered transformants with a super-repressed phenotype were recovered, but no transformants with an inducible phenotype (retaining signal recognition) were recovered. The super-repressed phenotype was associated with increased copy number and elevated levels of appropriately sized mRNAs. There was no apparent difference in the phenotypes of the transformants recovered with any of the plasmids encoding domain-swapped QUTR hybrid proteins. Figure 6 shows Northern-blot analysis of one constitutive (6A5) and two super-repressed (1A4 and 3C1) transformants of plasmid pNUFC286. These combined data imply that the QUTR DHQ domain has signal-recognition ability, but has no role in signal transduction.

Figure 7 Chain tracing of EPSPS from *E. coli*

The numbers show the six repeating folding units which make up the two halves of the molecule. The open arrows distinguish strands that are not directly connected to the rest of their folding unit. The extreme 5' end of the *qutR* gene is proposed to encode amino acids corresponding to two of the β -sheets of folding unit 6 and two β -sheets and two α -helices in folding unit 1.

Possible mechanism of signal transduction

The QUTA and QUTR proteins are proposed to have arisen via duplication and splitting of the *arom* gene (see Figure 1). This split is proposed to have occurred in the C-terminal region of the EPSPS domain, thereby producing a QUTR protein that has the C-terminal 88 amino acids of the EPSPS domain at its N-terminus. The crystal structure of *E. coli* EPSPS has been solved and the most striking feature is a 6-fold repetition of one folding unit or subdomain [40]. The N- and C-termini of EPSPS are located in subdomain 1 and form the antiparallel strands of one β -sheet. A topological diagram of these features is shown in Figure 7. The missing fifth of the EPSPS sequence located on the N-terminal *qutR* gene corresponds to the amino acid sequences forming two of the β -sheets of subdomain 6 and two β -sheets and two α -helices in subdomain 1. We speculate that a plausible mechanism for how QUTR recognizes and binds to QUTA is by reconstituting the amino acid sequences corresponding to subdomains 6 and 1.

This speculative model has the added attraction that it also offers an explanation for how the binding of QUTR could inactivate the transcription-activating function of QUTA. Previous genetic analysis of mutants in the *qutA* gene has identified a putative transcription-activating motif in the extreme N-terminus of the QUTA EPSPS domain [36]. The mutation in strain *qutA214* is dominant, preventing induction of the quinate-pathway enzymes and causing the inability to metabolize quinate. The mutation is a glutamate at position 457 changed to lysine in a region of high localized negative charge (Glu⁴⁵⁷-Asp-Gly-Leu-Glu-Glu). This glutamate residue is conserved in the equivalent QA-1F protein of *N. crassa* [2]. These amino acids are located in the sequence corresponding to the first β -strand of folding unit 1 in EPSPS. It is possible that reconstitution of the sequence corresponding to folding units 1 and 6 by the extreme N-terminus of QUTR occludes this area of localized negative charge, preventing it from functioning as a transcription-activating motif.

Conclusions

The data presented here show that the QUTR protein can be split into distinct regions involved in signal recognition and signal transduction. The N-terminal 88 amino acids are sufficient for signal transduction and the remaining 813 amino acids are involved in signal recognition. The C-terminal SDH-like function is essential for signal recognition at wild-type levels of transcription. At high levels of over-expression the DHQ-like domain can duplicate this signal-recognition function when it is part of a C-terminally truncated QUTR that lacks the SDH-like domain. It has been suggested that the basis for the evolution of the protein–protein recognition events necessary for signal transduction may be based on the specificity of proteases [25]. On the basis of the observations reported here we suggest that a possible mechanism for signal transduction in this relatively simple system is by reconstitution of a protein domain that was fragmented originally by splitting a duplicated copy of the AROM-encoding *arom* gene.

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