cudA: a Dictyostelium gene with pleiotropic effects on cellular differentiation and slug behaviour

Masashi Fukuzawa, Neil Hopper and Jeffrey Williams*
MRC Laboratory of Molecular Cell Biology and Department of Biology, University College London, Gower St, London WC1E 6BT, UK

*Author for correspondence (e-mail: jeff.williams@ucl.ac.uk)

SUMMARY

The Dictyostelium cudA gene encodes a nucleoplasmic protein that is essential for normal culmination. There are no functionally characterised homologues in other organisms but there is a related gene of unknown function in Entamoeba histolytica. The cudA gene is expressed by the prestalk cells that constitute the slug tip (the pstA cells), it is not detectably expressed in the band of prestalk cells that lies behind the tip (the pstO cells) but it is expressed in the prespore cells. This unusual pattern of expression suggests a role on both the stalk and spore pathways of differentiation and cudA− mutant cells are indeed defective in both stalk and spore formation. Furthermore, the slugs formed by cudA− cells continue to migrate under environmental conditions where normal slugs culminate immediately. This aspect of their behaviour can be reversed when the cudA gene is selectively expressed in the pstA cells. This shows that processes occurring in the pstA cells regulate entry into culmination.

Key words: culmination, Dictyostelium, cudA, prestalk cells

INTRODUCTION

During asexual development Dictyostelium cells undergo a remarkable transition, from unicellularity to multicellularity. Cells aggregate together in response to pulsatile emissions of cAMP from a signalling centre and form a cylindrical structure called the first finger or standing slug. This behaves like a multicellular organism, in which the constituent cells collaborate to ensure that the fruiting body is formed in an appropriate location. Under the environmental conditions that exist below the surface of the soil or leaf litter (low light levels, high humidity and high ionic strength) the first finger falls on to its side and migrates away as a slug. The slug is phototactic and thermotactic and these sensitivities direct it to the surface where culmination occurs.

The regulator of culmination is ammonia. This is produced in large amounts during development, by cellular catabolism, and experimentally induced depletion of ammonia causes migrating slugs to enter culmination immediately (Schindler and Sussman, 1977). Presumably, arrival at the surface, where there is a high light level, causes the slug to orient upwards off the substratum and so increases the rate of loss of ammonia by diffusion. Ammonia is thought to control entry into culmination by regulating vesicular pH (Davies et al., 1993) but cAMP dependent protein kinase (PKA) is also known to be involved, because expression of a dominant inhibitor of PKA under the control of a prestalk-specific promoter strongly favours slug migration over culmination and prevents stalk cell differentiation (Harwood et al., 1992). Furthermore, when the dominant PKA inhibitor is expressed under control of a prespore-specific promoter development is rendered hypersensitive to the inhibitory effects of ammonia (Hopper et al., 1993). This observation provides strong genetic evidence for a link between ammonia and PKA but the actual mechanisms that couple changes in vesicular pH to PKA are as yet unknown.

In a genetically tractable organism such as Dictyostelium one very powerful approach to tracing a signalling pathway is to isolate mutants. The ‘slugger’ mutants show delayed entry into culmination and, in some cases, they form defective fruiting bodies (Newell and Ross, 1982). They fall into 10 complementation groups and the development of mutants derived from all classes is hyper-sensitive to the inhibitory effects of ammonia (Gee et al., 1994). In a monolayer assay wild-type prestalk cells can be induced to differentiate into stalk cells by the addition of a membrane permeant cAMP analogue but some of the slugger mutants are insensitive to such induction (Inouye and Gross, 1993). Again, this suggests that PKA is involved in terminal stalk cell differentiation but the genes affected in these mutants have yet to be identified.

It has recently become possible to perform non-homologous gene disruption in Dictyostelium, using a method that facilitates the isolation of flanking genomic sequences (Kuspa and Loomis, 1992). We have applied this method of gene tagging (REMI, Restriction Enzyme Mediated Integration) to identify a slugger mutant. We describe the detailed phenotypic properties of the mutant and present the structure and expression pattern of the cognate gene. We have also used the mutant to characterise the cells that regulate the choice between slug migration and culmination.
Experiments in which the tips and bodies of a slagger mutant and a normal strain are reciprocally interchanged by grafting show that the tip region dictates entry into culmination (Smith and Williams, 1980). However, the tip is a crudely delineated sub-region of the slug with specific biological properties rather than a defined cell type. Here we analyse the role of specific cell types by directing expression of the cudA gene in different prestalk cell sub-populations in a cudA- background.

MATERIALS AND METHODS

Cell culture, transformation and development

The *D. discoideum* strain DH1 (a gift from P. Devreotes), a derivative of KA3 in which the *pyr5-6* gene is disrupted, was grown at 22°C in HL5 medium (Watts and Ashworth, 1970) supplemented with uracil at 200 μg/ml. Ax2 and DH1 cell strains transformed with *pyr5-6* vectors were grown in un-supplemented HL5 medium. The lacZ marker constructs ecmA0::lacZ (Jermyn and Williams, 1991), ecmA1::lacZ (Watts and Melton, 1980). However, the tip is a crudely delineated sub-region of the slug with specific biological properties rather than a defined cell type. Here we analyse the role of specific cell types by directing expression of the *cudA* gene in different prestalk cell sub-populations in a *cudA*- background.

**Materials and Methods**

**Cell culture, transformation and development**

The *D. discoideum* strain DH1 (a gift from P. Devreotes), a derivative of KA3 in which the *pyr5-6* gene is disrupted, was grown at 22°C in HL5 medium (Watts and Ashworth, 1970) supplemented with uracil at 200 μg/ml. Ax2 and DH1 cell strains transformed with *pyr5-6* vectors were grown in un-supplemented HL5 medium. The *lacZ* marker constructs *ecmA0::lacZ* (Jermyn and Williams, 1991), *ecmA1::lacZ* (Early et al., 1995), *ecmO::lacZ* (Early et al., 1993), *ecmB::lacZ* (Ceccarelli et al., 1991), *ST::lacZ* (Ceccarelli et al., 1991) and *pspA::lacZ* (Harwood et al., 1991) were introduced by calcium-phosphate transformation and clones were selected and maintained in 10 μg/ml G418 (geneticin; Gibco, BRL). For development, axenically grown cells (1-5x10⁵ cells/ml) were washed twice in KK₂ (16.5 mM KH₂PO₄, 3.8 mM K₂HPO₄, pH 6.2) and 2x10⁶ cells were spotted on to either 2% water agar or 2% KG₂ agar. Slugs were formed on water agar and harvested immediately after they started migration (normally at 16-18 hours of development). For development in filters, 2x10⁶ cells were spotted onto a nitrocellulose filter (Millipore) supported on a filter pad soaked with LPS buffer (KK₂ with 20 mM KH₂PO₄ and 2.5 mM MgCl₂), and allowed to migrate towards a dim, unidirectional light source.

**β-galactosidase staining**

Structures developing on agar plates were fixed and stained with X-gal as described by Dingermann et al. (1989). Specimens were normally stained for 2-3 hours at room temperature.

**REMI mutagenesis and molecular cloning**

REMI was performed (Kuspa and Loomis, 1992) using the plasmid pRHI30 (a gift from Robert Insall). The plasmid was linearized with BamHI and electroporated into DH1 cells along with the restriction enzyme *DpnII*. Transformants were selected for uracil auxotrophy in FM medium (Gibco, BRL) as described by Harwood et al. (1995). Genomic DNA isolated from the *cudA* mutant was cut with either *BclI* or *EcoRI*, self-ligated and transformed into electrocompetent *E. coli* cells. Ampicillin-resistant colonies were isolated, the plasmid DNA sequence was established. cDNA clones containing *cudA* sequences were isolated from a cDNA library and the 5’ end of the *cudA* mRNA was obtained from a DNA pool of the same cDNA library by PCR using a vector primer and a *cudA*-sequence-specific primer.

To disrupt the *cudA* gene, 15 μg of EcoRI-rescued plasmid DNA was relinearised with *EcoRI* and transformed into DH1 cells. The disruption frequency was 30–40%. One of these mutants (MF1) was used in all subsequent experiments where there was comparison with the parental DH1 strain. In some cases a *cudA* disruptant of Ax2 was used that was created by first inserting a blasticidin resistance vector (Sutoh, 1993) in place of the pRHI30 vector within the *EcoRI*-rescued plasmid. Then an internal deletion was created, within the coding region by replacing the 200 bp region immediately 5’ of the REMI insertion site with a 1.4 kb PCR-fragment extending from the ATG initiation codon (see Fig. 1A). All of the Ax2 disruptants showed exactly the same phenotype as the original REMI mutant in DH1 and so one of them (MF2) was used in all subsequent experiments.

**Nucleic acid analysis**

Genomic DNA from parental DH1 cells and from the original *cudA*-mutant was digested with restriction enzymes and electrophoresed on a 1% agarose gel. The DNA was then transferred onto a Hybond-N+ filter (Amersham) and hybridised with 32P-labelled *cudA* cDNA. Hybridisation was performed at 37°C in 5x SSC, 1× Denhardt’s solution, 20 mM sodium phosphate (pH 6.7), 1% SDS, 100 μg/ml salmon sperm DNA, and 50% formamide. The filter was washed in 1x SSC, 0.1% SDS at 65°C. For northern transfer analysispoly(A)+ RNA was isolated at different stages using a poly(A)-tract mRNA isolation kit (Promega). Samples (2 μg) of poly(A)+ RNA were separated on a 1.2% formaldehyde-containing gel and blotted onto a Hybond-N+ filter. Hybridisation was performed under the conditions described above for Southern transfer analysis except that the filter was washed in 2x SSC, 0.1% SDS at 65°C. To analyse prespore gene specific gene expression, total RNA was isolated and 10 μg was loaded per lane of a formaldehyde gel.

**In situ hybridisation**

The probe for in situ hybridisation was made by subcloning cDNA clone #51 (Fig. 1A) into pBluescriptII followed by transcription into RNA with T7 or T3 RNA polymerase. The probe was labelled with a DIG RNA labelling kit (Boehringer Mannheim) and in situ hybridisation was performed as described by Escalante and Loomis (1995). A sense probe was used as negative control.

**Bacterial expression of *cudA* and isolation of a monoclonal antibody**

In order to express CUD A as a histidine tagged fusion protein in *E. coli* the *cudA* cDNA sequence, extending from the start codon to the end of cDNA clone #16, was cloned into the vector pQE30 (Qiagen) to generate pQE*cudA*. For preparation of a fusion protein for immunisation the polyglutamine tract in the C terminus of the CUD A protein was eliminated, by cutting with *SacI* in the cDNA and with *XhoI* in the pQE30 vector. This was followed by end-filling and self-ligation to create pQE*cudA*, which is expressed to yield the N-terminal 326 amino acids of CUD A (ACUDA). These two expression constructs were transformed into *E. coli* strain M15 (Qiagen) and the fusion proteins were induced using IPTG. The fusion proteins were purified from bacterial lysates using a nickel affinity column. Mouse monoclonal antibodies were generated against the truncated CUD A fusion protein (ACUDA), and their specificity was confirmed by western blotting and immunohistochemical staining using both bacterially expressed CUD A and *Dicyostelium* cells overexpressing the *cudA* gene. One of the antibodies, mAb11, was used in all the experiments described.

**Western blotting**

Total cell lysates were prepared by boiling cells in SDS sample buffer and 5 μg of protein was loaded per lane of a 10% SDS-polyacrylamide gel. Nuclear extracts were prepared as described by Kawata et al. (1996), samples were denatured by boiling in SDS sample buffer and 10 μg was loaded per lane of a 10% SDS-polyacrylamide gel. Proteins were blotted onto Hybond-C filters (Amersham) and incubated sequentially with mAb11 and a 1 in 2000 dilution of goat anti-mouse IgG conjugated with peroxidase (Bio Rad). Signals were detected by chemiluminescence (ECL; Amersham).

**Immunohistochemical staining**

For individual cell staining, slugs were dissociated and cells were allowed to settle on a glass slide then fixed with methanol for 10 minutes. Whole mounts of slugs were placed onto a coverglass and similarly fixed with methanol. Specimens were incubated with mAb11, followed by FITC-conjugated goat anti-mouse IgG (Sigma).
For double staining, dissociated cell samples were first incubated with mAb11 and then with Texas-Red-conjugated goat anti-mouse IgG (Molecular Probes). After washing they were incubated with mAb17 (an IgM monoclonal antibody that recognises a nuclear-pore component; M. Fukuzawa unpublished results), followed by FITC-conjugated goat anti-mouse IgM (Sigma). False colours (green for Texas Red and red for FITC) were assigned to keep consistency with all the single staining analyses. Samples were visualised with a Leica DMRBE confocal microscope.

Construction of cudA expression constructs
A 1.5 kb, 5’ proximal region from the cudA gene was amplified from genomic DNA using the primers: 5’-CGGGATCCATGAATCAGAATTTTCAT-3’ and 5’-GCACCTTTCTACAATTACG-3’. The first of these two primers hybridises at the initiation codon (underlined in the oligonucleotide sequence) and the second hybridises at the position of the unique EcoRI site (see Fig. 1A). The amplified product was the size expected from the cDNA sequence, indicating there to be no intron(s) in this region. The insert from cDNA clone #16 was excised and subcloned into the EcoRI site of pBlue-scriptII to create pBS16. The 1.5 kb genomic PCR product was then excised with BamHI and EcoRI and inserted into the BamHI/EcoRI site of pBS16 to generate pBScudA. This contains the entire cudA coding region and the 3’ non-coding region from cDNA #16. The insert from pBScudA was then excised with BamHI/Xhol and inserted into the BglII/Xhol sites of ecmA/O: lacZ (Jermyn and Williams, 1991), ecmA: lacZ (Early et al., 1995), ecmO: lacZ (Early et al., 1993), pspA: lacZ (Harwood et al., 1991) so as to replace the lacZ gene and generate ecmA/O: cudA, ecmA:cudA, ecmO:cudA and pspA:cudA.

RESULTS
Isolation of the cudA- mutant and structure of the cudA gene
The cudA- strain was identified as showing aberrant terminal development and hence was designated a cud (culmination defective) mutant. Genomic DNA was isolated from the cudA- strain and two different restriction fragments containing vector DNA and flanking genomic DNA sequences, one bounded by EcoRI sites the other by BclI sites, were cloned in E. coli (Fig. 1A). We showed that the DNA which was isolated was responsible for the mutation by performing homologous gene disruption using the EcoRI fragment. About one third of the resultant transformant clones displayed the same phenotype as the original cudA- isolate (data not shown). Southern transfer analysis confirmed that they were disruptants of the cudA gene (Fig. 1B). We also made cudA- disruptants of the strain Ax2 in which an internal deletion was introduced within the cudA coding region (Fig. 1A) and, for one of the clones, disruption was also demonstrated by northern transfer analysis (Fig. 1C). In the disruptant there is a great reduction in the apparent level of the cudA transcript. A faint band is detectable in the original disruptant created with the dis-3 original REMI mutant DNA, and DNA from three cudA null clones created by transformation of EcoRI-rescue DNA (see A). Genomic DNAs digested with EcoRI or EcoRV were hybridised with the probe shown in A. (C) Northern analysis of the normal strain Ax2 and a cudA disruptant created with a blastidin resistance cassette. A faint band is detectable in the cudA- track.

that may act as a nuclear localisation signal (underlined in Fig. 2B). There is only one homologous gene in the databases (Fig. 2C), an Entamoeba histolytica gene of unknown function. The Entamoeba histolytica protein also contains a putative nuclear localisation signal and it is in the same relative position as in CUDA. The cudA gene encodes a 2.5 kb transcript that accumulates during aggregation and then maintains a relatively constant concentration thereafter (Fig. 3).

CUDA is a nuclear protein
The subcellular localisation of the CUDA protein was determined by immunochemical staining. A fusion protein was prepared containing CUDA sequences linked to an oligo-histidine tract. The fusion protein was purified from E. coli and a mouse monoclonal antibody was prepared against it. On a western blot of Dictyostelium proteins from the slug stage the antibody detects a protein of 90×10³ M₀, the size expected for
the CUDA protein (Fig. 4A). Immunohistochemical staining using cells dissociated from slugs shows localised staining in the majority of cells (Fig. 4B,C). Double staining with an antibody that recognises Dictyostelium the majority of cells (Fig. 4B,C). Double staining with an antibody that recognises Dictyostelium

The spatial pattern of cudA gene expression suggests a role on both pathways of differentiation

Immunohistochemical staining, using whole mounts of slugs, reveals a novel, and surprisingly complex, pattern of expression for the cudA gene (Fig. 5A). There is staining in the pstA region, staining in the prespore region but little or no staining in the pstO region. The cells from dissociated slugs that showed no staining (Fig. 4B) are presumably, therefore, pstO cells. In cudA− slugs there is no detectable staining (Fig. 5B; N. B. we assume that the putative cudA homologue (Fig. 1C) is not detected because it is expressed at a much lower level than cudA itself and immunohistochemical analysis is relatively insensitive).

Analysis by in situ hybridisation confirms the whole-mount staining data. The maximum concentration of cudA transcripts is present in the pstA zone and there is no detectable signal in the pstO region. There is expression throughout the prespore region but at a somewhat lower level than in the pstA region. At culmination there is strong expression throughout the papilla with an especially high level of staining at the prestalk-prespore boundary (Fig. 5C). Since this is the region where the pstO cells are located, the cudA gene must become activated in pstO cell derivatives at culmination (N.B. the low level of signal in the stalk and in the spores is most probably caused by failure of the anti-DIG antibody to penetrate the stalk tube).

CudA− strains display a strong ‘slugger’ phenotype

The development of cudA− cells appears normal up to and through the first finger stage and the migratory slugs that are formed resemble those of the parental strain (Fig. 6A). When, however, they are allowed to migrate on agar plates in the dark, or towards a low level unidirectional light source, cudA− slugs fail to culminate while DH1 aggregates form culminants within one or two days. When cudA− slugs are placed in overhead light they rear up, as if attempting to culminate (Fig. 6B), but immediately collapse back on to the subtratum and continue to migrate for many hours. After about 12 hours of migration they start to deposit very large clumps of cells in the slime trail (Fig. 6C). Eventually they rear up again, finally forming gnarled structures with a small, apparently undifferentiated, cell mass at the top (Fig. 6D,E).

---

**Fig. 2.** The CUDA protein sequence. (A) The ORF of cudA encodes a protein of 791 amino acids. By comparing the cDNA sequence to the genomic sequence (data not shown) we confirmed that this gene does not contain any introns. The REMI insertion in the original isolate is inserted at Asp573. The GenBank accession number for cudA is Y13119. (B) An alignment of CUDA protein and a related protein from Entamoeba histolytica. Conserved amino acids (asterisks) and conservative substitutions (dots) are indicated. A highly basic region is located between Lys317 and Arg325 (underlined) and this is the nuclear localisation signal.

**Fig. 3.** The developmental time course of cudA gene expression. Poly(A)+ RNA was prepared from the indicated stages of development and hybridised with a 32P-labelled cudA probe.
The above behaviour is observed using cells developing on agar under ionic conditions permissive for slug formation but the cudA− strain also shows a ‘slugger’ phenotype under more restrictive conditions. When placed on nitrocellulose filters in high ionic strength buffer the parental strain entirely fails to form migratory slugs and culmination occurs in situ (Fig. 6F). However, the cudA− first fingers go on to form slugs that move away from the site of deposition.

**CudA− terminal structures contain neither stalk nor spore cells but a large fraction of the cells express markers of stalk cell differentiation**

During culmination in a wild-type strain, cells in the prestalk zone synthesise a cellulose- and protein-containing extracellular matrix called the stalk tube. They do so both while moving upwards, towards the apex of the culmination, and as they change direction and move downwards towards the base. About one half of the cells in the back of the prestalk zone, the pstO cells, give rise to the upper cup, a structure that lies above the nascent spore head (Jermyn, 1996). There are cells that lie scattered in the posterior region of the slug called anterior-like cells (ALC) that share most if not all of the properties of the prestalk cells (Sternfeld and David, 1982; Devine and Loomis, 1985). At culmination some of the ALC differentiate to form the lower cup, a structure that lies below the nascent spore head, and some differentiate to form the basal disc (Jermyn, 1996; Williams et al., 1989).

High power microscopic examination of the terminal cudA− structures, and squashes derived from them, shows there to be no mature spores and no mature stalk cells (data not shown). Also, the cudA− terminal structures entirely fail to express spiA (data not shown), an early marker of spore formation (Richardson et al., 1991). The cells left behind in the slime trail do, however, show the extensive vacuolisation characteristic of stalk cells (Fig. 7A). Furthermore, cells within the terminal structures express an early marker of stalk cell differentiation. The ecmB gene encodes an extracellular matrix protein of the stalk tube. At culmination ecmB is expressed in all cells within the stalk tube, in the basal disc.

---

**Fig. 4.** Western blotting and immunohistochemical staining (B, C and D) with an anti-CUDA monoclonal antibody. (A) Western analysis using an anti-CUDA monoclonal antibody. Total cell lysate and nuclear extract from Ax2 derivatives were probed with mAb11 as described in Materials and Methods. The filter bearing total cell lysate was exposed overnight as the signal was very weak. The signal in the nuclear extract was obtained after a 15 second exposure of the film. By further exposure of the western of the nuclear extract, a weak band of the size of CUDA could be detected in cudA− slugs (data not shown). CUDA localisation in disaggregated slug cells. (B) Phase contrast view. Scale Bar, 5 μm. (C) Cells were stained with mAb11 as described in Materials and Methods. Note the presence of unstained cells. Scale Bar, 5 μm. (D) Double staining with anti-CUDA (mAb11; green) antibodies and anti-nuclear-pore (mAb17; red) antibodies. Scale Bar, 5 μm.

**Fig. 5.** Expression pattern of the cudA gene in developing structures. (A) A whole mount of an Ax2 slug stained with mAb11. Both the pstA and the prespore region are stained but we believe that the pstA staining looks relatively weak because the tip tapers down very quickly while most of the the prespore region is of a uniform thickness. Because Dictyostelium nuclei are small, relative to the cytoplasm the gaps between stained nuclei give the illusion of weak staining within the tip. (B) A whole mount of a cudA− slug (Ax2 background) stained with mAb11. (C) In situ hybridisation of a migrating slug. CudA is expressed somewhat more strongly in the pstA region than in the prespore region and we believe this to be the true pattern of gene expression because, in contrast to the immunostaining with mAb11, most of the cell is stained by in situ hybridisation. (D) In situ hybridisation of Ax2 mid culminants.
and in the upper and lower cups (Jermyn and Williams, 1991). The distal part of the ecmB promoter (the ST region) directs high level expression in cells within the stalk tube and very low level expression in basal disc cells and lower cup cells (Ceccarelli et al., 1991). Cells transformed with a construct containing the ST region fused to the lacZ gene show strong staining precisely as they enter the stalk tube. Hence ST: lacZ is a very early marker of commitment to the stalk cell pathway of differentiation. In a migrating slug formed from cudA- cells, ST: lacZ is expressed in just a few scattered cells, exactly as in normal slugs (Fig. 7B and data not shown) showing that expression of the ST fragment is correctly temporally regulated in cudA- cells. However, in the terminal structures formed by cudA- cells a large fraction of cells express ST: lacZ (Fig. 7C,D; NB because the terminally differentiating cells in these withered structures proved impossible to dissociate, it was not possible to determine the exact fraction of cells expressing the fusion gene).

An elevated rate of cellulose deposition is another feature of stalk cell differentiation and the cudA- structures show dispersed staining with calcofluor, a cellulose-specific reagent (Fig. 7E; NB spore cells also synthesise cellulose but it forms part of their cell walls and no spore cells are present within the cudA- structures). Expression of ST:gal and elevated cellulose deposition are both early events in stalk cell differentiation but the cudA- terminal structures also express staB a gene that is, under some circumstances, a marker of late stalk cell differentiation. When normal slugs enter culmination immediately (e.g. in cells developing on a nitrocellulose filter) the staB gene is expressed only in cells in the lower half of the stalk tube, i.e. in vacuolated cells that appear to have completed their differentiation into stalk cells (Robinson and Williams, 1997). The staB: lacZ construct is expressed throughout the lower part of cudA- terminal structures (Fig. 7F).

Thus when cudA- slugs attempt to culminate many cells enter the stalk cell pathway of differentiation. They express ST: lacZ, staB: lacZ and cellulose is accumulated. However, they do not vaculate. Interestingly, they also fail to fabricate a stalk tube: there is sometimes an approximately central core of ST: lacZ-expressing cells in the anterior region (Fig. 7D) but high power visualisation reveals no trace of a surrounding stalk tube.

Having characterised the terminal cudA- structures, we next determined whether there are defects in earlier development using prespore and prestalk-specific markers.

The CUDА protein is required for normal levels of expression of a sub-set of prespore-specific genes

The effect of the cudA- mutation on those genes that are selectively expressed in prespore cells varies markedly from gene to gene. The pspbA gene encodes a cell surface protein of unknown function that is present on the surface of prespore cells (Krefft et al., 1983) and the Dp87 gene encodes a matrix component of the spore mass (Nakao et al., 1994). Analysis by northern transfer shows that the level of expression of both of these genes in cudA- cells is two- to three-fold lower than in the control (Fig. 8A) but spatial patterning is normal; in a newly migrating slug derived from cudA- cells the pspbA: lacZ fusion gene is expressed in the rear, prespore region and in a few scattered cells in the prestalk region (Fig. 8B). The cotC gene encodes a component of the spore coat (Fosnaugh and Loomis, 1989) and its expression is much more severely reduced in a cudA- mutant. Expression of cotC is approximately ten-fold lower than in control slugs (Fig. 8A) and expression assessed using a cotC: lacZ construct is also greatly reduced (data not shown).

In cudA- cells expression of several prestalk cell-specific markers appears normal

The different sub-types of prestalk cell are defined by their patterns of expression of the ecmA and ecmB genes. The cap-site proximal region in the ecmA gene promoter directs expression in the the front half of the prestalk zone, in the pstA cells, and cap-site distal sequences direct expression in the back half of the prestalk zone, in the pstO cells (Early et al., 1993). EcmA/O: lacZ (a lacZ fusion construct containing the entire ecmA promoter), ecmO: lacZ and ecmA: lacZ constructs are all expressed normally in cudA- slugs (Fig. 9). In a newly formed slug the complete ecmB gene promoter directs expression in a cone of cells situated at the position in the slug tip where stalk tube formation is initiated at culmination (Jermyn et al., 1989) and there is such a concentration of ecmB-expressing cells in the tip of cudA- slugs (Fig. 9).
Synergy experiments indicate that cudA− prestalk cells are unable to enter the stalk tube at culmination

Although prestalk gene expression in cudA− cells appears normal the fact that the cudA gene is expressed in pstA cells and that stalk cells are not formed in the terminal cudA structures suggested that the cudA− prestalk cells might be defective in some other way. We therefore performed synergy experiments with wild-type cells. When marked cudA− cells are mixed with parental cells they become uniformly distributed throughout the slugs that are formed (data not shown). However, at culmination they are almost entirely excluded from the stalk (Fig. 10). They are also under-represented in the nascent spore mass and microscopic examination of squashes of the terminal structures show that they never form spores. The few scattered cells observed in the spore mass are most probably ALC and, consistent with this interpretation, the cudA− cells are greatly enriched in the upper and lower cups (Fig. 10), structures that derive from the ALC population (Jermyn and Williams, 1991).

Selective expression of the cudA gene in the pstA cells of the cudA− mutant reverts the slug behaviour

In order to further understand the cudA− phenotype we determined the consequences of expressing the cudA gene in different cell types. The cudA− mutant was transformed with fusion constructs containing the cudA coding region under the control of the actin15, pspA, ecmA/O, ecmO, and ecmA promoters. The actin15 gene is expressed during growth and early development (Cohen et al., 1986) and transformation with the actin15:cudA fusion construct reverts the mutant phenotype such that apparently normal mature culminants are formed (data not shown). When cudA− is expressed under the control of the pspA promoter, i.e. in prespore cells, there is no gross difference from the parental cudA− mutant (Fig. 11A). Again, the pspA:cudA-expressing strain forms a woidered finger-like structure when it attempts to culminate. Neither spore cells nor stalk cells are present (Table 1).

CudA− transformants expressing ecmA/O:cudA form outwardly normal fruit (Fig. 11B) but close microscopic examination shows that the spore head is semi-transparent. Such a ‘glassy’ spore head is typically observed when spore maturation is repressed and squashes of such structures confirm that very few mature spores are present but that stalk cell differentiation does occur at a high level (Table 1). Those few spores that are present may derive from ecmA/O:cudA-expressing cells that trans-differentiated into prespore cells at some point during development. Expression of the ecmO:cudA construct in cudA− cells yields similar structures to those observed with the ecmA/O:cudA construct (Fig. 11C and Table 1) but with the ecmA:cudA fusion construct the terminal structures comprise a very slender slug-like structure atop a stalk (Fig. 11D). Microscopic examination shows that these latter cells are indeed vacuolated cells, with the typical appearance of stalk cells, inside a stalk tube.

Finally we assessed the slugging behaviour of all four classes of transformant (Table 1). Cells expressing cudA from the pspA promoter show the same, slugger behaviour as cudA− cells. However, strains with the ecmA/O:cudA, ecmO:cudA and ecmO:cudA constructs all migrate on agar for the same approximate time as the parental strain, i.e. the cudA− slugger phenotype is reverted.

DISCUSSION

The sensing system that normally allows a slug to monitor environmental conditions is severely impaired in the cudA− mutant. The mutant slugs migrate on nitro-cellulose filters, a condition that prevents migration in the parental strain, and when kept in darkness on an agar surface they never culminate. In addition to its strong slugger phenotype, the cudA− strain displays a number of other defects.

Upon exposure to overhead light, cudA− slugs make an abortive attempt at culmination but then migrate again for many hours. Later in migration large numbers of stalk cells are left in the slime trail behind cudA− slugs. In wild-type strains such discarded cells are believed to derive from prestalk cells that prematurely initiated terminal stalk cell differentiation, became lost from the back of the slug and then differentiated into stalk cells (Sternfeld, 1992). The large numbers of stalk cells in the trail behind cudA− slugs suggests that premature terminal differentiation of prestage stalk cells occurs at an elevated rate. This incipient ‘stalkiness’ is consistent with several other features of the cudA− mutant. While there are no mature stalk cells and no stalk tube within the terminally differentiated cudA− structures a large proportion of cells within such structures express STl:Z and staB:Z: markers of stalk cell differentiation.

The cudA− mutant is also defective in prespore gene expression and entirely fails to make spore cells. It seems likely that the defect in spore maturation is a direct result of this failure to undergo normal prespore differentiation: i.e. it is a ‘dependent series effect’. Consistent with this notion, cudA− cells do not express spiA or produce spores when treated with 8 bromo cAMP (M. Fukuzawa and J. G. Williams unpublished results): a membrane permeant cAMP analogue that induces spore cell differentiation in wild-type cells. (Kay, 1989). The defect in prespore differentiation might also explain the pronounced tendency towards the stalk cell pathway of differentiation in the cudA− structures. If prespore to spore differentiation is blocked then the prestage to stalk cell pathway might be expected to act as the default pathway. It is therefore very important to understand how the CUDA protein functions in prespore-specific gene expression. In this respect, the behaviour of different prespore-specific genes in the cudA− mutant provides a clue.

Studies using a dominant inhibitor of PKA expressed under

<table>
<thead>
<tr>
<th>Table 1. Phenotypes of cudA-complemented strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promoter</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>pspA (prespore)</td>
</tr>
<tr>
<td>ecmA/O (prestalk)</td>
</tr>
<tr>
<td>ecmO (prestalk O)</td>
</tr>
<tr>
<td>ecmA (tip)</td>
</tr>
</tbody>
</table>

The symbol ++ shows that there was a level of complementation such that development was equivalent to that of the parental cells and a dash indicates that there was no complementation. The small numbers of spores formed in ecmA/O:cudA and ecmO:cudA transformants (+/−) may be due to transdifferentiation of cudA-expressing prestalk cells into prespore cells.
the control of a prespore-specific promoter, have shown that PKA is required for the transcription of three genes that encode spore coat proteins, cotA, cotB and cotC (Hopper et al., 1995). This is in marked contrast to the behaviour of two other prespore-specific genes, Dp87 and pspA (Hopper and Williams, 1994). Transcription of the Dp87 gene is only slightly reduced in the presence of the dominant inhibitor and transcription of the pspA gene is marginally enhanced. These observations suggest that different signal transduction pathways are utilised by different classes of prespore-specific genes. This conclusion is supported by analysis of the cudA-mutant, which shows similar extents of repression of the three classes of prespore-specific genes to that observed using the dominant inhibitor construct: i.e. cotC > Dp87 and pspA. It also suggests that the CUDA protein may lie in the PKA signalling pathway that directs spore coat protein gene expression.

The cudA gene is expressed in prespore cells and this is consistent with a role in the transcription of prespore genes. However, the gene shows an unusually complex pattern of gene expression that suggests that it might have additional functions. The cudA mRNA and protein are present within pstA cells but are not detectable within the pstO cells. The glycogen phosphorylase 2 gene is expressed in both prestalk and prespore cells and its promoter is responsive to DIF, the inducer of prestalk differentiation (Town et al., 1976; Morris et al., 1987; Williams et al., 1987) and to cAMP, the inducer of prespore differentiation (Yin et al., 1994). However, there is to our knowledge, no precedent for a promoter that is active within only one of the two sub-types of ecmA-expressing prestalk cells and in the prespore cells. Later, at culmination the cudA gene is expressed in the pstO cells. This occurs both in the subset of pstO cells that differentiate into upper cup cells, i.e. those that express the ecmB gene prior to entering the stalk tube, and in the sub-set that express the ecmB gene only after moving into the stalk tube.

The fact that the CUDA protein is located within the nucleoplasm in both pstA and prespore cells suggests that it functions as a transcriptional regulator in both cell types. There is, however, no recognisable DNA binding domain. If, therefore, it is a transcriptional regulator it must contain a novel form of DNA binding domain or it must function by interacting with other transcription factors. The notion that CUDA may be a transcriptional regulator is consistent with the very low level of expression of the cotC gene in cudA- slugs but we have no direct evidence that it acts as a transcriptional regulator in prestalk cells. The ecmA: lacZ, ecmO: lacZ, ecmB: lacZ and ST: lacZ constructs are all expressed. There could be subtle differences in the level of expression that, because of variation in copy number of the fusion genes would be difficult to estimate (to minimise this problem we use pooled populations derived from large numbers of transformant clones), but there is definitely not the major reduction observed for cotC.

**Fig. 7.** Analysis of cellular differentiation within cudA- structures. (A) High power (×40) view of cells within the slime trail behind a cudA- slug. The cells show the vacuolated appearance typical of mature stalk cells. (B-D) Structures derived from cells transformed with the stalk marker ST-lacZ: (B) slugs, (C) a terminal structure, (D) the anterior part of a terminal structure. (E) A terminal structure stained with calcofluor, a cellulose-specific reagent. (F) A terminal structure derived from cudA- cells transformed with staB-lacZ, a late stalk marker.

**Fig. 8.** Analyses of prespore gene expression in cudA- slugs. (A) Total RNA was prepared from migrating slugs derived from cudA- cells and control, DH1 cells, and expressions of three prespore-specific genes (psA, Dp87 and cotC) was analysed by northern transfer. The filters were reprobed using a probe for IG7, a gene which is expressed at a constant level during development (Jermyn et al., 1987). (B) Expression of pspA: lacZ in a cudA- slug.
Although we are unable to detect a defect in their gene expression the prestalk cells are clearly abnormal in that they cannot form a stalk tube when developing alone, they cannot enter the stalk tube when synergised with wild-type cells and they cannot differentiate into mature stalk cells unless they are shed from the aggregate into the slime trail. Further evidence for a defect in the prestalk cells of the cudA mutant is revealed when the CUDA protein is expressed under the control of different cell type-specific promoter fragments.

When CUDA is expressed under the control of a prespore-specific promoter there is very little effect. The transformants display a strong slugger phenotype and form terminal structures that closely resemble the parental, cudA−, mutant. The failure to form spores even though the defect in prespore cells is, presumably, corrected is consistent with previous studies where a defect in prestalk to stalk differentiation prevented spore maturation (Harwood et al., 1992; Shaulsky and Loomis, 1993). It may reflect the existence of a signal that passes from the prestalk cells to the prespore cells and induces their differentiation.

When the CUDA protein is expressed under the control of the ecmA/O prestalk-specific promoter, the slugger behaviour is corrected and outwardly normal looking fruit are formed but spore maturation does not occur. The same result is observed when the CUDA protein is expressed under the control of the ecmO sub-fragment of the promoter and this presumably reflects the fact that there is a continuous process of transdifferentiation of pstO cells into pstA cells during slug migration (Abe et al., 1994), so that, with increasing time of slug migration, expression of an ecmO:cudA construct becomes functionally equivalent to expression of an ecmA/O:cudA construct.

Expression of cudA under the ecmA sub-fragment of the ecmA promoter corrects the slugger behaviour but the terminal structures are quite different from those observed with the ecmA/O or ecmO fragments. EcmA/O:cudA transformants form a column, predominantly composed of stalk cells, or mature stalk, with no sorus-like structure of the kind found with the ecmA/O:cudA and ecmO:cudA transformants. It would appear therefore that expression of cudA in the pstO cells is an absolute requirement if a normally shaped and proportioned culminant is to be formed. However, in slugging assays, the ecmA:cudA cells behave like wild-type slugs.

The fact that the slugger behaviour is corrected when
expression is confined only to pstA cells accords well with results from grafting experiments, between a slugger mutant and a normal strain, which showed that the tip region dictates slugging behaviour (Smith and Williams, 1980). However, our result extends the previous observation because grafting experiments can give only an estimate of the physical location of the tip. The demonstration that the defect in entry into culmination is corrected when cuda is expressed in pstA cells indicates that the functionally defined tip is composed of cells that utilise the ecma-specific part of the ecma/O promoter, i.e. that they are likely to be pstA cells or some, as yet undetected, sub-set of the pstA cells.

This work was supported by BBSRC grant CAD05617.

REFERENCES


(Accepted 24 April 1997)