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TagA, a putative serine protease/ABC transporter of *Dictyostelium* that is required for cell fate determination at the onset of development

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SUMMARY

The *tag* genes of *Dictyostelium* are predicted to encode multi-domain proteins consisting of serine protease and ATP-binding cassette transporter domains. We have identified a novel *tag* gene, *tagA*, which is involved in cell type differentiation. The *tagA* mRNA accumulates during the first four hours of development, whereas TagA protein accumulates between two and ten hours of development and decreases thereafter. Wild-type cells express *tagA* in prespore cells and mature spores, defining *tagA* expression as prespore specific. However, *tagA* mutant cells that activate the *tagA* promoter do not sporulate, but instead form part of the outer basal disc and lower cup of the fruiting body. *tagA* mutant aggregates elaborate multiple prestalk cell regions during development and produce spores asynchronously and with low viability. *tagA* mutants produce about twice as many prestalk cells as the wild type as judged by a prestalk cell reporter construct. When mixed with wild-type cells, *tagA*$^{-}$ cells become overrepresented in the prestalk cell population, suggesting that this phenotype is cell-autonomous. These results suggest that TagA is required for the specification of an initial population of prespore cells in which *tagA* is expressed. Expression profiling uncovered a delay in the transcriptional program between 2 and 6 hours, coincident with TagA expression, revealing an early function for TagA. TagA also appears to play a general role in cell fate determination since *tagA* mutants express a spore coat protein gene (*cotB*) within vacuolated cells that form part of the stalk and they express a prestalk/stalk-specific gene (*ecmB*) within cells that become spores. The expression of TagA at two hours of development, the observed coincident delay in the transcriptional program and the subsequent mis-expression of cell-type specific genes provide evidence for cell fate determination beginning in some cells much earlier than previously believed.

Key words: differentiation, starvation, cell cycle, cell fate, *Dictyostelium*

INTRODUCTION

Peptide signals play a wide variety of roles in cell differentiation and multicellular development. Often these signals act at a distance from where they are produced and require membrane transporters either for export from the producing cells or for import into the responding cells. For example, the metazoan signaling peptide Hedgehog, which is involved in specifying axis formation, is exported from cells by the RND transporter Disheveled (Ma et al., 2002). The ATP binding cassette (ABC) superfamily of transporters comprises at least seven families whose members transport diverse substrates across different types of cellular membranes (Ambudkar and Gottesman, 1998). The ABCB family members are best known for conferring multiple drug resistance, but members of some ABCB subfamilies are capable of transporting peptides that lack classical export signals (Detmers et al., 2001). Homologs of these subfamily members such as the TAP proteins, as well as other ABC transporters that transport peptides, have been found in micro-organisms, plants and metazoans. CSF is a five amino acid peptide, found in *Bacillus subtilis*, which is derived from the C-terminal end of the 40 amino acid peptide encoded by the *phrC* gene (Lindsey et al., 2002; Stacey et al., 2002). The CSF peptide appears to regulate both competence and sporulation, since high levels of CSF inhibit competence and promote sporulation. The ABC transporter Spo0K is required to transport CSF into the cell from the extracellular environment (Solomon et al., 1996). The ABC transporter Ste6 transports a-factor, one of two peptides that determine mating compatibility in *Saccharomyces cerevisiae* (Kuchler and Thorner, 1992). In plants the developmental role of peptide signaling has been relatively well characterized (reviewed by Lindsey et al., 2002; Stacey et al., 2002). CLV3, a 96 amino acid peptide in *Arabidopsis* is involved in proper sizing of shoot apical meristems which form new leaves, and ENOD40-

encoded peptides of the legume family promote formation of nodules for colonization by nitrogen fixing bacteria. Loss of the *Arabidopsis* ABC transporter, AtMRP5 results in a shift from growth to development in the root. Mammalian ABC transporters have been shown to be capable of transporting hydrophobic peptides, although the biological relevance of this observation remains unclear (Sharom et al., 1998).

In *Dictyostelium discoideum*, unique members of the ABCB transporter subfamily, TagB and TagC, appear responsible for peptide signal export during development. These Tag proteins are required for cell differentiation in *Dictyostelium* and have the potential to carry out the processing and transport of peptide signals since they possess an N-terminal serine protease domain and a C-terminal transporter domain. The available genetic data suggest that the function of the Tag proteins is to transport signaling peptides that regulate the timing and nature of cell differentiation. During the development of *Dictyostelium*, equipotent cells differentiate into prespore cells and distinct populations of prestalk cells that later form the various tissues in the terminally differentiated fruiting body that consists of mature spores held atop a cellular stalk (Kessin, 2001). The TagB protein is required for cell-cell signaling that promotes spore encapsulation, and it is required for the differentiation of prestalk A (PstA) cells in a cell-autonomous fashion (Shaulsky et al., 1995). TagC is structurally similar to TagB and the phenotypic similarities between *tagC* null mutants and *tagB* mutants are so striking that it is presumed that TagB and TagC function as a heterodimer. The conserved amino acid residues known to be required for serine protease catalytic activity and for ABC transporter ATPase activity are required for TagB function (G.S., unpublished). In addition, TagC-null mutants fail to release the spore encapsulation-inducing peptide SDF-2 (Anjard et al., 1998). Thus, it is likely that Tag proteins work by the proteolytic processing and transport of signaling peptides, in a manner analogous to that in which STE6 transports the proteolytically processed, lipid-modified a-factor pheromone peptide of *S. cerevisiae* (Taglicht and Michaelis, 1998). Such a cell-cell signaling function is the obvious common feature of all of the transporters characterized as being involved in developmental signaling. A curious feature of the *tagB* mutant phenotype is the cell-autonomous defect in prestalk cell differentiation experienced by cells that would normally express the *tagB* gene in wild-type cell development (Shaulsky and Loomis, 1996). This novel feature suggests that the failure of a transporter to export a substrate/signal can affect the signal-producing cell as well.

Developing *Dictyostelium* aggregates contain several prestalk cell sub-populations that can be distinguished from the prespore cells and can be tracked with cell-type-specific reporter genes (Williams, 1997). Near the end of development, the PstAB cells initiate fruiting body formation by plunging through the prespore mass, forming a cellulosic stalk tube until they make contact with the substratum. The PstA cells enter the stalk tube following the PstAB cells and differentiate into stalk cells. As the prespore cells crawl up the elongating stalk they differentiate into mature spores. Terminal spore differentiation proceeds in a wave from top to bottom of the nascent sorus. This observation is consistent with a number of experiments that suggest a signal emanating from the PstA cells coordinates terminal spore differentiation with fruiting body morphogenesis (Harwood et al., 1993; Richardson et al., 1994; Shaulsky et al., 1995). The TagB and TagC transporters are expressed in PstA cells and so they are in the position to affect spore differentiation during this process (Shaulsky and Loomis, 1996) (G.S., unpublished data). The most plausible model is that TagC exports the encapsulation-inducing SDF-2 peptide, initiating the observed wave of spore differentiation as the PstA cells enter the stalk tube at the top of the prespore mass (Anjard et al., 1998). However, there is also a cell-autonomous requirement for both TagB and TagC in the formation of PstA-derived stalk cells. Cells that do not express a TagB or a TagC transporter cannot become stalk cells (Shaulsky et al., 1995). This aspect of Tag transporter function is not understood, but could be explained if the transport substrate acted as an inhibitor of PstA cell differentiation when retained inside the cell. Given an ABC transporter's capacity to maintain a concentration differential of a small molecule across a membrane, ABC transporter activity could alter cellular physiology in a way that would promote or inhibit a particular differentiation program. In theory, the flux of a signal through a single transporter could control the fates of the signal-producing cells as well as the responding cells. There is some tentative evidence for cell-autonomous functions of ABC transporters in cell differentiation. In *Dictyostelium*, the prespore-specific rhodamine transporter RhT may be required for the production or maintenance of prespore cells (Good and Kuspa, 2000). In mammalian systems, the ABC transporter encoded by the ABCB1 (*MDR1*) gene has been implicated in the maintenance of hematopoietic stem cells and in the regulation of programmed cell death (Smyth et al., 1998; Johnstone et al., 1999; Johnstone et al., 2000a; Johnstone et al., 2000b). Recently, the *ABCG2* gene has also been suggested to have a role in the maintenance of the undifferentiated state of stem cells (Zhou et al., 2001).

We have identified a novel member of the *tag* gene family, *tagA*. TagA mRNA is expressed in the first 2 hours following starvation, it becomes specific to prespore cells and is eventually expressed in mature spores. Inactivation of the *tagA* gene results in developing structures with enlarged or supernumerary prestalk regions with roughly twice the normal number of prestalk cells. Within these structures, the *tagA*$^{-}$ cells that activate the *tagA* promoter do not become spores but instead adopt a prestalk cell fate. Our results imply that the earliest cells to differentiate require TagA-mediated function to prevent their adoption of a prestalk cell fate, or for promoting their prespore cell fate.

MATERIALS AND METHODS

Cloning the *tagA* gene

Standard DNA and RNA protocols were performed as described previously (Sambrook et al., 1989). Polymerase chain reaction (PCR) amplification was carried out using primer TBR-5.1 encoding the amino acids VGPSGSG (5′-AAC TGC AGG THG GWC CWT CWG GWA GYG G; where W=A or T, R=A or G, Y=C or T and H=A or C or T) and primer TBR-3.1 encoding GGGLL(S/R)IA (5′-CCG GAT CCG CRA TWC TYT TYT TTT GWC CWC C). These sequences represent the conserved amino acid sequences within the nucleotide-binding domains (NBD) of *Dictyostelium* TagB and human P-glycoprotein (ABCB1). PCR amplification with wild-type genomic DNA was as follows: 5 cycles of 94°C (30 seconds), 37°C (30

seconds), 72°C (30 seconds), all with 1 degree/second ramping, followed by 25-30 cycles of 94°C (30 seconds), 55°C (30 seconds) and 72°C (30 seconds). Products were cloned into *Bam*HI-*Pst*I-linearized pGEM3 (Promega). One of the clones represented the NBD of *tagA*. Multiple cDNA fragments representing the *tagA*-coding sequence were isolated from a λ-ZAP (Strategene) cDNA library using the PCR product as a probe (Souza et al., 1998). The library was subsequently screened with probes derived from the identified cDNAs. All positive clones were converted to plasmids as described and their inserts were cloned into pGEM3 (Bai and Elledge, 1997). All cDNAs were confirmed to represent the *tagA* gene by sequencing and by mapping them using Southern analyses. The genomic *tagA* locus was isolated by screening size-selected fragments of the genome into pBluescript II KS(+/–). These fragments were confirmed to be *tagA* by sequencing and Southern analyses. Raw sequence data from the *Dictyostelium* genome sequencing project (http://dictygenome.bcm.tmc.edu/) were used to confirm the *tagA* contig and to design PCR primers that confirmed clone overlaps and allowed reconstruction of a complete *tagA* locus of 7.1 kb, including the 5.1 kb *tagA* coding sequence and 2 kb of upstream sequence (GenBank accession number AF263455).

Strain construction, cell growth and development

The *Dictyostelium* strains used in this study are described in Table 1. Ax4 cells were grown in HL-5 liquid medium (Sussman, 1987) supplemented with streptomycin (50 μg/ml) and penicillin (50 U/ml). Neomycin-resistant strains (neo^r) Ax4[*cotB/GFP*], Ax4[*ecmA/GFP*], Ax4[*act15/GFP*] and all respective derivatives, were grown in HL-5 liquid medium supplemented with 20 μg/ml G418 (Geneticin, Gibco). All strains were removed from drug-containing medium 36 hours prior to assay. Cells were plated for synchronous development on nitrocellulose filters as described previously (Sussman, 1987).

Transformation of *Dictyostelium* cells was performed according to the method of Manstein and Hunt (Manstein and Hunt, 1995) using a BTX 600 electroporation device, or by calcium-phosphate precipitation and glycerol shock (Nellen and Firtel, 1985). Insertional inactivation of the *tagA* gene was achieved by homologous recombination at the genomic locus after electroporation of linearized DNA fragments. The knockout vector was created by inserting a 1.5-kb fragment containing the blasticidin S resistance (*bsr*) gene, under the control of the actin 15 (*act15*) promoter and the actin 8 terminator (Adachi et al., 1994) into the *Cla*I site within the portion of the *tagA* cDNA predicted to encode the nucleotide-binding domain. The *bsr* cassette was excised from pBSRΔ*Bgl*Δ*Eco* with *Nar*I and *Acc*I and inserted into the *Cla*I site of *tagA* cDNA, destroying the *Acc*I, *Nar*I and *Cla*I restriction sites. Integration at the native locus was determined by Southern analyses.

An expression plasmid with the *tagA* coding region, under the control of its native promoter was constructed by substituting a 7.1 kb *Xba*I/*Hin*dIII *tagA* fragment, described above, for the *lacZ/act8* cassette in pDdGal16(H+) (Harwood and Drury, 1990). The resulting plasmid, p*tagA/tagA*, was transformed into *tagA*$^-$ cells by calcium-phosphate precipitation (Nellen and Firtel, 1985). The p*tagA*/*lacZ* expression plasmid was made with genomic DNA 2 kb upstream of the *tagA* coding region including the 5′ end of the coding sequence up to the first *Bam*HI site. This 2.2 kb *Eco*RI-*Bam*HI fragment was inserted into pDdGal16(H+) between its *Eco*RI and *Bgl*II sites. Staining of developmental structures and spores for β-galactosidase was carried out as described previously (Shaulsky and Loomis, 1993).

Spore and stalk cell assays

Sporulation was measured by harvesting cells from filters into 20 mM potassium phosphate buffer, pH 6.2, and treating them with 0.4% non-ionic detergent NP-40 for 10 minutes at 22°C. Cells were then washed with potassium phosphate buffer twice, and disaggregated by trituration with an 18-gauge needle. Refractile spores were counted by phase-contrast microscopy and plated on SM agar plates with bacteria. The number of colonies was used as an estimate of the number of viable spores in each sample. At least three independent determinations were carried out for each strain and are reported as the mean±s.e.m.

Table 1. *Dictyostelium* strains used in this study

Strain	Direct parent	Drug markers	Reference
Ax4*	Ax3	–	Knecht et al., 1986
Ax4 (*ecmA/GFP*)	Ax4	neo^{r†}	Good and Kuspa, 2000
Ax4 (*cotB/GFP*)	Ax4	neo^r	Good and Kuspa, 2000
Ax4 (*act15/GFP*)	Ax4	neo^r	This work
tagA$^-$	Ax4	bs^{r†}	This work
tagA$^-$ (*ecmA/GFP*)	Ax4 (*ecmA/GFP*)	neo^r, bs^r	This work
tagA$^-$ (*cotB/GFP*)	Ax4 (*cotB/GFP*)	neo^r, bs^r	This work
tagA$^-$ (*act15/GFP*)	Ax4 (*act15/GFP*)	neo^r, bs^r	This work
Ax2 (*ecmO/lacZ*)	Ax2	neo^r	Early et al., 1993
tagA$^-$ (*ecmO/lacZ*)	Ax4 (*ecmO/lacZ*)	neo^r, bs^r	This work
Ax4 (*tagA/tagA*)	–	neo^r	This work
tagA$^-$ (*tagA/tagA*)	*tagA*$^-$	neo^r, bs^r	This work
Ax4 (*tagA/lacZ*)	Ax4	neo^r	This work
tagA$^-$ (*tagA/lacZ*)	*tagA*$^-$	neo^r, bs^r	This work
Ax4 (*cotB/lacZ*)	Ax4	neo^r	Fosnaugh and Loomis, 1993
tagA$^-$ (*cotB/lacZ*)	Ax4 (*cotB/lacZ*)	neo^r, bs^r	This work
Ax4 (*ecmB/lacZ*)	Ax4	neo^r	This work
tagA$^-$ (*ecmB/lacZ*)	Ax4 (*ecmB/lacZ*)	neo^r, bs^r	This work

*Ax4 is used as the 'wild-type' laboratory strain. Ax4 is an axenic derivative of NC4 derived from Ax3 (Knecht et al., 1986).
†neo^r, neomycin resistance; bs^r, blasticidin S resistance.

Induction of prestalk gene expression was measured in submerged culture (Harwood et al., 1995) as modified previously (Wang and Kuspa, 2002). Vegetative cells were harvested at a density of 1-2×10^6/ml, washed once in 20 mM sodium phosphate buffer (pH 6.4) and three times in stalk buffer [10 mM Mes, 2 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$, 50 μg/ml streptomycin, 50 U/ml penicillin (pH 6.2)]. Cells were plated at 2.5×10^4 cells/cm^2 in stalk buffer supplemented with 5 mM cAMP. After 24 hours, cell cultures were washed free of cAMP with stalk buffer and the original buffer volume was replaced with stalk buffer with or without supplements. Cells were assayed for prestalk gene expression 24 hours later by fluorescence microscopy, via an *ecmA/GFP* reporter construct and scored for the production of stalk-like cells using phase-contrast microscopy. Cellulose deposition by the stalk-like cells was confirmed by staining with calcafluor (Harrington and Raper, 1968).

Protein and RNA expression assays

Antibodies against TagA protein were raised at Bethyl Laboratories, Inc. (Montgomery, Texas). The peptide LPSNSRNTRNADKLRN-RSET, representing amino acids 1627-1646 of the predicted TagA protein, was synthesized and conjugated to keyhole limpet hemocyanin via a cysteine residue added at the amino terminal end of the peptide. The conjugated peptide was used to immunize rabbits and the resulting polyclonal antibodies were affinity purified on a column conjugated with the peptide antigen.

Cells were harvested at various times during development and resuspended in 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 1 mM PMSF. Protein concentrations were determined with the BioRad protein determination kit (BioRad Laboratories, Richmond CA) and equal amounts of protein were resolved on 6% polyacrylamide gels. Protein was electrotransferred to a nitrocellulose membrane and detected with the affinity-purified anti-peptide antibody described above, followed by goat anti-rabbit antibody and visualized with the ECL kit according to the manufacturer's protocols (Amersham Life Sciences).

RNA was isolated from the wild type and the transformed strains

during vegetative growth and development. Spores and stalks were purified as described by Van Driessche et al. (Van Driessche et al., 2002). Spores and stalks purified by this procedure were estimated to be >99% pure by direct microscopic observation. Cells were harvested and suspended in TRIzol Reagent (Gibco BRL). RNA extraction was performed according the manufacturer's protocol. RNase protection assays were performed using a RPA III kit (Ambion) according to the manufacturer's protocol with probes synthesized from various fragments of *tagA* cDNA, or genomic DNA, templates using a riboprobe in vitro transcription system (Promega), as described in the text.

Expression profiling with microarrays

Expression profiling was carried out with DNA microarrays as described previously (Van Driessche et al., 2002). Cells (10^8) developing on two filters were harvested and processed to produce total RNA for each time point. The raw data from the microarray hybridizations were processed according to the procedure described in Van Driessche et al. (Van Driessche et al., 2002). Briefly, raw image files were quantified and the resulting data was subjected to a single array normalization procedure to remove spatial and intensity artifacts and to put the data on a common measurement scale (Yang et al., 2002). Replicate arrays from the same biological preparation were averaged, and the averages for two biological preparations were then averaged to yield the final data set. We compared the time patterns of gene expression in the *tagA* mutant cells and our previous wild-type data (Van Driessche et al., 2002). The 2,021 genes whose expression levels were altered dramatically during development were determined and ordered in the 'blue-yellow' plots as described previously (Van Driessche et al., 2002). Each gene's studentized score was compared against the linear contrast function y=x/12–1 to evaluate how strongly and consistently the gene appeared to change in relative expression across developmental time. The same gene order is used in all plots shown.

To compare microarray data for all genes from each time point of *tagA* mutant development with each time point of wild-type development we determined the time point for the wild type at which the expression pattern was most similar to that found in the *tagA*$^-$ mutant by using the Pearson correlation distance to make comparison. To measure the distance we used data from all of the genes that were not excluded from the analysis for quality control reasons (~6,000 genes).

RESULTS

Characterization of the *tagA* gene

Genomic sequence analysis has identified 68 ABC transporter genes from *Dictyostelium* (Anjard et al., 2002). We carried out a PCR screen of the *Dictyostelium* genome designed to identify genes of the ABCB transporter subfamily. A fragment of the *tagA* gene was among the PCR products that were cloned and this was used to screen cDNA and genomic DNA libraries to isolate a full-length copy of the gene. The predicted amino acid sequence of the 5.1-kb *tagA* coding region indicates that it is a member of the *tag* gene class of the ABCB subfamily (Fig. 1). This class includes the previously characterized *tagB*, *tagC* and *tagD* genes of *Dictyostelium* that are predicted to encode serine protease/ABC transporter proteins (Shaulsky et al., 1995; Anjard, et al., 2002). While the overall gene structure suggests it is a *tag* gene, the predicted amino acid sequence of the transporter domain is more similar to mammalian ABCB subfamily transporters than it is to the other *tag* transporters (Fig. 1D) (Anjard et al., 2002). In the most conserved region of the transporter domain, the NBD, the deduced amino acid sequence of TagA is 53% identical to that of human ABCB1 (MDR1 or P-glycoprotein) and 32% identical across the entire transporter domain.

To determine the timing of *tagA* expression during development an RNase protection assay was used to determine mRNA levels since we were unable to detect *tagA* mRNA on northern blots. A fragment of the *tagA* cDNA (PR 1 or PR 2, Fig. 1A) was used to protect *tagA* mRNA from digestion by RNase. This showed that the *tagA* gene is expressed at low levels in vegetative cells and accumulates to its highest levels in the first 2 hours of development (Fig. 2A, upper panel). Anti-peptide antibodies were raised to a predicted intracellular epitope within TagA and affinity purified using the immunizing peptide. Western blots stained with these antibodies detected a protein of an apparent molecular mass of 190 kDa, the size predicted for TagA (Fig. 2B). This protein was not detected in the *tagA* disruption mutant, but was detected in the same mutant transformed with a *tagA* expression plasmid (see below) indicating that the antibodies detect TagA protein. TagA accumulated to its maximum level between 6 and 10 hours and then persisted at lower levels to the end of development (Fig. 2B).

Inactivation of tagA alters morphogenesis and cell-type proportions

The *tagA* gene was inactivated in wild-type (Ax4) cells by inserting a selectable gene into the *Cla*I restriction site within the predicted NBD of the ABC transporter domain (Fig. 1A). The resulting mutant produced no detectable *tagA* mRNA by our RNase protection analysis and no detectable TagA protein (Fig. 2). When starved on nitrocellulose filters, the developmental morphology of *tagA*$^-$ cells appeared relatively normal until 12 hours after the onset of development. At 12 hours, the structures produced by the Ax4 strain were all hemispherical mounds, whereas the *tagA*$^-$ cell mounds had already formed a tip of prestalk cells. At 14 hours, when the Ax4 cells had formed tipped mounds, *tagA*$^-$ cells formed elongated finger-like structures with spiral-shaped tips or mounds with supernumerary tips (Fig. 3A). These structures went on to form fruiting bodies in an asynchronous fashion between 24 and 36 hours while Ax4 cells completed development by 26 hours (not shown).

The developing structures of *tagA* mutants suggested aberrant prestalk cell differentiation. To visualize the major cell types and examine possible defects in proportioning or morphogenesis, the *tagA* mutation was reproduced in strains expressing green fluorescent protein (GFP) under the control of either the prestalk-specific *ecmA* promoter or the prespore-specific *cotB* promoter. Slugs formed by the *tagA*$^-$[*ecmA/GFP*] mutants displayed an extended prestalk zone and a general increase in fluorescence resulting from additional GFP-positive cells in the prespore zone, consistent with increased numbers of *ecmA*-positive prestalk cells (Fig. 3B). The most dramatic difference between wild-type and *tagA* mutant fruiting bodies is in the lower cup region of the spore head. In wild-type cells, very little expression of the *ecmA* reporter construct is seen in the lower cup, presumably because of the lower number of *ecmA*-expressing PstB cells that contribute to this structure, whereas the *tagA* mutant fruiting bodies consistently displayed more GFP fluorescence in this region (Fig. 3C). The developmental morphology and the *ecmA/GFP* expression

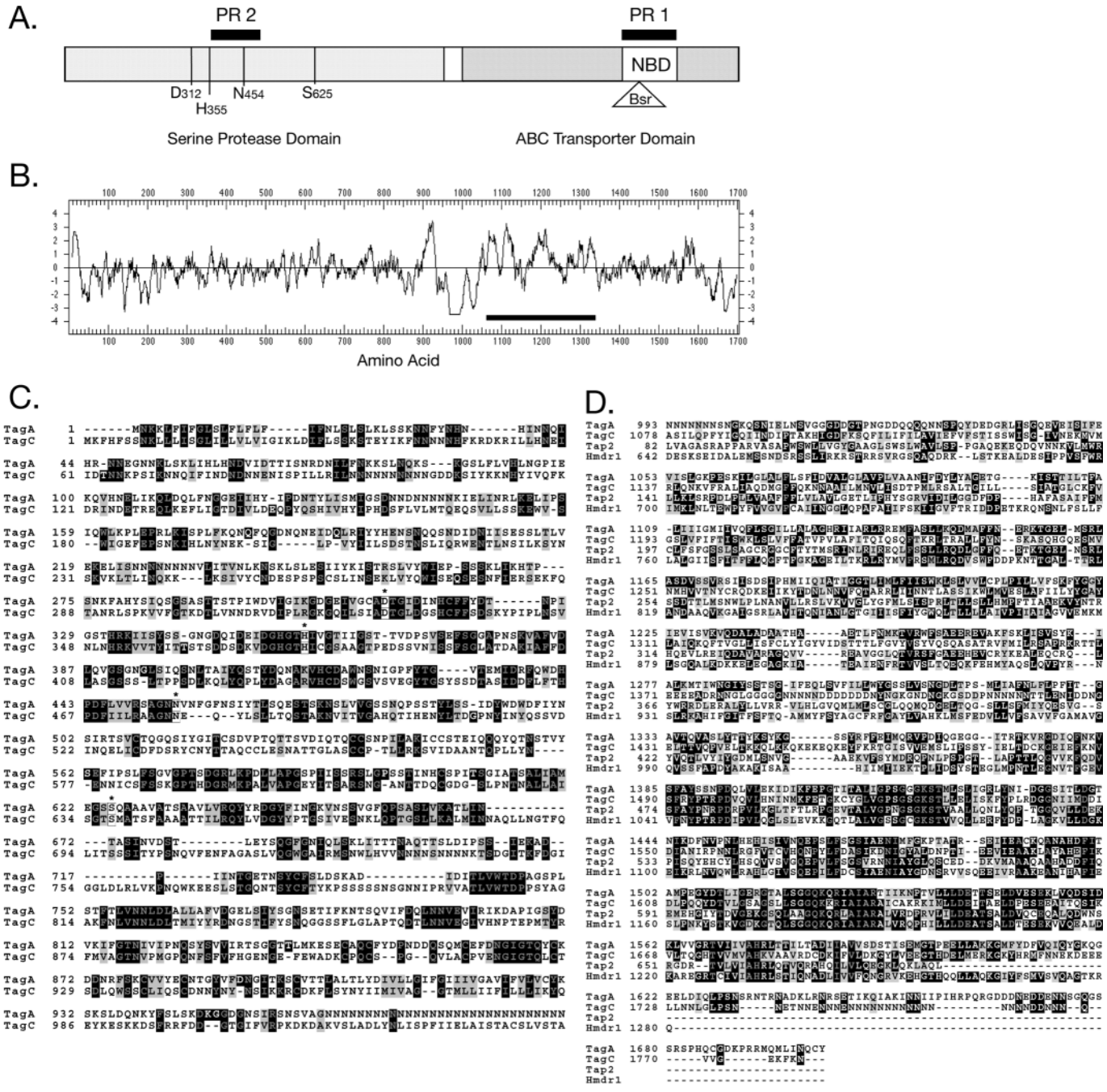

Fig. 1. The predicted domain structure of TagA**.** Similarity searches of the predicted amino acid sequence of the *tagA* gene suggests an amino-terminal serine protease domain and a carboxyl-terminal ABC transporter domain. A. Amino acid residues known to be required for protease activity are indicated by their single letter code and position. PR1 and PR2 represent regions of the gene that were used as probes for library screening and RNase protection assays. The position of a blasticidin resistance cassette (Bsr) insertion at the nucleotide binding site is also shown. B. Kyte-Doolittle hydropathy plot showing location of probable membrane-spanning regions within the ABC transporter domain (black bar). C. Sequence alignment of the deduced amino acid sequences for the predicted serine protease domains TagA and TagC. D. Sequence alignment of the deduced amino acid sequences for the predicted ABC transporter domains of *Dictyostelium* TagA, TagC, human ABCB.3 (Tap2) and human ABCB.1 (Hmdr1).

pattern suggested that the *tagA* mutant makes more prestalk cells than normal. We confirmed this by harvesting developing structures at various times, dissociating the cells and determining the percentage of *ecmA*-positive cells (Fig. 3D). This demonstrated that prestalk cell differentiation in the *tagA* mutant began at the same time as the wild type, but that the number of prestalk cells increased faster in the mutant and remained 2-3 times higher than wild-type throughout the second half of development. The expression pattern of the *cotB/GFP* reporter construct was found to be complementary,

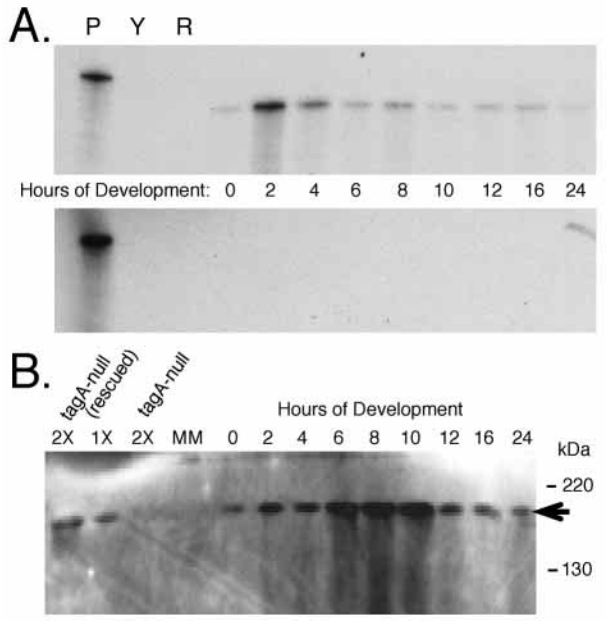

Fig. 2. Timing of TagA expression. **(**A) RNase protection assays were performed with total RNA collected from wild-type (upper panel) or *tagA* mutant (lower panel) cells. Lanes are: P, riboprobe without RNase treatment (1/10 the input for other lanes), Y, RNase digestion of probe incubated with yeast RNA, and (R) RNase digestion of the probe without RNA added, or (0-24 h) after hybridization to RNA samples collected across the 24 hours of development. (B) Western blot stained with a TagA antibody detects a protein of an apparent molecular mass of 190 kDa (arrow). Equal amounts of protein (10 μg) from vegetative (0 hours) or developing (2-24 hours) wild-type cells (Ax4) were loaded in each lane, along with molecular mass standards (MM). The *tagA* mutant and rescued mutant (*tagA*$^{-}$[*tagA/tagA*]) samples were mixtures of all vegetative and developmental time points. The amount of protein loaded in these lanes were equal (1×) or twofold (2×) the amounts of the developmental samples.

both spatially and proportionally, to that of *ecmA* (data not shown). 96% of all cells expressed one of these two constructs. We tested whether the excess *ecmA*-positive cells in the *tagA* mutant could be identified as PstO cells. Using wild-type cells expressing the *lacZ* reporter under the control of a PstO cell-specific promoter (Ax4[*ecmO/lacZ*]) and in the corresponding *tagA* mutant (*tagA*$^{-}$[*ecmO/lacZ*]) we observed no increase in the number of PstO cells in the *tagA* mutant (data not shown).

The propensity of *tagA* mutants to differentiate as *ecmA*-positive prestalk cells was examined in chimeras with wild-type cells to explore the possibility that this phenotype results from a perturbation of intercellular signaling. Chimeras were made with wild-type and *tagA* mutant cells at various ratios in which one of the strains was marked with the *ecmA/GFP* reporter to follow the prestalk population of each strain. Prestalk cells derived from the *tagA*$^{-}$ cells were consistently

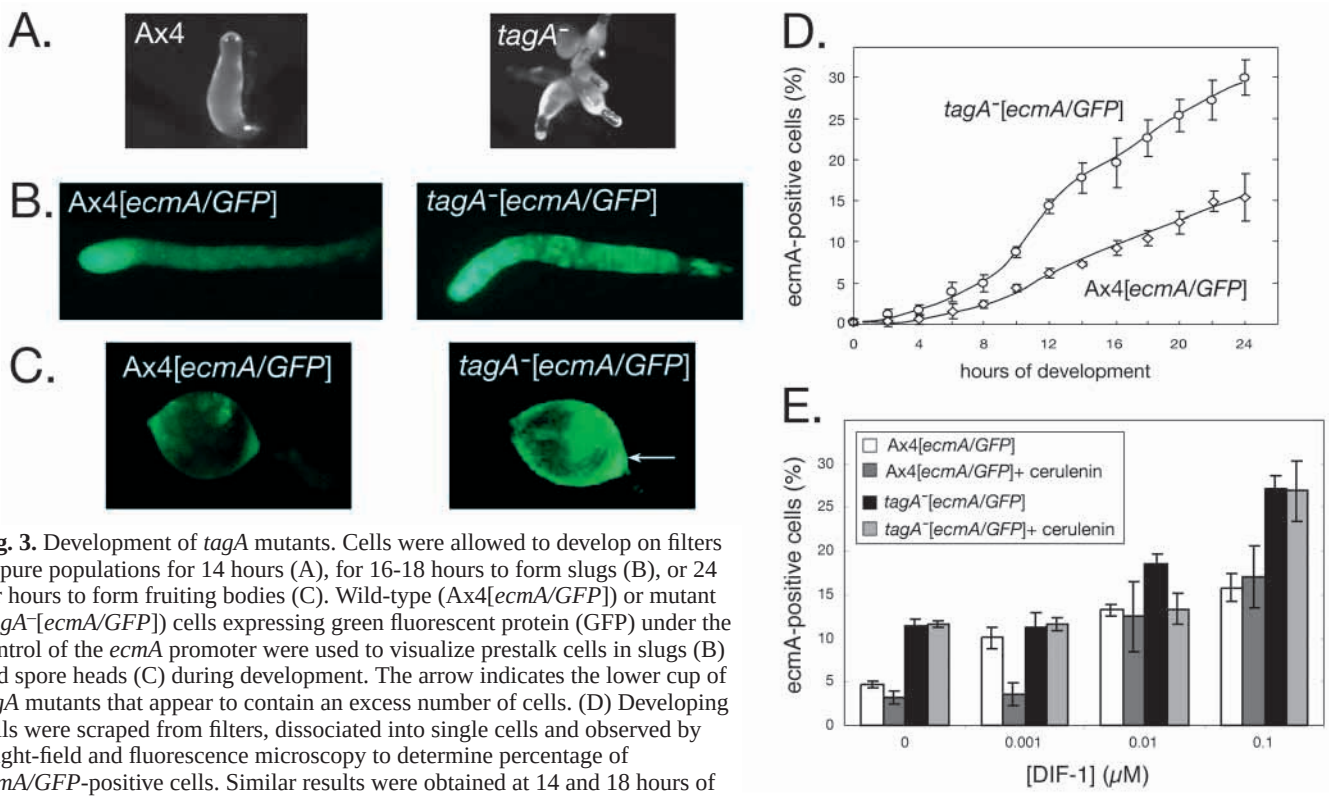

Fig. 3. Development of *tagA* mutants. Cells were allowed to develop on filters as pure populations for 14 hours (A), for 16-18 hours to form slugs (B), or 24 for hours to form fruiting bodies (C). Wild-type (Ax4[*ecmA/GFP*]) or mutant (*tagA*$^{-}$[*ecmA/GFP*]) cells expressing green fluorescent protein (GFP) under the control of the *ecmA* promoter were used to visualize prestalk cells in slugs (B) and spore heads (C) during development. The arrow indicates the lower cup of *tagA* mutants that appear to contain an excess number of cells. (D) Developing cells were scraped from filters, dissociated into single cells and observed by bright-field and fluorescence microscopy to determine percentage of *ecmA/GFP*-positive cells. Similar results were obtained at 14 and 18 hours of development whether an entire filter of cells was harvested for counting (D), or 10 individual developing structures were picked from filters, disrupted and counted. (E) Wild-type (Ax4) or *tagA*$^{-}$ cells were washed, plated at low density (1×10^4 cells/cm^2) in 24-well plates and incubated with 5 mM cAMP in stalk buffer for 24 hours. Cells were then washed free of cAMP and incubated with DIF or DIF + 5 μM cerulenin for another 24 hours and examined by fluorescence microscopy for the expression of prestalk-specific expression of GFP (*ecmA/GFP*). Three independent determinations were carried out for each condition and results are given as the mean±s.e.m.

Table 2. Propensity of *tagA*⁻ cells to differentiate as prestalk cells

Mixture*	Ratio	Total prestalk cells (%)†	Prestalk cells from marked strain (%)‡	% of prestalk cells that are marked§
1. Ax4+Ax4	1:1	15±1.2	6.9±0.92	46
	9:1	15±1.2	1.9±0.14	12
2. Ax4+*tagA*⁻	1:1	19±1.2	14±0.54	72
	9:1	15±1.4	2.8±0.36	18
3. *tagA*⁻+Ax4	1:1	20±1.5	7.0±0.60	35
	9:1	27±2.0	2.0±0.79	7.5
4. *tagA*⁻+*tagA*⁻	1:1	27±2.5	14±0.67	50
	9:1	27±2.5	3.1±0.30	11

*Data from two separate mixing experiments in which one experiment shows the total percentage of *ecmA*-positive prestalk cells (3rd column) and the other (4th column) shows the percentage of prestalk cells from a mixture in which only one of the two strains was marked (underlined in 1st column).

†The total prestalk cell population was determined by mixing the two strains where both strains express green fluorescent protein (GFP) under the promoter control of the prestalk specific gene *ecmA* (*ecmA/GFP*).

‡In these experiments, only the strain underlined in column 1 was marked with *ecmA/GFP* to determine the origin of the prestalk cells in the mixture.

§An estimate of the percentage of the prestalk cells that originated from the marked (underlined) strain in each mixture was obtained by dividing the percentage in the fourth column by the percentage in the third column. In control experiments 1 and 4, the expected percentage would be 50% and 10% for the 1:1 and 9:1 mixture ratios, respectively.

overrepresented in the prestalk cell population of the chimeras (Table 2). For instance, when only 10% of the cells were *tagA*⁻ 18% of the prestalk cells came from the mutant population. We also carried out an analogous set of experiments with the prespore reporter (*cotB/GFP*) and found that the *tagA* mutants were slightly underrepresented in the prespore population, as expected (data not shown). Finally, we demonstrated that *tagA* mutant cells participate in development of the chimeras by examining mixtures of control strains that expressed GFP under the control of the *actin15* promoter. Wild-type (Ax4[*act15/GFP*]) or mutant (*tagA*⁻[*act15/GFP*]) strains were mixed with unmarked cells to determine their representation within aggregates and both strains contributed at the expected percentage to the developing populations within each type of cell mixture described in Table 2 (data not shown). Since *tagA* mutant cells are able to enter aggregates as well as wild-type cells, these results suggest that the additional prestalk cells result from a cell-autonomous defect in cell differentiation.

The excessive prestalk cell differentiation in *tagA* mutants could result from a change in the way the mutant cells respond to signals in the mound, or by a cell-autonomous mechanism as suggested above. To explore this further, we examined the production of stalk cells by *tagA*⁻ cells at low cell density in submerged culture. In this assay, Ax4 cells have been shown to produce ~15% prestalk cells in response to DIF (Good and Kuspa, 2000). However, in our previous studies 4% of the cells consistently differentiated into prestalk cells (as defined by *ecmA* expression) without added DIF-1 and when most endogenous DIF-1 production was inhibited by addition of cerulenin (Kay, 1998; Good and Kuspa, 2000). As with development on filters, a higher percentage of *tagA*⁻ cells expressed *ecmA* in submerged culture compared to Ax4 cells (Fig. 3E). Without added DIF-1 nearly three times more *tagA*⁻ cells expressed *ecmA*. A similar percentage of *ecmA*-positive cells were formed when *tagA*⁻ cells were treated with cerulenin, suggesting that the endogenous DIF-1 synthesis is not required for this process (Fig. 3E). These results reinforce the idea that *tagA*⁻ cells have a cell-autonomous propensity to form excess prestalk cells.

PstB cells defined by the *ecmB* gene are scattered throughout the finger and slug structures prior to terminal differentiation (Williams, 1997). PstB cells at the bottom of the prespore region of the slug can contribute to the basal disk. In the absence of slug migration, a population of 'rearguard' cells will form the basal disc proper while the PstB cells will form the outer basal disc and the lower cup at the base of the sorus. Expression of the *ecmB* gene appears to be precocious and elevated in the *tagA* mutants (Fig. 4A). Additional *ecmB*-positive cells are also apparent in *tagA* mutant slugs but they do not appear to expand the volume of *ecmB*-positive tissues in the final fruiting body as we observed with *ecmA* expression (Fig. 4B).

Transcriptional profiling of tagA mutant cells during development

Information on the level of gene expression on a genomic scale can be used to measure the progression of cells through the developmental program and as a means of assessing the differences between wild-type and mutant cells (Hughes et al., 2000; Kim et al., 2001; Van Driessche et al., 2002). We compared the level of gene expression of 2,021 developmentally regulated genes during 24 hours of development in wild-type and in *tagA* mutant cells. The 2,021 genes that we focused on can be considered a 'universal phenotype' since they are not altered in their developmental regulation in different wild-type strains or in cells with different nutritional histories (Van Driessche et al., 2002). We need not be concerned whether the observed gene expression changes are a direct or indirect effect of the lack of TagA since we are only interested in deriving insights into TagA function by observing alterations in the transcriptional program. In Fig. 5A,B, the level of gene expression at each time point is presented relative to the mean level of each gene's expression throughout development in the respective strain. We found that the *tagA* mutant cells exhibited fairly normal regulation of gene expression. To further elucidate the differences between the strains, we compared the level of expression for each gene in the *tagA* samples with the mean level of that gene in the wild-type samples (Fig. 5C). This comparison revealed that the *tagA* mutant cells express the developmentally induced genes at a lower level than wild-type cells and they express the developmentally repressed genes at a higher level than the wild-type cells. In addition, the induction of developmental genes and the repression of growth phase genes were not as rapid in the mutant as in the wild type. These results indicate that *tagA* mutant cells fail to make the sharp transitions from growth to development and from the unicellular stage to the multicellular stage that are observed in wild-type cells (Van Driessche et al., 2002). We also compared the expression of the cell-type enriched genes defined by Van Driessche et al. (Van Driessche et al., 2002) in the *tagA* mutant with the wild-type expression. When the *tagA* mutant expression data is

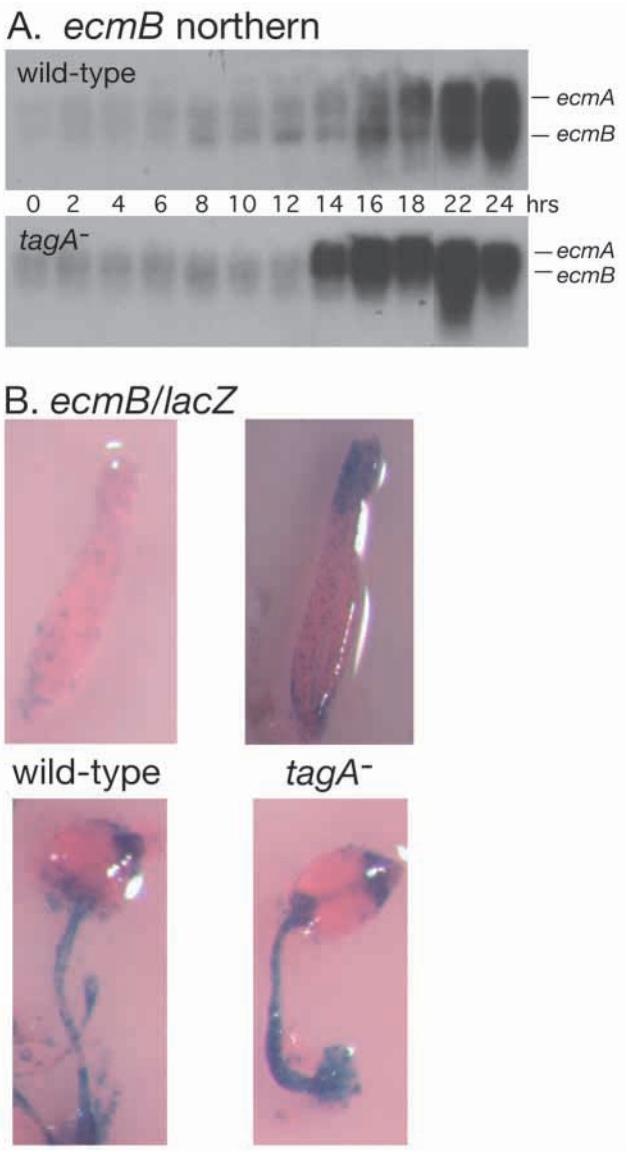

Fig. 4. Expression of ecmB during development. (A) Northern analysis of the developmental expression of the prestalk/stalk-specific *ecmB* gene. The upper band in both panels represents hybridization to the highly similar *ecmA* gene. (B) An *ecmB/lacZ* reporter gene was used to visualize *ecmB* expression at a cellular level. Structures were fixed at 16 hours (upper panels) and 24 hours (lower panels) of development and stained for β-galactosidase activity to visualize expression of the reporter gene. More cells appear to express *ecmB* in the *tagA* mutant, and many of the additional cells accumulate in the anterior of the slug, but their localization within fruiting bodies appears relatively normal.

normalized to the wild-type gene expression, most of the stalk-enriched genes appear to be overexpressed in *tagA* mutants while the spore-, prespore- and prestalk-enriched genes appear to be under-expressed (data not shown).

In order to compare the temporal progression of the mutant and the wild-type cells through development, we calculated the similarity in expression levels between each of the genes at every time point in the mutant samples with each of the genes at every time point in the wild-type samples. If there were few differences between the transcriptional programs of the two strains, one would expect this comparison to result in a diagonal line (Fig. 5D). The actual data indicate that the *tagA* mutants develop with normal timing only during the first 2 hours of development (Fig. 5D). The transcriptional program of the *tagA* mutant cells then appears to pause, as the samples collected at 4 and 6 hours of development are most similar to the sample collected from wild-type cells at 2 hours of development. After 6 hours of development, the overall trend of the plot suggests an accelerated rate of development up to 16 hours. At this time the transcriptional program appears to be arrested in the *tagA* mutants since all times from 16 to 24 hours in the *tagA* mutant are most similar to the 16-hour wild-type sample. The apparent 16-hour arrest in the developmental program of *tagA* mutants is consistent with their extended period of fruiting body formation (from 24 to 36 hours) and is reflected in the delayed spore production and poor spore viability. At 24 hours of development, wild-type cells produced $2.4\pm0.6\times10^7$ spores (87% of which were viable) while the *tagA* mutants produced $2.6\pm1.6\times10^6$ spores (13% of which were viable). These results indicate that *tagA*$^-$ mutants have a marked attenuation of development at the onset of TagA expression and again at culmination, suggesting that TagA performs critical functions at these two times.

Cell type specificity of gene expression is compromised in tagA mutants

Tissue-specific expression of the *tagA* gene was determined by expressing *lacZ* under the control of the *tagA* promoter in wild-type and mutant cells. This promoter is likely to be complete as it rescued TagA protein expression and it corrected the development of *tagA* mutant cells (Fig. 2B). Histochemical staining of the developing Ax4[*tagA/lacZ*] cells for β-galactosidase showed expression of the *tagA* gene in the prespore region of developing fingers and in the sori of fruiting bodies (Fig. 6A and data not shown). We isolated spores from mature fruiting bodies after 36 hours of development and stained them for β-galactosidase activity. We found that 82±8.0% of wild-type spores appeared to express the *tagA/lacZ* reporter construct as judged by their blue color in bright-field microscopy. In all developing structures observed, the tips of fingers and anterior (PstA) region of the slug showed no detectable *lacZ* expression. In the *tagA*$^-$[*tagA/lacZ*] strain, very faint staining could be observed in the prespore region during development, but only after 24 hours of staining. Upon culmination, weak β-galactosidase expression was evident only in cells of the outer basal disc and in the lower cup of the fruiting body (Fig. 6A). These *tagA*-expressing cells occupied the same position in the mutant fruiting bodies as the extra *ecmA*-positive cells described above. Interestingly, there was no detectable staining in the *tagA* mutant sori. Very few spores isolated from these fruiting bodies displayed any β-galactosidase activity as determined by staining for 48 hours (0.58±0.62%). To confirm this result we attempted an RNase protection assay on spore and stalk RNA with a probe that lies between the promoter and the insertion mutation in the *tagA* gene. The samples were harvested at a time that the *tagA* mRNA levels are predicted to be extremely low in wild-type cells (24 hours; Fig. 2) and less than ten percent of the *tagA* mutant cells are expected to express the gene (Fig. 6A). In spite of this, the assay consistently revealed an enrichment of *tagA* mRNA in the spores of the wild type, as expected, and a

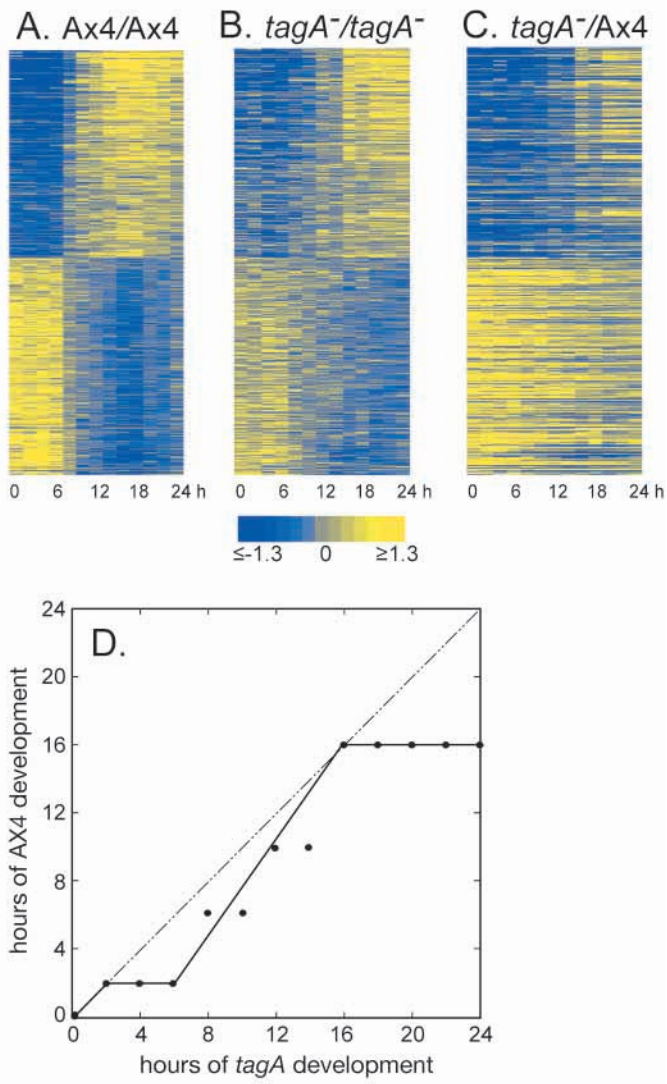

Fig. 5. Transcriptional profiling of *tagA* mutant cells. Wild-type (AX4) and mutant (*tagA*–) cells were developed for 24 hours. RNA samples were collected at 2-hour intervals and analyzed with a microarray of about 6,000 genes. The data from a selected set of 2,021 developmentally regulated genes were plotted to indicate the level of gene expression where the color scale represents the standardized $\log_2$ of the ratio between the test sample and the standard relative to the mean for each gene. Blue indicates lower than average level of gene expression for that gene and yellow indicates higher than average level of expression (Van Driessche et al., 2002). Each column represents a time point and each row represents a gene. (A) RNA from wild-type (Ax4) cells where the data are normalized with the gene means from the wild-type data (self-normalized). (B) RNA from *tagA*– cells where the data from every gene are self-normalized. (C) RNA from *tagA*– cells where the data from every gene are normalized to the corresponding gene mean in the wild-type (AX4) dataset. (D) The similarity (Pearson correlation) between all the genes at each time point in the AX4 dataset (y axis) and all the genes at each time point in the *tagA*– dataset (x axis) was calculated. For each time of *tagA*– development the most similar wild-type time point is plotted (solid line) in comparison with a theoretical plot between two identical time courses (broken line). The AX4 data set was published previously (Van Driessche et al., 2002) and was re-analyzed in the context of this experiment.

consistently higher signal in the stalk tissue relative to the spores produced by *tagA* mutant cells (Fig. 6B).

In an attempt to detect cell-cell signaling requiring TagA, we looked for the rescue of *tagA* expression in *tagA*– prespore cells by mixing *tagA*–[*tagA/lacZ*] cells with unmarked wild-type cells in a 1:1 ratio. Spores were isolated from the resulting fruiting bodies and 3.4±2.2% stained positive for *lacZ* expression. This modest six-fold increase in the staining of spores (from 0.58% when the mutant develops alone) indicates that *tagA* promoter could be activated above the threshold of detection by histochemical staining within the prespore cells of *tagA* mutants. This limited rescue of *tagA* expression by wild-type cells is suggestive of a positive feedback in *tagA* expression.

These results suggest that a fate change occurs in *tagA*-expressing cells within the *tagA*– cell population so that cells that would have become prespore cells develop into a cell type similar in character to PstB cells. Since the expression profiling suggested that the majority of *tagA*– cells are affected by the loss of TagA we examined the potential for additional alterations of cell specification by surveying the expression of archetypal cell-specific genes. The spore coat protein gene *cotB* is a reliable marker of prespore and spore cells and is coordinately regulated with several other spore coat protein genes (Fosnaugh and Loomis, 1991). The *cotB/lacZ* reporter construct used to visualize *cotB* expression showed a normal staining pattern in wild-type cells, with all the spores staining blue with X-gal and no staining of the stalk cells (Fig. 6C). TagA mutant spores stained as expected, but many of the vacuolated stalk cells were also stained, revealing that these cells had expressed the *cotB* gene at some time in development (Fig. 6C). The *spiA* gene is normally expressed exclusively within encapsulating prespore cells and is required for the long-term stability of dormant spores (Richardson and Loomis, 1992; Richardson et al., 1994). The *spiA* mRNA displayed slightly lower spore/stalk enrichment in the *tagA* mutant samples compared to the wild type (Fig. 6D). Intriguingly, the *ecmB* mRNA appears to be present in the spore RNA purified from *tagA* mutants in a much higher proportion than in the wild type. This contrasts with the expression pattern observed with the *ecmB/lacZ* reporter gene that showed little expression in *tagA* mutant prespore cells or spores (Fig. 4). This difference suggests that the promoter present in the *ecmB/lacZ* construct is not active in *tagA* mutant prespore cells or that the native *ecmB* gene has additional promoter elements that are missing in the artificial *lacZ* construct. Alternatively, the native *ecmB* mRNA may be more stable than *lacZ* mRNA in *tagA* mutant prespore cells. Nevertheless, the unexpected patterns of *cotB*, *ecmB* and *tagA* expression in *tagA* mutants indicate that *tagA* mutants produce terminally differentiated cells in spite of substantial mis-expression of cell-specific genes.

DISCUSSION

We have identified a new member of the *tag* gene family that is required for cell fate determination early in *Dictyostelium* development. TagA appears to prevent a discrete population of cells from becoming prestalk cells at the earliest stages of development. TagA expression defines a population of cells

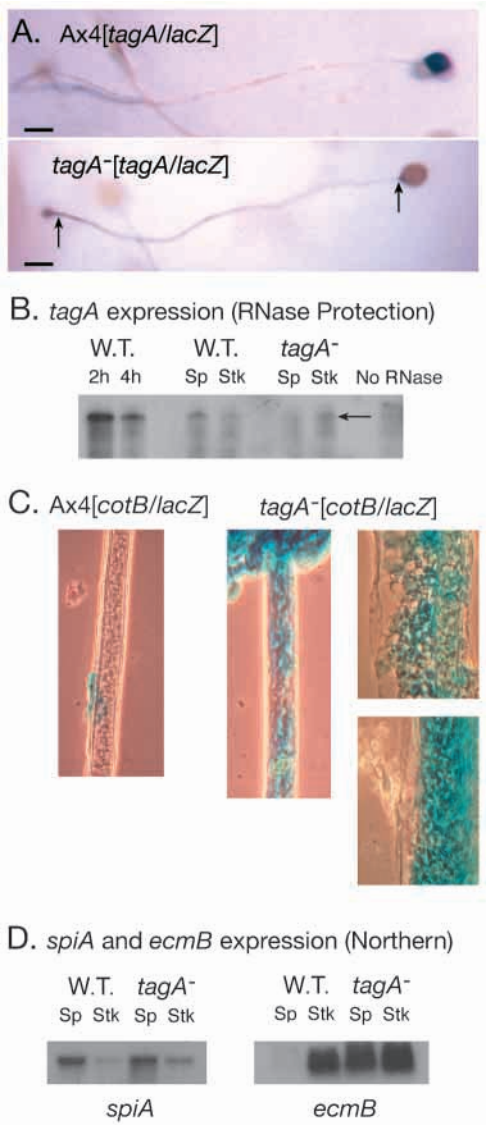

Fig. 6. Cell-type specific gene expression in *tagA* mutants. (A) Cells expressing a *lacZ* reporter gene under the control of the *tagA* promoter in a wild-type or *tagA* mutant background were plated as pure populations and stained with X-gal. The arrows indicate the stained regions in the mutant. Scale bars: 0.1 mm. (B) RNase protection analysis of *tagA* mRNA in purified spores and stalks reveals a reproducible enrichment of *tagA* mRNA in the stalk RNA of the *tagA* mutant. Controls are 2- and 4-hour developing wild-type cells and 10% of the input probe, not treated with RNase. (C) A *cotB/lacZ* reporter was used to visualize prespore/spore gene expression. An unstained wild-type stalk (left panel) is in stark contrast to the stained stalk cells of the *tagA* mutant (3 right panels). A portion of the *cotB*-positive sorus is shown above the *tagA* mutant stalk (middle). D. Northern analyses of spore and stalk RNAs with probes for the *spiA* (spore-specific) and *ecmB* (prestalk/stalk-specific) genes.

that normally differentiate into spores, but in the absence of TagA activity those cells become some type of stalk cell and form part of the outer basal disc and lower cup of the fruiting body. These cells behave like true PstB cells as determined by their localization in slugs and fruiting bodies. In addition, we observed a significant alteration in the cell-specific gene expression patterns. Three out of the four cell-type-specific genes that we examined showed significant expression in the other cell type in *tagA* mutants. These findings support the notion that TagA protein is fundamentally important for the specification or maintenance of the differentiated state of the cell types. However, the fact that not all *tagA* mutant cells become stalk cells and most still sporulate, suggests that there are overlapping mechanisms for the establishment and maintenance of cell type proportions that can compensate for the lack of TagA. Overlapping mechanisms that promote the production of spores would help to ensure the propagation of the species.

The analysis of the *tagA* gene suggests that overt prespore cell differentiation may occur as early as 2 hours of development. TagA mRNA and protein begin to accumulate to significant levels within the first 2 hours of development. The results obtained with the *tagA/lacZ* reporter construct indicate that TagA expression becomes spore specific. It is possible that the *tagA* promoter is active in all cells early, the β-galactosidase is turned over during aggregation and *tagA* expression becomes spore specific only later, but the more than 8-hour half-life of the β-galactosidase produced from this construct argues against this possibility (Detterbeck et al., 1994). Thus, it is possible that some cells require the expression of *tagA* in the first 2 hours of development in order to differentiate as prespore cells. In this regard, it is interesting that the first significant increase in the curve that describes the increase in *ecmA*-expressing cells in the *tagA* mutant extrapolates back to about 6 hours of development (Fig. 3). In addition, the global gene expression profiling of the *tagA* mutant revealed a delay in the developmental program beginning between 2 and 4 hours of development. Thus, the cell fate and gene expression changes that we observed in the mutant support the idea that the first critical time for TagA function is prior to 6 hours of development.

It has generally been accepted that cell-type-specific gene expression begins at about eight hours, as the mound forms during aggregation (Williams et al., 1989; Haberstroh and Firtel, 1990; Fosnaugh and Loomis, 1993). However, recent reports suggest that some form of cell-type divergence may occur much earlier. Iranfar and co-workers uncovered six genes with cell-type specific expression at the slug stage that initiate expression between 2 and 5 hours of development (Iranfar et al., 2001). Van Driessche and co-workers identified dozens of cell-type-enriched mRNAs that reach their highest expression levels from 2 to 6 hours of development (Van Driessche et al., 2002). More detailed analyses may reveal that these mRNAs become cell-type enriched much later in development through, for instance, their differential stability within the different cell types. It is also possible that these transcripts reflect an early divergence in the physiological state of cells within the starving population that influences cell-type specification.

Physiological differences, such as prior growth conditions and cytosolic pH, between vegetative cells have been observed to influence cell type divergence later in development (e.g., Leach et al., 1973; Maeda and Maeda, 1974; Gross et al., 1983). It is well documented that cell cycle phase at the time of starvation influences cell differentiation later in development (reviewed by Maeda, 1997). In fact, this influence can be observed as cell-autonomous cell-type specification in low cell density cultures and is under regulation by the RtoA protein

(Gomer and Firtel, 1987; Wood et al., 1996). It is likely that the influence of the cell cycle is to provide a bias in cell fate determination that is realized by later signaling through, for example, cAMP, DIF or calcium (e.g. Clay et al., 1995; Thompson and Kay, 2000; Azhar et al., 2001). A prespore-specific transporter has been characterized, RhT, that appears to be involved in prespore cell differentiation and whose activity can be detected prior to the expression of the spore coat protein gene, *cotB*, as the prestalk and prespore regions are coalescing in the early mound (Good and Kuspa, 2000). All of these physiological differences amongst cells can predispose a particular cell to one cell fate or another, but it is generally accepted that these biases do not determine cell fate and are reversible in different experimental contexts in vivo. The *tagA* gene is an example of a prespore-specific gene that is expressed at the onset of development and is also required for the sporulation of those cells that express it. Thus, *tagA* provides genetic evidence that some prespore cell differentiation occurs well before the aggregation stage and may provide a link between the physiological status of growing cells and the cell fate determination that occurs in the first few hours of development.

We used transcriptional profiling as a way of obtaining a global view of the physiological state of the *tagA* mutants during development. Monitoring global changes in gene expression allows the detection of mutation-induced deviations from an otherwise robust transcriptional program. The early pause in the transcriptional program that we observed between 2 and 6 hours of development in the *tagA* mutant coincides well with the onset of TagA RNA and protein expression in the wild type. The ablation of the normal transcriptional program precisely when TagA is first expressed reinforces the notion that *tagA* plays an important role in early development. It is important to note that we would not have observed this pause unless the majority of cells in the population had experienced a 4-hour delay in the transcriptional program. At least 70% of wild-type cells express *tagA* at some time during development, but only about ten percent of the cells express detectable levels of *tagA* in the mutant as judged by β-galactosidase staining. These facts together with the delay in the transcriptional program and the inappropriate expression of the *cotB* and *ecmB* genes suggests that most cells are affected by the loss of *tagA*, but most of them compensate for the loss and go on to make spores and stalk while a small percentage of cells are directed to an anomalous PstB-like state. The fact that the early delay in global gene expression in *tagA* mutants lasts for 4 hours suggests that the defect in the unicellular to multicellular transition stems from a failure to make an initial population of TagA-expressing cells in a timely fashion. The later delay in the transcriptional program in the *tagA* mutant, between 16 and 24 hours, suggests that TagA functions late or that the mutants lose synchrony as development proceeds. This fits with the observed morphological asynchrony, the 12-hour delay in the completion of development and low viability of *tagA* mutant spores.

Although we have yet to develop an assay for the biological function that is mediated by TagA, we were able to obtain indirect evidence of a putative TagA signaling event by monitoring deviation from the wild type in the expression of thousands of genes early in development. This is important given that the mutant organism can compensate for the lack of TagA, making it difficult to explore TagA function using cellular or morphological criteria. TagA is most similar in its predicted structure to TagC and TagB. TagC has been implicated in the cellular export of the peptide signal, SDF-2, thought to stimulate the terminal differentiation of prespore cells; there is also genetic evidence that TagB is required for this signaling event (Shaulsky et al., 1995; Anjard et al., 1998). The protease/transporter homology of TagA and the cell-autonomous phenotype of *tagA* mutants suggest that TagA exports a peptide that must be cleaved and removed from the cell for prespore cell differentiation to occur prior to aggregation. The export of a differentiation inhibitor was also proposed to explain the cell-autonomous specification of PstA cells by TagB and the maintenance of the undifferentiated state of stem cells in mammals (Shaulsky et al., 1995; Zhou et al., 2001).

It will be important to determine the regulatory pathways that *tagA* impinges on within the prespore cells that are most affected by loss of *tagA* function. There are two other genes whose inactivation results in the cell-autonomous production of PstB-like cells: one encodes the *Dictyostelium* homolog of glycogen synthase kinase 3 (GSK-3), *gskA*, and the other, *stkA*, encodes the Stalky protein that resembles a GATA family transcription factor (Harwood et al., 1995; Chang et al., 1996). When *gskA* mutant cells are co-developed with wild-type cells they produce PstB cells that occupy the lower cup and outer basal disk and they over-express the *ecmB* gene (Harwood et al., 1995). Both of these phenotypes are reminiscent of what we have observed in *tagA* mutants. Thus, one possibility is that TagA is needed to maintain active GSK-3 in a small cohort of prespore cells early in development. Stalky mutants also overproduce stalk cells, but *stkA* appears to function much later in development than either *tagA* or *gskA*. The function of *stkA* also appears to be independent of *gskA* (Chang et al., 1996). Future work will focus on identifying the signaling pathways controlled by TagA and the identification of the TagA substrate.

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