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Evidence that the *Dictyostelium* Dd-STATa protein is a repressor that regulates commitment to stalk cell differentiation and is also required for efficient chemotaxis

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SUMMARY

Dd-STATa is a structural and functional homologue of the metazoan STAT (Signal Transducer and Activator of Transcription) proteins. We show that *Dd-STATa* null cells exhibit several distinct developmental phenotypes. The aggregation of *Dd-STATa* null cells is delayed and they chemotax slowly to a cyclic AMP source, suggesting a role for Dd-STATa in these early processes. In *Dd-STATa* null strains, slug-like structures are formed but they have an aberrant pattern of gene expression. In such slugs, *ecmB/lacZ*, a marker that is normally specific for cells on the stalk cell differentiation pathway, is expressed throughout the prestalk region. Stalk cell differentiation in *Dictyostelium* has been proposed to be under negative control, mediated by repressor elements present in the promoters of stalk cell-specific genes. Dd-STATa binds these repressor elements in vitro and the ectopic expression of *ecmB/lacZ* in the null strain provides in vivo evidence that Dd-STATa is the repressor protein that regulates commitment to stalk cell differentiation. *Dd-STATa* null cells display aberrant behavior in a monolayer assay wherein stalk cell differentiation is induced using the stalk cell morphogen DIF. The *ecmB* gene, a general marker for stalk cell differentiation, is greatly overinduced by DIF in *Dd-STATa* null cells. Also, *Dd-STATa* null cells are hypersensitive to DIF for expression of *ST/lacZ*, a marker for the earliest stages in the differentiation of one of the stalk cell sub-types. We suggest that both these manifestations of DIF hypersensitivity in the null strain result from the balance between activation and repression of the promoter elements being tipped in favor of activation when the repressor is absent.

Paradoxically, although *Dd-STATa* null cells are hypersensitive to the inducing effects of DIF and readily form stalk cells in monolayer assay, the *Dd-STATa* null cells show little or no terminal stalk cell differentiation within the slug. *Dd-STATa* null slugs remain developmentally arrested for several days before forming very small spore masses supported by a column of apparently undifferentiated cells. Thus, complete stalk cell differentiation appears to require at least two events: a commitment step, whereby the repression exerted by Dd-STATa is lifted, and a second step that is blocked in a *Dd-STATa* null organism. This latter step may involve extracellular cAMP, a known repressor of stalk cell differentiation, because *Dd-STATa* null cells are abnormally sensitive to the inhibitory effects of extracellular cyclic AMP.

Key words: *Dictyostelium discoideum*, STAT, Tyrosine phosphorylation, Receptor signaling, Cell-type differentiation

INTRODUCTION

Dictyostelium discoideum cells form a multicellular organism by the chemotactic aggregation of up to 10^5 cells in response to extracellular cyclic AMP (cAMP) (Firtel, 1995; Chen et al., 1996). Upon aggregate formation, cells adopt one of two presumptive cell fates: approximately one-fifth differentiate into prestalk cells and the remainder into prespore cells (Firtel, 1995; Williams, 1995). Throughout the multicellular stages, the cell types exhibit a high degree of spatial patterning, which is independent of the size of the organism. By the migrating slug stage, the cell types have organized along an anterior/posterior axis. The prestalk cells, which normally differentiate as stalk cells, occupy the front one-fifth of the slug

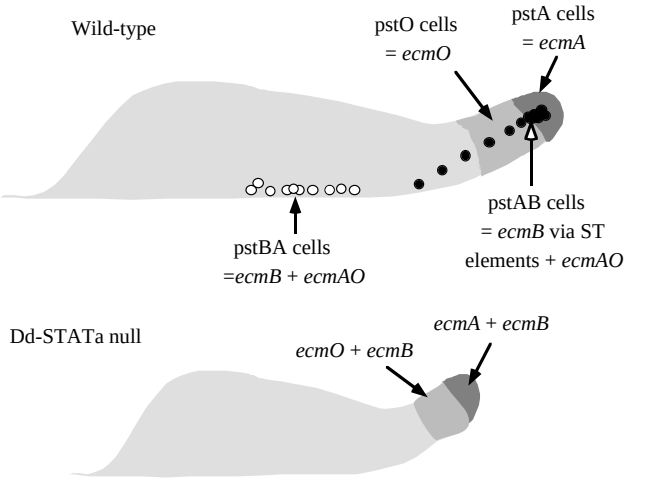


Fig. 1. Cartoon of the spatial localization of the individual cell types and the expression of cell-type-specific reporters. ST refers to the stalk-specific promoter element of *ecmB*.

and the prespore cells, the precursors of mature spores, occupy the rear. These are, however, presumptive fates; if the slug is cut into two halves, the cells within each half re-establish a properly proportioned slug and form a normal fruiting body through the inter-conversion of one cell type into another.

The migrating slug is an intermediate stage of development. In the laboratory, the organism can remain as a slug for extended periods of time, migrating towards unidirectional light, and will not initiate the formation of the fruiting body (culmination) until environmental conditions (e.g. humidity, directional light and ionic conditions of the medium) are optimal. During slug migration, a small fraction of the prestalk cells prematurely enter into the stalk cell pathway of differentiation, forming a cone-shaped mass of prestalk AB (pstAB) cells, which are periodically shed from the posterior of the slug (Fig. 1; Jermyn et al., 1989; Jermyn and Williams, 1991; Sternfeld, 1992). These prestalk cells are replaced by the trans-differentiation of prespore cells into prestalk cells, maintaining the cell-type proportions within the organism (Abe et al., 1994). Thus, *Dictyostelium* displays the defining feature of regulative development in higher organisms: cells achieve an intermediate level of differentiation but are held in this state until they are signaled to undergo terminal differentiation.

Recent studies of the signaling pathways regulating prestalk and stalk cell differentiation suggest a direct mechanistic link between prestalk and stalk cell differentiation (Kawata et al., 1996, 1997) that involves the morphogen DIF. DIF is a chlorinated hexaphenone that accumulates during development and is essential for prestalk cell differentiation (Kay, 1983; Morris et al., 1987; Town and Stanford, 1979; Williams et al., 1987). A large body of genetic and biochemical data indicate that prestalk cells are induced to form stalk cells at culmination by a rise in intracellular cAMP that serves to activate cAMP-dependent protein kinase (PKA; reviewed in Firtel, 1995; Williams, 1995). Paradoxically, extracellular cAMP is an inhibitor of stalk cell differentiation (Berks and Kay, 1988, 1990). This is surprising, since extracellular cAMP induces the activation of adenylyl cyclase through cell-surface G protein-coupled receptors and cAMP signals induce cells to synthesize cAMP (reviewed in Firtel, 1995). The precise mechanism of action of extracellular cAMP is unknown, but stalk cell differentiation is resistant to the inhibitory effects of cAMP in a null mutant of *gskA*, the gene encoding the *Dictyostelium* GSK-3, suggesting that GSK-3 lies on this pathway (Harwood et al., 1993).

The mode of action of DIF is unknown, although analysis of the promoter of *ecmA*, a DIF-inducible gene, identified a regulatory element that is necessary for DIF inducibility (Kawata et al., 1996). The promoter of the *ecmA* gene is modular; sequences proximal to the transcriptional start site are used by prestalk A (pstA) cells that occupy the front half of the prestalk region, while distal promoter sequences direct expression in prestalk O (pstO) cells that constitute the rear half of the prestalk region (Fig. 1; Early et al., 1993). The minimal regions that direct pstA and pstO expression each contain two copies of the sequence TTGA separated by a single A residue. In the case of the region directing pstO-specific expression, this sequence is essential for DIF inducibility and prestalk-specific gene expression (Early et al., 1993). The TTGA repeat is a high-affinity binding site for Dd-STAT, a *Dictyostelium* STAT protein (Kawata et al., 1997). Recently, two additional *Dictyostelium* STATs have been identified (M. Fukuzawa and J.G.W., unpublished observation). We have thus renamed the originally identified STAT, Dd-STAT, as Dd-STATa and will use that name henceforth.

Mammalian STAT proteins lie at the end of cytokine and growth factor signaling pathways and function as transcriptional regulators when homo- or heterodimerized (reviewed by Ihle and Kerr, 1995; Schindler et al., 1995; Darnell, 1996). STAT proteins contain an SH2 domain and a site of tyrosine phosphorylation. Dimerization occurs by the reciprocal interaction of the SH2 domain on one STAT with the phosphotyrosine in the tyrosine phosphorylation domain on the partner STAT. Dimerization is triggered by tyrosine phosphorylation of the STAT. This is most usually effected by a member of the JAK family of protein tyrosine kinases. Dd-STATa is highly homologous to metazoan STATs in the SH2, tyrosine phosphorylation and DNA binding domains, but the upstream steps in the Dd-STATa activation pathway are not fully understood.

The extracellular signal that activates Dd-STATa is cAMP and Dd-STATa displays complex changes in intracellular distribution during development that correlate with its tyrosine phosphorylation. It shows very low tyrosine phosphorylation and is not enriched in cell nuclei prior to aggregation. As the mound forms, it becomes tyrosine phosphorylated and highly enriched in the nuclei of all cells. However, by the migrating slug stage, nuclear enrichment is lost in all cells except pstA cells and a very few of the anterior-like cells (ALCs; Araki et al., 1998).

The discovery that transcriptional regulation mediated through the TTGA repeats forms the end point of the DIF signaling pathway revealed an interesting relationship with the pathway regulating stalk cell differentiation. This pathway had initially been defined using the *ecmB* promoter, which contains a distal activator region with the ability to direct transcription in prestalk cells prior to their commitment to stalk cell differentiation, and a proximal, repressor region that prevents premature expression of the gene in the prestalk cells of the slug or culminant (Ceccarelli et al., 1991; Harwood et al.,

1993). In constructs in which the repressor region is absent, the activator region directs reporter gene expression in prestalk cells prior to their moving into the stalk tube entrance, the place at which cells are thought to commit to terminal stalk cell differentiation. The *ecmB* repressor region contains two mutually redundant copies of an inverted repeat in which three out of four half-sites are TTGA and the fourth is TTGT (Harwood et al., 1993). Dd-STATa, purified to apparent homogeneity, is able to bind to both forms of the repressor, but with a tenfold lower affinity than to the *ecmO* activator, i.e. to TTGA direct repeats. These findings led to the combined activator-repressor model for stalk cell formation (Kawata et al., 1996). A single protein species, presumed to be Dd-STATa, was proposed to bind to TTGA direct repeats present in the promoters of all prestalk-specific genes. In this context, Dd-STATa would mediate DIF-induced gene expression by functioning as a transcriptional activator. Dd-STATa was also proposed to bind to inverted TTGA repeats present in the promoters of all stalk-specific genes, where it would function as a transcriptional repressor rather than an activator. The difference between the activator and repressor function of Dd-STATa was thought to be due to different conformations of the dimeric complex, depending on whether it bound to direct or inverted TTGA repeats. We have isolated a null mutation in Dd-STATa that allows us to directly examine the function of Dd-STATa. We show that Dd-STATa is not required for prestalk cell differentiation, but plays essential roles at several different stages of development and has the properties predicted for the repressor of stalk cell differentiation.

MATERIALS AND METHODS

Molecular biological, cell culture, and histochemical techniques

Cell culture, RNA extraction and northern blot hybridization, DNA extraction and Southern blot hybridization, cell transformation and β-galactosidase histochemical analyses have been described previously (Mann et al., 1998). DIF induction was performed exactly as described by Berks and Kay (1988). Induction with 8-Br-cAMP was done as described in Inouye and Gross (1993). Preparation of nuclear extracts and gel retardation analyses were performed as described previously (Kawata et al., 1997).

Isolation and molecular characterization of the Dd-STATa REMI mutation

The insertional mutant in *dstA* was isolated from a REMI mutational screen for genes required during multicellular development using a modified insertional vector carrying the Bsr dominant drug selectable marker (Sutoh, 1993). The vector with some 5′ and 3′ flanking DNA sequences was isolated by cleaving the genomic DNA with *Nde*I, recircularizing the DNA, and cloning it into *E. coli* as previously described (Aubry and Firtel, 1998). Sequencing of the DNA flanking the vector insert showed that the vector inserted in the *Dpn*II site in codons 81 and 82 of the ORF. The 5′ genomic sequence contained 321 bp upstream from the ATG translation initiation codon and a 188 bp intron in codon 31. The 3′ *Nde*I site is in the coding region and is centered in codon 113.

Chemotaxis assay

Log-phase vegetative cells were washed three times with Na/K phosphate buffer and resuspended at a density of $2\text{-}3\times10^6$ cells/ml in Na/K phosphate buffer and pulsed for 5 hours with 30 nM cAMP every 10 minutes (Devreotes et al., 1987; Mann and Firtel, 1987; Saxe III et al., 1991). Pulsed cells were plated in Na/K phosphate buffer at a density of 6×10^4 cells/cm^2 onto a plate with a hole covered by a 0.17 mm glass coverslip. An Eppendorf Patchman micromanipulator with a glass capillary needle (Eppendorf Femtotip) filled with 150 μM cyclic AMP (cAMP) solution was brought into the field of view of an inverted microscope. The response of the cells was followed by time-lapse video recording.

RESULTS

Identification of an insertional mutant that inactivates the Dd-STATa gene

The *Dd-STATa* null strain was identified in a screen for developmental mutants produced by the REMI method of insertional mutagenesis (Kuspa and Loomis, 1992). The DNA surrounding the vector was cloned and sequenced and a database search showed that the vector DNA inserted into the N-terminal region of *dstA*, the gene encoding Dd-STATa. In order to avoid complications from the secondary mutations that sometimes occur during REMI, the DNA rescued from the mutant was used to disrupt the *dstA* gene in two axenic strains, KAx-3 and Ax2. One KAx-3-derived clone and one Ax2-derived clone, each of which was shown by Southern blot analysis to have the vector inserted in the *dstA* gene (data not shown), were used in all subsequent experiments. Because very similar results were obtained with both axenic strains, we will in most cases distinguish between them only in the figure legends.

Several pieces of evidence show that the Dd-STATa protein is entirely absent from the gene disruptants, indicating that these are true null strains. First, western blot analysis, using a monoclonal antibody directed against the extreme C terminus of Dd-STATa protein, shows no detectable Dd-STATa protein (Fig. 2A). Second, in gel retardation assays, nuclear extracts from cells of the wild-type strain at the first finger stage yield a strong retarded band at the expected positions for a protein-DNA complex containing Dd-STATa and either an ecmA activator probe (Fig. 2B) or an ecmB repressor probe (Fig. 2C). Parallel experiments demonstrate that the bands at these positions are specifically competed with oligonucleotides containing the TTGA direct repeat (data not shown). This retarded band is entirely absent in nuclear extracts of the *Dd-STATa* null cells, also harvested at the first finger stage, although other much less intense bands are observed elsewhere on the gel (Fig. 2B). Lastly, RNA blot hybridization shows no detectable Dd-STATa mRNA in any of the gene disruptants (data not shown). Thus, we conclude that the isolates used are bona fide *Dd-STATa* null strains.

Dd-STATa null aggregates are delayed at the slug stage and form highly aberrant terminally differentiated structures

When *Dd-STATa* null cells are allowed to develop on water agar, phosphate-buffered agar or buffered filter pads, they form aggregates and slugs somewhat more slowly than the parental strains. Mounds form with an approximate 4-6 hour delay (Fig. 3Bb), compared to the parental wild-type strain (Fig. 3Aa), with no mound formation at 8 hours (Fig. 3Ba). The *Dd-STATa* null cells become temporarily arrested at the migratory slug stage (Fig. 3Bd-g,j), even under conditions that promote

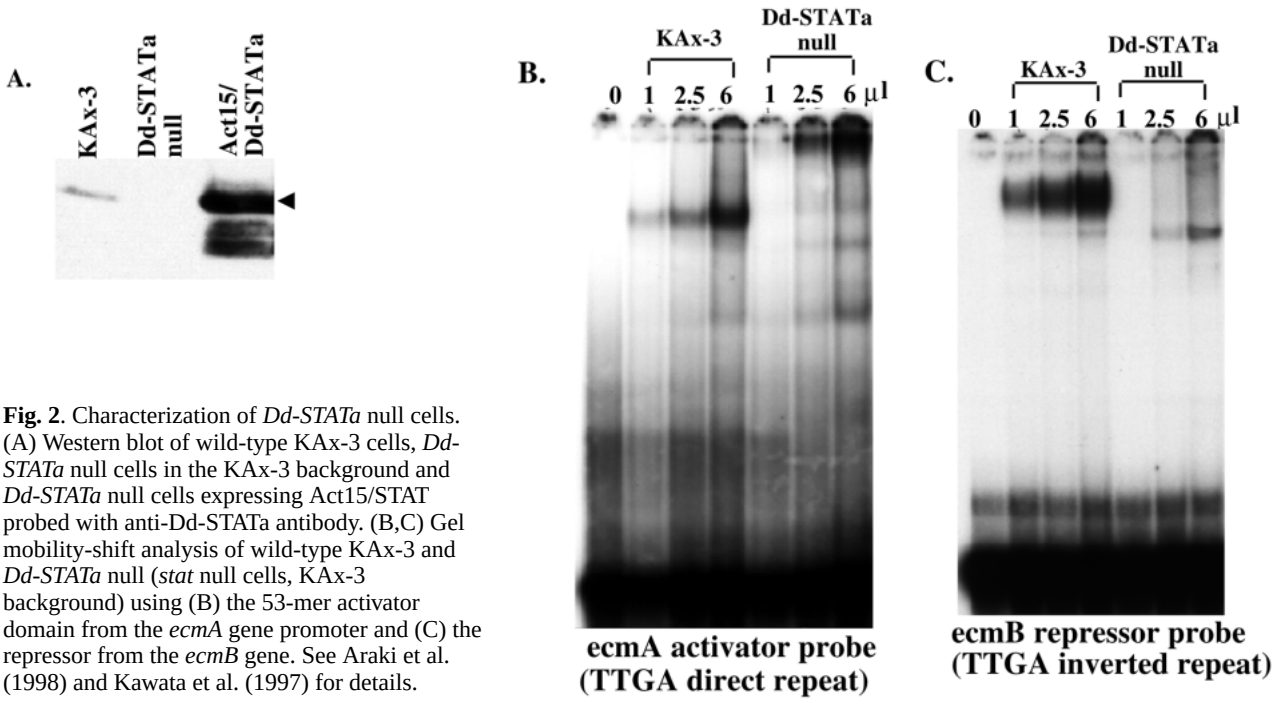


Fig. 2. Characterization of *Dd-STATa* null cells. (A) Western blot of wild-type KAx-3 cells, *Dd-STATa* null cells in the KAx-3 background and *Dd-STATa* null cells expressing Act15/STAT probed with anti-Dd-STATa antibody. (B,C) Gel mobility-shift analysis of wild-type KAx-3 and *Dd-STATa* null (*stat* null cells, KAx-3 background) using (B) the 53-mer activator domain from the *ecmA* gene promoter and (C) the repressor from the *ecmB* gene. See Araki et al. (1998) and Kawata et al. (1997) for details.

immediate culmination in wild-type strains (buffered filter pads with overhead light). A small fraction of the slugs eventually form upright, aberrant structures approximately 72 hours after plating (Fig. 3Bh,i,k,l), while wild-type strains form fruiting bodies within 24-26 hours (Fig. 3Ad).

As in wild-type strains, the first fingers of the *Dd-STATa* null strain topple over and commence migration but move only a little way from their site of formation and do not appear to be phototactically responsive. Time-lapse photomicroscopy shows that the slugs continually recapitulate the standing slug stage, perhaps in abortive attempts at culmination, before falling back onto the substratum (data not shown). Cells are discarded during migration as cell clumps (data not shown). As a result, the size of the slug becomes considerably reduced after a few days. In addition, *Dd-STATa* null slugs are often thicker than wild type and have stumpy tips, especially in Ax2-derived *Dd-STATa* null slugs (Fig. 3Bj). The terminal structures that are formed have a very small, irregularly-shaped bolus on top of a roughened column of cells (Fig. 3Bh,i,k,l). Such structures are formed infrequently when cells are developed on non-nutrient substrata. For unknown reasons, they are formed more frequently when cells are grown in association with bacteria on nutrient agar and allowed to develop after the bacterial food source is depleted (data not shown). The bolus contains prespore cells and spores of varying degrees of maturity. Some of the spores are fully mature, as they survive detergent treatment and are viable when plated onto a bacterial lawn (data not shown). However, the supporting, columnar structures contain few or no vacuolated stalk cells and there is no stalk tube, giving them a roughened appearance. Groups of stalk cells are present in the slime trail, so the block to stalk cell differentiation seems to operate only in the multicellular structures. The absence of a stalk tube surrounding the column of undifferentiated cells or within the bolus of prespore cells and spores suggests a failure to initiate and/or maintain the 'reverse fountain' movement pattern that shapes the fruiting body during normal culmination. Thus, while we do not understand how the spore mass is lifted up on the columnar structures in the null strain, it seems that these highly aberrant terminal structures are built from the bottom up rather than, as normally, from the top down.

Expression of Dd-STATa cDNA restores development to *Dd-STATa* null cells

Confirmation that the developmental arrest in *Dd-STATa* null cells results from the lack of Dd-STATa function was obtained by directing cDNA expression with a number of *Dictyostelium* promoters. Expression of Dd-STATa from the semi-constitutive *Act15* promoter, which produces high levels of Dd-STATa protein (Fig. 2A), leads to formation of an apparently normal fruiting body (Fig. 4). Overexpression of Dd-STATa directed by the *Act15* promoter does not affect the development of wild-type strains (data not shown). Expression of Dd-STATa from the *CP2* promoter (which is active late during aggregation) and the promoter of the *ecmA* gene (designated *ecmAO*, and known to direct expression in pstA and pstO cells), leads to a complete rescue of the slug-arrest and culmination phenotypes (data not shown). The fact that a prestalk-specific promoter, such as the promoter of the *ecmA* gene, directs rescue is consistent with the finding that Dd-STATa is enriched within the nuclei of cells in the pstA region of the slug and may therefore function predominantly in prestalk cells (Araki, 1998).

Early developmental defects in the *Dd-STATa* null cells revealed by time-lapse video microscopy and cAMP chemotaxis assays

As described above, *Dd-STATa* null cells exhibit a delay in forming mounds. When the size of the *Dd-STATa* null aggregates is compared to those of wild-type cells plated at the same density, Dd-STATa null aggregates are generally larger.

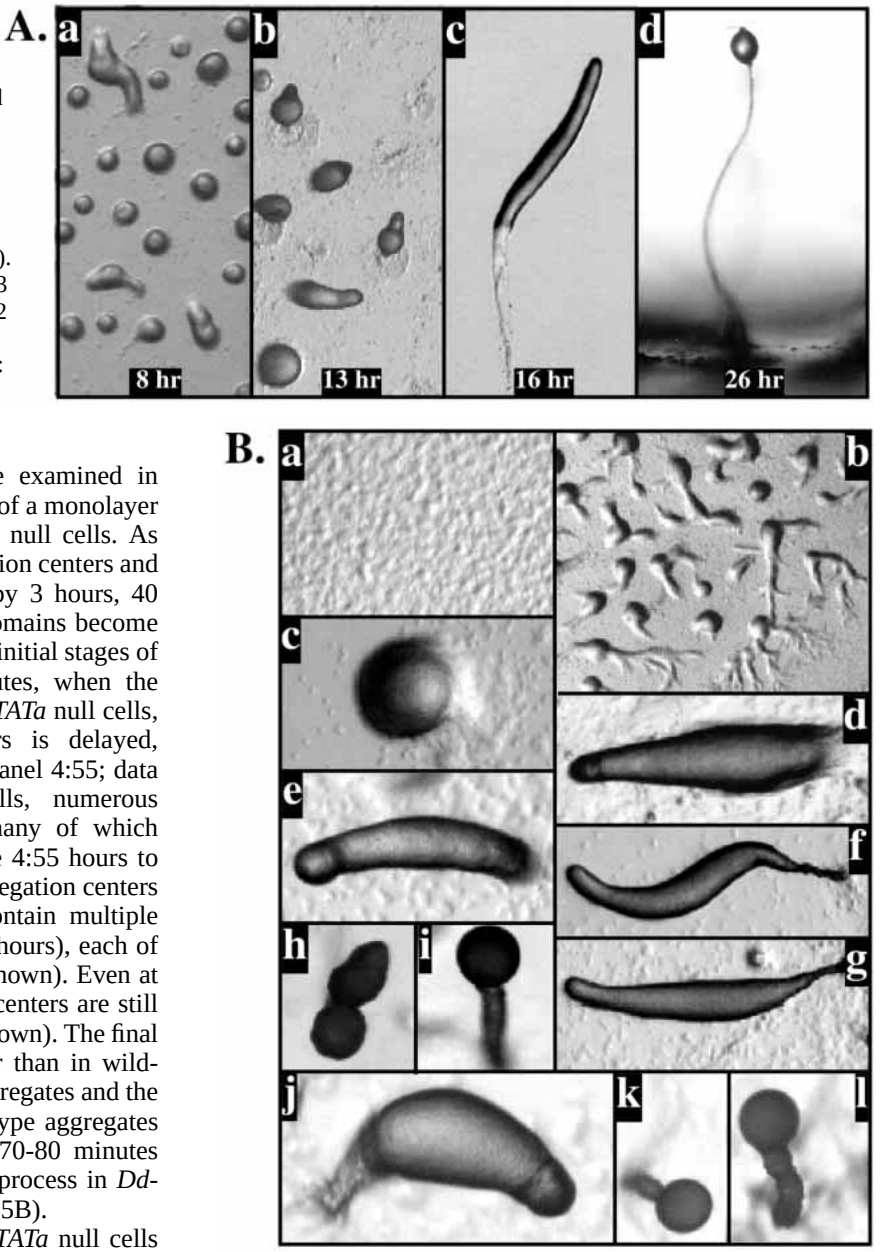


Fig. 3. Morphogenesis of wild-type and *Dd-STATa* null cells. Cells were developed on Na/KPO_4-containing agar. All photographs were taken on a Nikon SMZ-U zoom dissecting microscope equipped with a Sony 3CCD color video camera.
(A) Morphogenesis of wild-type KAx-3 cells is shown. (a) mounds (8 hours); (b) tipped aggregates, first fingers and very early slugs (13 hours); (c) migrating slug (16 hours); (d) mature fruiting body (26 hours). Magnifications: 0.75× (a,b), 4× (c), 5× (d).
(B) Development of *Dd-STATa* null cells. (a-i) KAx-3 background; (j-l) Ax2 background. (a) 8 hours; (b) 12 hours; (c) 16 hours; (d,e) 24 hours; (f) 36 hours; (g-i) 72 hours; (j) 28 hours; (k,l) 72 hours. Magnifications: 0.75× (a,b) 4× (c,f), 6× (d), 5× (e,g,j), 7× (h,i,k,l).

These potential aggregation aberrations were examined in more detail using time-lapse video microscopy of a monolayer of wild type (Ma et al., 1997) and *Dd-STATa* null cells. As described previously (Ma et al., 1997), aggregation centers and waves of cAMP are visible within the field by 3 hours, 40 minutes after plating (Fig. 5A), and defined domains become visible shortly thereafter (data not shown). The initial stages of chemotaxis are visible by 4 hours, 20 minutes, when the aggregation domains are well defined. In *Dd-STATa* null cells, the initial formation of aggregation centers is delayed, occurring after 4 hours, 30 minutes (Fig. 5B, panel 4:55; data not shown). In contrast to wild-type cells, numerous aggregation centers are observed initially, many of which become diffuse several minutes later (compare 4:55 hours to 5:02 hours). As chemotaxis initiates, these aggregation centers start to fuse into very large domains that contain multiple centers (Fig. 5B, 5:26 hours, 4:35 hours, 5:58 hours), each of which oscillates (observed in video; data not shown). Even at the later stages of aggregation, these multiple centers are still observed (Fig. 5B, 6:46 hours; video data not shown). The final stages of aggregation take significantly longer than in wild-type cells, presumably due to the size of the aggregates and the possibility of competing centers. While wild-type aggregates form loose aggregates within approximately 70-80 minutes after chemotaxis initiates (Fig. 5A), the same process in *Dd-STATa* null cells takes more than 4 hours (Fig. 5B).

The above aggregation phenotypes of *Dd-STATa* null cells suggest that the cells may have a defect in chemotaxis. To study this directly, we examined the ability of aggregation-competent wild-type and Dd-STATa null cells to move chemotactically toward a micropipette containing the aggregation-stage chemoattractant cAMP. As can be seen in Fig. 6A, wild-type cells become highly polarized and move towards the source of cAMP. In contrast, *Dd-STATa* null cells become less polarized and move more slowly (Fig. 6B). These results suggest that the *Dd-STATa* null cells aggregation-stage defect is due, at least in part, to a defect in chemotaxis.

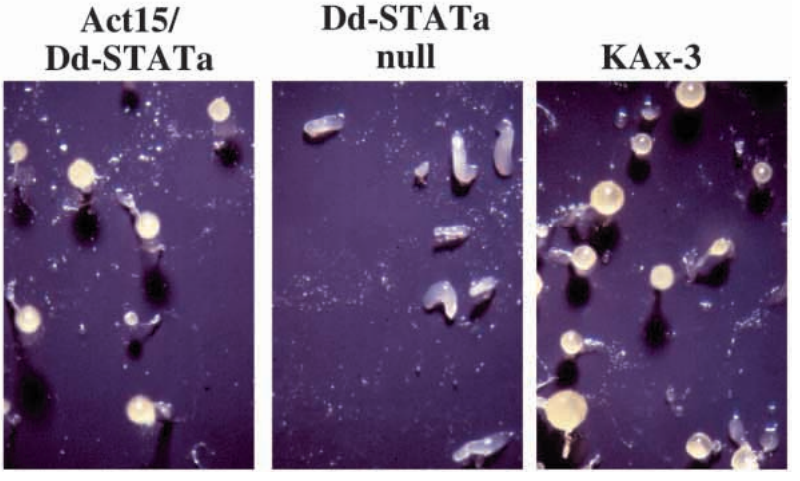


Fig. 4. Complementation of *Dd-STATa* null cells. KAx-3 cells, *Dd-STATa* null cells (*stat*–) in the KAx-3 background, and *Dd-STATa* null cells expressing Act15/STAT (Fig. 2A) were plated on black Millipore filters supported on buffer-saturated filter pads.

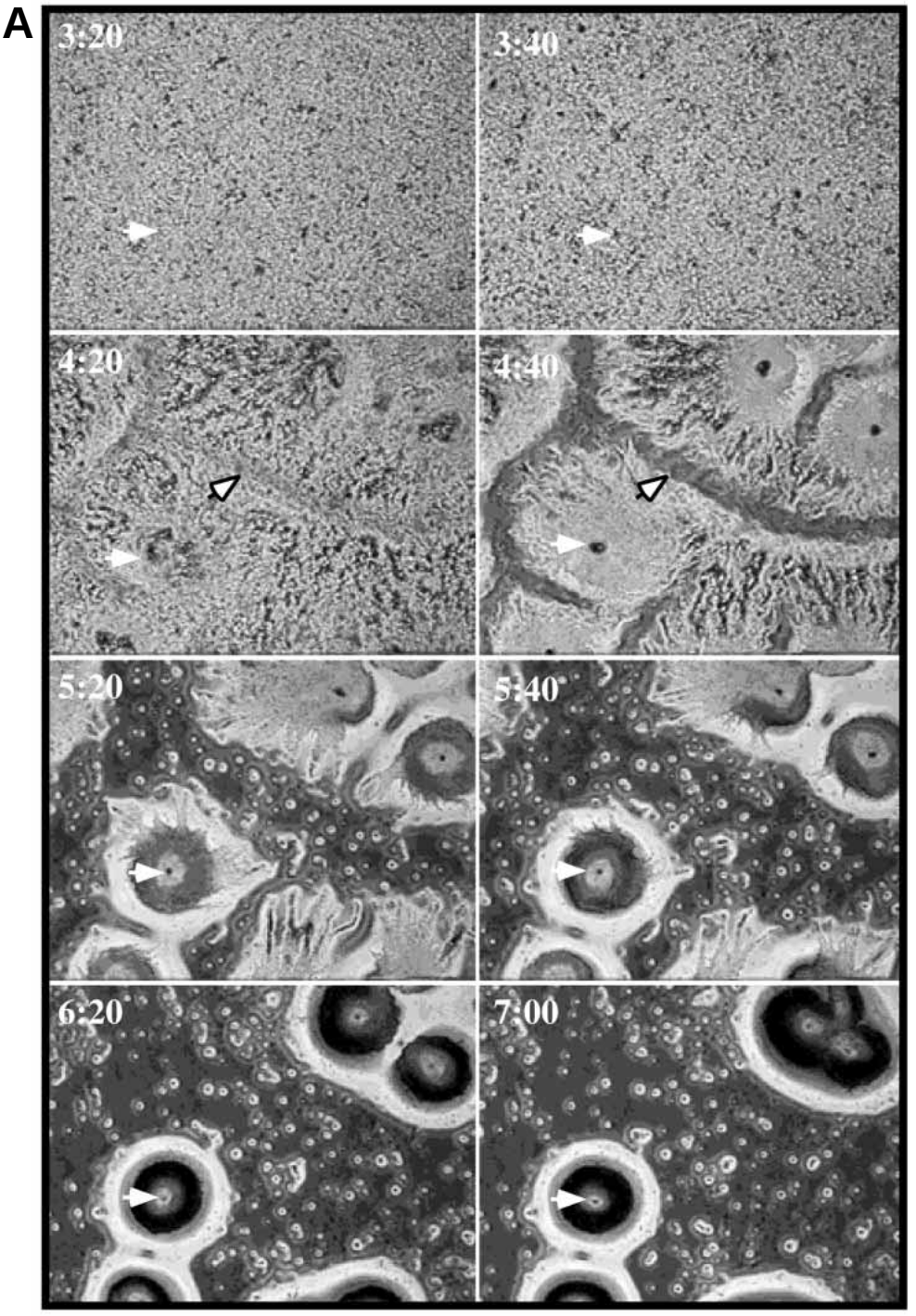
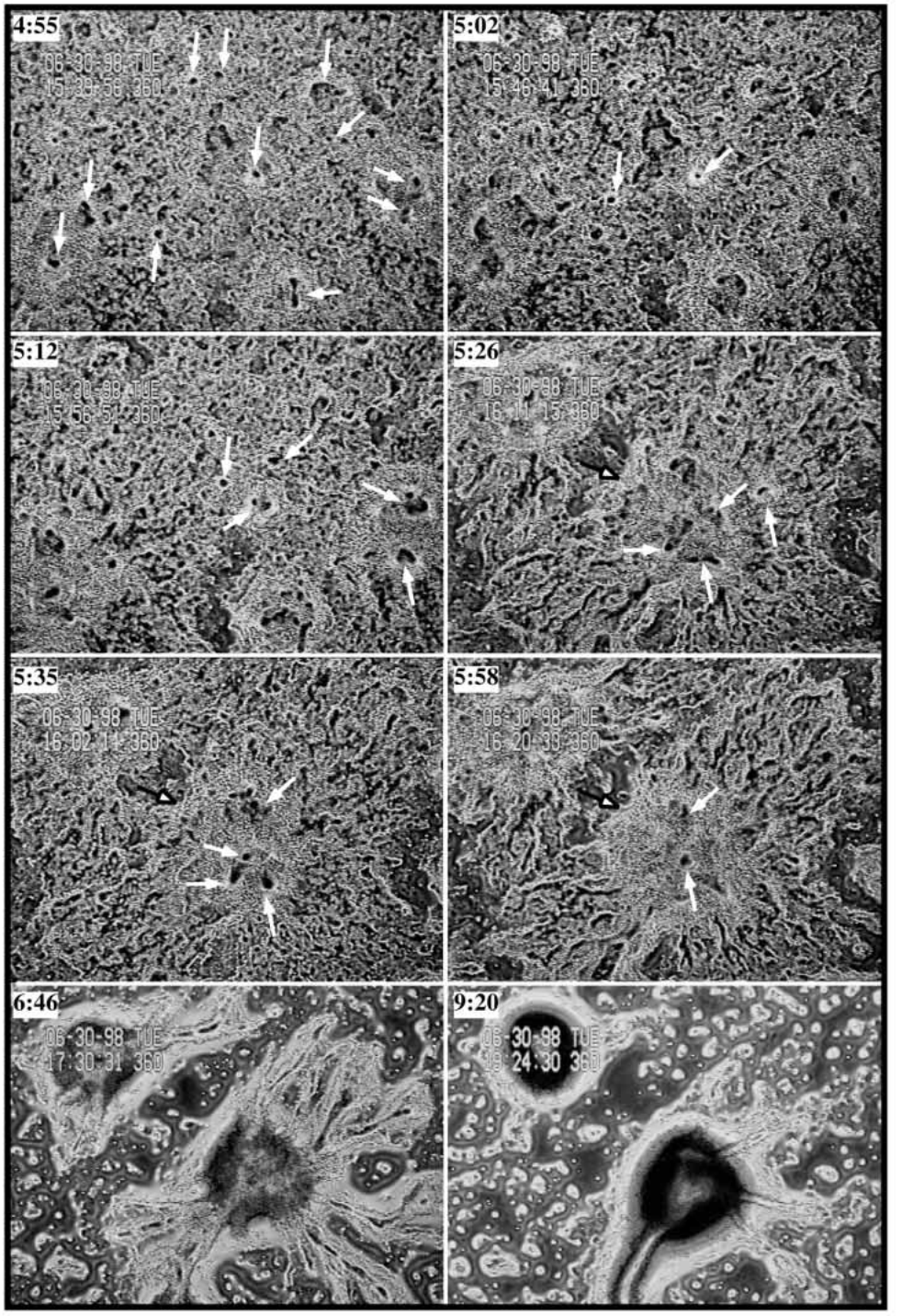


Fig. 5. Time-lapse video of aggregating cells. Wild-type (A) and *Dd-STATa* null (B) cells were plated as a monolayer on Na/KPO_4-containing agar and examined by time-lapse video phase microscopy as described in detail previously (Ma et al., 1997). Individual frames from the time-lapse videos were captured on a Scion imaging board. The numbers in the upper left-hand corner indicate the time after plating of the cells in hours and minutes. The solid arrows point to aggregation centers. The open arrows point to the outer edge of the forming aggregation domains. Not all of the aggregation centers are marked. Note the fusing of the aggregation domains in the *Dd-STATa* null field of cells starting in the panel labeled 5:26. As the aggregation domains for the *Dd-STATa* null cells became quite large for the lens (4× objective), it was necessary to move the position of the plate in order to record one of these forming, large centers. This was done starting at 5:20 hours after plating. The image at 5:26 hours represents the first image of the changed field of view that is shown in the montage. The first three panels show the same field of view. The last five panels show the same field of view.

Analysis of cell-type-specific markers in *Dd-STATa* null cells

Northern blot analysis of *Dd-STATa* null cells shows that the aggregation-stage marker *CsA* and the mound-stage (post-aggregation) markers *LagC* and *CP2* are expressed at an approximately normal level and with the same kinetics of induction as wild-type cells (data not shown). This is consistent with the fact that *Dd-STATa* null cells become markedly aberrant only after cell-type divergence. Hence, we investigated their phenotype in detail using reporters for the various prestalk and stalk cell subtypes and prespore cells.

The pattern of *SP60/lac*Z expression in wild-type slugs (Fig. 7A) and early *Dd-STATa* null slugs (Fig. 7Ba) is not distinguishably different, in both cases being detectable throughout the prespore zone. In wild-type slugs, the *ecmAO/lac*Z fusion gene (i.e. the complete promoter of the *ecmA* gene fused to *lac*Z) is expressed in pstA cells, pstO cells and ALCs (Figs 1, 7A; Early et al., 1993). *Dd-STATa* null slugs exhibit a wild-type pattern of expression with this marker at the first finger stage and very early during slug migration (Fig. 7Bb). Also, the *ecmA/lac*Z fusion gene, a marker of pstA cell differentiation, is expressed correctly in



Dd-STATa null slugs (Fig. 7Bc). At the time the first finger topples over, the *ecmO/lac*Z reporter, a marker of pstO cell differentiation, shows a normal pattern of expression in *Dd-STATa* null cells derived from Ax2 cells (Fig. 7Be). However, at earlier times in slug formation, there is a clear difference from the Ax2 parent. In Ax2-derived *Dd-STATa* null cells, there is *ecmO/lac*Z staining throughout the prestalk region (Fig. 7Bd). Since the pstO pattern is achieved by cell sorting of the pstA cells from the pstO cells (Early et al., 1993; Abe et al., 1994), we think that the most likely explanation for the uniform initial staining with *ecmO/lac*Z is a delayed segregation of the pstO and pstA populations that may be associated, in part, with the cell movement defects described above. One of the few reproducible differences between Ax2- and KAx-3-derived *Dd-STATa* null strains is also observed with *ecmO/lac*Z. Expression of *ecmO/lac*Z in the *Dd-STATa* null cells with a KAx-3 background occurs with a delay compared to wild-type Ax2 and KAx-3 strains and *Dd-STATa* null cells with an Ax2 background (Fig. 7Bf,g). A summary of the spatial distribution of prespore cells and the individual prestalk cell types is presented in Fig. 1. [Note that after prolonged slug migration of the *Dd-STATa* null cells, there are changes in some of these staining patterns (data not shown) that presumably result from the block to normal culmination, a condition that causes trans-differentiation of cells within the slug (Zhukovskaya et al., 1996).]

The fact that the *ecmO/lac*Z marker is expressed in the *Dd-STATa* null slugs was entirely unexpected, considering the major activity that binds the *ecmO* activator region *in vitro* is absent in *Dd-STATa* null cells (Fig. 2B). We therefore narrowed the promoter region, to include just the sequences that are minimally essential for prestalk-specific expression. When multimerized and placed upstream of basal promoter elements, the domain I region of the *ecmA* promoter directs weak *lac*Z reporter expression in pstA and pstO cells in wild-type strains (Fig. 8A; Kawata et al., 1996). As shown in Fig. 8B, this *lac*Z reporter is expressed in *Dd-STATa* null cells with a spatial pattern similar to that in wild-type cells. The fact that there is expression of this reporter in *Dd-STATa* null cells indicates that Dd-STATa is not required for gene expression from the DIF-responsive *cis*-acting element; i.e. the combined-activator repressor model of Kawata et al. (1996) is incorrect.

In synergy experiments, *Dd-STATa* null cells are excluded from the prestalk region

The delayed segregation of pstO and pstA cells in the *Dd-*

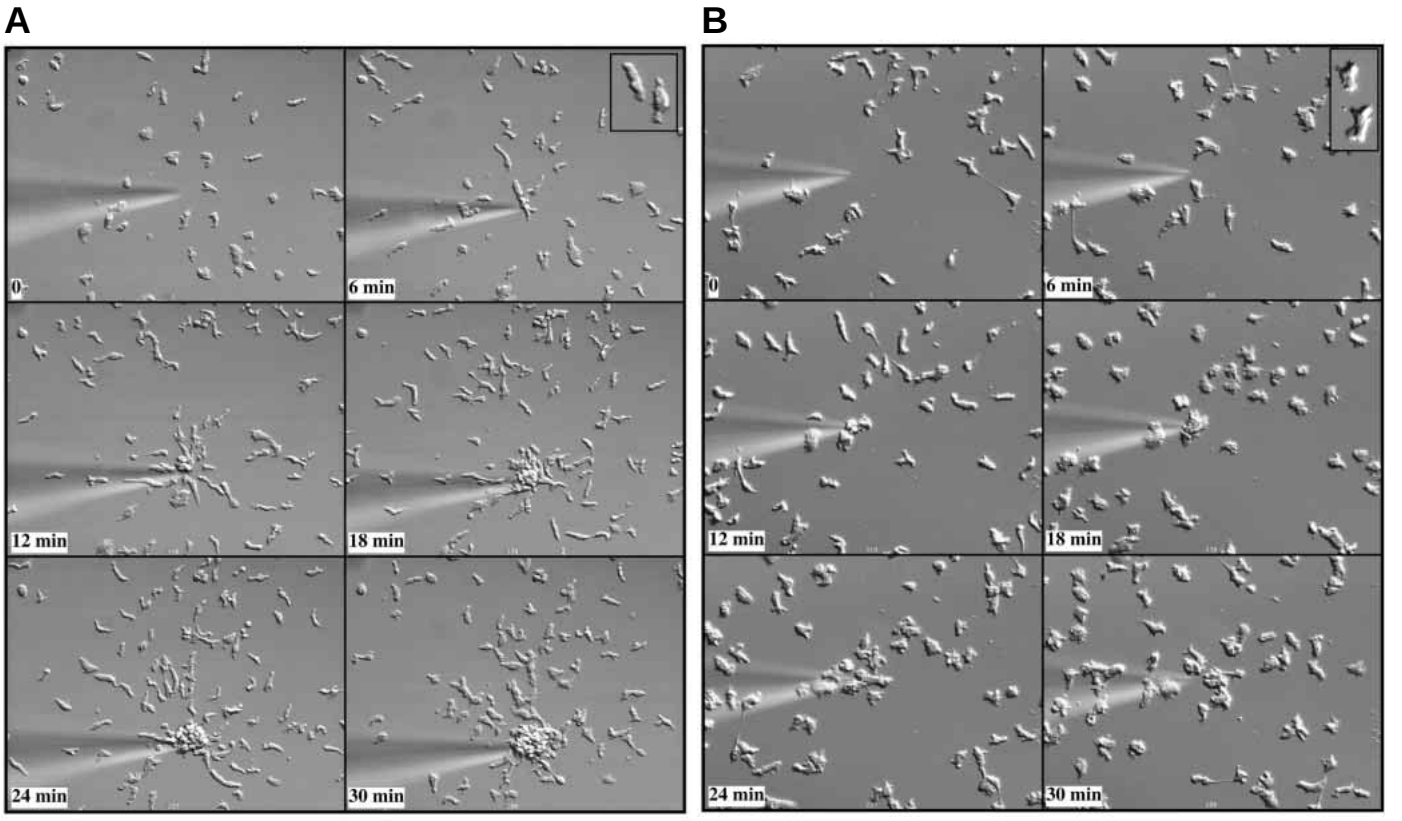


Fig. 6. Chemotaxis of wild-type and *STATa* null cells. Wild-type and *STATa* null cells were washed and pulsed for 4.5 hours with 30 nM cAMP every 10 minutes (see Materials and methods) and plated on a Petri dish with a hole in the center over which a glass coverslip has been glued in place for the chemotaxis assay. The tip of a micropipet containing 150 μM cAMP is visible. (A) Chemotaxis of wild-type cells. (B) Chemotaxis of *STATa* null cells. *STATa* null cells move very slowly and very few cells have accumulated at the tip after 30 minutes even though the initial density of the *STATa* null cells is the same as that of the wild-type cells (compare the '0 min' panels.**)** The enclosed boxes show a magnification of wild-type and *STATa* null cells. The *STATa* null cells are less polarized.

STATa null strain suggested that the null cells might exhibit a defect in cell sorting. To investigate this possibility and examine the full developmental potential of *Dd-STATa* null cells, we performed synergy experiments in which we examined the distribution of *Dd-STATa* null cells in chimeric slugs containing *Dd-STATa* null and wild-type cells.

Dd-STATa null cells tagged with *Act15/lacZ* were mixed with wild-type cells in different proportions and allowed to form chimeric slugs. The distribution of the *Dd-STATa* null cells in the chimeras was determined by histochemical staining for β-gal expression. Mixtures in which only 25% of the cells were wild-type formed normal-looking slugs and fruiting bodies, but staining revealed that the *Dd-STATa* null cells were entirely absent from the anterior prestalk region of the slug and were excluded from the stalk tube at culmination (Fig. 9A,B,E). The *Dd-STATa* null cells, however, differentiated into spores and basal disc cells (Fig. 9C,D). Similar results were obtained using either Ax2- or KAx-3-derived *Dd-STATa* null cells. Thus, a minority population of wild-type cells is able to rescue the potential of the *Dd-STATa* null cells to form spore cells but not to form prestalk cells (defining prestalk cells here as the cells that will enter the stalk tube at culmination). The wild-type cells do so by selectively forming the prestalk region of the slug and the stalk of the mature culminant.

Dd-STATa null cells ectopically express both *ecmB/lacZ* and *ST/lacZ* reporter constructs

The fact that *Dd-STATa* null cells are entirely excluded from the prestalk region in a synergy experiment with wild-type cells implies them to be defective. We find that they are indeed aberrant, in that they prematurely and ectopically express markers of stalk cell differentiation. In normal development, the *ecmB* gene is expressed in a cone of cells in the slug tip, the pstAB cells, and a band of cells at the front of the prespore region that are designated pstBA cells (Figs 1, 10Aa; Jermyn et al., 1989, 1996; Ceccarelli et al., 1991). The pstAB cells lie at the position where formation of the stalk tube is initiated at culmination and are periodically shed from the slug during migration, perhaps because of an abortive attempt at culmination (Sternfeld, 1992). The cells in the pstBA band are the precursors of the outer part of the basal disc and the lower cup, the structure that lies below, and perhaps helps support, the spore head (Dormann et al., 1996; Jermyn et al., 1996).

The pstAB cells are derived from pstA cells and are distinguished from their pstA precursors by expressing both the *ecmA* and *ecmB* genes. PstAB cells are believed to be committed to differentiate into stalk cells, but they do not do so while still within the slug. Instead, the core of pstAB cells periodically move from their anterior location, backwards through the slug, and are shed from the posterior, whereupon

Table 1. Comparison of the induction of stalk cell formation and a marker of stalk cell differentiation in Ax2 and *STATa* null cells

(A) The effect of 8-Br-cAMP on stalk cell formation in isolated prestalk cells

	8-Br-cAMP (mM)					
	0			10		
	Total	Stalk	%	Total	Stalk	%
Ax2	251	2	0.8	208	117	56.3
Corrected for prestalk purity			0.96			67.5
STATa null	235	0	0	417	114	27.3
Corrected for prestalk purity			0			47.9

Typical experiments are shown.

The assay conditions were those of Inouye and Gross (1993) except that cAMP was omitted from the stalk salts solution. Pooled prestalk regions of slugs were dissociated by titration through a 25-gauge needle, at 4°C in 20 mM potassium phosphate buffer, pH 6.1, containing 1 mM EDTA and 0.1 mM cAMP. Cell populations were sampled to establish the proportion of prespore cells, using a monoclonal antibody, MUD1, that recognizes a cell surface epitope on prespore cells (Krefft et al., 1983). The remaining cells were pelleted at approximately 700 ***g*** in a microcentrifuge and resuspended at a density of 2×10^4/ml in duplicate 3 cm Falcon tissue culture dishes containing 2 ml of stalk salts and 8-Br-cAMP at the concentration shown. Plates were scored after 24 hours. The percentage of stalk cells was in each case corrected for the proportion of prespore cells present.

(B) The effect of DIF and cAMP on the induction of the β-galactosidase reporter *ST/lac*Z

	Incubation time (hours)					
	23			48		
	Total	Blue	%	Total	Blue	%
Ax2						
–DIF/–cAMP	95	4	4.2	94	9	9.5
+DIF/–cAMP	61	44	72	143	96	67
+DIF/+cAMP	103	52	50.4	124	58	46
–DIF/+cAMP	86	18	20	87	25	28.7
STAT null						
–DIF/–cAMP	86	9	10.4	150	31	20.5
+DIF/–cAMP	63	35	55	112	79	70
+DIF/+cAMP	131	8	6.1	126	10	7.9
–DIF/+cAMP	98	0	0	78	0	0

(C) The effect of DIF and cAMP on stalk cell formation

	Incubation time (hours)					
	23			48		
	Total	Stalk	%	Total	Stalk	%
Ax2						
–DIF/–cAMP	113	0	0	240	3	2.1
+DIF/–cAMP	223	34	15.2	235	149	63.4
+DIF/+cAMP	119	0	0	230	69	30
–DIF/+cAMP	105	1	0.95	220	5	2.3
STAT null						
–DIF/–cAMP	119	0	0	200	1	0.5
+DIF/–cAMP	242	46	19	234	164	70.1
+DIF/+cAMP	100	0	0	230	1	0.43
–DIF/+cAMP	110	0	0	119	0	0

Typical experiments are shown.

The DIF assay conditions were essentially those of Berks and Kay (1988). Cells were harvested from axenic medium at less than 2×10^6 cells/ml and resuspended in stalk salts solution containing 5 mM cAMP, at a density of 10^5 cells/ml. 2-ml portions were dispensed into 3 cm diameter tissue culture dishes (Falcon). After 16 hours, the medium was aspirated off, and the cells washed twice with 1 ml stalk salts solution, then overlaid with 2 ml of fresh solution lacking cAMP. The stalk salts solution was augmented by various combinations, as shown above. DIF was added at 50 nM and cAMP at 5 mM. The plates were scored 23 and 48 hours after the initial harvesting. For scoring β-galactosidase staining, the cells were fixed in 1% glutaraldehyde for 5 minutes, washed twice in Z buffer, and stained overnight at 37°C. In the presence of a saturating level of DIF, *Dd-STATa* null cells express *ST/lac*Z just as efficiently as control wild-type cells. This holds true when considering both the fraction of cells that stain and the strength of staining of individual cells. [Note that the latter observation is important, because it suggests that the responding cells differentiate into pstAB cells rather than pstBA cells (i.e. lower cup or basal disc cells).] The basis for this interpretation derives from the strengths of expression of *ST/lac*Z in different tissues during normal development, which we estimate to be 50-100 times stronger in differentiating stalk cells than in the basal disc and lower cup cells. Therefore, if the *Dd-STATa* null cells were to differentiate into pstBA in response to DIF, we would expect their intensity of staining to be significantly lower than the control cells.

they very quickly differentiate into vacuolated stalk cells (Jermyn and Williams, 1991; Sternfeld, 1992). During culmination, differentiation of pstA to pstAB cells (i.e. expression of *ecmB*) occurs precisely at the stalk tube entrance, the place where cells are believed to become irreversibly committed to stalk cell differentiation. In *Dd-STATa* null cells, the pattern of expression of *ecmB* in the anterior of the slug is radically different from that in wild-type strains. In *Dd-STATa* null first fingers and slugs, the *ecmB* gene is expressed throughout the prestalk region (Fig. 10Ab,Ba), suggesting that Dd-STATa functions as the repressor of stalk cell differentiation (Ceccarelli et al., 1991). In the aberrant terminal structure formed by *Dd-STATa* null cells, *ecmB* is expressed in the supporting column (Fig. 10Bb,c).

Interpretation of results using the complete *ecmB* promoter is complicated by the fact that in the wild type, the *ecmB* gene is strongly expressed in multiple prestalk/stalk cell types, including the pstAB cells and pstBA cells (Figs 1, 10Aa). Different parts of the *ecmB* promoter direct expression in these different tissues in the mature fruiting body. We therefore analyzed the mutant further using *ST/lac*Z, a marker of the earliest known step in the terminal differentiation of prestalk cells into stalk cells: the formation of pstAB cells from pstA cells. Sequences distal to the cap site of the ecmB promoter direct expression in the upper cup cells, the structure that sits above the nascent spore head during culmination. Sequences proximal to the cap site, the ST (stalk) promoter region, direct strong expression in cells within the stalk tube (i.e. pstAB cells) and very weak expression in the stalk cells that form the outer basal disc and the lower cup (i.e. pstBA cells). With the important caveat that *ST/lac*Z is also very weakly expressed in basal disc and lower cup cells, the *ST/lac*Z reporter provides a marker of pstAB cell differentiation.

When transformed into wild-type Ax2 cells and analyzed at the slug stage or during formation of the terminal structures, *ST/lac*Z is expressed in a core of pstAB cells that lie at the position where the stalk tube will form at culmination (Fig. 11A). In contrast, in the *Dd-STATa* null slugs, *ST/lac*Z expressing cells are scattered throughout the prestalk region (Fig. 11B). This

result is consistent with our interpretation of the results using the complete *ecmB* promoter: if the Dd-STATa protein is indeed the stalk cell repressor protein then in its absence prestalk cells should express a marker for commitment to stalk differentiation (i.e. *ST/lac*Z) prematurely. (Note the expression of *ST/lac*Z in the *Dd-STATa* null slug is, however, very low and this forms part of the paradox analyzed below; why, if the repressor is absent, is there not uncontrolled stalk cell differentiation?)

Dd-STATa null cells ectopically express an *ecmA* promoter fusion gene containing an *ecm*B repressor element

In order to further test the notion that Dd-STATa is the stalk cell repressor, we used a *lac*Z fusion construct (*ecmAO-rep/lac*Z) in which an *ecmB* repressor element is placed downstream of the *ecmAO* promoter (Harwood et al., 1993). In wild-type slugs, this construct is expressed in pstO cells but not pstA cells (Fig. 10Ca), indicating the *ecmB* repressor elements limit expression directed by the proximal, pstA-specific part of the promoter. The repressor does not function on the more distal pstO-specific promoter elements, perhaps because of a distance effect (Harwood et al., 1993). In contrast, in *Dd-STATa* null strains, the *ecmAO-rep/lac*Z reporter is expressed throughout the entire prestalk region (Fig. 10Cb), indicating that the repressor activity is lost in *Dd-STATa* null strains.

Dd-STATa null cells are hyper-inducible by DIF in monolayer assay

In combination, the above observations suggest that Dd-STATa acts as the repressor protein that prevents stalk cell-specific gene expression until cells enter the stalk tube. We obtained further evidence consistent with this notion by analyzing stalk cell-specific gene expression in monolayer assay.

We first analyzed induction of the *ecmB* gene by DIF using northern transfer to quantitate mRNA levels. There is a major difference in the level of *ecmB* mRNA in cells exposed to DIF, with a greatly increased amount of *ecmB* mRNA accumulating in the *Dd-STATa* null cells (Fig. 12). We next analyzed the DIF response using cells transformed with *ST/lac*Z. Quantitative, biochemical analysis of β-galactosidase activity at different DIF levels shows that *Dd-STATa* null cells express *ST/lac*Z more efficiently than control cells, in that the dose response curve for DIF is shifted down by a factor of approximately three in the null mutant (Fig. 13; the 50% saturating DIF concentration is approximately 0.5 nM in the mutant and 1.5 nM in the parental strain).

Dd-STATa null cells can be induced to form stalk cells in monolayer assay using 8-Br-cAMP

If Dd-STATa is the repressor protein that regulates commitment to stalk cell differentiation, and if *Dd-STATa* null cells are hypersensitive to DIF for stalk-specific gene expression, why is there not uncontrolled, terminal stalk cell differentiation within the slug tip? It seems unlikely to reflect a loss of the potential to form stalk cells because, as noted above, cells that are discarded from the slug differentiate into stalk cells. However, we investigated this possibility further using a monolayer assay. When prestalk cells are

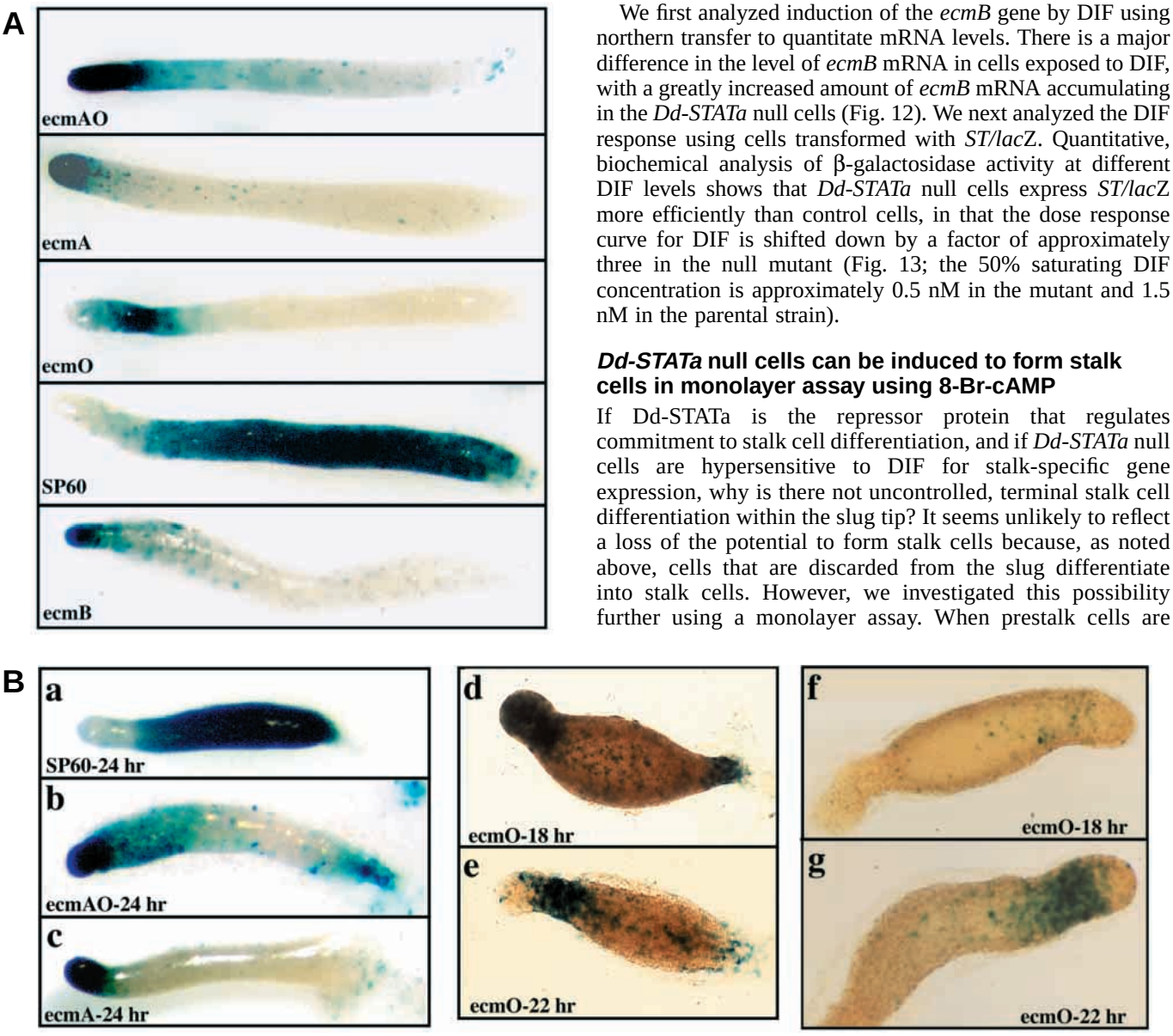


Fig. 7. Spatial patterning of cell types in wild-type and *Dd-STATa* null cells. (A) Spatial pattern of cell-type-specific *lac*Z reporter expression in wild-type KAx-3 slugs. Some of the data are taken from Yasukawa et al. (1998). (B) Cell-type-specific *lac*Z reporter analysis of *Dd-STATa* null strains. (a-c,f,g) KAx-3 background; (d,e) Ax2 background. For all panels, the reporter used and the developmental times are shown.

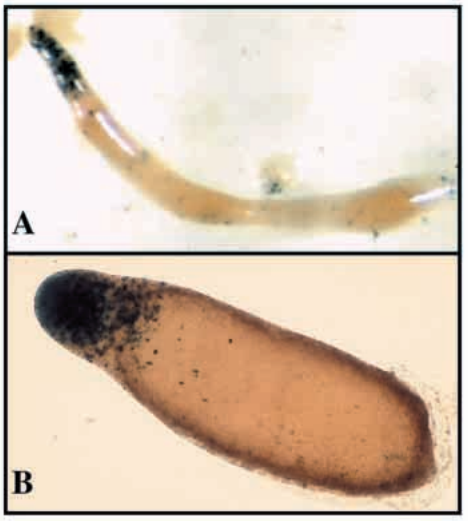


Fig. 8. Expression from the ecmO *cis*-acting element. Expression of a *lac*Z reporter driven by four copies of the ecmO 53-mer regulatory element (domain I in Kawata et al., 1996) is shown. (A) Wild-type cells (Ax2); (B) *Dd-STATa* null cells in the same background. Slugs are approximately 24 hours old.

surgically removed from the fronts of wild-type slugs and incubated in 8-Br-cAMP, a membrane-permeant cAMP analog, they are induced to differentiate into stalk cells (Inouye and Gross, 1993; Kubohara, 1993). The same holds true for *Dd-STATa* null cells, albeit at a slightly reduced level (Table 1A).

In a monolayer assay, *Dd-STATa* null cells are hypersensitive to the repressive effect of extracellular cAMP on stalk cell differentiation

The above experiments suggest that *Dd-STATa* null prestalk cells are able to differentiate first as pstAB cells and then as mature stalk cells under certain conditions, e.g. in the slime trail or in monolayer assay with 8-Br-cAMP, implying that conditions in the multicellular structures may be incompatible with such a differentiation. A possible cause of the block was revealed when we investigated the effect of extracellular cAMP on stalk cell differentiation. As noted in the Introduction, although increased PKA activity, presumably mediated by a rise in intracellular cAMP, is thought to cause terminal stalk cell differentiation, there is evidence to suggest that perceived extracellular cAMP levels must fall at culmination. This evidence comes from monolayer assays in which extracellular cAMP functions as an inhibitor of stalk cell differentiation if added to cells after they have become competent to respond to DIF. We find that extracellular cAMP at a concentration above 100 μM is a significantly more potent inhibitor of *ST/lac*Z expression (Table 1B) and terminal stalk cell differentiation (Table 1C) in the *Dd-STATa* null strain than in parental, Ax2 cells.

There is one potential artefact that needs to be considered in the above experiments. Developing *Dictyostelium* cells produce an extracellular cAMP phosphodiesterase (PDE), and degradation by PDE places a lower limit on the concentration of cAMP that can be assayed in the monolayer induction system. This might be a source of potential error if there were differential PDE levels in wild-type and mutant cells and could, perhaps, explain the slightly greater resistance of Dd-STATa null cells at cAMP concentrations lower than 100 μM. We therefore determined the behavior of the *Dd-STATa* null cells when treated with varying doses of Sp-cAMPS, a non-hydrolyzable cAMP analog. The Ax2 (parental) cells behave very similarly to cells dissociated at the tipped aggregate stage, in a study in which *ecmB/lac*Z was used to monitor total stalk cell-specific gene expression (Soede et al., 1996). Sp-cAMPS at concentrations as low as 5 mM totally inhibits stalk cell differentiation (Fig. 14), but stalk cell differentiation is

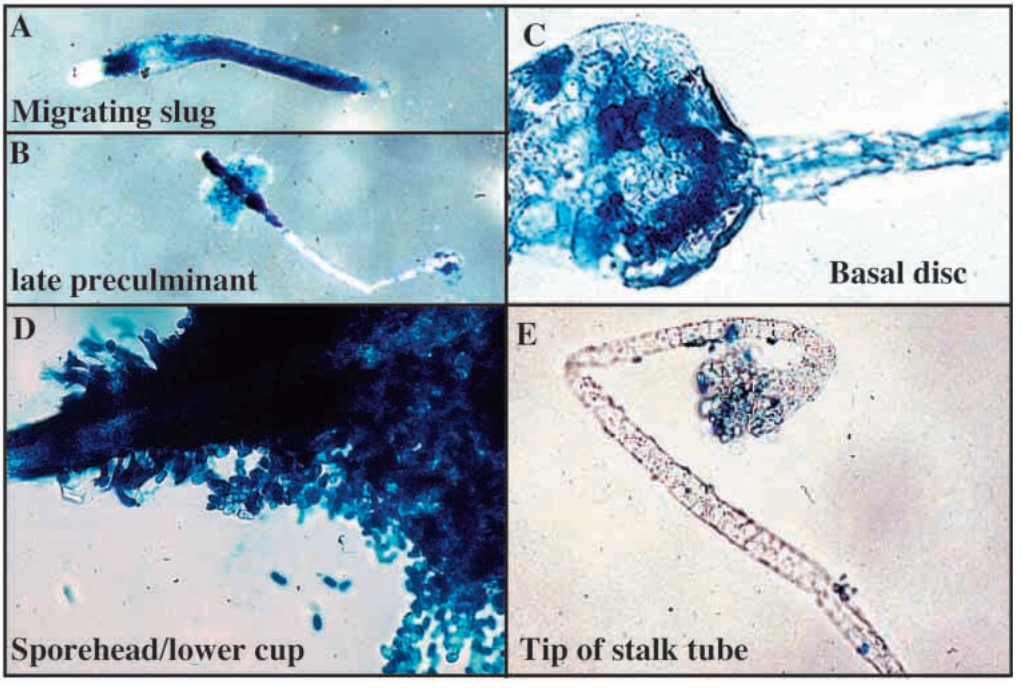


Fig. 9. Synergy of *Dd-STATa* null cells and wild-type cells. Three parts *Dd-STATa* null cells expressing *Act15/lac*Z, which marks all *Dd-STATa* null cells, were mixed with one part unlabeled wild-type cells and chimeric organisms were allowed to form. (A) Migrating slug; (B) late culminant; (C) basal disc of fruiting body with attached stalk; (D) enlarged view of lower cup and intersection of stalk and spore head containing stained spores; (E) tip of stalk tube from fruiting body. Note the lack of stain in the stalk.

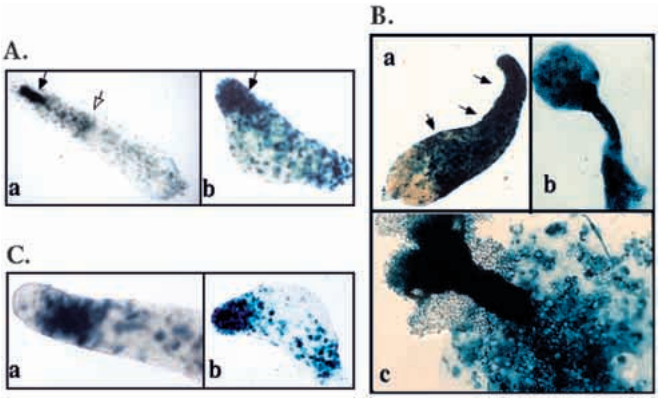


Fig. 10. Pattern of *ecmB/lacZ* staining. (A) Slugs of wild-type Ax2 and *Dd-STATa* null slug in Ax2 background. In wild-type strains, *ecmB/lacZ* expression is restricted to an interior core of cells in the anterior of the slug (solid arrow in a; see also Fig. 7A). In addition, staining is seen in the pstBA cells at the intersection of the prestalk and prespore domains (open arrow in a). In *Dd-STATa* null strains, expression is observed throughout the prestalk domain (solid arrow in b). (B) (a) Older *Dd-STATa* null slug (note extensive expression of *ecmB/lacZ* throughout all but the very posterior of the slug, marked with solid arrows); (b) terminal structure; (c) collapsed terminal structure with refractile, mature spores visible, and blue vacuolated stalk cells in slime trail. (C) Repressor function in Ax-2 wild-type (a) and *Dd-STATa* null (b) slugs. In a, only the anterior of the slug is shown. Staining is restricted to the pstO domain and some scattered anterior-like cells (ALCs).

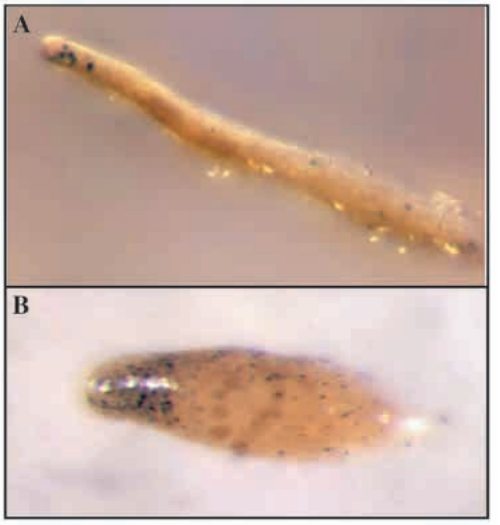


Fig. 11. *ST/lacZ* staining in Ax2 wild type (A) and *Dd-STATa* null (B) slugs.

partially recovered when the cAMPS concentration is raised to 100 mM. The *Dd-STATa* null cells differ radically in their behavior with Sp-cAMPS. Compared to Ax2 cells, they require a higher Sp-cAMPS concentration (about 20 μM) to totally inhibit stalk cell differentiation. However, the major difference with parental cells occurs at higher Sp-cAMPS concentrations, at which the *Dd-STATa* null cells show no trace of the 'recovery' of stalk cell differentiation observed with Ax2 cells at high Sp-cAMPS. In our hands this recovery occurs at

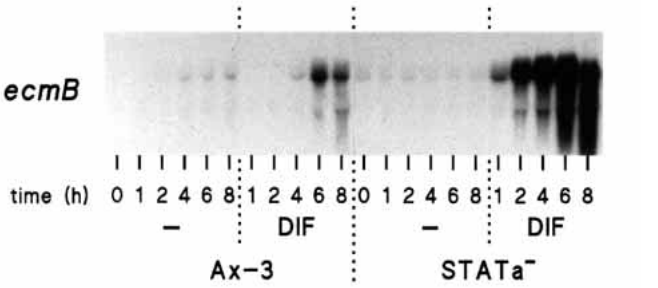


Fig. 12. Northern hybridization blot of DIF-induced RNA from Ax-3 wild-type and *STATa* null cells, probed with *ecmB* cDNA. Cells were harvested and developed for approximately 14 hours on non-nutrient agar until tipped mounds had formed. These were dissociated through a 19-gauge needle, and the cells resuspended in stalk salts (Inouye and Gross, 1993) at 3×10^6 cells/ml. They were divided into two samples, with 100 nM DIF added to one half, and both samples were shaken at 22°C and 150 rpm for 8 hours. RNA was isolated after 0, 2, 4, 6 and 8 hours of incubation and probed with *ecmB* cDNA.

cAMPS concentrations above approximately 50 mM. Because Sp-cAMPS is estimated to have an approximate 15- to 70-fold lower affinity for cAMP receptors than cAMP (Johnson et al., 1992), this result suggests that all cAMP concentrations greater than about 1 mM (if they could be maintained without degradation by endogenous cAMP) would totally repress stalk cell differentiation in *Dd-STATa* null cells while having only a marginal effect on Ax2 cells. This is totally consistent with the dose-response curves we obtain using cAMP rather than Sp-cAMPS (data not shown).

DISCUSSION

Dd-STATa is the repressor that regulates commitment to stalk cell differentiation

The *Dd-STATa* null mutant develops to the slug stage and expresses *ecmAO/lacZ*, a reporter fusion containing the promoter of the *ecmA* gene linked to the *lacZ* gene. This result shows that, although Dd-STATa binds avidly to the *ecmO* activator region in vitro, Dd-STATa is not essential for *ecmO* activation *in vivo*. We assume that some other protein binds to and activates transcription through the *ecmO* activator in vivo, aided perhaps by interactions with other transcription factors. We know, for example, that there is very likely to be an interaction between GBF, the transcription factor needed for the expression of most or all genes after aggregation (Schnitzler et al., 1994, 1995; Firtel, 1995), and the protein that binds the *ecmO* activator in vivo. The *ecmO* activator sequence synergizes very effectively with a G box, the GT-rich element that is the binding site for GBF (Kawata et al., 1996). Perhaps the in vitro binding data on Dd-STATa with the *ecmO* activation are misleading because they do not allow for the binding of, or transcriptional transactivation by, the partner proteins that are required for in vivo functioning.

The primary, direct evidence that Dd-STATa functions as the repressor that prevents precocious induction of stalk cell differentiation within the mound derives from our observations that *Dd-STATa* null cells are not responsive to the two repressor elements in the *ecmB* promoter, nor to a single repressor

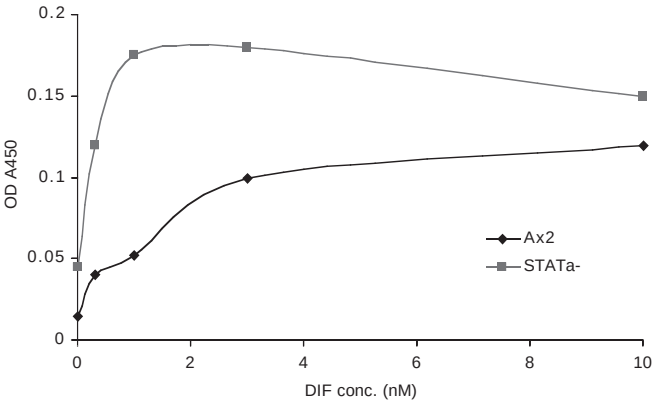


Fig. 13. Comparison of *ST/lac*Z induction by DIF in Ax2 wild-type and *Dd-STATa* null cells. Cells were harvested and washed twice in stalk salts, then diluted and incubated in stalk salts with 5 mM cAMP at 3×10^4 cells/cm^2 for 16 hours. They were rinsed twice and incubated in stalk salts (Birks and Kay, 1998) with DIF at the concentrations indicated. After 24 hours of induction, the cells were lysed in situ by freeze-thawing in 100 ml of Z buffer (Berks and Kay, 1988). Induction and enzymatic assay took place in the same well. To start the reaction 30 ml of 2.5× Z buffer and 20 ml of 10 mg/ml O-nitrophenyl-beta-D-galactopyranoside in 2.5× Z buffer ($MgSO_4$, Na_2HPO_4, NaH_2PO_4 and KCl) were added to each well. The reaction proceeded for 12-14 hours and was stopped by addition of 100 ml of 0.4 M Na_2CO_3. The reactions were transferred to a 96-well microtitre plate and the absorbance at 405 nm was determined with an LKB 1230 ARCUS plate reader.

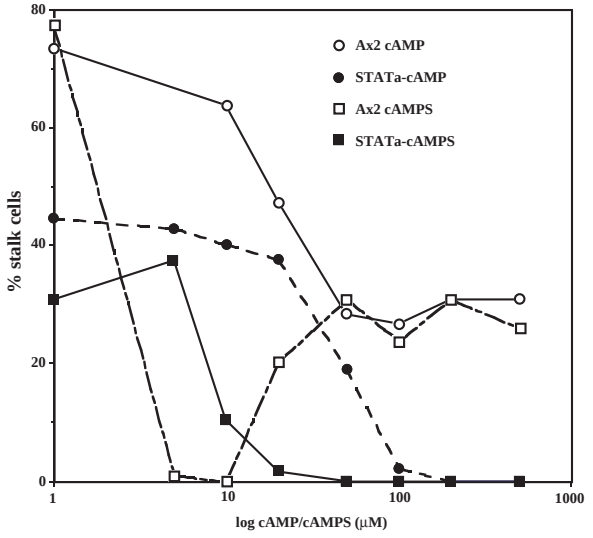


Fig. 14. The effect of increasing concentrations of cAMP and Sp-cAMPS on stalk cell formation with 50 nM DIF. Conditions were the same as Table 1B,C except that the cell concentration was 5×10^4 cells/ml, and stalk cells were scored 24 hours after DIF addition. A low cell density was used in order to negate the effect of endogenously produced DIF and this had an inhibitory effect on the absolute number of stalk cells formed, which varied between strains and between experiments. However, the inhibitory effect of cAMP and cAMPs was entirely reproducible.

element placed downstream of the *ecmAO* promoter. In both cases, we observe expression of the reporters throughout the pstA domain in *Dd-STATa* null cells. This is in contrast to the results in wild-type cells in which the repressor, but not a mutated repressor sequence that cannot bind Dd-STATa, prevents expression of the reporter in the pstA domain (Harwood et al., 1993). The result with the hybrid promoter (*ecmAO/ecmB* repressor) construct is important because it argues against the possibility that the ectopic *ecmB/lac*Z expression observed in the *Dd-STATa* null slugs using the *ecmB* promoter might be due to an effect of the *Dd-STATa* null mutation on the *ecmB* activator elements. This repressor function of Dd-STATa is consistent with our observation that Dd-STATa, which initially becomes nuclear-localized in all cells within the mound, is preferentially found in nuclei only in the pstA cells in the slug (Araki et al., 1998). This places Dd-STATa in the nuclei of just those cells where we expect it to function as a repressor, restricting stalk cell differentiation prior to culmination.

The above analyses indicate that the combined activator-repressor model for prestalk-stalk cell differentiation is, in its simplest form, incorrect. Dd-STATa is the repressor and there is a yet-to-be-identified prestalk activator protein. It remains to be seen whether the prestalk activator, also a TTGA binding protein, is a STAT, possibly one of the two newly identified STATs: Dd-STATb or Dd-STATc (M. Fukuzawa, T. Araki and J. G. W., unpublished observation). In gel retardation assays of wild-type and *Dd-STATa* null strains, we detect other bands using the *ecmO* activator probe. One of these has the correct competition pattern to contain the activator protein but is of such low abundance that we have not been able to determine whether it is tyrosine phosphorylated (T. Kawata, unpublished observation).

The fact that Dd-STATa is a repressor is somewhat unusual because, in most cases, STATs function as activators. However, in mammalian cells, there is one relevant case in which a STAT functions as a repressor. There are naturally occurring variants of STAT5 that lack the C-terminal transcriptional trans-activation domains common to most STAT isoforms. These variants act as dominant inhibitors of STAT5 signaling when overexpressed (Wang et al., 1996). This may be a particularly significant precedent, because there is no C-terminal transcriptional activation domain in Dd-STATa.

Of course, we cannot exclude the possibility that Dd-STATa may also have a separate activator function. Indeed such a function could account for the other *Dd-STATa* null phenotypes in which we find that absence of the Dd-STATa protein produces effects on cell movement and on the sensitivity of cells to various extracellular signals.

Dd-STATa is necessary for normal chemotaxis, aggregation and movement into the prestalk region in synergy experiments

Although Dd-STATa shows a major increase in tyrosine phosphorylation and nuclear localization at the mound stage, some tyrosine phosphorylated Dd-STATa is found during early development. Moreover, indirect immunofluorescence suggests that some Dd-STATa is nuclear during early development (Araki, 1998) and, consistent with some role in early development, we find that *Dd-STATa* null cells are defective in chemotaxis to cAMP. This could be the result of an intrinsic defect in cell movement or in the chemotactic signaling pathways that direct movement towards cAMP.

Defective chemotaxis could explain the delayed and aberrant aggregation of Dd-STATa cells. One clearly discernible difference between the parental and *Dd-STATa* null strains is the apparent rate at which the pstA and pstO populations segregate from one another; in the *Dd-STATa* null cells, segregation seems to be significantly slower. There is substantial evidence that during *Dictyostelium* development, cAMP signaling (which mediates chemotaxis during aggregation) persists in the slug, with the tip as the signaling center (reviewed by Siegert and Weijer, 1997). The pstA and pstO cells occupy and maintain their relative positions by differential chemotaxis to cAMP (Abe et al., 1994). Thus, one possible explanation for the delayed segregation into discrete pstA and pstO regions is that the *Dd-STATa* null cells are inefficient in chemotaxis to cAMP signals emanating from the tip. A defect in chemotaxis at later stages is also supported by the synergy experiments, in which we observe that *Dd-STATa* null cells are excluded from the pstA region of the migrating slug and from entering the stalk tube at culmination.

The *Dd-STATa* null strain is unable to undergo normal culmination and during migration there is a change in the proportion of cell types

The most striking feature of the development of the *Dd-STATa* null strain is the highly aberrant nature of the terminal structures. Microscopic examination reveals no trace of a stalk tube. At culmination in wild-type strains, *ST/lac*Z activation occurs exactly at the place where the prestalk cells move into the stalk tube and it is therefore a good cell-type marker for stalk tube formation. There is a low level expression of *ST/lac*Z in the Dd-STATa strain but, as would be expected from the absence of the repressor protein, this occurs in cells scattered throughout the prestalk region.

The lack of *ST/lac*Z expression in the majority of the prestalk cells (which express the whole *ecmB* promoter/*lac*Z construct) suggests that the most of the prestalk cells within the tip of *Dd-STATa* null slugs are pstBA cells, i.e. cells that at first expressed the *ecmB* gene and later expressed the *ecmA* gene (Fig. 1). This fact may explain their defective sorting behavior. Analysis of cell movement patterns in wild-type strains shows that pstA and pstO cells move apically within the mound, whereas pstBA cells move basally (Early et al., 1995; Williams et al., 1989). Thus, cells such as the *Dd-STATa* null cells, with phenotypic characteristics of both pstA/O and pstBA cells, might have a reduced ability to move to the tip.

Dd-STATa null cells show altered behavior in monolayer assay conditions

Some of the aberrations that are displayed during normal development of *Dd-STATa* null cells can be overcome when the strain is analyzed under monolayer assay conditions, in which differentiation is uncoupled from morphogenesis. In the monolayer assay, stalk cell differentiation and *ST/lac*Z expression are efficiently induced in the *Dd-STATa* null cells by DIF. Indeed, in the *Dd-STATa* null cells, expression directed by the entire endogenous *ecmB* promoter in the presence of a saturating amount of DIF is about tenfold higher than in parental cells and expression of *ST/lac*Z is also both about tenfold hypersensitive to DIF. These two results add weight to the notion that Dd-STATa is the stalk cell repressor because some models for such a regulatory system, composed of separate activator and repressor domains, predict a reduction in inducer threshold when the repressor function is eliminated. Increased sensitivity to DIF may also explain the increase in the pstO population and decrease in the prespore population during slug migration.

The absence of stalk cell formation, despite a hypersensitivity to DIF, could be explained if DIF levels were lower in the *Dd-STATa* null structures. However, we believe this to be unlikely because prestalk cell differentiation occurs normally. Rather, we suspect that the failure to undertake terminal stalk cell differentiation in the multicellular structure derives from hypersensitivity to the inhibitory effect of extracellular cAMP. All testable cAMP concentrations used (i.e. those above about 100 μM, but note that the actual levels will be lower than this because of PDE activity) permit stalk cell differentiation in Ax2 cells but totally repress stalk cell differentiation in *Dd-STATa* null cells (data not shown). This is not due to a difference in effective cAMP concentration caused by a difference in PDE production levels in wild-type and *Dd-STATa* null cells, because low concentrations of Sp-cAMPS repress stalk cell differentiation in both strains and Sp-cAMPS is refractory to degradation by PDE. Instead, the parental cells and the *Dd-STATa* null cells differ in that high Sp-cAMPS concentrations, and by implication high cAMP concentrations, do not bring about the ‘recovery’ of stalk-specific gene expression observed in Ax2 cells (Soede et al, 1996).

The recovery phenomenon is not yet understood and it is not therefore possible to explain why absence of the Dd-STATa protein should affect it. However, the concentration of Sp-cAMPS that causes reactivation, 50-100 mM, is equivalent to 1-2 mM cAMP and this is well below the extracellular cAMP concentration predicted to exist within the multicellular structures. The failure of Dd-STATa cells to show reactivation by extracellular cAMP could therefore entirely account for the failure of *Dd-STATa* null cells to express *ST/lac*Z at a high level and undergo normal culmination even in the presence of a predominance of wild-type cells.

We believe that, in combination, the above data strongly suggest that Dd-STATa is the repressor that prevents premature commitment to stalk cell differentiation. The further suggestion, that the Dd-STATa null cells fail to differentiate into stalk cells because of hypersensitivity to the inhibitory effect of extracellular cAMP, obviously implies that Dd-STATa cannot be the molecule that mediates the repressive effect of extracellular cAMP. Two pieces of evidence support this notion. The direct evidence is that an ecmB construct lacking the two repressor elements remains subject to cAMP repression (A. Ceccarelli, N. Zhukovskaya, Y. Yamada, A. Harwood and J. Williams, manuscript in preparation). The other evidence is that constitutive expression of the C subunit of PKA, a condition that would be expected to render the ecmB repressor elements constitutively inactive (because activation of PKA at culmination is believed to be the signal that negates the inhibitory effect of the repressor elements), leaves *ST/lac*Z sensitive to the inhibitory effect of extracellular cAMP (Hopper et al., 1993).

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