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RtoA links initial cell type choice to the cell cycle in *Dictyostelium*

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SUMMARY

In *Dictyostelium*, initial cell type choice is correlated with the cell-cycle phase of the cell at the time of starvation. We have isolated a mutant, *ratioA (rtoA)*, with a defect in this mechanism that results in an abnormally high percentage of prestalk cells. The *rtoA* gene has been cloned and sequenced and codes for a novel protein. The cell cycle is normal in *rtoA*. In the wild type, prestalk cells differentiate from those cells in S or early G_2 phase at starvation and prespore cells from cells in late G_2 or M phase at starvation. In *rtoA* mutants, both prestalk and prespore cells originate randomly from cells in any phase of the cell cycle at starvation.

Key words: cell fate, cell-type ratio, eukaryotic differentiation, *Dictyostelium*

INTRODUCTION

Development occurs by the progression of individual cells along pathways whose direction and final destination are influenced by both intrinsic and extrinsic factors. At each branch point along the differentiation pathway, a cell may be directed towards a particular fate by interactions with other cells or a cell may select its fate based on internal signals. The simplest case of cell fate determination is development involving only a single dichotomous choice. This situation is approximated by development in *Dictyostelium discoideum*, in which the mature organism, the fruiting body, is composed of two main cell types: spore cells and stalk cells.

Dictyostelium is a haploid eukaryotic amoeba that feeds on soil bacteria and divides by fission. When food sources become depleted and amoebae starve, the developmental program is initiated. Starving amoebae secrete a protein called conditioned medium factor (CMF) (Gomer et al., 1991; Jain and Gomer, 1994; Jain et al., 1992a; Yuen and Gomer, 1994). When enough amoebae in an area have starved, CMF concentrations reach levels sufficient to trigger aggregation (Yuen et al., 1995). Using relayed pulses of cAMP as a chemoattractant, approximately 10^5 amoebae form an aggregate (Roos et al., 1975). If necessary, a motile slug forms and migrates to a suitable environment for fruiting body development. In the fruiting body, a spore mass is elevated by a column of stalk cells, allowing wind or insects to efficiently disperse the spores (Loomis, 1975).

The precursors of the two main cell types can be identified in the aggregate and slug. Approximately 12% of the cells in a slug label with antibodies to cathepsin protease 2 (CP2), a prestalk marker (Gomer et al., 1986; Gomer and Firtel, 1987). These prestalk cells are initially scattered randomly throughout the aggregate but then form the anterior tip of the slug (Datta et al., 1986; Gomer et al., 1986). Antibodies to SP70, a prespore marker, label the posterior portion of the slug. Approximately 47% of the cells in a slug are prespore cells (Fosnaugh and Loomis, 1993; Gomer et al., 1986; Haberstroh and Firtel, 1990). The remaining cells in the slug, called null cells, express neither CP2 nor SP70 (Gomer et al., 1986). *ecmA*, a more general prestalk marker, is expressed after CP2, and the first *ecmA*-positive cells in the aggregate are a subset of the CP2-positive cells. The number of *ecmA*-positive cells increases, so that at approximately 20 hours of development, the CP2-positive cells and the *ecmA*-positive cells are almost completely overlapping sets. As the slug becomes a fruiting body, all of the CP2-positive cells and some of the null cells express *ecmA* (Clay et al., 1995; Early et al., 1993; Jermyn et al., 1987).

There have been two conflicting hypotheses to explain the initial differentiation of prestalk and prespore cells. According to one hypothesis, initial cell fate is controlled by the concentration of a morphogen in the immediate vicinity of a cell (Early et al., 1995; Kay et al., 1989). Prestalk differentiation, which is often measured by expression of a marker by the *ecmA* promoter, is proposed to result from exposure of cells to a high concentration of the chlorinated hydrocarbon DIF-1 (Differentiation Inducing Factor) (Kay, 1982). Two observations tend to contradict this theory. In slugs, high levels of DIF-1 are present in the prespore cell region and only low levels of DIF-1 are found in the prestalk cell region (Brookman et al., 1987). In addition, mutants that do not accumulate DIF-1 still produce both prestalk and prespore cells (Shaulsky et al., 1995; Shaulsky and Loomis, 1996), and several genes that are expressed in prestalk cells are insensitive to DIF-1 (Saxe III et al., 1996; Shaulsky and Loomis, 1996). A prediction based on the morphogen gradient theory is that cells exposed to the same concentration of DIF will have the same fate. Clay et al. (1995) demonstrated that this is not the case. Cells carrying the *ecmA* prestalk promoter fused to *lacZ* were plated at low density, starved, induced to develop and treated with DIF-1. Even though every cell was exposed to identical conditions, at most

only 25% of the cells expressed *ecmA*. These results suggest that initial cell fate is a cell-autonomous process and is not dependent on a morphogen gradient.

We and others have hypothesized that initial cell-type choice is a cell-autonomous process correlated with the cell-cycle phase of the cell at the time of starvation (Araki et al., 1994; Gomer and Firtel, 1987; McDonald and Durston, 1984; Ohmori and Maeda, 1987; Weijer et al., 1984a; Zimmerman and Weijer, 1993). Thus, prestalk cells develop from cells that were in the S phase or early G_2 phase of the cell cycle at starvation. Similarly, prespore cells develop from cells that were in the late G_2 or M phase of the cell cycle at starvation (*Dictyostelium* does not have a G_1 phase (Weijer et al., 1984b)). Several lines of evidence support this hypothesis. When a mixture of synchronized, labeled cells and asynchronous, unlabeled cells is starved and allowed to aggregate, cells in S phase or early G_2 at starvation sort to the anterior tip of the slug, the prestalk region, while cells in late G_2 at starvation sort to the posterior portion of the slug, the prespore region (Araki et al., 1994; McDonald and Durston, 1984; Ohmori and Maeda, 1987; Weijer et al., 1984a). In similar experiments, synchronized populations of cells starved in S or early G_2 formed slugs with abnormally low percentages of prespore cells; cells starved in late G_2 formed slugs with abnormally high percentages of prespore cells (Wang et al., 1988; Weijer et al., 1984a). Furthermore, when synchronized cells are starved in S or early G_2 and plated at low density, they form primarily prestalk or null cells, while those starved in late G_2 or M phase form mainly prespore or null cells (Gomer and Firtel, 1987). Finally, in experiments that did not require cell synchronization, we used time-lapse videomicroscopy of cells to trace the history of cells that differentiated at such low cell density that the effects of cell-cell communication were eliminated, and each cell was in an identical environment (Gomer and Firtel, 1987). By determining the cell-cycle position of each cell at the time of starvation, we demonstrated that cells in S or early G_2 differentiate into prestalk cells and cells in late G_2 differentiate into prespore cells. Under these conditions, approximately 12% of the cells express CP2 and 37% express SP70. We also found that for each differentiated cell there is a sister cell from the last division prior to starvation that initially does not differentiate but instead forms a null cell. Therefore, initial differentiation is also regulated by asymmetric cell division. When the length of S phase is increased by mild treatment of cells with DNA synthesis inhibitors, there is a concomitant increase in the percentage of CP2-positive or *ecmA*-positive prestalk cells in a low-cell-density differentiation assay (Gomer and Ammann, 1996). This indicates that initial cell-type choice is directly linked to the cell cycle and does not employ an independent timing mechanism.

Cell-cell interactions then modify the final ratios of cell types. For instance, when a null cell touches another cell, it sometimes becomes a prespore cell (Gomer and Firtel, unpublished observations). The prestalk-to-prespore ratio and final differentiation into stalk or spore cells can be influenced by a variety of extracellular factors such as adenosine, ammonia, oxygen and DIFs (Brookman et al., 1987; Gross et al., 1983; Kay et al., 1989; Kwong and Weeks, 1989; Schaap and Wang, 1986; Sternfeld, 1988; Williams et al., 1987; Xie et al., 1991). The different cell types can interconvert, indicating the action of a homeostasis mechanism (reviewed in Williams, 1988).

To examine the mechanisms of asymmetric cell division and cell-cycle-dependent cell-type choice, mutants with defects in these processes were identified in a shotgun antisense mutagenesis screen (Spann et al., 1996). Assuming that genes involved in these mechanisms are active at the time of starvation but are not required for cell division, the screen was designed to select for genes that are expressed in vegetative cells, and when repressed affect development but not growth. A cDNA library was placed downstream of a vegetative promoter in an orientation that resulted in the transcription of antisense RNA. The library was transformed into *Dictyostelium* and transformants were screened for developmental defects. The antisense cDNAs from transformants with normal growth but morphologically abnormal development were isolated using PCR, ligated into fresh vector and retransformed into *Dictyostelium*. In each case, the original mutant phenotype was repeated. The low cell-density differentiation assay was used to identify mutants with cell-autonomous defects in cell-type choice and to allow exclusion of those mutants with defects in cell-cell communication. One of the mutants identified in this screen has a delay in development at the mound stage. We find that this mutant has an abnormally high percentage of prestalk cells and a normal percentage of prespore cells. We have named this mutant ratioA (*rtoA*asr), the asr suffix designating antisense repeat. We describe here the disruption of *rtoA* by homologous recombination, which results in a mutant with the same phenotype as the antisense mutant. The sequence of *rtoA* indicates that RtoA is a novel protein with little similarity to any known protein. We show that the defect in *rtoA* that causes the differentiation of excess prestalk cells is not within the cell cycle itself but is within the mechanism that senses cell-cycle phase at starvation and uses this information to direct differentiation towards either prestalk or prespore cell development.

MATERIALS AND METHODS

Cell culture

Ax4 cells, an axenic *Dictyostelium discoideum* strain, were grown in shaking culture in HL-5 media supplemented with antibiotics (Gomer et al., 1991). For development, cells at mid-log phase (2×10^6 cells/ml) were washed in buffer and plated on filter pads (Jain et al., 1992b). Conditioned medium (CM) was prepared as described previously (Yuen et al., 1991). To determine the percentages of prestalk and prespore cells at low cell density, mid-log phase cells were collected, washed in PBM, resuspended in a 10% solution of CM and plated at 2500 cells/well in a 8-well glass bottom chamber slide (Nunc, Naperville, IL). After 6 hours of starvation in CM, cAMP was added to a final concentration of 300 μM and 12 hours later cells were fixed in ethanol. To determine the percentages of prestalk and prespore cells in aggregates, cells at mid-log phase were plated at 2×10^6 cells/cm^2 on water agar or fiter pads. After 15 hours, at the mound stage, cells were dissociated in PBM, plated in a chamber slide and fixed in ethanol. To label prestalk cells, slides were stained with anti-CP2 antibodies and, for prespore labeling, slides were stained with anti-SP70 antibodies (Gomer and Firtel, 1987).

Generation of gene disruption mutants

A genomic fragment containing *rtoA* was isolated based on modification of a method from Sambrook et al. (1989). Briefly, a library of 3.8-4.3 kb *Hin*dIII/*Eco*RI fragments in pBluescript (Stratagene, La Jolla, CA) was transformed into DH5α cells (Gibco, Grand Island,

NY). Colonies were grown on duplicate grid plates and, after 12 hours, one plate was overlaid with a ICN nylon membrane and incubated for 1 hour at 37°C. The membrane was probed with the 266 bp *rtoA* cDNA radiolabeled with ^{32}P-dCTP (NEN, Boston) using a Multiprime labelling kit (Amersham, Arlington Heights, IL). The hybridizations were performed at 60°C in 0.125 M Na_2HPO_4, pH 7.2, 0.25 M NaCl, 5% SDS, 0.001 M EDTA, 10% PEG (average relative molecular mass 8000) overnight. The washes were done with 0.05 M Na_2HPO_4, pH 7.2, 0.5% SDS one time at room temperature for 15 minutes, then 2× 15 minutes each at 55-60°C.

To generate a knock-out mutant, we utilized pDH29 (a gift from Dr Peter Devreotes, Johns Hopkins University), which was made by inserting the *pyr5-6* gene into the *Cla*I site of the polylinker of pJB1. Using the *Hin*dIII-*Eco*RI genomic fragment in pBluescript as a template, two PCR fragments were made. A primer complementary to *rtoA* coding sequence with a *Xho*I tail (5′-TGTGCTCGAGTGATGATGATTCAGATCC-3′) and T7 were used to make one fragment, and a primer complementary to *rtoA* coding sequence with a *Hin*dIII tail (5′-TGTGAAGCTTTCAAAAAGTACCTGTTGC-3′) and T3 were used to make the other. All DNA oligomers were synthesized by GibcoBRL (Gaithersburg, MD). These fragments were inserted into the *Xho*I site and *Hin*dIII-*Eco*RI site of pDH29, respectively. The resulting plasmid contains 1.9 kb of genomic sequence at the 5′ end of *rtoA*, the ura cassette replacing 340 bp of *rtoA* coding sequence, followed by 1.9 kb of genomic sequence at the 3′ end of *rtoA*. For transformation, this plasmid was cleaved with *Apa*I and *Eco*RI. 20 μg of this fragment was gel-purified using Geneclean (Bio101) and used to transform *Dictyostelium* DH1 cells (a gift of Dr Peter Devreotes). Transformation and selection of homologous recombination gene disruption transformants followed the protocol of Kuspa and Loomis (1992).

cDNA cloning

The 4.1 kb *Hin*dIII-*Eco*RI *rtoA* genomic fragment was used to probe a λgt11 library of *Dictyostelium* cDNA (Clontech, Palo Alto, CA) following Jain et al. (1992b). The cDNA inserts of positive clones were amplified by PCR using Pfu enzyme (Stratagene, La Jolla, CA). The PCR products were purified using the Wizard PCR kit (Promega, Madison, WI), cloned into the *Eco*RI site of pBluescript and sequenced. A 1.1 kb cDNA corresponding to the 3′ end of the gene was isolated. To obtain additional cDNA, a RNA PCR kit (Perkin Elmer) was used. The template was poly(A)+ RNA isolated with a FastTrack 2.0 kit (Invitrogen, San Diego, CA). Antisense primers immediately downstream of the intron (see Fig. 1A) (5′-CACTTGAAAGAGATTGCC-3′) and (5′-CCTGAATTTGAAGAGCTTGAAAC-3′) were used for first strand synthesis. The cDNA was then amplified using a series of primers upstream of the intron. Two primers that yielded product (5′-GTTTAAACCTGGATTTCATTCAC-3′) and (5′-GGAATTTATATAGAAAATGTGC-3′) correspond to sequence 610 bp and 670 bp from the *Hin*dIII site. A primer 500 bp from the *Hin*dIII site (5′-GGTCACCTTTTTCCCGATTG-3′), as well as primers further upstream failed to yield product.

Sequencing, Southerns and northerns

DNA was concentrated and desalted using a Microcon-100 micro concentrator (Amicon, Beverly MA). DNA sequencing was done with a Dye Terminator Chemistry Kit (Perkin Elmer, Foster City, CA) at the University of Texas Medical School at Houston core sequencing facility. Sequence was analyzed with software from the Genetics Computer Group (Madison, WI). Total genomic DNA was isolated from Ax4 cells using a Blood and Cell Culture DNA Kit (Qiagen, Chatsworth, CA) with the modification of using 5×10^8 to 1×10^9 cells/ml instead of 1×10^7 cells/ml as called for in the Qiagen protocol. Southern blots were done using Bio-Rad Zeta-Probe GT membranes (Bio-Rad, Hercules, CA). RNA isolation and northern blot analysis followed Jain et al. (1992b) with the exception that hybridization and washing were done as for the colony screening above. Northern blots were probed with a 390 bp *Bam*HI-*Eco*RI cDNA fragment corresponding to the 3′ end of *rtoA* or a 700 bp *Hin*dIII-*Fok*I genomic fragment from the 5′ end of *rtoA*. Blots were imaged with a Fujix BAS 1000 Bio-imaging Analyzer (Fuji, Tokyo).

Cell-cycle analysis

All procedures were at room temperature unless otherwise noted. 4′,6-Diamidino-2-phenylindole (DAPI) was from Sigma (St. Louis MO); RNAse A was from Sigma and was made DNase-free following Sambrook et al. (1989). The doubling time of cells was determined following Gomer and Ammann (1996). To determine the percentage of cells in M phase, 10^4 to 10^5 cells in 350 μl of HL5 were placed on an 18 mm^2 glass coverslip and allowed to settle for 10 minutes in a humid chamber. The medium was poured off and the cells were then covered with 150 μl of 70% ethanol. After 10 minutes, the cells were then air-dried for 1 hour. The cells were then stained for 15 minutes in the dark in (2 μg/ml DAPI, 0.1% NP-40 in PBS) and then rinsed for 5 minutes in PBS in the dark. The coverslip was then mounted in glycerol/para-phenylenediamine (Johnson and de C. Nogueira Araujo, 1981). The number of cells and mitotic figures were counted using a Microphot FX epifluorescence microscope (Nikon, Melville, NY) with a DAPI filter cube (Omega Optical, Brattleboro, VT) and a Zeiss 1.4 n.a. 63× objective. The percentage of cells in S phase was determined by pulse-labelling cells with BrdU, fixing and staining with anti-BrdU antibodies following Gomer and Ammann (1996) with the exception that the BrdU pulse was for 10 minutes and the cells were then allowed to settle on a slide for 10 minutes. For G_1/G_2 analysis by flow cytometry, 2×10^6 mid-log phase cells in shaking culture were collected by centrifugation at 800 ***g*** for 5 minutes, resuspended in 2 ml of PBM, and fixed by adding 5 ml of 95% ethanol with gentle vortexing. After 30 minutes, the cells were collected by centrifugation at 2000 ***g*** for 5 minutes and resuspended in 2 ml ice-cold TN (10 mM Tris HCl, 100 mM NaCl pH 7.4), collected by centrifugation and resuspended in 1 ml PI stain (made as 1 ml 500 μg/ml propidium iodide (Sigma) with 9 ml TN) on ice for 30 minutes. 20 μl of 5 mg/ml DNase-free RNase A was added 10 minutes after adding the stain. The DNA content of the cells was analyzed on an Epics profile analyzer (Coulter, Hialeah FL).

Videomicroscopy

Conditioned medium (CM), PBM buffer and Z buffer were prepared as described in Clay et al. (1995). Mid-log phase cells in shaking culture were diluted to 4×10^3 cells/ml in HL5 without antibiotics. 200 μl of the cells were placed in one well of a type 177402 eight-well glass slide (Nunc). A reference mark was scored on the bottom of the slide with a carbide scriber. The cells were observed with a 4× objective on a TMS inverted scope (Nikon, Melville, NY) with a green interference filter and a blue filter on the light source. An ITC-400 CCD camera (Ikegami, Maywood, NJ) was mounted on the microscope and connected to an EVT-801 WatchCorder (Sony, Melville, NY), which recorded 1 image every 8 seconds, and played back 60 images/second. Approximately 18 hours after being plated in growth medium, cells were starved by gently removing the growth medium and adding 200 μl of (10% CM in PBM). 6 hours after starvation, 1.2 μl of 50 mM cAMP was added to the well and the slide was shaken very gently for mixing. Cells were fixed at 12 hours after starvation by adding 200 μl of fix (1 ml 25% glutaraldehyde in 11.5 ml Z buffer). After 3 minutes, the liquid was removed and replaced with 200 μl of 95% ethanol; the slide was then air-dried 10 minutes later. After air-drying, the slides were stored in a dessicator at –20˚C. The cells were videotaped continuously from when they were first put down in growth medium until after they were fixed. To facilitate following the cells on the videotape, the position of the cells was marked on the monitor when the cells were starved or reagents were added, so that the slide could be returned to the same place after having been moved. Before staining for the CP2 prestalk and the SP70

prespore antigens by immunofluorescence following Gomer and Firtel (1987), the cells were covered with 53 mM $NaBH_4$ (EM Sciences, Cherry Hill, NJ) for 5 minutes to reduce nonspecific fluorescence from the glutaraldehyde.

RESULTS

rtoA was identified in a vegetative cell cDNA shotgun antisense mutagenesis screen designed to identify genes which, when repressed, affect development but not growth (Spann et al., 1996). Transformants with any sort of abnormal morphology were examined further to determine the percentage of prestalk and prespore cells using a low cell-density differentiation assay. The original and repeat antisense *rtoA* mutants (*rtoA*as and *rtoA*asr) had a developmental delay at the mound stage. Out of 58 shotgun antisense mutants and 200 REMI mutants (Kuspa and Loomis, 1992) with developmental defects, *rtoA*asr and another shotgun antisense transformant were the only mutants that consistantly had a prestalk percentage greater than 12%. In well over 2000 assays of wild-type cells, the percentage of prestalk cells has never exceeded 12%. In the parental strain Ax4, 9.32 ±1.41% of the cells expressed CP2, a marker of prestalk cell differentiation. In contrast, 14.92±2.64% of *rtoA*asr cells are CP2-positive; the difference is significant at P=0.025.

The *rtoA*asr phenotype resulted from the antisense expression of a 266 bp cDNA. Southern blot analysis, using the 266 bp antisense cDNA fragment as a probe, suggested that *rtoA* is a single gene and is contained within a ~4 kb *Hin*dIII-*Eco*RI genomic fragment. The antisense cDNA was used to probe a genomic *Hin*dIII-*Eco*RI restriction fragment library and a 4.1 kb genomic clone of *rtoA* was isolated. This clone was then used to probe a cDNA library and a 1.1 kb cDNA clone was isolated. The 1.1 kb cDNA fragment maps to an

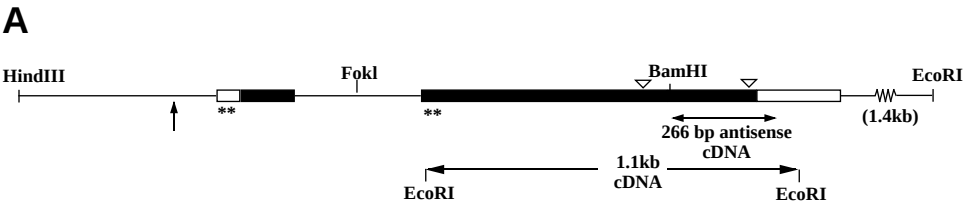

Fig. 1. Physical map and sequence of the *rtoA* gene and predicted amino acid sequence. (A) Map of the 4.1 kb *Hin*dIII-*Eco*RI genomic fragment containing the *rtoA* gene. The known transcript is indicated by boxes, open boxes represent untranslated sequence, closed boxes indicate the open reading frame. The triangles point to the boundaries of the sequence that was deleted and replaced with the pyr5-6 gene in the *rtoA* mutant. The locations of the 266 bp antisense cDNA and the 1.1 kb cDNA clone are indicated. Asterisks mark the locations of primers used to generate additional cDNA. Primers upstream of the solid arrow failed to yield cDNA in PCRs, suggesting that the transcription start site is downstream of this site. Relevant restriction sites are marked. (B) Sequence of *rtoA*. Lowercase letters indicate genomic sequence, uppercase letters indicate compiled cDNA sequence. Numbering is from the *Hin*dIII site of the genomic fragment. The open reading frame begins at nucleotide 669. There is an intron from nucleotide 831-1261. The potential transmembrane sequence (hydrophobic domain) at amino acid 22-50, ATP/GTP-binding motif at amino acid 300-307 (GSSSDGKT), and possible tyrosine phosphorylation site at amino acid 336-343 (RCISDNNY) are all underlined and labeled. Diamonds indicate starts of the serine-rich motifs. The probable AAUAAA poly(A) addition signal at nucleotide 2518 is underlined. Sequence that was deleted and replaced with the ura cassette in the gene disruption mutant is italicized. This sequence is available as GenBank accession number U48298.

B

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   1  aagcttcaattcttttcatgagaaatttaattggtgaaacttttcaagtttatcaagagg
  61  aaagagataaggaaccaaaagaagattgtgtcataaaattaatggaacctgctcatttac
 121  atactgcaattaaaaatactcccctatttaatttttattagtattattgcactttatagag
 181  attaaataatttcaaaaaaaaactaaattaaaacaattgatttctcatttttaaatttttt
 241  ttccaatatagtttaactttaaaatttttcaattagttaaagatgttttaaatttaaatt
 301  taaaaaattaaaaaataaaaagtttggattatttttttttattggataattggtttaggt
 361  aatataaaaaaagttaagtgaaaataagttgatttttggattagagaaagggtaggctt
 421  ttttttttgggtttatattataataaccatttttttaatttttaatttttaatttt
 481  aaaataaatttttttttttttttggtcacctttttcccaattgttttttttttttaaaat
 541  ttttttttttttttccaaattaattattctttatctgaattaccacattttcacacccaa

 601  tcaattctaGTTTAAACCTGGATTTCATTCACATAAAAAAAAAAGAAAAGAAAAAGAAAA

 661  AATCTGTTATGGTTTGGAATTTATATAGAAAATGTGCAAAAATGAAATTATTGACATTTA
                M  V  W  N  L  Y  R  K  C  A  K  M  K  L  L  T  F  T

 721  CTAATATTTCTTTTGTTATGATAAAAATGTGTCGTTTATTATTGTTGTTATTATTTTTAT
        N  I  S  F  V  M  I  K  M  C  R  L  L  L  L  L  L  F  L  L
                                                  hydrophobic domain

 781  TATCTTTTTTTTTTTATTATTATTTTAATTTTACAATAAAAAAAAAAAAACgggaattatt
        S  F  F  F  Y  Y  Y  F  N  F  T  I  K  K  K  N

 841  tattttaatttttaatttttaatttttatttttatattatttattttaaatccttttta
 901  ataaaataaaataaaataaaatgtaaccccaacaagggtacgattatcataatttaatat
 961  tattattaatatctttttttttatgttttattttgttttttatttactctataaaatggg
1021  ataatttttttttttttaatcattccatccacataattattatatattattatatatttt
1081  attatatatttattatatatttatttttatatttatttttatttttattgcttcata
1141  tttaaattttaaaaaatattatattttaattaattaattaaaaaaaaaaaaaaaaaaaaa
1201  aatgaaattaattgcttttattgcattttacttattctcattacaataattaacgcaca

1261  aGGTTCAATTGGCTCATCTAGCCAATCTCTTTCAAGTGAAGTTGATTCTTCAGATATTTC
        G  S  I  G  S  S  S  Q  S  L  S  S  E  V  D  S  S  D  I  S

1321  TTCTTCTGGATCAAATTCCACTGCAAGTTCAGAAGGTTCAGTTTCAAGCTCTTCAAATTC
        S  S  G  S  N  S  T  A  S  S  E  G  S  V  S  S  S  S  N  S

1381  AGGTTCTCAATCAACTTCAAATTCTGGCTCTGAAGCATCTGGTTCATCAAATTCCGGTTC
        G  S  Q  S  T  S  N  S  G  S  E  A  S  G  S  S  N  S  G  S

1441  TCAATCAACTTCAAATTCTGGCTCTGAAGCATCTGGTTCATCAAATTCTGGTTCTCAAAG
        Q  S  T  S  N  S  G  S  E  A  S  G  S  S  N  S  G  S  Q  S

1501  CTCAACTGATTCATCAAATTCTGGCTCTCAAGGTTCAACTGGTTCATCAAATTCTGGTTC
        S  T  D  S  S  N  S  G  S  Q  G  S  T  G  S  S  N  S  G  S

1561  TCAAAGTTCAACTGATTCATCAAATTCTGGTTCTCAAAGCTCAACTGATTCATCCAATTC
        Q  S  S  T  D  S  S  N  S  G  S  Q  S  S  T  D  S  S  N  S

1621  TGGTTCTCAAGGCTCAACTGGTTCATCAAATTCTGGCTCTGAATCATCAGGCTCATCAAA
        G  S  Q  G  S  T  G  S  S  N  S  G  S  E  ◊S  S  G  S  S  N

1681  CTCTGGATCCGAATCATCTGGTTCATCAAATTCAGGATCTGAATCATCATCAGGCTCATC
        S  G  S  E  ◊S  S  G  S  S  N  S  G  S  E  S  ◊S  S  G  S  S

1741  AAACTCTGGATCCGAATCATCGTCAGGCTCATCAAACTCTGGATCTGAGTCATCATCTGG
        N  S  G  S  E  S  ◊S  S  G  S  S  N  S  G  S  E  S  ◊S  S  G

1801  TTCATCAAACTCTGGATCTGAATCATCATCTGGTTCATCAAACTCTGGATCTGAATCATC
        S  S  N  S  G  S  E  S  ◊S  S  G  S  S  N  S  G  S  E  S  ◊S

1861  ATCTGGTTCTTCAAATTCTGGATCTGAATCATCATCAGGCTCATCCAACTCTGGATCTGA
        S  G  S  S  N  S  G  S  E  S  ◊S  S  G  S  S  N  S  G  S  E

1921  ATCATCATCAGGctcgtcaaattctggatctgaatcatctggatcatcaaactctggatc
        S  ◊S  S  G  S  S  N  S  G  S  E  ◊S  S  G  S  S  N  S  G  S

1981  tgaatcatcatctgatagtggatcctcatctgatggtaaaacaacctgtatttcattcca
        E  S  S  S  D  S  G  S  S  S  D  G  K  T  T  C  I  S  F  H
                                ATP/GTP binding

2041  tgacactctttcaatcaatactgtagatgatgatgaaattgaatgtactggtaaaggtga
        D  T  L  S  I  N  T  V  D  D  D  E  I  E  C  T  G  K  G  E

2101  aactagatgtatttccgataataactacaaatgtgctaccaaacaacgtcatggttcaat
        T  R  C  I  S  D  N  N  Y  K  C  A  T  K  Q  R  H  G  S  I
          tyrosine phosphorylation

2161  tgagtgctctgtaaatggctatattagatgcactggttcaaacatcaaatgtaaaattgg
        E  C  S  V  N  G  Y  I  R  C  T  G  S  N  I  K  C  K  I  G

2221  taacactgaatgttcaatccgtaattctcgggtagatattactgaaaaaccaatctttac
        N  T  E  C  S  I  R  N  S  R  V  D  I  T  E  K  P  I  F  T

2281  AAGAGGTCCACGTCACCATTAAATCAATTGTTCAAAAAGTACCTGTTGCTATATATAGTA
        R  G  P  R  H  H  *

2341  ATCATGTAAAATTTTGGTTTTTTTTATTATTTATTTTGGGAAAaaaaaaaaaaaaaaaaa

2401  aaaaggcaaccttttatttaatttaatttttttattaattttaaattaataatgggggaaaa

2461  aatgtttttttcttttttacccaatttctattaattaatttttccttttaataatttttaaaat

2521  aaaacaattctccttttttaaaaaaaaaaaaaaaaaaaaattttaaaaattgggaaattgg
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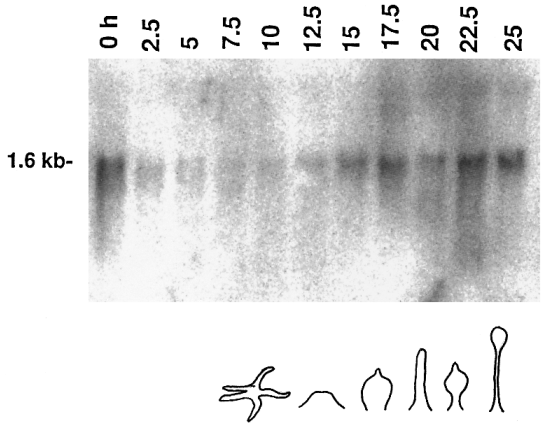

Fig. 2. *rtoA* is expressed as a 1.6 kb transcript in vegetative cells and during late development. A northern blot of total cellular RNA isolated from wild-type Ax4 cells at 2.5 hour intervals after starvation on filter pads was probed with a radiolabeled 400 bp *Bam*HI-*Eco*RI cDNA fragment from the 3′ end of the cDNA. The developmental stage at each time point is illustrated below the blot. A 1.6 kb transcript is detected in vegetative cells (0 hour). Expression levels are low from early aggregation to the loose mound stage (2.5 hours to 12.5 hours) and then increase at the tight-mound stage (12.5 hours), remaining high through the fruiting body stage (25 hours).

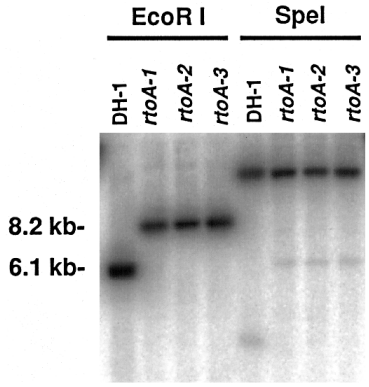

Fig. 3. There is a single integration event of the ura cassette in the *rtoA* gene in the *rtoA* gene-disruption mutants. DNA was isolated from wild-type Ax4 cells and three *rtoA* gene disruption mutants. The DNA was digested to completion with *Eco*RI or *Spe*I, size separated on a gel and transferred to a membrane. The Southern blot was probed with a radiolabeled 800 bp *Spe*I-*Eco*RI fragment of the *Hin*dIII-*Eco*RI genomic fragment. In wild-type cells, the *Eco*RI digests yield a labeled band at 6.1 kb. In the mutants, this band is increased to 8.6 kb by the replacement of 350 bp of *rtoA* coding sequence with the 2.9 kb *pyr5-6* gene. The insertion of the ura cassette does not alter the pattern obtained by SpeI digestion.

exon in the middle of the 4.1 kb genomic fragment (see Fig. 1A). To isolate additional cDNA, primers derived from coding sequence at the upstream end of the exon were used in a reverse transcriptase reaction with poly(A)+ RNA as the template. *rtoA* cDNA was then amplified using the same 3′ primers and a series of 5′ primers from genomic sequences upstream of the intron.

The resulting cDNAs span the splice site and compilation results in a 1350 bp cDNA. This cDNA includes the probable initiator AUG and a 1.2 kb open reading frame followed by an in-frame stop codon. Sequencing of the genomic fragment confirms the cDNA sequence and allows identification of the 3′ untranslated region, which contains a AAUAAA poly(A) addition signal. In the cDNA PCR described above, primers 500 bp downstream from the *Hin*dIII site do not yield cDNA while primers at 610 bp do. Therefore, although the exact transcription start site has not been identified, the *rtoA* message is predicted to be approximately 1.5 kb plus the poly(A) tail. The cDNA and predicted amino acid sequences of RtoA are depicted in Fig. 1B. The predicted protein is 400 aa in length with a calculated M_r of 39.8×10^3. The protein is acidic with a calculated pI of 4.9. The N terminus contains a hydrophobic region, which is predicted to form a transmembrane α-helical domain. There is a very acidic repetitive domain, which contains 10 perfect repeats of an 11-aa serine-rich sequence. A consensus ATP/GTP-binding motif and a possible tyrosine phosphorylation site are also present. Database searches show no significant homology with any known protein.

To examine the temporal expression pattern of *rtoA*, a northern blot of wild-type RNA isolated at intervals throughout development was probed with a 390 bp *Bam*HI-*Eco*RI cDNA fragment. Fig. 2 shows that *rtoA* is expressed in vegetative cells as a 1.6 kb transcript, in close agreement with the predicted message length of 1.5 kb from sequence analysis. As development begins, transcript levels decrease significantly. Only very low levels of *rtoA* expression are detectable from 2.5 hours to 12.5 hours after starvation, which corresponds to the early aggregation through mound stages of development. At the tight-mound stage (12.5 hours), transcript levels increase and then remain high through to the fruiting body stage at 25 hours after starvation.

To analyze the function of *rtoA*, a gene disruption mutant was made by replacing 340 bp of coding sequence with the *pyr5-6* gene. The knockout construct, which consists of 1.9 kb of 5′ and 1.8 kb of 3′ *rtoA* genomic sequence on either side of the ura cassette, was transformed into DH1 *ura*− cells. About 500 of the resulting *ura*+ transformants were screened for a developmental delay at the mound stage and 3 independent *rtoA* gene disruption mutants were isolated. Southern blot analysis of these mutants demonstrates that, in each case, there was a single integration event into the *rtoA* gene (Fig. 3). This was confirmed by northern blot analysis using the 5′ end of the gene as a probe. The wild-type 1.6 kb transcript is truncated to 1.0 kb in the mutants. As expected, the 600 bp at the 3′ end of *rtoA* are not expressed in the mutants (Fig. 4). This region includes the putative ATP/GTP-binding domain and possible tyrosine phosphorylation site.

The truncation of the *rtoA* transcript by insertion of the ura cassette gene is sufficient to recapitulate the phenotype of the antisense *rtoA* mutant. The *rtoA* knockout mutant stalls in development at the tight-mound stage, with ~90% of the aggregates never progressing further (Fig. 5B). The remaining ~10% of aggregates that do form fruiting bodies, do so after a lag time of roughly 48 hours compared to wild type. The *rtoA* fruiting bodies are aberrant with enlarged bases, twisted stalks, and small spore masses (Fig. 5C). Nevertheless, spores produced by *rtoA* are viable.

To determine if disruption of the *rtoA* gene affects cell-type choice, cells were starved, allowed to aggregate, and then dissociated and stained for the CP2 prestalk marker or the SP70 prespore marker. DH1 parental cells starved for 15 hours on either filter pads or agar differentiated into similar percentages of prestalk and prespore cells as previously described for Ax4 cells (Gomer et al., 1986; Gomer and Firtel, 1987). Compared

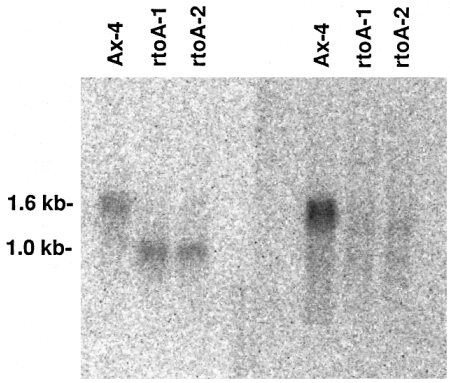

Fig. 4. The 1.6 kb *rtoA* message is truncated to 1.0 kb in the gene-disruption mutants. RNA isolated from vegetative wild-type Ax4 cells and three *rtoA* gene disruption mutants was probed with a radiolabeled 1.0 kb *Hin*dIII-*Fok*I fragment from the 5′ end of the *Hin*dIII-*Eco*RI genomic fragment (left panel). A 1.6 kb transcript is labeled in the wild type while in the mutants the transcript is truncated to 1.0 kb. In the right panel, the northern blot was probed with a radiolabeled *Bam*HI-*Eco*RI fragment from the 3′ end of the cDNA. The 1.6 kb transcript is labeled in wild-type cells but no transcript is labeled in the mutants.

to the DH1 cells, a similar percentage of *rtoA* cells differentiated into prespore cells, while a significantly higher percentage differentiated into prestalk cells (P<0.005 for cells on filter pads or agar) (Fig. 6). In the low cell-density differentiation assay (Fig. 7), *rtoA* knockout mutants also produced a significantly (P<0.005) higher percentage of prestalk cells than the parental DH1 cells. In the same assay, prespore cell percentages were roughly comparable to those in the parental strain. The conditions of this assay greatly limit cell-cell contact or intercellular communication through diffusible factors. Thus, each cell was exposed to identical conditions, and any variation in initial cell type choice must have been caused by cell-autonomous differences between mutant and wild-type cells.

One possible explanation for this difference is that the additional prestalk cells in the *rtoA* mutant result from an extension of S phase or early G_2, the part of the cell cycle in which cells normally differentiate into prestalk cells. To address this possibility, the cell cycle in mutant and wild-type cells was examined in detail (Table 1). The percentage of cells in M phase and in S phase, and cell-cycle length were all unchanged in the *rtoA* mutants when compared to both the wild-type Ax4 cells and the parental DH1 cells. In addition, *rtoA* cells had a cellular DNA content profile (determined by flow cytometry) similar to that of wild-type Ax4 cells. In both strains, most cells had a DNA content consistent with replicated DNA, which is expected since there is no detectable G_1 phase in *Dictyostelium* (Weijer et al., 1984a). These results indicate that the cell cycle is normal in *rtoA*; therefore, the differentiation of additional prestalk cells is not caused by an increase in the length of time amoebae spend in the S/early G_2 phase of the cell cycle. Similar results were obtained analyzing the cell cycle of *rtoA*asr cells and several other antisense transformants (data not shown).

To investigate whether the increased percentage of prestalk cells in *rtoA* is due to a defect in the cell cycle and asymmetric cell-division-dependent cell-type-choice mechanisms, the cell-cycle origin of prestalk and prespore cells was determined.

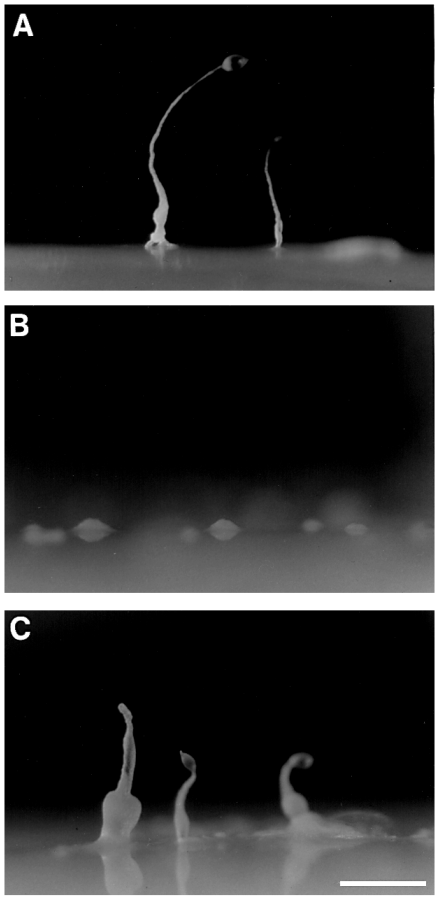

Fig. 5. The disruption of *rtoA* results in a partial developmental block at the tight-mound stage. (A) Wild-type Ax4 cells were plated with bacteria on agar. As the bacteria was consumed, development was triggered and, after 24 hours, fruiting bodies formed. A side view of normal fruiting bodies is shown. (B) *rtoA* cells form tight aggregates, but even after 72 hours, 90% of the aggregates have not progressed further. (C) The 10% of the *rtoA* aggregates that do progress further form fruiting bodies with enlarged bases, warped stalks and small spore masses. Bar in C is 0.5 mm.

Wild-type and *rtoA* cells were plated at low density and videotaped. While continuously videotaping, the cells were starved in the presence of CMF and treated with cAMP to allow development. The cells were then fixed and labeled with antibodies to CP2, a prestalk marker, or to SP70, a prespore marker. The history of each cell that differentiated was determined by following the cell on the videotape to identify the time of the last cell division prior to starvation. This information was used to calculate the cell-cycle phase of that cell at the time of starvation. The results are plotted in Fig. 8. In wild-type cells, the CP2-positive prestalk cells are in the S and early G_2 phases of the cell cycle at the time of starvation (Gomer and Firtel, 1987). In the *rtoA* cells, this restriction has been lost and CP2-positive prestalk cells originate randomly from throughout the cell cycle. The origin of prespore cells in the wild type is limited to the late G_2/ M phase. In the mutant, this regulation has also been disrupted with prespore cells originating from all phases of the cell cycle. Analysis of sister cells indicated that, as in wild type, the sister cells of the differentiated *rtoA* cells were null. These data suggest that the defect in *rtoA* does not affect

A

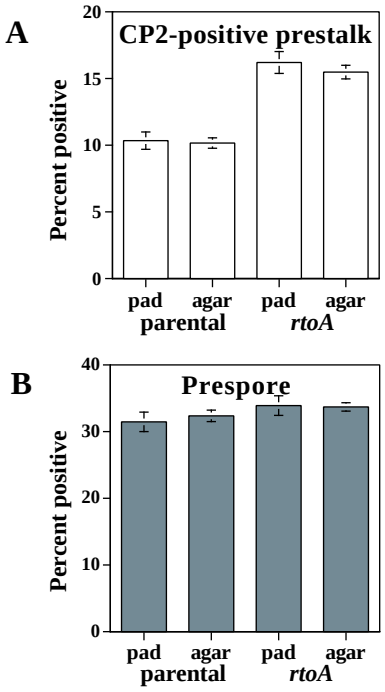

Fig. 6. The percentage of prestalk cells is increased in the *rtoA* mutant. DH1 parental and *rtoA* mutant cells were starved on filter pads (pad) or on 1.5% agar plates (agar) for 15 hours and then dissociated. The percentage of prestalk cells (A) was determined by counting the number of CP2-positive cells and the percentage of prespore cells (B) was determined by counting the number of SP70-positive cells. Results are the average of three separate experiments ± the SEM.

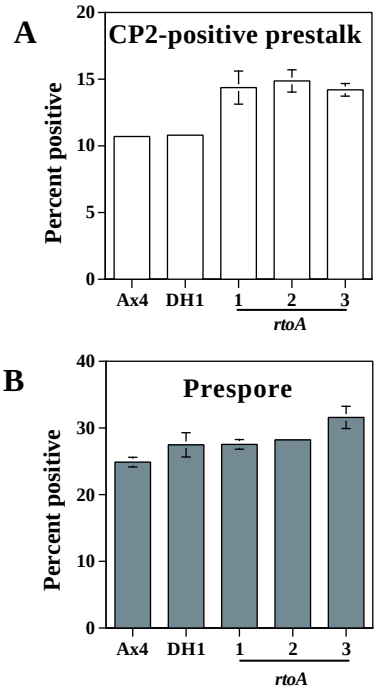

Fig. 7. The increased percentage of prestalk cells in the *rtoA* mutant is due to an alteration in a cell-autonomous mechanism. Wild-type Ax4, parental DH1 or three *rtoA* mutant cell lines were starved at low density in the presence of CMF, treated with cAMP, and then fixed for immunofluorescence. The percentages of prestalk (A) and prespore cells (B) was determined as in Fig. 6, and the results are the average of three separate experiments ± the SEM. No error bar indicates that the SEM was smaller than the plot symbol.

Table 1. The cell cycle in the *rtoA* mutant is normal

	Ax4	DH1	*rtoA*-1	*rtoA*-2
% M phase	0.93±0.09	0.91±0.11	0.94±0.09	0.92±0.17
% S phase	9.4±1.4	9.1±1.2	8.9±0.5	9.0±1.0
cell-cycle length	1.00	1.17±0.09	1.01±0.12	1.13±0.12

The percentage of cells in M phase, percentage in S phase and cell-cycle length (relative to Ax4 cells) for Ax4, parental DH1 and two mutant *rtoA* cell lines were determined as described in the methods. Results are the average of three separate experiments ± the standard deviation.

asymmetric cell division but does alter the cell-cycle-dependent initial cell-type-choice mechanism.

DISCUSSION

We have identified a mutant, *rtoA*, with a defect in the cell-cycle-phase-dependent mechanism that regulates initial cell-type choice in developing *Dictyostelium*. This defect results in the differentiation of an abnormally high percentage of prestalk cells in both aggregates and cells starved at low cell density. Unlike wild-type *Dictyostelium*, in which prestalk cells develop exclusively from those cells in S phase or early G_2 at the time of starvation, *rtoA* prestalk cells develop randomly from cells in any phase of the cell cycle at starvation. Although the percentage of prespore cells is normal in *rtoA*, the restriction of prespore differentiation to cells in late G_2 or M phase has also been lost in *rtoA*.

The *rtoA* gene codes for a novel protein with a predicted relative molecular mass of 39.8×10^3. Analysis of the predicted RtoA amino acid sequence indicates that RtoA may contain an N-terminal transmembrane domain, 10 perfect repeats of an 11 aa serine-rich motif, a consensus ATP/GTP-binding motif and a potential tyrosine phosphorylation site. The *rtoA* phenotype results from a truncation of the *rtoA* transcript from 1.6 kb to 1.0 kb. The 3′ portion of the message that has been deleted in the mutant contains the coding sequence for the putative ATP/GTP-binding motif and the putative tyrosine phosphorylation site, suggesting that some of these regions may be critical for normal RtoA function.

The *rtoA* mRNA is expressed in vegetative cells. This is as expected, since RtoA plays a role in mechanisms that need to be in place when the cells happen to starve. Transcript levels decrease dramatically as development begins, and remain low throughout the aggregation and loose mound stages. The message becomes more abundant again at the tight-mound stage and levels remain high throughout the rest of development and fruiting body formation. The re-accumulation of *rtoA* message and the developmental delay in *rtoA* mutants occur at the same developmental stage: the tight-mound stage. This suggests that RtoA may have dual functions, one in vegetative cells at starvation involving cell-type choice and a later function involving progression to the tipped mound stage. It is unlikely that the increase in prestalk cells from roughly 11% to 15% of the total would prevent development beyond the mound stage. Isolated slug tips, which are composed almost entirely of prestalk cells, can adjust cell-type ratios and produce fruiting bodies that are small but have normal morphology (Raper, 1940). In addition, when populations of synchronized cells are starved in S phase or early G_2 and allowed to develop, development proceeds to at least the slug stage, even though the percentage of prestalk cells in these slugs is abnormally high (Weijer et al., 1984a). These results indicate that homeostasis mechanisms operate during development to insure proper cell-type ratios and that even if initial cell-type ratios are abnormal, development will continue. Thus, loss of RtoA function at the mound stage, and not its role in cell-type choice, is the most likely explanation for the morphological phenotype.

During vegetative growth in *Dictyostelium*, each cell division is asymmetric: at starvation one daughter cell will dif-

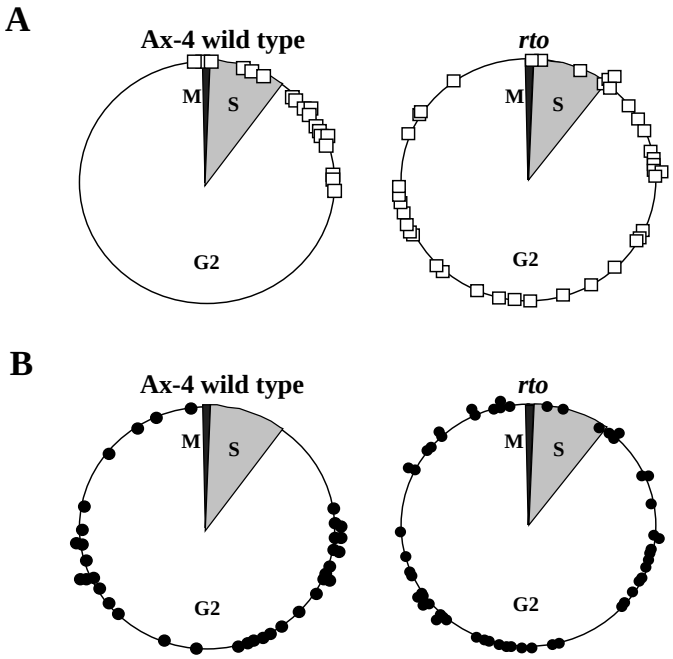

Fig. 8. Prestalk cells in the *rtoA* mutant originate randomly from cells in any phase of the cell cycle at starvation. Wild-type Ax4 cells (left) or *rtoA* cells (right) were plated at low density in submerged culture and videotaped. After at least 12 hours, the cells were starved in conditioned medium and 6 h later treated with cAMP. Cells were fixed 12 hours later for immunofluorescence labeling with (A) antibodies against the prestalk marker CP2 or (B) antibodies against the prespore marker SP70. The videotape was reviewed and the cell-cycle phase of each labeled cell at the time of starvation was determined and mapped (squares for prestalk cells, filled circles for prespore cells) on a diagram of the cell cycle. (A) In the wild type, CP2-positive prestalk cells originate from cells in S phase or early G_2 at starvation. In the *rtoA* mutant, CP2-positive prestalk cells originate randomly from cells in any phase of the cell cycle. (B) In the wild type, the SP70-positive prespore cells differentiate from cells in late G_2 or M phase at starvation. In the mutant, some SP70-positive prespore cells differentiate from cells in early G_2 as well.

ferentiate, while its sister, a null cell, will initially remain undifferentiated. We propose that each cell has a mechanism for monitoring the cell cycle. This information is used to choose between the prestalk and prespore pathways when starvation triggers development. This initial cell-type selection is linked directly to the cell cycle and does not use an independent timer. When the length of S phase is increased by mild treatment with S-phase inhibitors, the percentage of prestalk cells increases (Gomer and Ammann, 1996). The increase in prestalk cells in the *rtoA* mutant is not based on such an alteration of the underlying cell cycle. The length of S phase is normal in *rtoA*, as are all other aspects of the cell cycle examined, including the percentage of cells in M phase, the G_1/G_2 ratio and cell-cycle length.

Approximately 25% of the total cell-cycle length comprises the S and early G_2 phases. Therefore, the differentiation of half of the cells that are in these stages into prestalk cells accounts for the 12.5% prestalk cell differentiation. Likewise, half of the 75% of the cells that are in late G_2 or M phase at starvation will differentiate into prespore cells or 37.5% of the total. While the mechanism regulating asymmetric cell division is functioning normally in the *rtoA* mutant, the cell-cycle-phase-dependent cell-type-choice mechanism is totally disrupted. Both prestalk and prespore cells differentiate from cells in any phase of the cell cycle at starvation with no apparent bias towards a particular cell-cycle phase. However, the percentage of only prestalk cells is altered in the mutant, increasing from 10.7% to 14.5%: an increase of roughly 35%. Surprisingly, the percentage of prespore cells is not significantly different in the mutant compared to wild type. This may be due to the action of other differentiation control mechanisms. Because the choice to differentiate into a prestalk cell ultimately leads to death and is therefore an evolutionary dead end, selective pressure must be intense to maintain the proper cell ratios. One possible explanation for the changes in cell-type percentages in *rtoA* is that, in the absence of the cell-cycle-dependent regulation, a second mechanism becomes active. If this mechanism favors the differentiation of prespore cells at approximately 2:1 over prestalk differentiation and, if only one of each pair of daughter cells initially differentiates, then an increase from 12.5% to 16.7% in prestalk cells and a slight change from 37.5% to 33.3% in prespore cells would be expected in the mutant. The observed data fit this model within the range of experimental error: the ratio of *rtoA* prespore to prestalk cells is 2.00±0.08 and only one of each pair of daughter cells differentiates.

RtoA is probably directly involved in the initial cell-type-choice mechanism as opposed to downstream differentiation events, because in the *rtoA* cells there is no complete conversion to one cell fate. This is unlike the situation in a glycogen synthase kinase mutant (*gskA*) in *Dictyostelium* (Harwood et al., 1995). *gskA* plays a role in both the inhibition of prestalk and stalk cell differentiation and the induction of prespore and spore cell differentiation. In a *gskA* mutant, prestalk and stalk cell development dominates almost completely and very few spore cells form. GskA is probably acting downstream of the initial cell-type choice and homeostasis mechanisms and directly influencing differentiation.

Further characterization of RtoA will lead to a better understanding of the precise mechanisms that directly link cell-cycle phase at starvation to the choice of cell differentiation pathways. A key question that remains unanswered is which of the many physiological changes associated with the cell cycle is used as a signal on the pathway. Defining the role of RtoA may provide the answer.

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