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Mutual Relation between the Cell-Cycle Progression and Prespore Differentiation in *Dictyostelium* Development

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ABSTRACT—In *Dictyostelium discoideum* Ax-2, the cell-cycle progression from the early aggregate to mound stage has been proposed to have some connection with prespore differentiation. Hereupon, we examined the role of cell-cycle progression during the development on cell differentiation, using two kinds of cell-cycle inhibitors. Nocodazole, an inhibitor of microtubule formation, was found to inhibit greatly cell division around the mound stage as well as during the vegetative growth phase, when applied to exponentially growing Ax-2 cells. Essentially the same inhibition was attained by treatment of starved Ax-2 cells with calyculin A, an inhibitor of serine/threonine protein phosphatases. It is noteworthy that the nocodazole- or calyculin A-treated cells exhibit abnormal morphogenesis to form a stick-like multicellular structure on non-nutrient agar, and also that prespore differentiation as exemplified by the prespore-specific *Dp87* gene expression and prespore specific vacuole (PSV) formation was greatly suppressed. In contrast, the differentiation of prestalk (pstA) cells was scarcely affected by the drug treatments. Taken together these results seem to indicate that the cell-cycle progression around the mound stage is important for prespore differentiation.

INTRODUCTION

Dictyostelium discoideum Ax-2 cells grow axenically as free-living cells and multiply by binary fission in the presence of nutrients with a doubling time of about 7.5 hr (vegetative growth phase). The multicellular developmental program (differentiation phase) is induced by nutritional deprivation, and the starved cells gather together forming streams in response to pulsatile emissions of cAMP from the aggregation center. The cell aggregate assumes a hemispherical structure at the mound stage. Subsequently a tip arises at the apex of the mound, elongates and forms a migrating slug. At the slug stage, there is clear zonal differentiation along its long axis, with anterior prestalk cells and posterior prespore cells. During culmination, a fruiting body consisting of a spherical-shaped mass of spores and a supporting cellular stalk is formed. The growth and differentiation phases in this organism are temporally separated from each other and easily controlled by nutritional conditions, and the developental system is relatively simple as compared with that of other organisms. Thus, this organism is useful for basic studies on the growth/differentiation transition, cell differentiation, pattern formation, and a variety of other problems of current interests in developmental biology.

Previous studies have revealed that in the development of *Dictyostelium discoideum* Ax-2 cells the progression of S-phase is specifically reinitiated in prespore cells (Durston and Vork, 1978; Zimmerman and Weijer, 1993), and that the cell division actually occurs around the mound stage (Zada-Hames and Ashworth, 1978; Araki and Maeda, 1995). With respect to the cell-cycle progression of starved cells, Zada-Hames and Ashworth (1978) have showed that cell division is reinitiated during the mound-tipped aggregate stage but not in the aggregateless mutant (NP146). Recently, we have demonstrated using synchronized populations of Ax-2 cells that the cell-cycle progression around the mound stage is regulated in the cell mass, possibly coupling with prespore differentiation (Araki and Maeda, 1995). Alternatively, Cappucinelli *et al*. (1979) have claimed using nocodazole, an inhibitor of microtuble formation, that mitosis naturally noticed after starvation of Ax-2 cells is not indispensable for differentiation. Thus the two apparently opposite views have been proposed about the relationship between the cell-cycle progression and cell differentiation.

In this study, we attempted to elucidate the role of cell-cycle progression during development, especially around the mound stage, in cell differentiation, using two kinds of drugs for inhibiting the cell-cycle progression. One of the inhibitors used is nocodazole as a mitotic inhibitor that disrupts the spindle fibers (Cappucinelli *et al*., 1979). Another is calyculin A, a specific inhibitor of serine/threonine protein phosphatases (Ishihara *et al*., 1989) which immediately inhibits growth of vegetative cells and also arrests the starved Ax-2 cells at the early G2 phase (Akiyama and Maeda, 1992). The results obtained strongly suggested that the progression of cell cycle

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around the mound stage might be crucial for prespore differentiation.

MATERIALS AND METHODS

Chemicals

An inhibitor of the microtuble formation, nocodazole (methyl [5-(2-thienylcarbonyl)-1H-benzimidazol-2yl-] carbamate, Sigma), was dissolved in 100% dimetyl sulfone oxide (DMSO) at 200 μg/ml as the stock solution. Calyculin A (Wako Pure Chemicals), the inhibitor of serine/threonine protein phosphatases, was dissolved in 10% DMSO as the stock.

Strains and growth conditions

The cellular slime mold *Dictyostelium discoideum*, axenic strain Ax-2 (clone 8A and JW) and its transformants were used in this study. Ax-2 cells were transformed with a vector (*pEcmA-gal* or *pDp87-gal*) bearing a bacterial *β-galactosidase* gene under the control of cell-type specific promoters. The ecmA promoter directed specifically the gene expression in prestalk (pstA) cells (Early *et al.*, 1995), while the *Dp87* promoter in prespore cells (Ozaki *et al.*, 1993). Ax-2 cells were grown axenically in HL-5 medium supplemented with 1.5% glucose (Watts and Ashworth, 1970) and transformants in HL-5 medium containing 80 μg/ml G418 (Geneticin, Life Technologies Inc.). The transformants were transferred to G418-free HL-5 medium before use and grown for 24-48 hr to remove its effects on growth and differentiation, as suggested by Bühl and MacWilliams (1991). Ten ml of the cell suspension was cultured in a 200-ml Erlenmyer flask coated with Sigmacote (Sigma) at 22°C at 150 rpm on a rotary shaker.

Inhibition of the cell-cycle progression

Ax-2 cells growing exponentially in HL-5 medium were treated with various concentrations of nocodazole or calyculin A, and the subsequent changes in the cell number were determined using a haemocytometer. After the treatments, cells were harvested and washed twice by centrifugation (2,500 rpm, 90 sec) in 20 mM Na/K phosphate buffer, pH 6.4 (PB). The washed cell populations were then plated at a density of about 1.5×10^6 cells/cm^2 on white Millipore filters (HAWP; Nihon Millipore Ltd.) placed on 1.0% non-nutrient agar. This was followed by incubation of the starved cells at 22.0°C to allow development. To treat starved cells with the drugs, exponentially growing Ax-2 cells were harvested, washed as described above, and plated on 1.0% non-nutrient agar containing various concentrations of nocodazole or calyculin A. In another experiment, the washed cells were resuspended in PB at a density of 1.0×10^7 cells/ml and shaken at 150 rpm in a 25-ml Erlenmeyer flask coated with Sigmacote. After 6 hr of shake culture, various concentrations of the drug were added to the cell suspension, followed by 1 hr of shake culture. The treated cells were collected by centrifugation, resuspended in PB, and plated on 1.0% non-nutrient agar containing the respective concentrations of nocodazole or calyculin A at about 1.0×10^6 cells/cm^2 and incubated at 22°C to allow developmant. As controls, comparable concentrations of DMSO (the solvent of the drugs) were added to PB and 1% agar.

Measurements of temporal changes in the number of growing and developing cells

In the case of growing Ax-2 cells treated with the drugs or DMSO, the cell number was counted at 4 hr-intervals. The number of starved Ax-2 cells was determined as follows; Cells or cell masses on agar or filters were withdrawn every 2-4 hr and dissociated by pronase-BAL solution (1% pronase E (Merck) and 25 mM 2,3-dimercapto-1-propanol (BAL, Wako Co.) in 50 mM Tris-HCl buffer (pH7.0) for 15 min, with intermittent pippettings or vortex-shaking (Takeuchi and Yabuno, 1970). The dissociated cells thus obtained were briefly fixed with formaldehyde (final conc. 2.5%), followed by cell counts.

Histological detection of β-galactosidase activity

Cell masses were fixed and stained for β-gal activity according to a modification of the method previously described (Dingermann *et al.*, 1989). Filters with cell masses were floated on Z-buffer (containing 0.05% glutaraldehyde (GA) and 0.1% TritonX-100) for 5 min and then submerged in the same solution for 10 min. After two times of washings in Z buffer, the preparations were incubated in the staining solution (5 mM of both $K_3[Fe(CN)_4]$ and $K_4[Fe(CN)_3]$; 1 mM X-gal; in Z buffer) for β-gal activity. In the case of cell masses developed on agar, they were transferred to the poly-L-lysine coated coverslip, fixed

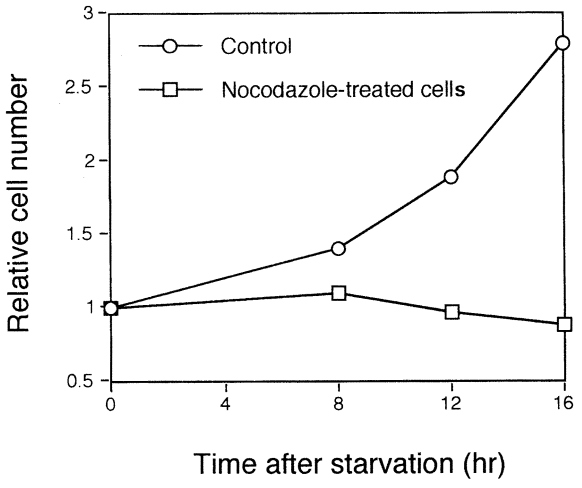

Fig. 1. Nocodazole-treatment of Ax-2 cells during the growth phase inhibits cell division around the mound stage. Exponentially growing cells were incubated in HL-5 medium for 12 hr with or without 3 μg/ml of nocodazole. The nocodazole-treated cells and control cells were separately harvested, washed, and allowed to develop on filters in the absence of nocodazole. This was followed by cell counts 8 hr (t_8), 12 hr (t_{12}), and16 hr (t_{16}) after starvation. It is evident that the nocodazole-treatment inhibits greatly cell division during the development (t_8-t_{16}). A representative result among five independent experiments is shown.

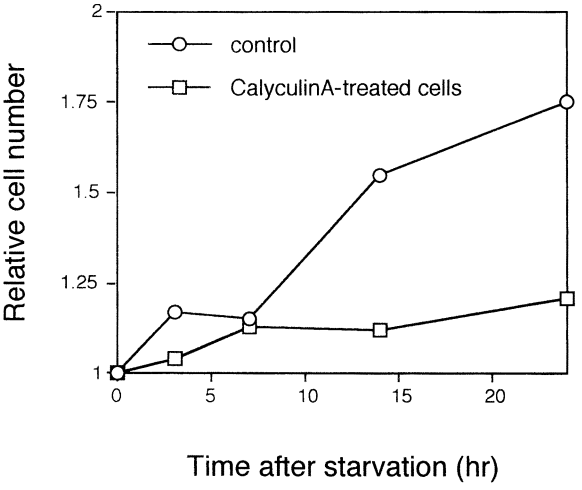

Fig. 2. Calyculin A-treatment of starved Ax-2 cells inhibits markedly cell division around the mound stage. Exponentially growing cells were harvested, washed, and allowed to develop on 1% non-nutrient agar containing 0.3 μM calyculin A or 0.3% DMSO (control). This was followed by cell counts as the case for Fig. 1. A representative result among four independent experiments is shown.

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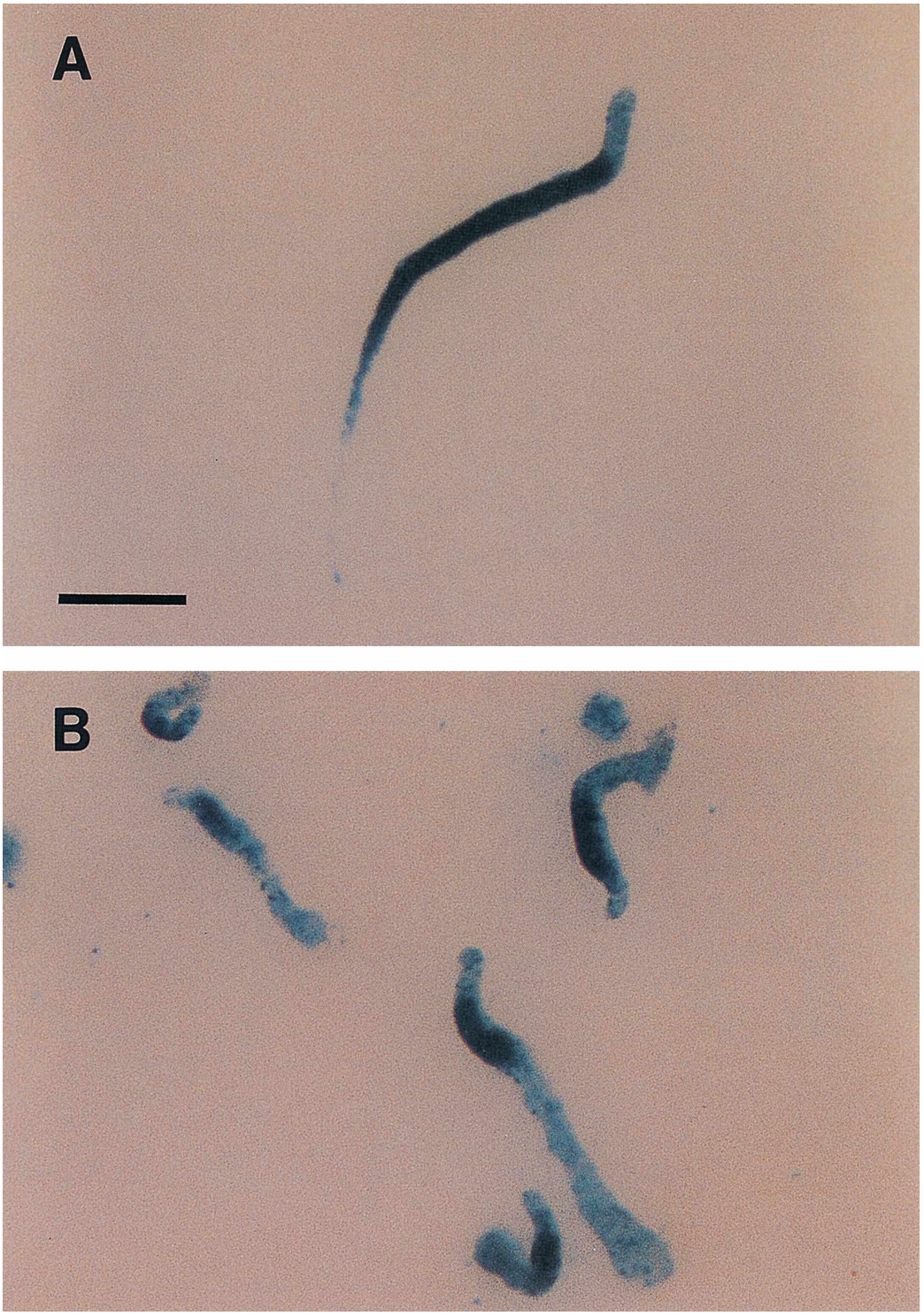

Fig. 3. Morphogenetical abnormality and suppression of the prespore-specific (*Dp87*) gene expression in the transformant treated with nocodazole. (**A**) The expression pattern of *Dp87* in migrating slugs formed without nocodazole-treatment. The *Dp87* gene is strongly expressed in the posterior prespore zone of migrating slugs. When cells were treated with 3 μg/ml of nocodazole for 12 hr during the growth phase, cell aggregates (mounds) were formed about 8 hr after starvation, as the case of control, but the cell mass elongates abnormally into the air and eventually formed a stick-like structure, as shown in (**B**). In such cell masses, the *Dp87* expression is greatly suppressed and limited to the anterior-most region of the original prespore zone of slugs (**B**). The scale bar represents 0.5 mm.

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for 10 min under a submerged condition, and stained as the case for filters, except for the fixation procedure. The staining was stopped by washing in Z buffer, and the stained cell masses were photographed in color.

Immunocytochemical detection of prespore-specific vacuoles (PSVs)

At 16-18 hr of starvation, cell masses were submerged and dissociated in pronase-BAL solution for15 min, with intermittent vortex-shaking. The dissociated cells were washed three times in BSS (Bonner's standard salt solution; Bonner, 1947), pre-fixed in ice-cold 50% methanol, and fixed in absolute methanol for 10 min on an ice-bath. The fixed cells were dried on cleaned coverslips. They were dipped in PBS (140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM K_2HPO_4, pH7.2) for 5 min and stained with the FITC conjugated anti-*D. mucoroides* spore IgG in a moisture chamber for 45 min at room temperature, according to the method of Takeuchi (1963). The samples were washed three times in PBS (5 min for each) and mounted in PBS containing 20% glycerol. The percentages of PSV-containing cells (prespore cells) were determined by immunofluorescent microscopy.

RESULTS

Inhibition of cell-cycle progression during development

When vegetative Ax-2 cells were treated with HL-5 medium containing 3 μg/ml of nocodazole for 12 hr and then starved on filtres, cell dividion was greatly inhibited enen in nocodazole-free medium at least during 24 hr of incubation (Fig. 1), as pointed-out by Cappucinelli *et al*. (1979). In practice it is quite difficult to withdrawn completely cells or cell masses developing on filters. Accordingly, no increase in number in the nocodazole-treated cells, as shown in Fig. 1, is not necessarily indicative of complete inhibition of cell division. Ax-2 cells treated with calyculin A during the vegetative growth phase stopped immediately advancing their cell cycle, as previously reported (Akiyama and Maeda, 1992). However, the treated cells exerted cell division around the mound stage as the case for non-treated control cells, when starved and incubated in the absence of calyculin A. Next, effects of nocodazole and calyculin A on starved Ax-2 cells were examined, with special emphasis on the cell-cycle progression around the mound stage. Exponentially growing cells were harvested, washed, and allowed to develop on non-nutrient agar containing 3 μg/ml of nocodazole or 0.3-0.5 μM calyculin A. As a result, cell division around the mound stage was found to be markedly inhibited in the calyculin A-treated cells (Fig. 2). In contrast, the nocodazole-treatment had no effect on the cell-cycle progression around the mound stage, and the treated cells exerted normal cell division and differentiation during subsequent development.

Abnormal morphogenesis and differenitation pattern induced by inhibition of the cell-cycle progression during developement

Mitotically arrested cells with 3 μg/ml nocodazole at the vegetative growth phase exhibited abnormal morphogensis when they were starved and incubated on agar or filters without nocodazole. The starved cells formed cell aggregates (mounds) after about 8 hr of incubation, as the case for 0.3% DMSO-treated control cells, but the mound elongated abnormally upword and eventually formed a stick-like structure (Fig. 3B). This is in somewhat contrast with the result reported by Cappucinelli *et al*. (1979) who have showed that mitosis naturally noticed during the developmental phase is not indispensable for morphogenesis and differentiation. Althouth the reason for this difference is presently unknown, the unique stick-like structure was also constructed by starved cells that had been treated with 0.3 μM calyculin A during development. The calyculin A-treated cells formed mounds after 12 hr of incubation, being slightly delayed as compared with control (0.3% DMSO-treated) cells. Subsequently, the mound elongated markedly and then formed a stick-like structure, as observed in the nocodazole-treated cells. Incidentally, the DMSO-treated control cells exhibited normal morphogenesis to form migrating slugs (Fig. 3A).

To examine the role of the cell-cycle progression in prespore differentiation, the transformant with the vector bearing a bacterial *β-galactosidase* gene under the promoter of the prespore specific gene *Dp87* was used. As previously reported by Ozaki *et al*. (1993), the *Dp87* gene was expressed in the posterior prespore cells in migrating slugs (Fig. 3A). In the stick-like structures induced by nocodazole-treatment, the prespore-specific *Dp87* expression was greatly suppressed and limited to the anterior-most region of the original prespore zone of migrating slugs (Fig. 3B). Such marked suppression of *Dp87* gene expression was also observed in the starving

Table 1. Effects of nocodazole and calyculin A on prespore differentiation

Treatments	Percentages of PSV-containing prespore cells (average ± S. D.)
Nocodazole-treated cells (3 μg/ml)	12.1 ± 5.3%
Control (0.3% DMSO-treated cells)	76.6 ± 3.7%
Calyculin A-treated cells (0.3 μM)	27.3 ± 11.8%
Control (0.3% DMSO-treated cells)	77.1 ± 5.7%

D. discoideum Ax-2 cells were treated with nocodazole and calyculin A, as described in Materials and Methods. After 16-18 hr of starvation, the resulting cell masses were dissociated, fixed, and stained with FITC-conjugated anti-spore IgG to detect specifically prespore cells. The number-ratios of stained cells (prespore cells) to non-stained cells (prestalk cells + undifferentiated cells) were determined counting at least 1,000 cells for each sample.

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cells treated with calyculin A (data not shown). A quantitative analysis, monitored by formation of the prespore-specific vacuoles (PSVs), revealed that the percentage of PSV-containing prespore cells was markedly reduced in mitotically arrested cells by nocodazole or calyculin A, as shown in Table 1. In the stick-like structure, the PSV-containing cells were found to locate in essentially the same region with *Dp87*-expressing cells, the anterior-most region of the original prespore zone of slugs (data not shown).

Effect of the cell-cycle arrest during development on another type of cell differentiation was examined using the transformant with the vector bearing a bacterial β-galactosi-

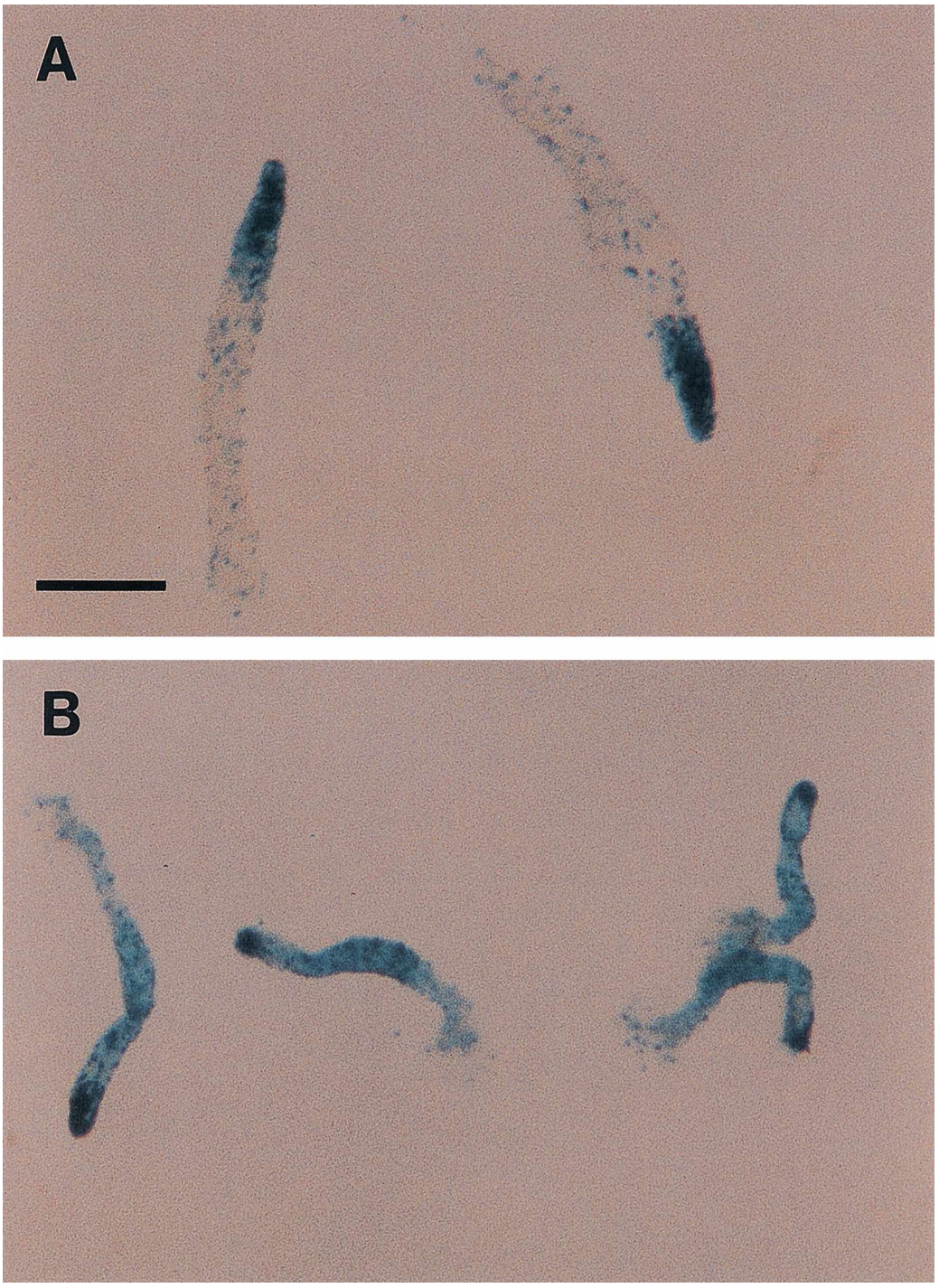

Fig. 4. Effect of calyculin A-treatment on the prestalk-specific (*ecmA*) gene expression in the transformed cells. (**A**) The expression pattern of *ecmA* in migrating slugs formed without calyculin A. The *ecmA* is expressed in the anterior prestalk region, particularly in a front half of the prestalk region. (**B**) In the cell masses formed in the presence of 0.3 μM calyculin A, the pattern of *ecmA* expression is basically similar to control (**A**), though some *ecmA*-expressing cells are scattered in the posterior region. The scale bar represents 0.5 mm.

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dase gene under the promoter of the prestalk cell-specific gene *ecmA* (Early *et al.*, 1995). In normal development, the *ecmA* gene was expressed in the anterior prestalk region of migrating slugs (Fig. 4A). In cell masses formed in the presence of calyculin A, the pattern of *ecmA* expression was somewhat different from control, and some *ecmA*-expressing cells were scattered in the posterior region, possibly because of failure of the cells to be predominantly sorted into the tip region (Fig. 4B). However, the ratio of pstA cells to total cells in the unique cell messes was estimated to be about 15% as the case for control, thus being scarcely affected by calyculin A. We could not test effect of nocodazole-treatment on the *ecmA* expression, because we failed to inhibit the cell-cycle progression of this transformant around the mound stage by nocodazole. Incidentally, a microscopical analysis showed that in the stick-like masses induced by nocodazole- or calyculin A-treatment mature stalk cells, which were highly vacuolated and surrounded by thick cell wall, were hardly noticed even after a prolonged time (>48 hr) of incubation.

Relationship between the cell-cycle progression and prespore differentiation

The cell-size of prespore cells in migrating slugs is considerably smaller as compared with that of prestalk cells, thus the prestalk cells ($\phi > 8$ μm) and prespore cells ($\phi \leqq 8$ μm) being distinguished on the size-difference within the probability above 90% (Abe and Maeda, 1989). In this connection, we have previously demonstrated using synchronized Ax-2 cells that mid-G2 phase cells destined for prespore cells exhibit cell division around the mound stage (Araki and Maeda, 1995). Actually, a good correlation between the cell-cycle progression and prespore differentiation was realized by preferential PSV-formation in smaller-sized cells (Table 2). Although there were some differences in inhibitory efficiency of the cell-cycle progression between the nocodazole- and calyculin A-treatments, the former was generally more effective in inhibiting the cell-cycle progression than the latter. At all events, the highr percentages of large cells (non-divided or mitotically arrested cells during the course of development) gave the lower rates of prespore differentiation (Table 2).

DISCUSSION

Using the two kinds of cell-cycle inhibitors (nocodazole and calyculin A) with different action mechanisms, it has been demonstrated that prespore differentiation is closely coupled with the cell-cycle progression around the mound stage, but that prestalk differentiation (*ecmA* expression) is scarcely affected by these drugs. Zimmerman and Weijer (1993) have reported that the cell-cycle progression is reinitiated around the mound stage only in differentiating prespore cells, and that prespore differentiation occurs just prior to the cell-cycle progression. Our results presented here, however, have showed that inhibition of the cell-cycle progression brings about

Table 2. Effects of nocodazole and calyculin A on cell division and prespore differentiation

		(A) Number-ratio of small to total cells (%)*	(B) Percentages of prespore cells (%)
Nocodazole-treatment**			
Experiment	Exp. No.1	12.0	3.9
	No.2	24.5	11.0
	No.3	27.1	13.3
Control	Exp. No.1	79.1	69.1
	No.2	75.5	73.4
Calyculin A-treatment***			
Experiment	Exp. No.1	25.9	18.1
	No.2	31.3	33.1
	No.3	47.6	35.0
Control	Exp. No.1	77.5	72.5
	No.2	75.5	73.4

* Small cells were arbitrarily defined as ones with less than 8 μm of cell diameter.
** Ax-2 cells were treated with nocodazole, and the ratios of prespore cells to total cells constituting cell masses were determined as described to the legend of Table 1.
*** 0.3 μM of calyculin A was added to the cell suspension after 6 hr of starvation and shake-cultured for 1 hr. This was followed by plating of the cells on non-nutrient agar containing 0.3 μM calyculin A, as noted in Materials and Methods. The number-ratios of small cells (presumably cells divided around the mound stage) to total cells were simultaneously counted under a phase-contrast microscope. At least 1,000 cells were counted for each sample. It is noteworthy that the values of (B) seem to be in proportion to those of (A).

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marked suppression of prespore differentiation. Although this seems to indicate that the cell-cycle progression is a prerequisite for prespore differentiation, PSVs were also found in only a few number of large (non-divided) cells as well as in small cells that had progressed their cell cycle, particularly in the case of calyculin A-treatment. Therefore, it is still uncertain if the cell-cycle progression is absolutely required for prespore differentiation and remains to be elucidated.

In the development of *Drosophila* nervous system, it has been demonstrated using aphidicolin that the *even-skipped* (one of pair-rule gene) expression is coupled to S-phase progression (Weigmann and Lehner, 1995). The S-phase progression is also required for the expression of gut lineage marker gene in *Caenorhabditis elegance* (Edgar *et al*., 1994). Moreover, cell fate determination in the neocortical neurons in mammals has been shown to be correlated with the progression through S-phase (McConnell and Kaznowski, 1991). In *Dictyostelium* cells, there is little or no G1-phase in the cell-cycle during development (Araki and Maeda, 1995) as well as during vegetative growth (Weijer *et al*., 1984; Maeda, 1986, 1993), thus the S-phase being tightly coupled with the preceding M-phase. The transcriptional activity is generally highest during and just after the S-phase, and therefore the S-phase progression may be of particular importance for prespore differentiation. This might be relate to the recent findings that 1) the duplication of mitochondrial DNA occurs in differentiating prespore cells but not in prestalk cells (Shaulsky and Loomis, 1995), and 2) the activity of mitochondrial respiration is higher in the prespore region than in the prestalk region (Matsuyama and Maeda, 1995).

Our previous data have showed that synchronized T1 cells and T7 cells interchange their relative positions in a cell mass during tip formation (Araki *et al*., 1994). In this connection, the chemotactic activity of Ax-2 cells to cAMP is acquired differently depending on the cell-cycle positions at the onset of starvation (Ohmori and Maeda 1987): T7 cells acquire these characters earlier than T1 cells, but the chemotactic activities of T1 cells and T7 cells are reversed at a certain developmental stage, presumably around the aggregation stream-mound stage. This reversion might cause the interchange of their relative positions of T1 and T7 cells in the cell mass during tip formation. In this connection, it seems to be possible that in response to the reentry of T7 cells (presumptive prespore cells) into the cell cycle around the mound stage some of the genes expressed at the vegetative growth phase might be re-expressed, thus resulting in their loss of once-acquired differentiation characters such as chemotactic ability of the cells to cAMP.

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