Cytological Studies of the Sexual Development in *Dictyostelium discoideum*

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ABSTRACT

Cells of *Dictyostelium discoideum* become sexually mature under submerged and dark conditions, and fuse with opposite mating-type cells to form zygote giant cells, which gather surrounding cells and finally develop into dormant structures called macrocysts. In the present study, I found that the multinuclear fused cells formed during this process frequently underwent cytokinesis driven by random local movements. The split cells were capable of re-fusion, and repeated cytokinesis. These radical behaviors continued until the extensive cell aggregation started around the giant cells. Thus, gamete fusion and initiation of zygote development do not coincide in the mating of *D. discoideum*. Analyses by confocal microscopy and flow cytometry indicated that the cessation of the random movement followed pronuclear fusion, and that microtubule organizing centers (MTOCs), abundant in the fused cells at the beginning, gradually decreased and only one of them remained within the developed macrocyst. Some of the genes known to control cell movement, such as *gefB* and *rasS*, increased shortly before the cessation of repeated fusion-cytokinesis and initiation of phagocytosis. These results suggest that the sequential molecular events are necessary in *D. discoideum* after gamete fusion to establish a new individuality of zygotes.

INTRODUCTION

Fertilization is the central event of sexual reproduction, where sequential cellular processes such as gamete encountering, cell recognition and fusion, and pronuclear fusion take place, and finally embryonic development is triggered to construct a new individual. For example, however sperm-borne oocyte-activating factor(s) in the mouse has been showed to be responsible for triggering zygote formation (Perry et al., 1999), the interplay between fertilizing sperm and egg factors that initiate oocyte activation is still unclear. It is thought to difficult to analyze that of mammals since it is complicated.

Soil amoeba, *Dictyostelium discoideum* proliferates as a unicellular amoeba in its vegetative state. Many features of these amoebic cells resemble animals both morphologically and biochemically. Unlike higher animal cells, however, they are haploid and therefore amenable to genetic manipulation, as well as to cell biological and molecular biological analyzes. *D. discoideum* has these features that make it especially well suited to model for studies of fundamental mechanisms of higher animals., In particular, the research in actin cytoskeleton has contributed to a general understanding of structure and function of cytoskeletal proteins (Noegel et al., 1995). Furthermore, the *Dictyostelium* mhcA- cells have proven extremely useful for investigating the role played by myosin II in varius cell functions (Manstein et al., 1989).

D. discoideum normally feeds on bacteria and proliferates by mitosis. Upon starvation, however, a hundred thousand cells gather, move as a multicellular slug, and finally culminate in a fruiting body composed of a spore mass and a stalk to raise it (Fig. 1, left). When the habitat environment is submerged and dark, and water-sensitive

spores being useless, cells of *D. discoideum* initiate a sexual process called macrocyst formation (Blaskovics and Raper, 1957; Nickerson and Raper, 1973) (Fig. 1, right). In this process, they sexually maturate and fuse with appropriate mating type cells to form zygote giant cells, which gather the surrounding cells by cAMP-mediated chemotaxis, phagocytose them for nutrition, and finally develop into macrocysts (O'Day, 1979; Szabo et al., 1982). The mating system in *D. discoideum* is polymorphic, having heterothallic (Erdos et al., 1973), homothallic, and bisexual strains (Robson and Williams, 1980). The offspring analysis revealed low frequencies of recombinants, suggesting poorly controlled meiotic recombination (Wallace and Raper, 1979). Thus, macrocyst formation in this organism represents prototypic features of the sexual reproduction system and is useful for analyzing its basic aspects. Moreover, the plant hormone ethylene induces macrocyst formation, while cAMP represses it (Amagai, 1992), which was first demonstrated in a related homothallic species, *Dictyostelium mucoroides* (Amagai, 1989).

Recent studies on macrocyst formation have often been dedicated to the analysis of gamete interactions. Several cell surface proteins involved in the gamete interactions have been identified in *D. discoideum*. For example, a cell surface protein, gp70, is presumed to function in mating type recognition (Urushihara et al., 1988). A light-induced inhibitory protein(s) was suggested by Habata et al. (1991). DE1, GG6, and HH9 antigens have also been implicated in sexual cell fusion (Aiba et al., 1992). However, the molecular basis of zygote development remains elusive, largely due to the lack of appropriate experimental systems. In the present study, I analyzed the post-fusion processes of macrocyst formation using the synchronous development system.

MATERIALS AND METHODS

Strains and culture conditions

Heterothallic strains of *D. discoideum*, KAX3 and V12, were used throughout the experiments. They were opposite mating type strains and cell fusion occurs between them. The strains were maintained as stock fruiting bodies on nutrient SM agar plates in association with *Klebsiella aerogenes* as a food source. KAX3 and their derivatives were also grown in HL5 medium (Cocucci and Sussman, 1970) containing streptomycin sulfate (50 μg/ml) with or without G418 (10 μg/ml). Transformants of V12 were grown with concentrated *K. aerogenes* on BSS (See below) agar plates containing 20 μg/ml of G418.

Sexual development

To obtain sexually mature fusion competent cells (FC-cells), growth-phase cells on SM agar plates were collected and cultured in Bonner's salt solution (Bonner, 1947) (BSS) containing concentrated *K. aerogenes* on a gyratory shaker for 15 h at 22 °C in darkness. At the end of the culture period, the bacteria were removed by repeated centrifugation in cold BSS, and the cells were resuspended in BSS and kept at 4°C until use. To initiate the sexual development, equal numbers of FC-cells of KAX3 and V12, or their transformants, were mixed and placed in a glass bottom dish (IWAKI, Japan) at a density of 2×10^5 cells/cm^2, and then incubated at 22°C. When appropriate, a glass coverslip or a plastic Petri dish (BIO-BIK, Japan) was used in place of a glass bottom dish, without affecting the developmental time-course. An assay for cell fusion in

suspension was conducted as first described by Saga et al (1983).

Quantification of cell motility

Cell motility was determined using a time-lapse recording of digital DIC (Differential interference contrast) images with an LSM510 inverted microscope (Zeiss, Germany) equipped with a 40 × 1.0 Plan-Apochromat objective. Cell centroids were marked and the mean centroid displacement per unit time was obtained using the public domain software Scion Image Beta 4.02 (http://www.scioncorp.com). For each experiment, approximately 50 cells in randomly selected fields were monitored, and the data were collected every 30 seconds for a 10 minute period.

Nuclear staining

Cells on a glass coverslip were fixed by the two-step procedure using formaldehyde and methanol (Fukui et al., 1987), and washed in phosphate buffered saline (140 mM NaCl, 2 mM KCl, 14 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4) (PBS) containing 0.05% Tween 20. They were treated with 1 mg/ml of RNaseA (Sigma, U.S.A.) in PBS for 1 h at room temperature, followed by incubation with 10 μg/ml of propidium iodide (PI) overnight at 4℃. Fluorescence images were obtained using an LSM510 inverted microscope (Zeiss, Germany) equipped with a 63 × 1.4 Plan-Apochromat objective.

Determination of cell size

Microscopic cell images of randomly chosen fields were analyzed to obtain the cell area using Carl Zeiss LSM510 Physiology / Time Series software version 2.8. At

least 100 cells were measured for each sample.

Flow cytometry

Approximately 1 x 10^7 cells were harvested from the culture, washed in BSS and pelleted by centrifugation. Their nuclei were prepared using a nuclei extraction kit NE-PER (PIERCE, U.S.A.), resuspended in 400 μl of PBS containing 200 μg/ml of RNaseA and 50 μg/ml of PI, and then analyzed by a FACS calibur (Becton Dickinson, U.S.A.) using an excitation wavelength of 488 nm. To ensure single-nucleus measurement, the gate was set using the FL2-A and FL2-W parameters of the doublet discrimination module. At least 20,000 nuclei were counted for each sample.

Generation of GFP-γ-tubulin expressing cells

The construct to express GFP-γ-tubulin (Asano et al., 2004) was a generous gift from Dr. A. Nagasaki at the National Institute of Advanced Industrial Science and Technology, Japan. This construct was introduced into KAX3 cells by the standard protocol of electroporation (Howard et al., 1988) with slight modifications. Cells (2×10^7) at growth phase were mixed with the DNA in 0.4 ml of an electroporation buffer and pulsed with an electric pulser (Biotechnologies & Experimental Research, Inc., U.S.A.). Transformation of V12 cells was carried out as follows. Growth phase cells on SM agar plates were harvested, washed three times in BSS to remove bacteria, and 1×10^7 cells were inoculated into a plastic Petri dish (ϕ = 9 cm) containing 10 ml of HL5 medium and incubated for 24 h at 22℃. Then, the cells were harvested and the DNA was introduced in the same way as for KAX3. After the recovery, the transformed V12 cells were mixed with the suspension of pre-grown *K. aerogenes* in BSS and spread on

a BSS agar plate containing 100 μg/ml of G418. Plaques of transformant clones were visible after 5 to 7 days of culture.

Immunofluorescence imaging

To detect α-tubulin in KAX3 and V12, cells were fixed as for nuclear staining and then incubated with 10 μg/ml of FITC-conjugated monoclonal anti-α-tubulin IgG (DM1A) (Sigma, U.S.A.). For transformants expressing GFP-γ-tubulin, the fixed cells were incubated with 10 μg/ml of monoclonal anti-α-tubulin IgG (Sigma, U.S.A.) followed by incubation with 50 μg/ml of TRITC-conjugated anti-mouse IgG. (Sigma, U.S.A.). All the above antibodies were diluted with 0.1% BSA in PBS, and the incubation was carried out overnight at 4℃. The confocal fluorescence images were obtained using an LSM 510 inverted microscope (Zeiss, Germany) equipped with 100 × 1.45 α Plan-Fluar objective.

RNA preparation and real-time RT-PCR

Total cellular RNA was prepared using an RNeasy Mini Kit (QIAGEN, Germany), followed by the treatment with an RNase-Free DNase Set (QIAGEN, Germany) to remove contaminating genomic DNA. The first strand cDNA was synthesized by SuperscriptII RT (Invitrogen, U.S.A.). The template concentration for PCR was adjusted by amplification using two primer sets for IG7 (Hopper et al., 1993) and FC-AL23 (Muramoto et al., 2003). Real-time RT-PCR was performed using an ABI 7900HT Sequence Detection System (Applied Biosystems, U.S.A.) in a 10 μl reaction volume containing SYBR Green PCR Master Mix (Applied Biosystems, U.S.A.). To ensure reproducibility, expression ratios were determined using cDNA templates synthesized

from two independently isolated RNA samples.

RESULTS

Cell fusion and cytokinesis in the early stages of sexual development

The characteristic steps of sexual development in the mated incubation of KAX3 and V12 are shown in Fig. 2. When FC-cells of KAX3 and V12 were mixed and incubated on a glass bottom dish, they moved around and fused immediately upon contact. Thus, large cells with multiple nuclei were frequently observed after 1 h of incubation. From around 12 h, the cell aggregation became remarkable, and large cells containing many endocytes in them were visible in the center of aggregates after 16 h of incubation. The precysts were formed at around 24 h. During the initial periods of sexual development, pairs of adjacent cells connected by long cytoplasmic bridges were noted (arrowhead in Fig. 2b). Analysis by time-lapse photography revealed that they were once fused cells now in the process of cytokinesis (Fig. 3). The fused cells did not behave as an established zygote but locally continued their independent movements in different directions. Sometimes the separating portions were pulled back, but mostly the cytoplasmic bridges were torn. The resultant split cells were capable of re-fusion, and again underwent cytokinesis. These radical behaviors of gametes ceased before 8 h of incubation. As shown in Fig. 4, the cell motility decreased during this period. When the FC-cells of KAX3 or V12 alone were incubated under submerged conditions, a decrease in cell motility was not observed.

Nuclear fusion during the sexual development

Since pronuclear fusion is an important step following gamete fusion, I investigated

the nuclear behavior by PI staining (Fig. 5A). Starting from the population of mostly mono-nucleated cells, giant cells containing 3 or more nuclei occupied a considerable fraction after 1 h of incubation. As the incubation proceeded, however, the number of nuclei in the cell gradually decreased, and returned to the original level at 8 h, about 10% of the cells being dinucleates and the rest, mono-nucleates (Fig. 5B). The counting of the nuclei in later stages was difficult due to the extensive cell aggregation. The observed decrease in the number of nuclei in the cell was most probably caused by the cytokinesis described above, and not by nuclear digestion, because the cell size distribution in the population decreased with incubation time (Fig. 5C). However, nuclear fusion also seemed to occur as judged from the existence of larger nuclei at 8 h (arrowheads). It was noted that a significant fraction of the cells were very small in size (cell area < 50 μm^2) at 8 h. To detect nuclear fusion, I isolated nuclei from the cells after various incubation periods and determined their DNA contents by flow cytometry. As shown in Fig. 6, an increase in the number of diploid nuclei was detected from 8 h. Thus, continuous cytokinesis following the initial extensive cell fusion resulted in the gradual decrease of nuclear numbers in a cell until around 8 h, when nuclear fusion started to occur.

Microtubule Organizing Centers during the sexual development

In consideration that microtubules and microtubule organizing centers (MTOCs) are generally closely associated with the nuclear behavior during cell division as well as fertilization, I monitored the microtubule distribution during the sexual development (Fig. 7). The counter-staining of α-tubulin and the nucleus revealed that the microtubule networks were evenly distributed in the cytoplasm of multinuclear cells at 1 h of sexual

development, with an MTOC-like structure localized close to each nucleus (Fig. 7A). The cytoplasmic bridges described earlier contained thick bundles of microtubules. When the cells expressing GFP-γ-tubulin were used, localization of the green fluorescence coincided with the center of the microtubule aster, indicating that the MTOC-like structures described above were actually MTOCs (Fig. 7B). The number of MTOCs in the cell corresponded to the number of nuclei (Fig. 7C). As the incubation proceeded, MTOCs became localized predominantly at the position between two adjacent nuclei. The microtubule network, originally abundant and evenly distributed in the cytoplasm, became faint and a thick accumulation at the peripheral regions was noted at the later stages of sexual development. In the macrocysts, only one MTOC was detected in the center (Fig. 7D), which was confirmed by serial sections of confocal microscopy. The peripheral regions of macrocysts were composed of the filaments strongly stained by anti-α-tubulin antibody.

Implication of cell movement to sexual development

To know whether the radical cell behavior described above is a regulated process or merely a parallel event, I first examined the effects of agents affecting cytoskeletal components. As shown in Table 1, latranculin A, an inhibitor of actin polymerization, completely suppressed the macrocyst formation at concentrations of 1 μM or higher when added at or before 12 h of development. When added later, however, negligible suppression was noted. Thus, active cell movement by the actin cytoskeleton is necessary for cell fusion, cytokinesis, and chemotaxis to the giant cells. Other agents inhibiting actin polymerization were also tried but they had no effects either on macrocyst formation or on cell motility. This was probably due to their poor

permeability into the cells judging from the fact that the treated cells did not round up. Thiabendazole, an inhibitor of tubulin polymerization, likewise suppressed macrocyst formation at 50 μM when added at or before 4 h of development. The results suggest that microtubules play an important role(s) during the period of cell fusion and cytokinesis, but not in the later processes. Since the cell fusion assayed in suspension was not affected by these agents (Table. 1, rightmost column), membrane fusion itself seems to be independent of actin and microtubules.

Next, I determined the expression patterns of genes related to cell movement and cytokinesis by quantitative PCR (Table 2 and Fig. 8). Among the 15 genes examined, 2 (*gefB* and *profilin I*) showed high (> 10 fold) and 3 (*cortexillin I*, *gapA*, and *rasS*) showed moderate (2 – 10 fold) increases. Another three (*dcof1*, *limB*, *talA*) showed suppression. The expression of *ABP-120*, *gefB* and *rasS* reached a maximum at around 16 h, when the cell aggregation was near completion and phagocytosis started.

DISCUSSION

Although gamete fusion is the critical step of sexual reproduction, zygote development is not its immediate consequence but requires sequential cellular processes. For example, in most mammals, the secondary division of meiosis completes after egg-sperm fusion and polar bodies are excluded prior to zygote formation. *Chlamydomonas* degenerates its flagella before development (Pan and Snell, 2000), and *Paramecium* duplicates micronuclei to exchange through a narrow cytoplasmic bridge (Hiwatashi and Mikami, 1989). Those requirements for post-fusion processes apparently depend on the extent to which the gametes are prepared in the organism. In the present study, I found that sexually competent gamete-phase cells of *Dictyostelium*, which were capable of instantaneous fusion to form multicellular giant cells, repeat their sequence of fusion and cytokinesis for several hours as if the fusion products were not determined for the new individuals of the next generation. Further observation revealed that when this chaos settled, at around 8 h of development, cells were either mono- or bi-nucleated, and that nuclear fusion began to take place thereafter. Thus, gamete fusion alone does not trigger zygote development in *D. discoideum*.

The ability of FC-cells for immediate and extensive cell fusion allowed me to detect the cytokinesis of fused cells in the present study. Although I experimentally induced the sexual maturation of cells and then mixed them, this can also happen in the field. A sexually mature growing population of one mating type may encounter the population of another mating type. At those front ends, a similar situation in my experimental system is expected, resulting in the fusion of more than two parents. Therefore, the

cytokinesis of giant cells seems to be a necessary step to adjust the number of nuclei to be fused in a cell. The steps of sexual development in *D. discoideum* are schematically shown in Fig. 9.

It has been previously reported that *D. discoideum* is different from *D. mucoroides* in zygote formation. Okada et al. (1986) found that multinucleated zygotes were formed in *D. discoideum* and that all of the nuclei in those zygotes but for the diploid one disappeared at an early stage of macrocyst formation. On the other hand, cell fusion takes place only between the two cells in *D. mucoroides*, and nuclear fusion follows (Amagai, 1989). The present study demonstrated that nuclear fusion in *D. discoideum* occurred in the dinucleate cells, suggesting the similarity of the process of zygote formation between the two species. Currently, I do not have the exact answer for the discrepancy between our results and those by Okada et al. for zygote formation in *D. discoideum*, but one possible explanation is their incubation of giant cells in suspension to measure the nuclear DNA content. Analysis of the nucleus in the later stages, which was not possible in the present study, might detect the cells containing diploid nuclei as well as haploid ones. In this connection, the possibility of zygote fusion in later stages of macrocyst formation is an interesting question to be examined, because it actually happens in *Physarum polycephalum* (Bailey et al. 1987).

The cells with a very low cytoplasmic content were present after the cytokinesis of the giant cells ceased at 8 h of incubation (Figs. 5A and 5C). Those cells had probably succeeded in budding out from the large multinuclear cells but have failed to carry the original amount of cytoplasm with them. In the literature, O'Day's group described such small cells in a mated culture of NC4 (a parental strain of KAX3) and V12, and claimed that those cells corresponded to the gametes from the timing of their appearance and

zygote detection. Since cell fusion occurs between the cells of normal size (Saga et al., 1983), I do not think it appropriate to call those small cells as gametes. The reason they did not detect the cytokinesis of giant cells is probably that the rate of cell fusion was low in their experimental system.

About the mechanism of cytokinesis of multinucleated cells, a recent report on the novel type of cytokinesis is worth to mention. Hibi et al. (2004) described that when large, multinucleate, myosin II-null cells of *Dictyostelium* were allowed to adhere to substrates, they quickly formed multiple leading edges and tore themselves into smaller fragments. This process was named Cytokinesis C, and Talin A was shown to be crucial for it. Cytokinesis C is similar to the cytokinesis following cell fusion described in the present study in that it is independent of cell cycle and that it requires cell adhesion to substrate and active cell movements. To analyze the mechanism of cytokinesis during sexual development, use of a myosin II-null and talinA-null mutant seems interesting.

In the multinucleate cells in general, the astral microtubules from adjacent MTOCs overlapped and generated anti-parallel microtubule arrays. The action of microtubule motors has been suggested to bring MTOC and nucleus together as during karyogamy in *S. cerevisiae* or apart as in *Drosophila* syncytial embryos. During the mating in S. *cerevisiae*, the kinesin motor Kar3p is required to bring the two nuclei together prior to nuclear fusion (Rose, 1996). Kar3p has been suggested to cross-bridge anti-parallel microtubules emanating from the two spindle pole bodies and to move towards their minus-ends. In *Drosophila* syncytial embryos, the centrosomes and their associated nuclei have been shown to migrate from the center of the embryo to the cortex at the end of division probably by the action of a plus-end directed motor acting on the anti-parallel arrays (Foe and Alberts, 1983). In the present study, MTOCs evenly

distributed in the cytoplasm in multinuclear fused cells at 1 hour became localized predominantly at the position between two adjacent nuclei, suggesting that zygote formation in *D. discoideum* was dependent on MTOC-mediated nuclear positioning.

Thiabendazole had no effects on macrocyst formation when added after 4 h of development, suggesting that nuclear fusion normally occurred. This may be suggesting that nuclear fusion itself is not required for microtubule and kinesin but other proteins. Consistent with this idea, in yeast, Kar5p is cluster near MTOC and required for nuclear fusion (Beh et al., 1997). In *D. discoideum* similar proteins in MTOC may also be involved in nuclear fusion. Unlike mating of yeast, however in *Dictyostelium* there are some nuclei from opposite mating type gamete in multinucleated cell. Is there nuclear selection of two parents? Moreover, which proteins provide membrane recognition and which catalyze the membranes? There issues are very interesting but currently I am unable to address these points.

Since the cell motility did not significantly decrease during the incubation of the KAX3 cells or V12 cells alone under submerged conditions, the cessation of movement is evidently a result of cell fusion and not of cell damage. Some of the genes related to cytoskeletal activities showed an interesting increase or decrease during sexual development. For example, the increase of *rasS* and *gefB* may be important for the cessation of cell movement and the initiation of phagocytosis, because these genes have been reported to be involved in the partial suppression of cell movement and enhanced phagocytosis (Chubb et al., 2000; Wilkins et al., 2000). To describe the zygote development in terms of gene expression, more careful examination is necessary. For example, zygote and peripheral cells should be separated when preparing their RNAs, and non-mated cultures should be used as controls. It should also be taken into

consideration that a differential usage or regulation of genes exists for asexual and sexual developmental processes. In fact, Muramoto et al. (2005) (recently identified a new regulator of the cAMP signaling pathway specific to sexual development. Among the estimated 12,000 genes in *Dictyostelium* (Glockner et al., 2002), some fractions are aimed at macrocyst formation in this organism.

Finally, When FC-cells of KAX3 and V12 were mixed at high density (approximately 1×10^6 cells/cm^2), macrocyst formation was defective since multinucleated cells appear difficult to divide by protrusive activities along periphery (data not shown). This suggested that cytokinesis following cell fusion is an important step for sexual development in *D. discoideum*. Thus, it is possible that the important factors for zygote formation are found by analyzing genes related to cytokinesis and cell movement. For this purpose, combination of individual and mass analyses (e.g., DNA array, two-dimensonal electrophoresis) is powerful tool. This will lead to a fundamental understanding of the mechanisms involved in zygote formation.

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Tables

Table 1. Effects of the cytoskeleton disrupting agents on sexual development of *D. discoideum*

Agent	Concentration	Macrocyst formation[a] in the presence of the agent added at						Cell fusion in suspension[c] (%)
		0 h	4 h	8 h	12 h	16 h	24 h	
LatranculinA[b]	0.5 μM	±	±	±	±	+	+	76.3 ± 3.2
	1 μM	-	-	-	-	+	+	78.0 ± 1.7
	5 μM	-	-	-	-	±	+	ND
Thiabendazole[b]	10 μM	+	+	+	+	ND	+	69.0 ± 1.6
	50 μM	±	±	+	+	ND	+	68.0 ± 3.1
	100 μM	±	±	±	+	ND	+	ND
DMSO	0.1%	+	+	+	+	+	+	72.0 ± 0.7

[a] Macrocysts were observed after 48 h of incubation. +: Macrocyst formation indistinguishable from the control without addition; ±: A small number of tiny macrocysts; -: No macrocysts.

[b] Stock solutions of latranculinA and thiabendazole were made in DMSO at the concentrations of 5 mM and 100 mM, respectively.

[c] Percentages of cells participated n cell fusion during 30 min of incubation on a reciprolal shaker. Means ± SDs of 3 independent experiments are shown.

Table 2. Expression of motility- and/or cytokinesis-related genes during the sexual development

Expression	Gene	Accession	Product	Function	Reference
Highly induced [a]	*gefB*	AF275723	RasGEFB	Motility	Wilkins et al., 2000
	profilin 1	X61581	Profilin	Both [c]	Haugwitz et al., 1994
Induced	*cortexillin 1*	AL49527	Cortexilin	Cytokinesis	Faix et al., 1996
	gapA	D88027	GAPA	Cytokinesis	Adachi et al., 1997
	rasS	Z14134	RasS	Motility	Chubb et al., 2000
Suppressed	*dcof1*	D37980	Cofilin I	Motility	Aizawa et al., 1996
	talA	U14576	Tal A	Cytokinesis	Niewohner et al.,1997
	limB	AF198250	LIM2	Motility	Chien et al., 2000
Other changes	*(No specific name)*	X15430	ABP-120	Motility	Cox et al., 1992
	racE	DDU41222	RacE	Cytokinesis	Larochelle et al., 1996
No change [b]	*(No specific name)*	Z50156	34-kD actin binding protein	Motility	Rivero et al., 1996
	(No specific name)	M25132	CAP34	Motility	Hug et al., 1995
	corA	X61480	Coronin	Both	de Hosots et al., 1993
	(No specific name)	U36936	Daip1	Both	Konzok et al., 1999
	countin	AF140780	Countin	Motility	Tang et al., 2002

[a] More than 10 fold increase.

[b] Changes less than to x 2 or half levels throughout.[c]

Figures and Legends

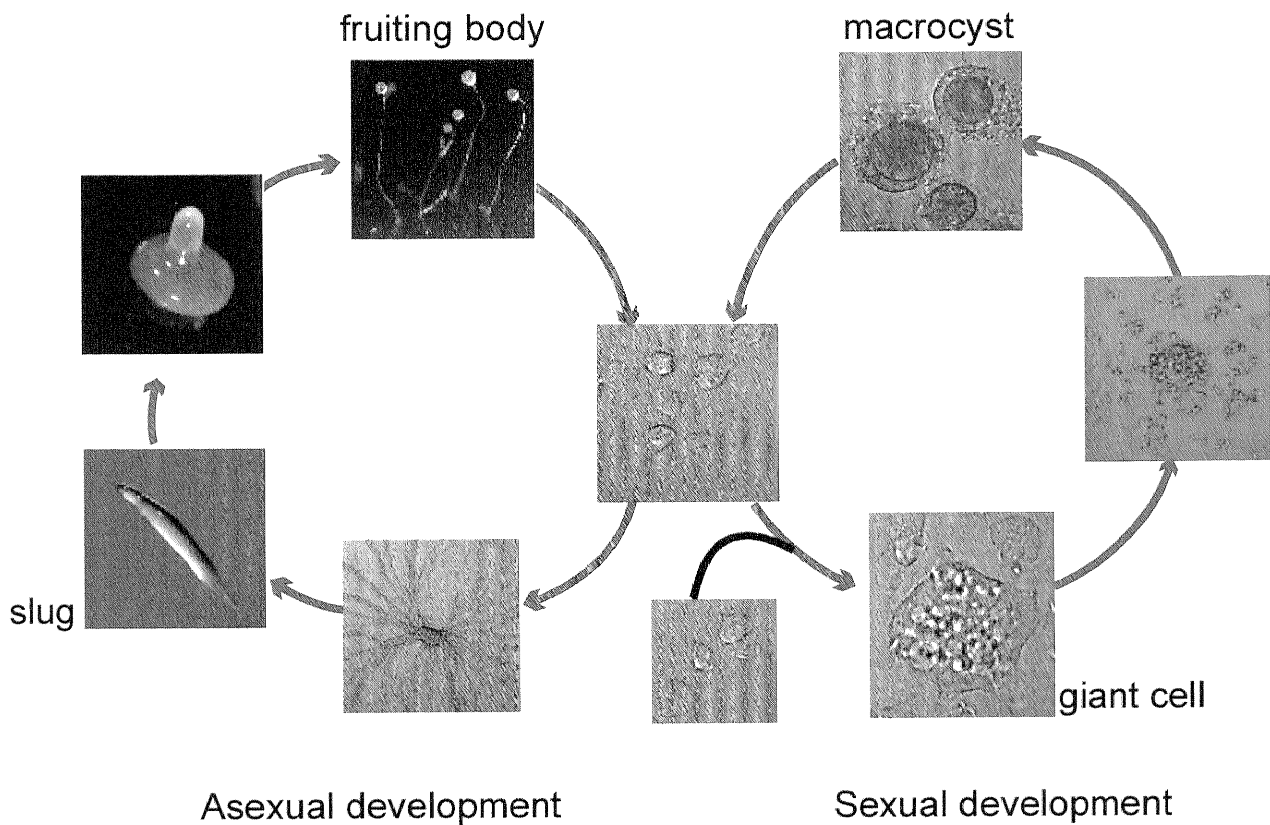


Figure 1 Two developmental modes in *Dictyostelium discoideum*

Haploid amoeboid cells of *D. discoideum* proliferate by fission. In the asexual development (left) under dry and starved conditions, cells aggregate and form fruiting bodies consisting of spore masse and stalk. In the sexual development (right) under the dark and wet condition, cells acquire fusion competency and fuse with complementary mating type cells. The fused cells become zygotic giant cells and develop into macrocysts.

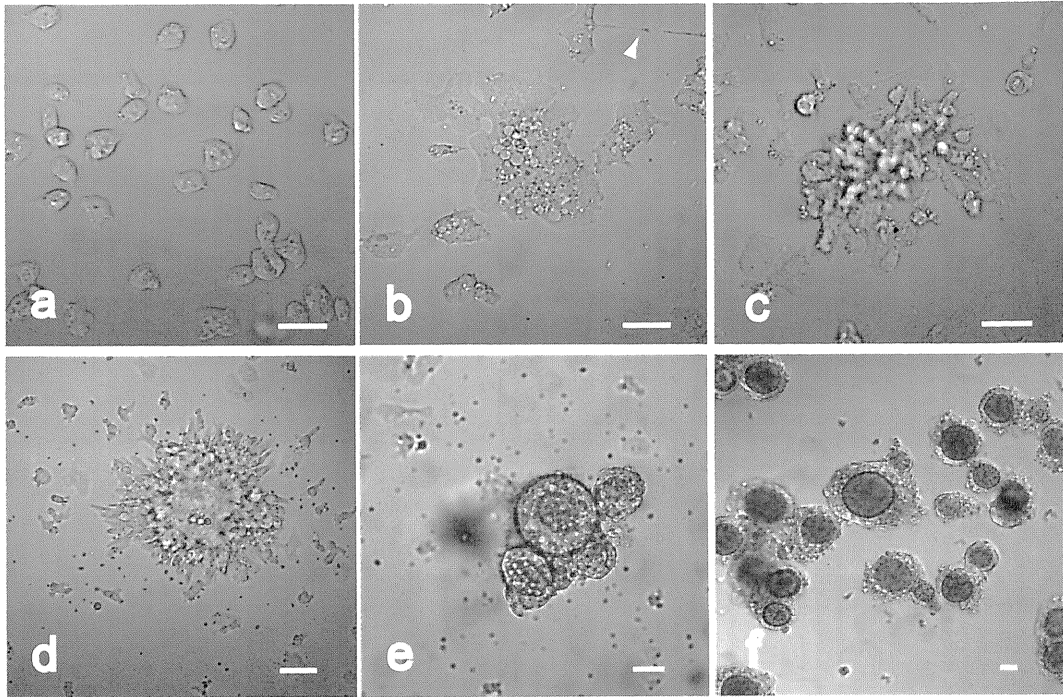


Figure 2. Sexual development of *D. discoideum*.

FC-cells of KAX3 and V12 were mixed and incubated as described in Materials and Methods. Photographs were taken for the characteristic stages at 0 h (a), 1 h (b), 12 h (c) 16 h (d), 24 h (e), and 48 h (f) of incubation. The arrowhead indicates a cytoplasmic bridge described in the text. The bar represents 20 μm.

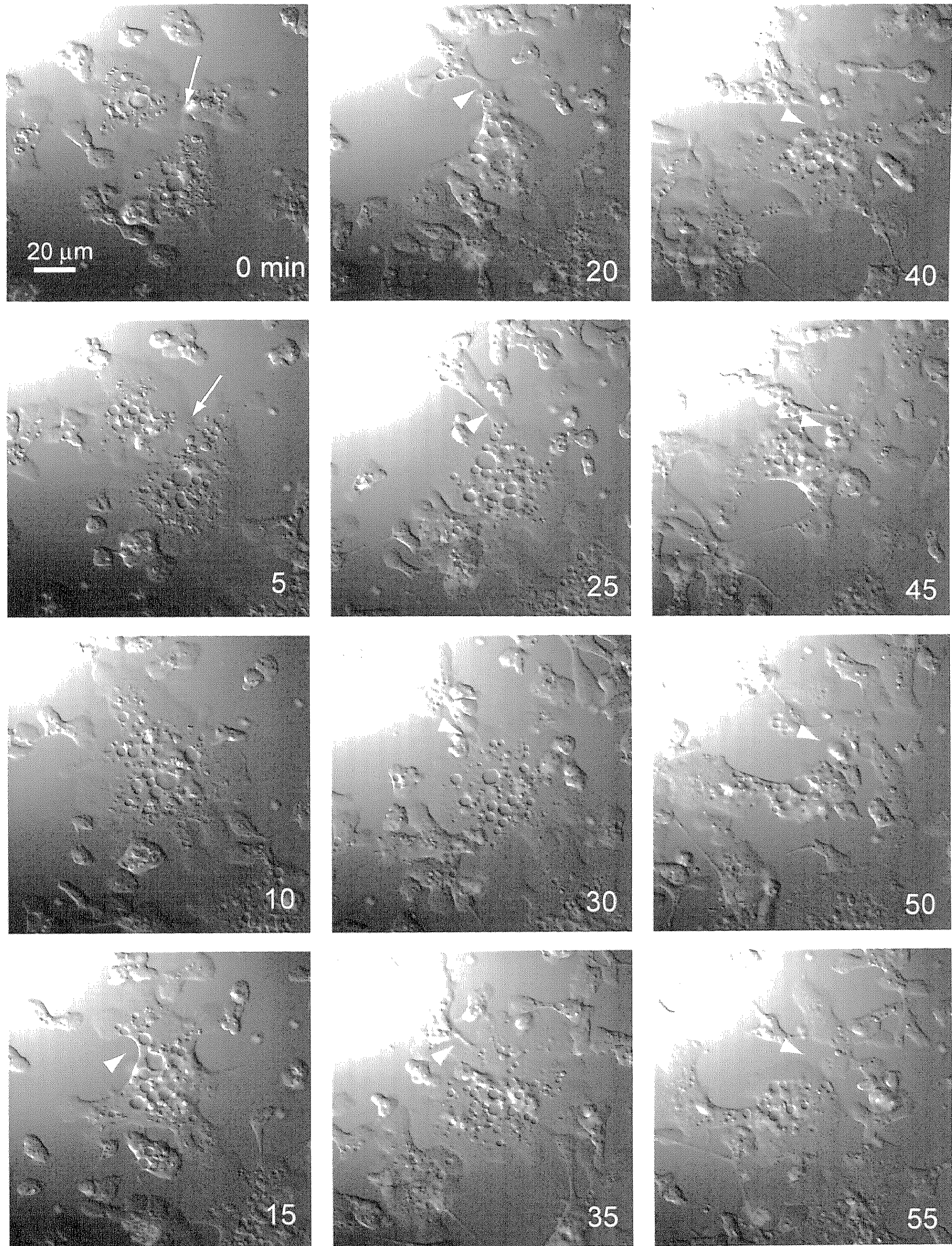


Figure 3. Cytokinesis of giant cells.

FC-cells of KAX3 and V12 were mixed and incubated as described in Materials and Methods. Time-lapse images were obtained at 5 min intervals starting from 1 h of incubation. The arrow indicates cell fusion, and the arrowhead, the point of cytokinesis.

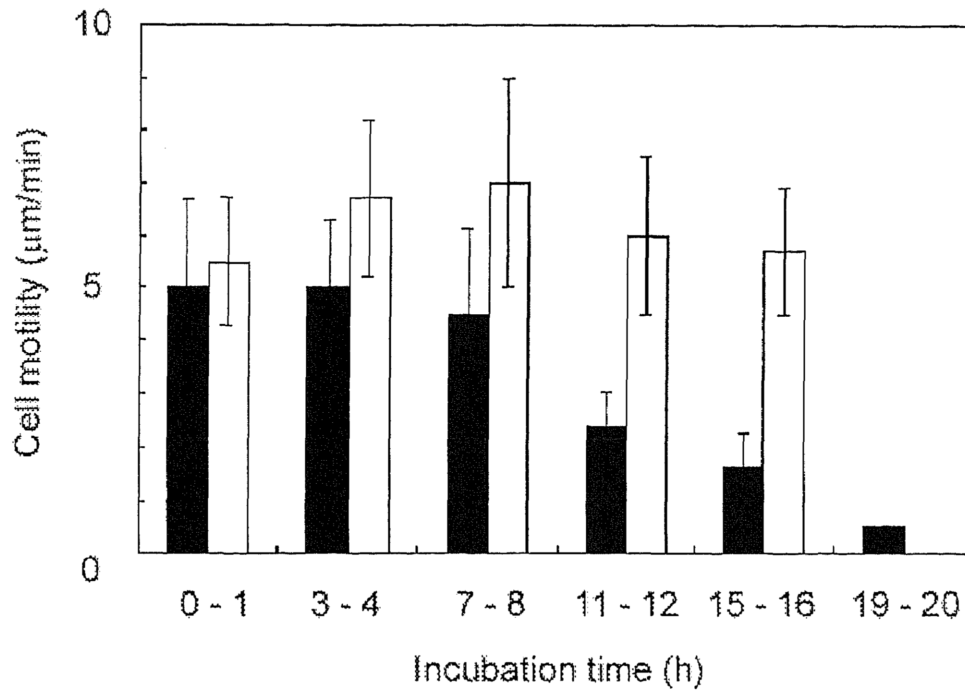
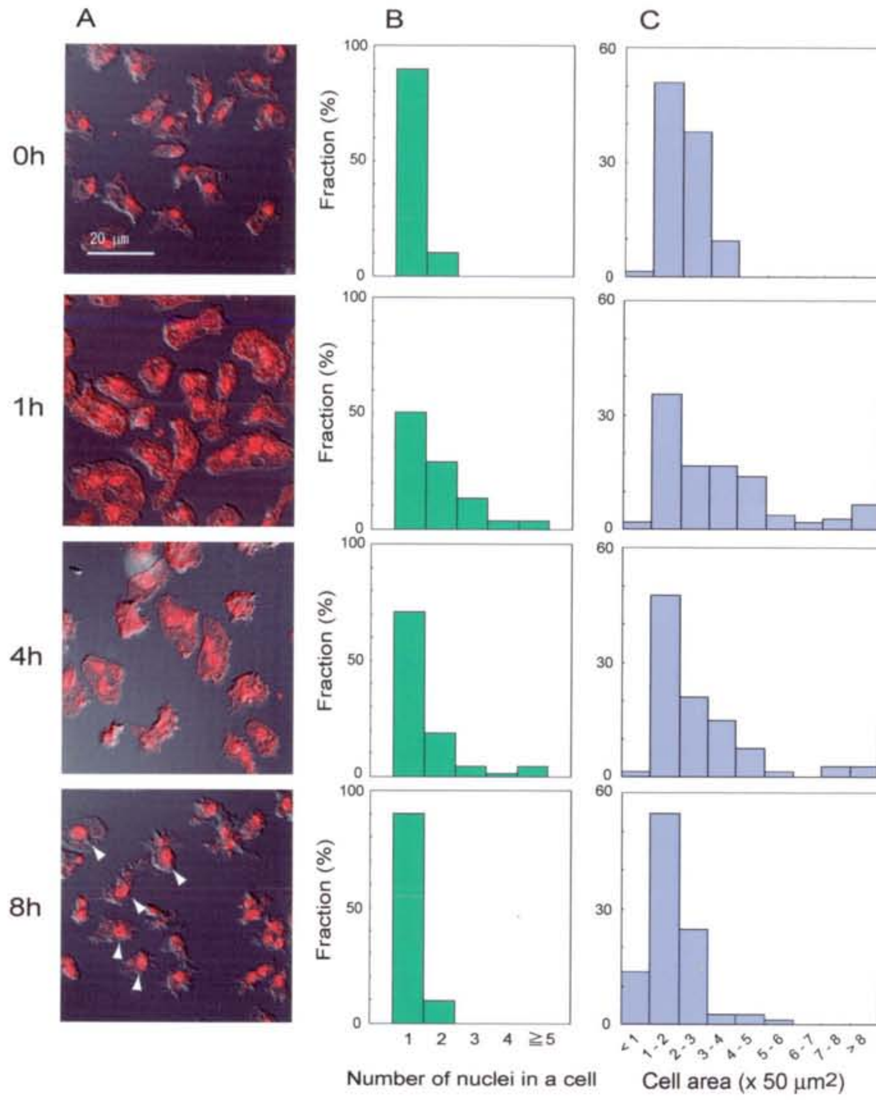


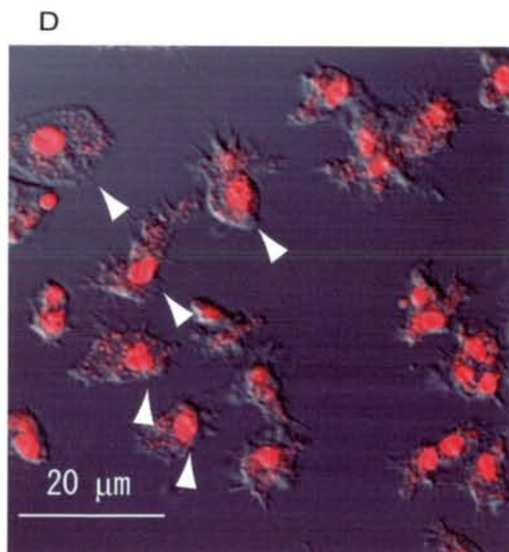
Figure 4. Change in cell motility during sexual development.

The motility is expressed as the movement of the cell center over the course of 10 min. Mated (KAX3 and V12) and non-mated (KAX3) incubation are shown by black and white boxes, respectively. At least 100 cells were measured. The bar represents SD.

Figure 5. Nuclear changes in the giant cells.

(A) Fluorescence images of the cells stained with PI are shown. The arrowhead indicates a large nucleus. **(B)** Percentages of cells containing nuclear numbers indicated on the ordinate are shown. **(C)** Percentages of cells with the areas indicated on the ordinate are shown. **(D)** Image of 8 h (A) is shown at a higher magnification.


A
B
C
0h
1h
4h
8h
20 μm
Fraction (%)
100
50
0
60
30
1 2 3 4 ≧5
Number of nuclei in a cell
<1 1-2 2-3 3-4 4-5 5-6 6-7 7-8 >8
Cell area (x 50 μm2)


D
20 μm

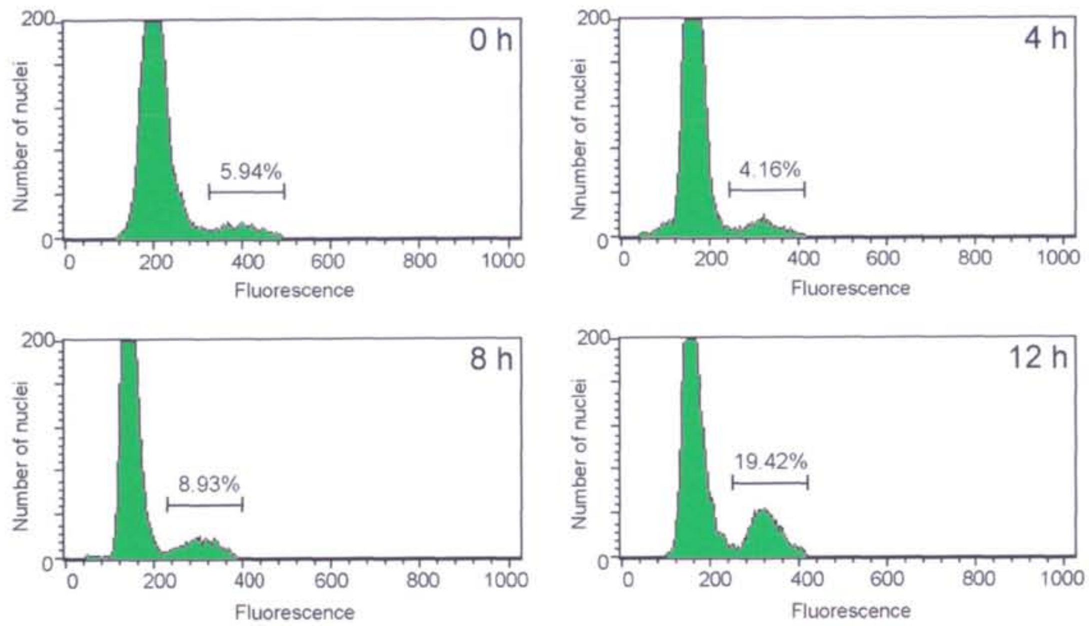


Figure. 6. Formation of diploid nuclei as determined by flow-cytometric analysis.

Nuclei were purified from the cells at the indicated periods of sexual development, stained with PI, and then analyzed by FACS, Twenty thousand nuclei were analyzed for each sample. The bar indicates the range of diploid nuclei.

Figure 7. Microtubule and MTOC distribution during sexual development.

FC-cells of either KAX3 and V12 **(A)** or GFP-γ-tubulin expressing KAX3 and V12 **(B, C, D)** were incubated. They were fixed at indicated periods of incubation and stained for the visualization of nuclei and microtubules. **A:** Nuclei were stained with PI (red), and microtubules with FITC-conjugated anti-α-tubulin monoclonal antibodies (green). **B:** Microtubules were stained red by double immuno-fluorescence. Only the merged images are shown. Yellow dots pointed by arrows indicate MTOC. **C:** Nuclei were stained with PI (red) to show close association of MTOC (green dots). Only the merged images are shown. **D:** The same as in **B** but fixed at 48 h. Microtubules in red (left), MTOCs in green (middle) and merged images (right) are shown. The arrow indicates the position of MTOC. The bar represents 20 μm.

0 h 1 h 4 h

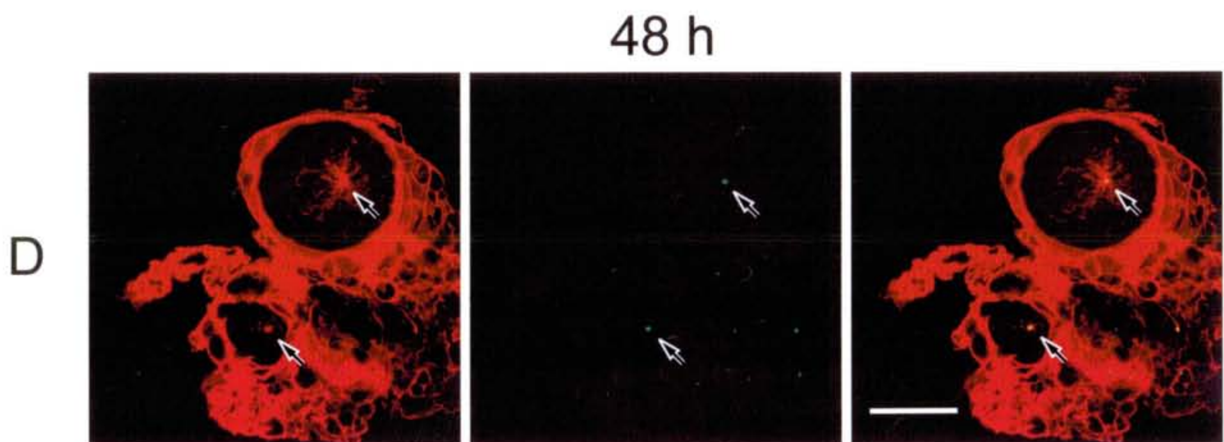
A

B

C

48 h

D



Color	A	B	C	D
Green	microtubule	γ-tubulin	γ-tubulin	γ-tubulin
Red	nucleus	microtubule	nucleus	microtubule

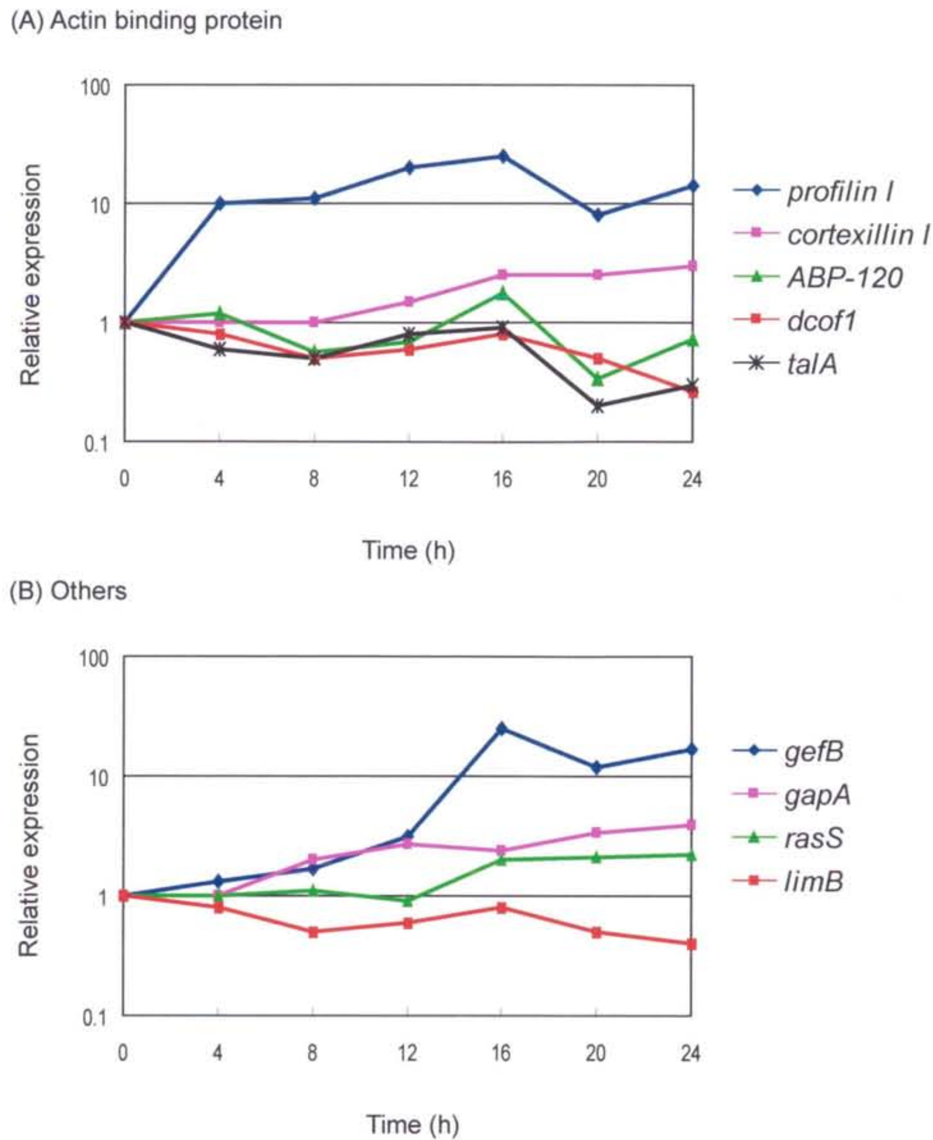


Figure 8. Expression patterns of genes related to motility and/or cytokinesis during sexual development.

FC–cells of KAX3 and V12 were mixed and incubated for sexual development. At the indicated periods of incubation, total RNA was extracted, reverse transcribed and used as templates for real-time PCR using specific primers for each gene. Results of the genes for actin binding proteins **(A)** and of others **(B)** are shown.

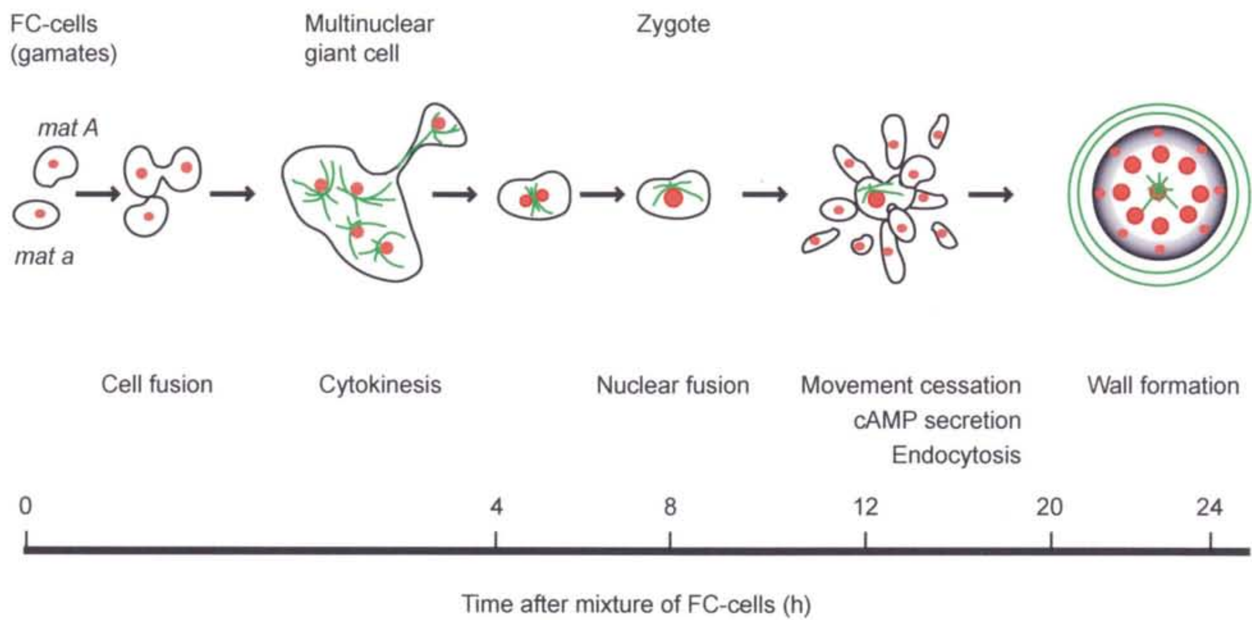


Figure 9. Schematic reresentation of sexual development in *D.discoideum.* Nuclei and microtubules are shown in red and green, respectively. *mat A* and *mat a* are camplementary mating types.