Cellular events during sexual development from amoeba to plasmodium in the slime mould *Physarum polycephalum*

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Time-lapse cinematography and immunofluorescence microscopy were used to study cellular events during amoebal fusions and sexual plasmodium development in *Physarum polycephalum*. Amoebal fusions occurred frequently in mixtures of strains heteroallelic or homoallelic for the mating-type locus *matA*, but plasmodia developed only in the *matA*-heteroallelic cultures. These observations confirmed that *matA* controls development of fusion cells rather than cell fusion. Analysis of cell pedigrees showed that, in both types of culture, amoebae fused at any stage of the cell cycle except mitosis. In *matA*-heteroallelic fusion cells, nuclear fusion occurred in interphase about 2 h after cell fusion; interphase nuclear fusion did not occur in *matA*-homoallelic fusion cells. The diploid zygote, formed by nuclear fusion in *matA*-heteroallelic fusion cells, entered an extended period of cell growth which ended in the formation of a binucleate plasmodium by mitosis without cytokinesis. In contrast, no extension to the cell cycle was observed in *matA*-homoallelic fusion cells and mitosis was always accompanied by cytokinesis. In *matA*-homoallelic cultures, many of the binucleate fusion cells split apart without mitosis, regenerating pairs of uninucleate amoebae; in the remaining fusion cells, the nuclei entered mitosis synchronously and spindle fusion sometimes occurred, giving rise to a variety of products. Immunofluorescence microscopy showed that *matA*-heteroallelic fusion cells possessed two amoebal microtubule organizing centres, and that most zygotes possessed only one; amoebal microtubule organization was lost gradually over several cell cycles. In *matA*-homoallelic cultures, all the cells retained amoebal microtubule organization.

Introduction

Sexual development in the myxomycete *Physarum polycephalum* involves the fusion of uninucleate, haploid, vegetative amoebae to form zygotes which give rise to giant, vegetative syncytia known as plasmodia. Two mating-type loci, *matB* and *matC*, influence the frequency of amoebal fusions, and a third locus, *matA*, controls the development of fusion cells (Youngman *et al.*, 1979, 1981; Kawano *et al.*, 1987); only cells heteroallelic for *matA* develop into plasmodia (Youngman *et al.*, 1981). One aim of the present study was to define the role of *matA* more precisely by comparing the sequence of cellular events during sexual development in *matA*-heteroallelic mixtures with the events that occur in *matA*-homoallelic mixtures.

Mutations associated with the *matA* locus give rise to apogamic strains which can form plasmodia asexually;

individual amoebae in such strains develop into haploid plasmodia (Anderson *et al.*, 1976; Bailey *et al.*, 1987). A second aim of our study was to compare the sequence and timing of events in sexual development with those already observed in an apogamic strain (Bailey *et al.*, 1987), in order to identify features common to both pathways of development. Time-lapse microcinematography of plasmodium development in the apogamic strain CL (Bailey *et al.*, 1987) showed that either one or both daughter cells from an apparently normal amoebal division could enter an extended cell cycle accompanied by continuing growth. During this cell cycle, ability to undergo the reversible amoeba–flagellate transformation was lost and the cell became irreversibly committed to development. At the end of the extended cell cycle, the large, uninucleate, haploid cell gave rise to a binucleate, haploid cell by mitosis without cytokinesis. This cell developed into a multinucleate plasmodium by rounds of synchronous mitoses unaccompanied by cytokinesis, and by fusion with other binucleate or multinucleate cells.

Abbreviations: DAPI, 4,6-diamidino-2-phenylindole; IMT, intermitotic time; MTOC, microtubule organizing centre.

In the present study, we used time-lapse microcinematography to examine the timing of cell fusion in relation to the amoebal cell cycle, and to determine whether an extended cell cycle with continuing growth occurred during sexual development. An earlier filming study (Holt & Huttermann, 1979; Holt *et al.*, 1979) had shown that nuclear fusion in *matA*-heteroallelic fusion cells occurred in interphase, about 2 h after amoebal fusion, but that nuclear fusion in *matA*-homoallelic fusion cells did not occur until mitosis, which was accompanied by cytokinesis. However, no information could be obtained from these films about the origins or final fates of individual cells because the cultures were filmed only for short periods.

Immunofluorescence microscopy was used to investigate the changes in microtubule organization that follow amoebal fusion in *matA*-heteroallelic and *matA*-homoallelic cultures, and to compare these with the extensive changes in microtubule organization that have been observed during apogamic development (Havercroft & Gull, 1983; Gull *et al.*, 1985; Blindt, 1987).

Methods

Strains. The amoebal strains used were LU859 (*matA2 matB1 matC1*; Cooke, 1974), CH508 (*matA2 matB3 matC2*; Youngman *et al.*, 1979) and LU648 (*matA1 matB1 matC1*; Cooke & Dee, 1975). Stocks were maintained on lawns of *Escherichia coli* on liver infusion agar at 26 °C (Bailey *et al.*, 1987). All experiments were done at 22 °C.

Time-lapse microcinematography. Filming was done in cavity slides using the equipment and conditions described by Bailey *et al.* (1987). Two mixtures of strains were filmed. The mixture CH508 × LU648 was heteroallelic for *matA* while the mixture CH508 × LU859 was homoallelic for *matA*; both mixtures were heteroallelic for *matB* and *matC*. Encysted amoebae were mixed together and inoculated into slide cultures at a starting density of 20–25 cells mm⁻². The cells had excysted and increased in number to 40–100 cells mm⁻² by the time filming began (18–27 h after inoculation) giving 35–60 cells within the film frame. Filming was continued for 32–34 h at one frame per 15 s. Clonal cultures of amoebae were set up using the same initial cell density and were filmed for the same length of time.

Film analysis. The intervals between cellular events were measured using an analysing projector as described by Bailey *et al.* (1987). In the slide cultures, the cells were flattened between an agar block and a coverslip. Bailey *et al.* (1987) demonstrated that, within a single slide culture, measurements of cell area could provide a reliable means of investigating cell growth; amoebal area approximately doubled during each cell cycle. In order to measure cell area, the films were projected onto a screen divided into 8800 squares, each corresponding to 7.30 µm² (1 arbitrary unit) in the slide culture. Since the amount of cell flattening varied from slide to slide, all measurements were taken from a single film. In the text, area measurements are given in arbitrary units. It should be noted that the arbitrary unit of area used by Bailey *et al.* (1987) was different (4.86 µm²) because a different magnification was used for the films. Statistical analyses were done using *t*-tests unless stated otherwise; significance levels are given in the text.

Immunofluorescence microscopy.

Media and reagents. Phosphate-buffered saline (PBS). This was prepared by adding 2.56 g NaH₂PO₄ and 22.49 g Na₂HPO₄ to 500 ml

water and adjusting the pH to 7.2–7.4; to this was added 87.66 g NaCl and the volume was made to 1 litre; this stock solution was diluted 1 in 10 with water before use.

Poly-L-lysine solution. A 50 µg ml⁻¹ solution in 10 mM-Tris/HCl buffer (pH 8.0) was stored at –20 °C. Poly-L-lysine-coated coverslips were prepared by soaking detergent-washed coverslips (Chance no. 1.5) in the poly-L-lysine solution for 10 min at room temperature and then allowing them to dry. The coated coverslips were stored in airtight containers.

DAPI/anti-fade. *p*-Phenylenediamine (100 mg; Sigma) was added to 10 ml 1 M-Tris/HCl (pH 7.5) and the volume was made up to 100 ml with glycerol. DAPI (4,6-diamidine-2-phenylindole, Sigma; 2.5 mg) was added to the glycerol solution which was subsequently stored at –20 °C.

Fixation and staining of cells. Encysted amoebae of two strains were harvested and mixed to give a suspension containing 2 × 10⁶ cells ml⁻¹ of each strain. Liver infusion agar plates, containing 3 mM-sodium citrate buffer, pH 5.0, were inoculated with 0.1 ml of cell suspension and 0.1 ml of a 1 in 3 dilution of formalin-killed bacteria (Bailey *et al.*, 1987). This mixture was spread to form a square of side 5 cm and incubated at 22 °C. At various times after inoculation, the cells were washed off five plates with 2 ml of water per plate, and the suspensions were combined. The cells were spun down and resuspended in water at 5 × 10⁶ ml⁻¹. Drops of suspension (20 µl) were placed on poly-L-lysine coated coverslips and the cells were allowed to settle for 40 min before being fixed by immersion in methanol at –20 °C for 10 s. The cells were rehydrated in PBS and 10 µl of the first antibody added for 1 h (anti-β-tubulin, Amersham; dilution 1:1000 in PBS); this antibody recognizes β-tubulin in a wide variety of cell types (Blöse *et al.*, 1982). After several washes in PBS, 10 µl of the second antibody was added for 1 h (fluorescein-isothiocyanate-conjugated goat-antimouse IgG, Tago Inc., USA; dilution 1:50 in PBS). The coverslips were then re-washed in PBS and mounted onto glass microscope slides using the glycerol/DAPI/anti-fade mix. Slides were examined using a Zeiss Axiophot microscope fitted with a ×100 Plan-neofluar objective. Amoebal strains grown alone and treated identically were used as controls.

A different preparation method was required for flagellates because flagella are quickly resorbed when the cells are placed on a surface. Cells were removed from culture plates as described above and resuspended at 5 × 10⁶ cells ml⁻¹ in water. To induce flagellate formation, 2 ml of suspension was shaken at 200 strokes min⁻¹ on a reciprocating shaker for 3 h at 26 °C. The cells were fixed in suspension by adding 0.1 vol formaldehyde (38%, w/v, stock solution). After 1 h, the cells were thoroughly washed in PBS, placed in methanol at –20 °C for 10 s, and finally rinsed in acetone for 5 min before being thoroughly washed in PBS. The cells were resuspended in PBS at 1–5 × 10⁶ cells ml⁻¹. Drops of suspension were placed on poly-L-lysine coated coverslips and allowed to dry; the cells were then stained as described above.

Results

Time-lapse cinematographic analysis of events preceding amoebal fusion

Three films were made of mixed cultures inoculated with a pair of amoebal strains carrying different *matA* alleles (CH508 × LU648) and two films of mixed cultures in which both strains carried the same *matA* allele (CH508 × LU859). Fusions between amoebae were observed during the course of all five films. Sexual

Table 1. *Timing of events*Times are given as the mean (h) $\pm$ SD; the number of observations, *n*, is given in parentheses.

Strains	(a) Amoebal IMT*	(b) Mitosis (M_1) to cell fusion†	(c) Cell fusion to mitosis (M_2)	(d) Cell fusion to split‡	(e) M_1 to M_2	(f) Binucleate to quadrinucleate
CH508 $\times$ LU648 (Heteroallelic for <i>matA</i>)	9.13 $\pm$ 1.46 (69)	3.46 $\pm$ 1.77 (98)	16.90 $\pm$ 1.44 (26)		20.58 $\pm$ 1.73 (37)	6.13 $\pm$ 0.62 (12)
CH508 $\times$ LU859 (Homoallelic for <i>matA</i>)	10.06 $\pm$ 1.44 (45)	(1) 3.30 $\pm$ 2.21 (72) (2) 3.94 $\pm$ 2.46 (19) (3) 3.12 $\pm$ 2.11 (48)	4.89 $\pm$ 2.00 (14)		9.35 $\pm$ 2.32 (20) 2.38 $\pm$ 1.23 (31)	8.15 $\pm$ 1.15 (12)

* Amoebal IMTs for clonal cultures were: LU648, 10.20 $\pm$ 2.02 (76); LU859, 10.34 $\pm$ 1.63 (29); CH508, 10.76 $\pm$ 1.52 (36).

† (1) All cells; (2) fusions where mitosis occurred in the fusion cell; (3) fusion cells in which cell fusion was followed by splitting into two uninucleates.

‡ Time from cell fusion to split – see text.

development occurred in the *matA*-heteroallelic cultures and by the end of each film the field of view was occupied by large plasmodia. In the films of *matA*-homoallelic cultures, plasmodium development did not occur.

Amoebal intermitotic times. The intermitotic time (IMT) was defined as the time between nucleolar disappearance in successive mitoses. The amoebal IMTs were compared for the three films showing sexual development; analysis of variance showed no significant differences between the films ($F = 0.51$; $P > 0.05$) and the data were therefore pooled for further analysis (Table 1*a*). The amoebal IMTs measured from the two films of *matA*-homoallelic cultures were also not significantly different ($P > 0.05$) and these data were pooled (Table 1*a*). The distribution of amoebal IMTs from each film appeared unimodal, suggesting that the two strains in each mixture did not show characteristically different IMTs. This conclusion was supported by the analysis of films of each amoebal strain cultured alone under the same conditions used for the mixed cultures; no significant differences were found between the IMTs in these clonal cultures ($P > 0.05$; Table 1*a*). There was, however, a significant difference ($P < 0.001$) between amoebal IMTs in *matA*-heteroallelic cultures and the amoebal IMTs for all other cultures.

Origins of fusing cells. An example of a pedigree traced from a film of sexual development is shown in Fig. 1. Out of 68 amoebal fusions observed in the films of sexual development, 66 involved pairs of uninucleate amoebae and two were multiple fusions, each of which involved three amoebae. Most of the amoebae participating in fusions (101/138) could be traced back to a previous amoebal division, and from 45 of these divisions it was

Fig. 1. Representative cell pedigree from a *matA*-heteroallelic culture. CF, cell fusion; NF, nuclear fusion; F, fusion of multinucleate cells; E, amoeba ingested by plasmodium. →, Cell left field. A, B, C, see text. Filming began at 0 h.

Fig. 2. Three representative cell pedigrees (a, b and c) from a *matA*-homoallelic culture showing examples of the different fates of binucleate fusion cells. CF, cell fusion; S, cell splits apart. →, Cell left field. D, E, see text. Filming began at 0 h.

possible to determine the fates of both daughter cells. There were 21 divisions from which both daughter cells mated (e.g. daughters of cell A in Fig. 1) and 24 divisions from which one daughter mated and the other completed an apparently normal amoebal cell cycle (e.g. daughters of cell B in Fig. 1). The mean IMT of the daughter cells that completed an apparently normal amoebal cell cycle was 8.92 ± 1.60 h ($n = 24$), not significantly different

from the mean for all amoebal cell cycles ($P > 0.05$; Table 1a). In 24 cases it was possible to measure the length of the cell cycle preceding the one in which fusion occurred; the mean length of this cycle (8.72 ± 1.18 h, $n = 24$) was not significantly different from the mean for all amoebal cell cycles ($P > 0.05$; Table 1a).

Fig. 2 shows examples of pedigrees traced from the two films of *matA*-homoallelic cultures. The events preceding amoebal fusion in these cultures were very similar to those seen in the *matA*-heteroallelic cultures. Forty-nine fusions were observed, of which two were multiple fusions involving three amoebae. Seventy-two of the amoebae that were seen to fuse were traced back to their origin at the previous amoebal division. Among 24 divisions from which the fates of both daughter cells could be determined, there were 17 divisions from which both daughter cells underwent cell fusion (e.g. daughters of cell E in Fig. 2) and seven divisions from which one daughter cell divided and the other fused (e.g. daughters of cell D in Fig. 2). The mean IMT of daughter cells that completed an apparently normal amoebal cell cycle (9.85 ± 1.48 h, $n = 7$) was not significantly different from the mean for all amoebal cell cycles ($P > 0.05$; Table 1a). The mean length of the cell cycle preceding the one in which cell fusion occurred (10.02 ± 1.45 h, $n = 23$) also showed no significant difference from the mean for all amoebal cell cycles ($P > 0.05$; Table 1a).

Age of amoebae at cell fusion. The films were analysed to determine whether the fusion of amoebae occurred at a constant time after mitosis. Fig. 3(a) shows the time from the start of mitosis (defined by nucleolar disappearance in prophase) to cell fusion in the *matA*-heteroallelic cultures; Fig. 3(b) shows the same information for the *matA*-homoallelic cultures. No fusions occurred for about 20 min following the start of mitosis (M in Fig. 3a, b); this period corresponds to the time taken to undergo mitosis and cytokinesis (D in Fig. 3a, b). Thereafter, amoebal fusions were distributed throughout a period roughly equal to the mean length of an amoebal cell cycle (marked as IMT on Fig. 3a, b; Table 1a, b). The data were further analysed to determine whether there was a consistent relationship between the ages of a fusing pair (Fig. 4). For example, if an amoeba could fuse only with another of similar age, the points in Fig. 4 would lie along line A. Alternatively, if the combined age of each pair of amoebae was constant, the points in Fig. 4 would lie along line B. The distribution of points in Fig. 4 suggests that fusions between amoebae occur irrespective of cell age.

Multiple amoebal fusions. In three of the four multiple fusions observed during filming, the origins of the fusing amoebae could be traced. In one fusion, in a culture heteroallelic for *matA*, a cell 4.9 h old fused simul-

Fig. 3. Ages of amoebae at cell fusion. Each bar represents the age of one amoeba at the time of cell fusion, measured from nucleolar disappearance at mitosis (M). The interval between M and D represents the mean time between the start of mitosis and the completion of cytokinesis. IMT represents the mean amoebal IMT. (a) *matA*-heteroallelic cells. (b) *matA*-homoallelic cells; this Figure represents the data on first fusions only (see text).

taneously with a pair of sister cells 1.9 h old. In another fusion, in a *matA*-homoallelic culture, a cell 4.7 h old fused simultaneously with sister cells 1.7 h old. The final fusion in which the origin of the fusing cells could be traced did not involve the fusion of sister cells; this fusion, also in a *matA*-homoallelic culture, began with the fusion of two cells 4.0 and 4.7 h old and was followed 2 min later by fusion with a third cell 4.3 h old.

Time-lapse cinematographic analysis of the events following amoebal fusion

In *matA*-heteroallelic cultures, nuclear fusion occurred in interphase about 2 h after cell fusion (2.05 ± 0.67 h, $n = 59$). In *matA*-homoallelic cultures, no interphase nuclear fusions were observed. Among the 45 *matA*-homoallelic fusion cells followed, two different fates were observed: 14 remained binucleate until both nuclei entered mitosis synchronously some hours later (Fig. 2*b, c*); the remaining 31 fusion cells split without mitosis to regenerate pairs of uninucleate cells (Fig. 2*a*). Of the 62 uninucleate cells formed by cell splitting, seven were later seen to fuse with different partners. In *matA*-homoallelic cultures there was no consistent relationship between the ages of amoebae at cell fusion and the fates of the binucleate fusion cells (Fig. 4).

The films showed that sexual development in *matA*-heteroallelic cultures included an extended cell cycle preceding binucleate plasmodium formation. The length of this cell cycle was calculated by adding the mean age of the pair of fusing amoebae to the time between cell fusion and the following mitosis at which the zygote

Fig. 4. Ages of pairs of mating amoebae. Line A, expected distribution if only amoebae of similar age are able to fuse; line B, expected distribution if the combined age for each pair of mating amoebae was similar. See text for further explanation. ×, Fusions in *matA*-heteroallelic cultures; ●, fusions in *matA*-homoallelic cultures where fusion ends with mitosis; ○, fusions in *matA*-homoallelic cultures where fusion ends in cell splitting.

became binucleate (Table 1*e*). The extended cell cycle was approximately 2.3 times the length of the mean amoebal cell cycle; the following cell cycle, leading to the formation of a quadrinucleate cell, was shorter than the mean amoebal cell cycle (Table 1*f*).

In *matA*-homoallelic cultures, no extended cell cycle was observed; for the 14 fusion cells that remained

binucleate until mitosis, the mean time between the mitosis preceding cell fusion and the synchronous mitoses in the fusion cell was not significantly different from the mean amoebal IMT ($P > 0.05$; Table 1*a, e*). For the 31 fusion cells that split to yield two uninucleates, the mean time between the mitoses preceding and following amoebal fusion was significantly shorter than the mean length of an amoebal cell cycle ($P < 0.001$; Table 1*a, e*).

When *matA*-homoallelic fusion cells entered mitosis, both nuclei proceeded synchronously. The nucleolus moved to the side of the nucleus before disappearing and shortly afterwards the prophase nuclei vanished. This sequence of events was different from that observed in zygotes, in which the nucleus remained visible throughout mitosis; metaphase plate formation and chromosome separation were clearly visible inside the zygotic nucleus. In seven *matA*-homoallelic fusion cells, nuclear fusion occurred during prophase and two uninucleate, presumably diploid daughter cells were formed after cytokinesis [Table 2, class (*a*); Fig. 2*c*]; two daughter cells, from one such division, could be followed until the next mitosis, at which time both divided into three. The mean IMT for these putative diploid amoebae (8.95 ± 0.27 h) was significantly shorter than the mean IMT for haploid amoebae ($P < 0.001$). In three fusion cells undergoing mitosis, nuclear fusion was not observed; each cell gave rise to two binucleate daughters [Table 2, class (*b*); Fig. 2*b*]. The binucleate daughter cells showed the same fates as newly formed fusion cells, either splitting into two uninucleates (three cases) or entering mitosis as a binucleate cell (one case; Fig. 2*b*). Two other *matA*-homoallelic fusion cells observed in mitosis divided to give three daughters [Table 2, classes (*c*) and (*d*); Fig. 2*c*].

Multiple amoebal fusions. In *matA*-heteroallelic cultures, it was possible to determine the fates of the three nuclei in each of the fusion cells formed by multiple amoebal fusions. Two of the nuclei in each cell fused together about 2 h after cell fusion, followed by fusion of the third nucleus 30–40 min later. The resulting zygotes became binucleate 16–17 h after cell fusion and one was observed to become quadrinucleate 5.2 h after becoming

Fig. 5. Areas of cells during sexual development. Mean area is given in arbitrary units ($\pm$ SD) for a sample of cells at each stage indicated on the pedigree. All measurements are taken from one film; 1 arbitrary unit = $7.30 \mu\text{m}^2$ (see Methods), n = number of cells measured. M, mitosis; CF, cell fusion; NF, nuclear fusion.

binucleate. Both cells eventually fused with larger plasmodia and the subsequent fates of their nuclei could not be determined.

In both the multiple fusions involving amoebae homoallelic for *matA*, the resulting trinucleate cells split to form a binucleate and a uninucleate. One binucleate cell subsequently separated into two uninucleates whose final fate could not be determined. The other binucleate cell entered mitosis and gave rise to two binucleate daughters whose subsequent fates could not be determined.

Cell growth and nuclear area

During amoebal cell cycles, cells approximately doubled in area (e.g. Fig. 5). In *matA*-heteroallelic cultures, amoebae that participated in mating were not significantly different in area, at birth, from amoebae that divided to yield daughter cells ($P > 0.05$; Fig. 5). At the time of mating, amoebal areas ranged from 7.5 to 21 arbitrary units; this large variation in area was consistent with the variation in cell age at fusion (Fig. 3*a*). During the extended cell cycle, zygotes continued to grow until, at the first plasmodial mitosis, they were approximately 3 times the area of amoebae in mitosis (Fig. 5). In contrast, *matA*-homoallelic fusion cells in mitosis were approximately 1.5 times the area of amoebae in mitosis. Cell area failed to double in the short cell cycle that ended with the

Table 2. Products of mitosis in binucleate fusion cells homoallelic for *matA*

Class	No. of cells undergoing mitosis	Daughter cells		Daughter nuclei
		uninucleate	binucleate	
(a)	7	2	0	2
(b)	3	0	2	4
(c)	1	2	1	4
(d)	1	3	0	3

Fig. 6. Changes in cell and nuclear area during sexual development. $\blacktriangleright$, Nuclei. Bar, 10 μ m. (a) A, haploid amoeba; FC, fusion cell with two small haploid nuclei; Z, diploid zygote just after nuclear fusion. (b) Z, zygote entering mitosis at the end of the extended cell cycle; note large size of cell and nucleus relative to those in (a). (c) Binucleate plasmodium; the two diploid nuclei are approximately the same size as the zygote nucleus in (a).

formation of a quadrinucleate plasmodium, increasing by a factor of only 1.6 (Fig. 5).

Increase in plasmodial size occurred both by growth of individual plasmodia and by fusions between plasmodia. Many fusions were observed between plasmodia with two or more nuclei, but no fusions were seen between uninucleate zygotes. Two zygotes fused with multinucleate plasmodia; both zygotes were undergoing mitosis at the time of fusion and completed mitosis after fusion.

Following nuclear fusion in *matA*-heteroallelic fusion cells, nuclear area increased during the remainder of the extended cell cycle; at mitosis, nuclear area was substantially greater than twice the nuclear area of a haploid amoeba entering mitosis (compare nuclear areas in Fig. 6a, b, c). Nevertheless, in quadrinucleate cells the area of each diploid nucleus was approximately twice the area of a haploid amoebal nucleus. In *matA*-homoallelic cultures, all the observed changes in nuclear area were consistent with the changes in ploidy assumed to have occurred at mitosis.

Microtubule organization during plasmodium development

Amoebae possess a complex array of cytoplasmic microtubules nucleated by a microtubule organizing centre (MTOC) which is closely associated with a pair of centrioles and linked to the nucleus. During amoebal mitosis, the MTOC and centrioles duplicate, divide, and migrate to form the two poles of the mitotic spindle. Plasmodia possess no centrioles, and microtubules are present only in the mitotic spindle which, unlike the amoebal spindle, is formed inside an intact nuclear membrane (Howard, 1932; Tanaka, 1973; Havercroft & Gull, 1983).

The changes in microtubule organization during plasmodium development were studied using immuno-

Table 3. *Numbers of cells with no, one or two MTOCs at different stages of development*

Stage of development	Number of interphase cells with:			Total scored
	No MTOC	1 MTOC	2 MTOCs	
Fusion cell	4 (16%)	8 (32%)	13 (52%)	25
Zygote	110 (33%)	212 (64%)	11 (3%)	333
Binucleate plasmodium	26 (58%)	10 (22%)	9 (20%)	45

fluorescence microscopy of cells stained with an anti- β -tubulin monoclonal antibody. The nuclei were stained with the DNA-specific fluorochrome DAPI. Counts were made of the number of MTOCs present in a sample of interphase fusion cells, zygotes and binucleate plasmodia (Table 3). Binucleate fusion cells were recognized by the presence of two nuclei similar in size to those of vegetative amoebae (Fig. 6a); the majority of these cells contained two MTOCs (Fig. 7a, b) although some possessed none or one. Zygotes were recognized as large cells containing a single large nucleus (Fig. 6b); only 3% of these possessed two MTOCs, the majority possessing one (Fig. 7c, d). Binucleate plasmodia were identified by the presence of two large nuclei within a large cell (Fig. 6c); most of these did not possess a MTOC (Table 3). Microtubules were present in all binucleate plasmodia, but in cells lacking a MTOC, the microtubules did not radiate from a single focus. Plasmodia with more than two nuclei were excluded from consideration because they could have been the result of fusion between smaller plasmodia.

In amoebae stained with the anti- β -tubulin antibody, a pair of small dots was seen closely associated with the MTOC; similar structures were present at the spindle poles of mitotic amoebae, suggesting that they were the centrioles. A stained cell population was used to

Fig. 7. Interphase cells from a *matA*-heteroallelic culture, double-stained with β -tubulin antibody to show microtubules (*a*, *c*, *e* and *g*) and with DAPI to show DNA (*b*, *d*, *f* and *h*). The dots in the cytoplasm in (*b*), (*d*), (*f*) and (*h*) are a mixture of mitochondria and ingested bacteria. Bar, 1 μ m. (*a*, *b*) Fusion cell with two MTOCs (►) and two small nuclei. (*c*, *d*) Zygote with one MTOC (►) and a single large nucleus. (*e*, *f*) Zygote with two pairs of centrioles in a cluster (►) and a single large nucleus. (*g*, *h*) Zygote with two separate pairs of centrioles (►) and a single large nucleus.

determine the number of pairs of centrioles present in cells at different stages of development. Out of 98 zygotes in which centrioles could be scored, 77 possessed two pairs of centrioles; these were either present as a group of four (62 cells; Fig. 7*e, f*) or as two separate pairs (15 cells; Fig. 7*g, h*). The remaining 21 zygotes each possessed only

Fig. 8. Mitotic cells from a *matA*-heteroallelic culture, double-stained with β -tubulin antibody to show microtubules (*a*, *c*, *e* and *g*) and with DAPI to show DNA (*b*, *d*, *f* and *h*). The dots in the cytoplasm in (*b*), (*d*), (*f*) and (*h*) are a mixture of mitochondria and ingested bacteria. Bar, 1 μ m. (*a*, *b*) Zygote in mitosis with no extranuclear MTOC; the small object to the right of the mitotic nucleus is the remains of an ingested amoeba with nucleus and microtubules still visible. (*c*, *d*) Zygote in mitosis with extranuclear MTOC or centrioles present at one pole of the spindle (►). (*e*, *f*) Zygote in mitosis with an extranuclear MTOC or centrioles (►); no ingested amoebae are present. (*g*, *h*) Binucleate plasmodium in mitosis; no extranuclear MTOC is present.

one pair of centrioles. Centrioles could not be observed in 71 of the zygotes examined.

Of zygotes in mitosis, 18 (35%) possessed an extranuclear structure labelled by the anti- β -tubulin antibody;

it was not possible to determine whether this structure was a MTOC or one or more pairs of centrioles. This extranuclear structure lay either on the side of the cell opposite the dividing nucleus (Fig. 8*e, f*) or at one pole of the spindle (Fig. 8*c, d*) and thus was evidently not nucleating the spindle. No extranuclear MTOC or centrioles were visible in the remaining 34 (65%) zygotes examined (Fig. 8*a, b*).

Of 29 binucleate plasmodia, ten possessed centrioles; three had one pair, six had two pairs and one had four pairs. No binucleate plasmodia in mitosis were observed to possess extranuclear centrioles or a MTOC (Fig. 8*g, h*).

Microtubule organization during mitosis in cultures homoallelic for matA

Filming analysis indicated that there were several possible outcomes for mitosis in a binucleate cell homoallelic for *matA* (Fig. 2, Table 2). Immunofluorescence microscopy of five binucleate fusion cells showed that the mitotic spindles lay in various orientations. In two cells the spindles lay end-to-end and in one of these only three spindle poles were observed (Fig. 9*a, b*). Since a cleavage furrow usually forms at the equator of a spindle when cytokinesis follows mitosis (Rappaport, 1971), the mitosis shown in Fig. 9(*a, b*) would be expected to produce three daughter cells [Fig. 2*c*; Table 2, classes (*c*) and (*d*)], of which one would contain either two haploid nuclei or one diploid nucleus. Two binucleate cells were observed in which the mitotic spindles were parallel (Fig. 9*c, d*); cytokinesis in such cells would be expected to produce two daughter cells, which might be diploid uninucleate or haploid binucleate [Fig. 2*b, c*; Table 2, classes (*a*) and (*b*)]. In the fifth cell the spindles were approximately perpendicular.

The mitotic spindle was also examined in putative diploid amoebae, of which 37 contained bipolar spindles and 12 contained tripolar spindles (Fig. 9*e, f*). The presence of tripolar spindles was consistent with our conclusion from filming analysis that diploid amoebae could divide to give three daughter cells.

A count of the number of MTOCs in interphase cells homoallelic for *matA* showed that 46 (67%) binucleate, haploid cells and nine (4.3%) putative diploid amoebae possessed two MTOCs.

Flagellate formation

In moist conditions, amoebae rapidly transform into flagellates in which the centrioles form the basal bodies for the one long (anterior) and one short (posterior) flagella; the posterior flagellum is usually pressed against the side of the cell and is difficult to observe. To

Fig. 9. Mitotic cells from *matA*-homoallelic cultures double-stained with β -tubulin antibody to show microtubules (*a, c* and *e*) and with DAPI to show DNA (*b, d* and *f*). The dots in the cytoplasm in (*b*), (*d*) and (*f*) are a mixture of mitochondria and ingested bacteria. Bar, 1 μ m. (*a, b*) Binucleate cell in synchronous mitosis; the spindles have fused at one pole. (*c, d*) Binucleate cell in synchronous mitosis; four sets of chromosomes and four spindle poles are visible. (*e, f*) Two uninucleate cells in mitosis; one spindle is bipolar while the other is tripolar.

determine the stage of sexual development at which the ability to undergo the amoeba-flagellate transformation was lost, flagellate formation was induced in a *matA*-heteroallelic population. A sample of each cell type was scored for the presence or absence of the long flagellum. Of the 500 amoebae scored, 87% were flagellates and all of these possessed one long flagellum (Fig. 10*a, b*). All five fusion cells observed were flagellates; four possessed one long flagellum and the other possessed two. Only 46 flagellates were observed amongst 420 zygotes; 41 possessed one long flagellum (Fig. 10*c, d*) and five possessed two. No flagella were observed on the 50 binucleate and multinucleate plasmodia scored. In a *matA*-homoallelic culture only 4% of cells failed to transform into flagellates; 6.2% of the population had two long flagella and the remainder had one.

Fig. 10. Flagellates from a *matA*-heteroallelic culture, double-stained with β -tubulin antibody to show microtubules (*a* and *c*) and with DAPI to show DNA (*b* and *d*). The dots in the cytoplasm in (*b*) and (*d*) are a mixture of mitochondria and ingested bacteria. Bar, 1 μ m. (*a*, *b*) Flagellate presumably derived from an amoeba. (*c*, *d*) Flagellate presumably derived from a zygote.

Discussion

Our filming analysis has provided support for the suggestion that *matA* has no effect on cell fusion (Youngman *et al.*, 1981). Amoebal fusions occurred frequently in cultures either heteroallelic or homoallelic for *matA*. In both types of culture, cell age at the time of cell fusion varied widely between a minimum of about 20 min and a maximum approximately equal to an amoebal cell cycle (Fig. 3); the age distributions were very similar in the two types of culture. In both *matA*-heteroallelic and *matA*-homoallelic cultures, the majority of amoebae fused in the first two-thirds of the cell cycle (6–7 h); during the remainder of the cell cycle, the number of cells available to fuse presumably declined as an increasing number of cells entered mitosis. Comparison of the age distributions of fusing cells in the two types of culture was complicated by the fact that the amoebal IMT was significantly shorter in the *matA*-heteroallelic mixtures than in all other cultures (Table 1*a*); this could have been a consequence of alterations in environmental conditions caused by the presence of developing plasmodia in *matA*-heteroallelic cultures. In *matA*-homoallelic cultures, a significant reduction in mean IMT was observed for cells which split apart after amoebal fusion but before undergoing mitosis (Table 1*a*, *e*); we can offer no explanation for this reduction.

In the sexual development of many organisms, cell fusion occurs in the G1 phase of the cell cycle, although there is evidence that *Tetrahymena* micronuclei are in the G2 phase at the time of cell fusion (Crandall, 1977; Doerder & De Bault, 1975). *Physarum* amoebae, like plasmodia, apparently have no G1 phase in their vegetative cell cycle (Turner *et al.*, 1981; Nygaard *et al.*, 1960). We cannot exclude the possibility that mating occurred only during a G1 phase in *Physarum*, but if this was so, the mean length of time for which an amoeba must have remained in G1 prior to mating was 3.5 h, about one-third of the length of an amoebal cell cycle (Fig. 3, Table 1). Such an extended G1 period seems unlikely since measurements of DNA content by flow cytometry indicate that there is no G1 peak in exponentially growing, clonal cultures (Fry & Matthews, 1987); such cultures should contain a significant proportion of mating competent amoebae (Shipley & Holt, 1982). If the cell cycle was normal in the mating populations that we observed, the amoebae must have been in the S or G2 phase at the time of cell fusion.

In agreement with the observations of Holt & Huttermann (1979), the first evidence that plasmodium development had been initiated in the *matA*-heteroallelic mixtures was the occurrence of nuclear fusion in interphase about 2 h after amoebal fusion; interphase nuclear fusion did not occur in *matA*-homoallelic mixtures. There is evidence that microtubules have an essential function in nuclear fusion in *Chlamydomonas* and other organisms (Dutcher, 1988; Oakley & Morris, 1980). Our immunofluorescence studies suggested that this might also be the case in *Physarum*, since changes in microtubule organization apparently began before nuclear fusion; although each member of a fusing pair of amoebae presumably contributed one MTOC to the fusion cell, two MTOCs were found in only 52% of *matA*-heteroallelic fusion cells and 3% of zygotes. In contrast, binucleate cells homoallelic for *matA* retained two clearly separate amoebal MTOCs and associated microtubules.

Comparison of the events in cell mixtures homoallelic and heteroallelic for *matA* revealed cellular events that were associated with *matA*-heteroallelicism: these included extension of the cell cycle, nuclear fusion in interphase, reduction in the number of MTOCs, and loss of ability to undergo the amoeba-flagellate transformation. These events were, therefore, directly or indirectly under the control of *matA*. Comparison of the events during sexual plasmodium formation with those previously described for apogamic plasmodium formation (Bailey *et al.*, 1987) indicates which events are essential for development; nuclear fusion is clearly not essential since it did not occur in apogamic strains. A striking similarity between sexual and apogamic development, revealed by our present and previous (Bailey *et al.*, 1987)

filming analyses, is the existence of an extended cell cycle, approximately 2.3 times the length of an amoebal cell cycle, ending with the formation of a binucleate cell by mitosis without cytokinesis. This extended period may be essential for development and may be required for the completion of a series of developmentally regulated events leading to the onset of the plasmodial type of nuclear division cycle and the suppression of cytokinesis. The fact that apogamic strains arise as a result of mutations at *matA* reinforces our suggestion that extension of the cell cycle is under the control of *matA*.

In sexual development, as was previously found in apogamic plasmodium formation (Bailey *et al.*, 1987), the extended cell cycle was accompanied by continuing growth, leading to an increased cytoplasm:DNA ratio in the cell entering mitosis. In both types of development, following the extended cell cycle, there was a short cell cycle, about 0.7 times the length of an amoebal cell cycle, ending in the formation of a quadrinucleate cell. At the end of this short cell cycle, a sexually formed, diploid, quadrinucleate plasmodium was, on average, 11 times the mean area of an amoeba at birth. In the apogamic strain CL, a haploid, quadrinucleate plasmodium was approximately 6 times the mean area of an amoeba at birth (Bailey *et al.*, 1987). These figures imply that the cytoplasm:DNA ratio, at this stage of development, was similar in both sexual and apogamic development. An increase and subsequent decrease in nuclear area occurred during the extended and shortened cell cycles in both sexual and apogamic development (Bailey *et al.*, 1987). In contrast, in *matA*-homoallelic fusion cells, there was no increase in the cytoplasm:DNA ratio; mitosis with cytokinesis occurred at the end of a period equivalent to the length of a normal amoebal cell cycle (Table 1a, e).

Our analysis of the timing of events in an apogamic strain (Bailey *et al.*, 1987) showed that cells became committed to development during the extended cell cycle, at a maximum of 13.5 h (slightly more than one amoebal cell cycle) before binucleate cell formation. From kinetic studies, Shipley & Holt (1982) suggested that, in sexual development, commitment to plasmodium formation coincided closely with the time of amoebal fusion and probably preceded nuclear fusion. The length of time between amoebal fusion and binucleate plasmodium formation, measured from our films, ranged from 13.6 to 20.2 h. Thus, if amoebal fusion coincides with commitment, binucleate plasmodium formation does not occur at a fixed time after commitment. There was a minimum interval of about 1.5 cell cycles between amoebal fusion and binucleate plasmodium formation; this interval could be related to the time required to complete a sequence of developmentally

regulated events, including the time required to reach a minimum cell size for the mitosis by which the zygote becomes binucleate.

In apogamic development, the ability to undergo the amoeba-flagellate transformation was lost during the extended cell cycle, 3–5 h before commitment, at about the time that the cell would have divided had it continued to cycle vegetatively (Blindt *et al.*, 1986; Bailey *et al.*, 1987). Our immunofluorescence studies of sexual development showed that all fusion cells and some zygotes could transform into flagellates when suspended in water. Thus in sexual development, unlike apogamic development, cells are still able to transform into flagellates at about the time of, or even after, commitment; this is consistent with previous studies in which mating was observed between flagellates (Ross, 1957). However, no zygotes in mitosis or binucleate plasmodia were observed with flagella, indicating that ability to undergo the amoeba-flagellate transformation was lost by the time of binucleate plasmodium formation. In contrast, in *matA*-homoallelic cultures, the majority of cells transformed into flagellates when suspended in water.

Even though many zygotes had two pairs of centrioles, very few of them possessed two long flagella. This agrees with observations on apogamic development which indicated that a change in centriolar function rather than a loss of centrioles was responsible for the loss of ability to undergo the amoeba-flagellate transformation and the failure to undergo amoebal mitosis (Blindt, 1987; Bailey *et al.*, 1987). In some zygotes, the two pairs of centrioles did not show the close association with the MTOC that they have in amoebae (Fig. 9); this provides further evidence in support of the suggestion that a change in centriolar function occurred during the extended cell cycle in both apogamic and sexual plasmodium development (Blindt, 1987; Bailey *et al.*, 1987).

The first observable change in microtubule organization detected by immunofluorescence studies of apogamic development occurred at the mitosis by which the developing uninucleate cell became binucleate; this mitosis appeared to be of the intranuclear plasmodial type (Blindt, 1987). Our films of sexual development suggested that the mitosis by which the zygote became binucleate might also be intranuclear since it resembled the mitosis observed in multinucleate plasmodia rather than mitosis in amoebae. Our immunofluorescence studies of sexual development showed that an extranuclear MTOC, presumably the remains of the amoebal MTOCs, was present in some zygotes at mitosis. In the following interphase, more binucleate plasmodia possessed two MTOCs than there were zygotes with two MTOCs, suggesting that the amoebal MTOC had duplicated even though it was not utilized during the first intranuclear plasmodial mitosis. The amoebal micro-

tubule organization was lost only gradually over several cell cycles. Similar observations have been made on apogamic development (Gull *et al.*, 1985; Blindt, 1987).

The isolation of diploid amoebae from mixtures of strains homoallelic for *matA* was first described by Youngman *et al.* (1981); such amoebae have been useful in genetic analysis by allowing dominance and complementation tests on mutants isolated in amoebae (Anderson & Youngman, 1985; Dee *et al.*, 1989). Holt & Huttermann (1979) observed that nuclear fusion occurred at mitosis in binucleate fusion cells homoallelic for *matA*, and that cytokinesis gave rise to pairs of uninucleate, presumably diploid daughters. Youngman *et al.* (1981) found that the frequency of diploid amoebae formed in *matA*-homoallelic mixtures was lower than the frequency of zygotes formed in *matA*-heteroallelic cultures; to account for this difference they suggested that a minority of the fusion cells homoallelic for *matA* might split apart before mitosis. In our films of *matA*-homoallelic cultures, the majority (75%) of fusion cells were observed to split apart before mitosis and some cells underwent two or even three rounds of successive fusion and splitting.

Our filming studies also showed that even when a *matA*-homoallelic fusion cell reached mitosis, this did not always lead to the formation of two diploid amoebae (Fig. 2, Table 2). Filming analysis indicated that putative diploid amoebae sometimes divided into three at mitosis, consistent with the observation of tripolar spindles in uninucleate cells during our immunofluorescence studies. The daughter cells from these divisions would presumably be aneuploid and would not be expected to survive; this may account for the reports that cultures of diploid amoebae have slower growth rates than cultures of haploid amoebae (Youngman *et al.*, 1981).

The immunofluorescence studies showed that synchronous mitosis in *matA*-homoallelic binucleate cells sometimes resulted in spindle fusion, although this involved only one pole of each spindle in some cases. Assuming that cytokinesis occurred at the equator of the spindle in each case (Rappaport, 1971), the different spindle orientations observed by immunofluorescence accounted for all the different types of products observed in the films (Figs 2 and 9). The occurrence of spindle fusion suggested that mitosis was of the amoebal type with breakdown of the nuclear envelope. Filming analysis of *matA*-homoallelic cultures showed that mitosis was always followed by cytokinesis, indicating that the characteristic coupling of these events that occurs in amoebae had not been lost.

All the cellular events observed in *matA*-homoallelic cultures can be explained by assuming that, although amoebal fusion occurred as a result of heteroallelism at *matB* and *matC*, all the cells in the culture retained

amoebal characteristics. Events associated with plasmodium development were observed only in cultures heteroallelic for *matA*, or in apogamic strains (Bailey *et al.*, 1987), which are able to develop as a result of mutation at *matA*. It is not clear which of these events is most closely related to the primary action of *matA*, and hence to the initiation of plasmodium development. Studies on mutants blocked at various stages of apogamic plasmodium formation, due to mutation at *matA* (Anderson *et al.*, 1989) and at several other loci, will play an important part in elucidating the genetic control of plasmodium development.

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