Stimulation of Mitosis Following Fusion of Plasmodia in the Myxomycete *Physarum polycephalum*

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SUMMARY

Rapid fusion after contact between multinucleate, naturally synchronous plasmodia of the same strain of *Physarum polycephalum* was demonstrated by autoradiography. Fusion between plasmodia of equal size at the same stage in the mitotic cycle resulted in synchronous mitosis with a delay. This delay was slight if the nuclei were about to undergo mitosis at the time of fusion, but increased in a roughly linear fashion to about 4 h in plasmodia fused at an early stage in the mitotic cycle. Fusion between plasmodia at different stages in the mitotic cycle also resulted in synchronous mitosis, division of nuclei late in the cycle being delayed and of those early in the cycle being accelerated. When both plasmodia were in mid-interphase, the amount of acceleration was proportional to the difference in stages at the time of fusion, a 5 h phase difference giving about 3 h acceleration. However, non-synchronous mitosis resulted when a plasmodium about to undergo mitosis was fused with one earlier in the nuclear cycle, the nuclei in the former plasmodium presumably having reached a stage at which retardation could not occur.

INTRODUCTION

When the myxomycete *Physarum polycephalum* is cultured as a multinucleate plasmodium, nuclear divisions are naturally synchronous and occur without cell division (Howard, 1932), a feature making the organism an attractive one for studying events in the mitotic cycle. In most eukaryotes, the mitotic cycle consists of a sequence of stages: a mitosis (M) to initiate the cycle, a phase of growth (G_1), a phase of DNA synthesis (S), a second wave of growth (G_2), and a mitosis (M) to terminate the cycle (Howard & Pelc, 1953). The mitotic cycle in plasmodia of *P. polycephalum* does not follow this sequence; lacking a demonstrable G_1 , the cell proceeds directly from M to S (Nygaard, Guttes & Rusch, 1960). When plasmodia are cultured with a mitotic cycle of 12 h, S has a duration of about 3 h; the cell spends the remainder of the cycle in G_2 .

Two earlier studies demonstrate mitotic stimulation through plasmodial fusion. Guttes & Guttes (1968) showed that when a small plasmodium containing labelled nuclei in S phase was fused with a larger plasmodium containing unlabelled nuclei in late- G_2 phase, the labelled nuclei divided together with the unlabelled nuclei shortly after fusion, i.e. division of S phase nuclei was stimulated. Rusch, Sachsenmaier, Behrens & Gruter (1966) reported that when two plasmodia of equal size but at different times in G_2 phase were fused by overlaying (i.e. placing one plasmodium above the other) followed by 90 min of starvation, all nuclei in the fused plasmodia divided synchronously at a time intermediate between the unfused controls.

The fusion technique of Rusch *et al.* (1966) has been modified for these studies. A simple procedure has been developed for preparing plasmodial pieces of uniform size. The demon-

stration of rapid fusion in the presence of medium has obviated the use of starvation. A method for measuring the effect of fusion, *per se*, upon mitosis has been incorporated into the experimental design. Fusion studies with plasmodia of equal size from different phases throughout the mitotic cycle have now been carried out in order to examine the effect of position in the cycle on mitosis and to determine the combination of plasmodia which would yield maximal stimulation.

METHODS

Preparation of plasmodial discs. *Physarum polycephalum* strain M₃CV, provided by Dr Harold P. Rusch, was cultured on an axenic medium (Chin & Bernstein, 1968). Synchronized plasmodia were formed by starving a thick slurry of microplasmodia from submerged culture on dry filter paper for 3 h. The first synchronous mitoses (MI) after fusion of microplasmodia occurred 7 h after addition of medium and the second 12 h later.

Approximately 15 ml of medium was added to a Petri dish containing the growing culture in order to float the plasmodium free of the filter paper support on which it had been synchronized and cultured. The growing edge of the plasmodium usually rose readily with the surface of the medium while the centre of the plasmodium tended to stick to the filter paper support. The plasmodium was gently teased free by sliding a curved spatula between the organism and the filter paper. With a little practice, cultures could be floated without tearing or submerging any part of the plasmodia. Excess medium was removed to lower the free-floating plasmodium back on to the filter paper, and filter paper and plasmodium were transferred together to a Petri dish. Replicate discs were cut from the growing edge of the plasmodium with a 12 mm cork borer. Medium was added again to refloat the culture and separate it from the filter paper. Replicate discs were teased free from the parent plasmodium with a needle. They were transferred by lifting them out of the medium with a curved spatula and sliding them off the spatula on to dry filter paper supported by glass beads in a Petri dish. Plasmodial fusion was initiated by placing one disc over another and adding growth medium immediately.

Autoradiography. Immediately after MI, nuclei of plasmodia were labelled by exposure to growth medium containing 5 μ c [$-\text{CH}_3-^3\text{H}$]thymidine (Schwarz Bio Research Inc., Orangeburg, New York, U.S.A.) 18 c/mmol/ml for 3 h. Samples for autoradiography were fixed in ethanol-acetic acid (3:1, v/v) for 2 h at 4 °C, embedded in paraffin and sectioned at 5 μ m. The sections were de-paraffinized on microscope slides, coated with Kodak NTB3 emulsion and exposed in the dark for 7 days at 4 °C before developing with Kodak Dektol.

Assessment of time of mitosis. Early prophase was clearly defined and readily observed and hence taken as indicating the time of mitosis. The nuclei were large. The nucleoli were breaking down but still stood out in clear contrast to the rest of the nucleoplasm, being crescent-shaped and occupying an eccentric position against the nuclear membrane. Wet mounts were prepared at regular intervals by mashing a small piece of plasmodium in a drop of medium with a spatula. This crude homogenate was lightly stained with methylene blue and examined immediately by phase-contrast microscopy. Mitotic synchrony was checked by examining 500 free-floating nuclei. In order to provide enough time for these determinations only 100 nuclei were counted when mitoses in separate samples occurred close together. Mitotic indices (i.e. fraction of nuclei in mitosis) were in excess of 0.95 except where otherwise noted. To ensure that nuclei proceeded through mitosis, fused cultures were randomly examined during the 90 min following early prophase. No evidence was obtained suggesting that nuclei did not continue through early prophase to complete mitosis.

Table 1. *The variation in time of mitosis in unfused replicate discs cut from plasmodia*

Expt	Time from excision to mitosis* (min)	No. of replicate discs	Variation in time of mitosis† (min)
1	128	6	10
2	270	8	18
3	437	8	11
4	454	6	24
5	481	8	14

* Interval from excision of discs to mitosis in the first of the replicate discs to undergo mitosis.

† Interval between mitosis in the first and last of replicate discs.

RESULTS AND DISCUSSION

Variation in time of mitosis in discs cut from one plasmodium. Discs were excised from a plasmodium and incubated on fresh medium. The time of the next mitosis in each disc was determined in order to measure the interval between the first and last disc to reach mitosis. The variation in timing of mitosis in discs from one plasmodium was short (20 min). Measurement of this interval with plasmodia cut at different stages between MI and MII showed that discs from one plasmodium remained synchronous at least over the last two-thirds of the mitotic cycle (Table 1). In terms of progress through the cycle in which they were prepared, the discs from one plasmodium can be considered to be identical.

Rate of plasmodial fusion. Fusion between plasmodia has been reported to occur within minutes after cell-to-cell contact is made (Carlile & Dee, 1967; Guttus & Guttus, 1968). It was desirable to confirm this under the conditions here employed.

Plasmodia were obtained by fusion of microplasmodia. Immediately following MI, the nuclei in one plasmodium were labelled during S phase by exposure to [$-\text{CH}_3-^3\text{H}$]thymidine for 3 h. At the end of the incubation period, the plasmodium was removed from the labelling medium and washed by refloating the plasmodium and supporting filter paper through two changes of growth medium without label. Incubation of the labelled plasmodium was continued subsequently on growth medium without label. Ten hours after MI, replicate discs from unlabelled and labelled plasmodia in late- G_2 were fused by placing discs from unlabelled plasmodia over discs from labelled plasmodia. Growth medium was added immediately following overlaying. Fusion samples were incubated for 30 and 60 min before fixation.

A microscopic study of autoradiographic sections revealed that fusion occurred within 30 min of overlaying and addition of growth medium. The presence of labelled nuclei in unlabelled plasmodia could be demonstrated in all sections from this time period. Fig. 1 is a representative section taken from one of these samples. The heavier labelling identifies the lower disc as the labelled plasmodium. This Figure shows a cytoplasmic bridge connecting the two plasmodia, as well as labelled nuclei interspersed among unlabelled nuclei in the previously unlabelled plasmodium. The pattern of labelled and unlabelled nuclei in the upper disc clearly gives the impression that the labelled nuclei have passed across the cytoplasmic bridge. It was not possible to distinguish parent plasmodia by the labelling pattern in samples fused for 60 min, indicating that cytoplasmic mixing had progressed extensively within this interval.

Effect of the fusion process on the time of mitosis. Replicate discs were excised from one plasmodium. Two discs were self-fused by placing one disc over another; unfused discs were

Fig. 1. Fusion of labelled plasmodium (D), with unlabelled plasmodium (C), within 30 min of overlayering on growth medium. This autoradiograph shows that a cytoplasmic bridge (B) connects the two plasmodia, and that labelled nuclei (L) from the lower plasmodium are already interspersed among the unlabelled nuclei (U) of the upper plasmodium. (a), Focused on grains; (b), focused on nuclei.

Table 2. Time in minutes from fusion to mitosis in fused plasmodial discs and unfused controls

Expt	Plasmodium				
	Unfused control A	Unfused control B	Experimental fusion culture A/B	Fused control A/A	Fused control B/B
1	105	350	240	122	410
2	155	370	245	180	415
3	190	480	325	225	515
4	198	420	318	245	450
5	210	305	280	250	350
6	235	275	295	283	333
7	238	338	300	285	357
8	255	825	675	275	1040
9	276	405	380	303	477
10	290	500	435	310	575
11	300	375	370	335	466
12	315	448	403	328	535
13	320	500	495	360	640
14	485	693	690	600	945

Fig. 2. The relationship between delay in mitosis caused by self-fusion and the time self-fusion took place in the mitotic cycle. Time of mitosis (abscissa) was measured from fusion to mitosis in unfused controls.

carried as controls. The time of the next mitosis in fused and unfused cultures was determined. Self-fused cultures always entered early prophase later than unfused controls (Table 2). The delay in mitosis was dependent upon the position of the replicate discs in the mitotic cycle. Fusion delays tended to be relatively high (~ 240 min) and variable in early-S and decreased roughly linearly to lower values (20 min) within a narrower range as the organism proceeded through the mitotic cycle (Fig. 2).

Effect of fusing plasmodia at different stages in the mitotic cycle. Cell fusions are currently being employed to study the events which control progress through the cell cycle (Harris, 1970; Rao & Johnson, 1970). In the present study, plasmodial fusions were extended through

Fig. 3. A schematic representation of the fusion experiment depicting: the excision of replicate discs from plasmodia A and B; the maintenance of discs from A and B as unfused controls; the overlayering of discs from the same plasmodium to form fusion controls, A/A and B/B; and the overlayering of discs from different plasmodia to form the experimental fusion culture, A/B.

the mitotic cycle, and the uniqueness of the slime mould was exploited to perform a series of fusions at intervals within one phase, G_2 .

A schematic representation of the complete fusion experiment is presented in Fig. 3. Each experiment utilized replicate discs cut from two plasmodia at different stages in the mitotic cycle. Those discs cut from the plasmodium later in the cycle, i.e. closer to mitosis, were designated A and those from the plasmodium earlier in the cycle B. Except where indicated cultures were in the interval between MI and MII. Since the precise location of a culture in the mitotic cycle is difficult to predict (Rao & Gontcharoff, 1969), the placement of plasmodia at the time of fusion was determined by measuring the intervals to subsequent mitoses in the unfused controls, A and B. Replicate discs from both A and B were fused by overlayering B with A to form the experimental fusion culture, A/B, as well as self-fused to form the fused controls A/A and B/B. In some experiments, B was placed over A to form experimental fusion cultures, B/A, as well as A/B. No significant differences in the timing of mitoses between B/A and A/B were observed. The time of mitosis subsequent to fusion was determined in the experimental fusion culture, A/B, as well as in the fusion controls, A/A and B/B, and in unfused controls, A and B.

Experimental fusions in one series were extended through the greater part of the mitotic cycle. Plasmodia were selected so that those late in the cycle, A, were positioned from early- G_2 to late- G_2 (105 min before MII in Expt 1, Table 2), while the early plasmodia, B, were positioned from early-S (30 min after early prophase of MII in Expt 8, Table 2) to mid- G_2 . The results of this series are presented in Table 2 and may be summarized, as follows: (i) nuclei in all plasmodial discs in this series, fused or unfused, divided synchronously; (ii) synchronous mitoses in the experimental fusion cultures, A/B, usually occurred at a time between the unfused controls, A and B, and between the fused controls, A/A and B/B. As a result of fusion and subsequent synchrony, nuclei from plasmodia early in the cell cycle were stimulated to divide sooner than they would otherwise have done, and nuclei from plasmodia late in the cycle were retarded; (iii) self-fused controls, A/A and B/B, consistently divided later than unfused controls, A and B respectively.

The amount of observed stimulation was determined as the difference in time of mitoses between the unfused early control, B, and the experimental fusion culture, A/B. A correction

Table 3. *Stimulation or delay in minutes resulting from plasmodial fusion**

Expt	Plasmodium					
	Interval between cultures A and B	B Observed stimulation (B-A/B)	A/A Delay due to fusion (A/A-A)	B/B Delay due to fusion (B/B-B)	A/B Estimated delay due to fusion $\frac{1}{2}[(A/A-A)+(B/B-B)]$	A/B Corrected stimulation†
1	245	110	17	60	39	149
2	215	125	25	45	35	160
3	290	155	35	35	35	190
4	222	102	47	30	39	141
5	95	25	40	45	43	65
6	40	-20	48	57	53	33
7	120	38	47	19	33	71
8	570	150	20	215	118	268
9	129	25	27	72	49	75
10	210	65	20	75	48	113
11	75	5	35	91	63	68
12	133	45	13	87	50	95
13	180	5	40	140	90	95
14	208	3	115	252	184	187

* Derived from data in Table 2.

† Observed stimulation+estimated delay due to fusion.

for fusion delay appeared to be desirable since fusion retarded mitosis. Fusion delays were not constant in fused controls throughout the mitotic cycle (Fig. 2) and could not be determined directly in experimental fusion cultures. The estimated delay due to fusion in A/B was taken as one-half the sum of the delays in fusion controls. Corrected stimulation was calculated as the sum of observed stimulation and estimated delay due to fusion. Maximal stimulation resulted from fusion of a plasmodium in early-S with a plasmodium in mid-G₂ (Table 3, Expt 8).

The analysis of the results from this experimental series is presented in Table 3; 190 min of corrected stimulation were obtained when cultures were separated by 290 min at the time of fusion (Expt 3). Inspection of these data shows that corrected stimulation is a direct function of the difference in phase in the mitotic cycle between cultures at the time of fusion – as long as both are in G₂ and not closer than 105 min to the next mitosis (Fig. 4). The linearity of the response suggests that one controlling event may be operative over this period. These calculations are presented in terms of stimulation because the acceleration of nuclei from B was always greater than the retardation of nuclei from A. Presentation of the same data in terms of delay would show that corrected retardation is complementary to corrected stimulation since estimated delay due to fusion displaces the point of synchronous mitosis but does not change the interval between A and B. A plot of corrected retardation against the interval between parent plasmodia, when plasmodia were in G₂, would also be linear. This information could be used to support the hypothesis that mitosis is controlled by the accumulation of a stimulator, as summarized by Rusch (1970). However the complementary retardation of nuclei suggests that a hypothesis in which mitosis is dependent upon the degradation of an inhibitor should also be considered.

In another experimental series, plasmodial fusions were arranged so that one member of the fusion pair was situated within the 105 min period before M (Table 4). A striking change in the pattern of results developed. Mitoses did not occur synchronously in the first division after fusion. Mitotic indices showed that only half of the total population of nuclei divided

Fig. 4. The relationship between mitotic stimulation and the interval (difference in phase in the mitotic cycle) between plasmodia at the time of fusion. All cultures were in G_2 and 105 min before M.

Table 4. *Experimental fusion with plasmodium A within 105 min of mitosis*

Expt	Time from fusion to mitosis (min)					Percentage A/B showing mitosis
	A	A/A	A/B	B/B	B	
a	68	82	86	535	426	47
b	77	90	101	395	357	30
c	105	124	130	470	375	46
d	63	73	77	830	675	52

together. This half-wave of mitosis occurred concurrently with synchronous mitosis in the fused late control, A/A. Those nuclei dividing at this time in the experimental fusion culture presumably had their origins in plasmodium A and could no longer be retarded by fusion with plasmodium B, although they were still sensitive to self-fusion.

The fate of the nuclei which did not divide in the first half-wave appeared to depend upon the location of the fusing members in the mitotic cycle. When B was in early- G_2 and A was well within the 105 min period before mitosis (Table 4, Expt a and b), no subsequent mitotic activity was observed although experimental fusion cultures were carried 1 h beyond mitosis in B/B. When B was in early- G_2 and A was at the beginning of this period before mitosis (Expt c), a second half-wave of mitosis was observed 60 min after the first half-wave. The following observations support the conclusion that these nuclei were not identical with those which divided in the first half-wave: at the time of the first half-wave, half of the nuclei were in early prophase, the other half were in interphase; 1 h later half of the nuclei were in reconstruction following division, the other half were in early prophase; 2 h later half of the nuclei were in interphase, the other half were in reconstruction. Finally, when B was in early-S and A was well into the period before mitosis (Expt d), in addition to the first half-wave a complete synchronous mitosis was also observed at the time of mitosis in B.

If the duration of a controlling event is indicated by the linear response to fusion through the forepart of G_2 , then the termination of that event is marked by the change in response

when fusions are extended within the 105 min period before mitosis. The nuclei making up the first half-wave are committed to mitosis and can no longer be retarded by fusion with early plasmodia. In one experiment, c, a second half-wave of mitosis was observed, suggesting that a transient material stimulatory for mitosis was present at the time of fusion (105 min before M). This point in the mitotic cycle may be critical, and efforts in this laboratory are being directed to its study.

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