Regular Article

Rapid response to nutrient depletion on the expression of mating pheromone, gamone 1, in *Blepharisma japonicum*

Mayumi SUGIURA[1], Mika YAMANAKA[1], Toshinobu SUZAKI[2] and Terue HARUMOTO[1*]

[1]*Department of Chemistry, Biology, and Environmental Sciences, Faculty of Science, Nara Women's University, Nara 630-8506, Japan*
[2]*Department of Biology, Graduate School of Science, Kobe University, Kobe 657-8501, Japan*

SUMMARY

　　Ciliate conjugation usually occurs when cells are moderately starved after rapid proliferation. In *Blepharisma japonicum*, at the stationary phase, a mating pheromone (gamone 1) is secreted by mating type I cells and stimulates mating type II cells to secrete another mating pheromone (gamone 2), which stimulates mating type I cells. Both stimulated cells are transformed to be able to form pairs. We showed previously that the gamone 1 transcription occurs only in the stationary-phase cells, not in logarithmic-phase cells. However, it is still unknown how rapidly cells response to starvation and express gamone 1. Here we show that gamone 1 expression is initiated by sudden artificial starvation, and we determined the time required for the initiation of transcription and secretion of gamone 1 in *B. japonicum*. Proliferating cells rapidly responded to artificial starvation, approx. 1.5 h at most was necessary for the initiation of the transcription of gamone 1 gene, and approx. 3 h was needed for the secretion of gamone 1. Gamone 1 expression tested in single cells suggests that gamone 1 expression may be correlated with the cell-cycle, most probably with the certain stage of G1 phase in *B. japonicum*. Our present findings provide an experimental system to investigate the environmental factors involved in the initiation of conjugation.

Key words: Conjugation, Starvation, Cell cycle

INTRODUCTION

Conjugation in ciliates, a process of sexual reproduction which generates new progenies, usually occurs when cells are moderately starved after rapid proliferation. Cells start interacting between complementary mating-type cells and initiate conjugation. In the case of *Blepharisma japonicum*, a mating pheromone (gamone 1), which is an approx. 30-kDa glycoprotein, is initially secreted by mating type I cells (Miyake, 1981a, 1981b, 1996, for review). The secretion of gamone 1 transforms mating type II cells to enable them to form conjugating pairs. Stimulated mating type II cells also start secreting another mating pheromone (gamone 2), which is a tryptophan-derivative compound resembling serotonin. The secretion of gamone 2 transforms mating type I cells so that they are able to form pairs. As a consequence of the interaction, the cells temporarily and partially fuse and undergo meiosis in the micronucleus, form gametic nuclei, exchange one of the gametic nuclei, form fertilization nuclei, break down old macronuclei, form macronuclear anlagen, etc., to complete the conjugation. Conjugation is a significant process as such in ciliates, however, the details of molecular mechanisms involved in the initiation of conjugation have not yet been elucidated. To reveal the environmental factors brought by starvation which trigger gamone 1 expression, the experimental system which controls gamone 1 expression is indispensable.

The genus *Blepharisma* is a suitable material to investigate the mechanisms underlying the initiation of conjugation for the following two reasons. (1) The mating pheromones gamone 1 and gamone 2 have been isolated and identified (Kubota et al., 1973; Miyake and Beyer, 1974). The gene encoding gamone 1 was isolated (Sugiura and Harumoto, 2001), and the expression of the gene can be evaluated at the transcriptional, protein and biological activity levels (Sugiura et al., 2005). (2) Gamone 1 induces pairs (homotypic

pairs) of type II cells at a very low concentration (16 U/ng gamone 1/ml) (1 unit [U] is the smallest amount of gamone activity that can induce at least one pair in 500–1000 cells that are suspended in 1 ml of a non-nutrient salt medium) (Miyake, 1981b; Miyake and Beyer, 1974). The biological activity of gamone 1 secreted even by a single cell can be evaluated.

We previously reported that the transcriptional level of gamone 1 is correlated with the starvation of cells (Sugiura et al., 2005). Gamone 1 transcription does not occur in cells at the logarithmic (log) phase; it occurs at the stationary phase. It is still unknown whether proliferating cells start transcribing gamone 1 in response to the sudden deprivation of a nutrient. If so, how rapid would the response be? Is gamone 1 expression correlated with cell-cycle stage of *Blepharisma*? To address these questions, we here investigated the gamone 1 expression in artificially starved cells and determined the time required for the initiation of transcription and secretion of gamone 1 in *Blepharisma japonicum*. Our present findings provide an experimental system to clarify in future the environmental factors for the initiation of conjugation.

MATERIALS AND METHODS

Cells and cell culture

B. japonicum strains R1072 (mating type I) and R48 (mating type II) were used. Cells were cultured at 25°C in WGP (Wheat Grass Powder, Pines) medium (Tokusumi and Takagi, 2000) inoculated with *Enterobactor aerogenes* 2 days before use.

Preparation of log-phase and stationary-phase cells and the induction of nutrient depletion

For the preparation of log-phase cells, cells were filtered through gauze and concentrated by mild centrifugation after 24-h cultivation, and they were washed three times with physiological balanced solution SMB(−) [Synthetic Medium for

Blepharisma without EDTA (Miyake, 1981b)] (designated SMB here) and then suspended in SMB. For the preparation of stationary-phase cells, cells were collected after 3–4 days' cultivation, and similarly suspended in SMB after washing. The cell density of both log-phase and stationary-phase cell suspension was adjusted at 7000 cells/ml.

Preparation of cell-free fluid (CFF) and the gamone 1 activity assay

A cell suspension of log-phase or stationary-phase type I cells was prepared and kept for the indicated time in SMB (nutrient-depleted condition) at 25°C. After incubation, CFF was obtained by removing cells from the cell suspension by mild centrifugation and filtration through a nylon net (5 μm × 5 μm). The obtained CFF contained gamone 1, which was secreted by the type I cells during each incubation time.

The gamone 1 activity is represented in units (U) measured as described (Kubota et al., 1977; Miyake, 1981b). R48 strain (mating type II) cells were used as tester cells for the gamone 1 activity assay.

Isolation of total RNA

Cells were collected by mild centrifugation after they were incubated in SMB for the indicated time. The collected cells were transferred to ice-cold SMB and incubated for 10 min to remove pigments (blepharismine), then suspended in TRIzol reagent (Thermo Fisher Scientific, Wilmington, DE). Total RNA was isolated by the acid guanidinium-phenol-chloroform method as described by Sugiura et al. (2005).

Northern hybridization analysis

To detect gamone 1 mRNA, full-length gamone 1 cDNA was labeled with digoxigenin (DIG) and used as probes. Total RNA (10 μg/lane) was fractionated on a 1% agarose gel containing 2.2 M formaldehyde and transferred to positively charged nylon membranes (Roche Molecular Biochemicals, Indianapolis, IN). Membranes were incubated with DIG-labeled gamone 1 probe in standard hybridization buffer [5×SSC; 0.1% (w/v) N-lauroylsarcosine; 0.02% (w/v) SDS; 1% blocking reagent]. After the hybridized membranes were washed, DIG was detected using a DIG Luminescent Detection kit (Roche Molecular Biochemicals). Details of the method were described by Sugiura et al. (2005). Transcriptional activity was evaluated as follows: the intensity of each band was quantified by the ImageJ (http://rsb.info.nih.gov/ij/) and normalized with loading controls (rRNA).

Gamone 1 expression assay in single cells

To examine the starting point of gamone 1 expression in individual cells, we prepared cells from log-phase cultures and starved them as described below. Log-phase cells were randomly selected and washed with SMB. A single cell was isolated to a drop of SMB (1 cell/20 μl SMB/drop) in a plastic dish. Approximately 24 drops (24 isolated cells) were prepared in a plastic dish. A 50-μl tester cell suspension (1000–2000 cells/ml) (moderately-starved mating-reactive type II cells) was then mixed with each drop, and the number of drops in which pair formations were observed was counted at the indicated time points.

To obtain cells shortly after cell division, dividing cells were isolated from log-phase cultures in WGP. After cell division, the cells were washed with SMB and a single cell was further isolated in a drop of SMB on a plastic dish. Then each cell was tested for the gamone 1 activity as described above.

For the observation of the frequency of cell division, randomly selected log-phase cells or cells just after cell division were isolated to a drop of SMB (1 cell/70 μl SMB/drop) in a plastic dish. Approximately 24 drops (24 isolated cells) were prepared in each sample. The number of drops in which the isolated cell divided was counted at the

indicated time points.

RESULTS

Gamone 1 expression was induced by sudden artificial starvation

Mating-type I cells (stock R1072) which inoculated in the fresh culture medium started proliferating (the culture 1 day after inoculation is here called log-phase culture), and 3–4 days after inoculation they reached the stationary phase. These log-phase and stationary-phase cells were transferred to the non-nutrient medium (SMB: Synthetic Medium for *Blepharisma*) and incubated. CFF was obtained at 30 min (0 day), 1, 3, or 10 days after incubation.

The gamone 1 activity of CFF was evaluated by mixing the CFF and type II cells as described in Materials and Methods. The incubated stationary-phase cells (Fig. 1, black bars) started secreting gamone 1 on day 0 (2^5 U/ml). The gamone 1 activity of incubated cells from the stationary phase increased gradually with the incubation time. The incubated cells from the proliferating cell population (Fig. 1, gray bars) which underwent forced starvation did not secrete gamone 1 on day 0, but they started secreting it by day 1 (2^{11} U/ml). The gamone 1 activity increased with the incubation time. Incubated cells from both the stationary phase and the log phase showed approximately the same gamone 1 activity at 1, 3 and 10 days.

The time required for the expression of gamone 1 induced by sudden artificial starvation

Proliferating log-phase cells incubated in SMB secreted gamone 1 within 1 day. We then investigated the time required for the initiation of transcription and the secretion of gamone 1 in incubated cells from the log phase in more detail. We tested CFF obtained from incubated cells in SMB at 2, 3, 4, 5, 8, 11, 14 and 26 h for gamone 1 activity (Fig. 2A, graph). At the start of the test (2 h), gamone 1 activity was not detected in CFF
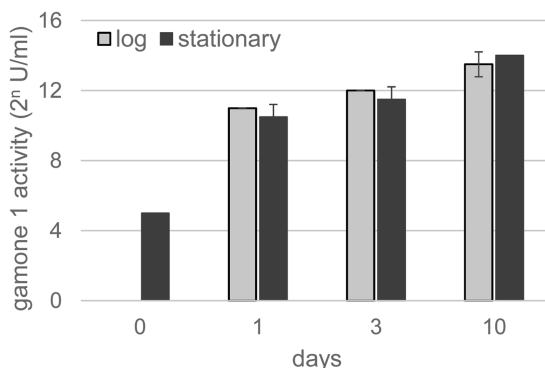

Fig. 1. Gamone 1 expression induced by artificial starvation. After the inoculation of cells to the fresh culture medium, cells were transferred to a non-nutrient medium (SMB) on the 1st day (log-phase cells) or 3rd day (stationary-phase cells) and incubated for the indicated time. The CFF obtained was bioassayed for gamone 1 activity. Gamone 1 activity was measured twice in each time point. Ordinate: the gamone 1 activity (n) (2^n U/ml). Mean ± s.d. Abscissa: the number of days after transfer of cells to SMB. Black bar: cells obtained from stationary phase. Grey bar: cells obtained from the log phase.

indicating that the incubated cells from the proliferating cell population did not secrete gamone 1 within 2 h. These cells started secreting gamone 1 within 3 h and the activity gradually increased to 2^{13} U/ml at 26 h (Fig. 2A, graph).

The transcriptional activity of gamone 1 gene was investigated in incubated cells from log phase (Fig. 2A, photo). Total RNA was isolated, northern hybridization was probed with gamone 1 cDNA, and the transcriptional activity was evaluated as described in Materials and Methods. After the transfer of log-phase cells to the non-nutrient medium (SMB), gamone 1 transcription was slightly detected at 2 h (arrow, Fig. 2A, photo) (transcriptional activity: 0.12), and the activity had greatly increased (51.8-fold) by 26 h (transcriptional activity: 6.22).

As gamone 1 transcription started within 2 h at most after the transfer of log-phase cells to the non-nutrient medium, we investigated the gamone 1 activity in the external medium and gamone 1 transcription in incubated cells from the log phase
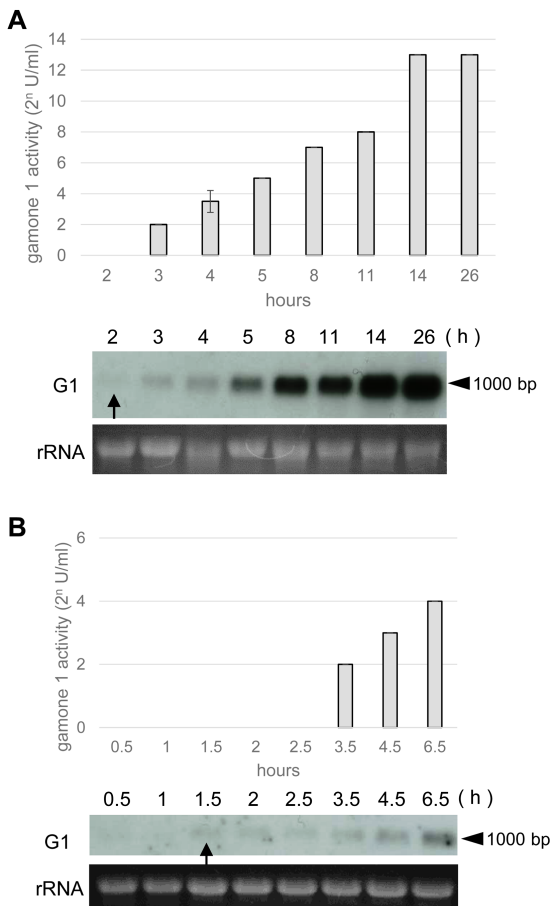
A

B

Fig. 2. The time required for the expression of gamone 1 induced by sudden artificial starvation in cells taken from the log-phase. Log-phase cells were transferred into SMB and incubated for 2, 3, 4, 5, 8, 11, 14 and 26 h (A), or 0.5, 1, 1.5, 2, 2.5, 3.5, 4.5 and 6.5 h (B). At each incubation time, the biological activity of gamone 1 released in CFF was estimated (Graphs), and transcriptional activity of the gamone 1 gene was assayed by Northern hybridization analysis (Blots). (Graphs) Ordinate: the gamone 1 activity (n) (2^n U/ml). Mean ± s.d. Abscissa: the time (hours: h) after the transfer of cells to SMB. (Blots) Northern blots were probed with cDNA of gamone 1. G1: gamone 1 transcript (1000 bp). rRNA: ethidium bromide-stained loading control. Arrow marks the detection of the transcript at 2 h (A) and 1.5 h (B).

every 30 min starting 0.5–2.5 h after the transfer (Fig. 2B). The arrow in the photo shows that gamone 1 transcription was not initiated at 0.5 and 1 h but was initiated by 1.5 h after the transfer of log-phase cells into the non-nutrient medium. Gamone

1 activity was not detected in the external medium at 0.5–2.5 h but detected by 3.5 h (Fig. 2B, graph). These results indicate that the cells started the transcription of gamone 1 gene at approx. 1.5 h and secrete gamone 1 at 3.0–3.5 h after the transfer to the non-nutrient medium.

Both gamone 1 transcriptional activity and gamone 1 activity in the external medium increased with time after the sudden deprivation of nutrients in incubated cells from the log phase. Two possible alternative scenarios explaining why the activities increased with time are as follows. (1) All of the cells in the population consistently and uniformly show the expression of gamone 1. The gamone 1 expression per cell is low at the beginning and gradually increases, and then the gamone 1 activity of the population increases with time, or (2) the cells in the population are not all consistent regarding the expression of gamone 1, as the population is a mixture of cells with and without gamone 1 activity. At the beginning, only a few cells have the activity, and the ratio of the cells with activity increases with time. To elucidate which of these scenarios is correct, we evaluated the gamone 1 activity of individual cells.

Gamone 1 expression in individual cells induced by sudden artificial starvation

We isolated proliferating type I cells and mixed them with tester cells (moderately starved type II cells: stock R48) in a small drop of SMB as described in Materials and Methods (Fig. 3A). The drops were observed if mating pairs were formed according to time (h) after mixing with tester cells. If an isolated type I cell secretes gamone 1, tester cells would form homotypic pairs. The ratio of cells that secrete gamone 1 was considered here as the ratio of the drops in which pair formation was observed among 22 or 24 drops.

In randomly isolated log-phase cells, gamone 1 secretion was first detected at 3 h, and the ratio of cells that secreted gamone 1 was zero at 0 h, 0.18 at 5 h, 0.27 at 10 h and 0.95 at 24 h (Fig. 3B).
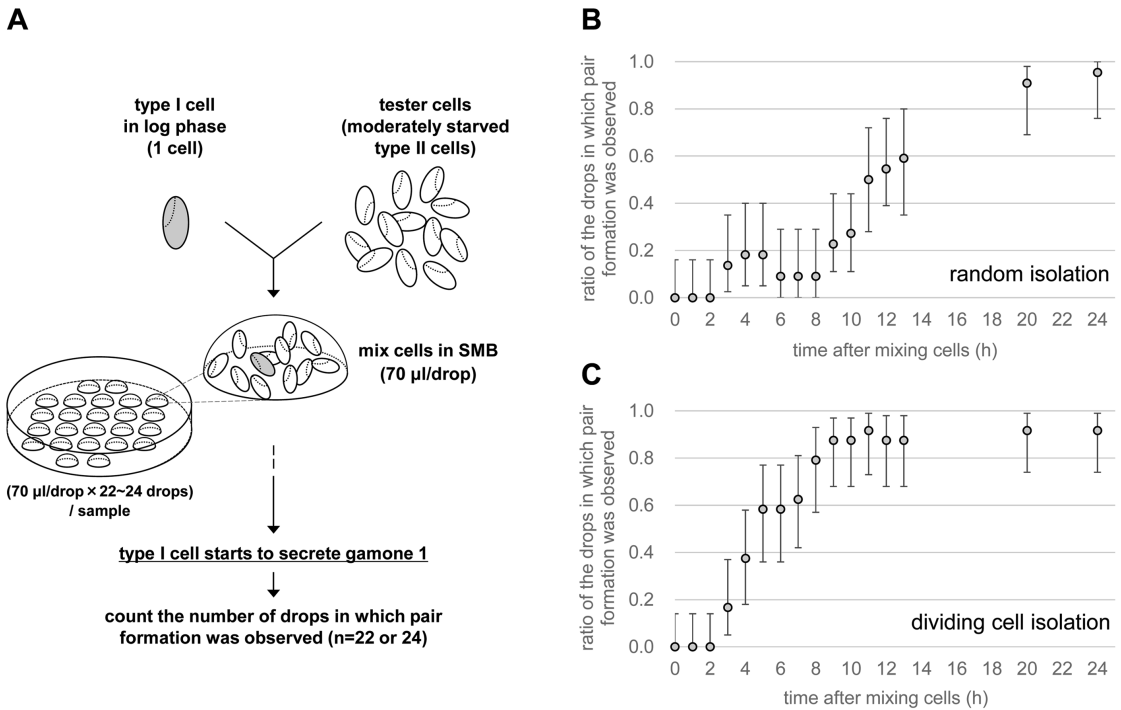
Fig. 3. Gamone 1 expression in single cells induced by sudden artificial starvation. (A) Scheme for the experimental design. Proliferating type I cells were isolated, transferred into a small drop of SMB, and mixed with tester cells (moderately-starved mating-reactive type II cells). The 22–24 drops (70 µl each) were incubated and observed for mating pairs. (B, C) Ordinate: the ratio of the drops in which pair formation was observed in 22 or 24 drops. Abscissa: the time after the cells were mixed. (B) Type I cells were randomly isolated from a proliferating cell population, and (C) type I cells shortly after division were isolated. Mean ± 95% confidence limits.

The number of cells that secreted gamone 1 increased with time, but it should be pointed out that even at 10 h, <30% cells secreted gamone 1. This result suggests that the individual cells were not synchronized for gamone 1 expression but rather had different timings for the expression.

  The proliferating cell culture likely consists of cells at various cell-cycle stages. It is plausible that the time required for gamone 1 expression depends on the cell-cycle stages in which the isolated cells were placed. These results are consistent with the idea that the isolated cells start gamone 1 transcription and secretion when the cells undergo the cell cycle and enter a certain stage in which gamone 1 transcription is possible.

  To investigate the correlation between gamone 1 expression and the cell cycle, we isolated cells shortly after division from proliferating log-phase cultures and tested them for gamone 1 expression (Fig. 3C). We compared these results with those of the randomly isolated cells from proliferating log-phase cultures (Fig. 3B). The gamone 1 expression of the isolated cells shortly after division showed a profile that differed from that of the randomly isolated cells. Fig. 3C shows that the ratio of cells that secreted gamone 1 increased with time from zero at 0 h to 0.58 at 5 h, 0.88 at 10 h, and 0.92 at 24 h. Thus, the gamone 1 expression began sooner in the isolated cells shortly after division than in randomly isolated cells. This result suggests that the individual cells shortly after division were relatively synchronized in the cell cycle, and started expressing gamone 1 at about the same time. Our next experiment was conducted to test
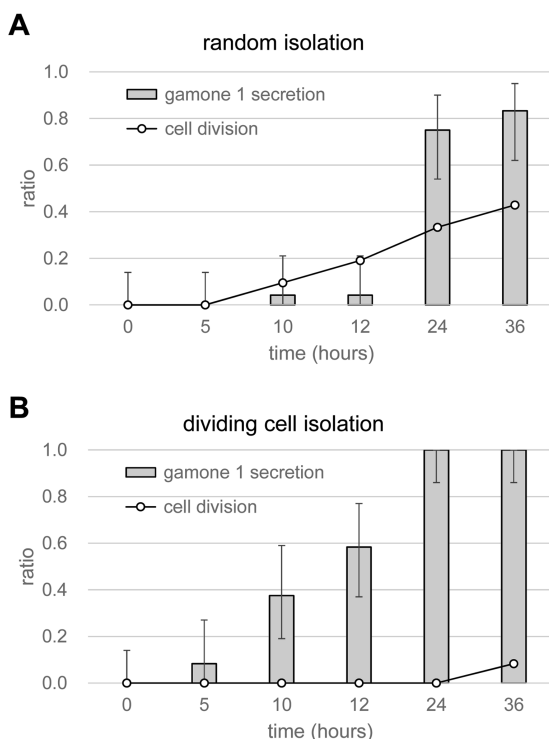
A

B

Fig. 4. Correlation between cell division and gamone 1 expression induced by sudden artificial starvation in single cells. Proliferating type I cells were isolated, transferred into a small drop of SMB, mixed and incubated with tester cells (moderately-starved mating-reactive type II cells) or incubated without mixing with tester cells. The 24 drops (70 µl each) mixed with tester cells were observed for mating pairs and the drops incubated without mixing with tester cells were observed for cell division according to the indicated time. (A) Type I cells were randomly isolated from a proliferating cell population, and (B) type I cells shortly after division were isolated. Ordinate: the ratio of drops in which mating pairs were observed (bars), and the ratio of cells that divided (lines). Mean ± 95% confidence limits. Abscissa: the incubation time (hours).

the correlation between gamone 1 expression and cell division.

We investigated the time required for gamone 1 expression and cell division in individual isolated cells taken randomly and in those taken from a divided cell population. Among the randomly isolated cells at 10–12 h, <10% expressed gamone 1, and nearly 80% of the cells expressed gamone 1 at 24 h (Fig. 4A, bar graph). Cell division was first observed at 10 h, and approx. 30%

of the cells completed division by 24 h (Fig. 4A, line graph). In contrast, in the isolated cells shortly after division, the ratio of cells expressing gamone 1 increased from 5 h and reached 0.6 at 12 h and 1.0 at 24 h (Fig. 4B, bar graph). Cell division was not observed by 24 h, and <10% cells had divided at 36 h (Fig. 4B, line graph). These data demonstrated that randomly isolated cells gradually divided from 10 h, and only a few cells expressed gamone 1 by 12 h, whereas the cells shortly after division did not divide by 24 h and started expressing gamone 1 earlier.

DISCUSSION

Fig. 1 indicates that the log-phase cells which did not secrete gamone 1 could rapidly respond to the artificial sudden starvation, start secreting gamone 1, and reach the same level of gamone 1 expression as in cells from the stationary-phase within 1 day. Fig. 2 indicates that gamone 1 transcription started about 1.5 h at most after the transfer of log-phase cells to the non-nutrient medium, and the transcriptional activity increased with time. Gamone 1 secretion was observed at 3 h in the external medium, and it also increased with time. Figs. 1 and 2 revealed that gamone 1 expression (transcription and secretion) is induced not only by gradual starvation from the log phase to the stationary phase, but also by a sudden deprivation of nutrients from log-phase cultures. An approx. 1.5-h incubation in the non-nutrient medium was required for the initiation of transcription of gamone 1 gene. Our findings clearly show that the gamone 1 transcription and secretion are strictly controlled by the environmental nutrient condition, as predicted in our previous report (Sugiura et al., 2005). These results also indicate that proliferating cells can be transformed to gamone-producing cells by artificial starvation, which enables to provide a useful experimental system to investigate the environmental factors to trigger conjugation in *B. japonicam*.

Fig. 3 showed that in the randomly isolated log-phase cells, the time required for gamone 1 expression was various; from 3 h to about 24 h, while in the cells shortly after division, the time was relatively synchronized; from 3 h to 10 h. The time required for gamone 1 expression was relatively shorter in cells shortly after division was also shown in Fig. 4. The Figure indicates that in the randomly isolated log-phase cells, the time required for cell division varied; the shortest was 10 h, and in the isolated cells shortly after division, the time was longer than 24 h. Together these findings, we suggest that in randomly isolated log-phase cells, some cells express gamone 1 only after cell division, and cell-cycle progression is necessary in these cells for gamone 1 expression. In cells shortly after division, we speculate that all cells can express gamone 1 without cell division. Our present results strongly indicate that gamone 1 expression is correlated with the cell cycle of *B. japonicum*. The cell cycle of *Blepharisma intermedium* (later classified as *Blepharisma japonicum var. intermedium*) was reported as approximately 16 h of G1, 7 h of S, and 1–2 h of G2 phase (Minutoli and Hirshfield, 1968). We suggest that cells start expressing gamone 1 at the certain point of the cell cycle, within several hours after cell division possibly at the G1 phase.

Fig. 5 shows a putative scheme of the correlation between gamone 1 expression and the cell cycle. Our present findings suggest that gamone 1 expression starts at a certain stage of the cell cycle (most probably G1 phase), and artificial starvation induces gamone 1 expression depending on the cell-cycle stage in which the cell is placed. For example, the cell at stage (a) in Fig. 5 (shortly after division) becomes able to express gamone 1 within several hours without subsequent division. A cell at (b) which is in putative G1 needs about 1.5 h for initiation of the transcription of gamone 1. The cells at (c) and (d) which are placed at more advanced stage do not express gamone 1 and take some time to reach the subsequent division stage,
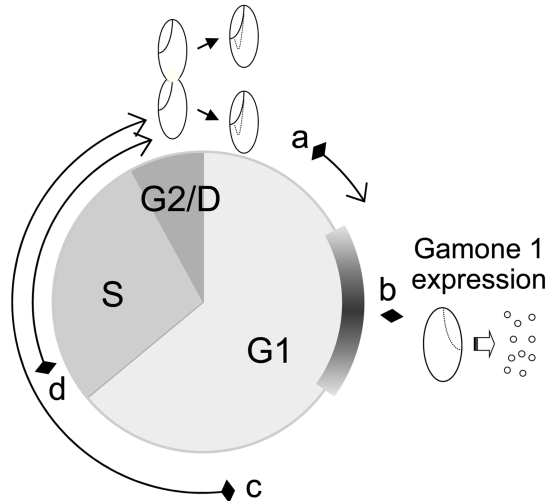

Fig. 5. The cell cycle and gamone 1 expression: A presumptive scheme. A presumptive cell cycle of *Blepharisma* is shown according to Minutoli and Hirshfield (1968). The present findings suggest that gamone 1 expression starts at a certain stage of the cell cycle (most probably G1 phase), and sudden artificial starvation induce gamone 1 expression depending on the cell-cycle stage in which the cell is placed. The cell at (a) shortly after division becomes able to express gamone 1 within several hours without subsequent division. A cell at (b) needs about 1.5 h for initiation of the transcription of gamone 1. The cells at (c) and (d) do not express gamone 1 and take some time to reach the subsequent division stage, D, and they express gamone 1 after cell division.

and express gamone 1 only after cell division. If cells are isolated randomly from a proliferating cell population (Figs. 3B and 4A), the cells are in various stages of the cell cycle, and the time required for gamone 1 expression would depend on every cell stage. If cells shortly after division are isolated (Figs. 3C and 4B), all of the cells would be at (a) (Fig. 5), and they express gamone 1 without subsequent division which is consistent with the results.

In ciliates, conjugation usually occurs when cells are moderately starved. In *Paramecium tetraurelia*, the putative mating type-determining gene, mtA, was recently identified (Singh et al., 2014), and northern blot analyses confirmed that it is expressed mating-type specifically and only in

moderately starved, sexually reactive E cells. The expression is limited to sexually reactive cells because transcripts could not be detected in exponentially growing cells or in over-starved cells. In *Tetrahymena thermophila*, expression of the mating type-specific gene was observed only in moderately starved mating-compatible cells, not in exponentially growing cells (Cervantes et al., 2013).

In *Blepharisma japonicum*, the present investigation clearly revealed that the transcription and secretion of the mating pheromone, gamone 1 is strictly correlated with starvation. Proliferating *Blepharisma* cells rapidly responded to sudden artificial starvation by the initiation of transcription and the secretion of gamone 1, in a relatively short time, approx. 1.5 h and 3 h, respectively. The results of our study also provide an evidence that the gamone 1 expression is correlated with a certain stage of cell-cycle. In *Euplotes raikovi*, mating pheromones are expressed throughout the cell cycle regardless of the log or stationary phase, and the mating pheromone is also involved in the proliferation of cells (Luporini et al., 2006; Vallesi et al., 1995). It is still unknown why these differences occur, and the regulation of gene expression in such mating type-specific genes, mating pheromones, or gamones may differ among species.

The deprivation of a nitrogen source combined with the presence of a nonfermentable carbon source leads cells to enter the developmental pathway of meiosis and sporulation in budding yeast *Saccharomyces cerevisiae* (Freese et al., 1982). The trigger molecules involving the initiation of conjugation in *Blepharisma* are unknown yet, however, our study provides an experimental system to reveal the environmental factors by the experiment *e.g.* adding certain nutrient into the artificially starved cells to inhibit gamone 1 transcription. The identification of the factors is now under investigation.

ACKNOWLEDGEMENTS

REFERENCES

Cervantes, M. D., Hamilton, E. P., Xiong, J., Lawson, M. J., Yuan, D., Hadjithomas, M., Miao, W. and Orias, E. (2013) Selecting one of several mating types through gene segment joining and deletion in *Tetrahymena thermophila*. PLOS Biol., 11, e1001518.

Freese, E. B., Chu, M. I. and Freese, E. (1982) Initiation of yeast sporulation of partial carbon, nitrogen, or phosphate deprivation. J. Bacteriol., 149, 840–851.

Kubota, T., Tokoroyama, T., Tsukuda, Y., Koyama, H. and Miyake, A. (1973) Isolation and structure determination of blepharismin, a conjugation initiating gamone in the ciliate *Blepharisma*. Science, 179, 400–402.

Kubota, T., Miyake, A. and Tokoroyama, T. (1977) Isolation and purification of conjugation-inducing substances in *Blepharisma*. *In:* Experimental Methods on Natural Organic Compounds - Extraction and Isolation of Physiologically Active Substances. Natori, S., Ikekawa, N. and Suzuki, M. (eds.). Kodansha, Tokyo, pp. 485–498.

Luporini, P., Vallesi, A., Alimenti, C. and Ortenzi, C. (2006) The cell type-specific signal proteins (pheromones) of protozoan ciliates. Curr. Pharm. Des., 12, 3015–3024.

Miyake, A. (1981a) Physiology and biochemistry of conjugation in ciliates. *In:* Biochemistry and Physiology of Protozoa. Levandowsky,

M. and Hutner, S. H. (eds.). Academic Press, New York, pp. 125–198.

Miyake, A. (1981b) Cell interaction by Gamones in *Blepharisma*. *In:* Sexual Interactions in Eukaryotic Microbes. O'Day, D. H. and Horgen, P. A. (eds.). Academic Press, New York, pp. 95–129.

Miyake, A. (1996) Fertilization and sexuality in ciliates. *In:* Ciliates: Cells as Organisms. Hausmann, K. and Bradbury, P. C. (eds.). Gustav Fischer Verlag, New York, pp. 243–290.

Miyake, A. and Beyer, J. (1974) Blepharmone: A conjugation inducing glycoprotein in the ciliate *Blepharisma*. Science, 185, 621–623.

Minutoli, F. and Hirshfield, H. I. (1968) DNA synthesis cycle in *Blepharisma*. J. Protozool., 15, 532–535.

Singh, D. P., Saudemont, B., Guglielmi, G., Arnaiz, O., Goût, J. F., Prajer, M., Potekhin, A., Przybòs, E., Aubusson-Fleury, A., Bhullar, S. et al. (2014) Genome-defence small RNAs exapted for epigenetic mating-type inheritance. Nature, 509, 447–452.

Sugiura, M. and Harumoto, T. (2001) Identification, characterization, and complete amino acid sequence of the conjugation-inducing glycoprotein (blepharmone) in the ciliate *Blepharisma japonicum*. Proc. Natl. Acad. Sci. U.S.A., 98, 14446–14451.

Sugiura, M., Kawahara, S., Iio, H. and Harumoto, T. (2005) Developmentally and environmentally regulated expression of gamone 1: the trigger molecule for sexual reproduction in *Blepharisma japonicum*. J. Cell Sci., 118, 2735–2741.

Tokusumi, Y. and Takagi, Y. (2000) Ectosymbiotic role of food bacteria for *Paramecium*: bacterial detoxification of paramecia-killing toxin contained in wheat grass powder. Zool. Sci., 17, 341–348.

Vallesi, A., Giuli, G., Bradshaw, R. A. and Luporini, P. (1995) Autocrine mitogenic activity of pheromone produced by the protozoan ciliate *Euplotes raikovi*. Nature, 376, 522–524.