Relationship between gametic differentiation and the cell cycle in the green alga *Chlamydomonas eugametos*

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The relationship between the vegetative cell cycle and sexual competence in the green alga *Chlamydomonas eugametos* was studied using synchronized cultures. The gametic stage was found to be a regular part of the cell cycle; it extended from the beginning of the cell cycle to about 2 h prior to the commitment point for cell division. All variations in the length of this period caused by external growth conditions were accompanied by corresponding changes in the period of mating competence. The period varied depending on the fluence rate, temperature, and nitrate or CO₂ concentration. We conclude that the length of the period in the cell cycle in which cells are mating competent is determined by the length of the precommitment period and that environmental factors do not influence mating competence directly, but rather affect the growth rate of the cells.

Introduction

Chlamydomonas eugametos is a heterothallic, biflagellate unicellular alga. It multiplies vegetatively by repeated divisions into 2ⁿ daughters per cell cycle. Sexual reproduction takes place between gametes which are similar in morphology to vegetative cells. When gametes of opposite mating type (*mt*⁺ and *mt*[−]) are mixed, they agglutinate to form clusters of cells. In response to physical contact with a partner the mating structure of each participating cell is activated and they fuse pairwise to form so-called vis-à-vis pairs. After some hours, the pairs fuse completely and develop into zygotes. A new vegetative cycle is started by germination of zygospores (van den Ende *et al.*, 1990).

Gametes are generally considered to be differentiated cells. As stated by Harris (1989), gametogenesis in algae often appears to be triggered by adverse environmental conditions. In *Chlamydomonas reinhardtii*, nitrogen depletion has been reported to be the most important inducing factor (Sager & Granick, 1954; Kates & Jones, 1964; Martin & Goodenough, 1975; Siersma & Chiang, 1971; Schmeisser *et al.*, 1973). Using synchronous cultures, Kates & Jones (1964) observed that cells resuspended in nitrogen-free medium produced abundant gametes only after a round of cell division. However, when cells were placed in nitrogen-free medium just after cell division, gametic activity was also

observed prior to the next cell division. This transient activity was not explained. *Chlamydomonas moewusii* was found to behave in a similar way (Kates & Jones, 1964).

C. eugametos is sexually compatible with *C. moewusii*, but nitrogen deprivation seems not to be the most important trigger for gametogenesis. For example, Tomson *et al.* (1985) found that in a partially synchronous batch culture, gametogenesis occurred at the end of the exponential phase of growth, even when adequate nitrogen was present. This observation led us to reinvestigate gametogenesis in *C. eugametos*, using cells grown in completely synchronous batch cultures. Unexpectedly, the gametic stage was found to be a normal part of the cell cycle, extending from cell hatching to the commitment point for cell division.

Methods

Culture strains. The *C. eugametos* strain UTEX 10 (*mt*[−]) was obtained from the Algal Collection at the University of Texas, Austin, USA. The *mt*⁺ strain, used to assess mating competence of the *mt*[−] strain, was a recombinant of UTEX 9 (*mt*⁺) and UTEX 10 (*mt*[−]), designated 17.17.2 (Schuring *et al.*, 1987).

Culture equipment and conditions. *C. eugametos* was grown in batch culture in 400 ml cylindrical vessels (length 50 cm, diameter 3.5 cm) at 30 °C, illuminated by six circular fluorescent tubes (32 W, Philips 'TL' E 32 W/84). The fluence rate at the surface of the culture vessels was approximately 50 W m^{−2}. The culture was aerated with 2% (v/v) CO₂

in air. The mineral nutrient solution was as described by Kates & Jones (1964), containing (l^{-1}): 1.0 g KNO_3 , 0.74 g KH_2PO_4 , 0.136 g $MgSO_4 \cdot 7H_2O$, 0.05 g $CaCl_2 \cdot 2H_2O$, and 1 ml of a solution of trace elements (Zachleder & Šetlik, 1982). Rapid synchronization was achieved by using cells flooded from the surface of a 3-week-old agar culture as inoculum (Mesland, 1976). Such cells were already partially synchronous. Under these conditions, cell division started at about the 16th hour and most of the cells divided into eight daughters. The cells were grown as a batch culture for one whole cell cycle and diluted to the initial density (10^6 cells ml^{-1}) at the beginning of the next light period, after which synchronized growth started. Synchronization itself was achieved by applying alternating light-dark periods, as follows. The dark period started as cells began their first protoplast fission. The length of the dark period was chosen to allow all cells to release their daughter cells. For the first two or three cycles, the culture was observed by light microscopy to set the correct length of both the light and dark periods. Once the culture was synchronous, the periods were kept constant (approx. 16 h light and 10 h dark).

Mating competence test. To test the mating competence of mt^- cells during synchronized growth, samples were taken and divided into two parts. One was fixed with 1.25% (v/v) glutaraldehyde to determine the cell density using a haemocytometer. One millilitre of the second part was mixed with one millilitre of a dense suspension of mt^+ gametes, obtained by flooding 3-week-old agar cultures, as described by Schuring *et al.* (1990). These cells had a density of 10^7 cells ml^{-1} , and about 80% of them were sexually competent, i.e. mated with gametes of the opposite mating type. The mixed suspension was placed in small Petri dishes (4 cm in diameter) and illuminated by two white fluorescent tubes ($15 W m^{-2}$) for 1 h at room temperature. After fixation with glutaraldehyde (1.25%, v/v), the number of vis-à-vis pairs was counted in a haemocytometer. The mating competence of the observed cells was expressed as described by Demets *et al.* (1987).

Commitment curves. To assess the time when the cells in a synchronous culture attain their commitment to cell division, samples were taken at 2 h intervals and spread on the surface of nutrient medium solidified with 2% (w/v) agar and incubated in the dark at 30 °C. Under these conditions, each cell will execute one cell division if it has passed the commitment point (Šetlik & Zachleder, 1984). After 20 h the cells had stopped dividing and had formed small colonies in which the number of released daughter cells could be counted. In this way, the percentage of cells which had divided into 2, 4, 8, 16 or more daughter cells was estimated. To construct the commitment curves, the percentages were plotted against the time of sampling.

Results

All experiments were performed with synchronously dividing cell populations that were routinely obtained by cultivating cells under conditions promoting the maximal growth rate (optimal temperature, irradiance, and CO_2 concentration). Synchronization was achieved by alternating light-dark periods, their length being determined by the time intervals the cells needed to traverse the various stages of the cell cycle. To ensure optimal irradiation, the cultures were repeatedly diluted at the beginning of each light period to the initial cell number (Fig. 2b). At the beginning of the light period the cells were small and ellipsoidal. During growth in the light

they became more rounded and at the end of the light period they were practically spherical and much larger (Fig. 1). From 10 h onwards, cells began to cluster, which is typical for *Chlamydomonas* species (Straley & Bruce, 1979; Demets *et al.*, 1985). Thereafter, most of the cells lost their flagella prior to mitosis. During the dark period, all cells divided into 8, 16 or 32 daughter cells.

Surprisingly, freshly released daughter cells (Fig. 1a) were sexually competent, i.e. they were able to produce vis-à-vis pairs with gametes of the opposite mating type, as shown in Figs. 1(d) and 2(a). Since the sexual behaviour in these cells was exactly the same as that of gametes obtained by classical means (i.e. flooding 3-week-old agar cultures), they must be genuine gametes, even though they grew as normal cells.

To investigate this further, samples were taken during the whole cell cycle to measure their sexual competence. At the same time their commitment to divide was assessed (Fig. 3). Seventy to eighty percent of the cells were sexually competent for most of the precommitment phase, but they rapidly lost their competence 1–2 h before the first commitment point. Thereafter, the cells were unable to mate until fresh daughter cells were hatched from the mother sporangia (see Fig. 6b).

In photoautotrophic organisms, the length of the precommitment phase is dependent on the growth rate (Šetlik & Zachleder, 1984; Donnan & John, 1984). This implies that if the growth rate is changed by varying the external conditions, the period of mating competence will change accordingly. This prediction was confirmed by showing that the period of mating competence could be extended by decreasing the light intensity from 50 $W m^{-2}$ to 35 and 25 $W m^{-2}$ (Fig. 3). A similar effect was produced by decreasing the temperature from 30 °C to 22 °C. Comparison of Figs 3(a) and 4(a), or 3(b) and 4(b), shows that the precommitment period was prolonged at low temperature, together with the period in which the cells were mating competent.

The effect of the CO_2 concentration on the length of the gametic phase was also studied. When a gas mixture of 2% CO_2 was used, the precommitment period was about 5 h at 30 °C and 50 $W m^{-2}$ (Fig. 3a) or 14 h at 22 °C and 35 $W m^{-2}$ (Fig. 4b). When the cells were aerated without a CO_2 supplement, their growth rate was slower and the precommitment period was prolonged to 17 h at 30 °C and 50 $W m^{-2}$ (Fig. 5a) and 22 h at 22 °C and 35 $W m^{-2}$ (Fig. 5b). The period of mating competence was again prolonged accordingly.

These results indicate that the length of the gametic period is determined by the length of the precommitment period and that environmental factors do not influence the gametic period directly but do so by affecting the growth rate of the cells.

This conclusion was reinforced by experiments in

Fig. 1. Cells of *C. eugametos* of different age in synchronous culture. Irradiance, 50 W m^{-2} , temperature, 30°C ; CO_2 concentration, 2% (v/v); cell density, 10^6 ml^{-1} . Sampling times after the onset of the light period (h): (a) 0 h, (b) 4 h, (c) 8 h. (d) Vis-à-vis pair of young daughter cells (arrow) from a synchronous culture of *C. eugametos mt*⁻, sampled directly after the onset of light with *mt*⁺ tester cells. Bars, 10 μm .

which cells were grown in medium containing 0.1 or 0.01 of the control nitrate concentration. The mating competence of control cells is illustrated in Fig. 3(a). In comparison, those grown in 0.1 nitrate had a prolonged precommitment period and the decrease in mating competence occurred much later (the 10th hour compared with the 5th hour; data not shown). The commitment curves and the curve recording the percentage of mating-competent cells were essentially the same as those recorded for low fluence rate (see Fig. 3c). The effect of nitrogen limitation was more pronounced for cells grown in 0.01 nitrate concentration. The mating

competence was about 80% for more than 20 h before slowly decreasing in the following 10 h. At the end of this period, about 10% of the cells were still mating competent. Most of them were unable to complete their cell cycle and were probably arrested before their first commitment point. Cells in nitrogen-free medium grew slowly for several hours and then stopped. Their initial mating competence (about 80%) remained unchanged for about 24 h, but then gradually decreased, probably because their energy reserves were exhausted. These results indicate that under conditions of nitrogen starvation the gametic period is related to the precom-

Fig. 2. Mating competence of newly released daughter cells (a) and variation in cell concentration (b) in a synchronous culture of *C. eugametos* in five consecutive cell cycles (conditions as in Fig. 1). Light and dark periods are marked by white and black bars.

mitment period of the cell cycle. The concentration of the nitrogen source in the medium plays only an indirect role as a regulator of the growth rate. In non-growing, non-cycling cells (nitrogen-free conditions), the high mating competence can be maintained as long as the cells remain motile and viable.

We therefore conclude that there is an inverse relationship between the growth rate and the length of the gametic period. Consequently, one would expect mating competence to persist if cells are arrested in the early growth phase by removing their energy source. To test this, subcultures were taken every 2 h from an illuminated parent culture and maintained in the dark at 30 °C with aeration. Thirty hours after the onset of the light period in the parent culture, cells in all the subcultures were assayed for mating competence. Cells darkened in their precommitment phase remained mating competent during the whole dark period (Fig. 6). However, those cells which had been sampled near their commitment point displayed a lower competence, suggesting that there is a period just before the commitment point in which the cells become exclusively vegetative. Cells which had been sampled after the commitment point again showed increased mating competence (Fig. 6a, curve 1), for they had completed their cycle in the dark and produced daughter cells which were mating competent.

When subcultures were kept in continuous light, the percentage of competent cells after 30 h was lower (50–60%) than in corresponding non-illuminated subcultures. The reason for this may be that in continuous light, newly formed daughter cells are larger, due to additional growth inside the mother cell wall prior to hatching. Consequently, such cells would arrive earlier at their

Fig. 3. Mating competence during the division cycle in a synchronous culture of *C. eugametos* grown at different irradiances: (a) 50 W m⁻², (b) 35 W m⁻², (c) 25 W m⁻². Other conditions as in Fig. 1. The graphs show percentage of mating competent cells (curve 1); percentage of cells committed to the first (curve 2), second (curve 3), third (curve 4), and fourth (curve 5) division of protoplasts; and percentage of cells releasing daughter cells (curve 6). Light and dark periods are marked by white and black bars.

Fig. 4. Mating competence during the division cycle in a synchronous culture of *C. eugametos* grown at 22 °C. (a) 50 W m⁻²; (b) 35 W m⁻². Other conditions as in Fig. 1. The graphs show percentage of mating competent cells (curve 1); percentage of cells committed to the first (curve 2), second (curve 3), third (curve 4) division of protoplasts; and percentage of cells releasing daughter cells (curve 5). Light and dark periods are marked by white and black bars.

Fig. 5. Mating competence during the division cycle in a synchronous culture of *C. eugametos* aerated with ambient air. (a) 30 °C, 50 W m⁻²; (b) 22 °C, 35 W m⁻². Other conditions as in Fig. 1. The graphs show percentage of mating competent cells (curve 1); percentage of cells committed to the first (curve 2), second (curve 3) division of protoplasts; and percentage of cells releasing daughter cells (curve 4). Light and dark periods are marked by white and black bars.

Fig. 6. (a) Committed mating competence (curve 1) and committed cell density (curve 2) in a synchronous culture of *C. eugametos*. Samples were derived at various times and subsequently kept in the dark. Values found at the end of the dark periods (30 h after start of the cell cycle) were plotted against the sampling time. (b) The course of commitment curves and actual mating competence of the cells in the main culture at the time of sampling. Culture conditions as in Fig. 3(b); designation of curves in (b) as in Fig. 3.

commitment point, resulting in a loss of synchrony and decline of overall mating competence.

Discussion

We define a gamete as a cell which has the ability to fuse with another cell of different mating type. Since in the formation of vis-à-vis pairs in *C. eugametos* fusion between cells of different mating type is involved (namely the formation of the plasma bridge), we consider cells which are able to produce vis-à-vis pairs as gametes.

The ability of a cell to fuse with another cell is of course dependent on the presence of a signalling system by which physical contact with a sexual partner is detected and by which cellular responses that allow cell fusion are initiated (van den Ende *et al.*, 1990). In some strains, this signalling system is activated by light. In the dark such cells are unable to fuse and then might be considered as 'pregametes'. The strain used in this study, however, was not light-requiring in this respect (Schuring *et al.*, 1987).

Once a vis-à-vis pair is formed the following developmental process, yielding zygotes, is autonomous. It involves differential gene expression and protein synthesis, but, as far as we know, is dependent on cell-cell interactions. So we felt justified in using vis-à-vis pair formation as parameter for gametogenesis.

The present results show that cells of *C. eugametos* can express both vegetative and gametic properties during a particular interval of the cell cycle, which starts at the release of daughter cells and ends some hours prior to the commitment point. Environmental conditions that slow the growth rate also lengthen the gametic phase. Moreover, cells that have passed their commitment point will continue to progress through the cell cycle and enter cell division even when the source of carbon, energy or nitrogen is withheld (Ballin *et al.*, 1988; Donnan & John, 1984; Donnan *et al.*, 1985). Such cells will accumulate in the precommitment phase of the next cell cycle and will all be gametic. In fact, one can predict that any treatment which leads to G1 arrest will result in the production of a high percentage of cells which are gametes.

Seemingly in contrast with the present results, Tomson *et al.* (1985) showed that in a partially synchronous batch culture, gametes were formed only at the end of the exponential growth phase. This observation can be explained in part by assuming that at the end of the growth phase the cells accumulate in the G1 phase and will thus be largely gametic in nature. It remains unclear why these authors did not observe any mating competence during the growth phase of their cultures.

The results presented in this paper are also at variance with those of Kates & Jones (1964), who used *C.*

reinhardtii and *C. moewusii*, the latter being closely related to *C. eugametos*. Using synchronous cultures, they observed that when freshly released daughter cells were suspended in nitrogen-free medium in the light, mating competence developed gradually over the following 12 h, followed by a rapid decline. Only after 21 h, when daughter cells were produced, did the cells become 100% gametic. While it is difficult to explain why the young daughter cells were sexually incompetent, the course of sexual development can be explained by assuming that the cells were first traversing a cycle and after cell division they were arrested in the gametic phase. However, when young daughter cells were incubated in nitrogen-free medium in the dark, no gametes were produced, in contrast to the present results. A possible explanation is that the time interval during which *C. reinhardtii* cells are mating competent starts at a later point than in *C. eugametos*; in other words, mating competence in freshly released daughter cells develops more slowly in *C. reinhardtii* than in *C. eugametos*. It would be interesting to see if the present results can be applied also to *C. reinhardtii*, since it has been reported that vegetative cells and gametes differ ultrastructurally (Martin & Goodenough, 1975). This is also borne out by the study of Treier *et al.* (1989), who showed that light-dependent RNA and protein synthesis is required for gametogenesis. The finding of Martin & Goodenough (1975) that gametes, induced by nitrogen deprivation, start growing normally on addition of a nitrogen source, and lose their gametic character only after 6 h (presumably when they pass the commitment point), reinforces our finding that cells of *Chlamydomonas* can have a dual vegetative and gametic character.

Mating competence is determined by the ability to confer sexual adhesiveness upon the flagella, primarily by incorporating agglutinin molecules into the flagellar membrane and next to activate the mating structure by which cell fusion can take place (van den Ende *et al.*, 1990). From earlier studies (Tomson *et al.*, 1985) it is evident that there is a diurnal fluctuation in sexual adhesiveness of the flagella (together with a fluctuation in the ability to form vis-à-vis pairs), while the overall agglutinin content of the cells is constant. The decline in sexual competence in the precommitment phase described in this paper could be due to this phenomenon. The subsequent clustering of cells and their loss of flagella prior to mitosis would of course result in a complete loss of sexual competence, even if the ability to synthesize agglutinins remains unchanged during the other parts of the cell cycle, a contention which has been put forward by Musgrave *et al.* (1986). It is clear, therefore, that studies at the molecular level are needed to assess whether gametogenesis requires differential gene expression in *Chlamydomonas*.

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