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REVIEW PAPER

Cell-cycle regulation in green algae dividing by multiple fission

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Abstract

Green algae dividing by multiple fission comprise unrelated genera but are connected by one common feature: under optimal growth conditions, they can divide into more than two daughter cells. The number of daughter cells, also known as the division number, is relatively stable for most species and usually ranges from 4 to 16. The number of daughter cells is dictated by growth rate and is modulated by light and temperature. Green algae dividing by multiple fission can thus be used to study coordination of growth and progression of the cell cycle. Algal cultures can be synchronized naturally by alternating light/dark periods so that growth occurs in the light and DNA replication(s) and nuclear and cellular division(s) occur in the dark; synchrony in such cultures is almost 100% and can be maintained indefinitely. Moreover, the pattern of cell-cycle progression can be easily altered by differing growth conditions, allowing for detailed studies of coordination between individual cell-cycle events. Since the 1950s, green algae dividing by multiple fission have been studied as a unique model for cell-cycle regulation. Future sequencing of algal genomes will provide additional, high precision tools for physiological, taxonomic, structural, and molecular studies in these organisms.

Key words: Cell division, cell size, DNA replication, green algae, growth, light, multiple fission cell cycle, nuclear division, temperature.

Introduction

Green algae represent a very diverse group of organisms forming the evolutionary base for land plants. Common features shared with land plants include chloroplasts containing *a* and *b* chlorophylls, and a rigid cell wall. Their body organization could be very simple, as in unicellular algae like *Chlamydomonas reinhardtii* or *Ostreococcus tauri*, or quite complex multicellular forms with defined tissues, as in the closest higher plant relatives, Charales.

The study of the green algal cell cycle has a long history dating back 60 years. Indeed, the first report describing synchronization of the green alga *Chlorella* was published by the group of Professor Tamiya in 1953 (Tamiya *et al.*, 1953), the same year that DNA structure was resolved by Watson, Crick, Wilkins, and Franklin (Franklin and Gosling, 1953a,b;

Watson and Crick, 1953a,b; Wilkins *et al.*, 1953a,b) and the cell cycle was first separated into four phases G1, S, G2, and M by Howard and Pelc (1953). In the original report, the Tamiya group used fractionated centrifugation to achieve a synchronized culture of *Chlorella*. However, very soon it was noted that some green algae form synchronous cultures in nature. Indeed, the algae dividing by multiple fission evolved so that they could grow autotrophically during the day when exposed to light and undergo DNA replication(s) and nuclear and cell division(s) in the dark. Since then, algal cultures have been synchronized by alternating light and dark periods. Such a method is not only very fast and reliable but, most importantly, it is natural, yields very high synchrony, and can be maintained almost indefinitely.

Traditionally, cell-cycle regulation has been studied in different model organisms from the genera *Chlorella*, *Chlamydomonas*, and *Scenedesmus*, all of which divide by a complex mechanism called multiple fission. Only recently, a novel model organism was added to this portfolio, the micro-alga *O. tauri*, discovered in 1995 (Chrétiennot-Dinet *et al.*, 1995) and shown to divide by binary fission. Here, we will discuss how multiple fission is performed in different species and how cell-cycle progression is coordinated with growth, how it is regulated and finally we will summarize the benefits of green algal studies in the era of cell-cycle genomics.

The multiple fission cell cycle

A common cell cycle consists of a growth phase, synthesis phase, second growth phase, and mitosis, followed closely by cell division. This classical cell-cycle scheme can be modified in some organisms, like the budding yeast, where the synthesis and mitosis phases overlap without an intervening second growth phase (Forsburg and Nurse, 1991) or under some conditions such as in embryonic development, where rapid cell cycles consist of only alternating S and M phases without any gap phases (Newport and Kirschner, 1982, 1984; Hormanseder *et al.*, 2013). However, the basic rule of one mother cell giving rise to two daughters is always kept. Most eukaryotic cells therefore divide by binary fission. In many green algae, the mother cell gives rise to more than two daughter cells, thus they divide by multiple fission. Generally, the mother cell divides into 2^n cells, where n is an integer from 1 up to 10. The number of daughter cells is dictated by external conditions, as will be discussed in more detail. The maximum number of daughter cells produced is fixed in different species and usually ranges from 8 to 16 (i.e. $n = 3–4$).

The cell cycle is generally perceived as a 'cycle' since the same sequence of events happens in mother and daughter cells. Speaking more precisely, however, the cell cycle is not a 'cycle' but rather a sequence of events repeating themselves in each cell (Cooper, 1979, 1984). According to Mitchison, each of such sequences consists of two coordinated processes, growth (G1 phase) and the DNA replication–division sequence, composed of DNA replication (S phase), G2 phase, nuclear division (M phase) and cytokinesis (C) (Mitchison, 1971). From now on, we will use the term 'DNA replication–division sequence' to describe the processes involving DNA replication, G2 phase, and nuclear and cellular division. Understanding the cell cycle as a sequence rather than a 'cycle' makes it easier to explain the mechanisms underlying the multiple fission cell cycle.

The formation of increasing numbers of daughter cells can be modelled by the increasing number of DNA replication–division sequences occurring within one cell cycle: in order to fit into one cell cycle, the individual sequences more or less overlap, depending on their number. Division by multiple fission is shared among even distantly related groups of chlorophycean algae while it is missing in their naked relatives (Floyd, 1978; Kirk, 1998). This seems to be a notable development since it allows the separation of light-dependent growth from light-independent DNA replication, nuclear, and cellular divisions and thus maximizes the exploitation of light energy for photosynthesis, permitting higher growth rates to be achieved compared with species dividing by binary fission (Rading *et al.*, 2011). The specific organization of the multiple fission cell cycle differs in distinct species; individual multiple fission cell-cycle patterns can be very simply split into consecutive and clustered types (Šetlík and Zachleder, 1984). However, this might represent an oversimplification since intermediate patterns also exist. We therefore prefer to describe cell-cycle patterns for each of the three model species separately.

Scenedesmus

Scenedesmus is a genus of the common nonmotile freshwater green chlorophycean alga from the order Sphaeropleales. Two of the genus species, *Scenedesmus obliquus* and *Scenedesmus quadricauda*, have been used as model organisms, mainly for study of photosynthesis, toxicity, and cell-cycle regulation. Members of the genus are characterized by the formation of coenobia. The coenobium arises upon division of a single mother cell when the daughter cells stay connected by a common cell wall. Therefore a coenobium is a special type of colony: a clonal colony formed by the descendants of the same mother cells. Such structures are also common among other distantly related green algae like the genera *Gonium*, *Eudorina*, and *Pandorina* and it has been hypothesized that such structures represent early phases of evolution of multicellularity (Kirk, 2004), indicating the importance of multiple fission for this process.

The key model organism for cell-cycle studies has, for decades, been *S. quadricauda* (Šetlík and Zachleder, 1984; Zachleder and Šetlík, 1990; Zachleder *et al.*, 2002). The coenobia of *S. quadricauda* can be four- or eight-celled and only under specific conditions are two-celled coenobia formed (Komárek and Růžička, 1969; Růžička, 1971), indicating that the cells normally divide twice or three times during a single cell cycle (Fig. 1). Cell-cycle progression starts with a period of growth, the G1 phase. During this phase, the cells increase their volume until they roughly double the volume of the daughter cell coenobia. This marks an important part of the cell cycle, called the commitment point (CP). Early experiments suggested, that when immediately put into the dark after passing through the CP, the cells are able to divide without any external energy supply while cells put into dark just prior to the CP are unable to do so. In the early works, the CP was noted as a 'point-of-no-return' (Moberg *et al.*, 1968), 'induction of division' (Šetlík *et al.*, 1972), 'transition point' (Spudich and Sager, 1980), or 'commitment point' (John, 1984, 1987); only recently has the last term been generally accepted. All of these terms illustrate the basic importance of this point (or short part of the cell cycle) for further cell-cycle progression. The CP exists not only in *S. quadricauda* but also in other green algae. As a key decision point in the cell cycle, it is the equivalent of START in budding yeast and restriction point in mammals (John, 1984). This fascinating part of the cell cycle will be discussed in more detail but now we return to *S. quadricauda* cells that have just reached the CP.
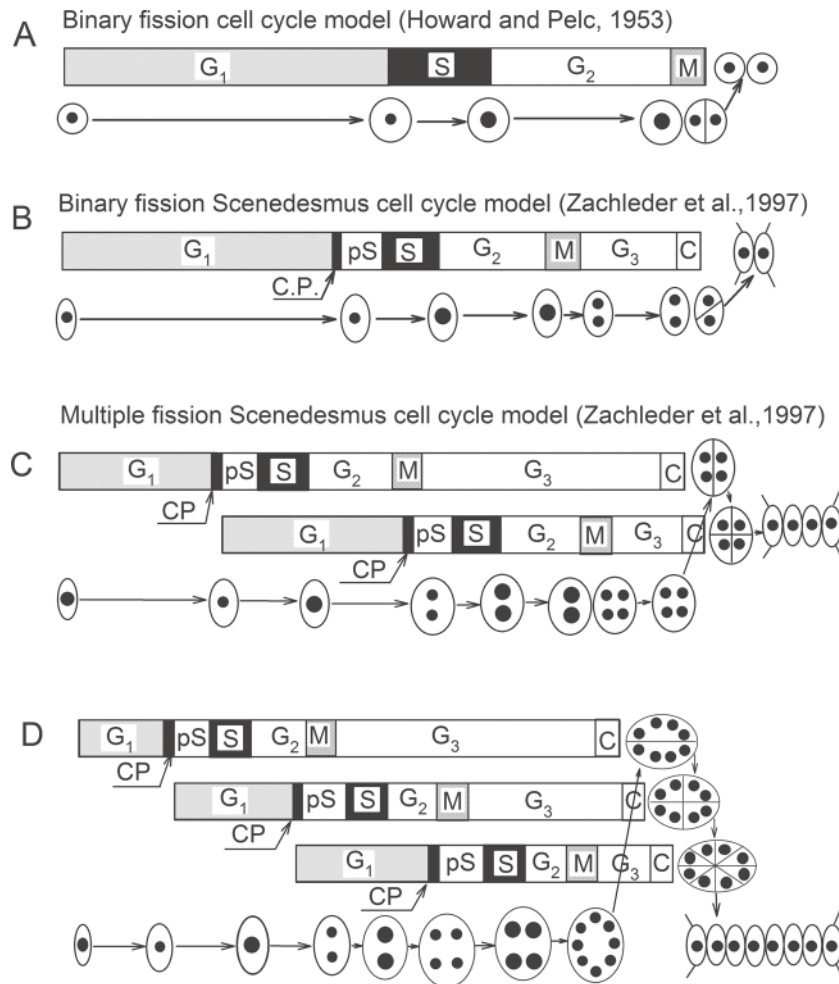
Fig. 1. Different types of cell-cycle phases of the classical cell-cycle model (A) and the multiple fission found in the alga *Scenedesmus* dividing into two (B), four (C), and eight (D) daughter cells. Classical cell-cycle model after Howard and Pelc (1953); *Scenedesmus* cell-cycle model after Zachleder *et al.* (1997). Individual bars show the sequence of cell-cycle phases during which growth and the DNA replication–division sequence take place. Whereas only single growth and the DNA replication–division sequence occurs during the cell cycle of cells dividing by binary fission (A, B), two partially overlapping growth and DNA replication–division sequences occur within a single cycle in cells dividing into four daughter cells (C) and three partially overlapping growth and DNA replication–division sequences occur within a single cycle in cells dividing into eight daughter cells (D). Two bars (C) and three bars (D) illustrate the simultaneous course of different phases from two and three, respectively, consecutively started sequences of growth and reproductive events. B, C, and D represent the cell-cycle patterns of the same species under different growth conditions. With improving growth conditions (increasing light intensity, optimum temperature), the same culture will increase the extent of overlapping and change the division pattern from B to C to D. Under optimal growth conditions, the culture will divide into eight daughter cells, as represented in D. Schematic pictures of the cells indicate their increasing size during the cell cycle and the black spots inside illustrate the size and number of nuclei. Large black spots indicate the doubling of the DNA. The small arrows pointing from the schematized cell pictures pinpoint the order of events as they occur within single cell. G_1, the phase during which the critical cell size is attained; it can be called a precommitment period because it is terminated when the commitment point (CP) is reached. CP, the stage in the cell cycle at which the cell becomes committed to triggering and terminating the DNA replication–division sequence. pS, the prereplication phase between the CP and the beginning of DNA replication; the processes required for the initiation of DNA replication are assumed to happen during this phase. S, the phase during which DNA replication takes place. G_2, the phase between the termination of DNA replication and the start of mitosis; processes leading to the initiation of mitosis are assumed to take place during this phase. M, the phase during which nuclear division occurs. G_3, the phase between nuclear division and cell division; the processes leading to cellular division are assumed to take place during this phase. C, the phase during which cell cleavage occurs. Modified from Zachleder V, Schläfli O, Boschetti A. 1997. Growth-controlled oscillation in activity of histone H1 kinase during the cell cycle of *Chlamydomonas reinhardtii* (Chlorophyta). *Journal of Phycology* 33, 673–681, with permission of the publisher.

After reaching the CP, a DNA replication–division sequence commences, which will ultimately lead to DNA duplication, mitosis and cell division. In this, the CP and the following processes copy the events of a classical binary fission cell cycle. Since attainment of the CP marks a distinct switch in the cell cycle, from growth to reproduction, we prefer to denote the

period immediately following the CP as a presynthetic phase (pre-S; Zachleder *et al.*, 1997). Historically, the pre-S phase is a part of the G1 phase in other organisms and is sometimes referred to as late G1 phase (Nasmyth, 1996; Sherr, 1996). The time of attaining the first CP in algae dividing by multiple fission is not only the start of a DNA replication–division sequence, but is also the time when a new growth phase starts. The second growth phase follows the same rules as the first G1 phase, where cells double their volume to reach four-times the volume of the daughter cells. After growth is finished, the second CP is reached. Cells put into the dark after passing the second CP will divide into four cells, implying that the second CP is switching on another DNA replication–division sequence that will again duplicate all the reproductive structures (DNA, nuclei, and cells).

In *S. quadricauda*, once a second CP is attained, two things can happen. Firstly, if the growth rate of cells is low due to environmental conditions (to be discussed), the cells will duplicate DNA, divide nuclei and cells, and produce a four-celled coenobium. Secondly, if the growth rate is sufficiently high and the environmental conditions are preferable, the cells will behave as they did once already and initiate a new G1 phase, this time a third one. Again, the rules are identical to the two preceding phases. The cells increase their volume two-fold for the third time, reaching roughly an eight-fold increase compared to the daughter cells; this leads, yet again, to attainment of a third CP and to DNA duplication, mitosis, and cell division. Thus, the cells divide three times, producing eight cells connected as an eight-celled coenobium.

The light-growth phase of the cell cycle is macroscopically evident as a stepwise increase in cell volume with the CP being attained roughly at each doubling. DNA replication and nuclear division of the first DNA replication–division sequence(s) overlap with growth and must somehow be coordinated, although the mechanism is, as yet, unknown. As already noted about the CP and dark, it is evident that all DNA replication–division sequences can be finished without any energy supply, in the dark. The dark phase is dedicated to nuclear and cellular division and finally to coenobia formation.

From this scheme, it is obvious that multinuclear phases are common during cell-cycle progression in *S. quadricauda* (Fig. 2). Cell division does not happen until the very end of the cell cycle, when the last nuclear division is finished. A G3 phase separating nuclear and cell division was therefore proposed for this organism (Zachleder *et al.*, 1997). Clearly the cells have a way of 'knowing' what is the last nuclear division since cell division happens immediately afterwards. A similar mechanism also exists in *Chlorella*, as will be described next. As yet, the signal is unknown, although we can speculate on this.

Chlorella

The genus *Chlorella*, of the class Trebouxiophyceae, is only distantly related to *S. quadricauda* of a different class, Chlorophyceae; however, it shares the property of division by multiple fission. *Chlorella ellipsoidea* was the species used in the seminal works by Tamiya's group (Hase *et al.*, 1957; Morimura, 1959; Morimura *et al.*, 1961; Tamiya *et al.*, 1961; Tamiya, 1964, 1966); they were the first to notice that when *Chlorella ellipsoidea* were grown under alternating light and dark periods (12/12 light/dark cycle) they grew only in the light, while cellular division or the separation of mother cells took place during the dark. Cell-cycle regulation is clearly based on the same principles as that of *S. quadricauda* and can be described similarly (Fig. 1); the cell-cycle organization, however, resembles more that of *Chlamydomonas reinhardtii* (Fig. 3). When new-born daughter cells are illuminated, they enter the G1 growth phase. Once the cells roughly double their cell volume, they attain the CP (Donnan *et al.*, 1985) and the first DNA replication–division sequence is allowed. Similarly to *S. quadricauda* cells, the attainment of the CP means not only switching on DNA replication and nuclear and cell division but also entry
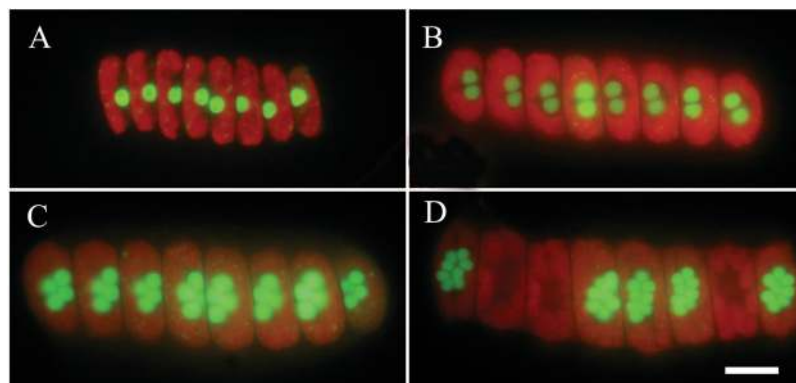
Fig. 2. Fluorescence photomicrographs of eight-celled coenobia of the chlorococcal alga *Scenedesmus quadricauda* during the cell cycle stained with 0.3% SYBR green I dye. (A) Uninuclear daughter coenobium; nuclei are visible as green spots; chloroplasts are visible in red colour which is an autofluorescence of chlorophyll. (B) Binuclear coenobium. (C) Tetranuclear coenobium. (D) Mother octonuclear coenobium; dividing cells remained unstained. Bar = 10 μm. Modified from Springer, Folia Microbiologica 50, 2005, 333–340, Visualization of DNA-containing structures in various species of Chlorophyta, Rhodophyta and Cyanophyta using SYBR green I dye, Vítová M, Hendrychová J, Cepák V, Zachleder V, Fig. 1. With kind permission from Springer Science and Business Media (this figure is available in colour at *JXB* online).
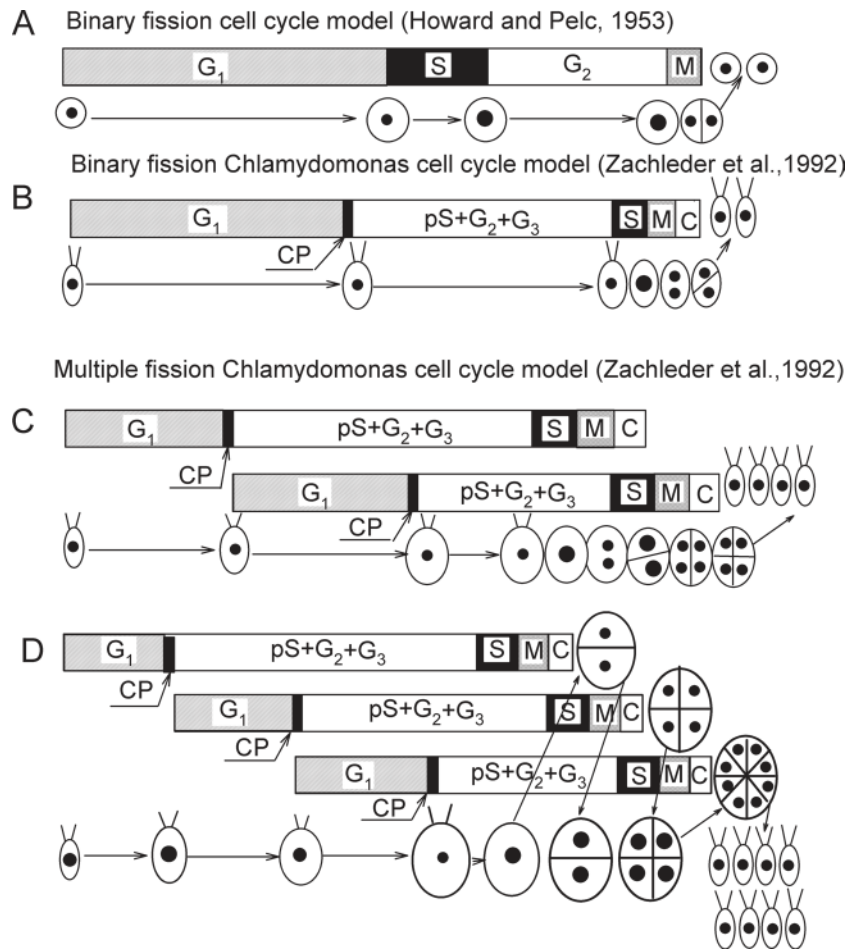
Fig. 3. Different types of cell-cycle phases of the classical cell-cycle model (A) and the multiple fission found in the alga *Chlamydomonas* dividing into two (B), four (C). and eight (D) daughter cells. Classical cell-cycle model after Howard and Pelc (1953), *Chlamydomonas* cell-cycle model after Zachleder *et al.* (1997). Individual bars show the sequence of cell-cycle phases during which growth and the DNA replication–division sequence take place. Whereas only single growth and the DNA replication–division sequence occurs during the cell cycle of cells dividing binary fission (A, B), two partially overlapping growth and DNA replication–division sequences occur within a single cycle in cells dividing into four daughter cells (C) and three partially overlapping growth and DNA replication–division sequences occur within a single cycle in cells dividing into eight daughter cells (D). Two bars (C) and three bars (D) illustrate the simultaneous course of different phases from two and three, respectively, consecutively started sequences of growth and reproductive events. B, C, and D represent cell-cycle pattern of the same species under different growth conditions. With improving growth conditions (increasing light intensity, optimum temperature), the same culture will increase the extent of overlapping and change the division pattern from B to C to D. The extent of overlapping can reach up to four overlapping growth and DNA replication–division sequences in the wild type grown under optimal growth conditions (Lien and Knutsen, 1979) and up to five sequences in the *mat3-4* mutant (Fang *et al.*, 2006), leading to production of 16 and 32, respectively, daughter cells. Schematic pictures of the cells indicate their increasing size during the cell cycle and the black spots inside illustrate the size and number of nuclei. Large black spots indicate the doubling of the DNA. Flagella are represented as two lines at the top of the cells, the cells resorb flagella before the first round of DNA replication starts and the newborn daughter cells regrow them when the division is completed. The small arrows pointing from the schematized cell pictures pinpoint the order of events as they occur within single cell. G1, the phase during which the critical cell size is attained; it can be called a precommitment period because it is terminated when the commitment point (CP) is reached. CP, the stage in the cell cycle at which the cell becomes committed to triggering and terminating the DNA replication–division sequence. pS, the prereplication phase between the CP and the beginning of DNA replication; the processes required for the initiation of DNA replication are assumed to happen during this phase. S, the phase during which DNA replication takes place. G2, the phase between the termination of DNA replication and the start of mitosis; processes leading to the initiation of mitosis are assumed to take place during this phase. M, the phase during which nuclear division occurs. G3, the phase between nuclear division and cell division; the processes leading to cellular division are assumed to take place during this phase. C, the phase during which cell cleavage occurs. Modified from Zachleder V, Schläfli O, Boschetti A. 1997. Growth-controlled oscillation in activity of histone H1 kinase during the cell cycle of *Chlamydomonas reinhardtii* (Chlorophyta). *Journal of Phycology* 33, 673–681, with permission of the publisher.

into the second G1 phase. Again the process repeats itself: a further increase in cell volume will bring about a four-fold increase in initial cell volume and attainment of the second CP, which will eventually lead to another round of DNA replication and nuclear and cell divisions. Depending on growth conditions, the cells will either immediately undergo cell division or will continue to grow by entering another G1 phase of the third DNA replication–division sequence. Should the cells enter the third growth sequence, they will eventually reach another doubling of cell volume, leading to attainment of yet another CP, and eventually to DNA replication and nuclear and cell division into eight cells.

The obvious question is 'how' the cell 'knows' that it should either undergo division directly after the second DNA replication–division sequence or continue to grow and enter into another DNA replication–division sequence. Data suggest the following explanation of the decision process between immediate division versus postponing division until after the next DNA replication–division sequence. Growth needs to be fast enough so that the following CP is attained, and the subsequent DNA replication–division sequence is switched on before the previous one is completed. Each initiated DNA replication–division sequence thus possesses an inhibitory effect on the previous one, preventing it from premature completion. This might be achieved either through a checkpoint or by some more general influence. Alternatively, the cells assess their size at a certain (as-yet unidentified) time of cell cycle, when they possibly sense whether the eight-fold increase in cell volume has already occurred (or is about to occur), in which case they postpone cell division until the CP is reached and another DNA replication–division sequence is switched on.

Similarly to *S. quadricauda* during its cell cycle, *Chlorella* cells contain two or four nuclei; the division into eight nuclei is again immediately followed by cell division so that octonuclear cells are almost undetectable. In contrast to *S. quadricauda* however, the multinuclear stages of the cell cycle are rather short and the nuclear division(s) occurs at the end of the light period, closely followed by each other. In this sense, the *Chlorella* cell cycle is similar to the clustered type of multiple fission cell cycle, as proposed by Šetlík and Zachleder (1984). However, the 'real' clustered type of multiple fission never contains multinuclear stages (as will be discussed).
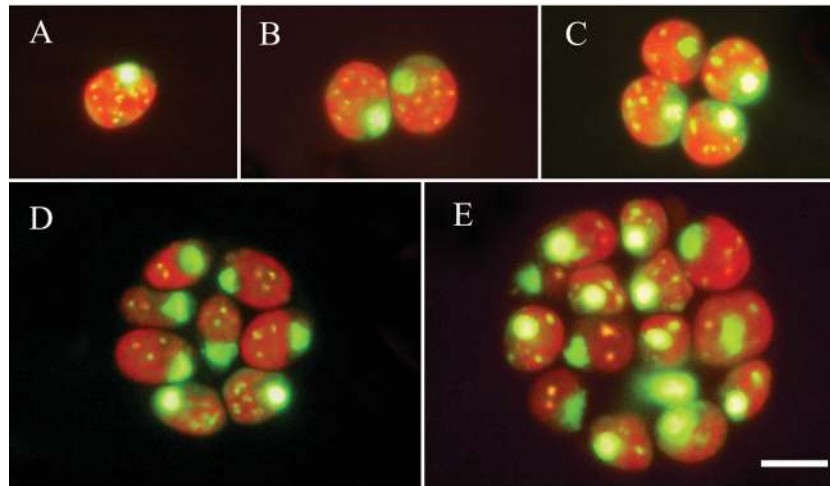
Chlamydomonas

The genus *Chlamydomonas* belongs to the same class of green algae, Chlorophyceae, as the genus *Scenedesmus*, but to a different order, Chlamydomonadales. The type species of the genus, *Chlamydomonas reinhardtii*, is one the most widely used model algal organisms. It has been mainly used to study the flagellar apparatus and photosynthesis, but also to study regulation of the cell cycle (Harris, 2001).

Cell-cycle organization in *Chlamydomonas reinhardtii* is traditionally described as consisting of a very long G1 phase (occupying up to 80% of the cell cycle) followed by several alternating rounds of S and M phases, where each nuclear division occurs soon after DNA replication and is immediately followed by cell division (Coleman, 1982; Craigie and Cavalier-Smith, 1982; Lien and Knutsen, 1976b, 1979). Indeed, this can be readily observed in light/dark synchronized *Chlamydomonas reinhardtii* cultures, and with the absence of any multinuclear stages, the *Chlamydomonas reinhardtii* cell cycle represents a clustered type of multiple fission cell cycle (Šetlík and Zachleder, 1984). One part of the *Chlamydomonas reinhardtii* cell cycle, the S/M phase, clearly resembles the early embryonic cell cycles where only S and M phases alternate in the absence of any intervening gap phases (Edgar, 1995; Edgar and Kiehle, 1988; Newport and Kirschner, 1982). However, this comparison might be somehow misleading because the rapid early embryonic cycles depend on the supply of proteins from the oocytes (Edgar, 1995; Edgar and Kiehle, 1988; Newport and Kirschner, 1982), whereas the *Chlamydomonas reinhardtii* S/M cycles depend on the preceding long G1 phase; the mechanisms providing the required proteins might therefore be quite different in embryonic cycles compared with single-celled *Chlamydomonas reinhardtii*. A careful analysis of the *Chlamydomonas reinhardtii* G1 phase revealed that it also contains at least one distinct regulatory CP (Moberg *et al.*, 1968; Spudich and Sager, 1980; John, 1984, 1987) and thus it can also be described by a scheme similar to that used here to describe *Scenedesmus* and *Chlorella* cycles.

Newly dark-born daughter cells enter G1 phase and start to increase cell volume until it approximately doubles and the cell attains the CP. This will allow for one DNA replication–division sequence to occur; however, this will not manifest itself until the very end of the cell cycle (Fig. 3). Analogous to the other multiple fission systems, the attainment of the CP also marks entry into growth phase of the next cell-cycle sequence. The growth phase will again culminate in the attainment of another CP once the volume reaches about four-fold of its initial value and another DNA replication–division sequence will be switched on. Similarly to the previously described models, another, the third, G1 phase leading to the third CP and the third DNA replication–division sequence can start (Fig. 3) (Knutsen and Lien, 1981; Donnan *et al.*, 1985). Wild-type *Chlamydomonas reinhardtii* cells can, under optimal growth conditions, reach up to four CPs and start up to four DNA replication–division sequences, dividing thus into 16 daughter cells (Knutsen and Lien, 1981; Vítová *et al.*, 2011b); mutant cells can accommodate even more overlapping sequences (Umen and Goodenough, 2001).

In contrast to the *Scenedesmus* and *Chlorella* models, no multinuclear stages are present during the cell cycle and thus no G3 phase separates nuclear and cellular division (Fig. 4). Moreover, there has even been discussion as to whether a G2 phase actually exists in *Chlamydomonas reinhardtii*. Indeed, if it is present, it must be very short since it is undetectable under normal conditions. However, it can be presumed that all the regulatory mechanisms running normally during G2 phase are present and occur, most probably, during the prolonged G1 phase. This would be in line with a continuum model proposed by Cooper (1979, 1984), which model predicts that there are no specific events for gap phases, only certain processes that need to be finished prior to progression

Fig. 4. Fluorescence photomicrographs of the volvocine alga *Chlamydomonas reinhardtii* during the cell cycle showing consecutive multiple division of protoplasts, stained with 0.3% SYBR green I dye. (A) Uninuclear daughter cell; the nucleus is visible as a green spot and chloroplast nucleoids as tiny green dots; the chloroplast is visible in red colour which is an autofluorescence of chlorophyll. (B) The first division of the protoplast; mother cell divided into two cells. (C) The second division of protoplasts; two cells divided into four cells. (D) The third division of protoplasts; four cells divided into eight cells. (E) The fourth division of protoplasts; eight cells divided into 16 cells. Bar = 10 μm. Modified from Springer and Folia Microbiologica 50, 2005, 333–340, Visualization of DNA-containing structures in various species of Chlorophyta, Rhodophyta and Cyanophyta using SYBR green I dye, Vítová M, Hendrychová J, Cepák V, Zachleder V, Fig. 2. With kind permission from Springer Science and Business Media (this figure is available in colour at *JXB* online).

to DNA synthesis and mitosis; the preparatory processes are happening continuously throughout the cell cycle. The value of G1 and G2 (and G3) phases is therefore only in semantics. The presence of gap phases is a manifestation of the fact that processes required for further progression in the cell cycle are not finished, while their absence suggests that processes necessary for further progression have already been completed earlier in the cell cycle (Cooper, 1979, 1984).

Macromolecular synthesis during cell cycle

Cells dividing by multiple fission are naturally synchronized by light/dark regimes present in their biotope. Actually, as aforementioned, division by multiple fission seems to be an adaptation to such naturally occurring cycles so that the light period is exploited for growth while the dark period serves to perform the necessary division processes. Therefore, it is not surprising that the metabolism of green algae dividing by multiple fission is, to a great extent, dependent on light.

The previous section discussed the involvement of growth in cell-cycle progression. Growth is manifested in several ways. The easiest one to observe is an increase in cell volume, detectable simply by microscopically following progression of the cell cycle. In an ideal, synchronized population, volume increases in steps with a slowdown, roughly at each doubling, where a new CP is attained. In a real population, the stepwise increase is not always as evident (Fig. 5). Clearly, the volume changes must be underlined by some macromolecular synthesis. There are two obvious candidates: an increase in total RNA and an increase in total protein. Both macromolecules increase continuously during the light period of the cell cycle and stabilize when the increase is stopped, usually during

the dark period (Fig. 6) (Iwamura, 1955; Kates and Jones, 1964; Wanka and Aelen, 1973; Baumgartel and Howell, 1977; Howell and Walker, 1977b; Zachleder and Šetlík, 1982). A very detailed analysis of global rates of protein synthesis, as well as synthesis of specific proteins during the cell cycle, was carried out by Howell and his collaborators in the 1970s (Howell, 1972; Howell *et al.*, 1975; Baumgartel and Howell, 1977; Howell and Walker, 1977a; Howell and Baumgartel, 1978). According to their analysis, about 20% of proteins are synthesized in a cell-cycle stage-specific manner while the remaining 80% are made at a rate proportional to general protein synthesis (Howell *et al.*, 1977). One example of such cell-cycle regulated proteins are α- and β-tubulins that accumulate coordinately during growth, reaching peak levels before or during division (Ares and Howell, 1982).

Apart from RNA and protein, other macromolecules also accumulate during the growth phase, the most prominent being starch and polyphosphate as carbon and phosphate stores, respectively. Both of these compounds are energetically rich and can serve as energy reserves. Starch and polyphosphates are both assimilated concomitantly with photosynthesis (Miyachi and Tamiya, 1961; Schmidt and King, 1961; Kulaev and Vagabov, 1967; Markarova and Baslavskaia, 1970a,b; Wanka, 1975; Voříšek and Zachleder, 1984). The number and size of starch and polyphosphate granules increase with the cell growth and both of them are rapidly degraded during the division process so that most of the store is spent. This is in line with the increase in activities of starch-degrading enzymes during cell division, in both *Chlorella* and *Chlamydomonas* (Wanka *et al.*, 1970; Spudich and Sager, 1980; John, 1984). Interestingly, starch is spent for the division process even if the cells are dividing in light, where energy could be provided by photosynthesis (Wanka,
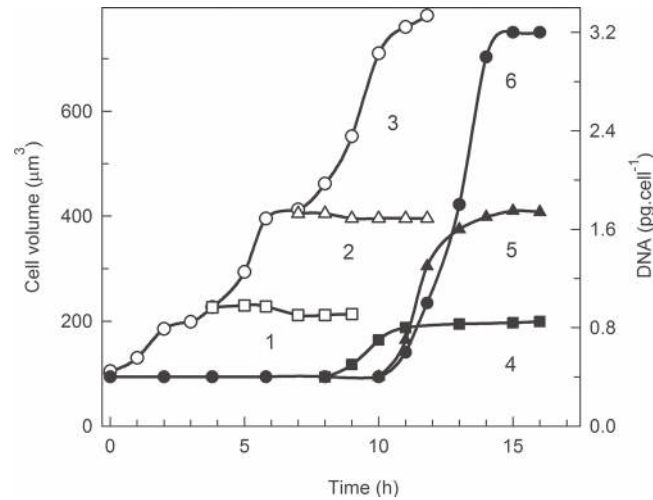
Fig. 5. Time courses of cell volume and DNA concentration in synchronous populations of *Chlamydomonas reinhardtii*. Cultures were grown at a high irradiance and placed in the dark after the first (4 h), second (7 h), and the third (12 h) commitment to trigger a DNA replication–division sequence. Curves 1–3: cell volume in subpopulations put in the dark after 4, 7, and 12 h, respectively; curves 4–6: DNA concentration per cell in subpopulations put in the dark after 4, 7, and 12 h, respectively. Modified from Zachleder V, Schläfli O, Boschetti A. 1997. Growth-controlled oscillation in activity of histone H1 kinase during the cell cycle of *Chlamydomonas reinhardtii* (Chlorophyta). *Journal of Phycology* 33, 673–681, with permission of the publisher.

1975; Vítová *et al.*, 2011b). This indicates a close relationship between cell-cycle progression and the energy stores.

All of the aforementioned synthetic processes depend on the energy provided by photosynthesis. The rate of photosynthesis (rate of oxygen evolution) increases during the light phase in both *Chlorella* and *Scenedesmus* (Berková *et al.*, 1972; Senger and Bishop, 1969; Senger, 1970; Kaftan *et al.*, 1999). In highly synchronized *Scenedesmus* cells, local maxima of oxygen production can even be attributed to attainment of the CP (Berková *et al.*, 1972). The rate of photosynthesis drops when the cells enter protoplast fission (*Scenedesmus*) or cell division (*Chlorella*). In light/dark synchronized cultures, this could be attributed to the dark transition. However, the decrease in photosynthesis significantly precedes the dark period. Moreover, the decrease is noticeable even if division occurs in the light. This indicates an intimate relationship between cell-cycle progression and photosynthesis; it also suggests the existence of a signal elicited from the nucleus (or cytoplasm) towards the chloroplast. A similar signal also has to be present to coordinate chloroplast and cellular divisions since the chloroplast division has to precede the cellular one. The nature of such signals is unknown.

Light and temperature affect cell-cycle progression

Light and temperature represent the two key natural components that affect the growth and subsequent cell-cycle progression of all autotrophic organisms. The effect of temperature is general for all organisms and is based on the fact that temperature greatly affects the entire metabolism; the temperature coefficient (Q_{10}) is 2 or more, indicating that a decrease or increase in temperature of 10 °C will change the rate of general metabolism by at least two-fold (John, 1984;

Vítová *et al.*, 2011a). The effect of light is more complicated since light can work both as an energy source and as a signal, a distinction is sometimes extremely difficult to discriminate. Early works studying the response of the cell cycle to light analysed the effect of different light intensities from the same light source as well as the effect of light of different wavelengths (red, blue, green, and UV light). Here, we will, for the sake of simplicity, focus only on the effect of light intensity and omit the effects of different wavelengths because such experiments are more prone to misinterpretation while partitioning the effect between the trophic and signalling roles of light.

The initial experiments analysing the effect of light and temperature were carried out by Morimura on cultures of *Chlorella ellipsoidea* (Morimura, 1959). During these experiments, he applied different light intensities to algal cultures grown at the same temperature. With increasing light intensity, the size of the mother cells increased significantly (Fig. 7) and was accompanied by an increase in the number of daughter cells. The light therefore had a clear trophic effect, enabling more CPs to be attained during the growth phase. Similar experiments were also carried out with *S. quadricauda* (Šetlík *et al.*, 1988; Zachleder and Šetlík, 1990; Zachleder, 1995), *Chlamydomonas eugametos*, and *Chlamydomonas reinhardtii* (John, 1984; Zachleder and van den Ende, 1992; Vítová *et al.*, 2011a,b). The conclusions from all of these experiments are the same: light intensity is a key component affecting growth rate under constant temperature. The higher the light intensity applied, the higher the growth rate that is reached and the more CPs that are attained during the growth phase. Irrespective of the applied light intensity (and thus growth rate), the cells always attained the CP at roughly the same volume, denoted as critical cell size (Šetlík *et al.*, 1972; Donnan and John, 1983; Fantes, 1977; Fantes and Nurse, 1977a, 1977b; John, 1984; Zachleder and Šetlík, 1990; Sveiczer *et al.*, 1996; Masui and
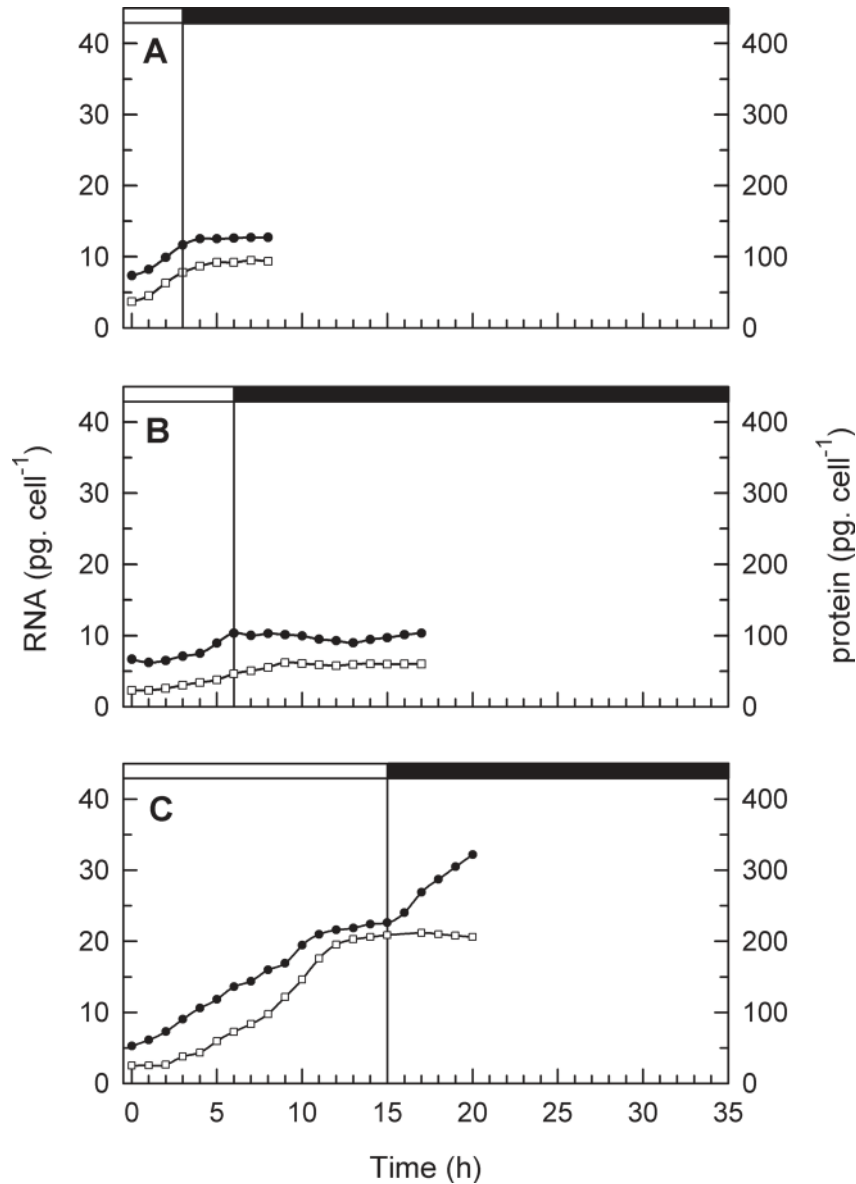
Fig. 6. The course of growth and reproductive processes in synchronous populations of *Scenedesmus quadricauda* grown for 3 h (A), 6 h (B), and 15 h (C) in light. Filled circles, RNA; open squares, protein. Black bars above the graphs indicate dark periods.

Wang, 1998; Wang *et al.*, 2000; Zachleder *et al.*, 2002). This caused very different lengths of the first G1 phase, denoted also as the precommitment phase (pre-CP). The length of the phase from the first CP until the first cell division commences, denoted as the post-commitment phase (post-CP), was more or less constant over a wide range of growth rates if the temperature was kept constant (for the effect of temperature see below). This might imply an involvement of a timer in the regulation of length of post-CP; however, this is not the case (as will be explained further). Instead, it was proposed the length of the post-CP phase is set by the time required to complete the processes of DNA replication–division sequence (Vítová *et al.*, 2011b). This is supported by the fact that the duration of the post-CP phase is prolonged with each additional DNA replication–division sequence started.

Independently from the length of the pre-CP phase, once cells reach a critical size and attain a CP, the DNA replication–division sequence is switched on. From this point, the DNA replication–division sequence is running. If the cells manage to attain another CP(s) before it is finished, they will undergo another DNA replication–division sequence(s) (and the post-CP period will be thus prolonged). This mechanism ensures that at very fast growth rates the maximum number of daughter cells is produced, but in contrast at very slow growth rates once the CP is reached and a single division is possible, it will be finished in the due time, which is limited only by the time required for completion of the sequence itself.

Changing temperature affects the entire metabolism of a cell. While increasing temperature generally speeds up metabolism, an increase in temperature significantly above the optimal will have the same effect as lowering the temperature. The first report describing the effect to temperature was again done by Morimura, using *Chlorella ellipsoidea*

(Morimura, 1959). He used a saturating light intensity to ensure light was not limiting for growth and incubated the algal cultures at different temperatures (9, 16, and 25 °C). He observed two interesting phenomena. As expected, growth rates at different temperatures differed and cell division occurred at quite different times. However, under all conditions, the cells divided into four daughter cells. This was caused by changing critical cell size, the lower temperature, the larger cell size; however, the lengths of the cell cycle differed extremely, from 30 to more than 300 hours (Fig. 8). Similar results were obtained by others who proved in other
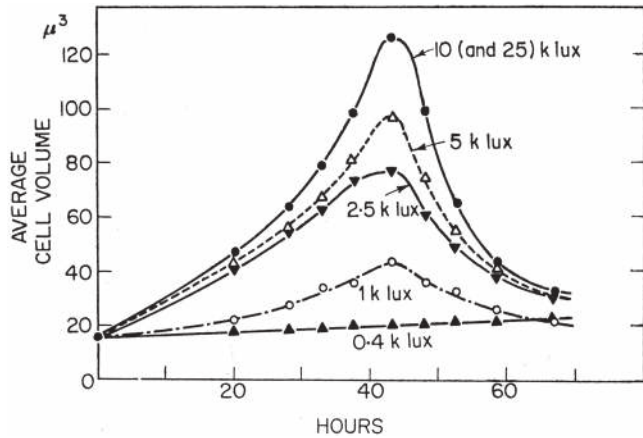
Fig. 7. Time courses of cell volume changes in synchronous populations of *Chlorella ellipsoidea* grown at 16 °C and under illumination with lights of varying intensities. Modified from Morimura Y. 1959. Synchronous culture of *Chlorella*. I. Kinetic analysis of the life cycle of *Chlorella ellipsoidea* as affected by changes of temperature and light intensity. *Plant and Cell Physiology* 1, 49–62.

green algae that not only the growth rate but also the sizer (sizing mechanism) was affected by temperature (Donnan and John, 1983; Donnan *et al.*, 1985; John, 1984; Vítová *et al.*, 2011a,b). This implies the critical cell size changes significantly with different growth rates, suggesting that the sizer comprises more than one component (as will be discussed). The temperature also significantly affected the length of the post-CP period; the duration of the post-CP phase almost doubled between 15 and 25 °C and between 18 and 28 °C (Vítová *et al.*, 2011a). This further supports that the duration of the post-CP phase is set by the time required for completion of the DNA replication–division sequence, which is, as any other metabolic process, temperature dependent.

Importantly, the experiments with light and temperature by Vítová *et al.* (2011a,b) clearly discredit involvement of any timer, including the circadian one in the cell-cycle regulation. The absence of circadian regulation (and other timers) in the cell cycle of *Scenedesmus* was confirmed by the experiments of Zachleder and Šetlík (Zachleder and Šetlík, 1990). However, in *Chlamydomonas*, this issue was a matter of longstanding discussion, with reports both supporting (Goto and Johnson, 1995) and excluding (Spudich and Sager, 1980; John, 1984; Zachleder and van den Ende, 1992) circadian control. We performed two comprehensive sets of experiments under a wide range of different light conditions that proved light intensity to be the sole regulator of cell-cycle progression under optimal temperature (Vítová *et al.*, 2011b). Similarly, over a wide range of temperatures, the length of the cell cycle ranges from 14 to 36 h, with the temperature coefficients between two temperatures differing by 10 °C ranging from 1.6 to 2.2. Such values contradict the temperature compensation hypothesis and thus confirm
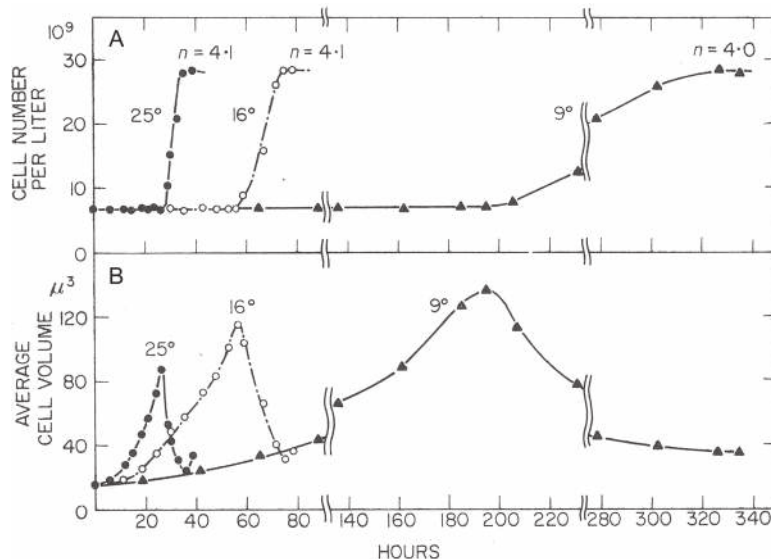
Fig. 8. Time courses of changes in cell number (A) and cell volume (B) in synchronous populations of *Chlorella ellipsoidea* grown at 9, 16, and 25 °C under illumination with light of saturating intensity (10 kilolux). Modified from Morimura Y. 1959. Synchronous culture of *Chlorella*. I. Kinetic analysis of the life cycle of *Chlorella ellipsoidea* as affected by changes of temperature and light intensity. *Plant and Cell Physiology* 1, 49–62.

no involvement of circadian timing in regulation of the cell cycle (Vítová *et al.*, 2011a).

When size matters

For the sake of simplicity in this review, it has thus far been presumed that all cells that start another of the overlapping sequences will always grow enough to be able to attain the CP and finish the entire DNA replication–division sequence. This, however, is not true in nature. While the cells always enter a new G1 phase after attaining the CP, they do not necessarily increase cell volume sufficiently to be able to attain the next CP. Such cells will produce bigger daughter cells, which will have a growth advantage in the next cell cycle and will eventually divide into more daughter cells, as compared to descendants of the cells that just managed to attain the CP in the previous cell cycle and divided into more smaller daughter cells (John, 1984). The most extreme situation is represented by cells that have just attained the CP and those that have not managed to do so. Such cells will not differ significantly in their sizes but they will produce different numbers of daughter cells that will differ in size by a factor of about two.

Clearly, cell size is of utmost importance for cell-cycle progression, and a mutation in the sizer will affect the cell-cycle pattern, as was shown by mutation of the retinoblastoma protein (Rb) homologue, MAT3, in *Chlamydomonas reinhardtii* (Umen and Goodenough, 2001). The dependence of further cell-cycle progression on a so-called 'critical cell size' led to a proposal for a sizer regulating cell-cycle progression in *Chlamydomonas* (Craigie and Cavalier-Smith, 1982; Donnan and John, 1983; Donnan *et al.*, 1985) and *Scenedesmus* (Šetlík *et al.*, 1972; Zachleder and Šetlík, 1990; Zachleder *et al.*, 2002). This is in line with what we have argued so far, that the CP is attained when cell volume has roughly doubled; the precise statement should be as follows: the CP is always attained when the cell reaches a critical cell size. The size control of cell-cycle progression and cell division has also been noted in other organisms, including yeasts and metazoans, and has been accepted as a golden rule of coordination between growth and cell cycle (Killander and Zetterberg, 1965; Zetterberg and Killander, 1965; Fantes *et al.*, 1975; Nurse, 1975; Fantes, 1977; Fantes and Nurse, 1977a,b; Johnston, 1977; Johnston *et al.*, 1977; Sveiczer *et al.*, 1996, 2004; Kondorosi *et al.*, 2000; Rupeš, 2002; Wells, 2002; Jorgensen and Tyers, 2004; Leslie, 2011).

However, we have already noted that the cell size required for attainment of the CP is modulated by temperature. This is in accordance with the differences in critical cell size in yeast, depending on growth conditions (Rupeš, 2002). Even though the critical size stays the same under constant conditions, this suggests that the determinant of critical cell size is not solely cell volume, protein content per cell (since that varies similarly to cell volume) (John, 1984), or RNA content per cell (RNA is a prerequisite for protein synthesis and also varies), but other compounds that accumulate concomitant with growth, the amount of which modulates the required critical

cell size. The light dependency of critical cell size indicates that synthesis of such a compound(s) is tightly linked to photosynthesis. On the other hand, temperature modulation suggests that accumulation is also temperature dependent. One of the candidate compounds is starch. Starch accumulates during the light phase and serves as an energy source both in the dark and for the DNA replication–division sequence.

We have previously suggested that the starch content per cell is crucial once the critical cell size is reached (Vítová *et al.*, 2011b). Here, we go a step further and hypothesize that starch is one of the determinants of critical cell size and that it is the amount of starch that is 'measured' before attainment of the CP is allowed. Should this be true, it would indeed be an elegant solution. As aforementioned, starch is used not only as an energy store in the dark but notably to provide the energy required for the DNA replication–division sequence, not only in dark but also in light when energy directly from photosynthesis could be supplied (Wanka, 1975; Vítová *et al.*, 2011b). The importance of starch for critical cell size should be evident from experiments under constant conditions when cell volume stays stable but the starch content changes. If, under such conditions, the CP is attained at a different cell volume but the same starch content, then the importance of starch levels for attainment of the CP should be clear. When dark periods of different lengths are inserted into the light period, starch that accumulated during the previous light period will be used by the cell to support metabolism in the dark but the cell size will not change. Depending on the length of the dark period, the starch reserves could be spent to only a certain extent or completely. Our initial experiments with an inserted dark period (Vítová *et al.*, 2011b) show that starch content varies less at the attainment of the first, second, and third CP than does cell volume, but more detailed experiments are clearly required to prove or disprove this hypothesis.

The other fascinating issue related to cell size is the question, 'how do the cells know how many CPs they have attained?' This might seem trivial in the case of the *Scenedesmus* cell cycle, when the cell undergoes all committed DNA replication–division sequences immediately. But it becomes more challenging when it comes to the *Chlamydomonas* cell cycle, where apparently 'nothing' happens until the very fast S/M phase. Two similar mechanisms were proposed in order to answer this fundamental question. Both of the mechanisms are based on a sizer. Craigie and Cavalier-Smith suggested that the cell measures its mother cell size, and based on that, decides how many times to divide (the number of division is also denoted as 'division number') (Craigie and Cavalier-Smith, 1982). This view was questioned by John (1984) who proposed that division number is rather determined by the cell size at commitment. We agree with John that attainment of commitment and thus the size at commitment (whatever should be the determinants of critical size) is the determinant of division number; this, however, does not answer the question of how the cells know how many CPs they have attained. One possible mechanism comes from a peculiar observation made by Howell (1972). He found that the amount of DNA encoding ribosomal RNA (rDNA) is not fixed in actively dividing *Chlamydomonas* cells. On the contrary, the amount

of rDNA increases during the cell cycle, with the first marked increase occurring at the time when the first CP is attained; the amount of rDNA returns to original once cell division is completed. Amplification of rDNA was suggested to be a compensatory mechanism providing additional templates for rRNA transcription in cells with high rates of rRNA synthesis, such as amphibian oocytes (Brown and Dawid, 1968; Gall, 1968). This mechanism could be a part of the processes switched on at the CP, allowing for additional rDNA copies to be replicated, thus promoting further growth that is not limited by rRNA transcription. The chromosomal copies of rDNA could also serve as a signal for how many times the DNA needs to be replicated in order to accommodate the correct number of CPs.

DNA replication and nuclear division

DNA replication occurs as a single wave, sometimes modulated by steps at the end of the light phase or at the transition from light to dark in both *Chlorella* (Iwamura, 1955, 1982; Wanka, 1962, 1967; Wanka and Geraedts, 1972) and *Chlamydomonas* (Sueoka, 1960; Knutsen *et al.*, 1974; Donnan and John, 1983; Knutsen and Lien, 1981; Lien and Knutsen, 1976a); several waves of DNA replication occur during the cell cycle of *S. quadricauda* (Šetlík *et al.*, 1988; Zachleder *et al.*, 1988, 2002). While DNA replication itself is light independent, the number of DNA replications is light dependent; the higher the light intensity, the more DNA is synthesized (Iwamura, 1955; Donnan and John, 1983; Šetlík *et al.*, 1988; Zachleder *et al.*, 1988). This is in line with the concept that presented earlier in this review, that light intensity sets the growth rate and thus affects the number of CPs attained and the number of DNA replication–division sequences initiated.

The ability of the cells to replicate DNA can be assessed in dark samples taken from light-grown cultures. Such 'committed DNA' shows a clear stepwise increase, even in the case of *Chlamydomonas* (Donnan and John, 1983), supporting the fact that DNA replication is indeed committed separately at each CP. Apart from nuclear DNA replication, one can also readily detect replication of chloroplast DNA. The two DNA entities are usually easily separable due to a different GC content, detected either by fractionation and differential centrifugation (Iwamura, 1966, 1970; Sueoka *et al.*, 1967; Iwamura and Kuwashima, 1969) or microscopically (Cepák and Zachleder, 1988; Zachleder *et al.*, 1990, 1995). The number of rounds of chloroplast DNA replication is also light dependent but they occur continuously throughout the cell cycle.

Interestingly, replication in the nucleus and chloroplast can be uncoupled from each other by the use of specific inhibitors of DNA replication (5-fluorodeoxyuridine; Zachleder, 1995) or chloroplast DNA replication (nalidixic acid; Zachleder *et al.*, 2004). The application of 5-fluorodeoxyuridine inhibits nuclear DNA replication while growth stays unaffected. This leads to the formation of a giant cell with a single genome (Zachleder, 1995). If the inhibitor is removed, the cell will rapidly undergo all the committed processes of

DNA replication–division: in the case of *S. quadricauda*, the cell-cycle progression will resemble that of *Chlamydomonas* (Zachleder *et al.*, 2002). In contrast, if chloroplast DNA replication is inhibited, the growth rate will be slightly slowed and all the processes in the nucleocytoplasmic compartment will run normally, with the number of CPs reflecting the slowdown in growth rate. Since the chloroplast usually contains multiple DNA molecules in structures called nucleoids, in the event of a block in chloroplast DNA replication, a growth defect is not very prominent (Zachleder *et al.*, 2004).

Thus far, this review has referred to DNA replication and nuclear division as a single unit, the DNA replication–division sequence. There are, however, some indications that this notion is an oversimplification. Under some conditions, the CP for DNA replication can be separated from the CP for nuclear division. This happens quite often in fast-growing cultures of *S. quadricauda*, where extensive overlapping, not only within one cell cycle but also between successive cell cycles, occurs. Under such conditions, the cells grow further after attaining the third CP and they may attain a critical cell size to only replicate DNA or to both replicate DNA and undergo nuclear division (Zachleder *et al.*, 2002). A similar situation also occurs in synchronously grown *Chlorella* cells, where some cells, when put into the dark, only replicate DNA while others also undergo nuclear division; nevertheless, none of those cells undergo cell division, indicating that cell division might also be regulated separately (Hase *et al.*, 1957; Morimura, 1959; Morimura *et al.*, 1961; Tamiya *et al.*, 1961; Tamiya, 1964, 1966). In contrast, such uncoupling of DNA replication and nuclear division was not observed in *Chlamydomonas reinhardtii* (Donnan and John, 1983). Uncoupling of S and M phase can be seen under certain conditions in budding and fission yeast. For budding yeast, the main growth phase is G1. However, when G1 cyclin Cln3 is overexpressed, thus overriding the requirement for growth in G1, growth also occurs during G2 and thus meets the minimal cell size requirement (Rupeš, 2002). On the contrary, for fission yeast, the main growth phase is G2. If mitotic entry is compromised by a *wee1* mutation and cells enter mitosis prematurely at a small cell size, then growth in G1 will also occur, ensuring that the cell will not fall below the minimum viable size (Mitchison *et al.*, 1997; Rupeš, 2002). All of these examples simply unmask the cryptic regulation that is present but usually hidden because it is not required. The key conclusion, therefore, is that individual components of the DNA replication–division sequence follow each other in an ordered way, where each previous one is a prerequisite for the next one. We must also keep in mind that, although completion of the previous process is merely a prerequisite for the next one, it is not guaranteed since each of the processes can have other requirements.

We have seen that the cell-cycle organization of *S. quadricauda* is quite different from those of *Chlamydomonas* and *Chlorella*. Namely, individual DNA replications and nuclear division are spread throughout the light and dark phases and are easily separated. *S. quadricauda* thus represents a suitable model to analyse the relationship between cell growth, DNA replication, and nuclear division, as well as between DNA

replication and nuclear division alone. Total RNA and protein in *S. quadricauda* are synthesized during the light period in doubling steps, reflecting the growth rate so that protein synthesis follows RNA synthesis with a certain delay. Using this system, Zachleder and Šetlík (1988) affected cultures by light deprivation or nitrogen or phosphorus starvation so that RNA and protein synthesis were uncoupled. The results clearly show that the extent of DNA replication is tightly linked with levels of RNA, while nuclear division is dependent upon protein synthesis. This not only supports the idea already discussed but also provides a hint into the mechanism that intricately intertwines the two dependent processes of the DNA replication–division sequence with two similarly dependent processes for growth.

Green algae in the limelight

The multiple fission cell cycle has probably evolved in fast-growing algae as an answer to alternating periods of day and night. Fast-growing algae can expand their volume more than twice during a single light period. However, division closely following the attainment of critical cell size would significantly hinder subsequent growth processes due to the cessation of photosynthesis. Thus, an ability to postpone a division process until the dark period would represent a major evolutionary advantage. Such a challenge was probably never faced by slow-growing algae such as *O. tauri*, which are unable to achieve more than one doubling of mass during a single light period. In the absence of evolutionary pressure, they kept the evolutionary original binary fission. Multiple fission provides a major advantage to cells since they can dedicate the entire light phase to growth and then perform the rest of the DNA replication–division sequence in the dark, in nature, at night. The first obvious benefit of this is 'not wasting' time for division when growth is possible. Secondly, UV irradiation, as a component of natural light during the day, can cause mutation(s) during DNA replication. Finally, as aforementioned, division processes severely interfere with growth. Division by multiple fission is therefore an extreme case of optimization of growth for unicellular organisms exposed to light/dark cycles of different lengths, as happens in nature.

A mathematical analysis of the consequences of binary or multiple fission on cell populations, size distribution, and growth rate suggest some interesting conclusions (Rading *et al.*, 2011). With an increasing growth rate, the size distribution of a population dividing by multiple fission does not change dramatically since the bigger mother cells just divide more times but will give rise to cells of approximately the same size. In contrast, the size distribution of populations dividing by binary fission will differ significantly with increasing growth rate since bigger mother cells will give rise to bigger daughter cells. The differences between binary and multiple fission become obvious with high growth rates. For binary division, the population growth rate will rapidly reach a plateau at the maximum level and further improving of the growth conditions will not affect population growth. In contrast, with multiple fission, population growth rate will

increase continuously, dependent on the growth conditions. This has clear implications for both the evolution of multiple fission and the ecology of its distribution. In habitats enabling higher growth rates, it would be advantageous to evolve division by multiple fission because the population growth rate of such an organism will be greater than that of organisms dividing by binary fission and the organisms dividing by multiple fission will soon dominate. This might be the reason why multiple fission arose several times during evolution. On the contrary, in habitats with only a limited maximum growth rate, division by binary fission is sufficient to cope with subtle changes in growth rates.

We have introduced green algae dividing by multiple fission and have shown their cell-cycle organization follows similar rules to those that have been postulated for binary fission. As expected, cell-cycle progression is regulated by similar mechanisms. Early works by John and Zachleder identified a CDK-like protein in *Chlamydomonas reinhardtii* (John *et al.*, 1989) and showed that its activity was related both to the attainment of the CP and to cell division (Zachleder *et al.*, 1997). Similarly, two distinct CDK-like activities were identified in *S. quadricauda*: one linked to the attainment of the CP while the other one present exclusively at the time of nuclear division (Bišová *et al.*, 2000). The complete sequencing of the *Chlamydomonas reinhardtii* genome allowed identification of cell-cycle regulators (Bišová *et al.*, 2005). In agreement with the functional analyses, the *Chlamydomonas reinhardtii* genome encodes a single CDK homologue of A-type CDK as well as a single homologue of B-type CDK. The other cell-cycle regulators include single homologues of A- and B- cyclins and four homologues of D-type cyclins, homologues of Rb/DP/E2F pathway, Wee1 kinase, and four putative Cdc25 homologues. Interestingly, all efforts to identify CDK inhibitors by informatics have failed but that could be due to the known sequence divergence of these proteins.

Taken together, it is clear that binary fission cell-cycle regulation can be studied in organisms undergoing multiple fission. There are several obvious advantages: (1) highly synchronous cells are obtained by natural means of light/dark alternation; (2) cell-cycle progression of unicellular algae can be studied in the absence of developmental cues that might mask regulation of the cell cycle; (3) the amount of material required for any analysis is not limited since synchronization can be done at high cell densities and in large volumes; and (4) cell-cycle regulators are mostly present in single copies, simplifying a reverse genetics approach. In our opinion, however, the main advantage of the green algal cell cycle lies in its flexibility. Taking the different cell-cycle patterns of *Scenedesmus*, *Chlorella*, and *Chlamydomonas*, at first sight, the quite different *Chlorella* cell cycle could be changed to that of *Chlamydomonas* by simply applying high temperature (Šetlík *et al.*, 1975) while the *Scenedesmus* cell cycle could be made to resemble that of *Chlamydomonas* by addition of a nuclear DNA synthesis inhibitor, 5-fluorodeoxyuridine (Zachleder, 1995). During the past decades, many different treatments have been tested for their effect on cell-cycle progression (Hase *et al.*, 1959; Howell *et al.*, 1975; Tamiya *et al.*, 1962; Šetlík *et al.*, 1981; Voříšek and Zachleder, 1984; Bišová

et al., 2003; Zachleder, 1994; Zachleder *et al.*, 2002, 2004; Oldenhof *et al.*, 2004a,b, 2006), varying from different light conditions and temperatures to nutrient starvation, exposure to various chemical compounds such as heavy metals, and addition of specific inhibitors of eukaryotic and prokaryotic proteosynthesis and DNA synthesis.

We have seen that algae offer opportunities to study relationships between growth and the cell cycle, as well as between different processes within the cell-cycle sequence. Temperature can be used to temporarily uncouple different cell-cycle processes similarly to the use of cell-division temperature-sensitive mutants without the adverse effect of the mutation. Often, conditions that require the use of specific inhibitors in plant and animal systems require only a slight change in the growth conditions of algae, within the physiological range, without addition of any external compounds. We strongly believe that the combination of these advantages with the immense flexibility of cell-cycle patterns (Zachleder *et al.*, 2002) and the completion of genomic sequences of more organisms will mean that green algae that divide by multiple fission will become unique models for a systems biology approach to studying the cell cycle.

Conclusions

We have discussed division by multiple fission as it is performed by selected green algae. One of our aims was to bring attention to works of different groups from as far back as the 1950s. We believe that, while being largely unknown to the present day reader, they have much to say, especially with regard to coordination of the cell cycle and growth which has become very popular recently. We have presented different sets of data in order to support our strong belief that green algae dividing by multiple fission represent a unique tool not only for the study of coordination between growth and cell-cycle progression but also to study the coordination of individual processes of the cell cycle.

What are the challenges of the field? We think that one hindrance to research on the multiple fission cell cycle is the lack of genomic sequences of more than a few species. However, this is becoming less and less a problem with next-generation sequencing becoming available. We are convinced that, when more genomic sequences are available, green algae and their cell cycles will become a prime tool for a systems biology approach to study cell-cycle regulation.

Lastly, for many decades efforts have been made to understand the regulation of multiple fission. However, we are convinced that a deep understanding of the cell cycle is impossible without mathematical modelling similar to that performed by Tyson and Novak for yeast or mammalian cell cycles (Novak and Tyson, 1995, 1997, 2004; Tyson and Novak, 2008). Such a modelling would not only provide grounds for testing but would also unravel features common to binary and multiple fission that are not obvious now. We hope that, just as the size distribution after multiple fission attracted recent attention, so will the regulation of multiple fission.

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