Circadian changes in cyclic AMP levels in synchronously dividing and stationary-phase cultures of the achlorophyllous ZC mutant of *Euglena gracilis*

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Summary

Oscillations in adenosine 3′,5′-cyclic monophosphate (cyclic AMP) level have been proposed to be part of the biochemical feedback loop(s), or ‘clock(s)’, believed to underlie circadian rhythmicity. This possibility has been examined for a cellular circadian oscillator in synchronously dividing (or nondividing) cultures of the photosynthesis-deficient ZC mutant of the alga *Euglena gracilis* Klebs (Z). We have demonstrated a bimodal, autonomously oscillating, circadian variation of cyclic AMP content in this unicell. Rhythmic changes of the cyclic AMP level, which may reflect the transition of the cell population through the different phases of the cell division cycle (CDC) in division-phased cultures, also persisted after the culture medium had become limiting and the cells had stopped dividing. We have also shown that the free-running, circadian oscillation of cyclic AMP content displayed by nondividing cells in continuous darkness could be phase-shifted by a light signal (a property inherent to most circadian systems), in a manner that could be predicted from the phase-response curve previously obtained for the cell division rhythm in the ZC mutant. These results suggest a possible role for cyclic AMP, either as an element of the coupling pathway for the control of the CDC by the circadian oscillator, or as a ‘gear’ of the clock itself.

Key words: biological clock, cyclic AMP, cell cycle, circadian rhythm, *Euglena gracilis*, rhythm, ZC mutant.

Introduction

A variety of eukaryotic organisms ranging from unicells to higher plants and animals exhibit circadian rhythms. In microorganisms, these periodicities include rhythms in cell shape, cell division, phototaxis, photosynthesis capacity, enzyme activities, and many others (reviewed by Edmunds, 1988). Adenosine 3′,5′-cyclic monophosphate (cyclic AMP), which plays a pivotal role in the regulation of a number of cellular functions, could be a possible second messenger facilitating the regulation of some of these processes by a circadian clock(s). Oscillations in cyclic AMP level have also been proposed to be part of the biochemical feedback loop itself that is believed to underlie circadian rhythmicity (Cummings, 1975). Circadian variations in cellular cyclic AMP levels indeed have been observed in *Neurospora crassa* (Hasunuma et al. 1987), *Tetrahymena pyriformis* (Dobra and Ehret, 1977), and *Acetabularia mediterranea* (Vanden Driessche et al. 1979).

One of the cellular events modulated by the circadian oscillator in the algal flagellate *Euglena gracilis* is the progression of cells through the different phases of their division cycle (Edmunds, 1964). Genetic experiments on the budding yeast *Saccharomyces cerevisiae*, as well as physiological studies in mammalian cells, have shown that transient changes in cyclic AMP level are necessary for the transit of these cell types through the different phases of the cell division cycle (CDC) (reviewed by Whitfield et al. 1987). Indeed, there is increasing evidence that both a transient rise and the ensuing fall in the level of cyclic AMP are required for the initiation of DNA synthesis. A second cyclic AMP surge is observed during *G*₂, which may be correlated with the onset of mitosis (Boynton and Whitfield, 1983). A clock-controlled variation of cyclic AMP level – that is, the periodic repetition of a cyclic AMP signal – thus may participate in the 'gating' of DNA synthesis and cell division to a certain phase of the circadian cycle.

Circadian rhythms of cell division perhaps have been most extensively studied in the unicell *E. gracilis* (Edmunds and Laval-Martin, 1984). The use of achlorophyllous (organotrophic) mutants that are entrainable by light–dark cycles (LD) circumvents the dual use of light (as a substrate for growth, and as a time cue for the circadian clock) inherent in photosynthetic organisms.
and makes possible the study of rhythms that persist under conditions of constant darkness (DD). For example, cell division in the achlorophyllous ZC mutant of *E. gracilis* Klebs (strain Z) can be synchronized, or entrained, to a 24-h period by LD: 12,12 (12 h light, 12 h dark). Transferred to DD, the cultures exhibit a persistent circadian rhythm of cell division, which is free-running with a period (T: average period of free-running rhythm in constant conditions) of approximately 25 h (Carré *et al.* 1989). Using this cellular system, we have investigated the possible role of circadian rhythmicity in cyclic AMP level in the control of the CDC by the clock. Variations in the cyclic AMP level were studied in order to establish: (1) their relation to the cell division rhythm in synchronously dividing cultures entrained by LD: 12,12; and (2) their possible independence from the CDC in nondividing cultures that have reached the stationary growth phase. Finally, the endogenous nature of the oscillation (as opposed to a variation driven by the LD cycle) was demonstrated in both dividing and stationary cultures displaying free-running circadian rhythmicity in DD.

### Materials and methods

**Organism and culture conditions**

The achlorophyllous ZC mutant of *Euglena gracilis* Klebs (strain Z) was used in all experiments. This strain, obtained from Dr R. Calvayrac (Laboratoire des Membranes Biologiques, Université Paris VII, France) was derived from the wild-type by action of 2.5×10⁻³ M-diuron, [3-(3,4)-dichlorophenyl-1,1-dimethyl urea (DCMU)] in a 33-mM-lactate medium (pH3.5), under illumination and anoxia (Calvayrac and Ledoigt, 1976; Carré *et al.* 1988).

Axenic, aerated, magnetically stirred, 4-l batch cultures were grown in environmental chambers, on a modified Cramer and Myers’ medium supplemented with vitamins B₁ and B₁₂ (Edmunds and Funch, 1969) and containing ethanol (0.1 %, v/v) as the sole carbon source. Cysteine and methionine (10⁻² M), which improve the coupling of cell division to the circadian oscillator (Edmunds *et al.* 1976), were also added to the cultures. Illumination (3000 lx) was provided by clock-programmed, cool-white fluorescent lamps. Cell number was monitored every 2 h by a miniaturized fraction collector and a Coulter Electronic Particle Counter (Edmunds, 1964).

Cultures growing in the infradian mode (average generation (doubling) time of a population of cells, g >24 h) were obtained at 16.5±(0.5)°C. Cell division could then be entrained to a 24-h period by imposition of LD: 12,12. Typically, the cells divided during the dark intervals, and the onset of mitosis occurred at the onset of darkness (12 h after light onset). The circadian rhythm of cell division persisted after transfer of the culture to DD, with a period (τ) of 25±(2) h (Carré *et al.* 1989). Cell extracts for cyclic AMP measurements were prepared every 2 h. For cells entrained by a strong Zeitgeber (LD: 12,12), extracts were prepared from two out-of-phase cultures during a 12-h time span. This system could not be used in DD because of the variability of τ, and the cyclic AMP rhythm had to be monitored over 34-h time spans. The onset of cell division (taken as the phase-reference point, ϕ₀) was considered to fall at CT 12 (onset of cell division in a dividing culture; CT, circadian time).

### Phase-shifting experiments

A culture synchronized by LD: 12,12 until it reached the stationary phase was transferred into DD. It was then divided into two daughter cultures shortly before the experiment. Approximately at CT 17, one of them was submitted to 1 h of illumination (3000 lx). Changes in cyclic AMP level were compared during the 16 h following the light pulse, then 3 days later in both the control and the experimental cultures.

### Cyclic AMP extraction

Every 2 h, approximately 10×10⁶ cells were harvested by centrifugation (10 min, 7700 × g) of the cell suspension. The pellet was resuspended in 0.7 ml of supernatant. A known volume of cell suspension was extracted in trichloroacetic acid (TCA) at 7.5 % (w/v) final concentration for 20 min at 4°C. Pelletable material was eliminated after centrifugation (10 min, 39 000 × g). The supernatant was then extracted five times with an equal volume of water-saturated diethyl ether, the ether phases being eliminated by aspiration. After the fifth extraction, the remaining ether was boiled off at 80–90°C in a water bath until bubbling stopped. The extracts were frozen in liquid nitrogen, and stored at −40°C until the day measurements were performed.

### Cyclic AMP measurements

The amounts of cyclic AMP were measured by the competitive protein-binding assay of Gilman (1970). Assays were carried out in test tubes kept in iced water, into which were successively injected: 50 µl of cell extracts (or of standard solutions of cyclic AMP), then 100 µl of an incubation mixture containing 40 µg of protein kinase inhibitor (Sigma) and 3 pmol (60 000 disintegrations min⁻¹) of ³H-labeled cyclic AMP (Amersham) in sodium acetate (500 mm, pH 4.0). The binding reaction was started by the addition of either 50 µl of a protein mixture (containing approximately 10 µg of protein kinase (Sigma) and 40 µg bovine serum albumin (BSA)) or 50 µl of water (for the measurement of nonspecific binding). The samples were incubated at 0°C for 90 min, and the reaction was stopped by dilution with 1 ml of potassium phosphate buffer (20 mm, pH 6.0). After filtration under mild suction through cellulose ester Millipore filters (HAWP025), the filters were rinsed three times, air dried, and then transferred to scintillation vials and dissolved in 1 ml ethylene glycol monomethyl ether (Sigma). A standardized volume of 5 ml of scintintive (Fisher) was added; the vials were agitated overnight and then allowed to refrigerate for a few hours before being counted for 10 min in a Packard Tri-Carb liquid scintillation spectrometer (model 3320). The cyclic AMP concentrations per 10⁶ cells were extrapolated from a standard curve, simultaneously obtained by exactly the same procedure applied to known quantities of cyclic AMP (ranging from 0.5 to 20 pmol).

As a control of the specificity of the assay in cell extracts, the hydrolysis of cyclic AMP by cyclic AMP phosphodiesterase was also assayed. Cell extracts (100 µl) were incubated (10 min at 30°C) in the presence of 0.025 unit of cyclic AMP phosphodiesterase (SIGMA) in 100 µl of 50-mm-Tris–HCl (pH7.5) containing 0.2 mg BSA. In order to take into account the destruction of cyclic AMP during the 10-min incubation period at 30°C, cyclic AMP concentrations then were compared with those of identical extracts incubated at 30°C for 10 min with 100 µl of Tris buffer (50 mm, pH 7.5) alone.

### Results

**Average cyclic AMP levels in dividing and nondividing cells**

The average cyclic AMP level was first determined for the

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ZC mutant during both the exponentially dividing phase and the stationary phase of growth. Dividing cultures contained an average of 10±1 pmol of cyclic AMP per 10⁶ cells, a value that agrees with the one reported for the wild-type strain by Keirns et al. (1973), but is greater than that found by Nicolas and Nigon (1974) and by Carell and Deardfield (1982). The possibility that substances were present that cross-react with cyclic AMP for binding to protein kinase, which are frequently found in plant tissue (Bressan et al. 1976), was ruled out by the fact that the cyclic AMP measured was completely hydrolyzed in the presence of phosphodiesterase (results not shown). The average cyclic AMP level became significantly higher (18±2 pmol per 10⁶ cells) when the culture reached stationary phase and the cells had stopped dividing.

**Oscillations of cyclic AMP level in synchronously dividing cultures**
The concentration of cyclic AMP was measured every 2 h over a 12-h time span in two, synchronously dividing cultures (entrained by LD: 12,12) that were 180° out of phase. Under such a strong Zeitgeber, a clearly bimodal, 24-h oscillation of cyclic AMP level was observed, ranging between 5 and 20 pmol per 10⁶ cells (Fig. 1A). Minimum values were found at CT 6 and 20 (that is, at the middle of the light and the dark periods). The first cyclic AMP surge to be observed was 0–2 h after the beginning of the light interval (CT 0–2), a period during which most cells were in the G₁ phase of the CDC (Edmunds, 1964). A second peak, of higher amplitude, occurred at the beginning of the dark period (CT 12–14), a time that corresponded to the onset of mitosis for the ZC mutant.

**Oscillations of cyclic AMP level in nondividing cells**
The bimodal oscillation in the cyclic AMP level persisted with an identical phasing after the culture conditions became limiting, and the cells had stopped dividing (Fig. 1B). Some characteristics of the oscillation, however, were modified. Cellular cyclic AMP content was higher than in dividing cells, and the range of the rhythm lay between 12 and 30 pmol per 10⁶ cells (values as high as 40 pmol per 10⁶ cells sometimes were found). Two cyclic AMP surges were observed, at exactly the same phases (CT 0–2 and CT 12–14) as previously observed in synchronously dividing populations. Interestingly, the maximum cyclic AMP levels observed at CT 12–14 were comparable in dividing and in nondividing cells, whereas those at CT 0–2 were twice as high in stationary-phase cultures.

**Persistence of the cyclic AMP rhythm in constant conditions**
A basic test for the endogenous nature of a diurnal rhythm is to determine whether it will persist in conditions held constant with respect to the major environmental timing cues (light, temperature). Transferred to DD, the ZC mutant of *E. gracilis* typically exhibits a persisting circadian rhythm of cell division, free-running with a τ of 25±2 h (Carré et al. 1989). The bimodal rhythm in cellular cyclic AMP also free-ran in DD with a similar period, both in dividing and in stationary cultures. The changes in cyclic AMP concentration in a

**Fig. 1.** Entrained and free-running circadian oscillations of the cyclic AMP level in synchronously dividing and in nondividing cultures of the chlorophyllous ZC mutant of *Euglena gracilis* (strain Z), grown at 16.5°C on a mineral medium supplemented with ethanol (0.1%). A. Diurnal variations of the cyclic AMP level in cultures exhibiting rhythmic cell division, entrained to a 24-h period by a LD: 12,12 cycle. B. Persistence of the bimodal 24-h oscillation of the cyclic AMP level after the cells had ceased dividing. C. Variations of the cyclic AMP level in a culture displaying a free-running rhythm of cell division in DD and just entering the stationary growth phase. For experiments A and B, cell extracts were prepared every 2 h over a 12-h time span from two cultures entrained by out-of-phase LD cycles, thus scanning the entire circadian period. Results were double-plotted (open symbols) as a function of the circadian time. In experiment C, cell extracts were prepared every 2 h over a 34-h time span. The onset of the last burst of cell division (also corresponding to the first peak on the graph) was used as a reference for CT 12, and the interval between the first and the third peak gave the estimated value (27 h) for τ. In order to facilitate the comparison with curves A and B, the results were then normalized to a 24-h period.

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Phase-shifting of the free-running circadian oscillation of the cyclic AMP level by a 1-h light signal in the achlorophyllous ZC mutant of *Euglena gracilis* (strain Z). A culture, entrained by LD: 12:12 until the stationary growth phase was attained, was placed in DD and subdivided into two flasks. About 29 h after transfer to DD (at approximately CT 17), one of the two daughter cultures was given a 1-h light pulse (3000 lx). The time course of changes in the cyclic AMP level was compared in the control (O) and the light pulse (3000 lx), given at approximately CT 17, on the circadian rhythm in cyclic AMP level: cyclic AMP concentration continued to decrease in a parallel fashion with that of the control. The next cyclic AMP surge, however, was delayed by 4 h, and after 3 days, a steady-state phase-shift (Δφp) of 5 h had been achieved.

**Discussion**

This paper demonstrates a bimodal, autonomously oscillatory, circadian variation in cyclic AMP content in the achlorophyllous ZC mutant of *Euglena gracilis* (strain Z). This cyclic AMP rhythm seems to be driven by the same clock as the cell division rhythm, although it can be divorced from the CDC in stationary cultures.

In cultures in which the cell division rhythm was synchronized by LD:12,12, the level of cyclic AMP exhibited a bimodal oscillation (Fig. 1A). Maximum cyclic AMP levels corresponded to the beginning of the light interval period (CT 0–2) and to the onset of darkness (CT 12–14). This finding is consistent with results obtained in other organisms: bimodal circadian oscillations in cyclic AMP level have been found in *Neurospora crassa* (Hasunuma et al. 1987), *Tetrahymena pyriformis* (Dobra and Ehret, 1977), and *Acetabularia mediterranea* (Vanden Driessche et al. 1979). Diurnal variations in cyclic AMP levels, adenylate cyclase and phosphodiesterase activity also have been observed in brain and heart tissue of rats maintained in LD: 12:12 (Lemmer et al. 1987; Lemmer, 1989). Thus, cellular cyclic AMP content seems to be under control of a circadian oscillator in a variety of organisms.

In our working conditions (dividing cultures maintained in LD: 12,12), the rhythmic variation in cyclic AMP level might have other possible causes. In the first place, transient cyclic AMP surges might be caused by the periodic shifts in environmental conditions (for example, from light to darkness). Although light signals do not seem to affect the cyclic AMP level in *Aplysia* eyes (Eskin et al. 1984), they induce transient variations in the cyclic AMP content of *N. crassa* (Hasunuma et al. 1987), which have been proposed to participate in the phase-resetting of the circadian oscillator. The endogenous nature of the cyclic AMP oscillation in *Euglena* cells, however, was demonstrated after the cultures were transferred to DD: the bimodal rhythm in cyclic AMP level persisted in the absence of any environmental timing cue (Fig. 1C). The rhythm was free-running with a circadian period, which was approximately that (25 h) measured for the rhythm of cell division of the ZC mutant free-running in DD (Carré et al. 1989).

The bimodal oscillation in the cyclic AMP level that was observed in synchronously dividing cultures also could have been attributed to the timing of the cell division rhythm. Bimodal variations in cyclic AMP level, indeed, have been observed during the CDC of many different cell types, including microorganisms such as diatoms, *Tetrahymena pyriformis* and *Saccharomyces cerevisiae*, and multicellular systems such as amphibian blastema cells, regenerating amphibian limb cells and specialized mammalian cells (reviewed by Boynton and Whitfield, 1983). The first peak, occurring during G1 phase, is one of the events leading to the initiation of DNA synthesis, whereas the second peak, occurring during G2, seems to be correlated with the initiation of mitosis (Boynton and Whitfield, 1983; Whitfield et al. 1987). Similar variations in cyclic AMP content have also been reported for *E. gracilis* cells in which DNA synthesis and cell cycle progression had first been blocked by vitamin B12 deprivation and then released (synchronously) by replenishing the medium with the vitamin (Carell and Dearfield, 1982). In our system, the first cyclic AMP surge (observed at the beginning of the subjective day) also occurred at a time when most cells were in the G1 phase (Edmunds, 1964) and needed a transient cyclic AMP signal to progress into the S phase.
Similarly, the second peak of the oscillation (at the beginning of the subjective night) corresponded to the onset of cell division and may be the signal required for the cells to undergo mitosis. Thus, the variations in the cyclic AMP level observed in *Euglena* cultures exhibiting a circadian rhythm of cell division could simply reflect the transit of the cells through the different phases of their CDC.

We tested this hypothesis by examining cultures of the ZC mutant that had reached the stationary phase of growth for possible variations in cyclic AMP level. The average cyclic AMP content (18±2 pmol per 10^6 cells) found in nondividing cells was much higher than that measured in dividing cells (10±1 pmol per 10^6 cells), as is typical of quiescent animal cells (Pardee et al. 1978). The cyclic AMP levels, however, were not constant: nondividing cultures, in which the circadian oscillator either was entrained by LD:12,12 (Fig. 1B) or was free-running in DD (Fig. 1C), also exhibited a bimodal rhythm, the phasing of which was identical to that observed in synchronously dividing cultures. Further, cyclic AMP content oscillated at a level much higher in stationary cultures than in dividing cultures—a level that may be above the threshold compatible with cell proliferation. Interestingly, the peak in cyclic AMP level observed at CT 12–14 was comparable in dividing and in nondividing cells, whereas cyclic AMP levels measured at CT 0–2 were much higher in stationary cultures. This observation suggests possible differences in the regulation of the enzymes responsible for cyclic AMP metabolism (adenylate cyclase and cyclic AMP phosphodiesterase) in proliferating and stationary cell populations. In sum, these results demonstrated that the circadian oscillation of the cyclic AMP level can be uncoupled from the cell division rhythm and, therefore, must be under the control of an endogenous, autonomously oscillating pacemaker.

Phase-shiftability of the oscillation by light signals is an important characteristic of circadian systems. This property was also verified for the circadian rhythm in cyclic AMP in nondividing cultures of the ZC mutant. A 1-h light signal, given at CT 17, did not immediately perturb the cyclic AMP oscillation. The next cyclic AMP surge, however, was delayed by 4 h (Fig. 2). A transient change of cyclic AMP level in response to light pulses has been proposed to participate in the transduction of the signal to the circadian oscillator controlling the conidiation rhythm in *N. crassa* (Hasunuma et al. 1987). Such a rapid perturbation of the cyclic AMP level occurring during the first minutes following the light signal cannot be ruled out in the case of the ZC mutant of *E. gracilis*, even though no significant difference was seen 30 min after the pulse in comparison to the unperturbed control culture. On the assumption that the circadian oscillator is reset immediately to a new CT following the light signal, the fact that the rhythm in cyclic AMP level was not immediately shifted to the new phase suggests that it is probably not an element, or 'gear' of the clock (as defined by Goto et al. 1985), but rather a 'hand' lying downstream from it. The steady-state phase-shift measured 3 days after the pulse was a delay of approximately 5 h, a value that could have been predicted from the phase-response curve for 1-h light signals derived previously for the circadian rhythm of cell division in the ZC mutant (Carré et al. 1989). This result strongly suggests that both the cyclic AMP oscillation and the cell division rhythm are under the control of the same circadian clock.

In conclusion, we have demonstrated in dividing as well as in nondividing cells a bimodal circadian rhythm in cyclic AMP content, which appears to be controlled by the same endogenous pacemaker as the CDC. How can cells integrate this circadian oscillation of their cyclic AMP levels into their requirement for transient increases of cyclic AMP at several control points of the CDC? We suggest that for cells growing in the infradian mode (g >24 h), the periodic changes in cyclic AMP (at levels lower, nevertheless, than the critical threshold above which the progression of the CDC is suppressed) may be signals for competent cells to initiate the sequence of events leading either to DNA synthesis or to mitosis only at specific phases of the circadian cycle. The tight coupling between the circadian oscillator and the CDC thus afforded would ensure temporal regulation of the latter and an orderly progression of the events, or 'landmarks,' of which it is composed.

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