CHAPTER

Biochemistry and Circadian Regulation of Output from the *Gonyaulax* Clock:
Are There Many Clocks or Simply Many Hands?

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Abstract

The unicellular dinoflagellate *Gonyaulax* exhibits numerous circadian-controlled processes, many of which are amenable to study and biochemical analysis. Their peaks (acrophases) fall roughly into three classes. Bioluminescence flashing and the abundance of proteins involved in bioluminescence are greatest in the middle of the night phase; cell division and a bioluminescent glow occur around the transition from night to day phase; while motility and photosynthesis peak during the day phase, along with the synthesis of proteins associated with photosynthesis. Although these several rhythms are typically in synchrony, exhibiting the same free-running periods and other properties, there are conditions under which they can dissociate and exhibit different periods. Biochemical correlates of several rhythms have been identified and, for some, circadian changes in the rates of synthesis of several proteins have been shown to be associated with the rhythms. In each of the cases studied, the circadian regulation of protein synthesis has been found to be controlled at a translational level, rather than by the abundance of the relevant mRNAs.

Introduction

The unicellular protist *Gonyaulax polyedra* (now *Lingulodinium polyedrum*) is a member of the dinoflagellates,[1] a group of organisms most closely related to a group of cellular parasites known as Apicomplexans. The dinoflagellates have a variety of life-styles, including free-living, parasitic and symbiotic; while many are autotrophic, some species are heterotrophs. The majority of the autotrophs have plastids surrounded by three membranes that contain the unusual carotenoid peridinin, whose presence confers the characteristic reddish color to the algae that gave rise to their original botanical classification as the Pyrrophyta (Greek *pyrrhos*, flame-colored).

The dinoflagellates, like their apicomplexan cousins, have a series of flattened sacs termed alveoli around the cell just underneath the plasma membrane. In the armored dinoflagellates, like *Gonyaulax* (Fig. 1), the alveoli are filled with cellulosic plates, whose patterns are used for taxonomic purposes. Their outer armor has horizontal and vertical grooves, in which lie their two flagella. This orientation gives rise to a characteristic spiraling swimming movement, and their classification as dinoflagellates (Greek *dinos*, whirling). The dinoflagellates are perhaps best known for their unusual nuclear features such as permanently condensed chromosomes, absence of histones, and the fact that the nuclear membrane remains intact through mitosis.

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The Circadian Clock in Eukaryotic Microbes, edited by Fred Kippert.

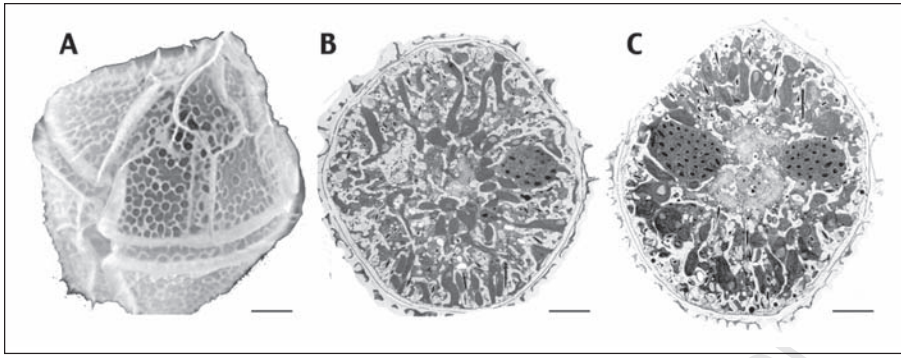

Figure 1. The unicellular protist *Gonyaulax*. A) Scanning electron micrograph of a *Gonyaulax* cell. (Photo courtesy of N. Nassoury.) B) Transmission electron micrograph (TEM) of day phase *Gonyaulax*. C) TEM of night phase *Gonyaulax*. Scale bars one micron. TEMs reprinted from: Fritz L, Morse D, Hastings JW. The circadian bioluminescence rhythm of *Gonyaulax* is related to daily variations in the number of light-emitting organelles. J Cell Sci 1990; 95:321-8.

The dinoflagellate chloroplast genome is similarly unusual, with many species encoding their plastid proteins on a series of single gene minicircles.[2]

Dinoflagellates have contributed considerably to an understanding of how the circadian clock controls circadian rhythms as well as to the basic principles of phase resetting common to circadian oscillators. In this chapter, we discuss the biology and biochemistry of several circadian rhythms in these algae and the molecular mechanisms whereby they are controlled by the clock. In a second chapter, we discuss the effects of various types of stimuli on the clock.

Desynchronization Suggests More Than One Oscillator

It has long been appreciated that in a given organism many different processes may exhibit circadian rhythmicity. Some authors have tabulated such rhythms,[3] but such lists are seemingly endless, documented to number in the hundreds for the human, so the possibility that there would be different and separate mechanisms for regulating each seems remote. Moreover, the concept of a single "master oscillator" controlling the timing of the many different functions in an organism has always seemed attractive. In this perspective, the clock mechanism is distinct from the measured rhythms, or "hands" of the clock, and ultimately the hands could be removed without affecting operation of the underlying oscillator.

Indeed, early studies with *Gonyaulax* paved the way to this paradigm. For example, the single clock model is supported by the observation that total light emitted after stimulation to exhaustion is rhythmic and has the same period and unusual temperature coefficient (less than 1.0) as the rhythm of spontaneous luminescence.[4] In addition, the biochemical components of the luminescence system are evidently not themselves part of a putative feedback loop generating the rhythm because the phase of the rhythm remained the same after the luminescent system was depleted by exhaustive stimulation.[5] In another test, after photosynthetic electron transport was specifically inhibited for some hours by DCMU, the phase of the photosynthesis rhythm, which resumed after removal of the drug, was the same as in untreated cells.[6]

In a different approach, the relative phase angles of rhythms for four different processes were evaluated after 17 days in constant conditions.[7] No differences were detected, indicating that under constant conditions the periods were all the same. Two other canonical features of circadian rhythms, namely the PRC and the temperature coefficient of the period were also the same for different rhythms.

While these results were confirmed in other experiments, challenges to the paradigm emerged in the early 1990s. First, different free running periods were reported for the glow and flashing

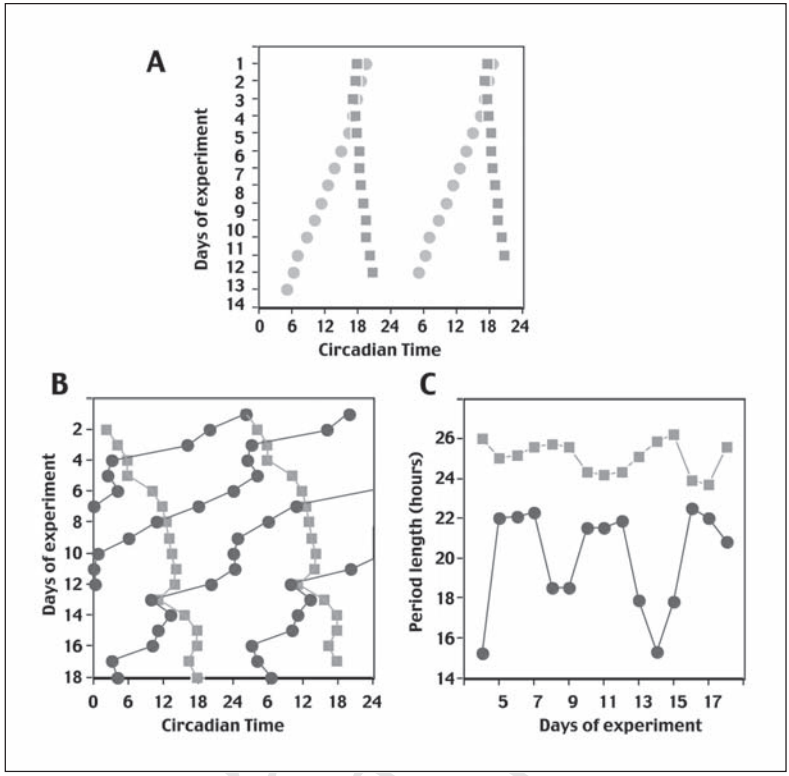

Figure 2. Evidence for several clocks. A) The acrophases for flashing (squares) and glow (circles) rhythms in a culture maintained in constant dim light for two weeks. The free-running period of the glow rhythm is shorter than that of the flashing rhythm in this experiment. Data redrawn from.[8] B) Acrophases of bioluminescent flashing (squares) and cell aggregation (circles) rhythms in a culture kept in constant dim red light. The period of the flashing rhythm is somewhat greater than 24 hours and that for the aggregation rhythm much shorter than 24 hours. C) Period changes indicative of relative coordination during the course of the experiment shown in B, analyzed by calculating the spectral analysis for 4-day windows across the time series of the unsmoothed data. Data for B and C redrawn from reference 9.

rhythms of bioluminescence,[8] and for the flashing and aggregation rhythms[9] (Fig. 2A,B). However, the conditions responsible for causing the dissociation of the different rhythms were not well established. One possibility was that the color of the light to which the culture is exposed was responsible; another relates to the method of measurement, which requires a minute of darkness every half hour. Such dark pulses do have a significant effect on the period with a white background light,[10] and might differ for the two rhythms, which have been found to differ in sensitivity to dark pulses given in a constant dim red background light.[11]

A noteworthy feature of the experiment shown in Figure 2B is that even when the two putative oscillators are running independently they still appear to interact in a kind of transient coupling, as indicated by the temporary compromises in the periods of both rhythms (Fig. 2C). Rhythms of sleep/wake and body temperature in humans are also known to similarly dissociate and interact. This has been referred to as relative coordination,[12] and different oscillators (X and Y) have been postulated to control the two rhythms.[13,14]

A second type of challenge has come from studies using brief applications of nitrate, an important nutrient, to generate a phase response curve. As discussed below, *Gonyaulax* has a

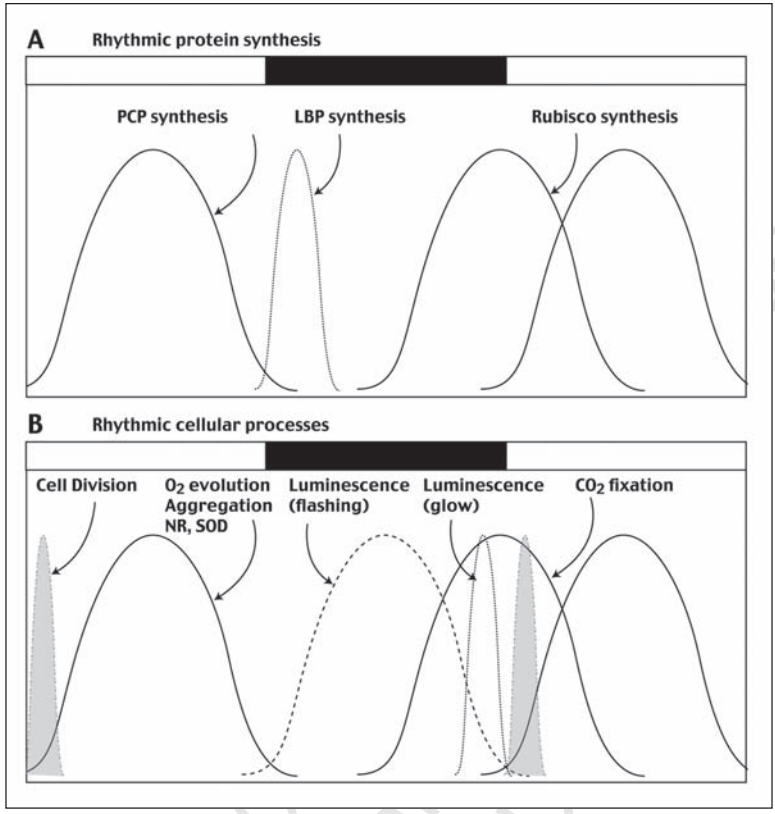

Figure 3. Three different phases of physiological rhythms and circadian protein synthesis. A) In vivo labeling of proteins and 2D PAGE analysis shows there are three principal phases of protein synthesis: those involved in bioluminescence (such as LBP), those involved in photosynthetic CO_2 fixation (such as ribulose 1,5-biphosphate carboxylase) and those involved in photosynthetic O_2 evolution (such as peridinin-chlorophyll-a-binding protein). Data redrawn from reference 16. B) The three main acrophases of physiological rhythms are those peaking near the dark to light transition (CO_2 fixation, luminescent glow and cell division), those peaking during mid-day (photosynthetic O_2 evolution) and those peaking during mid-night (bioluminescence).

nightly maximum in its nitrate reduction rhythm. The addition of nitrate to nitrate-starved cells causes phase delays, and these are of different magnitudes for the two rhythms, and the two exhibit markedly different free running periods thereafter.[15] The fact that the action of nitrate can be blocked by MSX, an inhibitor of glutamine synthase, which is involved in nitrate metabolism suggests that the nitrate metabolism rhythm may indeed impact on the clock mechanism driving the rhythm.

Some evidence suggests that even more than two oscillators might be present. When the global patterns of circadian controlled protein synthesis in *Gonyaulax* are examined, it appears that they can be placed into three different groups (Fig. 3A). These correspond to the start, middle, and end of night phase,[16] suggesting that three oscillators might be involved in the different rhythms, which also appear in three main groups (Fig. 3B). Some of the proteins associated with each of these three groups have now been identified and shown to be associated with specific rhythms. For example, synthesis of the luciferin binding protein falls into the early night group, at a time appropriate for initiation of the bioluminescent flashing rhythm.[17]

The synthesis of the enzyme Rubisco, which catalyses a key step in carbon fixation, begins at mid-night phase, a time when in vivo carbon fixation rates are observed to rise,[18] and the onset of PCP synthesis occurs at later night phase, a time when oxygen evolution rates begin to increase. This correlation between the temporal regulation of protein synthesis and the physiological rhythms in which the protein participates suggests that three different oscillators might regulate protein synthesis in *Gonyaulax*.

While none have yet been identified in *Gonyaulax*, clock genes and their protein components have now been identified and characterized in several other systems.[19,20] The identity of the components vary, but all systems include a gene whose product feeds back to repress its own transcription. How the clock mechanism regulates its hands (i.e., the rhythms) with their different phases, is a key open question in circadian biology. In many cases this is presumed to involve clock controlled genes (CCGs), so perhaps clock gene products functioning as transcription factors might regulate CCGs directly. Interestingly, it has recently been reported that two different clock controlled genes in *Arabidopsis* are transcribed with different circadian periods in constant conditions.[21] *Arabidopsis*, a model plant system where the molecular mechanisms of the clock are slowly falling into place, may thus help to understand how multiple clocks might function in regulating the different *Gonyaulax* rhythms.

The Several Rhythms of Gonyaulax

Among the many different rhythmic processes reported in *Gonyaulax* are three aspects of in vivo bioluminescence (total stimulatable emission, spontaneous flashing frequency and spontaneous glow),[5,22] photosynthesis,[6] cell aggregation (motility),[23] and the timing of cell division[24,25] (Fig. 3B). In addition, several rhythms (e.g., bioluminescence, photosynthesis and cell division) have some defined biochemical components, changes in which might constitute the point of regulation. A number of other biochemical components have been shown to exhibit rhythms, such as nitrate reductase,[26] superoxide dismutase,[27] and melatonin,[28] without clear knowledge as to what physiological rhythms, if any, might be associated with the particular changes.

Measurements have been automated for different rhythms, including bioluminescence, aggregation and photosynthesis,[10,29] and some (photosynthesis and bioluminescence) have been shown to occur in single cells.[30,31] Single cells are models of minimal complexity where some of the complications found in multicellular organisms are absent,[32-34] and this brings into sharp focus the cellular basis of circadian behavior.

Bioluminescence

Bioluminescence is a beautiful and dazzling phenomenon. At least 30 different systems are known, widely distributed among different groups of organisms ranging phylogenetically from bacteria to fish, with most having no similarity in reaction components.[35] However, all involve the oxidation of a substrate (termed a luciferin) by molecular oxygen catalyzed by a luciferase enzyme specific to each system.

In the *Gonyaulax* system, the luciferin is a linear tetrapyrrole structurally related to chlorophyll.[36] It is highly susceptible to nonluminescent autoxidation, and is sequestered in the cell by a luciferin binding protein (LBP). The binding of luciferin is strong to LBP at a basic pH and to luciferase (LCF) at acidic pH.[37] This and other considerations suggest that flashing is regulated by changes in pH.

The cellular organization of the two proteins supports this idea, as they are found together in discrete light emitting organelles termed scintillons,[38,39] which are present in the cell at night but absent during the day[40] (Fig. 4A,B). Isolated and purified scintillons contain only LCF, luciferin and LBP, and can be stimulated to emit a flash by a drop in pH.[41] In the cell, scintillons are cytoplasmic protrusions into the acidic vacuole, almost completely surrounded by the vacuolar membrane,[42] which conducts an action potential that triggers bioluminescence.[43,44] The mechanism is proposed to involve an opening of proton channels thereby decreasing the pH of the scintillon.

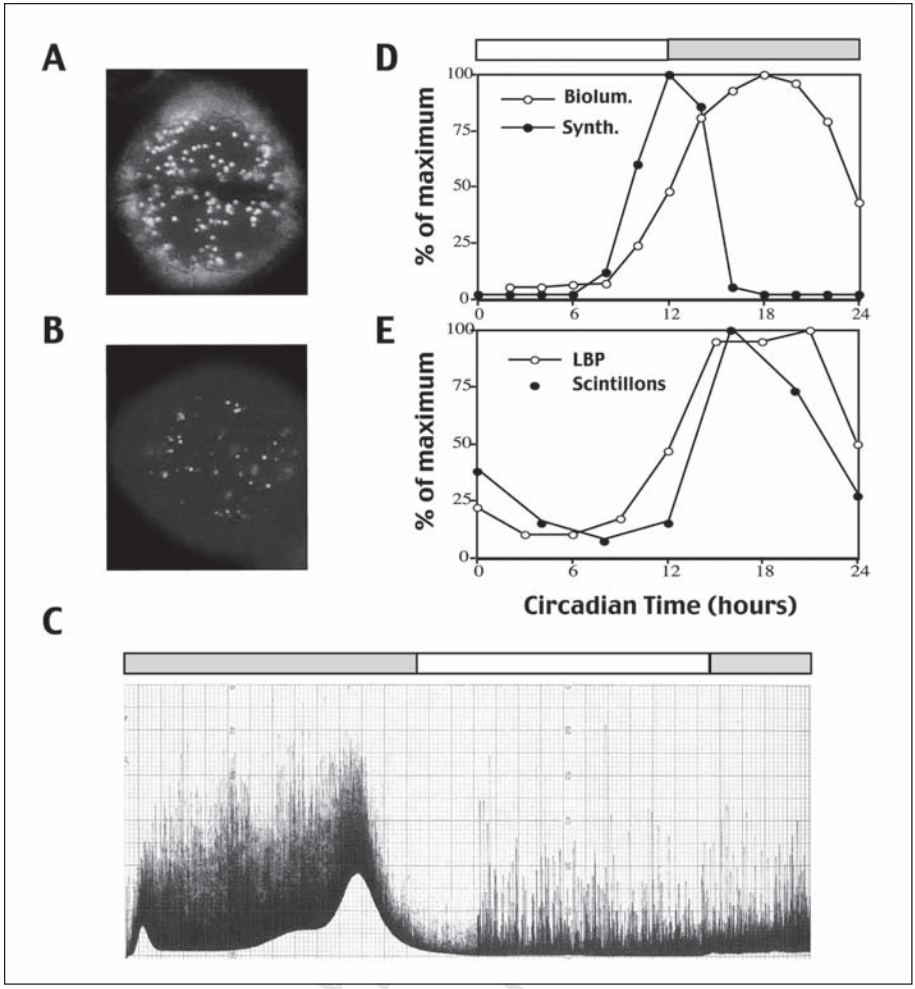

Figure 4. Bioluminescence. A,B) Fluorescence micrographs of single night and day phase cells, respectively, showing that the scintillons (the bioluminescence organelles) are much more abundant at night. (Photos courtesy of L. Fritz.) C) Chart recording of spontaneous luminescence from a culture in DD. The vertical lines result from brief and bright flashes, while the glow is seen as changes in baseline light emission. Night and day phases based on prior light dark cycle indicated above by dark (night) and light bars. D) Circadian changes in bioluminescence capacity and rate of synthesis of LBP and, E) cellular levels of LBP and number of scintillons. Cultures maintained in LL, with night and day phases indicated above D. Data redrawn from references 40, 51.

Bioluminescence, being easily measured, is the most extensively studied of the *Gonyaulax* rhythms.[32,45] Light emission occurs in two different modes (Fig. 4C). Flashing, the display that is visible to the naked eye consists of brief (0.1 sec) intense (peak intensity, 10^9 quanta/sec/cell) bursts of light. These flashes appear in strip chart recording of bioluminescence as vertical lines (amplitudes are truncated by the slowness of the pen). Both spontaneous flashing and that in response to stimulation are greatest in the middle of the night phase, and the latter is believed to decrease predation by startling or diverting predators.[46,47] Each cell typically produces spontaneously only a single flash (~10^8quanta) per night.[45]

The second mode is called glow; it consists of a low intensity emission that gradually rises to a peak (~10^4 quanta sec^{-1} $cell^{-1}$) and then declines to zero over a period of several hours at the end of the night phase. The glow cannot be detected by the eye (it is masked by flashes) but is visible as a steady change in the baseline light emission on the chart recorder trace. It is believed to result from the daily breakdown of scintillons and not to have ecological significance. The total amount of light produced each cycle from the glow is about 10^7 quanta $cell^{-1}$.

Several different kinds of measurements of luminescence have been used to follow rhythmicity. It was first measured by total stimulatable light.[48] Many aliquots were dispensed and measured individually at intervals (1 to 4 hours) thereafter. Samples were stimulated mechanically for a minute by stirring or bubbling air to exhaustion, by injecting acetic acid, and the total light emitted determined, after which the sample was discarded. Total light is greatest in the middle of the night phase, and corresponds to about 10^8 quanta per cell.[49] This may reflect the amount of luciferin bound to LBP, which has been estimated to be 2 x 10^8 molecules in night phase cells.[37] The amount of luciferin,[50] the amount of LBP[51] and the number of scintillons[40] are all rhythmic.

In measuring stimulatable light emission it was noted that light emission was exhausted more rapidly from night-phase than from day-phase cells. This suggests that the cells are more sensitive to stimulation during the night, which thus constitutes a rhythm in its own right that must also be clock controlled with a separate signal transduction pathway.

Both flashes and glow occur spontaneously. The origin of spontaneous flashing is not known; it does not increase with increased surface area in the vial, where cells might collide, nor does it increase with culture density, which might increase the number of cell-to-cell collisions.[45] Also, it is not less in cultures maintained on a vibration-free table. The flashing frequency (number of flashes per unit time) is greatest in the middle of the night phase. Although the traces of flashes from a night-phase culture appear very numerous, by calculation each cell emits only one flash over the course of the night. Since the flashes are distributed over a wide envelope of time, this means that the circadian time at which individual flashes occur in the different cells is very different.[31]

For the glow, the timing appears to be quite precise, to within a few minutes per day.[52] Its acrophase, however, is very different from that of flashing, falling at the very end of the night phase. Since this is very close to the time when the maximum number of cell divisions occur (see below), it was thought at first that the glow might represent a "leak" in the biochemical regulation at that time. However, later experiments definitively disproved this possibility. Using cultures of newly divided (and thus synchronized, but slow growing) cells, there were no divisions 24 hrs later but the glow peak at that time was undiminished.[53] This clearly showed that the two phenomena are unlinked. It is postulated that the glow derives from a breakdown of the scintillons, whereby the biochemical components are released into the acidic vacuole.

Flashing and total light both involve LCF and their rhythms are phased similarly, but their signal transduction pathways from the clock may be very different. The rhythm of total stimulatable light emitted is much more directly linked to the bioluminescence biochemistry, as it correlates with the number of scintillons (Fig. 4D,E), which varies ten-fold each cycle.[40] Since scintillons contain LBP and LCF, it is not surprising that the cellular levels of each protein also change ten-fold between day and night.[51,54,55]

This daily rhythm of LBP has been extensively studied, and LBP synthesis in vivo increases over 50 fold for several hours at the start of the night phase (Fig. 4D), resulting in rising LBP levels (Fig. 4E).[51] LCF abundance also exhibits a robust rhythm, and the control of both is clearly at a translational level, as the amount of mRNA is constant over a 24h period.[51,56] However, their lifetimes (12 hours or longer),[57] and compartmental or structural changes over time could alter the ability of the message to be translated. The in vitro synthesis rates in a heterologous (rabbit reticulocyte) system are the same with *lbp* mRNAs isolated at different time points, indicating that a cytoplasmic or repressor is involved in recalculating the translation of *lbp* mRNA.

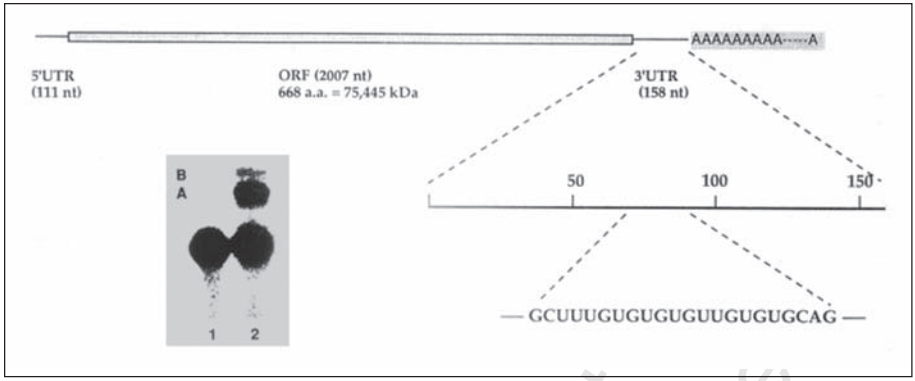

Figure 5. *Gonyaulax* proteins bind the 3'UTR of LBP RNA. A schematic view of LBP mRNA shows a 22-nucleotide region rich in UG in the 3'UTR. The electrophoretic mobility of this UG-rich region (inset, lane 1) can be reduced by binding to proteins extracted from *Gonyaulax* (inset, lane 2). Data reprinted from: Mittag M, Lee D-H, Hastings JW. Circadian expression of the luciferin-binding protein correlates with the binding of a protein to the 3' untranslated region of its mRNA. Proc Natl Acad Sci USA 1994; 91:5257-5261.

To investigate this, the complete *lbp* cDNA, including the 5′ and 3′ UTRs, was cloned and sequenced.[58] Mobility shift assays were used to look for protein factor(s) binding to the untranslated regions (UTRs) of the mRNA. No proteins were found to bind the 5' UTR, but a protein was detected in *Gonyaulax* extracts that binds specifically to a 22 nucleotide region of the *lbp*3′ UTR containing seven U(U)G repeats (Fig. 5). The binding activity of the ~45 kDa dimeric protein cycles on a daily basis and is greatest during the day phase,[59] suggesting that it may function as a clock-controlled repressor preventing the translation of *lbp* mRNA during the day. This might be expected to act at the level of initiation, and thus involve the 5' region of the message. Indeed, differential translational initiation of *lbp* mRNA appears to occur due to an open reading frame in the 5' UTR.[60] Although the distinctive UG repeat has not been found in genes coding for any other *Gonyaulax* clock-controlled proteins sequenced to date, including LCF, a protein from *Chlamydomonas* that binds to this sequence has been isolated and characterized,[61,62] suggesting that it has a more universal occurrence.

If the bioluminescent glow derives from the degradation of scintillons, its rhythm must also involve signals from the clock mechanism different from that for flashing. Scintillon breakdown has not yet been visualized at the ultrastructural level, although the formation of scintillons can be observed as small protein aggregates that originate in the Golgi area as a dense material and migrate towards the cell periphery to form an association with the vacuolar membrane.[63]

Rhythms in Photosynthesis and Chloroplast Ultrastructure

Photosynthesis is arguably the most important biological process on earth. The liberation of oxygen, which is highly reactive, and the accumulation of it and organic compounds resulting from reduction of carbon dioxide, were both key in evolution and the development of aerobic metabolism. Its optimization in all respects, including temporal, can be viewed as a feature selected for in evolution.

The chloroplasts in *Gonyaulax* experience daily changes in shape, position in the cell, and in biochemistry. Like most dinoflagellate plastids, they are surrounded by three membranes instead of the usual two or four,[64] and containing the unusual carotenoid peridinin.[65,66] They are more or less oriented radially and are up to 50% longer in day than in night phase cells.[67] Night phase plastids are more central and form pyrenoids, characterized by the presence of widely separated thylakoid stacks.[67-69]

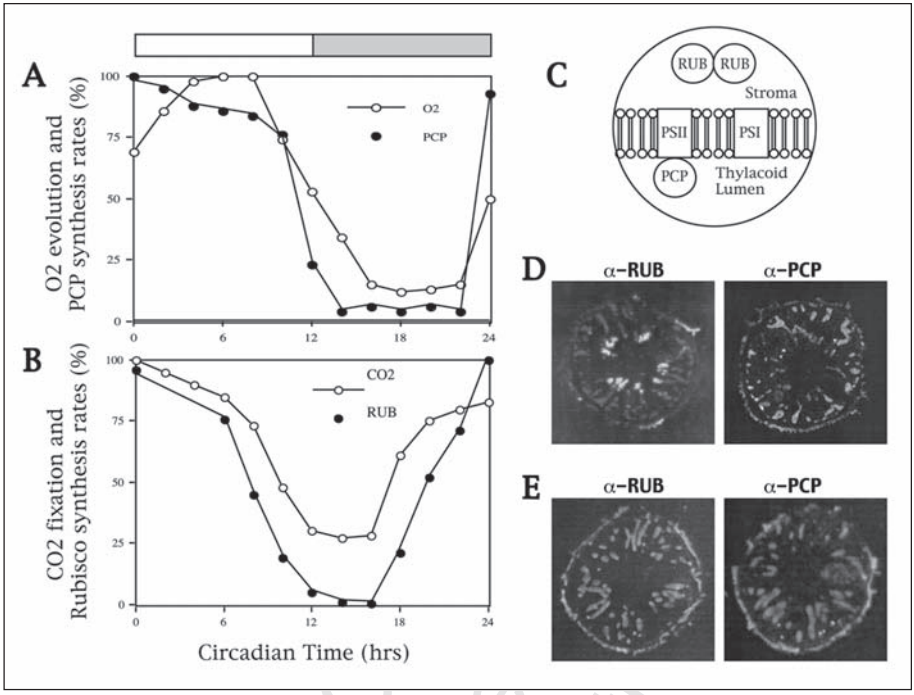

Figure 6. Photosynthesis. A) Circadian changes in rates of oxygen evolution and in vivo synthesis rates for the light harvesting protein PCP in cultures maintained in constant light and temperature, with night and day phases indicated as in Figure 3. B) Circadian changes in rates of carbon fixation and the synthesis of Rubisco, showing that the acrophase of the rhythm occurs about six hours earlier than for oxygen evolution. C) A schema of a thylakoid membrane inside the chloroplast, showing Rubisco located in the stroma and PCP inside the thylakoid lumen. D) An immunofluorescence labeling study of cells that fix carbon well stained with either anti-Rubisco (left) or anti-PCP (right). The bulk of the PCP is near the cell periphery while the bulk of the Rubisco is nearer the cell center. E) An immunofluorescence labeling study of cells that fix carbon poorly stained with either anti-Rubisco (left) or anti-PCP (right). Both proteins are distributed evenly throughout the plastids. Data redrawn from references 16, 18. (Photos in D,E, courtesy L. Fritz.)

Both oxygen evolution[30] and carbon fixation[6] are rhythmic in *Gonyaulax* (Fig. 6A,B). It is clear that the release of O_2 following removal of electrons from water requires absorption of light energy by photosystem II (PSII). The carotenoid peridinin, used to capture solar energy in the blue-green range of the spectrum, is found mostly bound to a unique soluble light-harvesting peridinin-chlorophyll *a*-protein (PCP) which has no similarity to other light harvesting proteins.[70-72] The soluble PCP is found inside the thylakoids of chloroplasts (Fig. 6C), where it feeds energy primarily into PSII.[73] PCP was originally thought to be involved in the O_2 evolution rhythm because alterations of electron flux through PSII correlate with the rhythm, but PCP levels do not change appreciably over the daily cycle[74] suggesting the rhythm be caused by another factor, such as changes in the molecular organization of PSII complexes.[75]

It is interesting, in light of the above, that the synthesis of PCP in vivo is circadian regulated, with its acrophase the same as that of the rhythm of oxygen evolution (Fig. 6A).[16,74] This control of protein synthesis rate also occurs at the translational level, as PCP mRNA levels do not vary over the cycle.[74] It is possible that a positive cis-acting translation factor functions in mediating clock control of PCP synthesis, as no protein can be produced from PCP mRNA in heterologous systems in vitro. Translational control is a recurring theme in *Gonyaulax*.[76]

Increased O_2 evolution in day phase cells is accompanied by an increase in reactive oxygen species such as superoxide anion $O_2^{\cdot-}$ derived from the single electron reduction of oxygen by PSII. Superoxide dismutase (SOD) protects against $O_2^{\cdot-}$ so it is thus not surprising that the amount and activity of a plastid SOD isoform changes over the circadian cycle by almost four-fold in *Gonyaulax*, also regulated translationally.[77]

Progress has been made in understanding the mechanism of circadian CO_2 fixation. The rate-limiting step is catalyzed by the enzyme ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) in all organisms,[78] so it is a likely candidate for clock control. Rubisco can bind both O_2 and CO_2 with O_2 acting as a competitive inhibitor of CO_2, and resulting in photorespiration, whereby the algae oxidize sugars instead of fixing carbon. This phenomenon is prominent in dinoflagellates containing type II Rubisco, which is much more oxygen sensitive than type I,[79-81] and prior to its discovery in dinoflagellates, had been found only in anaerobic bacteria. Cyanobacteria engineered to express only a form II Rubisco grow poorly,[82] and it is still not clear why this relatively inefficient form of the enzyme has been evolutionarily conserved by dinoflagellates.

The circadian control over CO_2 fixation is not due to changes in the amount of the Rubisco enzyme.[18,83] However, there are changes in the sub-organellar distribution of Rubisco correlated with the rhythm. When CO_2 fixation rates are low, Rubisco is distributed uniformly over the chloroplast stroma; when high, it is found only in pyrenoids (Fig. 6D,E).[18] The increased spacing between thylakoid membranes also reduces the relative amount of PCP in the pyrenoid making it a region specialized for CO_2 fixation not O_2 evolution. A lower rate of oxygen evolution in the pyrenoid should allow CO_2 to compete more efficiently for the active site of Rubisco.

It is not known how Rubisco moves from the peripheral regions of the plastid to the regions of the pyrenoid, or what maintains this sequestration of the enzyme. However, it is interesting that the circadian rhythm of bioluminescence in the dinoflagellate *Pyrocystis lunula* is associated with the movement of the luminous organelles from one cellular location to another.[84] This movement appears to require the actin cytoskeleton, as does movement of the chloroplasts.[85]

It is striking that CO_2 fixation and O_2 evolution rhythms have different acrophases in cells kept in constant light, with maximum carbon fixation rates preceding maximum oxygen evolution by almost 6 hours (Fig. 6A,B). This suggests that in the normal light-dark cycles, carbon fixation may begin in the dark before oxygen can be generated at all. It also suggests that the two rhythms may be regulated by different clock mechanisms.[18] It is thus interesting to note that the changes in Rubisco synthesis rates in vivo track the CO_2 fixation rhythm, while PCP synthesis parallels the O_2 evolution rhythm (Fig. 6A,B). As was found for PCP, Rubisco synthesis is also under translational control in *Gonyaulax*.[86]

Swimming Behavior and Nitrate Reduction

Motility is virtually always rhythmic in motile organisms having circadian clocks. In *Gonyaulax* this is exhibited in its swimming behavior and is complex. As mentioned above, the period of this rhythm may differ under certain conditions by several hours from that of the bioluminescence flashing rhythm,[9] implicating a different cellular clock as its driving mechanism.

In culture flasks or dishes, the cells form patterns at and below the cell surface during the day phase (Fig. 7A) and then sink down to form a lawn at the bottom of the flasks at night.[87] The patterns formed during the day depend on several factors, including the shape of the container and cell density as well as the direction and intensity of the light source.[23]

This rhythm is related to diel vertical migration (DVM) in the water column, where cells rise toward the surface near the end of the night and sink down 10 meters or more several hours before dusk (Fig. 7B).[88,89] DVM may be physiologically important in the ocean since nitrate is generally depleted near the surface, where temperature and light intensity are higher.[90,91] DVM thus allows for nitrate uptake during the night and photosynthesis during the day.

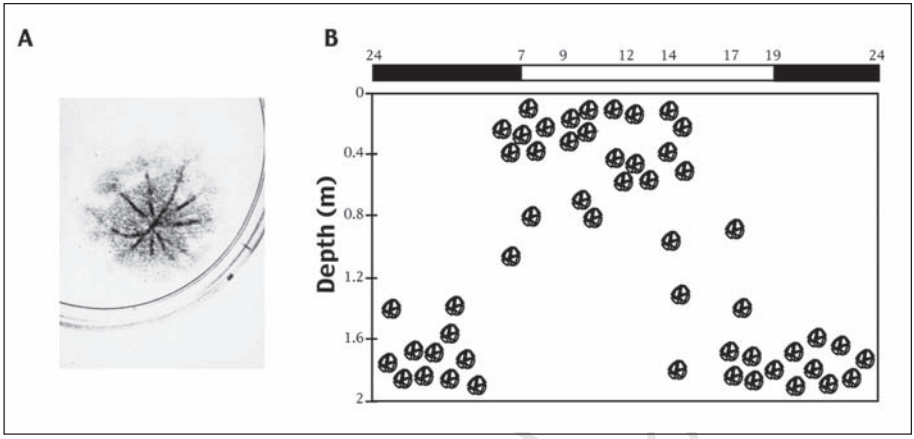

Figure 7. Cell motility. A) Cells growing in a Petri plate photographed during the day phase showing a characteristic ribbon pattern of swimming cells. (Photo courtesy T. Roenneberg.) B) The distribution of *Gonyaulax* cells over the day (LD 12:12; dark bars night) in a 2 meter-deep laboratory vessel (vertical scale). In the ocean, these vertical movements may take place over a 10 m range. Data is redrawn from reference 90.

In the laboratory, regulation of the DVM has a circadian component but is also dependent on both phototaxis and gravitaxis. During subjective day, *Gonyaulax* exhibits positive phototaxis to medium intensity light and negative phototaxis to high intensity,[23,88] while a uniformly negative phototaxis occurs during night phase. This presumably allows the algae in nature to find a preferred level of daytime illumination by regulating their position in the water column.[90] However, *Gonyaulax* also have a negative gravitaxis during the day (i.e., they move toward the surface) that is absent during the night. Changes in the buoyant density of cells could also be involved in the movement of the cells over such large distances.

Both experimental and theoretical studies suggest that nitrate also plays a role in regulating the DVM.[90,92,93] In nitrate-depleted media, cells descend earlier during the day than do nitrogen sufficient cells,[90] a result that may be due to its action in shortening of the circadian period.[94] In fact, application of nitrate to nitrate-starved cells at the time of the night-to-day transition provokes a phase delay in their ascent.[15] These observations suggest that algae encountering a high nitrate concentration late at night would delay their vertical rise in the water column. This delay could allow the algae to balance their need for reduced nitrogen with their need for photosynthate. Experimentally, cells starved for nitrate also tend to descend faster in the water column, thereby extending the duration of their exposure to higher nitrate levels.

NR activity in *Gonyaulax* is circadian, with peaks of both activity and protein at midday.[26,95] Furthermore, immunological studies show that the *Gonyaulax* enzyme is localized in the chloroplasts[95] where photosynthesis takes place. However, as nitrate concentrations are often low at or near the surface, *Gonyaulax* must also be able to use stored photosynthate as an energy source. In support of this, several studies suggest that nitrate assimilation can occur in darkness.[90,96,97] *Gonyaulax* thus has a specialized circadian biochemistry that favors nitrate metabolism during the day phase, yet a circadian behavior that allows the cells to encounter nitrate at higher concentrations during the night.

Cell Division

G. polyedra is reported to have roughly 200 pg of DNA per nucleus,[98] which is 40 times that of the human nucleus. The nuclear envelope does not break down during mitosis, and the spindle traverses the nucleus in a membrane-bound conduit.[99,100] The attachment between

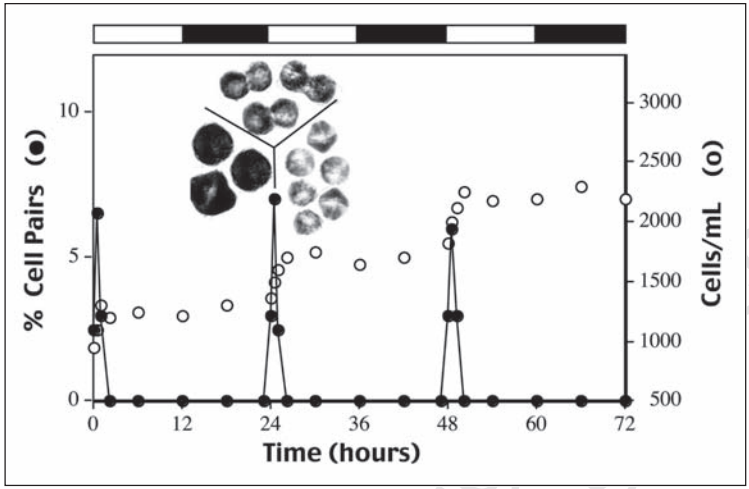

Figure 8. Cell division. The number of cells undergoing division, as measured by paired cells (left ordinate, solid circles), exhibits a rhythm gated by the circadian clock to occur at roughly an hour after subjective dawn in *Gonyaulax*. The number of cells in the population (right ordinate, open circles) increased only by about 30% at each step, as individual cells take up to three days to double their mass. Data redrawn and extrapolated from reference 24. An inset at the top of the graph shows at the same magnification the large premitotic cells (lower left), the paired cells characteristic of cell division (top), and the smaller post-mitotic daughter cells (lower right).

chromosomes and the spindle is thus facilitated by or traverses the nuclear envelope. Nonetheless, *Gonyaulax* appears to have a typical eukaryotic cell cycle with G1, S, G2 and M phases.[25,101]

Cell division in *Gonyaulax* exhibits a circadian rhythm with an acrophase about an hour after light onset in a 12:12 light-dark cycle. Mother and daughter cells remain attached for about an hour after mitosis (Fig. 8 inset, top), allowing a rhythm to be measured in the number of "pairs" (Fig. 8, left ordinate). As a consequence of the rhythm, the cell number exhibits a staircase-like pattern with time (Fig. 8, right ordinate). This rhythm is entrainable and can be phase shifted by light; it persists in constant light with a temperature-overcompensated period similar to other *Gonyaulax* rhythms.[24] In the one study reported it failed to dissociate from the other rhythms.[7]

Since the doubling time is 2 to 3 days, cell division does not occur for every cell every dawn. Mitosis is thus said to be "gated" by the circadian clock. It is as if the clock opens a window of opportunity for mitosis at dawn, during which time those cells that are of a sufficient size and DNA content can divide. It is likely that clock control is exerted at the G1/S transition rather than at the G2/M transition.[25,101] In *Gonyaulax*, a roughly 4 hour S phase starts roughly 12 hours before M phase,[25] suggesting that the length of G2 will be constant. This has been confirmed in *Amphidinium*, where adverse growth conditions lengthen only the amount of time spent in G1.[102]

There may be a strong evolutionary force for phasing DNA replication to occur during the dark, where damage due to ultraviolet radiation may be avoided. This hypothesis, termed "escape from light",[103] receives support from the fact that DNA replication and cell division take place at night in many microorganisms with a near 24-h cell division cycle.[104] It has also been shown that in *Chlamydomonas*, which is known to have a clock-controlled cell division cycle, the light sensitivity is not only rhythmic but also in phase with the times in which S/G2 is expected to occur.[105] However, in some dinoflagellates, the acrophase of the cell division rhythm differs;[106] for *Prorocentrum micans* it is in the middle of the day phase, so the explanation of light avoidance is not applicable in all cases.

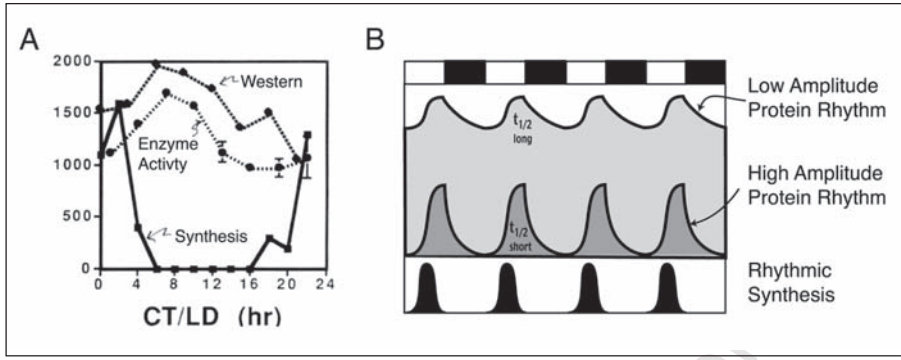

Figure 9. Synthesis and abundance of GAPDH. A) The circadian rhythm in GAPDH synthesis is high amplitude, being virtually undetectable during midday. However, the amplitude of either the protein amount or the enzyme activity is much reduced due to the relatively long half-life of the protein in the cell. Data reprinted from: Fagan T, Morse D, Hastings JW. Circadian synthesis of a nuclear encoded chloroplast Glyceraldehyde-3-phosphate dehydrogenase in the dinoflagellate *Gonyaulax polyedra* is translationally controlled. Biochemistry 1999; 38:7689-7695. B) Rhythmically synthesized proteins with low degradation rates ($t_{1/2}$ long) have smaller amplitude rhythms than do proteins with fast degradation rates ($t_{1/2}$ short) when total protein is measured.

Circadian Regulation of Transcription and Translation

The molecular nature of the putative core oscillator may be elucidated by knowledge of how it controls an "output" system, which may involve the regulation of gene expression at transcriptional, translational or post-translational levels.[107-110] For many overt rhythms it is difficult to specify the biochemical identity of clock controlled component(s), but in *Gonyaulax,* the synthesis rates of enzymes that catalyze the rate limiting steps of various reactions appear to be direct targets of the circadian clock. As described above, this occurs for enzymes in photosynthesis and bioluminescence rhythms, but what about the patterns of synthesis for proteins more generally?

In fact, it appears that circadian regulation of a large number of proteins in *Gonyaulax* occurs at the translational level. Pulse labeling of *Gonyaulax* proteins at times 12 hours apart, followed by 2D gel analysis, showed that many are preferentially synthesized at one phase or the other, and regulated translationally.[76] Determined at more frequent time points,[16] ten of these were found to fall into three time frames, with about tenfold differences from peak to trough; three of these are shown in Figure 3. But while their synthesis rates are strongly circadian controlled, the cellular concentrations of some of the proteins do not exhibit pronounced rhythms, such as glyceraldehyde-3-phosphate dehydrogenase (Fig. 9A). These observations are explicable if the protein has a long turnover time, as in this case the daily pulse of synthesis would add to the total cellular content of the protein by only a few percent (Fig. 9B).[111]

Transcriptional control is a predominant theme for circadian regulation in both models and experimental results.[19,20] The advent of the microarray technique provided the opportunity to search for such possibilities on a global scale in dinoflagellates. This was carried out with a different dinoflagellate, *Pyrocystis lunula*, whose luminescence is also circadian controlled.[112] About 3,500 cDNAs were prepared and used to compare the abundance of transcripts at circadian times separated by 12 hours.[113] Using ratios of ≥ 2 or ≤ 0.5 as a cutoff, about 3% of the genes screened were identified as circadian-controlled, and appeared to fall in a distinct class, rather than in the tail of a normal distribution (Fig. 10). More than 50% of these could be identified with diverse known genes. Light exposures at times expected to induce phase shifts in the rhythm revealed 30 differentially expressed genes, including some potentially participating in photic entrainment and others in pathways connecting a central oscillator to output

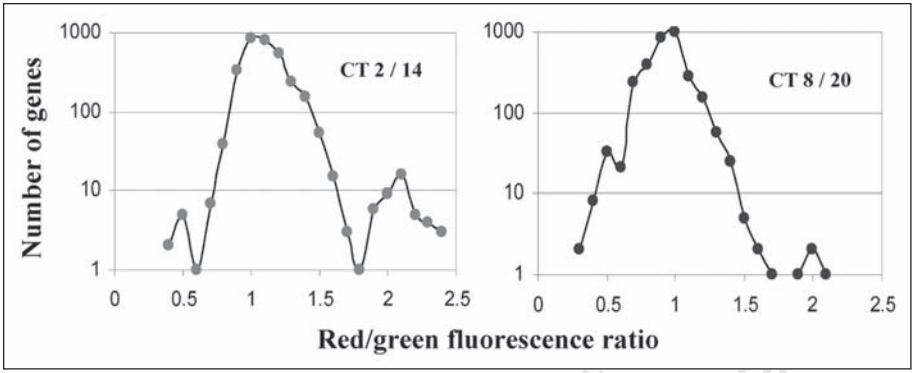

Figure 10. Clock control of transcription in dinoflagellates. Relative expression levels of dinoflagellate (*P. lunula*) genes at circadian times 12 hours apart. Distributions of genes (ordinates, number of genes in bins of 0.1 ratio units) as a function of the fluorescence ratios for CT 2/14 (top) and CT8/20 (bottom). Genes with a ratio close to 1 are the most frequent. Data reprinted from: Okamoto OK, Hastings JW. Novel dinoflagellate circadian-clock genes identified through microarray analysis of a phase shifted clock. J Phycol 2003; 39:1-9.

rhythms. Five genes, which may represent core clock genes, appeared in both screens but there were no similarities with clock genes from other organisms.

Circadian control may also occur at the post-translational level.[114] Translocation and compartmentalization may be involved.[115,116] In *P. lunula*, it has been shown that the luciferase is not synthesized and destroyed each day,[117] and its message is not circadian.[113] Instead, it is inactivated in day phase by translocatation to a different cellular compartment where it is no longer coupled to the flash generation mechanism.[84,85,118] The circadian control must therefore be mediated via protein(s) different from those involved in the *Gonyaulax* regulation, which underscores the diversity of mechanisms, even within a narrow phylogenetic group.

Conclusion

It is difficult to escape the conclusion that a model with a single master biochemical oscillator is inadequate to explain many aspects and features of the many different rhythms in *Gonyaulax*. At the same time, it is not reasonable to suggest that each different rhythm has a separate driving oscillator. Indeed, different rhythms evidently interact in a kind of coupling, as illustrated in (Fig. 2C) for the flashing and aggregation rhythms, where a compromise phase is adopted transiently before each rhythm continues with a separate period. One of the key questions concerns how a cellular oscillatory mechanism can give rise to two (or more?) different periods for different clock-controlled processes, analogous perhaps to a clutch mechanism with different slippage for different processes. Continuing with the analogy, signal transduction pathways for different processes may be viewed as clutches. The several rhythms in *Gonyaulax*, especially those of bioluminescence and carbon fixation, provide systems where answers to these questions may be forthcoming in future investigations.

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