The *Gonyaulax* Clock at 50: Translational Control of Circadian Expression

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The unicellular circadian clock of *Gonyaulax polyedra* (now renamed *Lingulodinium polyedrum*) has provided important insights concerning circadian rhythmicity. Many, perhaps most, of its key systems are circadian-controlled, ranging from bioluminescence and photosynthesis to motility, cell division, and the synthesis of many proteins, favoring the “master clock” concept. But different rhythms may have different free-running periods and different phase angles under different T cycles, observations not easily accommodated in a single oscillator model. *Gonyaulax* has a feature significantly different from that of other known systems, namely, that clock control of protein synthesis occurs at the translational level. With one mRNA, this involves a protein binding to a 22-nucleotide region in the 3'-untranslated region (3'UTR), but no similar regions have been found in other mRNAs. Pulses of protein synthesis inhibitors cause phase shifts, whereas inhibitors of protein phosphorylation administered chronically cause period changes. In *Gonyaulax* and other systems, low temperature results in arrhythmicity. A return to a permissive temperature results in a reinitiation of the rhythm, with the phase established by the time of increase, similar to the effect of bright light. Evidence for cellular communication via substance(s) in the medium has been obtained.

INTRODUCTION

I am pleased to participate in this second Cold Spring Harbor Symposium on Biological Clocks and thank the organizers for the opportunity to say a few words on the *Gonyaulax* clock, 50 years after my first publications with the late Beatrice Sweeney (Hastings and Sweeney 1957; Sweeney and Hastings 1957). At the time of the first Symposium in 1960, it was rare to have meetings on a specialized topic; the Cold Spring Harbor series were pioneering in that respect and highly productive.

At Harvard, the long-standing custom of a 15-minute chapel service every morning at 8:45 is still with us. Classes cannot be scheduled during this time. The service includes a 5-minute talk given by a member of the University. Mine have always been secular; in one I commented on how an unexplained phenomenon may require a lengthy exposition, whereas when understood, it can often be described very briefly. I recalled that in the 1960s I took four lectures to describe and explain oxidative phosphorylation, but in the 1980s, subsequent to Mitchell's discoveries, it took only a part of one lecture.

The biological clock field is in many respects still in a pre-Mitchell stage, with models having complicated schemes and arrows going in many directions. Clock genes and proteins, along with many other discoveries, have contributed much to understanding circadian mechanisms, but there is still a long way to go. Studies of the unicellular dinoflagellate *Gonyaulax* have also contributed significantly to progress in understanding the circadian mechanism. These include results that suggested the mechanism of temperature compensation (Hastings and Sweeney 1957), basic features of phase shifting by light and the first PRC (Hastings and Sweeney 1958), the action spectrum for light phase shifting (Hastings et al. 1961), and phase shifting by drugs and drug PRCs, as well as drugs affecting period (for summary, see Hastings 2001).

I review here three aspects of the *Gonyaulax* clock that are not understood in terms of a generalized circadian mechanism: translational control of the synthesis of proteins, the reversible loss of rhythmicity at low temperature, and evidence for cellular communication of circadian phase.

TRANSLATIONAL CONTROL

During the past decade, luciferases have been widely used as reporters for tracking circadian changes in gene expression. *Gonyaulax* was effectively the pioneer in this regard; it is a bioluminescent organism whose light emission depends on its own unique luciferase, and the synthesis of this luciferase is circadian-controlled.

In lab cultures, rhythms of luminescence can persist in constant dim light for many cycles—20 or 30—with a period that may be greater or less than 24 hours, depending on conditions of light, temperature, drugs, or other factors. The cells also exhibit rhythms in many other processes, with different phase angles: cell division, photosynthesis, cell aggregation, and the synthesis of many proteins (Hastings and Sweeney 1959; Hastings 1961).

The bioluminescence comes from small (0.4 μ m) organelles enveloped in the vacuolar membrane called scintillons where the two proteins involved are localized (Nicolas et al. 1987, 1991). These are luciferase (LCF) and the luciferin (substrate)-binding protein (LBP), which are unreactive at pH 8 (Morse et al. 1989a). A mechanically initiated action potential in the membrane allows protons to enter the scintillons and a rapid flash (100 msec) occurs.

As visualized in Figure 1, the scintillons are synthesized and destroyed each day (Fritz et al. 1990), as are LCF and LBP, as shown by western blots (Johnson et al. 1984; Morse et al. 1989b). New protein synthesis is usually associated with new transcription, but *Gonyaulax*

Figure 1. Fluorescence in vivo of *Gonyaulax* cells during night phase (left) and day phase (right) showing scintillations by their luciferin fluorescence (excitation wavelength, 405 nm) and their greater numbers at night. Background red is from the fluorescence of chlorophyll. (Photo by Carl H. Johnson.)

was a surprise; a daily approximately 5-hour bout of LBP synthesis occurred while the message for that protein remained constant (Morse et al. 1989b). Indeed, several *Gonyaulax* mRNAs have been found to have lifetimes of many hours (Rossini et al. 2003).

The luminescence-related proteins are not the only ones to be circadian-controlled at the translational level. Milos et al. (1990) showed on two-dimensional gels of extracts made at day and night phases that many proteins exhibited circadian changes in amount. However, equal amounts of protein were synthesized by in vitro translation from poly(A)⁺ RNA extracted from cells at day and night phases, indicating that the message itself is present and fully capable at all times of the cycle. Morse and colleagues (Markovic et al. 1996) used pulse labeling to track the in vivo synthesis of a dozen or so of these circadian-controlled proteins and found that they fell into three acrophases, and later identified several of them.

One of them, GAPDH, like LBP, has constant message levels over the circadian cycle but illustrates nicely an additional point of interest in circadian biology (Fagan et al. 1999). Not all of the GAPDH is destroyed in a single cycle, so the rhythm of abundance is not as marked as it is with LBP or LCF (Fig. 2). Thus, synthesis might be strongly circadian, but if the protein has a lifetime of many days, its abundance would not exhibit a readily detectable rhythm.

Translational control is known to be an important regulatory mechanism in both bacteria and eukaryotes. It can be mediated by a variety of mechanisms, prominent among which are protein factors able to bind to either the 5'UTR or 3'UTR of an mRNA and act as either activators or repressors. In *Gonyaulax*, Mittag et al. (1994) made use of gel retardation and found proteins binding to a 22-nucleotide region in the 3'UTR of LBP containing seven UG repeats. They further reported that the binding activity cycles on a daily basis under constant conditions. A recent attempt to repeat these observations has not been successful (Lapointe and Morse 2008).

Further studies of translational control of circadian processes has been made by Mittag with her own group in Germany. She found that the 22-nucleotide sequence

Figure 2. Synthesis rate, specific enzyme activity, and protein levels estimated by western analysis for GAPDH (synthesis rates from Markovic et al. 1996) and westerns in relative units. Specific activities in nanomoles of NADPH formed per minute per milligram protein. (Modified, with permission, from Fagan et al. 1999 [© American Chemical Society].)

mRNA from *Gonyaulax* binds three proteins from extracts of the unicellular green alga *Chlamydomonas* in a circadian-dependent way (Mittag 1996). The most abundant, designated CHLAMY-1, was shown to be a heterodimer with three lysine homology motifs on one subunit and three RNA-recognition motifs on the other. A role in the circadian mechanism was implicated by the fact that the pattern of the photo-accumulation rhythm was altered in cells in which the individual subunits were either overexpressed or silenced (Iliev et al. 2006).

Evidence for translational control of circadian expression in a dinoflagellate has been obtained from microarray studies with a related luminous species, *Pyrocystis lunula*, about

Figure 3. Expression ratios at circadian times 12 hours apart (top, CT2 and CT14; bottom, CT8 and CT20) for individual genes of *Pyrocystis lunula*, in bins of 0.1 ratio units. (Reprinted, with permission, from Okamoto and Hastings 2003 [© Wiley-Blackwell].)

Figure 4. Like luciferase genes in all dinoflagellate species studied, those of *Noctiluca* occur in tandem (top), with different intergenic regions in different species (Liu and Hastings 2006). The *Noctiluca* gene contains both LCF-like and LBP-like regions in a single gene; the relationship to those in *Gonyaulax* is indicated at the bottom. The LCF-like domain shares a 56% sequence identity but is shorter at the amino terminus by 60-amino-acid residues than an individual domain of *Gonyaulax* LCF, where there are three such domains. The LBP-like region of 591-amino-acid residues has four repeat domains and is 41% identical to the comparable region of *Gonyaulax* LBP. (Reprinted, with permission, from Liu and Hastings 2007 [© National Academy of Sciences].)

3% of the approximately 2800 unique cDNAs screened were preferentially expressed by a factor of 2 or more at times of day 12 hours apart (Fig. 3), but none by a factor greater than 3 (Okamoto and Hastings 2003). About 50% of those sequenced could be identified with diverse known genes. Similar studies with *Gonyaulax* are in progress.

In the last decade, my lab has been especially concerned with the structures of the LBP and LCF genes and proteins, both of which have unusual repeat-domain components: LBP has four repeats and LCF three, and each of the latter is able to function independently as a luciferase (Li et al. 1997). A crystal structure has been obtained for one of the luciferase domains showing the putative substrate pocket and helices believed to be responsible for activity regulation by pH (Schultz et al. 2005). Luciferase genes are very similar in seven different photosynthetic species (Liu et al. 2004); in an eighth, *Noctiluca miliaris*, sequences are similar but arranged quite differently (Fig. 4) (Liu and Hastings 2007).

LOSS OF RHYTHMICITY AT A LOW TEMPERATURE

Gonyaulax luminescence in cells kept at temperatures below about 12°C exhibits no rhythm whatsoever, but if then returned at any time to a permissive temperature, the rhythm resumes with a typical pattern, with the phase determined by the time at which the temperature was increased (Njus et al. 1977). An explanation for this has not been obtained. It could be that a critical clock protein is cold-sensitive; for example, the subunits might dissociate at a low temperature.

Although there are many reports of loss of rhythmicity in the older literature (cited in Sweeney and Hastings 1961), I know of no recent studies of this kind. Do cyanobacterial rhythms exhibit this feature in vivo, and if so, is the in vitro rhythm of KaiC phosphorylation also reversibly lost at a low temperature? Such studies might provide important new insights into the circadian mechanism.

CELLULAR COMMUNICATION OF CIRCADIAN PHASE

It has often been considered that the circadian clock might be affected by humoral factors produced by the cells themselves. Some years ago, we performed experiments to investigate this possibility (Broda et al. 1985; Hastings et al. 1985). Two out-of-phase cultures were mixed and the luminescence rhythm was measured for about 10 days thereafter (Fig. 5). The maxima of the two came closer and closer over the 10 days, finally merging into a single peak. But in mixed cultures in which the medium was replaced every 4 days, the rhythms of the two remained distinct. Factors in the medium, either secreted or taken up, are implicated in this effect.

Even earlier than this, we had investigated with Neil Krieger the possibility that extracts of the suprachiasmatic nucleus could affect the *Gonyaulax* rhythm. The results were positive: A dramatic shortening of the period resulted. But cortical extracts gave the same effect, so it was judged not to be specifically related to the clock system.

Figure 5. Phase plots of peaks of luminescence (glow) of two out-of-phase cultures measured separately in different vials but plotted on the same graph (left); cultures were mixed 50/50 and measured from a single vial (center) and mixed without medium and then replaced with fresh medium every 4 days. (Reprinted, with permission, from Hastings et al. 1985 [© Springer Science and Business Media].)

Roenneberg et al. (1988) pursued this matter and showed that muscle extracts had the same effect and finally discovered that it was due to the phosphagen creatine. He pursued the matter further and demonstrated an endogenous period-shortening substance, which he chemically identified as a cyclopropane carboxylic acid and named it gonyauline (Roenneberg et al. 1991). Synthetic gonyauline was prepared and shown to have similar activity.

Progress with the *Gonyaulax* system has been hampered for lack of a genetic system. However, it remains a valuable model system and promises to be of even greater value in the future after the structure and sequences of its genome are available. It is not widely appreciated that there is 40 times more DNA per *Gonyaulax* cell than in the human cell, so significant progress in determining sequence has been made only recently.

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