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Commentary

Oscillating molecules and how they move circadian clocks across evolutionary boundaries

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Circadian clocks are all over the place, at least organismically speaking. In addition to being well known in systems such as the avian pineal gland and the mammalian suprachiasmatic nucleus (1), it is now appreciated that an organism need not be a complex metazoan in order to run a daily pacemaker. We know this from a host of studies that have documented bona fide circadian pacemakers in microbial organisms such as *Neurospora, Gonyaulax*, and *Euglena* (reviewed in refs. 1 and 2). As chronobiologists have extrapolated their investigations of these rhythms downward (starting with a higher plant in 1729; ref. 3), so have some of them extrapolated their thinking about cellular-level (including microbial) clocks back upward to higher forms. Thus, a pacemaker entity that mediates 24-hr rhythms would not have to be composed of an intercellular network or the like.

If biological complexity is not the hallmark of a circadian system, such clocks could have arisen early in evolution. Yet it seemed to have been assumed that daily rhythms could not cross the dread eukaryotic–prokaryotic boundary. As usual, that kind of investigatory and intellectual barrier fell (4, 5). Circadian rhythms in cyanobacteria were discovered and have been routinely, if not saliently, reported upon for 8 years (4–6). In these prokaryotes, and in eukaryotic microbes as well, the pacemakers retain their defining features (e.g., refs. 7 and 8): they "free-run" in constant conditions with periods close to a day; they have their phases set by light (the environmental factor that is principally responsible for daily resetting of circadian clocks to precisely 24-hr periods in natural light/dark cycles; ref. 1), and they manifest temperature compensation.

Although we seem to make good use of our internal alarm clock, and developing fruit flies are smart enough to emerge from metamorphosis during a salutary window of time, why would a bacterium need a clock to conduct its dreary affairs (borrowing the words of a review writer from long ago; ref. 9)? An interesting rationale accompanied one of the first reports of a metabolic circadian rhythm in a cyanobacterium: The cells need to temporally compartmentalize incompatible biochemical reactions—involving photosynthesis and nitrogen fixation—into different halves of the day (5). A subsequent study showed that a nitrogenase rhythm, which underpins that of prokaryotic nitrogen fixation, is mediated by daily fluctuations in the amount of that catalytic factor, this being in turn controlled by a circadian rhythm of the enzyme-encoding mRNA (10).

All of this and more is presented in a paper on cyanobacterial rhythms that appears in the current issue of these *Proceedings*; it resulted from experiments in which Kondo *et al.* (11) molecularly analyzed the rhythmicity of a factor involved in photosynthesis. The primary manner in which the rhythm of this "photosystem II" gene product was monitored involved a reporter-transgenic strain of *Synechococcus*: an upstream regulatory region of the gene was fused to luciferase-encoding sequences, which permitted the circadian rhythmicity of the transcription rate to be tracked in real time as daily oscillations of bioluminescence (11). Similar observations had been made in higher plants, where rhythmicity involving chlorophyll a/b-binding protein (*Cab*) mRNA was shown, by the creation of analogous transgenic strains, to be transcriptionally mediated (12). This approach recently culminated in the demonstration that circadian cycling of a glow rhythm can be monitored "on-line" in *Arabidopsis*; the experiments involved fusing a *Cab* gene promoter to a firefly luciferase reporter (13). Clock-controlled changes in the levels of mRNAs transcribed from a wide variety of genes were, in the meantime, observed in organisms ranging from fungi to mammals (reviewed in refs. 14–16).

The technology used to detect glowing organisms could in principle permit one to determine whether individual cyanobacterial cells generate daily rhythms of bioluminescence (see refs. 17 and 18 for general aspects of the pertinent methodology). Rhythmicity at this level has been demonstrated for the real (not reporter-based) glow oscillations that are put out by the eukaryotic alga *Gonyaulax* (ref. 19; see also ref. 20). Thus, communication among cells in such microbial cultures is not necessary for the individual organisms to operate a circadian clock. However, such cross-talk can communicate rhythm-phase information when separate *Gonyaulax* cultures are mixed (21). Indeed, the individual circadian cells of a metazoan pacemaker structure would also need to talk to each other (at least in free-running conditions), to synchronize the overall output from the clock. Nevertheless, as was previewed in our introductory remarks, higher eukaryotes may have daily clocks running within each of their circadian pacemaker cells. For instance, dispersed avian pineal cells, with minimal cell-to-cell contact, manifested circadian periodicities of melatonin release (22). Although this publication could not rule out intercellular (humoral) communication being somehow required for the circadian rhythmicity in question, a subsequent study showed that pacemaker cells from the eye of a mollusc continued to manifest their daily electrophysiological rhythm when monitored as single-cell isolates (23). Some years before the metazoan search for a clock-within-a-cell culminated in these very recent molluscan experiments, a multicellular model for the circadian clock of *Drosophila* appeared. It was based on the dramatic effect of rhythm mutations, at a genetic locus called period (*per*) (reviewed in ref. 24), on intercellular communication (25). But these results could not be reproduced (26), and that model for the action of the *per* "clock gene" was abandoned (27). Thus, much of the current thinking about clocks revolves around circadian pacemaking being mediated intracellularly. (This view is not unanimous, however; e.g., ref. 28.)

So what would a so-called clock gene, defined by mutations that alter or eliminate circadian rhythms (24, 29, 30), be doing? Consistent with the aforementioned intracellular notions is a belief that oscillations in the expression of products encoded by clock genes may be rather close to the central pacemaker mechanism. This idea has stemmed from monitoring levels of *per* gene products over the course of a day: Both *per* mRNA and the *per*-encoded protein undergo circadian fluctuations in their abundances (reviewed in ref. 31); the mRNA cycling

involves intra-day modulation of *per*'s transcription rate (32). Since *per* mutations affect the amplitude, period, and phase of this transcript oscillation (33), there is a feedback loop in which the *per* gene product influences transcription of the nucleic acid encoding it. This led to the proposal that the molecular loop is part of the clock itself (32–34). It has not escaped our attention that *per* could also influence the fluctuating transcription of "output genes" (compare refs. 14–16) in a similar if not identical manner. Remarkably, recent information on the expression of a *Neurospora* clock gene, frequency (*frq*) (reviewed in ref. 35), indicates that a situation similar to that of *per* obtains in this distant eukaryote; that is, *frq* mRNA levels also undergo a circadian oscillation that is sensitive to a mutation in the *frq*-encoded protein (previewed in ref. 30).

We might pause to note that a variety of lines of evidence have revealed organisms to have more than one circadian clock (for example, those that run autonomously in different tissues; ref. 1). This includes the recent demonstration that two separate clocks exist in *Gonyaulax*, hence almost certainly within a given cell of this algal species (36). Keeping in mind what the fly's and the bread-mold's clock genes seem to be doing, this explanation for the bi-clock algal cell suggests itself: Two gene-expression feedback loops—between which there would be no cross-talk, and each involving a separate *per*- or *frq*-like gene—could readily permit independent pacemakers to run with noticeably different periodicities (as they do; ref. 36).

Molecular mechanisms of this sort could be very old evolutionarily and (therefore?) universal. Feedback control of oscillating transcription rates can operate in "anything," whether or not the biological entity has a nucleus or is within a multicellular structure.

How to go forward from these suppositions? One hopes to delve into the cellular and molecular mechanisms of other tractable clock systems, by doing things like isolating rhythm mutants and seeing how they may influence mRNA fluctuations of (at least) clock output functions. In fact, as Kondo *et al.* (11) point out (also see ref. 13), the oscillating luciferase reporter they have created can be used in screenings that could lead to isolation of the first bacterial rhythm mutants. Understanding the cell biochemistry of the clock genes so identified may be facilitated by the genetic, molecular, and metabolic manipulability of this prokaryotic system. Knowledge about the action of such factors—as to how they may help control central pacemaking, influence rhythmic outputs, or both—could soon catch up to the accelerating understanding of the two eukaryotic clock genes that are in hand molecularly (29–35). Then we can, as it were, take a time-out to see just how universal are the components comprising circadian clocks.

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