Molecular control of circadian rhythms

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Circadian rhythms are virtually ubiquitous in eukaryotes and have been shown to exist even in some prokaryotes. The generally accepted view is that these rhythms are generated by an endogenous clock. Recent progress, especially in the *Drosophila*, *Neurospora* and mouse systems, has revealed new clock components and mechanisms. These include the mouse *clock* gene, the *Drosophila timeless* gene, and the role of light in *Neurospora*.

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Introduction

The circadian clock (pacemaker or oscillator) is considered to be endogenous, as opposed to driven, because rhythmic oscillations persist with near 24-hour periodicity under constant environmental conditions. It is connected to the environment, however, as the rhythm is (usually) entrained by the 24-hour light/dark cycle, the major environmental zeitgeber or time cue. The clock is also connected to downstream outputs, namely the biochemical and behavioral fluctuations that are generally observed as rhythmic phenomena. Pacemaker locations, the mammalian suprachiasmatic nucleus (SCN) for example, can often be anatomically defined [1–3]. Some systems contain multiple clocks, and they may 'run' independently even within a single-celled organism [4]. Although substantial progress has been made in understanding how the pacemaker influences many downstream molecules and processes, including gene expression (clock-controlled genes) [5–10], this review will focus narrowly on the pacemaker mechanism itself.

The current prevailing view is that a complete fully autonomous clock is probably housed within single metazoan cells. Pioneering efforts by Takahashi and colleagues [11] indicated that pinealocytes keep circadian time with little intercellular contact. Physiological studies in individual *Bulla* pacemaker neurons showed definitively that this clock is functional without cell–cell contact [12]. A very recent landmark study [13••] recorded circadian rhythms from rat SCN neurons. The results suggest that the SCN contains a large population of autonomous single-cell oscillators; functional synaptic connections are neither necessary for the rhythms nor sufficient to synchronize them. Perhaps melatonin participates in synchronizing the SCN neurons [14].

This emphasis on an intracellular pacemaker has been reinforced by numerous inhibitor studies suggesting that the clock requires ongoing RNA and protein synthesis [15–17]. This conclusion has been strengthened significantly by molecular genetic studies in *Drosophila* and *Neurospora*, in which the predominant foci have been the well studied clock genes *period* (*per*) and *frequency* (*frq*), respectively. For many years, genetic evidence has been accumulating that these genes encode important components of the pacemakers [18–21]. More recent molecular experiments support this view and suggest that it is the rhythmic synthesis of the gene products (PER and FRQ) that is intimately tied to the clock mechanism.

The *Drosophila* and *Neurospora* paradigms

Some remarkable parallels exist between the *Drosophila* and *Neurospora* systems. Firstly, both *per* and *frq* mRNA levels undergo robust circadian oscillations that depend on the presence of their translation products [22,23••]. Moreover, missense mutations alter the periods of the RNA fluctuations and the rhythmic outputs in parallel [22,23••]. In the *Drosophila* system, *per* RNA cycling has been linked to rhythmic changes in *per* transcription [22], and robust cycling of PER itself has been observed [24,25,26•,27•]. Assuming that the same situation exists in *Neurospora*, the observations suggest that both PER and FRQ participate in feedback loops that influence their own transcription [22,23••]. Taken together, the genetic and molecular evidence suggests that the molecular fluctuations, as well as these feedback loops, are at the heart of their respective circadian oscillators.

This postulate has received recent strong support from related experiments in both systems. In *Drosophila*, a gene fusion construct placing *per* under the control of a rhodopsin promoter was introduced into other-

Abbreviations

bHLH—basic helix-loop-helix; ***frq***—*frequency*; **HS**—heat-shock; ***per***—*period*; **PRC**—phase-response curve; **SCN**—suprachiasmatic nucleus; ***tim***—*timeless*.

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wise wild-type flies [27•]. The high-level constitutive *per* expression in the eye stopped the clock in a tissue-specific manner, meaning that the rhythmic *per* expression in the eye was eliminated without affecting the molecular rhythms in the brain or the behavioral rhythm in locomotor activity. The *per* eye RNA was pegged at trough levels, suggesting not only that the *per* feedback loop is intracellular but also that PER inhibits its own synthesis. To address the importance of the PER fluctuations in a more dynamic fashion, a construct placing *per* under the control of a heat-shock promoter (HS–*per*) was introduced into an otherwise wild-type strain [28••]. Consistent with the notion that PER is a clock component, exposure to a 1 hour 37°C heat pulse caused stable phase shifts of the rhythms in the fly. The magnitude and direction (advance or delay) of the phase shift were dependent on time, that is, on the state of the clock at the time of the heat shock. The measurements created a HS–*per* phase-response curve (PRC) analogous to the more traditional light PRC that describes the temporal variation in the response of the clock to a pulse of light. A comparison of the HS–*per* PRC with the normal fluctuations of PER indicates that these are integral to the clock mechanism. In circadian parlance, PER is not only a clock component, but also a state variable [29].

Similar conclusions have been drawn in the *Neurospora* system. Dunlap and colleagues [23••] transformed wild-type *Neurospora* with a recombinant DNA construct expressing the FRQ gene product from a strong regulated promoter. High-level FRQ expression from this transgene stopped the clock and pegged endogenous *frq* RNA at its trough levels, suggesting that FRQ inhibits its own transcription, meaning that the *frq* feedback loop is negative. The clock was stopped at the (circadian) time that corresponds to these minimal *frq* RNA levels, as inhibiting expression from the transgene restarted the clock from this setpoint. The observations indicate that the *frq* feedback loop is a clock component, so *frq* RNA is also a state variable.

Yet the similarities of the two systems should not obscure the differences. Levels of *per* mRNA peak at night, whereas *frq* RNA levels peak in the morning. In the absence of PER, *per* RNA levels are at 50% of peak values, whereas in the absence of FRQ, *frq* mRNA levels are very high. In general, the *frq* system corresponds more simply to a transcriptional negative feedback loop [27•].

Drosophila biochemistry and effects of the rhythm mutants

In the fly system, there are indications that one or more post-transcriptional mechanism also contributes to PER cycling [30]. A comparison of the *per* RNA and protein curves suggests that the latter cannot easily derive from the former without some form of post-transcriptional regulation; PER translation and/or PER degradation must be temporally regulated [27•]. Also, the two classic missense mutations per^L and per^S both affect the PER cycle in a manner suggesting an effect on post-transcriptional processes [25,26•,31•]. The notion is that the mutations may have only an indirect effect on the transcriptional feedback loop.

In addition, the original biochemical characterization of PER cycling by western blot analysis indicated that PER is subject to temporal phosphorylation [26•]. This appears to be progressive, the phosphorylation becoming more and more extensive until the early morning, when protein levels decrease dramatically. These observations might relate to recent experiments indicating that protein kinase A is involved in *Drosophila* circadian rhythms [32]. The temporal phosphorylation of PER might influence its degradation, which is another post-transcriptional mechanism that may be under temporal control (M Dembinska, R Stanewsky, JC Hall, M Rosbash, unpublished data).

Temporal phosphorylation might also regulate PER activity, namely its effect on its own transcription. This is an attractive proposition because oscillatory negative feedback loops require a substantial delay between the synthesis of a factor and its inhibitory activity; otherwise, the fluctuations stop and the system comes to equilibrium. Consistent with the delay proposal is a recent report indicating that the nuclear localization of PER is itself under temporal control: PER remains cytoplasmic throughout its accumulation phase and then undergoes nuclear entry during a narrow time window [31•]. This temporal control of nuclear entry was visible only in the brain neurons most likely to encode the circadian pacemaker and was not detected in other PER-expressing tissues, such as photoreceptor cells. Also, mutations at the per^L site retarded the timing of nuclear entry in parallel to their effects on period. (The subcellular localization of PER had previously been described as nuclear in most adult tissues, but had been assayed carefully only at peak times of expression [33].) It is tempting to speculate that PER temporal phosphorylation is related to this gating of nuclear entry. Indeed, two research groups have independently based computer models of *per* RNA and protein fluctuations on the possible link between phosphorylation and nuclear entry (A Goldbeter, personal communication; LF Abbott, H Zeng, M Rosbash, unpublished data).

These recent observations suggest that the circadian pacemaker encompasses PER temporal fluctuations as well as all of the mechanisms that underlie them. Although FRQ has not yet been biochemically characterized, a similar conclusion for the *Neurospora* system would not be surprising. Yet, the biochemical activities of PER and FRQ remain unknown. Their participation in transcriptional feedback loops does not make matters more certain, because their effects on transcription could be quite indirect: progress around the cycle requires that

an increase in PER is eventually followed by a decrease in *per* transcription. The ambiguity in biochemical function is also a result of the fact that the primary sequences of the gene do not provide an unambiguous link with defined biochemical activities for the product [19,34]; absolutely no clues are available in the case of FRQ, and there are only hints in the case of PER.

PER and PAS

The PAS domain is the only link between PER and proteins with a known biochemical activity, the domain being shared with a number of DNA-binding transcription factors of the basic helix-loop-helix (bHLH) family [35–38]. *In vitro* experiments have defined PAS as a protein–protein interaction domain [39], and more recent experiments show that it contributes to the interaction between the aryl hydrocarbon receptor and the ARNT protein, two mammalian bHLH-PAS proteins that undergo ligand (dioxin) gated heterodimerization [40,41•]. PER contains no known DNA-binding motif, and the region of the protein occupying the location corresponding to the bHLH region of other PAS-containing proteins is not even conserved between related *Drosophila* species [42]. It is more likely, therefore, that PER influences transcription indirectly by contacting other proteins through its PAS domain. As PER is nuclear for at least part of its cycle [31•,33], the influence of PER on transcription may even be through an interaction with a bona fide PAS-containing transcription factor. Recent experiments in mammalian cell culture indicate that such a scenario is feasible [41•]. Caution is dictated, however, by the very recent identification of the first prokaryotic PAS-containing protein, the *Bacillus subtilis* kinase KinA [38]. The presence of PAS in a protein kinase indicates that even if the real *in vivo* target of PER is a PAS-containing protein, it might not be a transcription factor, so the effect of PER on transcription may be even more indirect than has been imagined.

There are hints that other interactions with PER might also be relevant. The PAS region of the aryl hydrocarbon receptor interacts with heat-shock protein 90 and dioxin [43•,44•], so an interaction of *per* PAS with heat-shock protein 90 or with small molecules cannot be excluded. PER has recently been identified and characterized in the giant silkmoth, *Antheraea pernyi* [45]. Moth PER can also rescue the arrhythmic per^{01} strain of *Drosophila* [46], consistent with the evident primary sequence conservation. The moth sequence significantly improves the previously available evolutionary comparisons between *Drosophila* species [42] and has focused attention on a ~40 amino acid region of the protein that (in addition to PAS) is highly conserved.

The C domain and temperature compensation

The ~40 amino acid conserved region may be relevant to a particularly intriguing aspect of clockology, namely the fact the circadian periods are generally temperature-compensated (jargon for temperature-insensitive). The 40 amino acids are included within a larger domain of PER (the C domain) that has been shown to interact with PER PAS *in vitro* as well as in a two-hybrid assay [47•]. The interaction between these two domains is suggestive of a PER intramolecular interaction and has been studied in the context of the per^L mutation, which affects the PAS domain. The per^L mutation lengthens the period in flies and also compromises temperature compensation (i.e. the period lengthens even further with increasing temperature). The mutation also renders PER–PER interactions temperature-sensitive in yeast, suggesting a relationship between the yeast and fly assays. In contrast to the PER–PER interaction assay, the interactions between PAS and the C domain (*in vitro* or in yeast) are strengthened by the per^L mutation. The experiments suggest that the temperature compensation of circadian period may be a result of temperature-independent PER activity, which is based in turn on a competition between intermolecular and intramolecular interactions with similar temperature coefficients. There is also evidence that the glycine–threonine repeat region of PER contributes to temperature compensation (B Kyriacou, personal communication).

timeless

Among the most exciting developments in the field of *Drosophila* circadian rhythms over the past year has been the emergence of *timeless* (*tim*) as the second bona fide rhythm gene from this organism [48••,49•]. In addition to the existence of arrhythmic and period-altering alleles ([48••]; A Rothenfluh-Hilfiker, MW Young, personal communication), the mutant gene has been shown to interact with the *per* system, and this has attracted much attention. In a tim^0 (arrhythmic, presumably null allele) background, there is no detectable *per* RNA cycling [48••]. Moreover, a PER–β-galactosidase fusion protein that usually generates prominent staining of photoreceptor cell nuclei is mislocalized to the cytoplasm with little or no effect on fusion protein levels [49•]. The fusion protein mislocalization in the absence of the *tim* protein (TIM) may be related to the temporal control of PER nuclear entry [31•]. However, the precise effects of tim^0 on the fusion protein might be an idiosyncrasy of the reporter system, because there is almost no wild-type PER detectable in a tim^0 background [50•]. Taken together, these observations suggest that TIM has a post-transcriptional effect on PER; without TIM, PER does not accumulate and therefore cannot engage in the feedback loop necessary for the *per* RNA fluctuations [50•]. It will be interesting to see whether

the TIM protein sequence is biochemically informative and whether the post-transcriptional effect of TIM on PER is based on a direct TIM–PER interaction. Future biochemical studies should also reveal proteins that interact in a clock-significant manner with either PER or TIM.

Where is the *Drosophila* clock?

The location of the *Drosophila* clock has been of interest for some time. Where is the pacemaker for *Drosophila* locomotor activity rhythms? PER is expressed in a number of adult neural and non-neural tissues [24,25,51–53]. Its function in most of these tissues is unknown, as they are unnecessary for locomotor activity rhythms. There is, however, strong circumstantial evidence that ~50 PER-expressing brain neurons (the 'lateral neurons') include the likely location of the circadian pacemaker that governs this rhythm [52,54]. Two very recent papers lend strong support to the hypothesis that these are indeed pacemaker cells.

Helfrich-Forster [55] has shown that pigment-dispersing hormone, a molecule known to be involved in insect as well as crustacean and mollusc circadian rhythms, is coexpressed with PER in a subset of the lateral neurons. One imagines that this hormone is released in rhythmic fashion and is involved in communicating with the output pathway [55]. Vosshall and Young [56] expressed PER from a *glass* promoter known to drive gene expression in photoreceptor cells. The transgene rescued the rhythms of the arrhythmic per^{01} host, and PER was expressed in the brain within or near the lateral neurons as well as in the eyes and ocelli. As the eyes and ocelli were shown to be irrelevant for the behavioral rescue, the results are also consistent with a subset of the lateral neurons being the location of the locomotor activity clock. As the *glass* promoter is active in these cells, they may also have some direct photoreceptor function important perhaps for light entrainment.

How does light affect the clock?

The role of light in circadian rhythms has been addressed recently by Dunlap and colleagues [57•], who discovered that *frq* RNA levels strongly and rapidly increase within minutes after a light pulse, suggesting that this is the earliest response of the clock to light. The fact that *frq* RNA levels are highest in the morning suggests that the *frq* RNA response is relevant to the entraining features of the normal 24-hour cycle, as well as to the effects of phase-shifting light pulses.

In *Drosophila*, *per* RNA is highest in the evening, almost completely out of phase with the *frq* RNA curve. Consequently, light might have the opposite effect in flies. Indeed, PER levels are very low under constant light conditions [25,50•]. A more complicated *Drosophila* story can be anticipated, however, as the locomotor activity rhythm PRC is profoundly affected by the *per* mutations [58].

Other clock mutants

Despite all this progress, there is much more to come from future studies. As yet, *per*, *tim*, and *frq* are the only well characterized rhythm genes, and we expect additions to this list from both *Drosophila* and *Neurospora*. For example, if PER temporal phosphorylation is truly important for the clock mechanism, we should find clock mutants that encode the relevant kinases. We still do not understand how the clock cycle is connected to its downstream targets: what the clock controls directly. Mutants that define this output pathway will also be important.

Clock genetics is burgeoning in a number of other systems. In hamsters, a very interesting and well characterized clock mutant has been identified called *tau* [59]. In addition, the recently identified mouse mutant *clock* is of great interest and is hopefully the first in a series of behavioral mutants that will be obtained by assaying individual mutagenized mice [60••]. Given the state of the mouse genome project [61], *clock* should be easier to clone than *tau*. Like the *Drosophila per* mutants, the effects of *tau* and *clock* are semi-dominant. A number of *Arabidopsis* mutants have been identified by imaging mutagenized plants transformed with a cycling *CAB*–luciferase reporter construct, in which the firefly luciferase gene is under the control of a CAB (chlorophyll *a/b* binding protein) promoter [62•]. The luciferase reporter has also helped elucidate the complicated but interesting effects of the photoreceptor pathways on the period of the *Arabidopsis* clock [63]. A similar strategy has been used to identify mutant clock genes in the photosynthetic bacterial system [6,64•,65]. We anxiously await the cloning and characterization of these clock genes, as identifying their products may provide some clue as to what kinds of biochemical pathways are important.

Evolutionary considerations

Comparisons between different organisms may also address an important evolutionary question: how similar are their clocks? In different systems, clock components may be the same or closely related, meaning that circadian pacemaker molecules could be as conserved as cyclins or other cell cycle molecules. However, it may be relevant that PER and FRQ manifest little if any primary sequence similarity [34] and that homologs have not been identified in mammals. A second

possibility is that the components are different but the molecular mechanisms (e.g. transcriptional feedback loops) conserved. A third is that different circadian oscillators exist that are unrelated except by functional convergence. Although the initial question is a proper subject for a more speculative review, the near future will hopefully render it moot. As more clock components and more mechanisms become defined and the field of circadian rhythms continues its demystification process, the answers should become apparent.

Conclusions

The past year has seen significant advances in our understanding of how the *Drosophila* and *Neurospora* clocks tick, as well as the identification of likely clock components. The *timeless* gene has come of age as a bona fide character in the *Drosophila* rhythm story, and the *clock* gene will probably do the same in mammals. Elucidation of the nature of several rhythm genes from plants and bacteria is around the corner.

Note added in proof

The studies referred to in the text as A Goldbeter, personal communication and LF Abbott, H Zeng, M Rosbash, unpublished data have now been accepted for publication [66,67].

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