

Common threads in eukaryotic circadian systems

Jay C Dunlap

Within the past 18 months, common regulatory patterns have emerged among eukaryotic circadian systems – extending from fungi through to mammals. Heterodimeric complexes of PAS-domain-containing transcription factors play positive roles in clock-associated feedback loops, and classic clock proteins like FREQUENCY (FRQ), PERIOD (PER), and TIMELESS (TIM) appear as negative elements. Post-transcriptional control governs the amount and type of FRQ and makes the clock responsive to temperature.

Addresses

Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755, USA; e-mail: jay.c.dunlap@dartmouth.edu

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Abbreviations

<i>Clk</i>	<i>Clock</i>
<i>cyc</i>	<i>cycle</i>
<i>dbt</i>	<i>double-time</i>
<i>frq</i>	<i>frequency</i>
<i>oli</i>	<i>oligomycin resistance</i>
<i>per</i>	<i>period</i>
<i>tim</i>	<i>timeless</i>
<i>wc</i>	<i>white collar</i>

Introduction

Circadian rhythms and the cellular oscillators that underly them are extremely common among eukaryotes — and with good reason. With the exception of a few fast-growing microbial eukaryotes, such as the yeast *Saccharomyces* and some creatures that live deep inside caves or at the bottom of the ocean trenches, dawn means food (if you are green and fix carbon, or if you move and hunt with your eyes), predation (if you are hunted), and changes in all the geophysical variables that naturally accompany the sun's effect on the earth's surface (warming, winds, etc.). It's a big deal when the sun comes up, and most eukaryotes are adapted to anticipate this change and to adjust their lives and metabolism to it.

As might be expected given this omnipresent and ancient evolutionary pressure, there appear to be common elements in the ways in which eukaryotic circadian oscillators are built and the components that are used to build them [1]. In this review, I focus first on the nature of these common elements that have appeared over the past few years. As the study of rhythm genetics began in *Drosophila* and *Neurospora* and has been fleshed out using the molecular genetics of chiefly these systems complemented more recently by work in mammals, and as all of what we know about how (at least) mammalian clocks are either run or reset closely parallels the behavior of one or both of these two models (and chiefly because within the page limit set

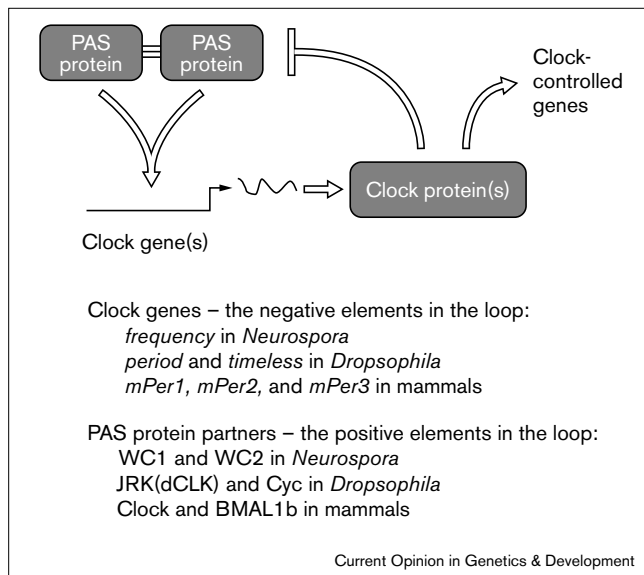
here there isn't room to do any justice at all to recent work in mammals or plants), here I concentrate mostly on fungi and flies — with just the odd reference to mammalian work. Similarly, I will only discuss the oscillator and how it is synchronized to light and temperature, leaving circadian regulation of output for another time. The punchline as it now seems to be emerging — minus all the interesting details (and in case you are in a hurry) is in the first Figure and next two paragraphs.

How does it work? A generic feedback loop circadian oscillator

A circadian system can be made up of one or more interconnected feedback loops. Of these, one or more may take the lead but every time this core loop regulates one of its inputs, for instance by regulating a photoreceptor (e.g. [2]), and every time an output from the core influences an input (e.g. [3]), another loop is added. All these loops are by necessity interconnected and therefore affect each other and, in common, give rise to the exact characteristics of classic circadian properties such as period length, temperature compensation, and resetting by light or temperature — and perhaps even sustainability. Many of these outer loops will be organism-specific whereas some, including one or a few at a core, may be more universal. I'll come back to these subjects in more detail later but here it suffices to say that a variety of data now suggest that one core among the eukaryotes may look like this (Figure 1).

Several facts emerge from this simple picture. First, there is a feedback loop that involves both positive and negative elements and that is centered on the transcription and translation of clock genes and clock proteins. The positive element in the loop is the transcriptional activation of a clock gene(s) through binding of paired transcriptional activators on the clock gene promoter; they are paired by virtue of interaction via PAS domains. Functionally similar PAS-domain containing DNA-binding clock elements have now been described in the three best molecularly studied eukaryotic clock systems: *Neurospora* [4••], *Drosophila* [5••–7••], and mice [8••]. Transcription of the clock gene gives rise to a message the translation of which (subject to additional regulation) generates a clock protein(s) that provides the negative element in the feedback loop. The negative element in the loop feeds back to interfere with or block the clock gene's activation so the amount of clock gene mRNA declines and eventually the level of clock protein also declines. This robust daily cycling clock gene mRNA [9–11,12••,13••] and clock protein [14•,15,16], is characteristic of these eukaryotic circadian systems. Although not all of the details of all of the above have been described yet in all systems from fungi through humans, some of these elements are known in all of the systems examined, and the threads of similarity

Figure 1



Common elements in the design of eukaryotic circadian oscillators. A general regulatory scheme seen in the three best-understood eukaryotic circadian oscillatory systems, fungi (*Neurospora*), insects (*Drosophila*), and mammals (rodents). Paired heterodimeric PAS proteins act as positive elements to turn on clock genes. Clock proteins are negative elements in a feedback loop, apparently acting to negate the activation of the positive elements. The functionally similar elements in the different systems are listed.

among all systems suggests that this emerging theme may reflect a common mechanistic core for at least one lineage of eukaryotic circadian oscillators.

The biologically interesting details

So, that's the bottom line, but of course the interesting biology is in the detail — the myriad ways in which the core has been adapted to different systems to provide different adaptations. These are reflected chiefly in, first, the way in which external signals from the environment act to synchronize the core with the daily light/dark cycle and second, especially, the different kinds of processes that are regulated on a daily basis by the clock.

The first mutations in clock genes were identified in *Drosophila* (the *period* [*per*] gene) and in *Neurospora* (the *frequency* [*frq*] gene) in the early 1970s ([17,18]; for review, see [19,20]) and were cloned in the 1980s [21–23]. Progress in understanding how circadian oscillators work has been closely tied to understanding how these genes are regulated.

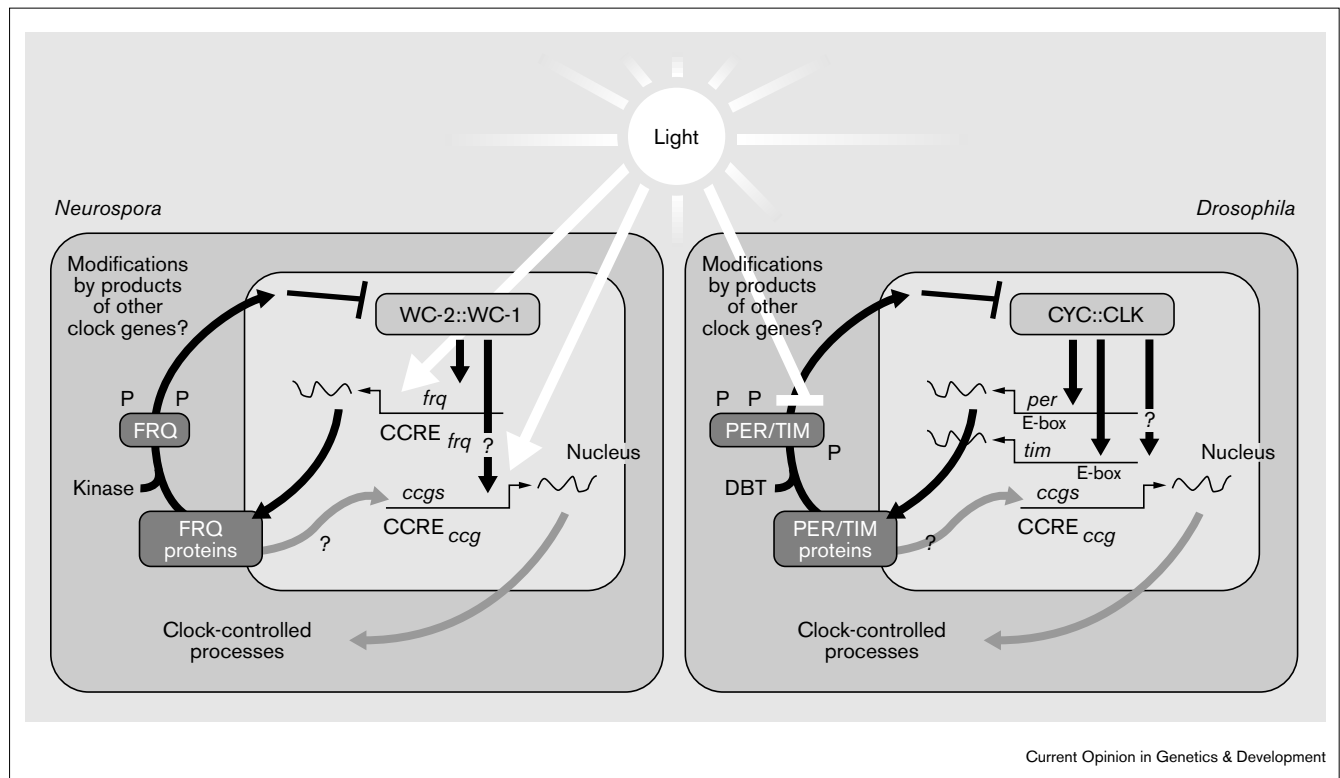
frq is a clock gene that encodes central components of a circadian clock [9,20,24]. The circadian oscillator in *Neurospora* includes an autoregulatory feedback cycle [9], wherein *frq* gives rise to transcripts that encode two forms of FRQ, a long form of 989 amino acids and a shorter form of 890 amino acids resulting from alternative initiation of translation at an internal ATG codon [14•,25]. Both *frq*

RNA and FRQ cycle in their amounts [9,14•], and FRQ acts to depress the level of the *frq* transcript [9], possibly by interfering with the normally required activation of the gene by a heterodimeric activator composed of WC-1 and WC-2 [4••]. Importantly in this negative feedback oscillator, rhythmic change in the amount of *frq* transcript is essential for the overt circadian rhythm (no level of constant *frq* expression supports the rhythm) and step changes in *frq* expression reset the clock [9].

Using Figure 2 as a guide, we can imagine the *Neurospora* clock cycle starting at midnight (0 hrs). *frq* and FRQ levels are low but *frq* transcript is beginning to rise, a process that will take ~10–12 hrs to reach peak. This late-night increase in *frq* is the result of action by a heterodimeric pair of transcription factors encoded by *white collar-1* (*wc-1*) and *wc-2* [4••]; these positive elements are the PAS proteins in the *Neurospora* system. WC-1 and WC-2 heterodimerize via their PAS domains [26] and are believed to activate transcription from their target genes by binding to promoter elements within these genes. After a short lag that represents a regulated part of the circadian cycle [27•], FRQ protein begins to appear [14•]; FRQ enters the nucleus soon after its synthesis [14•,28•] where it may interact with WC-1 and WC-2. Whatever the mechanism, we know that in reconstruction experiments — where in a *frq-null* strain *frq* is driven from a regulatable heterologous promoter — the part of the feedback loop extending from the onset of *frq* transcription through the complete decline in *frq* mRNA levels can take place in just 6 hrs; the inhibition part of the loop is fast so that for most of the day, *frq* transcript levels are low and FRQ levels are higher. *frq* mRNA levels peak in the mid-morning [9,29] ~4 hrs before the peak of total FRQ which occurs in the early afternoon [14•]. As soon as either form of FRQ can be seen, it is already partially phosphorylated. Midday (1200 hrs) finds the amount of FRQ in the nucleus falling but the total amount in the cell rising, and the amount of partially phosphorylated FRQ (both forms) is also increasing. During the afternoon, *frq* levels fall and the level of FRQ, now becoming extensively phosphorylated, declines through the early night, consistent with a model in which phosphorylation triggers FRQ turnover.

The *Drosophila* oscillator follows a similar pattern but with a reversed phase. *per* and *timeless* (*tim*) mRNA levels begin to rise late in the subject day [10], their increase being the result of activation by the PAS protein heterodimer of *Drosophila* CLOCK (dCLK or CLK) and another fly protein CYCLE (CYC) [5••–7••]. In nicely executed experiments, this part of the feedback loop has been reconstructed in insect S2 tissue culture cells [5••]. CYC is normally expressed in these cells but co-expression of CLK serves to activate *per* and *tim*, and simultaneous expression of PER blocks this activation but has no effect on *per* gene expression in the absence of CLK [5••]. This is wholly consistent with the model in Figure 1 where the negative elements (the clock gene products) act on the

Figure 2



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Feedback loops within circadian oscillators. The details of the feedback loops found in the *Neurospora* and *Drosophila* clocks are shown. Bold lines trace a core loop of the oscillators, a feedback loop including the *frq* gene and proteins and the WC-1 and WC-2 proteins in *Neurospora* and the *per* and *tim* genes and PER, TIM, CYC, and CLK, proteins in *Drosophila*. All of these proteins are thought to act in the nucleus but other proteins including the kinase (encoded by *dbt* in the fly) and potentially the gene products of other clock genes, act in the cytoplasm. Light acts through the WC proteins to reset the *Neurospora* clock by inducing *frq* and to induce the transcription of other genes including the *ccgs* (clock-controlled genes). Light acts in *Drosophila* to reset the clock by initiating the turnover of TIM which destabilizes PER. *ccgs* are output genes, regulated by the clock but not a part of this feedback loop. CCRE, clock-control regulatory element.

positive elements (the activators of PAS proteins) rather than acting directly on the clock gene promoter. It is likely that PER and TIM enter the nucleus soon after their synthesis, just as FRQ does, as PER and TIM mRNA levels begin to decline within 3 hrs of dusk, hours before a mass movement of PER and TIM into the nucleus that is seen around midnight [30]. Through the night, PER and TIM become increasingly phosphorylated [15,31] apparently through the action of the *Drosophila* homolog of mammalian casein kinase 1 ϵ , the clock element identified as *double-time* (*dbt*) in another forward genetic screen for clock genes [32*,33**]. PER and TIM finally turn over during the early part of the subjective day.

In both *Neurospora* and *Drosophila*, the genetics of these clock elements have tied the cell and molecular biology of the loop described above to the overt rhythms in the organism. *frq* [18], *per* [17], *tim* [34], *Clk* [6**], *cyc* [7**], and *dbt* [33**] were all identified in forward genetic screens for mutations affecting the clock, and period effects are now known for *wc-2* also (M Collett, personal communication). Clock roles for *Clk* and *cyc* were identified independently through molecular biological means [5**] as were *wc-1* and

wc-2 [4**]. A particularly satisfying aspect of the work to date is the remarkable degree of functional conservation — clock genes as negative elements, PAS protein heterodimeric transcriptional activators as positive elements in a transcription/translation-based negative feedback loop — among circadian systems separated by billions of years of evolution. Although extended sequence (as distinct from functional) conservation of clock elements across the entire eukaryotic span is limited to the PAS and transcriptional activation domains, several *Drosophila* genes have true mammalian sequence homologs — PER as PER1 [12**,13**], PER2 [35*,36*], and PER3 [35*], CLK as CLOCK [8**], CYC as CLOCK's partner BMAL1 [37**,38**], and DBT as casein kinase 1 ϵ [32*,33**] — suggesting true conservation of these clock feedback loops.

Although this really does make a nice 'just-so' story — a plausible core oscillator bolstered by genetics showing that loss-of-function of *frq* or *wc-1*, or *wc-2*, or *per*, *tim* or *cyc* results in a clock that either will not run at all or cannot run in a sustained manner — there are many reasons to believe that it will not be as simple as just this. PER cycling persists in the *Drosophila* eye, albeit weakly, in the absence of

per mRNA cycling [39•], a conclusion consistent with other studies in insects showing evidence for a post-transcriptional loop [40,41,42•]. In PER-expressing presumptive clock neurons in the moth brain, PER appears always non-nuclear [43]. Antisense clock gene transcripts have been detected in the same moth [43] and in *Neurospora* [44], suggesting additional regulation. Regulated translational control gives rise to multiple forms of FRQ (see below) [14•,45••], a process that may also occur with TIM [46], and a *per* transgene that perfectly rescues behavioral rhythmicity is blatantly hypophosphorylated [47]. The *frq* and mammalian *per* transcripts peak in the day in the brain, whereas *Drosophila* has a night-phase clock. Finally, a number of mutant genes with strong effects on period length exist, particularly in *Neurospora*, that are not yet cloned and placed in the scheme (see below).

Further, it is important to keep a view of the core oscillator in the context of the whole cell and the attendant aspects of physiology, development, and metabolism that it controls. Cells are rife with feedback oscillators (as I have noted before [19]) — for instance, as the natural result of feedback regulation of metabolic pathways via endpoint control — and it seems impossible that the clock would not also influence some of these, and therefore be connected to them. Further, there is evidence for clock regulation of input in some systems (e.g. [48]), feedback of output back to input [3], or both [2] — so in a very real sense the whole organism with all of its inter-regulated metabolism must be considered the ‘circadian system’ in that elimination of any part of it ought to (and does) affect the rest. However, all of it is not required for building a circadian oscillator. An expectation from this would be that if the core oscillator is removed genetically (for instance, by a loss-of-function mutation in *frq* or *per*) residual oscillations might be expected to remain that would have lost many of their true circadian characteristics, including persistence, temperature and nutritional compensation, and homeostasis of periodicity. Such oscillations are predicted theoretically [49••] and have in fact been described in both *Neurospora* [50,51] and *Drosophila* [52,53] in null mutants of *frq* and *per* respectively.

What might these loops be? The short answer is that we do not know, although one would argue from first principles that unbiased forward genetic screens ought to identify them if they are indeed important for the operation of the clock; indeed there are a number of hints, both in genes with period effects that have not yet been cloned and in genes with small effects that are. Among the *Neurospora* genes identified in forward screens, *prd-1*, *prd-2*, *prd-3*, *prd-4*, *prd-6* [54•] and *chr* (reviewed in [20,55]) have yet to be cloned, although this will get much easier within the year as the physical map of *Neurospora* is completed. Among known genes, *oligomycin resistance (oli)*; a mitochondrial ATPase subunit [56]), *arg-13* (a mitochondrial arginine carrier [57]), and *spe-3* (spermidine synthase [58,59]) have been cloned and suggest a connection between mitochondrial function and rhythmicity, although the period

effects in all these cases are small. In contrast, methionine starvation of *cys-9* strains devoid of thioredoxin reductase shortens the period by 5 hrs [60], an effect that is difficult to interpret mechanistically at present. Similarly, the *cel* and *chol-1* mutants which affect lipid synthesis are reported to be defective in temperature compensation [61,62]. Since, as mentioned above, it is impossible to imagine that the single feedback loops described in either *Drosophila* or *Neurospora* comprise the entire oscillator, and given experimental evidence for residual (albeit non-circadian) rhythmicity in the absence of canonical clock genes like *frq*, it is likely that future insights into oscillator function will come from the cloning of some of these uncharacterized genes and the molecular dissection of the functions and the ways in which they affect the clock.

Influence of environmental factors on the rhythm

Clocks function in organisms that live in the real world and the operation of these clocks is influenced by external cues in ways that keep the clock adaptive under various environmental conditions. Reflecting the biological niches of the organisms studied, the two principal time-giving agents in most circadian systems are light and temperature, although a number of other cues have been described (e.g. [63,64]) in other organisms.

Light resets the *Neurospora* clock by acting rapidly through the WC-1 and WC-2 proteins to induce *frq* [4••,29]. As *frq* mRNA and FRQ levels normally cycle with a phase that is strictly correlated with biological time (i.e. subjective dawn always corresponds to low *frq* transcript and low protein, and the peak in *frq* mRNA means late morning), any abrupt change in *frq* levels is tantamount to an abrupt change in time. Hence, in the late night and early morning when *frq* mRNA levels are rising, induction of *frq* rapidly advances the clock to a point corresponding to midday, whereas through the subjective evening and early night when *frq* is falling, induction rapidly sends the clock back in time to peak levels (corresponding to midday), yielding a phase delay [29]. A similar phasing of expression is seen in the mammalian putative clock genes *per1* and *per2* [12••,13••,35•] and, as a result, it is not surprising that a mechanism quite similar to that seen for *Neurospora* appears to hold for light resetting of the mammalian clock [35•,36•,65•]. Based upon the same logic, as the *Drosophila* clock is phase-reversed with respect to the light/dark cycle compared to the fungal and mammalian clocks, one might expect the mechanism of resetting also to be different — which indeed it is. Light results in the rapid turnover of TIM protein, and as TIM is required to stabilize PER, PER also disappears. Thus, in the late day and early evening when PER and TIM are increasing, light results in a delay back to the low point of PER and TIM, and in the late night and early subjective morning, light-induced destruction of PER and TIM results in their premature disappearance and thereby advances the clock into the next day [16,31,66,67].

Ambient temperature influences rhythmicity in several ways: first, temperature steps reset the clock in a manner similar to light pulses; second, there are physiological temperature limits for operation of the clock; but, third, within these limits the period length is more or less the same ('temperature compensation'). Compensation remains a hard nut to crack and is being approached through both theoretical (e.g. [68]) and molecular [69] routes, the latter of which interestingly demonstrates the influence of natural selection on the sequence of the clock gene *per*. Temperature-resetting responses have now been studied in *Neurospora* in some depth and, unlike the case with light where transcriptional regulation is key, temperature effects are mediated through translational control so far as they are understood. As noted above, *frq* transcripts give rise to both a long and short form of FRQ as a result of alternative in-frame initiation of translation. Although either form alone is sufficient for a functional clock at some temperatures, both forms are necessary for robust overt rhythmicity. Temperature regulates the total amount of FRQ and the ratio of the two FRQ forms by favoring different initiation codons at different temperatures and when either initiation codon is eliminated, the temperature range permissive for rhythmicity is reduced. This novel adaptive mechanism extends the physiological temperature range over which the clock can function [14•,45••].

The resetting of the clock by temperature steps also reflects post-transcriptional regulation. Although *frq* transcript oscillations at different temperatures are close to superimposable, FRQ amounts oscillate around higher levels at higher temperatures — the lowest point in the curve (late night) at 28°C is higher than the highest point in the curve (late day) at 21°C — so the 'time' associated with a given number of molecules of FRQ is different at different temperatures. A shift in temperature thus corresponds to a shift in the state of the clock (literally a step to a different time) although initially no synthesis or turnover of components occurs. Following the step, relative levels of *frq* and FRQ are assessed in terms of the new temperature, and they respond rapidly and proportionally. Hence, unlike light which acts via a photoreceptor outside the loop, temperature changes reset the circadian cycle instantaneously and from within [70••]. Exposure of *Drosophila* to elevated (heat-shock) temperatures results in the turnover of PER and TIM and phase delays in the early evening, although it has little effect in the late night [71•]. Surprisingly too, contrary to expectations in the field, non-extreme temperature changes in *Neurospora* can have a stronger influence on circadian timing than light [70••] but in all cases light and temperature cues reinforce each other to keep clocks synchronous in the real world.

Conclusions

There is now an awareness of the genuine molecular common ground among circadian systems; a very similar circadian clock-associated feedback loop is found in organisms from a eukaryotic evolutionary lineage extending from

fungi through mammals. Here, heterodimeric transcriptional activation complexes drive expression of clock genes and proteins that, after a lag, appear to negate their own activation, giving rise to an oscillation. Light acts in *Neurospora* and mammals through transcriptional means to induce the negative elements — and in *Drosophila* through post-translational means to degrade the negative elements, in this way resetting the clock and thereby synchronizing it to the daily light/dark cycle. In *Neurospora*, temperature-influenced translational regulation of FRQ synthesis sets the physiological temperature limits over which the clock operates and appears to mediate temperature-entrainment of the clock. Although the overall pattern can be seen, many details are lacking in all the systems analyzed, and immediate progress will be tied to filling out the loop by establishing its biochemical bases, for instance establishing the negative step in the loop. Longer-term progress will be tied to understanding the role of this feedback loop in the various circadian systems, and in identifying and describing additional loops — within the core oscillator or connecting the core with input and output — that may be coupled to create a complete circadian system.

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