Cell, Vol. 96, 271–290, January 22, 1999, Copyright ©1999 by Cell Press

Molecular Bases for Circadian Clocks

Review

Jay C. Dunlap
Department of Biochemistry
Dartmouth Medical School
Hanover, New Hampshire 03755

Life is a cyclical chemical process that is regulated in four dimensions. We distinguish parts of the cycle: development describes the changes from single cell to adult, and aging the changes from adult to death. Birth to death, a cycle, and there are cycles within cycles—circannual rhythms, menstrual cycles, semilunar cycles, and daily 24 hr or circadian cycles.

Twice a year we get a reminder of the importance of our internal circadian biological clocks. Daylight savings: in October we fall back just an hour, and yet we wake up an hour early on Monday anyway and think meals are late—but only for a day, until our clocks are reset. The reminder is about the way we process environmental information and time, namely that we use external time cues (light and temperature changes that track the day *without*) to set an internal clock that guides the day *within*. This internal clock is the lens through which we survey acute external factors; it takes the lead in determining what we perceive as time.

It used to be that research in chronobiology moved along at a gentlemanly pace. It was a field in that it shared a common set of problems, a common vocabulary, and a series of common assumptions: only eukaryotes had real clocks and they probably evolved just once, since the basic properties of the rhythms were generally the same. Any cell in fungi, plants, or protists could be a clock cell, but only neurons kept time in organisms that had them. Input to the clock was readily separable from how the clock itself worked. But within the past few years progress in understanding how clocks work in this assemblage of organisms has been increasing exponentially, coming to a crescendo during the final half of 1998 in an eruption of data that has largely disproven the assumptions and permanently changed the face of the field. The dust is still settling, but what we now see, albeit in broad outline, is probably the outline of how a large part of biological timing works at the molecular level. It's been quite a ride.

How'd we get this far? One ought naturally to be able to consult reviews, but there have been so many concerning the molecular analysis of rhythms that clearly what is needed here is a review of the survey of the reviews. This brings to mind a short story by H. L. Mencken in which peace of mind was brought to the literary populace in the early 1900s only through the synthesis and condensation of all of the pertinent literary critiques each week into reviews of reviews and ultimately into a grand review word (the first week being something like MIFLHMP) that readers could read, be satisfied that they were up to date, and enjoy their evenings being at home, content (Mencken, 1919). For such a telegraphically quick review of the molecular basis of the currently understood transcription/translation feedback loop (we'll get to this) circadian oscillators, the review word for the late 80s and early 90s would have been PERFRQT, reflecting the *Drosophila period* gene and the *Neurospora frequency* gene (the fruits of the first decades of genetic and molecular genetic analysis of clocks) and the fact that the *Drosophila timeless* gene, *tim*, was still in the process of arriving. This era was spent convincing ourselves that such genes really were the key to understanding how clocks work. Flies and fungi were PERFRQT systems for working out basic tools, paradigms, and approaches—gene products whose expression levels themselves oscillate, the importance of negative feedback, criteria to begin to distinguish which oscillatory gene products might contribute to the action of an internal timer as distinct from being output (reviewed in Dunlap, 1996), and a universal appreciation of the importance of genetics. If overall this left us with a less than PERFRQT understanding of timing in general, at least many found optimism in the sense that we were, finally, asking the right questions. This naturally segued into an interlude where light resetting was explained by two different mechanisms, through transcriptional induction of oscillator components in *Neurospora* (Crosthwaite et al., 1995) or protein turnover in *Drosophila* (reviewed in Young, 1998). But by mid 1997 the word was PASWCCLK (the first clock components with known biochemical functions [transcriptional activators], the first mammalian clock gene, and the first protein domain [PAS] conserved among clock molecules from different phyla) and then MPERMPER (mammalian orthologs and paralogs of model system clock genes), and then in mid 1998 the already ungainly CYCBMALJRKDBT (and a grand unifying theory for clocks within the animal/fungal clade of the crown eukaryotes; e.g., Dunlap, 1998b), and for the close of 1998 [WHAT WORD?]. So if you can be satisfied by intoning the four words in a dimly lit room, then enjoy your evening; and if not, read on to find out who's who in the phylogeny of timers.

Clocks in Cells

It is now common to begin from a general assertion that, at the most basic level, circadian oscillators (but not systems) will be describable as a circular list of causes and effects that closes within the bounds of a single cell, even in the most complicated systems like the vertebrate suprachiasmatic nucleus (SCN) (reviewed in Block et al., 1995; Welsh et al., 1995; Herzog et al., 1998). Events that happen outside the cell, or interactions of the cell with surrounding cells (e.g., Liu et al., 1997a) or the environment will influence the behavior of the clock-in-cell, but they are not necessary to describe its progress. However, events outside clock cells will affect the clock's progress, thus giving rise to the distinction between the circadian oscillator and the circadian system. Here, the oscillator is taken to mean the minimal set of molecular causes and effects sufficient to describe circadian cycles as they might operate (i.e., what you'd want to add in a reconstruction experiment to make it go). There are three general questions which, if answered in terms of genetics and biochemistry, would

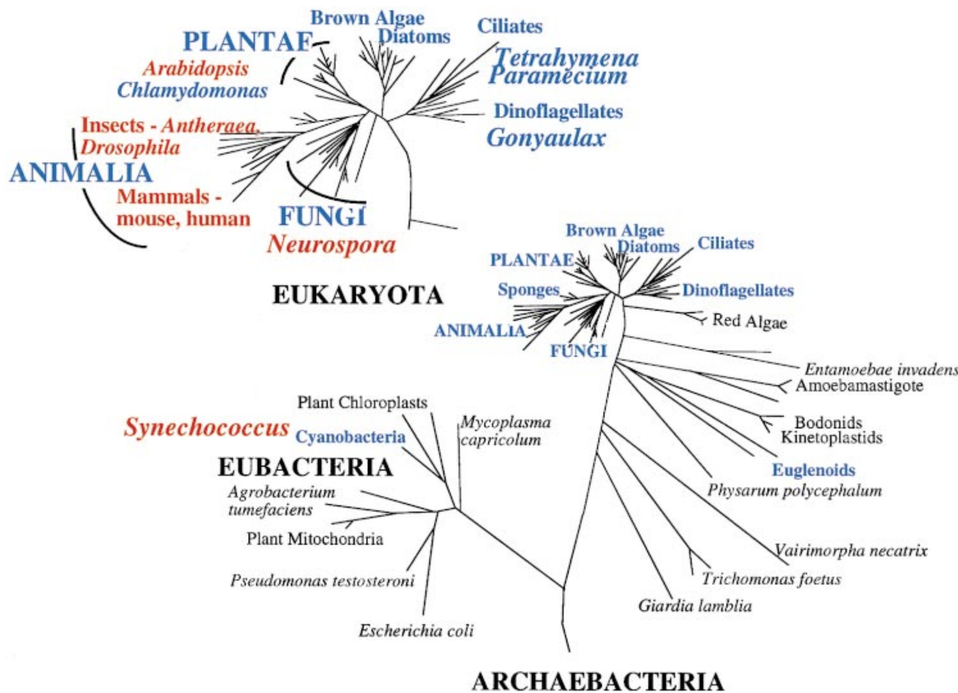


Figure 1. Circadian Systems in the Universal Tree of Life

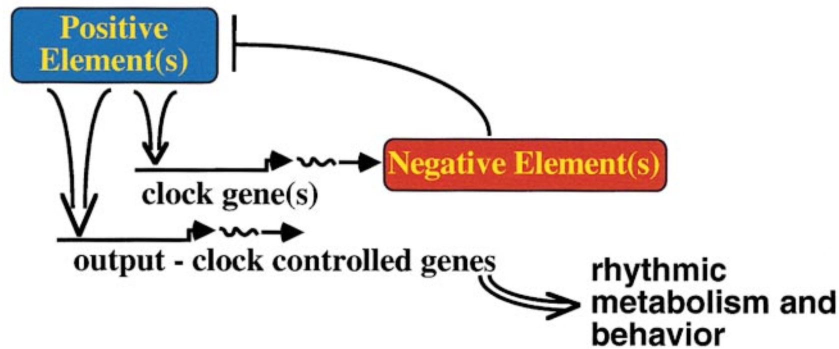
Shown is an unrooted universal phylogenetic framework reflecting a maximum-likelihood analysis for the relationships among living things. Line segment lengths correspond to evolutionary distance as measured by rates of change in small subunit rRNA genes (Sogin, 1994). The three major assemblages of organisms, Archaebacteria, Eubacteria, and Eukaryota, diverge from a single ancestor. The portion of the tree representing the "Crown Eukaryotes" that emerged (relatively) rapidly about half a billion years ago is reproduced in greater detail in the upper left. Shown in blue are phylogenetic groups where circadian rhythms have been described and/or that correspond to the well-studied experimental circadian systems, and in red are given the names and placements of those systems where the genetic and molecular analysis of clock mechanism has progressed significantly.

adequately describe this clock. The first question revolves around "How does the clock work," meaning what is the biochemical and genetic basis for the oscillator that lies at the base of the observed rhythms. A second set of questions concerns *input*—how this intracellular oscillator is brought into synchrony with the geophysical cycles of the extracellular, and extraorganismal, world. Third, given a synchronizable intracellular clock, how is the "molecular time" generated by the clock then transduced within the cell to bring about changes in the behavior of the cell, and thereby bring about changes in the behavior of the organism; this is *output*. It is clear, especially in vertebrates, that there is feedback from the output behavior back to the clock (e.g., Mrosovsky et al., 1989), from the clock to input photoreceptors (e.g., Fleissner and Fleissner, 1992), and from output to input ion channels surrounding and affecting the clock but not being necessary for its basic timing (e.g., Block et al., 1995). The ensemble of these interactions will be needed to perfectly model the circadian systems of real life; however, this narration would circumscribe more than what is my goal here, which is simply to cartoon the core oscillator(s) in clock cells.

A General Biology of Time

Some 30 years ago, Colin Pittendrigh penned for a Harvey lecture that "a truly general biology is an evolutionary biology" (Pittendrigh, 1961). By this he meant that evolution provides a great perspective for viewing any biological problem, one that emphasizes that the organization one sees in a system now is strongly dependent both upon physical necessities (which lead to convergent evolution) and upon previous choices made during evolution that delimit later options. That circadian clocks are adaptive is apparent (and recently proven [Ouyang et al., 1998]), but one of the aspects of chronobiology that so fascinates chronobiologists is the extent to which the problem itself, really one of basic self-controlling intracellular regulation, keeps luring students of time back out of the lab to consider the real world of light/dark cycles, of cyclic food availability, and of predation. We remain a long way from a true evolutionary biology of biological timing, but Figure 1 may provide a frame of reference through which to discern some trends. First, clocks have been sought in all three kingdoms, albeit only sporadically within the Archaebacteria (where none have been found). They exist in some cyanobacteria (reviewed in Golden et al., 1998) but apparently not in most Eubacteria, are found frequently among the Eukaryota, and are nearly ubiquitous among the taxa that emerged during the Cambrian phylogenetic explosion and that comprise the "Crown Eukaryotes" of the lineage, the Plantae, Fungi, and Animalia (Sogin, 1994). At first glimpse, the sightings of rhythmicity on this tree suggest the possibility of more than one but not dozens

Common Elements in the Design of Circadian Oscillators



Positive elements in circadian loops:	Negative elements in circadian loops:
kai A in Synechococcus	kaiC in Synechococcus
WHITE COLLAR-1 & WC-2 in Neurospora	FREQUENCY in Neurospora
CLK & CYC in Drosophila	PERIOD and TIMELESS in Drosophila
CLOCK & BMAL1 (MOP3) in mammals	PER1, PER2, PER3 (& TIMELESS?) in mammals

Figure 2. Common Elements in the Design of Circadian Oscillatory Loops

of independent origins for timing. However, support for such a conjecture in sequence data may be hard to come by because, perhaps reflecting their close interface with the environment, clock genes are among the most rapidly evolving genes in the organism. Be that as it may, I'll use the Tree of Life to provide some perspective on how much and how little we know, and to focus the discussion about how living things keep time.

If trends exist in the logic and molecules underlying the assembly of biological timing systems across all phyla, these trends ought to reflect the evolutionary histories of the organisms. In the accepted pattern of evolution within the terminal branches of the Tree of Life, animals and fungi share a common lineage that separated from the plants perhaps 700 million years ago, the fungi and animals subsequently diverged within a hundred million years after that, and insects from the lineage that gave rise to mammals millions of years after that. Applying the available molecular data on clocks to this phylogenetic framework, we can see common elements that may be conserved in the logic of the oscillators, in the sequences of molecules used in the oscillators, as well as in their functions within oscillatory loops—elements that are common to all living clocks, common just to the fungi and animals, common only to animals, and unique to mammals. Figure 2 is meant to provide a view of what some of the common elements might be in the logic underlying the assembly of circadian oscillators, and Table 1 a list of who's who at the molecular level.

The nature of an oscillation is that it describes a system that tends, in a regular manner, to move away from equilibrium before returning. To achieve this, all that is needed is a process whose product feeds back to slow down the rate of the process itself (a negative element), and a delay in the execution of the feedback (Figure 2). Thus, biological oscillators could be built using a number of different regulatory schemes—a metabolic pathway or an ion flux should work as well as transcription and/or translation—or kinds of delay, which could result from a threshold phenomenon preventing immediate feedback (a relaxation oscillator such as a pipette washer) or from hysteresis (a slowness of response yielding an overshoot when approaching equilibrium) or nonlinearity (as when multiple components must find each other prior to executing feedback). A further necessity for a biological oscillator is a positive element, a source of excitation or activation that keeps the oscillator from winding down. Intriguingly, all known circadian oscillators use loops that close within cells (none require cell–cell interactions), and that rely on positive and negative elements in oscillators in which transcription of clock genes yields clock proteins (negative elements) which act in some way to block the action of positive element(s) whose role is to activate the clock gene(s).

Figure 2 shows such an oscillator schematically and includes the names of some of the cognate elements identified in different circadian systems currently under study. This picture could be taken as implying that circadian oscillators will be simple transcription/translation feedback loops, but they will not; this is just what is in common about what has been described so far in the feedback loops that are generally (but not universally [Lakin-Thomas, 1998; Roenneberg and Merrow, 1998]) taken as one of the core oscillatory loops of circadian systems. It didn't have to be this way, and in fact in the premolecular era (when clock models outnumbered data), most models did not incorporate transcription but instead were centered on various aspects of metabolic regulation (see models within Hastings and Schweiger, 1976). The positive element in the loop in Figure 2 is the transcriptional activation of a clock gene(s). In the cyanobacterium *Synechococcus* this is through the action of the *kai*A gene product, and in Crown Eukaryotes (with examples here from *Neurospora*, *Drosophila*, and mouse) it is apparently through the binding of transcriptional activators, paired by virtue of interactions via PAS domains, on the clock gene promoters. Functionally

Table 1. Circadian Clock Genes: Roles, Products, and Regulation

System	Gene	Clock Role	Protein Product(s)	Regulation	Phenotype of Mutants
Synechococcus[a]	kaiA	positive element	no structural motifs identified	CR—RNA peaks ~CT 9–12; no protein data	long period (30 hr, 33 hr), ARR
	kaiB	unknown	no structural motifs identified	CR—RNA peaks ~CT 9–12; no protein data	short period (21 hr, 22 hr)
	kaiC	negative element	ATP and GTP binding sites	CR—RNA peaks ~CT 9–12; no protein data	14 alleles; long, short, and ARR
Neurospora[b]	frq	negative element	two proteins made from single open reading frame via temperature-responsive translational control; rhythmically phosphorylated	CR—RNA peaks ~CT 4, and is induced by light; protein peaks ~CT 8–12;	long period (24 hr, 28 hr) alleles show loss of temperature compensation; short period alleles (16 hr, 19 hr); ARR alleles
	wc-1	positive element; required to activate frq transcription	transcription factor: Zn finger DNA binding domain, GLN-rich activation domain, PAS domains mediate heterodimerization with WC-2	transcriptionally induced by light; relatively constant expression in the dark	null mutants and DNA binding mutants are photoblind; null mutants ARR, with low frq expression
	wc-2	positive element; required to activate frq transcription	transcription factor: Zn finger DNA binding domain, acidic activation domain, PAS domain mediates protein–protein interactions with WC-1	not induced by light; protein always present in the dark	null mutants photoblind and ARR, low frq expression; partial loss of function yields long period length, altered temperature compensation
Drosophila[c]	per	negative element	PAS domains mediate interaction with negative element TIM; rhythmically phosphorylated	CR—RNA peaks ~CT 14; protein peaks ~CT 19	several long period alleles showing loss of temperature compensation; short period alleles; several ARR
	tim	negative element	no PAS domains; interacts with PER; phosphorylated	CR—RNA peaks ~CT 14; protein peaks ~CT 19	long period length, short period, and ARR alleles
	dbt	facilitating element	sequence homolog of casein kinase 1ϵ; required for development; regulates accumulation of PER	constitutive	long period length, short period, and ARR alleles
	Clk (Jrk)	positive element	transcription factor: bHLH DNA-binding domain, GLN-rich activation domain; PAS domains mediate heterodimerization with CYC; molecular relative of mammalian CLOCK	CR in RNA is sometimes unimodal peaking at ca. CT23 or bimodal with a second peak near dusk	null mutants ARR, show low per and tim expression; no light-induced "startle" response
	cyc	positive element	transcription factor; bHLH DNA-binding domain, GLN-rich activation domain; PAS domains mediate heterodimerization with CYC; molecular relative of mammalian BMAL1/MOP3	constitutive	null mutants ARR, show low per and tim expression; no light-induced "startle" response
Mouse[d]	Per1	(putative) negative element	contains PAS domains which mediate interaction with other mammalian PER proteins; significance of interactions with negative element TIM controversial; molecular relative of insect per	clear CR in mRNA expression with peak around CT4 in SCN, around CT10 in retina and peripheral tissues; transiently induced by light	no mutants available
	Per2	(putative) negative element	contains PAS domains which mediate interaction with other mammalian Per proteins; significance of interactions with negative element TIM controversial; molecular relative of insect per	clear CR in mRNA expression with peak around CT8 in SCN, around CT14 in retina and peripheral tissues; transiently induced by light	no mutants available
	Per3	(putative) negative element	contains PAS domains which mediate interaction with other mammalian Per proteins; significance of interactions with negative element TIM controversial; molecular relative of insect per	clear CR in mRNA expression with peak around CT6 in SCN, a broad peak ~CT10–14 in retina and peripheral tissues	no mutants available
	tim	facilitating (negative?) element	clear sequence homolog of insect TIM	not rhythmically or weakly rhythmically expressed	no mutants available

(Continued)

Table 1. (Continued)

System	Gene	Clock Role	Protein Product(s)	Regulation	Phenotype of Mutants
	Clock	positive element	transcription factor: bHLH DNA-binding domain, GLN-rich activation domain; PAS domains mediate heterodimerization with BMAL1/MOP3; molecular relative of insect dCLK	not rhythmically expressed	one allele with long period length; homozygote has very long period, grading to ARR; reduced light induction of Per1
	bmal1/ mop3	positive element	transcription factor: bHLH DNA-binding domain, GLN-rich activation domain; PAS domains mediate heterodimerization with CLOCK; molecular relative of insect CYC	rhythmically expressed in rats; may not be rhythmically expressed in mice	no mutants available

Contents of this table were restricted to those genes and proteins with known or expected roles in circadian oscillatory loops.

CT, circadian time; a formalism for comparing subjective time from organisms having different endogenous periodicities. By convention, CT 0, subjective dawn, and CT 12, subjective dusk. CR, circadian rhythm observed in level of expression. ARR, arrhythmic.

[a] A cyanobacterial system displaying a transcription/translation–based negative feedback oscillator.

[b] A fungal system displaying a negative feedback transcription/translation–based oscillator and using heterodimeric PAS domain–containing transcription factors as positive elements.

[c] An insect system displaying a negative feedback transcription/translation–based oscillator, using heterodimeric PAS domain–containing proteins as positive elements, and having paired negative elements.

[d] A mammalian system displaying a negative feedback transcription/translation–based oscillator, using heterodimeric PAS domain–containing proteins as positive elements, and a gene family of three similar negative elements.

similar PAS domain–containing DNA-binding clock elements (or putative clock elements) have been described in the three best molecularly studied eukaryotic clock systems, *Neurospora* (Crosthwaite et al., 1997), *Drosophila* (Allada et al., 1998; Darlington et al., 1998; Rutila et al., 1998), and mouse (Gekakis et al., 1998; Hogenesch et al., 1998; see Reppert, 1998). These positive elements drive transcription of the clock gene(s) giving rise to a message(s) whose translation generates a clock protein(s) that provides the negative element in the feedback loop. These are the *kai*C gene product in *Synechococcus*, FRQ in *Neurospora*, PER and TIM in flies, and (presumably) PER1, PER2, and PER3 in mammals (and maybe also mammalian TIM). The negative element in the loop feeds back to block the clock gene's activation so the amount of clock gene mRNA declines, and eventually the level of clock protein also declines. Since as the loop cycles it generates cyclical inhibition of transcription factors (the positive elements), the action of these positive elements on other clock-controlled genes provides an appealing idea for the escapement by which time information from the oscillator might drive output by virtue of regulating target clock-controlled genes (*ccg*s) (Honma et al., 1998; Dunlap et al., 1999); confirmation of this mode of *ccg* regulation within the SCN has just appeared (Jin et al., 1999). This robust daily cycling of clock gene mRNA (Hardin et al., 1990; Aronson et al., 1994a; Sehgal et al., 1995; Sun et al., 1997; Tei et al., 1997; Ishiura et al., 1998), clock protein (Siwicki et al., 1988; Myers et al., 1996; Garceau et al., 1997), and clock-controlled gene RNA and protein (reviewed in Dunlap, 1998a; Hall and Sassone-Corsi, 1998; Loros, 1998) is characteristic of circadian systems. Evidence supporting this loop as a core of circadian oscillators lies both in the internal consistency of the underlying genetics—all genes identified in screens for circadian clock-affecting genes in cyanobacteria, *Neurospora*, *Drosophila*, and mice, whose functions are known can be nicely fit into this framework—and in the fact that environmental effects upon these components has in several cases been shown to underlie resetting of the clock cycle by environmental cues of light and temperature. (A potential caveat here might have been that the original rhythm-mutant screens targeted nonessential genes; however, more recently screens in flies and fungi have not been biased against lethals and yet they continue to turn up new mutations in old loci. Perhaps we may be closing in on a full list.) Although not all of the details of all of the above have been described yet in all systems from cyanobacteria through fungi through humans, many of these elements are known in all of the systems examined, and the threads of similarity among all systems suggest that this emerging theme may reflect a common mechanistic core for most if not all lineages of circadian oscillators.

Molecular Bases for Circadian Oscillatory Loops

Within the past 2 years enormous progress has been made in describing the molecular details of circadian systems in five groups of organisms that appear at different places in the Tree of Life: cyanobacteria, plants, fungi, insects, and mammals. Building on the conceptual framework developed above we can describe what may be central aspects of the molecular bases for keeping

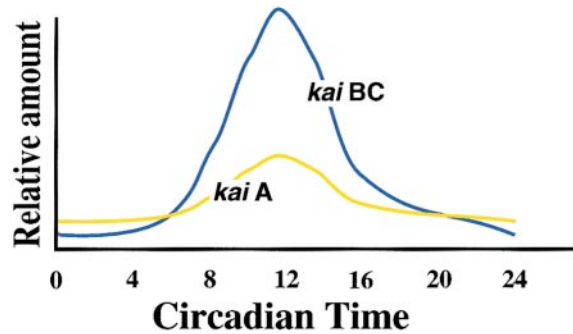
time in at least four of these groups—cyanobacteria, fungi, insects, and mammals. I'll follow the assembly and operation of circadian oscillatory loops from the simplest to the most complex, drawing attention to similarities as the complexity increases, since these similarities may identify the choices made during the evolution of circadian timing systems. Because (to my mind) the state of the oscillator in plants is still in flux, this will be revisited last of all.

Cyanobacteria—an Intracellular Feedback Loop Involving Transcription and Translation

The circadian system in *Synechococcus* is the first non-eukaryotic clock to be described. The identification of clock mutants (Kondo et al., 1994), and their cloning and analyses this year (Ishiura et al., 1998) which revealed completely novel genes connected by similar regulatory mechanisms, represents a major advance in our understanding of biological timing systems.

The circadian clock in *Synechococcus* spp. regulates a variety of aspects of the life of this nonfilamentous cyanobacterium, including cell division, amino acid uptake, nitrogen fixation, photosynthesis, carbohydrate synthesis, and respiration (reviewed in Golden et al., 1997). The particular utility of a timing system is manifested in the last two of these, since the nitrogenase enzyme required for nitrogen fixation is poisoned by the oxygen evolved from photosynthesis. Although the growth rates of wild-type and clock-mutant strains are similar (Kondo et al., 1994), the adaptive significance of this cyanobacterial clock was recently confirmed by demonstrating that strains have a competitive advantage in an environment where the period length of their clock most closely approximates that of the periodic environment in which they compete (Ouyang et al., 1998).

Mutations in genes affecting the operation of the *Synechococcus* clock were obtained in a reporter screen assay (Kondo et al., 1994). Beginning from the knowledge that photosynthesis is clock-regulated, a photosynthetic system II gene promoter (*psbAI*) was fused to bacterial luciferase and used to drive rhythmic bioluminescence (Golden et al., 1997). With some clever engineering and computer programming, this colony-based assay was readily adapted to screening on plates for colonies with long or short period rhythms, a screen that turned up more than 50 mutants with period lengths ranging from 14 to 60 hr. The genes corresponding to the mutant alleles were cloned by complementation with a wild-type library and shown to comprise a cluster of three genes known as the *kai* genes (from the Japanese *kai* for cycle) (Table 1, Figure 3). Expression of these genes is driven from two promoters, one for *kai*A and one driving expression of both *kai*B and C. Virtually all of the known period mutations in *Synechococcus* can be complemented by a wild-type copy of the *kai* gene cluster, and given the number of independent hits on these genes, the data suggest that the genetics (at least from this type of nonconditional screen) is saturated. One other modifier locus (Kutsana et al., 1998) has recently been identified and, through modified screens, the identification of additional genes is anticipated (Golden et al., 1998; Ishiura et al., 1998).



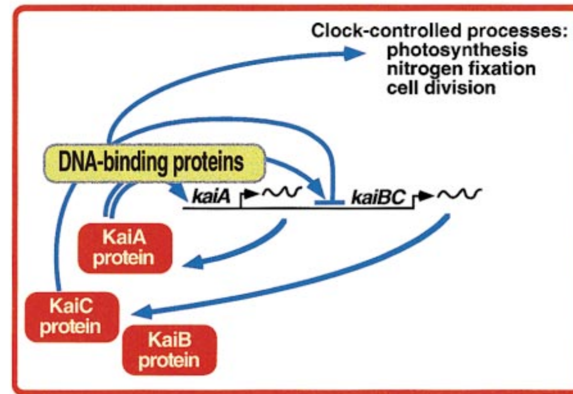


Figure 3. Identity and Regulation of Elements in the *Synechococcus* Clock

(Top) Temporal regulation of the *kai* genes. Yellow, positive element; blue, negative element.

(Bottom) Elements in the regulatory network comprising the core oscillator in *Synechococcus*.

The functions within the oscillator of the different genes in the cluster can be inferred from their regulation and from the phenotypes of alleles; deletion or overexpression of either *kai*A or *kai*BC results in arrhythmicity but not in the same way (Ishiura et al., 1998). *kai*A is rhythmically expressed with an RNA peak late in the subjective day, around CT 9–12 (Figure 3). (CT, circadian time, is a formalism for normalizing subjective biological time under constant conditions among organisms with different endogenous period lengths. By convention CT 0 corresponds to subjective dawn, and CT 12 to subjective dusk.) Loss-of-function mutations of *kai*A result in arrhythmic expression from the *kai*BC promoter, and overexpression of *kai*A yields constant superelevated expression *kai*BC and, again, arrhythmicity. These data suggest a role for the *kai*A gene product as an activator of transcription. *kai*B and C are expressed more or less in synchrony with *kai*A with a sharp RNA peak around CT 12, and inactivation of either or both genes yields arrhythmicity and elevated expression from the *kai*BC promoter. Overexpression of *kaiC* results in arrhythmicity and in severely dampened expression of *kai*BC but not of *kai*A, and pulsatile production of *kai*C resets the oscillation (Ishiura et al., 1998). These data are consistent with a role for the *kai*C gene product as a negative element in the oscillator; no role can currently be assigned to *kai*B. Limited data on protein–protein interaction among the gene products is consistent with their

expected roles in the clock such that a model can be envisioned (Figure 3B, Ishiura et al., 1998).

An outstanding question for the cyanobacterial system is, How is transcription actually affected by the products of the *kai* cluster? Surprisingly, the sequences of the *kai*-encoded proteins have not shed significant light on this. No protein structural motifs were found in either kaiA (33 kDa) or B (11.4 kDa); kaiC (58 kDa) has several putative ATP/GTP-binding sites, but none of the proteins carry motifs suggesting a role in nucleic acid binding. However, the proteins clearly do affect RNA levels, so an attractive possibility is that they might act to regulate the activity of polymerase itself or the activity of an essential regulator of polymerase, such that mutants in the essential gene would have been missed in the nonconditional screen. Cyanobacterial circadian rhythms can be synchronized ("entrained" in the circadian lexicon) to temperature and light-dark cycles (reviewed in Golden et al., 1997), and there are elements of an action spectrum for this photoresponse and for light effects on the levels of several photosynthesis-related genes (Tsinoremus et al., 1994) suggesting the action of a photoreceptor. However, expression of the *kai* genes is not acutely affected by light, and currently there is no information on how entrainment might operate for this oscillator.

Output in the *Synechococcus* system reveals an embarrassment of riches—virtually the whole genome is under circadian control (Liu et al., 1995). The expression of most genes is in synchrony with that of the *kai* cluster, although not all waveforms are the same and there are genes expressed in antiphase with this (Liu et al., 1995, 1996). Interestingly, even noncircadianly regulated promoters from other organisms (including simply an *E. coli* consensus promoter) become rhythmically expressed in *Synechococcus*. This leads one inescapably to the conclusion that rhythmic gene transcription here is the default brought about through a general non–gene-specific mechanism (effected, for instance, by large-scale changes in DNA organization, energy charge, or polymerase activity) (Golden et al., 1997), which would still allow for the presence of *cis* elements modifying the regulation of individual genes. Indeed, an example of this was found where disruption of a sigma factor gene specifically affected the expression of a subset of genes without affecting the clock itself (Tsinoremas et al., 1996), thus fulfilling the very definition of a *ccg* (Loros et al., 1989; Loros, 1998). It must be remembered also that widespread rhythmic transcription does not necessarily mean that all proteins will cycle in activity or amount.

Synechococcus provides a clear example of a minimal system that nevertheless fulfills all the requirements to be called truly circadian—an oscillation with a period length of about a day that is entrainable to environmental cues of light and temperature and that is compensated such that the period length remains approximately the same when measured at different ambient temperatures within the physiological range of the organism. The logic of its assembly employs the positive and negative elements demanded by theory. Perhaps of note is that, although a variety of different cellular processes might have been chosen as the basis for the feedback loop, evolution settled upon an oscillator built upon regulators affecting core elements of the Central Dogma such that rhythmic expression of clock genes is central to the oscillator. The most parsimonious conclusion may be to assume, as suggested elsewhere (Baranaga, 1998; Golden et al., 1998), that this oscillator arose independently from those of eukaryotes and that this convergent choice of transcription was dictated by necessity. However, the cyanobacterial clock does have "a familiar ring" to it (Baranaga, 1998); it may also be that the use of rhythmic transcription represents the legacy of an evolutionary choice made by a progenitor cell eons past when rhythmicity first evolved. But in any case, cyanobacteria go one up on the quip from the mid 1980s (before it was clear that all circadian oscillators were cellular) that you don't need a brain to have a clock—they do it all, and without a nucleus.

Although several classical experimental circadian systems (including *Euglena*, *Gonyaulax*, *Tetrahymena*, and *Paramecium*) are found among the eukaryotic protists below the crown eukaryotes in the Tree of Life, few molecular details are presently available concerning the clocks in these systems, so the next step takes us within the crown and after the divergence of plants from the animals and fungi.

Neurospora—a Fungal System Displaying a Negative Feedback Transcription/Translation–Based Oscillator and Using Heterodimeric PAS Domain–Containing Transcription Factors as Positive Elements

Neurospora, with *Drosophila*, represents a salient model system in which the tools and paradigms necessary for the molecular dissection of circadian timing systems were developed. When cultures are grown on a solid substrate, the clock controls the pattern of asexual development in the region of the growing front; aerial hyphae (leading to the production of vegetative spores) arise through development from mycelia laid down in the late night through early morning, whereas mycelia laid down at other times of day are determined not to develop. Although the clock runs (for instance in liquid culture) in the absence of this rhythmic change in growth habit, it remains the most obvious manifestation of the *Neurospora* clock. The circadian nature of this developmental switch was noted 40 years ago (Pittendrigh et al., 1959), and the first clock-mutant strains, alleles of the *frequency* (*frq*) gene, appeared in the early 1970s (Feldman and Hoyle, 1973). At present some 30 distinct rhythm altering mutations exist defining 14 genes, most of which have not been cloned. The first cloned and best understood is *frq*.

frq is a clock gene that encodes central components of an oscillatory loop within the circadian clock of *Neurospora* (Aronson et al., 1994a, 1994b; Dunlap, 1996). The oscillator includes an autoregulatory feedback cycle (Aronson et al., 1994a) in which *frq* gives rise to transcripts encoding two forms of the FRQ protein, a long form of 989 amino acids (lFRQ) and a shorter form of 890 amino acids (sFRQ) resulting from alternative initiation of translation at an internal ATG codon (Nakashima and Onai, 1996; Garceau et al., 1997). Although both FRQ

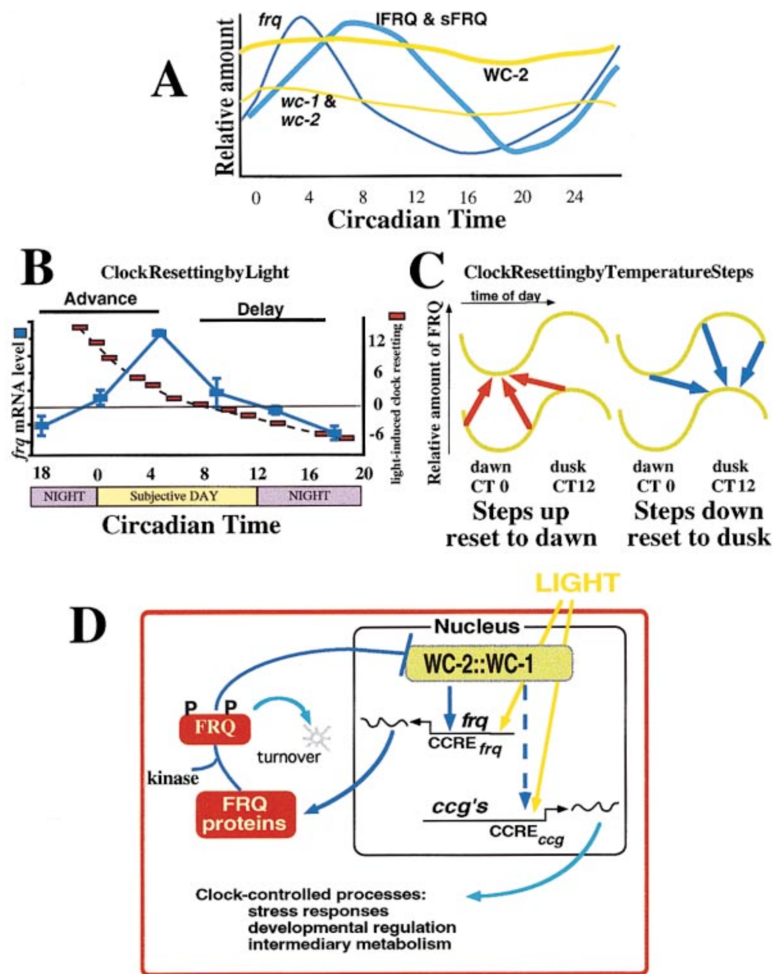


Figure 4. Identity and Regulation of Elements in the *Neurospora* Oscillator and Their Roles in Entrainment

(A) Temporal regulation of the *frq* gene and the large (lFRQ) and small (sFRQ) proteins. Here, as in subsequent figures, shades of blue denote negative elements in the oscillator, and yellow denotes positive elements. Thin lines correspond to mRNA and thick lines to protein. Care has been taken concerning the relative amplitude of the oscillation and in the timing of peaks.

(B) How light resets the *Neurospora* clock. Light rapidly transcriptionally induces the *frq* gene. If *frq* mRNA levels are already slowly rising, this rapid induction results in an advance into the day phase; if *frq* mRNA levels are slowly falling, this rapid increase results in a delay back to the day phase.

(C) How temperature resets the *Neurospora* clock. Yellow lines follow the cycle of FRQ protein levels through the day at low temperature (lower curve) and at higher temperatures (upper curve) within the physiological range; red arrows track the effect of temperature steps up, and blue arrows track steps down. For steps up, all the points on the lower temperature curve are low compared to the high-temperature curve, so the clock is reset to the time corresponding to the low point in FRQ—near to subjective dawn. For steps down, the reverse is true: all the points on the higher temperature curve are high compared to the low-temperature curve, so the clock is reset to the time corresponding to the high point in FRQ—late day to subjective dusk.

(D) Elements and control logic in the circadian oscillatory loop of *Neurospora*. Arrows denote positive regulation, and lines terminating in bars denote negative regulation. CCRE, circadian clock regulatory element.

forms are required for robust rhythmicity across the physiological range, a functional distinction between the forms has yet to be discovered (Liu et al., 1997b). The levels of both *frq* RNA and FRQ cycle (Figure 4A, Aronson et al., 1994a; Garceau et al., 1997), and FRQ acts to depress the level of the *frq* transcript (Aronson et al., 1994a), very likely by interfering with the normally required activation of the gene by a heterodimeric activator composed of WHITE COLLAR-1 (WC-1) and WC-2 (Crosthwaite et al., 1997). In this negative feedback loop, rhythmic change in the amount of *frq* transcript appears essential for the overt circadian rhythm (no level of constant *frq* expression supports the rhythm), and abrupt changes in *frq* expression reset the clock (Aronson et al., 1994a).

Using Figure 4 as a guide, we can follow the progress of the *Neurospora* clock cycle starting from midnight. At this time *frq* RNA and FRQ levels are low, but *frq* transcript is beginning to rise, a process that will take about 10–12 hr to reach peak. This increase in *frq* is the result of action by a heterodimeric pair of transcription factors encoded by *wc-1* and *wc-2* (Crosthwaite et al., 1997); these positive elements are the PAS proteins in the *Neurospora* system. WC-1 and WC-2 have bona fide PAS dimerization domains; they homo- and heterodimerize in vivo and in vitro with each other and with canonical vertebrate PAS proteins such as AHR, all via their PAS domains (Ballario and Macino, 1997; Ballario et al., 1998); they bind specifically to elements in the promoters of genes that they transcriptionally activate (although this has yet to be shown specifically for *frq*). After a lag that represents a regulated part of the circadian cycle (Merrow et al., 1997), FRQ proteins begin to appear just before dawn (Garceau et al., 1997) and soon enter the nucleus (Garceau et al., 1997; Luo et al., 1998) where they interact with the WC proteins. In important confirmations of predictions from the model, FRQ interacts with WC-2 in vitro, and a partial loss-of-function allele of *wc-2* displays both a long period length and altered temperature compensation. (Temperature compensation refers to the characteristic, universal and defining among circadian clocks, that the endogenous period length is relatively constant when measured at temperatures across the physiological range.) The *wc* transcripts and the WC-2 protein are always present in the cell (no data are available yet on WC-1); their levels may show slight circadian variations but not significant cycling. *frq* mRNA levels peak in the midmorning (Aronson et al., 1994a; Crosthwaite et al., 1995) about 4 hr before the peak of total FRQ in the early afternoon (Garceau et al., 1997). As soon as either form of FRQ can be seen, they are already partially phosphorylated. Midday

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 FRQ, now becoming extensively phosphorylated, declines through the early night, consistent with the hypothesis that phosphorylation triggers FRQ turnover (as it apparently does with PER; Price et al., 1998).

Why does this feedback loop oscillate with a 22 hr period length? There are two issues here, the nature of the required time lag between transcription and negative feedback, and the origin of the long 22 hr time constant. We know from reconstruction experiments (where in a *frq*-null strain a transgenic *frq* is driven from a regulatable heterologous promoter) that the part of the feedback loop extending from the onset of *frq* transcription through the complete decline in *frq* mRNA levels occurs relatively rapidly, requires fewer than 25 molecules of FRQ per nucleus, and can take place in as little as 6 hr; however, nearly 14 hr are required for FRQ to become phosphorylated and to turn over, so for most of the day *frq* transcript levels are low and FRQ levels are at least somewhat elevated (Merrow et al., 1997). Thus, it appears that the long time constant arises in part due to the kinetics of turnover of both forms of FRQ. The finding (Luo et al., 1998) that FRQ enters the nucleus within a few hours after synthesis suggests that prenuclear events have relatively less to do with the long time constant than do posttranslational events; this is not the case in *Drosophila* (see below). However, it is clear that essential actions of FRQ for the clock happen in the nucleus (Luo et al., 1998) and that these events are separated in time from the onset of *frq* transcription, so this time difference must be sufficient to initiate an oscillation rather than yielding an equilibrium. Factors that affect either nuclear entry or FRQ turnover should affect both the period length of the clock and the ability of the loop to oscillate. If FRQ fails to enter the nucleus (as seen in Luo et al., 1998) or if it could not turn over within a day, the loop should cease to oscillate but would instead simply reach an equilibrium and act to moderate the level of *frq* and FRQ expression.

A number of additional clock genes and clock-affecting genes have been described in *Neurospora*, some of whose actions can be understood in terms of the *frq*/FRQ feedback cycle and others of which will point the way to novel interactions, regulations, or possibly even additional feedback loops that contribute to create the whole circadian system. Among those identified in forward genetic screens for period length mutants, *prd-1*, *prd-2*, *prd-3*, *prd-4*, *prd-6* (Morgan and Feldman, 1997), *chr* (reviewed in Dunlap, 1996; Dunlap et al., 1998), and *rhy-1* (Chang and Nakashima, 1998) have yet to be cloned (although this will get much easier within the year as the physical map of *Neurospora* is completed). The extant clock models would predict that a partial loss of RNA polymerase I function might result in modest period lengthening, which is the case (Onai et al., 1998). Mutations resulting in small period effects in genes affecting mitochondrial metabolism include *oli* (a mitochondrial ATPase subunit; Dieckmann and Brody, 1980), *arg-13* (a mitochondrial arginine carrier; Liu and Dunlap, 1996), and *spe-3* (spermidine synthase; Susuki et al., 1996; Katagiri et al., 1998). Methionine starvation of *cys-9* strains (lacking thioredoxin reductase) shortens the period by 5 hr (Onai and Nakashima, 1997); both *cys-4* and *cys-12* strains display slightly shortened periods when starved for cysteine (reviewed in Dunlap, 1996), and the *cel* and *chol-1* mutants that affect lipid metabolism are reported to be defective in temperature compensation (Mattern et al., 1982; Lakin-Thomas et al., 1997), although presently these effects are difficult to interpret mechanistically. It seems likely that cloning of some of these genes and molecular dissection of their functions and the ways in which they affect the clock may reveal unexpected regulatory relationships between cellular metabolism and the operation of the circadian oscillators that operate within the cell.

These molecular feedback oscillators at the core of circadian clocks operate in cells that live in the real world, so they must be synchronized with (entrained to) real world cycles; the goal of entrainment by light is to move the day phase of the clock (subjective day) so that it coincides with the day phase of the external world. Therefore, the molecular basis of entrainment by light is that the same photic cue must have opposite effects on the timing mechanism depending on whether light is perceived in the early evening (when delays are needed) or late in the night (when advances into the next day are needed). For this reason, with clock components that peak in the daytime, photic induction of the components will reset the clock quite well (Figure 4B). In *Neurospora*, light acts rapidly through the WC-1 and WC-2 proteins to transcriptionally induce *frq* (Crosthwaite et al., 1995, 1997); only WC-1 is required for this transient induction, although both proteins are required for the clock to run. Since *frq* mRNA and FRQ levels normally cycle with a defined phase (i.e., subjective dawn always corresponds to rising *frq* transcript and low protein, and the peak in *frq* mRNA means late morning), any abrupt change in *frq* levels yields an abrupt change in subjective time. Hence, in the late night and early morning when *frq* mRNA levels are rising, rapid 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 (Crosthwaite et al., 1995). This resetting model appears also to work quite well for the presumptive components of the mammalian circadian oscillator that also peak in the day, since similar results are seen in the induction of the mammalian clock genes *per1* and *per2* (Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997).

The other major zeitgeber for entrainment of most clocks is temperature, a factor that 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 (Bunning, 1973). Third, within these limits the period length is relatively constant, a characteristic known as temperature compensation. Compensation remains a hard nut to crack in all circadian systems, and it is being approached through both theoretical (e.g., Ruoff et al., 1996) and molecular routes (see below, Sawyer et al., 1997). Temperature resetting responses

are known in a variety of organisms, including *Neurospora* (Francis and Sargent, 1979) and *Drosophila* (Zimmerman et al., 1968; Winfree, 1972) and are becoming understood. Unlike the case with light where transcriptional regulation is key, in *Neurospora* temperature effects appear to be mediated largely through translational control. *frq* transcripts give rise to both a long and short form of FRQ as a result of alternative in-frame initiation of translation that favors the short form of FRQ at low temperatures and the long form at high temperatures. Although either form alone is sufficient for a functional clock at some temperatures, both forms are necessary for robust biological rhythmicity. Temperature thus regulates both the total amount of FRQ and the ratio of the two FRQ forms by favoring different initiation codons at different temperatures; 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 functions (Garceau et al., 1997; Liu et al., 1997b).

Resetting of the clock by temperature steps also reflects posttranscriptional regulation in *Neurospora* (Figure 4C). *frq* transcript oscillates between similar limits at different temperatures, but FRQ amounts clearly oscillate around higher levels at higher temperatures—the lowest point in the curve (near subjective dawn) at 28°C is higher than the highest point in the curve (late day to dusk) at 21°C—so the "time" associated with a given number of molecules of FRQ is different at different temperatures. Thus, a shift in temperature, prior to any adjustments in FRQ levels, corresponds to a shift in the state of the clock (literally a step to a different time), although initially no synthesis or turnover of clock components occurs. After the step, relative levels of *frq* and FRQ are assessed in terms of the new temperature, and they respond rapidly and proportionally. In this way, unlike light, which acts via a photoreceptor outside the loop, temperature changes reset the circadian cycle instantaneously and from within (Figure 4C, Liu et al., 1998). Temperature changes seen at dusk and dawn in the natural environment approximate step changes, and surprisingly such nonextreme temperature changes in *Neurospora* (and in a variety of other organisms) can have a stronger influence on circadian timing than light (Liu et al., 1998). In all cases, though, light and temperature cues reinforce each other to keep clocks synchronous in the real world.

The term "ccg" (for clock-controlled gene) was coined to describe output regulatory targets of the oscillator in *Neurospora*, genes whose transcription was modulated on a daily basis but which when mutated did not at all affect the progress of the clock (Loros et al., 1989). Over a dozen such *ccg*s have been identified in *Neurospora*, and they contribute to the rhythmic control of a variety of cellular processes (Loros, 1998). Among those with known or suspected functions, *ccg-1*, *ccg-9* (trehalose synthase), and *ccg-12* (copper metallothionene; Bell-Pedersen et al., 1996b) contribute to clock regulation of stress responses, *ccg-2* (the *Neurospora* hydrophobin; Bell-Pedersen et al., 1992), *ccg-4*, *ccg-6*, *con-6*, and *con-10* (Lee and Ebbole, 1998a, 1998b) contribute to clock regulation of development, and *ccg-7* (glyceraldehyde 3-phosphate dehydrogenase; Shinohara et al., 1998) occupies a key position in the glycolytic pathway at the core of intermediary metabolism. Several *ccg*s have been shown by nuclear run-on analysis to be regulated at the level of transcription, and in *ccg-2* the circadian clock regulatory element (CCRE) has been localized to a short region near to the start of transcription separable from parts of the promoter conferring light, nutritional, or developmental regulation (Bell-Pedersen et al., 1996a).

Drosophila—an Insect Circadian System Displaying a Negative Feedback Transcription/Translation–Based Oscillator Using Heterodimeric PAS Domain–Containing Transcription Factors as Positive Elements and Having Paired Negative Elements

Drosophila, as with *Neurospora*, represents a paradigmatic molecular circadian system whose development has been central to our understanding of how clocks work at the molecular level. Beginning even in the premolecular era, seminal work by Pittendrigh on *Drosophila pseudoobscura* laid out many of the formalisms that are still in use for describing how clocks work. The originally observed rhythmic output in *D. pseudoobscura* and the one that drove most of the early research was pupal eclosion (emergence) which takes place in a tightly defined window of time near subjective dawn. More recent work has used the daily crepuscular (dawn and dusk) rhythm in locomotor activity (reviewed in Hall, 1995). Chronogenetics began in *Drosophila* in the work of Konopka, *per* was the first clock gene cloned, and a great deal of what we know about rhythms has arisen from the study of this gene, its regulation, and its products (reviewed in Hall, 1998; Young, 1998). The genetic analysis of this locus and others in the fly is ongoing (e.g., Hamblen et al., 1998). Evolutionarily, with animals comes tissue specialization of function; both mosaic and transplantation studies have localized the tissues and cells driving behavioral rhythmicity to two clusters of lateral neurons in the fly brain (Hall, 1995, 1998), but recent studies showed surprisingly that biochemically similar *period/timeless*–based cell-autonomous clocks are found in separate fly body parts (Hege et al., 1997; Plautz et al., 1997b) and that virtually every body part has a clock (Plautz et al., 1997a). How do these clocks work?

A good place to start is with a simple description of the core transcription/translation feedback loop. *per* and *tim* mRNA levels begin to rise in the subjective day (Hardin et al., 1990; Sehgal et al., 1995) and are translated into protein (Figure 5). *per* is now expected to encode a single major protein species (Cheng et al., 1998) (in contrast to a prior report; Citri et al., 1987), and aspects of PER structure reveal the influence of natural selection to fine tune the ability of the protein to operate in a clock in different environments (Sawyer et al., 1997; Peixoto et al., 1998). Although recent experiments suggest that posttranscriptional regulation contributes to this increase in *per* and *tim* RNA (So and Rosbash, 1997; Stanewsky et al., 1997), the increase is seen largely as the result of activation by a heterodimer of CLK (*Drosophila* CLOCK, also called JRK; Allada et

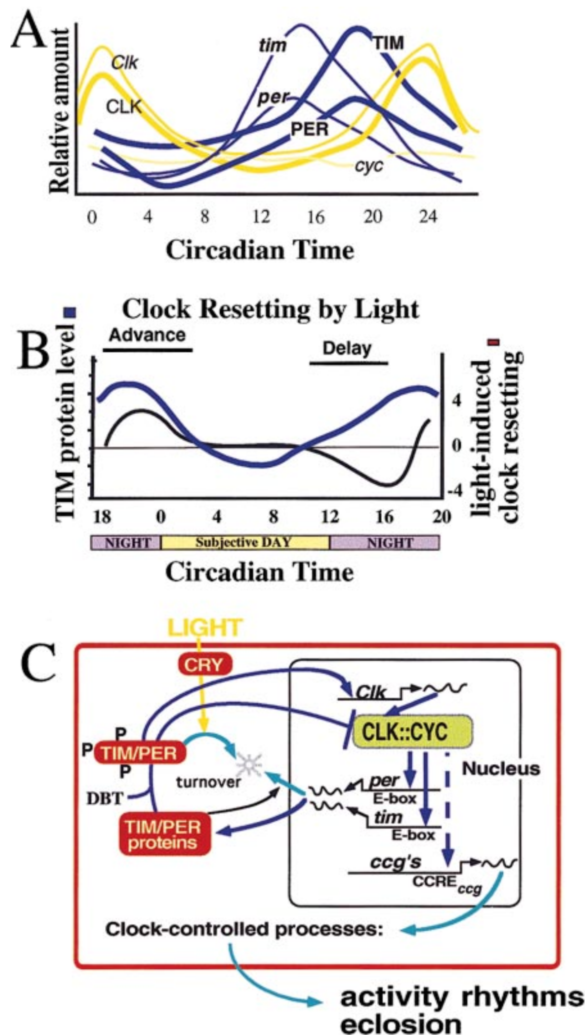


Figure 5. Identity and Regulation of Elements in the *Drosophila* Oscillator and Their Roles in Entrainment

(A) Temporal regulation of the *per*, *tim*, *Clk*, and *cyc* genes and proteins. Care has been taken concerning the relative amplitude of the oscillation and in the timing of peaks.

(B) How light resets the *Drosophila* clock. Light results in the rapid destruction of TIM whose loss destabilizes PER. If TIM levels are already slowly rising, this rapid loss results in a delay back to the previous day phase; if TIM levels are slowly falling, this rapid loss results in an advance into the next day phase.

(C) Elements and control logic in the circadian oscillatory loop of *Drosophila*. Arrows denote positive regulation, and lines terminating in bars denote negative regulation.

al., 1998) and CYC (for CYCLE; Darlington et al., 1998; Rutila et al., 1998). These proteins have PAS domains like PER, WC-1, and WC-2 that probably mediate their heterodimerization as is the case in a large number of PAS domain–containing proteins (Crews, 1998) including the WC-1/WC-2 heterodimer (Ballario et al., 1998), but they utilize bHLH domains instead of Zn fingers to bind the DNA of the E boxes in clock gene promoters (Hao et al., 1997). *Clk* is rhythmically expressed, sometimes with a double peak (Figure 5A; Bae et al., 1998; Darlington et al., 1998), but *cyc* appears to be constantly expressed (Rutila et al., 1998); this part of the feedback loop has been reconstructed in insect S2 tissue culture cells (Darlington et al., 1998). CYC is normally found in these cells, but coexpression of CLK serves to activate the *per* and *tim* genes; simultaneous expression of PER and TIM (but at a large molar excess) blocks this activation and has no effect on *per* or *tim* gene expression in the absence of CLK (Darlington et al., 1998). This is wholly consistent with the model in Figure 5C where the negative elements PER/TIM act on the positive element proteins themselves (the PAS protein activators, CLK/CYC) rather than acting directly to suppress their own promoters. PER and TIM are presumed to act in the nucleus, and their nuclear localization requires their heterodimerization (e.g., Saez and Young, 1996). *per* and *tim* mRNA levels begin to decline within 3 hr of dusk presumably due to nuclear entry of PER/TIM heterodimers in a sufficient number to execute their function, although this is several hours before the mass movement of PER and TIM into the nucleus reportedly seen around midnight (Curtin et al., 1995). Also by midnight the level of *Clk* mRNA is beginning to rise, and a recent study suggests that the timing here may be more than coincidental (i.e., that PER and/or TIM may enhance *Clk* transcription or stability [Bae et al., 1998]) in contrast to their normally negative functions. While activating *Clk* expression, PER/TIM are believed to block the activation by the CLK/CYC heterodimer and thereby turn down the level of their own expression. Molecular support for this has also recently appeared in a study demonstrating molecular interactions between CLK and PER/TIM (Lee et al., 1998). Beginning as soon as they are made and continuing through the night, PER and TIM become increasingly phosphorylated (Edery et al., 1994; Zeng et al., 1996) probably through the action of the *Drosophila* homolog of mammalian casein kinase 1ε, a clock element identified as *double-time* (*dbt*) in another forward genetic screen for clock genes (Kloss et al., 1998; Price et al., 1998). This phosphorylation affects the initial rate of PER accumulation and appears necessary for PER's turnover, since turnover is delayed and hypophosphorylated PER hyperaccumulates in *dbt* partial loss-of-function mutants (Price et al., 1998). PER and TIM finally turn over during the late night and early part of the subjective day (again, a cyclically regulated process; Dembinska et al., 1997) about when CLK levels are peaking.

Why does this feedback loop oscillate, and from where does the long 24 hr time constant arise? Data from *Neurospora* suggested the importance of postnuclear events for the long constant, but data from *Drosophila* suggest that a large part of the long time constant arises from a lag in protein accumulation prior to nuclear entry and action. As noted above, PER and TIM heterodimerize. This interaction serves two functions, the stabilization of PER (which is unstable in the absence of TIM) and the promotion of nuclear entry of the complex (Rosbash et al., 1996; Saez and Young, 1996; Hall, 1998; Young, 1998). The bimolecular nature of the interaction results in a lag in the accumulation of the complex, so that the first evidence of nuclear function (suppression of *per* and *tim* RNA levels) is seen a few hours after dusk even though transcripts began to appear by midday. Because a part of this equation is the phosphorylation-induced instability of unpartnered PER, loss-of-function

mutations in the kinase should slow or stop the oscillator, as indeed they do (Price et al., 1998). The feedback loop still works but fails to oscillate, instead settling at an equilibrium of low *per/tim* transcription, low TIM, and elevated PER (Price et al., 1998).

These scenarios for *Neurospora* and *Drosophila* describe the data well and leave one with the impression that the transcriptional feedback is essential for the clock in both cases. This was implied for the *Neurospora* *frq*/FRQ feedback loop in experiments in which the regulatable *qa-2* promoter was used to drive constant levels of *frq* expression (Aronson et al., 1994a) that could not rescue rhythmicity in a strain lacking a clock-regulated *frq*. However, a number of recent experiments in *Drosophila* have suggested that transcriptional rhythms in both *per* and *tim* may not be required for the fly clock. The first clues came in work (Frisch et al., 1994) in which a *per* construct lacking the *per* promoter and first intron was shown capable of rescuing rhythmicity in a *per*-null strain; these data suggested that promoter-mediated regulation of *per* was not required, but left the caveat that there was an internal promoter or enhancer that could drive rhythmic expression, or that the successful inserts happened to be in otherwise naturally rhythmic genes. Using nonrhythmic promoters, a construct driving *per* expression under the control of the *glass* promoter was shown capable of rescuing rhythmicity in a *per*-null strain (Vosshall and Young, 1995), and a *rhodopsin* promoter-*per* transgene driving constant expression of *per* in the *Drosophila* eye has been shown to allow *per* rhythmicity in the eye but not in the rest of the fly (Cheng and Hardin, 1998). Most recently nuclear runons were used to show that the rate of transcription in the promoterless construct of Frisch is in fact constant, thereby establishing that some aspect of posttranscriptional regulation is sufficient to close a regulatory loop (So and Rosbash, 1997). Finally, there remains the surprising finding that in the embryo of a related insect, the moth *Antheraea*, a brain-based clock runs in the apparent absence of obvious PER nuclear entry or cycling (Sauman and Reppert, 1996) (although it may be that abundant cytoplasmic PER expression is here obscuring a *Drosophila*-like PER/TIM nuclear entry and action). Overall, that *per* and *tim* transcriptional rhythms are robust and present in most insects is not in doubt, and it may be that so long as a mechanism exists to generate a delay (PER/TIM association) and to suppress PER accumulation in the absence of TIM (phosphorylation-induced turnover), the whole loop will cycle so long as *tim* cycles.

Because the *Drosophila* clock components peak and are active chiefly at night, the photic transcriptional induction model seen in *Neurospora* could not work for entrainment, and so flies use the alternative, namely light-induced turnover of clock components. For entrainment in the *Drosophila* clock, light acts through eye and extraocular pathways (Stanewsky et al., 1998; Suri et al., 1998; Yang et al., 1998) to result in the rapid turnover of TIM protein, and since TIM stabilizes PER, PER also disappears. Thus, in the late day and early evening, a time when PER and TIM are increasing, a light-induced decrease in PER/TIM results in a delay, back to the low point of PER and TIM in the day. Conversely in the late night and early subjective morning when PER/TIM levels are normally falling, the same light-induced destruction of PER and TIM results in their premature disappearance and thereby advances the clock into the next day (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996; Suri et al., 1998; Yang et al., 1998). It is not known for sure whether the PAS heterodimer CLK/CYC mediates any light effects in *Drosophila* as do WC-1/WC-2 in *Neurospora*, but mutations in either *Clk* or *cyc* eliminate the normal "wakeup" response, the increase in activity seen in flies 30 to 60 min after exposure to light (Allada et al., 1998; Rutila et al., 1998).

Recently the nature and regulation of the photoreceptors required for this response have been clarified. *Drosophila* utilizes both rhodopsin and a homolog of the flavin-mediated blue light photoreceptor-associated *cry* genes (Emery et al., 1998; Stanewsky et al., 1998). In cry^b, a point mutation of an amino acid residue required for flavin association in CRY results in no PER or TIM cycling in either constant darkness or in a light/dark (LD) cycle. However, whereas pulses of light do not entrain, full photoperiod LD cycles still do drive cycling in the ventral-lateral neurons in the fly brain, and (importantly) temperature cycles can entrain behavioral rhythms that will continue under constant conditions. These and other data suggest that CRY is the cell-autonomous photoreceptor for body clocks in the fly and may mediate nonparametric entrainment (i.e., entrainment by short discrete light pulses; discussed in Crosthwaite et al., 1995; Pittendrigh, 1961), but that the lateral neurons receive photic information both through the blue light CRY pathway and through the eye-mediated rhodopsin pathway (which may mediate entrainment by gradual changes in light, known as parametric entrainment). CRY is thus involved in light perception but is not required for operation of the clock. As regards temperature influences, studies of natural populations suggest that parts of the PER protein have coevolved to optimize temperature compensation (Sawyer et al., 1997). Entrainment by temperature changes occurs in *Drosophila* as in *Neurospora* where exposure to an elevated temperature within the physiological range results in strong resetting (Winfree, 1972; Wheeler et al., 1993; Tomioka et al., 1998) and heat shock (a short duration step to 37°C) results in the turnover of PER and TIM. Unexpectedly though, this yields only small phase delays in the early evening with no apparent effect on phase in the late night (Sidote et al., 1998). With the availability now of tools to identify and follow rhythmic expression of CLK, it will be of interest to revisit these studies to see whether the temperature effects can be better understood as affecting (or not affecting) cellular levels of this activator.

In terms of output from the oscillator, several clock-controlled genes and/or genes mediating output are known in *Drosophila* (reviewed in Hall, 1995). These include a *Dreg-5* and *Crg-1* whose functions remain obscure, and *lark* (McNeil et al., 1998; Newby and Jackson, 1996), which encodes an RNA-binding protein that acts like a repressor of eclosion. *lark* mRNA expression is not rhythmic, but protein levels cycle with a peak late in the day around CT 8, thus implicating translational regulation in rhythmic control as is known in Gonyaulax (Mittag et al., 1997). That *lark* performs an essential

embryonic function (Newby and Jackson, 1993) prior to eclosion suggests that its role may be more generalized than simply in regulating the timing of that behavior.

Mouse—a Mammalian System Apparently Using a Negative Feedback Transcription/Translation–based Oscillator, Using Heterodimeric PAS Domain–Containing Proteins as Positive Elements, and a Gene Family of Three Similar Negative Elements

The past 2 years have seen genuine progress in our understanding of the molecular basis of the mammalian circadian system. Classical genetics and molecular genetics yielded *CLOCK*, (Antoch et al., 1997; King et al., 1997b) and clever molecular screens identified *Per1* (Tei et al., 1997) and CLOCK's activator partner (BMAL1/MOP3; Gekakis et al., 1998; Hogenesch et al., 1998), but the avalanche of mammalian clock genes has arisen from analysis of genomics data as illuminated by the paradigms and molecules identified in model systems. Although the dust is still settling on this chronobiological "year of the genome project," the fascinating story that seems to be emerging is that the usual suspects are all there (sometimes more than once), but they're not always behaving as we would have expected (see Table 1). Moreover, whereas the year began with the expectation that the cells and tissues having autonomous circadian oscillators described a select few, the expectation now is that autonomous oscillations can be found in many tissues if you just know where and how to look (Balsalobre et al., 1998). Rewardingly, the mammalian oscillator has clearly taken its cues from its position in the evolutionary tree; it is gratifyingly similar to its closest well-studied relatives, the insects, and contains aspects of logic and protein structure clearly conserved from the fungi and perhaps beyond. As with the other systems herein described, a good place to start is at the beginning of the circadian day.

There are three different *per* gene relatives in the mouse (*Per1*, Sun et al., 1997; Tei et al., 1997), (*Per2*, Albrecht et al., 1997; Shearman et al., 1997; Takumi et al., 1998a) and (*Per3*, Takumi et al., 1998b; Zylka et al., 1998b); all three are related by sequence to the *Drosophila per* gene and contain PAS domains but no bHLH or other putative DNA-binding domains. (Similar genes are found in many animals; where necessary for this discussion mouse genes will have an "m" prefix; rat, "r"; human, "h"; and *Drosophila*, "d.") Transcript levels for the first of these, *mPer1* begin to increase in the late night before subjective dawn. (Phases of timed events are generally delayed in body clocks as compared to the SCN as shown in Figure 6A. Because of the body of information demonstrating the dominant role of the SCN in determining the characteristics of organismal timing, I'll follow times in the SCN here and take up the body clock differences later.) This increase is the result at least in part of activation by a heterodimer of CLOCK and BMAL1 (=MOP3) (Gekakis et al., 1998; Hogenesch et al., 1998), the mammalian equivalent of the PAS domain–protein heterodimer that acts as the positive element in the circadian loop in the crown eukaryotes; bHLH domains bind the DNA of E boxes at least in *mPer1* gene promoter. In vitro studies have demonstrated a strong interaction between MOP3/BMAL1 and MOP4, but the role (if any) of such interactions in the clock is undescribed (Hogenesch et al., 1998). The sequence of the first mammalian clock gene cloned, *Clock*, revealed a protein bearing sequence and functional similarities to the WC-1 protein (paired PAS domains, DNA-binding [bHLH instead of Zn finger] and Gln-rich transcriptional activation domains), and like *wc-1*, *wc-2*, and *cyc* (but unlike *Drosophila Clk*), the abundance of *Clock* is not circadianly regulated in mammals (Sun et al., 1997; Tei et al., 1997; although it is in zebrafish, Whitmore et al., 1998). *bmal1* is reported to be weakly circadianly regulated in the SCN antiphase to the *mper*s (Honma et al., 1998; Oishi et al., 1999). Parts of the feedback loop have been reconstructed in mammalian cells in culture (Gekakis et al., 1998), where CLOCK and BMAL1 together activate transcription from an E box in the *mPer1* promoter, and the activation is blocked by the dominant-negative action of the canonical *Clock* allele that possesses a truncation in its transcriptional activation domain—a nice molecular confirmation and explanation of its expected genetic defect (King et al., 1997a). Not long after *mPer1* levels start to rise, the levels of *mPer3* and then *mPer2* also increase (Figure 6A), and the three genes peak at different times in the day, *mPer1* first at CT 4–6 (the same time as *frq* transcript), next *mPer3* in a broad peak see between CT 4 and 8, and lastly *mPer2* with a peak late in the day around CT 8. The clear difference in timing of *mPer2* suggests that activators in addition to CLOCK/BMAL (or posttranscriptional effects) may affect *mPer2* expression, consistent with the distinct paucity of E box–like sequences in the gene. Tools to identify the PER proteins from mammals have yet to appear, but since the genes are truly similar to their *Drosophila* counterparts we can infer some of what the proteins will do based on the well understood story in flies. Thus, specifically, we expect (1) the PERs to enter the nucleus and by virtue of interactions via their PAS domains, (2) to disrupt the BMAL1/CLOCK activation of their own promoters and thereby to shut themselves off, (3) the proteins to become phosphorylated and turn over, (4) the ever present BMAL1/CLOCK heterodimer to turn the mPERs on again, and the cycle to repeat itself. It is much to soon to know the details of this, such as whether a PER interacts with the activator complex before or after it contacts the E box, but these details will doubtless emerge and it is likely that some details will be different from that seen in insects. In support of this, the mammalian *tim* gene (just one?) has been cloned, but again the story may be complicated. In several functional assays it performs somewhat like its fly counterpart, interacting with mammalian PER, weakly dampening transcriptional activation by BMAL1/CLOCK after transfection, and in insect cells helping dPER into the nucleus (Sangoram et al., 1998; Takumi et al., 1999). However, completely unlike the case in *Drosophila*, mPER–mPER interactions appear in all cases much stronger than any mPER–mTIM interactions (Zylka et al., 1998a; Takumi et al., 1999), and *mTIM* mRNA cycles weakly if at all in abundance (Sangoram et al., 1998; Zylka et al., 1998a; Takumi et al., 1999). Reviewing this now is like trying to hit a moving target; it's a good bet

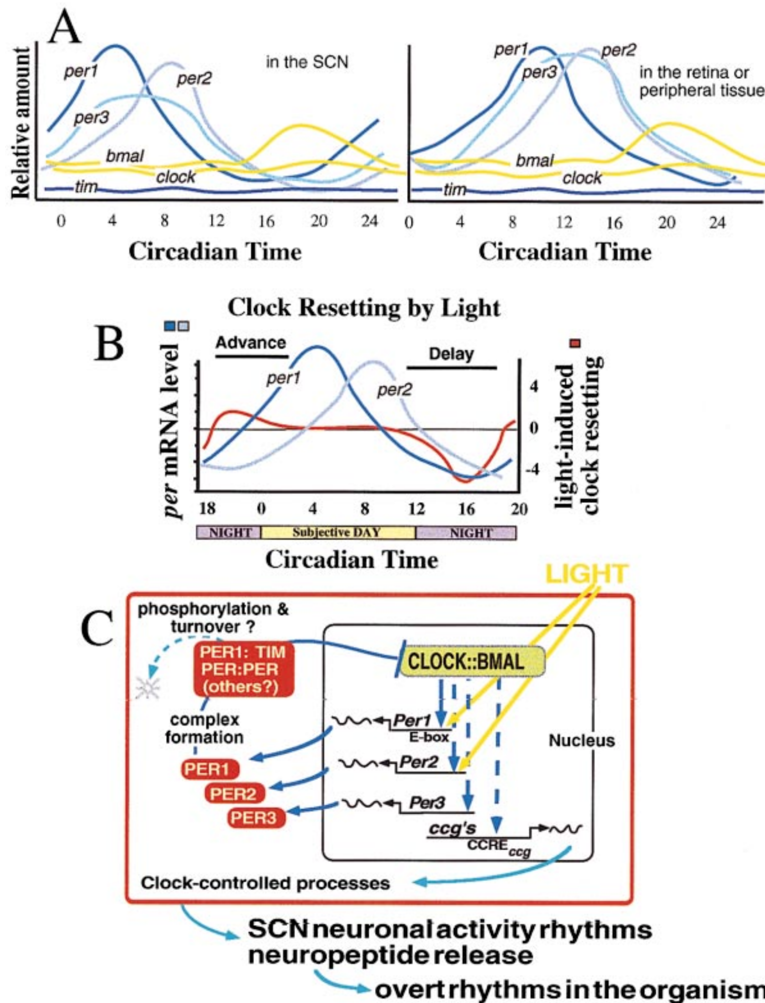


Figure 6. Identity and Regulation of Elements in the Mammalian Oscillator and Their Roles in Entrainment

(A) Temporal regulation of the *Per1*, *Per2*, *Per3*, *tim*, *Clock*, and *bmal1(mop3)* genes.

(B) How light resets the mammalian clock. Light results in the induction of *Per1* and *Per2* but to different extents at different times, so the effect of light on the clock components and therefore on the clock is very much influenced by the time of day; see text for details.

(C) Elements and control logic in the circadian oscillatory loop of mammals. Arrows denote positive regulation, and lines terminating in bars denote negative regulation. Dashed lines indicate possible regulatory connections.

that soon there will be several more reports of *mTim* causing the plot to thicken further, but at this point it seems likely that heterodimeric PER–PER interactions will play a major and novel role in the mammalian clock.

So what's the deal—why three *Pers* and a single (barely?) rhythmic *mTim*? It seems plausible that if mTIM does have a role, it is to facilitate the action of the PERs independently, and having three separate cycling mTIMs was a redundancy evolution found unnecessary. So in Table 1, I have listed mTIM, tentatively, as a facilitator in the same way as DBT in flies. An *mTim* knockout will neatly solve this (if there is really only one *mTim*); the world awaits. Clearly though, the regulatory action will be with the distinct regulation and interactions of the mPERs—which cells within the SCN express which one(s) and what they do, which cells coexpress more than two (e.g., Takumi et al., 1998a) and to what extent they talk to one another. It may be that the low level of mTIM is rate limiting for mPER nuclear entry and, by requiring an mPER to build to a critical concentration in order to heterodimerize, it would thus serve to keep the time of nuclear entry confined to a discrete window of time. Then again, since mPER–mPER interactions appear stronger than mPER–mTIM, it may be that mTIM's role is as a default or cytoplasmic anchor for undimerized mPER. Or perhaps mTIM protein levels will not track RNA levels at all and, like *lark* (see above), be rhythmic after all. Stay tuned.

Because the identified molecular components that are circadianly regulated in the SCN all peak during the daytime, the transcriptional induction model for resetting seen in *Neurospora* appears to apply neatly to entrainment in the SCN, although again there will be wrinkles. Light yields an acute induction of *mPer1* with a peak 60 min after lights on and of *mPer2* with a peak about 90 min after lights on (Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997; Takumi et al., 1998a; Zylka et al., 1998b), but interestingly *mPer3* is not light induced (Takumi et al., 1998b; Zylka et al., 1998b). Unlike *Neurospora*, light induction is gated so that *mPer1* and *mPer2* are induced only late in the day (*mPer2*) or at night (both), thereby here imposing an additional layer of autoregulation on the mammalian circadian system where clock output makes a loop back to affect input. Consistent with predictions based on the evolutionary conservation of the PAS domain heterodimers as positive elements and the role of the *Neurospora* positive elements in light regulation, the *Clock* mutation also attenuates light induction of the *mPers* (Shearman and Weaver, 1999). Light information is

thought to be perceived only by the eyes (Foster, 1998) (see, however, Campbell and Murphy, 1998) and to proceed rapidly via glutamatergic pathways to reset the oscillator itself, an event that happens within 2 hr based on behavioral tests (Best et al., 1999) and in good agreement with the molecular data. Just as expression of the *mPer* genes is not uniform across the SCN, light induction is not uniform among all cells and is not the same for *mPer1* and *mPer2* (e.g., Shigeyoshi et al., 1997; Takumi et al., 1998a, 1998b); sorting out the cellular and molecular connections in this process will be challenging. Light induces the MAP kinase pathway (Obrietan et al., 1998) and several immediate early genes (Morris et al., 1998) in the SCN in a circadianly gated manner, actions that may drive or merely parallel clock-specific effects involved with entrainment (reviewed in Schwartz et al., 1995). Interestingly, although the SCN is believed not to be photoresponsive, a gene encoding a putative blue light photoreceptor (*Cry1*) is rhythmically expressed there and knockouts of a related gene (*Cry2*) result in a 1 hr period lengthening and partial reduction in *mPer1* light induction (Miyamoto and Sancar, 1998; Thresher et al., 1998). Paradoxically, however, they yield increased phase shifting in response to a long (6 hr) light treatment, a duration that could work through a parametric pathway. It may be that the mammalian *Cry* genes chiefly mediate a nonparametric response as suggested above for the fly *cry* gene, and that a long 6 hr pulse is convolving parametric and nonparametric effects. In any case the data are consistent with a role for CRY2 in contributing to light perception and an interaction of this protein with the oscillatory machinery, but by analogy to *Drosophila*, CRY2 would not be expected to play an essential role in the oscillator. Clocks can also be reset pharmacologically, the most trendy such drug being melatonin (Reppert, 1995), which interacts with a family of receptors in the SCN and elsewhere.

Ultimately, the reason we are interested in mammalian clocks is that they regulate our own lives; there exist in mammals more clock-controlled properties than you could shake a stick at. Beyond simple description of more *ccg*s and clock-controlled properties, current research is directed at understanding the initial steps connecting the oscillatory mechanism with output. Within the SCN, the initial steps might be expected to include control of *ccg*s by the clock-regulated positive-regulating complex of BMAL1 and CLOCK (Figures 2 and 6D) (Jin et al., 1999), a scenario supported by the finding that CLOCK/BMAL1 heterodimers activate a target gene (vasopressin) (Jin et al., 1999). Some such target genes might in turn affect the regulation of genes and proteins further downstream, and these could in turn affect the operation of the oscillator itself without being required for its operation. One recent example of such a gene would be *dbp* (encoding the D box–binding protein), which is required for circadian regulation of the albumin gene (Wuarin and Schibler, 1990) and is rhythmically expressed in both the SCN and liver. Disruption of *dbp* has no effect on its own regulation, affects downstream genes, results in less overall locomotor activity, and in a 30 min period shortening of the clock (Lopez-Molina et al., 1997). Similarly, in the pineal gland the CREM (cyclic AMP response element modulator) product, ICER, is rhythmically expressed and participates in a transcriptional autoregulatory loop that also controls the amplitude of oscillations of serotonin N-acetyl transferase (AANAT), the rate-limiting enzyme of melatonin synthesis (Foulkes et al., 1996). This circadian regulation of autoregulatory feedback loops (a circadian loop in the SCN driving an output loop) is a theme also seen in plants (see below). As the first mammalian clock gene homologs appeared, it became clear that they were being expressed not just in the SCN but in a variety of body tissues, and further that their peaks there were delayed roughly 4–6 hr with respect to the SCN (Figure 6B, Zylka et al., 1998b); although the body rhythms require the SCN (Sakamoto et al., 1998), clearly body rhythms were not being immediately controlled by the SCN. The stunning confirmation of this was the report (Balsalobre et al., 1998) that Rat-1 fibroblasts and H35 hepatoma cells in culture expressed serum-shock-entrainable rhythms in the expression of *rPer2*, *rdbp*, and *tef*. Temperature-compensated self-sustained circadian clocks were already known in the retina (Tosini and Menaker, 1998), but the implication of the cell culture data is clearly that oscillators may run in many peripheral tissues and be synchronized in a heirarchical manner by the SCN clock via humoral means (Oishi et al., 1998) or perhaps by temperature (as suggested in Liu et al., 1998).

Plants

As with animals, there is an enormous biology describing rhythmic phenomena in plants, both in terms of clock-controlled genes and behaviors and in terms of the essential role of the clock in photoperiodic responses such as flowering (Sweeney, 1987). However, the molecular underpinnings of plant circadian oscillators has remained obscure until recently. Although clock mutants (as yet uncloned) exist in *Arabidopsis* (reviewed in Millar and Kay, 1997), most progress recently has been tied either to analysis of *ccg*s in plants (in particular in regulation of the proteins such as the LHCB complex of photosystem II (Carré, 1996) or catalase (McClung, 1997)) or to analysis of mutant strains identified in screens for flowering mutants (e.g., Hicks et al., 1996; Schaffer et al., 1998). The past 2 years have been a watershed in research on the molecular basis of plant circadian rhythms, beginning the dissection of the complex network of photoreceptors involved in light perception and revealing the identities of components of autoregulatory loops involved in output and possibly in the oscillator itself (Millar, 1998).

The analysis of circadian output has driven the preponderance of recent research. One of the best studied circadianly regulated genes is one that encodes components of the light harvesting chlorophyll a/b complex LHCB (or CAB) whose expression peaks in the midday, 4–6 hr after dawn. As with many of the *Neurospora ccg*s, *lhcb* is acutely induced by light (to an extent that is gated by the clock; Millar and Kay, 1996) and is also subject to circadian regulation in the dark. Among the best candidates for genes encoding clock components in plants is *toc-1*, a gene repeatedly identified in a screen using clock-regulated luciferase fused to the *lhcb* promoter such that out of phase or arrhythmic plants could

be identified (Millar et al., 1995). The *toc-1* mutation is known to affect multiple circadian outputs (Somers et al., 1998b). Analysis of the *lhcb1*1* (CAB) promoter for sequences conferring light and/or clock regulation has revealed that a short fragment supporting clock regulation also drives light induction and is bound by a number of DNA-binding protein complexes, but that a minimal clock-regulated fragment is no longer light responsive (Carré and Kay, 1995; Wang et al., 1997). One of these is CCA1, a *myb*-related protein whose transcript level cycles in a circadian manner and whose DNA binding can be modulated by CK2 (casein kinase II) phosphorylation (Sugano et al., 1998; Wang and Tobin, 1998). Its overexpression results in loss of rhythmic expression of several known *ccg*s that peak at different times during the day, loss of photoperiodic control (indicative of loss of circadian regulation) (Wang and Tobin, 1998), and loss of rhythmicity in *LHY* expression. *LHY* (late elongated hypocotyl) in turn, is a gene identified in a screen for flowering mutants that also encodes a *myb*-like factor whose level also oscillates in a circadian manner (Schaffer et al., 1998). Based on analysis of overexpressing alleles, both *LHY* and *CCA1* encode components of mutually regulatory negative feedback loops (overexpression dampens their own and each others expression) that affect multiple circadian outputs. As the authors carefully point out, these data are consistent with (but not confirmatory of) roles for these genes within circadian oscillators, and, if so, the oscillators in plants would be very different from those seen in the other members of the eukaryotic crown. However, a caveat to the anointment of *CCA1* and *LHY* as clock components is that these phenotypes are based on (necessarily dominant) overexpressing strains. There are abundant precedents for feedback of output loops on input loops and on oscillators themselves (see above), so it is possible that the dominant nature of these alleles is obscuring a still functional clock that would be unmasked in a null allele. Such conditional arrhythmicity has previously been seen in another flowering mutant, *elf3*, shown to have a functional clock despite loss of rhythmicity in some outputs (Hicks et al., 1996), and light and clock regulation of plant transcription factors is known (e.g., Zheng et al., 1998). Additionally there exists an excellent precedent for an autoregulatory negative feedback loop involved in circadian output in plants (the CCR2 (=AtGRP7) gene; Heintzen et al., 1997). It may be that such a driven oscillator in an output serves to maintain a robust output rhythm at any desired phase by affording the organism flexibility in the timing of various outputs driven from a single central clock. Are CCA1 and LHY involved in input or output loops that feed back so that the clock will still run in their absence (as might be predicted from evolutionary arguments requiring PAS-containing components), or are they really clock components, the avatars of a novel eukaryotic clock mechanism? Indeed, the world awaits.

In terms of circadian input, plants make their living using light and are equipped by nature with a staggering armament of proteins and chromophores with which they optimize its detection and use. Period length is modulated by light fluence (photons per unit time) in constant dim light, and this assay has been used to identify the range of action of specific photopigments—phytochrome A (PHYA) for low fluence red and blue light, PHYB for high fluence red, CRY1 with PHYA for low fluence blue, and CRY1 for high fluence blue—interestingly leaving no apparent role for CRY2 (Somers et al., 1998a). Evidence that PHYA phosphorylates CRY1 is testament to the degree of cross-regulation in this complex system (Ahmad et al., 1998).

Conclusions

Looking back now at the regulatory loops described, it is clear that nature is using some well-conserved themes but is mixing and matching them in a variety of ways. In *Neurospora* and flies, all the negative elements cycle (FRQ in the former case and PER/TIM in the latter), but in mammals only the (not one but three distinct) *per* genes cycle robustly in expression. With the positive elements, none cycle strongly in *Neurospora*, one (*Clk*) does in flies, and a different one (*bmal1*) does in mammals. Similarly, and perhaps more surprisingly, the phases at which the elements act are shifted in different systems, so that positive and negative elements drive a day-phase clock in *Neurospora* and in mammals, whereas flies use a subset of the same components as mammals to build a night phase clock, a shift that dictates a complete change in the entire logic and mechanism of clock resetting by light. But through this variation, a core in circadian regulation of transcription (as in Figure 2) runs as a theme, as do PAS domain heterodimeric activators in the eukaryotes, as do PER and TIM in the animals.

The molecular bases of circadian rhythms has emerged as an enticing and tractable puzzle in recent years. Clocks represent at once both a nearly ubiquitous aspect of cellular regulation and also a molecular regulatory process that has clear and immediate effects on organismal behavior. Research has now blossomed to the point that it is all but impossible to embrace it all in a single review. Thus, the pioneering genetic studies in the clocks of model systems in the early 1970s have borne great fruit in giving rise to a field of study, again demonstrating that a fascinating question and an honest genetic approach win their own friends.

Acknowledgments

I thank Drs. J. Hall, S. Kay, T. Kondo, J. Loros, H. Okamura, S. Reppert, and M. Young for helpful discussions, sharing of unpublished data, and for critical reading of the manuscript and M. Sogin for help with Figure 1. This work was supported by grants from the National Institutes of Health (GM 34985, MH44651, and MH01186) and the Norris Cotton Cancer Center core grant at Dartmouth Medical School.

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